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Tiffany Russell April 9, 2021

Modeling Dysregulation of Neurogenesis and Neurodevelopment in Tuberous Sclerosis Complex with Human Brain Organoids

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Abstract

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Tuberous sclerosis complex (TSC) is a genetic disorder in humans that manifests in the growth of benign tumors primarily in the brain as well as in other body systems. The neurological pathologies of TSC include cortical tubers, subependymal nodules (SENs), and subependymal giant cell astrocytomas (SEGAs), all of which are abnormal growths. These pathologies can result in symptoms such as epilepsy, autism spectrum disorder, attention deficit hyperactivity disorder, and other cognitive and learning disabilities in patients with TSC. TSC is caused by a mutation in one or both tumor suppressor genes *TSC1* and *TSC2*; specifically, the mutation that results in an individual having TSC is a loss-of-function mutation in either or both genes. The *TSC1* gene codes for a protein called hamartin (TSC1) and the *TSC2* gene codes for a protein called tuberin (TSC2). Both hamartin and tuberin are responsible for inhibiting the mechanistic target of rapamycin complex 1 (mTORC1) protein complex, which is crucial in the regulation of cell growth through several mechanisms. When *TSC1* and *TSC2* are mutated and hamartin and tuberin are respectively not produced correctly, mTORC1 is upregulated, which allows for the growth of tumors observed in individuals with TSC. Brain organoids generated from iPSCs serve as small-scale models of the brain and are useful in studying morphological differences in neurogenesis and neurodevelopment between individuals with TSC and individuals without TSC. This study investigated the impact that TSC has on the developing brain in regards to neural progenitor cell proliferation and soma size using human brain organoids. The hypothesis was that the loss of *TSC1* and *TSC2* function will result in abnormal hyperproliferation and soma

overgrowth in neural progenitor cells (NPCs) in TSC cortical organoids. This hypothesis was supported by the data collected through fluorescence microscope analysis; it was observed that (1) a significantly higher proportion of NPCs were proliferating in the TSC organoids than in the control organoids and (2) NPC soma sizes were significantly larger in TSC organoids in comparison to control organoids.

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TABLE OF CONTENTS

Abstract

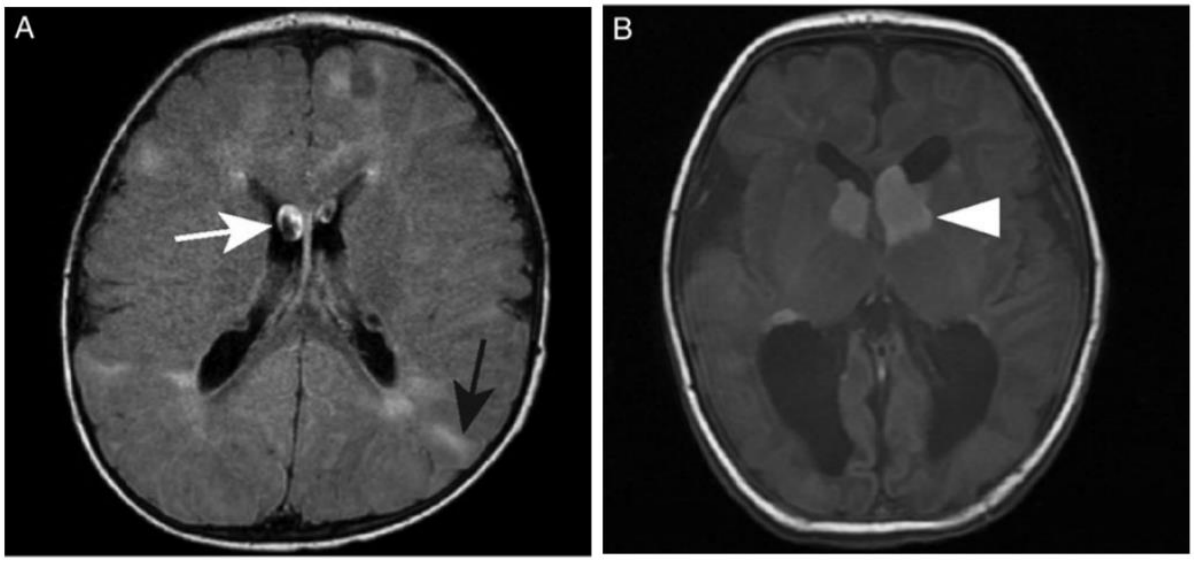
Tuberous sclerosis complex (TSC) is a genetic disorder in humans that manifests in the growth of benign tumors primarily in the brain as well as in other body systems. The neurological pathologies of TSC include cortical tubers, subependymal nodules (SENs), and subependymal giant cell astrocytomas (SEGAs), all of which are abnormal growths. These pathologies can result in symptoms such as epilepsy, autism spectrum disorder, attention deficit hyperactivity disorder, and other cognitive and learning disabilities in patients with TSC. TSC is caused by a mutation in one or both tumor suppressor genes *TSC1* and *TSC2*; specifically, the mutation that results in an individual having TSC is a loss-of-function mutation in either or both genes. The *TSC1* gene codes for a protein called hamartin (TSC1) and the *TSC2* gene codes for a protein called tuberin (TSC2). Both hamartin and tuberin are responsible for inhibiting the mechanistic target of rapamycin complex 1 (mTORC1) protein complex, which is crucial in the regulation of cell growth through several mechanisms. When *TSC1* and *TSC2* are mutated and hamartin and tuberin are respectively not produced correctly, mTORC1 is upregulated, which allows for the growth of tumors observed in individuals with TSC. Brain organoids generated from iPSCs serve as small-scale models of the brain and are useful in studying morphological differences in neurogenesis and neurodevelopment between individuals with TSC and individuals without TSC. This study investigated the impact that TSC has on the developing brain in regards to neural progenitor cell proliferation and soma size using human brain organoids. The hypothesis was that the loss of *TSC1* and *TSC2* function will result in abnormal hyperproliferation and soma overgrowth in neural progenitor cells (NPCs) in TSC cortical organoids. This hypothesis was supported by the data collected through fluorescence microscope analysis; it was observed that (1) a significantly higher proportion of NPCs were proliferating in the TSC organoids than in the

