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TITLE: A Transgenic Model for Learning Defects: Role of NF1 in Drosophila Visual-Spatial Learning

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<b>14. ABSTRACT</b> Learning difficulties and attention deficits are observed in 40-50% of children with Neurofibromatosis Type 1 (NF1). Additionally, many NF1 patients exhibit specific deficits in visuo-spatial tasks, such as the Judgment of Line Orientation task. Spatial learning is also disrupted in heterozygous mouse Nf1 mutants, and can be rescued by reducing Ras activity either genetically or pharmacologically. Conversely, olfactory learning defects observed in Nf1 mutant fruit flies are cAMP-dependent rather than Ras-dependent. Visuo-spatial learning in flies depends on the Central Complex region of the brain, which is distinct from the Mushroom Body region that is essential for olfactory learning. We hypothesize that visual learning defects in Nf1 mutant flies will be Ras dependent, and that this fruit fly model can be exploited to assay potential therapeutic treatments for NF1 cognitive deficits. We propose to assay visual learning in Nf1 mutant flies, and in flies where Ras activity is disrupted in specific regions of the adult brain. This report describes the generation of transgenic fly lines to localize RNAi knockout of NF1, Ras and MAPK, and to express RasGAP defective NF1 isoforms. We have also assayed olfactory learning acuity in Nf1 mutant flies that have been fed drugs that reduce Ras activity, or affect downstream targets of Ras/NF1, such as farnesyl transferase inhibitors, statins or rapamycin. Construction of the visual learning apparatus has been completed and visual learning of wild type flies has been assayed.					
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## Table of Contents

	<u>Page</u>
<b>Introduction.....</b>	<b>4</b>
<b>Body.....</b>	<b>4-11</b>
<b>Key Research Accomplishments.....</b>	<b>12</b>
<b>Reportable Outcomes.....</b>	<b>12-13</b>
<b>Conclusion.....</b>	<b>14</b>
<b>References.....</b>	<b>15</b>
<b>Appendices.....</b>	<b>na</b>

## **INTRODUCTION:**

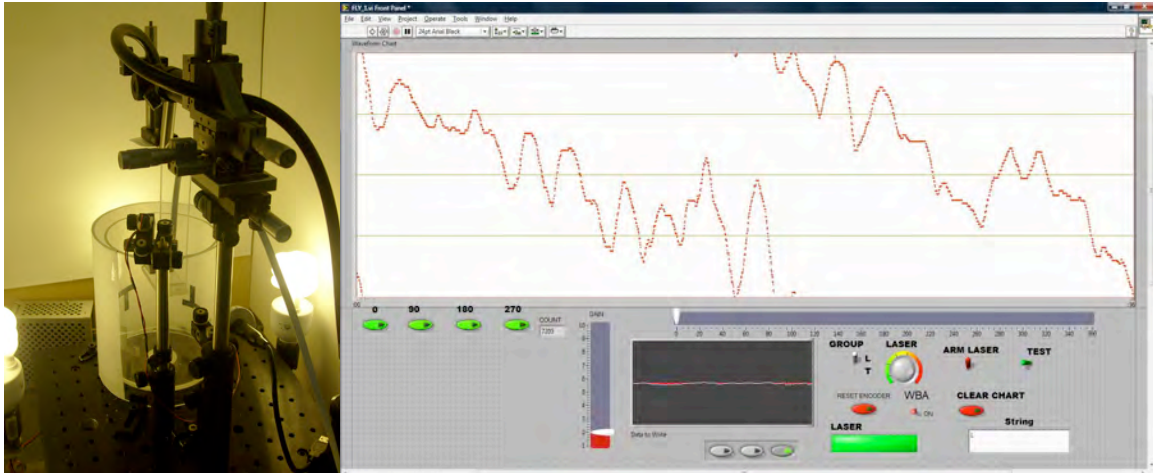
Learning difficulties and attention deficits are observed in 40-50% of children with Neurofibromatosis Type 1 (NF1). Additionally, many NF1 patients exhibit specific deficits in visuo-spatial tasks, such as the Judgment of Line Orientation task. Spatial learning is also disrupted in heterozygous mouse *Nf1* mutants, and can be rescued by reducing Ras activity either genetically or pharmacologically. Conversely, olfactory learning defects observed in *Nf1* mutant fruit flies are cAMP-dependent rather than Ras-dependent. Visuo-spatial learning in flies depends on the Central Complex region of the brain, which is distinct from the Mushroom Body region that is essential for olfactory learning. We hypothesize that visual learning defects in *Nf1* mutant flies will be Ras dependent, and that this fruit fly model can be exploited to assay potential therapeutic treatments for NF1 cognitive deficits. We propose to assay visual learning in *Nf1* mutant flies, and in flies where Ras activity is disrupted in specific regions of the adult brain. We will use the Gal4/Gal80ts/UAS (TARGET) system to localize RNAi knockout of NF1, Ras and MAPK, or to express RasGAP defective NF1 isoforms with precise temporal and spatial control. These flies will also be assayed for MAPK and AC activity. In addition, we will assay visual learning acuity in *Nf1* mutant flies that have been fed drugs that reduce Ras activity, or affect downstream targets of Ras/NF1, such as farnesyl transferase inhibitors, statins or rapamycin.

