CELLULAR MECHANISMS MEDIATING THE ACTIONS OF NERVE GROWTH FACTOR IN SENSORY NEURONS

Kellie Adrienne Park

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

August, 2007

Accepted by the Faculty of Indiana University, in partial
fulfillment of the requirement for the degree of Doctor of Philosophy.

_____
Michael R. Vasko, Ph.D., Chairperson

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

Doctoral Committee

_____
Grant D. Nicol, Ph.D.

_____
Gerry S. Oxford, Ph.D.

_____
Lawrence A. Quilliam, Ph.D.

July 26, 2006

DEDICATION

This thesis is dedicated to my Mom and Dad. You taught me by example what it means to work hard. Your love, confidence, and never-ending support have made this achievement possible. I will never be able to express how much I appreciate all you have done and continue to do for me.

ACKNOWLEDGEMENTS

I would like to acknowledge three important mentors in my academic life and family and friends that have made possible my ability to obtain a Ph.D. First, I would like to thank Dr. Michael Vasko. I aspire to be a scientist like him. He is question-driven, with logic well-rooted in an extensive knowledge of the literature. More importantly, his fairness toward others, strong work ethic, and confidence in strong science make him a good role model for my future. Second, I would like to thank Dr. Walter Bruyninckx. He helped me to build a strong foundation in biology, and those fundamentals provided a solid platform on which to achieve a great deal in science. I appreciate the invitation to go to the lab of Sean Colgan to perform research in Boston. Most of all, I appreciate that he believed in me as a student at Hanover College. Third, I would like to thank Katie (Forness) Goodman. Her classes provided my first memories of biology as a discipline. Her love of the subject is tangible and contagious. She inspired me to go into the field of science, and to this day I appreciate her guidance and support.

I am especially thankful for the members of the lab, including Eric Thompson, Jill Fehrenbacher, Chunlu Guo, Carla Faletti, Neilia Gracias, and the work-studies, who provide endless support at each level of day-to-day work that is performed in the lab. I would like to thank Dr. Judith Richter, Brad Bohnstedt, and Brian Quinn, who performed significant experiments that are included in this document to enhance the strength of the interpretation of data presented in this thesis. Outside of the lab, I would like to thank Zach Miller, who helped to keep me positive during such a challenging three years of doctoral work. I could not

have had a better friend to provide the necessary conversations that dispelled fears that I could not write a thesis. I would like to thank my sister, Kari, and best friend, Kendra Gordon, for their support and love. Their friendships gave me necessary distractions and interactions while doing research and especially while writing. I would like to thank my Committee, Wade Clapp, Maureen Harrington, and the staff of the Departments of Pharmacology and Toxicology and the Graduate Office for support through the long process of the combined degree program. Lastly, I would also like to thank INGen and NIH for funding the research in this thesis through predoctoral fellowships.

ABSTRACT

Kellie Adrienne Park

CELLULAR MECHANISMS MEDIATING THE ACTIONS OF NERVE GROWTH FACTOR IN SENSORY NEURONS

Nerve growth factor (NGF) is a neurotrophin upregulated with injury and inflammation. Peripheral administration of NGF causes hyperalgesia and allodynia in animals. Blocking NGF signaling reverses these effects. At the cellular level, chronic exposure of sensory neurons to NGF enhances expression the neurotransmitter, calcitonin gene-related peptide (CGRP). Acute exposure to NGF increases capsaicin-evoked CGRP release from sensory neurons in culture. Thus, NGF increases peptide release from neurons by: (1) increasing expression of peptides, and/or (2) altering their sensitivity. The increase in peptide outflow by either mechanism could contribute to development of hyperalgesia and allodynia. The signaling cascades mediating the actions of NGF in sensory neurons are unclear. Therefore, experiments were designed to determine which pathways regulate changes in iCGRP content and evoked release from primary sensory neurons in culture.

The Ras/MEK/ERK cascade was identified as a possible regulator of iCGRP expression in response to NGF. To test this pathway, it was manipulated in neurons by (1) expression of dominant negative or constitutively active isoforms of Ras, (2) farnesyltransferase inhibition, (3) manipulation of the RasGAP, synGAP, and (4) blocking MEK activity. When the pathway was blocked, the NGF-induced increase in iCGRP expression was attenuated. When the Ras pathway was activated, iCGRP expression increased. These data

indicate that Ras, and downstream signaling kinases, MEK and ERK, regulate the NGF-induced increases in CGRP in sensory neurons.

To determine which pathway(s) regulate the increase in capsaicin-evoked iCGRP release upon brief exposure to NGF, the Ras/MEK/ERK pathway was manipulated as described above, and pharmacological inhibitors of the PI3 kinase, PLC, and Src kinase pathways were used. There were no differences observed in NGF-sensitization when the Ras and PI3 kinase pathways were inhibited, suggesting these two pathways were not involved. However, when the Src kinase inhibitor PP2 was used, the NGF-induced increase in release was completely blocked. Furthermore, the PKC inhibitor, BIM, also inhibited the sensitization by NGF. This data indicate Src and PKC regulate of sensitivity of sensory neurons in response to brief exposure to NGF. Thus, there is differential regulation of iCGRP content and evoked release from sensory neurons in response to NGF.

Michael R. Vasko, Ph.D., Chairperson

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

18. Effect of caRas expression on content

19. FTI-276 increases the ratio of unprenylated Ras to prenylated Ras in neurons

20. Expression of Ras in neuronal cultures is not altered by treatment with vehicle or FTI-276 (5μM) nor by treatment with 30 or 100 ng/ml NGF for 7 days

21. FTI-276 reduces the NGF-induced increase in iCGRP content in neurons exposed to 100ng/ml NGF

22. Cell viability of cultures from dorsal root ganglia treated with FTI-276 for 7 days is not affected

23. The inhibitor, FTI-276, increases the capsaicin-evoked release of iCGRP from sensory neurons in culture

24. Dorsal root ganglion neurons in culture express the neuronal RasGAP, synGAP

25. Compared to littermate controls, dorsal root ganglion neurons from synGAP heterozygous mice have increased levels of iCGRP expression in response to NGF

26. NGF augments capsaicin-evoked release of iCGRP from wildtype neurons and from neurons with a heterozygous mutation of synGAP

27. Rat sensory neurons in culture exposed to synGAP siRNA (100nM) have reduced expression of the synGAP protein

28. The level of RasGTP in neurons treated with synGAP siRNA in increased compared to neurons treated with NeuroPorter® alone

29. The NGF-induced sensitization of capsaicin-evoked iCGRP release (not normalized to content) is greater in neurons treated with synGAP siRNA

30. Seven-day treatment with NGF (100 and 250ng/ml) increases the level of pERK relative to total ERK in sensory neurons in culture

31. Ten-minute treatment with NGF does not alter the level of pERK relative to total ERK in sensory neurons in culture

32. The MEK inhibitor, U0126 (1μM), significantly decreases NGF-induced upregulation of iCGRP content in sensory neurons in culture

33. Cell viability of cultures from dorsal root ganglia treated with U0126 for 7 days is not affected

34. U0126 blocks the NGF-induced increase in iCGRP content in neurons derived from synGAP heterozygous mice

35. U0126 does not block the NGF-induced increase in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture

36. U0126 does not block the PDBu-induced increase in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture

37. U0126 blocks the increase in pERK observed after 7 day treatment with 100ng/ml NGF

38. U0126 (1μM) blocks PDBu-induced upregulation of pERK in sensory neurons in culture

39. The effects of PD 98059 and U 73122 on NGF-induced increases in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture

40. Neither LY294002 (3μM) nor wortmannin (3nM0 inhibits the NGF-induced increase in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture

41. The effect of BIM (100nM) on the NGF-induced increases in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture

42. The effect of BIM (30nM) on the NGF-induced increases in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture

43. The NGF-induced sensitization of capsaicin-evoked iCGRP release (not normalized to content) does not appear to be different in neurons treated with control or PKCα siRNA

44. The effect of PP2 (10μM) and PP3 (10μM) on the NGF-induced increases in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture

45. The effect of ceramide on 30nM capsaicin-evoked iCGRP release from sensory neurons in culture

46. The effect of sphingosine-1-phosphate on the NGF-induced increases in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture

47. The effect of DMS (10μM) on NGF-induced increases in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture

48. The effect of neutral sphingomyelinase inhibitor (N-SMase inhibitor, 15μM) on NGF-induced increases in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture

LIST OF ABBREVIATIONS

cap	capsaicin
caRas	constitutively active Ras
dnRas	dominant negative Ras
DMS	*N,N*-dimethylsphingosine
DTT	dithiothreitol
HRP	horseradish peroxidase
MPL	methylpyrrolidinone
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDBu	4-beta-phorbol-12,13-dibutyrate
Ptn	protein
PVDF	polyvinylidene fluoride
RBD	Raf-1 binding domain
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SP	substance P
S1P	sphingosine-1-phosphate
TBS	Tris buffered saline
TBST	TBS with 0.1% Tween-20
TBST-M	TBST with 5% non-fat dry milk

INTRODUCTION

Pain Perception and Pain Pathways in Normal Mammalian Physiology

Physiological Pain Perception

Pain is a sensory modality defined by the International Association for the Study of Pain® (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage…" (IASP Task Force on Taxonomy, 1994). Pain is a prevalent symptom of an acute injury and of many chronic disease. It affects approximately one quarter of the US population (75 million individuals) each year and is one of the most common symptoms that causes people to seek medical attention (Brownlee and Schrof, 1997; Gureje et al., 1998; Goodman, 2003; Woolf, 2004). Pain can be either acute (defined as pain for less than three months), persistent (defined as three to six months), or chronic (greater than six months) (Bonica JJ and Loeser JD, 1990; Portenoy RK and Kanner RM, 1996).

Pain results from a noxious stimulus and from injury to somatic and/or visceral tissues. Pain is not uniform, but has many different qualities (Portenoy RK and Kanner RM, 1996). For instance, pain resulting from activation of somatic primary sensory neurons is usually well-localized. Somatic pain is usually described as stabbing, lancinating (lightening-like), aching, squeezing, or throbbing. Pain resulting from visceral primary sensory neurons is not well-localized due in past to fewer nociceptors per volume of tissue, and is often described as diffuse and is usually dull, achy, or cramping. If visceral pain is

sharp or stabbing, the pain usually results from organs with capsules, or coverings of fiberous tissue that are heavily innervated (Bonica JJ and Loeser JD, 1990).

Dorsal Root Ganglion Neurons

The cell bodies of primary sensory neurons, which are responsible transducing sensory input from the cutaneous and visceral sites to the spinal cord are located in dorsal root ganglia. These ganglia are found in the intervertebral foramen of the vertebral column. These cells are the basis for sensing light touch, vibration, position in space, themosensation, itch, and nociception. Different subsets of neurons transduce each of these modalities, although some neurons are polymodal.

A specific subset of sensory neurons, nociceptors, or neurons that transduce noxious stimuli into electrical and chemical signals are the focus of this thesis (Besson and Chaouch, 1987). Under non-pathological conditions, this subset of sensory neurons has the ability to respond to noxious thermal, chemical, or mechanical stimuli. These neurons have small diameter cell bodies whose axons are lightly myelinated (A-δ fibers) or unmyelinated axons (C-fibers). Besides the presence of myelin, the definitive way to distinguish between these two types of nociceptive neurons is by conduction velocity: C-fiber action potentials travel at 0.2-1.5m/s, whereas A-δ fiber action potentials travel much faster at 5-30m/s (Kandel and Schwartz, 2000).

Primary sensory neurons are pseudounipolar. They have two fused processes that form a single bifurcating axon as it leaves the cell body. The peripheral

branch of the axon innervates cutaneous tissues and viscera. The peripheral ends of the axon terminate in a branching pattern in structures such as the dermis and epidermis of the skin. The central branch travels proximally and synapses on second order neurons in the dorsal horn of the spinal cord.

The peripheral ends of nociceptive neurons are referred to as “free endings” because they are unmyelinated and uncoupled to specialized structures. This distinguishes nociceptive neurons from non-nociceptive neurons. Non-nociceptive sensory neurons have special sensory organs, such as Pacinian corpuscles and Meissner’s corpuscles, at their peripheral endings which transduce mechanical stimuli such as pressure and light touch (Shepherd, 1994).

Stimuli that cause primary nociceptive neurons to generate action potentials are harmful to tissues. These stimuli include temperatures greater than 43˚C, high mechanical pressure, and chemical activators, such as hydrogen ions (low pH). Individual nociceptive neurons may respond to specific stimuli (i.e., only respond to mechanical or thermal stimulation), or may respond to multiple types of stimuli (i.e., mechanical, thermal, and chemical stimuli) (Lang et al., 1990). Neurons that respond to multiple types of stimuli are generally C-fibers and are termed polymodal.

The mechanisms by which noxious stimuli active nociceptive neurons continue to be studied. Some types are noxious stimuli are known to activate specific types of receptors, such as the transient receptor potential/vanilloid 1 (TRPV1) receptor on the cell surface of neurons. TRPV1 a non-selective cation channel activated by heat (>43˚C), protons, and by capsaicin, the active

ingredient in hot chili peppers (Caterina et al., 1997; Tominaga and Caterina, 2004). When the channel is activated by a stimulus, it allows the influx of Na^+ and Ca^{2+}. If the influx of ions is sufficient to cause the neuron to depolarize, it can release neurotransmitters to signal to the second order neurons. Upon depolarization, action potentials are generated and travel the length of the axon. Once the action potential reaches the central neuronal endings, they cause voltage sensitive Ca^{2+} channels open to allow cations to enter. Calcium entry into the neuronal ending causes fusion of synaptic vesicles to the membrane. The neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) and other transmitters, such as glutamate, are released into the extracellular space within the spinal cord (Li et al., 1996; Kandel and Schwartz, 2000). Within the spinal cord, CGRP and SP interact with receptors on the second order neurons of the dorsal spinal cord (dorsal horn neurons). Dorsal horn neurons may depolarize and from these neurons nociceptive signals travel on to higher brain regions, such as the thalamus, periaqueductal gray, reticular formation, and the somatosensory cortex of the cerebrum discussed below.

The Spinal Cord and Higher Brain Regions in Pain Signaling

The gray matter of the spinal cord is divided into 10 lamina that are numbered dorsally to ventrally. The sensory neurons that conduct nociceptive signals terminate on lamina I, II and V of the dorsal horn. Spinal cord neurons in lamina I, also called the lamina marginalis, receive input mostly from Aδ-type primary sensory neurons. The axons of the neurons of the spinal cord then cross

over to the contralateral side, and travel rostrally to the thalamus and on to the cerebral somatosensory cortex. This pathway is referred to as the neospinothalamic tract. This tract transmits noxious stimuli to the brain rapidly, and pain is perceived immediately such as pain upon contact with a hot stove. Spinal neurons of lamina II, also referred to as the substantia gelatinosa, receive inputs mostly from the C-type primary sensory neurons. Axons from these spinal neurons also cross to the contralateral side of the spinal cord, but they terminate rostrally in areas of the brainstem such as the medulla and pons. This pathway is referred to as the paleospinothalamic tract. This tract transmits pain that is characterized as burning, aching, and poorly localized pain. This tract is associated with pain that develops slowly and is perceived longer after the immediate pain of an injury ceases (Simone et al., 1989; Baumann et al., 1991).

