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TITLE: Investigation of Cell Types Responsible for Seizure Generation in a Model of Neurofibromatosis Type 1

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14. ABSTRACT

Up to 20% of people with neurofibromatosis type 1 (NF1) have epilepsy, but the mechanism is unknown. The purpose of this research is to determine how a mutation in the *Nf1* gene leads to increased seizure susceptibility. We hypothesize increased seizure susceptibility in the Nf1+/- mouse neocortex is due to increased interneuron activity.

Aim 1: Determine whether loss of neurofibromin in interneurons or excitatory neurons is sufficient to increase seizure susceptibility. We are using electrophysiological and optogenetic techniques to determine effects of Nf1 mutation in interneurons or excitatory neurons at the synaptic and circuit levels. Work is in progress investigating rates of spontaneous seizures and susceptibility to kainic acid induced seizures in mice with selective loss of neurofibromin in different cell types. We are also beginning work to determine whether activation of interneurons or excitatory neurons in NF1 cortical slices will lead to increased interictal- or ictal-like events,

Aim 2: Pharmacologic rescue of increased seizure phenotype. Future work will test whether MEK or mTOR inhibitors can reverse increased seizure susceptibility in NF1 *in vivo* and *in vitro*.

15. SUBJECT TERMS
Neurofibromatosis type 1, seizures, epilepsy, interneurons

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1. Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant neurocutaneous disorder with a prevalence of approximately one in 2,500 people worldwide. Up to 20% of NF1 patients have epilepsy, but the mechanism behind epilepsy in NF1 is unknown. The purpose of this research is to determine how a mutation in the *Nf1* gene leads to increased seizure susceptibility. We will examine the roles of excitatory versus inhibitory neurons in NF1 seizure susceptibility using *in vitro* and *in vivo* electrophysiological and optogenetic studies. NF1 also leads to increased mTOR activity which is partly responsible for the GABAergic abnormalities documented in this condition. We will therefore perform pharmacological studies to determine the effectiveness of mTOR inhibition as a treatment strategy for seizures in NF1. This work will comprise the first animal studies investigating the mechanism of seizures in NF1 and could lead to novel therapeutic strategies targeting inhibitory neurotransmission or mTOR activity, which may be helpful not only for patients with NF1, but also those with other epileptic conditions.

2. Keywords

neurofibromatosis type 1; seizures; epilepsy; vGlut; parvalbumin; optogenetics; kainic acid; EEG

3. Accomplishments

- **What were the major goals of the project?**

The major goals of the project as stated in the approved SOW are listed below. The particular tasks with a projected timeline falling within the current reporting period (months 1-12 of the project) are highlighted in yellow.

Specific Aim 1: Determine whether loss of neurofibromin in interneurons or excitatory neurons is sufficient to increase seizure susceptibility.

Major Task 1: Determine whether reduced neurofibromin expression in interneurons or excitatory neurons leads to hyperexcitability in vitro.

Subtask 1: Await approval from ACURO for animal work to commence. Months 1-3 **COMPLETE**

Subtask 2: Expression of opsins via intracerebroventricular injections of viral vectors. Months 4-6 **COMPLETE**

Subtask 3: Characterize 4-AP induced seizures in brain slices from mice with targeted loss of neurofibromin in PV+ and vGlut+ cells. Months 13-15

Subtask 4: Characterize biophysical properties of PV+ cells and vGlut+ cells in response to optogenetic depolarization or hyperpolarization. Months 16-18

Subtask 5: Determine effects of photostimulation of PV+ or vGlut+ cells on 4-AP ictal activity. Months 19-21

Major Task 2: Determine whether loss of neurofibromin in interneurons or excitatory neurons is sufficient to increase seizure susceptibility *in vivo.*

Subtask 1: Implant mice with intracranial EEG electrodes. Months 4-5

Subtask 2: Determine if loss of neurofibromin in PV+ or vGlut+ cells leads to spontaneous recurrent seizures via continuous video-EEG recordings. Months 11-15

Subtask 3: Determine if loss of neurofibromin in PV+ or vGlut+ cells alters susceptibility to kainic acid-induced seizures. Months 6-10

Projected milestones:

Determine if neocortical slices from mice with reduced neurofibromin expression in PV+ cells or vGlut+ cells are more susceptible to 4-AP induced seizures, and the specific role of neurofibromin in these cells in initiating or aborting interictal- and ictal-like activity. **24 months**

Publish manuscript on the role of neurofibromin in PV+ and vGlut+ cells in NF1-associated seizures at the synaptic and circuit levels. **24 months**

Specific Aim 2: Pharmacologic rescue of increased seizure phenotype.

