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Deciphering Circuit-level Mechanisms Underlying Intrinsic
Epileptogenicity of Cortical Tubers in TSC

PRINCIPAL INVESTIGATOR:

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14. ABSTRACT Cortical tuber (CT), the neuropathological hallmark of tuberous sclerosis complex (TSC), is a major type of pediatric cortical dysplasia characterized by cortical dyslamination and constituent dysplastic cell types such as balloon cells and cytomegalic neurons. It is generally believed that seizures associated with TSC arise from CTs, and dysplastic cell types residing in CTs may be culprits to blame in seizure generation. However, we currently know little about how these abnormal cell types interact with other CT cells to drive seizure activity. By applying the multi-patch recording on slices prepared from surgically resected, epileptogenic CTs, we examine the connectivity of these dysplastic cell types to identify the potential "epileptic" cell types that may be critical for CT seizure generation. Single-cell RNA-sequencing is also applied to CT to uncover the transcriptomic landscape of CT at the level of cell types and to unravel genetic mechanisms underlying the unique phenotypes of abnormal cell types specific to CT. Our unbiased profiling of the cellular constituents of CTs from TSC cases so far uncovered the specific cellular populations in CT and suggests cytomegalic interneurons may be a critical player in seizure generation in CT.					
15. SUBJECT TERMS Tuberous sclerosis complex, connectivity, electrophysiology, single-nucleus RNA-sequencing, balloon cells, cytomegalic neurons, Patch-seq, epilepsy, GABA, interneurons					
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1. INTRODUCTION:

Tuberous sclerosis complex (TSC) is a complex genetic disorder often associated with intractable epilepsy, autism, and intellectual disability, characterized by focal cortical dysplasia known as cortical tubers (CTs) as well as tumors in other organs. It is generally believed that seizures associated with TSC arise from CTs, and dysplastic cells such as cytomegalic neurons and balloon cells residing in CTs may be culprits to blame in seizure generation. The ability of CTs to generate ictal discharges in TSC patients thus may rest on the aberrant connectivity of these abnormal cell types that are not present in normal analogous cortices, and deciphering their connectivity patterns may help to identify the potential “epileptic” cell types that may be critical for seizure generation. To this end, we apply a high-throughput multi-patch recording method to surgically resected, epileptogenic CTs to interrogate the connection abnormalities of CT local circuit. Massive, multi-patch recordings of distinct cell types from the same CT slices enables us to unravel the specific connectivity patterns of abnormal cell types characteristic of CTs, in addition to their intrinsic electrophysiological properties. The study will provide the first insight into circuit-level mechanisms underlying CT epileptogenesis, with the potential to identify the “epileptic” cell types that are critical for seizure generation in TSC patients. Single-cell RNA-sequencing is also applied to CT to reveal the transcriptomic landscape of CTs at the level of cell types and unravel the genetic mechanisms underlying the abnormal phenotypes of potential “epileptic” cell types.

2. KEYWORDS:

Tuberous sclerosis complex, connectivity, slice electrophysiology, single-cell RNA-sequencing, balloon cells, cytomegalic neurons, Patch-seq, epilepsy, GABA, interneurons

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1 (specified in proposal): Generate a morphological taxonomy of cell types in CTs and map their connections	Timeline	% of completion
Major Task 1: identifying the potential “epileptic” cell types by revealing the connectivity and intrinsic electrophysiological properties of major cell types in CTs (we need at least 25 TSC tissue cases and 5 control cases)	Months 1-24	
Subtask 1: Simultaneous multi-cell patch recording on CT slices	1-24	~90%
Subtask 2: Biocytin staining, NeuN staining, morphology reconstruction.	2-24	~90%
Subtask 3: Data Analysis	3-24	~80%
Milestone(s) Achieved: Revealing the connectivity pattern of major cell types in CT; Identifying the most likely candidate for “epileptic” cell types	11-24	~90%
Local IRB/IACUC Approval	0	

Specific Aim 2: Derive transcriptomic signatures of potential “epileptic” cell types in CTs using single-cell RNA-seq	12-36	
Major Task 2: to test if the potential “epileptic” cell types have specific transcriptional profiles (we need at least 9 TSC tissue cases and 5 control cases)	12-36	
Subtask 1: single-nucleus RNA-seq and Patch-seq	12-36	~45%
Subtask 2: Transcriptomic data analysis and Bioinformatics analysis	13-36	~35%
Subtask 3: Immunocytochemistry and in situ hybridization	21-36	~30%
Milestone(s) Achieved: Mapping out the transcriptomic signatures of the potential “epileptic” cell types, such as cytomegalic interneurons.	23-36	~30%

What was accomplished under these goals?

