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TITLE: Effects of Pharmacologic and Genetic Inhibition of Alk on Cognitive Impairments in NF1 Mutant Mice

PRINCIPAL INVESTIGATOR: Jacob Raber, PhD

CONTRACTING ORGANIZATION: Oregon Health & Science University
Portland, OR 97239

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Effects of Pharmacologic and Genetic Inhibition of Alk on Cognitive Impairments in NF1 Mutant Mice

Concordant with studies in flies, we found enhanced retention of spatial memory in Alk mutant mice. Retention of spatial memory is a hippocampal dependent function. We also demonstrated expression of Alk throughout the adult murine hippocampus. The behavioral phenotype of Alk mutant mice is the opposite of the behavioral phenotype of NF1 mutant mice. Therefore, in this pilot project, we tested the hypothesis that the genetic interaction between Alk and Nf1 in mice is similar to the behavioral phenotypes of Alk and NF1 mutations in flies and that pharmacologic or genetic inhibition of Alk in NF1 mutant mice will attenuate or even rescue learning impairments in mice. We are excited to report that we have observed the predicted improvement in hippocampal-dependent cognitive function using both genetic and pharmacologic inhibition of Alk in heterozygous NF1 mutant mice. Our findings are consistent with the hypothesis that pharmacologic or genetic inhibition of Alk in NF1 mutant mice rescues their cognitive impairments.

14. ABSTRACT

15. SUBJECT TERMS

cognitive performance, pharmacological inhibition, spatial memory, hippocampus

16. SECURITY CLASSIFICATION OF:

<table>
<thead>
<tr>
<th>a. REPORT</th>
<th>b. ABSTRACT</th>
<th>c. THIS PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unclassified</td>
<td>Unclassified</td>
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</tr>
</tbody>
</table>
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction</td>
<td>4</td>
</tr>
<tr>
<td>2. Keywords</td>
<td>4</td>
</tr>
<tr>
<td>3. Accomplishments</td>
<td>4</td>
</tr>
<tr>
<td>4. Impact</td>
<td>13</td>
</tr>
<tr>
<td>5. Changes/Problems</td>
<td>14</td>
</tr>
<tr>
<td>6. Products</td>
<td>15</td>
</tr>
<tr>
<td>7. Participants &amp; Other Collaborating Organizations</td>
<td>16</td>
</tr>
<tr>
<td>8. Special Reporting Requirements</td>
<td>n/a</td>
</tr>
<tr>
<td>9. Appendices</td>
<td>n/a</td>
</tr>
</tbody>
</table>
1 INTRODUCTION:

In previous experiments, we studied the expression of Alk and the effects of Alk mutations on learning and memory in mice. Concordant with studies in flies, we found enhanced retention of spatial memory in Alk mutant mice. Retention of spatial memory is a hippocampal dependent function. We also demonstrated expression of Alk throughout the adult murine hippocampus. The behavioral phenotype of Alk mutant mice is the opposite of the behavioral phenotype of NF1 mutant mice. In this pilot project, we tested the hypothesis that the genetic interaction between Alk and Nf1 in mice is similar to the behavioral phenotypes of Alk and NF1 mutations in flies and that pharmacologic or genetic inhibition of Alk in NF1 mutant mice will attenuate or even rescue learning impairments in mice. We are excited to report that we have observed the predicted improvement in hippocampal-dependent cognitive function using both genetic and pharmacologic inhibition of Alk in heterozygous NF1 mutant mice. Our findings are consistent with the hypothesis that pharmacologic or genetic inhibition of Alk in NF1 mutant mice rescues their cognitive impairments.

KEYWORDS: neurofibromatosis; Alk; cognition; intervention; mouse model; hippocampus; pharmacological inhibition; spatial memory

2 ACCOMPLISHMENTS:

- **Major goals of the project**

Specific Aim (months 1-24). Determine the effects of genetic and pharmacologic inhibition of Alk on retention of spatial memory in heterozygous Nf1 mutant mice.

