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14. ABSTRACT

Our purpose is to examine the role of neurofibromin in modulating the survival of embryonic sensory and sympathetic neurons. To understand how reduced neurofibromin levels might impact the survival responses to activity-mediated signaling (mimicked with KCl) and to neurotrophins, we used dissociated cultures of Nf1+/- and exon23a-/- sensory and sympathetic neurons in an NGF withdrawal paradigm. Reduction or elimination of neurofibromin through targeted mutation leads to a diminished apoptotic response when NGF is removed, and also results in an improved response to activity-mediated survival signaling. Thus, Nf1-deficient neurons may be more sensitive to signaling interactions in the developing nervous system, and may be more resistant to environmental insults (low levels of survival factors, hypoxia, DNA damage) that promote apoptotic death. To begin to address possible mechanisms of enhanced survival in Nf1-deficient neurons, we are examining the contributions of Egln3 and SDHD to modulating apoptosis in precursors and neurons of the peripheral nervous system.

15. SUBJECT TERMS
NF1, sympathetic neuron, sensory neuron, neuronal apoptosis, activity-mediated survival signaling, neurotrophins, NGF, EglN3, succinate dehydrogenase, DNA damage, DNA repair

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INTRODUCTION

Absence of neurofibromin (Brannan et al., 1994; Jacks et al., 1994) leads to the neurotrophin-independent survival of embryonic sensory and sympathetic neurons (Vogel et al., 1995; Vogel and Parada, 1998). Although much of the pro-apoptotic action of neurofibromin can be attributed to its negative regulation of Ras signaling (Vogel et al., 2000), other interactions of this large protein are undoubtedly important in regulating the survival response. Over the past year, we have completed characterizing responses of neurofibromin-deficient (*Nf1*+/-, *Nf1*-/-, and *exon23a*-/-) dorsal root ganglion (DRG) sensory neurons and superior cervical ganglion (SCG) sympathetic neurons to a variety of survival and apoptotic stimuli. Through collaboration with Dr. Patricia Dahia (UTHSCSA) we have also expanded the project to include non-Ras interactions of *Nf1* in the context of neuronal survival in the peripheral nervous system.

BODY

Neurofibromin deficiency: effects on activity-mediated survival signaling and sensitivity to neurotrophins (Task 1. Characterize responses of *Nf1*+/+ and *Nf1*+/- DRG and SCG neurons to activity-mediated survival signaling *in vitro*, and Task 3. Characterize synergy of neurotrophin- and activity-mediated survival signaling in *Nf1*+/+ and *Nf1*+/- DRG and SCG neurons.)

We have completed our experiments with embryonic sensory (DRG, trigeminal) and sympathetic (SCG) neurons, isolated at 4 different stages (E13, E15, E17, E18), and acquired sufficient data for statistical analyses; this was the "sticking point" for publication of this work. We expect to submit the first of two manuscripts later this year**. Figures 1 and 2** show representative examples of data and experimental paradigm, with neurons isolated from mouse embryos harboring an exon23a targeted mutation that eliminates type II neurofibromin.