control organoids and (2) NPC soma sizes were significantly larger in TSC organoids in comparison to control organoids.

Introduction and Background

What Is Tuberous Sclerosis Complex?

Tuberous sclerosis complex (TSC) is a genetic disorder in humans that manifests in the growth of benign tumors primarily in the brain as well as in other body systems.[1] The name of the disease stems from the Latin word *tuber*, meaning "root-shaped growth," and the Greek word *skleros*, meaning "hard," because of the signature tuber-like tumors found in TSC patients. Specifically, the neurological pathologies of TSC include cortical tubers, subependymal nodules (SENs), and subependymal giant cell astrocytomas (SEGAs), all of which are abnormal growths.[2] These pathologies can result in symptoms such as epilepsy, autism spectrum disorder, attention deficit hyperactivity disorder, and other cognitive and learning disabilities in patients with TSC.



MacKeigan JP, Krueger DA. 2015. Major neurological features of TSC. Neuro-Oncology. 17(12):1550-1559. doi:10.1093/neuonc/nov152

The images above from a publication by MacKeigan and Krueger in 2015 shows the brains of two individuals with TSC.[2] Image A was generated from a CT scan, and it displays cortical

tubers and radial migration lines denoted with the black arrow and SENs denoted with the white arrow. Image B was generated from an MRI scan, and it displays SEGAs denoted by the white arrow. These growths occurred as a result of each individual having TSC, which demonstrates the significant impact that TSC can have on the brain.

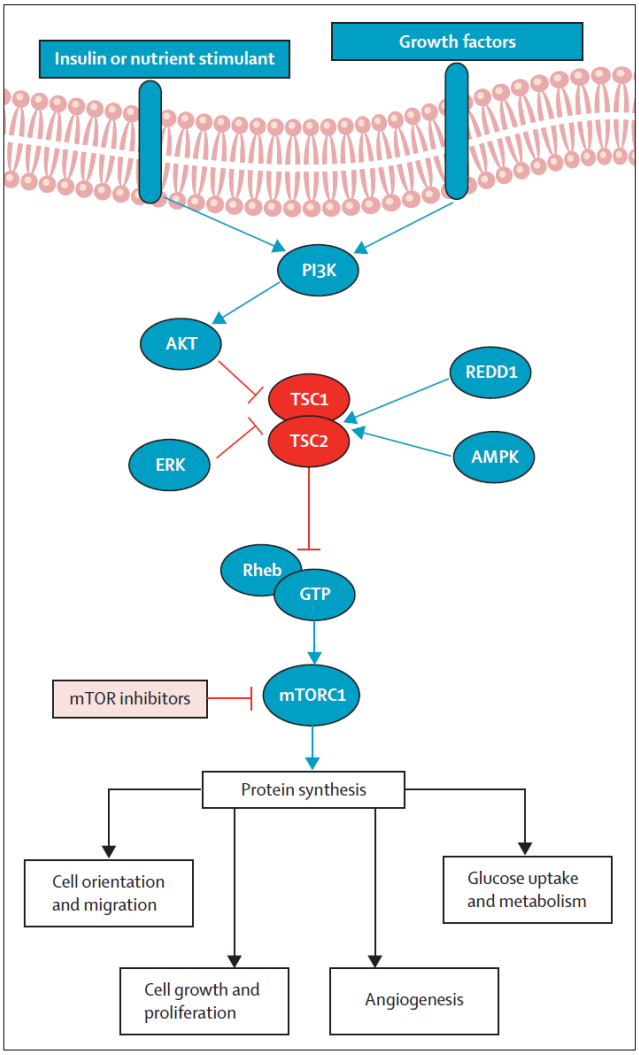
In regards to the epidemiology of TSC, the disease is present across all races, ethnicities, and sexes out of the approximately 2 million cases worldwide.[3] Prognosis for individuals with TSC can vary from a normal life expectancy in those with milder forms to the occurrence of fatal complications, such as status epilepticus or bronchopneumonia, in those with more severe forms.[4] Similarly, in severe TSC cases, sudden unexplained deaths due to epilepsy complications have been recorded.[5] Since TSC is a genetic disease, there are no treatments available that can cure patients, only chronic treatments to prevent complications.

