AWARD NUMBER: W81XWH-18-1-0207

TITLE: Characterization of a novel humanized mouse model of Neurofibromatosis

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CONTRACTING ORGANIZATION: Oregon Health and Science University

REPORT DATE: Dec 2019

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
Dec 2019	Annual	1 Sept 2018 –31 Aug 2019

4. TITLE AND SUBTITLE

Characterization of a Novel Humanized Mouse Model of Neurofibromatosis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-18-1-0207

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of this study was to characterize a mutant mouse that bears an NF1 mutation that is identical to one found in humans. No human samples exist to determine the nature of how this particular mutation affects the NF1 gene and protein. This mutation is particularly important to understand as it does not lead to the neurofibroma formation found in most patients, but it can lead to learning challenges, developmental delay and in some cases epilepsy. This study was able to determine that this particular mutant NF1 protein neither affects the most common pathways associated with the function of the NF1 protein nor leads to some inflammatory aspects of the disease. These are critical findings as they reveal that the protein has additional functions that are linked to a very specific region of the protein and that this mutation may be acting in very distinct ways from the effects of complete loss of the NF1 protein. This study also found a link between this mutation and programmed cell death in a region of the brain critical for learning and memory. This information will allow new studies to more clearly define the molecular basis for the neurological aspects of Neurofibromatosis with this new understanding bringing the potential for new therapeutic targets.

15. SUBJECT TERMS

Brain development, Point-mutation, Mouse model, Signaling, Neuron, Neurofibromatosis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	16	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION

The hypothesis of this application was that new insights into Neurofibromatosis (NF) type 1 and its underlying molecular basis could be gained by using a newly developed mouse model that incorporates a specific human mutation. The subject of this work is the characterization of a human mutation of the Neurofibromatosis 1 (NF1) gene called R1809L in the context of the developing brain. Patients with the R1809L mutation lack some NF disease symptoms typically associated with loss of neurofibromin 1 (NF1) production such as tumor formation. The major symptoms observed in R1809L patients are neurological in nature including learning disabilities and developmental delay which are also found in around half of NF1 patients that have other mutations in the gene. The R1809L mutation affects only one amino acid among the approximately 2800 amino acids that make up the NF1 protein. This very slight alteration in the protein has profound effects on brain function that can include learning deficits, reduced IQ and in some cases seizures. The scope of this work includes characterizing a new mutant mouse which also has the R1809L mutation in its NF1 gene. This model allows testing of various aspects of brain development and cell signaling which are known to be affected in NF but cannot be directly tested in patient populations. As no biological samples are available from these patients, this new model allows us to query signaling pathways and binding partners that are known to be associated with NF1 but may be altered in this mutant form of NF1. Furthermore, it can allow us to identify new pathways and partners in the affected tissue rather than a heterologous system.

2. KEYWORDS

Brain development, Point-mutation, Mouse model, Signaling, Neuron, Neurofibromatosis

3. ACCOMPLISHMENTS

What were the major goals of the project?

The major goals of this project were:

1.) to determine if the canonical Ras-Erk signaling pathways was affected by the R1809L mutation

2.) to determine if brain development was affected by this mutation

3.) to determine how protein-protein interactions with the NF1 protein may be affected by the R1809L mutation.

What was accomplished under these goals?

Overall Project Summary

All work presented is unpublished

Aim 1: To elucidate the effect of the R1809L mutation on brain patterning and signaling.

In Aim1.1, we proposed to use immunohistochemical markers to determine how the R1809L mutation alters the development and morphology of the brain.

Observation 1: One of our first discoveries during the breeding of these animals was that R1809L homozygous animals were not viable postnatally. The physiologic basis of this compromised survival is not clear as all organ systems are impacted by the mutation. We were able to harvest brain tissue from one affected animal just after birth. Our analysis of this animal using the programmed cell death marker cleaved caspase 3 CCas3 indicated that most of the brain was normal except the developing hippocampus that displayed significant amounts of CCas3 staining (**Figure 1**) relative to littermate controls which exhibit very little hippocampal cell death at this stage. While the selective nature of this effect is interesting, it is currently unknown why alterations in the NF1 protein might lead to such an outcome and future may focus on the hippocampus of these animals, a known center of learning and memory processing. **(unpublished data)**