## **BODY:**

During this project period (June 2007 – December 2008) this research resulted in the generation of transgenic fly lines for RNAi knockout of NF1, and other Ras pathway genes, and for expression of mutant NF1 proteins. Pharmacologic testing of flies was performed using lovastatin, rolipram, and rapamycin, including assays of survival, climbing ability and olfactory learning. We have finished construction of the visuo-spatial learning apparatus and testing of wild type flies. All figures shown in this report (except **Figure 4**) contain new data collected since the preceding Annual report.

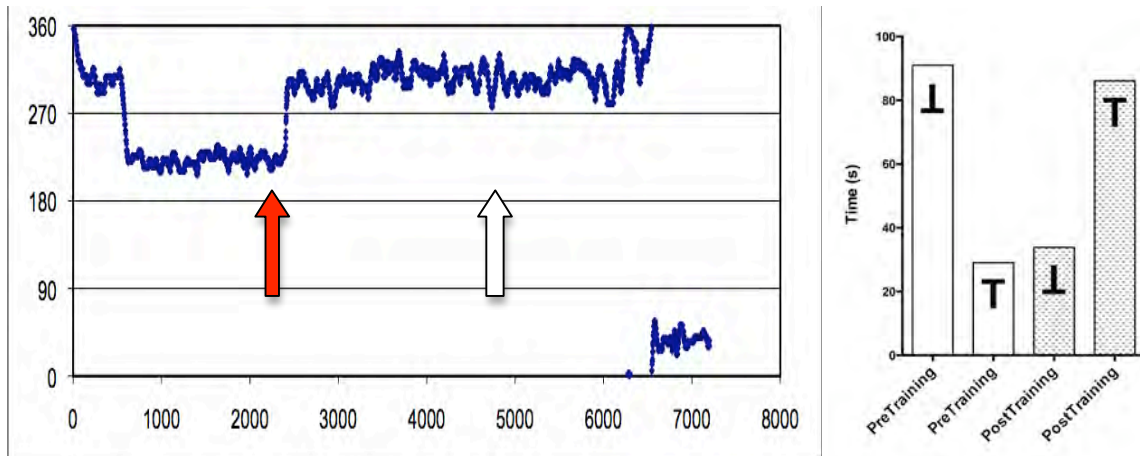
### **Task 1. Assay *Nf1* mutant flies for visual learning defects.**

In order to assay visual learning in flies, we have designed and built an apparatus that incorporates a wing-beat analyzer (WBA), in place of the torque meter used to gauge the fly's direction of flight by the Heisenberg lab (Wolf & Heisenberg, 1992). The WBA has been used extensively for analysis of *Drosophila* flight dynamics in the Dickinson lab (Fry et al., 2008), and offers more precise capture of the flies' motion than the torque meter. We have retained Heisenberg's rotating panorama for presentation of visual cues, since it provides better control of the visual field and is less prone to overheating the flight arena than the LED display field used by Dickinson. Successful implementation of this modified apparatus will facilitate research, both by our lab and others, in the field of visual learning. The WBA is a custom built piece of equipment, which led to some delays in its procurement, since we had to wait until other WBA orders were filled. Consequent delays in construction of the flight arena, and writing and testing of software to run the apparatus were unavoidable. The apparatus has now been completed (**Figure 1A**), and software to control the flight arena and analyze results has been developed using Matlab as shown in this screen shot (**Figure 1B**).



**Figure 1.** (A) Fly flight arena. (B) Screen shot of software that analyzes the wing beat of the fly that is tethered in the center of the arena and rotates the drum according to which visual cue the fly is trying to fly towards (an upright **T** or an upside down **T**).