Neurotransmitters in Pain Signaling

The primary neurotransmitters released by small diameter sensory neurons are excitatory amino acids (EAAs) and the neuropeptides, SP and CGRP (Budai, 2000). Several lines of evidence support the notion that neurotransmitters are involved in nociceptive signaling in mammals. First, studies show that peripheral noxious stimulation of sensory neurons, such as intense pinching of skin, causes release of SP and CGRP into the dorsal horn of the spinal cord (Kuraishi et al., 1985; Holzer, 1988). Secondly, injection of SP into the intrathecal space at the level of the lumbar spinal cord causes a concentration-dependent increase in mechanical hyperalgesia (Matsumura et al., 1985). Thirdly, administration of antagonists at the receptors for these neuropeptides

reduces allodynia induced by capsaicin (Sun et al., 2003; Sun et al., 2004). Fourthly, expression of the neuropeptides is limited to the small- and medium- diameter sensory neurons of the dorsal root ganglion, which correlates to the subset of neurons responsible for transducing noxious stimuli (Pohl et al., 1990; Hiruma et al., 2000).

There also are many studies that delineate the mechanisms by which these neurotransmitters signal to second order neurons. Once EEAs are released from primary sensory neurons, they interact with either ionotropic or metabotropic receptors of the spinal neurons. The ionotropic glutamate receptors are the N-methyl-D-aspartate (NMDA) receptors, (R,S)-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors, and kainic acid (KA) receptors. When bound to agonist, these channels to open and allow influx of Na^+ and efflux of K^+. In addition, activated NMDA receptors cause Ca^{2+} entry into the cell. Ionotropic receptor activation and sufficient flux of ions into spinal neurons causes their depolarization.

The EEAs released from primary sensory neurons also activate metabotropic glutamate receptors. The metabotropic glutamate receptors consist of a family of G protein-coupled receptors and are divided into groups I, II, and III. The divisions are based on amino acid sequence homology to one another, on which ligands they bind, and on which G protein they activate. The group I metabotropic receptors are coupled to the activation of the enzyme phospholipase-C β (PLC-β), which increases intracellular phosphoinositides and the release of Ca^{2+} from intracellular stores, whereas group II and III metabotropic receptors are linked to the inhibition of adenylyl cyclase and the

decrease of cAMP (Budai and Larson, 1998). The metabotropic receptors are thought to be important in nociceptive signaling as they are localized to small diameter primary sensory neurons. Furthermore, when the receptors are activated, they increase sensitivity to noxious heat in behavioral studies and prolong hypersensitivity induced by CFA injection into the paw (Bhave and Gereau, 2004; Adwanikar et al., 2004).

In addition to the release of glutamate, other important neurotransmitters such as substance P (SP) and calcitonin gene-related peptide (CGRP), are released from sensory neurons once they are activated. Substance P is a tachykinin derived from an mRNA transcript which is spliced to generate SP and another tachykinin, neurokinin A. When released from the terminals of sensory neurons, these two peptides bind to two different GPCRs localized on dorsal horn neurons that are linked to the PLC signaling pathway (Helke et al., 1986; Guard and Watson, 1987; Helke et al., 1990; Severini et al., 2002). Activation of this pathway in neurons increases intracellular Ca^{2+} concentration and activates protein kinase C (PKC).

Calcitonin gene-related peptide (CGRP) also is a transmitter involved in signaling the presence of noxious stimuli. Like SP, CGRP binds to G protein-coupled receptors on spinal neurons when it is released from sensory neurons. There are at least three known types of CGRP receptors: CGRP receptor 1 and 2 and an atypical type (Quirion et al., 1992; van et al., 1997). Based on pharmacological studies in spinal neurons, CGRP receptor 1 is the likely mediator of nociceptive signaling (Sun et al., 2004). In spinal neurons, CGRP

binds to its receptor and activates adenylyl cyclase to increase cAMP (Parsons and Seybold, 1997). The binding of CGRP to its receptors does not cause significant depolarization, but augments the response of spinal neurons to EAAs through activation of signaling molecules like PKA (Sun et al., 2004). Interestingly, CGRP receptors also are present on primary sensory neurons themselves. In nodose ganglion neurons, activation of the CGRP receptor alters Ca^{2+} currents (Wiley et al., 1992). Also, treatment of dorsal root ganglion neurons with CGRP increases tetrodotoxin resistant sodium currents, indicating the neuropeptide is potentiating neuronal activity (Natura et al., 2005). These data suggest that CGRP signals in an autocrine manner to primary sensory neurons to modulate their function.

The importance of CGRP and SP in relation to one another in nociceptive signaling is not known. It is unclear if they are released differentially under specific circumstances *in vivo*. In studies where CGRP and SP are quantified simultaneously, the peptide neurotransmitters display similar trends in the outcomes measured. For instance, the two neuropeptides are generally co-expressed in the same neurons (Zhang et al., 1995). Furthermore, alterations in expression and release of these two neurotransmitter peptides in neurons appear to be similar after various treatments, such as decreased expression in neurons from diabetic mice or increased release after incubation with various inflammatory mediators (Lindsay et al., 1989; Sango et al., 1994; Zhang et al., 1995; Barber and Vasko, 1996; Frayer et al., 1999; Belanger et al., 2002). However, a notable exception was published by Bowles *et. al.* They found that

animals injected with nerve growth factor (NGF, 0.1mg/kg) for 7 days increased the level of CGRP release but not SP release into the spinal cord (Bowles et al., 2004). These data may indicate that CGRP is the relevant neuropeptide effector that mediates the responses to NGF in animals when they are treated systemically with NGF.

Sensitization of Neurons in the Pain Pathway

Peripheral sensitization and central sensitization

Detection of noxious stimuli is necessary for survival. However, too much activity in the nociceptive pathways can also be detrimental. The development of persistent or chronic types of pain that occur in the absence of a noxious stimulus or even in the absence of systemic pathology is referred to as pathological pain. Pathological pain serves no purpose that is understood to date and it causes a great deal of suffering and decreased quality of life (Woolf and Decosterd, 1999).

Pathological pain develops from alterations at all levels of nociceptive signaling, including changes in the activity of primary sensory neurons, the activity of neurons in the spinal cord, and activity of neurons in brain(Costigan and Woolf, 2000; Julius and Basbaum, 2001). In general, when neurons become more responsive to a noxious stimulus, they are *sensitized* (Castellucci and Kandel, 1976; Lewin et al., 1993; Hingtgen and Vasko, 1994; Stucky et al., 1998; Devor, 2006). When primary nociceptive neurons are sensitized, they respond to

lower intensity or non-noxious stimuli, such as heat below 43˚C or lower threshold mechanical stimuli. Sensitization can result in pain perception in the absence of any noxious stimulus if the threshold for activation of the neurons occurs at physiological temperatures or pH.

The pathological consequences of increased sensitivity of nociceptive neurons is the development of hyperalgesia and/or allodynia (Basbaum, 1999; Richardson and Vasko, 2002). The IASP defines hyperalgesia as “an increased response to a stimulus that is normally painful,” while allodynia is “pain due to a stimulus which does not normally provoke pain” (IASP Task Force on Taxonomy, 1994). Examples of these two conditions are common. For instance, passive or active movement of a joint, such as the knee, is usually non-painful. In an individual with arthritis of the knee, it is thought that neurons surrounding the inflamed joint are sensitized and thus simple movement evokes a painful sensation. In another example, a pin prick of the skin is usually painful. However, a pin prick to inflamed skin results in a more intense pain sensation than normally experienced. This is an example of hyperalgesia.

The allodynia and hyperalgesia observed in humans can be reproduced in animals. Injection of inflammatory mediators into the knee joint of rats causes a sustained inflammation. This inflammation causes a decrease in the nociceptive thresholds with passive movement of the joint (Coggeshall et al., 1983; Schaible et al., 1987; Schaible et al., 1990; Donaldson et al., 1995). There is an increase in the activity of primary sensory neurons innervating the inflamed joint compared to non-inflamed animals with a concomitant increase in activity of dorsal horn nociceptive neurons.

While highlighting the increased activity of neurons from these animals, these studies also demonstrate that the modulation of responsiveness to a stimulus can occur in neurons of the peripheral or central nervous system (Woolf, 2004).

Peripheral sensitization refers specifically to the hypersensitivity of primary sensory neurons (Levine JD and Reichling DB, 1999). This hypersensitivity can result from exposure of the sensory neuron to a sensitizing agent (Hingtgen et al., 1995; Evans et al., 1996; Nicol et al., 1997; Southall and Vasko, 2001). Sensitizing agents, which are often inflammatory mediators such as prostaglandins and bradykinin, bind to receptors on the surface of cellular membranes, and activate intracellular signaling cascades. Activation of the signaling cascades causes transcriptional alterations in proteins or post-translational modifications of ion channels and other proteins that regulate the sensitivity to a stimulus.

At least two post-translational mechanisms have been shown to regulate the sensitivity of sensory neurons in response to inflammatory mediators (Planells-Cases et al., 2005). First, the increased capsaicin sensitivity of sensory neurons in response to NGF may result, in part, from a change in the phosphorylation state of TRPV1 that alters the receptor's localization in the cell. This was proposed by Zhang *et al.* They propose that NGF activates PI3 kinase, which then interacts with another kinase, Src, which phosphorylates TRPV1 (Zhang et al., 2005). Upon phosphorylation, TRPV1 moves from intracellular vesicles to the surface of membranes. This change increases sensitivity to capsaicin. Also, phosphorylation of the TRPV1 channel by specific kinases alters the capsaicin-evoked currents. Jin *et al*. showed that capsaicin-currents in dorsal

root ganglion neurons could be blocked by inhibition of Src kinase or enhanced by inhibition of phosphatases (Jin et al., 2004). These results demonstrate the importance of the phosphorylation state of ion channels in regulating sensitivity of cells to a stimulus.

With respect to translational changes, neurons also can be sensitized as a result of an increased expression of proteins, such as increases in sodium channels and TRPV1, or a decreases in expression of some potassium channel subtypes (Winter et al., 1988; Okuse et al., 1997; Gould, III et al., 1998; Waxman et al., 1999; Ji et al., 2002; Jiang et al., 2003). For instance, the expression of sodium channels, Nav1.8 and 1.9, which are localized on small diameter primary sensory neurons, are increased with nerve injury (Waxman et al., 2000). Overall, the altered expression of receptors and/or ion channels at the surface of membranes in response to inflammatory mediators or neuronal injury can cause neurons to be more excitable to a given stimulus.

Neurons localized in the dorsal spinal cord also can become sensitized, and this phenomenon is termed central sensitization (Woolf, 1983; Ji et al., 2003). High frequency firing from peripheral nociceptive neurons can increase the firing rate of dorsal horn neurons. Multiple mechanisms are thought to regulate this sensitization, including increases in NMDA receptors at the membrane. As with increased TRPV1 or sodium channels at the surface of primary sensory neurons, an increased density of NMDA receptors will increase the responsiveness of spinal neurons to glutamate (Woolf, 1983; Ji et al., 2003).

Inflammation and sensitization

The physiology of inflammation is complex. With acute and chronic inflammation, there is increased vascular permeability and extravasation of leukocytes, including polymorphonuclear cells (PMNs) and macrophages (Springer et al., 2003). With acute inflammation, the purpose of leukocyte invasion is to defend against infection and to resolve tissue damage. With chronic types of inflammation, it is less clear why white blood cells are invading tissues. The extravasation of leukocytes and their inappropriate activation in tissues leads to complications of chronic inflammation, including edema, loss of function, and pain.

Chronic inflammation, such as rheumatoid arthritis, is characterized by hyperalgesia and allodynia. The hyperalgesia and allodynia may result from sensitization of the primary sensory neurons that innervate the tissues with ongoing chronic inflammation. However, the exact cellular mechanisms mediating the changes in sensitivity of sensory neurons in response to inflammation are not known. The neuronal signal transduction cascades that predominate in regulating these effects have not been determined.

With inflammation, there is release of endogenous chemicals, including but not limited to serotonin, histamine, acetylcholine, bradykinin, prostaglandins, cytokines, and nerve growth factor (NGF) from the tissues. These inflammatory mediators are derived from multiple cell types such as lymphocytes, keratinocytes, and neurons themselves. These substances activate and sensitize nociceptors in a paracrine or autocrine fashion (Falcini et al., 1996; Basbaum, 1999; Woolf and Salter, 2000; Richardson and Vasko, 2002; Obata et al., 2002).

Animal models of inflammatory pain have helped to build concepts that explain the mechanisms of development of hyperalgesia and allodynia. A common method to induce inflammatory pain is injection of an immunogenic or inflammatory mediator, such as Complete Freund's Adjuvant (CFA), formalin, endotoxin, PGE_2 or NGF, into the paw of mice or rats (Ferreira et al., 1978; Donnerer et al., 1993; Woolf et al., 1994; Honore et al., 2000). After various times, ranging from minutes to days, the nociceptive behaviors in the animal are measured. These behavioral measurements test allodynia or hyperalgesia. Von Frey hairs are commonly used to quantify allodynia and mechanical hyperalgesia (Reeh et al., 1986; Ahlgren and Levine, 1993; Ahlgren et al., 1997). The hairs are stiff nylon fibers that poke the surface of the inflamed paw. The fibers bend when a calibrated force is applied to the paw. When the animal withdraws the paw, it is assumed that the force is noxious. A decrease in the amount of force needed to cause paw withdraw indicates development of mechanical allodynia. A force that once was non-painful prior to injection now has become painful.

Thermal hyperalgesia is tested in a similar manner to mechanical hyperalgesia. The paw is injected with an inflammatory mediator. A source of heat, such as an intense beam of light, is imposed on the paw of the animal. The latency for withdraw of the paw is measured. If the time for withdraw decreases significantly, the assumption is the animal has developed thermal hyperalgesia.

Nerve Growth Factor

Many types of inflammatory mediators cause hyperalgesia and allodynia (Hong and Abbott, 1994). While the relative importance of each of these mediators in inflammatory pain is not known, NGF recently has moved to the forefront. Nerve growth factor was discovered in the 1950s by Levi-Montalcini *et al.* as a trophic factor essential for the survival of sensory and sympathetic neurons (Levi-Montalcini, 1964; Levi-Montalcini et al., 1996; Aloe, 2004). In laboratory animals and humans, NGF is upregulated with inflammation (Lewin and Mendell, 1994; Woolf et al., 1994; Safieh-Garabedian et al., 1995; Falcini et al., 1996; Nonogaki et al., 1996). Nerve growth factor can be produced by a variety of cells in the body. These cells include keratinocytes, melanocytes, smooth muscle cells, salivary glands, thyroid and parathyroid tissues, and reproductive tissues (Levi-Montalcini et al., 1996). Leukocytes, such as mast cells, monocytes, macrophages, and T-lymphocytes also produce NGF, which implicate this growth factor in immune and inflammatory functions (Leon et al., 1994; Lambiase et al., 1995; Lambiase et al., 1995; Bonini et al., 1996). During injury, Schwann cells produce increased levels of NGF in the peripheral nervous system (Lindholm et al., 1987).

