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14. ABSTRACT: The long term goals of this research are to understand the mechanisms by which <i>NF1</i> and its partners control growth using the Drosophila peripheral nerve as our assay system. This system is advantageous because we can apply a number of powerful molecular genetic methodologies that are not available in other systems. This project addresses four specific aspects of growth control, two of which were begun during the first twelve months of funding, and the third was begun during this funding year. Our major findings continue to be generated from aim #4. This year we found that co-overexpression of <i>PI3KCAAX</i> and <i>Akt</i> within peripheral glia conferred a striking increase in perineurial glial thickness compared with overexpression of each transgene individually. We also found that overexpression of the transcription factor <i>FOXO</i> within peripheral glia strongly suppressed the growth promoting effects of <i>PI3KCAAX</i> . This result suggests that <i>PI3K</i> activates perineurial glial growth by inhibiting <i>FOXO</i> . Finally, we found that the increase in perineurial glial nuclei number conferred nonautonomously by <i>RasV12</i> is mediated by both <i>PI3K</i> and <i>Raf</i> . This result suggests that perineurial glial cell growth can be genetically uncoupled from perineurial glial cell number.						
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INTRODUCTION

Over the last several years, my lab has been developing the *Drosophila* peripheral nerve as a system with which to identify and study the signalling pathways controlling growth of the perineurial (outer) glial layer. To accomplish this goal, we apply the various molecular genetic methodologies uniquely available in *Drosophila*; we hope that these methodologies will enable us ultimately to identify all of the relevant genes that interact with *NF1* to control growth, and place *NF1* and these partner genes in as complete a mechanistic context as possible. Then this mechanism could be tested and refined in systems more similar to humans but more difficult to work with (e.g. the mouse). Because all of the experiments are performed on the acutely dissected third instar larva, there are no complications or caveats associated with experimentation on cell culture systems, and we assay the entire nerve cross section as it exists within the whole organism. We thought that a more complete mechanistic understanding of growth control within peripheral nerves would greatly facilitate the ability to design drugs able to combat neurofibromas. Within this larger context, I proposed four different tasks to investigate various aspects of the genetic control of growth within peripheral nerves. These tasks involve elucidation of the relationship among Neurofibromin, pushover, and protein kinase A, as well as the identification of signalling pathways downstream of Ras that affect growth within peripheral nerves. For this funding period (months 13-24) I completed work on task #1, began work on task #3, and continued work on task #4.

BODY

Task one (completed): Does Neurofibromin activate PKA? Several experiments were proposed in the grant application to address this possibility, and many of these were completed during the first year of funding. However, as discussed in last year's report, the completed experiments gave inconclusive and in some cases conflicting results, making it impossible to place the data in a mechanistic framework. Last year, I reported that a large amount of larva to larva variability in nerve thickness was making it difficult to obtain meaningful data, and suggested the possibility that genetic background effects might be obscuring the genetic effects under investigation. To address this possibility, we re-tested the effects of the *NF1^{P2}* null mutation on perineurial glial growth in larvae expressing *Ras^{V12}* in the peripheral glia, but this time used stocks in which each genetic element was isogenized (by five successive back-crosses) to our isogenic wildtype stock. We found that in an isogenic genetic background, the *NF1^{P2}* mutation actually enhanced, rather than suppressed, the growth promoting effects of *Ras^{V12}*. In particular, perineurial glial thickness in *NF1^{P2}; gli-Ras^{V12}* larvae was 3.1 +/- 0.2 μm (n=13), which is significantly thicker than glial thickness in *NF1⁺; gli-Ras^{V12}* (2.2 +/- 0.1). This unexpected result is definitive, as this result controls for genetic background effects. The enhancement of *Ras^{V12}* by *NF1^{P2}* could be the result of loss of *NF1* in the perineurial glia, or the peripheral glia. We will distinguish between these possibilities by determining if expression of *NF1⁺* specifically in the peripheral glia can rescue this phenotype. All of the necessary mutations and transgenes have been isogenized and are available for stock construction and testing.

