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TITLE: “NF1 Is an Effector and Regulator of the GPCR Signaling in the Nervous System

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14. ABSTRACT Neurofibromatosis type 1 (NF1) is a monogenic dominant disorder caused by the mutations in the NF1 protein. In addition to developing tumors, NF1 patients suffer from prominent neuropsychiatric symptoms that include cognitive impairments, motor coordination problems, attention difficulties and pain. The focus of this proposal is on molecular mechanisms by which NF1 protein affects signal transduction in the key neuronal pathways and, as such, underlies development of neuropsychiatric problems. During the initial funding period we have achieved significant progress in many of the proposed directions and are on track to continue successful implementation of proposed studies during the next period. Specifically, we have generated all necessary animal models, tools and reagents and applied them to investigate impact of NF1 on: (1) cAMP signaling in brain tissues and cultured cells, and 2) Ras signaling in neurons, revealing its key role in transducing signals from opioid receptors. These findings will likely have an impact on understanding signaling disruptions in neurofibromatosis and development of innovative therapeutic strategies.					
15. SUBJECT TERMS neurofibromatosis, nervous system disorders, receptor signaling mechanisms, NF1					
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TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	3
2. Keywords	3
3. Accomplishments	3 - 9
4. Impact	9 - 10
5. Changes/Problems	11
6. Products	12 - 13
7. Participants & Other Collaborating Organizations	14 - 15
8. Special Reporting Requirements	15
9. Appendices (None)	15

- 1. INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Neurofibromatosis type 1 (NF1) is a monogenic dominant disorder caused by the mutations in the NF1 protein. In addition to developing tumors, NF1 patients suffer from prominent neuropsychiatric symptoms that include cognitive impairments, motor coordination problems, attention difficulties and pain. Research over the last decade established that NF1 protein plays critical role in the nervous system by controlling MAPK signaling, by a virtue of its RasGAP activity and production of second messenger cAMP, by unknown mechanism. The focus of this proposal is on molecular mechanisms by which NF1 protein affects signal transduction in the key neuronal pathways and, as such, underlies development of neuropsychiatric problems. Our preliminary data indicate that NF1 associates with the $\beta\gamma$ subunits of the heterotrimeric G proteins and this interaction is dynamically regulated by the neurotransmitter activated dopamine G protein Coupled Receptor (GPCR). The study is designed pursue two aims: (1) to understand the mechanism of AC activity regulation by NF1 and (2) to test whether NF1 provides the critical link for the activation of the MAPK pathway by the GPCRs.

- 2. KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Neurofibromatosis type 1, NF1 protein, cAMP, Ras signaling, MAPK pathway, G protein coupled receptors, neuronal signal transduction

- 3. ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?

Specific Aim 1. To understand the mechanism of AC activity regulation by NF1	Timeline
Task 1: <i>Probe the contribution of NF1 to the effects of G protein subunits on cAMP production</i>	Months
A. Regulatory approval of DoD ACURO protocol Status: Fully completed	1-3
B. Generate Nf1 ^{flx/flx} :: CAG-CreERT2 double transgenic line Status: Fully completed	2-4
C. Breed sufficient numbers of experimental mice and their control littermates, induce recombination, Status: In progress. Ongoing activity with no milestones.	4-36
D. Harvest tissues, prepare membranes, run AC assays Status: In progress. ~50% completed. Please refer to specific description of results below.	5-8
E. Perform overexpression studies in MEF cells Status: In progress. MEF cells were obtained from a collaborator and successfully grown. Initial experiments were performed. Please refer to specific description of	7-10