Genetic Basis and Neurological Impacts

TSC is caused by a mutation in one or both tumor suppressor genes *TSC1* and *TSC2*; specifically, the mutation that results in an individual having TSC is a loss-of-function mutation in either or both genes.[6] TSC is an autosomal dominant disorder, meaning that it can be inherited if only one parent has a mutated copy of either gene. However, only about one-third of individuals with TSC inherited a *TSC1* or *TSC2* gene mutation from a parent, whereas the majority of TSC cases occurred because of a spontaneous germline mutation—a mutation in the egg or sperm cell. According to a publication by Sancak et al. in 2005, *TSC2* is more often the mutated gene responsible for an individual having TSC, with the ratio of *TSC2* mutations to *TSC1* mutations being 3.4 to 1.[7] Similarly, the mutation frequency per nucleotide of the *TSC2*

gene is higher than that of the *TSC1* gene. The most common type of mutation to occur in *TSC1* and in *TSC2* is the insertion or deletion of a single base pair, which occurs in 99% of *TSC1* mutations and 75% of *TSC2* mutations. These mutations can cause the protein resulting from transcription and translation to be truncated, thus dramatically reducing its function or rendering it non-functional. The variability in the genetic cause of TSC is what leads to the variability in the severity of the symptoms displayed across TSC cases, although individuals with a mutation in the *TSC2* gene have generally been observed to have a more severe TSC phenotype than individuals with a mutation in the *TSC1* gene.[8]

The *TSC1* gene, which is located on chromosome 9q34, codes for a protein called hamartin (also called TSC1) and the *TSC2* gene, which is located on chromosome 16p13.3, codes for a protein called tuberin (also called TSC2).[6,9,10] Both hamartin and tuberin are responsible for inhibiting the mechanistic target of rapamycin complex 1 (mTORC1) protein complex.[11] mTORC1 is crucial in the regulation of cell growth in the way that it responds to the presence of adequate amounts of resources (nutrients, growth factors, and energy) required for cell growth. When *TSC1* and *TSC2* are mutated and hamartin and tuberin are respectively not synthesized correctly, mTORC1 is upregulated, which allows for the growth of tumors observed in individuals with TSC. The diagram shown below from a publication by Curatolo et al. displays the pathway of how hamartin and tuberin (labeled as TSC1 and TSC2, respectively) inhibit mTORC1.[12]



Curatolo P, Moavero R, de Vries PJ. 2015. Pathophysiology of tuberous sclerosis. The Lancet. 14(7):733-745. https://doi.org/10.1016/S1474-4422(15)00069-1

In the presence of resources necessary for cell growth, phosphatidylinositol-3 kinase (PI3K) activates the cascade of reactions leading to cell growth.[12] PI3K activates protein kinase B (AKT), which inhibits the TSC1-TSC2 (hamartin and tuberin) protein complex along with extracellular signal-regulated protein kinase (ERK). Conversely, regulated in development and DNA damage responses (REDD1) and AMP-activated protein kinase (AMPK) activate the TSC1-TSC2 protein complex when resources are absent. When the TSC1-TSC2 protein complex is activated, the protein complex composed of Ras homologue enriched in brain (Rheb) and guanosine triphosphate (GTP) is inhibited. The Rheb-GTP protein complex is responsible for

activating mTORC1, which stimulates the synthesis of proteins that encourage cell growth, thus mTORC1 is inhibited when the Rheb-GTP protein complex is inhibited by TSC1-TSC2, preventing cell growth. The mutations in the *TSC1* and *TSC2* genes characteristic of TSC lead to the aforementioned truncation of hamartin (TSC1) and tuberin (TSC2), respectively, which reduces the TSC1-TSC2 protein complex's ability to inhibit mTORC1 from stimulating the protein synthesis that leads to cell growth.

In the 2013 publication by Magri et al. that investigated the neuropathology of TSC using mouse models, the upregulation of mTORC1 was observed to result in megacephaly, improper migration and organization of cortical neurons, enlargement of neuronal soma (cell body), and the presence of multiple nuclei in neuronal soma.[13] These neurological abnormalities in the TSC-affected mice stemmed from the signature mutations of the *TSC1* and *TSC2* genes that cause TSC. The truncated hamartin and tuberin proteins synthesized when *TSC1* and *TSC2* are respectively mutated form a TSC1-TSC2 complex that is less able to regulate mTORC1, resulting in the inappropriate growth, division, and organization of neurons in the cortex.

