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TITLE: Characterization of a novel humanized mouse model of Neurofibromatosis

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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

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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 phenotype 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

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.

This sub-aim was hindered by limited numbers of animals and COVID-19 restrictions at our Institution. However, we expect to complete this aspect of the study in the next reporting period as discussed in section 5.

We proposed in **Aim 1.2** to determine if signal transduction pathways reported to be altered by NF1 loss also displayed signaling perturbations in the R1809L mutant brain using Western blot analysis. We have extended our study into embryonic ages, probing signaling in the developing cerebral cortex of control and mutant animals. We tested two timepoints, one that is dominated by neurogenesis (embryonic day 14.5, E14.5, **Figure 1A**) and another that also includes astrogliogenesis (E18.5, **Figure 1B**) These data do not reveal a significant difference between control and mutant samples at either age, consistent with the notion that the PH domain mutation (R1809L) may be acting independently of the Ras GAP domain.

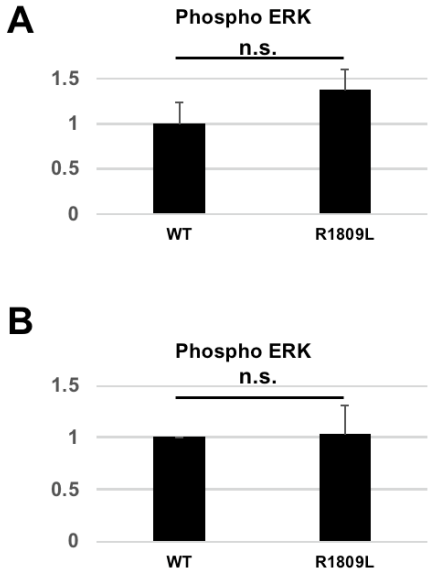


Figure 1. Western blot analysis of littermate control and R1809 mutants for the MAP Kinase activation at E14.5 (**A**) and (**B**) E18.5. n=3 per condition

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 if the mutant PH domain affected previously reported interaction partners of NF1: serotonin receptor 6 (5HTR6), GPCR trimeric G-protein subunits (Gβ and Gγ), LIM Kinase 2 (LIMK2), and Valosin Containing Protein (VCP). The challenges associated with the co-purification of NF1 with these candidate molecules has led us to consider a different approach to test if their interaction depends on the PH (wild-type or mutant). This new approach is known as proximity biotin ligation (BIOID) and is described in our plans for the next reporting period.

Aim 2.2 focused on an unbiased screen for novel NF1 interacting proteins that require an intact PH domain. The challenges faced by Aim 2.1 would have also negatively affected this aim as well, thus the above mentioned BIOID strategy will also be employed to accomplish this sub-aim as well.

Key Research Accomplishments

Conclusion

We have further characterized our new neurofibromatosis mouse model that bears a point mutation associated with learning deficits and developmental delay in patients, continuing our examination of the signaling pathways associated with dysregulated or lost NF1 function. It had not previously been tested whether a heterozygous R1809L mutation affects growth pathways, but the lack of tumor formation in patients indicated this may be the case. Our data demonstrate that this is indeed the case and denote potentially dissociable function for the Sec14-PH domain of NF1. While Ras-Erk signaling has been implicated in learning and memory studies, our results indicate that the Sec14-PH domain of NF1 is likely playing a distinct role in cognitive function. These findings are consistent with our earlier findings that the Ras-Erk signaling pathway appears unaffected in the adult brain in the context of a heterozygous R1809L mutation.

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 complete our histologic analysis of developmental brain morphology with embryonic timepoints (embryonic days 14.5 and 18.5) and post-natal days 0 and 8. We will pay particular attention to histochemical results at E14.5 as a slight, but not significant, trend of increased phospho-Erk was observed at this age in the Western blotting data and staining may reveal a subpopulation of cells is producing this signal. We will also use staining to determine the distribution of the R1809L form of NF1 using primary neuronal cultures from the mutant mice.

We will use the BIOID method to proximity label (biotinylate) and purify interactors, confirming interaction with Western blotting to determine if their binding specifically requires the region of NF1 containing the R1809L mutation and if this amino acid change disrupts the interaction.

We will also complete the proteomic approach for Aim 2.2 of wild-type and mutant NF1 (R1809L) complexes to determine in an unbiased way if any novel protein interactors are altered in the endogenous setting using the BIOID method discussed in Section 5.

We 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-2021	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-2021	45%
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-2021	25%
Subtask 2: Determine the biochemical status of NF in neurons Cell lines used: 9 adults including 3 timed-pregnant females	Aug-2021	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 pathway are likely perturbed in this particular mutation of the NF1 protein, revealing novel aspects of NF function related to brain develop and function. This conclusion was only possible through the use the new mouse model of NF as no patient samples exist to be analyzed for 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

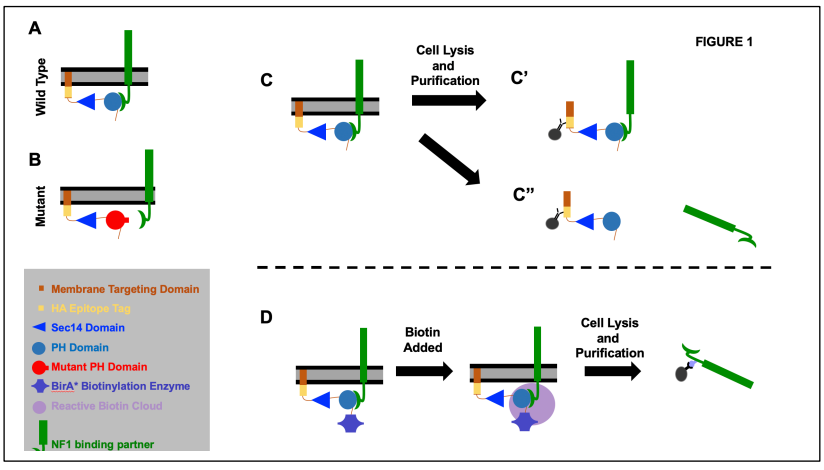
We encountered unexpected challenges in completing Aim 2.1 owing to technical issues regarding the co-purification of our NF1 fragment with previously reported interaction partners. We have decided to change our approach to a methodology known as BIOID that is already used successfully in the laboratory. This method requires very little optimization as the purification conditions would be identical regardless of which partner protein being tested due to eliminating the need to maintain intact complexes with NF1. This fact will allow us to streamline our testing and successfully complete this Aim despite COVID-19 limitations.

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

We encountered delays on this project due to a research pause in the first phase of the COVID-19 pandemic outbreak at our Institution and subsequent modified operations (limited times on campus) coupled with limited genetic animal models that are central to our studies. We have developed a productive workplan to optimize our time at the bench within the limited operations requirements and established a breeding colony capable of supplying the required samples for completion of this study.

As described in the changes in approach section above, we have decided to use a distinct strategy to address the goals of Aim 2. The figure below illustrates the region of the Neurofibromatosis 1 (NF1) protein that has been used in previous studies and that we are using to capture potential interaction partners that relate to the function of this segment of NF1 (**Figure 1A**). Our hypothesis is that a particular NF1 mutation (R1809L) disrupts specific protein interactions with the PH domain of NF1 (**Figure 1B**). Our original approach in Aim 2.1 was to test proteins reported to bind NF1 for which the binding site had been localized near the Sec14-PH domain region. We proposed to isolate and detect binding partners using affinity purification via a hemagglutinin (HA) epitope tag incorporated in the NF1 domain and high-affinity anti-HA anti-sera coupled to agarose beads (**Figure 1C,C'**). While this technique can be very successful, it depends critically on the conditions used to disrupt the cells or tissues expressing the target proteins. Should a protein-protein interaction be weak or transient, it can be difficult to detect the interaction, as the proteins may dissociate during the lysis and purification steps (**Figure 1C"**).

Our attempts to purify these reported interactions have been unsuccessful, but this may be due in part to the different conditions used in each study that detected each NF1 interaction. We would like to take a different approach to detect these interactions that is called proximity-dependent BIOtin IDentification (BIO-ID). This method uses a bacterial protein called biotin ligase (BirA) that usually creates reactive biotin and deposits it on specific bacterial proteins that require this modification for their function. A mutant form of this protein (BirA*) has been discovered that retains its ability to produce



reactive biotin, but is no longer able to transfer it to specific proteins. Instead, biotin is simply released to react with any nearby proteins containing exposed lysine residues. The mutant enzyme is very amenable to fusion with proteins of interest, allowing it to be used to map a protein's closest neighbors. Essentially, a cloud of reactive biotin is produced that envelopes proteins near the enzyme, covalently tagging them with biotin allowing subsequent selective purification using beads coupled to a protein called Streptavidin that has high affinity for biotin (**Figure 1D**). Purified proteins can then be identified via either Western blotting or mass spectrometry.

We have used this approach for other projects in my laboratory to successfully purify protein complexes that either confirmed known interactions or revealed novel binding partners. We believe this approach will have a greater likelihood of success as it does not depend on the maintenance of NF1 complexes with binding partners. Using this approach, the binding partners are the purification targets, and the strength of the biotin-streptavidin interaction allows stringent washes to remove any non-specific proteins during purification. Our paradigm is ideal for this approach as we would have wild-type NF1 fused to BirA* to act as a positive control so that loss of partner protein labeling by mutant NF1-BirA* fusion protein would be indicative of a weaker or disrupted interaction. For sub-aim 2.1, we currently have the expression vectors for all the candidate interactors, and could proceed with testing each interaction by co-expression of NF1-BirA fusion proteins and streptavidin purification much as originally described, but substituting BIO-ID for co-immunoprecipitation. We could also apply this BIO-ID approach for sub-aim 2.2 by expressing either the wild-type or mutant NF1-BirA* fusions and purifying all biotinylated proteins and subjecting them to mass spectrometry analysis to determine which are found in only samples that express the wild-type version of NF1 but not the mutant. This unbiased approach would allow us to discover novel interactors that require the intact PH domain of NF1 and would expand our understanding of how the 1809 mutation impacts cognitive function in affected patients.

Changes that had a significant impact on expenditures

This delay associated the above-mentioned research pause and COVID-19 limitations 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: 2

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 PI received funding unrelated to this project and this funding does not impact this project. The PI is now a co-investigator on an NIH R01 (5R01NS111948) focused on the mechanobiology of cortical development and recently was approved for internal bridge funding from his Institution to support an related project focused on membrane trafficking during brain development.

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

9. APPENDICES

Nothing to Report