Task three: Does pushover (push) act in the PKA pathway or a parallel pathway to control perineurial glial growth? Work on this task was scheduled to start at month 30. We decided to begin some stock constructions early so that the stocks would be available on schedule. To address the question posed in this task, we first needed to construct a double mutant defective in both *push* and a *PKA* null allele (*PKA^{H2}* is the allele chosen). Because *PKA* and *push* are both on chromosome 2, this construction requires recombination between the two genes. These genes are located about 3 map units. This distance is short enough to make acquisition of the desired recombination event difficult, but not so short as to make it impossible. We crossed *push* flies to *PKA^{H2}* flies and set up about 35 lines carrying

recombinant chromosomes. We tested them all, and found that none of them carried the necessary recombination event. I don't think that this failure reflects an underlying problem with the experimental procedure, but rather it likely reflects random nature of obtaining recombinants. We'll try again and set up more fly lines (about 60) carrying recombinant chromosomes. I am optimistic that we will succeed in obtaining the desired line this time.

We also have begun constructing the lines that will identify the cell type (neuronal or glial) in which *push* must function to control peripheral nerve growth. As described in the grant proposal, we will express our *UAS-push^{RNAi}* construct in motor neurons and peripheral glia to identify the cell type within which *push* knockdown confers the growth phenotype. The construction of *push^{RNAi}; D42-GA:4* (motor neuron *GAL4* driver) is in its final stage of construction and soon will be evaluated for the nerve growth phenotype. Construction of the other required lines will begin in the next few months.

Task four: Identification of additional Ras signalling components regulating perineurial glial growth. I proposed to conduct experiments for this task during the entire period of the award. As was the case during the first year of funding, progress on this task during the second year was greater than progress in the other tasks. In this task, I proposed to determine if Ras acted through the Raf-MAP kinase pathway, or the PI3 kinase, to exert its non-autonomous, growth promoting effects. After this identification was successfully completed, I then proposed to follow the identified signalling pathway further downstream by testing the effects of altering the activity of known downstream components. Last year we provided several lines of evidence that PI3 kinase activity mediates the nonautonomous, growth promoting effects of Ras. This year we have completed the experiments that prove this conclusion. Further, last year we found that the kinase downstream of PI3 kinase (called Akt or PKB) is also necessary for this growth promoting effect. This year we found that Akt activity is sufficient for this growth-promoting effect as well. This year we have provided evidence suggesting that the transcription factor FOXO mediates the effects of PI3K/Akt as a negative regulator. Finally, this year we have begun investigating the signalling pathways that mediate the nonautonomous effect of *Ras^{V12}* on perineurial glial nuclei number. We found that the effects of *Ras^{V12}* are mediated by both Raf and PI3K. The experimental details for these findings are described below.

Last year we showed that the heteroallelic loss of function PI3 kinase mutations *PI3K^A* (a deletion of PI3 kinase) and *PI3K^{2HI}* (a strong hypomorph) significantly suppressed the growth-promoting effects of *Ras^{V12}* expression. This result demonstrated that PI3 kinase activity is necessary for the growth-promoting effects of *Ras^{V12}*. To demonstrate that PI3 kinase activity is necessary in the peripheral glia, we co-expressed both *Ras^{V12}* and a dominant-negative *PI3K* mutation specifically in the peripheral glia. We found that expression of the dominant-negative *PI3K* significantly suppressed the effects of *Ras^{V12}* on perineurial glial growth. These data were presented in Figure 2 from last year's report and are included in this report in Figure 1 to enable comparisons with additional experiments. These results demonstrated that PI3 kinase activity is required in the peripheral glia. However, these results left open the possibility that at least part of the growth-suppressing effects of *PI3K^A/PI3K^{2HI}* might be due to loss of PI3K activity in the perineurial glia as well.

This year we tested this latter possibility. In particular, we introduced the *PI3K^A/PI3K^{2HI}* heteroallelic combination into larvae expressing *PI3KCAAX* in the peripheral glia and found that this combination, which strongly suppressed the effects of *Ras^{V12}*, had no significant effect on the ability of *PI3KCAAX* to increase perineurial glial growth nonautonomously (Figure 1 below). This result, combined with results presented last year, demonstrate that PI3K activity in the peripheral glia is both necessary and sufficient to increase perineurial glial growth.