Major Task 1: Determine if MEK or mTOR inhibition in vitro will normalize increased inhibition.

Subtask 1: Determine alterations in Ras/PI3K/MEK/mTOR pathways via Western blots. Month 24

Subtask 2: Determine effect of the MEK inhibitor U0126 on interictal and ictal-like activity in neocortical brain slices from mice with neurofibromin deficiency in PV+ or vGlut+ cells. Month 25

Subtask 3: Determine effect of the mTOR inhibitor everolimus on interictal and ictal-like activity in neocortical brain slices from mice with neurofibromin deficiency in PV+ or vGlut+ cells. Month 26

Major Task 2: Determine if MEK or mTOR inhibition in vivo will decrease seizure susceptibility.

Subtask 1: Implant mice with intracranial EEG electrodes. Months 25-26

Subtask 2: Administration of U0126 or everolimus. Months 26-27

Subtask 3: Determine if U0126 or everolimus treatment has an effect on spontaneous recurrent seizures via continuous video-EEG recordings. Months 27-31

Subtask 4: Determine if U0126 or everolimus treatment alters susceptibility to kainic acid-induced seizures. Months 28-32

Projected milestones (with timelines if stated in SOW):

Determine if MEK and/or mTOR inihibitors can reverse NF1-associated increased seizure activity in neocortical brain slices. **36 months**

Determine if MEK and/or mTOR inhibitors can reverse NF1-associated seizure susceptibility in young adult mice. **36 months**

Publish manuscript on the mechanistic role of the Ras/PI3K/MEK/mTOR pathway in increased seizure susceptibility in NF1. **36 months**

- **What was accomplished under these goals?**

The current reporting period covers the first 12 months of this project. In accordance with the approved SOW, the work done to date falls exclusively under Specific Aim 1.

Specific Aim 1: Major Task 1, Subtask 1 was approval from ACURO. This was complete July 22, 2022.

Specific Aim 1: Major Task 1, Subtask 2 was optimizing expression of opsins via intracerebroventricular injections of viral vectors. We have performed intracerebroventricular injection of viral vectors to express channelrhodopsin-2 (ChR2) (pAAV-EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA; addgene plasmid #20297) or enhanced halorhodopsin from Natronomonas (eNpHR) (pAAV-double floxed-eNpHR-EYFP-WPRE-pA; addgene plasmid #20949) in Cre expressing PV+ cells or vGlut2+ cells at P1. Representative sections from mice with selective Nf1 mutation in either PV+ or vGlut+ cells are shown in Figure 1.

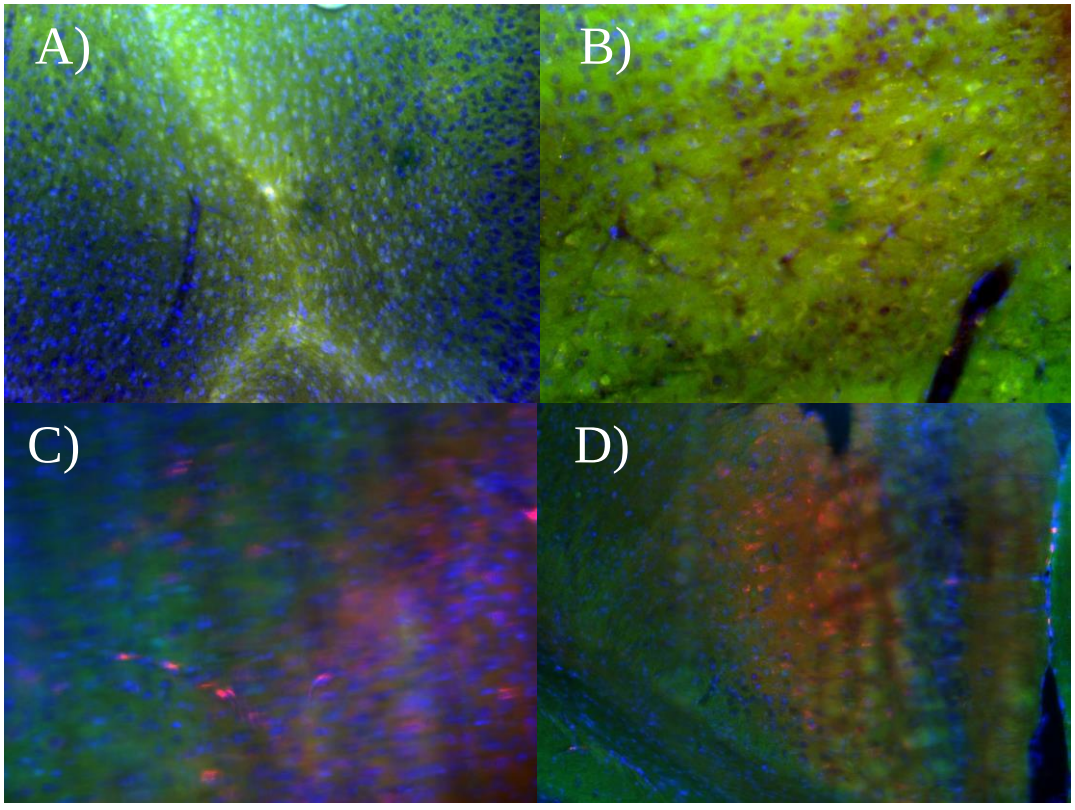

Figure 1. Representative sections through the neocortex at post-natal day 28 from mice who underwent intracerebroventricular injection of various viral vetors at post-natal day 1. A) $Nf1^{flox/flox}PVCre^{+/-}$ mouse injected with pAAV-double floxed-eNpHR-EYFP-WPRE-pA. Cells labelled green express the opsin eNpHR, with a blue DAPI counterstain. B) $Nf1^{flox/flox}vGlutCre^{+/-}$ mouse injected with pAAV-double floxed-eNpHR-EYFP-WPRE-pA. Cells labelled green express the opsin eNpHR, with a blue DAPI counterstain. C) Nf1flox/floxPVCre+/- mouse injected with pAAV-EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA. Cells labelled red express the opsin ChR2, with a blue DAPI counterstain. D) $Nf1^{flox/flox}vGlutCre^{+/-}$ mouse injected with pAAV-EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA. Cells labelled red express the opsin ChR2, with a blue DAPI counterstain.

Specific Aim 1: Major Task 2, Subtask 1 was to implant mice with intracranial electrodes. To date we have implanted a total of 43 mice from the various groups with intracranial EEG electrodes. Young adult male and female mice (age 3-4 months) were implanted with pairs of recording electrodes in the CA3 region of the hippocampus and contralateral somatosensory neocortex, with a reference electrode at a frontal area.

Specific Aim 1: Major Task 2, Subtask 2 was to determine if loss of neurofibromin in PV+ or vGlut+ cells leads to spontaneous recurrent seizures via continuous video-EEG recordings. After implantation with intracranial electrodes as described above, mice underwent continuous video-EEG monitoring for five days to detect spontaneous seizures or interictal activity. EEG signals are recorded using amplifiers (MP160, BIOPAC Systems) with a sampling rate of of 1000Hz, bandpass filtered with a high-pass of 0.5Hz and low-pass of 3.0 kHz. A webcam is anchored next to each cage to monitor behavior. EEG and auto-synchronized video data are acquired, stored, and analyzed using AcqKnowledge software (BIOPAC Systems). Seizures are identified by ictal discharges lasting at least 10s with concurrent behavioral seizure on video. This work was projected to be performed between months 11-15 and is still in progress. Data files from mice who already underwent continuous video-EEG recordings are currently being analyzed.

Specific Aim 1: Major Task 2, Subtask 3 was to determine if loss of neurofibromin in PV+ or vGlut+ cells alters susceptibility to kainic acid-induced seizures. The projected timeline for Subtask 3 completion was 10 months, however we have been unable to meet this timeline due to turnover of personnel in the lab. Mr. Ting Wang, research assistant, performed these studies but left the lab unexpectedly in April 2023 to move to Australia. Therefore, this portion of the work has been on hold. It will be continued by Ms. Avery Cameron who is joining the Reid lab as a graduate student in Sept 2023. The data collected and analyzed to date are presented in Figure 2.

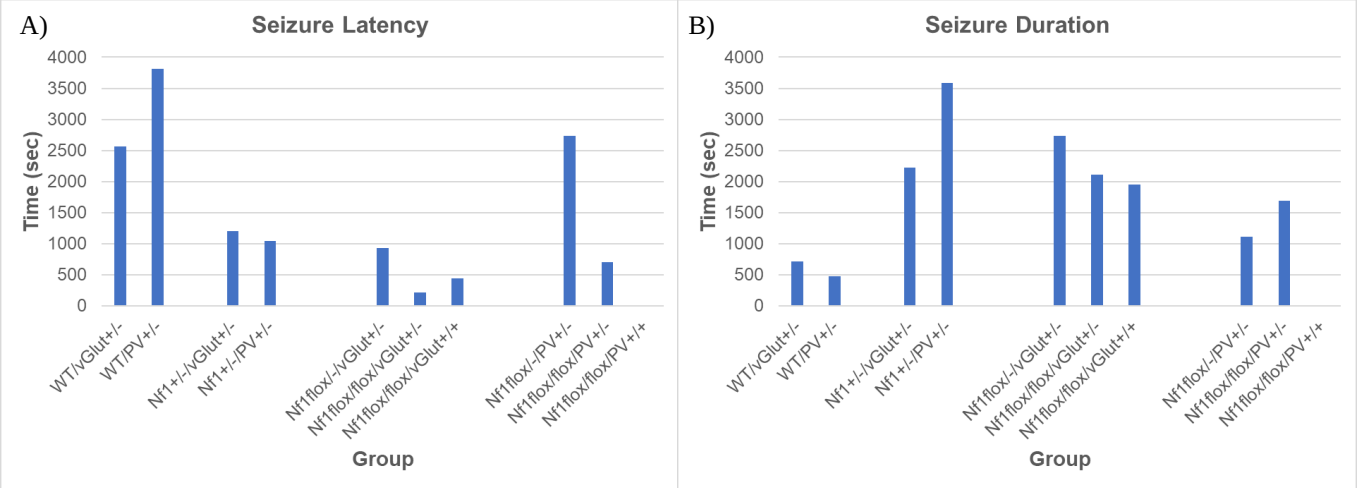

Figure 2. Graphs showing average latency to seizure onset (A) and average seizure duration (B) in groups of mice with selective loss of neurofibromin in different cell types. This data set is not currently complete, more animals are required in each group (some groups do not yet have any animals tested). Therefore no error bars are presented and no statistical tests have been performed.

- **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?**

The next reporting period (months 13-24) will focus on completing Specific Aim 1.

Specific Aim 1: Major Task 1, Subtask 3 is just underway. The Valiante lab is beginning to characterize 4-amino-pyridine (4-AP) induced seizures in brain slices from the various mouse lines with different neurofibromin mutations. They will characterize rates and duration of 4-AP induced seizures, power changes, latency to ictal onset, and IIS spike size as a function of time from 4-AP perfusion to look for differences between groups. After that is complete, they will begin work on Major Task 1, Subtasks 4 and 5. pPhotostimulation with a 30 ms pulse at a frequency of 20 Hz with a 473 nm (ChR2) or 589 nm (eNpHR) continuous wave will be done with a solid-state laser source (Mightex) in standard aCSF. The effects of photostimulation on biophysical properties of PV+ cells or vGlut2+ cells they innervate will be determined with whole cell recordings. We will record extracellular field potentials to determine whether depolarization or hyperpolarization of PV+ interneurons or vGlut2+ excitatory cells leads to altered evoked responses, interictal- or ictal-like events in any groups in the absence of 4-AP. We will then apply low dose 4-AP (starting with 10 uM) to the bath and repeat photo-stimulation in the early phase, when 4-AP generates interictal but not ictal-like phenomena, to determine the effect of PV+ cell or vGlut2+ cell depolarization or hyperpolarization on triggering ictal-like events. Finally, we will perform photostimulation during 4-AP ictal activity to determine effect on aborting ictal-like events.

We will also complete Specific Aim 1: Major Task 2. When Ms. Avery Cameron begins her MSc degree in Sept, 2023 we will continue to perform continuous video-EEG recordings to determine the rates of spontaneous seizures in the different groups with selective loss of neurofibromin in different cell types. In addition, we will complete the kainic acid seizure susceptibility testing.

Once Specific Aim 1 is complete, we will have determined if neocortical slices from mice with reduced neurofibromin expression in PV+ cells or vGlut+ cells are more susceptible to 4-AP induced seizures, and the specific role of neurofibromin in these cells in initiating or aborting interictal- and ictal-like activity. We aim to have a manuscript submitted on the role of neurofibromin in PV+ and vGlut+ cells in NF1-associated seizures at the synaptic and circuit levels by the end of the next reporting period.

4. Impact

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

Nothing to report.

- **What was the impact on other disciplines?**

Nothing to report.

- **What was the impact on technology transfer?**

Nothing to report.

- **What was the impact on society beyond science and technology?**

Nothing to report.

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**

There has been personnel turnover in the Reid and Valiante labs which has impacted progress. Research assistant Dr. Chiping Wu took and early retirement from the lab in May 2022, just prior to the start of these projects in July 2022. It took some time to train Dr. Mingdong Yang, who was already working in the lab, how to perform many of the technical aspects that Dr. Wu had been performing, such as the intracerebroventricular injections in post-natal day 1 mice (Specific Aim 1: Major Task 1, Subtask 2) and the intracranial electrode implantations (Specific Aim 1: Major Task 2, Subtask 1). However, she is now proficient in these areas.

In addition, research assistant Mr. Ting Wang was working on Specific Aim 1: Major Task 2 and left the lab with short notice in April 2023 to move to another country. We did not have any other personnel in the lab at that time that could immediately take over those projects. However, Ms. Avery Cameron will be starting an MSc degree in the Reid lab Sept 11, 2023 and will take over the projects. She will require some training from other lab personnel before she is able to independently complete this work, but it is expected it will be completed within the next reporting period and that we will still meet timelines for the overall completion of Specific Aim 1.

- **Changes that had a significant impact on expenditures**

There has been turnover in the Reid and Valiante labs which has impacted salary expenditures. There has been a delay in hiring a suitable graduate student, therefore the plan to fund a graduate student in years 1 and 2 has now shifted to Years 2 and 3. Ms Avery Cameron has been hired and will start an MSc degree with Dr. Reid working on this project Sept 11, 2023. In addition, it was deemed difficult to find an appropriately trained post-doctoral fellow to perform the in vitro physiology work, therefore this work will now be done by a research associate already working in the Valiante lab, Dr. Homeira Moradi. The salary for the portion of her time dedicated to this project will be paid by this grant.

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

Nothing to report.

- **Significant changes in use or care of human subjects.**

Not applicable.

- **Significant changes in use or care of vertebrate animals.**

Nothing to report.

- **Significant changes in use of biohazards and/or select agents**

Nothing to report.

6. Products

- **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:	Aylin Reid
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	ORCID 0000-0001-6855-9669
Nearest person month worked:	4
Contribution to Project:	Dr. Reid has overseen the personnel involved in this project, submitted all required regulatory paperwork, helped with troubleshooting and developing techniques for opsin expression, educated lab personnel on how to perform kainic acid testing and how to review EEGs.
Funding Support:	University Health Network.

Name:	Mingdong Yang
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	6
Contribution to Project:	Dr. Yang has performed husbandry of the breeding colonies and genotyping of the various mouse lines. She has performed intracerebroventricular injections of viral vectors and intracranial electrode implantations.
Funding Support:	This award

Name:	Ting Wang
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	n/a

Nearest person month worked:	6
Contribution to Project:	Mr. Wang assisted with animal husbandry and genotyping. He set-up the continuous EEG recordings and performed kainic acid seizure susceptibility testing.
Funding Support:	This award

Name:	Marawan Sadek
Project Role:	Research assistant
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	2
Contribution to Project:	Mr. Sadek set up video-EEG recordings and analyzing EEG recordings from kainic acid seizure susceptibility testing.
Funding Support:	This award

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

Nothing to report.

- **What other organizations were involved as partners?**

Nothing to report.

8. Special Reporting Requirements

None

9. Appendices

None