Flies are mounted on a fine needle using UV-activated glue applied to their necks while under cold anesthesia. Flies are rested for 15-30 minutes prior to mounting in the flight arena. Both wild type flies and NF1 mutants are able to fixate on the visual cues used in our flight arena (an upright **T** shape versus an upside down **T**; data not shown). A 2 minute pre-test period establishes the flies preference for the upright **T** or upside down **T** shape, expressed as the amount of time the fly stays in either of the two quadrants containing that cue. This is followed by 2 min training period during which the laser is activated whenever the fly enters the punishment quadrant, defined by the preferred shape. Flies are then tested for 2 min to observe whether they learned to associate the laser with the punishment quadrant.



**Figure 2.** (A) Trace recording of fly's flight path. Y-axis (degrees) X-axis (sample number - 20 per second). Red arrow – Training Starts; white arrow - Training Ends. (B) Graphical representation showing time (s) spent in each quadrant.

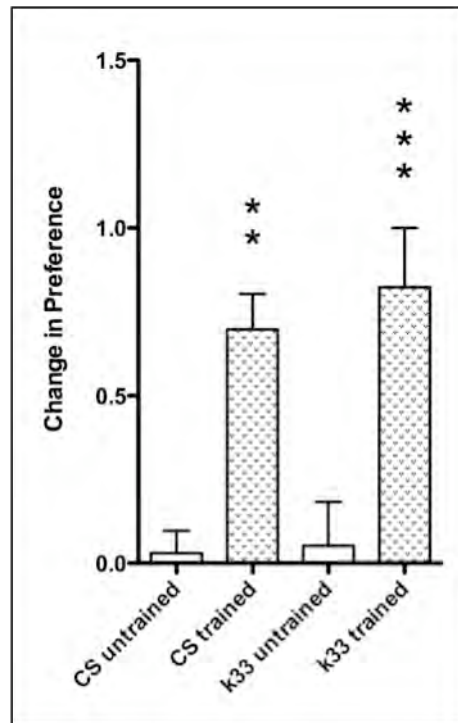
A representative trace recording for an individual fly is shown above (**Figure 2A**). This fly initially flew in an upright **T** quadrant (270-360) for about 30 s, then it flew in the upside down **T** quadrant (180-270) for the next 90 s, demonstrating a “preference” for that shape. The fly was then punished with the laser (red arrow) for approaching the upside down **T**. It immediately began flying toward the upright **T** and remained in that quadrant for the remainder of the 2 min punishment period. After the training period (white arrow) it continued to fly towards the upright **T** for most of 2 min test period. The graph shows the number of seconds spent flying towards the upside down **T** or upright **T** before and after the laser training period (**Figure 2B**). The fly is only punished if it actually flies into the punishment quadrant.

We have begun exhaustive preliminary testing of wild type and mutant control flies. We have also obtained fly lines with mutations that are known to affect visual learning including *rutabaga* and *ignorant* flies (Putz et al., 2004; Liu et al., 2006), to use as controls to test the visual learning apparatus. Preliminary results with wild type flies show that we are able to train individual flies to avoid either the upright **T** or upside down **T** visual cues (**Figure 3**).

Preference is calculated as follows:  

$$\left[ \frac{\text{Time in Preferred Quadrant} - \text{Time in Non-Preferred Quadrant}}{\text{Total Time}} \right] \text{ prior to training} - \left[ \frac{\text{Time in Punished Quadrant} - \text{Time in Non-Punished Quadrant}}{\text{Total Time}} \right] \text{ after training}.$$

A positive change in preference indicates learning, which is exhibited as decreased preference for the shape that was punished. Without training the flies exhibit no change in preference. Statistical significance is assessed using Multiple ANOVA with Tukey-Kramer post-hoc analysis. We are collecting more data on wild type and mutant control flies using a variety of visual cues, including line segments of different orientations and elevations, prior to assaying NF1 mutants. We will begin to prepare a manuscript for publication that describes our visual learning assay within the next 6 months, and hope to follow it up with a manuscript describing the effect of NF1 mutations on visual learning within the next 12 months.



**Figure 3.** Visual learning in wild type flies (CS and k33). Flies learned to avoid the visual cue that was punished by laser, as shown by preference for the alternate cue (n=12, 8, 11, 9; \*\*p<0.001; \*\*\*p<0.0001)

**Task 2. Disrupt NF1 expression in subregions of the adult fly brain to ascertain the anatomical location of visual learning deficits.**

We have obtained an NF1-RNAi transgenic *Drosophila* line that can be used to knock out expression of NF1 in specific tissues or subregions of the brain, using the UAS-Gal4 system to control expression of a double stranded NF1 RNA fragment. This transgenic line was part of a collection of RNAi lines that covers the entire *Drosophila* genome (Dietzl et al., 2007) which is available from the Vienna *Drosophila* Resource Center (VDRC). The NF1-RNAi construct (VDRC #13892) is directed against sequences found in all NF1 isoforms (**Table 1**), however, the transgene insertion in this particular line disrupts a vital gene, causing homozygous lethality, which limits its usefulness. Accordingly, we have used genetic crosses with a line carrying the delta23 transposase to move the transgene to other locations in the genome and establish new lines. We were unable to precisely locate the original transgene insertion site by inverse PCR, however, we have utilized conventional genetic mapping to identify several new lines where the transgene has moved to another chromosome. These lines are no longer lethal, indicating precise removal of the transgene and restoration of the essential gene that was interrupted. Because of all the genetic manipulations that were necessary, it has taken much longer than we expected to get the RNAi knockout of NF1 up and running.

**Table 1: Anti-NF1 DS-RNAi constructs**

Construct (VDRC#)	Primer 1	Primer 2	Fragment Size (bp)	Isoform(s) Targeted
Nf1 (#13892)	CGCGAATTCCCATTCTGCCAC TCGCCCTTC	GCGTCTAGAGACCCACTTTGA TAGCCACCTTTGT	380	Nf1-RB, Nf1-RC, Nf1-RD
Nf1-N	GTCTAGACGAGGAGATGTCCA CCCAAC	AGAATTCTCATCTACTTCC ACAAGC	349	Nf1-RB, Nf1-RC, Nf1-RD
Nf1-NB	AGAATTCATTCTCATCGGAA TCATTAG	GTCTAGAATCGTTTGGAGTGT TGGGAC	300	Nf1-RB
Nf1-ND	GTCTAGACCACTGAATGCGAA AGAAGG	AGAATTCATGTGCAACATGCC GTCTTC	349	Nf1-RD

In addition to the problems of lethality with the NF1-VDRC line, it also has a potential off-target effect due to a small region of sequence similarity with the Neurotactin gene. We have therefore made three additional UAS-RNAi constructs to knock out expression of NF1 (**Table 1**). One of these targets a region in the middle of NF1 that is common to all known isoforms, while the other two constructs are directed against NF1 isoforms with different COOH terminal domains. We also utilized a new vector, pValium, that only recently became available (Ni et al., 2008). This vector facilitates cloning of the NF1 PCR fragments in a head-to-head, or tail-to-tail, orientation in order to produce double stranded NF1 RNA when expressed in flies under control of the Gal4-UAS system. This vector further allows us to insert the transgenic constructs in defined locations in the fly genome to avoid any effect of transgene insertion site on expression of the constructs. This greatly reduces the time needed to make and screen transgenic lines, and also stops insertional lethality issues.

We ran into several problems with insertion of the second fragment into these clones that we think are due to a DNA hairpin interfering with replication in bacteria as

well as in PCR reactions. We switched to the In-Fusion Advantage PCR Cloning system (Clontech) to increase the yield of transformants with the correct insert. This system uses a site specific recombinase to clone inserts without ligation in any desired vector at any chosen site. The constructs with two inserts in head-to-head or tail-to-tail orientation have been verified by PCR and sequencing, and injected into flies to generate transgenic lines.

We have several transgenic fly lines that express Gal4 in the whole brain or mushroom bodies (elav, armadillo, scabrous, MB247, 201y, OK107 and c747). We have also obtained a number of lines that express in the central complex region that is required for visual learning, including the fan-shaped body (107y, 210y, c5 and c205) and the ellipsoid body (7y, 52y and c232). In addition, we now have several lines for the expression of Gal80ts under control of the tubulin promoter (McGuire et al., 2003), as well as some recently published Gal4ts lines (Mondal et al., 2007).

All of the obtained RNAi and Gal4 lines have been outcrossed for 5 generations to our standard behavioral control line in order to remove any effect of background mutations that can affect behavior. The fly lines have been remade into homozygous state, and have been tested for effects on survival, olfaction and shock reactivity. We are currently testing the olfactory learning ability of the RNAi lines in the absence of a Gal4 driver to check that insertion of the RNAi construct does not affect learning. Then we can assay the effect on olfactory learning when the RNAis are expressed in the brain, particularly the mushroom bodies. Now that the visual learning apparatus is completed we can begin pre-testing these RNAi lines for visual learning defects. We can then assay the effect on visual learning of expressing these RNAi lines in the central complex region of the brain.

**Task 3. Use localized RNAi knockout or expression of mutated NF1 proteins to assay the Ras/NF1-dependence of visual learning:**

In addition to the NF1-RNAi lines described above, we have obtained two Ras-RNAi lines, two MAPK-RNAi lines, an EGFR-RNAi line, three TSC-RNAi lines and an S6K-RNAi line from the VDRC (Dietzl et al., 2007), as shown in **Table 2**. Some of the VDRC lines are lethal, and all are inserted randomly into the genome. This could potentially affect expression levels of the RNAi constructs, or interfere with other genes that are important for Drosophila behavior. The lines have been outcrossed and re-homozygosed, and have been tested for survival and sensory deficits, prior to assays of olfactory or visual learning ability, with or without Gal4 controlled transgene expression in specific brain regions.

We have made additional UAS-RNAi constructs to knockout expression of the TSC1 and TSC2 genes (**Table 2**), in order to take advantage of the new pValium vector system, and generate lines that are inserted in a defined location to avoid insertion site effects. These plasmids have been verified by PCR and sequencing and injected into flies to generate transgenic lines. We are also making additional RNAi lines directed against Ras1, Ras2, MAPK, and S6K that will utilize the pValium vector system.

**Table 2: Other Ras/MAPK Pathway DS-RNAi constructs**

Construct (VDRC#)	Primer 1	Primer 2	Fragment Size (bp)	Isoform(s)/Targeted
Ras1 (#12553)	CGCGAATTCACGGAATACAAA CTGGTCGTCGTTG	GCGTCTAGAGGGCACCTCTTC GGCATCCT	327	Ras85D-RA
Ras2 (#1517)	CGCGAATTCGCTTCGATGAGA TCCCCAAGTTCC	GCGTCTAGAGCAGCACTTCCT CTTGCCCTTCTT	349	Ras64B-RA
MAPK (#4697)	CGCGAATTCGGATCTACGGAA GTTCCCTCAATCTAATGCT	GCGTCTAGAAAACATCTCTCA TTTGGTCTATGCTATCAACTC	301	rl-RA, rl-RB, rl-RC, rl-RD, rl-RE, rl-RF
EGFR (#1654)	CGCGAATTCGGAGCACGCAAA TCGCCAAG	GCGTCTAGAGCCAAAGGTCAG CAGTCCCAAA	284	Egfr-RA, Egfr-RB
Tsc1 (#11836)	CGCGAATTCCTTGAGGTGTTCG GGCATTG	GCGTCTAGAGGACGGGCAGCG ATAGGTTG	336	Tsc1-RA
Tsc2 (#1454)	CGCGAATTCCTTACCACAAGCC CACCCGACA	GCGTCTAGAGGGTAAAACGAG GGCGTGGAA	252	Gig-RA
S6K (#6646)	CGCGAATTCGGCAAAGGTGG TTATGGCAAAGT	GCGTCTAGACGGGCTTCAGAT CGCGGTAG	374	S6k-RA
Tsc1-T1	AGAATTCAGTCCGCATAACGA GATTGC	GTCTAGAGAGACATCCGGGAA ACTGAAC	316	Tsc1-RA
Tsc2-T2	AGAATTCGAAGGATCGTCTGC ATCACCTGC	GTCTAGAGAGCCTCGATCGTC TTAATGC	304	Gig-RA

Pre-existing lines that express GAP-defective human NF1 transgenes have been extensively tested for rescue of *Nf1* mutant body size and olfactory learning and memory defects, and biochemical assays have been performed (Hannan et al., 2006; Ho et al., 2007). We have also generated a number of additional human NF1 constructs that carry clinically identified missense mutations affecting regions of the NF1 protein outside the GAP domain, as well as both PKA and MAPK phosphorylation site mutants (**Table 3**).

Transgenic flies carrying insertions of human NF1 mutants on the second chromosome have been crossed into the *Nf1*<sup>-/-</sup> mutant background. These lines have been tested for effects on mutant body size, and we are now assaying for olfactory learning and memory defects, when the transgene is expressed in the nervous system under Gal4-UAS control. Lines without second chromosome insertions have been crossed to the delta23 transposase line to move the transgenes from their current insertion sites, and new lines are being mapped currently.

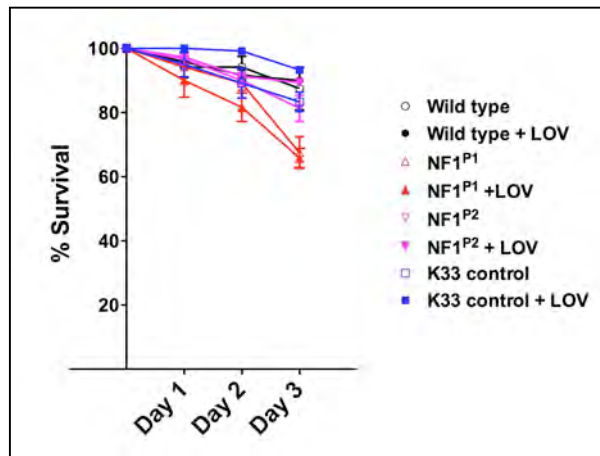
**Table 3. Human NF1 mutant lines.**

Mutation	Effect	2 <sup>nd</sup> Chromosome Lines
S818A	PKA site	F302, F303, F307, F310
991delM	Patient mutant	C604, C609
K1105R	Ubiquitin site	-
R1391S	GAP domain	E117a, E117b, E117c
1658delIY	Sec1p domain	-
L1932P	Patient mutant	T201, T205, T207
2366delNF	Patient mutant	-
S2739A	MAPK site	-

**Task 4. Rescue visual learning defects pharmacologically with farnesyl transferase inhibitors, statins or rapamycin.**

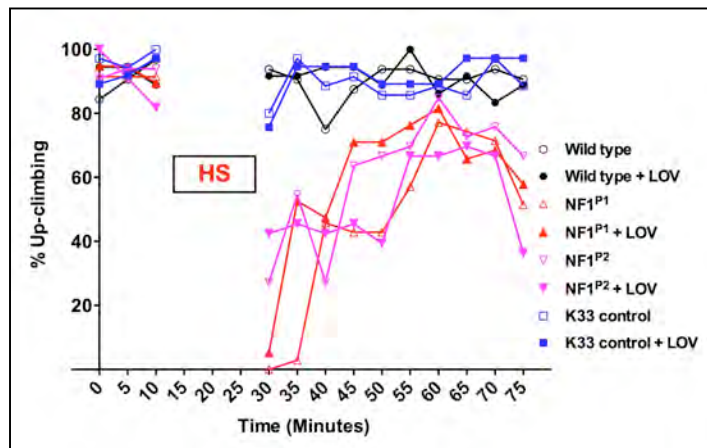
These studies have focused on three drugs approved for use in humans that target pathways or molecules downstream of NF1 – lovastatin, rapamycin and rolipram. Lovastatin, which is commonly prescribed for cholesterol reduction, has been shown to rescue spatial learning defects in *Nf1* mutant mice (Li et al., 2005). Rapamycin is an immune suppressive, anti-proliferative drug that inhibits mTOR (mammalian target of rapamycin), a protein which is regulated by NF1 and TSC proteins, and may affect cognitive functions via the S6K pathway. Rolipram is a cAMP-specific phosphodiesterase (PDE4) inhibitor marketed for its anti-inflammatory and anti-psychotic effects. Inhibition of PDE4, which is normally responsible for decreasing cAMP concentrations, may facilitate learning.

Adult flies were exposed to drugs dissolved in food (2-3 days; chronic treatment), or added to 4% sucrose solution on filter paper (18-24 hours; acute treatment), and drug uptake was monitored by co-ingestion of food dye. There was no significant effect of any of the three drugs on mortality or locomotor activity, for either wild type or NF1 mutant flies, over a range of concentrations from 100-750 mg/ml, as shown here for survival on 100mg/ml lovastatin for three days (**Figure 4**). We have also shown that there is no effect on survival of flies after chronic exposure to drug at concentrations up to 0.75 mg/ml (not shown).



**Figure 4.** Acute exposure to lovastatin (100 mg/ml) does not affect survival of NF1 mutant flies (NF1<sup>P1</sup>, NF1<sup>P2</sup>) or control flies (wild type, k33).

We also assayed flies for recovery of climbing ability after 20-30 minutes heat shock (HS) (**Figure 5**). In this assay, NF1 mutants show greatly reduced mobility after HS, and do not regain normal activity levels up to 30 min later, while control flies recover rapidly within 5 min of HS (**Figure 5**). This activity has been shown to be cAMP dependent (Tong et al., 2007). Levels of phospho-MAPK or phospho-S6K in



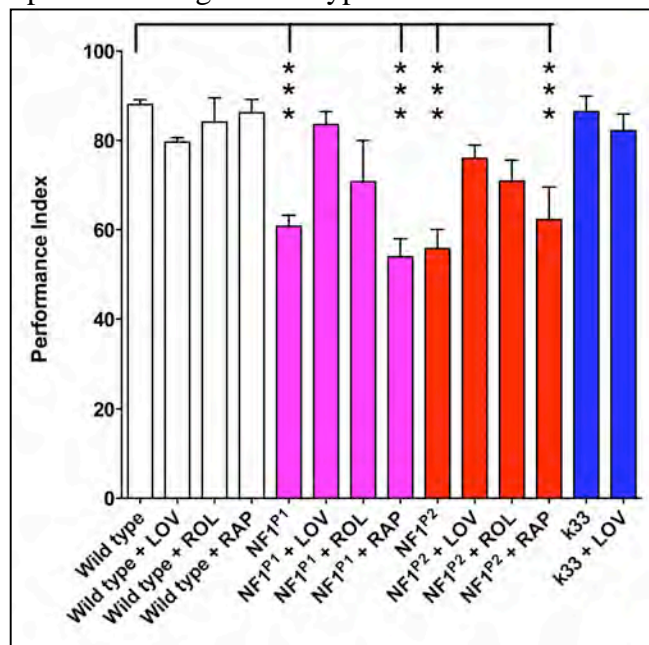
**Figure 5.** Acute exposure to lovastatin (100 mg/ml) does not affect post-heat-shock recovery of climbing ability in NF1 mutant flies (NF1<sup>P1</sup>, NF1<sup>P2</sup>) or control flies (wild type, k33).

treated versus control flies will be measured using Western blot analysis, while cAMP levels will be assayed using an ELISA based kit.

Adult olfactory learning assays were performed as described previously (Guo et al., 2000; Ge et al., 2004; Ho et al., 2007). Briefly, an odor (benzaldehyde BA or methylcyclohexanol MCH) is paired for 1 min with electric shock (60Hz), flies are rested for 1 min then exposed to the other odorant for 1 min without shock. After a further 1 min rest interval, flies are given a choice between the two odors, and their odor preference recorded. A performance index (PI) is calculated from the responses of flies to reciprocal training regimes that pair each odor with shock. PI=100 indicates full learning of the odor-shock association, PI=0 indicates random distribution. Approximately 150 flies are tested in each trial and the operator is blind to both genotype and drug treatment. Essential behavioral controls including locomotor activity, electric shock reactivity and olfactory acuity were not affected by drug treatment (data not shown).

Significant rescue of olfactory associative learning was observed in two different NF1 mutants (NF1<sup>P1</sup> and NF1<sup>P2</sup>) after chronic or acute exposure to 100 µg/ml lovastatin or rolipram, but not to rapamycin, as shown here for acute treatment (**Figure 6**). Statistical significance is assessed using multiple ANOVA with Tukey-Kramer post-hoc analysis. Learning was not affected by exposure to drug in wild type control flies (22020u), or in the parental control flies (k33). The performance of NF1 mutants exposed to drugs was

increased to levels that are indistinguishable from wild type or k33 parental control flies. In order to achieve full rescue of learning defects we will use higher drug concentrations and/or extended exposure times. The rescue of learning in adult NF1 mutants by lovastatin was not expected. We speculate there may be crosstalk between Ras and cAMP pathways, possibly mediated by the growth factor stimulated NF1/Ras dependent AC-X that we have also identified (Hannan et al., 2006). Rapamycin does not show any effect on learning which is consistent with its affecting the Rheb/mTOR pathway downstream of PI3K/TSC. We have begun preparing a manuscript that describes the olfactory learning results which we will submit to Learning & Memory.



**Figure 6.** Rescue of olfactory learning defects in NF1 mutant adults by acute exposure to lovastatin (LOV) and rolipram (ROL) but not rapamycin (RAP) at 100 mg/ml. (\*\*\*p<0.0001; n=21,5,5,11,121,7,5,11,13,6,5,7,9,7)

## **KEY RESEARCH ACCOMPLISHMENTS:**

- Design and construction of new visual learning apparatus
- Assays of visual learning ability of wild type flies
- Generation of transgenic lines for expression of mutant NF1 proteins
- Assays of survival and locomotor activity in *Nf1* mutant flies treated with lovastatin, rapamycin, and rolipram
- Demonstration of rescue of olfactory learning deficits in *Nf1* mutant flies treated with lovastatin, and rolipram but not rapamycin

## **REPORTABLE OUTCOMES:**

### **Manuscripts:**

Shilyansky C., Li W., Legius E., **Hannan F.**, Wiltgen B., Hardt M., Krab L., Elgersma Y., Hunter-Schaedle K., Acosta M. & Silva A.J. (2008). Molecular and cellular mechanisms of learning disabilities: a focus on neurofibromatosis type I. In: Animal and Translational Models of Behavioral Disorders, Vol 2, R.A. McArthur & F. Borsini, eds. Elsevier, In press.