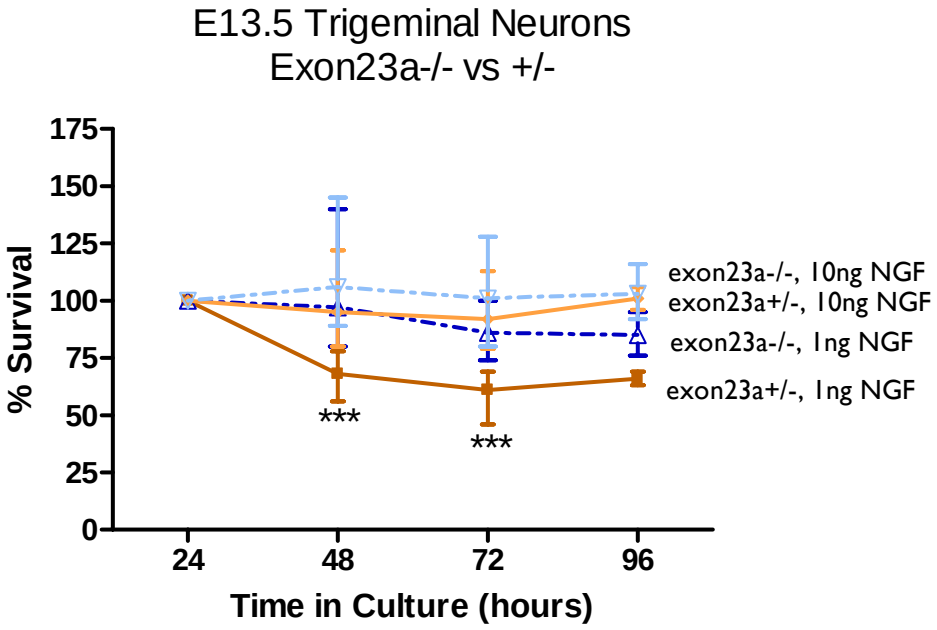

Figure 1. Embryonic trigeminal sensory neurons that lack the exon23a variant of neurofibromin (type II neurofibromin) are more sensitive to the survival-promoting effects of nerve growth factor (NGF). Cohorts of dissociated neurons, maintained in optimal and suboptimal concentrations of nerve growth factor (NGF), were monitored each day over a period of 4 days.

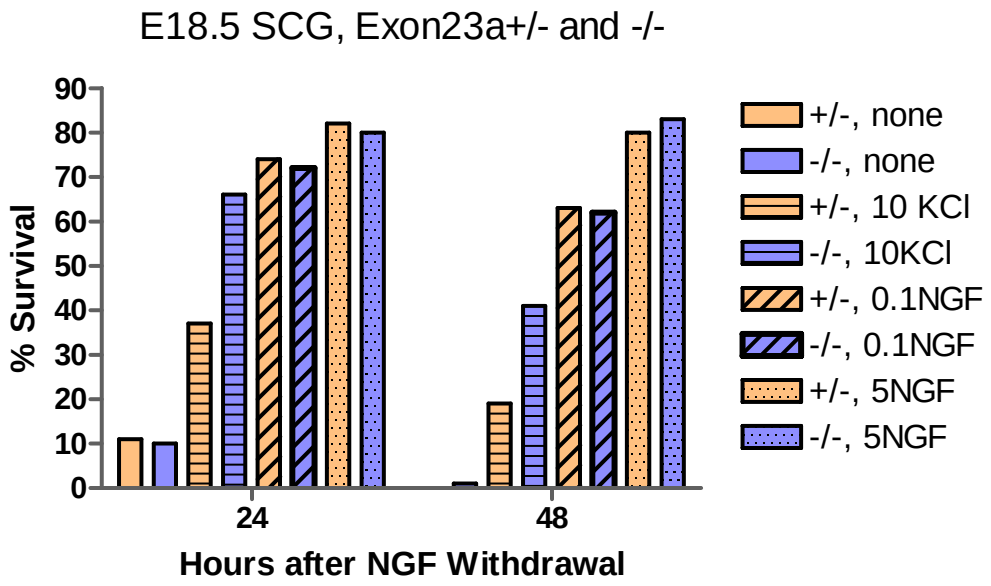

Figure 2. Embryonic SCG neurons that lack type II neurofibromin are more sensitive to the survival-promoting effects of KCl (depolarization). NGF was withdrawn from cohorts of dissociated E18.5 neurons after 72 hours in culture, and survival was monitored at 24 and 48 hours after neurotrophin removal.

Our results with E18 DRG and SCG neurons indicate that the differential response of *Nf1*+/+ and *Nf1*+/- neurons may become more significant in late embryonic, and possibly postnatal, development.

Differential response of *Nf1*-/- neurons to neurotrophin withdrawal and other apoptotic stimuli (Task 4. Characterize the role of neurofibromin in mediating neuronal apoptosis following neurotrophin withdrawal.)