results below.	
Task 2: Examine effect of NF1 on dynamics of cAMP signaling in primary neuronal cultures	
A. Establish neuronal cultures, transfection protocols and neurotransmitter applications Status: Fully completed.	3-6
B. Perform the evaluation of NF1 KO cultures, pharmacological manipulations, and genetic blockade experiments Status: Studies initiated. First experiments are being conducted.	6-12
Task 3: Examine contribution of Raf-1 to NF-1 dependent AC regulation.	
A. Setup AC sensitization experiments in primary neuronal cultures. Status: Experiments pending during the next funding period.	12-15
B. Perform the evaluation of NF1 KO cultures, pharmacological manipulations, and genetic blockade experiments examining AC activity. Status: Experiments pending during the next funding period.	15-20
C. Perform biochemical characterization of cultures by Western blotting and RT-PCR Status: Experiments pending during the next funding period.	15-20
Task 4: Analyze complex formation between NF1 and AC.	
A. Test effects of Spred1 knockdown and overexpression in NF1 KO primary cultures Status: Experiments pending during the next funding period.	15-20
B. Test effects of Spred1 knockdown and overexpression in RGS9 KO cultures Status: Experiments pending during the next funding period.	20-24
B. Perform co-immunoprecipitation and pull-down experiments between components of putative NF1/Spred1/RGS9-2/AC/Gbg complex Status: Initial observations are confirmed. Additional experiments are planned during the next funding period.	1-6
<i>Summarize findings, troubleshoot experiments, perform additional replicates, consider alternative design.</i>	1-24
Specific Aim 2. To test whether NF1 provides the critical link for the activation of the MAPK pathway by the GPCRs	
Task 1: Study the role of NF1 in mediating Gbg effects on ERK1/2 phosphorylation in neurons	
A. Perform Gbg overexpression studies in cultured neurons. Analyze the results by monitoring pERK regulation by Western blotting. Status: These experiments have not been started.	24-30
B. Conduct Gbg functional blockade and control experiments. Status: These experiments have not been started.	24-30
Task 2: Probe the effect of Gbg on GTPase Activating Protein (GAP) activity of NF1	
A. Perform activated Ras pull-down experiments from primary cultures comparing NF1 KO and WT and effects of Gbg overexpression Status: In progress. Please refer to specific description of results below.	6-12
B. Use BRET sensors to probe Ras activation in transfected HEK293 cells. Status: In progress. Please refer to specific description of results below.	1-6

C. Conduct biochemical experiments with recombinant proteins studying GTPase activity of Ras and its regulation by Gbg and NF1 Status: In progress. Most experiments are done. New directions are planned. Please refer to specific description of results below.	15-20
Task 3: <i>Test contribution of NF1 to GPCR-mediated signaling to ERK1/2</i>	
A. Analyze changes in pERK1/2 phosphorylation in response to GPCR activation in the presence or absence of NF1 in primary neurons. Status: In progress. ~50% completed. Please refer to specific description of results below.	30-36
B. Monitor the dynamics of the FRET response using Ras-activation sensor. Status: In progress. ~50% completed. Please refer to specific description of results below.	30-36
<i>Summarize findings, troubleshoot experiments, perform additional replicates, consider alternatives.</i>	1-36
<i>Prepare manuscript for publication, perform data analysis for the entire project, generate report</i>	30-36

What was accomplished under these goals?

Much of the work during the initial period of funding focused on generating necessary animal models, tools and reagents. These efforts were very successful allowing us to complete many tasks ahead of schedule. As a result we have achieved significant progress in many of the proposed directions and are on track to continue successful implementation of proposed studies during the next period. The detailed account of the accomplishments is provided below.

Aim 1 is to understand the contribution of NF1 to regulation of cAMP signaling. Working towards this goal we have successfully generated a conditional mouse model where NF1 elimination is achieved globally in a temporary resolved function by activating Cre-recombinase with tamoxifen. We have bred sufficient numbers of these mice and used their brain tissues to culture neurons and obtain membrane preparations for the studies on cAMP regulation. Thus Task 1 (A, B, C) is completely accomplished. We used NF1flx/flx:CreERT2(+) mice as an experimental group and their NF1flx/flx:CreERT2(-) littermates as controls. First we measured total cAMP levels in different brain regions of mice. We found that deletion of NF1 did not alter cAMP levels in this model in 3 brain regions that we examined (**Figure 1**). Next, isolated brain membrane and studying enzymatic aspects of cAMP production by measuring adenylyl cyclase (AC) activity in response to its stimulation by G α s-GTP γ S and forskolin. Surprisingly, the results indicated increased basal AC activity and enhancement of its sensitivity to Gs stimulation (**Figure 2**). We are currently in process of measuring AC activity across different brain regions. We further cultured primary neurons derived from NF1flx/flx mice and achieved deletion of NF1 by infecting differentiated cultures with AAV carrying Cre recombinase (Task 2 A and B). We used these cultures along with uninfected controls to measure cAMP production both basally and in response to challenge with morphine or forskolin. In accordance with the results obtained in the whole brains, we did not detect significant changes in cAMP responses (**Figure 3**). In each case we confirmed NF1 deletion by Western blotting and found the protein levels substantially reduced relative to control cells.