1. Continuous collection of TSC tissue for proposed studies: During the pandemic, there is a significant decrease in the number of TSC and control surgical cases due to surgery cancellation and delay. We were still collecting fresh brain tissue samples whenever available despite restricted access to lab and hospital. Due to restricted access to lab space and electrophysiological rig in this period, electrophysiology recordings on these tissue specimens was significantly limited. In the past fiscal year, we have collected 6 TSC and 7 control fresh tissue samples. Most of the tissue samples were frozen for immunostaining and single-nucleus RNA-sequencing (snRNA-seq, see below). As the institute and hospital was gradually reopening with more working capacity allowed, we were resuming more and more electrophysiology recordings on each TSC tissue sample we collect.

2. Developing snRNA-seq protocol on frozen human tissue to profile cell types and states in CTs. Given the limited resource of fresh tissue during the pandemic, we have been optimizing a single-nucleus RNA-sequencing (snRNA-seq) protocol on frozen human tissue. Different from the single-cell RNA-sequencing protocol (scRNA-seq), the snRNA-seq protocol does not rely on the fresh live tissue but has a comparable capability in capturing single-cell transcriptomic information (Bakken et al., 2018). This approach thus enables us to take advantage of those frozen TSC tissue samples collected by the lab of Dr. Anderson, one of the co-investigators of this project, in the last five years. In addition, this novel high-throughput approach allows us to use the frozen disease-free tissue from the NIH tissue bank, an ideal control for TSC tissue, enabling a single-cell-resolution comparison to uncover cell types and states specific to TSC.

We have adopted an snRNA-seq protocol used by many others (Habib et al., 2017; Mathys et al., 2019) for human frozen tissue we collected. Briefly, the tissue is homogenized in ice-cold Nuclei EZ lysis buffer (#EZ PREP NUC-101, Sigma) and nuclei were isolated. Debris will be removed using the Nuclei PURE Prep Isolation Kit (#NUC201-1KT, Sigma Aldrich). Droplet-based snRNA-seq libraries will be prepared using the 10X Genomics Chromium Single Cell kit according to the manufacturer’s protocol. Paired-end libraries will be sequenced on an Illumina Nextseq500 at a minimum depth of 10k-50k reads per nuclei. Reads passing initial quality control will be mapped to the human genome and then transcripts per nuclei will be counted. Individual nuclei will be filtered with upper limits for the number of mitochondrial reads and unique molecular identifiers (UMIs) per nucleus to remove poor quality cells and doublets. Data will be processed through a combination of the CellRanger pipeline by 10X Genomics, a custom pipeline developed by our lab, and the Seurat package in R. Nuclei will be grouped into discrete clusters using the Louvain algorithm, and cluster visualizations will be created with UMAP (Fig.1).

We first used age-matched tissue samples that come from autopsy cases with a normal neurological history, obtained from the NIH tissue bank. We have profiled the transcriptomes of 61,083 nuclei from pediatric cortical tissues (3-5 year-old, five samples), with ~7,000 uniquely transcripts from ~3000 genes detected for each nucleus. We differentiated the neuronal population from the non-neuronal population (Fig. 1A) using the neuronal marker gene (RBCOX3), resulting in 32,817 neurons. The non-neuronal population could be grouped into astrocytes, oligodendrocytes or oligodendrocyte precursors (OPC), microglia, and endothelial cells with respective known marker genes (Fig.1B) (Lake et al., 2016). We differentiated excitatory neurons and inhibitory neurons by their marker genes, SATB2 and GAD1 respectively (Fig. 1A, B) (Lake et al., 2016). The excitatory and inhibitory neuronal population could be further grouped into several subclusters and types (Fig. 1B). Based on previously identified laminar markers, we will assign each excitatory cluster to the putative layers, which can be further confirmed or refuted by Patch-seq (see below).