This year we also continued studying genes acting downstream of PI3K. Last year we showed that loss of function mutations in Akt, a kinase activated by PI3K activity, suppressed the growth-promoting effects of PI3K in a dose-dependent manner (see Figure 3 from last year's

Figure 1: PI3K activity is required in the peripheral glia to promote perineurial glial growth

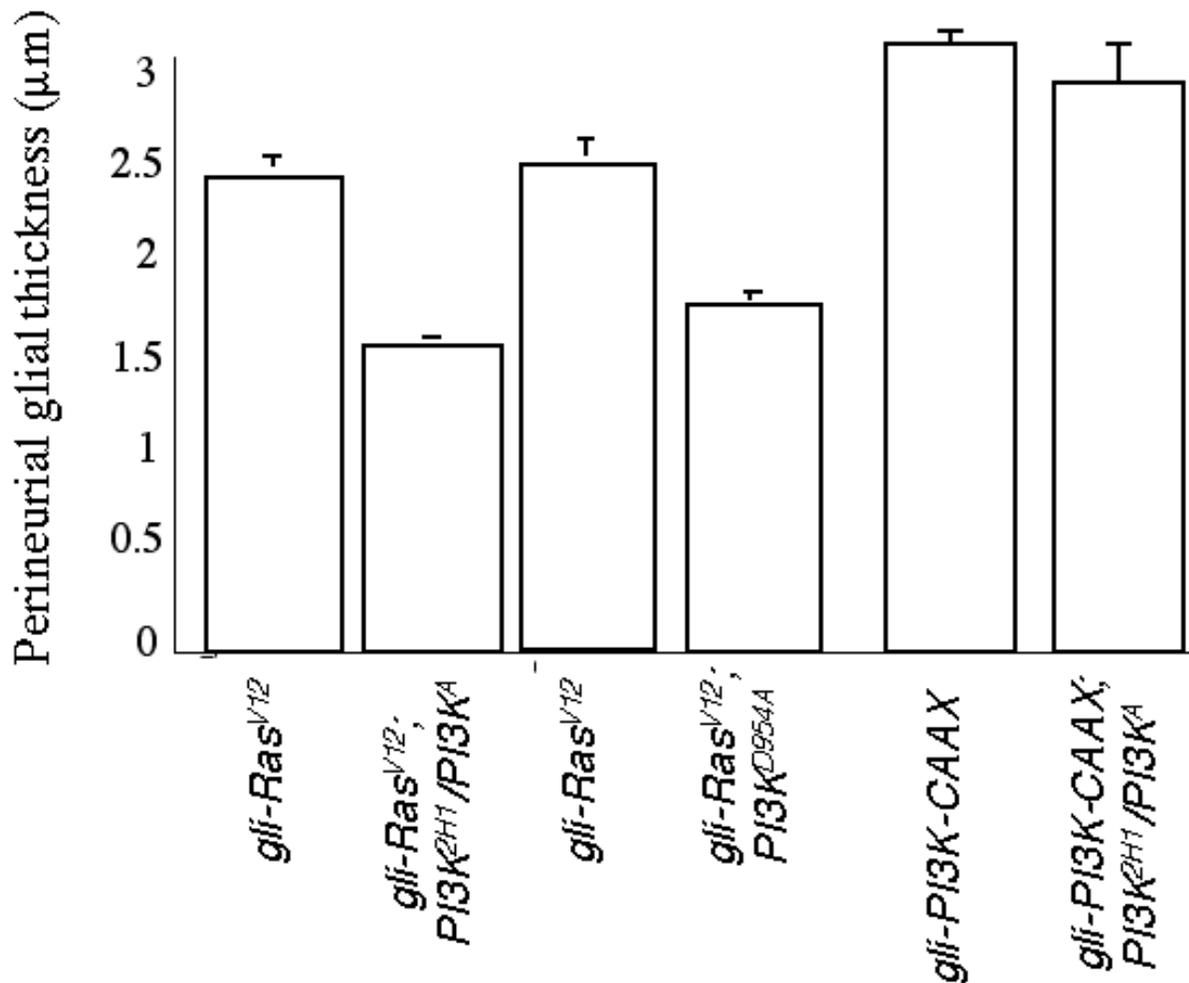


Figure 1: Y-axis: perineurial glial thickness (microns) from larval peripheral nerves of the indicated genotypes (X-axis). Values indicate means +/- SEMs. For *gli-Ras^{V12}*, (lane #1), n=50, vs *gli-Ras^{V12}; PI3K^A/PI3K^{2H1}*, n=85, p< 0.0001. For *gli-Ras^{V12}* (lane #3), n=72 vs. *gli-Ras^{V12}-PI3K^{DN}*(n=49), p< 0.0001. For *gli-PI3K-CAAX* (lane#5), n=42 vs. *gli-PI3K-CAAX; PI3K^A/PI3K^{2H1}*, n=11, p=.43 Reduction of PI3K activity in the peripheral glia (via expression of the dominant-negative *PI3K^{D954A}* transgene) or globally (via introduction of the heteroallelic loss-of-function combination *PI3K^{2H1}/PI3K^A*) strongly suppress the growth-promoting effects of *Ras^{V12}* expression (first four lanes), but not *PI3K-CAAX* expression (last two lanes).

report). This result demonstrated that Akt activity is necessary for the growth-promoting effects of PI3K activity. This year, we tested the possibility that Akt activity is sufficient to increase perineurial glial growth, and can do so nonautonomously. Although we found that overexpression of *Akt⁺* in the peripheral glia had no effect on perineurial glial growth, co-overexpression of *PI3KCAAX* and *Akt* conferred significantly greater perineurial glial growth than overexpression of either transgene combined (Figure 2A below). In particular, perineurial glial thickness in larvae overexpressing both *PI3KCAAX* and *Akt* was over 6 µm. This result supports the idea that *Akt* acts in the peripheral glia to control perineurial glial growth as a downstream effector of *PI3K*.

