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14. ABSTRACT This project examines pathogenic NF1 missense mutations. We have modeled these mutations in Drosophila transgenes in order to investigate their molecular and cellular consequences in vivo. We have examined their ability to rescue Ras signaling in neurons in Drosophila deficient for endogenous NF1. Further, we have assessed their ability to rescue NF1 mutant developmental and adult-specific defects, including organismal growth, climbing ability and sleep/circadian behaviors. These assays have identified previously unexplored regions of the neurofibromin protein that are required for correct function in Drosophila. We will use these insights for subsequent CRISPR/Cas9 gene editing of human induced pluripotent stem cells (iPSCs) to model specific missense mutations of interest and examine their functional consequences in derived neurons.					
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INTRODUCTION

Neurofibromatosis type 1 is a common disorder with autosomal-dominant inheritance affecting 1 in ~3,500 (1). Symptoms include near universal benign but often disfiguring peripheral nerve associated tumors known as neurofibromas, as well as malignant tumors, including usually fatal peripheral nerve sheath tumors (2). Non-tumor symptoms include skeletal and vascular abnormalities, pigmentation defects, reduced overall growth and cognitive deficits, the latter seen in 50-70% of children with NF1 (3,4). In part reflecting higher rates of vascular defects and cancer, the life expectancy of NF1 patients is reduced by 15-20 years (5). No effective therapy for any NF1 symptom yet exists. The *NF1* gene encodes the protein neurofibromin, a 320 kDa protein whose only widely accepted function is to serve as a Ras GTPase Activating Protein (RasGAP) for H-, K-, N-Ras and R-Ras1, 2 and 3 (6). RasGAPs promote the conversion of active Ras-GTP into inactive Ras-GDP by stimulating the low intrinsic rate of Ras-GTP hydrolysis (7). In patients with *NF1*-driven tumors, targeting Ras pathway components such as MEK or ERK is a reasonable therapeutic option. However, given that Ras is subject to highly robust regulation (8,9), this may explain why despite considerable effort, no effective therapy for Ras-driven cancers has yet emerged. Even so, chronically blocking Ras may never be an appropriate strategy for treating the many serious but non-life threatening symptoms of NF1, such as cognitive problems in children. This has motivated our work to find the precise pathways responsible for NF1 symptoms, in the hope that this will allow better-targeted therapy. The mutational spectrum of *NF1* ranges from single nucleotide substitutions to large deletions. To date, 2571 different *NF1* mutations have been reported in the Human Gene Mutation Database. Pathogenic missense mutations occur throughout *NF1*, both within and outside of the centrally located region encoding the GAP-related domain (GRD) in neurofibromin - the defined RasGAP catalytic domain. This suggests that parts of the highly conserved neurofibromin outside of the central GRD are also essential for its function. Clinical studies so far have identified just two hypomorphic (incomplete loss-of-function) alleles that appear to correlate with specific NF1 symptoms - a three bp deletion resulting in loss of a single residue (M991Δ) and missense mutations of R1809 - each of which are outside the central Ras-GAP domain. Although patients with these mutations fulfill diagnostic criteria for NF1, including pigmentation defects and cognitive deficits, they show a complete absence of cutaneous or plexiform neurofibromas [10,11]. We believe that additional hypomorphic alleles may also contribute to the highly variable expressivity of NF1. Determining genotype-phenotype correlations has been hampered due to complexity of NF1 disease phenotypes in different tissues, age and sex dependency of symptoms, impact of environmental factors and genetic heterogeneity, suggesting the role of modifier genes [12]. This work aims to shed light on this issue by studying the functional consequences of selected *NF1* missense mutations in both fruit fly (*Drosophila*) and human systems. We hypothesize that studying these mutants and correlating their genotype with resulting molecular and cellular phenotypes may lead to a better understanding of the function of neurofibromin protein and pathways critical for NF1 symptom development. Our approach has been to firstly model NF1 patient mutations in *Drosophila* in order to assess their function, followed by selecting the most informative of these to develop isogenic human iPSC models using CRISPR/Cas9 gene editing. These *NF1* mutant iPSC lines will then be used to derive neurons (and other cell types pertinent to NF1 e.g. Schwann cells) with which to look for

molecular/cellular correlates with different *NF1* genotypes. We hypothesize that the combination of these approaches may reveal important novel insights into neurofibromin function that may be important for disease outcome.

KEYWORDS

Neurofibromatosis type-1, missense mutations, genotype-phenotype correlations, *Drosophila*, neurons, Ras signaling, induced pluripotent stem cells

ACCOMPLISHMENTS

What were the major goals of the project:

During this reporting period, the SOW focused on Aim 1: Test pathogenic *NF1* mutations for altered function in a *Drosophila* model

Aim 1: Test pathogenic *NF1* mutations for altered function in a *Drosophila* model

- a) We will generate a series of *Drosophila dNf1* transgenic lines bearing conserved pathogenic mutations from NF1 patients to enable tissue-specific expression and assessment of function *in vivo*.
- b) We will test *Drosophila* neurofibromin bearing conserved patient mutations for altered Ras signaling.
- c) We will functionally test *Drosophila* neurofibromin mutants for abnormal synaptic development.
- d) We will test ability of *dNf1* missense mutants to rescue mutant cognitive defects.

Specific Aim 1: To test pathogenic <i>NF1</i> mutations for altered function in a <i>Drosophila</i> model	Predicted Timeline	Actual Timeline
Major Task 1: Generate series of mutant <i>Drosophila dNf1</i> transgenic lines	Months	Months
Mutagenesis of <i>dNf1</i> cDNA, DNA sequencing and subcloning of transgenic constructs	1-4	1-6
Injection of mutant <i>dNf1</i> transgenic constructs, setting up fly stocks, crossing into appropriate genetic backgrounds and expansion of stocks	4-7	7-9
Major Task 2: Test transgenes for Ras signaling rescue		

Setting up crosses	7-8	10-12
Preparation of lysates and western blot analyses		
Major Task 3: Test transgenes for rescue of abnormal synaptic development		
Setting up crosses, dissections, staining and confocal imaging	7-10	11-12
Analysis of images, statistical analysis	11-12	-
Major Task 4: Test transgenes in <i>Drosophila</i> cognitive assays		
Setting up crosses and assaying associative learning and memory	7-12	11-12
Statistical analysis	10-12	-

What was accomplished under these goals?

a) Generate *Drosophila* NF1 transgenic lines bearing conserved pathogenic missense mutations.

To test whether conserved missense mutations disrupt *dNf1* function *in vivo*, we selected 43 from 99 disease-associated missense mutations reported in the literature that are in residues conserved between human and *Drosophila* neurofibromin (shown in Figure 1). Pathogenic mutations within the GAP-related domain (GRD) have been well studied and are known/predicted to affect Ras binding or GAP activity (21). Therefore, as a control, we modeled a known GAP-deficient mutation (R1276P) as well as several missense mutations from NF1 patients that have recently been described as having an inability to bind to the Spred1 protein (13,14). However, we have concentrated our efforts on generating *dNf1* transgenes that bear disease-associated mutations in conserved protein segments outside of the GRD. We have focused on regions that contain clusters of mutations, suggesting they may harbor novel functions.

Mutations were generated using the Q5 Site-Directed Mutagenesis kit (NEB) with specifically designed primers. To minimize the chance of inadvertently introducing extraneous mutations during the PCR-based strategy, we used relatively small fragments of the *dNf1* cDNA for the mutagenesis procedure and subsequently reintroduced the successful mutants (determined by sequencing) in these intermediate clones back into the full-length *dNf1* cDNA context. Finally the mutant *dNf1* cDNAs were subcloned into a *UAS*-transgenic vector with an in-frame eGFP tag at their C-terminus. This permitted the expression of each transgene to be assessed using anti-GFP antibodies and for possible subcellular localization studies. All of the *UAS-dNf1* transgenic constructs were prepared using Qiagen Midi-prep plasmid DNA kits for making transgenic flies. We utilized the services of Rainbow Transgenic Inc. for fly embryo

injections. Transgenes were integrated into the fly genome using the *phiC31 attB40* site on chromosome 2 to give consistent protein expression under the control of the *Gal4/UAS* system, as well as to facilitate crossing into the *dNf1* null mutant background. We anticipated that tagging would not interfere with neurofibromin function based upon previous experiments using transgenic dNf1-SBP tagged at its C-terminus (Walker, unpublished data).

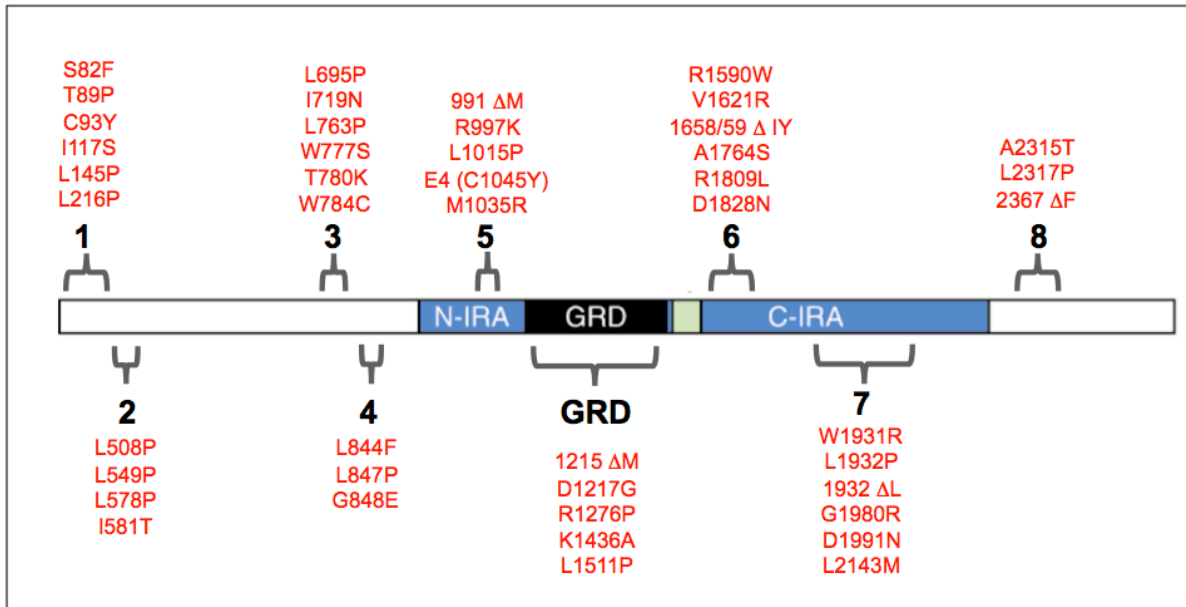


Figure 1. Location of NF1 patient missense mutations conserved in *Drosophila*. These were selected from a survey of published and unpublished missense mutations from NF1 patients that are predicted to result in amino acid substitutions or small deletions. Missense mutations near splice sites, or mutations predicted to create novel splice sites were excluded from consideration. Residues conserved between human and fly neurofibromin are indicated and clustered into regions (1-8). These mutations will be made in dNf1 cDNA using the Q5 mutagenesis kit and subsequently used to generate *UAS-dNf1* transgenic fly lines. GRD: GAP-related domain; IRA: regions with homology to yeast IRA (Inhibitory Regulator of the RAS-cAMP pathway) proteins; Green box: Sec14-like and pleckstrin homology (PH)-like domain. The position of the EMS-generated hypomorphic allele dNf1^{E4} (Y1045C) is also indicated.

After screening for successful transformants we have established two independent lines for each transgene. These were balanced using the CyO balancer chromosome to maintain stably integrated stocks. Each transgenic line was then crossed into the *dNf1*^{E1} mutant background using standard techniques. The *UAS-dNf1* mutant transgenes were crossed to a different *Gal4* driver lines enabling tissue/cell-specific expression. These included the *Ras2-Gal4* that drives expression in a subset of neurons and is sufficient for rescue of *dNf1* mutant phenotypes including organismal growth and cognitive deficits and the *n-syb-Gal4* driver, which gives pan-neuronal expression.

Firstly, we determined whether the transgenic lines correctly expressed full-length eGFP-tagged neurofibromin. It is possible that certain missense mutations result in misfolded/ unstable protein or that transgenic constructs were corrupted during the mutagenesis process and therefore result in truncated proteins. Each *UAS-dNf1* transgene was crossed to the *Ras2-Gal4* driver line, the adult progeny were collected and decapitated. Protein lysates were made and submitted to SDS-PAGE and transferred to nitrocellulose membranes. Expression of each mutant transgenic neurofibromin was verified using western blotting with anti-eGFP antibodies (Figure 2). Our preliminary results suggest that the majority of the transgenes express at comparable levels. However, we need to repeat these initial western blots to ensure that the flies collected were of the correct genotype and normalize protein loading.

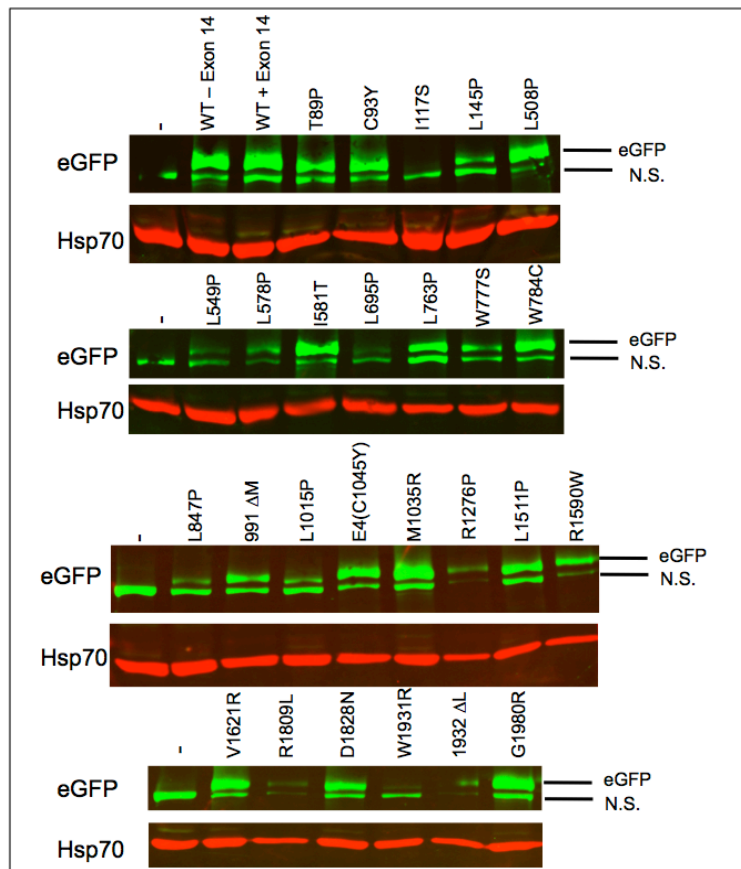


Figure 2. Expression of selected *UAS-dNf1* transgenes. Mutant transgenes were crossed to the *Ras2-Gal4* line to drive expression in neurons. Heads from the resulting progeny were removed from adult flies, crushed in lysis buffer and subjected to SDS-PAGE and western blotting using anti-eGFP. Anti-Hsp70 was used as a loading control. In some cases (I117S) it is possible that the incorrect flies were collected for lysate preparation. We are in the process of repeating these western blots with the entire collection of mutant transgenes. The first lane of each blot contains lysate from a line of flies that contain the *Ras2-Gal4* driver but no transgene. The lower band in these anti-eGFP blots is a non-specific cross-reacting protein (N.S)

b) Test *Drosophila* neurofibromin bearing conserved *NF1* patient mutations in Ras signaling.

Our next step was to assess the effect of mutations on Ras-GAP activity. We performed western blot analysis of phospho-ERK (pERK) levels. *dNf1* null mutant flies have elevated pERK levels (3-4-fold compared to wild type) in both larval CNS and adult brain extracts (15). Aberrant up-regulation of pERK signaling can be restored to normal by reintroducing a neuronally-driven *UAS-dNf1* wild-type transgene. Lysates were prepared from adult fly heads expressing transgenic mutant proteins driven using *Ras2-Gal4* neuronal driver. These were assayed for

pERK levels compared to wild type flies, as well as *dNf1* mutants with and without wild type transgenic *dNf1* expression (Figure 3). As predicted the R1276P mutation in the GRD abolished RasGAP activity (mutation of a catalytically essential arginine residue) and is unable to rescue pERK levels. However, we were surprised to find that several missense mutations outside of the GRD also failed to fully restore pERK signaling. Notably mutations in Regions 5 (L1015P, C1045Y and M1035R) and 6/7 (R1809L, D1828N, W1931R) did not rescue the elevated pERK levels. We hypothesize that they may recruit other factors required for correct Ras-GAP activity or affect subcellular localization such that the mutant neurofibromin is inappropriately placed in the neuron.

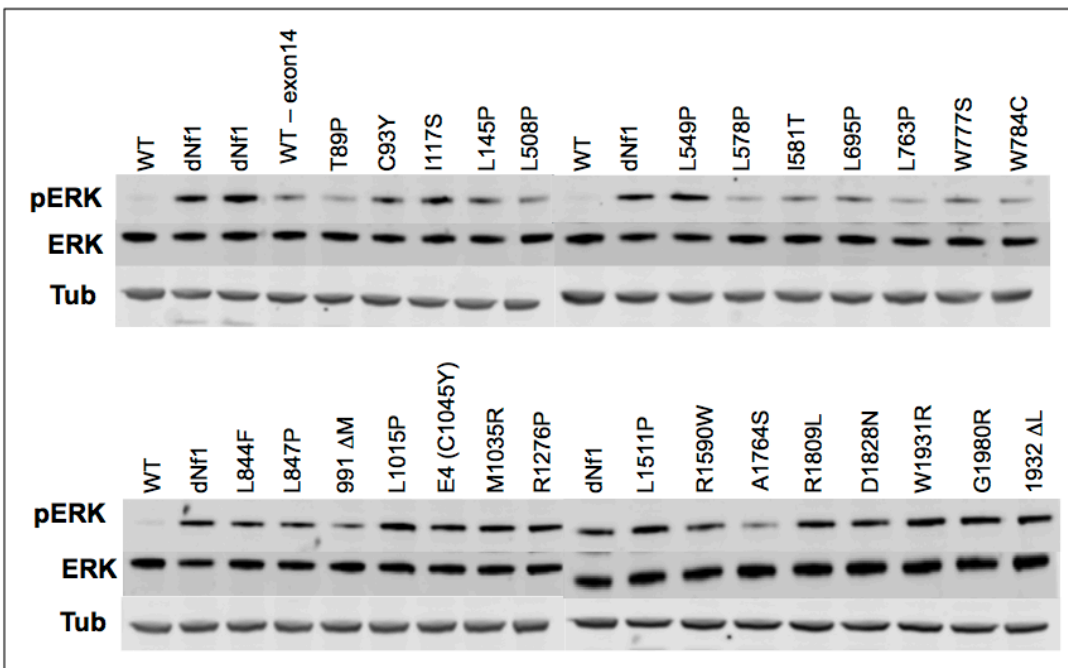


Figure 3. Testing ability of mutant *dNf1* transgenes to rescue Ras signaling in *dNf1* mutant flies. Western blotting was used to show that *dNf1* mutant flies have enhanced signaling through the MEK/ERK pathway in adult neurons compared to wild type flies (WT). This can be rescued by expressing *UAS-dNf1* in neurons using the *Ras2-Gal4* driver (WT-exon 14). Similarly, the ability of *UAS-dNf1* bearing missense mutations found in NF1 patients to rescue elevated pERK levels was tested. Activated ERK (pERK), total ERK (ERK) and beta-tubulin – loading control. We have quantified pERK levels using imaging software (data not shown).

c) Functional testing of *Drosophila* neurofibromin mutants for synaptic development.

We had intended to test our mutant transgenes for their potential to rescue a recently described larval neuromuscular junction (NMJ) overgrowth phenotype of *dNf1* mutants (17,18). However, for reasons given in **Changes/Problems** below (Page 18), these experiments had to be delayed, although are now underway with new personnel.

d) Test ability of *Drosophila* neurofibromin missense mutants to rescue *dNf1* cognitive defects.

We also proposed to test the collection of *dNf1* mutant transgenes for their ability to rescue the *dNf1* mutant cognitive phenotype in olfactory *associative* learning assays (16). Due to advice from colleagues at Brandeis University, we decided to adopt a different, improved experimental set up for this assays. As detailed in **Changes/Problems** below (Page 18), we have now obtained this new apparatus and are poised to conduct these experiments.

Alternative Assays:

Due to the temporary delay in being able to conduct the NMJ and cognitive assays (discussed in Changes/Problems below), we decided to test our transgenes in three other functional *in vivo* assays to determine whether they can compensate for loss of endogenous *dNf1*. The first of these is an organismal size assay (developmental growth) that we have used with previous success (ref), while climbing and sleep behavioral assays have been used by other groups.

(i) Test ability of *Drosophila* neurofibromin missense mutants to rescue *dNf1* growth defects

We have previously shown that *dNf1* controls systemic growth non-cell-autonomously by inactivating neuronal Ras (15). Full-length *UAS-dNf1* transgenes are able to rescue this phenotype when driven during larval development either pan-neuronally or in specific subsets of neurons. Further, this small size phenotype can be phenocopied by expressing transgenically activated Ras and Raf in neurons during development. To date, rescue of the small size *dNf1* phenotype correlates with rescue of Ras/pERK signaling.

We therefore tested our collection of mutant transgenes for the developmental rescue of organismal size. Each transgene was expressed in neurons of *dNf1* mutants using the *Ras2-Gal4* driver and pupal length measurements made to assess the effects on growth (Figure 4). As expected we found that the R1276P mutation (Ras-GAP deficient) failed to rescue the pupal size of *dNF1* mutants. Additionally, other mutations within the GRD (M1215Δ and L1511P), each of which have been shown to have an inability to bind the Spred1 protein, also failed to rescue the *dNf1* growth phenotype. Other missense mutations in Region 5 were similarly defective in their rescuing potential, while those in Region 2 were partially defective. We note that the mutations in Region 5 were also shown not to rescue the Ras/pERK phenotype (Figure 3). However, the mutations in Region 2 while only partially rescuing the pupal size, did not display an ability to rescue pERK levels.

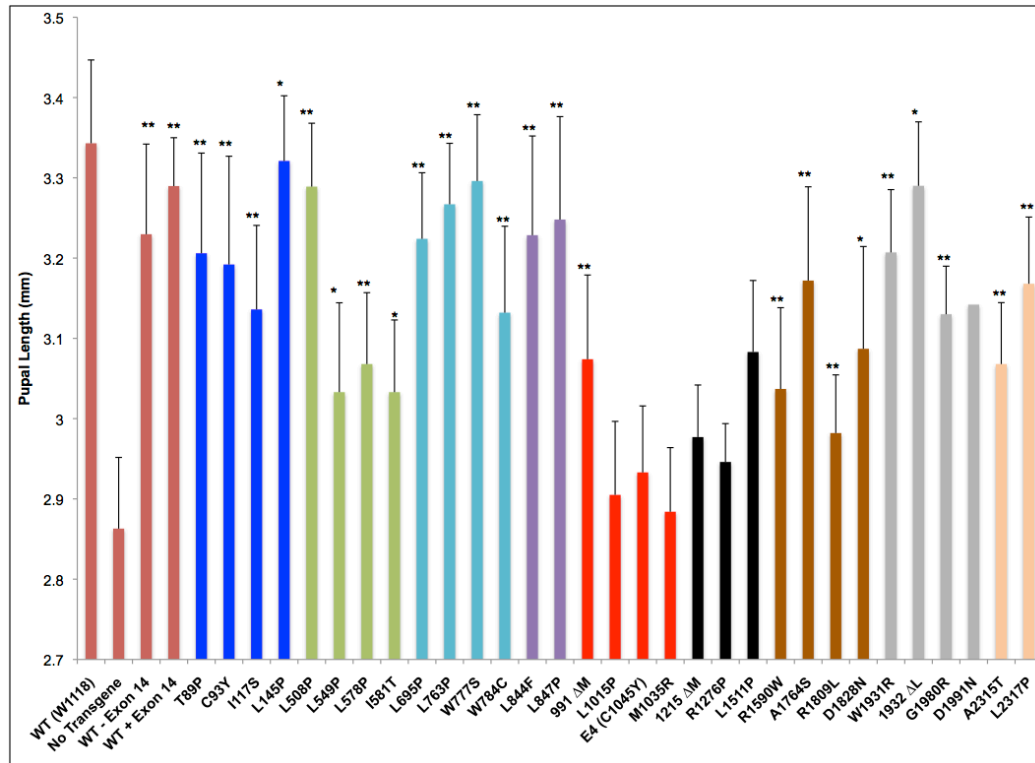


Figure 4. Testing transgenes for ability to rescue *dNf1* mutant larval growth defect. Female pupal lengths (mm) for the indicated genotypes. Control measurements include wild-type (w^{1118}) and *Ras2-Gal4/+; dNf1^{E1}/dNf1^{E2}* (“No Transgene”). All other genotypes indicate the mutation within the *UAS-dNf1* transgene being drive by the *Ras2-Gal4* driver in the trans-heterozygous *dNf1^{E1}/dNf1^{E2}* mutant background. Colors refer to the different regions of neurofibromin. Error bars denote standard deviations (based on measurements of at least 20 pupae), and * and ** *p*-values < 0.01 and < 0.001, respectively.

(ii) Test ability of *Drosophila* neurofibromin missense mutants to rescue *dNf1* climbing defects

dNf1 mutant flies display defective climbing behavior in a negative geotaxis assay (39). Although we have shown that both size and climbing phenotypes are dependent on the RasGAP function of dNF1 ((14) and our unpublished data), it is possible that *dNf1* mutations that do not affect Ras signaling activity could still fail to rescue these defects (e.g. due to altered protein interactions). We therefore tested our transgenes driven pan-neuronally for their ability to rescue this phenotype.

Vials of equal numbers of male flies (aged-matched) were set up in front of a video camera. Flies were knocked down by administering a rapid tap to the vials and their subsequent motion was filmed and later analyzed (Figure 5). The fraction of flies that were able to climb above a defined line within a 3 second window after having been tapped to the ground was assessed.



We found that missense mutations within the GRD are unable to rescue the climbing defect of *dNf1* mutants, confirming that Ras-GAP activity is likely required for correct behavior in this assay. While the E4 mutant was similarly defective, the other Region 5 mutants appeared to be able to rescue this phenotype. However, we also noted that mutations in Region 3, several of which are able to restore pERK levels (Figure 3) were also unable to rescue climbing behavior. This intriguing result suggests that residues in this region may also play a role in neurofibromin function in adult neurons.

Figure 5. *dNf1* mutant climbing/locomotor defect. Image of two vials of flies (*dNf1* and WT) after flies have been knocked down.

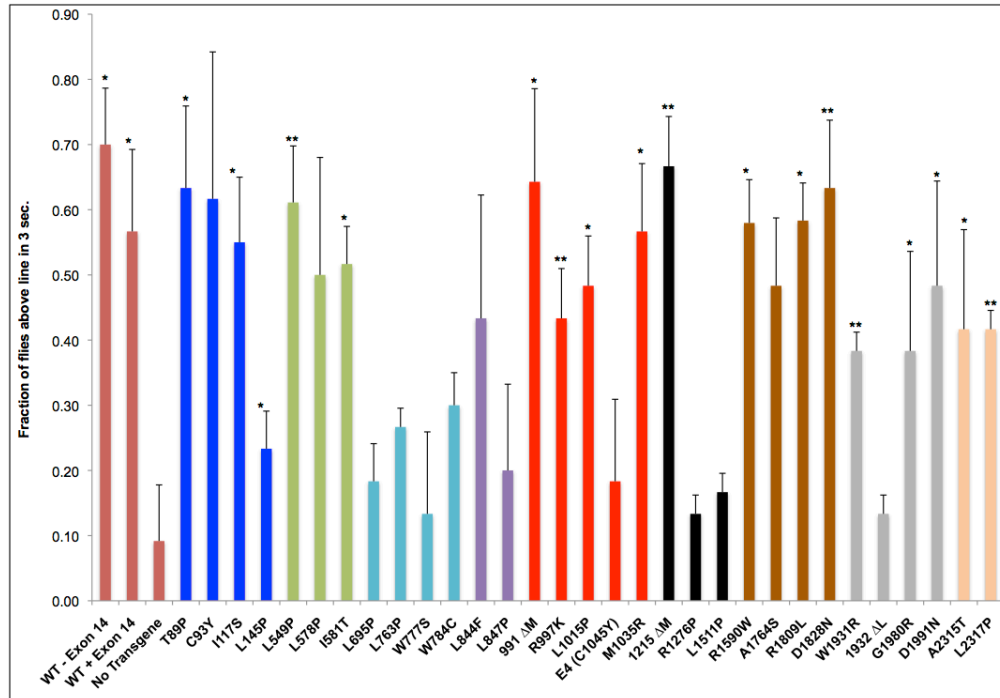


Figure 6. Testing transgenes for ability to rescue the *dNf1* climbing/locomotor defect. Male adult flies of similar age of the indicated genotypes were tested for their ability to climb above a defined height, 3 seconds after being banged to the bottom of a vial. The *dNf1* control measurement (“No Transgene”) is *n-syb-Gal4/+; dNf1^{E1}/dNf1^{E2}*. All other genotypes indicate the mutation within the *UAS-dNf1* transgene being drive by the pan-neuronal *n-syb-Gal4* driver in the trans-heterozygous *dNf1^{E1}/dNf1^{E2}* mutant background. Colors refer to the different regions of

neurofibromin. Error bars denote standard deviations (based on measurements of sets of 20 flies in three independent tests), and * and ** *p*-values < 0.05 and < 0.01, respectively.

(iii) Test ability of *Drosophila* neurofibromin missense mutants to rescue *dNf1* sleep/circadian defects

With the delay in being able to test rescue of cognitive function in *dNf1* mutant flies, we decided to turn our attention to the sleep/circadian deficits of *dNf1* mutants that have recently been described (19). These display a number of complex defects in different aspects of sleep behavior.

We used the TriKinetics *Drosophila* Activity Monitoring (DAM) system to quantify locomotor activity of individually housed flies. Each fly was maintained in a vial with food at one end and a cotton plug at the other in a 12h:12h Light:Dark cycle. 32 of these vials are then kept in one monitor in such a way that an infrared beam passes through the center of each vial. Every time the fly walks and crosses the midline, the beam breaks which is recorded to an attached computer. We examined the locomotor activity of 8 flies per genotype recorded every 30s over a one week period. For each 30s epoch, a fly was defined to be sleeping if it was active for the 2 minutes prior and after, for a total of 4.5 minutes of inactivity.

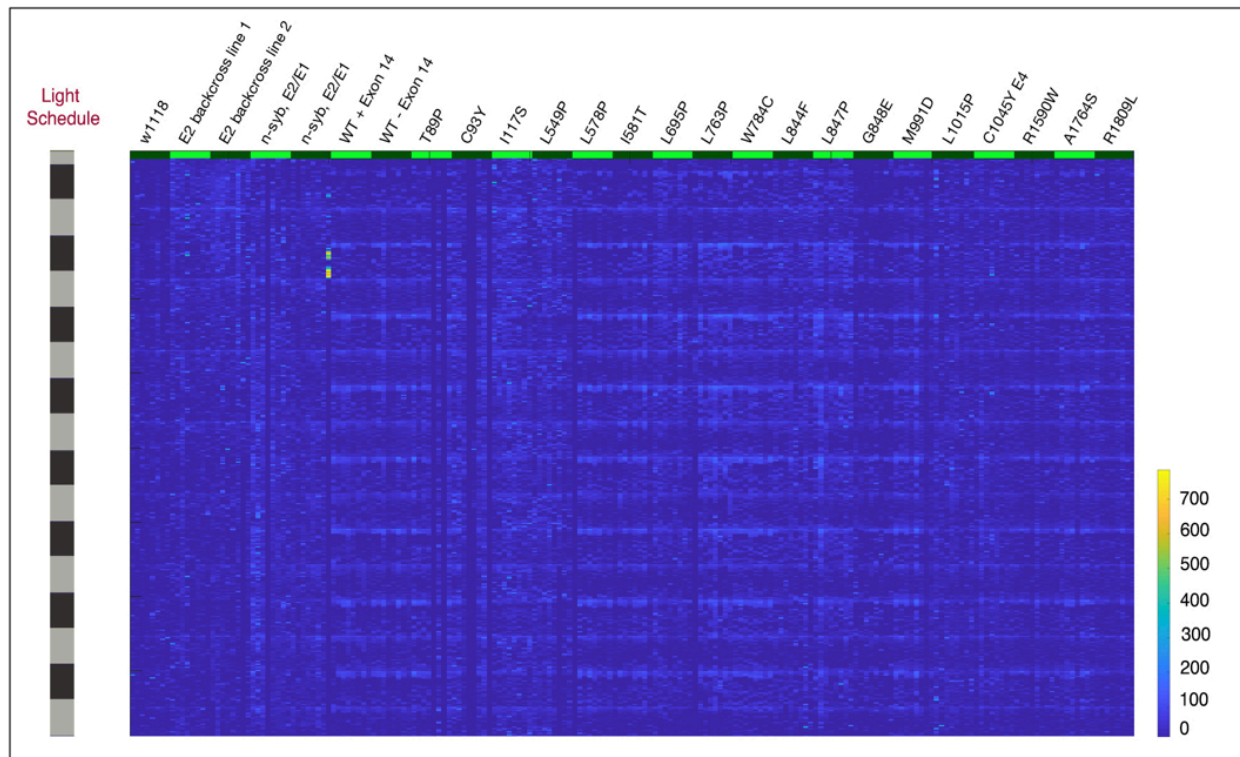


Figure 7: Testing ability of mutant transgenes to rescue defective *dNf1* sleep activity. Heat map for the beam-break counts (a surrogate of locomotor activity) from the DAM system binned into 60 min intervals. Columns correspond to individual flies and rows to time. The gray/black bar to the left indicates the LD12:12 lighting schedule. 8 flies of each genotype were used, and the alternating light and dark green are an aid to the visual distinction of the genotype groups.

Control flies include a wild-type strain (w^{1118}), two $dNf1^{E2}$ mutant lines that have been backcrossed 5 times to the w^{1118} strain and $n\text{-syb-Gal4/+}; dNf1^{E1}/dNf1^{E2}$ (i.e. no transgene). All other flies contain the indicated mutant $UAS\text{-}dNf1$ transgene in the $n\text{-syb-Gal4/+}; dNf1^{E1}/dNf1^{E2}$ background.

The data in Figure 7 indicates that the wild type $UAS\text{-}dNf1$ transgene is able to rescue the increased nocturnal activity of $dNf1$ mutants. Additionally many of the missense mutant transgenes are also able to restore normal sleep/waking activity. However, flies expressing several other mutant transgenes are clearly still defective. The I117S mutant's inability to rescue may be accounted for by its low expression level (Figure 2). However, L549P (Region 2) and L847P (Region 4) are expressed and fail to rescue the activity phenotype. Quantification of the genotypic group-level average activity patterns (Figure 8) and 'normalized sleep' (Figure 9) reveals the complexity of the data that we have collected and we are still analyzing it to examine different aspects of sleep and circadian behaviors

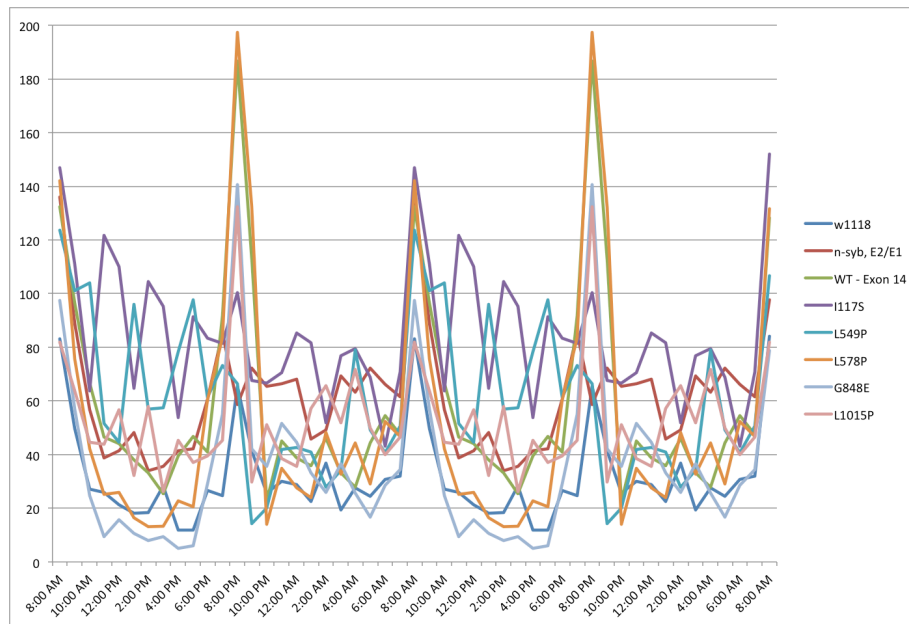


Figure 8: Quantification of the genotypic group-level average activity patterns from the TriKinetics DAM system. Indicated data are for one selected day from lights-on time to the next lights-on time. Data are double plotted (i.e. the same data are plotted twice over) to aid visualization.

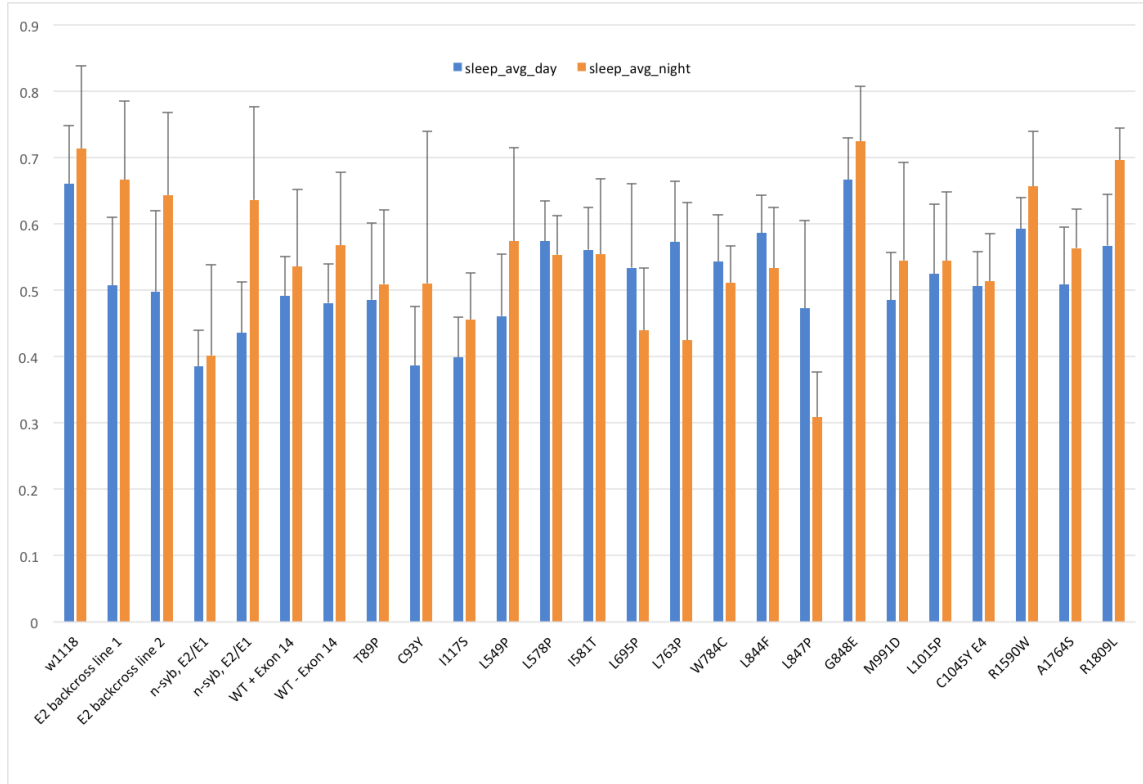


Figure 9: Normalized sleep i.e. the fraction spent sleeping during lights on (blue) and lights off (orange) periods for different genotypes. Each genotype had upto 8 flies. Dead flies were censored for these analyses.

In sum, the four functional *in vivo* assays for neurofibromin (Ras/pERK, organismal growth, climbing behavior and sleep/activity behavior) have allowed us to identify individual mutations or even clusters of mutations outside the GRD in NF1 that result in neurofibromin protein with impaired activity. Previously, we have established that the *dNf1*^{E4} (Y1045C) mutation (Region 5) behaves as a temperature-sensitive hypomorph in another assay (systemic growth) resulting in a partial reduction in organismal size without affecting Ras/pERK signaling (12). We note that so far, two other mutations in this region located next to Y1045C also fail to rescue the *dNf1* size defect and elevated pERK levels. Such clustering of mutations highly suggests that this region of neurofibromin, distinct from the GAP domain, is functionally important (Figure 10). Further, the location of patient mutations in this region is between two potential PKA phosphorylation sites in both human and fly neurofibromin, which could potentially serve a regulatory role. Overall, we hypothesize that these missense mutations may compromise neurofibromin function by reducing its ability to regulate Ras activity, affect protein stability or alter its ability to function in neurons.

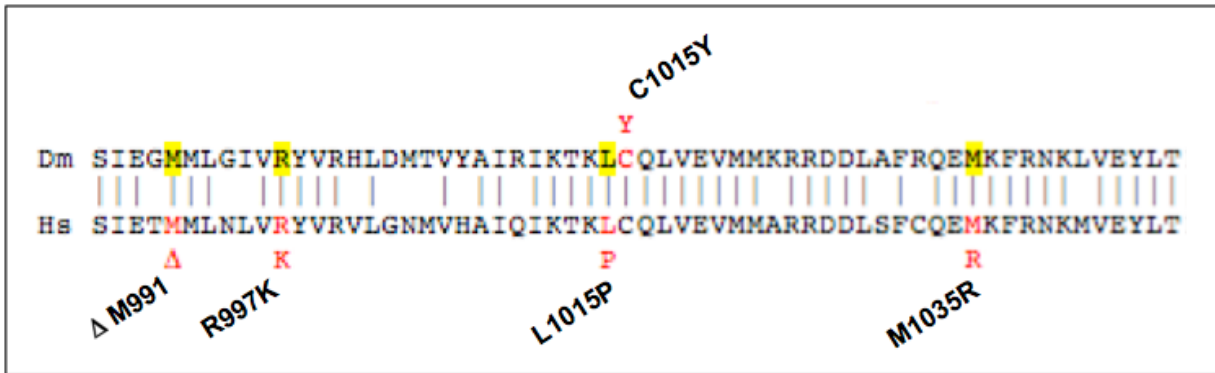


Figure 10. Missense mutations in Region 5. The E4 C1045Y mutation is hypomorphic (partial loss of function) - found in a fly mutagenesis screen to look for new mutations. Since this is in the same region of neurofibromin that is mutated in Nf1 patients (other annotated mutations) - this region is clearly of importance in dNf1 function and will be highlighted for further studies.

At the conclusion of our functional studies (including NMJ development and cognitive assays) we will prioritize mutations for further experiments based upon the following criteria: (i) mutations in similar regions that give the clearest indication of inability to rescue fly *dNf1* phenotypes in functional assays; (ii) mutants that give disparate results in different assays, e.g. fail to rescue cognitive function or NMJ overgrowth, but are able to rescue Ras/ERK signaling.

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

Nothing to Report.

How were the results disseminated to communities of interest?

Invited seminars were given at Brandeis University and the University of Massachusetts, Boston in February and May 2017 respectively. At the recent Children's Tumor Foundation (CTF) conference in Washington DC (June 2017) we initiated a collaboration with Dr. Annette Schenck's laboratory at the Radboud University Medical Center, Netherlands. They have recently reported that flies with RNAi knock down of NF1 show defects in habituation assays. We plan to provide our transgenic flies to the Schenck lab to test in their assays.

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

The next reporting period concentrates on Specific Aim 2 (see below). However, we will also conclude our *Drosophila* experiments from Specific Aim 1. These include testing the few

outstanding missense mutations in Ras signaling and functional assays. We are also in place to test the mutant transgenes in the cognitive assays and neuronal morphology (NMJ) assays (see below in Changes/Problems). We anticipate that these will take an additional two months to complete. Together, the results of these assays will help us to focus on specific missense mutations, which we plan to engineer into hiPSCs using CRISPR/Cas9 for deriving neurons for subsequent functional analyses. This work will be performed in collaboration with Dr. S. Haggarty's laboratory.

Given our interesting results from the *Drosophila* functional assays, at present we have decided to focus our attention on selected NF1 patient missense mutations found in Regions 3 and 5. We hypothesize that these regions are involved in regulating the Ras-GAP activity of neurofibromin, possibly by altering its subcellular localization. We have already started designing specific CRISPR/Cas9 sgRNAs to permit generating the knock in of specific missense mutations. We have also gained familiarity with the iPSC differentiation protocols used by the Haggarty Lab and do not foresee any obvious technical problems with Aim 2.

Aim 2: Generate of isogenic *NF1* iPSC lines using CRISPR/Cas genome editing

a) We will introduce the most informative *NF1* missense mutations validated in Aim 1 using CRISPR/Cas gene editing into human iPSCs to create a panel of otherwise isogenic knock-in cell lines.

b) We will differentiate iPSCs with *NF1* missense mutations into neural crest (NC) progenitors/neural progenitor cell (NPC) lines, from which we ultimately derive neurons as determined by lineage specific markers.

Specific Aim 2: Generation of isogenic <i>NF1</i> iPSC lines using CRISPR/Cas genome editing	Predicted Timeline	Site
Major Task 1: Introduce most informative <i>NF1</i> missense mutations from Aim 1 using CRISPR/Cas gene editing into human iPSCs to create a panel of otherwise isogenic knock-in cell lines.		
Design and preparation of <i>NF1</i> targeting constructs	13-14	Haggarty/Walker Labs (MGH)
CRISPR/Cas gene editing of hiPSCs; transfection, drug selection, picking clones, sequence analysis	15-20	Haggarty/Walker Labs (MGH)
Expansion, validation, karyotyping, analysis of appropriate markers	21-22	Haggarty Lab (MGH)
Major Task 2: Directed differentiation of iPSCs to neural crest and CNS lineages		
Differentiation protocols to make neurons	23-27	Haggarty Lab (MGH)

IMPACT

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

Nothing to Report

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

CHANGES/PROBLEMS

Changes in approach and reasons for change

Our original proposal included conducting functional tests of the transgenes bearing missense mutations in both synaptic development and cognitive function. *dNf1* mutant larvae display a neuromuscular junction (NMJ) overgrowth phenotype, while *dNf1* mutant adult flies exhibit significant learning defects in olfactory *associative* learning assays (18,17). We have previously demonstrated that the larval NMJ defects and the associative *learning* phenotype of *dNf1* mutants can be rescued by targeted re-expression of dNf1 within subsets of neurons.

However, during the course of the first year, we had to change our approach for these functional assays for several reasons. Firstly, our resident expert in NMJ dissection, staining and analysis left the laboratory. Secondly, on consultation with fellow *Drosophila* researchers at Brandeis University, we learned of a superior experimental set up for cognitive assays over the apparatus we originally proposed to use (Fly Training Machine (CeExplorer Labs Co.)). We therefore decided to purchase custom-made apparatus (designed and produced by Francisco Mello of Brandeis University), which enables multiple genotypes to be tested concurrently and is generally easier to use and generates higher quality data. This equipment was made to order and took several months to build. During this time, we also had to seek out a dedicated temperature-controlled room in which to conduct these sensitive assays, which we have now

accomplished. Together these situations resulted in a delay in setting up the NMJ and cognitive assays, such that we were unable to complete them during the course of the reporting period. However, as detailed below, we have now resolved these issues and still intend to conduct both of these assays as planned.

In the interim, we conducted three other functional assays on our series of mutant transgenes. These were discussed above and include the *dNF1* growth defect (resulting in reduced body size), reduced ability of *dNF1* mutants to climb and defects in sleep/circadian activity of *dNF1*-deficient flies. All these phenotypes reflect a requirement of dNF1 in either larval (growth) or adult (climbing and sleep) neurons and so we deemed adequate replacements of the NMJ and cognitive assays.

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

We intend to conduct the NMJ and cognitive experiments in the next few months. We have now recruited a new Research Technician, formerly of Dr. Davie Van Vactor's lab at Harvard Medical School, who has extensive experience in NMJ dissection and analysis. As discussed above, we now have the improved apparatus for the cognitive experiments (Figure 11) in a dedicated temperature-controlled room. We have also taken the opportunity to visit the labs of Dr. Leslie Griffiths and Dr. Michael Rosbash at Brandeis University and observed their set up

and procedures for the cognitive assays. We believe that we now have everything in place to conduct these two functional assays to conclude our Specific Aim 1.

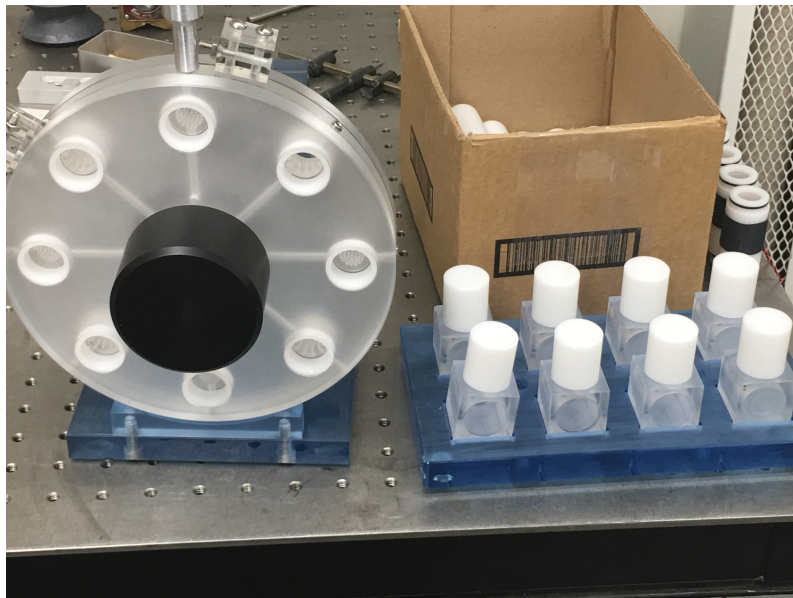


Figure 11. *Drosophila* cognitive testing apparatus. This set up was designed and built by Francisco Mello of Brandeis University. It allows multiple genotypes to be tested at the same time for either aversive or appetitive olfactory learning and memory.

Changes that had a significant impact on expenditures

Nothing to Report

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

Nothing to Report

PRODUCTS

A series of *Drosophila* transgenic

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	<i>James Walker</i>
Project Role:	<i>PI</i>
Nearest person month worked:	<i>8</i>
Contribution to Project:	<i>Dr. Walker designed the study, supervised Mr. Scanlon and Ms. Connolly as well as conducting the experiments</i>

Name:	<i>Stephen Haggarty</i>
Project Role:	<i>Co-PI</i>
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Dr. Haggarty provided supervision and gave advice on functional assays as well as design of subsequent CRISPR gene editing of iPSCs</i>

Name:	<i>Laura Connolly</i>
Project Role:	<i>Undergraduate Student (University of Bath, UK)</i>
Nearest person month worked:	<i>6</i>
Contribution to Project:	<i>Ms. Connolly generated the transgenic constructs – mutagenesis, DNA subcloning and DNA preparation for injections</i>

Name:	<i>Garrett Scanlon</i>
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Project Role:	<i>Undergraduate Student (Northeastern Univeristy)</i>
Nearest person month worked:	6
Contribution to Project:	<i>Mr. Scanlon helped to generate the transgenic fly stocks, maintained fly stocks, performed genetic crosses and conducted functional assays and data analysis</i>

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

SPECIAL REPORTING REQUIREMENTS

N/A

APPENDICES

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