We have applied the same protocol on TSC tissue and the snRNA-seq data of two samples from one

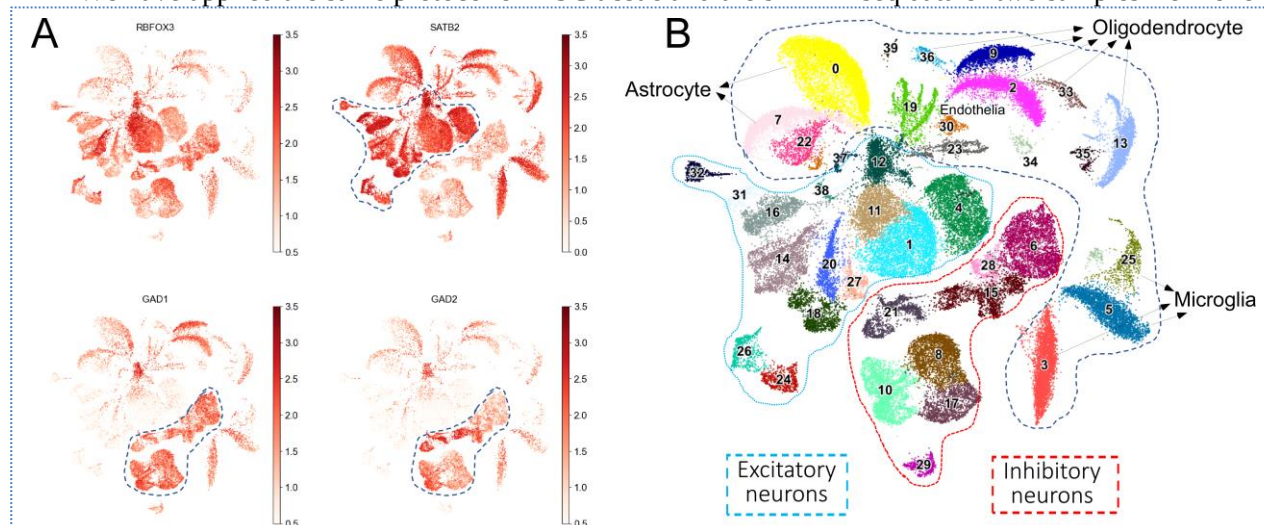


Fig 1: snRNA-seq data from normal pediatric tissue. (A) scRNA-seq of 61,000 nuclei from pediatric frontal cortex reveals the overall molecular heterogeneities of cell types in this brain region. Using neuronal marker gene RBCOX3, the neuronal population can be differentiated from no-neuronal population. Excitatory cell population and inhibitory neurons can be differentiated from each other by the expression of marker gene SATB2, GAD1 and (B) The clusters and subclusters belonging to oligodendrocyte or its precursors, astrocyte, microglia, and endothelial cells, as well as excitatory and inhibitory neurons.

case of TSC has been processed simultaneously with two age-matched control cases (**Fig.2A**). The data quality of TSC cases is comparable with control cases, with ~7,000 uniquely transcripts from ~3000 genes detected for each nucleus. After we labeled major cell types in CT with marker genes, we found that the cellular composition of CT is strongly skewed toward the non-neuronal population, especially toward the microglia-like population (**Fig.2C**). In addition, gene expression profiles for each major cell type, including excitatory neurons are significantly different from their counterparts from control tissue (green color for CT), indicating the TSC1/2 mutation changes the gene expression landscape across all major cell types. We will continue this line of research in the extended period of this grant to generate a comprehensive taxonomy of molecular cell types in CT, as well as a census of cell types in control tissue. As illustrated in the Year 1 Technical Report, this molecular cell atlas will be used as a comprehensive reference against which to compare the Patch-seq data to identify those clusters corresponding to balloon cells and cytomegalic interneurons. In addition, we will use machine learning and bioinformatics to recapitulates cell-type-specific marker genes and cell-type-specific changes in CTs.