We propose that target contact, and concomitant exposure to neurotrophins, initiates the development of neurotrophin dependence in peripheral neurons, even if they lack neurofibromin. Environmental cues encountered by growing axons *en route* to the target undoubtedly influence acquisition of neurotrophin dependence; in cultures of ganglia isolated from developing embryos, some neurons may have extended axons towards or even contacted the peripheral target, whereas others have not yet developed axons. Over the past year, we have completed some experiments to determine whether the degree of prior NGF exposure influences the rate or extent of apoptosis among *Nf1*-/- neurons, following withdrawal of the neurotrophin. We have also compared the survival response to depolarization (KCl) for Nf1-/- and wild-type sensory neurons (DRG and trigeminal, **Figures 3 and 4**). Our experiments indicate that the duration of NGF exposure does not influence the proportion of *Nf1*-/- neurons that undergo apoptosis following withdrawal; rather, we believe that (unidentified) environmental cues encountered at or en route to target tissues control the acquisition of neurotrophin dependence. In addition, our results with *Nf1*-/- SCG neurons are consistent with differing mechanisms for acquisition of neurotrophin dependence for sensory and sympathetic neurons. Now that we have sufficient data for statistical analyses, we can complete a short manuscript describing the differing apoptotic responses to NGF, and survival responses to depolarization, for *Nf1*-/- trigeminal, DRG, and SCG neurons. We are continuing this project, to address possible mechanisms for apoptosis, as a collaboration with Dr. Patricia Dahia (see below).

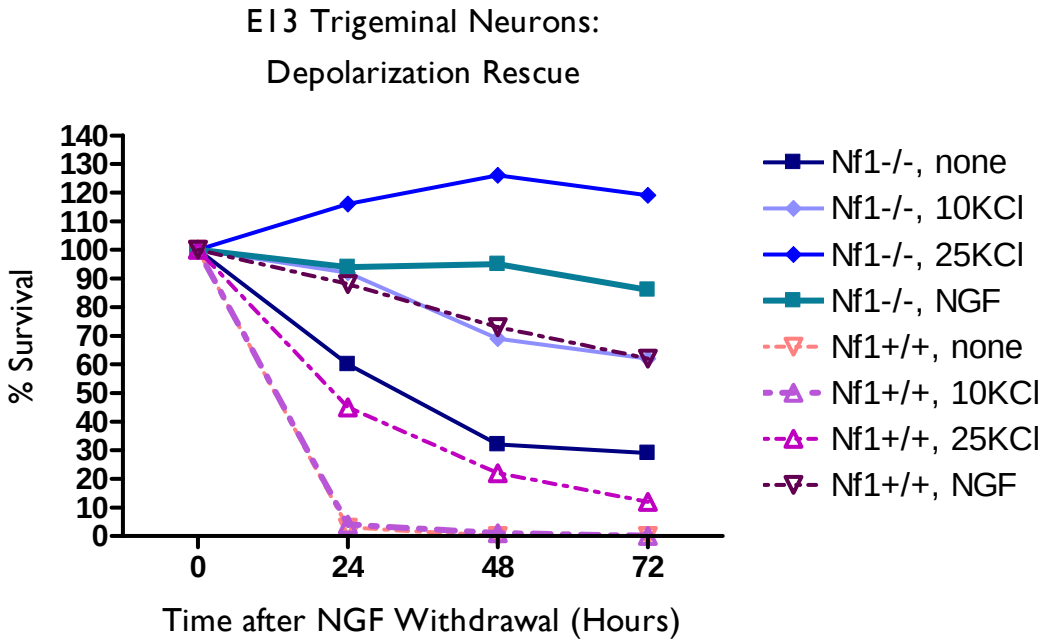

Figure 3. Prolonged survival of NGF-deprived E13 *Nf1*-/- trigeminal neurons, using KCl-mediated depolarization. Following NGF withdrawal at 48 hours, the medium was supplemented with NGF, or with 10mM or 25 mM KCl, and survival of neuronal cohorts monitored over a period of several days.

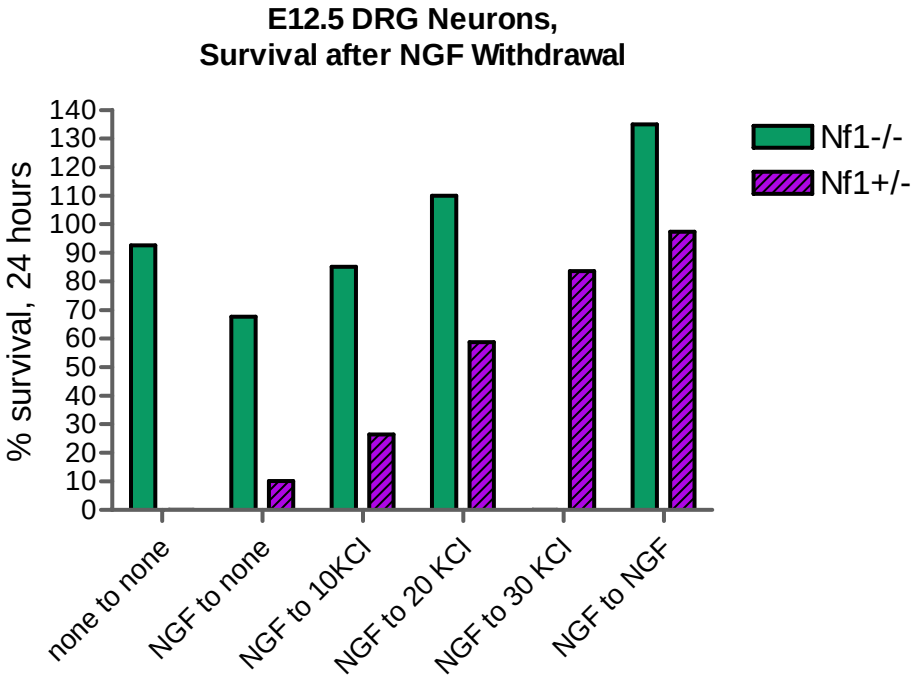

Figure 4. Survival of DRG sensory neurons at 48 hours after NGF withdrawal. 20 mM KCl is sufficient to promote the survival of 100% of *Nf1*-/- neurons, following removal of NGF.

Egln3 and neuronal apoptosis

Recently, Lee and colleagues (2005) examined the role of familial pheochromocytoma genes, including succinate dehydrogenase (*SDH*) and *Nf1*, in modulating neuronal apoptosis following neurotrophin withdrawal. The prolyl hydroxylase EglN3 acts downstream of c-Jun, and is required for apoptosis in PC12 cells and in primary SCG neurons, following withdrawal of NGF. We have initiated a collaboration with the laboratory of Dr. Patricia Dahia at UTHSCSA, to 1) examine levels of EglN3 mRNA and protein in sympathoadrenal

precursors, sensory neurons, and sympathetic neurons isolated from *Nf1*-deficient mouse embryos and deprived of neurotrophins, and 2) to use a targeted mutation in the succinate dehydrogenase D (SDHD) gene to examine the contributions of this respiratory chain protein to tumor progression and neuronal apoptosis in the context of NF1. This work arises from the experiments accomplished as part of Task 4, in which we found that sensory and sympathetic neurons differ in their responses to NGF withdrawal; we are currently applying for funding for these projects.

DNA damage sensitivity and DNA repair in the peripheral nervous system

Differential sensitivity to DNA damage and capacity for DNA repair have been proposed to contribute to the variable expressivity of NF1 (Wiest et al., 2003). To examine this issue in the context of the peripheral nervous system, a tissue that is subject to degenerative changes during the aging process, we have initiated experiments to examine DNA damage and repair in Schwann cells, fibroblasts, and neurons isolated from *Nf1*+/- and wild-type mice throughout the lifespan. A recently-awarded pilot project grant from the Nathan Shock Center and the NIA will allow us to obtain preliminary data for future funding applications. In addition, we were able to complete mutant frequency and mutation spectrum analyses required for a manuscript that will be published in *Mutation Research*, and which includes data for CNS and peripheral nerve tissues.

KEY RESEARCH ACCOMPLISHMENTS

- Completed experiments on effects of neurofibromin reduction (*Nf1*+/-, *exon23a*-/-) on activity-mediated survival signaling and neurotrophin sensitivity in embryonic sensory (DRG, trigeminal) and sympathetic (SCG) neurons
- Completed experiments on responses of *Nf1*-/- sensory (DRG, trigeminal) and sympathetic (SCG) neurons to NGF withdrawal and activity-mediated survival signaling
- Initiated collaboration on role of EglN3 and SDHD in apoptosis in *Nf1*-/-, +/-, and +/+ sympathoadrenal precursors and sensory neurons (with Dr. Patricia Dahia, UTHSCSA); examined Egln3 mRNA levels in trigeminal and DRG neurons following neurotrophin withdrawal
- Obtained funding to expand experiments on DNA damage sensitivity and repair in the peripheral nervous system, in the context of Nf1 deficiency

REPORTABLE OUTCOMES

Manuscripts and Abstracts

- June 2006: Children's Tumor Foundation, Molecular Biology of NF1, NF2, and Schwannomatosis Meeting, platform presentation. "DNA repair capacity in Schwann cells and sarcoma lines isolated from Nf1-deficient mice"
- Miller, S.J., Li, H., Rizvi, T.A., Huang, Y., Johansson, G., Bowersock, J., Sidani, A., Vitullo, J., **Vogel, K.S.**, Parysek, L.M., DeClue, J.E., and Ratner, N. (2003) Brain lipid binding protein in axon-Schwann cell interactions and peripheral nerve tumorigenesis. Molecular and Cellular Biology 23, 2213-2224
- Ling, B.C., Wu, J., Miller, S.J., Monk, K.R., Shamekh, R., Rizvi, T.A., Decourten-Myers, G., **Vogel, K.S.**, DeClue, J.E., Ratner, N. (2005) Role for the epidermal growth factor receptor in neurofibromatosis-related peripheral nerve tumorigenesis. Cancer Cell 7, 65-75
- Garza, R., Hudson, R.W., Walter, C.A., and **Vogel, K.S.** (accepted, pending revisions) A mild mutator phenotype arises in malignancies associated with neurofibromatosis type 1. (*Mutation Research: Fundamental and Molecular Mechanisms of Carcinogenesis*)
- Tominaga, K., Perreira-Smith, O., and **Vogel, K.S.** (in preparation). Mrg15 haploinsufficiency reduces DNA double-strand break repair capacity in a mouse model for MPNST.
- Brannan, C.I., and Vogel, K.S. (in preparation) Reduction in neurofibromin expression modulates the response to neurotrophin- and activity-mediated survival signaling in sensory and sympathetic neurons.

Cell Lines and Animal Models

- Combined targeted mutations in *Nf1* and *Mrg15*
- Combined targeted mutations in *Nf1* and *SDHD* (initiated September 2006)

Funding Obtained and Pending

- Nathan Shock Center on Longevity and Aging Studies, "DNA Damage and Repair in Nf1-Deficient Schwann Cells throughout the Lifespan", $30,000

- USMRMC NFRP, "Rapid Assessment of DNA Repair Capacity in *Nf1*-deficient Schwann Cells and Astrocytes", Pending
- San Antonio Cancer Institute, Genomic Integrity and Tumor Development Program, "EglN3 and SDHD in peripheral nervous system development and tumorigenesis", Pending

Employment and Training Opportunities

- Rene Garza, Research Assistant
- Robert Hudson III, Senior Research Assistant (currently second-year dental student at UTHSCSA)
- Sienna Skye Edwards, middle school student; science fair projects at the state and national level

CONCLUSIONS

Importance and Implications

Our results to date support the emerging idea that neurofibromin expression and *Nf1* haploinsufficiency influence the behavior of both peripheral and central neurons. Loss of neurofibromin, with the resulting abnormalities in Ras and PI3 kinase signaling, has profound effects on the neurotrophin dependence and sensitivity of embryonic sensory neurons (Vogel et al., 1995; Klesse and Parada, 1998; Vogel et al., 2000). Behavioral experiments with *Nf1*+/- and *exon23a*-/- mice indicate that neurofibromin function in CNS neurons modulates learning and memory (Silva et al., 1997; Costa et al., 2001). We have shown that *Nf1* haploinsufficiency affects both neurotrophin- and activity-mediated survival signaling for sensory and sympathetic neurons, at least by embryonic day 15 in the mouse; the differences between *Nf1*+/- and +/+ neurons appear to become more significant with age. Our results may have implications for two areas: 1) the pathogenesis of learning disabilities in children with NF1, and 2) therapeutic strategies or targets for prolonging neuron survival, or for increasing neuronal response to protective agents, following injury or damage.

Neurotrophin withdrawal experiments involving E12.5 *Nf1*-/- and +/- SCG neurons revealed a difference between sympathetic and sensory neurons at this early stage. Whereas a proportion of neurofibromin-deficient DRG neurons die after NGF withdrawal (given prior NGF exposure), none of the NGF-deprived SCG neurons underwent apoptosis, even after a period of several days. We propose to continue this line of investigation, with particular emphasis on involvement of the EglN3 and SDHD gene products, in *Nf1*-/- sensory and sympathetic neurons; this work will also have relevance to the biology of familial pheochromocytoma.

"So what" section

The learning disabilities associated with NF1 constitute a highly variable phenotype, and in addition represent a controversial topic of research and clinical interpretations. Using mice that harbor targeted mutations in *Nf1*, Silva and colleagues have demonstrated that aberrant or reduced regulation of Ras signaling by neurofibromin may contribute to certain aspects of the spatial learning disorder (Silva et al., 1997; Costa et al., 2001). More recently, these researchers have proposed that the excessive Ras activity in *Nf1*+/- neurons leads to increased GABA-mediated inhibition and defects in long-term potentiation (Costa et al., 2002). Our results in Tasks 1 and 3, for both sensory and sympathetic neurons, are consistent with the interpretation that *Nf1*+/- neurons may respond aberrantly to electrochemical (ion gradients) and neurotrophin stimuli, which could potentially affect neuronal function and synaptic transmission. To relate these *in vitro* results to the complex issue of NF1-related learning disorders, it may be of interest to use computer modeling to characterize possible consequences of additional neurons (particularly inhibitory ones) in a circuit, or of aberrant signaling by neurons within a given circuit. Our results with *Nf1* haploinsufficient neurons also point to neurofibromin as a possible therapeutic target following neuronal injury; reduction in neurofibromin activity may prolong neuron survival, or enhance the response to protective agents.

Both neurotrophin signaling and activity-mediated processes are required to achieve correct target innervation patterns and synaptic plasticity in the peripheral nervous system (Davies, 2003). For many types of embryonic neurons, activation of the intracellular signaling pathways required for these processes often correlates with the timing of target contact. To date, our experiments indicate possible differences in the role of neurofibromin in acquisition of neurotrophin dependence, for embryonic sensory and sympathetic neurons. Both molecular mechanisms and the importance of target contact can be addressed readily in our *in vitro*

system, and should contribute to our understanding of how neurotrophin signaling pathways are established during development.

Recent experiments with cancer cells have led to the concept of "oncogene addiction" (reviewed by Jonkers and Berns, 2004). For example, Buzzai and colleagues (2005) reported that cancer cells with activated Akt have increased glucose uptake and metabolism, and are susceptible to apoptosis following glucose withdrawal because they can no longer metabolize nonglycolytic bioenergetic substrates. Loss of, or reduction in, neurofibromin function leads to activation of Ras signaling in both cancer cells and in postmitotic neurons, thus potentially creating a type of "oncogene addiction". Moreover, aberrant activation of Akt and other signaling pathways in Nf1+/- and Nf1-/- neurons may lead to defects in metabolism and synaptic transmission, and contribute to the learning disorders associated with NF1. In this context, the combination of Nf1 and SDHD mutations in embryonic neurons will be of interest.

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