Multiple studies correlate NGF and inflammation. Synovial fluid aspirated from the joints of adults with rheumatoid arthritis have detectable levels of NGF, where fluid from non-inflamed joints do not (Aloe et al., 1992). Similarly in children, elevated serum NGF is directly correlated to the severity of juvenile chronic arthritis (Falcini et al., 1996). Finally, NGF regulates the expression of neuropeptides SP and CGRP both *in vitro* and *in vivo* suggesting that NGF may

be linked to nociceptive signaling (Lindsay and Harmar, 1989; Verge et al., 1995; Ruiz and Banos, 2005).

In *in vitro* studies, injection of NGF into the forearm of adult humans induces hypersensitivity to noxious stimuli (Petty et al., 1994). Cutaneous injection of NGF into rats also causes the development of hypersensitivity (Lewin et al., 1993). Injection of bacterial endotoxin causes a dose dependent increase in serum NGF in rats (Safieh-Garabedian et al., 2002). There is upregulation of the SP content of dorsal root ganglion neurons 4-5 days after CFA injection into the paw (Donnerer et al., 1992), and the thermal hyperalgesia that develops in the paw with CFA-injection is reduced when signaling by NGF signaling is neurtralized by NGF blocking antibody (Woolf et al., 1994). These studies together demonstrate that NGF is increased with inflammation and is regulating the sensitivity of sensory neurons. A current major unresolved issue is which cellular mechanisms and neuronal signaling cascades regulate the increase neuronal sensitivity in response to NGF.

Nerve growth factor sensitization of sensory neurons in vivo

NGF is synthesized as a proneurotrophin. The mature, fully active protein is a homodimer composed of two13kDa peptides, which is referred to as beta-NGF (McDonald et al., 1991; McDonald and Blundell, 1991). The naturally occurring form of NGF is referred to as 7S NGF. It is a multimeric protein with two alpha, one beta and two gamma subunits. Often the 2.5S form will be used in

experiments. This form is 9 amino acids shorter than the beta form and lacks the other subunits of the multimer.

It is well-established that NGF regulates the expression of neuropeptides in sensory neurons both *in vitro* and *in vivo* (Lindsay and Harmar, 1989; Lindsay and Harmar, 1989; Noguchi et al., 1995; Verge et al., 1996; Schuligoi and Amann, 1998; Miki et al., 1998; Fehrenbacher, 2005). Injection of 0.1 mg/kg NGF subcutaneously into rats for 7 days increases the CGRP immunoreactivity in dorsal root ganglion neurons (Verge et al., 1995). When the sciatic nerve is transected, SP and CGRP immunoreactivity decrease, but intrathecal administration of NGF reverses the decrease presumably by increasing their transcription (Verge et al., 1995). NGF treatment of rat paws increases SP and CGRP expression in dorsal root ganglion neurons innervating inflamed tissues (Donnerer et al., 1992). Finally, treatment of neurons in culture with NGF increases neuropeptide expression in dorsal root ganglion neurons (Lindsay and Harmar, 1989; Fehrenbacher, 2005).

In addition to its actions on neuropeptide expression, there is evidence that NGF also increases the sensitivity of sensory neurons. This increase is not based on translational changes, but on post-translational modifications of proteins in cells. Shu and Mendell demonstrated that the decreased response in firing cause by successive capsaicin stimulation of neurons could be reversed by 10 minute treatment with NGF (Shu and Mendell, 1999). Bonnington and McNaughton measured changes in intracellular Ca^{2+} concentrations in response to NGF (Bonnington and McNaughton, 2003). They found after 2 minute exposure of sensory neurons to NGF there was an increase in the Ca^{2+} entry upon stimulation. Zhang and Nicol demonstrated that a brief

exposure of neurons to NGF increased the number of actions potentials measured in response to a ramp of depolarizing current (Zhang and Nicol, 2004). A study by Fehrenbacher showed that brief exposure to NGF increases the capsaicin-evoked release of iCGRP from neurons in culture (Fehrenbacher, 2005). These results show that acute administration of NGF sensitizes senosry neurons and the time course of action implies signaling through some post-translational mechanisms.

Based on the concept of a dichotomy between NGF-induced changes in post-translational events and changes in transcription, a model emerges that may explain at least two ways by which NGF can alter the release of neuropeptides from sensory neurons (Fig. 1). Figure 1 demonstrates a primary sensory neuronal ending, a synaptic cleft, and the membrane of a dorsal horn neuron, onto which the sensory neuron is releasing a reproducible amount of neuropeptide. With long term exposure to NGF, neurons express more neuropeptide, and this has been confrimed in the literature (see above, and left panel Fig. 1). With more neuropeptide packaged into vesicles, a depolarizing stimulus would result in an increased release of neuropeptide into the synaptic cleft. Malcangio *et. al.* provided proof of this concept (Malcangio et al., 1997). Animals were treated with NGF for two weeks, and the evoked release of neuropeptides was measured. They demonstrated a significant increase in the amount of CGRP released from the dorsal horn of the spinal cord. Fehrenbacher *et. al.* obtained similar results *in vitro* when dorsal root ganglion neurons were exposed to NGF for 7 days, and released more iCGRP when exposed to capsaicin (Fehrenbacher, 2005).

The right panel of Figure 1 illustrates the effect of acute exposure to NGF where there is also an increased evoked-release of neuropeptides from sensory neurons that

is not a result of altered content. This concept is based on the findings of Fehrenbacher (Fehrenbacher, 2005) that when measuring release as a percentage of total content, NGF still augments capsaicin-evoked releasef. The exact signaling cascades that regulate these changes in content and stimulated release of neuropeptides in response to NGF are not known. Consequently determining the signaling pathways that control the changes in sensitivity of sensory neurons to NGF is the focus of this thesis.

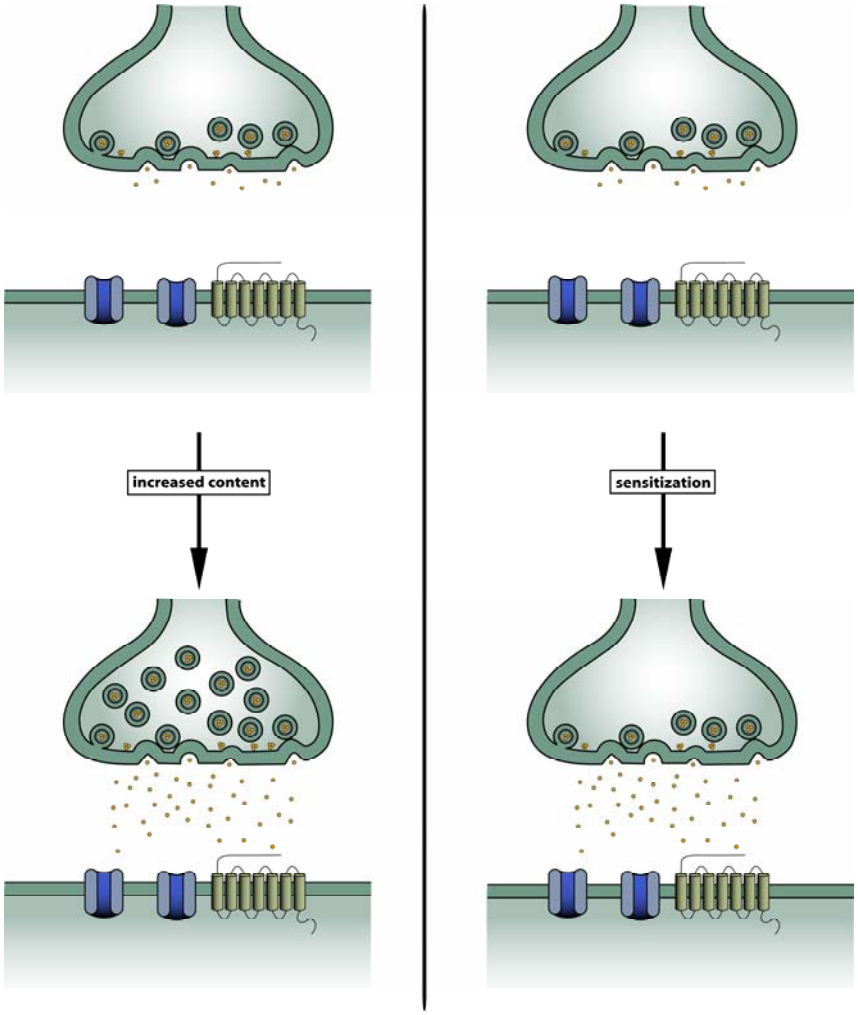


Figure 1. Model representing two possible mechanisms for increased release of neuropeptide in response to NGF exposure. The left panel represents a sensory neuronal ending filled with vesicles signaling to the cell surface of a second order neuron before (top) and after (bottom) NGF exposure for 7 days. After days of exposure to NGF, there is increased expression and therefore increased cellular content. When sensory neurons are exposed to a noxious stimulus, there is an increased release of peptides (Fehrenbacher, 2005; Malcangio et al., 1997). The right panel represents what occurs in a sensory neuron exposed to NGF for 10 minutes, and then stimulated with capsaicin. Upon stimulation, the neurons release more peptide, or they are sensitized. This sensitization cannot be a results of alterations in content as 10 minutes exposure to NGF does not change CGRP expression. Therefore, there is both an acute and chronic effect of the neurotrophin on sensory neurons.

Nerve growth factor receptors

Nerve growth factor has two known membrane receptors, the TrkA and p75 receptors (Fig. 2). Both receptor types are found on primary sensory neurons of adult animals (Verge et al., 1989; Bothwell, 1995; Kaplan and Miller, 1997). Nearly all (92%) of sensory neurons that express CGRP also express the TrkA receptor (Averill et al., 1995). NGF binds to the extracellular portion of these receptors to elicit its effects. Once bound to ligand, the NGF receptors alter cell function through intracellular signaling via a variety of different cascades described below.

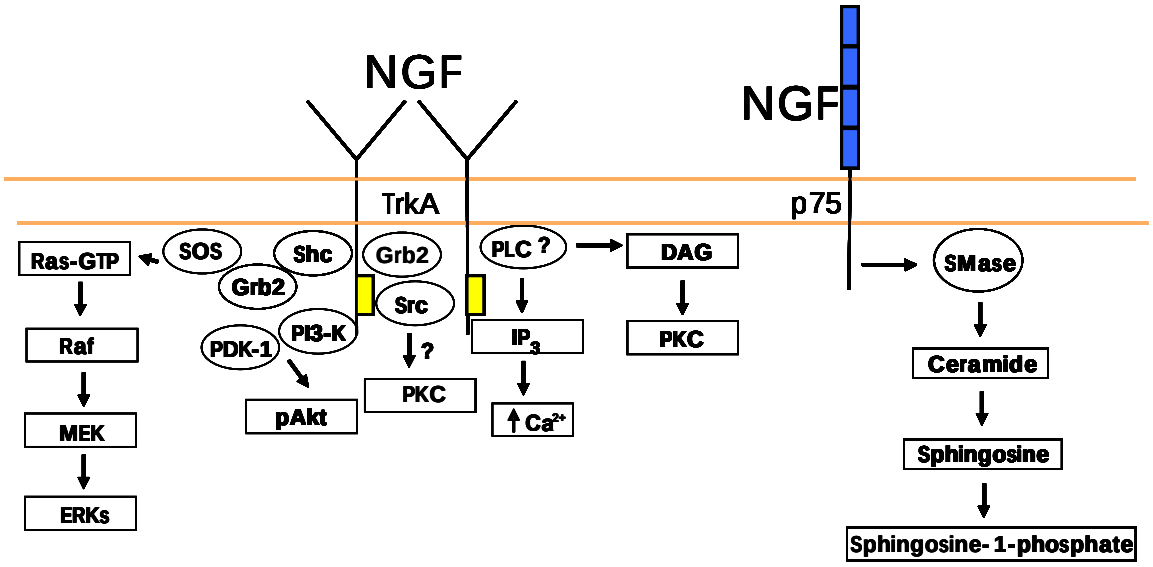


Figure 2. Possible signal transduction cascades activated in sensory neurons by NGF.

TrkA receptor and downstream signaling cascades

The TrkA receptor for NGF is a tyrosine kinase receptor (Kaplan et al., 1991; Kaplan et al., 1991; Kaplan et al., 1991). The active receptor is a homodimer, and each subunit contains an extracellular ligand-binding domain, a transmembrane domain, and an intracellular catalytic domain (Martin-Zanca et al., 1986). Upon NGF binding, the receptors dimerize and transphosphorylate tyrosine residues of its partner to activate the kinase domain on each molecule. The kinase domain autophosphorylates specific tyrosine residues that act as binding sites for adapter proteins and enzymes described in detail below. These adapter proteins trigger the activation of signaling molecules in the cell (Cunningham et al., 1997). The TrkA receptor actives multiple downstream cascades in neurons, including the Ras/MEK/ER pathway, the phosphatidylinositol 3-Kinase (PI3 kinase) pathway, the phospholipase C (PLC) pathway, and Src kinase signaling (Sofroniew et al., 2001).

Ras/MEK/ERK signaling

One consequence of activation of the TrkA receptor is an increase in the activity of the small G-protein, Ras, with subsequent phosphorylation of the MAP kinase, ERK (Kaplan and Miller, 1997; Katz and McCormick, 1997). The pathway is activated when tyrosine residue Y490 of the cytoplasmic domain of TrkA is autophosphorylated after NGF binding to the receptor. Once phosphorylated, the adapter proteins, Shc, Grb-2, and Son of Sevenless (Sos) are recruited to the receptor complex at the near the membrane surface (Basu et al., 1994). Sos is a guanine nucleotide exchange factor which binds RasGDP and exchanges the low-energy GDP for GTP to activate Ras (Katz and McCormick, 1997). Once Ras is bound to GTP (RasGTP) it binds and activates a series of downstream kinases. It first activates Raf, a serine/threonine kinase. This kinase, in turn, phosphorylates and activates MEK, which then phosphorylates and activates ERK. This kinase has numerous functions in the cell, including regulation of transcription of many types of proteins in the cell (Katz and McCormick, 1997). ERK is implicated in the regulation of the sensitivity of sensory neurons (Aley et al., 2001; Zhuang et al., 2004).

The Ras pathway has been implicated in regulation of acute sensitizing effects of NGF. Bron *et al.* examined the effects of the combination of NGF and GDNF only in sensory neurons cultured without NGF for 4-5 days (Bron et al., 2003). After 15 minutes of exposure to the neurotrophin, NGF, they observed increased cell-surface TRPV1, increased pERK levels as measured by

immunoreactivity, and increased cobalt uptake in neurons exposed to capsaicin. This increase was blocked by dominant negative Ras. The increases in TRPV1 immunoreactivity and pERK level in neurons also if the neurons were incubated with a MEK inhibitor (U0126). Conversely, the increase in these two parameters could be reproduced by expression of constitutively active Ras (12V) into their neuronal preparation.