With our institute is opened with a full capacity, we expect to generate a transcriptomic cell atlas reference specific to TSC using snRNA-seq in the next 6 months.

3. Patch-seq in human brain slices

In addition to performing drop-seq on frozen tissue, we also performed Patch-seq as originally proposed whenever the tissue is available. We use a modified patch-clamp recording protocol to improve RNA recovery (Cadwell et al., 2016; Cadwell et al., 2017). After 5-10 mins of the whole-cell recording of firing patterns and synaptic events of human cortical neurons in CTs, as well as dye filling and imaging (biocytin or fluorescent dyes) in human brain slices, RNA is collected from each neuron and complementary DNA (cDNA) is generated as described in detail in our previous study (Cadwell et al., 2016). Only high-quality cDNA samples yield ≥ 2 ng, average length ≥ 1500 bp) are sequenced. After 18 amplification cycles, sequencing libraries are constructed from the cDNA using Tn5-mediated tagmentation (SMART-seq v4 (Clontech) (Picelli et al., 2014). Quality control is performed on both the amplified full-length cDNA and the

library using an Agilent Bioanalyzer. cDNA libraries are sequenced using an Illumina HiSeq 3000 or 4000. Reads are aligned to the human genome using STAR (Dobin et al., 2013), (normalized, and quantified as transcripts per million (TPM).

Mapping the transcriptome collected by Patch-seq to the reference transcriptomic atlas from the droplet-based approach: We will follow our previous mapping methods to map the transcriptomic data across two