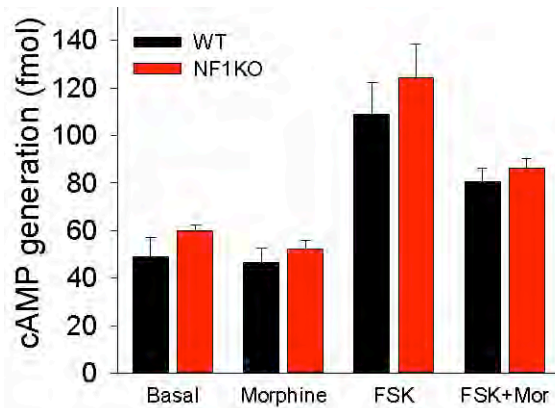


Figure 1. Effect of NF1 elimination on cAMP levels in different brain regions.

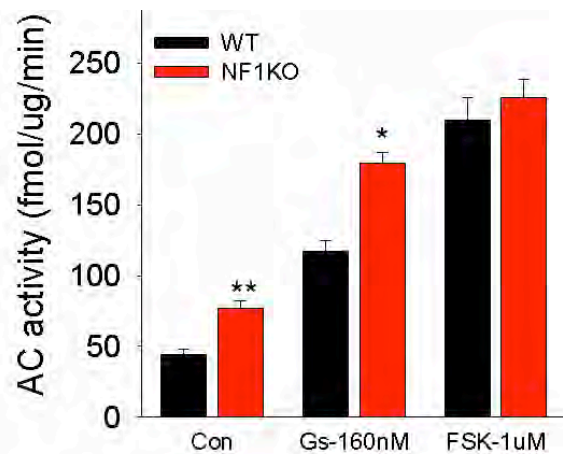


Figure 2. Activity of adenylyl cyclase (AC) in the striatal membrane preparations when stimulated by either Gs or forskolin (FSK) in comparison between wild-type (WT) and NF1 knockout (NF1 KO) tissues.

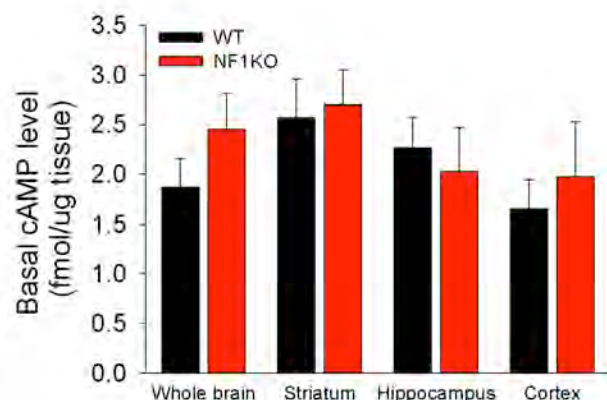


Figure 3. Effect of NF1 ablation on cAMP concentration in striatal cultures.

We have further obtained MEF cells with NF1 deletion and studied cAMP regulation in this model (Task 1E). Using this model, we performed the same studies as with primary cultures examining AC activity. In this MEF model, in agreement with the published studies, we have observed a significant decrease in AC activity, both basally and stimulated by Gs-GTP or FSK (**Figure 4**). While many of the experiments still remain to be performed, analysis of the data thus far strongly suggests that regulation of cAMP signaling may be: (1) cell specific and (2) sensitive to developmental timeframe of inactivation. The second point may be specifically relevant to pathophysiology of neurofibromatosis as it is increasingly viewed as a neurodevelopmental disorder. Therefore, going forward we would like to switch from using pan-neuronal tamoxifen inducible model to a model where NF1 is eliminated in specific populations (striatal medium spiny neurons) earlier in development. In line with this goal, we began generating another mouse model, this time containing non-inducible but conditional elimination of NF1 specifically in the striatum. This was achieved by crossing the NF1^{flx/flx} mice with Rgs9-Cre driver line. Characterization of cAMP signaling in this model is planned to be performed in the next year.

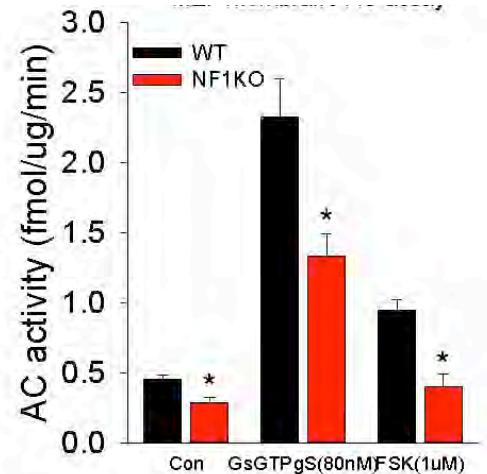


Figure 4. Activity of adenylyl cyclase (AC) in the membrane preparations from MEF cells when stimulated by either Gs or forskolin (FSK) in comparison between wild-type (WT) and NF1 knockout (NF1 KO) cells.