In the work by Aley *et al.*, they injected epinephrine into the paws of rats to induce hyperalgesia (Aley et al., 2001). After hyperalgesia developed, the authors extracted the dorsal root ganglion neurons from the animals and used immunohistochemistry to measure the level of ERK phosphorylation in the cells. They found an increased level of phosphorylation of the ERK 1 and 2 in cells after minutes of exposure to the sensitizing agent. Furthermore, they found that animals develop hyperalgesia during this time, which can be blocked by MEK inhibitors. In these experiments they use NGF as the positive control to demonstrate that after 5 minutes exposure to 50 ng/ml NGF there is a rapid rise in ERK phosphorylation. What is not clear from this study is whether ERK and MEK regulate the acute development of hyperalgesia in response to NGF.

Zhuang *et al.* studied the development of hyperalgesia in the presence of NGF (Zhuang et al., 2004). They showed that 15 minutes after NGF injection into the paw, the animals developed hyerpalgesia as measured by paw withdrawal latency. The time to paw withdrawl from a noxious stimulus decreased if hyperalgesia had developed. However, if they pretreated the area of the paw before injection of NGF with an ERK inhibitor PD 98050 (10 µg), they are able to

block the development of hyperalgesia. While the results from these experiments are interesting, they are not well-controlled. For instance, the authors did not use this concentration of ERK inhibitor injected into the paw alone, and then test for paw withdrawal latency. Therefore, it is not clear that the differences are a result of the drug or of inhibition of NGF-induced increases in ERK activity.

PI3 kinase signaling

A second signaling cascade linked to TrkA signaling is the PI3 kinase pathway (Alessi et al., 1997). PI3 kinases are heterodimers composed of an 85-kDa regulatory subunit and a 110 kDa catalytic subunit. When NGF binds to the TrkA receptor, the regulatory subunit of PI3 kinase binds directly to the cytoplasmic domain of the neurotrophin receptor. The regulatory domain recruits its catalytic domain to the membrane. Once at the membrane, PI3 kinase phosphorylates phosphoinositides to produce phosphoinositide-3,4-diphosphate (PI-3,4-P_2) or phosphoinositide-3,4,5-triphosphate (PI-3,4,5-P_3). These two phospholipids recruit Akt, a serine/threonine kinase, to the membrane (Stephens et al., 1998). In addition to Akt, PI-3,4-P_2 and PI-3,4,5-P_3 recruit 3-phosphoinositide-dependent kinase (PDK1) to the membrane. PDK1 phosphorylates and activates Akt (Alessi et al., 1997).

The PI3 kinase pathway is implicated in regulation of sensitivity of sensory neurons by NGF (Bonnington and McNaughton, 2003; Zhuang et al., 2004; Zhang et al., 2005). PI3 kinase is expressed in sensory neurons from adult animals (Bartlett et al., 1999) and PI3 kinase is activated by NGF (Reynolds et al., 1998). In the study by Bonnington and McNaughton, they used neurons from neotnatal mice, and measured the percentage of cells that were sensitized with 100 ng/ml NGF for 2 minutes. The endpoint measured was the ratio of Ca^{2+} entry (measured with preloaded dye) during capsaicin stimulation compared to baseline. Sensitization was defined as an increase in the ratio by approximately 2

standard deviations above the mean. Using this endpoint, they were able to block NGF-sensitization in a majority of the cells tested by using 20 nM wortmannin, a PI3 kinase inhibitor. In a later study by this same group, they used heterologous expression systems to determine whether NGF exposure increased TRPV1 expression at the membrane surface in a PI3 kinase-dependent manner. They concluded that TRPV1 translocation accounts for the capsaicin-specific sensitization with NGF. These experiments were not repeated using dorsal root ganglion neurons to determine if TRPV1 did translocate to the surface of primary cells with brief exposure to NGF.

In another study, Zhuang *et al.* used adult sensory neurons in culture to examine various signaling pathways that might regulate responses to NGF in sensory neurons (Zhuang et al., 2004). They discovered that neurons exposed to NGF for 10 minutes had a significant increase in pERK levels. This change was dependent on PI3 kinase because when preincubated with LY294002 (100μM), another PI3 kinase inhibitor, subsequent exposure to NGF did not alter the levels of pERK in neurons in culture. The authors also showed the PI3 kinase inhibitor blocked changes in heat hyperalgesia that develop with NGF injection into the paw after 15 minutes.

Phosphorylation of Akt in sensory neurons after stimulation with NGF also has been reported (Bron et al., 2003; Zhuang et al., 2004). Bron *et al.* showed that after 3 days exposure to 50 ng/ml NGF, there is a significant increase in Akt phosphorylation in isolated sensory neurons as measured by Western blotting (Bron et al., 2003). Zhuang *et al.* use a much shorter time course, and show that

within 2 minutes of exposure to 100 ng/ml NGF, Akt phosphorylation increases (Zhuang et al., 2004). In addition, they show that when injected into the paw of an animal, NGF causes hyperalgesia within 15 minutes. The development of hyperalgesia is blocked by administration of the PI3 kinase inhibitor, LY294002. However, after 6 hours the NGF-induced hyperalgesia has disappeared in the group of animals not treated with LY294002, and Zhuang *et al.* did not measure phosphorylation of Akt at this time point. Therefore, the question arises whether Akt phosphorylation, which is shown to be active by Bron *et al.* for 3 days after NGF exposure, is truly responsible for this early hyperalgesia. Overall these studies are compelling and suggest that the PI3 kinase pathway is important in acute changes in sensory neurons in response to NGF, and that it may be important in regulating hypersensitivity that develops in animals in response to NGF. The experimental conditions described above are highly variable, and it is difficult to determine whether altering culture conditions or treatment conditions would yield consistent results. Based on their findings, the experiments contained in this thesis test the PI3 kinase pathway to determine if it is involved in the regulation of sensitivity of sensory neurons in culture.

PLC signaling

A third signaling cascade activated by NGF binding to the TrkA receptor is the PLC pathway. The isoform, PLC-γ, is recruited and binds to autophosphorylated TrkA receptor at tyrosine 785 (Vetter et al., 1991). This complex catalyzes the hydrolysis of phosphatidylinositol 4,5-diphosphate (PI 4,5-P_2) to inositol 1,4,5-P_3 (IP_3) and diacylglycerols (DAGs). Inositol 1,4,5-P_3 binds to multiple IP_3 receptors on the endoplasmic reticulum and causes increases in intracellular Ca^{2+}. DAGs bind to and activates multiple proteins, including protein kinase Cs (PKCs), Muncs, guanine nucleotide exchange factors, and DAG kinases (Rhee, 2001; Yang and Kazanietz, 2003). Each of these signaling molecules downstream of DAGs is expressed in sensory neurons. Of these downstream effectors, PKC is implicated in the increased sensitivity of sensory neurons (Barber and Vasko, 1996; Gold et al., 1998; Frayer et al., 1999; Cesare et al., 1999; Premkumar and Ahern, 2000). Work from the Vasko laboratory indicates that activation of PKC either in the sensory neuronal endings of the spinal cord slice or in sensory neurons in culture increases release of both SP and CGRP.

There are other correlative studies that test the actions of PKC on the TRPV1 channel. Using electrophysiological studies, it was determined that PKC phosphorylation of the TRPV1 channel increases its activity (Hall et al., 1995; Cesare and McNaughton, 1996; Vellani et al., 2004). In a heterologous expression system (oocytes), NGF application caused increased currents through TRPV1 channels in response to noxious heat and capsaicin (Chuang et

al., 2001). This increased current could be mimicked by addition of PLC to the preparation and blocked by the addition of anti-PLC antibody. However, it remains to be determined whether PLC or its downstream signaling proteins regulate the sensitization of sensory neurons in response to NGF.

Src kinase signaling

A fourth pathway implicated in TrkA signaling in sensory neurons is the Src kinase cascade. Src is a non-receptor tyrosine kinase that is associated with NGF activation of the TrkA receptor (Wooten et al., 2001). Src kinase associates with the adapter protein, Grb2 (Meakin et al., 1999; Tsuruda et al., 2004). Grb2 is the same adapter that is necessary in the activation of GEFs and the Ras protein in the Ras/MEK/MAPK signaling pathway.

There may be competition for a Grb2 binding at the activated TrkA receptor by the Ras and Src kinase cascades. Several factors, such as the compliment of proteins present in sensory neurons, the simultaneous exposure to other growth factors, or the levels of individual growth factors influence which pathway will become activated in the cell in response to agonist binding. Ohmichi *et al.* showed that about half of the Shc in PC12 cells was associated to Grb2 in response to NGF, and this proportion of bound Shc changed depending on the exposure of the preparation to other growth factors (Ohmichi et al., 1994). Their results support the notion that lend evidence that signaling pathways diverge, such as at the binding site of the adapter protein Grb2, and the level of activity of the pathways downstream is very sensitive to slight variations in the external milieu.

Once Grb2 binds to the TrkA receptor, Src kinase can bind at its SH3 binding domain. The kinase has been shown in PC12 cells to signal to a variety of proteins, including the mitogen activated protein kinase, JNK, or atypical forms of PKC, including PKCζ and PKCι (Wooten et al., 1994; Seibenhener et al.,

1999). Studies of Src in mammalian sensory neurons have just started to emerge. In mouse sensory neurons, blocking Src kinase activation by the inhibitor PP2 blocks the NGF-induced increase in Ca^{2+} influx that occurs with capsaicin stimulation (Zhang et al., 2005). These studies suggest that the acute effects of NGF, such as altering sensitivity to capsaicin stimulation, could be mediated by the Src kinase signaling cascade.

In another study by Igwe *et al.*, they showed that IL-1β-induced secretion of SP could be inhibited by inhibitors of Src kinase (Igwe et al., 2003). Furthermore, increase in expression of SP by IL-1β also could be inhibited by Src kinase. These data suggest that Src kinase, again, may be involved in regulation of acute changes in neurotransmitter release, but also may be involved in regulation of expression of neuropeptides over a longer time course.

p75 receptor and downstream signaling cascades

The p75 neurotrophin receptor ($p75^{NTR}$) is a member of the tumor necrosis factor alpha (TNF-α) family of receptors that binds NGF among other neurotrophins (Barrett, 2000). When $p75^{NTR}$ binds NGF, it activates either apoptotic or survival promoting signaling pathways, but it is not clear what defines the outcome with receptor activation (Huang and Reichardt, 2003). Although it has no intrinsic enzymatic activity, through a series of adapter proteins, $p75^{NTR}$ is known to activate neutral and acidic sphingomyleinase (N-SMase and A-SMase, respectively) (Dobrowsky et al., 1994; Dobrowsky et al., 1995). Early studies on the sphingomyelin signaling cascade showed that ceramide acts as a second messenger (Hannun, 1994), and can activate phosphatases, PI3kinase and atypical PKC isoforms (Dobrowsky and Hannun, 1994; Roux, 2001) Ceramide is further metabolized by ceramidase to form sphingosine (Roux et al., 2001). Sphingosine can be phosphorylated by sphingosine kinase, an enzyme present in dorsal root ganglion neurons (Toman et al., 2001; Toman et al., 2004), to form sphingosine-1-phosphate (S1P). Sphingosine 1-phosphate is a known ligand of S1P receptors (Taha et al., 2004).

There are indications that this pathway is important in regulation of the sensitivity of sensory neurons. Both ceramide and S1P have been shown to regulate the excitability of sensory neurons in response to NGF, and inhibiting the $p75^{NTR}$ with a blocking antibody inhibits NGF-induced increases in excitability (Zhang et al., 2002; Jiang et al., 2003; Zhang and Nicol, 2004; Zhang et al., 2006). It has been shown in both hippocampal and sympathetic neurons that

NGF treatment increases intracellular ceramide (Brann 1999; Song and de Chaves, 2003). In adult sensory neurons, exposure to ceramide increases the excitability of sensory neurons. Conversely, blocking the $p75^{NTR}$ with an antibody blocks the ceramide-induced increases in actions potentials, but this inhibition can be overcome with increases in ceramide concentrations used (Zhang et al., 2002; Zhang and Nicol, 2004).

Sphingosine can be phosphorylated by sphingosine kinase to form spingosine-1-phosphate (Toman and Spiegel, 2004). Sphingosine 1-phosphate activates a number of enzymes implicated in regulating sensitivity of neurons, including phospholipase D (PLD) and PKC isoforms (Natarajan et al., 1994; Meacci et al., 1999; Roux et al., 2001). However, it is not known if this pathway regulates the NGF-induced increase in iCGRP release from sensory neurons. It is important to understand because the focus of most literature regarding acute effects of NGF focus on the TrkA neurotrophin receptors, and the $P75^{NTR}$ pathway may be a completely separate, but equally important means to control the sensitivity of sensory neurons.

Signaling pathways implicated in regulation of neuropeptide content

In studying release of neuropeptides from sensory neurons, it is important to consider the expression levels of SP and CGRP because increased content will lead to increases in stimulated release (Malcangio et al., 1997; Fehrenbacher, 2005).

It is well established that NGF increases expression both SP and CGRP in neonatal and adult dorsal root ganglion cells in culture (Adler et al., 1984; Lindsay and Harmar, 1989; Lindsay et al., 1989). In these studies, neurons were removed and cultured immediately with NGF. They found NGF (25-100ng/ml) increased cellular content of CGRP and substance P (SP), and increased in the mRNA, after 1-3 days in culture. Furthermore, if NGF was not added, there was a significant loss of peptides after 2-4 days. These data indicate NGF may be necessary for the maintenance of expression of these peptides.

Multiple studies demonstrate that sciatic nerve transaction in the rat caused decreases in the content of neuropeptides in dorsal root ganglion. Application of NGF to the stump of the nerve fiber reverses the loss of peptide and increase its expression in DRG (Fitzgerald et al., 1985; Goedert et al., 1981; Inaishi et al., 1992; Verge et al., 1995). The increase in expression of peptides in the dorsal root ganglion neurons is also observed with CFA injection or NGF injection into the plantar surface of rat paws (Donnerer et al., 1992; Leslie et al., 1995).

To study the cellular mechanisms of regulation of CGRP expression in response to NGF, the promoters for the CGRP gene must be understood. Knowledge of its structures helps to gain insight into the cellular mechanisms of regulation of CGRP expression in response to NGF (Tverberg and Russo, 1992; Tverberg and Russo, 1993; Durham and Russo, 2003c). The CGRP gene has a minimal promoter region of approximately 150 bases upstream of the gene that is necessary for the activation of CGRP transcription by NGF. Mutation of this

promoter region blocks transcription activity (Watson and Latchman, 1995; Watson et al., 1995; Freeland et al., 2000). The region contains a cyclic AMP response element (CRE), which is activated by cAMP response element binding protein (CBP). When CBP is phosphorylated by the p42/44 MAP kinase in response to NGF, CBP can induce transcription of a variety of proteins (Liu et al., 1998). However, speculating how NGF might control transcription of proteins in DRG neurons based on activity in PC12 cells, may not be appropriate.