Then we began to test effects of genes acting downstream of *Akt*. One such gene is *FOXO*, a transcription factor that negatively regulates expression of PI3K-Akt-induced genes. *Akt* inhibits *FOXO* activity by phosphorylating *FOXO* and causing phospho-*FOXO* to be sequestered in the cytoplasm (Puig et al., 2003). If PI3K-Akt promotes perineurial glial growth by inhibiting *FOXO*, then we predict that we should be able to overcome this growth promotion by *FOXO* overexpression. To test this possibility, we co-expressed *PI3K* and *FOXO* (using both of two independent *UAS-FOXO* constructs that were kindly provided by Marc Tatar) in the peripheral glia. We found that overexpression of *FOXO* suppressed the effects of *PI3KCAAX* overexpression on perineurial glial growth (Figure 2B below). This result is consistent with the possibility that PI3K nonautonomously promotes perineurial glial growth by inactivating *FOXO*.

We also studied the signalling pathways downstream of *Ras* that nonautonomously promote perineurial glial nuclei number. We previously found that expression of *Ras^{V12}* in peripheral glia increased perineurial glial nuclei number. We found that this increase is mediated by both *Raf* and *PI3K*: expression of either constitutively active *Raf* (*Raf^{F20}*) or *PI3KCAAX* in the peripheral glia increased perineurial glial nuclei number, although to a somewhat lesser extent than for *Ras^{V12}*

Figure 2A: Akt activity is required in the peripheral glia to promote perineurial glial growth