Using Induced Pluripotent Stem Cells to Investigate TSC

Cell samples, such as skin fibroblasts, can be taken from individuals with and without TSC, and they can be subsequently reprogrammed into induced pluripotent stem cells (iPSCs).[14] iPSCs have the potential to differentiate into any type of human cell, regardless of the original cell type. Because the cell samples obtained from patients with TSC have DNA with a *TSC1* or *TSC2* gene mutation, the iPSCs generated from the samples also have the *TSC1* or *TSC2* gene mutation, and the cell type into which the iPSCs are differentiated thusly has the same mutation. Therefore,

these iPSCs can be differentiated into neurons and develop into 3-D cortical organoids, which act as small-scale models of the brain. Brain organoids are useful in studying morphological differences in neurogenesis and neurodevelopment between individuals with TSC and individuals without TSC. Previously, animal models have been utilized to evaluate the impact that TSC has on the brain, such as in the aforementioned publication by Magri et al. in 2013.[13] However, using human brain organoids, the impact of TSC on the brain can be observed specifically in humans without the risk of having to extrapolate results from an animal model to humans. Because the commonalities in the role of the TSC1-TSC2 complex in inhibiting mTORC1 in animals and humans, the hyperproliferation of neurons and overgrowth of the soma that have been observed in animal model brains could similarly be observed in human brain organoids.

Hypothesis

The loss of *TSC1* and *TSC2* function will result in abnormal hyperproliferation and soma overgrowth of neural progenitor cells (NPCs) in TSC cortical organoids. If abnormal hyperproliferation is present in the TSC organoids, a higher proportion of NPCs—cells with the capability to develop into neurons—will be actively dividing in the TSC organoids than in the control organoids, indicating hyperproliferation of neurons in TSC organoids. If soma overgrowth is present in the TSC organoids, the average size of the NPCs in the TSC organoids will be larger than the average size of the NPCs in the control organoids.

Methods

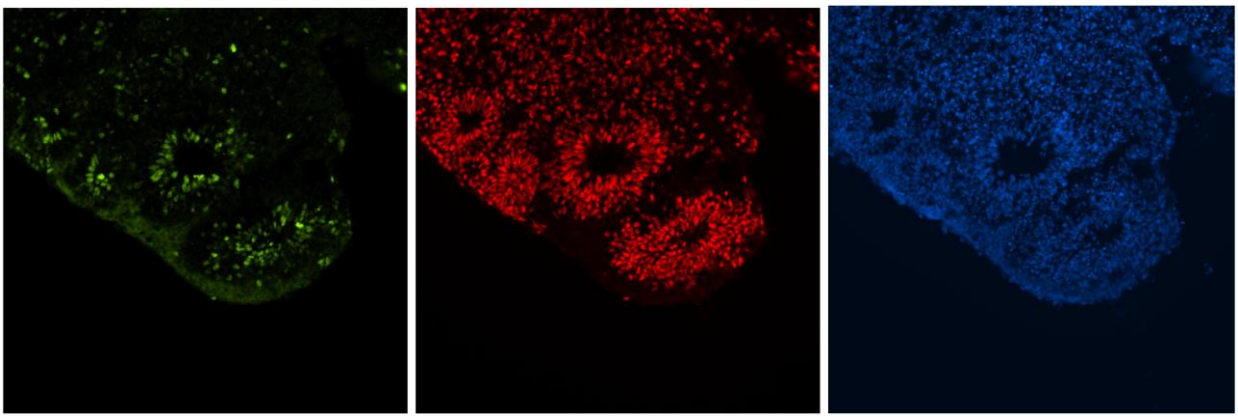
Organoid Preparation

In order to generate the iPSCs needed for organoid growth, skin fibroblasts from individuals with and without TSC were first obtained then reprogrammed into iPSCs. Then, those iPSCs were differentiated into 3-D cortical organoids with the *TSC1* or *TSC2* gene mutation (experimental group) and cortical organoids with the wild type (WT) *TSC* genes (control group), using established protocol.[15] There were two time point groups: organoids that were 28 days into development and organoids that were 42 days into development. Similarly, the process of fixing the organoids was completed through established protocol in preparation for immunofluorescent staining.[15] After being fixed, the organoids were sectioned into 5 μm-thick slices with a Leica Cryostat machine. The slices were then adhered to microscope slides and labeled with the type of organoid (TSC or control), time point group (day 28 or day 42), and slide number, and the slides were kept refrigerated until they were stained. The *TSC2* gene mutation is more common than the *TSC1* gene mutation and the two mutations both result in the TSC phenotype, thus a majority of samples had the *TSC2* gene mutation.