It is known that in primary sensory neurons, that NGF signals to a variety of pathways. In PC12 cells, the Ras/MEK/Erk cascade is essential to differentiation of these cells, including reduction of proliferation and increased neurite outgrowth (Ng and Shooter, 1993; Basu et al., 1994; Ganju et al., 1998; Freeland et al., 2000; Rong et al., 2003). These physiological changes which occur in response to NGF are attributed to Ras (Wood et al., 1992). A question that remains is which intracellular signaling pathways in DRG neurons regulate the change in CGRP content upon exposure to NGF. The Ras/MEK/ERK cascade is implicated because other MAPKs have been shown to regulate the activation of the promoter region in PC12 cells and increase transcription. However, no studies directly test whether Ras induces the change in CGRP content in sensory neurons through the MEK/ERK cascade.

GTPases and GTPase Activating Protein (GAPs)

Overview of function of GTPases and GAPs

Functions of GTPases and GAPs in neurons: Ras

The Ras family proteins are small GTP-binding proteins (20-40kDa) that cause tumor formation with inappropriate activation (Takai et al., 2001). However, their normal functions in neurons are still being determined. The mechanisms of Ras activation, inactivation, and downstream effectors are well-established. In cells, Ras cycles from an active to inactive state depending on whether it is bound to GDP or GTP (Fig. 3).

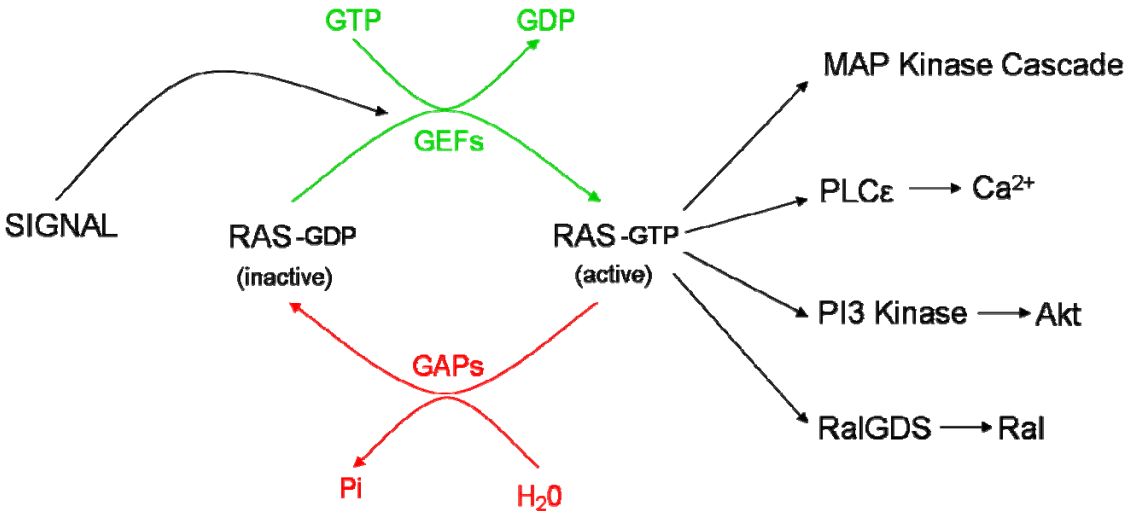


Figure 3. The Ras cycle. Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs).

Ras activation is involved in the effects of neurotrophic factors on sensory neurons. Ng *et. al.* demonstrated exposure of primary sensory neurons in culture to NGF (10-50 ng/ml) increases Ras-GTP in cells after a five-minutes (Ng and Shooter, 1993). Bron *et. al.* microinjected a mutant, constitutively activated Ras or a mutant dominant negative Ras isoform into mouse sensory neurons, and this resulted in an increase and decrease of TRPV1 expression, respectively (Bron et al., 2003). Bonnington and McNaughton used an inhibitor of Ras activation, the Sos inhibitory peptide, and MEK inhibitor, U0126, which did not block sensitization of sensory neurons exposed to NGF as measured by Ca^{2+} uptake in cells (Bonnington and McNaughton, 2003).

These studies did not explore changes that might occur in neurotransmitter content and release. Release of neurotransmitter is the primary means of communication by sensory neurons to the CNS. Studies on activation of Ras in sensory neurons and how this protein affects CGRP content and release may be very important in understanding how Ras signaling may regulate nociception.

Regulators of Ras activity and downstream effectors in neurons

Major regulators of Ras activity in cells are GTPase activating proteins (GAPs) They accelerate hydrolysis of GTP and turn off Ras signaling (Scheffzek et al., 1997; Bernards, 2003). Mutations in a specific GAP, called neurofibromin, result in the human disease neurofibromatosis (NF) (Donovan et al., 2002). Patients with NF develop certain types of cancers, benign tumors (neurofibromas), and learning disabilities, which are attributed to deregulated Ras activity. Discrete dermal neurofibromas arise

specifically from the Schwann cell surrounding neurons and are associated with pain and itching (Packer et al., 2002).

A recently discovered, neuronal-specific GAP, called synGAP, is known to be an important negative regulator of Ras in neurons (Komiyama et al., 2002b; Kim et al., 2003). SynGAP is present in neurons of the central nervous system, such as hippocampal and cortical neurons, but it may be present and functioning in sensory neurons as well. Thus, manipulation of this regulatory protein, SynGAP, could be used as a mechanism to alter Ras signaling and measure changes that might occur in neurotransmitter content and release from sensory neurons.

In addition to enzymes that act directly on Ras to modify its function, such as GEFs and GAPs, post-translation modification of Ras, namely farnesylation, must occur in order for Ras to be localized at the membrane and have activity (Sebti and Hamilton, 1997). The enzyme that regulates the addition of the lipid moiety, farnesyl, to the Ras protein is farnesyltransferase (FT). Farnesyltransferase is an enzyme that transfers the hydrocarbon chain to the C-terminal end of the Ras protein. The lipid moiety is attached to a cysteine residue in a specific sequence, CAAX (Sebti and Hamilton, 1997). The last three residues, AAX are cleaved from the protein, and the cysteine residue is methylated to stabilize the protein. The farnesyl group then intercalates into the membrane and Ras is in the correct orientation for activation by GEFs.

RasGTP activates specific downstream effectors in neurons, including the Raf/MEK/ERK pathway, phosphoinositol-3-kinase (PI3K), and phospholipase Cε (Cullen and Lockyer, 2002). Multiple studies implicate these pathways in regulation of sensitivity of sensory neurons (see above).

Evidence indicates the MAPK cascade regulates expression of CGRP in sensory neurons. Durham *et. al.* identified increases in MAPK activity increased CGRP expression in sensory neurons of the head and neck (Durham and Russo, 2000; Durham and Russo, 2003). In other studies, Ma *et. al.* found that chronic exposure of primary sensory neurons in culture to morphine increased both MAPK activation and CGRP content. By selectively blocking MAPK in the presence of morphine, they blocked increases in CGRP suggesting MAPK controls CGRP expression (Ma et al., 2001). However, there are few studies on the control of CGRP expression by other downstream effectors of RasGTP, such as Akt (PKB) and PKC. Many studies show mediators both upstream and downstream of Ras are involved in expression changes, but the field lacks a direct link between Ras and changes in both peptide release and content. The experiments in this thesis explore whether Ras activation and its downstream effectors control changes in the release of peptide through changes in content in primary sensory neurons in culture.

Mechanism of action of dominant negative/ constitutively active isoforms of Ras

As described above, GAPs and GEFs regulate the activity of Ras (Bollag and McCormick, 1991; Bollag and McCormick, 1991; Donovan et al., 2002). Mutation of the one of the isoforms, H-Ras, at critical residues has allowed the development of dominant negative or constitutively active isoforms of the protein. The dominant negative isoform, termed Ras S17N, or just 17N, has a serine converted to an asparagine at residue 17 (Feig and Cooper, 1988; Stacey et al., 1991; Segal et al., 1993). This mutation causes 17N Ras to bind with greater affinity to GDP. When the

mutated protein binds to GEF, it does not allow the exchange of GDP for GTP. Ras17N and the GEF form a stable, but inactive complex that inhibits native Ras activation. Thus, expression of this mutant isoform is dominant although native Ras is still expressed. The S17N mutation of H-Ras also blocks the GTP exchange for the other isoforms, K-Ras and N-Ras (Matallanas et al., 2003).

The constitutively active isoform, Ras61L, has a glutamine to leucine mutation. This mutation disrupts the GTPase activity of the protein. Once Ras is activated by its GEF, the mutated GTPase remains active because GTP hydrolysis by the protein cannot occur. These two mutations provide a specific tool to increase and decrease the activity of Ras in neurons. The mutated proteins will be used to determine if Ras signaling affects the acute sensitizing actions of NGF or the upregulation of expression of CGRP and SP in sensory neurons.

Specific Aims of the Thesis

The studies in this thesis will investigate the intracellular signaling pathways that regulate the observed effects of NGF on sensory neurons, namely the increased expression of peptide neurotransmitters with long exposure, or the increase in peptide release with just brief exposure to NGF. Understanding which signaling pathways control these responses of sensory neurons may lead to new ideas about future drug targets and therapeutics to attenuate acute and/or chronic pain that develops with inflammation and injury. Therefore, the specific aims are:

1. To determine if the Ras signaling pathway mediates NGF-induced increases in CGRP content in sensory neurons.

2. To determine which signaling effectors downstream from NGF receptors regulate the ability of NGF to augment capsaicin-evoked release of CGRP that cannot be accounted for by changes in CGRP content.

MATERIALS AND METHODS

Materials

Unless otherwise specified, all chemicals were from Sigma-Aldrich (St. Louis, MO). Nerve growth factor (7S) was purchased from Harlan (Indianapolis, IN). LY294004, wortmannin, PP2, and PP3 were from EMD Biosciences (San Diego, CA). C-2 ceramide and D-*erythro*-sphingosine-1-phosphate were from Avanti Polar Lipids (Alabaster, AL). Tissue culture plates and 96-well plates for absorbance measurements were from Becton-Dickinson (San Jose, CA). F-12 media, horse serum, antibiotics, proteinase K, OptiMEM I®, and Normocin-O™ were from Invitrogen (Carlsbad, CA). Bovine IgG protein, Bradford reagent, pre-cast polyacrylamide gels, PVDF membranes, Laemmli buffer, HRP-conjugated goat anti-mouse and anti-rabbit antibodies were from Bio-Rad (Hercules, CA). CGRP was from Tocris (Ellisville, MO). Radiolabeled NaI (NaI^{125}) for iodination of CGRP and Western Lightning® chemiluminescent HRP substrate solution were from Perkin-Elmer (Shelton, CT). Radiographic film was from RPS imaging (Michigan City, IN). LI-COR Odyssey blocking solution was from LI-COR Biosciences (Lincoln, NE). NeuroPorter® was from Gene Therapy Systems (San Diego, CA). Plasmids, antibiotics, and chemically competent *E.coli* were from Invitrogen (Carlsbad, CA). Restriction enzymes described in this section were from New England Biolabs (Ipswich, MA).

Use of Laboratory Animals

The Animal Care and Use Committee at Indiana University School of Medicine, Indianapolis, IN approved all procedures used in these studies. Adult male Sprauge-Dawley rats were purchased from Harlan (Indianapolis, IN). Mice with a heterozygous mutation of sygGAP were generously donated by Dr. R. Huganir (Johns Hopkins University) and bred in the Indiana University Laboratory Animal Research Center.

Isolation of adult sensory neurons from rats and mice

Adult dorsal root ganglion cultures were prepared as previously described (Lindsay et al., 1989; Southall et al., 2002). Adult male (150-175 g) Sprauge-Dawley rats and age and sex-matched adult (10-15 weeks) mice were euthanized by CO_2 asphyxiation. Dorsal root ganglia from the cervical to sacral levels of the entire spinal column were dissected, incubated in F-12 media containing 0.01% collagenase for 2 hours in 3% CO_2 at 37ºC, and mechanically dissociated. Approximately 15,000 cells were plated into each well (22 mm diameter) of a 12-well culture plate or 7,500 cells were plated into each well (16 mm diameter) of a 24-well culture plate. All culture plates were precoated the day prior to DRG isolation. Wells were first treated with poly-D-lysine (1 mg/10ml; 70,000-150,000 MW) for 1 hour, rinsed once with ddH_2O, and then exposed to laminin (1 mg/ml) overnight. The cells were maintained in F-12 media

supplemented with 10% horse serum, 2 mM glutamine, 100 µg/ml normocin-O™, 50 µg/ml penicillin, 50 µg/ml streptomycin, 50µM 5-fluoro-2'-deoxyuridine, 150µM uridine, and the indicated amount of NGF in ng/ml. Cells were placed in an incubator at 3% CO_2 at 37ºC. Growth medium was changed every other day. Cells were used 7-11 days after plating.

In Vitro Release of iCGRP from Culture of DRG Neurons

Release studies were performed on cells as previously described (Hingtgen and Vasko, 1994; Vasko et al., 1994). The neuronal cultures were washed once with HEPES buffer consisting of 25 mM HEPES, 135 mM NaCl, 3.5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 3.3 mM D-glucose, and 0.1% bovine serum albumin, pH 7.4. The cells were maintained at 37˚C, and then incubated for successive 10 min intervals with 0.4 ml of the same buffer in the absence or presence of drugs. Basal or resting release was determined by exposing the cells to HEPES buffer alone, or buffer in the presence of inhibitory or activating drugs as indicated for each group of experiments. Drugs included U0126 (1µM), PDBu (10 or 100nM, as indicated), LY294004 (3µM), wortmannin (3nM), PD98059 (20 and 200µM), U73122 (15µM), PP2 (5µM), or bisindolylmalemide I (30 or 100nM). Drugs not soluble in water were prepared in methylpyrrolidinone (MPL). The maximal concentration of MPL was 0.1% by volume as this concentration was the lowest measured concentration of vehicle that had no affect on iCGRP release from sensory neurons in culture. In all instances, the maximum

concentration of drug used in experiments was included in a standard curve to assure that the drug did not influence the results of the radioimmunoassay. Cells were then exposed for a second 10 min incubation to NGF (100ng/ml) in the presence or absence of inhibitory drugs. To determine evoked release, the second incubation was followed by a 10 min incubation with the same concentration of a drug and/or NGF in the presence of 10 or 30nM capsaicin as indicated. Cells then were exposed to HEPES buffer without drugs for 10 min incubations to reestablish resting release. During incubations, the cells were maintained at 37°C. After each incubation, the buffer was removed to measure the amount of iCGRP using radioimmunoassays (RIAs) as described below.

For chronic treatment of cells with the farnesyltransferase inhibitor, FTI-276, 10 mM stock was prepared in MPL and 10 mM DTT, which was stored at -80˚C covered in argon. Neuronal cultures were treated for 4 days with vehicle (0.05% MPL with 5 mM DTT) or with FTI-276 (5μM), changing the media every other day. Subsequently, neurons were exposed to the indicated concentration of NGF in the presence or absence of FTI-276 for 4-5 more days. *In vitro* iCGRP release experiments were then performed as described above.

In some experiments, release values were normalized to total content. In order to correlate the iCGRP release from a single well to the iCGRP content of that well, the content of a single well was determined. After the release experiment, each well was incubated with 400 μl 0.1N HCl in ddH_2O for 10 min at room temperature on an orbital shaker. An aliquot from each well was diluted

and assayed for the iCGRP content of that well using radioimmunoassay as described above. The release value then was normalized to the content.