Task 1 (1-12 months). *Actual completion date: July 2017.* Breeding of the appropriate mice for genetic studies. The first set of experiments compares four genotypes in a series of behavioral and cognitive tests. The four genotypes are 1) wild type, 2) homozygous Alk mutants, 3) heterozygous Nf1 mutants and 4) homozygous Alk mutants that are also heterozygous Nf1 mutants (double mutants). Each group will consist of 15 three-months-old male mice. A second, complimentary set of studies will be performed employing pharmacologic inhibition of Alk to determine the efficacy and specificity of this strategy. In these experiments six groups of 15 male mice roughly three months old will be tested. The groups will consist of treated and untreated mice of the following genotypes 1) wild type 2) homozygous Alk mutants and 3) heterozygous Nf1 mutants. The NF1 mutant mice for breeding will be purchased from JAX. We anticipate that we need to order at the very least 2 mice with the mutation for breeding. The Alk mutant mice for breeding we will receive from the University of Toronto. To breed the mice will require B6 mice from Jax and 129 mice from Taconics. We will purchase 5 female and 5 male mice from each vendor, all 8 weeks old. To generate and maintain breeding cages for the NF1 mutant mice for 1.5 year will take on average of 2 breeding cages, each with two females and one male. To generate and maintain breeding cages for the Alk mutant mice for 1.5 year will take on average of 4 breeding cages, each with one female and one male. To generate the 150 male mice or 300 mice of offspring by crossing NF1 and Alk heterozygous mice, estimating an
average of 4 male mice per litter will require 38 litters. We anticipate that it will take 5 breeding cages, each with 2 females and 1 male.

Task 2 (12-18 months). Actual completion date: July 2017. Behavioral and cognitive testing of the four genotypes.

Behavioral and cognitive testing of the four genotypes. Behavioral and cognitive testing will be performed on four groups of 15 male mice of four genotypes 1) wild type, 2) Alk/Alk mutants, 3) Nf1/+ mutants and 4) Alk/Alk; Nf1/+. Thus, the total number of animals employed will be 60. The tests employed will assay sensorimotor function on the rotorod, measures of anxiety in the elevated zero maze, and retention of spatial memory in the water maze.

Task 3 (12-24 months). Actual completion date: July 2017. Administration of TAE684 to appropriate genotypes and behavioral and cognitive testing. For the pharmacologic experiments two groups of each of three genotypes will be tested. The two groups consist of treated and untreated animals. The three genotypes are 1) wild type, 2) Alk/Alk homozygous mutants, and 3) Nf1/+ heterozygotes. The total number of animals will therefore be 6X15=90. All mice will be male approximately 3 months old. The schedule for behavioral and cognitive testing is identical to that for the behavioral testing of various genotypes.

Task 4 (12-24 months). This task has not been started yet. As described in more detail below, this project involved much more breeding, mice, and behavioral and cognitive testing than initially anticipated. A total of 221 mice were tested. Immunoblot analysis of Alk phosphorylation in TAE684 treated mice.

Brains of wild type, Alk/Alk, and Nf1/+ mice treated with TAE684, for a total of 90 mice as described in Task 3 will be harvested at the end of behavioral and cognitive testing. The brains will be homogenized in lysis buffer, cleared by centrifugation and subjected to immunoblotting to detect total Alk and phospho-Alk.

- Accomplished under these goals

The objective of this project was to determine the effects of genetic and pharmacologic inhibition of Alk on retention of spatial memory in heterozygous NF1 mutant mice. The objective was met and the main results of the major activities are described below.

Data of the Genetic Study

The number of mice and nomenclature of the genotypes for the genetic study are indicated in Table 1.
Table 1. Number of mice and genotype nomenclature of the genetic study.

<table>
<thead>
<tr>
<th>Label</th>
<th>Alk Genotype</th>
<th>NF1 Genotype</th>
<th>Females</th>
<th>Males</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>11</td>
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<tr>
<td>NF1</td>
<td>WT</td>
<td>NF1-/+</td>
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<td>WT</td>
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<td>12</td>
<td>23</td>
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<tr>
<td>2xMut</td>
<td>Alk/-</td>
<td>NF1-/+</td>
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<td>16</td>
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<tr>
<td>2xHet</td>
<td>Alk/-+</td>
<td>NF1-/+</td>
<td>10</td>
<td>15</td>
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</table>

**Spatial learning in the water maze**
The mice were trained to locate a platform in three distinct locations during hidden platform water maze training (Hidden Location 1: sessions 1-4; Hidden Location 2: sessions 5-6; Hidden Location 3: sessions 7-8). Subsequently, the mice were trained to locate a visible platform (Visible Location: sessions 9-10). The time to reach the hidden target location (latency) is shown in Fig. 1.
The data were analyzed, for each platform location separately, using a repeated-measures ANOVA with genotype and sex as between group factors. For training to locate the first platform location, there was a significant NF1 x Alk genetic interaction ($F(1,111) = 7.490, p = 0.007$) and a significant effect of Alk ($F(2,111) = 7.456, p = 0.001$). Compared to WT mice, NF1 mice showed impairments in their ability to reach the platform ($p = 0.048$, Tukey-Kramer). Reducing Alk in the 2xHet and 2xMut mitigated this impairment. For training to locate the second platform location, there was a significant effect of Alk ($F(2,111) = 6.096, p = 0.003$), and a trend towards a NF1 x Alk genetic interaction ($F(1,111) = 3.629, p = 0.059$). For training to locate the third platform location, there was a significant NF1 x Alk x session interaction ($F(1,111) = 10.218, p = 0.002$), a significant effect of Alk ($F(1,111) = 10.218, p = 0.002$). Interestingly, the protective effect was more pronounced in 2xHet than 2xMut mice. These effects in the water maze could not be attributed to the pattern of genotype differences in swim speeds seen in the water maze (Fig. 2).

Contextual fear learning and memory

Next the mice were tested for acquisition and extinction of hippocampus-dependent contextual fear. During training (day 1), there was an effect of genotype on motion prior to the first tone (baseline motion, Fig. 3). There was a significant Alk x NF1 interaction for baseline motion ($F(1,111) = 5.816, p = 0.018$). WT mice moved more than 2xHet ($p = 0.012$) and 2xMut ($p = 0.014$) mice. There was no effect of genotype on freezing in response to the tone during
training (not shown). There was no effect of genotype on freezing between the tone-shock pairings during training (not shown), indicating that all genotypes acquired the task equally well.

![Baseline Motion](image)

**Fig. 3.** Baseline motion in the contextual fear conditioning test in the genetic study. *p < 0.05 versus WT mice. For details, see text.

Next, we determined the ability of the mice to suppress the memory when placed daily in the same environment as that used for learning the tone-shock association. The data are shown in Fig. 4. There was a significant Alk x NF1 x sex x day interaction ($F(3.455,383.522) = 3.6984$, $p = 0.009$) and a significant Alk x day interaction ($F(6.910,383.522) = 2.172$, $p = 0.037$). Freezing levels decreased more in WT than NF1 mice. This effect was mitigated in 2xHet and 2xMut mice.

![Contextual Extinction](image)

**Fig. 4.** Extinction of contextual fear. For details, see text.

Consistent with this pattern, when the difference in freezing levels between days 1 and 7 were compared as measure of extinction of a contextual fear memory, there were significant differences in freezing levels on day 1 vs day 7 in all groups except the NF1 mice (Fig. 5, Sidak’s multiple comparisons).
A home cage sensor system, as described 1 was used to assess circadian activity levels in NF1 mice on an Alk wild-type (WT) (NF1, n = 6 mice) and Alk heterozygous knockout (2xHet, n = 3 mice) background, HomAlk (n = 10 mice) and HetAlk (n = 10 mice). NF1 mice on an Alk WT background showed lower activity levels during the dark phase, when the mice are active, than 2xHet mice (Fig. 6). The difference between activity levels during the dark and light phase was much smaller in NF1 mice on an Alk background than 2xHet mice. Although the number of mice in the latter genotype was especially small, these data indicate that genetic Alk inhibition improves circadian activity levels in NF1 mice. These data are especially intriguing in light of the circadian alterations seen in NF1 patients 2-7.