Immunostaining and Fluorescence Microscope Imaging

After being fixed and sectioned, the organoid sections were stained with multiple antibodies and green fluorescent protein (GFP), which allows the individual neurons to be seen with a fluorescence microscope. For immunostaining, the microscope slides were washed three times for five minutes each with a phosphate-buffered saline (PBS) solution before permeabilization, which punctures the cell membrane such that the stain will be able to access the internal components of the cells. The slides were permeabilized with 0.2% Triton-X in PBS for 30

minutes. To prevent the stain from binding to nonspecific binding sides, each slide was then blocked with a blocking medium consisting of 10% donkey serum in PBS and 0.1% Triton-X for 30 minutes. The antibodies used to stain the organoids (DAPI, Ki-67, MAP2, and SOX2) were diluted using 5% donkey serum in PBS and 0.1% Triton-X, and the slides were treated with the antibody staining solution. Once the organoids have been stained, they are photographed under a fluorescence microscope. Different fluorescent shades illuminate different neurons depending on certain characteristics based on the stain that can adhere to the cell, made visible by the GFP. The DAPI antibody stain is a marker for the nuclei of the cells present in the organoid, so the fluorescence microscopy photographs can display a baseline of the number of cells present. The Ki-67 antibody stain is used as a marker for proliferating cells, meaning that only the cells that are actively dividing are visible in fluorescence microscopy photographs and cells that are quiescent, or not dividing, are not. The MAP2 antibody stain is a marker for microtubules, which are prevalent in the axons and dendrites of neurons, making structures with microtubules visible under the fluorescence microscope. However, the photographs of the MAP2+ cells were not used for this specific investigation. The SOX2 antibody stain is a marker for NPCs, thus the NPCs present in the organoids are visible in fluorescence microscopy photographs. Each area of the organoids is photographed four times under a different fluorescent shade for each image, which captures individual images of the cells positive for the four aforementioned antibody stains. Below is an example of the visibility of the Ki-67+ cells (left), SOX2+ cells (middle), and DAPI+ cells (right) with the fluorescence microscope images. This specific organoid is a control group cortical organoid from day 42.



Cell Counting and Measurement

ImageJ was used to process the images of the organoids. Abnormal hyperproliferation of neurons was evaluated by counting the numbers of the DAPI+, Ki-67+, and SOX2+ cells in each perspective of the organoids captured. The means of the proportions of Ki-67+/SOX2+ cells out of all NPCs (SOX2+) in control and TSC cortical organoids at both time points as well as the means of the proportions of SOX2+/DAPI+ cells out of all DAPI+ cells in control and TSC cortical organoids at both time points were calculated and compared between experimental groups. Soma overgrowth was evaluated by tracing the area (in microns^2) of a random selection of NPCs (SOX2+ cells). The means of the NPC sizes in TSC and control cortical organoids at both time points were calculated and compared between experimental groups.

Statistical Analysis

Statistical significance was determined using an unpaired t-test with a p-value of $p<0.05$ indicating a significant difference. The determination of presence of outliers in each data set was performed using Grubbs' test for outliers.

Results

TSC Cortical Organoids Demonstrated Significantly Higher Proportions of Proliferating Neural Progenitor Cells Than Control Cortical Organoids

The proportions of actively proliferating NPCs were quantified by counting the number of Ki-67+/SOX2+ cells and dividing this number by the population of all SOX2+ cells. At both day 28 and day 42 time points, the TSC cortical organoids had a statistically significantly higher proportion of actively proliferating neural progenitor cells than did the control cortical organoids. In the day 28 group, the average proportion of Ki-67+/SOX2+ cells out of all SOX2+ cells was 0.576 (N=15) for the control cortical organoids, whereas the average proportion of Ki67+/SOX2+ cells out of all SOX2+ cells was 0.817 (N=17) for TSC cortical organoids ($p<0.0001$). In the day 42 group, the average proportion of Ki-67+/SOX2+ cells out of all SOX2+ cells was 0.493 (N=29), whereas the average proportion of Ki67+/SOX2+ cells out of all SOX2+ cells was 0.692 (N=42) for TSC cortical organoids ($p<0.0001$). For both day 28 and day 42 time points, the proportions of NPCs (SOX2+/DAPI+) present out of all cells (DAPI+) in control and TSC cortical organoids were not significantly different.

TSC Cortical Organoids Demonstrated Significantly Larger Neural Progenitor Cell Soma Sizes Than Control Cortical Organoids

The average soma sizes in square microns of the TSC and control cortical organoid NPCs at each time point were determined by tracing the perimeter of a random sample of SOX2+ cells and finding the mean in each group. At both day 28 and day 42 time points, the TSC cortical organoids had statistically significantly larger soma sizes than did the control cortical organoids. In the day 28 group, the average NPC soma size was 43.02 μm^2 (N=30) for control cortical

organoids, whereas the average NPC soma size was 57.03 μm^2 (N=37) for TSC cortical organoids ($p<0.0001$). In the day 42 group, the average NPC soma size was 40.55 μm^2 (N=29) for control cortical organoids, whereas the average NPC soma size was 55.37 μm^2 (N=43) in TSC cortical organoids ($p<0.0001$).

Figures

Figure 1: Difference in Proportion of Proliferating Neural Progenitor Cells Out of All Neural Progenitor Cells Between Control and TSC Cortical Organoids

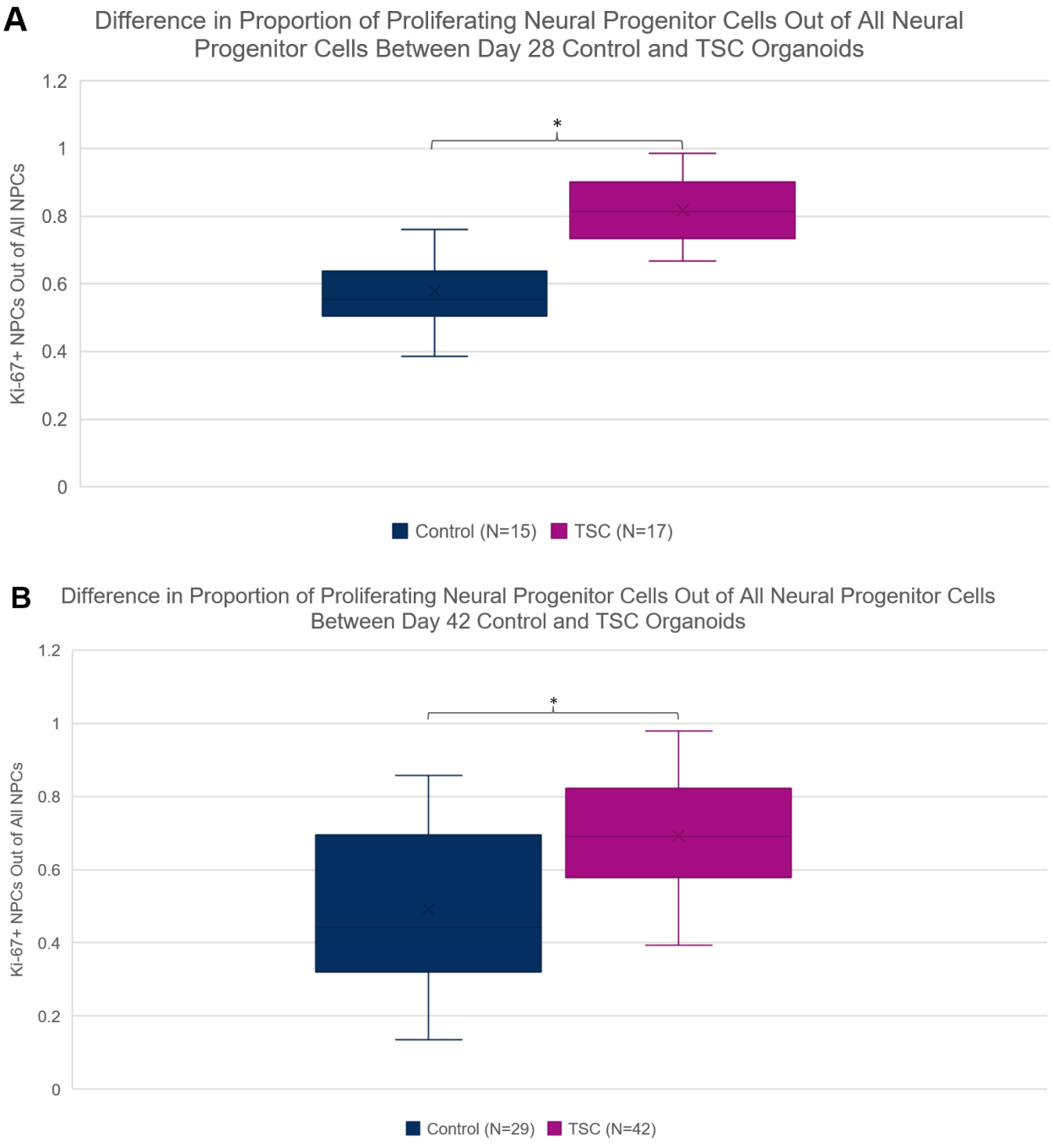


Figure 1: Difference in Proportion of Proliferating Neural Progenitor Cells Between Control and TSC Cortical Organoids. (A) Difference in proportion of proliferating neural progenitor cells out of all neural progenitor cells between day 28 control (navy blue) and TSC (magenta) organoids. Data distribution is displayed with a box and whisker plot. An asterisk indicates the difference in proportion was statistically significant. (B) Difference in proportion of proliferating neural progenitor cells out of all neural progenitor cells between day 42 control (navy blue) and TSC (magenta) organoids. Data distribution is displayed with a box and whisker plot. An asterisk indicates the difference in proportion was statistically significant.