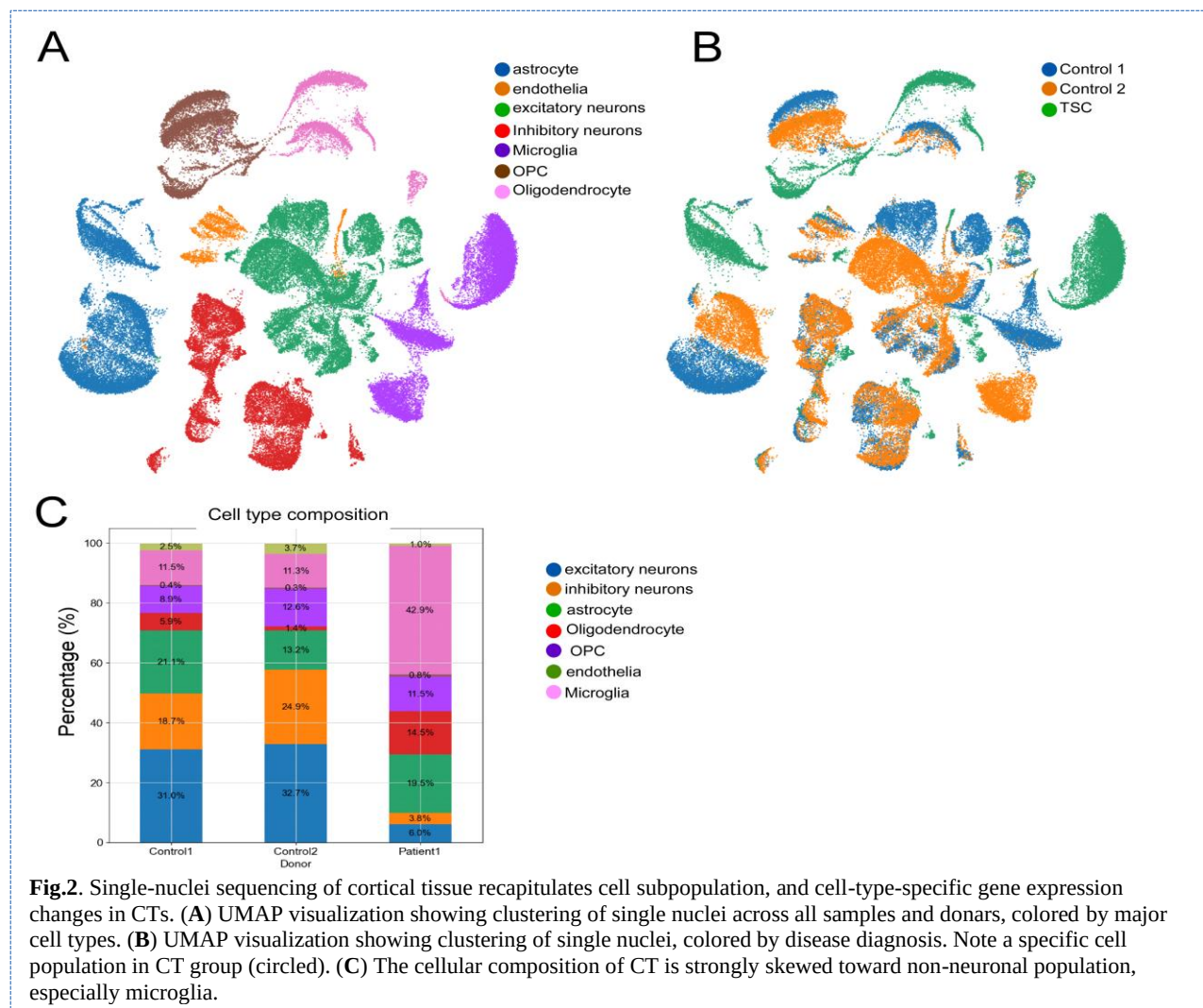


Fig.2. Single-nuclei sequencing of cortical tissue recapitulates cell subpopulation, and cell-type-specific gene expression changes in CTs. **(A)** UMAP visualization showing clustering of single nuclei across all samples and donors, colored by major cell types. **(B)** UMAP visualization showing clustering of single nuclei, colored by disease diagnosis. Note a specific cell population in CT group (circled). **(C)** The cellular composition of CT is strongly skewed toward non-neuronal population, especially microglia.

scRNA-seq protocols (Scala et al., 2018; Cadwell et al., 2019). Using the count matrix of reference scRNA data from dissociated cells, we select 3000 “most variable” genes as described previously (Kobak et al., 2018). We then log-transformed all counts with $\log_2(x+1)$ transformation and averaged the log-transformed counts across all cells in each of the identified clusters, to obtain reference transcriptomic profiles of each cluster ($N \times 3000$ matrix). Out of these 3000 genes, 2575 are present in the mm10 reference genome that we used to align reads in our patch-seq data. We apply the same $\log_2(x+1)$ transformation to the read counts of Patch-seq cells, and for each cell computed Pearson correlation across the 2575 genes with all clusters. Each cell is assigned to the cluster to which it has the highest correlation.

We have collected 16 Patch-seq samples from balloon cells and a few samples from excitatory neurons so far. We need to collect Patch-seq samples from cytomegalic neurons, particularly cytomegalic interneurons in the next reporting period. Then we will use the method described above to identify the transcriptomic cluster(s) corresponding to each abnormal cell type, especially balloon cell and cytomegalic interneurons, and understand the gene expression profiles that underlie their unique morphology, electrophysiology, and connectivity.

4. Slice Electrophysiology for connectivity analysis

In addition to performing RNA-seq on cortical tissue, we also performed multi-patch recording on both control and TSC tissue as originally proposed whenever the tissue was available.

In this fiscal year, we have collected more data to confirm our preliminary observations from the previous reporting periods on the connectivity patterns of different cell types in CTs. We confirmed that balloon cells do not form any synaptic connections with any cell types in CTs as reported in the first Annual

Report. We also have more data to characterize immature excitatory cells, in addition to prominent phenotypes of cytomegalic cells and balloon cells (Fig 3).

Compared to the relatively control tissue, the immature excitatory cells, regardless of the depth they reside in, barely have extended apical dendrites. Their dendritic trees are also simpler than the relatively normal cells with a fewer number of spines. In addition, these immature excitatory neurons barely form an excitatory synaptic connection with each other or other cell types. This may be the reason why there are sparser spontaneous excitatory synaptic events in cortical tubers. However, these cells do receive the inhibitory synaptic events (Figure not shown).