Fig. 6. Circadian activity levels in the home cage. A. NF1 mice (orange bars) showed lower activity levels during the dark phase than NF1 mice with reduced Alk expression (2xHet, black bars). The mean activity levels during the light and dark periods are shown, based on circadian activity data from three subsequent 24 hr periods. B. Detailed activity levels, averaged over 2 hour-periods. NF1 mice (in orange) showed lower activity levels during the dark phase than NF1

Fig. 5. Extinction of a contextual fear memory in the genetic study. #p < 0.0001 versus day 7. For details see text.
mice with reduced Alk expression (2xHet, in black). The dark periods are indicated with black lines and are separated by dashed blue vertical lines. AU: arbitrary units. For details, see main text.

Data of the Pharmacological Study

The number of mice and nomenclature of the genotypes for the pharmacological study are indicated in Table 2.

Table 2. Number of mice and genotype nomenclature for the pharmacological study.

<table>
<thead>
<tr>
<th>Label</th>
<th>Alk Genotype</th>
<th>NF1 Genotype</th>
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<td>WT Drug</td>
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<td>WT</td>
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<tr>
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<td>NF1 -/+</td>
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<td>NF1 Drug</td>
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<td>Vehicle</td>
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<tr>
<td>HomAlkD</td>
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<td>WT</td>
<td>Alk Inhibitor</td>
<td>7</td>
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</table>

In our pharmacological studies, we used the lowest effective dose of an Alk inhibitor (1.8 mg/kg) with activity against central nervous system tumors. This dose was more than 10 fold lower than the maximal effective dose of (20 mg/kg). We treated 2-3 month old mice daily for 3.5 weeks. The drug or vehicle was made up in a flavored gelatin mixture and delivered in 1 cm x 1 cm squares to allow easy daily administration without the risk of injury due to repeated oral gavage and as voluntary oral administration would be the most relevant administration route for human patients. In the water maze, spatial memory retention was assessed 24 hours following
training of the first (sessions 1-4) and second (sessions 5-6) platform location. In the probe trial following training for the first platform location, NF1 mice showed spatial memory retention and spent more time searching in the quadrant that contained the platform during training than any other quadrant. However, when the mice were trained to locate a novel platform location, NF1 mice showed impairments in spatial memory retention in the second probe trial (platform removed, Probe 2) and these impairments were mitigated by the Alk inhibitor (Fig. 7). The Alk inhibitor, at least under these treatment conditions, did not affect spatial memory retention of WT or homozygous Alk knockout mice, who did show spatial bias also after vehicle administration (Alk) (Fig. 7).

We are excited to find this low dose efficacious, as these studies have the ultimate objective of motivating treatment of humans with neurofibromatosis. To this end minimization of drug toxicity is paramount. We are encouraged that this low dose is sufficient to rescue completely the memory impairment in NF1 mice. In summary, this pilot project was very successful and based on these timely and exciting findings a full proposal was submitted and two manuscripts with the data generated are being worked on.

- **Opportunities for training and professional development the project has provided**

Tessa Marzulla was trained and selected in a very competitive program to be trained as a genetic counselor at the University of Michigan. Sydney Weber was trained and accepted as a graduate student at UT Austin and OHSU. She decided to join the graduate program at OHSU. Amelia Mulford was trained and received a job offer from Genetic Alliance in DC (http://www.geneticalliance.org). As she would like to become a genetic counselor, this is an ideal environment for her to fully prepare her for the next step in her career.

- **How were the results disseminated to communities of interest?**

As indicated above, two manuscripts containing data from this project are currently being worked on. Outreach activities enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities during this project are described below.
**Undergraduate Students:**

Erin Bidiman, 2012 and 2013: Role mentor and supervisor for training of Lewis & Clark undergraduate student for research as part of HHMI funded training.

Alicia Callejo-Black, 2013: Role mentor and supervisor for training of Lewis & Clark undergraduate student for research as part of CRT (still partially HHMI funded) funded training.

Tara Kugelman, 2014 and 2015: Role mentor undergraduate student of Lewis & Clark for research as part of Murdock Trust funded training.

Amelia Mulford 2014: Role mentor and supervisor for training of Lewis & Clark undergraduate student for research as part of HHMI/Rogers funded training.