Figure 2: Difference in Size of Neural Progenitor Cell Somas Between Control and TSC Cortical Organoids

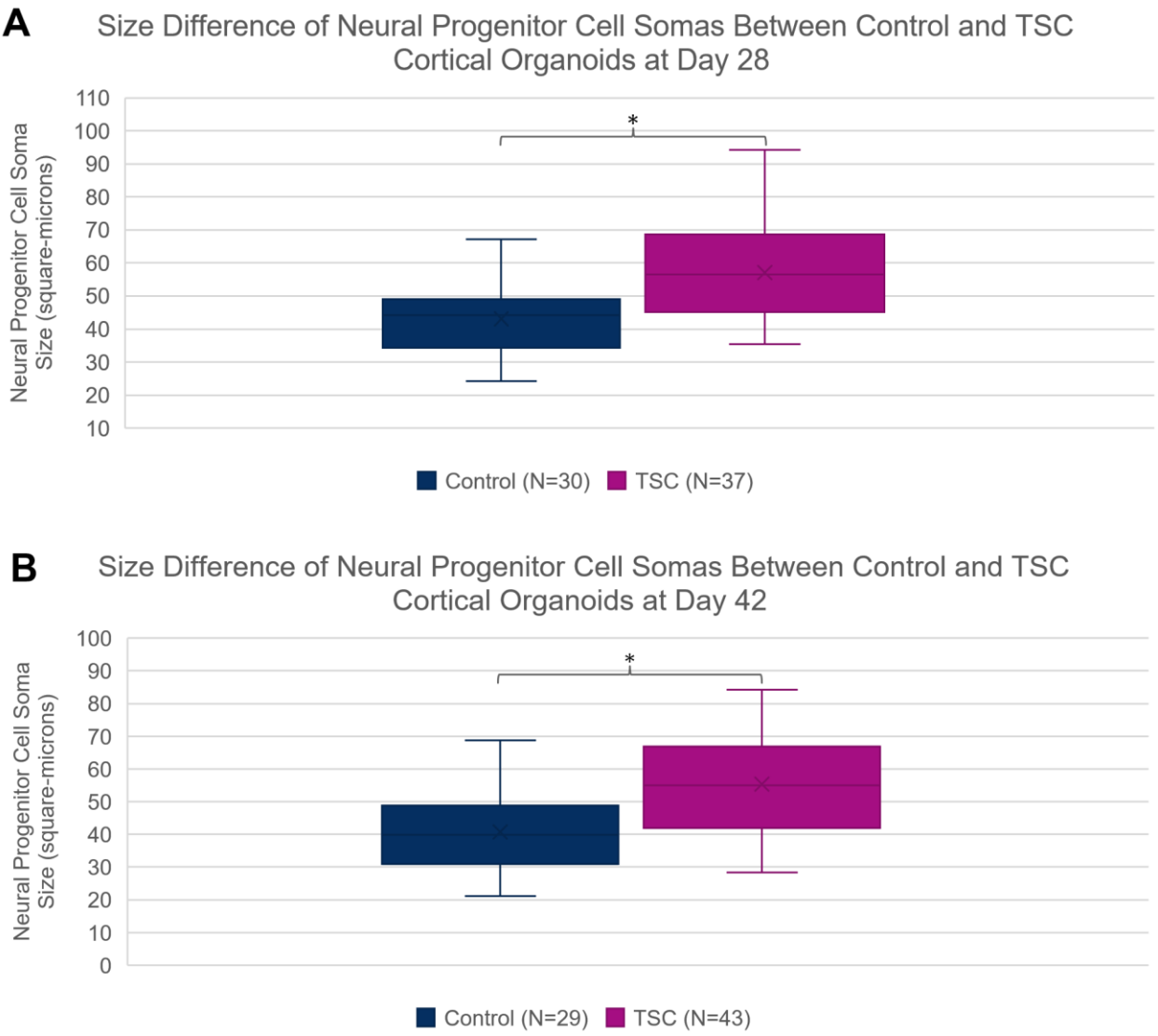


Figure 2: Difference in Size of Neural Progenitor Cell Somas Between Control and TSC Cortical Organoids. (A) Difference in size of neural progenitor cells (SOX2+) between control (navy blue) and TSC (magenta) organoids at day 28. Data distribution is displayed with a box and whisker plot. An asterisk indicates the difference in proportion was statistically significant. (B) Difference in size of neural progenitor cells (SOX2+) between control (navy blue) and TSC

(magenta) organoids at day 42. Data distribution is displayed with a box and whisker plot. An asterisk indicates the difference in proportion was statistically significant.

Discussion

At both day 28 and day 42, statistically significantly higher proportions of actively proliferating NPCs were observed in TSC cortical organoids in comparison to control cortical organoids. Similarly, at both day 28 and day 42, statistically significantly larger NPC soma sizes were observed in TSC cortical organoids in comparison to control cortical organoids. Therefore, the hypothesis set forth, which predicted that cortical organoids having the *TSC1* and *TSC2* gene mutations will result in hyperproliferation of NPCs and larger NPC soma sizes when compared to cortical organoids with the WT *TSC1* and *TSC2* genes, was supported.