We will continue this line of research in the extended period to complete and confirm the connectivity patterns of all major cell types in CTs. Importantly, during the extended period, we will collect more data from the control cases against which to compare the data from CTs. These comparisons will help understand the unique connectivity pattern of abnormal cell types in CTs.

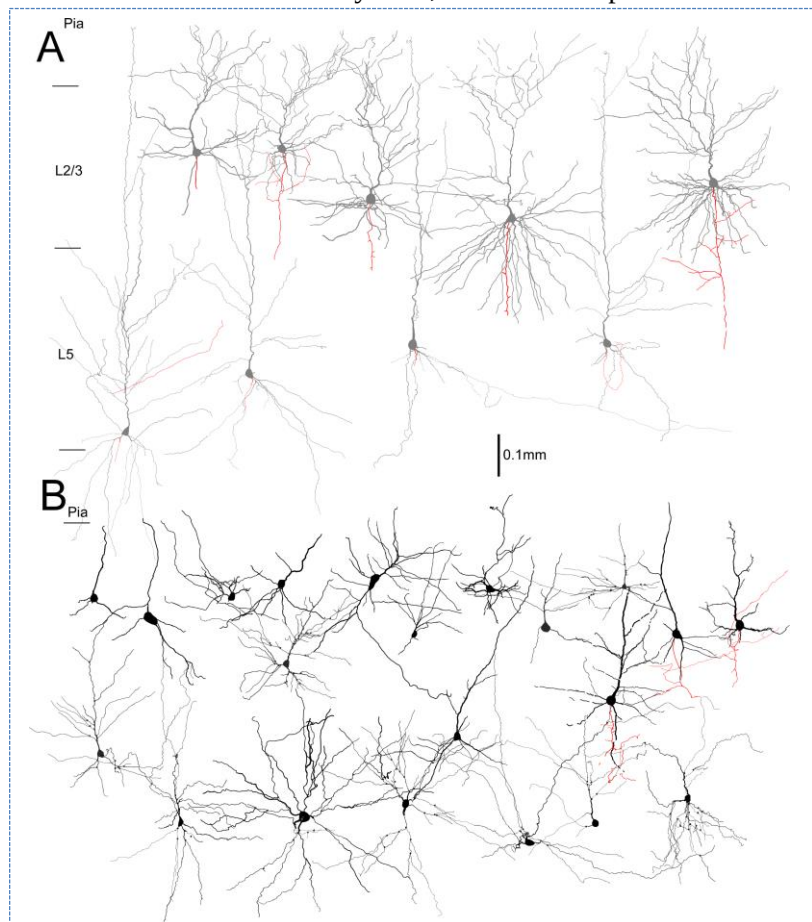


Fig.3. Immature excitatory neurons in CTs compared to their control counterparts. **(A)** L2/3 and L5 pyramidal neurons of temporal cortex with normal cortical architecture. **(B)** immature excitatory neurons from different depth of cortical tubes resected from temporal lobe. Note their apical dendrites are missing or under development.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

We plan to do the following: 1) oral or poster presentations at national and international TSC meetings; 2) regular meetings with general pediatricians and neurologists with interest in epilepsy in general, TSC in specific; 3) contributions to internal and external activities of national and international TSC-patient support groups, such as Tuberous Sclerosis Alliance; 4) discussions with academic and non-academic drug-discovery groups.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Our project delivers 1) for the first time, the comprehensive taxonomy of cell types and states in cortical tubers in TSC by transcriptomic profiling of cortical tubers in TSC at the single-cell resolution; 2) for the first time, the connectivity pattern of each abnormal cell type in CT, 3) translational potential of identifying epileptic cell types as a target for developing biomarker and treatment for TSC patients.

These research outcomes impact 1) our understanding of the epileptogenesis and ictogenesis of cortical tubers in TSC, 2) how TSC1/2 mutation interferes with the development of cortical cell types, and 3) any future discussion by clinicians in terms of biomarker identification.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?