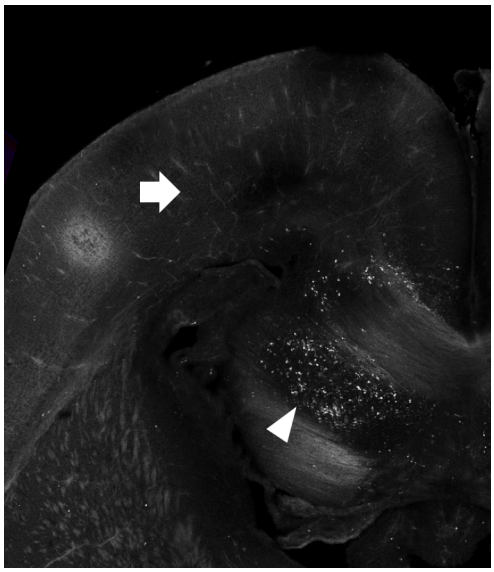

Figure 1. Immunostaining of activated caspase 3 staining in the P0 brain of a homozygous R1809L mutant. An arrowhead indicates the increased staining in the hippocampal formation and an arrow indicates the cerebral cortex where very little caspase staining is observed.

Observation 2: While we have not yet been able to assess all of the proposed timepoints, we were able to sample the post-natal day 60 (P60) time point using 3 control and 3 R1809L heterozygous animals. In comparing these animals, we do not observe a gross difference in gross brain size. This suggests that early developmental events in brain patterning have not been affected by this mutation, but completion of our embryonic studies will be required to confirm this. **(unpublished data)**

Observation 3: Previous studies have noted the striking finding that neural-selective deletion of NF1 results in significant increase in reactive astrocytes as detected by glial fibrillary acidic protein (GFAP) staining. We do not detect this effect in our P60 animals (**Figure 2A,B**). This finding reinforces the notion that this particular mutation has very selective effects on brain organization and does not lead to a neuro-inflammatory state as seen in NF1 gene deletion models.

Aim1.2: We proposed to determine if signaling transduction pathways previously reported to be altered by NF1 loss also displayed altered dynamics in the R1809L mutant brain. We used Western blotting to query several intracellular pathways including: MAP kinase (phospho-Erk), PKA (phospho-PKA), PI3K (phospho-Akt), and mTOR (phospho-p70 S6 kinase). We were able to analyze these cellular components in P60 brain tissue (n=3 for each genotype), with studies exploring embryonic time points upcoming. **(unpublished data)**

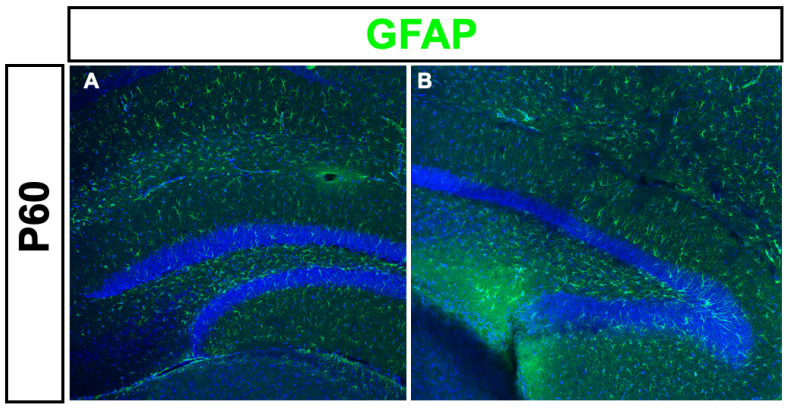

Figure 2. Immunostaining of glial fibrillary acidic protein (GFAP) in the hippocampal regions of a control litter mate **(A)** or a heterozygous R1809L mutant **(B)**.

Observation 4: Increased phosphorylation has been previously observed in each of these pathways in either NF1 KO cells and conditionally-deleted brain tissue. Quantification of our results indicate that no significant difference exists in any of these signaling pathways (Figure 3A-D). These data highlight the stark differences between complete loss of NF1 in neural tissue and the effect of the R1809L mutation. The data also further support the idea that this alteration does not affect the Ras GAP activity of the NF1 as this would have been reflected in the assessed pathways. **(unpublished data)**

Aim 2: To define the effect of R1809L on Sec14-PH domain interacting partners and NF1 localization in multiple brain cell types. It is known that NF1 serves as a scaffold for several proteins positioning them to act on incoming signals via intracellular signaling and cellular remodeling. We hypothesized that the R1809L mutation may alter the function or localization of NF1 by perturbation of these interactions resulting in altered brain patterning or function.

In Aim 2.1, we proposed testing previously reported interaction partners of NF1 that had been described as binding near the site of the mutation currently being investigated, arginine 1809 to leucine. We obtained tagged versions of each of the interactors: serotonin receptor 6 (EGFP-5HTR6), GPCR trimeric G-protein subunits (YFP-Gβ and YFP-Gγ), LIM Kinase 2 (LIMK2), and Valosin Containing Protein (VCP-EGFP). Our initial expression tests with each of these proteins revealed a number of unexpected challenges.

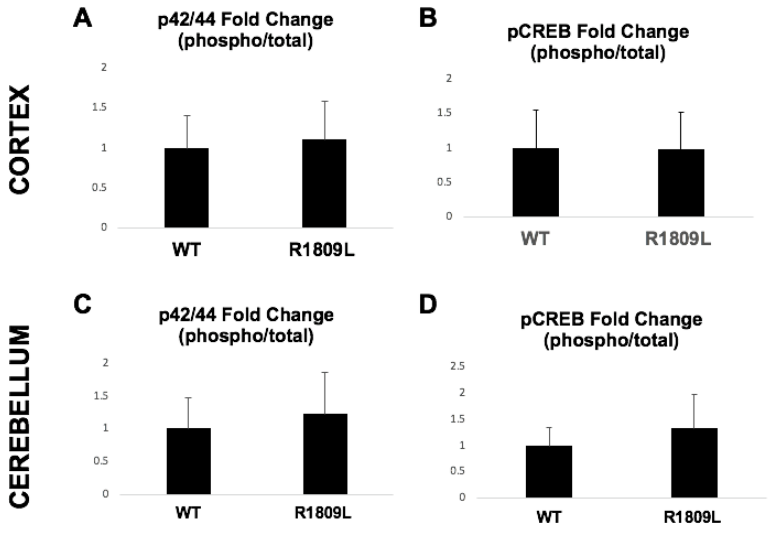

Figure 3. Western blot analysis of signaling for the MAP Kinase (p42/p44) and PKA (pCREB) pathways in the cerebral cortex (**A,B**) and cerebellum (**C,D**) of littermate control and R1809 mutants harvested at P60.

5HTR6 exhibited a strong sensitivity to denaturation conditions that led it an indistinct smear on Western blot analysis. We are now optimizing our denaturing conditions using decreased denaturing temperatures and alternative denaturing agents to resolve this. YFP-Gβ was well expressed, but did not demonstrate significant binding to the wild-type Sec14-PH domain. We are reducing the stringency of the co-

immunoprecipitation conditions to determine if this interaction may be very sensitive to salt concentrations etc. Expression of YFP-Gγ yielded very low protein production. This may reflect decreased stability when expressed in the absence of Gβ. We are currently testing this possibility by co-expressing Gβ and Gγ together. It is also possible that the interaction with NF1 requires both proteins to complexed. These Gβ/Gγ co-expression experiments will be used to test this possibility. LIMK2 exhibited low levels of expression and we believe this relates to the expression vector supplied. We are sub-cloning the cDNA into a standard mammalian expression vector to overcome this obstacle. The most confounding issue arose from initial experiments with VCP. Control co-immunoprecipitation experiments indicate that VCP has a strong affinity for the anti-rabbit magnetic beads that we used for isolating the Sec14-PH domain portion of the NF1. In absence of the Sec14-PH domain, strong VCP binding was observed. This non-specific binding was not seen for the other partner proteins. We are testing alternative affinity purification materials (protein A/G-agarose beads) to determine if this non-specific binding is unique to the anti-rabbit magnetic beads. If so, we will conduct the experiments with VCP using this alternative purification matrix. **(unpublished data)**

Key Research Accomplishments

Conclusion

We have developed a new mouse model of neurofibromatosis that incorporates a point mutation that when present in human patients leads to learning deficits and developmental delay.

We tested whether this mutation affected brain structures in ways similar to the total loss of expression of the Neurofibromatosis 1 gene in other studies where the gene has been removed entirely.

We determined that this mutation does not affect the signaling pathways previously reported to be altered in brain tissue from NF1 mutant mice that lack expression of the entire NF1 protein. This is a key distinction since the mutation we are studying alters only one amino acid in the protein. **(unpublished data)**

We were able to confirm that the Ras-Erk signaling pathway does not show indications of being altered in adult brains with the R1809L mutation. Similarly, other signaling pathways do not appear to be significantly affected by the mutation. We also do not detect histological changes in brain tissue that have been previously reported with complete loss of the NF1 gene in brain tissue. These findings indicate that the cognitive challenges experienced by R1809L NF1 patients do not arise from the pathway most commonly associated with NF1 protein function (Ras-Erk signaling). While this pathway is linked to learning, our results indicate that alternative aspects of NF1 function must be considered to understand the source of the learning and other cognitive effects of NF1 dysfunction. **(unpublished data)**

Our characterization of the new mouse model indicates that this mutation does not affect the Ras GAP function of the NF1 protein. This finding is consistent with the lack of hyperproliferative cells in patients with mutations at this site in the protein. The similarities between this patient cohort and our model indicates that it holds the potential to help understand the molecular basis of learning deficits associated with altered NF1 protein function in the context of the R1809L mutation. **(unpublished data)**

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 will finish our analysis of the signaling pathways and developmental brain morphology for embryonic timepoints (embryonic days 14.5 and 18.5).

We will complete our optimization of the purification and Western blotting conditions of the known NF1 interactors that may target the region of NF1 containing the R1809L mutation. This will allow us to determine if any of these interactions are affected by this alteration in NF1.

We will determine the distribution of the R1809L form of NF1 using primary neuron cultures from mutant and control mice.

We will complete the proteomic analysis described in Aim 2.2 for wild-type and mutant NF1 (R1809L) complexes to determine which protein-protein interactions are altered in the endogenous setting of the developing and adult brain. Any detected differences would be confirmed by immunoblotting from neural or glia co-IPs.

We also plan to submit our findings for publication and present this work at local and national neuroscience meetings.

STATEMENT OF WORK

Site 1: Oregon Health and Sci Univ[GSU]
3181 SW Sam Jackson Pk Rd
HRC5N
PI: Anthony Barnes, PhD
(if applicable)

Research-Specific Tasks:

Specific Aim 1: To understand mutant brain development and signaling	**Completion Target Date**	**% Complete**
Major Task 1: To elucidate the effect of the R1809L mutation on brain patterning and signaling.		
Subtask 1: Characterize Brain Development in Novel NF1 model Mouse line used NF1: R1809L 80 adult animals (including 6 timed-pregnant females).	Aug-2020	35%
Subtask 2: Define the altered signaling associated with this NF1 mutation Mouse line used NF1: R1809L 15 adult animals (including 6 timed pregnant females)	Aug-2020	25%
Milestone(s) Achieved: Delineation of specific effects on brain development and signaling by the R1809L mutant		
Specific Aim 2: Biochemical Effects of NF1 mutation		
Major Task 2: To define the effect of R1809L on Sec14-PH domain interacting partners and NF1 localization in multiple brain cell types.		
Subtask 1: Identify the effect of NF1 binding proteins and localization of mutant NF1 Mouse line used NF1: R1809 6 timed pregnant females	Aug-2020	25%
Subtask 2: Determine the biochemical status of NF in neurons Cell lines used: 9 adults including 3 timed-pregnant females	Aug-2020	0 %
Milestone(s) Achieved: Mapping of the NF1 interactome		

4. IMPACT:

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

The findings made during this study establish that the R1809L mutation in NF is distinct from other mutations that affect the function of the NF1 protein. Specifically, this mutation does not affect the well-studied cellular pathways linked to neurofibroma formation. Instead, our study suggests that alternative pathways are likely perturbed in this particular mutation of the NF1 protein, revealing novel aspects of NF1 related to brain development and function. This conclusion was only possible through the use of this new mouse model of NF as no patient samples exist to be analyzed to determine the nature this mutation.

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

The expansion of our mutant mouse colony progressed unexpectedly slowly, but has now reach a sustainable level. The reasons for this are unclear but since the mutation was generated via CRISPR/Cas9 there may have been due to off-target effects on the genome.

Changes that had a significant impact on expenditures

This delay in scaling up the mouse colony resulted in reduced expenditures for the mouse costs as well as reagents for some experiments.

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

Nothing to Report

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

This project characterized a novel mouse model of Neurofibromatosis type 1. Animal models of this disease historically have centered on the loss of the NF1 gene. This model is molecularly identical to a mutation found in the human population.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name: Dr. Anthony Barnes

Project Role: Principle Investigator

Researcher Identifier (e.g. ORCID ID): 0000-0002-4598-9591

Nearest person month worked: 12

Name: Dr. Sarah Santiago

Project Role: Post-doctoral researcher

Researcher Identifier (e.g. ORCID ID): 0000-0002-7224-3429
Nearest person month worked:12

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

Nothing to Report

9. APPENDICES

Nothing to Report