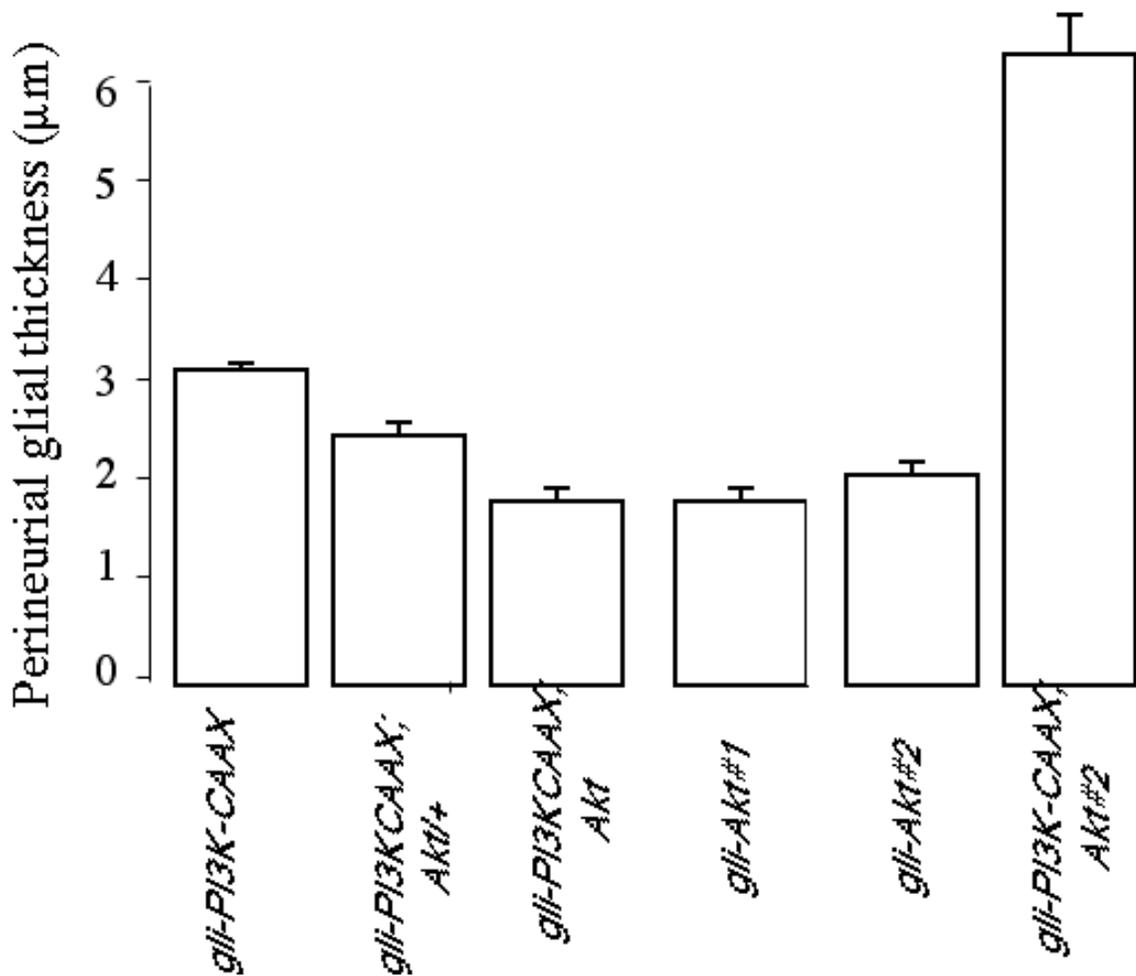


Figure 2B: FOXO mediates the nonautonomous effects of PI3K on growth

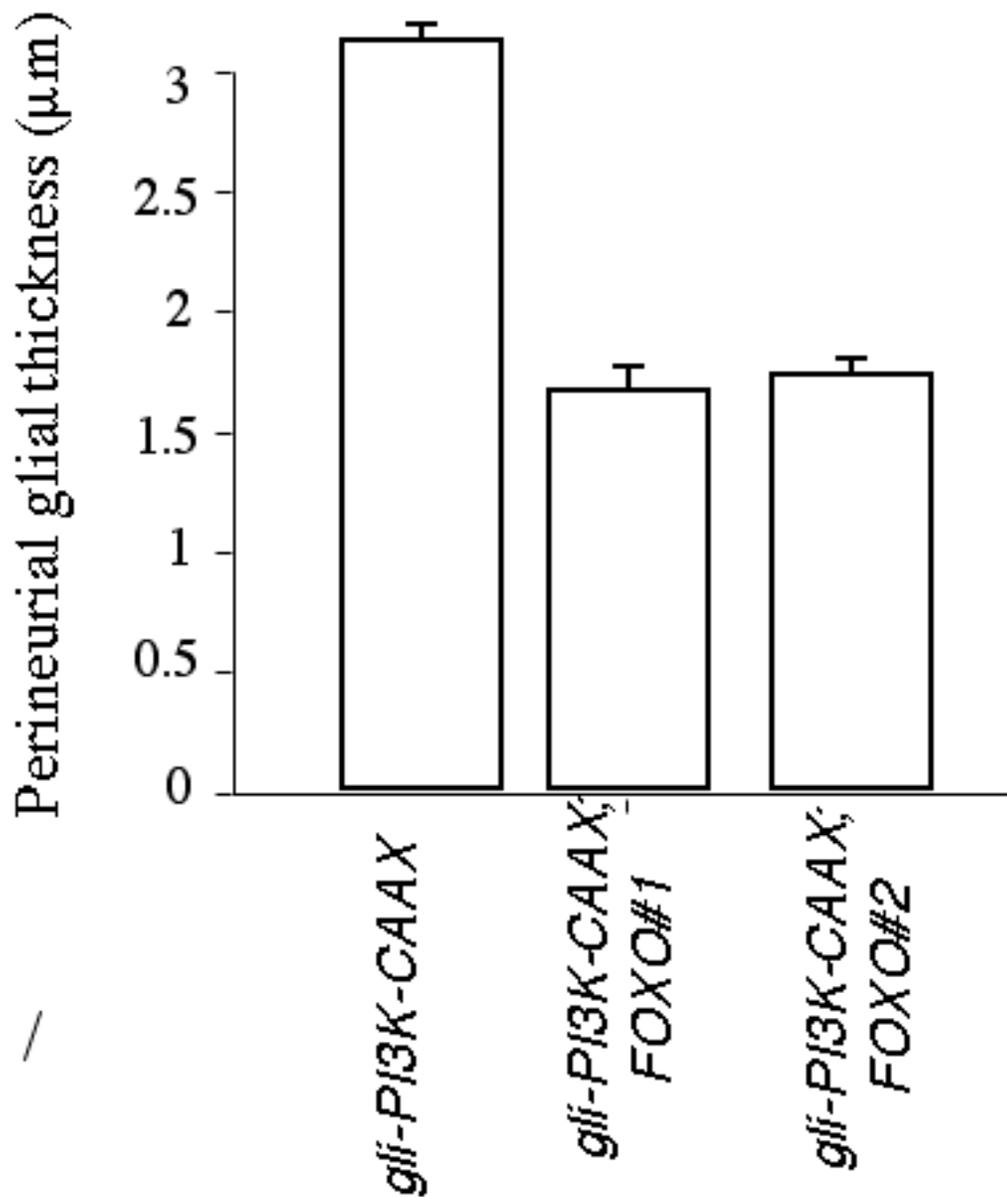


Figure 2: Y-axis: perineurial glial thickness (microns) from larval peripheral nerves of the indicated genotypes (X-axis). Values indicate means +/- SEMs. A. For *gli-PI3KCAAX*, (lane#1), n=42, vs. *gli-PI3KCAAX; Akt⁴²²⁶/+* (lane #2), n=29, p=0.0024; and vs. *gli-PI3KCAAX; Akt⁴²²⁶* (lane#3),n=29, p<0.0001. *gli-Akt #1* (lane#4), and *gli-Akt #2* (lane#5) exhibit perineurial glia of wildtype thickness. However, *gli-PI3K-CAAX; Akt #2* (lane #6), n=28 is significantly different from *gli-PI3K-CAAX* (lane#1): the p value between these genotypes is <0.0001. B. For *gli-PI3KCAAX*, (lane#1), n=42, vs. *gli-PI3KCAAX; FOXO #1*, (lane#2), n=29, p<0.0001, and vs. *gli-PI3KCAAX; FOXO #2*, (lane#3), n=30, p<0.0001

expression (Figure 3 below). Co-expression of *Raf^{f20}* and *PI3KCAAX* further increased mean perineurial glial nuclei number, although the resulting value was not significantly different from the values obtained

when either *Ras*^{V12}, *Raf*^{gof}, or *PI3K-CAAX* were expressed alone (data not shown). These results support the notion that Ras exerts its effects on nuclei number via both Raf and PI3K.

Finally, we found that larvae homozygous for the *NF1*^{P2} mutation (following five backcrosses to our isogenic, wildtype, stock) exhibited an increase in perineurial glial number equal to that conferred by expression of *Ras*^{V12} in glia (40.2 +/-4.2 nuclei per mm of nerve). This result is consistent with the possibility that loss of *NF1* in the peripheral glia is increasing perineurial glial nuclei number by activating Ras. However, it is also possible that *NF1* is required in the perineurial glia to control perineurial glial nuclei number. This issue will be examined during experiments planned for the upcoming year (see Future Goals).

Our results strongly suggest that the peripheral glia produces factors that control both perineurial glial cell size and cell number. These two processes appear to be regulated somewhat differently: cell growth is regulated primarily by PI3K, with Raf contributing only a minor amount, whereas cell number is regulated by both PI3K and Raf.