Aim 2 is to understand the role of NF1 in transducing GPCR to the activation of MAPK signaling cascade.

Although the experiments in this direction were not planned until year 2 and year 3 of the project, we did initiate these studies and achieved a substantial progress much ahead of the schedule.

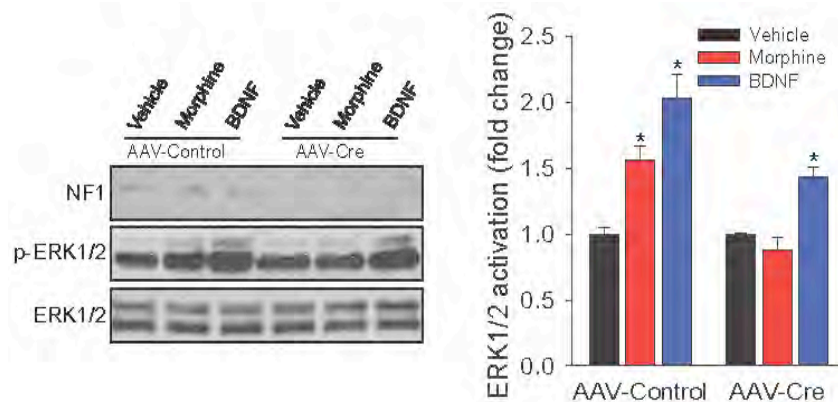


Figure 5. Effect of NF1 deletion in cultured striatal neurons on MAPK signaling. Cultured neurons from NF1^{flx/flx} mice were infected with either AAV-Cre deleting NF1 or control AAV. Neurons were stimulated with morphine or BDNF.

Using primary striatal neurons obtained from conditional NF1^{flx/flx} we analyzed the role of NF1 in controlling morphine-induced changes in Ras activation and regulation of its signaling to downstream protein kinases (Task 3). We infected neurons with the AAV virus encoding Cre-recombinase achieving efficient NF1 elimination (**Figure 5**).

In parallel, neurons were infected with a control AAV virus.

We found that application of morphine to control cultures indeed induces increase in ERK phosphorylation.

In contrast, this increase in ERK activation was lost upon NF1 knockdown. Interestingly, the effects of BDNF on regulation of ERK remained the same suggesting that NF1 is specifically involved in controlling opioid receptor signaling. Studies are ongoing to examine activation status of the Akt-mTOR pathway also regulated by Ras. In a complementary set of experiments we have examined Ras activation in primary striatal cultures upon NF1 inactivation. These studies were done in collaboration with Dr. Ryohei Yasuda in neighboring Max Planck Institute for Neuroscience (**Figure 6**). In these experiments we used FLIM-FRET reporters for Ras activation transfected into striatal neurons. We found that application of morphine to cultures resulted in robust activation of Ras, whereas inactivation of NF1 led to complete loss of morphine-induced Ras activation. These results are consistent with the key role of NF1 in transmitting the signal from opioid receptors to Ras.

In order to understand the mechanistic basis of G protein mediated regulation of NF1, we performed biochemical studies examining the effect of purified G $\beta\gamma$ on the GAP activity of NF1 (Task 2). We successfully setup Ras GTPase assays in a single turnover format and used them to study kinetics of GTP hydrolysis by Ras in the absence or presence of NF1. Addition of the NF1 fragment containing the GAP-related domain (GRD)/Sec14/PH module greatly accelerated GTP hydrolysis on N-Ras; from $0.015 \pm 0.003 \text{ min}^{-1}$ to $0.215 \pm 0.025 \text{ min}^{-1}$ (**Figure 7**). The G $\beta\gamma$ significantly inhibited the NF1's GAP activity but