Charity Miltenberger, 2015: Role mentor undergraduate student of Concordia University for research as part of Murdock Trust funded training.

Tara Kugelman, 2015: Role mentor undergraduate student of Lewis & Clark for research as part of Miller training award.

Dana Button, 2015: Role mentor undergraduate student University of Portland

Colton Erickson, 2015: Role mentor and supervisor for training of Lewis & Clark undergraduate student for research as part of HHMI/Rogers funded training.

Betty Wu, 2016: Role mentor and supervisor for training of Lewis & Clark undergraduate student for research as part of HHMI/Rogers funded training.

**Highschool Students:**

Jaclyn Lanz, Spring and Summer 2013, intern St Mary’s Academy.

Meredith Datena, Summer 2013, Summer Intern Saturday Academy Program.

Matthew Bernis, Summer 2013, Summer Intern Saturday Academy Program.

Claire McLaughlin, Spring and Summer 2014, Intern St Mary’s Academy.

Hwan Dong, Spring 2014, Intern Catlin Gabel high school.

Chi Phan, Summer 2014, Intern Saturday Academy Program

Collin McCormack, Summer 2014, Intern HHMI/Rogers program.

Anna-Maria Hartner, Summer 2015, Intern Saturday Academy Program

Dania Ruud, Spring 2015, Intern St Mary’s Academy
Shelby Spohn, Intern St Mary’s Academy
Dhara Brown, Spring 2015, Intern St Mary’s Academy
Hime Worku, Summer 2015, Intern HHMI/Rogers program.
Mikala Capage, Summer 2016, Summer Intern Saturday Academy Program.
Sara Curelaru, Summer 2016, CELS Scholar Beaverton Health & Science High School.
Joanne Lee, Summer 2016, Summer Intern Saturday Academy Program.

Other Outreach Activities:
2013, 2014, 2015 Judge for the Graduate Student / Post-Doctoral Fellows Poster Competition
NASA HRP Investigators Workshops, Galveston, TX
2015 Title: “The Science of Fear and Anxiety”. 25 minute lecture about fear prior to
featuring the movie, An American Werewolf in London. October 14 edition of
OMSI’s monthly series called, Reel Science where popular movies are paired
with an expert.

• What do you plan to do during the next reporting period to accomplish the
goals?
Nothing to Report.

3 IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes,
or any change in practice or behavior that has come about as a result of the project
relative to:

Impact on the development of the principal discipline(s) of the project
The findings that a low drug dose is efficacious in a NF1 animal model are of high impact and
are of great promise for the ultimate objective of therapeutic treatment of humans with
neurofibromatosis. As minimization of drug toxicity is paramount, to know that a low dose is
efficacious is a critical finding. It is exciting that this low dose is sufficient to rescue completely
the memory impairment in NF1 mice. In our pilot project, the length of treatment was limited to
3.5 weeks. In addition, in our pilot study we did not assess potential effects of the Alk inhibitor
on behavioral performance, including circadian (sleep/wake) activity of the mice. This is
important as Alk in flies has very recently been implicated in the regulation of sleep. Therefore,
the results described above are subject to refinement in more comprehensive studies. We
recently submitted a proposal containing more definitive and detailed studies to justify clinical
trials in humans of Alk inhibition to treat NF1.
Impact on other disciplines

Based on the exciting results of this study, we started to explore the potential of cognitive testing in patients with lung cancer that are treated with Alk inhibitors. In addition, we started to explore potential aorta phenotypes in Alk heterozygous and homozygous mutants.

Impact on technology transfer
Nothing to Report.

Impact on society beyond science and technology

Increased understanding of the role of Alk in cognitive performance is likely to have an impact on public knowledge and cognitive performance and cognitive skills beyond mitigating cognitive impairments in neurological conditions.

4 CHANGES/PROBLEMS:
• Changes in approach and reasons for change
There were no changes in approach during the reporting period.