Figure 3: Ras nonautonomously increases perineurial glial nuclei number via Raf and PI3K

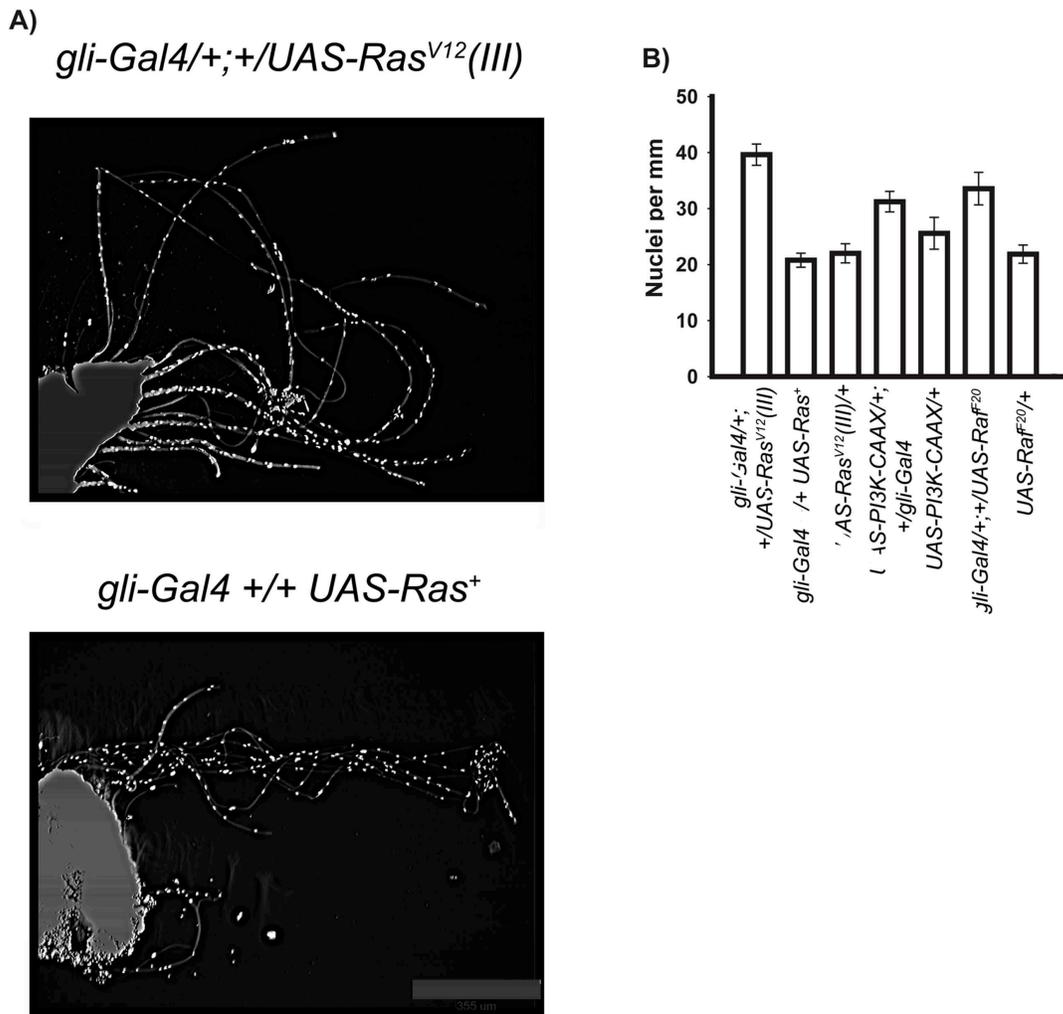


Figure 3: A) Images of fluorescent images of peripheral nerves from larvae carrying *gli-GAL4* and *UAS-Ras*^{V12} (left panel) and *gli-GAL4* and *UAS-Ras*⁺ (right panel). The large, nucleus-rich structure on the right of the right panel is the ventral ganglion. B. Means and SEMs of perineurial glial nuclear

density from larvae of the indicated genotypes. Means were calculated by measuring the nuclear density (number of nuclei/ mm) of individual nerves, and then averaging these values for each nerve. At least 19 nerves containing at least 340 nuclei were averaged for each data point. The following combinations had statistically significant differences: *gli-Ras^{V12}* vs. *gli-Ras⁺*: $p < 0.0001$; *gli-PI3K-CAAX* vs. *PI3K-CAAX*: $p = 0.0184$; *gli-Raf^{F20}* vs. *Raf^{F20}*: $p = 0.0005$