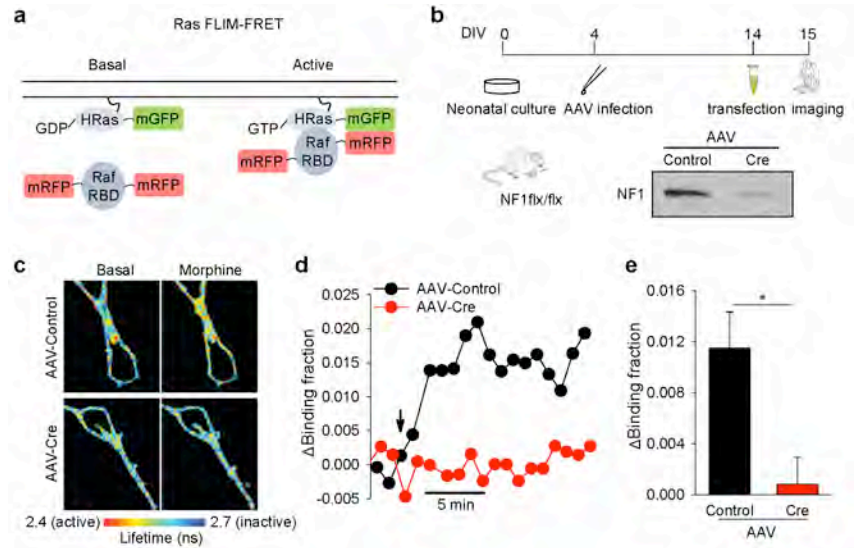


Figure 6. Effect of NF1 deletion in cultured striatal neurons on Ras activation. **A**, Schematics of Ras FRET sensors. **B**, Experimental outlines of Ras FLIM-FRET assay. *Insert*, Significant reduction of NF1 protein level after AAV-Cre infection. **C**, Representative fluorescence lifetime images before and after the application of morphine. **D**, Representative traces of changes in binding fraction in response to morphine. **E**, Quantification of the peak changes in binding fraction before and after morphine stimulation. * $p < 0.05$

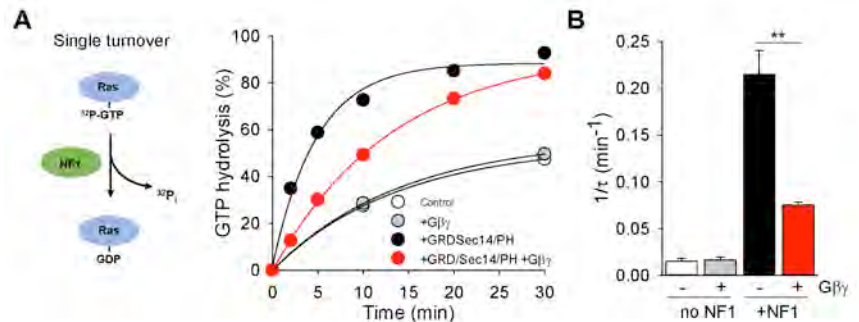


Figure 7. G $\beta\gamma$ inhibits GTPase accelerating protein (GAP) activity of NF1. **A**, *Left*, Diagram of single turnover principle of Ras GTP hydrolysis assay. *Right*, Effects of recombinant G $\beta\gamma$ on NF1 fragment (GRD/Sec14/PH)-stimulated GTPase activity of N-Ras. **B**, Quantification of the effect of G $\beta\gamma$ on GTPase activity of NF1.

had no effect on the rate of basal GTP hydrolysis by Ras in the absence of NF1. These results indicate that opioid receptors transduce signal to Ras activation via inhibiting NF1 GAP activity by binding to G protein $\beta\gamma$ subunits.

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

Dr. Keqiang Xie, a postdoctoral fellow on the project presented the results obtained in these studies at Gordon Conference on G protein and phosphorylation mediated networks held in 2014 in Mane and at Gordon Conference on Molecular Pharmacology held in California in 2015. Both conferences are top venues in the field. Dr. Xie was selected for an oral presentation at the former conference, attesting the significance of the findings for the field. His participation in the conference proved valuable for his career development.

The project also provided an opportunity for an undergraduate student Maxwell A. Kassel from Florida Atlantic University to perform research internship in our laboratory at Scripps. Mr. Kassel learned modern techniques used in biomedical research and current state of signal transduction research on molecular basis of neurofibromatosis.

How were the results disseminated to communities of interest?

In addition to presentations to the immediate field of specialists by Dr. Xie mentioned above, Dr. Martemyanov disseminated the key results of the study to broader scientific community by delivering public talks at National Physics Laboratory in London, UK; Mount Sinai School of Medicine in New York and University of Texas in Houston.

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

We will continue to follow the plan outlined in the Statement of Work. Specifically, we plan to continue studying mouse models of neurofibromatosis where NF1 is specifically eliminated in the striatum (Nf1^{flx/flx}:RGS9-Cre). Plans include examining the role of NF1 in regulation of signaling to cAMP and MAPK pathways, as detailed in the proposal. Furthermore, we plan to expand on understanding the biochemical mechanisms of G $\beta\gamma$ -NF1 interaction and its implications for the regulation of Ras catalytic activity. We are also in the middle of summarizing the results reported above for publication and hope to be able to submit the manuscript during the next period.