Hyperproliferation of Neural Progenitor Cells in TSC Cortical Organoids

There was a significantly higher proportion of actively dividing NPCs observed in the cortical organoids with *TSC1* and *TSC2* gene mutations than in the cortical organoids with WT *TSC* genes. This result can be explained by the lack of mTORC1 inhibition that is characteristic of the *TSC1* and *TSC2* gene mutations. When either hamartin or tuberin are truncated due to a TSC-associated gene mutation, the TSC1-TSC2 complex that is usually formed from the two proteins is less effective at inhibiting mTORC1.[11] In the presence of certain resources such as nutrients and growth factors, mTORC1 is responsible for repressing a gene that inhibits cell proliferation, *SIRT4*.[16] The TSC1-TSC2 protein complex is integral in preventing mTORC1 from inappropriately repressing *SIRT4*. Thus, when the proteins hamartin and tuberin involved in the TSC1-TSC2 complex are incorrectly synthesized, mTORC1 is not as effectively inhibited from inducing cell proliferation. The proportion of proliferating NPCs in TSC organoids being higher than that in control organoids indicates that the inhibition of mTORC1 by the TSC1-TSC2 protein complex is crucial in preventing neural progenitor cells from actively producing new

neurons when proliferation is not suitable in current environment, possibly due to a lack of nutrients, growth factors, or other resources.

The hyperproliferation of NPCs in the TSC cortical organoids could partially explain how the neurological pathologies that result of *TSC1* and *TSC2* gene mutations develop in the cortices of individuals with TSC. When mTORC1 is uninhibited, the cellular division is not regulated as effectively, which is partially responsible for the generation of the TSC-associated abnormal masses through increased frequency and less regulation of cell division in the brains of those with TSC. Furthermore, the significantly higher proportion of hyperproliferating NPCs demonstrated in TSC cortical organoids compared to the control cortical organoids illustrate how early in neurodevelopment the *TSC1* and *TSC2* gene mutations impact the growth of the brain. As early as in the group of organoids that were 28 days into development, the presence of mutated *TSC1* and *TSC2* genes had a significant impact on development, as indicated by the difference between the proportion of proliferating NPCs in the TSC organoids and in the control organoids. Proliferation of NPCs is seen to be heavily influenced by the presence of a mutation in the *TSC1* or *TSC2* gene early in development.

Enlarged Neural Progenitor Cell Somas in TSC Cortical Organoids

The somas of the NPCs in the TSC organoids were significantly larger than those in the control organoids, which supports the hypothesis in this investigation. The result of mTORC1 stimulating the synthesis of proteins involved in growth and development has many facets—cell orientation and migration, cell growth and proliferation, angiogenesis, glucose uptake and metabolism, and more.[12] Specifically, mTORC1 has been previously observed to be involved in

shifting the mechanism of glucose metabolism in cells from oxidative phosphorylation to glycolysis.[17] As opposed to oxidative phosphorylation, the utilization of glycolysis in glucose metabolism aids in the acquisition of biomass in cells. This impact of mTORC1 on glucose metabolism serves as an explanation for the result observed in this investigation in regards to soma size. The truncation of hamartin or tuberin stemming from the TSC-associated mutations in genes *TSC1* and *TSC2* leads to the upregulation of mTORC1.[11] Thus, since mTORC1 is active in the shift of glucose metabolism to glycolysis that leads to the acquisition of biomass in cells, then the increased NPC soma size observed in TSC cortical organoids demonstrates that a lack of inhibition of mTORC1 leads to overgrowth of the cell body in neural progenitor cells.

Similar to the hyperproliferation of NPCs, the significantly larger somas of the NPCs observed in TSC cortical organoids in comparison to control cortical organoids demonstrate the early impact that TSC has on neurodevelopment. The enlargement of NPC somas was observed in TSC organoids 28 days into development, further illustrating how TSC influences the structure and function of the brain as it progresses through development.

Future Directions

A future investigation that could be pursued that expands on this study would be to utilize the MAP2 antibody stain to observe the impacts of the *TSC1* and *TSC2* gene mutations on axon and dendrite length in cortical organoids. The MAP2 stain serves as a marker for microtubules, which are highly prevalent in the axons and dendrites of neurons. The fluorescence microscope images that display MAP2+ neurons could be measured in both TSC and control organoids, which would determine if there is a significant difference between the length of the neural branches between cortical organoids with the *TSC1* and *TSC2* gene mutations and the cortical organoids with the WT *TSC1* and *TSC2* genes.

A second future investigation could be to observe the impacts of an mTOR pathway inhibitor on cortical organoids with the *TSC1* and *TSC2* gene mutations as a potential basis for a drug therapy for individuals with TSC. Partially inhibiting the mTOR pathway to mimic the role that the TSC1-TSC2 protein complex has in inhibiting mTORC1 from stimulating cell growth could serve as a treatment for TSC patients. Using human brain organoids in the development of such a drug would alleviate the need to use animal models because the success of a drug observed in animal models does not always equate to the same success when the drug is used in humans.

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