FUTURE GOALS FOR TASK #4;

First, I plan to determine if the increase in perineurial glial nuclei conferred by the *NFI^{P2}* mutation is the result of loss of *NFI* in the peripheral glia, rather than the perineurial glia. To address this question, we first will determine if nuclei number is rescued by expression of *NFI⁺* specifically in the peripheral glia. The necessary transgenes are available and have been isogenized, and the stock needed is currently under construction. If we find that *NFI* is required in the peripheral glia, then I propose to test the role of putative upstream activators of *NFI* (such as *amnesiac*) in this process. The necessary stocks are in hand to perform these experiments as well.

Next, I plan to complement our studies on Akt/FOXO by seeing if another prominent Akt-dependent pathway (the Tsc1/Tsc2-Tor pathway) also contributes to the regulation of perineurial glial growth. I propose to see if expression within the peripheral glia of transgenes that activate this pathway increase perineurial glial growth, and also to co-express (with PI3K) transgenes that inhibit signalling through this pathway to see if this expression suppresses the effects of PI3K activation on perineurial glial growth. Flies carrying all necessary transgenes are available, and many have already been provided or promised to us.

We will also evaluate the role of signalling molecules downstream of Raf and PI3K in the regulation of perineurial glial nuclei number. We will be performing experiments that are conceptually similar to what we have done to understand the control of perineurial glial growth. In particular, we will express transgenes encoding wildtype, constitutively active, or dominant-negative versions of signalling molecules downstream of Raf (such as Erk and the transcription factors Creb, pointed and yan) and PI3K (including the molecules described above). Then the effects of this overexpression on nuclei number will be evaluated as described above. From these experiments, we will gain a much clearer idea of the molecular mechanisms that measure cell growth and cell number within peripheral nerves.

KEY RESEARCH ACCOMPLISHMENTS

PI3K nonautonomously increases perineurial glial growth via activation of the kinase Akt and the consequent inhibition of the transcription factor FOXO.

The constitutively active Ras^{V12} nonautonomously increases perineurial glial cell number by activating both Raf and PI3K.

Mutations in *NFI*, similar to peripheral glial Ras^{V12} expression, increase perineurial glial cell number.

REPORTABLE OUTCOMES

None

CONCLUSIONS

Task one is completed and our conclusions are disappointing. When we tested carefully isogenized lines, we were unable to reproduce previous observations concerning the effects of altered *NFI* and *PKA* activity on perineurial glial thickness in the presence of Ras^{V12} expression. In fact, with

isogenized lines, we found that the *NFI^{P2}* null mutation actually enhanced, rather than suppressed, the growth-promoting effects of Ras^{V12}. I conclude that the effects described previously were the result of genetic background effects rather than a real biological phenomenon.

In contrast, our experiments within task #4 continue to be successful. We have confirmed and extended the observations reported last year that the kinase Akt mediates the nonautonomous growth-promoting effects of PI3K activity. In addition, we showed that overexpression of the transcription factor *FOXO*, which is phosphorylated and inhibited by Akt, suppresses the growth-promoting effects of PI3KCAAX overexpression. These results identify a Ras-PI3K-Akt-FOXO pathway as a major regulator of nonautonomous perineurial glial growth. We have also found that the control of perineurial glial nucleus number is regulated nonautonomously by somewhat different mechanisms from perineurial glial growth. In particular, we found that alteration of Raf activity has little effect on perineurial glial growth, but these alterations confer significant effects on perineurial glial number. This year we will identify the molecules that act downstream of Raf and PI3K in glial nucleus number and see if similar or different molecules mediate the growth effect and the nuclei number effect. Finally, we have found that the *NFI^{P2}* mutation increases perineurial glial nucleus number, to the same extent as *Ras^{V12}* expression. This is the first peripheral nerve effect of *NFI^{P2}* (in an otherwise wildtype background) that we have found and we are particularly interested in pursuing this effect more completely this year.

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