• Actual or anticipated problems or delays and actions or plans to resolve them
This project required way more breeding, litters, and behavioral and cognitive testing than originally anticipated. In addition, as a minimal number of mice was provided to us for breeding of the NF1 and Alk mutant mice and mice needed to be backcrossed, this further expanded the time window required to get this project in full force going. As a result, this stretched out over a much longer period of time. More specifically, this project involved complicated breeding and two mutant mouse models that needed to be imported, after processing all the required paper work for it. Also, as the mice we requested needed to be bred and become available first, this delayed the shipment of these mice. We received only 4 male and 3 female Alk KO mice on the C57BL/6J background on 9/25/2013 from Dr. Liliana Attisano at the University of Toronto and only 2 male NF1+/- mice, on the 129 genetic background, on 10/09/2013 from Dr. Nancy Ratner at the Cincinnati Children's Hospital. The complicated breeding scheme combined with the delayed shipment of the mice has prolonged this project and as a result we requested to continue and finish this project in Yr 3, which was approved last year.

• Changes that had a significant impact on expenditures
The increased breeding effort and numbers of mice for behavioral and cognitive testing had an impact on the per diem charges. However, we made sure that we did not increase the charges to this grant and stayed with the exact amounts budgeted and approved.

• Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
All procedures with vertebrate animals and the numbers of mice used for this project were approved by the OHSU IACUC committee.

N/A

• Significant changes in use or care of vertebrate animals.
N/A

• Significant changes in use of biohazards and/or select agents
N/A
5 PRODUCTS:
Nothing to Report.

• Publications, conference papers, and presentations
As indicated above, two manuscripts with the data generated as part of this project are being worked on.

• Technologies or techniques
N/A

• Inventions, patent applications, and/or licenses
N/A

• Other Products
N/A

References
What individuals have worked on the project?

Jacob Raber, Joseph Weiss, Tessa Marzulla, Sydney, Weber, and Amelia Mulford.

<table>
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<th></th>
<th>Yr 1 effort</th>
<th>Yr 2 effort</th>
<th>Yr 3 effort</th>
<th>Contribution to Project:</th>
</tr>
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<tr>
<td>Jacob Raber, PI</td>
<td>5% 0.6 cal</td>
<td>4.85% 0.6 cal</td>
<td>6.94% 0.8 cal</td>
<td>Functioned as the PI of this project. He was responsible for the overall design and coordination of all proposed experiments and supervised all experiments for this project. He supervised Tessa, Sydney, and Amelia, who assisted him in carrying out the experiments and analyze the data obtained. Dr Raber wrote up research results for progress reports and is currently working on writing up the results for the corresponding manuscripts.</td>
</tr>
<tr>
<td>Joseph Weiss, Co-I</td>
<td>5% 0.6 cal</td>
<td></td>
<td></td>
<td>Functioned as the Co-I of this project. He contributed to the experimental design and interpretation of experimental results of proposed experiments. He also assisted Dr. Raber with the write up for progress reports and is assisting Dr. Raber with the write up for publications.</td>
</tr>
<tr>
<td>Tessa Marzulla, RA</td>
<td>15.5% 1.86 cal</td>
<td>1.26% 0.15 cal</td>
<td></td>
<td>Tessa assisted with breeding and genotyping of the mice for this project, with the pharmaceutical treatments, BrdU injections, and behavioral and cognitive testing.</td>
</tr>
<tr>
<td>Sydney Weber, RA</td>
<td></td>
<td>13.52% 1.6 cal</td>
<td>(Feb – Jun 2016)</td>
<td>Sydney assisted with breeding and genotyping of the mice for this project after Tessa went back to school. Sydney also assisted with the pharmaceutical treatments, BrdU injections, behavioral and cognitive testing, and statistical analyses and preparation of figures for this project. Finally, Sydney assisted with the perfusion of the mice and dissection of brain region following cervical dislocation.</td>
</tr>
<tr>
<td>Amelia Mulford, RA</td>
<td></td>
<td>15.53% 1.86 cal</td>
<td>(May – Jun 2016)</td>
<td>Amelia assisted Sydney with breeding and genotyping of the mice for this project. Amelia also assisted Sydney with the pharmaceutical treatments, BrdU injections, perfusion of mice, and dissecting of brain regions.</td>
</tr>
</tbody>
</table>
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.