The outcomes of our project impact 1) parents/guardians of children and teenagers with TSCs; 2) TSC patient support-groups, 3) academic and non-academic drug-discovery groups.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

In response to the Covid-19 Pandemic, Baylor College of Medicine (BCM) instituted college-wide measures to help limit the spread of the virus and perform responsible conduct of research. Starting March 23, 2020, limited access to research facilities was implemented with phased increases of access as recovery efforts commenced. Following the OMB Flexibility guidelines, researchers were retained on grants during this period when they had both continuity support and direct activities in support of the grant.

The new fiscal year of the project started in April 2020 when the school and hospital remained closed with limited access to the on-campus facilities and resources. During this phase, a number of surgeries have been canceled and delayed which has significantly limited the tissue availability for the proposed studies. The facilities at BCM were ordered to be closed, and the frozen financial budget and limited access to the fund have delayed us in purchasing the necessary equipment and reagents for proposed experiments. In addition, restricted access to the lab space and facilities prevented our teams from doing proposed electrophysiology recordings in a full capacity. All these have significantly affected the progress of the project. When the school moved to Phase III fully reopening on Nov. 2020, we were back on the track working on the Specific Aim 1-2. However, because of delays in the visa application and travel restriction, the postdocs and students we hired for this project could not enter the USA, and we are short-staffed for this project.

Action and Plan to resolve:

We implemented several strategies and actions to overcome the above-mentioned issues to ensure the specific aims continue to be studied as originally proposed.

- 1) To accommodate the social distancing required by Covid-19, we invested more efforts in establishing and optimizing the data analysis pipeline while simultaneously collecting data for Specific Aim 1&2. We hoped that once the pandemic is more under control, we then could make full use of this optimized analysis pipeline to expedite data analysis so that research goals in Aim 1&2 can be accomplished without a significant delay.
- 2) To deal with being short-staffed, one strategy is to recruit the postdoc or more senior scientists from the US instead if the visa and travel still remain as an issue for international hiring. In addition, we have requested the emergency visa interview for an international postdoc candidate, and the request was approved and the visa was approved recently. We aim to have sufficient personnel to conduct the experiments required to accomplish the goals.
- 3) To deal with the shortage of fresh tissue samples during the pandemic, we have developed and apply a new single-nucleus RNA-sequencing (snRNA-seq) approach to take advantage of the frozen TSC tissue collected prior to this pandemic. Dr. Anderson, one of co-investigators of this project, has been collecting tissue samples from TSC and other control cases in the last five years, which provides us abundant tissue samples for snRNA-seq. Therefore our single-cell sequencing can move forward as expected without relying on the availability of fresh human tissue. This approach helps expedite the progress of this project, specially during the pandemic.
- 4) Given that the social distancing required by Covid-19 has delayed us in completing the aims, we have consulted with SPO and requested a no-cost extension (NCE) to provide another year to accomplish the goals. The NCE has been approved.

Changes that had a significant impact on expenditures

No

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

No

Significant changes in use or care of vertebrate animals

No

Significant changes in use of biohazards and/or select agents

No

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to Report

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

Nothing to Report

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Xiaolong Jiang
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	000-001-8066-1383
Nearest person month worked:	1
Contribution to Project:	Dr. Jiang has performed slice electrophysiology , supervised the progress of the whole project
Funding Support:	R01MH120404; R01MH114830; R01MH109556
Name:	Andrew McKinney
Project Role:	Graduate student
Researcher Identifier (e.g. ORCID ID):	N/A

Nearest person month worked:	1
Contribution to Project:	Mr. McKinney has performed slice electrophysiology and connectivity study, and data analysis.
Funding Support:	T32 MH 312008
Name:	Junzhan Jing
Project Role:	Postdoc
Researcher Identifier (e.g. ORCID ID):	000-003-4647-0932
Nearest person month worked:	1
Contribution to Project:	Dr. Jing has performed slice electrophysiology and connectivity study, single-cell RNA-sequencing, and data analysis.
Funding Support:	R01 MH120404, R01 MH109556
Name:	Qianqian Ma
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Dr. Ma has performed tissue collection and help with slice electrophysiology and data analysis
Funding Support:	R01 MH120404, R01NS110767

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

The active support has been changed as below:
R01 MH122169 was awarded to PI on April 1 2020
R01 NS110767 was awarded to PI on June 1, 2020

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: