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PRINCIPAL INVESTIGATOR: Andre Bernards, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, MA 02114-2698

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### 6. AUTHOR(S)
Andre Bernards, Ph.D.

Email – abernard@helix.mgh.harvard.edu

### 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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ABSTRACT

The protein that is defective in patients with neurofibromatosis type 1 (NF1) functions as a GTPase Activating Protein (GAP) for Ras, yet homozygous loss of a highly conserved Drosophila NF1 ortholog results in several phenotypes that are insensitive to manipulating Ras signal transduction, but rescued by increasing signaling through the cyclic AMP-dependent protein kinase A (cAMP/PKA) pathway. To study how NF1 modulates this pathway and to evaluate whether the cAMP/PKA pathway represents a valid therapeutic target in NF1, the aims of this project are to perform a comprehensive structure-function transgenic analysis to assess the in vivo importance of protein domains. We also aim to investigate whether different phenotypes reflect roles for NF1 as a GAP for either conventional Ras ortholog Ras1 or for R-Ras ortholog Ras2, to determine whether a neuroendocrine defect explains the non-autonomous Nfl growth deficiency, and to perform other experiments to determine why growth is restored by increasing PKA activity. Finally, with a long term goal of manipulating human NF1 expression levels, we aim to identify cis-acting elements and their cognate transcription factors that drive Drosophila Nfl expression in larval CNS neurons and in peripheral nerves.
Introduction

The protein that is defective in patients with Neurofibromatosis type 1 (NF1) functions as a GTPase Activating Protein (GAP) for the Ras signaling protein, yet loss of a conserved Drosophila NF1 ortholog results in several phenotypes that are insensitive to manipulating Ras signal transduction, but rescued by increasing signaling through the cyclic AMP-dependent protein kinase A (cAMP/PKA) pathway. To study how NF1 modulates this pathway and to evaluate whether the cAMP/PKA pathway represents a valid therapeutic target in NF1, we proposed to perform a comprehensive structure-function transgenic analysis to assess the in vivo importance of protein domains. We also proposed to investigate whether different phenotypes reflect roles for NF1 as a GAP for either conventional Ras ortholog Ras1 or for R-Ras ortholog Ras2, to analyze whether a neuroendocrine defect explains the non-autonomous Nf1 growth deficiency, and to perform various other experiments to determine why growth is restored by increasing PKA activity. Finally, we have found that some NF1 mutants rescue only when expressed at relatively high levels. Thus, with a long term goal of manipulating human NF1 expression, we proposed to identify cis-acting elements and their cognate transcription factors that drive Drosophila Nf1 expression in larval CNS neurons and in peripheral nerves.

Body

Our Statement of Work described the following aims and sub-aims for year one:

Aim 1
1.1.1 Generate transgenics for two smaller N-terminal and one smaller C-terminal deletion mutants.
1.1.2 Generate transgenics for proteasomal degradation motif mutant.
1.1.3 Generate transgenics expressing N-terminal (~1-900) protein segment.
1.1.4 Generate full length construct for ERK phosphorylation site mutant.
1.1.5 Generate human NF1-GRD-HA and NF1-GRD^{R1276P}-HA transgenics.
1.1.6 Provide characterized transgenics to collaborators for phenotypic rescue analysis.
1.1.7 Map and balance new transgenes.

**Aim 2**
1.2.1 Analyze whether tissue-specific expression of UAS-Rasl or UAS-Ras2 causes defects and/or enhances Nfl phenotypes.
1.2.2 Analyze effects of tissue-specifically expressing UAS-Rasl^{N17} and UAS-Ras2^{N19} in wild-type and Nfl backgrounds.
1.2.3 Generate transgenics for short-hairpin pWIZ-Ras2 construct.
1.2.4 Test whether induction of four UAS-GRD-HA transgenes affects wing size, eye development, or overall development/viability.
1.2.5 Clone Ras2 genomic segment in preparation for gene targeting.

**Aim 3**
1.3.1 Analyze CNS gene expression profiles of dnc^{l} and Nfl:dnc^{l} 3rd instar larvae.
1.3.2 Analyze CNS gene expression profile of dCREB2 hypomorphic 3rd instar larvae.
1.3.3 Induce tissue-specific expression of UAS-AKH and analyze Nfl size rescue.
1.3.4 Generate and characterize AKH and AKH receptor promoter driven GAL4 lines.
1.3.5 Characterize UAS-dCREB2 isoform transgenics.

**Aim 4**
1.4.1 Generate and characterize transgenics containing 17.3 kb genomic Nfl transgene
1.4.2 Provide transgenic line to Michael Stern to test rescue of glial thickening phenotype.
1.4.3 Generate Nfl promoter – GFP reporter constructs.

The following describes progress made towards achieving these goals.

**1.1.1 Generate transgenics for two smaller N-terminal and one smaller C-terminal deletion mutants.**

In our original proposal we described that large segments of Drosophila neurofibromin are dispensable for rescuing the Nfl pupal size phenotype. Thus, neurofibroma mutants that carry in-frame deletions of amino acids 492-1094 upstream of and including part of the N-IRA homologous region, of amino acids 1611-1769 representing the Sec14 domain, or of residues 1770-2265 in the C-IRA domain efficiently rescued the size defect when expressed in Nfl mutants. By contrast, a transgene that lacked the GAP catalytic domain (amino acids 1219-1580) did not rescue, whereas a transgene representing just the GAP domain was sufficient for rescue. These and other observations support our conclusion that a functional GAP domain is necessary and sufficient for size rescue. However, we also observed that proteins that lacked residues 12-493 or that were truncated beyond amino acid 2347 were expressed, but failed to rescue. Since the GAP domain by itself is sufficient for rescue, we hypothesized that both N and C-terminal segments may control the proper localization of neurofibromin, regulate its stability, or its GAP activity. We noted that the 12-493 segment consists of two evolutionary conserved regions separated by a non-conserved spacer, and proposed to generate two smaller N-terminal deletions that individually removed the conserved segments. To further analyze the importance of these individual conserved domains, we generated transgenics that lack residues 12-284 or 191-492. The rescuing potential of these transgenes has not yet been established. The C-terminal truncation mutant lacks an evolutionary conserved segment that in human neurofibromin interacts with the cytoplasmic segment of syndecans (Hsu et al., 2001). Although in a directed two-hybrid experiment Drosophila neurofibromin and syndecan showed no interaction, we generated a Drosophila Nfl transgene that lacks residues 2672 to 2750, corresponding to the C-terminal syndecan binding site in human neurofibromin. A single transgenic line expressing this mutant was obtained, and the transgene crossed into the Nfl mutant background.
Interestingly, this 78 amino acid deletion mutant is expressed, but fails to rescue the Nfl size defect. To confirm that deletion of residues 2672-2750 results in a functionally deficient protein, we recently generated 10 more transgenic lines. The transgene integration sites of these lines are being mapped, after which several lines with transgenes on the second chromosome will be used to confirm that the 2672-2750 segment is essential for in vivo size rescue.

Immunoblot analysis of 3rd instar larval CNS or adult fly head extracts detected elevated levels of MEK and ERK phosphorylation in NF1 mutants compared to wild-type controls. Thus, we routinely test whether transgenic size rescue correlates with a reduction in P-ERK level. So far we have found a perfect correlation between the two phenotypes. However, it will be interesting to analyze whether the 78 amino acid C-terminal deletion mutant, which has an intact GAP domain but fails to rescue, affects P-ERK levels. If it did not, this would suggest an important role for the C-terminus of neurofibromin in regulating its localization and/or GAP activity. If the mutant did reduce P-ERK levels, this would argue that ERK regulation may not be directly involved in NF1 size control. To further analyze the importance of inappropriate ERK activation in NF1 growth control, we crossed several Ras1, MEK, and ERK null alleles into the NF1 mutant background. None of these mutants affects NF1 pupal size and we are in the process of testing whether they affect larval CNS P-ERK levels. We also obtained a transgenic line expressing a UAS-MKP3 construct. MKP3 is a Drosophila phosphatase that interacts with and has high specificity for the Drosophila rolled ERK ortholog (Kim et al., 2002). We are in the process of analyzing whether ubiquitous or neuronal expression of UAS-MKP3 down-modulates P-ERK and modifies NF1 pupal size. Finally, we tested whether mutations in other Ras signaling components, such as PI3-kinase, Ral, and RaGDS, modified NF1 pupal size. None of these mutants modified the NF1 size defect.

1.1.2 Generate transgenics for proteasomal degradation motif mutant.

The group of Dr. Karen Cichowski previously reported that human neurofibromin undergoes rapid proteasomal degradation when quiescent cells are stimulated by serum or by various growth factors (Cichowski et al., 2003). Her group identified a conserved KYFTLF motif (residues 1095-1100 in human neurofibromin), which is required for serum-induced degradation. When expressed in mammalian cells, a Drosophila neurofibromin segment that includes this motif showed similar serum-stimulated degradation. Thus, to determine whether proteasomal degradation of neurofibromin is important in vivo, we generated a substitution mutant in which the FTLF residues in the degradation motif (Drosophila NF1 residues 1145-1149) were altered into four alanines. Three lines expressing this transgene were generated and crossed into an Nfl mutant background. In all cases the mutant protein efficiently rescued the Nfl size defect, arguing that the FTLF box is not essential for function. We also tested whether heat shock induced expression of the mutant protein in a wild-type background affected pupal size, or resulted in any obvious phenotypes. No obvious defects were noted. Thus, proteasomal degradation may regulate the activity of neurofibromin, but a mutant that lacks the identified proteasomal degradation motif appears fully functional and does not cause obvious dominant phenotypes upon overexpression. It remains possible that this mutant will show subtle defects when tested in other assays.

1.1.3 Generate transgenics expressing N-terminal (~1-900) protein segment.

We speculated in our original proposal that N-terminal or C-terminal segments of neurofibromin might interfere with the proper localization or regulation of the protein when overexpressed. We had generated five transgenic lines expressing a C-terminal 455 amino acid segment, and had proposed to similarly generate a transgene expressing residues 1-920 followed by a HA-tag. The idea was to determine whether these transgenes acted in a dominant negative manner when expressed in wild-type flies. We have since analyzed flies expressing the C455 fragment and found no obvious effects on wing or male pupal size. In one line there was a slight but statistically significant reduction in female pupal size, but this effect was small and not observed in smaller male pupae. Routine cloning problems have so far prevented the generation of the 1-920-HA transgene.

1.1.4 Generate full length construct for ERK phosphorylation site mutant.
Serum or growth factor stimulation of several mammalian cell types induces the rapid proteasomal degradation of neurofibromin, followed by its reappearance 30 minutes later (Cichowski et al., 2003). When neurofibromin reappears, it migrates more slowly, which is believed to reflect ERK-mediated phosphorylation. An interesting hypothesis is that ERK phosphorylation activates the GAP activity or stabilizes neurofibromin in the continued presence of growth factors. The only evolutionary conserved, canonical PXSiTP ERK phosphorylation site is located near the C-terminus of human and Drosophila neurofibromin, and preliminary evidence from Dr. Cichowski's lab suggested that this site is indeed phosphorylated by ERK. To test the relevance of this site we changed serine 2741 in the Drosophila NF1 PPSP motif into an alanine and generated a full length transgene. Although not proposed in our original application, we also generated full length transgenes in which serine 2741 was altered into phospho-mimicking aspartate or glutamate residues. As usual, transgenes were extensively mapped and relevant segments sequenced to confirm mutations and guard against unexpected changes. Six transgenic lines harboring the heat-shock inducible S2741A mutant were generated, and none rescued the size defect when induced in a Nft mutant background. However, in immunoblots no NF1 protein expression was detected in any of these six lines. Transgenic lines for the phospho-mimicking mutants have been obtained, but not yet analyzed. We are currently resequencing the injected S2741A transgene and we are exploring whether the S1741A change affects protein stability.

1.1.5 Generate human NF1-GRD-HA and NF1-GRD^{R1276P}-HA transgenics.

In our hands a Drosophila NF1-GRD-HA transgene that includes only the GAP catalytic domain rescued the mutant size defect, whereas two similar transgenes harboring missense mutants that affect GAP activity did not rescue. We obtained similar results with wild-type and GAP deficient full length NF1 transgenes. These results underline our conclusion that GAP activity is necessary to rescue the cAMP-sensitive mutant size defect. Yi Zhong's group at Cold Spring harbor Laboratories, however, continues to argue that GAP activity is not essential for size rescue, since in their experiments GAP deficient human NF1-GRD transgenes rescued efficiently. Although we used Drosophila transgenes and Zhong used human constructs, this is obviously a major discrepancy. One possibility is that the human mutants down-modulate Ras by interacting with and sequestering the GTPase. To investigate this further we had proposed to generate our own wild-type and GAP deficient (R1276P) human NF1-GRD transgenes. We did generate both transgenes, but in anti-HA immunoblots detected no expression in several transgenic lines. Thus, to properly compare the reagents used, we recently decided to instead exchange transgenes with the Zhong lab. Beyond ongoing attempts to reproduce Zhong's result and investigate its mechanistic basis, we performed several experiments to further determine the importance of GAP activity in the rescue of NF1 phenotypes. As indicated in our proposal, neuronal expression of Gap1, using the available Gap1^{EP45} line, did not rescue the NF1 size defect. However, in recent experiments expression of a full length transgene representing the Drosophila p120GAP ortholog (termed vap for vacuolar peduncle) does appear to rescue. If confirmed this would provide strong support for our conclusion that GAP activity is necessary and sufficient for rescue. Beyond confirming that vap rescues, we will also determine whether NF1, Gap1, and vap act as GAPs for the same GTPases. Drosophila Ras1 is the sole ortholog of mammalian conventional H-, K- and N-Ras, while Ras2 is closely related to three mammalian R-Ras paralogs (R-Ras1, R-Ras2/TC21, and R-Ras3/M-Ras). In order to test Ras2 in GAP assays we produced the protein in the baculovirus system, since the GST-Ras2 protein that we had planned to use was entirely insoluble. Similar to results obtained with the human protein, a bacterially produced Drosophila NF1-GRD protein potently stimulates the intrinsic GTPase activity of Ras1 and Ras2 (Figure 1). We recently also generated maltose binding protein (MBP) fusion proteins representing the catalytic domains of Gap1 and vap. We are in the process of testing their in vitro GTPase specificities. We have also created our own full length UAS-Gap1 transgene. We will generate transgenic lines that express this transgene at various levels, to further confirm that Gap1 is unable to substitute for NF1 in growth regulation.
Figure 1. A Drosophila NF1-GRD MBP fusion protein stimulates the GTPase activity of Ras1 and Ras2 in vitro. The graphs show the percentage radioactivity remaining after a 10 minute incubation of 6 nM GTP-$\gamma$32P loaded Ras1 or Ras2 in the presence or absence of NF1-GRD protein. No GAP activity was detected in similar assays with Drosophila RaIA or Rap1.

1.1.6 Provide characterized transgenics to collaborators for phenotypic rescue analysis.

Beyond exchanging NF1 transgenes with the lab of Yi Zhong, we have so far provided transgenic flies expressing various NF1 mutants to collaborators Drs. Scott Waddell (U. Mass) and Michael Stern (Rice U.).

1.1.7 Map and balance new transgenics.

This is routinely performed for any new transgene.

Aim 2

1.2.1 Analyze whether tissue-specific expression of UAS-Rasl or UAS-Ras2 causes defects and/or enhances Nfl phenotypes.

We generated transgenics expressing wild-type UAS-Ras2, activated UAS-Ras2va14, and dominant negative UAS-Ras2N19. Transgenic lines expressing the corresponding Ras1 mutants were obtained from the Bloomington Stock Center. We had proposed to test whether over-expression of wild-type Ras1 or Ras2 enhances Nfl defects, based on a published report that overexpression of Ras1 enhances the brain degeneration phenotype of the Drosophila vap mutant (Botella et al., 2003). However, this latter result has been called into question (David Hughes, personal communication), and overexpression of either UAS-Rasl or UAS-Ras2 under the control of several GAL4 drivers did not modify the Nfl size defect.

1.2.2 Analyze effects of tissue-specifically expressing UAS-Rasl17 and UAS-Ras2N19 in wild-type and Nfl backgrounds.

Although not originally proposed, we generated several Drosophila lines that express the yeast GAL4 transcription factor under the control of the Ras1 or Ras2 transcriptional promoters. Ras1-GAL4 drives UAS-GFP expression ubiquitously without discernable pattern, presumably reflecting the ubiquitous expression of Ras1. By contrast, Ras2-GAL4; UAS-GFP flies express the GFP reporter in highly specific subsets of cells in the ventral ganglion and the midbrain sections of the larval CNS (Figure 2). Several independent Ras2-GAL4 lines differ in expression level, but not in expression pattern. This pattern faithfully recapitulates the endogeneous Ras2 mRNA expression pattern (Salzberg et al., 1993). Interestingly, Ras2-GAL4 drives very little if any UAS-GFP expression outside of the larval CNS, arguing for a nervous system specific role for this R-Ras ortholog. Expressing the cell death inducing UAS-reaper transgene under the control of UAS-Ras2 caused early lethality, arguing that Ras2 expressing cells are essential for normal development.

Figure 2; Ras2-GAL4 driven UAS-GFP expression in third instar larval CNS. No obvious UAS-GFP expression is seen in imaginal discs or in any other larval tissue. Driving UAS-NF1 with Ras2-GAL4 drivers substantially rescues the NF1 size defect. Driving activated Ras1 or Ras2 mutants with Ras2-GAL4 drivers results in small pupae.

Importantly, expression of UAS-NF1 under the control of the two strongest Ras2-GAL4 drivers rescues the NF1 size defect. Further supporting the idea that Ras2 expressing cells play an important role
in NF1-mediated growth regulation, expressing activated UAS-Ras2mut14 under the control of Ras2-GAL4 results in small pupae, which do not eclose. A similar result was obtained when Ras2-GAL4 is used to drive expression of the activated UAS-Ras1mut12 mutant. We are in the process of determining whether expression of dominant negative UAS-Ras1N17 or UAS-Ras2N19 mutants under the control of Ras2-GAL4 rescues the NF1 size defect. Initially we only obtained a single transgenic line harboring the dominant negative UAS-Ras2N19 mutant. Expressing this mutant under the control of Ras2-GAL4 did not modify the NF1 size defect. However, we recently generated several more transgenic lines that are currently being tested. In parallel we are testing whether UAS-Ras1N17 modifies pupal size when driven by Ras2-GAL4.

1.2.3 Generate transgenics for short-hairpin pWIZ-Ras2 construct.

As proposed, we generated several transgenic lines expressing a pWIZ-Ras2 gene knockdown construct. However, ubiquitous expression under the control of the Act5C-GAL4 driver did not cause any obvious phenotype in wild-type flies, and expression in NF1 mutants did not modify pupal size. Similar results were obtained using specific neuronal GAL4 drivers. However, in control RNA blots ubiquitous expression of pWIZ-Ras2 did not reduce the level of endogenous Ras2 mRNA. Although not proposed, we also generated two different pWIZ-NF1 short-hairpin constructs to test whether their expression would cause dominant small fly phenotype that might be useful in genetic screens. We again tested several lines for each construct, but none showed any NF1-like phenotype. Researchers in adjacent labs have successfully used the pWIZ short hairpin gene knockdown approach to suppress several non-neuronal genes. Thus, we believe that our failure to suppress Ras2 or NF1 may a problem with the pWIZ RNAi approach in neuronal cells. For the time being we have abandoned this experiment.

1.2.4 Test whether induction of four UAS-GRD-HA transgenes affects wing size, eye development, or overall development/viability.

At the time of proposal submission we had identified four Drosophila RasGAP-related proteins (NF1, Gap1, vap and SynGAP/CG32560). We have since identified a fifth evolutionary conserved potential RasGAP, CG1657, which interestingly also includes a Vps9 putative RabGEF domain. We had proposed to generate hybrid transgenes that included the catalytic domains of Gap1, vap and SynGAP in the context of full length NF1 backbones, as well as UAS-GRD-HA catalytic domain transgenes for the three initially identified non-NF1 RasGAPs. Our proposal was to use these transgenes to test for functional redundancy. Preliminary data suggested that hybrid NF1-Gap1-NF1 and NF1-vap-NF1 proteins did not rescue. However, while we have confirmed this result for the NF1-vap-NF1 hybrid, we have since found that the Gap1 hybrid is poorly expressed. Similarly, while the UAS-NF1-GRD-HA protein is expressed and rescues NF1 phenotypes, we have since found that in anti-HA immunoblots of transgenic fly lysates, none of the other three catalytic domains is detectable. Thus, we have changed tactics and focused on expressing full length RasGAPs. We previously used an available GAL4-inducible Gap1EP45 line to show that Gap1 does not substitute for NF1 in growth regulation. However, since a full length p120GAP ortholog does appear to rescue, we recently generated a full length UAS-Gap1 transgene to confirm that Gap1 does not rescue. The Gap1EP45 line was generated through random insertion of an enhancer-promoter containing transposon upstream of the Gap1 gene. We have confirmed that expression of Gap1EP45 in the eye causes a rough eye phenotype that can be suppressed through heterozygous loss of Ras1, as had been reported (Rorth, 1996). However, additional UAS-Gap1 lines may express higher transgene levels, allowing a more rigorous test of functional redundancy.

1.2.5 Clone Ras2 genomic segment in preparation for gene targeting.

Before embarking on the labor intensive process of generating a specific Ras2 mutant through gene targeting, we decided to first obtain more definitive evidence that Ras2 is the relevant target for NF1 in organismal growth control. Thus, rather than generating a Ras2 genomic clone in preparation for gene targeting, we first generated and analyzed pWIZ-Ras2 transgenics (see section 1.2.3). Although not originally proposed, we also generated and characterized Ras2 promoter driven GAL4 (Ras2-GAL4) transgenics (section 1.2.2). The Ras2-GAL4 drivers are expressed in a highly specific subset of cells in
the larval CNS, and driving UAS-NF1 in this pattern rescues NF1 pupal size. However, by crossing two deficiencies that include Ras2 into an NF1 background, we found that heterozygous loss of Ras2 does not modify the NF1 size defect. Thus, we are not yet convinced that Ras2 is the relevant target for NF1 in growth regulation, and we first plan to perform additional GAP assays comparing the catalytic activities of vap, Gap1, and NF1 against several Drosophila Ras related proteins, before attempting to generate specific Ras2 mutants.

Aim 3

1.3.1 Analyze CNS gene expression profiles of dnc1 and Nfl;dnc1 3rd instar larvae.

Microarray analysis of total RNA from developmentally synchronized, wandering stage 3rd instar wild-type and NF1 larvae revealed >2-fold changes in the expression of 169 genes. Several of these genes showed highly robust, up to 60-fold expression changes. However, similar experiments with RNA from dissected 3rd instar larval CNS identified only few genes whose expression was altered by at most 4.5-fold. For many genes these changes in expression were confirmed either by RNA blot or real time RT-PCR analysis. Among the genes that are misexpressed in NF1 CNS is dopa decarboxylase (ddc), whose expression is reduced 4.5-fold, the gene encoding the adipokinetic hormone precursor (akh), whose expression is increased 2-fold. To begin to dissect the mechanism underlying the non-cell-autonomous NF1 growth defect, we had proposed to analyze which CNS genes are restored to wild-type expression in genetically rescued flies. Specifically, we proposed to compare CNS expression profiles of dnc1 and NF1;dnc1 wandering stage 3rd instar larvae. Underlying this experiment was our observation, confirmed by others (Williams et al., 2001), that loss of the dnc cAMP phosphodiesterase rescued NF1 pupal size. However, when crossed into our most recent isogenized NF1/E2 mutant background, the dnc1 allele available in our lab no longer modified pupal size. We believe this reflects a problem with the dnc1 mutant, since dnc1 fly extracts showed no increase in cAMP, as had been reported (Byers et al., 1981). A dnc1 stock subsequently obtained from the Bloomington stock center also behaved abnormally and did not modify NF1 pupal size. Thus, as described below, we have focused on more direct experiments to reveal the mechanism responsible for the non cell-autonomous NF1 growth deficiency.

1.3.2 Analyze CNS gene expression profile of dCREB2 hypomorphic 3rd instar larvae.

NF1 and hypomorphic dCREB2 mutants show similar reductions in postembryonic size, and microarray analysis of whole 3rd instar NF1 and dCREB2 larval RNA showed similar changes in gene expression. Thus, since NF1 is required in CNS neurons to rescue overall size, and since only few genes are misexpressed in NF1 CNS, we proposed to compare the CNS gene expression profiles of developmentally synchronized, wandering stage 3rd instar NF1, dCREB2 and wild-type larvae. This analysis, performed in triplicate, showed that multiple genes are up or down regulated in dCREB2 CNS. Thus, while overall 3rd instar larval NF1 and dCREB2 gene expression profiles are very similar, only few genes show relatively small expression changes in NF1 larval CNS, while many more genes show more robust expression changes in dCREB2 CNS.

Alternatively spliced dCREB2 isoforms function as transcriptional activators and repressors (Yin et al., 1995), which may explain why many genes are either up- or down-regulated in dCREB2 CNS. The fact that NF1 and dCREB2 CNS expression profiles differ significantly is not compatible with the idea that NF1 is a major upstream regulator of adenylyl cyclase/PKA/dCREB2 signals. Further supporting this conclusion, ELISA assays detected identical cAMP levels in extracts of dissected wild type and NF1 CNS. These results do not rule out that NF1 acts as a regulator of the adenylyl cyclase/PKA/dCREB2 pathway in specific cells in the CNS. However, our results are also compatible with the hypothesis that NF1 alters the expression or secretion of a neuroendocrine factor, which activates the adenylyl cyclase/PKA/dCREB2 pathway in other cells, either in the CNS or elsewhere. Thus, the microarray analysis of dCREB2 CNS has
provided important clues, but have not yet revealed the precise mechanism whereby NF1 causes a cAMP-sensitive overall growth defect.

1.3.3 Induce tissue-specific expression of UAS-AKH and analyze Nf1 size rescue.

Adipokinetic hormone is an eight amino acid hormone that regulates insect energy metabolism in preparation for strenuous activity, such as flight. The akh gene codes for the 79 amino acid adipokinetic hormone precursor and is expressed in specific neuroendocrine cells of the Drosophila ring gland (Noyes et al., 1995). Microarray analysis of CNS RNA identified the akh gene as being down regulated by approximately 4-fold in NF1 mutants, which was confirmed by real-time RT-PCR analysis. Intriguingly, the akh and Ras2 genes are immediate genomic neighbors, but Ras2 expression does not appear to be altered in NF1 mutants. To analyze whether NF1 phenotypes are sensitive to increasing akh expression, we generated UAS-akh transgenics. Induction of this transgene with several neuronal GAL4 drivers, including the akh-GAL4 driver (see below) did not modify the NF1 size defect. Thus, adipokinetic hormone does not appear to be involved in the NF1 size defect.

1.3.4 Generate and characterize AKH and AKH receptor promoter driven GAL4 lines.

We generated a transgene that has the putative akh transcriptional promoter inserted upstream of the GAL4 coding region. Transgenics harboring this construct were obtained and their transgenes mapped. These flies were subsequently crossed to UAS-GFP or UAS-NF1 transgenics. As expected, the akh-GAL4 driver induced UAS-GFP expression in a very small number of cells in the neuroendocrine larval ring gland (not shown). Driving UAS-NF1 in this highly specific pattern did not modify NF1 pupal size. Expressing UAS-NF1 under the control of several other ring gland drivers also does not affect NF1 pupal size. CG3441/Nplpl is the only neuropeptide gene whose expression is increased (by about 2-fold) in NF1 CNS. Although not proposed, we generated several UAS-CG3441 transgenics as well as several lines that harbor a CG3441 promoter-driven GAL4 construct. The latter lines drive UAS-GFP expression in highly specific cells in the CNS ventral ganglion and the midbrain region (Figure 3). This pattern strongly resembles the endogenous Nplpl expression pattern (Verleyen et al., 2004). Recent results indicate that CG3441-GAL4 driven UAS-NF1 expression does not alter NF1 pupal size. However, Drosophila neuroendocrinologist Dr. Paul Taghert has generated CG3441 hypomorphic mutants and identified a behavioral phenotype. We are interested in determining whether this phenotype resembles the uncoordinated phenotype of NF1 mutants (The et al., 1997). If these phenotypes are related, it will be interesting to test whether altered CG3441 expression plays any role. We have started a collaboration with Dr. Taghert to analyze this and other possibilities.

Figure 3; CG3441-GAL4 driven UAS-GFP expression in 3rd instar larval CNS.

All experiments proposed under this aim have the broad goal of identifying the precise mechanism whereby loss of NF1 in the larval CNS causes an overall growth defect. As described in our grant proposal, several of our preliminary results argued against the idea that NF1 mutants are small because they are nutritionally deprived. Insulin is a major neuroendocrine growth regulator in Drosophila, but several additional results argued that insulin signaling was normal in NF1 mutants. However, to further analyze a potential role for insulin, we analyzed the life span of NF1 mutants, given that some mutants with attenuated insulin signaling have a significantly increased life span (Clancy et al., 2001; Tatar et al., 2001). However, the life span of NF1 mutants and isogenic controls was very similar. Several insulin pathway mutants also develop more slowly. Interestingly, when we analyzed developmental timing, NF1 mutants were found to undergo significantly accelerated development. We performed this experiment by comparing trans-heterozygotes for two NF1 EMS alleles (NF1F1/NF1F2) to isogenic control flies. In these experiments the NF1 mutants pupariated at least 10 hours before controls. Wild-type flies undergo a
developmental transition 70 hours after egg laying (Beadle et al., 1938). Thus, Drosophila larvae die when transferred to a sucrose-only diet before this time. When larvae are transferred to sucrose after 70 hrs, they continue to develop and pupariate at a reduced size. Unlike control flies, we found that NF1 mutants survive and continue to develop when transferred to sucrose as early as 60 hours after egg laying. Thus, by at least two measures NF1 mutants develop more rapidly. Initially we were very excited about this finding, because most growth in insects occurs during larval development, and because accelerated development appeared to provide a unique explanation for the small size of NF1 mutants. However, we have since found that accelerated development is a dominant phenotype specific to the NFIE2 allele. Thus, neither NFIEI nor the original NF1P mutant exhibits accelerated development, and the accelerated development of NFIE1/NFIE2 trans-heterozygotes is not rescued by expressing an NF1 transgene. While we remain interested in determining whether accelerated development is a dominant phenotype of the NFIE2 early truncation mutant, or is caused by an unrelated second mutation, we no longer believe that accelerated development provides an explanation for the reduced size phenotype of NF1 mutants.

1.3.5 Characterize UAS-dCREB2 isoform transgenics.

We had proposed to analyze whether the size defect of dCREB2 mutants is cell autonomous or non-autonomous. We also proposed analyzing whether dCREB2 and NF1 are required in the same or in different cells to rescue phenotypes. For these experiments we made transgenics representing two dCREB2 isoforms with opposite roles in transcription and olfactory learning. UAS-dCREB2a (activator) and UAS-dCREB2b (antagonist) constructs were made, and several transgenic lines obtained for each construct. The UAS-dCREB2a transgene has been analyzed most extensively so far. This transgene expressed under the control of the widespread 69B-GAL4 driver rescued the lethality of dCREB2 females. The same driver and transgene combination also partially rescued the pupal size defect of dCREB2 mutant males (dCREB2 male pupal size 2.40 mm, rescued to 2.65 mm by 69B-GAL4; UAS-dCREB2a. Average wild-type male pupal size is 2.86 mm). The hsp70-GAL4 driver also allowed partial male pupal size rescue in the absence of heat shock induction. By contrast, the elav-GAL4 neuronal or repo-GAL4 glial drivers did not rescue. Finally, expression of UAS-dCREB2a under the control of two neuronal drivers (elav-GAL4 and c23-GAL4) did not modify the NF1 size defect. Obvious follow-up experiments to determine whether widespread expression of UAS-dCREB2 rescues the NF1 size defect are in progress.

Aim 4

1.4.1 Generate and characterize transgenics containing 17.3 kb genomic Nfl transgene.

At the start of this project a technician was hired to perform these experiments. Unfortunately he left the lab to enter medical school nine months later, before generating this transgene.

1.4.2 Provide transgenic line to Michael Stern to test rescue of glial thickening phenotype.

For reasons explained in section 1.4.1, the generation of this transgenic line has been delayed.

1.4.3 Generate Nfl promoter – GFP reporter constructs.

We did generate two constructs that contained approximately 50% and 100% of the genomic region between the 5'-end of NF1 and the 3'-end of the upstream CG8968 gene, inserted upstream of the EGFP (enhanced green fluorescent protein) coding region. We also generated constructs in which the same putative NF1 promoter segments were inserted upstream of the GAL4 coding region. Transgenics harboring in which a UAS-GFP reporter is expressed under the control of the two NF1-GAL4 drivers do not recapitulate the highly distinctive endogenous NF1 expression pattern. The same is true for transgenics harboring the NF1 promoter driven EGFP reporter constructs. Thus, the genomic segment between the 5' end of NF1 and the 3'-end of CG8968 does not appear to include all promoter/enhancer elements required for the neuronal specific expression of NF1. Thus, we will renew our efforts to generate the 17.3 kb genomic transgene and determine whether this region includes all regulatory elements.
required for endogenous expression. We note that during the past year three transcription factors have been implicated in regulating the expression of mammalian neurofibromin (Zhu et al., 2004; Chong et al., 2005; Yang et al., 2005). We will continue to monitor this literature and analyze whether Drosophila orthologs of mammalian transcription factors play similar roles.

**Key Research Accomplishments:**

- Identified 78 amino acid evolutionary conserved C-terminal neurofibromin segment that appears essential for size rescue.
- Established that Drosophila NF1 has GAP activity towards Ras1 and Ras2.
- Generated and characterized Ras1-GAL4 and Ras2-GAL4 transgenic driver lines.
- Established that UAS-NF1 expression under the control of the Ras2-GAL4 driver rescues the NF1 size defect.
- Established that Ras2-GAL4 driven expression of activated mutants of either Ras1 or Ras2 mimics the NF1 size defect.
- Established that ablation of Ras2 expressing CNS cells results in small pupae that do not eclose.
- Obtained preliminary evidence that expression of full length UAS-vap rescues NF1 pupal size.
- Generated and characterized UAS-akh and akh-GAL4 transgenic lines.
- Established that manipulating akh expression does not modify NF1 size defect.
- Established that expressing UAS-NF1 in akh pattern, or in other ring gland cells, does not modify NF1 size defect.
- Generated and characterized UAS-CG3441 and CG3441-GAL4 lines.
- Established that expressing UAS-NF1 in CG3441 pattern does not modify size defect.
- Identified and characterized accelerated development phenotype specific to NF1E2 early truncation mutant.
- Identified genes that are misexpressed in dCREB2 larval CNS.
- Generated and characterized transgenic lines expressing wild-type and mutant dCREB2 isoforms.

**Reportable outcomes**

**Publications:**

None so far.

**Abstracts:**


**Conclusions:**

We achieved most aims identified for year 1, and several aims planned for year 2. We continue to work towards at least one major manuscript that shows that the role of NF1 in organismal growth control is non-cell-autonomous, and that defective GAP activity is the proximal cause of this cAMP-sensitive NF1 phenotype. Submission of thus manuscript has been delayed, because our results argue that GAP activity is essential for in vivo rescue, whereas Zhong et al find that GAP activity is not required. Much of our effort in the past few months had been aimed at resolving this important discrepancy.
References:


