RET-DEPENDENT AND RET-INDEPENDENT MECHANISMS OF GFL-INDUCED ENHANCEMENT IN THE CAPSAICIN STIMULATED-RELEASE OF iCGRP FROM SENSORY NEURONS

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in the Department of Pharmacology and Toxicology,
Indiana University

December 2009

Accepted by the Faculty of Indiana University, in

partial fulfillment of the requirements for the degree of Doctor of Philosophy

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DEDICATION

This thesis is dedicated first to my wife, Alicia, for her love, support, and patience with the constant scientific thinking and long hours spent in the lab. I would also like to dedicate this thesis to my parents, who inspired me to work hard, aspire for greatness, and chase my dreams.

ACKNOWLEDGEMENTS

I would like to first and foremost thank my mentor Dr. Cynthia Hingtgen, who has, through her patience and enormous amount of effort helped to form me into a quality scientist. She has challenged me to think through scientific problems, to ask scientifically relevant questions, and to pursue those questions rationally and efficiently. She has given me the freedom to pursue and develop my project in the way I desired, guided me in the appropriate ways to think about my thesis work, and the opportunity to participate in collaborative work. Dr. Hingtgen also encouraged me to be an expert in my own work and have a mastery of all literature relevant to my thesis work. She taught me the value of presenting my work in written and oral formats on a laboratory, local, and national level. I would also like to thank Dr. Hingtgen for her editorial work on my early written documents that has allowed me to become a published and able scientific writer. Dr. Hingtgen's willingness to discuss not only scientific issues but clinical issues as well, and the chances she provided me to accompany her to the clinic periodically have added invaluably to my doctoral education. Dr. Hingtgen has such integrity and displays an ethical compass I aspire to be able to mimic. I cannot thank her enough for being the type of mentor that few graduate students can have the pleasure of working for.

I must give much credit to my doctoral thesis committee for all of their guidance and suggestions for direction of my thesis and critical experiments to conduct. In particular, I would like to thank Dr. Andy Hudmon for suggesting I shift the focus of my thesis to the more impactful studies evaluating Ret-independent mechanisms of GFL sensitization. Dr. Michael Vasko's help with technical difficulties and insightful questions forcing me to think about scientific problems more critically and deeply than I ever imagined possible was priceless. I would like to thank Dr. Theodore Cummins for focusing my work and helping to keep my thesis work in perspective. Finally, I would like to thank Dr. Nikolai Broustovetski for his practical advice on certain technical aspects of my thesis work.

Aside from my committee, I would like to thank the entire Department of Pharmacology and Toxicology for challenging me during seminars and for teaching some of the best didactic courses I have taken. There are three members of this department I would like to especially acknowledge. Dr. Grant Nicol helped me to think more globally about my thesis work and to examine all the GFLs, which lead to some of the most exciting data in the thesis. I am indebted to Dr. Rajesh Khanna for helping me optimize the conditions of my Western blots, which allowed me to easily finish these data sets in a reasonable time period. Dr. Khanna also brought me on board on a couple of quite exciting collaborative projects and helped me to think about how some of my thesis work could be applied to new questions and problems. Finally, I learned many valuable things from Dr. Weiguo Zhu, who helped me with understanding other effects of the GFLs, was always willing to lend a hand with thinking about GFL signaling, and for keeping me updated on all his interesting work on GFLs.

Other members of my scientific life I am thankful to include Dr. Joseph Hingten, Dr. James Trofatter, Dr. Alan Schmetzer, Dr. Sonny Boyd, Dr. Matthew Asay, and Dr. Sacha Malin. I appreciate all Dr. Joseph Hingtgen did for me as an aspiring scientist during my summers spent at the Institute of Psychiatric Research. I am also grateful to him for introducing me to my mentor-to-be, Dr. Cynthia Hingtgen. Dr. James Trofatter was an integral part of peaking my interest in science and teaching me basic wet lab skills. Dr. Alan Schmetzer is responsible for connecting me with Dr. Trofatter, which began my scientific career. I want to also thank Dr. Schmetzer for showing me how rewarding both medicine and science can be. Dr. Sonny Boyd and Dr. Matthew Assay, two members of my scientific experience at the University of Notre Dame, helped me to learn the lesson of how hard I had to work in the lab to be successful. Finally, I want to thank Dr. Sacha Malin for our impromptu conversations, her advice about GFLs and Ret-independent signaling, and her career advice.

There are members of the Hingtgen lab I would like to thank. Shannon Roy has been an absolute joy to work with. I cannot thank her enough for all of her help in acclimating me to the lab, for maintaining a large breeding colony, and for putting up with the craziness and messiness that invariable comes with being in close contact with me. I'd like to thank Leslie Sitzman for her help in the lab and for long conversations about life and science. I'd also like to thank Rena Meadows for helping me to learn how to teach someone in the lab and for bringing a fresh perspective to the lab, as well as her work to help me finish some final experiments. I'd also like to acknowledge Dr. Patrick sheets, Andrew Piekarz, Neilia Gracias, and Brian Jarecki for advice on how to survive the graduate school experience and for being a friendly face to turn to on a daily basis. Neilia also was very valuable as a resource for many of the techniques used in this thesis, and I appreciate all of our long conversations about career plans and life lessons.

I'd like to thank other members of my family, my grandmother, Barb, and my in-laws, Bob and Cindy. They have always made me feel that my accomplishments in science mattered and for encouraging me in all my endeavors. I must thank my dog, Ed, who greeted me with a tail wag and excited attitude each day and was able to cheer me up after tough days in the lab.

The MSTP Program at Indiana University has been very supportive as well, especially Jan Receveur, Dr. Maureeen Harrington, and Dr. Wade Clapp. I am so thankful for all of their help, advice, and direction. I feel I have received one of the best educations and career direction in the country thanks to these people. I'd also like to thank INGEN for funding a portion of my work, the Department of Pharmacology and Toxicology for funding a portion, and the Children's Tumor Foundation (CTF) for funding most of my work. The CTF is a wonderful organization and it has been a pleasure to work with them.

Finally, I would like to thank the Lord for giving me the ability to do all that I do and the drive to accomplish anything.

ABSTRACT

Brian S. Schmutzler

RET-DEPENDENT AND RET-INDEPENDENT MECHANISMS OF GFL-INDUCED ENHANCEMENT IN THE CAPSAICIN STIMULATED-RELEASE OF iCGRP FROM SENSORY NEURONS

The glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) are peptides implicated in the inflammatory response. They are released in increased amounts during inflammation and induce thermal hyperalgesia. Whether these molecules directly affect the sensitivity of primary nociceptive sensory neurons is unknown. This information could provide a link between increased inflammation-induced release of GFLs and their ability to promote inflammatory hyperalgesia. These molecules bind to one of four GFRα receptor subtypes, and this GFL-GFRα complex often translocates to the receptor tyrosine kinase, Ret. The focus of this dissertation was to determine whether GFLs modulate the stimulated-release of calcitonin gene-related peptide (CGRP). Isolated sensory neurons and freshly dissociated spinal cord tissue were used to examine the enhancement in stimulated-release of CGRP, a measure of sensitization. Exposure of isolated sensory neurons to GDNF, neurturin, and artemin, enhanced the capsaicin stimulated-release of immunoreactive CGRP (iCGRP). Sensitization by GFLs occurred in freshly dissociated spinal cord tissue. Persephin, another member of the GFL family, did not enhance stimulated-release of iCGRP. These results demonstrate that specific GFLs are mediators of neuronal sensitivity. The intracellular signaling pathways responsible for this sensitization were also evaluated. Inhibition of the mitogen activated protein kinase (MAPK)/extracellular signal-related kinase 1/2 (Erk 1/2) pathway selectively abolished the enhancement of CGRP release by GDNF. NTN-induced sensitization was abolished by inhibition of the phosphatidylinositol-3-kinase (PI-3K) pathway. Reduction in Ret abolished the GDNF-

induced sensitization, but did not fully inhibit NTN or ART-induced sensitization. Inhibition of other cell surface receptors (neural cell adhesion molecule (NCAM), and Integrin β-1) had distinct effects on the sensitization capability of each of the GFLs. Ret and NCAM inhibition in combination abolished ART-induced sensitization. It was necessary to inhibit Ret, NCAM, and Integrin β-1 to prevent the NTN-induced sensitization. These data demonstrate that the GFLs use distinct signaling mechanisms to induce the sensitization of nociceptive sensory neurons. The work presented in this thesis provides the first evidence for these novel and distinct Ret-independent pathways for GFL-induced actions and provides insight into the mechanism of sensory neuronal sensitization in general.

Cynthia M. Hingtgen, M.D., Ph. D.

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LIST OF ABBREVIATIONS

- 5-HT: Serotonin
- 6-OH DA: 6-hydroxydopamine
- ALS: Amyotrophic lateral sclerosis
- ART: Artemin
- ART-OE: Artemin over expressing mice
- BK: Bradykinin
- Cap: Capsaicin
- CFA: Complete Freund's Adjuvant
- CGRP: Calcitonin gene-related peptide
- CLR: Calcitonin receptor like receptor
- CNS: Central nervous system
- DAGs: Diacylglycerols
- DRG: Dorsal root ganglia
- ELISA: Enzyme-linked immunosorbent assay
- Erk: Extracellular signal-related kinase
- FSR2: Fibroblast growth factor receptor substrate-2
- GAS1: Growth arrest specfici receptor-1
- GDNF: Glial cell line-derived neurotrophic factor
- GFL: Glial cell line-derived neurotrophic factor family ligand
- GFRα: GDNF family receptor alpha
- GPI: Glycosyl phosphatidylinositol
- GTP: Guanosine-5'-triphosphate
- KCl: Potassium chloride
- kDa: Kilo Dalton

- IB4: Isolectin B4
- LPS: Lipopolysaccharide
- MAP-2: Microtubule-associated protein-2
- MAPK: Mitogen-activated protein kinase
- MDCK: Madine Darby canine kidney
- MEN: Multiple endocrine neoplasia
- NCAM: Neural cell adhesion molecule
- *Nf1*+/-: Neurofibromatosis type 1 heterozygous mouse
- NF-M: Neurofilament-medium
- NGF: Nerve growth factor
- NTN: Neurturin
- PGE_2: Prostaglandin E_2
- PI-3K: Phosphoinositide-3 Kinase
- PKC: Protein kinase C
- PLC-γ: Phospholipase C- γ
- PNS: Peripheral nervous system
- PSP: Persephin
- RAMPs: Receptor activity modifying proteins
- RIA: Radioimmunoassay
- Scram: Scramble siRNA
- SEM: Stardard error of the mean
- siRNA: Small interfering RNA
- SFKs: Src family kinases
- SP: Substance P
- TGFβ: Transforming growth factor-β

- TH: Tyrosine hydroxylase
- TM: Transmembrane
- TNFα: Tumor necrosis factor-α
- TrkA: Tyrosine kinase receptor A
- TRPV1: Transient receptor potential vanilloid 1
- VTA: Ventral tegmental area

I. INTRODUCTION

The following thesis will provide evidence of the ability of the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) to sensitize sensory neurons. The GFLs are released in increased amounts during inflammation, and direct injection of the GFLs into the periphery induces inflammatory hypernociception. However, the connection between these two phenomena has not been elucidated. Enhancement in stimulated-release induced by GFLs could provide evidence that may explain this inflammatory hypernociception. The intracellular signaling pathways by which the GFLs induce this sensitization are also determined. Interestingly, each of the GFLs use distinct compliments of pathways to accomplish their sensitization. I have also identified novel Ret-independent signaling pathways by which two of the GFLs, neurturin and artemin, induce their sensitization of sensory neurons. The characterization of the effects of the GFLs on sensory neuronal sensitization, and the novel pathways used by the GFLs identified in this thesis, may provide insight into general mechanisms of enhanced pain perception. This insight may lead to better and more effective treatments for chronic pain syndromes and other disorders modulated by the GFLs, such as optic nerve degeneration.

A. The history of the actions of the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs)

The glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) were identified originally in the rat B49 glioma cell line, though the function of the molecules were not known and the molecules were not named (Schubert et al., 1974). When one of these molecules was found to promote the survival and growth of embryonic midbrain dopaminergic neurons, it was further characterized and named glial cell line-derived neurotrophic factor, or GDNF (Lin et al., 1993). GDNF release from central nervous system glia, specifically astrocytes and microglia, is critical for embryonic substantia

nigra neurons to survive (Lin et al., 1993). Removal of this peptide results in death of nearly all of the neurons in the substantia nigra (Lin et al., 1993). Neurturin (NTN) was next identified as a relative of GDNF, purified, and cloned (Kotzbauer et al., 1996). NTN had potent survival effects on CNS neurons as well (Kotzbauer et al.,1996) Persephin (PSP) was the third GFL identified due to its homology to GDNF. Using degenerate PCR, Milbrandt et al., 1998 identified, purified, and cloned PSP. PSP was also found to support the survival of ventral midbrain dopaminergic neurons and motor neurons (Milbrandt et al., 1998). Finally, artemin (ART) was identified and cloned in the same manner and also found to have survival effects on midbrain dopaminergic neurons (Baloh et al, 1998).

Trophic effects of the GFLs on spinal motor neurons and central noradrenergic neurons have also been observed (Arenas et al., 1995;Henderson et al., 1994;Milbrandt et al., 1998). GDNF is critical for motor neuron survival and growth (Zurn et al., 1994). Additionally, injury to embryonic and adult motor neurons induces degeneration and neuronal death. GDNF reduces the number of neurons that degenerate and die by greater than 50% after induction of an injury (Li et al., 1995). NTN is trophic for developing motor neurons only, not adult motor neurons (Garces et al., 2001). However, unlike GDNF, NTN does not affect survival of central noradrenergic neurons, but induces neurite outgrowth of these neurons in embryonic and adult mice (Holm et al., 2002) there is no compelling evidence for actions of ART on either motor neurons or noradrenergic neurons. Finally, PSP promotes survival and differentiation of developing motor neurons, but does not seem to have effects on noradrenergic neurons (Milbrandt et al., 1998). These observations have led to speculation that the GFLs could be used as a treatment for amyotrophic lateral sclerosis (ALS), as well as a number of other diseases of the central nervous system (Airaksinen and Saarma, 2002).

The GFLs have effects on the peripheral nervous system, specifically sensory neurons. GDNF promotes neurite outgrowth in adult sensory neurons (Blesch and Tuszynski, 2003). Implantation of fibroblasts genetically modified to secrete high levels of GDNF led to sensory neuron regeneration, neurite outgrowth, and re-myelination of the regenerating sensory neurons after spinal cord transection (Blesch and Tuszynski, 2003). Application of GDNF selectively activates axonal growth in these injured sensory neurons and the phenotype of these injured sensory neurons may be changed to a more immature, “growth capable” neuron (Mills et al., 2007). NTN maintains adult sensory neurons (Baudet et al., 2000), in part by preventing growth cone collapse of neurites mediated by semaphorin 3A (Wanigasekara and Keast, 2006). Semaphorin 3A protein is involved in growth cone guidance during development and neuronal regeneration (Kolodkin et al., 1993). ART not only supports sensory neuron growth, survival, and axonal growth by providing a growth and guidance signal (Baloh et al., 1998;Paveliev et al., 2004), but also alters the sensitivity of ion channels in sensory neurons in such a way as to increase the sensitivity of these neurons to noxious stimuli (Elitt et al., 2006), such as heat. There are reports of analgesic properties of GDNF as well, particularly through its ability to prevent or reverse some of the increased sensitivity of sodium channels in response to neuronal injury (Boucher et al., 2000). However, these studies were conducted using a neuropathic pain model, which induces a different compliment of changes in sensory neurons that inflammatory pain. For this reason, and because this manuscript is focused on the inflammatory pain models, GDNF will be referred to as a molecule that is pronociceptive and not analgesic through the rest of this manuscript. The effects of PSP on sensory neurons are not as well studied and by no means extensive. However, in the few studies conducted on sensory neurons, PSP has been unable to affect the properties of these neurons (Paveliev et al., 2003).

The effects of the GFLs have also been identified in cells outside of the nervous system. Uteric kidney bud branching, a critical step in kidney development and differentiation, is dependent upon the actions of GDNF through its signaling receptor, Ret (Schuchardt et al., 1994). In spermatogenesis, GDNF and neurturin (NTN), another of the GFLs, play a crucial role. Both GDNF and NTN are necessary for proper DNA synthesis in spermatogonia (Viglietto et al., 2000). Proper levels of GDNF secretion are necessary for proper spermatagonia development. Reduced levels of GDNF result in an inability of the testes to generate the necessary amount of spermatogenic stem cells (Meng et al., 2000). A large number of undifferentiated spermatogenic stem cells and few differentiated spermatogonia result from lower than normal levels of GDNF or NTN (Meng et al., 2001a;Meng et al., 2001b). Neither ART nor PSP has any documented effects outside of the nervous system.

There are a number of possible clinical applications of the GFLs. Aberrant GDNF responses of neurons in the ventral tegmental area (VTA), the area of the brain responsible for addictive behaviors, have been identified in mice with compulsive, addictive behaviors towards cocaine and alcohol (He et al., 2005;Messer et al., 2000). A reduction in GDNF levels or prevention of GDNF signaling in this brain region enhanced addictive behaviors, whereas injection of GDNF or use of pharmaceuticals that increase GDNF levels reduced or abolished these same addictive behaviors (He et al., 2005;Messer et al., 2000). Recently, of particular interest clinically, is the fact that GDNF is able to aid the maintenance and growth of dopaminergic cells in the substantia nigra, the cell type whose death is responsible for Parkinson's disease (Akerud et al., 2001;Kordower et al., 2000). GDNF and NTN are in clinical trials for treatment of Parkinson's disease, with limited success (Evans and Barker, 2008). There are problems with efficient delivery of GFLs to the substantia nigra, off-target toxicity, and degradation of the GFLs in the brain (reviewed by (Abdel-Salam, 2008). The ability of ART and PSP

to protect against or reverse substantia nigra neuronal death has not been evaluated. The knowledge of the primary actions of the GFLs has broadened from solely central nervous system neuronal development to include promotion of kidney and gonadal development, as well as prominent effects on mature peripheral sensory neurons.

B. Structure of the GFLs

The GFLs are a set of small peptides distantly related to the TGFβ super-family of molecules (Eigenbrot and Gerber, 1997). They exist naturally as homodimers and include GDNF, neurturin (NTN), artemin (ART), and persephin (PSP; Eigenbrot and Gerber 1997; Wang et al., 2003). While the amino acid sequence homology of the GFLs and TGFβ is low, less than 20%, there is a high degree of structural similarity between these molecules (Kotzbauer et al., 1996;Lin et al., 1993;Milbrandt et al., 1998;Saarma, 2000). In particular, both the GFLs and the other TGFβ super-family members contain seven cysteine residues in approximately the same spacing, leading to a protein folding of the peptides into a “cysteine-knot” motif with an interlinking disulfide bond (Eigenbrot and Gerber, 1997). This interlinking disulfide bond results in the GFL monomers existing naturally as homodimers.

Each of the GFLs is similar to the other GFLs in size and three-dimensional structure. Specifically, the size of the monomer of each of the GFLs is between 10 and 16 kDa. GDNF has a molecular weight of ~16 kDa (Okragly and Haak-Frendscho, 1997); NTN, ~11.5 kDa (Kotzbauer et al., 1996); ART, ~12 kDa (Baloh et al., 1998); and PSP, ~10 kDa (Milbrandt et al., 1998). The structure of the monomer of each of the GFLs is also similar, due in part to the nearly identical (more than 80%) amino acid sequence homology of the GFLs (Wang et al., 2006). The basic structure is referred to as a finger, heel, finger conformation. Each of the fingers is composed of several β-sheets with short interruptions, and between the two finger motifs exists the heel motif, composed of an α-helix in a perpendicular orientation to the finger motifs (Wang et al., 2006). This

structural conformation allows each monomer to come in contact with another monomer. The heel motif from one monomer complexes with the finger region of the other, and vice versa, and a cysteine knot is created from disulfide binds between these two regions (Wang et al., 2006). Overall, this structure results in homodimerization and efficient binding of each of the GFLs to its GDNF family receptor alpha subtype, which does not occur with the GFL monomers.

C. GFLs' actions through GDNF family receptor α (GFRα) subtypes

Although the GFLs are in the TGFβ superfamily of molecules, their signaling is more closely related to the signaling of cytokines. Each of the GFLs has its own GDNF family receptor α (GFRα) subtype to which it preferentially binds, allowing for ligand-receptor specificity. All of the different GFL-GFRα complexes can subsequently initiate signaling through a common cell surface signaling molecule, Ret.

The GFRα molecules are receptors with a sequence of 300-500 amino acids, a hydrophobic core on the carboxy end, a number of cysteine residues, and several glycosylation sites (Airaksinen and Saarma, 2002;Treanor et al., 1996). This structure is strikingly similar to other cytokine receptors, including interferon – α and β, which also indicates the GFLs may be more closely related to cytokines than the TGFβ molecules (Bazan, 1990). Additionally, because there is an Ala-Ser-Ser amino acid sequence immediately preceding the C-terminus, the GFRα receptors are linked to the outer plasma membrane through a glycosyl phosphatidylinositol (GPI)-attachment (Treanor et al., 1996). The GPI-anchor localizes the GFRα receptors to lipid rafts (Poteryaev et al., 1999). This localization allows activated GFRα-1 to interact directly with Src family kinases (SFKs), verified using co-immunoprecipitation, and induce subsequent signaling through the mitogen activated-protein kinase (MAPK) signaling pathway, the phospholipase C-γ (PLC-γ) pathway, and the cyclic AMP (cAMP) pathway (Poteryaev et

al., 1999). Lack of GPI-attachment prevents GFRα-1 localization to lipid rafts and disrupts the signaling of GDNF through this receptor (Treanor et al., 1996).

1. GFL-GFRα binding and translocation

The first of the GFRα subtypes discovered was GFRα-1, the preferred receptor for GDNF (Treanor et al., 1996). This receptor was cloned from midbrain, dopaminergic neurons, but is present in nearly all neuronal types (Treanor et al., 1996). The next receptor discovered in this family was the GFRα-2 receptor, initially named TGF-β-related neurotrophic factor receptor 2 (TrnR2), which has nearly 50% sequence homology to GFRα-1 (Baloh et al., 1997). GFRα-2, as it was later renamed, binds both GDNF and NTN, and similar signaling pathways are activated by both factors (Buj-Bello et al., 1997;Sanicola et al., 1997). NTN induces Ret phosphorylation through GFRα-2 30 to 100 times more efficiently than GDNF through GFRα-2 (Klein et al., 1997;Sanicola et al., 1997). Ret is the receptor tyrosine kinase through which the GFL- GFRα complexes initiate their signaling. This receptor will be described in depth in Section I.D.

GFRα-3, the preferential receptor for artemin, was first identified as an orphan receptor with no known ligand (Jing et al., 1997). Artemin was later purified, and GFRα-3 was identified as the primary and preferential receptor for this molecule (Baloh et al., 1998). The secondary and tertiary structure of GFRα-3 is less similar to GFRα-1 and GFRα-2 than the structure of these receptors are to each other (Airaksinen et al., 1999;Nomoto et al., 1998;Worby et al., 1998). Despite its later discovery, there has been more characterization of the structure and function of this GFRα subtype than any of the others. The tips of both of the finger motifs of ART bind in the pocket of a triangle structure in GFRα-3 created by three alpha helices in a tip to tail conformation (Wang et al., 2006). While the sequence and proposed structure of each of the GFRα receptors is known, only the crystal structures of GFRα-1 and GFRα-3 have been completed (Airaksinen et al., 1999;Wang et al., 2006;Parkash et al., 2008). These two receptors are

similar, but GFRα-1 has a smaller bend angle and, therefore, a smaller area of interface with Ret (Parkash et al., 2008). The larger area of interface between GFRα-3 and Ret is hypothesized to account for the often higher potency of ART compared to the other GFLs (Parkash et al., 2008). GFRα-2 and GFRα-4 likely have similar crystal structures and characteristics of binding to their preferential GFL. However, these studies are yet to be completed.

The fourth, and last, GFRα receptor identified was GFRα-4, the preferential receptor for persephin (Lindahl et al., 2001). Initially, this receptor was cloned in the chicken and thought to be present only in this avian species (Thompson et al., 1998). However, a gene encoding for this receptor was later identified in mice (Gunn et al., 1999). Identification of the presence of this receptor on adult rodent neuronal tissues followed (Lindahl et al., 2000). GFRα-4 was found to be present on developing neuronal cells, as well (Mason, 2000), which led to the possibility that GFRα-4 could play a role development and maintenance of neuronal tissues. It was later determined that persephin, through binding to GFRα-4, is unable to induce axonal outgrowth in cultured sensory neurons (Paveliev et al., 2004). This is presumably as a result of the lack of either the intracellular portion of the GPI-anchor or presence of a non-functional domain 2 (D2) on GFRα-4 (Lindahl, et al., 2000), determined by sequencing and structural analysis. The GPI-link is responsible for keeping the GFRα receptors anchored in the membrane and interacts with intracellular portions of Ret (see Sariola and Saarma, 2003 for a review). GFRα-4 may also exist primarily in a soluble form that is unable to induce the typical signaling pathways and actions of the GFRα receptors (Lindahl et al., 2001). This soluble form of GFRα-4 could be acting as a sink for Ret, in essence binding Ret molecules and making them unavailable for activation by other GFLs. The D2 portion of the GFRα receptors interacts directly with Ret to initiate Ret autophosphorylation

(Lindahl et al., 2000). Presence of a non-functional D2 or lack of a GPI-anchor results in a GFRα-4 receptor that is unable to induce its actions through Ret.

2. GFL-GFRα initiation of signaling

The GFRα receptors are localized to lipid rafts by the GPI-anchors (Poteryaev et al., 1999) and recruit Ret into the lipid raft after binding with a GFL homodimer (Tansey et al., 2000). The action of the GFRα receptors is initiated when a GFL homodimer approaches two GFRα receptors, of the same isoform, and causes them to homodimerize (Trupp et al., 1998b). This GFL-GFRα complex aids in the translocation of the Ret receptor tyrosine kinase to lipid rafts, by an unknown mechanisms, and causes a dimerization of Ret initiating a number of intracellular signaling pathways (Trupp et al., 1998a). This recruitment leads to the activation of Ret and subsequent signaling described below. There is extensive characterization of this process with GDNF. Interestingly, mutated forms of GDNF that lack the ability to efficiently promote translocation of GFRα to Ret are still able to initiate Ret autophosphorylation (Eketjall et al., 1999). A weak Ret-GFRα-1 association is present in cells exposed to these mutated forms of GDNF, indicating that Ret and the GFRα receptors can associate prior to GFL-GFRα binding (Eketjall et al., 1999). Whether GFL-GFRα complex translocates to Ret or the GFRα-Ret complex is preassembled, it is clear that the GFRα receptors are critical components in GFL-induced signaling and function.

3. GFL-GFRα specificity

While the GFLs preferentially bind to a specific GFRα receptor; GDNF to GFRα-1, NTN to GFRα-2, ART to GFRα-3, and PSP to GFRα-4; there is evidence of lower affinity binding of the GFLs to GFRα receptors other than their preferred subtype (Airaksinen and Saarma, 2002). GFRα-1 and GFRα-2 have similar binding affinities for both GDNF and NTN (Cik et al., 2000). GFRα-1 binds GDNF with a K_d of 0.63 to 5.0 nM and NTN with a K_d of 1.0 to 3.0 nM. GFRα-2 binds GDNF with a K_d of 1.0 to 3.0 nM and NTN with

a K_d of 0.6 to 3.0 nM (Baloh et al., 1998; summarized in Table 1). Both GDNF and NTN are able to activate the MAPK, PI-3K, and PLC-γ pathways (Kaplan and Miller, 2000;Yang et al., 2001) and mediate their survival and neurite outgrowth actions on neurons through binding to either GFRα-1 and GFRα-2 (Baloh et al., 1997;Buj-Bello et al., 1997;Sanicola et al., 1997). However, NTN-induced Ret autophosphorylation and activation of the MAPK through GFRα-2 is five to 20 times more potent than through GFRα-1 (Baloh et al., 1998;Klein et al., 1997). There is also evidence of actions that ART can initiate Ret dimerization and autophosphorylation through GFRα-1, but at concentrations of ~10 nM (Bespalov and Saarma, 2007).

The GFRα-3 receptor maintains the most fidelity to its preferential ligand, ART (Baloh et al., 1998). ART is the only GFL that binds to GFRα-3 at near physiologic concentrations (K_d of GDNF for GFRα-3: > 100 μM, K_d of NTN for GFRα-3: > 100 μM, K_d of ART for GFRα-3: 3.1 nM, and K_d of PSP for GFRα-3: not tested) and the primary GFL that can induce biological actions through GFRα-3, including differentiation and proliferation of cell lines and survival of neurons (Airaksinen et al., 1999;Baloh et al., 1998;Sariola and Saarma, 2003).

GFRα-4 binds PSP with a much higher affinity than any of the other GFLs (K_d of GDNF for GFRα-4: > 50 μM, K_d of NTN for GFRα-4: > 50 μM, K_d of ART for GFRα-4: not tested, nM, and K_d of PSP for GFRα-4: 1.0 to 6.0 nM (Lindahl et al., 2000;Milbrandt et al., 1998). NTN is able to enhance the survival of superior cervical sympathetic ganglia (SCG) neurons through GFRα-4, but only at high concentrations (100 μM) and when added in addition to PSP at a concentration of 5 ng/mL (Enokido et al., 1998). Because PSP and GFRα-4 do not modulate the function of adult, mammalian sensory neurons, there will be only minor discussion of this GFL in future sections. For this reason, from this point forward when referring to the GFLs as a group, PSP will not be included, unless otherwise indicated.

Table 1

Affinity of GFLs for specific GFRα subtypes

Receptor	GDNF	NTN	ART	PSP
GFRα-1	0.63 - 5.0 nM [1, 2, 3]	1.0 - 3.0 nM [3,6,7,8,12]	> 10 nM [1, 3, 10]	1:1,000 [14]
GFRα-2	1.0 - 3.0 nM [2, 4, 5, 7]	0.6-3.0 nM [3, 6, 9, 10, 12]	> 100 nM [1,3,10]	1:800 [15]
GFRα-3	> 100 μM [3,9,11,12]	> 100 μM [3, 6, 9, 10, 12]	3.1 nM [3]	Not Tested
GFRα-4	> 50 μM [13, 14, 15]	> 50 μM [13, 14, 15]	Not Tested	1.0 - 6.0 nM [13, 14, 15]

1. Airaksinen and Saarma, 2002; 2. Cik et al., 2000; 3. Baloh et al., 1998; 4. Kaplan and Miller, 2000; 5. Yang et al., 2001; 6. Baloh et al., 1997; 7. Buj-Bello et al., 1997; 15. Enokido et al., 199811. Airaksinen et al., 1999; 12. Sariola and Saarma, 2003; 13. Lindahl et al., 2000; 14. Milbrandt et al., 1998; 8. Sanicola et al., 1997; 9. Klein et. Al, 1997; 10. Bespalov and Saarma, 2007;

The GFLs bind directly to the GFRα receptors. Figure 1 illustrates this binding and signal initiation profile in diagrammatic form for the primary ligand/receptor interactions. However, the intracellular signaling pathway initiation by the GFLs and the physiological functions of the GFLs most often require additional steps and/or receptors.

D. Ret receptor tyrosine kinase and the GFLs

The Ret receptor tyrosine kinase was first identified as a transforming gene after human lymphoma cDNA was transfected into NIH 3T3 cells and two unlinked segments of this cDNA were linked via recombination (Takahashi et al., 1985). While the nucleotide sequence of the 5' end of Ret is unique, the 3' end of the gene shows 40-50% homology to other known tyrosine kinase receptors (Takahashi and Cooper, 1987). Further analysis of the amino acid sequence of Ret demonstrates its homology (25-30%) to the tyrosine kinases Src, Abl, Met, and Kit, particularly at the carboxy-terminus (Takahashi and Cooper, 1987). Not surprising is that the carboxy-terminus of the Ret receptor is where the tyrosine kinase activity resides, and the amino-terminus is the ligand binding end. Additionally, there is a highly hydrophobic portion of the molecule that forms the transmembrane domain (Takahashi et al., 1988). Overall, the intracellular signaling pathway induction and structural changes of Ret are similar to many of the other receptor tyrosine kinases that directly bind their ligand (for example, TrkA and NGF), except that it lacks a direct ligand binding site (Treanor et al., 1996).

Low levels of Ret were identified in adult rat tissue, with the brain, testis, and thymus having the highest levels (Tahira et al., 1988). The lungs, heart, spleen, and small intestine showed a low amount of Ret reactivity (Tahira et al., 1988). However, it is now clear that Ret is present on many adult mammalian tissues, including CNS neurons, PNS neurons, renal tissue, thyroid tissue, and enteric neurons (Quartu et al., 2007;Yang et al., 2006a;Yang et al., 2006b;Yoong et al., 2005).

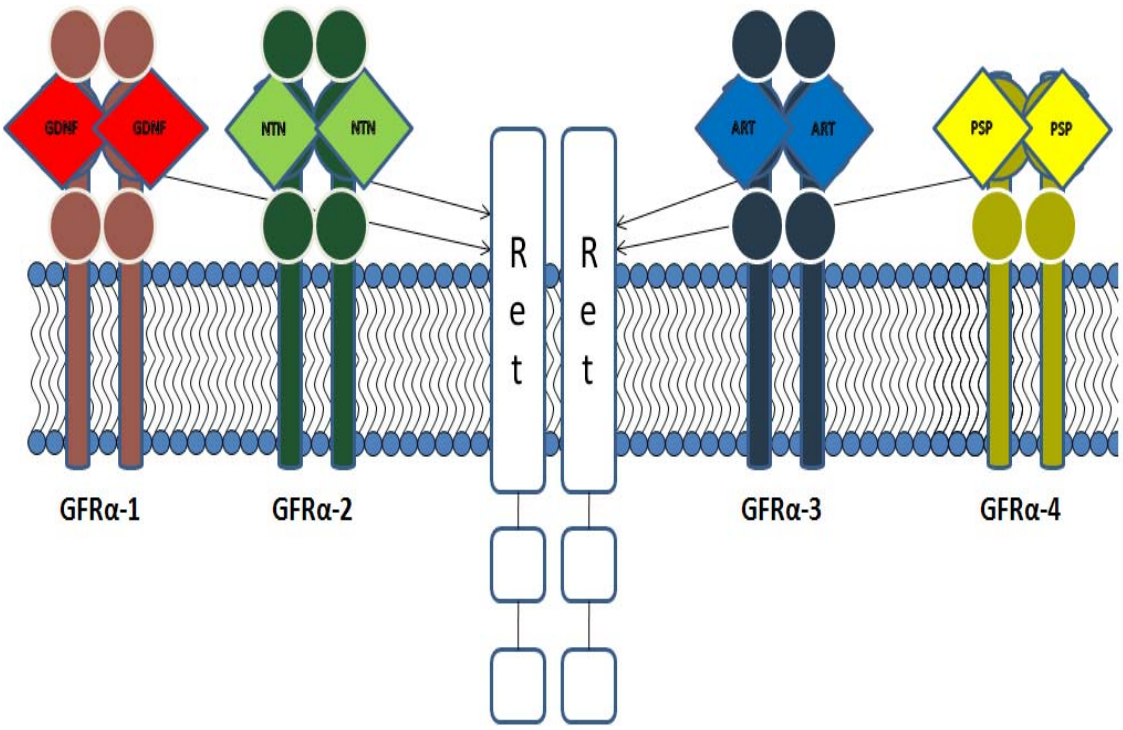

Figure 1. Diagrammatic representation of the binding of GFLs to their preferential GFRα receptor subtype and consequent interaction with Ret. Each of the GFLs, as a homodimer, binds preferentially to a GFRα receptor subtype, which homodimerizes as well. This GFL-GFRα complex translocates to the receptor tyrosine kinase, Ret, which autophosphorylates and initiates downstream signaling events. Adapted from Sariola and Saarma, 2003.

There are two isoforms of Ret (Tsui-Pierchala et al., 2002b); a short form that has nine amino acids after the first conserved 1062 amino acids, and a long form that has fifty one (Myers et al., 1995;Tahira et al., 1990;Ishizaka et al., 1989). The Ret 9 short form has 12 tyrosine phosphorylation sites, and the Ret 51 long form has 14 of these sites (Alberti et al., 1998;Carter et al., 2001). These extra two phosphorylation sites may be important in more robust and efficient activation of the PLC-γ signaling pathway (Tsui-Pierchala et al., 2002c). The differences in these two isoforms have been extensively studied, especially in renal and digestive system development. However, no consensus exists as to the possible roles of each of the different Ret isoforms in GFL-induced function in many mammalian cell types, including neurons. There is evidence of some differential functions of the two Ret isoforms in development and maintenance of certain cell types. Ret 9 appears to be necessary for early kidney development and enteric ganglia formation in the kidney, while Ret 51 is neither necessary nor sufficient for these processes (de et al., 2001). However, Ret 51 may be involved in later kidney development, including survival and tubulogenesis in the collecting duct system (Lee et al., 2002). Few studies have been conducted to evaluate the role of each Ret isoform in neuronal function. NGF-induced survival, somal growth, and NGF-dependent gene expression in adult, sympathetic neurons can be mediated by the long, Ret 51 isoform of Ret (Tsui-Pierchala et al., 2002a). Specifically, NGF induced Ret 51 phosphorylation in the absence of GFLs in adult sympathetic neurons. If the Ret phosphorylation was prevented by inhibition of NGF signaling through TrkA, the NGF-induced increased production of tyrosine hydroxylase (TH), microtubule-associated protein-2 (MAP-2), and neurofilament-medium (NF-M) was abolished. Additionally, the expression of these neuronal proteins did not increase in response to NGF in sympathetic neurons lacking Ret. While this Ret 51, is likely involved in the functions of GFLs on adult sympathetic

neurons, Ret 9 cannot be excluded as an important receptor for adult mammalian sensory neuronal function.

The gene coding for Ret was localized to chromosome 10q11.2 (Ishizaka et al., 1989), which put this gene in close proximity to the gene locus for a set of thyroid cancers, known as the multiple endocrine neoplasia type 2, MEN 2 (Yamamoto et al., 1991;Hofstra et al., 1994). MEN 2 syndrome is characterized by medullary thyroid carcinomas, pheochromocytomas, and other hyperplasias and neoplasias (Marini et al., 2006). This observation, in addition to the presence of a mutated form of constitutively active Ret in several cancer cells types, including neuroblastomas (Tahira et al., 1990;Takahashi et al., 1991), leukemias (Takahashi et al., 1991), pheochromocytomas (Yoshimoto et al., 1995), and thyroid carcinomas (Grieco et al., 1990;Santoro et al., 1990), has led to interest in Ret as a cancer-causing or modifying receptor.

Ret was an orphan receptor until it was identified as a critical receptor for the actions of GDNF in the substantia nigra (Trupp et al., 1996), kidney development (Durbec et al., 1996), and peripheral nervous system development (Durbec et al., 1996). Ret and the ligand binding receptor for GDNF, GFRα-1, associate, and the actions of GDNF described above are unable to occur in the absence of either GFRα-1 or Ret (Treanor et al., 1996). There is no direct binding of GDNF to Ret, nor can GDNF activate the phosphorylation of Ret in the absence of GFRαs (Treanor et al., 1996). Additionally, GFRα-1 associates with Ret weakly in the absence of GDNF and strongly in the presence of GDNF, indicating that both GFRα-1 and Ret are necessary and critical for GDNF to function and that GDNF facilitates the association of GFRα-1 and Ret (Treanor et al., 1996).

Each of the other GFLs, NTN, ART, and PSP, have a similar system for Ret activation, initiated by binding of the GFL with its specific GFRα, translocation of the GFL-GFRα complex to Ret, activation of Ret via autophosphorylation (Coulpier et al.,

2002), and initiation of downstream signaling (Baloh et al., 1998;Buj-Bello et al., 1997;Masure et al., 2000). The GFRα resides in a lipid raft, and when the GFL binds, the GFL-GFRα complex recruits Ret to the lipid raft (Paratcha et al., 2001;Tansey et al., 2000). Disruption of this recruitment, or of the lipid raft structure, results in decreased GFL-induced neuronal differentiation and survival (Tansey et al., 2000), reduced neurite outgrowth (Paratcha et al., 2001), abolition of Ret downstream signaling (Paratcha et al., 2001), and axonal expansion and growth cone lengthening (Paratcha et al., 2001).

The intracellular signaling pathways by which Ret initiates its functions are diverse and numerous. Downstream signaling initiated by Ret is accomplished by one of two primary mechanisms. Within lipid rafts, Ret signals through the Src homology 2 domain containing (SHC) protein, which then activates the common signaling molecule Grb2 (Paratcha et al., 2001). Outside of lipid rafts, Ret activation results in initiation of the FGF receptor substrate 2 (FSR2) signaling pathway (Paratcha et al., 2001). Signaling initiation by either route activates many of the classic intracellular signaling pathways of receptor tyrosine kinases, including MAPK-Erk 1/2 (Trupp et al., 1999b;Worby et al., 1996), PI-3K-Akt (Maeda et al., 2004;Segouffin-Cariou and Billaud, 2000;Trupp et al., 1996), Jun NH2-terminal protein kinase (Chiariello et al., 1998), p38 MAPK (Watanabe et al., 2002), and PLC-γ (Borrello et al., 1996).

E. Ret-independent GFL-induced signaling

There is emerging evidence of GFL-induced, Ret-independent signaling pathways. GDNF activates Src family kinases (SFKs), phosphorylates CREB, and upregulates fos in raphe nucleus and motor neuron cell lines lacking Ret (Trupp et al., 1999c). Additionally, GDNF is able to activate Src-family kinases and PLC-γ in a Ret deficient neuroblastoma cell line (Poteryaev et al., 1999). Embryonic dorsal root ganglia (DRG) neurons from mice genetically modified to express no Ret have provided evidence that GDNF can signal in a Ret-independent manner (Poteryaev et al., 1999). The Ret-

independent pathways include the MAPK-Erk 1/2 and pCREB pathways (Poteryaev et al., 1999). This Ret-independent, GDNF-induced signaling is specifically through GFRα-1 (Pezeshki et al., 2001). NTN is unable to accomplish these actions through either GFRα-1 or GFRα-2 (Pezeshki et al., 2001). These studies indicate that Ret-independent signaling is present in neurons and that while the signaling pathways activated by Ret-dependent and Ret-independent mechanisms are the same, the pathways may be activated in different manners or in different cellular compartments.

GDNF-induced, Ret-independent signaling can be accomplished through GFRα-1 directly initiating signaling cascades or via an interaction of GFRα-1 with other cell surface receptors. The activation of SFKs by GFRα-1 has been demonstrated in neuroblastoma cell lines (Poteryaev et al., 1999). Neural Cell Adhesion Molecules (NCAMs) were the first candidate cell surface receptors identified as possible GFRα-1 binding partners and signaling activators. NCAM signals through the Fyn/FAK pathway, a SFK pathway, in the RN33B raphe nucleus cell line (Paratcha et al., 2003). GFRα-1 binds directly to NCAM (Cao et al., 2008a), and the GFRα-1-NCAM signaling mechanism has functional consequences on axonal outgrowth of hippocampal and cortical neurons (Paratcha et al., 2003), as well as neurite outgrowth of dopaminergic, midbrain neurons (Cao et al., 2008a). There is also evidence that GFRα-1 can bind with Integrin β-1 in substantia nigra dopaminergic neurons and initiate the Shc and FAK signaling pathways in an Integrin β1-dependent manner (Cao et al., 2008b). However, there is no functional evidence of GDNF-induced, Integrin β1-dependent actions, nor have any studies evaluated the role of Integrin β-1 on sensory neuronal sensitization. There is precedence, however, for other extracellular matrix adhesion proteins, such as fibronectin, induce sensory neuronal sensitization (Jeske et al., 2009). Finally, nucleotide and amino acid sequence homology analysis has identified growth arrest specific (GAS-1) receptor, a tumor suppressor protein, as another possible co-receptor for the GFRα-1-

GDNF complex (Schueler-Furman et al., 2006). Whether GAS1 is a functional co-receptor for the GFRα-1-GDNF complex has yet to be determined.

F. The GFLs in inflammation

One way inflammation is incited is when an object creates a wound in a tissue, such as the skin. The results of this wound include the classic inflammatory signs and symptoms: rubor (redness), calor (heat), tumor (swelling), dolor (pain), and functio laesa (loss of function). These signs and symptoms are due to vasodilatation and extravasation of inflammatory cells from the blood vessels into the area of the injury (Darwin, 1875). The inflammatory cells responsible for the initiation of inflammation include mast cells (BENDITT et al., 1955), neutrophils (PAGE and GOOD, 1958), and other peripheral blood leukocytes (Hartman and SCHRECK, 1958). When these cells are activated by substances released after the injury is incited, they release a number of molecules known as inflammatory mediators (Spector, 1958). Among the classic inflammatory mediators are histamine, bradykinin, and prostaglandins (ROCHAESILVA, 1964). Recently, growth factors, specifically nerve growth factor (NGF), have been identified as potent inflammatory mediators (Bienenstock et al., 1987).

There is also evidence of a role for GFLs in the inflammatory process. Application of several substances that induce inflammation, including IL-1β, TNFα, and lipopolysaccharide (LPS), to glial cells from the enteric nervous system increases the level of GDNF four to five fold, from 50 pg/mL to as high as 300 pg/mL (von Boyen et al., 2006). Glial cells supporting neurons of the enteric system in the myenteric plexus are known to be responsive to inflammatory mediators. Additionally, inflammatory mediators cause several types of inflammatory cells to release the GFLs. Macrophages in culture, when activated by LPS, release increased amounts of GDNF compared to those not treated with this inflammatory substance (Hashimoto et al., 2005). Most compelling, the levels of artemin are increased when inflammatory inducers are injected *in vivo*. The

levels of artemin mRNA were increased up to 1000 times after injection of Complete Freund's Adjuvant (CFA), and artemin levels increase in the dental pulp after inflammation induction (Baloh et al., 1998;Malin et al., 2006). Inflammation associated with breast cancer increases the level of GDNF (Esseghir et al., 2007). Since the levels of the GFLs are dramatically increased during inflammation, they may be responsible, in part, for changes in the properties of the sensory neurons associated with inflammatory processes.

G. Sensitization by GFLs

The exposure of neurons to different types and durations of stimuli results in changes in their properties. One neuronal property often changed under these circumstances is the sensitivity of the neurons. This change in neuronal sensitivity was first demonstrated in the whole animal by Sherrington (1906) using the scratch reflex paradigm in dogs. In this paradigm, electrical or mechanical stimulation is applied to the shoulder of the dog, and the natural reflex arc is for the dog to scratch its shoulder with the hind limb. The intensity, amplitude, and rhythm of the hip muscle flexion are then measured. Repeated, high-frequency electrical or mechanical stimulation to the skin of the shoulder resulted in a progressive reduction in the flexion amplitude of the hip muscle (Sherrington, 1906). Additionally, the muscle contractions are unpredictable and weak, compared to the initial stimulation (Sherrington, 1906). Sherrington was also able to elucidate the location of this decrease in the reflex, since full and strong contractions occurred when electrical stimuli were applied to the muscle. He concluded that the neuron itself, with its endings likely present in the shoulder skin, was responsible for the decreased response, later called habituation or desensitization. Not surprisingly, the opposite of this habituation also occurred with low-level, sub or near threshold stimulation (Sherrington, 1906). Specifically, this type of stimulation to the shoulder skin of the dog resulted in a lowering of the threshold for hip flexion and scratching.

Sherrington initially called this phenomenon “bahnung,” and it was later termed sensitization.

Since Sherrington’s initial identification of sensitization, other definitions of sensitization have been added. These include an increase in the activity of the neuron to a given stimulus, measured in several different ways, or a decrease in the threshold for activation to given environmental (i.e. mechanical) or chemical (i.e. changes in pH) alterations. Some of the assays used to measure changes in neuronal sensitivity have included complex biological assays, such as long-term potentiation (Hawkins et al., 1993) and complex behavioral assays, such as thermal latency (Hargreaves et al., 1988) in rodents, and other simple behavioral assays in less complex organisms (Bliss and Lomo, 1973). Electrophysiological changes, or changes in the electrical properties of the neuron, have been useful in determining the chemical, mechanical, and other stimuli capable of or necessary for producing neuronal sensitization.

The primary mode of communication between neurons is through chemical neurotransmitter release. When a neuron is activated or stimulated, chemical neurotransmitters are released from pre-synaptic neurons and cause responses in nearby post-synaptic neurons and non-neuronal cells (Katz et al., 1962b). Depending upon the specific neurotransmitter and the neurotransmitter receptor, the release of neurotransmitters and the subsequent response to these substances can be either activation or inhibition. This activation or inhibition can be of another (post-synaptic) neuron, non-neuronal cells, or the releasing neuron itself (autocrine actions). The frequency and duration of the neuronal stimulation is dependent upon the amount of quantal neurotransmitter release. Since neurotransmitter release is the primary mode of neuronal communication, an enhancement in the release of neurotransmitters in response to a given stimulus can be used as an effective and important measure of neuronal sensitization.

The focus of our laboratory is to determine how certain functions, specifically the sensitivity, of primary afferent, small-diameter, peptidergic sensory neurons are altered by molecules released during inflammation. We are also interested in the cellular mechanisms of this sensory neuronal sensitization. The specific subset of primary afferent neurons of which are particularly interested are the small-diameter, and typically nociceptive sensory neurons. These neurons respond to noxious stimuli and are activated by inflammation and injury (Bartho et al., 1990;Coderre and Melzack, 1987;Kocher et al., 1987;Weihe et al., 1988). The studies in this thesis will be conducted using agents that are present in increased amounts during inflammation, the GFLs, and stimuli that activate these small-diameter, nociceptive sensory neurons.

1. Behavioral sensitization

The fact that the levels of the GFLs are increased during inflammation has led to the study of the ability of the GFLs to cause inflammatory hyperalgesia. Specifically, injection of each of the GFLs, GDNF, NTN, and ART, into the paw of rodents dramatically reduces the thermal withdraw latency evaluated with a Hargreaves test (Malin et al., 2006). This thermal hyperalgesia occurs within 30 minutes to one hour and is long-lasting. Mechanical hyperalgesia is another result of GDNF injection into the paw of rodents. Using the Ugo Basile analgesymeter, in which a mechanical force applied to the paw is linearly increased at 16 g/s, it was found that GDNF lowered the threshold for paw withdraw (Bogen et al., 2008). This hyperalgesia is accomplished when 10 ng/mL GDNF is injected and it is prevented by inhibition of PLC-γ, Src family kinases, PI-3K, MAPK-Erk, and CDK5. The limitations of this study are two-fold. First, injection of these inhibitors into the paw of a rodent affects all of the neuronal and non-neuronal cells in the area, which means these modified behavioral responses may not be as a result of modulation of nociceptive sensory neurons. Secondly, the inhibitors used in these experiments are non-specific and inhibit many other signaling pathways at the

concentrations used, making interpretation of this data difficult. Despite these obvious limitations in an in vivo system, these studies suggest that the MAPK-Erk, PI-3K, CDK, Src family kinase, and PLC-γ pathways are involved in GDNF induced changes in sensory neurons. Additionally, mice genetically modified to over express artemin in the skin displayed hyperalgesia to both noxious heat and cold, when compared to wild-type litter mates (Elitt et al., 2006). Complete Freund's Adjuvant (CFA) induces inflammation and results in inflammatory hyperalgesia upon injection. When a blocking antibody to GDNF is injected into the paw of rats in addition to CFA, the CFA-induced mechanical hyperalgesia is attenuated (Fang et al., 2003). These studies give insight into the possible physiological and biochemical mechanisms of GFL-induced sensitization.

2. Sensitization of sensory neurons by GFLs

There is direct evidence of effects of GFLs on sensory neurons, both alterations in channel properties and changes in the overall threshold of activation of these neurons. Injection of GDNF into the dorsum of the paw resulted in enhanced small-diameter, nociceptive neuronal responses to mechanical stimuli measured with an *in vivo* electrophysiological preparation (Bogen et al., 2008). Interestingly, application of each of the GFLs, GDNF, NTN, and ART, enhanced the calcium influx through TRPV1 in sensory neurons in culture in response to capsaicin, a specific exogenous ligand for this channel (Malin et al., 2006). These observations demonstrate that the GFLs are able to directly modulate properties of small-diameter sensory neurons in such as way as to make them more excitable. It is yet to be established whether this change in excitability results in an increased release of neurotransmitters involved in neurogenic inflammation and pain signal propagation from these neurons.

a. Electrophysiological and ion channel sensitization by GFLs

There are a number of studies that demonstrate that the GFLs are able to modulate the electrophysiological properties of nociceptive sensory neurons. C-fibers from animals over expressing GDNF in the skin have dramatically reduced thermal and mechanical thresholds (Albers et al., 2006). When GDNF is injected in to the receptive field of C-fibers in the skin, those C-fibers have reduced thermal thresholds and unaffected mechanical thresholds, determined by using the single-fiber electrophysiological technique (Bogen et al., 2008). Each of these previous studies was conducted using *in vivo* or *ex vivo* preparations. GDNF and neurturin also directly affect the electrophysiological properties of TRPV1 in isolated sensory neurons in culture by increasing the capsaicin-induced calcium influx through the channel (Malin et al., 2006). Artemin shows similar characteristics in its ability to alter the electrophysiological properties of nociceptive sensory neurons. Mice over expressing artemin in the skin have enhanced firing rates of C-fiber in response to noxious heat (Elitt et al., 2006). However, and in contrast to GDNF, C-fibers from artemin over-expressing mice did not respond more robustly to mechanical stimuli than wild type mice (Elitt et al., 2006). Interestingly, artemin enhanced calcium influx in intact C-fibers in response to capsaicin, a stimuli specific for TRPV1, but not other stimuli, including ATP (Elitt et al., 2006). Similar to GDNF and neurturin, artemin enhances calcium influx through TRPV1 channels in isolated sensory neurons in culture, but the potency of artemin is higher (producing a response at 1 ng/mL) than that of GDNF or neurturin (producing a response at 10 ng/mL; Malin et al., 2006). The GFLs, therefore, sensitize specific electrophysiological properties of nociceptive sensory neurons.

b. GFLs may enhance release of neuropeptides

There are four basic criteria that must be met in order that a molecule can be classified as a neurotransmitter (reviewed by (Paton, 1958)). These criteria include: the molecule is present and produced or activated in the pre-synaptic neuron, activation of the pre-synaptic neuron causes the release of this molecule from that neuron, exposure of the post-synaptic neuron to the molecule has the same effect as stimulating the pre-synaptic neuron, and substances that inhibit release of the molecule from the pre-synaptic neuron must also prevent the biological effect of activation of the post-synaptic neurons. While this set of criteria were established using studies with acetylcholine, it is now clear that any molecule that meets all of these criteria, regardless of its chemical makeup or structure, can be classified as a neurotransmitter.

Among the neurotransmitters known to be present in and responsible for the actions of sensory neurons are glutamate, substance P (SP), and calcitonin gene-related peptide (CGRP), as well a numerous others (Hokfelt et al., 1975;Schoenen et al., 1989;Wiesenfeld-Hallin et al., 1984). However, the peptide transmitters, including CGRP, are important transmitters used by the small-diameter sensory neurons (Garry et al., 1989;Kangrga and Randic, 1991;Wiesenfeld-Hallin et al., 1984). Neuropeptide release is critically important for sensory neuronal signal propagation and function, as described previously. It could follow, then, that increases in the release neuropeptides like CGRP, would alter the signal propagation centrally and peripherally. Additionally, quantifying the changes in the release of this neuropeptide is a reliable measurement of changes in sensory neuronal function, in particular the subset of small diameter sensory neurons containing CGRP. The peptide also fulfills the four criteria detailed previously for neurotransmitters in these neurons. For these reasons, I have chosen to focus the experiments in this thesis on the neuropeptides, specifically CGRP.

CGRP is present and colocalized in small-diameter sensory neurons and spinal cord neurons (Skofitsch and Jacobowitz, 1985;Wiesenfeld-Hallin et al., 1984). It is involved in different components of the neurogenic inflammation response. CGRP mediates vasodilatation (Escott et al., 1995;Messlinger et al., 1995;Brain et al., 1985). Not only is this neuropeptide present in small-diameter sensory neurons, but its release profile is nearly identical to that of Substance P (SP; another neuropeptide) in response to noxious stimuli (Franco-Cereceda et al., 1987;Hingtgen and Vasko, 1994a;Hingtgen et al., 2006;Schicho et al., 2005). Because of the better stability of CGRP and the reliability of the release levels of CGRP in release assays, I will focus on CGRP for release assays in this thesis.

CGRP production results from alternative splicing of the calcitonin gene in neurons, creating a 37-amino acid small peptide (Amara et al., 1982;Nelkin et al., 1984;Rosenfeld et al., 1983). Two isoforms of CGRP exist, CGRPα and CGRPβ (Holman et al., 1986). While these two isoforms may have differential functions in several tissues, there is no evidence that this is true in sensory neurons (Beglinger et al., 1988;Beglinger et al., 1991). In terms of its role as a neurotransmitter, CGRP fits all four criteria. CGRP is present in sensory neurons, specifically small diameter sensory neurons (Fang, 1987;Gibson et al., 1984). Stimulation of sensory neurons via mechanical, electrical, or chemical means results in increased release of CGRP from small-diameter sensory neurons. (Franco-Cereceda et al., 1987;Wahlestedt et al., 1986). Injection of CGRP into the thecal space mimics the nociceptive behavioral effects of small-diameter sensory neuronal stimulation (Gamse and Saria, 1986), and injection of CGRP in the periphery induces neurogenic inflammation in a similar fashion as stimulation of small-diameter sensory neurons (Brokaw and White, 1992). Finally, inhibition of the actions of CGRP in the central and peripheral terminals prevents the nociceptive behaviors and neurogenic

inflammation induced by small-diameter sensory neuronal activation, which will be described in more detail below.

One of the most dramatic effects of CGRP is its ability to induce and enhance neurogenic inflammation by dilating blood vessels (Brain et al., 1985;Girgis et al., 1985). In fact, the combination of the direct vasodilatory effects of CGRP on blood vessels and the indirect vasodilatory effects of CGRP through increasing histamine release from mast cells, modulation of the effects of SP on blood vessels, and the autocrine function of CGRP causing the release of additional vasodilators from sensory neurons makes CGRP one of the most potent and important vasodilators (Cruwys et al., 1992;Piotrowski and Foreman, 1986). Injection of the Fab fragment of a goat anti-human CGRP antibody prevents the neurogenic inflammation induced by both CGRP and capsaicin, as measured by a prevention of the increased blood flow induced by these molecules (Buckley et al., 1992).

CGRP also produces hyperalgesia, increased behavioral responses to a given noxious stimulus, another form of sensitization. CGRP injection into the thecal space enhances rodent responses to noxious mechanical stimulation, which may be through direct interaction of CGRP with second order neurons or may be through enhancement of SP production or reduction of SP degradation (Oku et al., 1987). Strikingly, injection of a CGRP blocking antibody into the thecal space completely abolished mechanical allodynia (Ambalavanar et al., 2006). Additionally, CGRP antisera reduces arthritic hyperalgesia and recombinant herpes virus encoding antisense CGRP attenuates capsaicin-mediated thermal hyperalgesia (Kuraishi et al., 1988;Tzabazis et al., 2007). These studies provide evidence for the importance of CGRP in both neurogenic inflammation and inflammatory hyperalgesia.

The receptors for CGRP have been characterized recently. The CGRP receptors are heterodimers composed of a Family B, seven trans-membrane (TM) G-protein coupled receptor (GPCR), known as calcitonin receptor like receptor (CLR) and an accessory single TM receptor, RAMP (Receptor Activity Modifying Proteins; McLatchie et al., 1998). There are two distinct CGRP receptors that are differentiated by their affinity for CGRP. CGRP-R1 is the high affinity receptor, and CGRP-R2 is the low affinity receptor (Juaneda et al., 2000). Additionally, endothelial cells on blood vessels express the CGRP receptors and respond to CGRP by relaxing, which leads to vasodilatation (Hirata et al., 1988). While the expression profiles of these receptors are different, there is a lack of evidence for any functional differences of the receptors in sensory neurons.

The functional characteristics of CGRP, its role in neurogenic inflammation and inflammatory hyperalgesia, and the availability of a highly sensitive radioimmunoassay (RIA) for this neuropeptide, allowing direct quantification of the release of CGRP from sensory neurons in culture and spinal cord slices, have contributed to my decision to use changes in CGRP release as the measurement for sensitization in this thesis.

The rationale behind examination of the small diameter sensory neurons, and the release of CGRP specifically, is two-fold. First, while it is clear that the GFLs are released in increasing amounts during inflammation (Baloh et al., 1998;Malin et al., 2006;von Boyen et al., 2006), and that these molecules induce hyperalgesia *in vivo* (Bogen et al., 2008;Malin et al., 2006), it is yet to be determined whether these two phenomena can be connected through GFL-induced sensitization of sensory neurons. Evaluating the ability of the GFLs to enhance the release of the neuropeptide CGRP would further enhance the likelihood that the GFLs released during inflammation are causing hyperalgesia via modulation of nociceptive sensory neurons. Secondly, because CGRP induces neurogenic inflammation in the periphery (Buckley et al., 1992), GFL-induced enhancement in CGRP release could cause increased release of the GFLs and

other inflammatory mediators that could further sensitize the sensory neurons within this inflammatory milieu. One could hypothesize that this autocrine, feed-forward system could also contribute to the inflammatory hyperalgesia induced by the GFLs.

1. Sensitization of neurotransmitter release

While many neuropeptide expressing neurons are responsive to GFLs (Bennett et al., 2006;Price et al., 2005), and the GFLs increase the content of CGRP and TRPV1 in sensory neurons (Price et al., 2005;Priestley et al., 2002;Ramer et al., 2003), it is unclear whether the GFLs directly modulate the release of CGRP from nociceptors in the DRG. Several concentrations of GDNF were able to enhance the potassium and capsaicin-stimulated release of CGRP in a trigeminal ganglia preparation, but at least a portion of this effect was due to the long-term exposure to GDNF increasing the content of CGRP in the ganglia (Price et al., 2005). Similarly, a 3 day exposure artemin exposure was able to induce the recovery of substance P release from injured sensory neurons (Bennett et al., 2006). However, there has been no exploration into whether short-term exposure of sensory neurons to the GFLs enhances the stimulated release of neuropeptides and this information would be important in defining a role for GFLs in the initiation of inflammatory hyperalgesia.

a. Agents that stimulate sensory neurons

In order to evaluate the ability of the GFLs to enhance the release of CGRP from sensory neurons, appropriate stimuli that evoke the release of this peptide must be chosen. General depolarization of sensory neurons, and resulting transmitter release, can be accomplished with the use of electrical stimuli or a high concentration of extracellular potassium (Hodgkin, 1950;Katz et al., 1962a). Among common physiological stimuli that activate sensory neurons, specifically small diameter sensory neurons, are noxious heat, a number of chemicals, and extreme mechanical pressure (Cooper and Diamond, 1977;Davis et al., 1993;Woolf et al., 1985). A number of

neurotransmitters, including acetylcholine, and other endogenous molecules, including bradykinin, can also induce the activation of small diameter sensory neurons (Fjallbrant and Iggo, 1961;Fock and Mense, 1976). Finally, capsaicin, the neurotoxin found in high levels in chili peppers of the *Capsicum* genus, is a selective, exogenous activator of a subset of small diameter sensory neurons (Bowie et al., 1994;Holzer, 1991;Toh et al., 1955). While capsaicin is not an endogenous, physiologic stimulus, it is a selective activator of small-diameter sensory neurons which it a powerful tool to dissect the reaction of nociceptive sensory neurons in a heterogeneous population of dorsal root ganglia (DRG) neurons. Sensory neuronal stimuli will be described below, with particular emphasis on high extracellular potassium and capsaicin, as these are the primary stimuli used in experiments in this manuscript.

1. Capsaicin

Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is a small, pungent molecule found in peppers of the genus *Capsicum* and has been study extensively for its actions on sensory neurons (TOH et al., 1955). The effects of capsaicin include a “spicy” taste when present in food and several actions on primary afferent sensory neurons. The specific effects of capsaicin on small diameter sensory neurons is concentration dependent, with a low concentration causing activation and a large concentration causing inhibition and even neuronal death (Chard et al., 1995;Hiura and Ishizuka, 1989).

Low concentrations of capsaicin selectively excite polymodal sensory neurons, those sensitive to many stimuli including thermal, chemical, and mechanical (Kenins, 1982;Szolcsanyi et al., 1988;Welk et al., 1983) . This polymodal, capsaicin-sensitive set of sensory neurons is primarily composed of small-diameter sensory neurons, both slow-conducting, unmyelinated C-fibers (Heyman and Rang, 1985) and lightly myelinated Aδ-fibers (Such and Jancso, 1986). Larger diameter fibers, not generally accepted as

mediating neurogenic inflammation and pain except in the case of allodynia (Yamamoto et al., 2008), are not sensitive to capsaicin. In addition to stimulating the subset of sensory neurons responsible in large part for neurogenic inflammation and pain propagation, the majority of capsaicin sensitive sensory neurons contain neuropeptides, including CGRP (Hingtgen and Vasko, 1994a;Hingtgen et al., 2006;Holzer, 1988;Skofitsch and Jacobowitz, 1985).

Capsaicin stimulates small-diameter sensory neurons through binding to and activation of a ligand-gated, non-selective cationic channel. The receptor for capsaicin was originally identified using radiolabeled capsaicin-related molecules (Szallasi and Blumberg, 1990). This receptor was later cloned and given the name transient receptor potential vanilloid 1 (TRPV1), due to its ability to transiently allow the passage of cations, such as sodium and calcium (Caterina et al., 1997;Oh et al., 1996). This influx of cations causes depolarization and neurotransmitter release (Bevan and Szolcsanyi, 1990;Wood et al., 1988). TRPV1 is found predominantly on a subset of small-diameter, nociceptive sensory neurons (Caterina et al., 1997;Helliwell et al., 1998). This receptor also responds to endogenous and commonly encountered noxious stimuli, including acidic shifts in pH and noxious heat above 43° C, making activation of this receptor by capsaicin a surrogate for natural nociceptive stimuli encountered by sensory neurons. Moreover, TRPV1 colocalizes nearly completely with CGRP in the dorsal root ganglia (Aoki et al., 2005;Price and Flores, 2007;Yu et al., 2008), which is further evidence of its role in neurogenic inflammation and pain propagation. Interestingly, the inhibition of neuronal activity induced by high concentrations of or prolonged exposure to capsaicin are a result of desensitization of TRPV1, likely through calcium-induced mechanisms (Mondadi et al., 2004). The mechanisms of TRPV1 modulation and the resulting changes in sensitivity will be discussed below.

Capsaicin is able to elicit responses from a subset of small-diameter sensory neurons, those which are responsible for neurogenic inflammation and pain propagation, specifically through the TRPV1 cationic channel. This attribute makes capsaicin a useful and powerful tool for the study of the sensitivity of these neurons. While repeated stimulation of sensory neurons by capsaicin leads to desensitization (termed tachyphylaxis) REF, this substance has a well established ability to elicit the release of CGRP at low concentrations in many different tissues and model systems. These attributes make capsaicin a primary tool for use in my peptide release experiments.

2. Potassium

Potassium ion concentrations are maintained at a low level in the extracellular compartment and a high concentration inside the neurons. This concentration gradient is responsible for the homeostasis of the membrane potential. When the concentration is increased outside of the neuron, this homeostasis is no longer present, fewer potassium ions are able to exit the neuron through potassium channels, and a positive charge is created at the neuronal membrane. This change in potential is called depolarization, and depolarization results in many voltage gated ion channels changing their properties so as to further depolarize the membrane. In this way, potassium can be used as a general depolarizing stimulus in CGRP release experiments. The use of a high extracellular concentration of potassium to activate sensory neurons and induce release of CGRP is extensive (Hingtgen and Vasko, 1994a;Hingtgen et al., 1995;Hingtgen et al., 2006;Mason et al., 1984;Saria et al., 1986). High extracellular potassium, at concentrations approximately 10 times physiological extracellular concentrations, will be used as a general depolarizing stimulus in my release experiments.

b. Agents that sensitize sensory neurons

1. NGF

Recently, the growth factor family of molecules has been identified as being released during inflammation and sensitizing sensory neurons (for a comprehensive review see (Mendell et al., 1999)). Nerve growth factor (NGF) was the first of these growth factors for which increased levels during inflammation (Weskamp and Otten, 1987) and sensitization of sensory neurons was demonstrated. NGF sensitizes nociceptive behavioral responses elicited by small-diameter sensory neurons, electrophysiological characteristics of these neurons, and the release of neuropeptides from these neurons.

The initial sensitizing effects of NGF on small diameter sensory neurons were evaluated using behavioral methods. Long-term administration of NGF from birth to adulthood (5 weeks old) led to increased sensitivity of these animals to two classic nociceptive stimuli, noxious heat and noxious mechanical pressure (Lewin et al., 1993). Later, injection of NGF directly into the paw also caused enhanced behavioral responses to noxious heat and mechanical stimuli, using the hot plate test and Von Frey hairs (Della et al., 1994). This phenomenon has been further characterized and appears to be as a result of the interaction of NGF with TRPV1. Specifically, NGF increases the insertion of TRPV1 into the membrane of small diameter sensory neurons (Zhang et al., 2005) and phosphorylates this channel by interacting with either or both of its receptors, TRKA and p75, which in turn activates a number of intracellular signaling pathway (Doya et al., 2005;Zhang and Nicol, 2004;Zhu and Oxford, 2007;Zhuang et al., 2004). The NGF-induced modulation of TRPV1 by these pathways is directly responsible for the hyperalgesia observed.

NGF also modulates the electrophysiological characteristics of small diameter sensory neurons, resulting in enhanced sensitivity of these neurons. NGF applied to the receptive skin fields of an attached skin-saphenous nerve preparation increased the

thermal sensitivity of small diameter C and Aδ fibers (Rueff and Mendell, 1996). In fact, a number of these neurons that were initially non-responsive to the noxious heat became responsive after NGF administration. NGF is also able to reverse the capsaicin-induced desensitization of sensory neurons and to increase the responsiveness of small diameter sensory neurons to a second application of capsaicin (Shu and Mendell, 1999). This effect of NGF is likely due to TRPV1 insertion in the cell membrane in a protein kinase C (PKC)-dependent fashion (Bonnington and McNaughton, 2003) and phosphorylation and subsequent increased activation of TRPV1 in a PKA-dependent fashion (Shu and Mendell, 2001).

Finally, enhancement in the release of neuropeptides, another means by which to measure sensory neuronal sensitization, is accomplished by NGF. Exposure of sensory neurons to NGF for 10-20 minutes enhances the stimulated release of both SP and CGRP in response to several stimuli, including potassium (Malcangio et al., 1997b;Malcangio et al., 1997a), capsaicin (Hingtgen et al., 2006), anandemide and arachidonyl-2-chloroethylamide (Price et al., 2005). This effect of NGF is present in the spinal cord (Malcangio et al., 1997b;Malcangio et al., 1997a), trigeminal ganglia (Price et al., 2005), and DRG (Hingtgen et al., 2006). For this reason, NGF will be used as a positive control of sensitization.

2. GFLs

The purpose of this introduction has been three fold. First, evidence was provided that many forms of sensory neuronal sensitization, including behavioral, electrophysiological, and neuropeptide release, are accomplished by inflammatory mediators. Second, it was clearly demonstrated that known behavioral and electrophysiological sensitizers of sensory neurons, especially NGF and PGE_2, also sensitize these neurons to stimulated release. Finally, the literature demonstrates that

the GFLs may have similarities to these other inflammatory mediators that sensitize sensory neurons.

Thus far, this introduction has established that a number of substances released during inflammation, including the GFLs, change the sensitivity of sensory neurons. Evidence has been presented above that each of the GFLs is released in increased amounts during inflammation, cause hyperalgesia, increase the excitability and other physiological properties of sensory neurons, and increase the levels of neuropeptides and ion channels important in sensory neuronal sensitization. Each of these characteristics of GFLs add to the likelihood they are important modulators of sensory neuronal sensitivity and nociception. However, the question still remains: do these GFLs mediate this inflammatory hyperalgesia by altering electrophysiological properties of the neurons eventually leading to neuropeptide release, neurogenic inflammation, and nociception.

Finally, a function of this introduction was to justify the use of isolated DRG neurons in culture as a model system for the studies outlined below. Many of the studies examining GFL-induced sensitization have used mice (Albers et al., 2006;Bennett et al., 2006;Elitt et al., 2006;Malin et al., 2006), and in order to evaluate the release of CGRP and other phenotypes of sensory neurons, it is advantageous to use isolated sensory neurons (Malin et al., 2006). This particular preparation will allow me to determine the effects of GFLs on the sensory neurons themselves, without the complications that often exist as a result of the presence of other neuronal cell types (i.e. interneurons) and molecules in behavioral assays, which limits the complicating factors in this preparation. Since small diameter, nociceptive sensory neurons are the type of cell that releases the majority of CGRP (Traub et al., 1990), the measure of changes in the release of CGRP is a valid functional endpoint for the evaluation of GFL-induced sensitization of nociceptors. Most importantly, this model systems allows me to manipulate the

intracellular signaling pathways within the sensory neurons, pharmacologically and genetically, to determine the pathways responsible for GFL-induced sensitization.

H. Potential intracellular signaling pathways of GFL-induced sensitization

There are a number of candidate pathways by which GFLs may be able to sensitize sensory neurons. The GFLs activate several signaling pathways, in both a Ret-dependent and independent manner, including the MAPK, PI-3K, PLC-γ, Src, and Fyn pathways (see (Sariola and Saarma, 2003) for a review). Interestingly, these same pathways are implicated in behavioral and electrophysiological sensitization of sensory neurons by other known sensitizers of nociceptive sensory neurons.

1. Ras

Activation of Ret by the GFLs results in recruitment and interactions of intracellular proteins that lead to activation of guanine nucleotide exchange factors (GEFs;(Fukuda et al., 2002). GEFs convert Ras-GDP to Ras-GTP, its active form (Shih et al., 1980b). Ras-GTP recruits the kinase Raf to the membrane, which results in a cascade of phosphorylation of several proteins. There is a genetically modified mouse available, a mouse with half the amount of functional neurofibromin protein (*Nf1* +/-), which is a potent guanine triphosphatase (GTPase) for Ras (Xu et al., 1990). This protein accelerates the de-activation of Ras by hydrolyzing Ras-GTP, the active form of Ras, to Ras-GDP, the inactive form (Shih et al., 1980a). One eventual downstream effector of Ras activation is Erk, one of the mitogen activating protein kinase (MAPK) proteins (Bron et al., 2003). Ras-GTP signaling is also one mechanism by which the PI-3K pathway is initiated. *Nf1* +/- mice have increased Ras activity (Kim et al., 1995), and therefore increased MAPK/Erk 1/2 and PI-3K pathway activation.

2. MAPK pathway

Although the classic MAPK-Erk pathway was originally identified as a growth promoting pathway, it is generally accepted that this pathway is often critical in sensory neuronal sensitization (see reviews in (Cheng and Ji, 2008;Obata and Noguchi, 2004). The well characterized sensory neuronal sensitizer, NGF, activates Erk and exerts some of its sensitizing effects through the MAPK-Erk pathway (Averill et al., 2001). Erk activation is necessary for NGF-induced thermal hyperalgesia and NGF-induced potentiation of capsaicin currents (Zhuang et al., 2004). Blocking activation of Erk with pharmacological inhibitors attenuates, at least in part, these NGF mediated sensitizing effects (Zhuang et al., 2004). Similarly, after inflammation created by CFA, PGE_2-induced sensitization is Erk dependent (Dina et al., 2003). While the exact mechanisms of Erk-activated sensory neuronal sensitization are unclear, there are two intriguing possibilities. First, activated Erk may aid in increased insertion of TRPV1 into the cell membrane (Zhang et al., 2005). TRPV1 insertion would lead to enhanced nociceptive responses to noxious heat and pH. MAPK/Erk 1/2 pathway inhibitors prevent the NGF-induced enhancement in capsaicin currents (Zhu and Oxford, 2007). Since Erk is a kinase, activated Erk may mediate this enhancement by directly phosphorylating TRPV1 and increasing the ion fluxes through this channel (Zhu and Oxford, 2007). The PKC pathway also plays a role in NGF-induced sensitization (Dina et al., 2003;Shu and Mendell, 2001) leading to controversy about whether Erk is the primary intracellular signaling pathway by which NGF sensitizes sensory neurons or whether Erk activation is secondary to induction of other intracellular signaling pathways, such as the PKC pathway.

While GFLs activate the MAPK-Erk pathway in sensory neurons (see (Sariola and Saarma, 2003) for a review), whether GDNF-induced sensitization is Erk dependent is unknown. Erk is necessary for GDNF-dependent increases in TRPV1 surface

expression in nociceptive sensory neurons (Bron et al., 2003). GDNF-mediated voltage gated calcium channel insertion in the cell membrane, leading to increased neuronal excitability, is Erk dependent as well (Woodall et al., 2008). Most strikingly, inhibition of Erk activation with the pharmacological inhibitor U0126 abrogated GDNF-induced mechanical hyperalgesia (Bogen et al., 2008). However, none of these studies have evaluated the role of GDNF-induced actions directly on sensory neuronal sensitization and neurotransmitter release through the MAPK-Erk pathway. The evidence for the role of the involvement of the MAPK-Erk pathway in sensitization by growth factors makes this pathway a candidate for GFL-induced enhancement of the release of CGRP.

3. PI-3K pathway

The PI-3K pathway, which mediates many of its sensitizing effects through several intracellular signaling pathways, including the PIP_2 and DAG-activated pathways (reviewed by (Manning and Cantley, 2007)), is activated by inflammatory mediators, including NGF (Zhu and Oxford, 2007). Activation of PI-3K is important for initiation of some nociceptive behavioral responses. The exploratory test is one such nociceptive behavioral test. Rodents experiencing nociception, when placed in a new environment, do not explore their new surroundings as completely as mice not experiencing nociception. Recovery of normal exploratory behavior can be completed by inhibition of the PI-3K pathway by the PI-3K inhibitor, Wortmannin, a potent but non-selective inhibitor of PI-3K pathway activation (Sun et al., 2007). Additionally, NGF sensitizes capsaicin currents, and this sensitization is abolished by the PI-3K pathway inhibitor, LY294002 (Zhu and Oxford, 2007). This sensitization of capsaicin currents by NGF is mediated by phosphorylation of the channel by the downstream effectors of the PI-3K pathway, since mutation of sites known to be phosphorylated by these effectors to residues that cannot be phosphorylated prevents this sensitization (Zhu and Oxford, 2007).

Proper spermatogonia development and neuronal survival and outgrowth, mediated by GFLs is PI-3K dependent (Lee et al., 2007). However, there is little information on the role of this pathway in GFL-induced sensory neuronal sensitization in the literature. In fact, only one study evaluating this phenomenon is available. GDNF-induced mechanical hyperalgesia is also PI-3K pathway dependent (Bogen et al., 2008), which does not provide direct evidence of the mechanism of GDNF-induced sensory neuronal sensitization, since there are complications of these behavioral assays (as described previously). The PI-3K pathway inhibitors, Wortmannin and LY294002, prevented the decrease in the mechanical threshold of paw withdrawal upon plantar injection of GDNF (Bogen et al., 2008). This pathway is yet another candidate pathway for examination for the mechanism of GFL-induced enhancement in the release of CGRP.

4. PLC-γ

Inflammatory mediators initiate the PLC pathways through G-protein coupled receptors and receptor tyrosine kinase receptors to induce sensory neuronal sensitization. Activation of the PLC-γ pathway is critical for NGF and bradykinin-induced thermal hyperalgesia. Both pharmacological inhibition of the PLC-γ pathway and treatment with a blocking antibody preventing activation of PLCs attenuates this hyperalgesia (Chuang et al., 2001b). One mechanism for PLC-γ in thermal hyperalgesia and modulation of sensory neuronal sensitivity is by releasing TRPV1 from PIP_2 inhibition, although this mechanism is controversial and may be indirect (Lukacs et al., 2007). In fact, it appears that phosphatidylinositols may have a dual regulation of TRPV1, resulting in both activation and inhibition of channel activation (Lukacs et al., 2007). However, the mechanisms is complex and not well defined. The use of carrageenan (CARR), an inflammatory mixture injected into the paw, to induce mechanical hyperalgesia was also used to evaluate the role of the PLC pathway in inflammatory hyperalgesia. A pharmacological inhibitor of the PLC-γ pathway, U73122,

and an antisense PLC oligonucleotide prevented the CARR-induced hyperalgesia (Joseph et al., 2007). Additionally, a PLC activator, lysophosphatidylcholine (LPC), was able to mimic the CARR and PGE_2 mediated hyperalgesia. Modulation of transient receptor potential A1 (TRPA1), another ion channel present in nociceptive sensory neurons and responsible for some of the signals leading to sensitization and hyperalgesia, by bradykinin is PLC-dependent as well (Wang et al., 2008a). The PLC modulation of TRPA1 seen in heterologous expression systems is recapitulated in behavioral assays, with bradykinin mediated chemically-induced hyperalgesia being prevented with PLC inhibitors.

The role of the PLC pathway in bradykinin (BK) and PGE_2 mediated sensory neuronal sensitization and hyperalgesia is well accepted. It is unclear whether NGF-induced sensitization occurs through this or different signaling mechanisms than BK and PGE_2. Application of a PLC inhibitor, polylysine, and PIP_2 applied to excised inside-put patches from heterologous expression systems enhanced TRPV1 responses (Stein et al., 2006). Enhancement of capsaicin induced currents through TRPV1 were also unaltered by co-transfection of an activity deficient PLC-γ in a heterologous expression system (Zhu and Oxford, 2007). In another study, neomycin, a potent but non-selective inhibitor of the PLC-γ pathway, did not change the enhanced calcium ion influx induced by NGF in neonatal neurons (Bonnington and McNaughton, 2003). This same study also showed that U73122 actually enhanced the NGF-induced calcium influx. A controversy still remains as to the role of the PLC-γ pathway in NGF-induced sensory neuronal sensitization.

The PLC-γ pathway, through diacylglycerols (DAGs), initiates protein kinase C (PKC) activation, which is important in some forms of sensitization (Sikand and Premkumar, 2007). PKC is another mediator of sensory neuronal sensitization. Activated PKC levels increase during many types of inflammation, including ultraviolet radiation (Matsui and

DeLeo, 1990). An exogenous stimulator of PKC, phorbol esters, causes sensitization of nociceptors to thermal stimulation, which is prevented by an inhibitor of PKC, staurosporine (Leng et al., 1996). PKC is involved in neuronal sensitization to a number of other stimuli, including capsaicin, PGE_2, and bradykinin (Cesare et al., 1999;Gold et al., 1998;Sluka et al., 1997). This sensitization is accomplished in several ways. PKC enhanced the overall inward ionic current (Cesare et al., 1999), tetrodotoxin-resistant inward sodium currents (Gold et al., 1998), and TRPV1-dependent inward sodium and calcium currents in response to heat (Sluka et al., 1997). The role of PKC activation in NGF-induced sensory neuronal sensitization is less clear. In one study, BIM (inhibitor of PKC α, β_1, β_2, γ, δ, and ε) and staurosporine (a potent but non-selective inhibitor of PKC α, β_1, β_2, γ, and λ), two inhibitors of PKC, reduced the NGF-induced sensitization (Bonnington and McNaughton, 2003), while in two other studies the same inhibitors and others inhibitors of PKC did not change the NGF-mediated enhancement in TRPV1 currents (Chuang et al., 2001a;Shu and Mendell, 2001). Neuropeptide release is also augmented by PKC. Both capsaicin and potassium-stimulated release of SP and CGRP are enhanced by the PKC activator, PDBu (Barber and Vasko, 1996). Although controversy exists, PKC is probably an important mediator of some forms of sensory neuronal sensitivity in response to some specific sensitizers.

The GFLs also activate the PLC-γ pathway in sensory neurons and neuron-like cell lines (Lee et al., 2006;Mikaels-Edman et al., 2003). In both of these cases, GDNF and neurturin, through the PLC-γ pathway, were able to induce neurite outgrowth. Strikingly, the PLC-γ pathway inhibitor, U73122, abrogated GDNF-induced mechanical hyperalgesia *in vivo* (Bogen et al., 2008). Whether GFL-induced hyperalgesia or sensitization is induced through PKC activation is still unclear. Therefore, the PLC-γ pathway, and possibly subsequent PKC activation, may be one pathway by which GFLs enhance the stimulated release of CGRP.

5. Src family kinases

Src family kinases, which in neurons include Src, Fyn, Lck, Hck, and Yes (Jeong et al., 2008b;Omri et al., 1996;Pyper and Bolen, 1989;Ramseger et al., 2009;Wang and Yu, 2005), are non-receptor tyrosine kinase molecules for which emerging evidence suggests a role in sensory neuronal sensitization. Ionotropic and metabotropic glutamate receptors can be modulated by Src family kinases, resulting in central sensitization in the dorsal horn of the spinal cord (Kawasaki et al., 2004). Inhibition of calcium influx through NMDA channels by pharmacological Src inhibition prevents spinal dorsal horn sensitization and the subsequent inflammatory mechanical hyperalgesia (Guo et al., 2004). Src also plays a role in the sensitization of primary afferent neurons in the DRG. Inhibition of Src family kinases by the inhibitor PP2 prevents the enhanced NMDA responses of primary afferent neurons from rats with experimental colitis (Li et al., 2006). The osmomechanical stimuli transducer on sensory neurons, TRPV4, is also modulated by Src family kinases (Wegierski et al., 2008). Pharmacological inhibition of the Src family kinase (SFK) pathway by PP1 attenuates nociceptive behaviors induced by injection of a hypertonic solution in the paw, a model of inflammatory pain (Alessandri-Haber et al., 2005). Specifically, the increases in paw flicks in rats and paw licking and shaking in mice after injection of a hypertonic solution is prevented by concurrent application of PP1 (Alessandri-Haber et al., 2005;essandri-Haber et al., 2008). Additionally, SFKs are necessary for initiation of mechanical hyperalgesia after a neuropathic injury. SFK constitutive activation by the YEEI peptide mimics the mechanical hyperalgesia as a result of neuropathic pain, and this hyperalgesia in both cases is mediated by the TRPV4 receptor (Alessandi-Haber 2008). SFKs also bind to TRPV1 at a specific residue, Y200, and mediated the NGF-induced insertion into the cell membrane and enhanced sensitivity of this channel in neurons (Zhang et al., 2005). These effects were prevented with a general SFK inhibitor, PP2, and by infection with

dominant negative Src. Some of the other Src family kinases, such as Lck and Fyn, also modulate the ion channels in sensory neurons (Wang and Yu, 2005). Application of a solution containing a 4 mM potassium activated the Na^+K^+ pump, and the SFK inhibitor PP1, as well as specific blocking antibodies to Lck and Fyn, prevented this channel's activation (Wang and Yu, 2005).

The Src family kinase signaling pathways are activated by GFLs. Specifically, Src family kinases are necessary for GDNF dependent neuronal outgrowth and survival in both neuronal cells and neuron-like cell lines (Encinas et al., 2001;Paveliev et al., 2004;Poteryaev et al., 1999;Trupp et al., 1998a;Trupp et al., 1999a). Injection of the SFK inhibitors PP2 and SU6656 into the paw of a rodent abrogated the mechanical hyperalgesia induced by GDNF (Bogen et al., 2008). Many of the GDNF-induced, Src-dependent actions appear to be Ret-independent in nature, since blocking the Ret receptor and using Ret-deficient cell lines does not alter the effects of the GDNF (Bogen et al., 2008;Paratcha et al., 2003;Paveliev et al., 2004;Poteryaev et al., 1999;Trupp et al., 1999a). There are three possible mechanisms for this GDNF-induced, Ret-independent function. These include: GDNF-induced SFK activation through GFRα-1, GDNF-induced SFK activation indirectly through the Integrin β-1 receptor, or GDNF-induced SFK activation indirectly through the NCAM receptor (Cao et al., 2008a;Cao et al., 2008b;Paratcha et al., 2003;Poteryaev et al., 1999). These NCAM-induced effects are mediated through one specific SFK pathway, the Fyn pathway (Cao et al., 2008a;Cao et al., 2008b). Application of artemin to isolated DRG promotes neurite outgrowth, which is prevented by pharmacological inhibition of SFK pathways with SU6656 (Jeong et al., 2008). Actin polymerization, another measure of the ability of DRG to efficiently grow neurites, was dependent upon Src and Hck (Jeong et al., 2008b). It is, therefore, necessary and intriguing to evaluate the possible role of Src

family kinases in both Ret-dependent and Ret-independent GFL-induced enhancement in the release of CGRP.

I. Potential mechanisms of GFL-induced enhancement in release of CGRP

There are several potential mechanisms for enhancement in the release of CGRP induced by GFLs, which will be described in the following sections in detail. First, GFLs may increase the content of CGRP, thereby providing a larger releasable pool upon stimulation. Second, The GFLs may change the phosphorylation state of a number of proteins, including intracellular proteins, ion channels, TRPV1, or proteins important in vesicle fusion and neurotransmitter release. The GFLs may also enhance the insertion of important proteins into the cell membrane, such as TRPV1. Finally, even acute (10 minute) exposure of sensory neurons to the GFLs could increase the expression of ion channels important in sensitization, including sodium channels and TRPV1.

There is precedent for growth factor application increasing the content of neuropeptides like CGRP. Long-term exposure to NGF can change the content of CGRP in sensory neurons when administered *in vivo* or to neurons in culture (Bowles et al., 2004;Donnerer et al., 1996;Schuligoi and Amann, 1998). Additionally, long-term exposure to GDNF (5 day exposure) increases the content of CGRP in trigeminal primary afferent neurons (Price et al., 2005). It is possible, then, that the GFLs are increasing the amount of CGRP present in DRG sensory neurons, and this could account for any GFL-induced enhancement in the stimulated release of CGRP. However, this mechanism is unlikely, since the sensory neurons in the preparation used to evaluate stimulated release are only exposed to the GFLs for 20 minutes. I will verify that the short term exposure to GFLs do not change the total amount of CGRP in the sensory neuron cultures.

Inflammatory mediators that enhance the release of CGRP often alter the phosphorylation state of intracellular proteins, ion channels, or synaptic vesicle proteins.

For example, NGF is able, through its intracellular signaling pathways, to phosphorylate two important synaptic vesicle proteins, synaptotagmin-4 and synapsin I (Mori et al., 2008;Raiteri et al., 2003). Phosphorylation of these proteins leads to enhanced release of glutamate and neuropeptide Y. Pathways activated by NGF also phosphorylate and enhance the activity of a number of sodium and other ion channels in a number of cell types (Good et al., 2008;Leung et al., 1994). One important ion channel whose activity is enhanced by phosphorylation through NGF-activated signaling pathways is TRPV1. The PI-3K and MAPK pathways, activated by NGF, are critical in TRPV1 phosphorylation and activation (Zhu and Oxford, 2007). Since GFLs activate many of the same pathways responsible for NGF-induced phosphorylation (see previous sections) and activation of synaptic proteins and ion channels, the GFLs may similarly enhance the release of CGRP by these mechanisms.

One unique and interesting effect of NGF on sensory neurons is the ability of this growth factor to induce increased membrane expression of TRPV1 very rapidly (Zhang et al., 2005). This effect of NGF is mediated by the PI-3k and Src family kinase pathways. Since GFLs activate both of these pathways, rapid membrane insertion of TRPV1 is yet another possible mechanism for GFL-induced enhancement in the stimulated release of CGRP.

J. Hypothesis and specific aims

Evidence presented above demonstrates that the GFLs, GDNF, NTN, and ART, are released in increased amounts during inflammation and are important in inflammatory processes, including inflammatory hyperalgesia. The behavioral consequences of the GFLs may be a result of sensory neuronal sensitization. GFLs can increase many diverse and interconnected intracellular signaling pathways, including MAPK, PI-3K, PLC-γ, PKC, and Src family kinases. These pathways, either through direct modulation of ion channels and synaptic proteins or through increased insertion of cell membrane

proteins, can increase the release of neurotransmitters, including the neuropeptides, in many neuronal cell types in the CNS and PNS. This GFL-induced sensitization, in my experiments, can be expressed as enhancement in CGRP release from sensory neurons. Because GFLs may accomplish their sensitization through compliments of signaling pathways and mechanisms different than other known sensory neuronal sensitizers, or may exert their effects on a distinct population of nociceptive sensory neurons, these growth factors are particularly intriguing. The hypothesis of this thesis is that select GFLs enhance the release of CGRP from isolated sensory neurons, and that this release is mediated by distinct intracellular pathways for each of the GFLs. There is emerging evidence that the GFLs can mediate their actions indirectly through mechanisms not requiring the classic Ret initiated pathways. Therefore, GFLs could mediate some of their sensitization effects through Ret-independent mechanisms. To examine these hypotheses, the following specific aims will be addressed:

1. Determine which of the GFLs augment stimulus-evoked release of the neuropeptide transmitter CGRP and the extent to which this augmentation occurs.
2. Determine the intracellular signaling pathways responsible for the enhancement in release of CGRP by each of the GFLs and whether these pathways are similar or distinct.
3. Determine the contribution of Ret-dependent signaling to the augmentation of stimulus-evoked release of CGRP by GFLs from sensory neurons.
4. Determine the contribution of Ret-independent signaling to the augmentation of stimulus-evoked release of CGRP by GFLs from sensory neurons.

II. Methods

A. Materials

Cell culture supplies were purchased from Sigma (St. Louis, MO, USA). Capsaicin was purchased from Sigma Chemical Company (St. Louis, MO, USA) and was first dissolved in 1-methyl,2-pyrrolidinone (Aldrich Chemical Co., Milwaukee, WI, USA) to a concentration of 10 mM. It was then serially diluted to a concentration of 50-500 nM in the appropriate release buffer as noted below. Horse serum, F-12 medium, L-glutamine, and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Collagenase, poly-D-lysine, laminin, 5-fluoro-2-deoxyuridine, uridine and standard laboratory chemicals were from Sigma (St. Louis, MO, USA). Antibody to calcitonin gene-related peptide (CGRP) was generously provided by Michael J. Iadarola (NIH). Nerve growth factor, NGF, was purchased from Harlan Bioproducts for Science, Inc. (Indianapolis, IN, USA) in the 7S, gel filtered form and lyophilized for long-term storage. Calcitonin gene-related peptide (CGRP), a peptide containing only residues 27-37, was obtained from Bachem (Torrance, CA, USA) and generously iodinated by Dr. Michael Vasko. The CGRP peptide had a residue 27 replaced with a tyrosine for iodination. CGRP standard peptide, residues 27-37, was purchased from Tocris Bioscience (Ellisville, MO, USA). Recombinant human glial cell line-derived neurotrophic factor (GDNF), neurturin, artemin, and persephin were purchased from Peprotech (Rocky Hills, NJ, USA). Pharmacological inhibitors were purchased from Calbiochem (Darmstadt, Germany), unless otherwise indicated. The siRNA constructs were either purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or developed by Eric L. Thompson in Dr. Michael L. Vasko's laboratory and created by Dharmacon, Inc (Lafayette, CO, USA) as indicated. All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise indicated.

B. Animals

The mice used for all experiments, C57BL/6 mice, were purchased from Harlan Laboratories (Indianapolis, IN) and/or bred and housed in the Indiana University Laboratory Animal Research Center (LARC). Mice were housed in group cages in a light-controlled room at a constant temperature of 22° C. All mice were adults, between three and six months in age. Food and water were available at the convenience of the animals. Mice heterozygous for the *Nf1* mutation on a C57BL/6J background, developed by Dr. Tyler Jacks (Jacks et al., 1994), were bred and housed in the Indiana University Laboratory Animal Research Center (LARC), as well. All procedures described and conducted were done so under approval from of the Animal Care and Use Committee of Indiana University School of Medicine.

C. Isolation and culture of adult mouse sensory neurons

Dorsal root ganglia (DRG) from adult mice were used to establish sensory neuronal cultures. The DRG were removed from adult mice in a manner similar to that previously published (Hingtgen, et al., 2006). Specifically, adult mice were euthanized with CO_2, and the spinal column removed. The spinal column was cut to expose the spinal cord, which was removed. Then, DRG were extracted using microdissection and placed in Puck's solution. DRG were digested in 0.1% collagenase in two separate 30 minute incubations at 37° C. Additionally, cells were digested in F12 media containing DNAse (1-3 µg/3 mL) for one minute at room temperature. The preparation was dissociated by mechanical agitation. Cells were plated in wells of 24-well Falcon culture dishes coated with poly-D-lysine (0.1 mg/mL) and laminin (0.25 mg/mL) at a density of 30,000-50,000 cells/well. Cultures were maintained at 37° C in a 5% CO_2 atmosphere in F12 media supplemented with 2 mM glutamine, 50 µg/mL penicillin and streptomycin, 10% heat-inactivated horse serum and mitotic inhibitors (50 µM 5-fluoro-2-deoxyuridine and 150 µM uridine). NGF, at a concentration of 30 ng/mL, was added to this media. Growth

medium was changed every 2-3 days, and the added NGF removed 48 hrs prior to all experiments unless otherwise indicated.

D. Counting of sensory neurons prior to plating

In order to ensure similar amounts of cells are being plated for each experiment, the amount of cells/mL were determined. Prior to plating the DRG, 30 µl of DRG suspended in F12 Media was mixed with 30 µl of Trypan Blue solution (Sigma). Then, 25 µl of this mixture was placed on a counting chamber, and the number of neurons in each of five square portions of the chamber was counted. Based on volume calculations, the total number of cells in the entire DRG preparation was determined. In all cases, the density of cells/well when plated was 30,000-50,000. Counting of cells was conducted approximately every 3-6 months, unless the content of CGRP (method for determining content is below in Section II.F) was outside of the consistent range. The details of how this consistent range was determined are described below. In this case, cells from the next three harvests were counted.

E. Release of calcitonin gene-related peptide (CGRP) from sensory neurons grown in culture

Measurement of stimulus-evoked release and content of immunoreactive CGRP (iCGRP) from isolated sensory neurons was accomplished as previously published (Hingtgen et al., 2006). The measured amount of peptide is referred to as iCGRP because it is identified by a polyclonal antibody directed at CGRP. After 5-7 days in culture, culture media was removed from the sensory neurons in culture and the basal or resting release of iCGRP measured from cells incubated for 10 minutes in Hepes buffer consisting of (in mM): 25 Hepes, 135 NaCl, 3.5 KCl, 2.5 CaCl2, 1 $MgCl_2$, 3.3 dextrose, and 0.1% (w/v) bovine serum albumin, pH 7.4, and maintained at 37° C. The cells were incubated in Hepes buffer containing stimulus (capsaicin or high potassium) for 10 minutes, and then incubated again with Hepes buffer alone to reestablish resting release

levels. The concentrations of capsaicin (50 nM) and potassium (30 nM and 50 nM) were chosen because they lie on the low end of the highly sloped portion of the concentration response curve for iCGRP release. The use of these concentrations allow for evaluation of enhancement in release of CGRP after exposure to sensitizing molecules. The amount of iCGRP released in each incubation was measured by radioimmunoassay (RIA). GFLs were added in the basal incubation period (10 minutes) and in the stimulated incubation period (10 additional minutes). The neurons were exposed to GFLs for a total time of 20 minutes. A minimum of three different preparations were used for each condition, including growth factor application and stimulus.

Inhibition of intracellular signaling pathways and surface receptors was accomplished using both small interfering RNA (siRNA) manipulations and pharmacological inhibitors. All siRNA molecules were directed toward particular regions of mouse RNA for each specific protein and most were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Those siRNA molecules directed toward Src and used as a scramble (designed as a scramble for APE1) were developed by Eric L. Thompson in Dr. Michael Vasko's laboratory and ordered from Dharmacon, Inc. (Chicago, IL). Table 2 provides all pertinent details about the siRNA molecules used. When using siRNA to inhibit specific protein production, these molecules were added two days after DRGs were plated. Metafectine Pro (Biontex Laboratories, Martinsried, Planegg, Germany), the transfection agent, was diluted to a titer of 1:250 in each well in Optimem reduced serum media (Invitrogen, Carlsbad, CA). The siRNA molecules were also diluted in Optimem. The Metafectine and siRNA dilutions were allowed to sit at room temperature for two minutes then mixed at a 1:1 ratio and allowed to incubate at room temperature for 20 minutes. The mixture was added to each well so that the final concentration of the siRNA was 100 nM. The following day, F12 media containing NGF and normocin was added to the wells to a final volume 1.0 mL. Twenty four hours later, all the media was removed from the

Table 2

Details of siRNA molecules

	Source	Concentration	Specific sequence vs. pooled	Sequence (if known)
Fyn siRNA	Calbiochem, Inc.	100 nM	Pooled	N/A
Integrin β-1 siRNA	Calbiochem, Inc.	100 nM	Pooled	N/A
NCAM siRNA	Calbiochem, Inc.	100 nM	Pooled	N/A
Ret siRNA	Calbiochem, Inc.	100 nM	Pooled	N/A
c-Src siRNA	Eric L.Thompson/Dharmacon, Inc,	100 nM	Specific sequence	GCCTACTGCCTCT CTGTATGGCCCA AGTCATGAAGAAA
Scramble siRNA	Eric L.Thompson/Dharmacon, Inc,	100 nM	Specific sequence	CCAUGAGGUCAGCA UGGUCUGAAGGUA CUCCAGUCGUACCAG

wells and 500 µl of normal growth media (F12 media supplemented with F12 media supplemented with glutamine, penicillin and streptomycin, horse serum and mitotic inhibitors as indicated above) was added. F12 media without supplemented NGF was added to the cells 48 hours prior to conducting experiments. Pharmacological inhibitors were added acutely. They were only present in the first basal and stimulated condition at concentrations indicated below.

F. Content of CGRP in sensory neurons grown in culture

After the release protocol, the remaining peptide content in each well was determined by exposing the cells to 2 N acetic acid for 10 minutes. Aliquots of this incubation were diluted in Hepes and iCGRP was determined by RIA. The remaining peptide content was added to the content from each of the incubation to give the total content of CGRP. The release of iCGRP is expressed as percent of the total content per 10 minute incubation, unless otherwise indicated.

G. Release of CGRP from spinal cord slices

Stimulus-evoked release and content of iCGRP from spinal cord slices was accomplished as previously published (Chen et al., 1996;Southall et al., 1998). Briefly, adult mice were euthanized with CO_2 and the entire spinal cord was removed from each animal. It was weighed and chopped into 300 µm cross-sections using a McIllwain Tissue Chopper. The chopped spinal cord from each animal was placed into its own individual chamber and perfused at a rate of 0.1 mL/minute for 20 minutes with Hepes buffer supplemented with 200 mM ascorbic acid, 100 µM Phe-Ala, and 20 µM bacitracin (all used as peptidase inhibitors to prevent the breakdown of CGRP during the process; Chen et al., 1996). The perfusion buffer was aerated with 95% O_2/5% CO_2 and maintained at a pH of 7.4. Serial 10 minute collections (1.0 mL of perfusate) were obtained from each spinal cord. Initially, the tissue was perfused with Hepes buffer alone or Hepes buffer containing 10 ng/mL growth factor for 30 minutes. The perfusate was

changed to Hepes buffer containing 500 nM capsaicin ± 10 ng/mL growth factor for 30 minutes to measure stimulated release. The tissue was perfused for 60 minutes with Hepes buffer after the stimulus exposure to allow a return to resting levels of peptide release. Aliquots from each 10 minute collection period were assayed for iCGRP using RIA. After the protocol was completed, the remaining iCGRP content of the tissue was determined by homogenizing the spinal cord tissue in 0.1 N HCl and serially diluting the supernatant with Hepes buffer and 1.0 M MES. The content was added to the amount of iCGRP released during the entire perfusion to obtain the total peptide content. The release of iCGRP is expressed as percent of the total content per 10 minute perfusion.

H. Radioimmunoassay for quantification of release of iCGRP

Samples containing CGRP were removed from the plates and diluted in Hepes buffer to a final volume of 300 µl. Radioactive CGRP, diluted to a range of 900,000 to 1,100,000 counts per minute (CPM), and CGRP antibody, at a concentration of 1:1,000,000, were added to the samples, centrifuged for 5 minutes, and allowed to incubate overnight at 4° C. A charcoal suspension was added to the samples and the combination centrifuged for 20 minutes. The samples were decanted and the supernatant placed on a Packard γ-Counter to be counted for 5 minutes each. The supernatant was counted because it contains the CGRP bound to antibody. The pellet contains the charcoal, which absorbs the radioactive peptide not bound to the CGRP antibody. The samples were plotted on a standard curve, created with CGRP peptide standards at the same time as the samples, and the fmol of iCGRP determined. The minimum amount of iCGRP detected by the RIA is 5 fmol with a 95% confidence interval (Chen et al., 1996).

I. Isolation of protein samples from sensory neurons in culture

Sensory neurons from DRG from adult mice plated on 12 well Falcon plates were maintained in culture for 5-7 days. Cells were then washed with Hepes buffer and

treatments added as indicated below. After treatment, the cells were washed once with PBS, then 500 µl of PBS added to each well. The wells were then scraped and the cells transferred in solution to ependorf tubes. The tubes were centrifuged at 16,000 rpm for 20 minutes. The supernatant was removed and the remaining pellet was either placed on dry ice and transferred immediately to a freezer at -80° C or protein content quantified immediately.

For protein quantification, DRG pellets were resuspended in 50 µl of general lysis buffer (1mM Na pyrophosphate, 50 mM Hepes, 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, and 1 mM Na orthovanadate) supplemented with proteinase inhibitor mixture (aprotinin, leupeptin, pepstatin, PMSF; Calbiochem, Sand Diego, CA, USA). The resuspended protein was incubated for 15 minutes on ice with frequent vortexing. The suspension was sonicated 3 times for 10 seconds each at 45 watts. The suspension was then centrifuged at 4,000g for 2 minutes. The supernatant was removed and stored at -20° C. The protein was quantified using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) and read on a Wallac plate reader at 595 nm for 1.0 s.

J. Western blots of protein from sensory neurons grown in culture

A total of 40 µg of the protein samples were mixed with loading buffer (Ambion, Austin, TX, USA) containing β-mercaptaethanol to a final volume of 60 µl and denatured at 70º C for 10 minutes. The samples were then incubated at room temperature for 15 minutes and loaded into wells of precast 10% SDS-PAGE gels (Biorad, Philadelphia, PA, USA) containing 10 lanes. The samples were run on the gels, which were connected to a Biorad power source, for 2 hours at 115 mV at room temperature. While the gel was running, filter papers (Biorad, Philadelphia, PA, USA), fiber pads (Biorad, Philadelphia, PA, USA), and PVDF transfer membranes (Millipore, Darwinweg, the Netherlands) were soaked in 1X transfer buffer (25 mM Tris at pH 7.5; 192 mM Glycine; 5-20 % methanol). Prior to soaking in transfer buffer, the PVDF membranes were soaked in 100% methanol

for 1 min and washed extensively with ddH_20. SDS-PAGE gels were placed on transfer membranes within a transfer cartridge and transferred in a Biorad system at 100 mV for 1 hour at room temperature with an ice pack in the apparatus.

After transfer, the membranes were removed from the apparatus and placed in 10% powered skim milk (EMD, Gibbstown, NJ, USA) in 1X TBS (20 mM Tris PH 7.5; 150 mM NaCl as a blocking solution for 1 hour at room temperature. Then, the blocking solution was discarded and the membranes placed in 5% milk in TBST (TBS containing 0.1% Tween-20) containing primary antibodies at concentrations of 1:200 to 1:1,000 (Table 3). The membranes were incubated in this solution overnight at 4° C. Several short washings and three 10 minute washings were accomplished with TBST after the overnight incubation. Secondary antibody, at concentrations from 1:4,000 to 1:25,000, in 5% milk in TBST was applied to the membrane for 1 hour at room temperature. A similar set of washings was done after the secondary antibody exposure, then the membranes were blotted dry and placed in the combination of solutions for enhanced chemiluminescence (ECL; Thermo Scientific, Rockford, IL, USA) for 3 minutes. The membranes were placed in clear plastic sheets and inserted into X-ray cartridges (Soyee Products, New York City, NY, USA). Once in complete darkness, X-ray films (Midwest Scientific, St. Louis, MO, USA) were placed inside the cartridge and exposed for 5 seconds, 30 seconds, 1 minute, 5 minutes, and 15 minutes. Films were first placed in Kodak/GBX developer (Thermo Fisher Scientific, Pittsburgh, PA, USA) for 30 seconds per side, then washed in cold water for 1 minute per side. The films were fixed in Kodak/GBX fixing solution (Thermo Fisher Scientific, Pittsburgh, PA, USA) for 30 seconds per side, washed again, and allowed to dry for 2 hours.

Table 3

Primary antibody titers

Antibody target	Source	Monoclonal vs. polyclonal	Company	Titer	Secondary antibody	Secondary antibody titer
α-*tubulin*	mouse	momoclonal	Sigma	1:1,000	Goat anti-mouse	1:10,000
Akt	rabbit	polyclonal	Cell Signaling Technologies	1:800	Goat anti-rabbit	1:10,000
c-Src	mouse	momoclonal	Santa Cruz Biotechnology	1:500	Goat anti-mouse	1:10,000
Erk	rabbit	polyclonal	Cell Signaling Technologies	1:900	Goat anti-rabbit	1:10,000
Fyn	mouse	momoclonal	Santa Cruz Biotechnology	1:300	Goat anti-mouse	1:25,000
Integrin β-*1*	mouse	momoclonal	Santa Cruz Biotechnology	1:300	Goat anti-mouse	1:25,000
NCAM (p140)	rabbit	polyclonal	Santa Cruz Biotechnology	1:500	Goat anti-rabbit	1:25,000
p-Akt	mouse	momoclonal	Cell Signaling Technologies	1:500	Goat anti-mouse	1:10,000
p-c-Src	mouse	momoclonal	Santa Cruz Biotechnology	1:300	Goat anti-mouse	1:25,000
p-Erk	mouse	momoclonal	Cell Signaling Technologies	1:500	Goat anti-mouse	1:10,000
p-Fyn	goat	polyclonal	Santa Cruz Biotechnology	1:200	Donkey anti-goat	1:25,000
p-Ret	rabbit	polyclonal	Santa Cruz Biotechnology	1:300	Goat anti-rabbit	1:25,000
p-SFK	rabbit	polyclonal	Cell Signaling Technologies	1:500	Goat anti-rabbit	1:10,000
Ret	rabbit	polyclonal	Santa Cruz Biotechnology	1:500	Goat anti-rabbit	1:25,000
SFK	rabbit	polyclonal	Cell Signaling Technologies	1:500	Goat anti-rabbit	1:10,000

Secondary antibody dilutions of 1:25,000 were used with the Super Signal Femto ECL kit

K. Densitometric evaluation of immunoreactive bands

The dried films were scanned as JPEG files and densitometric measurements made with Un-Scan It (Orem, UT, USA). Immunoreactive bands of interest were normalized to α-tubulin bands.

L. Statistical analysis

All differences in iCGRP release, total content, and immunoreactive band densities were compared with analyses of variance (ANOVAs) and Dunnett's post hoc analysis or Student t-tests, as indicated. A p value of <0.05 was used to indicate statistical significance between treatment and non-treatment groups.

III. RESULTS

A. GFLs at the highest concentrations used do not disrupt the radioimmunoassay

The experiments necessary to evaluate the role of the GFLs in the enhancement of the release of CGRP require the use of the RIA method. Since the GFLs are peptides, there is a possibility that the CGRP antibody could non-specifically bind to the GFLs and disrupt the RIA (Sheffield et al., 1977a). For this reason, each of the GFLs was added separately to Hepes buffer and the standard curve portion of an RIA performed with this buffer. The GFLs were added at the highest concentration used for experiments, 500 ng/mL. Figure 2 shows that none of the GFLs at 500 ng/mL altered the standard curves. Consequently, the GFLs do not interfere with the RIA.

B. Criteria used to determine a valid experiment evaluating the release of iCGRP

In order to ensure results from release experiments were reliable and to ensure that all data from experiments were consistent, several criteria for a valid CGRP release experiment were established. First, the basal or resting outflow of iCGRP during the initial 10 min incubation period had to be above the detection limit of the RIA. The basal release or resting outflow of iCGRP during the 10 min incubation period following the stimulation period also had to be above the detection limit of the RIA (5 fmol; Chen et al., 1996). It was necessary for the basal values of iCGRP to be above the detection limit of the RIA so that the stimulated value of iCGRP could be compared to the basal value. Additionally, having basal values of iCGRP above the detection limit of the RIA indicates that the neurons are producing enough CGRP to allow reliable detection of CGRP levels. Second, it was necessary for fmol of iCGRP released in response to the stimulus to be at least twice that of the basal level. For example, if the basal release were 10 fmol, the stimulated release would have to be greater than 20 fmol for the release to be

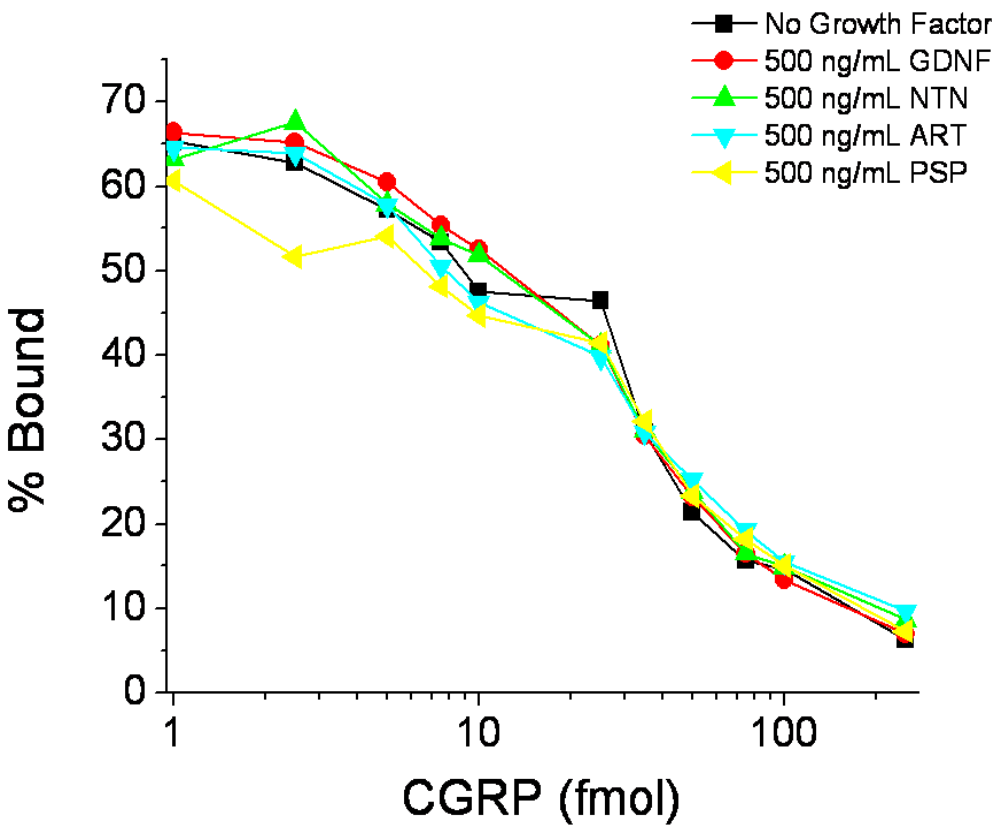

Figure 2. GFLs in the Hepes buffer do not interfere with the RIA. Standard curves in which the Hepes buffer solution contained no GFL (black), 500 ng/mL GDNF (red), 500 ng/mL NTN (green), 500 ng/mL ART (cyan), or 500 ng/mL PSP (yellow), are shown. This is the highest concentration used in experiments evaluating the release of iCGRP. Standard curves were run simultaneously with the same radiolabeled CGRP peptide (^{125}I-CGRP), antibody to CGRP, and CGRP standard peptide. N=1.

deemed valid. A doubling in stimulated-release over resting release of iCGRP indicates that the DRG neurons are responsive to the stimulus, and that the stimulus is in fact present in the stimulated condition. It is also well established in DRG in culture that concentrations of capsaicin from 30 nM to 50 nM and 30-50 mM KCl stimulate the release of iCGRP at least two-fold (Hingtgen and Vasko, 1994b;Hingtgen and Vasko, 1994a;Hingtgen et al., 1995;Hingtgen et al., 2006).

Because of the natural variability in cell density per well when plating the DRG neurons, most data from experiments involving GFL modulation of the release of iCGRP were reported as the amount of iCGRP released in 10 minutes as a percent of total iCGRP content in the well. For this reason, it was critical to establish a range of iCGRP content values that were deemed acceptable. A low content of iCGRP would indicate that the DRG neurons are not producing amounts of iCGRP that can be reliably measured. It could also indicate that there is a low density of cells, below the 30,000-50,000 per well necessary. High content of iCGRP could indicate that there a too many neurons per well, which could change the properties of these neurons. Examining content values over 161 samples, a mean (1312.98 fmol) and standard deviation (494.32 fmol) was determined. Incorporating values within two standard deviations of the mean would provide a range in which 95% of the values fall and would eliminate outliers in the 2.5% on the high or low end of the values. Therefore, total contents of iCGRP between 324.34 fmol and 2301.62 fmol were determined to represent valid experiments from sensory neuronal cultures. After this criterion was established, all data sets from each well were judged upon these three criteria. If any one was not met, the entire data set from the well was not used. At least three wells of a no treatment condition, a condition in which no growth factors or inhibitors were added, were present in each experiment.

C. Effects of GFLs on isolated sensory neurons

1. Characterization of GFL-induced enhancement in capsaicin stimulated-release of iCGRP

The levels of the GFLs are increased during inflammation (Aloe et al., 1992) and treatment of isolated sensory neurons with GFLs increases intracellular calcium levels in response to capsaicin (Malin et al., 2006). While the local levels of the GFLs near the sensory neurons in intact animals have not been established, levels in whole brain (Kirik et al., 2000) and in plasma (Onodera et al., 1999) are in the high pg/mL to low ng/mL range. Additionally, the concentrations of the GFLs used in previous experiments on freshly dissociated sensory neuronal preparations and sensory neurons in culture are between 1 ng/mL and 100 ng/mL (Malin et al., 2006;Price et al., 2005). These concentrations correspond to 0.0667 nM to 6.67 nM for GDNF, 0.0847 nM to 8.47 nM for NTN, 0.0833 nM to 8.83 nM for ART, and 0.0971 nM to 9.71 nM for PSP.

To evaluate the ability of GDNF to modulate the stimulated release of iCGRP, GDNF was applied in the basal and capsaicin stimulated (50 nM capsaicin) conditions. First, the absolute value of fmol of iCGRP amounts of stimulated release was measured. As seen in Figure 3, GDNF at 1 ng/mL and 10 ng/mL significantly increased capsaicin stimulated-release by 50-100%, using an ANOVA with Dunnett's post hoc testing (No GFL: 148.63 ± 12.42 fmol, 100 ng/mL NGF: 217.44 ± 31.20 fmol, 1.0 ng/mL GDNF: 227.82 ± 23.01 fmol, 10 ng/mL GDNF: 208.75 ± 38.99 fmol). Additionally, this enhanced release is similar to that seen with NGF, a growth factor with a well-established role in neuropeptide release modulation (Hingtgen et al., 2006). An enhancement in the stimulated-release of iCGRP, without a change in basal release, is what is defined as sensitization in this thesis. In fact, any statistically significant enhancement in stimulated release of iCGRP, no matter the amount, is defined as sensitization.

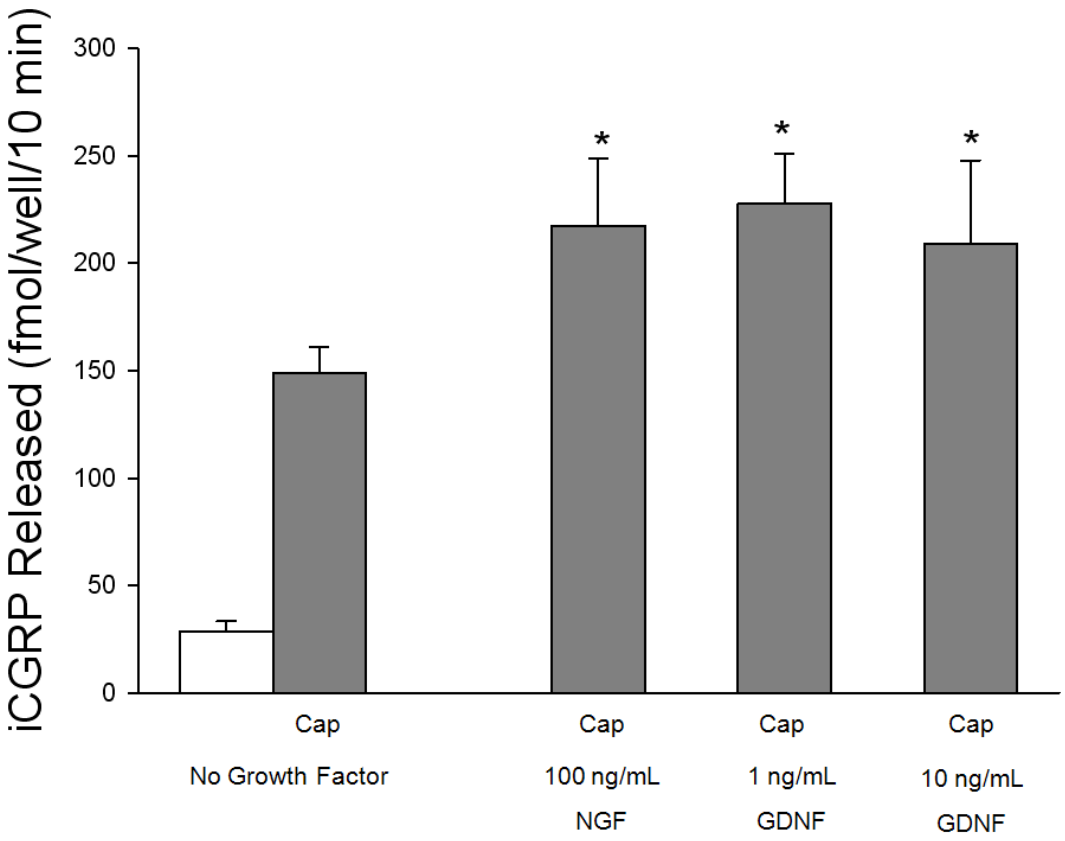

Figure 3. GDNF enhances the capsaicin stimulated-release of iCGRP in fmol from isolated sensory neurons. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bar) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 12-15 wells per condition). NGF, at a concentration of 100 ng/mL, and GDNF, at concentrations of 1 ng/mL and 10 ng/mL) were included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor exposure time was 20 minutes. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no growth factor condition using an ANOVA with Dunnett's post-hoc test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

Individual preparations of isolated sensory neurons in culture were exposed to different concentrations of GDNF for 10 minutes prior to and during a 10 minute capsaicin-stimulated period. In the absence of GDNF, basal release of iCGRP was 7.15 ± 1.25 fmol/well and capsaicin-stimulated release was 78.84 ± 6.21 fmol/well (mean ± SEM). When expressed as the percent of the total content of iCGRP in the well, these values correspond to 0.45 ± 0.11% per 10 minutes in the basal condition and 4.19 ± 0.93% in the capsaicin-stimulated condition (Figure 4). This release of iCGRP is consistent with a previous report, which indicates that capsaicin stimulated-release of iCGRP is approximately 5% of total content per 10 minutes (Hingtgen et al., 2006). From this point forward, all release data will be expressed as iCGRP as a percent of total content of iCGRP. This normalization is necessary because of the variability in the amount of cells plated between each well and between preparations. When 1 or 10 ng/mL GDNF was added, capsaicin-stimulated release of iCGRP was significantly enhanced (No GFL: 4.19 ± 0.93%, 0.1 ng/mL GDNF: 6.74 ± 1.87%, 1.0 ng/mL GDNF: 8.45 ± 1.05%, 10 ng/mL GDNF: 8.35 ± 1.16%, 100 ng/mL GDNF: 6.34 ± 1.15%; Figure 4). There was no change in the basal release of iCGRP with exposure to GDNF. Other studies have identified a similar profile of the concentration-related actions of GDNF. Specifically, application of 10 ng/mL GDNF to trigeminal ganglia for 5 days in culture increases the capsaicin stimulated-release of CGRP from, and the total content of CGRP in, TRPV1-postive neurons (Price et al., 2005). However, 100 ng/mL GDNF did not increase the release of CGRP (Price et al., 2005). There are several possibilities for why treatment with 100 ng/mL GDNF did not enhance the release of iCGRP. First, compensatory pathways may be activated by higher concentrations of GDNF that are not activated by 10 ng/mL GDNF. These compensatory pathways may prevent the GDNF from sensitizing the sensory neurons. In a neuroectodermal cell line, 100 ng/mL GDNF increased p-Akt levels and blunted the ability of the MAPK pathway to activate

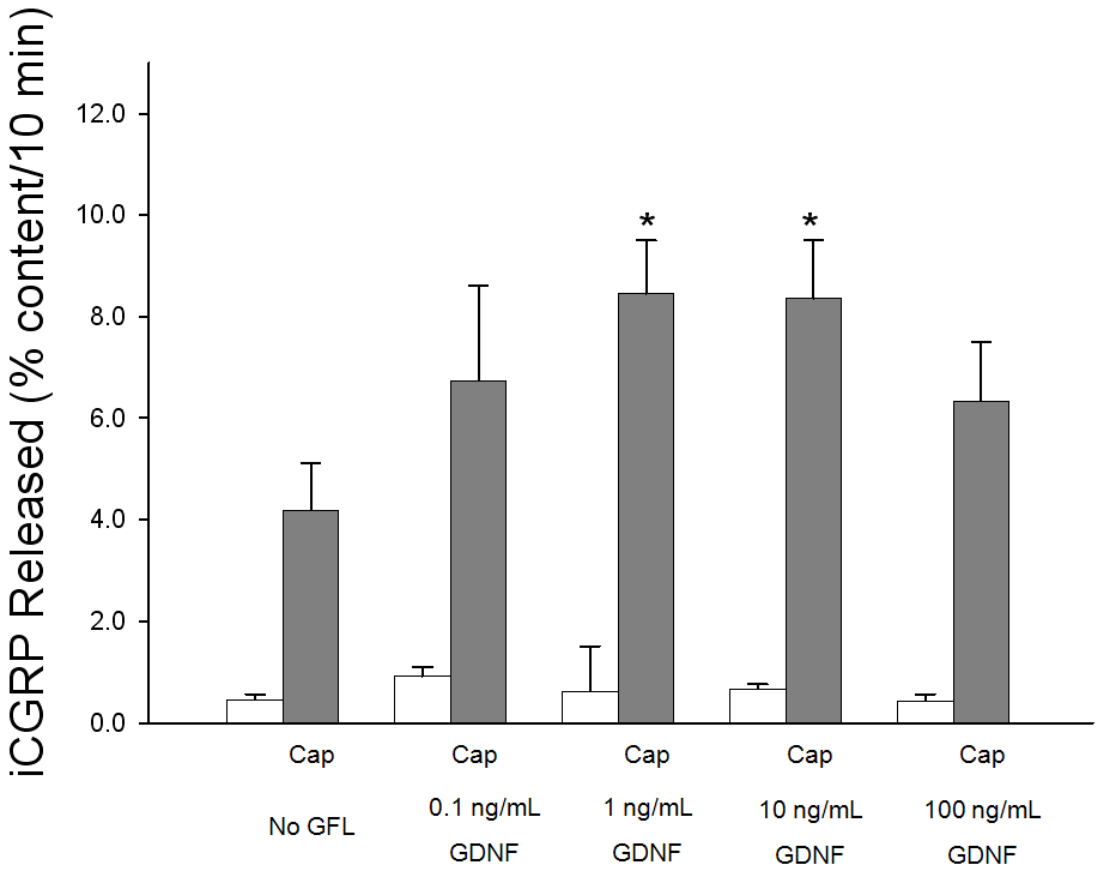

Figure 4. GDNF enhances capsaicin-stimulated release of iCGRP from isolated sensory neurons. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bars) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 9 wells per condition). GDNF (at concentrations from 0.1 ng/mL to 100 ng/mL) was included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor exposure time was 20 minutes. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no GFL condition using an ANOVA with Dunnett's post-hoc test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

transcription factors, which may also explain why 100 ng/mL GDNF was unable to sensitize the DRG neurons (Mograbi et al., 2001).

GDNF was used as a representative GFL to characterize, in general, the enhancement in stimulated-release of iCGRP by the GFLs, including the optimum concentration to use to examine the effects of sensitization. Then, to determine if the actions of GFLs on the TRPV1 receptor result in increased functional output, such as enhanced transmitter release, the ability of the other GFLs to modulate the stimulated-release of iCGRP from isolated sensory neurons was measured. Studies of the effects of the GFLs on sensory neuronal sensitization were conducted with concentrations of the GFLs between 0.1 ng/mL and 500 ng/mL to remain close to the physiological range and to correspond to concentrations used in similar experiments.

After establishing that GDNF is an effective sensitizer of sensory neurons from the DRG, the ability of the other GFLs, NTN, ART, and PSP, to enhance the stimulated release of iCGRP was evaluated. As seen in Figure 5, 10 ng/mL NTN and ART also significantly enhanced the capsaicin-stimulated release of iCGRP (No GLF: 4.30 ± 0.58%, NTN: 10.60 ± 1.70%, ART: 10.00 ± 1.57%). These data indicate that GDNF, NTN, and ART are all able to sensitize sensory neurons and enhance the release of iCGRP to a similar degree.

Unlike the other GFLs, PSP did not alter the capsaicin-stimulated release of iCGRP (PSP: 3.40 ± 0.52%; Figure 5). This may result from a lack of functional GFRα4 in adult DRG neurons, and the fact that PSP binds specifically to GFRα4 (Enokido et al., 1998; Paveliev et al., 2004). Even at concentrations as high as 500 ng/mL, PSP was unable to enhance the capsaicin-stimulated release of iCGRP (Table 4). It is unlikely that the PSP was inactivated or altered since the same batches of PSP rescued neurons from damage by 6 hydroxydopamine (personal communication with the lab of Dr. Richard Nass). Therefore, since concentrations of PSP above physiological levels were unable

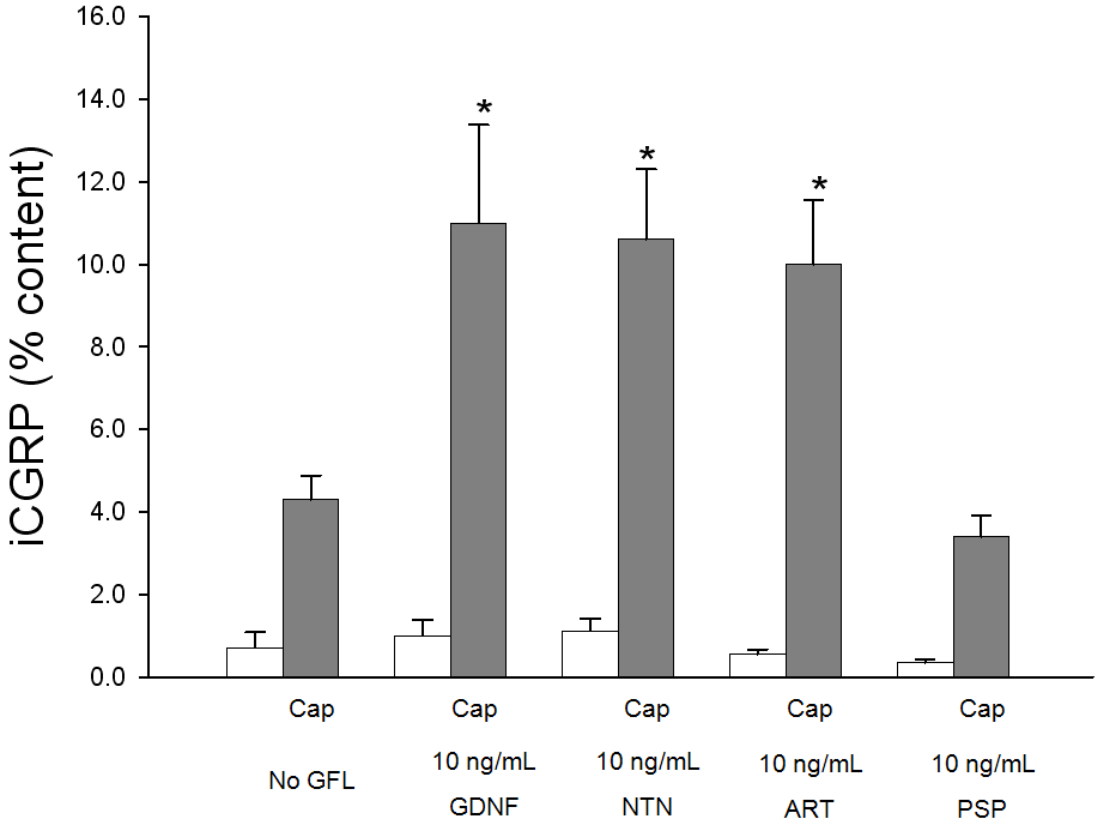

Figure 5. GFLs enhance capsaicin-stimulated release of iCGRP from isolated sensory neurons. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bars) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 9-22 wells per condition). GDNF, neurturin (NTN), artemin (ART), or persephin (PSP), at 10 ng/mL, was included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor exposure time was 20 minutes. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no GFL condition using an ANOVA with Dunnett's post-hoc test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

Table 4

Persephin Does Not Enhance the Capsaicin-Stimulated Release of iCGRP

	Basal	50 nM Capsaicin
No GFL	0.67 ± 0.13	5.28 ± 0.46
10 ng/mL PSP	0.68 ± 0.18	5.94 ± 0.88
100 ng/mL PSP	0.68 ± 0.16	4.97 ± 0.41
500 ng/mL PSP	1.23 ± 0.16	5.59 ± 0.64

All values are mean ± SEM % content iCGRP released, n = 9 wells per condition

to enhance capsaicin-stimulated release of iCGRP, PSP is not a molecule that sensitizes adult mammalian sensory neurons.

To ensure that GDNF, NTN, and ART did not directly alter the resting release of iCGRP, sensory neurons were exposed to the GFLs for two consecutive 10 minute incubations in the absence of any stimulus. No enhancement in the release of iCGRP was observed with these treatments (Table 5).

When sensory neurons are exposed to the GFLs for several days, the levels of CGRP are increased (Ramer et al., 2003; Price et al., 2005). To ensure that the GFL-induced enhancement in capsaicin-stimulated release of iCGRP was not the result of an increase in the total content of iCGRP, we measured iCGRP content at the end of each experiment. There was no change in the total content of iCGRP after the 20 minute exposure to the GFLs (No GFL: 1487 ± 154 fmol/well, GDNF: 1322 ± 108 fmol/well, NTN: 1500 ± 128 fmol/well, ART: 1320 ± 102 fmol/well, and PSP: 1518 ± 177 fmol/well, n = 9-22 wells per condition).

2. GFLs do not enhance the potassium-stimulated release of iCGRP

Previous studies have focused primarily on GFL-induced changes in response to capsaicin in isolated DRG neurons. To determine whether responses to stimuli other than capsaicin could be enhanced by GFLs, a general depolarizing stimulus, high extracellular potassium, was used. Exposure to HEPES buffer containing 50 mM high extracellular potassium (KCl) for 10 minutes caused a release of iCGRP of 6.99 ± 1.06%. Acute treatment with GDNF, NTN, or ART (10 ng/mL) 10 minutes prior to and throughout the stimulus period did not alter KCl-stimulated iCGRP release (Figure 6; No GFL: 6.99 ± 1.06%, GDNF: 6.95 ± 0.92%, NTN: 6.63 ± 0.61%, and ART: 6.74 ± 1.11%). In addition, treatment with 100 ng/mL GDNF was unable to enhance KCl-stimulated release of iCGRP. PGE_2 is a well established sensory neuronal sensitizing

Table 5

GFLs alone do not increase the release of iCGRP

GFL	No GFL mean % content iCGRP ± SEM n = 9 wells	10 ng/mL GFL, 10 minutes mean % content iCGRP ± SEM n = 9 wells	10 ng/mL GFL, 20 minutes mean % content iCGRP ± SEM n = 9 wells
No GFL	0.55 ± 0.35	0.43 ± 0.11	0.76 ± 0.20
10 ng/mL GDNF	0.96 ± 0.12	0.67 ± 0.14	0.50 ± 0.19
10 ng/mL NTN	0.41 ± 0.06	0.44 ± 0.04	0.70 ± 0.15
10 ng/mL ART	0.91 ± 0.30	0.68 ± 0.20	0.54 ± 0.16

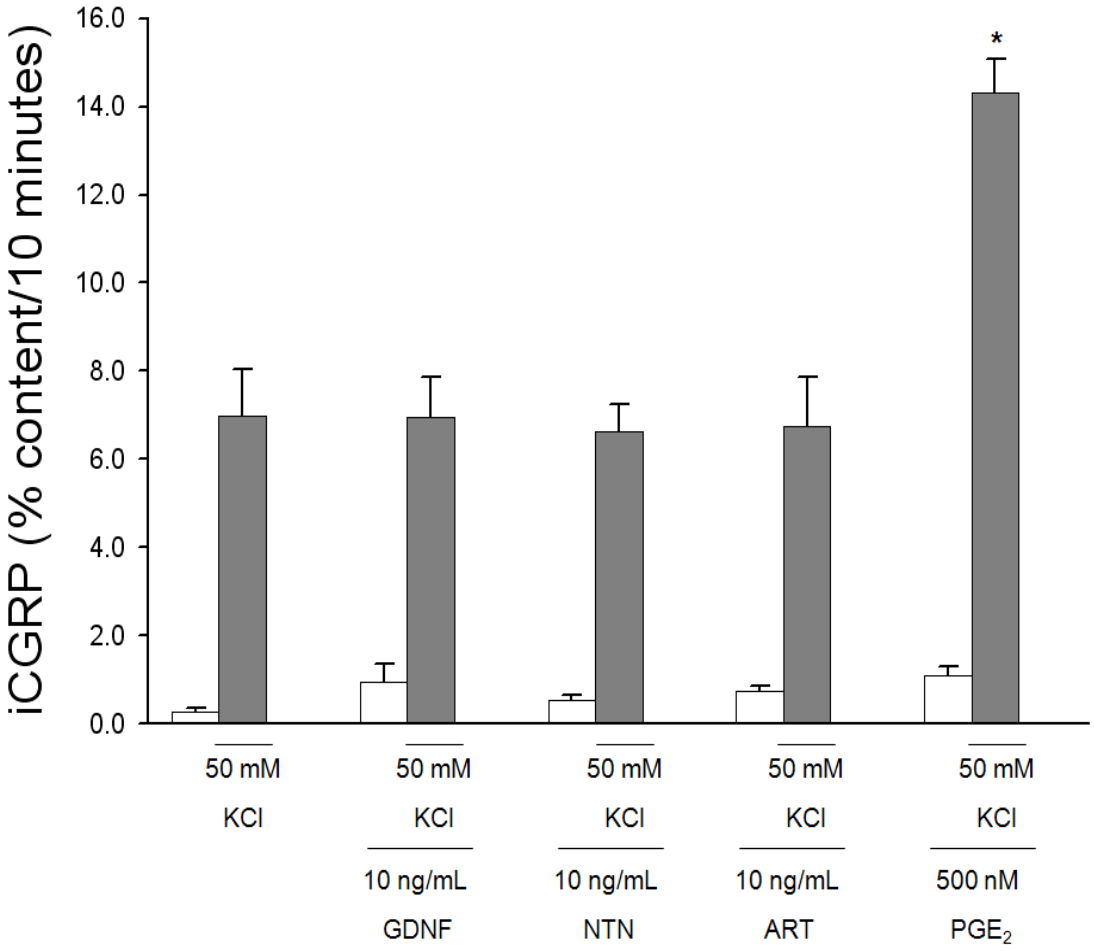

Figure 6. GFLs do not enhance the 50 mM potassium-stimulated release of iCGRP from isolated sensory neurons. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bars) or Hepes buffer containing 50 mM potassium (KCl; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 9-12 wells per condition). GDNF, neurturin (NTN), or artemin (ART) at 10 ng/mL, was included in the 10 minutes prior to and throughout potassium exposure. PGE_2 was present in the basal and stimulated conditions at a concentration of 500 nM. Total growth factor and PGE_2 exposure time was 20 minutes. There were no significant differences in iCGRP release between treatment groups and the no GFL condition. A significant enhancement in iCGRP release was observed with PGE_2 using ANOVA with Dunnett's post-hoc test ($p<0.05$). In all cases, release stimulated by potassium was significantly higher than basal release.

agent (Martin et al., 1987; (Mense, 1981). It is known to sensitize sensory neurons to many stimuli, including high extracellular potassium (Southall and Vasko, 2000). Accordingly, PGE_2 enhanced the potassium-stimulated release of iCGRP by nearly 2 fold (Figure 6; 14.3 ± 0.79%). Additionally, the GFLs were not able to induce sensitization when 30 mM KCl was used as the stimulus (Figure 7). This lower concentration was used because there was a possibility that the amount of iCGRP released in response to 50 mM KCl may be the maximum amount of iCGRP that could be released by the cells, preventing the possibility of any sensitization occurring. These data suggest that GDNF, NTN, and ART, unlike PGE_2, sensitize sensory neurons through an interaction with TRPV1 and not by mechanisms independent of the stimulus.

3. **Presence of growth factors in the culture media**

NGF treatment of sensory neuronal cultures increases the expression of TRPV1 (Xue et al., 2007) and increases the amount of TRPV1 insertion into the plasma membrane (Stein et al., 2006). Additionally, sensory neuronal exposure to NGF in culture increases the expression of CGRP and other neuropeptides (MacLean et al., 1989;Sango et al., 1994). Therefore, there is a possibility that some of the media components, specifically the NGF, are affecting the responses of the sensory neurons to either or both the capsaicin and the GFLs. To address this concern, iCGRP release was measured from neurons grown in the presence or absence of NGF to determine whether this change in the media components would alter the capsaicin-stimulated release and the GFL-induced enhancement in this release. The fmol of capsaicin-stimulated iCGRP released when NGF was omitted from the culture media was ~25% less than when NGF was present (No added NGF: 97.39 ± 10.42 fmol/well, Growth in 30 ng/ml NGF: 127.83 ± 11.24 fmol/well). While the amount of capsaicin-evoked release was enhanced by 10 ng/mL GDNF in both conditions, the absolute level of iCGRP released was again ~25%

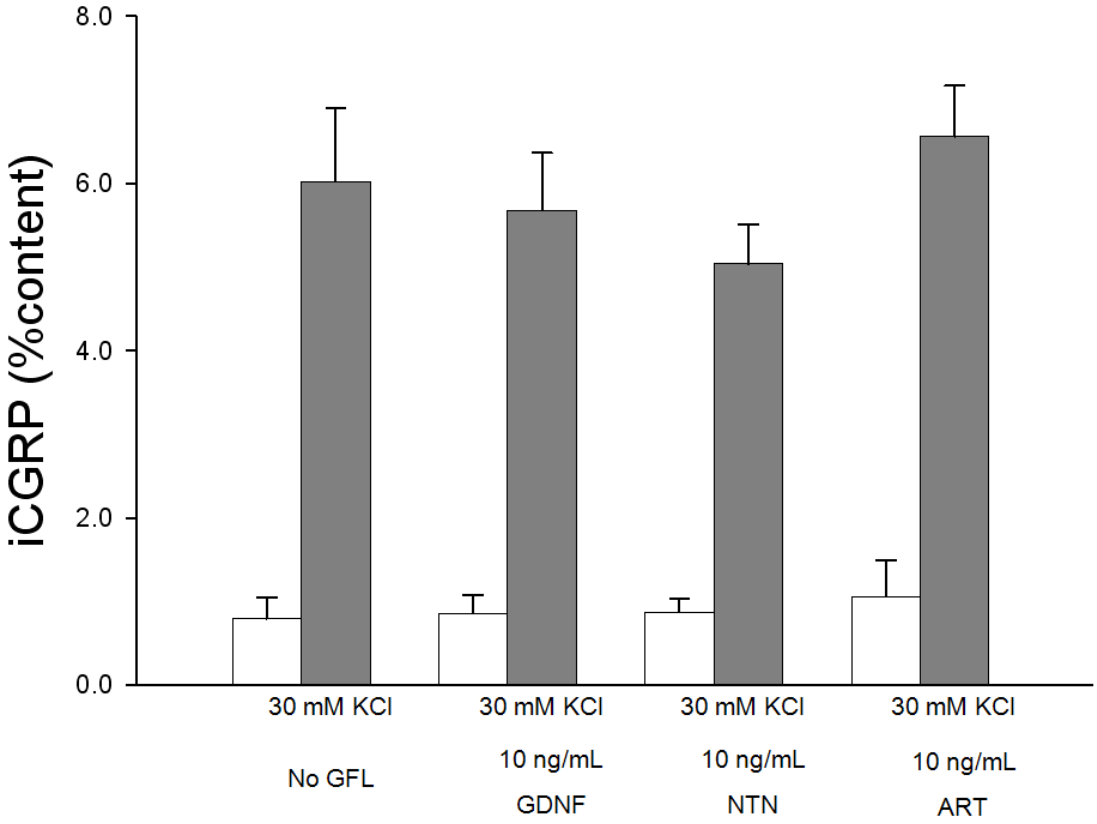

Figure 7. GFLs do not enhance the 30 mM potassium-stimulated release of iCGRP from isolated sensory neurons. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bars) or Hepes buffer containing 30 mM potassium (KCl; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 9-12 wells per condition). GDNF, neurturin (NTN), or artemin (ART) at 10 ng/mL, was included in the 10 minutes prior to and throughout potassium exposure. Total growth factor exposure time was 20 minutes. There were no significant differences in iCGRP release between treatment groups and the no GFL condition. In all cases, release stimulated by potassium was significantly higher than basal release.

less in the cells that were not exposed to NGF (No added NGF: 162.54 ± 8.45, Growth in 30 ng/ml NGF: 219.27 ± 21.86 fmol/well). However, when NGF was present in the culture media, the total content of iCGRP was also ~25% higher than when NGF was omitted. The presence or absence of NGF in the culture media did not change the magnitude of capsaicin-stimulated release of iCGRP or the GDNF-induced enhancement of peptide release as percent of total content (Figure 8). The reduction in absolute levels of iCGRP released from sensory neurons maintained in culture in the absence of added NGF, but an absence of change in the percent of the total content of iCGRP released, is consistent with previous observations (Park et al., 2006).

When GDNF was added to the media instead of NGF, the profile of stimulated release was altered. Specifically, the stimulated release was significantly higher (6.810 ± 0.559%, n=15 wells) than when no growth factor (4.970 ± 0.344%, n=11 wells) or NGF (4.650 ± 0.677, n=11 wells) was present in the growth media (Figure 9). When GDNF was then added in the buffer during the basal and capsaicin-stimulated conditions, there was still an enhancement in the stimulated release of iCGRP (11.500 ± 1.25%, n=15). Taken together, these data indicate that the presence of NGF in the culture media does not alter the stimulated release of iCGRP normalized to total content of iCGRP. Instead, it is likely that the NGF is increasing the overall content of CGRP in the neurons, making more peptide available for release. However, unlike NGF, when GDNF is present in the culture media, stimulated-release of iCGRP is altered, suggesting that GDNF is altering components of the release process and not just increasing the amount of peptide available.

D. Effects of GFLs on freshly dissociated spinal cord slices

Sensitization of the central terminals of primary sensory neurons, which synapse onto second order neurons in the spinal cord, is also important during inflammation and

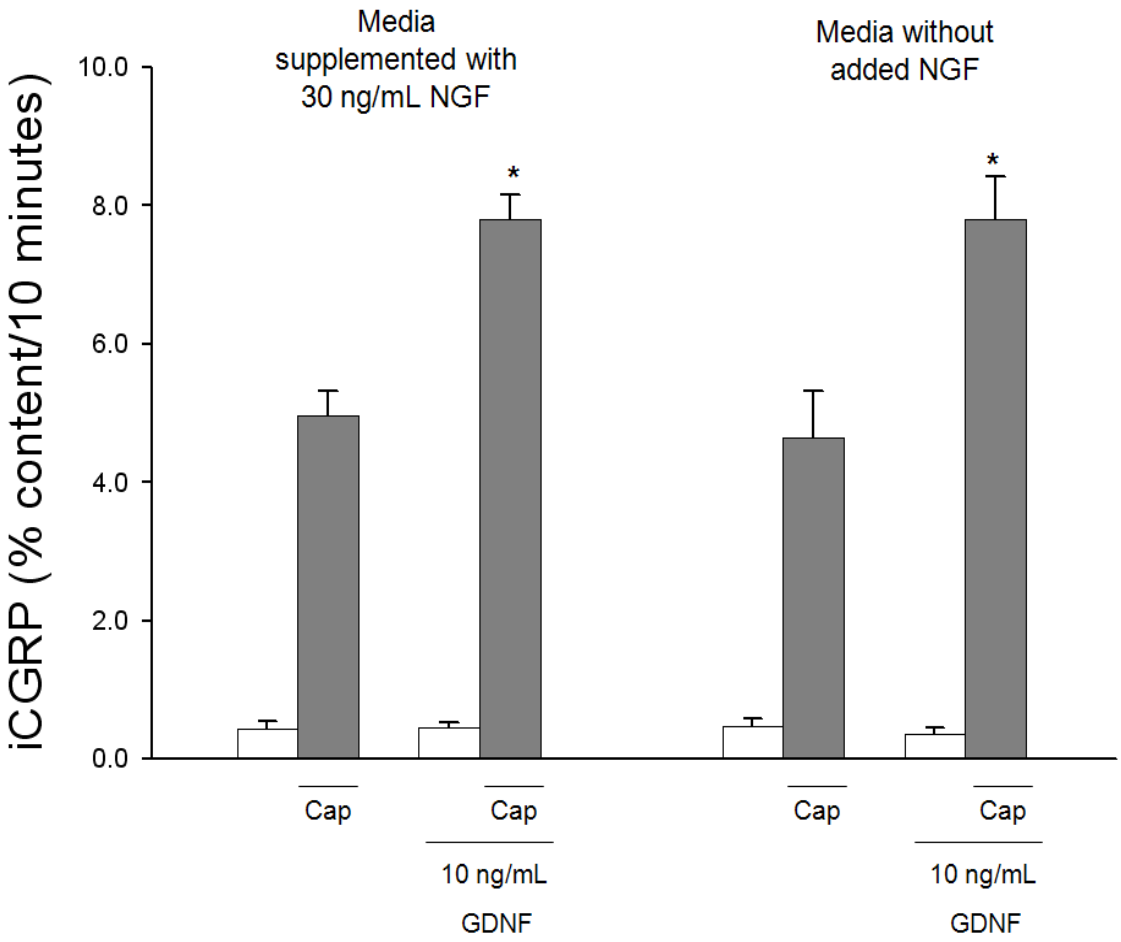

Figure 8. NGF in culture media does not change the stimulated release of iCGRP and the GDNF-induced enhancement in release. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bars) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 9 wells per condition). 10 ng/mL GDNF was included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor exposure time was 20 minutes. NGF was added in the culture media at a concentration of 30 ng/mL or omitted from the culture media. Asterisks (*) indicate statistically significant differences in iCGRP release between GDNF treatment group and the no GFL condition using an t-test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

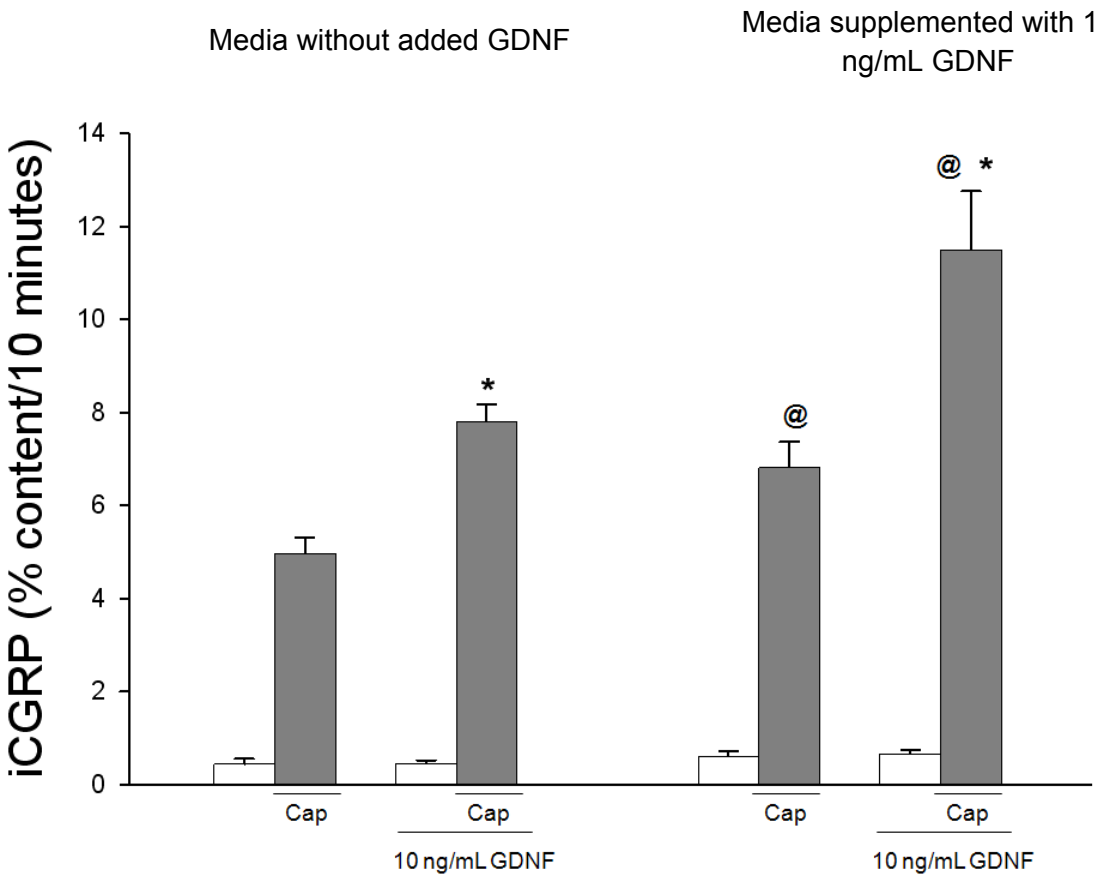

Figure 9. GDNF in culture media changes the stimulated release of iCGRP and the GDNF-induced enhancement in release. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bars) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 9 wells per condition). 10 ng/mL GDNF was included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor exposure time was 20 minutes. GDNF was added in the culture media at a concentration of 10 ng/mL or omitted from the culture media. Asterisks (*) indicate statistically significant differences in iCGRP release between GDNF treatment group and the no GFL condition using a t-test ($p<0.05$). Ampersands (@) indicate statistically significant differences in iCGRP release between different media conditions using a t-test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

propagation of the pain signal. The GFRα receptors are present on the central terminal of primary afferent neurons (Josephson et al., 2001), and GFLs are released by astrocytes in the spinal cord (Nomura et al., 2002;Nosrat et al., 1996). In order to examine the actions of GFLs in sensitization of the central terminals of sensory neurons, iCGRP release from spinal cord slices was measured.

Figure 10A compares capsaicin-stimulated release of iCGRP in the absence or presence of 10 ng/mL GDNF. The fmol of iCGRP in each 10 minute collection fraction were normalized to the total iCGRP content in the spinal cord, as described in the Materials and Methods Section II.G. In the three basal fractions, iCGRP release was similar for both treatment and control conditions. The capsaicin-stimulated release of iCGRP was significantly enhanced by exposure to GDNF for 30 minutes prior to and throughout the stimulus period (No GFL: 0.94 ± 0.12%, 1.46 ± 0.18%, and 1.28 ± 0.15%; 10 ng/mL GDNF: 1.14 ± 0.091%, 2.29 ± 0.084%, and 1.96 ± 0.25%). The profiles of increased release of iCGRP were similar when the spinal cord slices were exposed to NTN and ART. Evoked release was determined by subtracting the three basal fractions of iCGRP release from the three capsaicin-stimulated fractions. As demonstrated in Figure 10B, GDNF, NTN, and ART all were able to significantly enhance the capsaicin-evoked release of iCGRP from the spinal cord slices by two to three fold (No GFL: 2.50 ± 0.42%, GDNF 4.05 ± 0.43%, NTN: 6.18 ± 0.28%, ART: 5.88 ± 1.94%). The total content of iCGRP per mg of protein in the spinal cord slices was not changed by exposure to GFLs (No GFL: 298.41 ± 44.28 fmol/mg, GDNF: 275.46 ± 25.13 fmol/mg, NTN: 220.71 ± 84.84 fmol/mg, ART: 227.58 ± 98.63 fmol/mg). These data indicate that the GFLs are able to sensitize the central terminals of the sensory neurons to capsaicin stimulation.

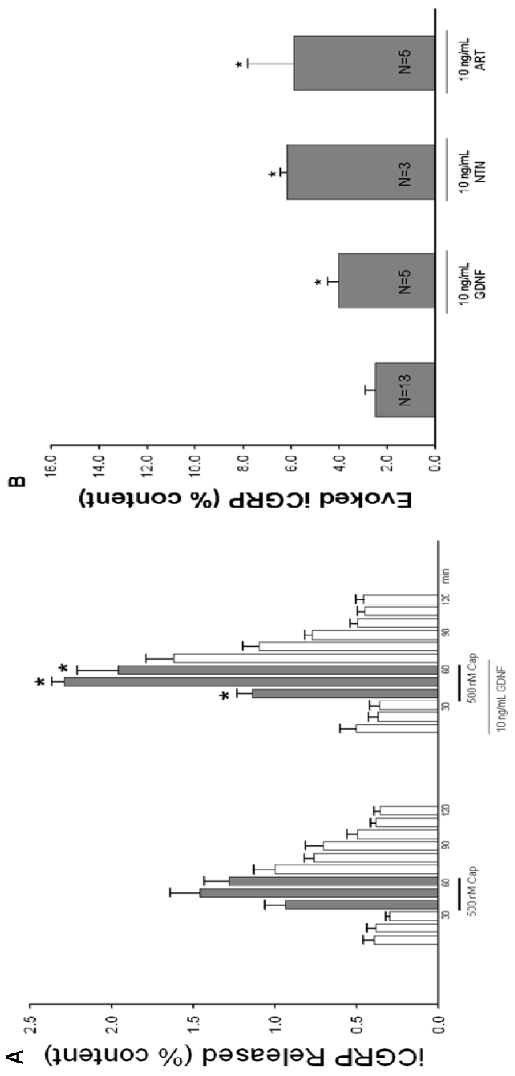

Figure 10. GFLs enhance capsaicin-stimulated release of iCGRP from spinal cord slices. A) Peptide release from spinal cord slices stimulated by three 10 minute exposures to Hepes buffer alone (open bars) or Hepes buffer containing 500 nM capsaicin (dark bars) is expressed as mean percent total peptide content of iCGRP in the spinal cord slice ± SEM (n = 3-10 animals per condition). GDNF, neurturin (NTN), or artemin (ART), at 10 ng/mL, was included in the six 10 minute incubations indicated by lines with growth factor name below, for a total exposure time of 60 min. B) Evoked release, or release due to capsaicin stimulation alone, is compared between growth factor treatment and no GFL groups. The evoked release was obtained by subtracting peptide release during the three basal fractions from that during the three capsaicin-stimulated fractions in each treatment group. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no growth factor condition using an ANOVA with Dunnett's post-hoc test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

E. Distinct signaling pathways are responsible for GFL-induced enhancement in the release of iCGRP

Having established that selected GFLs sensitize sensory neurons, as measured by an enhancement in the stimulated release of iCGRP, the intracellular signaling pathways each of the GFLs use to accomplish this sensitization were evaluated. The intracellular signaling pathways activated by the GFLs and implicated in sensory neuronal sensitization by other known sensitizers are detailed in the Introduction, Section I.H. The role of each of these pathways in the sensitization by each of the individual GFLs was undertaken.

1. Ret-Dependent pathways

Most of the evidence for the actions of the GFLs on adult, mammalian neurons indicates that Ret-dependent pathways are involved in GFL-mediated actions (Durbec et al., 1996). Ret autophosphorylation, induced by GFLs binding to their preferential GFRα receptor subtype and translocating to this receptor, initiates many “classically” Ret-dependent signaling pathways. These include the MAPK/Erk 1/2 pathway, the PI-3K/Akt pathway, and the PKCε pathway (Bogen et al., 2008;Encinas et al., 2001;Mikaels-Edman et al., 2003;Woodall et al., 2008). First, it was determined if the GFLs activated these pathways. Then, the role of these pathways in enhancement of release of iCGRP was elucidated with the use of different inhibition techniques. Western blots for phospho-specific antibodies were used to verify the alterations in signaling cascades with GFL treatment or the use of inhibitors of the signaling cascades. The Western blots were conducted with separate sets of DRG cultures than ones used for the release of iCGRP. This was necessary, since the total content of iCGRP was determined after lysing the DRG with acetic acid and this treatment likely affects the signaling pathways within the

cells. In all cases, however, the cultures used for release experiments and those used for Western blots were treated in the same manner.

a. The role of the Ras signaling cascade in GFL-induced sensitization

Because *Nf1+/-* mice have increased activity of the Ras signaling cascade (Martin et al., 1990;Xu et al., 1990) these mice were used to evaluate the role of the Ras signaling cascade in GDNF and ART-induced sensitization of sensory neurons.

The stimulated-release of iCGRP in dissociated DRG from *Nf1+/-* mice is ~1.25 times higher than in wild-type mice (wild-type: 3.92 ± 0.32% of total content of iCGRP, *Nf1+/-*: 4.98 ± 0.36% of total content of iCGRP; Figure 11A). When 10 ng/mL GDNF was added in the basal and capsaicin-stimulated condition, sensitization occurred, with the amount of iCGRP released greater in *Nf1+/-* mice than in wild-type mice (wild-type: 6.08 ± 0.48% of total content of iCGRP, *Nf1+/-*: 7.41 ± 0.59% of total content of iCGRP; Figure 11A). However, when comparing the fold change of stimulated-release in DRG neurons from wild-type and *Nf1+/-* mice in the absence and presence of GDNF, there is no difference in the fold change between the genotypes (wild-type: 1.41 ± 0.13, *Nf1+/-*: 1.48 ± 0.08; Figure 11B). A similar profile was seen in the spinal cord slice release experiments, where evoked release of iCGRP from spinal cord slices from *Nf1+/-* mice was ~2 times higher than in wild-type mice (wild-type: 1.38 ± 0.65%, *Nf1+/-*: 2.69 ± 0.43%; Figure 12). The evoked release of iCGRP in both the wild-type and *Nf1+/-* mice doubled in the presence of 10 ng/mL GDNF (wild-type: 3.20 ± 0.28%, *Nf1+/-*: 5.91 ± 0.27%; Figure 12), and the fold change in each genotype was not different (wild-type: 2.32 ± 0.11, *Nf1+/-*: 2.20 ± 0.09), suggesting that GDNF-induced sensitization is not dependent on Ras activity.

The responses of sensory neurons from wild-type and *Nf1+/-* DRG to ART were compared, as well. When 10 ng/mL ART was added in the basal and capsaicin stimulated condition, sensitization occurred, with the absolute amount of iCGRP

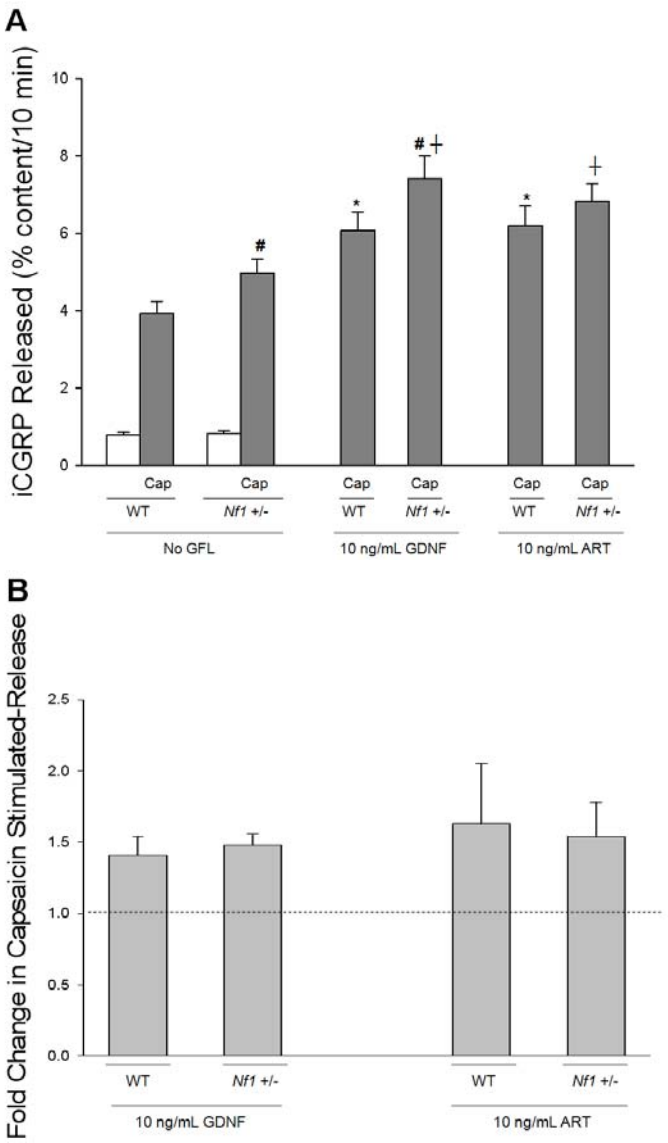

Figure 11. Comparison of responses of wild-type and *Nf1* +/- DRG sensory neurons to GDNF and ART. *Nf1* +/- sensory neurons have increased stimulated-release of iCGRP in the absence of GFL exposure. A) GDNF and ART enhance the stimulated-release of iCGRP in both wild-type and *Nf1* +/- sensory neurons. B) The fold change in enhancement in stimulated-release of iCGRP induced by GDNF and ART in each genotype is shown. The fold change in stimulated-release is induced by GDNF and ART in *Nf1* +/- neurons is not different than in wild-type neurons. The # represents statistically significant differences in stimulated-release between genotypes using a t-test ($p<0.05$). Asterisks (*) represent statistically significant differences between treatment conditions in the wild-type sensory neurons using an ANOVA with Dunnett's post hoc test ($p<0.05$). The crosses (┼) represents statistically significant differences between treatment conditions in the *Nf1* +/- sensory neurons using an ANOVA with Dunnett's post hoc test ($p<0.05$). In all cases, stimulated-release was significantly higher than basal or resting release. N = 3-12 wells/condition.

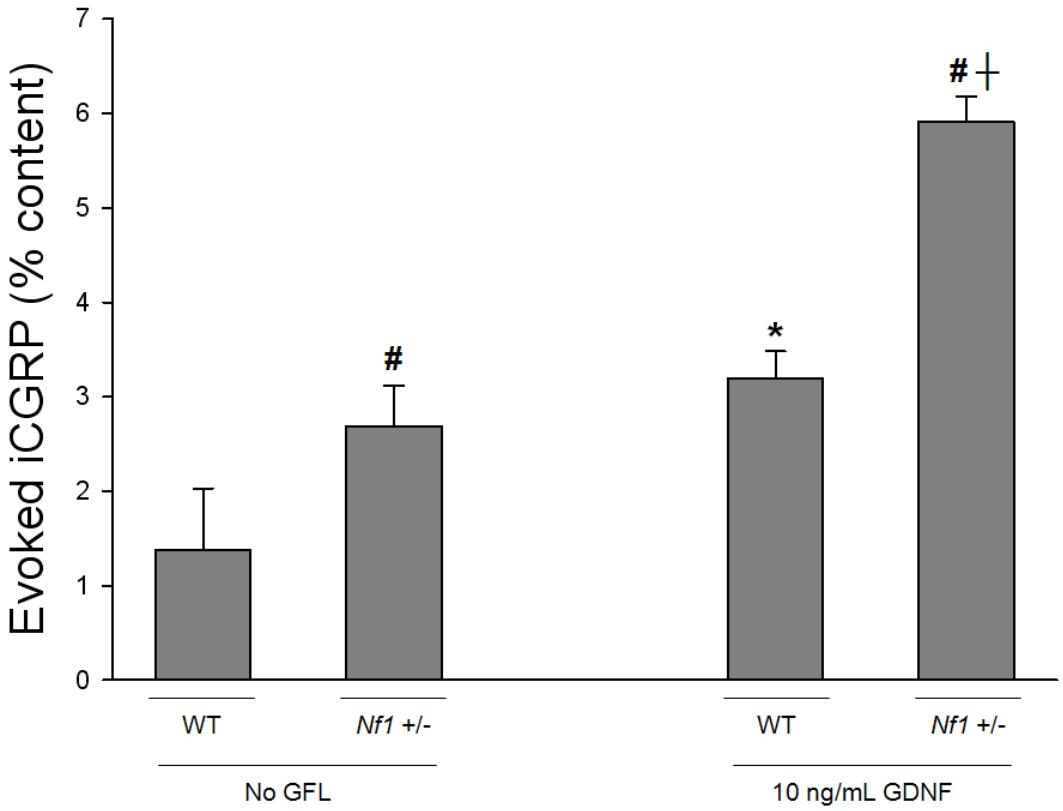

Figure 12. GDNF enhances capsaicin-stimulated release of iCGRP from wild-type and *Nf1* +/- spinal cord slices. Evoked release, or release due to capsaicin stimulation alone, is compared between GDNF treatment and no GFL groups in both genotypes. The evoked release was obtained by subtracting peptide release during the three basal fractions from that during the three capsaicin-stimulated fractions in each treatment group. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no growth factor condition using an t-test ($p<0.05$). The # symbol indicates statistically significant differences in iCGRP release between genotypes in each condition using a t-test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release. N = 3-9 animals per condition.

released greater in *Nf1*+/- mice than in wild-type mice (wild-type: 6.21 ± 0.50% of total content of iCGRP, *Nf1*+/-: 6.83 ± 0.46% of total content of iCGRP; Figure 11A). However, similar to the responses of DRG from *Nf1*+/- mice to GDNF, when comparing the fold change of stimulated-release in DRG neurons from wild-type and *Nf1*+/- mice in the absence and presence of ART, there is no difference in the fold change between the genotypes (wild-type: 1.63 ± 0.42, *Nf1*+/-: 1.54 ± 0.24; Figure 11B). Although the use of *Nf1*+/- mice is not a direct way to determine if Ras activity is important in the actions of GFLs, this data would suggest that Ras is not critical for ART-induced sensitization of sensory neurons. For this reason, no further experiments involving *Nf1*+/- mice were conducted.

b. Intracellular signaling pathways responsible for GDNF-induced sensitization

GDNF activates both the MAPK/Erk 1/2 and PI-3K pathways (Bron et al., 2003). Both of these pathways can be activated by Ras, among other signaling cascades (Klinghoffer et al., 1996;Thomas et al., 1992). For this reason, studies to evaluate the role of each of these pathways in GDNF-induced sensitization were conducted. Additionally, there are commercially available, specific inhibitors of the MAPK/Erk 1/2 and PI-3K pathways, as well as inactive control compounds for these inhibitors.

For the MAPK/Erk 1/2 pathway, PD98059 is a compound that irreversibly and specifically inhibits phosphorylation of Erk 1/2 by MEK 1/2 (Davies et al., 2000;Hotokezaka et al., 2002;Pang et al., 1995), although the exact mechanism is unknown. U0126 is another compound that has potent inhibitory effects on the MAPK/Erk 1/2 pathway, reportedly by preventing phosphorylation, and therefore activation, of MEK 1/2 (Davies et al., 2000;Favata et al., 1998). However, this inhibitor may also prevent phosphorylation of proteins by PRAK and PKBα (Davies et al., 2000). There is an inactive analogue compound for U0126, which is U0124. It is of similar

structure and size, but without the ability to inhibit phosphorylation of any tested intracellular signaling proteins (Duncia et al., 1998).

A potent and specific inhibitor of the PI-3K/Akt pathway exists as well. The compound LY294002 has an IC_{50} for PI-3K activity of between 1.40 and 10 μM and does not inhibit a similar protein, PI-4K (Davies et al., 2000;Vlahos et al., 1994). LY294002 has an inactive analogue, LY353011, which has an NH-group in place of the oxygen group on the morphine-ring portion of the molecule (Ding et al., 1995;Vlahos et al., 1994). This molecule has no effect on PI-3K dependent cell activities, including superoxide release in neutrophils and inhibition of voltage gated potassium currents in pancreatic beta cells (El-kholy et al., 2003;Vlahos et al., 1994).

Using the inhibitors described above in the basal and capsaicin-stimulated condition, the role of each of these pathways, MAPK and PI-3K, in GDNF-induced sensitization was examined. The phosphorylation of the MAPK pathway by GDNF, as a surrogate for activation, and prevention of this phosphorylation by the inhibitors in isolated DRG neurons was determined using protein from cell lysates and Western blotting techniques, probing for immunoreactive phospho-Erk (p-Erk) and total Erk with specific antibodies (see Table 3 for details of antibody sources and titers; Cell Signaling, Danvers, MA, USA). Exposure of DRG to 10 ng/mL GDNF for 10 minutes increases p-Erk levels compared to DRG exposed to Hepes buffer alone (Figure 13A). Total Erk and alpha tubulin (used as a protein loading control) levels were not affected. Phosho-Erk density was compared to total Erk density, then normalized to alpha tubulin density. Once a p-Erk level was established for the no treatment condition each treatment condition was normalized to this value. The control condition was given a value of one. The GDNF-induced increase in p-Erk was prevented by 10 μM PD98059 and 1.0 μM U0126, but not by 10 μM U0124 (Figure 13A and B). For these experiments, inhibitors were added to the DRG in culture for a total of 20 minutes. However, GDNF did not increase the

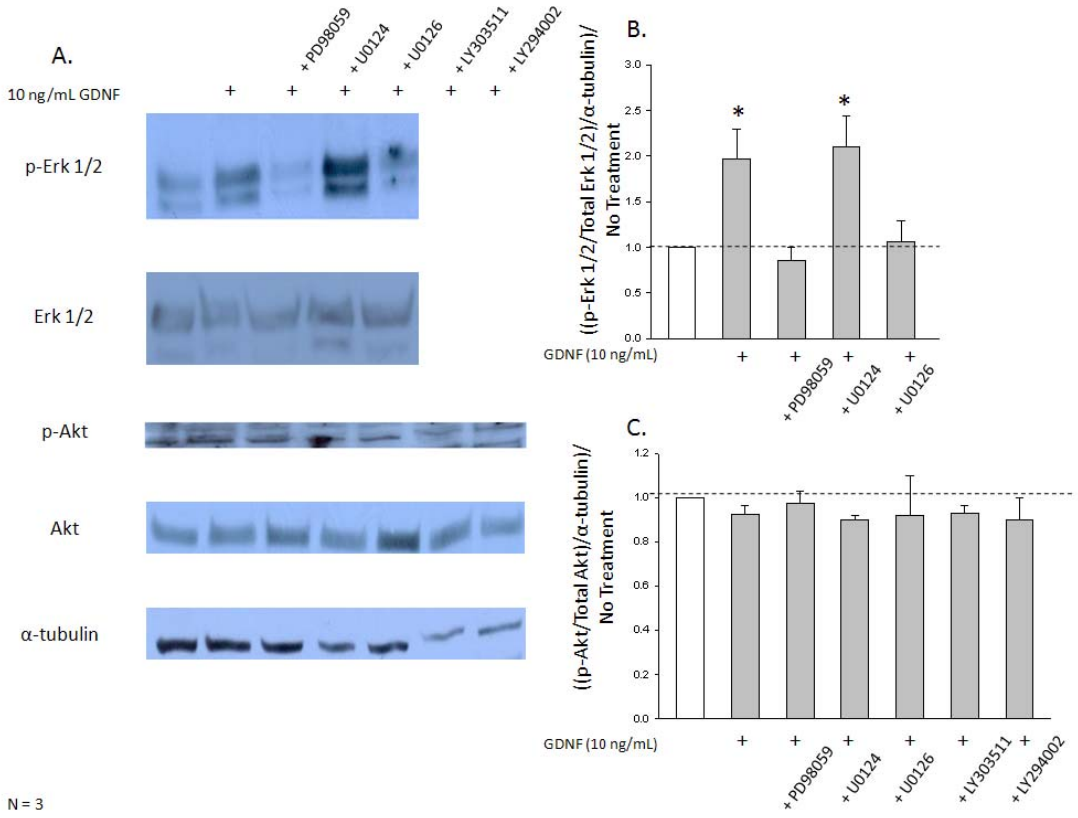

Figure 13. Signaling pathways activated by GDNF. A) This representative Western blot demonstrated changes in levels of p-Erk 1/2, Erk 1/2, p-Akt, Akt, and α-tubulin in DRG in response to a 10 minute incubation with 10 ng/mL GDNF and the subsequent prevention of these changes by inhibitors of the MAPK pathway (10 μM PD98059 and 1 μM U0126) and the PI-3K pathway (10 μM LY294002). Inactive control compounds for these pathways were added as well (1 μM U0124 for MAPK and 10 μM LY303511 for PI-3K). B) Densitometric analysis of three separate Western blots like that in A probing for MAPK pathway components. The level of p-Erk was divided by total Erk levels then normalized to α-tubulin levels in each condition. Asterisks (*) indicate statistically significant differences between treatment conditions and the no GDNF and no inhibitor condition using an ANOVA with Dunnett's post hoc testing ($p<0.05$). C) Densitometric analysis of three separate Western blots like that in A probing for PI-3K pathway components. The level of p-Akt was divided by total Akt levels then normalized to α-tubulin levels in each condition.

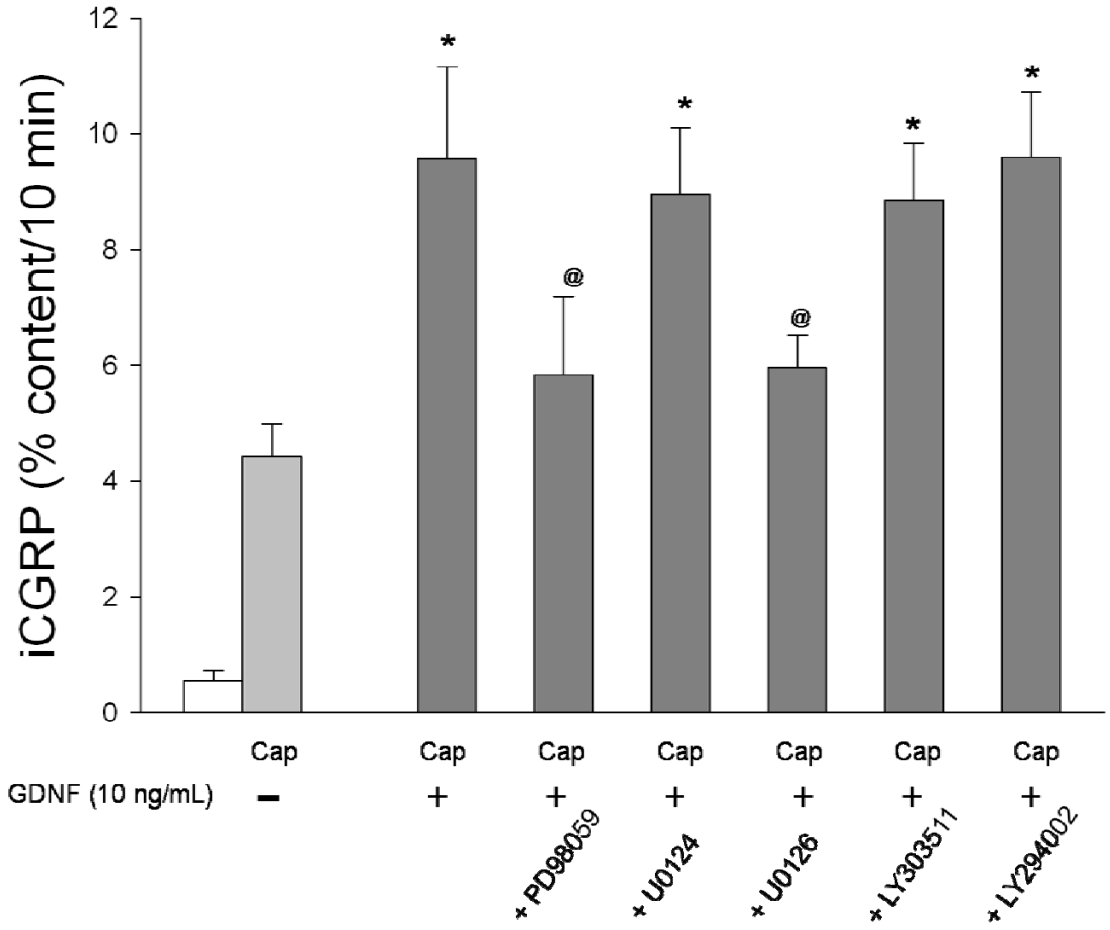

Figure 14. GDNF-induced enhancement in the stimulated-release of iCGRP is mediated by the MAPK/Erk 1/2 pathway. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bar) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 12-18 wells per condition). GDNF and inhibitors (10 μM PD98059, 1 μM U0126, 10 μM LY294002, 1 μM U0124, and 10 μM LY303511).were included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor and inhibitor exposure time was 20 minutes. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no GDNF condition using an ANOVA with Dunnett's post-hoc test ($p<0.05$). Ampersands (@) indicate statistically significant differences between the 10 ng/mL treatment condition and the condition containing the inhibitor listed below the graph using t-tests ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release. N = 12-18 wells/condition.

amount of immunoreactive phospho-Akt (p-Akt; Figure 13 A and C). These data indicate that not only does GDNF increase p-Erk levels in DRG, but also that the MEK inhibitors PD98059 and U0126 inhibit this increase at the concentrations used. These inhibitors were then added to the DRG in culture during the basal and capsaicin-stimulated incubations to determine the significance of the MAPK/Erk 1/2 and PI-3K/Akt pathways in sensitization of sensory neurons by GDNF. As seen in figure 14, the enhancement in stimulated release of iCGRP induced by 10 ng/mL GDNF was prevented by the MEK inhibitors PD98059 (10 μM) and U0126 (1.0 μM), but not by the inactive control U0124 (1.0 μM). The PI-3K inhibitor, LY294002 (10 μM), and the inactive control for this compound, LY303511 (10 μM), did not affect the GDNF-induced sensitization (Figure 14). These pharmacological manipulations did not alter the total content of iCGRP (Figure 15). Taken together, these data indicate that GDNF-induced sensitization of sensory neurons occurs through activation of the MAPK/Erk 1/2 pathway and not through the PI-3K/Akt pathway.

c. Intracellular signaling pathways responsible for NTN-induced sensitization

Immunoblots were performed with NTN as the GFL inducing signaling MAPK and PI-3K pathway activation. NTN activates a myriad of signaling pathways, including the MAPK/Erk 1/2 and PI-3K/Akt pathways (Althini et al., 2004;Soler et al., 1999). When dissociated DRG were exposed to 10 ng/mL NTN for 10 minutes, both p-Erk 1/2 and p-Akt levels were increased (Figure 16A). PD98059 (10 μM) and U0126 (1.0 μM) prevented the NTN-induced increase in p-Erk, while the inactive analogue U0124 (1.0 μM) did not affect NTN increases in p-Erk (Figure 16 A and B). Unlike GDNF, exposure of DRG to NTN increased p-Akt levels, and this increase was prevented by LY294002 (10 μM) and not LY303511 (10 μM; Figure 16 A and C). Interestingly, NTN-induced enhancement in the release of iCGRP was abolished only by LY294402, the PI-3K/Akt pathway inhibitor (Figure 17). Neither of the MAPK/Erk 1/2 inhibitors, nor the inactive

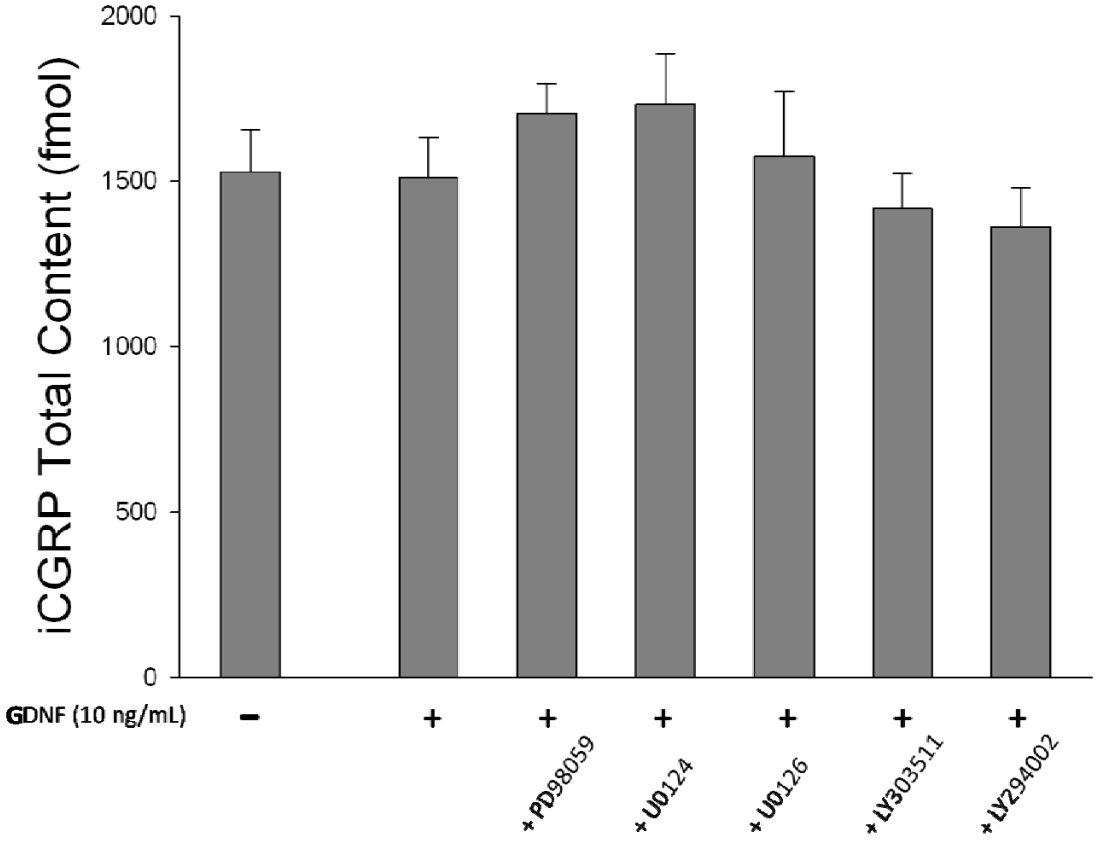

Figure 15. GDNF and pharmacological inhibitors do not change total content of iCGRP in DRG. Twenty minute exposure of DRG to 10 ng/mL GDNF and pharmacological inhibitors listed below the graph (10 μM PD98059, 1 μM U0126, 10 μM LY294002, 1 μM U0124, and 10 μM LY303511) did not change the total content of iCGRP, compared with an ANOVA. N = 12-18 wells/condition.

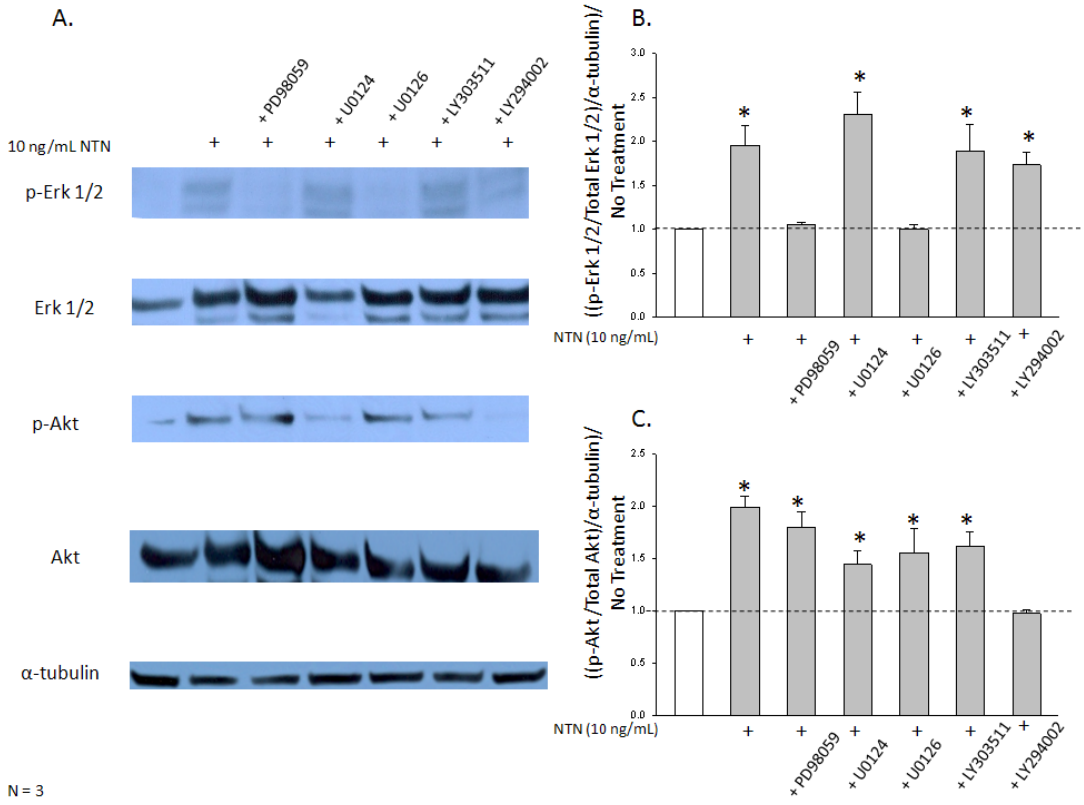

Figure 16. Signaling pathways activated by NTN. A) This representative Western blot demonstrated changes in levels of p-Erk 1/2, Erk 1/2, p-Akt, Akt, and α-tubulin in DRG in response to a 10 minute incubation with 10 ng/mL NTN and the subsequent prevention of these changes by inhibitors of the MAPK pathway (10 μM PD98059 and 1 μM U0126) and the PI-3K pathway (10 μM LY294002). Inactive control compounds for these pathways were added as well (1 μM U0124 for MAPK and 10 μM LY303511 for PI-3K). B) Densitometric analysis of three separate Western blots like that in A probing for MAPK pathway components. The level of p-Erk was divided by total Erk levels then normalized to α-tubulin levels in each condition. Asterisks (*) indicate statistically significant differences between treatment conditions and the no NTN and no inhibitor condition using an ANOVA with Dunnett's post hoc testing ($p<0.05$). C) Densitometric analysis of three separate Western blots like that in A probing for PI-3K pathway components. The level of p-Akt was divided by total Akt levels then normalized to α-tubulin levels in each condition. Asterisks (*) indicate statistically significant differences between treatment conditions and the no NTN and no inhibitor condition using an ANOVA with Dunnett's post hoc testing ($p<0.05$).

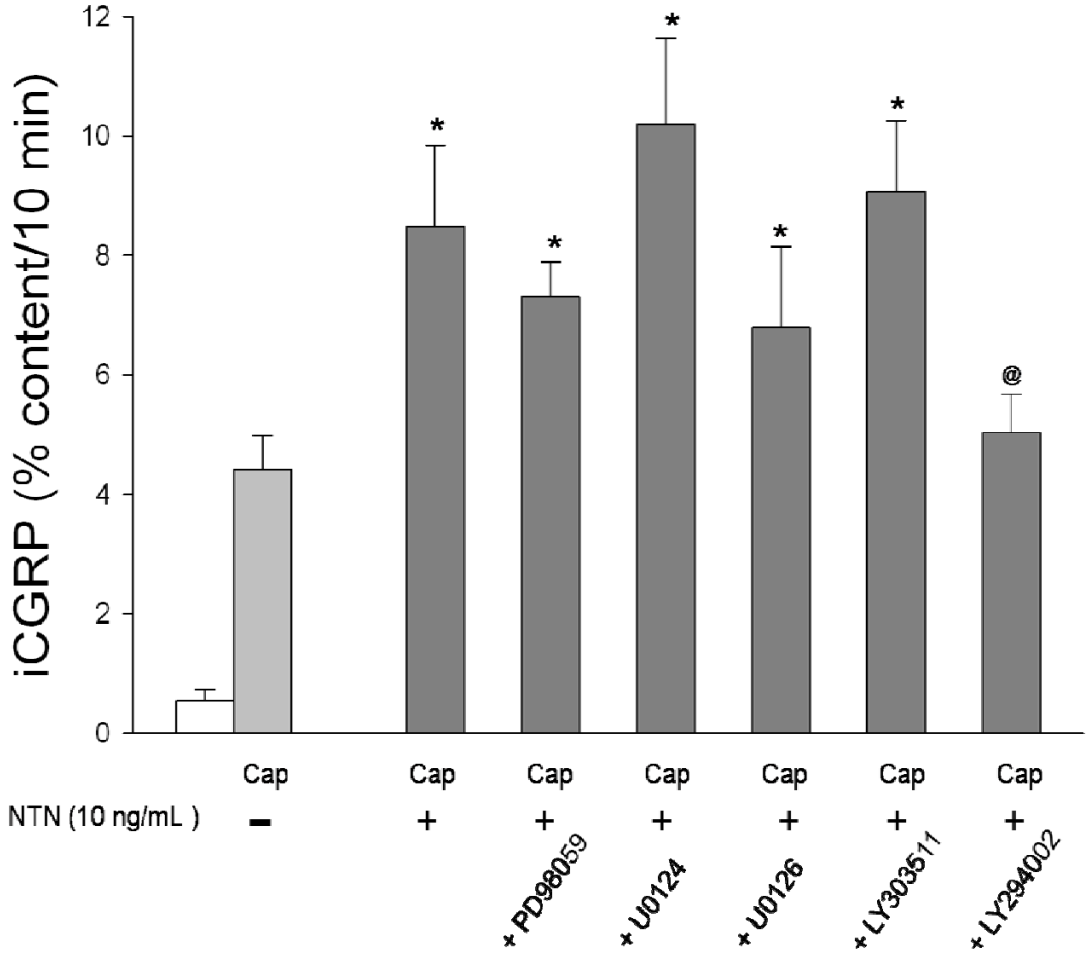

Figure 17. NTN-induced enhancement in the stimulated-release of iCGRP is mediated by the PI-3K pathway. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bar) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 12-18 wells per condition). NTN and inhibitors (10 μM PD98059, 1 μM U0126, 10 μM LY294002, 1 μM U0124, and 10 μM LY303511) were included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor and inhibitor exposure time was 20 minutes. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no NTN condition using an ANOVA with Dunnett's post-hoc test ($p<0.05$). Ampersand (@) indicates statistically significant differences between the 10 ng/mL treatment condition and the condition containing the inhibitor listed below the graph using t-tests ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release. N = 12-18 wells per condition.

control compounds, significantly prevented sensitization (Figure 17). The MAPK inhibitors did reduce the amount of enhancement in iCGRP release induced by NTN by 25%, and a potential explanation for this phenomenon will be discussed more fully in the discussion section of this document (Section IV.F). However, since I have defined sensitization as a statistically significant increase from control in stimulated-release of iCGRP, inhibition of the MAPK pathway by these inhibitors does not prevent NTN-induced sensitization. These pharmacological manipulations did not alter the total content of iCGRP (Figure 18). Therefore, it appears that NTN accomplishes a significant portion of its sensitization of DRG neurons through the PI-3K pathway.

d. Intracellular signaling pathways responsible for ART-induced sensitization

The signaling pathways activated by ART were also examined using Western blots to measure changes in p-Erk and p-Akt. These pathways are activated by ART and responsible for altering many functions in sensory neurons (Hauck et al., 2006;Jeong et al., 2008a;Soler et al., 1999). A 10 minute exposure to 10 ng/mL ART, similar to NTN, increased both p-Erk and p-Akt levels when compared to the no growth factor condition (Figure 19 A, B and C). The inhibitors of the MAPK pathway (10 μM PD98059 and 1.0 μM U0126) prevented the ART-induced increases in p-Erk, while the inactive control compound, U0124 (1.0 μM), did not affect the ART-induced increase in p-Erk (Figure 19 A, B). LY294002 (10 μM), the inhibitor of the PI-3K-induced phosphorylation of Akt, prevented the ART-induced increases in p-Akt, while the inactive control compound, LY303511 (10 μM), did not affect the ART-induced increase in p-Akt (Figure 19 A, C).

Despite the ART-induced activation of the MAPK and PI-3K pathways, the enhancement in the release of iCGRP was not affected by inhibition of the MAPK/Erk 1/2 or PI-3K/Akt pathways. Specifically, inhibition of the MAPK/Erk 1/2 pathway by PD98059 and U0126 did not prevent the ART-induced enhancement in the capsaicin

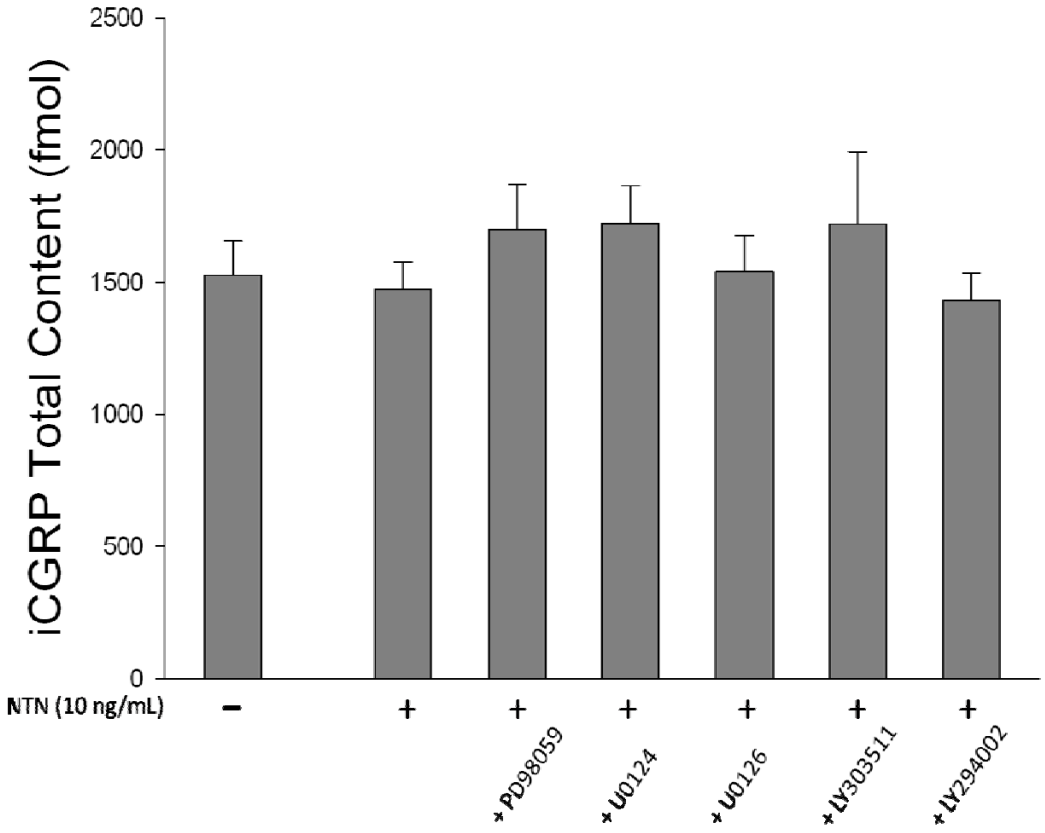

Figure 18. NTN and pharmacological inhibitors do not change total content of iCGRP in DRG. Twenty minute exposure of DRG to 10 ng/mL NTN and pharmacological inhibitors listed below (10 μM PD98059, 1 μM U0126, 10 μM LY294002, 1 μM U0124, and 10 μM LY303511) the graph did not change the total content of iCGRP, compared with an ANOVA. N = 12-18 wells per condition.

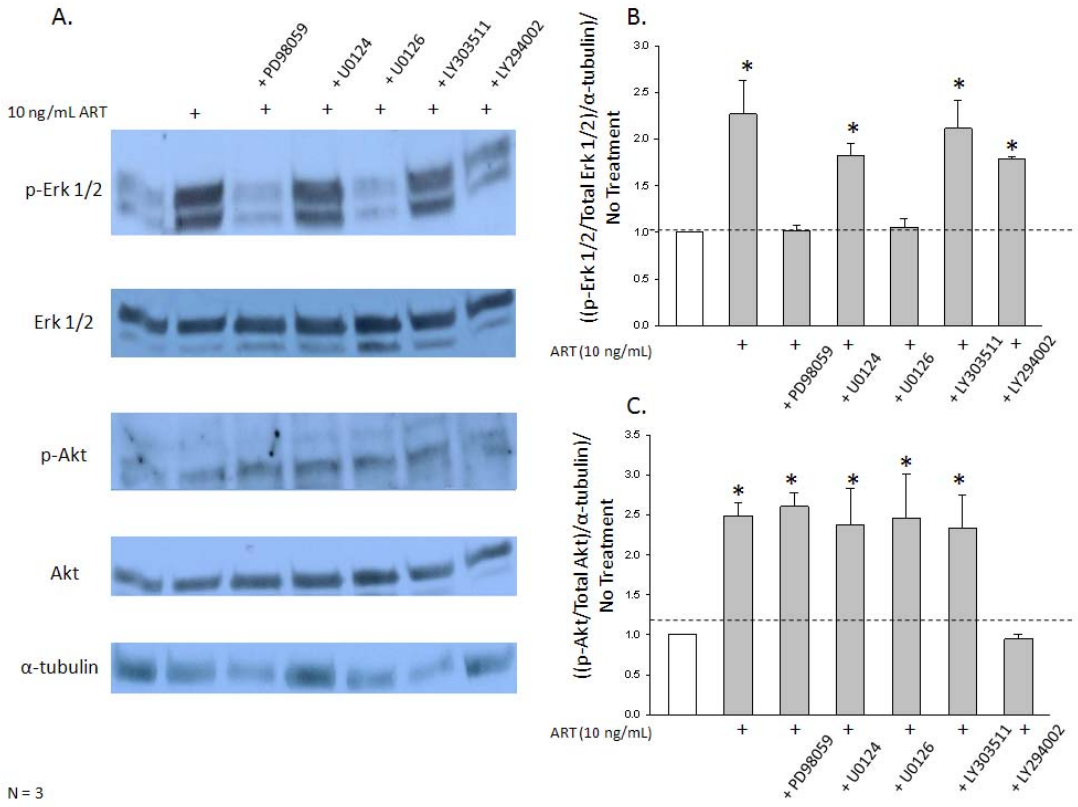

Figure 19. Signaling pathways activated by ART. A) This representative Western blot demonstrated changes in levels of p-Erk 1/2, Erk 1/2, p-Akt, Akt, and α-tubulin in DRG in response to a 10 minute incubation with 10 ng/mL ART and the subsequent prevention of these changes by inhibitors of the MAPK pathway (10 μM PD98059 and 1 μM U0126) and the PI-3K pathway (10 μM LY294002). Inactive control compounds for these pathways were added as well (1 μM U0124 for MAPK and 10 μM LY303511 for PI-3K). B) Densitometric analysis of three separate Western blots like that in A probing for MAPK pathway components. The level of p-Erk was divided by total Erk levels then normalized to α-tubulin levels in each condition. Asterisks (*) indicate statistically significant differences between treatment conditions and the no ART and no inhibitor condition using an ANOVA with Dunnett's post hoc testing ($p<0.05$). C) Densitometric analysis of three separate Western blots like that in A probing for PI-3K pathway components. The level of p-Akt was divided by total Akt levels then normalized to α-tubulin levels in each condition. Asterisks (*) indicate statistically significant differences between treatment conditions and the no ART and no inhibitor condition using an ANOVA with Dunnett's post hoc testing ($p<0.05$).

stimulated-release of iCGRP (Figure 20). Similarly, inhibition of the PI-3K pathway by LY294002 did not prevent the ART-induced enhancement in the capsaicin stimulated-release of iCGRP (Figure 20). It was still possible that ART-induced sensitization occurred by activating both pathways, and so inhibition of either pathway alone was not sufficient to prevent sensitization. To ensure that the MAPK and PI-3K pathways were each sufficient but not both necessary for ART-induced enhancement in iCGRP release, both the MAPK inhibitor PD98059 (10 μM) and the PI-3K inhibitor LY294002 (10 μM) were used. When these inhibitors were used in combination, there was still no effect on ART-induced sensitization (Figure 20). These pharmacological manipulations did not alter the total content of iCGRP (Figure 21). Overall, the results of these experiments indicate that the mechanism of ART-induced sensitization of capsaicin stimulated iCGRP release is independent of both the MAPK and PI-3K pathways.

e. Involvement of the Src family kinase pathway in GFL-induced sensitization

There is evidence that the Src family kinases (SFKs) play an important role in sensory neuronal sensitization (Slack et al., 2008;Zhang et al., 2005). Additionally, each of the GFLs activates one or more of the SFKs (Encinas et al., 2001;Jeong et al., 2008a). The commercially available and most widely used antibody for measuring levels of SFKs by Western blot is a pan SFK antibody, which cannot distinguish between each of the SFKs. The most commonly used inhibitor for each of the SFKs is PP2, which is a pan SFK inhibitor. The details of this inhibitor will be described below. The studies of the role of c-Src and SFK pathways in GFL-induced sensitization began with a broad evaluation of the ability of SFK pathways, in general, to mediate GFL-induced sensitization.

PP2 is potent inhibitor of the SFK pathway. It has an IC_{50} for c-Src, Lck, and Fyn activity of between 4 nM and 7 nM and does not inhibit proteins from other

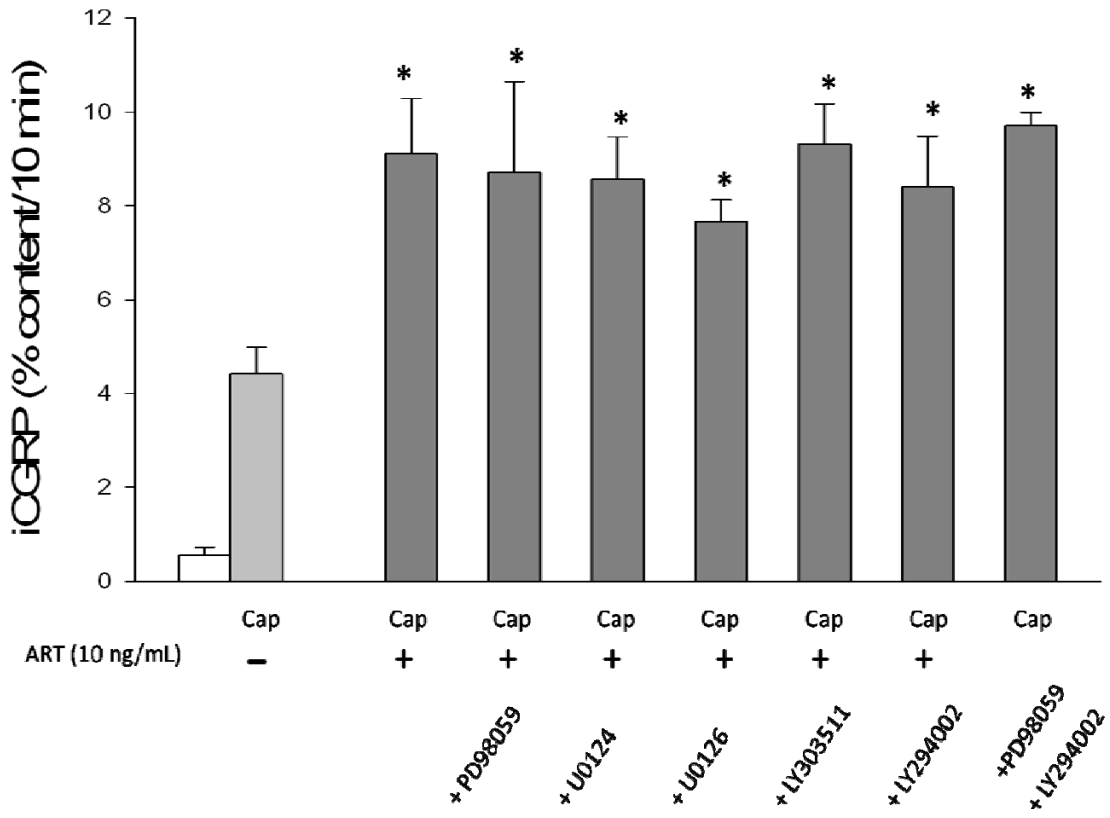

Figure 20. ART-induced enhancement in the stimulated-release of iCGRP is mediated by neither the MAPK nor the PI-3K pathway. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bar) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 12-18 wells per condition). ART and inhibitors were included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor and inhibitor exposure time was 20 minutes. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no ART condition using an ANOVA with Dunnett's post-hoc test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

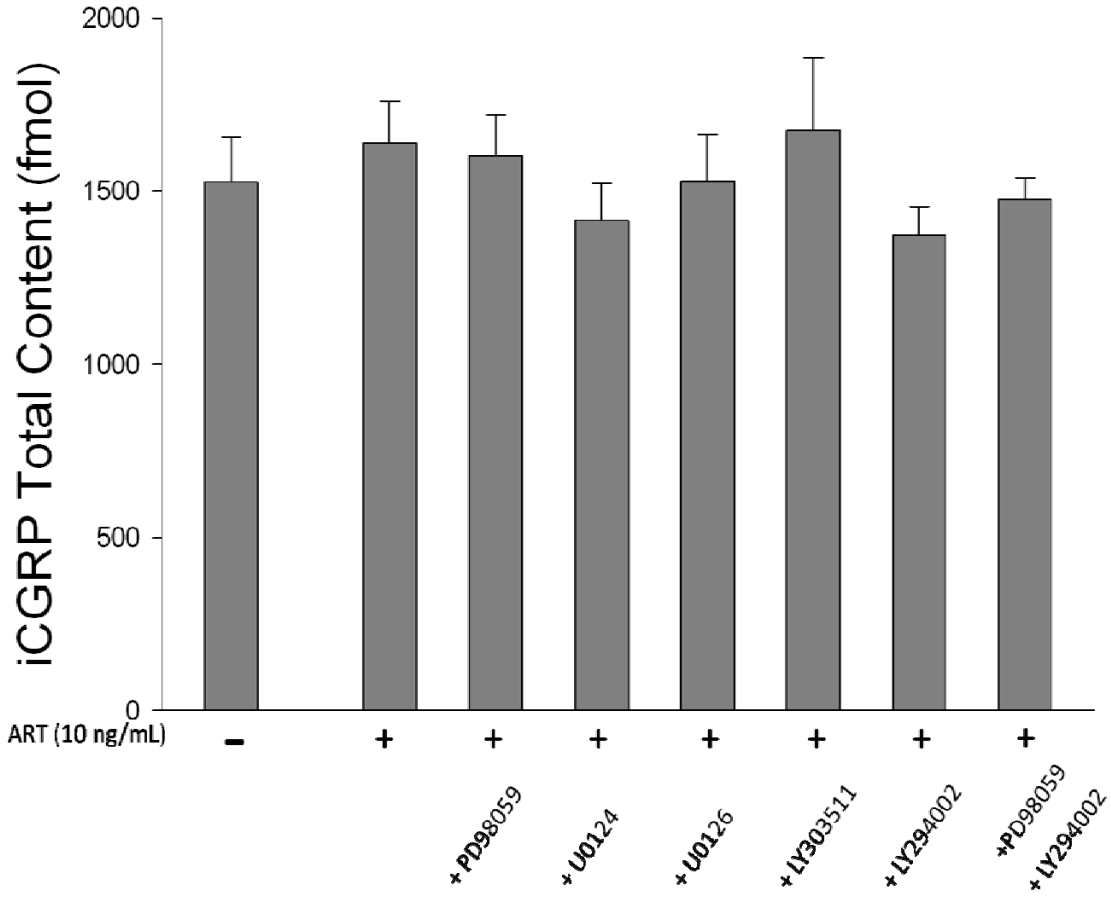

Figure 21. ART and pharmacological inhibitors do not change total content of iCGRP in DRG. Twenty minute exposure of DRG to 10 ng/mL ART and pharmacological inhibitors listed below the graph (10 μM PD98059, 1 μM U0126, 10 μM LY294002, 1 μM U0124, and 10 μM LY303511) did not change the total content of iCGRP, compared with an ANOVA. N = 12-18 wells per condition.

phosphorylation-dependent, related signaling pathways, JAK-2 and EGF-R (Hanke et al., 1996). PP2 has an inactive analogue, PP3, which has several modifications of the PP2 molecule, most importantly the removal of a benzene ring-linked chloride ion (Tisdale and Artalejo, 2006). This molecule has no effect on SFK-dependent cell activities, including GDNF-induced, SFK-dependent neurite outgrowth in neuronal cell lines (Encinas et al., 2001) and rescue of hippocampal neurons from hypoxia-ischemia injury (Jiang et al., 2008).

To evaluate the role of SFKs in GFL-induced sensory neuronal sensitization, DRG in culture were first exposed to each of the GFLs and the amount of the active form of SFKs, phospho-SFKs (p-SFK), were measured. Each of the GFLs increases p-SFK levels, and the SFK inhibitor, PP2 (10 μM) prevented this increase (Figure 22 A and B). The inactive analogue of PP2, PP3, did not prevent, but did reduce the GFL-induced increase in SFKs (Figure 22 A and B).

These pharmacological agents, PP2 and PP3, were then added to the DRG in the basal and capsaicin stimulated conditions of the CGRP release assay to determine the role of this GFL-activated pathway in the GFL-mediated enhancement in the stimulated release of iCGRP. PP2 abolished the sensitization of stimulated release by GDNF, NTN, and ART, while the inactive control, PP3, did not affect any of the GFL-induced sensitization (Figure 23). The SFK inhibitor, PP2, did not alter the total content of iCGRP (Figure 24). These experiments indicate that activation of SFKs is critical for GFL-induced sensitization. The SFKs are also reported to be initiated prior to the MAPK/Erk 1/2 and PI-3K pathways and to activate these pathways (Encinas et al., 2001;Jeong et al., 2008a). When the Neuro2A neuroblastoma cell line was stimulated with GDNF or NTN, PP2 inhibited activation of the MAPK/Erk 1/2 and PI-3K pathways and prevented survival of granule and sympathetic neurons, actions dependent upon these two cellular signaling pathways (Encinas et al., 2001).

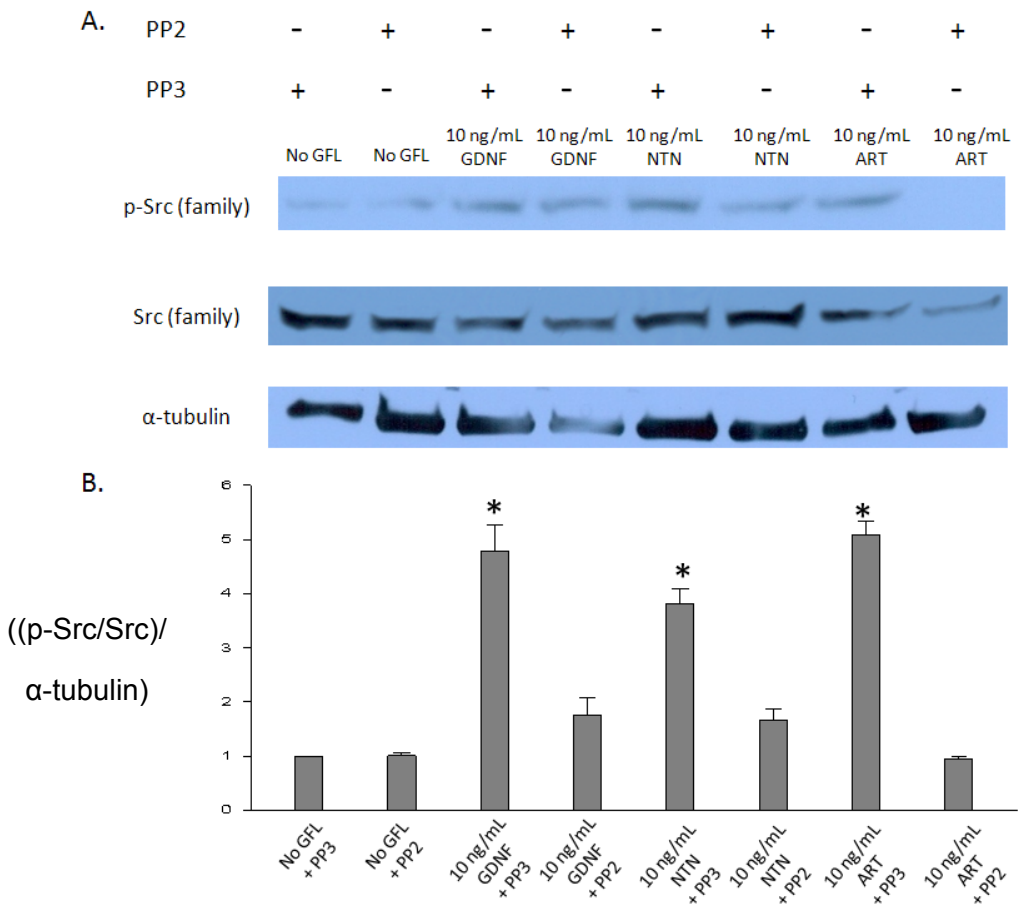

Figure 22. The GFLs activate SFKs and PP2 prevents that activation. A) This representative Western blot demonstrated changes in levels of p-SFKs, SFK, and α-tubulin in DRG in response to a 10 minute incubation with 10 ng/mL GDNF, NTN, and ART and the subsequent prevention of these changes by an inhibitor of the SFK pathway (10 μM PP2). The inactive control compound for this pathway was added as well (10 μM PP3). B) Densitometric analysis of three separate Western blots like that in A probing for SFKs. The level of p-SFK was divided by total SFK levels then normalized to α-tubulin levels in each condition. Asterisks (*) indicate statistically significant differences between treatment conditions and the no GFL and no inhibitor condition using an ANOVA with Dunnett's post hoc testing ($p<0.05$). N = 12-18 wells per condition.

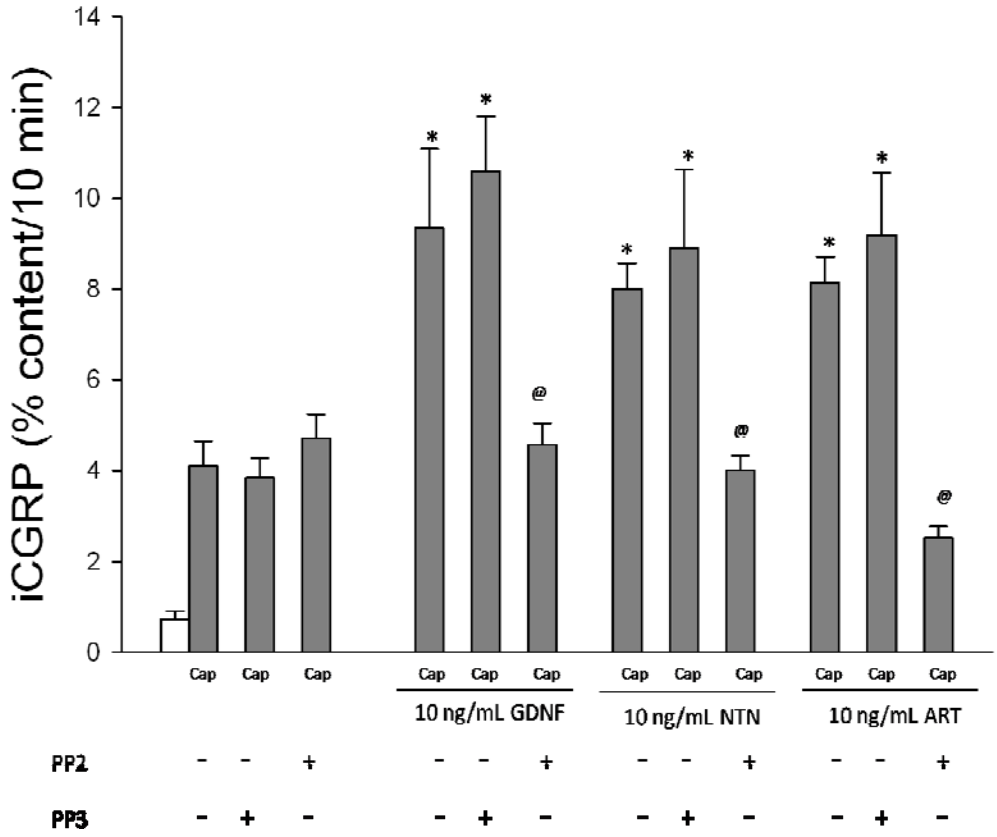

Figure 23. GFL-induced enhancement in the stimulated-release of iCGRP is mediated by SFK pathway. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bar) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 12-18 wells per condition). GFLs and inhibitors (10 μM PP2 and 10 μM PP3) were included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor and inhibitor exposure time was 20 minutes. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no GFL condition using an ANOVA with Dunnett's post-hoc test ($p<0.05$). Ampersands (@) indicate statistically significant differences between the GFL treatment and the PP2 treated condition using a t-test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

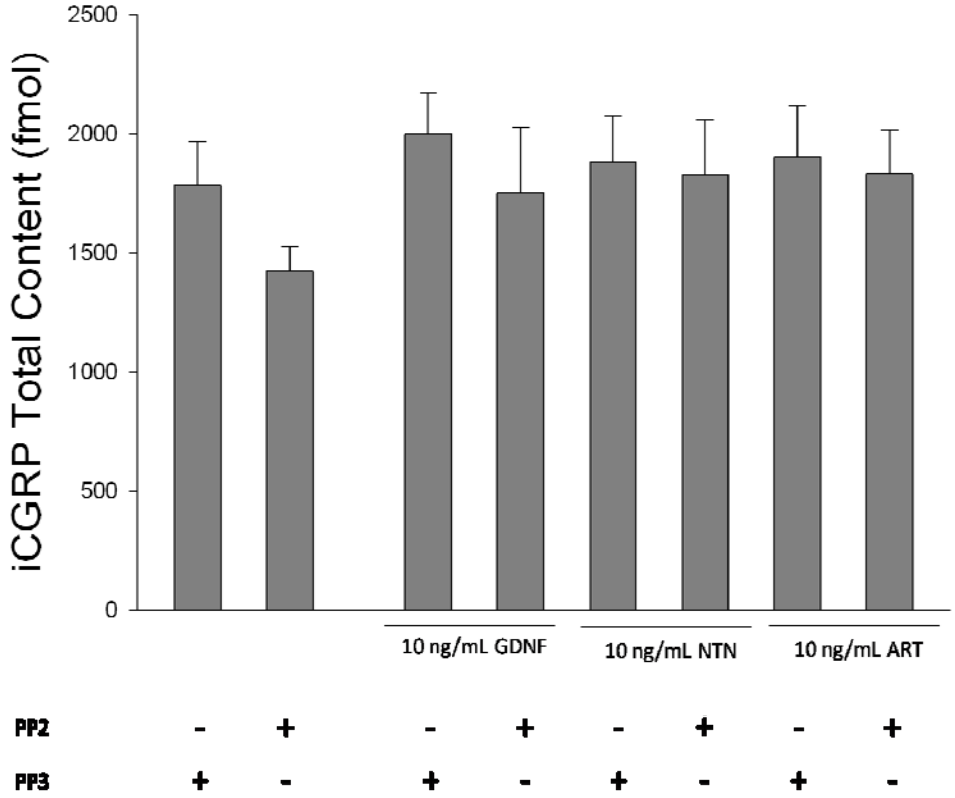

Figure 24. GFLs and the pharmacological inhibitors PP2 and PP3 do not change total content of iCGRP in DRG. Twenty minute exposure of DRG to 10 ng/mL GFLs and PP2 or PP3 (10 μM PP2 and 10 μM PP3) did not change the total content of iCGRP, compared with an ANOVA. N = 12-18 wells per condition.

However, PP2, like many pharmacological inhibitors, prevents phosphorylation of many proteins. These include Src (Nagao et al., 1998), the other SFKs Fyn and Yes (Encinas et al., 2001), and Ret (Encinas et al., 2001). Since the GFLs mediate many of their effects through Ret, some of the attenuation of GFL-induced sensitization seen with PP2 application could be a result of Ret inhibition. For this reason, siRNA targeted to c-Src (refer to Table 1 for details) was used as a tool to more specifically evaluate the role of the c-Src pathway in GFL-induced sensitization. The c-Src siRNA (100 nM) was added to the DRG in culture two days after plating and remained in the culture media for 48 hours. Figure 25 shows that the c-Src siRNA reduces c-Src expression by ~80% and does not change Fyn levels. Scramble siRNA (an siRNA designed as a scramble for APE1; Table 1), did not affect c-Src or Fyn levels (Figure 25). When the amount of capsaicin–stimulated release of iCGRP from DRG neurons exposed to c-Src siRNA was evaluated, the GDNF, NTN, and ART-induced sensitization observed previously was still present (Figure 26). However, the amount of enhancement of stimulated-release of iCGRP by the GFLs was reduced when the neurons were exposed to c-Src siRNA compared to when this siRNA was absent from the culture media, both in the control and scramble siRNA conditions (Figure 26). Approximately 30% of the original level of c-Src protein remained after siRNA treatment and the remaining c-Src could be responsible for the sensitization remaining after treatment with c-Src siRNA. Treatment with siRNA did not alter the total content of iCGRP (Figure 27). Interestingly, c-Src siRNA did not affect Ret levels or increases in p-Ret induced by GDNF (Figure 28), while PP2 did prevent Ret activation by ART (Figure 29). This is an important point, since it has been unclear until now which portion of the Ret-SFK pathway PP2 is inhibiting. There is evidence that GDNF-induced initiation of SFKs can phosphorylate Ret in a *trans* fashion, meaning that the SFKs are directly phosphorylating Ret instead of direct Ret activation by the GFL-GFRα complex inducing autophosphorylation (Kato et al., 2002). The other possibility is

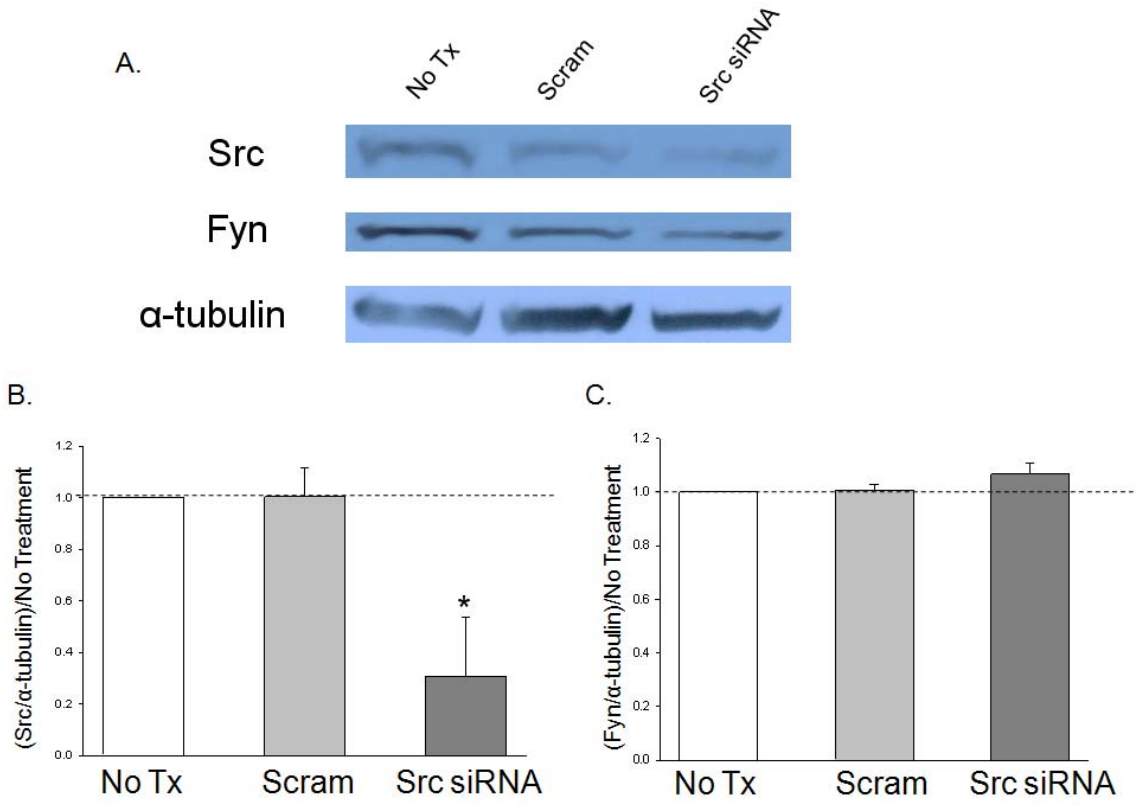

Figure 25. Src siRNA decreases c-Src levels. A) This representative Western blot demonstrates reduction in levels of c-Src and no change in Fyn levels in DRG in response to Src siRNA treatment. B) Densitometric analysis of three separate Western blots like that in A probing for c-Src. The level of c-Src was divided by α-tubulin levels and normalized to the no treatment condition. C) Densitometric analysis of three separate Western blots like that in A probing for Fyn. The level of Fyn was divided by α-tubulin levels and normalized to the no treatment condition. Asterisk (*) indicates statistically significant differences between treatment conditions and the no treatment condition using an ANOVA with Dunnett's post hoc testing ($p<0.05$). N = 3.

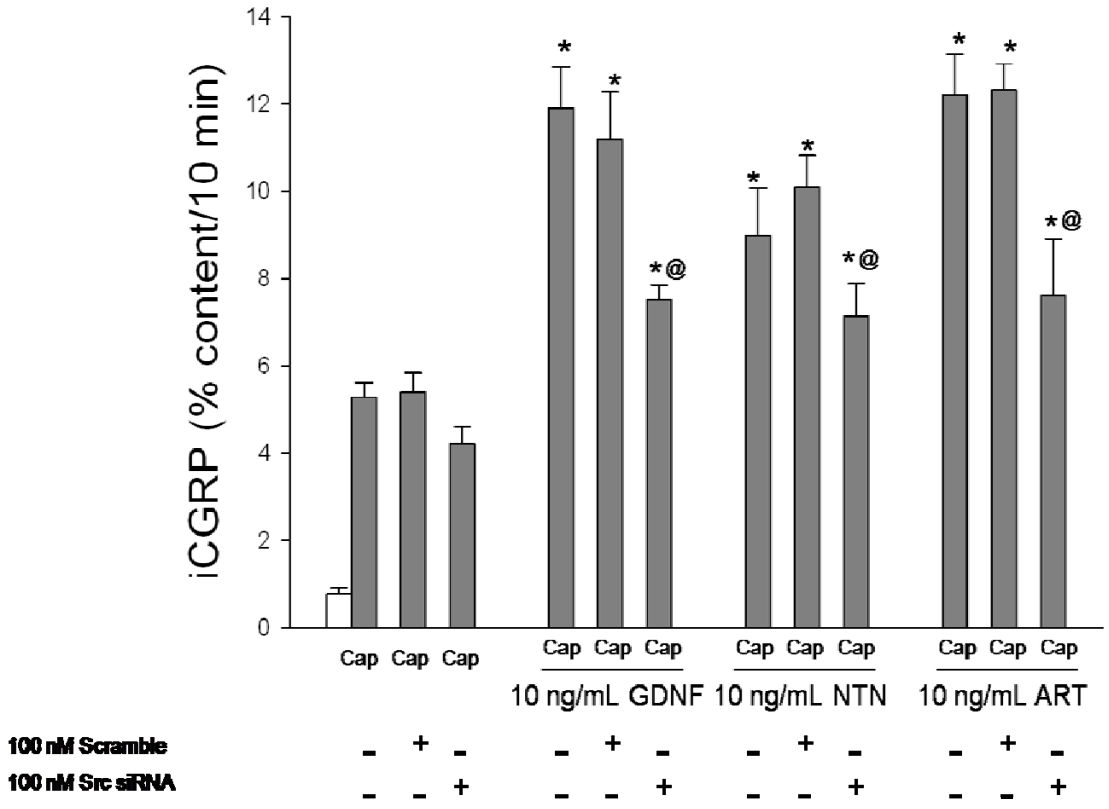

Figure 26. GFL-induced enhancement in the stimulated-release of iCGRP is mediated by the Src kinase pathway. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bar) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 12-18 wells per condition). GFLs were included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor and exposure time was 20 minutes. An siRNA designed as a scramble for APE1 was used as an siRNA transfection control. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no GFL condition using an ANOVA with Dunnett's post-hoc test ($p<0.05$). Ampersands (@) indicate statistically significant differences between the GFL treatment and the siRNA treated condition using a t-test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

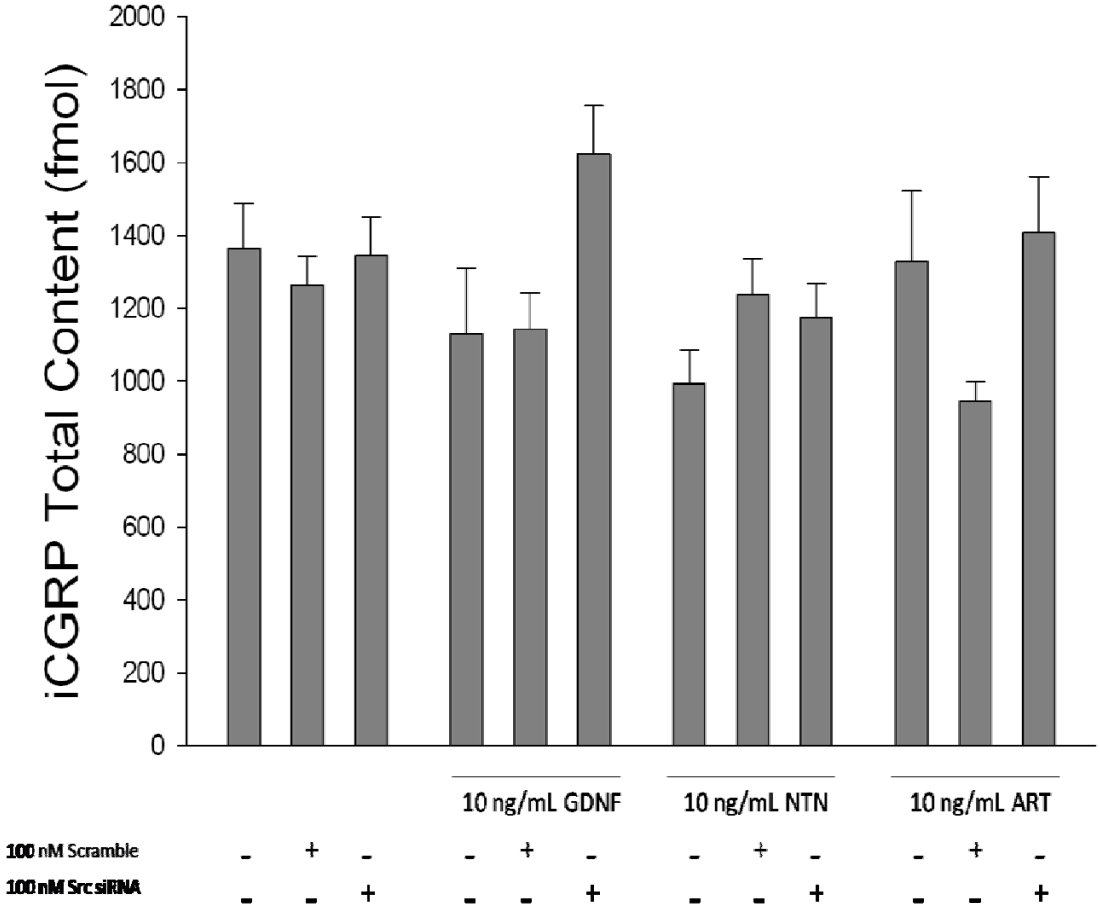

Figure 27. GFLs and Src siRNA do not change total content of iCGRP in DRG. Twenty minute exposure of DRG to 10 ng/mL GFLs, scramble siRNA, (100 nM) and/or Src siRNA (100 nM) did not change the total content of iCGRP, compared with an ANOVA.

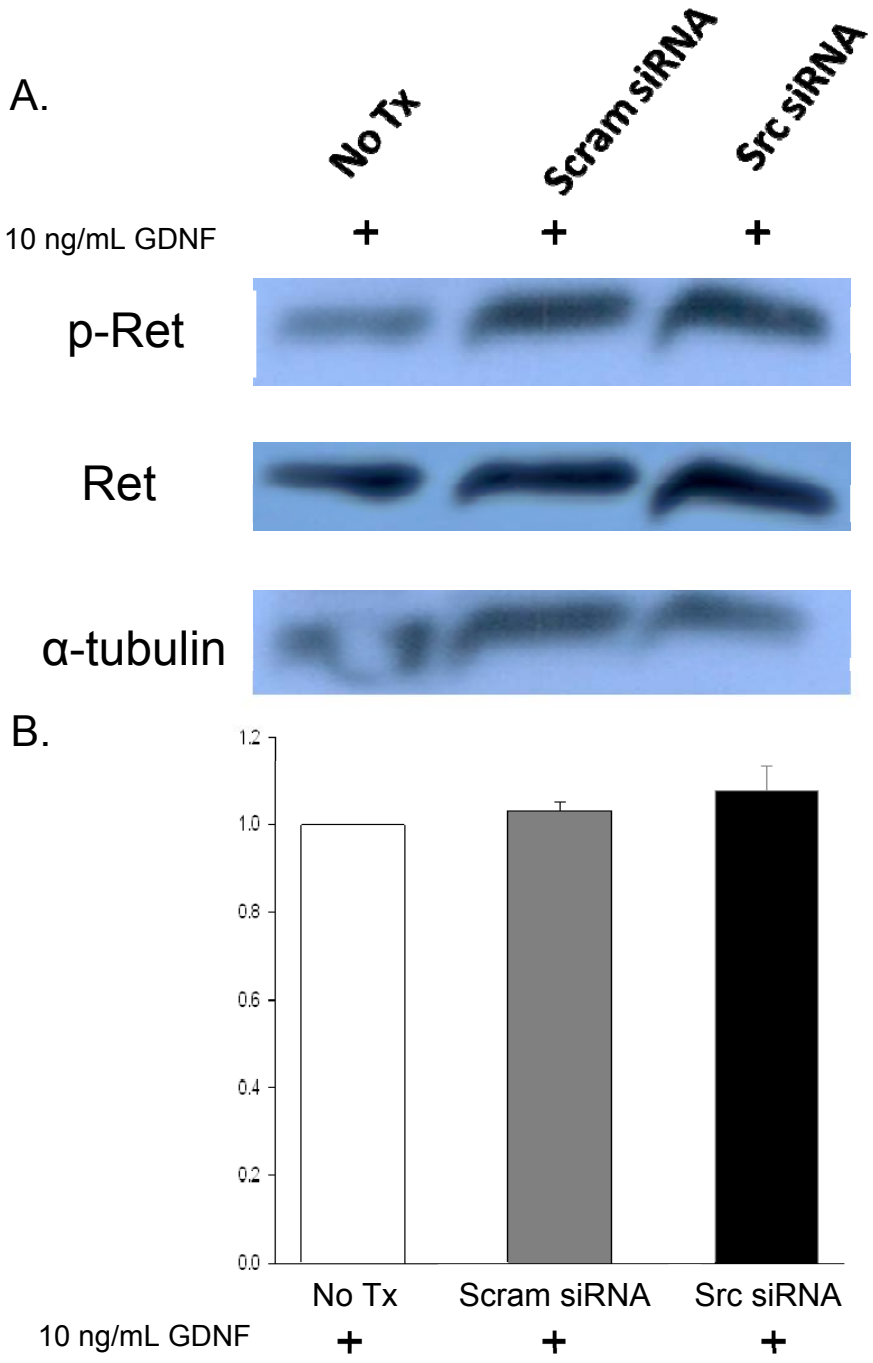

Figure 28. Src siRNA does not prevent Ret phosphorylation by GDNF. A) This representative Western blot demonstrates that the increase in p-Ret induced by 10 ng/mL GDNF is not prevented by either scramble siRNA or Src siRNA. B) Densitometric analysis of three separate Western blots like that in A probing for p-Ret. The level of p-Ret was divided by the level of Ret and then by α-tubulin levels and normalized to the no treatment condition.

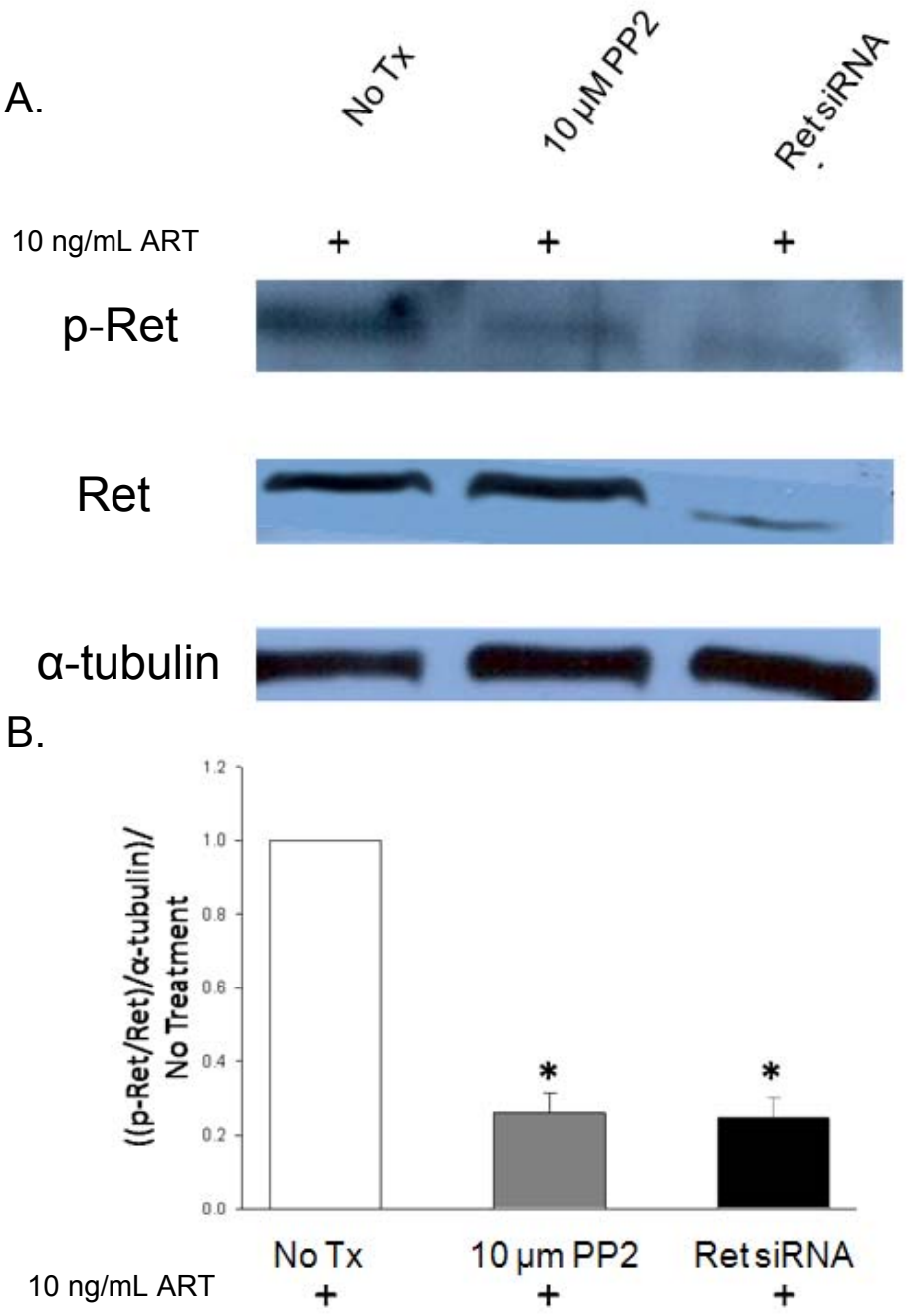

Figure 29. PP2 prevents Ret phosphorylation by ART. A) This representative Western blot demonstrates that twenty minute exposure of DRG to 10 ng/mL ART increased p-Ret levels, and this increase was prevented by both Ret siRNA and the SFK inhibitor PP2. B) Densitometric analysis of three separate Western blots like that in A probing for p-Ret. The level of p-Ret was divided by the level of Ret and then by α-tubulin levels and normalized to the no treatment condition. Asterisks (*) indicate statistically significant differences between treatment conditions and the no treatment condition using an ANOVA with Dunnett's post hoc testing ($p<0.05$). N = 3.

that autophosphorylation of Ret resulting from the activity of the GFL-GFRα complex is inhibited directly by PP2 (Encinas et al., 2001). Figures 28 and 29 indicate that PP2 may be directly preventing Ret phosphorylation.

These experiments demonstrate three key characteristics of c-Src and SFK function in GFL-mediated sensitization of sensory neurons. First, Src likely is an important component of the enhancement of stimulated release by GFLs. GFL-induced sensitization was not abolished by inhibition of the Src pathway. However, since the enhancement in stimulated-release of iCGRP was significantly reduced by Src pathway inhibition, this pathway is likely involved in this enhancement mechanism. Secondly, because the increase in p-Erk levels after stimulation with GDNF and the increase in p-Akt levels after stimulation with NTN are prevented by Src siRNA, Src is likely upstream of these effectors in the respective pathways. Finally, these data demonstrate conclusively that PP2 is specific neither to Src nor the SFKs, but actually directly inhibits Ret autophosphorylation induced by the GFLs.

I have now demonstrated that each of the GFLs, GDNF, NTN, and ART, use distinct, classically Ret-dependent pathways to accomplish enhancement in the stimulated release of iCGRP, thereby sensitizing the sensory neurons of the DRG. GDNF sensitizes DRG neurons through the MAPK/Erk 1/2 pathway. NTN-induced sensitization is PI-3K pathway dependent. The ART-induced sensitization is not accomplished through the MAPK or the PI-3K pathways. However, the PKCε pathway is a possible signaling pathway responsible for sensory neuronal sensitization by ART (personal communication, Dr. Weiguo Zhu). This working set of pathways model is illustrated schematically in Figure 30.

2. Ret-Independent pathways

There are several recent studies that indicate there may be a Ret-independent component to GFLs' actions on many cell types, including neurons

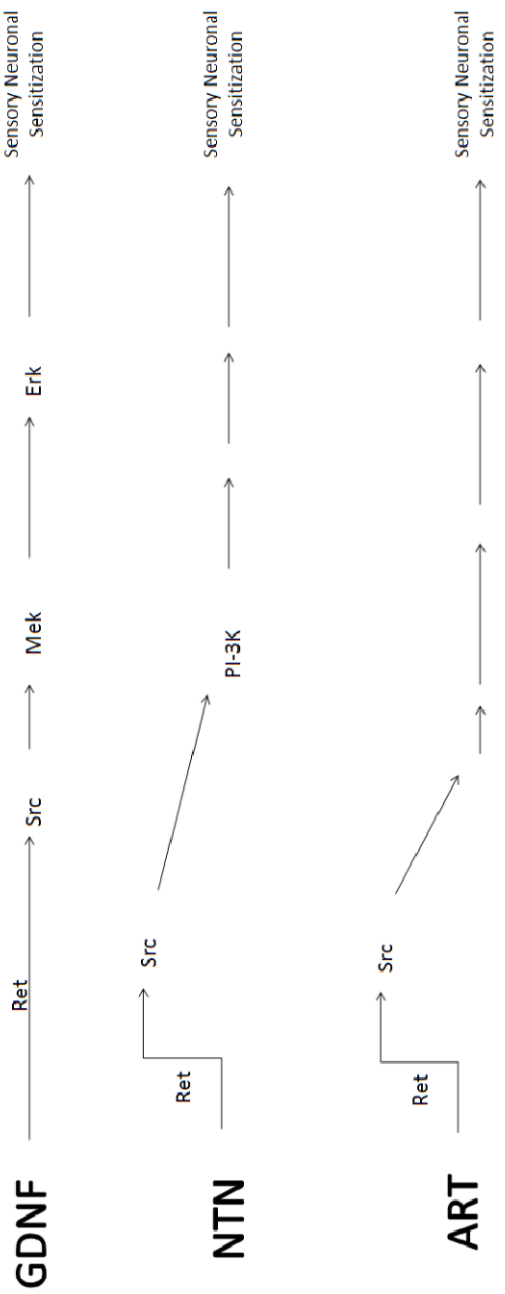

Figure 30. Schematic of working model of the distinct pathways of sensitization by the GFLs. Each of the GFLs uses primarily specific and distinct classically Ret-dependent pathways to accomplish sensitization.

(Cao et al., 2008a;Cao et al., 2008b;Paratcha et al., 2003;Sjostrand et al., 2007). The fact that PP2 inhibits both Ret and SFKs (Figure 29), and that c-Src siRNA did not completely prevent GFL-induced sensitization (Figure 26), led to the consideration that there may be Ret-independent and/or a non-Src SFK set of pathways by which the GFLs accomplish their sensitization. Other SFKs, in particular Fyn, are not activated by Ret, but are activated by one of the proposed Ret-independent, GFL-activated pathways through the NCAM receptor (Cao et al., 2008a;Paratcha et al., 2003). Therefore, whether there are in fact Ret-independent pathways for GFL-induced sensory neuronal sensitization was determined. Then, the specific cell surface co-receptors and signaling pathway initiators were explored.

a. NTN and ART, not GDNF, exhibit Ret-independent mechanisms of enhancement in stimulated-release of iCGRP

To determine the contribution of GFL-induced Ret-dependent signaling pathways to the enhancement in stimulated-release of iCGRP, Ret levels were reduced with the use of a specific pool of siRNA molecules directed at Ret, since no specific pharmacological inhibitors of the phosphorylation of this molecule exist. This pool of siRNAs (100 nM) reduced the amount of Ret protein in the DRG by ~85% compared to the no treatment condition, while the scramble siRNA used as a control for siRNA transfection did not alter Ret levels (Figure 31). In addition, a purified protein of a portion of Ret, residues 31-330, was used as a positive control. As shown in Figure 31, the immunoband present in the lane loaded with the purified Ret control protein is at the same location as the bands for the DRG treatment conditions probed with Ret antibody. This indicates that the Ret antibody is likely labeling the endogenous Ret protein in the samples. To ensure that this siRNA did not affect other possible pathways of GFL-induced sensitization, SFK levels were measured in the presence and absence of Ret siRNA. Figure 32 demonstrates that

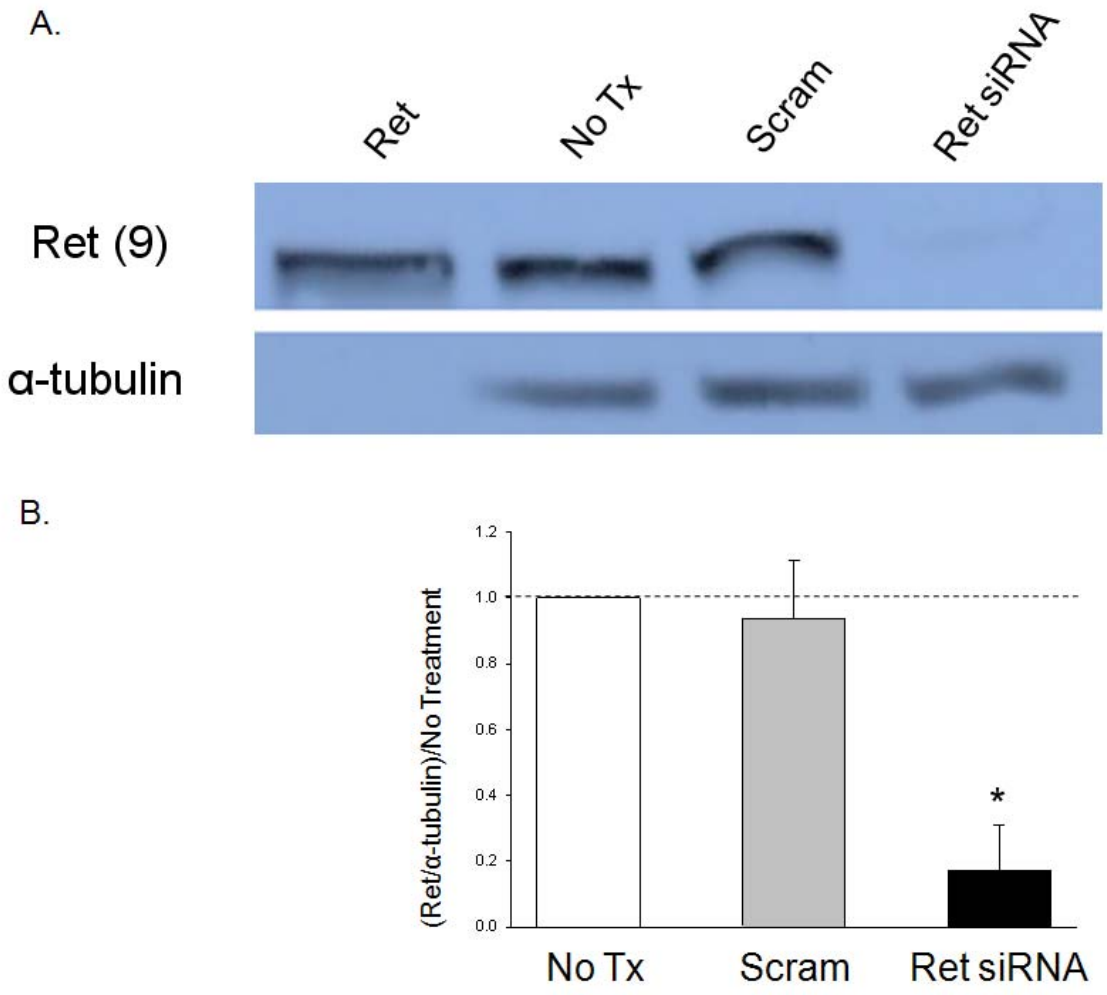

Figure 31. Ret siRNA reduces levels of Ret in DRG. A) This representative Western blot demonstrates that exposure of DRG to Ret siRNA decreases Ret levels, while scramble siRNA does not change Ret levels. Additionally, the purified Ret control protein is present on the immunoblot with a band the same size as Ret from DRG. B) Densitometric analysis of three separate Western blots like that in A probing for Ret. The level of Ret was divided by α-tubulin levels and normalized to the no treatment condition. Asterisk (*) indicates statistically significant differences between treatment conditions and the no treatment condition using an ANOVA with Dunnett's post hoc testing ($p<0.05$).

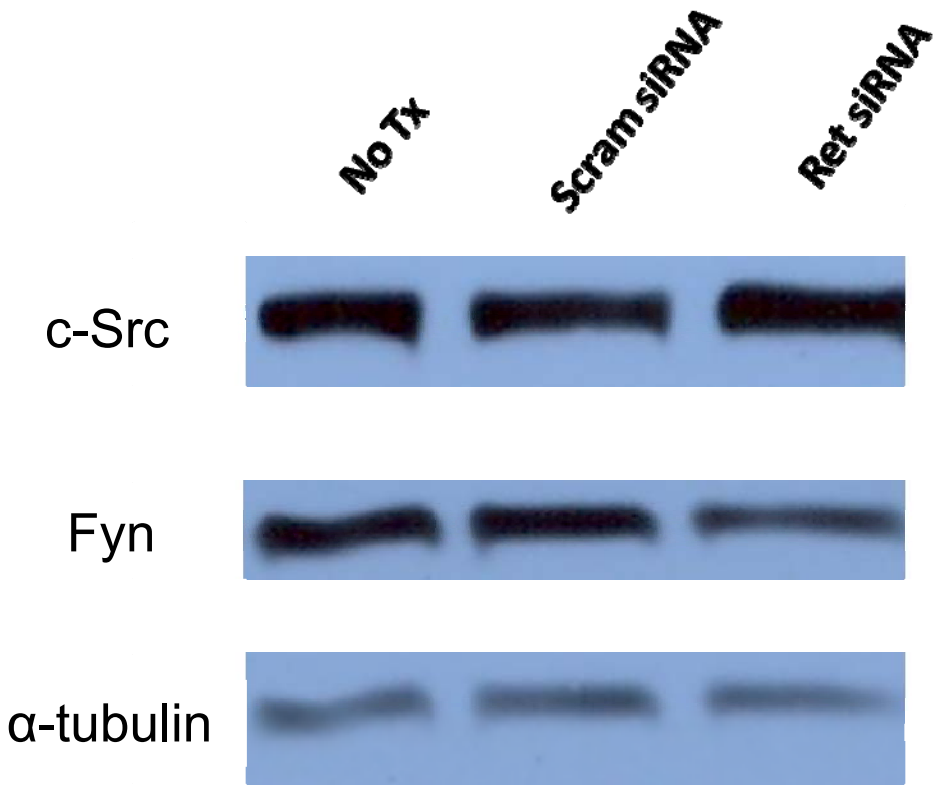

Figure 32. Ret siRNA does not change levels of c-Src or Fyn in DRG. This Western blot demonstrates that exposure of DRG to scramble siRNA (100 nM) and Ret siRNA (100 nM) does not change c-Src or total Fyn levels.

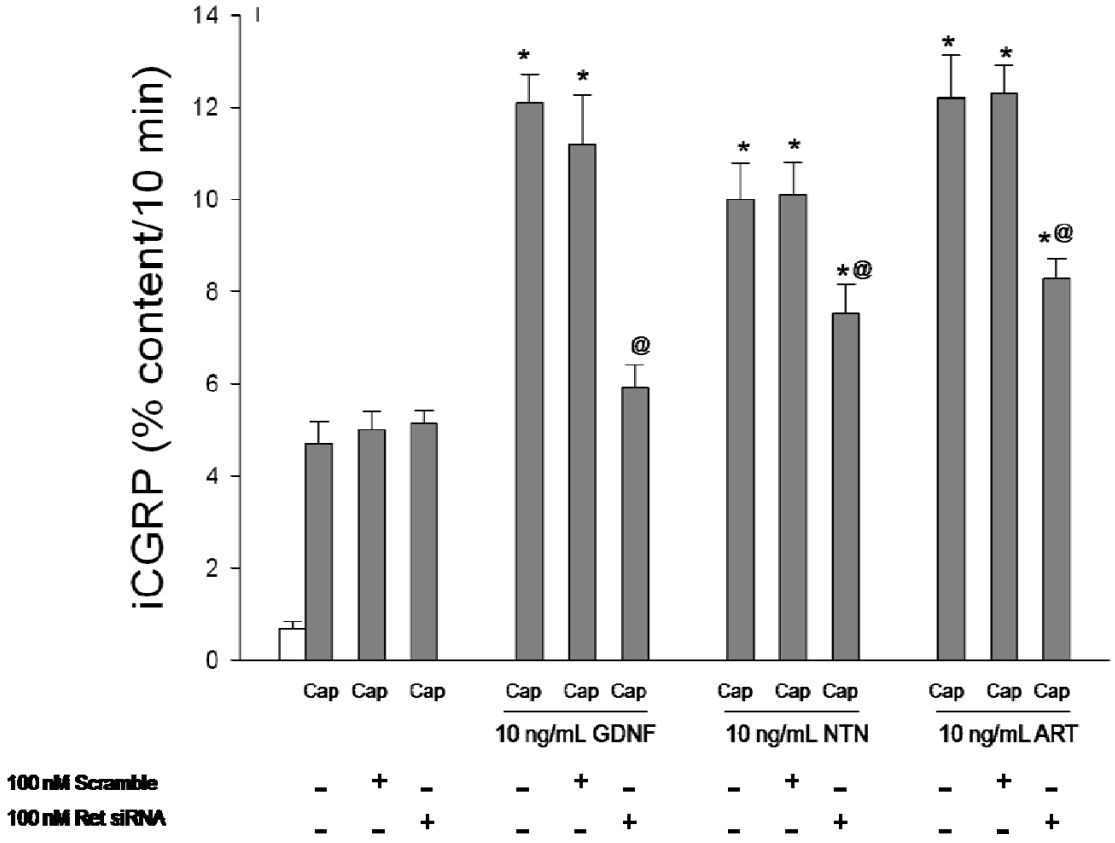

Figure 33. GDNF-induced enhancement in the stimulated-release of iCGRP is mediated by Ret-dependent pathways. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bar) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 12-18 wells per condition). GFLs were included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor exposure time was 20 minutes. An siRNA designed as a scramble for APE1 was used as an siRNA transfection control. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no GFL condition using an ANOVA with Dunnett's post-hoc test ($p<0.05$). Ampersands (@) indicate statistically significant differences between the GFL treatment and the siRNA treated condition using a t-test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

Ret siRNA has no effect on SFK levels, c-Src levels, and Fyn levels, with a single experiment.

Once the effectiveness of the Ret siRNA was established, it was then added to DRG in the culture media in the same way as previously described for c-Src siRNA. The basal and stimulated-release of iCGRP was then measured in the presence and absence of GFLs. The goal was to determine whether Ret was necessary for each of the GFLs' ability to induce an enhancement in the stimulated-release of iCGRP. Interestingly, while GDNF was not able to sensitize sensory neurons when Ret siRNA was added, NTN and ART were still capable of enhancing release (Figure 33). The enhancement in stimulated-release of iCGRP by NTN and ART, while still present, was significantly reduced (Figure 33). The total content of iCGRP was not affected by these manipulations (Figure 34). Therefore, NTN and ART are still capable of sensitizing sensory neurons when Ret signaling is reduced. Taken together, these data demonstrate that Ret is responsible for some of the enhancement in the stimulated-release of iCGRP induced by NTN and ART. While the remaining sensitization could be a result of incomplete reduction in Ret levels, it seems likely that some of the enhanced release of iCGRP must be due to Ret-independent mechanisms.

b. ART-induced enhancement in stimulated-release of iCGRP is mediated by Ret-dependent and NCAM-dependent mechanisms

The role of one of the other possible binding partners of the GFL-GFRα complexes, NCAM, in the NTN and ART-induced sensitization of DRG neurons was next explored. NCAM is a large membrane protein composed of an intracellular (NCAM140) and extracellular (NCAM180) component tightly linked to one another. No pharmacological inhibitors of this cell surface receptor exist, so NCAM levels were decreased using a pool of siRNA directed towards NCAM p140 (Table 1). NCAM p140 is the intracellular portion of this protein responsible for initiation of intracellular signaling pathways,

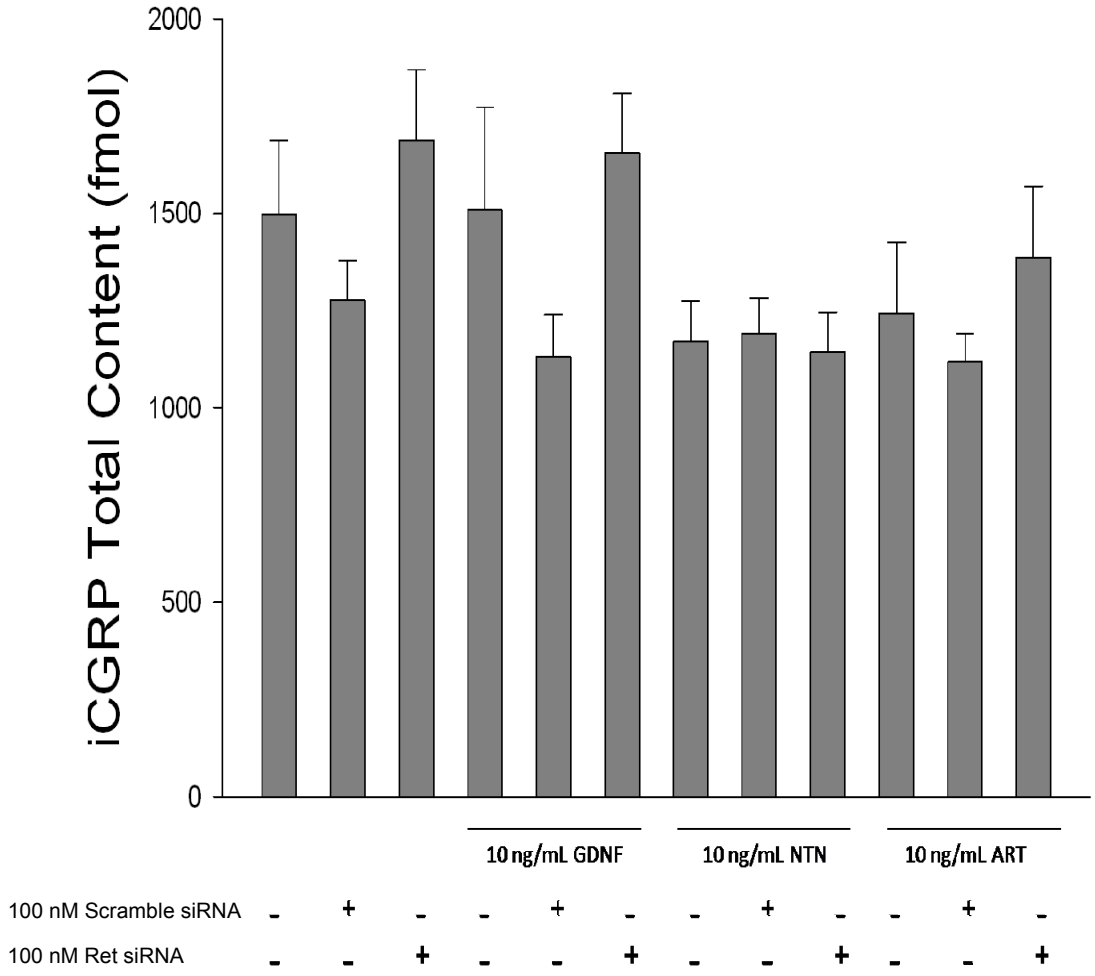

Figure 34. GFLs, scramble siRNA, and Ret siRNA do not change total content of iCGRP in DRG. Twenty minute exposure of DRG to 10 ng/mL GFLs, scramble siRNA (100 nM), and Ret siRNA (100 nM) did not change the total content of iCGRP, compared with an ANOVA. N = 12-18 wells per condition.

specifically the Fyn kinase pathway (Beggs et al., 1997). NCAM p180 is the portion of the molecule exposed to the extracellular matrix and likely not involved in GFL-GFRα complex signaling initiation (Beggs et al., 1997). Importantly, Fyn is not activated by Ret autophosphorylation (Cao et al., 2008a), making this signaling pathway a good downstream effector to use as an indicator of NCAM activation. This is necessary because there is no direct way to measure NCAM activity.

First, the effect of NCAM siRNA on the level of NCAM was determined. NCAM siRNA applied to the DRG reduced NCAM p140 levels by ~75% compared to non-treated control cells and scramble siRNA treated cells, while not affecting NCAM p180levels (Figure 35). The amount of NCAM present in non-treated DRG and scramble siRNA treated DRG were not different.

With the molecular effects of NCAM siRNA established, the role of NCAM activation in the NTN and ART-induced enhancement in the stimulated-release of iCGRP was examined. When NCAM siRNA was added to the DRG, the NTN and ART-induced sensitization remained, although the absolute level of enhancement in stimulated-release of iCGRP was reduced (Figure 36). NCAM siRNA did not affect the GDNF-induced sensitization. The total content of iCGRP was not affected by these manipulations (Figure 37). This data indicates that NCAM plays a role in NTN and ART-induced sensitization, but that it is not the only mechanism by which these GFLs induce sensitization.

To more fully address the possibility of more than one pathway being responsible for GFL-induced sensitization, NCAM and Ret siRNA were added in combination to the DRG in culture. In this case, NCAM siRNA (50 nM) and Ret siRNA (50 nM) were added to the DRG in culture on 2 days and 5 days after plating. This treatment regimen was followed to ensure the total amount of siRNA present in the culture media was consistent (100 nM). The basal and stimulated-release of iCGRP was then measured in the

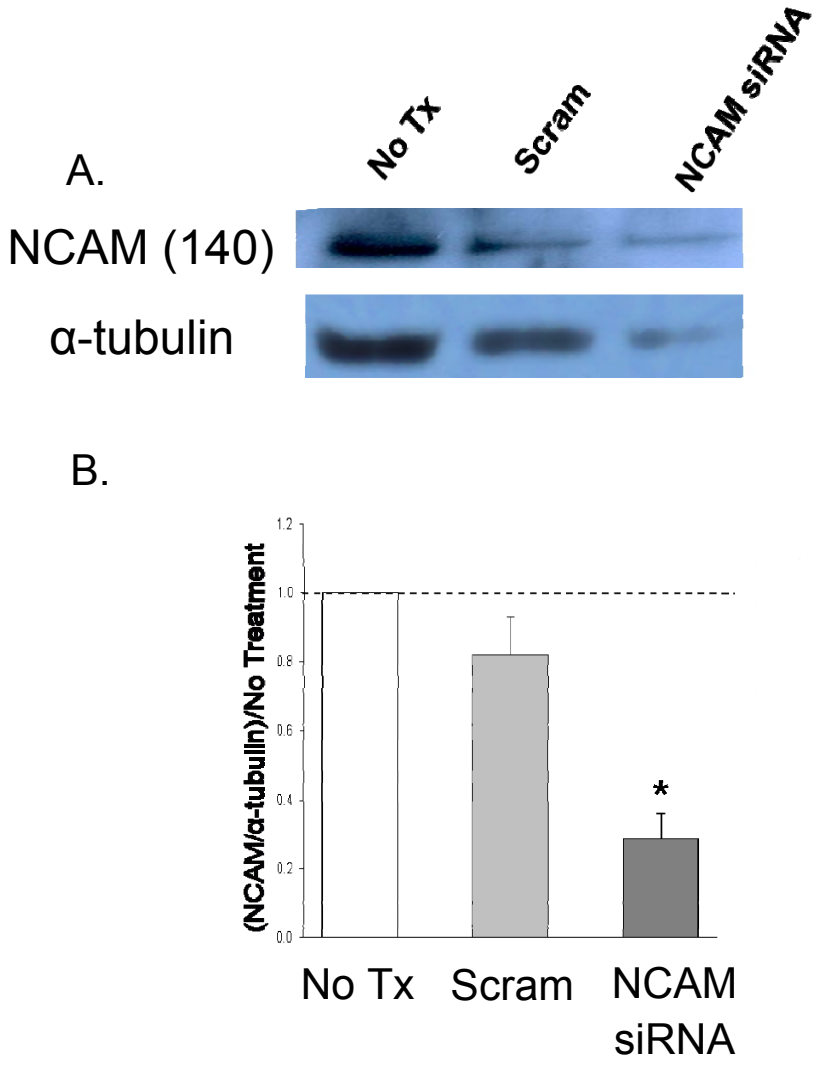

Figure 35. NCAM siRNA reduces levels of NCAM in DRG. A) This representative Western blot demonstrates that exposure of DRG to NCAM siRNA decreases NCAM levels, while scramble siRNA does not change NCAM levels. B) Densitometric analysis of three separate Western blots like that in A probing for NCAM p140. The level of NCAM was divided by α-tubulin levels and normalized to the no treatment condition. Asterisk (*) indicates statistically significant differences between treatment conditions and the no treatment condition using an ANOVA with Dunnett's post hoc testing ($p<0.05$). N = 3.

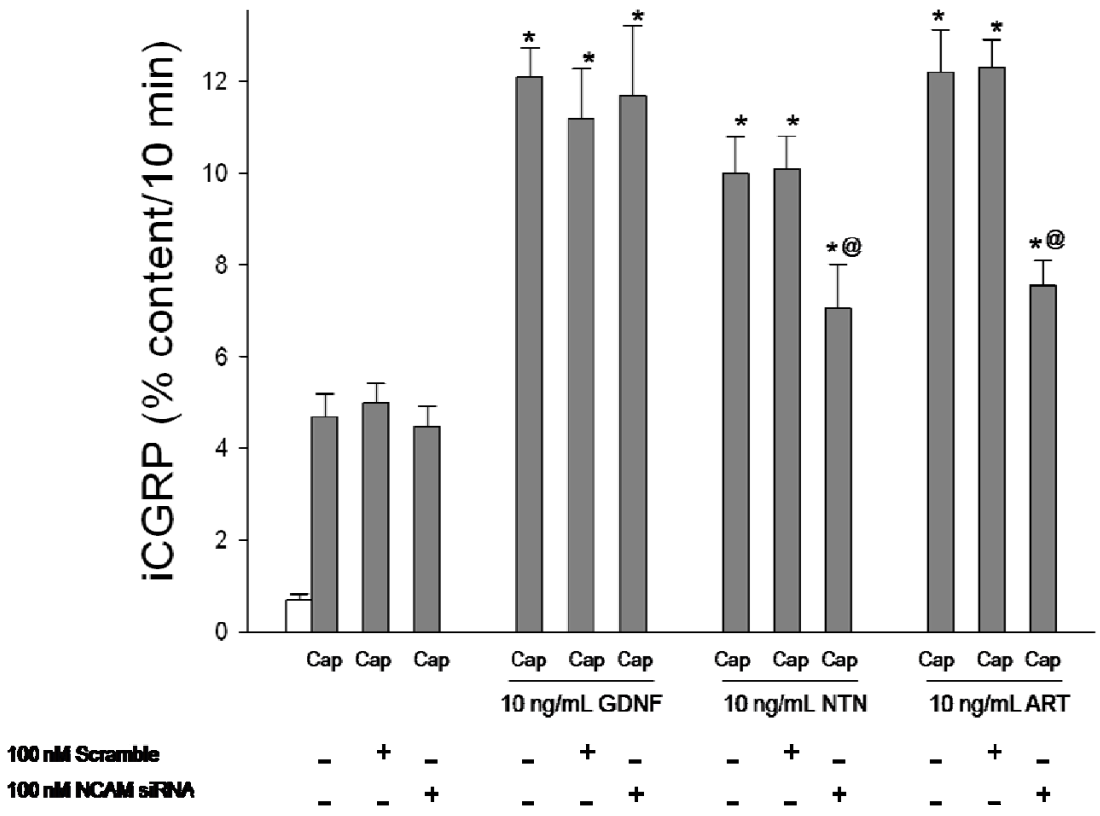

Figure 36. NTN and ART-induced enhancement in the stimulated-release of iCGRP is mediated, in part, by NCAM-dependent pathways. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bar) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 12-18 wells per condition). GFLs were included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor exposure time was 20 minutes. An siRNA designed as a scramble for APE1 was used as an siRNA transfection control. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no GFL condition using an ANOVA with Dunnett's post-hoc test ($p<0.05$). Ampersands (@) indicate statistically significant differences between the GFL treatment and the siRNA treated condition using a t-test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

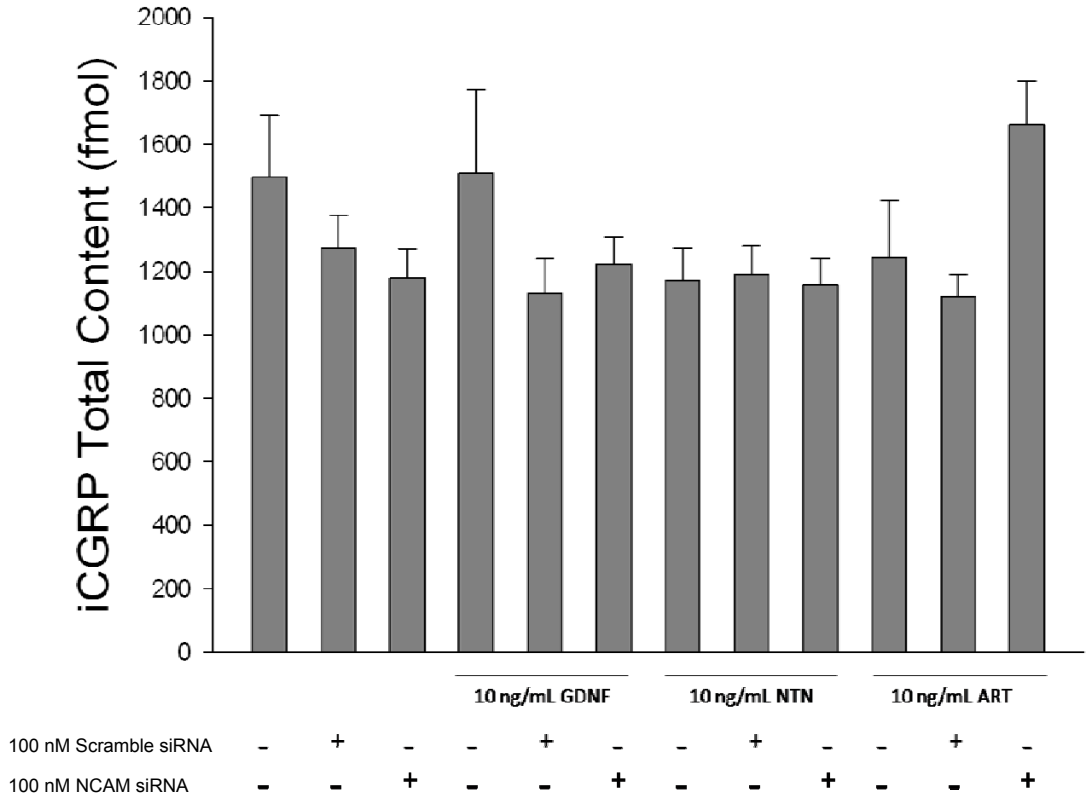

Figure 37. GFLs, scramble siRNA, and NCAM siRNA do not change total content of iCGRP in DRG. Exposure of DRG to 10 ng/mL GFLs, scramble siRNA (100 nM), and Ret siRNA (100 nM) did not change the total content of iCGRP, compared with an ANOVA. N = 12-18 wells per condition.

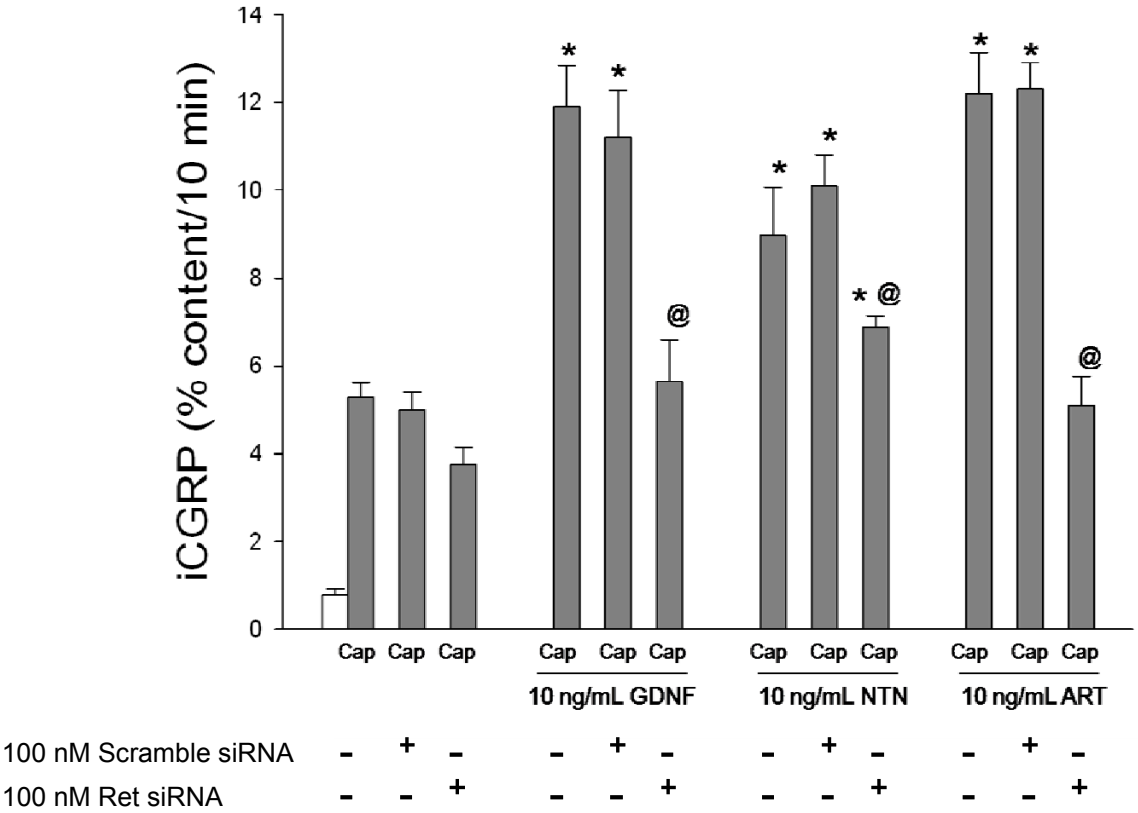

Figure 38. ART-induced enhancement in the stimulated-release of iCGRP is mediated by Ret-dependent and NCAM-dependent pathways. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bar) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 12-18 wells per condition). GFLs were included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor exposure time was 20 minutes. An siRNA designed as a scramble for APE1 was used as an siRNA transfection control. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no GFL condition using an ANOVA with Dunnett's post-hoc test ($p<0.05$). Ampersands (@) indicate statistically significant differences between the GFL treatment and the siRNA treated condition using a t-test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

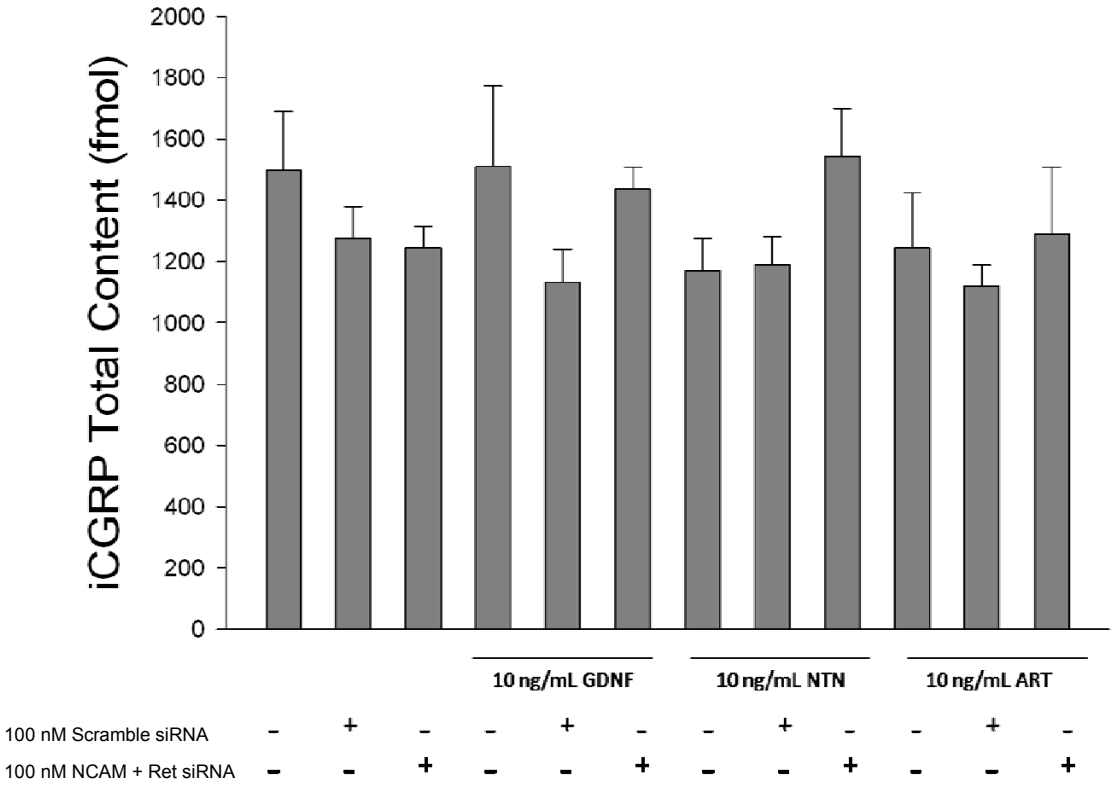

Figure 39. GFLs, scramble siRNA, and NCAM + Ret siRNA do not change total content of iCGRP in DRG. Exposure of DRG to 10 ng/mL GFLs, scramble siRNA, (100 nM) and NCAM + Ret siRNA (100 nM) did not change the total content of iCGRP, compared with an ANOVA. N = 12-18 wells per condition.

presence of GFLs. GDNF-induced sensitization was abolished with this siRNA treatment regimen, presumably because the enhancement in stimulated-release of iCGRP accomplished by GDNF is Ret-dependent (Figures 38 and 33). The ART-induced sensitization, while not eliminated by Ret siRNA treatment alone, was completely abolished by NCAM and Ret siRNA treatment in combination (Figure 38). The total content of iCGRP was not affected by these manipulations (Figure 39). NTN-induced sensitization was not prevented by NCAM and Ret siRNA treatment in combination. However, the absolute level of enhancement in stimulated-release of iCGRP in response to NTN was significantly lower than in the absence of the treatment of the two siRNA in combination (Figure 38).

To further evaluate the role of the NCAM-initiated signaling cascade, manipulations of Fyn kinase were conducted. Since there are no commercially available specific pharmacological inhibitors of Fyn, Fyn siRNA (100 nM) was used to reduce the level of Fyn in the DRG. Fyn siRNA treatment reduced Fyn levels by ~80% compared to non-treated DRG and DRG treated with scramble siRNA (Figure 40). There was no difference in Fyn levels between non-treated and scramble siRNA treated DRG (Figure 40), and Fyn siRNA did not affect the level of the other SFK, c-Src (Figure 41).

The ability of Fyn siRNA to alter the GFL-induced sensitization was then evaluated using the CGRP release assay. When the DRG cultures were treated with Fyn siRNA, the release profile mimicked the NCAM siRNA treatment profile. GDNF-induced sensitization was not affected, while NTN and ART-induced sensitization were still present, but the absolute amount of NTN and ART responsive enhancement of stimulated-release of iCGRP was reduced (Figure 42). When the DRGs were treated with both Ret siRNA and Fyn siRNA, the ART-induced sensitization was abolished, while the NTN-induced sensitization was still present (but the absolute amount of NTN responsive enhancement of stimulated-release of iCGRP was reduced; Figure 44). The

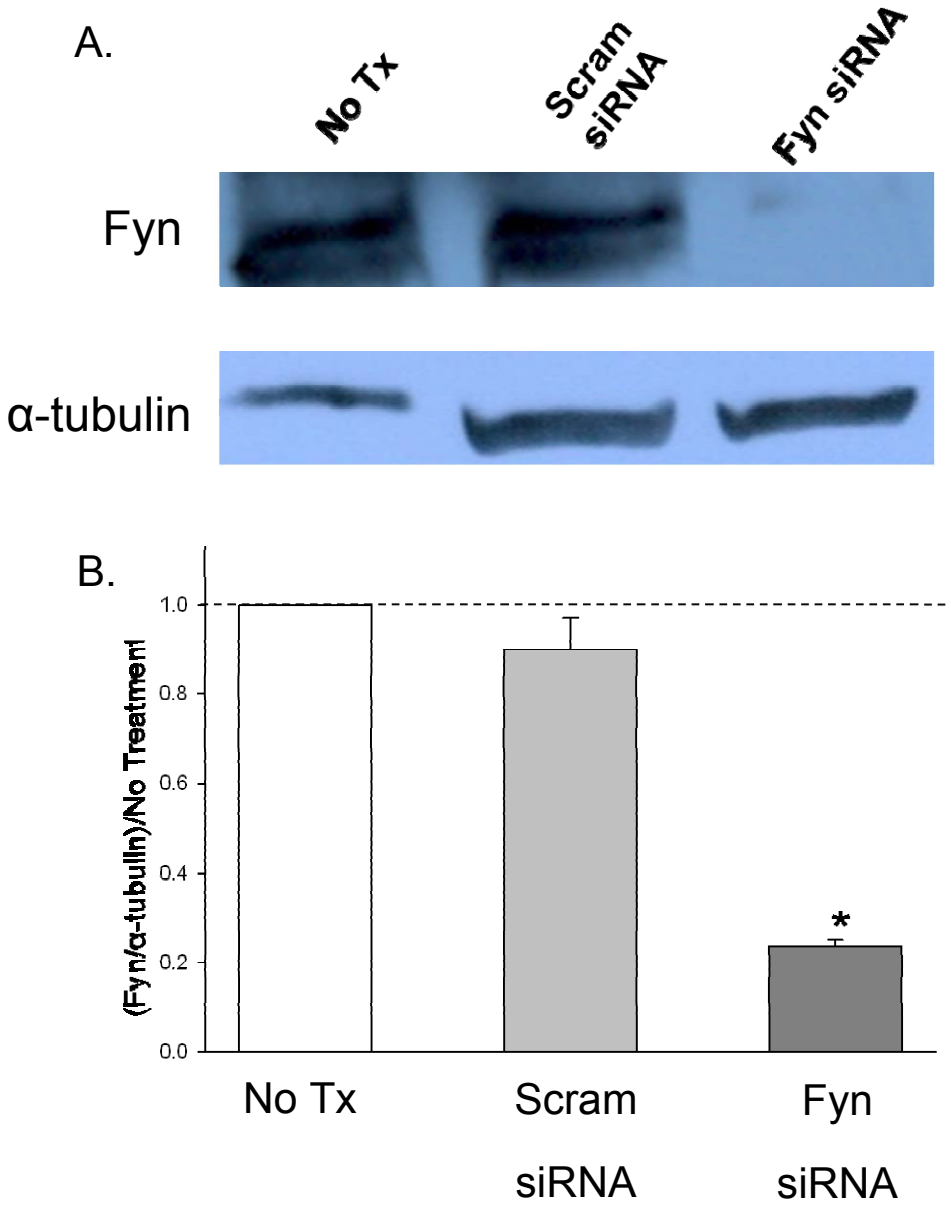

Figure 40. Fyn siRNA reduces levels of Fyn in DRG. A) This representative Western blot demonstrates that exposure of DRG to Fyn siRNA decreases Fyn levels, while scramble siRNA does not change Fyn levels. B) Densitometric analysis of three separate Western blots like that in A probing for Fyn. The level of Fyn was divided by α-tubulin levels and normalized to the no treatment condition. Asterisk (*) indicates statistically significant differences between treatment conditions and the no treatment condition using an ANOVA with Dunnett's post hoc testing ($p<0.05$). N = 3.

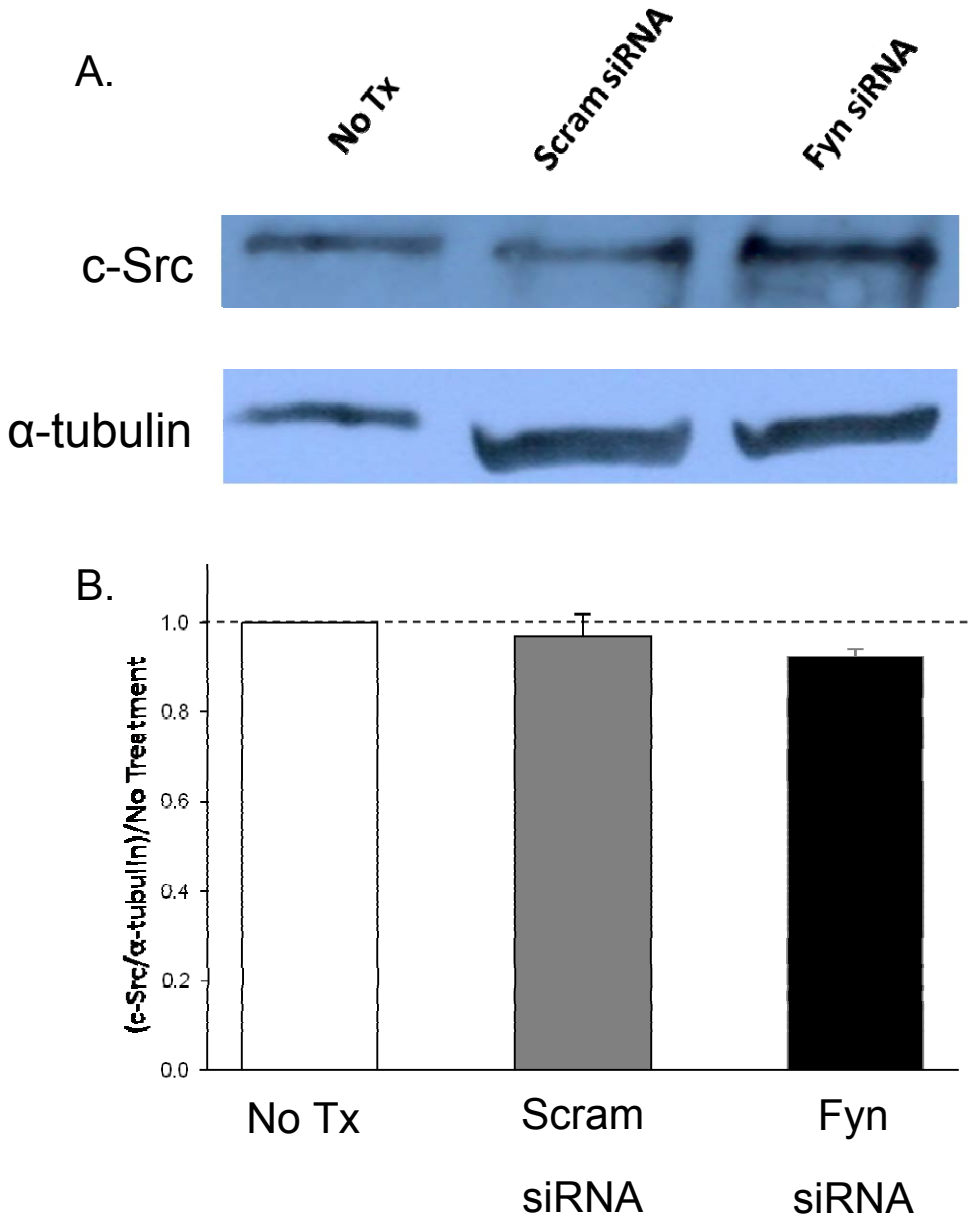

Figure 41. Fyn siRNA does not affect c-Src in DRG. A) This representative Western blot demonstrates that exposure of DRG to Fyn siRNA (100 nM) and scramble siRNA (100 nM) do not change c-Src levels. B) Densitometric analysis of three separate Western blots like that in A probing for c-Src. The level of c-Src was divided by α-tubulin levels and normalized to the no treatment condition. N =3.

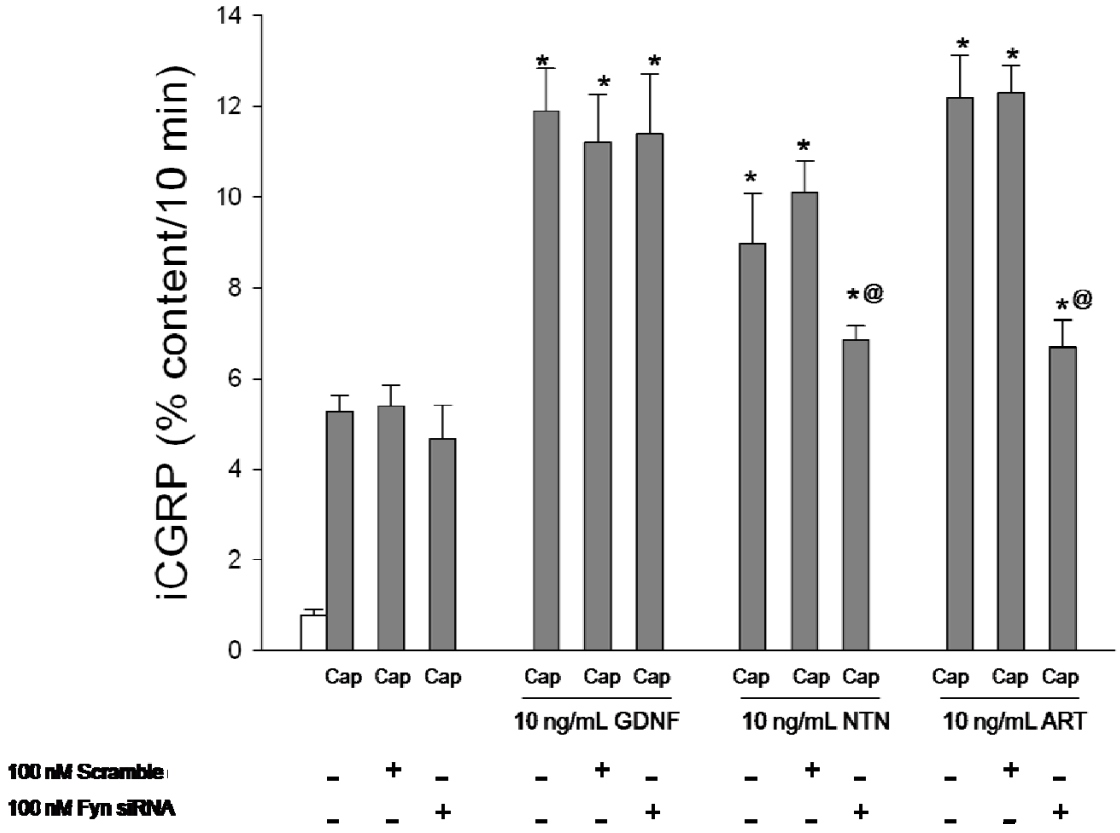

Figure 42. ART-induced enhancement in the stimulated-release of iCGRP is mediated, in part, by Fyn-dependent pathways. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bar) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 12-18 wells per condition). GFLs were included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor exposure time was 20 minutes. An siRNA designed as a scramble for APE1 was used as an siRNA transfection control. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no GFL condition using an ANOVA with Dunnett's post-hoc test ($p<0.05$). Ampersands (@) indicate statistically significant differences between the GFL treatment and the siRNA treated condition using a t-test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

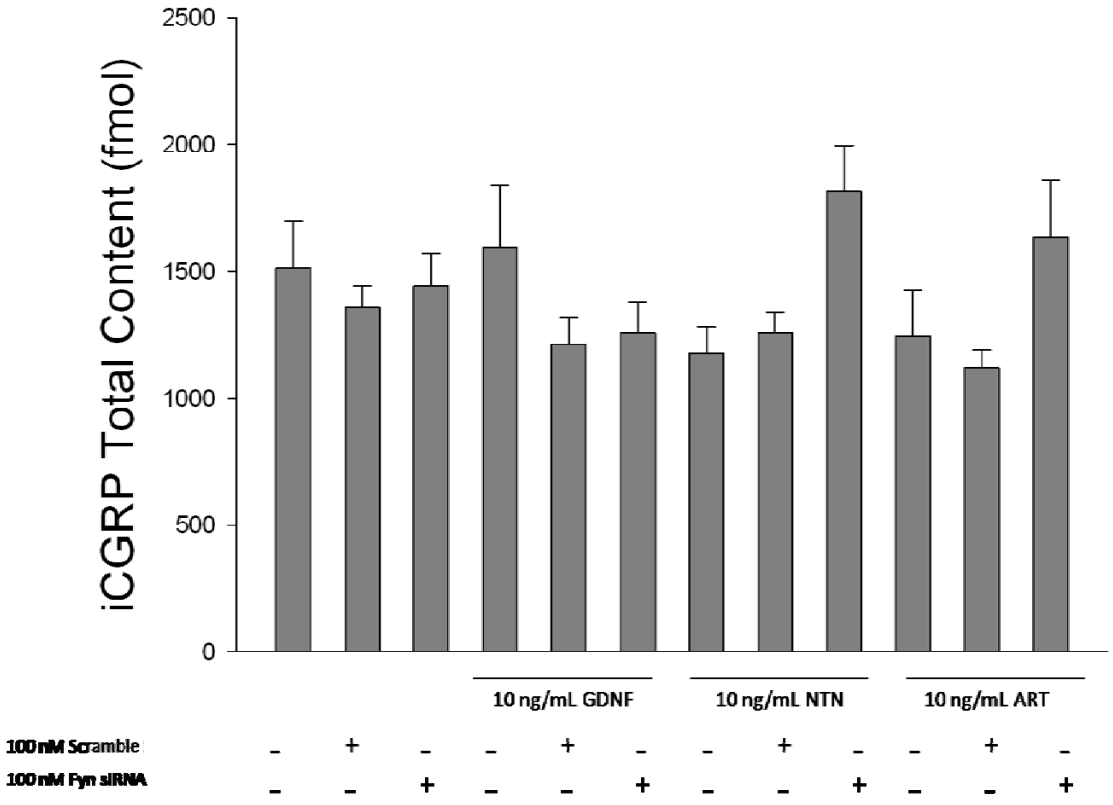

Figure 43. GFLs, scramble siRNA, and Fyn siRNA do not change total content of iCGRP in DRG. Exposure of DRG to 10 ng/mL GFLs, scramble siRNA (100 nM), and Fyn siRNA (100 nM) did not change the total content of iCGRP, compared with an ANOVA. N = 12-18 wells per condition.

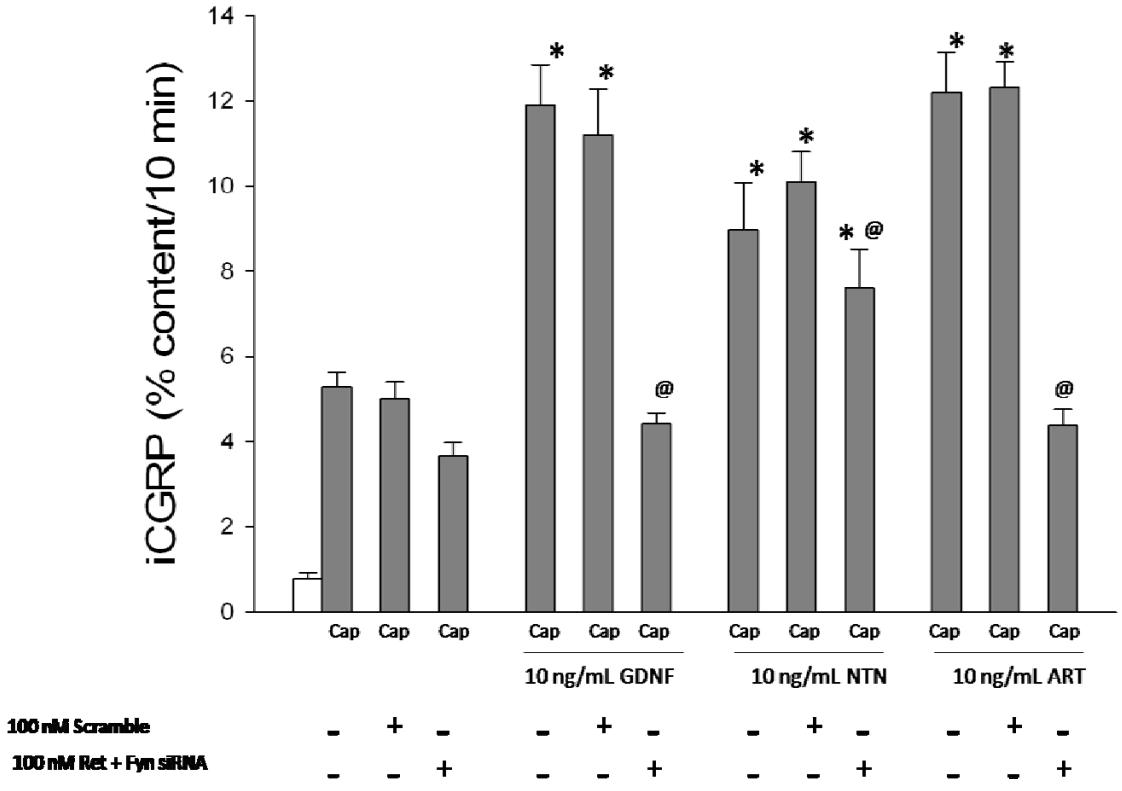

Figure 44. ART-induced enhancement in the stimulated-release of iCGRP is mediated by Ret-dependent and Fyn-dependent pathways. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bar) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 12-18 wells per condition). GFLs were included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor exposure time was 20 minutes. An siRNA designed as a scramble for APE1 was used as an siRNA transfection control. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no GFL condition using an ANOVA with Dunnett's post-hoc test ($p<0.05$). Ampersands (@) indicate statistically significant differences between the GFL treatment and the siRNA treated condition using a t-test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

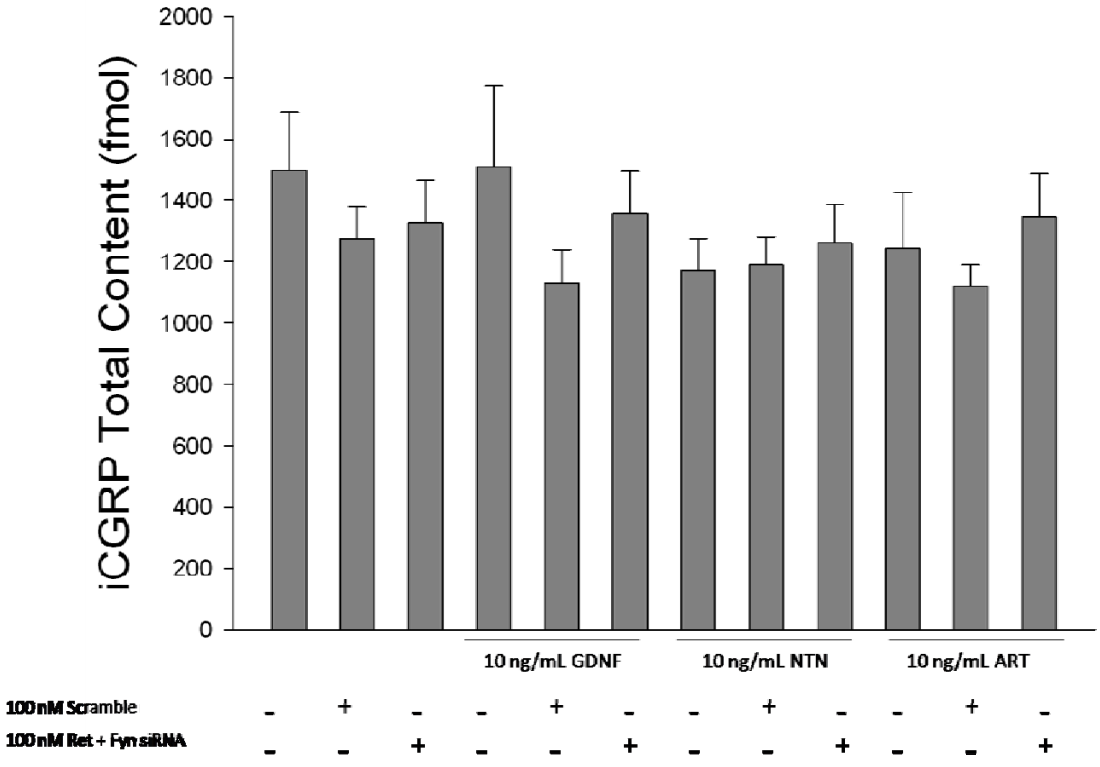

Figure 45. GFLs, scramble siRNA, and Ret + Fyn siRNA do not change total content of iCGRP in DRG. Exposure of DRG to 10 ng/mL GFLs, scramble siRNA (100 nM), and Ret + Fyn siRNA (100 nM) did not change the total content of iCGRP, compared with an ANOVA.

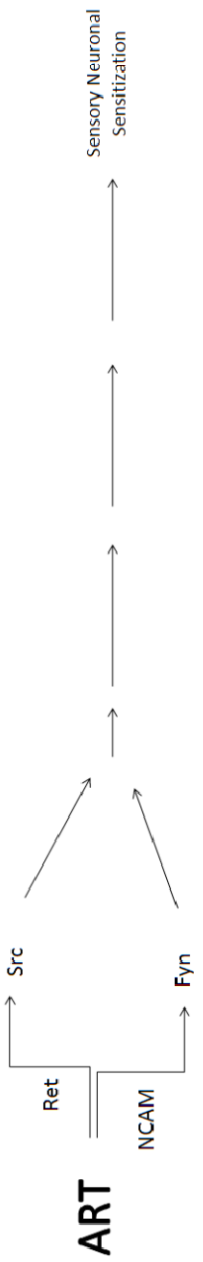

Figure 46. Schematic of working model of the pathway of sensitization by ART. ART uses Ret-dependent and NCAM-dependent signaling pathways to accomplish its sensitization through Src and Fyn respectively.

total content of iCGRP was not affected by these manipulations (Figures 43 and 45). Together, this data indicate that ART-induced sensitization is accomplished in a Ret-dependent manner as well as a Ret-independent manner through activation of the NCAM receptor and subsequent initiation of the Fyn kinase pathway (represented in schematic form in Figure 46).

c. NTN-induced enhancement in stimulated-release of iCGRP is mediated by Ret-dependent, NCAM-dependent, and Integrin β-1-dependent mechanisms

Finally, the role of the other receptor reported to be a binding partner of the GFL-GFRα complex, Integrin β-1, was determined (Cao et al., 2008b). There is no pharmacological inhibitor of Integrin β-1, so again a pool of siRNA molecules (100 nM; Table 1) directed at this receptor was used in order to inhibit its function. Integrin β-1 is a cell adhesion molecule (De Strooper et al., 1989), similar to NCAM. It is part of a larger complex of Integrins (De Strooper et al., 1989). While there is no current evidence in the literature of a functional connection between Integrin β-1 and GFLs, the fact that these two sets of molecules bind is intriguing in terms of this receptor's ability to promote GFL function. Therefore, the role of Integrin β-1 in GFL-induced sensory neuronal sensitization was evaluated. This receptor may play a role in NTN-induced sensitization, since inhibition of both Ret and NCAM abolished the GDNF and ART-induced sensitization, but not the NTN-induced sensitization (Figure 38).

The efficiency of inhibition of Integrin β-1 by the pool of siRNA was verified with a Western blot probing for the Integrin β-1 intracellular fragment, which has a molecular weight of 130 kDa and is the direct signaling portion of the molecule (Mocsai et al., 2002). Figure 47 A is a representative Western blot of Integrin β-1 levels after treatment of DRG with standard media, scramble siRNA, or Integrin β-1 siRNA, which shows that Integrin β-1 siRNA reduces the level of this receptor by ~90%. The average of three independent blots indicates a knock down of Integrin β-1 by ~72% (Figure 47 B).

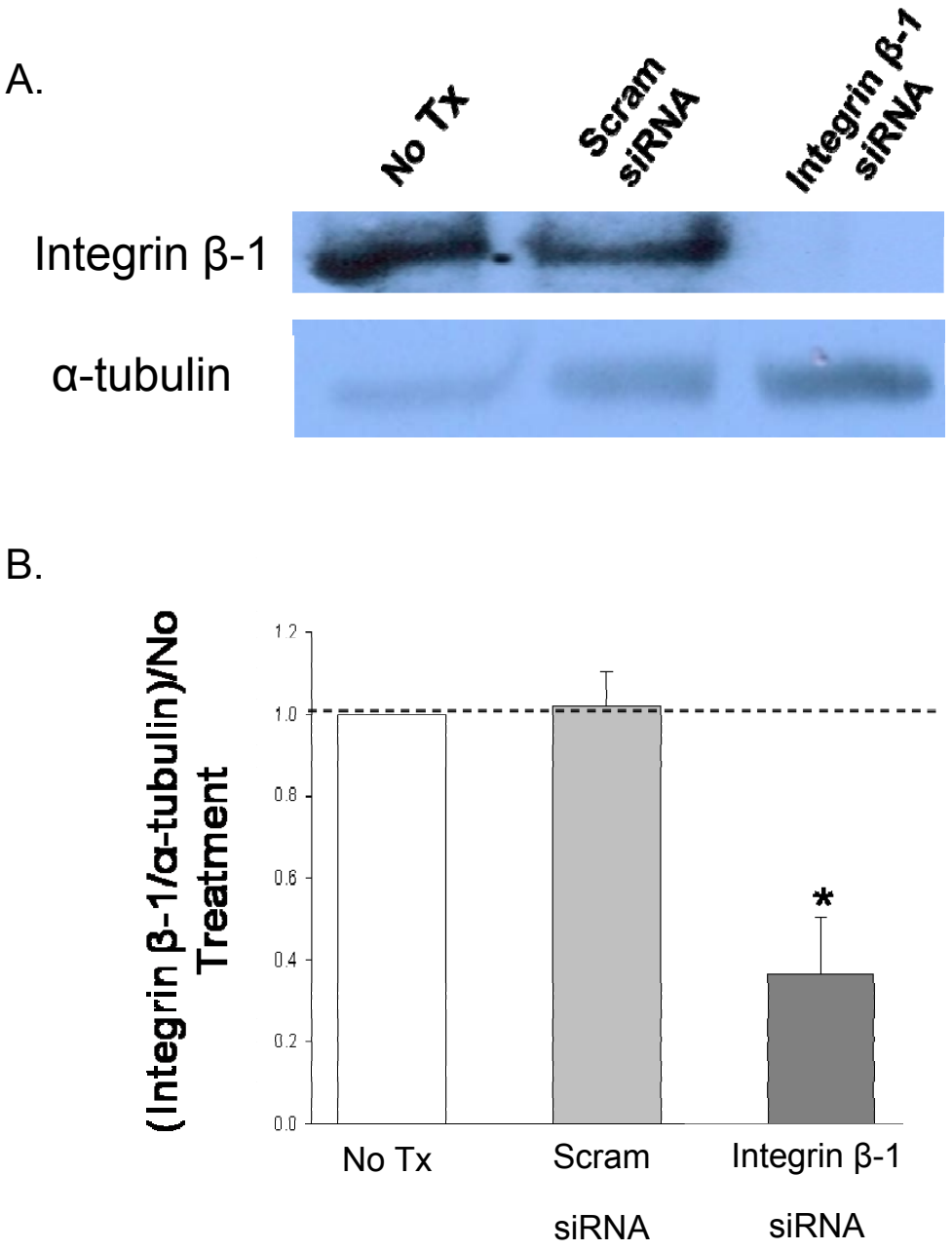

Figure 47. Integrin β-1 siRNA reduces levels of Integrin β-1 in DRG. A) This representative Western blot demonstrates that exposure of DRG to Integrin β-1 siRNA decreases Integrin β-1 levels, while scramble siRNA does not change Integrin β-1 levels. B) Densitometric analysis of three separate Western blots like that in A probing for Integrin β-1. The level of Integrin β-1 was divided by α-tubulin levels and normalized to the no treatment condition. Asterisk (*) indicates statistically significant differences between treatment conditions and the no treatment condition using an ANOVA with Dunnett's post hoc testing ($p<0.05$). N = 3.

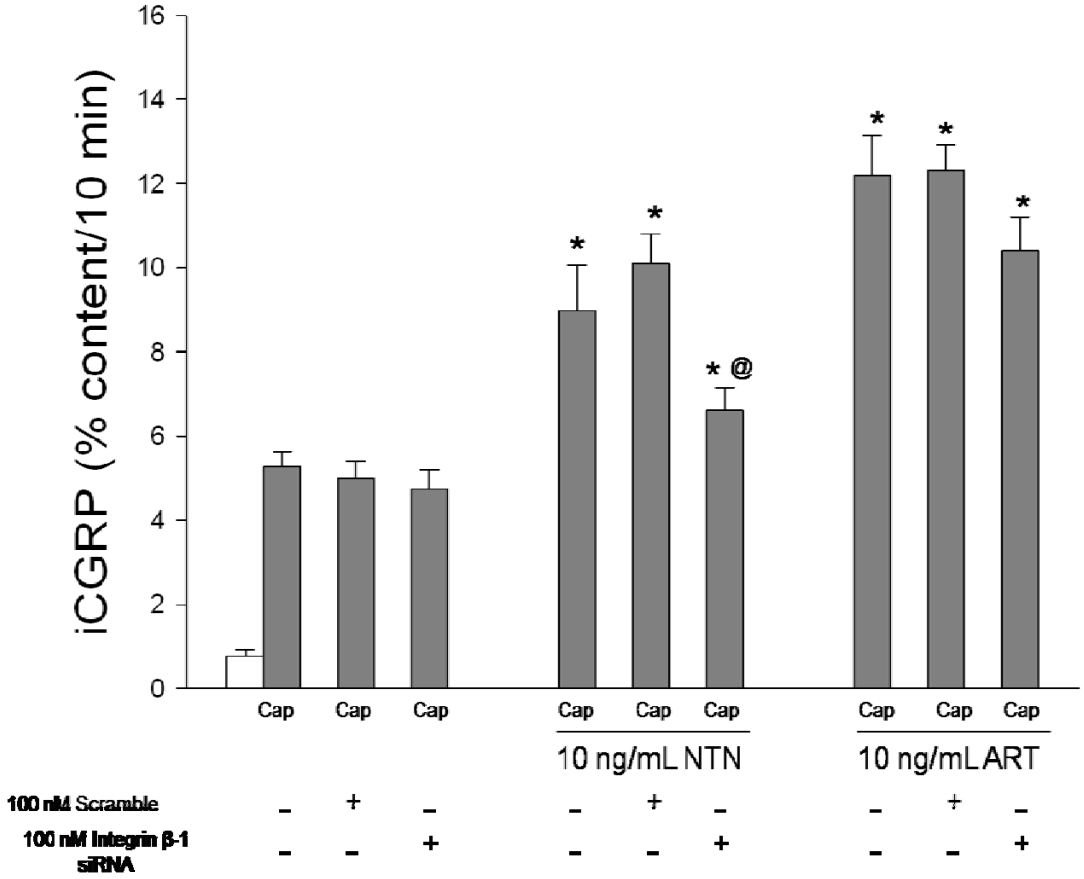

Figure 48. NTN-induced enhancement in the stimulated-release of iCGRP is mediated, in part, by Integrin β-1-dependent pathway. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bar) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 12-18 wells per condition). GFLs were included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor exposure time was 20 minutes. An siRNA designed as a scramble for APE1 was used as an siRNA transfection control. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no GFL condition using an ANOVA with Dunnett's post-hoc test ($p<0.05$). Ampersands (@) indicate statistically significant differences between the GFL treatment and the siRNA treated condition using a t-test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

Having established the ability of this pool of siRNA molecules to knock down Integrin β1, this inhibition technique was used to evaluate the role of this receptor in NTN-induced sensitization. When Integrin β-1 siRNA was added to DRG cultures, the NTN-induced sensitization remained, although the absolute level of enhancement in stimulated-release of iCGRP was reduced (Figure 48). Integrin β-1 siRNA did not affect the ART-induced sensitization. The total content of iCGRP was not affected by these manipulations (Figure 49). This data indicates that Integrin β-1 plays a role in NTN-induced sensitization, but that it is not the only mechanism by which NTN induces sensitization.

An alternative possibility is that the ~30% of Integrin β-1 remaining after siRNA treatment is sufficient to sustain NTN-induced sensory neuronal sensitization. To determine whether NTN is able to sensitize sensory neurons through Integrin β-1 only or if NTN can initiate sensitization through a combination of all of the receptors tested thus far, the basal and stimulated-release of iCGRP was measured from DRG after exposure to siRNA directed at Ret, NCAM, and Integrin β-1. In this case, DRG were transfected with all three siRNA (33 nM each) on day 2, 4, and 6 after plating. This treatment regimen was followed to ensure the total amount of siRNA present in the culture media was consistent (100 nM). When all three pools of siRNA were added to the DRG in culture, the basal-release of iCGRP was not affected while the NTN-induced sensitization of stimulated-release was abolished (Figure 50). The total content of iCGRP was not affected by these manipulations (Figure 51). With this set of data, it is now clear that NTN induces sensitization through several pathways. There is a Ret-dependent component, which contributes to about half of the NTN-induced enhancement in the capsaicin stimulated-release of iCRGP. NCAM-dependent and Integrin β-1-dependent activation account for approximately one fourth of the NTN-induced enhancement in the capsaicin stimulated-release of iCGRP. However, it is

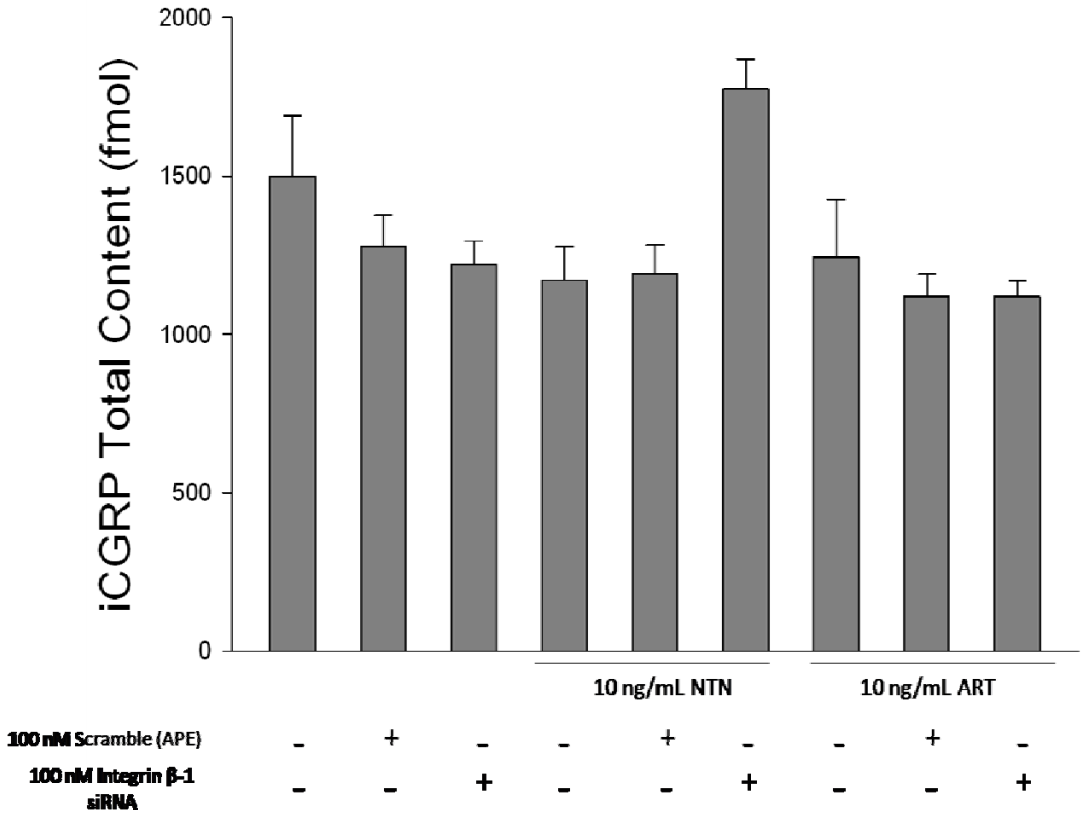

Figure 49. GFLs, scramble siRNA, and Integrin β-1 siRNA do not change total content of iCGRP in DRG. Exposure of DRG to 10 ng/mL GFLs, scramble siRNA (100 nM), and Integrin β-1 siRNA (100 nM) did not change the total content of iCGRP, compared with an ANOVA. N = 12-18 wells per condition.

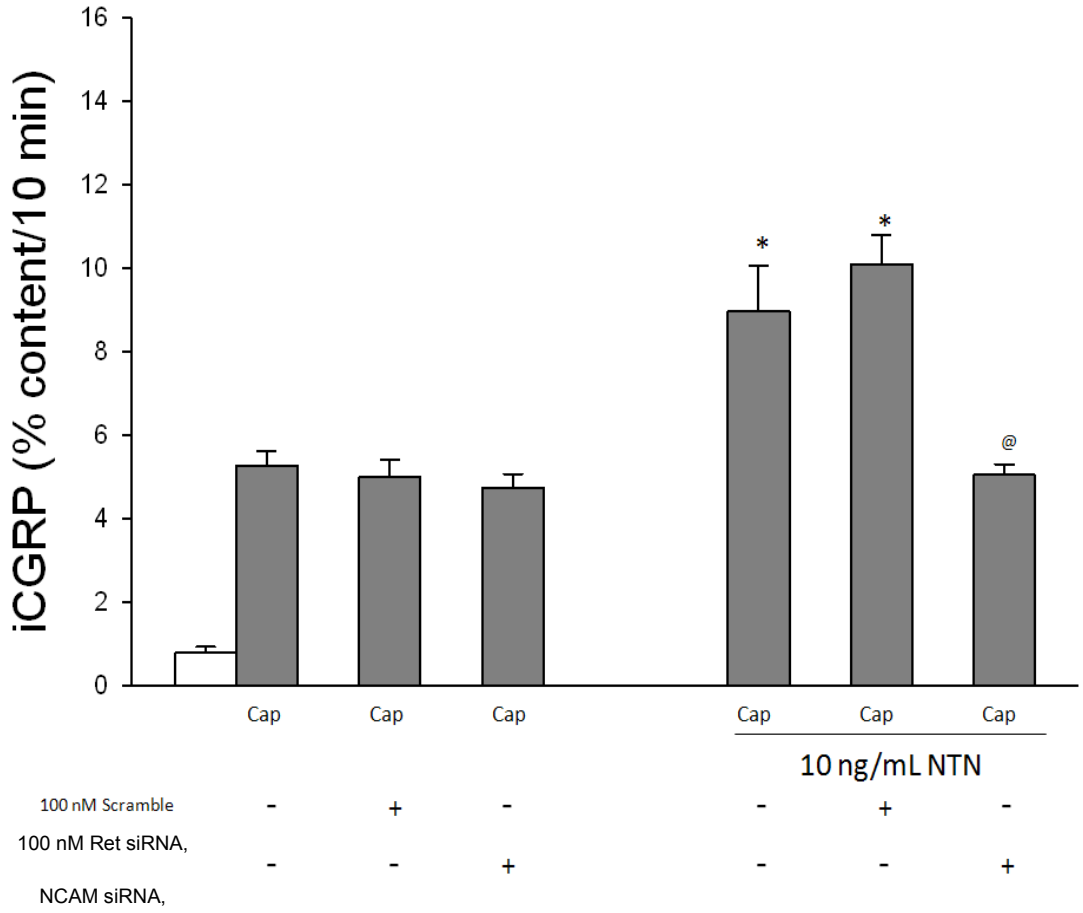

Figure 50. NTN-induced enhancement in the stimulated-release of iCGRP is mediated by Ret-dependent, NCAM-dependent, and Integrin β-1-dependent pathways. Peptide release elicited by a 10 minute exposure to Hepes buffer alone (open bar) or Hepes buffer containing 50 nM capsaicin (Cap; dark bars) is expressed as mean percent total peptide content of cells in each well ± SEM (n = 12-18 wells per condition). GFLs were included in the 10 minutes prior to and throughout capsaicin exposure. Total growth factor exposure time was 20 minutes. An siRNA designed as a scramble for APE1 was used as an siRNA transfection control. Asterisks (*) indicate statistically significant differences in iCGRP release between treatment groups and the no GFL condition using an ANOVA with Dunnett's post-hoc test ($p<0.05$). Ampersands (@) indicate statistically significant differences between the GFL treatment and the siRNA treated condition using a t-test ($p<0.05$). In all cases, release stimulated by capsaicin was significantly higher than basal release.

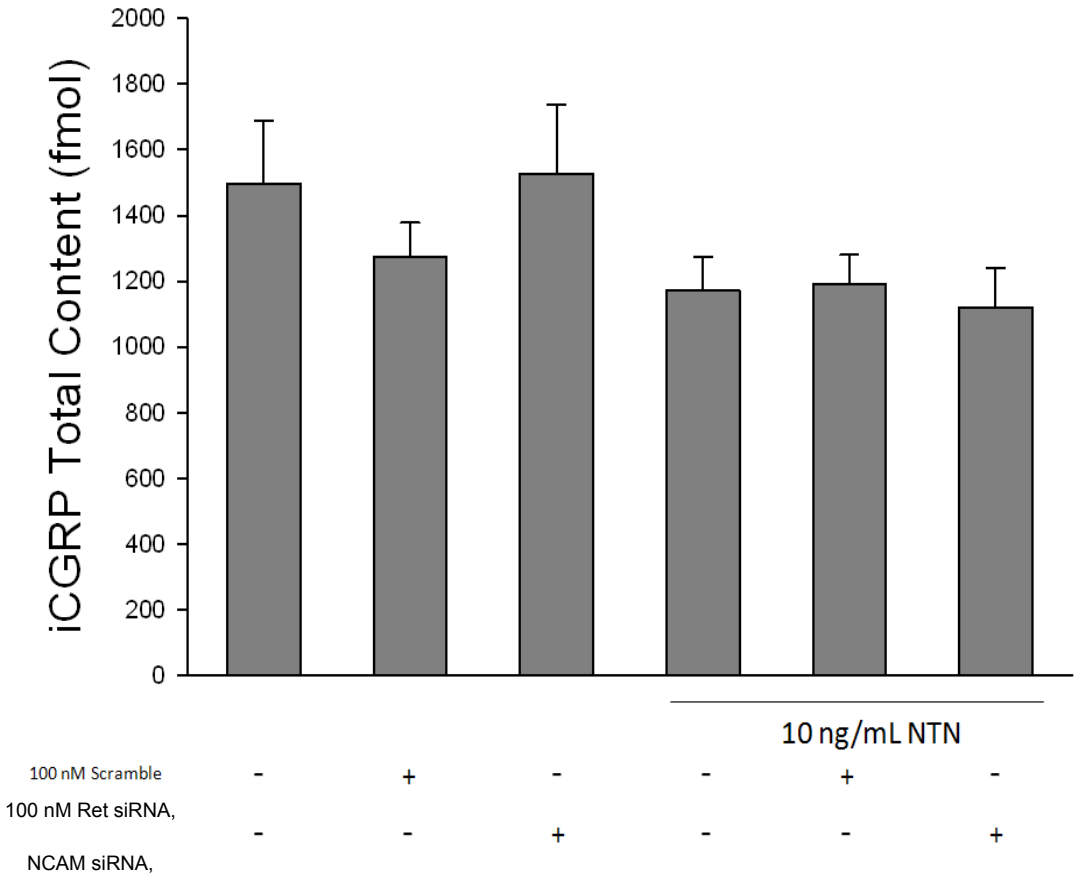

Figure 51. NTN, scramble siRNA, and Ret, NCAM, and Integrin β-1 siRNAs do not change total content of iCGRP in DRG. Exposure of DRG to 10 ng/mL GFLs, scramble siRNA (100 nM), and Ret, NCAM, and Integrin β-1 siRNA (100 nM) did not change the total content of iCGRP, compared with an ANOVA. N = 12-18 wells per condition.

necessary to inhibit all three of these pathways to eliminate NTN-induced sensitization, which is a novel observation for the mechanism of the GFL-induced actions on sensory neurons. Whether Integrin β-1 affects the expression of the GFRαs is unknown, but this could affect the interpretation of the data above.

3. The distinct Ret-dependent and Ret-independent pathways of GDNF, NTN, and ART-induced sensitization

The experiments detailed above demonstrate that each of the GFLs have distinct, though overlapping, compliments of signaling initiation pathways for the induction of sensory neuronal sensitization. GDNF accomplishes its sensitization in a Ret-dependent manner through the MAPK/Erk 1/2 pathway. NTN accomplishes its sensitization through the PI-3K pathway in both a Ret-dependent manner and a Ret-independent manner via the NCAM and Integrin β-1 receptors. ART induces sensitization in a Ret-dependent and Ret-independent manner, via the NCAM receptor. Actions of ART may be mediated through PKCε activation (Dr. Weiguo Zhu, personal communication). The pathways of sensitization by each of the GFLs are represented schematically in Figure 52.

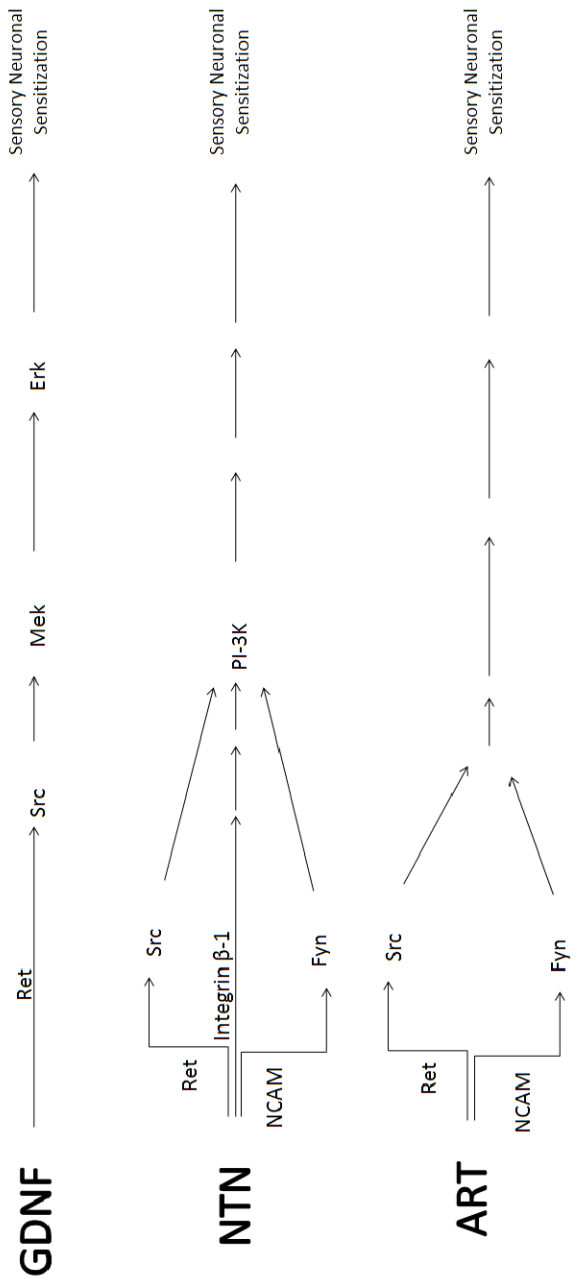

Figure 52. Schematic of working model of the pathways of sensitization by GDNF, NTN, and ART.

IV. Discussion

The work presented here demonstrates that the GFLs, GDNF, NTN, and ART, enhanced the release of the neuropeptide CGRP from adult sensory neurons grown in culture and freshly dissociated spinal cord tissue. These GFLs did not alter the total content of CGRP in the neurons, nor did PSP, the other molecule in this family, have any effect upon the release of CGRP from sensory neurons. Additionally, each of the GFLs uses different and distinct sets of intracellular signaling pathways to accomplish sensitization. GDNF, through the Ret receptor, activated the MAPK/Erk 1/2 pathway, and pharmacological inhibition of this pathway with PD98059 and U0126 prevented the GDNF-induced sensitization. NTN activated both the MAPK/Erk 1/2 and PI-3K pathways. However, NTN-induced sensitization was abolished only by the PI-3K inhibitor, LY294002, not by the MAPK inhibitors listed above. ART-induced sensitization was neither MAPK nor PI-3K dependent, despite the fact that ART activated both of these pathways. Intriguingly, inhibition of the receptor tyrosine kinase, Ret, eliminated GDNF-induced sensitization, but not NTN or ART-induced sensitization. ART-induced sensitization was abolished by Ret and NCAM inhibition, while NTN-induced sensitization was only prevented when Ret, NCAM, and Integrin β-1 were all inhibited. These data show the involvement of Ret-dependent and Ret-independent pathways in the sensitization of sensory neurons by the GFLs and the role of distinct compliments of intracellular signaling pathways for each of the GFLs (schematically represented in Figure 52).

A. Characterization of radioimmunoassay (RIA) and effects of GFLs on the RIA

The majority of the studies in this manuscript consisted of results from radioiummunoassays to measure changes in release of iCGRP. For this reason, it was necessary to extensively characterize the RIA in terms of criteria required for a successful release, as well as to ensure that the GFLs did not disrupt the RIA. This

characterization was necessary to eliminate the possibility that results seen in studies conducted were due to some artifact of the RIA.

The criterion set for a valid and successful release included: 1) values of iCGRP in the basal conditions had to be above the level of detection of the RIA and greater than 5 fmol, 2) stimulated values had to be at least twice the initial basal values, and 3) total content values of iCGRP had to be within 2 standard deviations of the mean (1312.98 ± 494.32 fmol; Section III.B.). All data that did not meet these criteria, while very few, was discarded. These criteria were established to ensure that the data was reliable and reproducible. The rationale for each criterion is listed below.

First, in order to compare stimulated values to basal or resting values of iCGRP released, the basal values have to be present. There are three primary reasons these values could be absent. The cells may not be healthy, and therefore, not producing or releasing the normal amount of iCGRP. There are many components of the culture media that could account for this problem. However, the most likely explanation is that the NGF added to the culture media has lost its potency, since NGF is necessary for increased transcriptional production of CGRP (Sango et al., 1994;Watson et al., 1995;Xu and Hall, 2007). The NGF can become less potent due to the labile nature of the protein (Eng et al., 1997), or it may be that the batch of NGF was inherently compromised. In either case, less potent NGF can lead basal values to be absent, resulting in data being discarded.

Next, doubling of iCGRP release was necessary for the stimulated-release to be considered adequate. The concentrations of capsaicin and potassium used in the experiments in this thesis have been well characterized as stimuli that at least double the release of iCGRP (Hingtgen and Vasko, 1994b;Hingtgen and Vasko, 1994a;Hingtgen et al., 1995;Hingtgen et al., 2006). In experiments where this doubling did not occur, the data set was discarded. Again, one primary reason this doubling may

not occur is lower potency NGF, since NGF is responsible for the increased transcription and insertion in the cell membrane of the TRPV1 channel (Stein et al., 2006;Xue et al., 2007). Another possibility is that the stimulus itself, either capsaicin or potassium, has been compromised. Finally, low concentration or an absence of calcium in the Hepes buffer could be responsible for a lack of iCGRP release. Whatever the underlying reason for as lack of doubling of iCGRP in response to the stimulus, the data was discarded. There were three wells exposed to control conditions in each experiment, and if these three wells did not meet the criteria for a valid release, the entire experiment was considered invalid.

Finally, while the density of the sensory neurons per well were controlled prior to plating (see Section II.D.), the variability in healthy, viable cells in each well can be substantial. For this reason, most of the CGRP release studies conducted were reported as iCGRP released as a percent of total content of iCGRP. A 95% confidence interval was established with the use of 161 individual samples, and all content values that did not fall within this range (324.34 fmol to 2301.62 fmol) were discarded. This criterion allowed more reliable comparison between experiments conducted by different scientists across long periods of time, if necessary. These criteria will continue to be used in the Hingtgen laboratory.

Additionally, the GFLs, even at the highest concentrations used in release experiments, did not alter the RIA. A protein-antibody interaction between endogenous CGRP and the anti-CGRP antibody is necessary for the RIA to function properly. Other proteins present in the buffer can disrupt this interaction, thereby interfering with the ability to detect the level of iCGRP using the RIA (Sheffield et al., 1977b). I was able to determine that none of the GFLs were disrupting the standard curve of the RIA, which indicates the results of the experiments are valid and not an artifact of GFL disruption of

CGRP-antibody interactions. This observation was critical in the interpretation of the results in this thesis.

The RIA has one distinct advantage compared to other methods of measuring the release of iCGRP (i.e. ELISA). The RIA is more sensitive than the ELISA, which allows for easier and more quantifiable changes as well as comparisons of small changes in release of iCGRP (Koskela and Leinonen, 1981). Therefore, this fact in combination with the expertise using this technique in the Hingtgen laboratory, is the impetus for the use of an RIA for all studies evaluating the release of iCGRP.

B. GFL-induced enhancement in the capsaicin-stimulated release of iCGRP

Initial experiments showed that addition of the GFLs at the highest concentrations used for sensitization experiments did not themselves stimulate the release of iCGRP (Table 4). This observation is important in the interpretation of the subsequent data indicating that GFLs induce sensitization. Other known sensitizers of sensory neurons, PGE_2 and PGI_2, can themselves stimulate sensory neurons at high concentrations (Hingtgen and Vasko, 1994a;Hingtgen et al., 1995). Having established that GFLs are not stimulators of sensory neurons, the sensitizing role of these molecules could be evaluated. At the time of this thesis, there was little evidence in the literature of GFL-induced sensory neuronal sensitization, and the work in this thesis is the first to evaluate the actions of GFLs on neurotransmitter release in sensory neurons.

As seen in Figure 3, GDNF increased the stimulated-release of iCGRP to comparable levels as NGF, a well-established sensitizer of sensory neurons. This data provides evidence that GFLs are another set of growth factors, similar to NGF, that have potent sensitizing effects on sensory neurons. Once this phenomenon had been established, a concentration-response curve of GDNF sensory neuronal sensitization was conducted (Figure 4). Although this was not a complete concentration-response, it did allow selection of a concentration of GDNF that maximally sensitized stimulated

release of iCGRP, and this concentration was used in proceeding experiments with GDNF and the other GFLs. Interestingly, application of 1.0 ng/mL and 10 ng/mL GDNF resulted in maximal sensitization, while 100 ng/mL GDNF did not sensitize the sensory neurons. Other studies have indicated a similar profile of the actions of the GFLs in similar systems (Price et al., 2005). Specifically, 10 ng/mL GDNF increased the content of CGRP and the capsaicin stimulated-release of CGRP from TRPV1 positive neurons in the trigeminal ganglia (TG), while 100 ng/mL GDNF did not induce these effects. In this study, 1.0 ng/mL GDNF increased CGRP content and capsaicin-stimulated release, but to a lesser extent than 10 ng/mL GDNF. There are three possible explanations for this loss of sensitization at 100 ng/mL GDNF. First, high levels of GDNF (100 ng/mL) may activate compensatory intracellular signaling pathways in the sensory neurons, which may work to decrease the amount of p-Erk or other molecules in the MAPK/Erk 1/2 pathway and prevent sensitization. Secondly, 100 ng/mL GDNF may be a high enough concentration of GDNF to bind to GFRα-2, the receptor for NTN, and activate additional compensatory pathways, such as the PI-3K pathway (see Section I.C.3 for a description of the non-specific binding of GFLs to GFRα receptors). In neurons responsive to GDNF, which may be different than neurons responsive to NTN, PI-3K activation may inhibit sensitization. Finally, GDNF may be shifting the capsaicin concentration-response curve leftward. Capsaicin exhibits a concentration-response curve in the shape of an inverted U, where very high concentrations of capsaicin actually have no effect or even decrease the response (Dray et al., 1989;Wood et al., 1988). GDNF at higher concentrations may shift this curve, resulting in lower concentrations of capsaicin (50 nM) inducing responses typically seen at much higher concentrations (500 nM). While not tested, this could be easily examined in the future by using incremental concentrations of capsaicin and GFLs and constructing multiple concentration-response curves and performing a probit analysis.

After determination of the ability of GDNF to sensitize sensory neurons and the concentrations at which this sensitization occurs, the other GFLs were examined at similar concentrations. NTN and ART enhanced the capsaicin stimulated-release of iCGRP to comparable levels as GDNF when added at a concentration of 10 ng/mL. These data demonstrate that GFLs are sensitizers of sensory neurons. However, the possibility remained that the total exposure time (20 minutes) of the sensory neurons to GFLs may have changed the content of iCGRP in the sensory neurons. I eliminated this possibility by comparing the content of iCGRP in the no treatment and GFL present conditions, and no difference existed between these conditions.

While capsaicin induces activation of small-diameter sensory neurons through TRPV1 (Chard et al., 1995;Hiura and Ishizuka, 1989), there are several other stimuli that induce activation of these neurons. High concentrations of potassium were used as a general depolarizing stimulus. Interestingly, the GFLs were not able to sensitize the potassium-evoked release of iCGRP in DRG neurons (Figures 6 and 7). A similar profile of NGF-induced sensitization of capsaicin-evoked release, but not general depolarization (in this case by electrical stimulation) stimulated-release, has been observed in rodent sensory neurons (Malcangio et al., 1997b) Taken together, these data strongly suggest that the GFLs modulate sensory neuronal sensitivity, at least in part, through modulation of TRPV1.

Many of the pathways activated by GFLs alter the phosphorylation profile of TRPV1 in such a way as to increase the sensitivity of this channel (Zhang et al., 2008). Some examples of these pathways are the MAPK-Erk 1/2 pathway, the PI-3K pathway, the SFK pathway, and the PKC pathway. Other investigators have found that the acute exposure of the DRG to GFLs enhance the calcium influx through TRPV1 in response to capsaicin (Malin et al., 2006). The modulation may be through specific changes in phosphorylation states of the channel (Dr. Weiguo Zhu, personal communication).

Essentially, GFLs activate the effector pathways mentioned above and phosphorylate specific residues on TRPV1. This phosphorylation changes the channel properties and/or kinetics so that it is more responsive to capsaicin and its endogenous activators, heat (above 42° C) and acidic shifts in pH. Therefore, TRPV1 allows influx of more sodium and calcium ions, which depolarizes the cell, activates other intracellular pathways important in sensory neuronal sensitization, and induces calcium-dependent neurotransmitter vesicle docking and release.

Not only do the GFLs modulate the responses of sensory neurons maintained in culture, but they also sensitize sensory neurons from freshly dissociated spinal cord tissue (Figure 10). The sensitization profile is similar in spinal cord tissue and DRG maintained in culture. The combination of these studies indicates that the responses seen in DRG in culture are not an artifact of this culture technique, since spinal cord slices are freshly dissociated and not exposed to the culture conditions. This similarity validates the DRG in culture as a model for sensory neuronal sensitization and allowed me to use the DRG culture system to undertake an in depth study of the mechanisms of GFL-induced sensitization. The fact that the GFLs were able to induce sensitization in the spinal cord tissue was also a novel observation. This GFL-induced sensitization is in contrast to NGF, which does not sensitize capsaicin stimulated-release in spinal cord tissue (Malcangio et al., 1997). This difference in sensitization profile could be a result of the presence of GFRα receptors on the central terminal of the primary sensory neurons and a lower level of expression or lack of TrkA on these terminals. Additionally, GFLs are released in the CNS by microglia and astrocytes in adult mammals (Sandhu et al., 2009), while NGF is released primarily by glial cells of the PNS (He et al., 2009).

PSP, the other molecule in the GDNF family, did not alter the responses of sensory neurons in culture to capsaicin (Figure 5 and Table 3). Since there is an abundance of evidence that the specific receptor for PSP, GFRα4, lacks the appropriate components

to initiate Ret autophosphorylation in adult mammalian sensory neurons (Lindahl et al., 2001) and induce signaling pathways, it is not surprising that PSP was unable to induce sensitization. This set of experiments supports the current theory of PSP as not critically important in the activity of fully developed sensory neurons (Lindahl et al., 2000). Even at a concentration of 500 ng/mL, well above the K_D of GFRα-4 for PSP, PSP has no sensitizing effects. A good positive control would have been to use a different pure activator of GFRα-4. However, no such molecule exists. Therefore, there is no available positive control for GFRα-4 activation. The data presented in this document, therefore, support the assertion that PSP does not have effects on sensory neurons through any of the GFRα subtypes.

The studies outlined in this thesis provide a mechanism for GFL-induced hyperalgesia (Bogen et al., 2008;Malin et al., 2006) associated with the increase in GFLs during inflammation (Hashimoto et al., 2005;Malin et al., 2006;von Boyen et al., 2006). These studies could also explain one mechanism by which hyperalgesia is induced by direct injection of the GFLs (Bogen et al., 2008;Malin et al., 2006). Specifically, GFLs released in increased amounts during inflammation may modulate TRPV1 and other aspects of sensory neuronal function. This modulation could allow these neurons to transmit information more easily (by releasing more of the nociceptive neuropeptide, CGRP, in response to a given stimulus). Increased release of CGRP would initiate neurogenic inflammation (Brain et al., 1985;Girgis et al., 1985)., which would intensify the responses of the primary sensory neurons. CGRP is also involved in the propagation of the nociceptive pathway (Ambalavanar et al., 2006), so increased release of CGRP could result in increased responses to a given noxious stimulus. Therefore, the behavioral responses that these sensory neurons mediate would be enhanced by increased stimulus-evoked release of CGRP.

C. NGF and GDNF in the culture media have different effects on sensory neurons

NGF and GDNF both have effects on DRG when the sensory neurons are exposed to these molecules in the culture media for several days. Some of the known effects are increases in CGRP content (MacLean et al., 1989;Price et al., 2005;Sango et al., 1994), increase in TRPV1 expression and insertion in the membrane (Stein et al., 2006;Xue et al., 2007), as well as modified morphology and increased trafficking of other channels and proteins important in sensory neuronal sensitivity (Anand et al., 2006;Kerr et al., 2001). While 30 ng/mL NGF in the culture media increased the absolute level of iCGRP released and the total content of iCGRP, the enhancement in the stimulated release of iCGRP as percent of total content in response to 10 ng/mL GDNF was not changed. Long-term exposure to NGF may change certain characteristics of the sensory neurons, but these do not alter the responses of these neurons to acute treatment with GDNF.

In contrast to growth in NGF, maintaining the DRG in 10 ng/mL GDNF increased stimulated release compared to cultures maintained in the absence of GDNF. For the cultures grown in GDNF, acute exposure to GDNF induced an even greater enhancement in the stimulus-evoked release of iCGRP than those grown in NGF alone or in the absence of growth factors. These responses are not likely due to changes in morphology, CGRP content, or insertion of TRPV1 into the membrane. A more likely explanation for the increased enhancement in stimulated release in response to GDNF is that GDNF present in the culture media increases the presence of either GFRα-1 and/or Ret. Addition of 10 ng/mL GDNF in the culture media increases Ret expression by 12 times in a neuroblastoma cell line (Peterson and Bogenmann, 2004). These increases could result in greater responsiveness of the sensory neurons to GDNF and could explain how recurrent, acute inflammatory pain can be converted to chronic pain syndromes.

D. Ret-dependent signaling pathways of GFL-induced sensory neuronal sensitization

The GFLs classically signal through Ret, and the role of three prominent pathways activated by Ret are known to induce sensory neuronal sensitization. These pathways are the MAPK/Erk 1/2 pathway, the PI-3K pathway, and the Src kinase pathway. Each of the GFLs activated distinct complexes of these pathways and used different pathways to accomplish their sensitization.

1. GDNF-induced sensitization is Ret-dependent and through the MAPK/Erk 1/2 pathway

The intracellular signaling mechanism of GDNF-induced sensitization appears the most clear of all the GFLs. In many cell types, GDNF has been shown to activate all three of the pathways mentioned above (Bron et al., 2003;Poteryaev et al., 1999). Initially, I expected that this multi-pathway activation would occur in the sensory neurons in culture as well. Surprisingly, GDNF activated the MAPK/Erk 1/2 pathway and the Src pathway but not the PI-3K pathway (Figures 13 and 22). In accordance with the molecular data, MAPK/Erk 1/2 pathway inhibitors and SFK inhibitors prevented the GDNF-induced sensitization, and Src siRNA reduced the GDNF-induced enhancement in the stimulated-release of iCGRP, while inhibition of the PI-3K pathway did not (Figures 14, 23, and 26). Other studies have observed increased PI-3K activation with exposure to GDNF (Bron et al., 2003). However, this study used DRG from large (greater than 250 g) Sprague-Dawley rats, whereas the studies in this document were conducted on mouse DRG. Bron et al., 2003 exposed the rat DRG to high concentrations of GDNF (50 ng/mL and higher) and measured intracellular p-Akt increases. The high concentration of GDNF used could have initiated non-specific effects of GDNF through the GFRα-2 receptor (Baloh et al., 1997;Buj-Bello et al., 1997;Sanicola et al., 1997). In addition, it is possible that the signaling responses in rat and mouse tissue are different.. Because Erk

1/2 is generally considered an effector of the MAPK pathway (Turner and Cantrell, 1997;Xing et al., 1998), and Src is often upstream of the MAPK/ERK 1/2 pathway (Irigoyen and Nagamine, 1999), it is likely that Src is being activated by GDNF through Ret and initiating the MAPK/Erk 1/2 pathway (diagrammed in Figure 30). Complicating this pathway is the fact that Src can be activated by PI-3K. There is also evidence that GDNF can activate SFKs in a Ret-independent manner (Poteryaev et al., 1999). However, these studies were conducted on DRG from embryonic Ret-deficient mouse DRG and two neuronal cell lines, that lack Ret expression (NIH3T3 and SHEP neuroblastoma), stably transfected with GFRα-1. These cells could have different compliments of signaling pathways and cell surface receptors than the wild type adult mouse DRG that were used in the studies in this thesis. The developmental, species, and genetic differences between these preparations could account for the seemingly discrepant signaling mechanisms for GDNF.

There was also a possibility that GDNF was activating this pathway sequence in a Ret-independent manner (Pezeshki et al., 2001;Poteryaev et al., 1999). These studies were all conducted on either embryonic cell cultures or cell lines (described above). However, when Ret was inhibited using a specific siRNA, the GDNF-induced sensitization was abolished (Figure 33). The use of other siRNA inhibitors of other receptors, NCAM, or Ret-independent signaling pathways, Fyn, did not affect the GDNF-induced sensitization (Figures 36 and 42). The differences between previous reports of GDNF-induced, Ret-independent actions and the data presented here could simply be the result of the different cell types used. It is possible and likely that embryonic neurons and cell lines differ drastically in their responses to GFLs. The primary reason for this difference is that embryonic neurons and cell lines are experiencing not only the modulatory effects of the GFL, but also the growth promoting effects. This additional set of growth effects could completely change the way the cells respond to the GFLs in

terms of their modulatory effects. The data presented in this thesis indicate that GDNF accomplishes its sensitization in a completely Ret-dependent manner through the MAPK/Erk 1/2 pathway.

2. Ret-dependent, NTN-induced sensitization is through the PI-3K pathway

There is evidence for NTN activation of MAPK, PI-3K, and Src and SFK pathways (Althini et al., 2004;Hauck et al., 2006;Soler et al., 1999). NTN, unlike GDNF, robustly activated all three of these pathways, as measured by the production of phosphorylated downstream effector proteins (Figure 16). Therefore, it would seem likely that NTN could accomplish its sensitization through all three of these pathways. However, NTN-induced sensory neuronal sensitization was prevented by inhibition of the PI-3K and Src and SFK pathways, not the MAPK pathway (Figures 17, 23, and 26). NTN is, therefore, likely activating Src and the PI-3K pathways sequentially to accomplish enhancement in the stimulated-release of iCGRP (diagrammed in Figure 30).

NTN exhibited an interesting and intriguing difference with GDNF in its initiation of sensitization. Using Ret siRNA to knock down this receptor led to a partial reduction in the NTN-induced enhancement in the capsaicin stimulated-release of iCGRP (Figure 33), and sensitization was still present. This Ret-independent, NTN-induced sensitization will be discussed further below. The PI-3K pathway is the critical pathway for NTN-induced sensitization, whether through Ret-dependent or Ret-independent initiation.

One other interesting phenomenon observed in the case of NTN-induced sensitization is the dissociation of pathway activation and sensitization. Specifically, NTN activates MAPK/Erk 1/2, as measured by the production of p-Erk, but inhibition of this pathway does not affect NTN-induced sensitization. This dissociation may be a result of different pathways having differential activation of downstream proteins. For example, the PI-3K activated by NTN may be phosphorylating TRPV1, while the MAPK/Erk1/2 may be phosphorylating other proteins and/or altering gene expression.

3. ART-induced sensitization is through neither the MAPK/Erk 1/2 nor the PI-3K pathway

ART also activates the MAPK, PI-3K, and Src and SFK pathways (Althini et al., 2004;Hauck et al., 2006;Soler et al., 1999). ART caused each of these pathways to be activated in the sensory neurons in culture, as measured by phosphorylation of downstream effector proteins (Figure 19). It would be expected that inhibition of any of these pathways, or several of them in combination, would prevent ART-induced sensitization. However, only Src inhibition was able to reduce the amount of ART-induced enhancement in the stimulated-release of iCGRP (Figure 26). No inhibitors of the MAPK/Erk 1/2 or the PI-3K pathways prevented the ART-induced sensitization (Figure 20). There is the possibility that both of these pathways are sufficient, but neither is necessary, for ART to induce its sensitizing effects. Essentially, ART could use either of these pathways to accomplish its sensitization, but neither one of them individually when inhibited will prevent the ART-induced sensitization. There is no evidence in the literature for the need for dual pathway activation to induce the effects of ART. There is evidence, however, of the need for both MAPK and PI-3K activation for neuronal protection by GDNF (Villegas et al., 2006). The MAPK/Erk 1/2 pathway and PI-3K pathways also modulate each other (Zhuang et al., 2004). Addition of GDNF to B92 glial cells prevented the damage and death of these cells by high concentrations of ethanol. GDNF activated both the MAPK and PI-3K pathways in this preparation and the inhibition of either pathway, individually, did not reverse the effects of GDNF. When both pathways were inhibited at the same time, the GDNF could no longer prevent the cell damage and death. With this study in mind, inhibitors of both the MAPK and PI-3K pathways were used to evaluate ART-induced sensitization. The use of one inhibitor of the MAPK/Erk 1/2 and one inhibitor of the PI-3K pathway in combination did not affect the enhancement in stimulated-release of iCGRP induced by ART (Figure 20). These

data demonstrate that Src is a likely pathway important in ART-induced sensitization, but that neither the MAPK nor the PI-3K are necessary for this sensitization. While this seems to contradict previous studies identifying either or both of these pathways as important in alteration of sensory neuronal function by ART (Hauck et al., 2006;Jeong et al., 2008a;Soler et al., 1999), all of these studies were conducted on neuronal cell lines, embryonic neurons, or motor neurons, not adult sensory neurons. Since the responses in embryonic tissue, immortalized cells or motor neurons may be very different from those in adult sensory neurons, the conclusions from experiments in this document may differ from that in the literature. There is new evidence for the importance of the PKC pathway for NGF and ART induced sensitization (Shu and Mendell, 2001;Sikand and Premkumar, 2007). Furthermore, unpublished observations suggest a critical role for both the SFK and PKC pathways in ART-induced modulation of the TRPV1 channel, whereas the sensitization of TRPV1 by ART was not affected by the MAPK/Erk 1/2 inhibitors, PD98059 and U0126, and the PI-3K pathway inhibitor, LY294002 (unpublished observation, Dr. Weiguo Zhu).

Ret-independent, ART-induced sensitization was also observed. Ret siRNA did not abolish ART-induced sensitization, although it did reduce the enhancement in the release of iCGRP (Figure 33). This Ret-independent, ART-induced sensitization will be discussed in greater detail in later sections (Section IV. D.3).

4. Direct Ret inhibition by PP2

One controversy confronted in this thesis is whether PP2 is an inhibitor of Ret. PP2 was originally used as a specific inhibitor of the kinase activity of the SFKs, c-Src and Lck (Hanke et al., 1996). Since that time, it has been erroneously used as a specific c-Src inhibitor, as well as a SFK inhibitor. However, PP2 was eventually identified as a potent Ret inhibitor (Encinas et al., 2001). This effect was either due to inhibition of Ret phosphorylation by some intracellular signaling pathway induced directly by GFRα-2 (as

described above) or due to direct inhibition of Ret autophosphorylation. The novel mode of Ret activation thru action of the GFRα receptor was identified and termed *trans* activation. This *trans* activation can occur when GFLs through their cognate GFRα receptor initiate SFK signaling to phosphorylate Ret (Kato et al., 2002). Other cytokines can activate Ret through intracellular signaling pathways in the same *trans* fashion (Kato et al., 2002). It has, therefore, been unclear whether PP2 was preventing Ret phosphorylation through inhibition of the Src *trans* activation or direct inhibition of Ret autophosphorylation. Figures 28 and 29 in combination provide evidence that PP2 is inhibiting Ret autophosphorylation. Since c-Src siRNA did not affect GDNF-induced Ret phosphorylation, c-Src *trans* activation of Ret is not the likely mechanism of Ret phosphorylation by the GFLs. Additionally, c-Src siRNA did not eliminate GDNF-induced sensitization. Since there is no evidence in the literature of Ret-induced activation of any SFKs besides c-Src and GDNF accomplished its sensitization in a Ret-dependent manner, GDNF-induced sensitization of sensory neurons is unlikely to be a result of phosphorylating Ret in a *trans* fashion. PP2, on the other hand, completely prevented the ART-induced Ret phosphorylation. This prevention of Ret phosphorylation was as effective as the prevention of ART-induced Ret phosphorylation by Ret siRNA. ART was chosen as the representative GFL for these experiments because sensitization by the other GFLs does not follow this SFK-mediated pattern.

Overall, the data presented in this thesis demonstrate three important characteristics of the sensitization of sensory neurons by GFLs. First, PP2 can no longer be used as an SFK-specific inhibitor, especially when evaluating the actions of the GFLs, since it is clearly also an inhibitor of Ret autophosphorylation. Second, each of the GFLs uses different compliments of intracellular signaling pathways to accomplish sensory neuronal sensitization. GDNF uses the MAPK/Erk 1/2 pathway, NTN uses the PI-3K pathway, and ART does not use either the PI-3K or MAPK Erk 1/2 pathways to elicit sensitization of

sensory neurons. Finally, there is both Ret-dependent and Ret-independent GFL-induced sensitization on sensory neurons.

E. Ret-independent signaling pathways of GFL-induced sensory neuronal sensitization

Ret is the classic signaling partner of the GFL-GFRα complex, but there is an increasing amount of evidence from the literature that some of the GFLs can signal independently of Ret in primary neurons and cell lines lacking Ret, ureteric buds from Ret deficient mice, and in Madine Darby canine kidney (MDCK) cells, which lack Ret (for a review see Sariola and Saarma, 2003). Although GDNF-induced, Ret-independent sensitization was not observed in this thesis, one of the other possible Ret-independent signaling mechanism for the actions GDNF on ureteric budding is directly through the GFL-GFRα complex (Enomoto et al., 2004;Popsueva et al., 2003). GDNF protection of substantia nigra neurons from damage by 6-hydroxy dopamine (6-OH DA) is mediated by the NCAM receptor (Cao et al., 2008a;Chao et al., 2003;Paratcha et al., 2003). Although there is no evidence for GFL-induced effects in any cell system through the Integrin β-1 receptor, the GDNF-GFRα complex does bind to Integrin β-1 in substantia nigra neurons (Cao et al., 2008b). The GAS1 (growth arrest-specific receptor 1) receptor, a tumor suppressor gene receptor, binds to the GFL-GFRα-1 complex and induces Ret phosphorylation (Cabrera et al., 2006;Lopez-Ramirez et al., 2008).

1. GDNF lacks Ret-independent signaling mechanisms of sensory neuronal sensitization

Previous studies in the literature have identified only GDNF-induced, not NTN or ART-induced, actions through Ret-independent pathways. GDNF exhibited no Ret-independent effect in the induction of sensory neuronal sensitization in any studies in this document. This difference can likely be accounted for by the use of different cell types. The studies that found GDNF-induced, Ret-independent effects were done

primarily in cancer and neuronal cell lines (Enomoto et al., 2004;Paratcha et al., 2003). GDNF promoted ureteric kidney budding in mouse embryos independently of Ret or any other cell surface receptor, and embryonic substantia nigra neurons were protected from 6-OH DA damage in a Ret-independent, NCAM-dependent manner (Cao et al., 2008a;Cao et al., 2008b). Some of the demonstrated effects of GDNF in these cells were likely due to the growth promoting effects of GDNF, which were not likely to play a role in my studies of the acute effects of GDNF on sensory neuronal sensitization. For example, the most striking evidence for GDNF-induced, NCAM-dependent effects is the protection of substantia nigra CNS neurons from 6-OH dopamine damage. These neurons were exposed for to GDNF for several days and later treated with 6-OH DA. The protection of these neurons by GDNF was NCAM-dependent, since addition of an NCAM blocking antibody eliminated these protective effects (Cao et al., 2008a). It is clear from the data presented in this thesis that GDNF does not exert its sensitization actions through Ret-independent mechanisms in peripheral sensory neurons.

2. NTN accomplishes sensitization through at least two Ret-independent mechanisms

There is no evidence in the literature for NTN-induced, Ret-independent effects in any cell type. Although NTN binds to the NCAM receptor (Paratcha et al., 2003), there are no studies showing a functional effect of NTN through an NCAM-dependent mechanism. Additionally, no studies to evaluate the NTN-induced effects through an Integrin β-1-dependent mechanism have been published. The current work showing a NTN-induced, Ret-independent sensitization is the first to demonstrate that NTN can have effects through Ret-independent mechanisms.

NTN-induced enhancement in the stimulated-release of iCGRP was reduced by Ret siRNA exposure, but the sensitization was not eliminated (Figure 33). A similar effect with NCAM siRNA was seen (Figure 36). One complicating factor in inhibition of NCAM

is that NCAM activates many intracellular signaling pathways. Despite this possible complication, when Ret and NCAM siRNA were used in combination, NTN-induced sensitization was still present, but the amount of enhancement in the stimulated-release of iCGRP was dramatically reduced (Figure 39). The effects of NCAM were reproduced by Fyn siRNA exposure (Figures 42 and 44), which further supports the partial role of NCAM in NTN-induced sensitization. NCAM directly activates Fyn, while there is no evidence that Ret does. In addition, exposure of DRG to Integrin β-1 siRNA reduced NTN-induced enhancement in the stimulated-release of iCGRP, but did not eliminate sensitization (Figure 48). However, when the DRG were exposed to Ret, NCAM, and Integrin β-1 siRNA in combination, the NTN-induced sensitization was completely abolished (Figure 50). Addition of all three siRNAs could affect the integrity of the neuronal cell membranes and/or the ability of the supporting glia to secrete factors necessary for neuronal function. Since addition of all three of these siRNAs did not alter content of iCGRP, nor did it change the resting release of iCGRP, it is unlikely that the combination of siRNA treatments altered the baseline functions of the sensory neurons.

There are two possible explanations for reduction of sensitization by reducing expression of Ret and NCAM in combination or Ret or NCAM individually. The first possibility is that the 15-30% of Ret and NCAM protein remaining after siRNA treatment could be enough to allow for NTN-induced sensitization. There were two ways to address this issue; add higher concentrations of siRNA molecules or add the siRNA molecules in combination. Adding these siRNA molecules in combination allows evaluation of the possibility that NTN is capable of using any or all of these receptors depending on availability. Clearly, NTN is able to use these alternate receptor-pathway combinations when the others are eliminated. NTN probably can use these pathways to promote its effects, which is the second possibility for why each of the siRNA molecules

individually did not prevent NTN-induced sensitization. This seems to be the more likely scenario.

The data would suggest that NTN is capable of using three distinct receptors to initiate its effects through a common intracellular signaling cascade, PI-3K, to induce this sensitization. NTN-induced, Ret-dependent effects are likely through the c-Src kinase pathway. Ret autophosphorylation activates c-Src, while there is no evidence that Ret autophosphorylation can activate Fyn or Syk. NCAM activation induces the Fyn kinase pathway. Integrin β-1 activation results in Syk kinase cascade initiation. The role of Src and Fyn in NTN-induced sensitization was evaluated in this thesis, while Syk kinase function in NTN-induced sensitization was not. However, these studies could be conducted at a later point. The downstream effector of NTN-induced sensitization, whether the actions are initiated through Ret-Src, NCAM-Fyn, or Integrin β-1-Syk, is PI-3K. PI-3K activation likely modulates the TRPV1 phosphorylation state and thereby alters the sensitivity of the channel to noxious stimuli. This NTN-induced, Ret-independent pathway of sensitization is a completely novel pathway and is the first demonstration of GFL-induced, Ret-independent pathways of sensory neuronal sensitization.

3. ART accomplishes sensitization through Ret-dependent and Ret-independent, NCAM-dependent mechanisms

Several studies indicate that ART-induced sensitization occurs, in part, through Ret-independent mechanisms (Bennett et al., 2006;Zihlmann et al., 2005). Therefore, there is precedent in the literature for ART-induced, Ret-independent actions in sensory neurons. The normal electrophysiological functions of injured C-fibers are recovered by exposure to ART. Interestingly, this recovery occurs on C-fibers that express GFRα-3 but not Ret, demonstrating these effects of ART are Ret-independent (Bennett et al., 2006). Originally, ART was shown to increase the tyrosine hydroxylase (TH)

immunoreactivity, the neurite outgrowth, and the neurite branching of neurons from the rat ventral mesencephalon (Zihlmann et al., 2005). However, it was then found that some DRG sensory neurons damaged by axotomy were ART responsive and GFRα-3 positive, but lacked the Ret receptor (Bennett et al., 2006). The GFRα-3 positive, Ret negative neurons had increased transcription of ART-dependent genes, increased release of substance P (SP), and enhanced physiological properties (Bennett et al., 2006). Together, these studies suggested a role for Ret-independent actions of ART in sensory neurons.

ART-induced sensitization was not prevented by either Ret siRNA or NCAM siRNA alone, but the enhancement in the release of iCGRP was significantly reduced by each of these manipulations (Figures 33 and 36). Fyn siRNA had the same effect as NCAM siRNA (Figure 42). When Ret and NCAM or Ret and Fyn siRNA were used in combination, the ART-induced sensitization was abolished (Figures 38 and 44). Exposure of the DRG to Integrin β-1 siRNA did not affect ART-induced enhancement in the release of iCGRP (Figure 48). The effects of all of these manipulations show that ART uses both the Ret-dependent pathway through Src and the NCAM-dependent pathway, likely through Fyn, to accomplish its sensitization. A direct evaluation of the connection between NCAM activation and Fyn kinase initiation was not conducted, but these studies could be done in the future. ART-induced sensitization is not mediated by the MAPK/Erk 1/2 or the PI-3K effector pathways.

F. Physiological and pathophysiological significance of the distinct and novel signaling pathways of GFL-induced sensitization

The data presented in this thesis demonstrate that each of the GFLs use distinct pathways to sensitize sensory neurons. One question that remains, however, is what physiological and pathophysiological significance there could be for seemingly similar molecules having different pathways of sensitization. This is interesting for the GFLs,

because all of these molecules were originally thought to mediate their actions through the Ret receptor and similar sets of intracellular signaling pathways. Additionally, why NTN and ART can signal in a Ret-independent manner and GDNF cannot is yet to be determined. There are three possible explanations for these differences in signaling between GFLs. First, each GFL is modulating the responses of sensory neurons that are innervating different tissues, and therefore may have different compliments of receptors and signaling pathways. Second, each of the specific GFRα receptors is localized on different portions of the cell membrane where different compliments of receptors and pathways are present. Finally, each of the GFLs may cause different structural changes in their specific GFRα receptor subtype that allow different interactions with Ret, NCAM, and Integrin β-1.

1. Each of the GFLs may modulate different populations of sensory neurons in the DRG

The DRG is a heterogeneous population of sensory neurons; in particular there are sensory neurons that innervate the skin, the viscera, and the musculoskeletal system. There is an abundance of evidence for each of the GFLs having specific and/or preferential populations of sensory neurons of which they modulate responses. GDNF has long been touted as a potential treatment for amyotrophic lateral sclerosis (ALS). GDNF not only has potent effects on motor neuron survival and maintenance (Ribotta et al., 1997;Yamamoto et al., 1996) but also participates in the recovery of damaged proprioceptive sensory neurons innervating muscle tissue (Buj-Bello et al., 1995). There is no evidence in the literature that NTN, ART or PSP have this effect on proprioceptive sensory neurons. Additionally, when afferents from muscle, skin, and viscera were labeled, GDNF was only able to modulate the responses of TRPV1 to capsaicin in motor afferents, which contain small amounts of CGRP (Malin et al., 2009). These results were recapitulated in an *ex vivo* preparation where the muscle is removed with the attached

motor afferents and responses of the afferents were evaluated. GDNF modulated the electrophysiological responses of these neurons making them more sensitive to noxious stimuli (personal communication, Dr. Sacha Malin, University of Pittsburgh). These data could indicate that GDNF is modulating this population of DRG primary sensory neurons, possibly due to increased expression of the GFRα-1 receptor on this population.

Similar to GDNF, NTN appears to have a specific subset of sensory neurons that it preferentially modulates. When sensory neurons from the colon were retrogradely labeled and treated with each of the GFLs, NTN produced the most robust modulation of capsaicin-induced calcium influx through TRPV1 (Malin et al., 2009). NTN caused a greater peak influx of calcium through TRPV1 and a larger area under the curve of calcium influx than GDNF or ART. This data suggests that NTN preferentially modulates the responses of sensory neurons innervating the colon and other viscera. Again, these effects of NTN are presumably through increased expression of GFRα-2 on this neuronal subtype compared to the other sensory neuronal populations, although this has not been evaluated.

ART may preferentially modulate the responses to noxious stimuli of afferents innervating the skin. ART is the most effective GFL at altering the function of sensory neurons innervating the skin, robustly modulating TRPV1 responses to capsaicin in greater than 80% of a population of these neurons (Malin et al., 2009). GDNF and NTN had effects on far fewer (approximately 40%) of these neurons. Additionally, ART over expression (ART-OE) selectively in skin keratinocytes led to increased excitability of the C-fibers (a subset of nociceptive sensory neurons) innervating the skin and behavioral hypersensitivity to noxious heat (Elitt et al., 2006). The level of ART and its receptor, GFRα-3, are increased in the skin after inflammation is induced by CFA (Malin et al., 2006), and ART-OE mice have increased calcium influx through TRPV1 in response to capsaicin compared to wild-type mice (Wang et al., 2008b). ART was able to elicit

enhanced responses of TRPV1 in afferents from skin and viscera as well. However, fewer of these neurons responded to ART, and the responses of these afferents to ART were less robust than in skin afferents. Together, these data indicate that ART is an important modulator of sensory neuronal sensitivity of primary afferents innervating the skin.

In the DRG cultures used for experiments described in this thesis, there is a heterogeneous population of neuronal subtypes; skin afferents, motor afferents, and visceral afferents. The sensitization of the sensory neurons in this preparation is equal for each of the GFLs, which could be due to modulation of each of the different populations of sensory neurons by the different GFLs. It is also possible that each of the subtypes of primary afferents has distinct sets and abundances of receptors (Ret, NCAM, and Integrin β-1) and signaling pathways available for the action of sensitization (MAPK/Erk 1/2, PI-3K, SFKs, Fyn, and PKC). For instance, muscle afferents may express only Ret and GFRα-1 receptors and preferentially use the c-Src kinase signaling cascade through the MAPK/Erk 1/2 pathway for sensitization, while visceral afferents may express GFRα-2, Ret, NCAM, and Integrin β-1 receptors and preferentially use the c-Src, Fyn, and Syk kinase signaling cascades through the PI-3K pathway for sensitization. ART, on the other hand, may express only Ret, NCAM, and GFRα-3 receptors and preferentially use the Fyn kinase and c-Src kinase signaling cascade through the PKC pathway for sensitization. This could be tested by immunohistochemical staining of the DRG for each of these receptors and determination of their colocalization, although, this colocalization does not necessary correlate with function in these neurons.

These differences in GFL responsiveness of different subtypes of primary afferents could be important in physiologic and pathophysiologic function. The joint and muscular pain of diseases like rheumatoid arthritis may be mediated by the actions of GDNF on

musculoskeletal afferents (Fang et al., 2003;Liu et al., 2006). Colonic and other visceral pain may be mediated by NTN, since GFRα-2 is highly expressed in SP containing neurons from the myenteric ganglia, and GFRα-2 deficient mice displayed dysfunction in visceral pain (Rossi et al., 2003). Pain from the skin may be mediated by ART, as discussed above. Direct injection into the paw with any of the GFLs induces inflammatory hyperalgesia (Malin et al., 2006), which could argue against the idea of a specific GFL mediating pain only from a particular tissue. However, there is a complex interplay of glial cells, hematopoietic cells, and connective tissue which may complicate the ability to determine the effects of individual GFLs on sensory neurons *in vivo*. Overall, it is clear that each of the GFLs may play important roles in pain of different physiologic origin.

2. Differential localization of signaling components of GFL-induced sensitization

A growing number of studies have probed the concept of receptor and signaling component localization in GFL-induced function, specifically the role of lipid rafts in this signaling. When the GFL-GFRα complex binds its GFL, this complex recruits Ret into lipid rafts and initiates signaling (Tansey et al., 2000). This activation and signaling is interrupted by lipid raft disrupters and leads to a dampening of the effects of GFLs on survival and neurite outgrowth (Pierchala et al., 2006). Additionally, there are different pathways activated by Ret when it is inside lipid rafts versus outside lipid rafts (Pierchala et al., 2006). Inside lipid rafts, Ret signals through the Src homology 2 domain containing (SHC) and Grb2 (Paratcha et al., 2001). Outside of lipid rafts, Ret signals through FGF receptor substrate 2 (FSR2; Paratcha et al., 2001). Each of these pathways initiators activate different intracellular signaling pathways (Paratcha et al., 2001). Finally, NCAM and Integrin β-1 can signal both within lipid rafts (Schaeren-Wiemers et al., 2004;Vassilieva et al., 2008) and outside of lipid rafts. It is reasonable, then, to hypothesize that each of the GFLs could be activating different receptors and signaling

pathways due to localization of these receptors and pathways with individual GFRα receptors.

3. Different GFRα subtypes may alter the structure of Ret and the resulting activation of Ret-dependent pathways

The final possibility for different signaling pathway activation and resulting sensitization by each of the GFLs is that each specific GFL-GFRα complex may be altering the structure of Ret in such a way as to activate different compliments of signaling pathways. Specifically, it has been shown that when the ART-GFRα-3 complex translocates to Ret, it activates the MAPK pathway more slowly and less robustly than when the GDNF-GFRα-1 complex translocates to this receptor (Parkash et al., 2008). This may be because different tyrosine residues are available depending on the Ret configuration. Different Ret configurations are induced by the specific GFL-GFRα complex because of the different angles of the GFL-GFRα complexes (Parkash et al., 2008). Different Ret confirmations lead to activation of different signaling cascades and signaling effectors by changing the relationship of the two Ret molecules in the homodimer in such a way as to alter which tyrosine molecules are available for autophosphorylation (Parkash et al., 2008). Different patterns of autophosphorylation could lead to different intracellular signaling molecules binding to Ret and being activated and could explain, in part, the differential compliments of pathways used by each of the GFLs to elicit sensory neuronal sensitization.

Whether the differential compliments of signaling pathways used by the GFLs to accomplish their sensitization is due to different GFL sensitize populations, differential localization of receptors and signaling pathways, or different structural alterations in Ret by the receptor complex, it is clear that there is some difference in signaling cascade induction. While not the focus of this thesis, this aspect of GFL-induced sensitization warrants further investigation.

V. SUMMARY AND CONCLUSIONS

The results of this thesis can be summarized by the following statements:

1. The GFLs, GDNF, NTN, and ART, enhance the capsaicin stimulated-release of iCGRP from isolated sensory neurons and freshly dissociated spinal cord tissue.

2. The GFLs do not modulate the potassium stimulated-release of iCGRP.

3. Chronic exposure of DRG to GDNF in the culture media increases the capsaicin stimulated-release of iCGRP and the sensitization produced by acute exposure to GDNF.

4. GDNF induces sensitization of isolated sensory neurons in a Ret-dependent manner through the MAPK/Erk 1/2 pathway.

5. NTN-induced sensitization of isolated sensory neurons involves Ret, NCAM, and Integrin β-1. NTN-induced sensitization is mediated by the PI-3K pathway.

6. ART induces sensitization of isolated sensory neurons via Ret and NCAM activation of an unidentified pathway, possibly the PKC pathway.

Each of the GFLs used different compliments of Ret-dependent and/or Ret-independent pathways to accomplish sensory neuronal sensitization. The demonstration of Ret-independent signaling in sensory neurons is novel. Additionally, NTN-induced sensitization through Integrin β-1 is a novel signaling mechanisms for NTN in any cell type. It remains to be determined the mechanism of activation of these different pathways by each of the GFLs and the exact physiological reason behind these

differences. The data presented in this thesis indicates novel and distinct pathways of GFL-induced sensory neuronal sensitization and adds a layer of complexity to the present knowledge of the actions and signaling pathways of the GFLs. Finally, the effector of this GFL-induced enhancement in the stimulated-release of iCGRP, and the mechanisms by which the signaling pathways modulate this or these effectors, remains to be elucidated. However, the data presented in this thesis provide insight into the signaling pathways of GFLs and the mechanisms of the induction of sensory neuronal sensitization in general.

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University of Notre Dame, Notre Dame, IN

B.A. Psychology, B.S. Science Pre-Professional Studies 2004

- Eli Lilly Endowment Scholarship (4 Year Scholarship for Tuition, Fees, and Books)
- Suma Cum Laude (College of Science and College of Arts and Letters)
- Psi Chi Psychology Honor Society

Honors, Awards, and Fellowships:

- Society for Neuroscience Annual Meeting "Hot Topics".....2009

- Judy Boyd White Award for Graduate Student 2008

  Research Presentations the Indiana University

  School of Medicine Chapter of Sigma Xi
- Invited Speaker; Children's Tumor Foundation 2008

  Annual Neurofibromatosis Conference
- Indiana University School of Medicine Graduate School 2008

  Educational Enhancement Grant Travel Award
- Central Society for Clinical Research (CSCR) 2008

  Midwest Trainee Travel Award
- NIH F30 NRSA Fellowship, Submitted 8/2008 2008

  Priority Score 197
- Children's Tumor Foundation 2007-2009

  Young Investigator's Award Fellowship
- Graduate Student Organization Travel Award.....2007
- INGEN Scholarship for Indiana University 2004-2006

  School of Medicine MD/PhD Program
- Indiana University School of Medicine.....2004-2005

  Graduate School Scholarship

Teaching Experience:

Instructor – Physics - Medical College Admissions Test 2004-2006

Responsible for all lesson planning and teaching

University of Notre Dame, Notre Dame, IN

Teaching Assistant for Sophomore Level 2002-2004

Biology Laboratory Course

Responsible for teaching and overseeing the laboratory techniques of the students. Some test administration and grading duties.

Related Experience:

Indiana University School of Medicine, Indianapolis, IN

Combined Degree Student Committee, Co-President 2007-Present

Act as a conduit of information from MD/PhD students to program directors and vice versa. Started a monthly student seminar series, attend and give input at MD/PhD program business meetings, organize volunteer and social events.

American Physician Scientists Association (APSA)

Institutional Representative 2005-2009

Public Relations Committee and Membership Committee

Conveyed information from this student-run organization, which is focused on MD/PhD programs and training, to the MD/PhD program at Indiana University School of Medicine, as well as provided information to the national MD/PhD community about Indiana University School of Medicine's MD/PhD program; I have also been responsible for helping with press releases and information distribution, as well as contacting deans and students at schools not yet members of APSA to inform them of the benefits of APSA membership.

University of Notre Dame, Notre Dame, IN

Student Research Assistant 2002-2003

Worked with full-time laboratory members (Professors and Post-Doctoral Fellows) to clone GAD 65 from frogs and investigated the interaction of soy isoflavones with liver enzymes.

Indiana University School of Medicine

Research Assistant 2001-2003

Worked independently to genotype several hundred mice in an attempt to correlate specific SNPs with compulsive behaviors (phenotypic changes).

Publications and Papers:

Publications

Schmutzler BS, Roy SR, Hingtgen CM. (2009) Glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) enhance capsaicin-stimulated release of CGRP from sensory neurons. *Neuroscience* 161: 148-156.

Chi XX, **Schmutzler BS***, Brittain JM, Wang Y, Hingtgen CM, Nicol GD, Khanna R. (2009) Regulation of N-type voltage-gated calcium channels (CaV2.2) and transmitter release by collapsin response mediator protein-2 (CRMP-2) in sensory neurons. *J Cell Sci.* In Press.

Schmutzler BS, Roy SR, Hingtgen CM. (2009) Ret-dependent and Ret-independent mechanisms of GFL-induced sensitization of isolated sensory neurons. *Neuron* In Preparation.

Posters

Hingtgen CM, Roy SL, **Schmutzler, BS**. Stem cell factor and nerve growth factor sensitize sensory neurons from wild-type and Nf1 haploinsufficient mice. Program No. 393.22. 2005 Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience, 2005. Online.

Hingtgen CM, Roy SL, **Schmutzler BS**, Clapp DW. Stem Cell Factor and Nerve Growth Factor Sensitize Sensory Neurons – Implications for Pain Signaling in the Tumor Environment. Presented at NNFF International Consortium for the Molecular Biology of NF1 and NF2 and Schwannomatosis; June 5-8, 2005; Aspen, CO.

Schmutzler BS, Roy SL, Hingtgen CM. Glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) sensitize sensory neurons from wild-type and *Nf1* haploinsufficient mice. Presented at ASCI/AAP and APSA Annual Meeting; April 13-15th, 2007; Chicago, IL and NF International Annual Meeting; June 10-13, 2007; Park City, Utah.

Schmutzler BS, Hingtgen CM. Glial cell line-derived neurotrophic factor (GDNF) enhances stimulus-evoked neuropeptide release from isolated sensory neruons. Presented at Society for Neuroscience Annual Meeting; November 3rd-7th, 2007; San Diego, CA.

Schmutzler BS, Hingtgen CM. Glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) enhance the release of the neuropeptide CGRP. Presented at Central Society for Clinical Research and AAP/ASCI Meetings; April 24th-27th, 2008; Chicago, IL.

Schmutzler BS, Hingtgen CM. Glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) enhance the release of the neuropeptide CGRP.

Presented at the Gill Symposium for Neuroscience; May 21st, 2008; Bloomington, IN.

Schmutzler BS, Hingtgen CM. Glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) enhance the release of the neuropeptide CGRP via different intracellular signaling pathways. Presented at Society for Neuroscience Annual Meeting; November 16th, 2008; Washington, DC.

Schmutzler BS, Hingtgen CM. Glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) enhance neuropeptide release via different Ret-dependent and Ret-independent intracellular signaling pathways. Presented at Society for Neuroscience Annual Meeting; October 15th-22nd, 2009; Chicago, IL.

Memberships:

- American College of Physicians
- Sigma Xi
- Student Research Committee
- Children's Tumor Foundation
- Society for Neuroscience
- American Physician Scientist Association
- Indiana University Combined Degree Student Committee