### **Meeting Abstracts:**

Pharmacologic Rescue of Behavioral Deficits in *Drosophila Nf1* Mutants. Childrens Tumor Foundation National Neurofibromatosis Conference, Bonita Springs, FA, June 2008.

### **Presentations:**

Pharmacologic Rescue of Behavioral Deficits in *Drosophila Nf1* Mutants. Childrens Tumor Foundation National Neurofibromatosis Conference, Bonita Springs, FA, June 2008.

Pharmacological Rescue of Learning Deficits in a *Drosophila* Model of Neurofibromatosis Type 1. 20th Annual Graduate Student Research Forum, March 2008, Valhalla, NY.

Modeling Disease in the Fruit Fly *Drosophila melanogaster*. Club Neuron, New York Medical College, Valhalla, NY, November 2007.

Modeling Human Disease in the Fruit Fly *Drosophila*. New York Eye & Ear Infirmary Founders Meeting, Harvard Club, New York, NY, October 2007.

**Funding Applied For:**

Children's Tumor Foundation Drug Discovery Initiative

Children's Tumor Foundation Young Investigator Award

Cold Spring Harbor Drosophila Neuroscience Course

DoD CDMRP NFRP Concept Award

DoD CDMRP NFRP Exploration - Hypothesis Development Award

DoD CDMRP TSCRIP Concept Award

Genetics Society of America DeLill Nasser Award

Howard Hughes Medical Institute Early Career Scientist Award

March of Dimes Research Program

National Institutes of Health Small Business Innovation Research R43/R44

Tuberous Sclerosis Alliance Innovator Award

**Personnel Receiving Pay:**

Frances Hannan, Ph.D.

Peter O'Brien

## CONCLUSIONS:

Research using the fruitfly *Drosophila* to study the function of the NF1 protein has provided remarkable insights for the NF1 research field. Almost a decade ago, examination of fly *Nf1* mutants revealed a previously unsuspected role for NF1 in the regulation of AC activity (Guo et al., 1997). Subsequent studies have shown that this pathway is also affected in mice, and that human NF1 can control AC activity when it is expressed in flies. Expression of the human NF1 mutations and deletions in *Drosophila* has proven invaluable for defining the mechanism of NF1/Ras-dependent AC activation, and for definition of a functional domain outside the GRD of NF1 that controls NF1/Gs $\alpha$ -dependent AC activity and body size (Hannan et al., 2006). We have also been able to show that NF1 Ras-GAP activity is essential for protein synthesis dependent long-term memory in flies, while the C-terminal region contains sequences that are necessary for olfactory learning (Ho et al., 2007). This is the first time that a single protein has been shown to differentially affect these very distinct phases of memory formation. This underscores the crucial role of NF1 learning and memory.

We are uniquely placed to investigate the role of the AC and Ras pathways in the processes of learning and memory using our human NF1 mutants to disrupt the AC or Ras/MAPK pathways. We are also poised to examine the differential contribution of Ras versus AC pathways to both olfactory and visual learning, using the new technique of RNAi mediated knockout of genes in specific brain regions. This should also provide insight into the role of major neuron specific isoforms of NF1 in olfactory versus visual learning. It is critical that future creation of transgenic fly lines utilize vectors such as pValium, which allow insertion of constructs into defined locations in the fly genome, avoiding position effects on expression, and lethality of insertions, as well as allowing much more rapid generation and manipulation of transgenic lines.

Our demonstration that lovastatin and rolipram can rescue olfactory learning deficits in *Nf1* mutant flies is the first to show that drugs affecting the NF1/Ras or cAMP pathways in mammals can also be effective in fruit flies. These are exciting basic research results, of enormous general interest, which will stimulate further basic research into NF1 function in mammals, and generate new ideas for the development of therapeutic agents. This also opens up the tantalizing possibility of using our fruitfly system to screen for novel compounds that may be effective against symptoms of NF1 in humans, including cognitive deficits in children with NF1.

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