- 4. 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:

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

We think that our findings have a significant impact on our understanding of the mechanisms of cellular signaling. The main result of this study is the demonstration of a novel signaling mechanism for transmitting GPCRs activation to positive regulation of kinase signaling networks. On the basis of our findings, we propose that NF1 is a direct G protein effector. Inhibition of its Ras GAP activity by the G $\beta\gamma$ subunits released upon activation of opioid receptors in the striatal neurons results in the activation of small GTPase Ras. In addition to advancing basic understanding of cellular signaling, these findings have implications on design of novel therapeutic strategies for the treatment of neurofibromatosis. The loss of the GPCR-mediated activation of Ras in the striatal neurons changes the current paradigm that largely focuses on the upregulation of Ras signaling as a primary consequence of NF1 loss and provides a theoretical basis for Ras inhibition as therapeutic strategy for neurofibromatosis treatment. While our results confirm the well-documented increase in the basal levels of active Ras associated with NF1 dysfunction, they additionally reveal that the signal-regulated Ras activation (e.g. through GPCR activation) is abolished. Thus, insufficiency of signaling through Ras may contribute to some of the neuropsychiatric manifestations seen in type 1 neurofibromatosis. From this perspective, NF1-mediated opioid signaling to Ras in the striatum may prove helpful for understanding related aspects of the disease, such as procedural learning difficulties and neuropathic pain, as well as for exploring opioid receptors as possible therapeutic targets for neurofibromatosis.

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:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

We did not experience any unforeseen challenges or problems.

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

We do not anticipate problems or delays.

Changes that had a significant impact on expenditures

We did not have any changes with impact on expenditures.

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

Not applicable.

Significant changes in use or care of vertebrate animals

Not applicable.

Significant changes in use of biohazards and/or select agents

No significant changes.

- 6. PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

Publications, conference papers, and presentations

Report only the major publication(s) resulting from the work under this award.

Journal publications.

Nothing to report.

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

Nothing to report

Other publications, conference papers and presentations.

Dr. Martemyanov presented some of the results stemming from this support during the following invited lectures:

09.2014	Max Planck Institute for Neuroscience, Jupiter, FL
09.2014	National Physics Laboratory, London, UK
09.2014	Department of Genetics, Université Pierre et Marie Curie, CNRS, Paris, France
10.2014	External Seminar Series, the Scripps Research Institute, La Jolla, CA
11.2014	Department of Neurology, Mount Sinai Medical School, New York, NY
02.2015	Department of Integrative biology and Pharmacology, University of Texas, Houston, TX

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:	Kirill Martemyanov
Project Role:	Associate Professor
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1 calendar month
Contribution to Project:	Dr. Martemyanov participated in design and analysis of all experiments as well as securing necessary regulatory permits.
Funding Support:	DOD, Institutional Funds and NIH
Name:	Keqiang Xie
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	9 calendar months
Contribution to Project:	Dr. Xie performed most of the experiments described in this report including measurements of cAMP and MAPK signaling
Funding Support:	DOD, Institutional Funds and NIH
Name:	Natalia Martemyanova
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3 calendar months
Contribution to Project:	Ms. Martemyanova generated mouse models of NF1 as detailed in the proposal.
Funding Support:	NIH
Name:	Maxwell Kassel
Project Role:	Undergraduate Intern
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1 calendar month
Contribution to Project:	Mr. Kassel provided technical help performing some of the experiments.
Funding Support:	Institutional Funds

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

K02 Career Development Grant - DA026405 National Institute on Drug Abuse Grants Officer: Paul Hillary (PI: Martemyanov) This grant was successfully renewed.	04/01/15-03/31/20 \$112,500 301-435-1887	0 calendar months ph44X@nih.gov
R01 DA036596-01 National Institutes of Health Grants Officer: Paul Hillery (PI: Martemyanov) This grant was listed as pending and has been awarded.	09/15/14-07/31/19 \$250,000 301-435-1887	3.0 calendar months ph44x@nih.gov

What other organizations were involved as partners?

Max Planck Florida Institute for Neuroscience
One Max Planck Way
Jupiter, FL 33458

Collaboration

We have collaborated with Dr. Yasuda to help measure the effect of NF1 ablation on Ras activation in striatal neurons.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

- 9. APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.