Radioimmunoassay of Immunoreactive Calcitonin Gene-Related Peptide

The amount of immunoreactive CGRP (iCGRP) was measured using radioimmunoassay as previously described (Vasko *et. al.*, 1994). Samples with known amounts of CGRP, or standards (0-250 fmol), were prepared for each assay. Standards were prepared in duplicate. Experimental samples containing unknown amounts of CGRP were also prepared in duplicate. The volumes of all standards and unknown samples were 300 µl. Added to each sample was 25 µl of a 1:70,000 dilution of CGRP antibody (a generous gift from Dr. M. Iadarola, NIH) and 25 µl of ^{125}I-[Tyr^{0}] CGRP. The samples were allowed to equilibrate at 4˚C for 16-20 hours. After equilibration, 0.5 ml of a 0.1 M phosphate buffer (pH 7.4) containing 1% charcoal was added to each sample, and the mixture was centrifuged at 1,500 x *g* for 10 minutes using a Beckman-Coulter Allegra™ 6R Centrifuge (Fullerton, CA). The charcoal absorbs and binds any CGRP not bound to antibody. The samples were centrifuged at 3000 RPM for 10 min to pellet the charcoal. The supernatant contains CGRP bound to antibody. The supernatant was decanted to new tubes, and the radioactivity was measured in these samples using gamma scintillation spectrometry with a Cobra II Auto-Gamma from Perkin-Elmer (Shelton, CT). The amount of iCGRP in experimental samples was determined by 4 point least squares linear regression analysis using the CGRP standards. The amount of iCGRP was normalized to the protein level in the aliquot. The concentration of protein in an aliquot of that sample was measured prior to radioimmunoassay as described below for Western blotting.

Western Blotting and Densitometry

To collect cells for lysis and Western blotting, they were scraped from wells into ice-cold lysis buffer which was a modified RIPA buffer using a rubber policeman and placed into eppendorf tubes. The RIPA buffer contained the following: 50 mM Trizma base, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 µg/ml pepstatin, 1 ug/ml leupeptin, 1 ug/ml aprotinin, 1 mM Na_3VO_4, and 25 mM NaF. Cells were pelleted in a microcentrifuge tube at 14000 x *g* at 4˚C, and the supernatant was removed. The cells were resuspended in 25 µL of ice-cold RIPA buffer, and then cells were sonicated at 4˚C for 15 seconds on ice using a 550 Sonic Dismembrator from Fisher Scientific (Pittsburg, PA). A dilution of each sample lysate was prepared in ddH_2O.

To measure the protein level in each sample, standard concentrations (0-500 mg/ml) of bovine IgG protein were prepared in ddH_2O for absorbance measurements. Samples of the standards or samples of the unknowns were mixed in clear plastic 96-well plates with 1:4 dilution of Bradford reagent according to the manufacturer's instructions. When protein reacts with the Bradford reagent a color change occurs in direct proportion to the amount of protein present in the sample. After mixing, samples were allowed to incubate in the plate at room temperature for 2-3 minutes. The absorbances of the samples were measured at 595nm using a SpectraFluor Plus spectrophotometer with Magellan 5.03 software from Tecan (Crailsheim, Germany). A standard curve

was generated using the absorbance readings from the known protein samples, and linear regression analysis was used to calculate the protein concentrations from the absorbance measurements of the unknown samples.

Samples were loaded onto a 10% precast polyacrylamide gel and electrophoresis was performed at 75V for 1hr. Proteins were transferred onto a PVDF membrane at 100V for 1hr. The membrane was washed twice with ddH_2O and then incubated in freshly prepared TBST-M for 30 minutes at room temperature with constant agitation. The membrane was incubated overnight at 4°C with agitation using primary antibodies as necessary in freshly prepared TBST-M. The membrane was washed with TBST two times for 10 minutes each. The membrane was incubated for 1 hour at room temperature with agitation in 1:1000 dilution of HRP-conjugated goat anti-mouse antibody in TBST-M. The membrane was washed 3 times using TBST for 10 minutes each. To detect RasGTP or total Ras present on PVDF membranes, Western Lightning® chemiluminescent HRP substrate solution was used. Membranes were incubated with the solution for 1 minute and then signal was detected on radiographic film. Signals present on film were scanned into the Adobe Photoshop 7.0 program (Mountain View, CA) using a Hewlett-Packard Scanjet 5470c scanner (Palo Alto, CA), and densitometric analysis was performed using QuantityOne® software from Bio-Rad (Hercules, CA). The signal from the protein of interest, in this case, Ras, was normalized to the signal from the loading control such as actin.

To measure the amount of farnesylated Ras and unfarnesylated Ras, cells from neuronal cultures were collected for Western blotting as described above.

However, instead of using a 10% polyacrylamide gel, a 15% gel was used to resolve the unfarnesylated Ras from farnesylated Ras proteins. Electrophoresis was performed at 75V over 2 hours or until the Ras proteins had nearly reached the end of the gel as indicated by a pre-stained protein ladder (Invitrogen). All other methods for Western blotting were identical. Using the 15% gel, Ras appears as two bands. The band at the lower molecular weight represents prenylated Ras, whereas the band at the higher molecular weight is unprenylated Ras. Densitometry was used to measure both bands, and a ratio of the two was determined. The ratio was normalized to signal from lysates of cells that were not treated with FTI-276. The PVDF membranes from these experiments were also probed for actin. The expression of actin was quantified using densitometry. The total density of both Ras bands was normalized to the signal for actin to determine if there were changes in Ras expression. The results were reported as a fraction of the non-treated controls.

Measurement of RasGTP in Neuronal Cultures

Ras activity assays were conducted using a Ras Activation Assay Kit from Upstate Biotechnology (Lake Placid, NY) (Chen et al., 1997). Cells were exposed to NGF ranging from 0 to 250 ng/ml for 7 days. Neurons were then collected into Eppendorf tubes on ice by scraping them from plates with a rubber policeman into ice-cold Mg^{2+} lysis buffer: 125 mM HEPES, pH 7.5, 750 mM NaCl, 5% Igepal CA-630, 50 mM $MgCl_2$, 5 mM EDTA, 10% glycerol, 10 µg/ml aprotinin, 10 µg/ml

leupeptin, 1 mM Na_3VO_4, and 25 mM NaF (0.5 ml of lysis buffer per 150 mm tissue culture plate). The cells were gently pipetted up and down to completely lyse them in suspension, and then lysates were cleared of nuclear debris by centrifugation at 4˚C and 14,000 x *g* for 5 minutes. Supernatants were transferred to new Eppendorf tubes and kept on ice. For positive and negative controls, either 2.5 µl of 10 mM GTPγS or 2.5 µl of 100 mM GDP, respectively, was added to 250 µg of protein from control samples. The samples were incubated at 30˚C while gently rocking for 30 minutes to allow the Ras binding to either GTPγS or GDP to reach equilibrium, and then the reaction was stopped by addition of 60 mM $MgCl_2$. To determine the amount of RasGTP in each sample, 10 µl of the GST fusion protein, RBD, bound to glutathione agarose beads was added. The samples were gently mixed on a rocker at 4˚C for 45 min. The agarose beads were pelleted for 10 sec at 14,000 x *g* and 4˚C. The supernatant was removed and discarded. The beads of each sample were washed three times with 100 µl of the Mg^{2+} lysis buffer. The samples then were boiled in 40 µl of Lamelli buffer which contained: 125 mM Tris-HCl, 10% glycerol, 10% SDS, 1 M DTT to release bound RasGTP from the beads. After boiling, the beads were pelleted briefly for 1 min at 14,000 x *g* and the supernatant was transferred to a new tube for assay by Western blotting.

Western blotting was performed as described above for on samples incubated with RBD agarose to detect the portion of Ras bound to GTP. A second aliquot of sample that had not been incubated with RBD was used for Western blotting to detect total Ras. Mouse anti-Ras antibody (provided in the kit)

was diluted to 1 µg/ml in freshly prepared TBST-M. HRP-conjugated goat anti-mouse secondary antibody was used at a 1:1000 dilution of in TBST-M. Detection and quantification were performed as described above. The RasGTP was measured relative to the total Ras protein in each sample. The values for each sample were normalized to the value for the sample incubated with GTPγS (positive control).

Development of Lentivirus Expressing Dominant Negative or Constitutively Active Ras Isoforms

The dominant negative (dnRas, S17N isoform) and the constitutively active (caRas, Q61L isoform) were generous gifts of Dr. L. Quilliam, Biochemistry Department, Indiana University School of Medicine. Fragments containing the dnRas and caRas sequences were amplified by PCR from the original plasmids with restriction sites added that were compatible with the multiple cloning site of the pIRES2-EGFP vector. The sites added were *EcoRI* and *SacII*. The PCR primers used to add the two restriction sites were 5'-CCGGAATTCACCATGACGGAATATAAGCTG-3' and 5'-ATTAGGCCGCGGTCAGGAGAGCACACACTT-3'. The PCR protocol was 95°C for 3 minutes, followed by 30 cycles with the following conditions 94°C for 1 minute; 57°C for 1 minute, and then 65°C for 2.5 mintues. These cycles were followed by an elongation phase of 65°C for 10 minutes. Products were resolved on a 1% agarose gel. Figure 4 shows an agarose gel demonstrating PCR

products at approximately 0.6 kb (Ras mutant) and at approximately 6.0 kb (undigested plasmid).

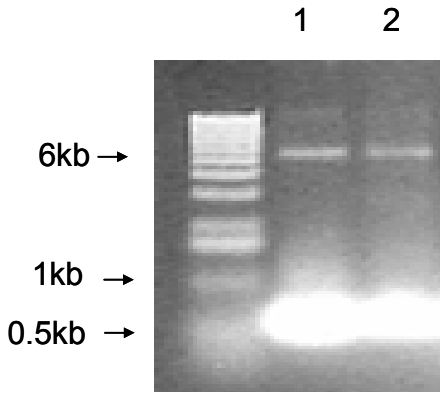


Figure 4. PCR amplification of Ras mutants inserted into the pIRES2-EGFP vector. The left lane is a 1kb ladder. Lane 1 is the Ras PCR product amplified from vector containing the dnRas isoform. Lane 2 is the Ras PCR product amplified from vector containing the caRas isoform. The bright band in lanes 1 and 2 are PCR products resulting from the amplification of the Ras insert (574bp), while the faint band above 6kb is likely the uncut supercoiled plasmid template.

After digestion with *EcoRI* and *SacII* enzymes, the PCR fragment was inserted into the pIRES2-EGFP vector via ligation using T4 DNA ligase according to the manufacturer's instructions. The plasmids were then transformed into OneShot® Top10 *E. coli* following the manufacturer's instructions. Colonies were selected based on kanamycin resistance. Plasmid DNA was isolated from bacterial cultures grown from original kanamycin-resistant colonies using a Hurricane® Mini-prep kit (Gerard Biotech, Oxford, OH). The appropriate Ras fragment was sequenced to ensure there were no mutations generated during PCR or during insertion of constructs into plasmids. The sequencing primers were 5'-TAACAACTCCGC CCCATTGACG-3' and 5'-GACGGCAATATGGTGGAAAATA-3'. Sequencing was performed at the

Biochemistry Biotechnology Facility of the IU School of Medicine, and the results were analyzed using Chromas 2.31 chromatogram viewer software (Technelysium, Australia).

The dnRas-IRES-EGFP or a caRas-IRES-EGFP constructs were subcloned into an entry vector, pENTR 1A. First, the two constructs were cut from the pIRES2-EGFP vector using *EcoRI* and *NotI*. The pENTR 1A vector was cut with the same enzymes. The digested products were resolved on a low melting point 0.8% agarose gel. The fragments were gel purified using a S.N.A.P. ™ Gel Purification Kit (Invitrogen) per manufacturer's instructions. The individual Ras constructs were inserted into separate pENTRA 1A vectors via ligation using T4 DNA ligase according to the manufacturer's instructions. The plasmids were then transformed into OneShot® Top10 *E. coli* per manufacturer's instructions. Colonies were selected by kanamycin resistance, selected as described above, and plasmid DNA was isolated from cultures using a Hurricane® Mini-prep kit (Gerard Biotech, Oxford, OH).

The Gateway® LR Clonase™ II reaction was used to move the isolated dnRas-IRES-EGFP or the isolated caRas-IRES-EGFP construct into the lentiviral transfer vector, pCSCGW. The construct was transformed into STBl3 *E. coli* and then selection was performed using zeocin resistance. Plasmid was isolated using the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA). An aliquot of plasmid was used for PCR to ensure the Ras insert could be amplified from the destination vector (Fig. 5). The same primers and PCR protocol from the above amplification of Ras were used. Products were resolved on a 1% agarose gel.

With confirmation that the destination vector contained the Ras insert, the remaining plasmid was used for production of lentivirus containing dnRas-IRES-EGFP or containing caRas-IRES-EGFP (Vector Production Facility, IU School of Medicine).

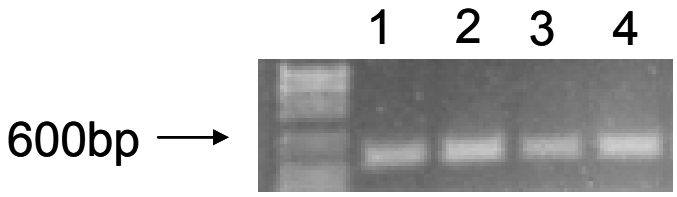


Figure 5. PCR amplification of Ras mutant isoforms inserted in the pCSCGW destination vector. The left lane is a 100 bp ladder, with the 600 bp position indicated by the arrow. Lanes 1 and 2 are amplified from vector containing the dnRas isoform, while lanes 3 and 4 are amplified from vector containing the caRas isoform. These data indicate that the Ras mutants are inserted into the destination vector used to produce the lentivirus.

To determine the quantity of lentivirus particles contained in solution produced by the Vector Production Facility, IU School of Medicine, the QuikTiter™ Lentivirus Quantitation Kit (HIV p24 ELISA) was used per manufacturer's instructions (CellBioLabs, San Diego, CA). Because of the similarity between human immunodeficiency virus (HIV) and lentivirus, the quantification of lentivirus is based on quantification of the HIV-1 p24 core protein (Naldini, 1998). There are approximately 1.25×10^7 lentivirus particles per 1 ng p24 core protein, and there are approximately 10^3 lentivirus particles per plaque forming unit (pfu). A standard curve of known amounts of p24 protein was generated. Serial dilutions of viral solutions with unknown amounts of particles were prepared. All samples including standards were prepared in duplicate. Samples were loaded into wells pre-coated with anti-p24 antibody and allowed to

equilibrate with gentle rocking at 4˚C overnight. The wells were washed, and cells were incubated with biotin-conjugated goat anti-p24 antibody for 1 hour at room temperature on an orbital shaker. Wells were washed and incubated with the provided streptavidin-enzyme conjugate for 1 hour at room temperature on an orbital shaker. Wells were washed and substrate solution was added to each well for 20 minutes at room temperature on an orbital shaker. The reaction was stopped by adding an acid solution provided in the Lentivirus Quantitation Kit and the absorbances of the wells were measured at 450 nm. Using the colorimetric ELISA assay and the standard curve, the number of viral particles in the unknown samples was determined using linear regression analysis, and the pfu were calculated.

Lentivirus Infection

Cells were incubated in F-12 media with or without added NGF for 2 days after dissection from animals. After 2 days, cultures were exposed to lentivirus diluted in media at a final concentration of 150 pfu for 48 hours. After 2 days, media containing virus was removed and fresh F-12 media with or without added NGF was added to wells. Media was changed every other day for 7 days. Cells were visualized using a Nikon TE2000 inverted microscope with a 75W Xenon lamp (Japan) coupled to an Olympus camera with MagnaFire SP 2.1B software (Fort Gibson, OK). A filter to view green fluorescene was employed to determine if cells were producing EGFP. After 7 days of infection with lentivirus expressing

EGFP or EGFP and Ras mutants, the expression levels of Ras were determined in neuronal cultures. Cells were collected and Western blotting was performed as described above for Ras protein in lysates. Immunoblotting was performed using mouse anti-Ras antibody (1:1000 dilution) (Upstate, Lake Placid, NY) or mouse anti-actin antibody (1:2000) (Upstate) in freshly prepared TBST-M. Secondary antibody was a 1:5000 dilution of HRP-conjugated goat anti-mouse antibody (Bio-Rad) in TBST-M. Densitometry was used to quantify the signals as described below. The Ras signal was normalized to the actin signal, and values were reported as a ratio of uninfected samples.

Genotyping synGAP mice

To determine if mice were homozygous or heterozygous for synGAP, each animal was genotyped. Genomic DNA was isolated by digesting 3 mm whole tail tissue in 100 µL ddH_20 in the presence of 100 µg/ml proteinase K at 55 ºC for 3 hr. The whole cell lysate was used as template. The following primers sets were added to each PCR reaction to determine which alleles of the synGAP genes were present in each mouse, (1) synGAP forward primer 5'-ACCTCAAATCCACACTCCTCTCCAG-3', (2) synGAP reverse primer, 5'-AGGGAACATAAGTCTTGGCTCTGTC-3', and (3) the primer for neomycin resistence gene insert in mutants 5'-ATGCTCCAGACTGCCTTGGGAAAAG-3' (Invitrogen). PCR cycles started with DNA denaturation at 95ºC for 2 minutes followed by 35 cycles of the following three conditions: 95ºC for 1 minute, 60ºC

for 1 minute, and then 72ºC for 20 seconds. These cycles were followed by an elongation phase of 72ºC for 5 minutes. PCR products were resolved on a 2% agarose gel. If there was a single fragment at 500 bp this indicated the wildtype condition, while a fragment at both 300 and 500 bp indicated truncation of one allele of synGAP and the heterozygous condition (Fig. 6). Images were acquired using a Kodak EDAS 290 camera with Kodak ID 3.6 software.

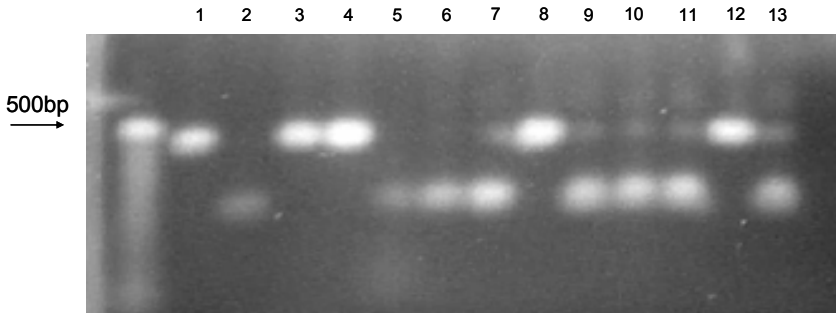


Figure 6. Sample agarose gel demonstrating PCR products from the DNA of 13 different animals. Genomic DNA was used as PCR template, and PCR products were resolved on a 2% agarose gel. The left lane demonstrates a base pair ladder, with the bright band at approximately 500 bp. Lanes containing a single band at 500bp indicates the wildtype condition, with two normal alleles for the synGAP gene. Lanes containing a band at 300bp (with or without a faint band at 500bp) indicate the heterozygous condition, as animals have one normal wildtype allele and one truncated allele for the synGAP gene.

CellTiter 96® AQueous One Solution Assay for Cell Viability

To determine if treatment for 7 days with FTI-276 affects cell viability, which in turn could affect the measured levels of iCGRP, we measured the relative numbers of metabolically active cells in culture in the presence or absence of drug. The CellTiter 96® AQueous One Solution Assay from Promega

(Madison, WI) was used to measure cell viability. In this assay, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) which is a tetrazolium compound. MTS is taken up by cells and bioreduced to a colored formazan product by dehydrogenase enzymes and NADPH or NADH in metabolically active cells. The formazan product is soluble in tissue culture medium, and its quantity as measured by the absorbance at 492 nm is directly proportional to the number of living cells in culture. Absorbance was measured using a SpectraFluor Plus spectrophotometer with Magellan 5.03 software from Tecan (Crailsheim, Germany).

In these experiments, coated wells containing no cells were used as blanks for the spectrophotometer. As a positive control for cell death, cells were killed by 10 min exposure to 4% paraformaldehyde in PBS. Other controls included wells treated with media alone or vehicle (0.1% MPL). The remaining wells were treated with 1, 10, or 30μM FTI-276 for 7 days. Cells then were incubated with the MTS substrate for 4 hours according to the manufacturer's instructions. Absorbance in wells exposed to FTI-276 was normalized to the values for wells of cells treated with media alone.

These procedures were repeated for cells treated for 7 days with the MEK inhibitor, U0126. At 492 nm there was very little absorbance in wells with no cells (blank) and the absolute absorbance values were similar to those in experiments where cells were treated with FTI-276, indicating that there was little to no cell

death. Therefore, dead cells and blank wells were omitted and values were normalized to treatment with media alone.

LI-COR Quantitative Immunohistochemistry

To confirm whether U0126 inhibited MEK activity in rat or mouse dorsal root ganglion neurons, neuronal cultures were exposed to either no added NGF or 30 or 100 ng/ml NGF for 7 days in the presence of the 100nM or 10μM U0126. Stock solutions of 10mM U0126 were prepared in MPL before each use, and then diluted in media. As a vehicle control, groups of cells were treated with vehicle 0.01% MPL alone. The media in the absence and presence of U0126 was changed every other day. After 7 days, the cells were fixed at the end of the treatment course with 4% paraformaldehyde in PBS for 20 min. To quantitate the relative amounts of pERK relative to the total ERK protein, cells were permeabilized by washing 3 times for 5 min each with 0.5% Triton X-100 in PBS. Cells were incubated overnight at 4°C with a 1:1 dilution of PBS and LI-COR blocking solution to decrease non-specific antibody binding. Cells were treated overnight at 4°C with primary antibodies to either p-ERK and ERK diluted 1:100 together in the blocking buffer. Primary antibodies, the mouse monoclonal anti-pERK and rabbit anti-ERK, were from Cell Signaling (Beverly, MA). Cells were washed five times in TBST. Some wells were not incubated with primary antibody to determine non-specific staining of secondary antibodies. Secondary antibodies, goat anti-mouse antibody conjugated to IRDye™ 680 (Invitrogen, Carlsbad, CA) and goat anti-rabbit

antibody conjugated to IRDye™ 800 (Rockland Inc, Gilbertsville, PA), were diluted simultaneously at 1:800 in 1:1 LiCor blocking solution in PBS. Cells were incubated in secondary antibodies for 2 hours in the dark. Cells were washed 5 times in 0.5% tween-20 in PBS in the dark. Plates of cells were then scanned for infrared signal using an Odyssey Imager from LI-COR (Madison, WI). Focus offset was 3.0 mm using a 24-well template. Scan intensity was set at 5 for both 700 nm and 800 nm wavelength channels, while scan quality was set at a resolution of 169 μm. Scans for the signals from both secondary antibodies were performed simultaneously. The background signal from cells incubated with secondary antibody only was subtracted from the signal from cells incubated with primary antibody. Signal intensity at 800 nm wavelength (total ERK) was normalized to the most intense signal for each experimental group to control for differences in staining intensities between experiments. The signal intensity at 700 nm (pERK) was divided by the signal at 800 nm to normalize for differences in total ERK. Data were expressed as a ratio of pERK to total ERK relative to the control group of cells.

Small interfering RNA (siRNA) Treatment of Neurons

After neurons were grown for 2 days in culture dishes, the standard F-12 media was replaced with F-12 media without antibiotics. On the fourth day in culture, growth media was aspired and replaced with 1 ml of OptiMEM I® serum-free media. Cells were incubated with 100nM of the 21-mer oligos double-strand

siRNA to synGAP, Ape1 (or scramble siRNA), or PKCα in 10 µl of the transfecting reagent, Neuroporter®. The Ape1 siRNA sequences were: 5'-GUCUGGUAAGACUGGAGUACC-3' and 5'-UACUCCAGUCUUACCAGACCU-3'. The synGAP siRNA sequences were: 5'-AAAGCGGAAGAAGGACAAGUU-3' and 5'-CUUGUCCUUCUUCCGCUUUUU-3'. The PKC-α siRNA sequences were: 5'-GGACGACACGGAAUGACUUUU-3' and 5'-AAGUCAUUCCGUGUCGUCCUU-3'.

After a 24 hour incubation, 0.5 ml of fresh media without antibiotics was added to each well. After an additional 24 hours, the medium containing siRNA was aspirated and replaced with 1 ml of F-12 media containing antibiotics and the media was changed every other day. After 10 days of treatment, cells were exposed to the standard iCGRP release protocol as indicated (and see above for methods), and then cells were collected for analysis of lysates by Western blotting. The protocol for Western blotting was performed identically to those methods described above with the following alterations. For immunoblotting for synGAP, rabbit anti-synGAP antibody (Upstate, Lake Placid, NY) was used at a dilution of 1:500 in TBST-M. Goat anti-rabbit secondary antibody (Bio-Rad, Hercules, CA) was used at a dilution of 1:2000 in TBST-M. For immunoblotting for PKCα, mouse anti-PKCα (Upstate) was used at a dilution of 1:1000 in TBST-M. Goat anti-mouse secondary antibody (Bio-Rad) was used at a dilution of 1:5000 in TBST-M.

Immunocytochemistry

Cells were fixed by incubating in 4% paraformaldehyde in PBS for 20 min at room temperature, and then permeabilized by washing five times with 0.5% Triton-X 100 in PBS. Cells were incubated overnight at 4˚C with blocking buffer (1:1 dilution of Odyssey Blocking Buffer in PBS). Cells were incubated overnight at 4˚C on an orbital shaker with goat anti-peripherin primary antibody from Santa Cruz Biotechnology (Santa Cruz, CA) diluted 1:250 in blocking buffer. Cells were washed with TBST five times for 10 min each wash. Cells were incubated for one hour in the dark at room temperature on an orbital shaker with a 1:1000 dilution of donkey anti-goat secondary antibody conjugated to AlexaFluor488™ or AlexaFluor585™ from Invitrogen (Carlsbad, CA). Cells were washed 5 times for 10 min each with TBST protected from light. Fluorescent images of neurons in Figure 7 were acquired on a Leica DM LB microscope (Bensheim, Germany) with a Krypton-Argon laser attached to a Spot CCD camera from Diagnostic Instruments (Sterling Height, MI). Images were collected and overlaid using Adobe Photoshop 7.0 (Adobe, Mountain View, CA). Fluorescent images of neurons stained for synGAP in Figure 24 were acquired at the Indiana Center for Biological Microscopy using a Bio-Rad MRC-1024MP Laser Scanning Confocal/Multiphoton scanner (Hercules, CA) attached to a Nikon Diaphot inverted microscope with a Nikon X60 1.2-NA water-immersion objective (Fryer, Huntley, IL). Fluorescence excitation was provided by a Krypton-Argon (488, 568, 647 nm) laser. Images were collected in black and white to maximize

resolution and pseudocolored and overlaid using Adobe Photoshop 7.0 (Adobe, Mountain View, CA).

Statistical Analysis

All data was analyzed using Prism 3.0 by GraphPad. All graphs are presented as the mean ± S.E. Stastistical significance was established using one-way ANOVA with Tukey's posttest, or two-way ANOVA with a Bonferroni's posttest as indicated. The posttests were chosen because they were the most stringent, and would produce the fewest number of Type I errors (rejecting the null hypothesis of no difference among groups when, in fact, the null hypothsis is true). N = number of wells per condition from at least three separate harvests of cells from dorsal root ganglion of animals. The significance level was set at $p < 0.05$.

RESULTS

Cellular Signaling Regulating the NGF-induced increase in iCGRP expression in primary sensory neurons

NGF exposure for 7 days increases the cellular content of iCGRP in rat sensory neurons in culture

Previous work demonstrated that NGF increases substance P and CGRP content in sensory neurons (Lindsay and Harmar, 1989; Noguchi et al., 1995; Verge et al., 1996; Schuligoi and Amann, 1998; Miki et al., 1998). These increases in neuropeptide content are important in physiological responses in the whole animal (Winter, 1988). Therefore, we characterized the effects of NGF on the expression of iCGRP in sensory neurons in culture (Lindsay et al., 1989; Hingtgen and Vasko, 1994; Burkey et al., 2004). As can be seen in Figure 7A, after 7-10 days, neuronal cultures derived from dorsal root ganglia are a mixed population of cells. The arrowhead points to a representative cell body of a sensory neuron, whereas the arrow points to a support cell. In general, the cell bodies of neurons are larger than non-neuronal cells in culture and appear granular.

To visualize the neurons in culture, cells were incubated with goat anti-peripherin antibody followed by a fluorescently labeled secondary antibody. The primary antibody is selective for the neuronal-specific protein, peripherin, and this antibody staining is used to distinguish neurons from non-neuronal cell types (Ferri et al., 1990). Using a fluorescent microscope, the neuronal cell bodies and an extensive network of neuronal processes can be seen in Figure 7B.

To confirm that long-term exposure to NGF increases expression of iCGRP in adult sensory neurons in culture, cells were grown for 7 days in the absence or presence of NGF. As can be seen in Figure 8, cells grown in the absence of added NGF contain 3.4 ± 0.3 fmol iCGRP per µg protein. The expression of the iCGRP in sensory neurons significantly increases to 6.6 ± 0.5 when grown in 30 ng/ml NGF and 8.1 ± 0.6 fmol when grown in 100 ng/ml NGF. The expression of iCGRP was not different when comparing the two concentrations of added growth factor. These studies agree with literature demonstrating an upregulation of CGRP *in vivo* in sensory neurons after administration of NGF either by endoneurial injection or injection of the neurotrophin into the hindpaw (Schicho and Donnerer, 1999; Ruiz and Banos, 2005).

Because previous studies of primary sensory neurons *in vitro* reported total peptide content as the amount per well of cells, rather than per µg of protein as reported here, the effects of NGF on total protein expression were examined. As shown in Figure 9, the overall expression of protein in sensory neuronal cultures did not change with increasing concentrations of NGF. The protein level in 0, 30, and 100ng/ml of added NGF was 2.7 ± 0.7, 3.0 ± 0.8, and 3.2 ± 0.7 µg per µl of buffer respectively. These results show that the effects of NGF on iCGRP content are not secondary to a non-specific change in protein expression in cells.

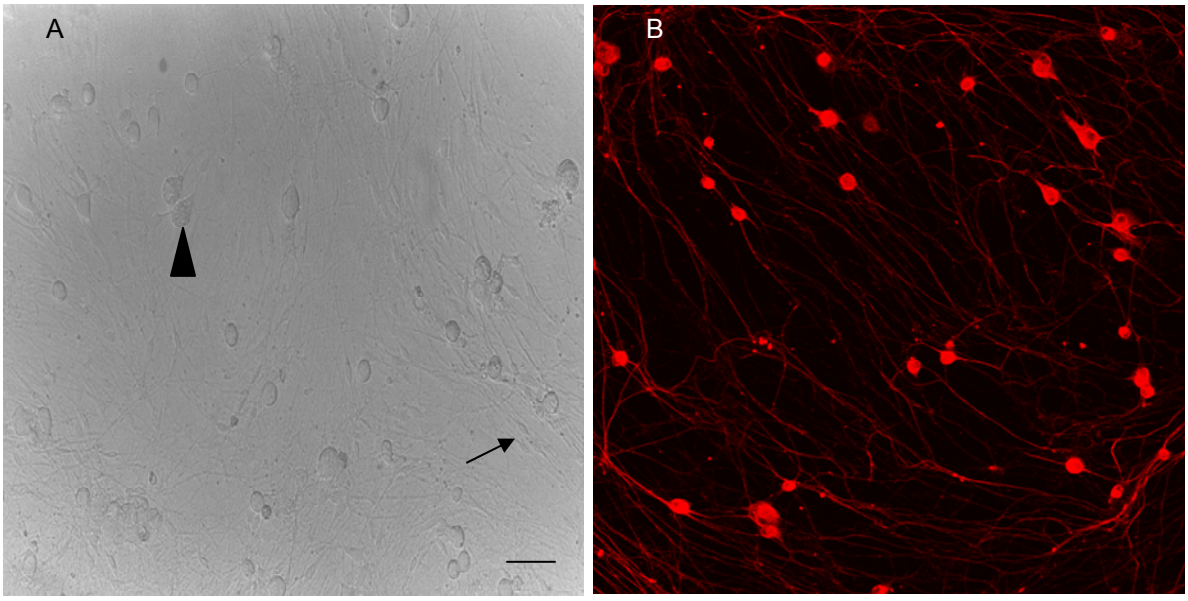


Figure 7. Isolated rat dorsal root ganglion neurons after 7 days in culture grown in 30 ng/ml NGF as described in Methods. (A) Brightfield view demonstrating cell bodies of sensory neurons and non-neuronal cells (— 50μm). The arrowhead points to a cell body of a sensory neuron, whereas the arrow point to a non-neuronal cell. (B) Fluorescent microscope images of sensory neurons in culture after antibody staining for peripherin, a neuronal-specific protein, which allows visualization of cell bodies and neuronal processes.

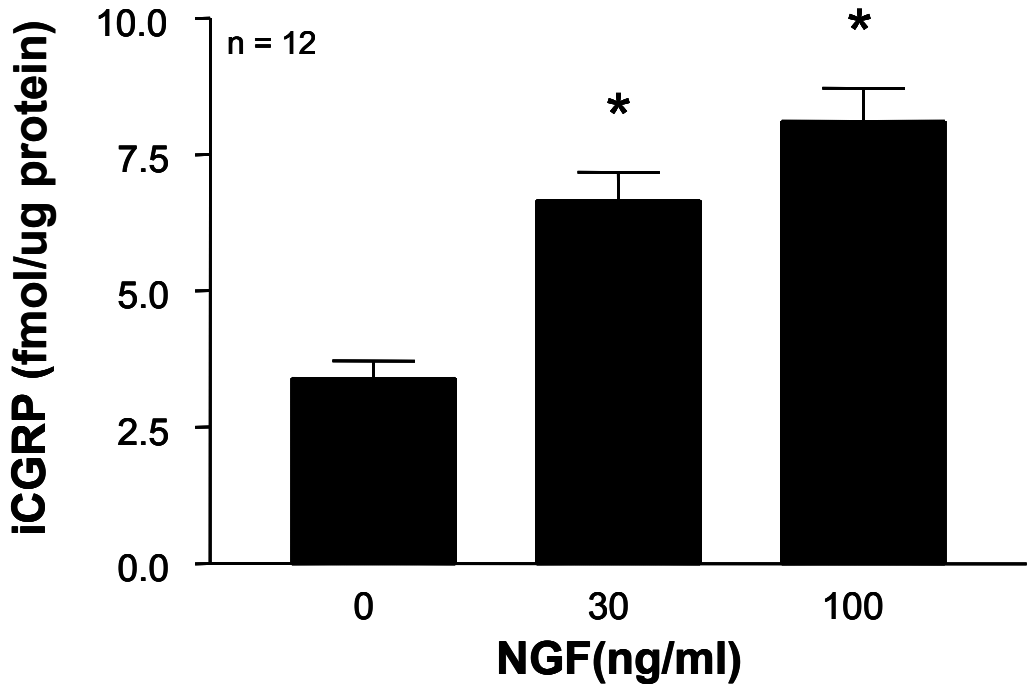


Figure 8. NGF (30ng/ml) and (100ng/ml) for 7 days increases expression of iCGRP in isolated sensory neurons in culture compared to cells with no added NGF (0 NGF). An (*) indicates a significant increase in iCGRP expression compared to 0ng/ml NGF ($p<0.05$, n=12, from 12 separate experiments using 3 wells of cells per condition. Data was analyzed using one-way ANOVA with Tukey's post-hoc test). The content of iCGRP measured after growing cells in 30 and 100ng/ml NGF were not significantly different.

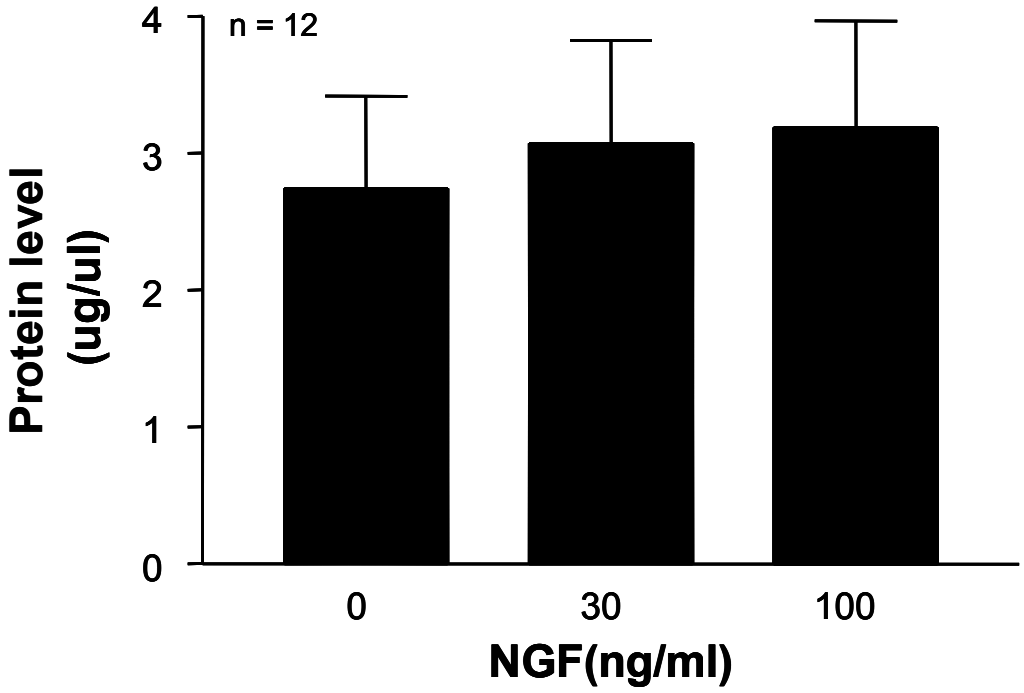


Figure 9. NGF does not alter total protein expression in sensory neuronal cultures. No difference in total protein expression was observed at any concentration (n=12, from 12 separate harvests, using one-way ANOVA).

Activation of Ras and the MEK/ERK pathway in neuronal cultures exposed to NGF for 7 days

In PC12 cells, effectors downstream of Ras, such as ERK, are involved in changes in the activity of promoters of neuropeptides, such as CGRP (Durham and Russo, 2003a; Dai et al., 2002; York et al., 1998). Based on these data, studies were performed to determine whether the Ras/MEK/ERK cascade mediated the ability of NGF to increase the content of iCGRP in sensory neurons.

For this work, studies examined whether growing neurons in the presence of varying concentrations of NGF altered the level of RasGTP and pERK measured in sensory neuronal cultures. Then the Ras pathway was manipulated in cultures of isolated sensory neurons by using pharmacological inhibitors of the Ras pathway, by overexpressing mutant Ras isoforms into neurons by lentivirus infection, by using siRNA directed against synGAP, and by using neurons from mice lacking synGAP. Employing multiple methods to manipulate Ras strengthened the interpretation of the role of Ras in NGF-induced alterations of iCGRP release and expression in sensory neurons.

Neuronal cultures were exposed to increasing concentrations of NGF for 7 days. Cells were collected, lysed, and the RasGTP levels were measured as outlined in Methods. As positive and negative controls, an aliquot of the cell lysate was incubated with either GTPγS or GDP, respectively, to maximize and minimize the amount of RasGTP in samples. The RasGTP measured in samples was normalized to the maximum amount of RasGTP detected in the positive control.

As shown in Figure 10, there was no change in the ratio of RasGTP to total Ras in neurons grown in 1, 3, 10 or 30 ng/ml NGF compared to no added NGF. Neurons in no added NGF demonstrated a ratio of RasGTP to Ras of 0.35 ± 0.11, while the ratios for neurons grown in 10 and 30 ng/ml NGF were 0.34 ± 0.03 and 0.42 ± 0.09, respectively. The value for the negative control was 0.34 ± 0.04. However, when NGF was increased to 100 or 250 ng/ml, there was a significant increase in the ratio. Neurons grown in 100 ng/ml NGF had a ratio of 0.57 ± 0.08 and neurons grown at 250 ng/ml NGF had a ratio of 0.71 ± 0.11. These studies indicated that after 7 days exposure to 100 or 250 ng/ml NGF, more RasGTP is present in cells in culture. It is interesting when no NGF is added to the F-12 media that the level of RasGTP in cells is similar to the negative control. This suggests that in a quiescent state, the Ras pathway is not activated in these cells.

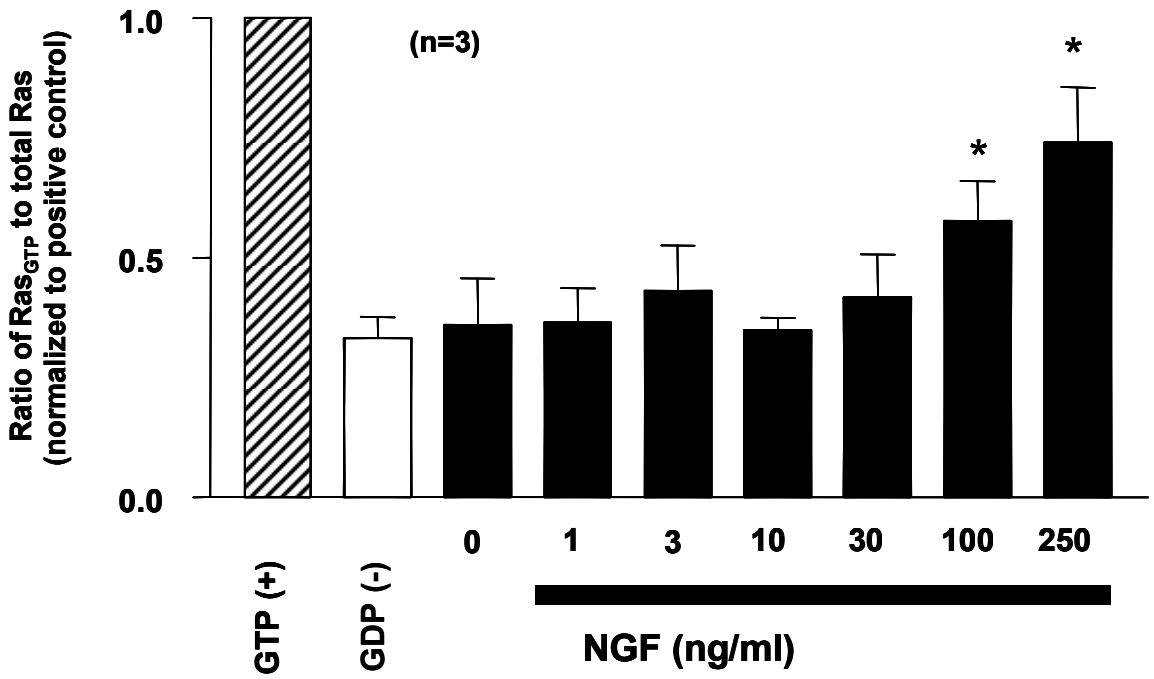


Figure 10. NGF exposure for 7 days increases RasGTP levels in sensory neurons in culture. Neurons were exposed to increasing concentrations of NGF as indicated for 7 days. The amounts of RasGTP and total Ras were measured in cell lysates. As a positive control, samples were spiked with GTPγS to maximize RasGTP levels. All values were normalized to the maximum amount of RasGTP possible . An (*) indicates a significant increase in RasGTP compared to 0 NGF ($p<0.05$, n= 3 from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

To further explore whether the Ras/MEK/ERK pathway regulates the NGF-induced increases in iCGRP content in sensory neurons, the phosphorylation of ERK was measured in cultures grown in various concentration of NGF for 7 days. After treatment, neurons were immediately fixed and the level of phosphorylated ERK (pERK) relative to the total amount of ERK was measured using Li-Cor as described in Methods. The relative levels of pERK compared to total ERK are reported. Due to variability of the absolute values from experiment to experiment, the ratios were normalized to the values measured in neurons exposed to no added NGF after each experiment. As shown in Figure 11, 30 ng/ml NGF did not significant increase the ratio of pERK to total ERK, with a normalized value of 1.20 ± 0.02. However, the ratio significantly increased when neurons were grown in 100 or 250 ng/ml NGF, with a ratio of 1.63 ± 0.08 and 1.66 ± 0.10, respectively. The total amount of ERK did not change in with increasing concentrations of NGF (personal communication, Dr. Judith Richter). These experiments demonstrated that chronic exposure to NGF increases the levels of pERK in neuronal cultures.

The data showing an increase in pERK levels at 100 and 250 ng/ml NGF are analogous to data showing an increase in RasGTP at these same concentrations of NGF (Fig. 10). These data together strongly suggest that the Ras/MEK/ERK pathway is activated by 7-day exposure to the higher concentrations of NGF.

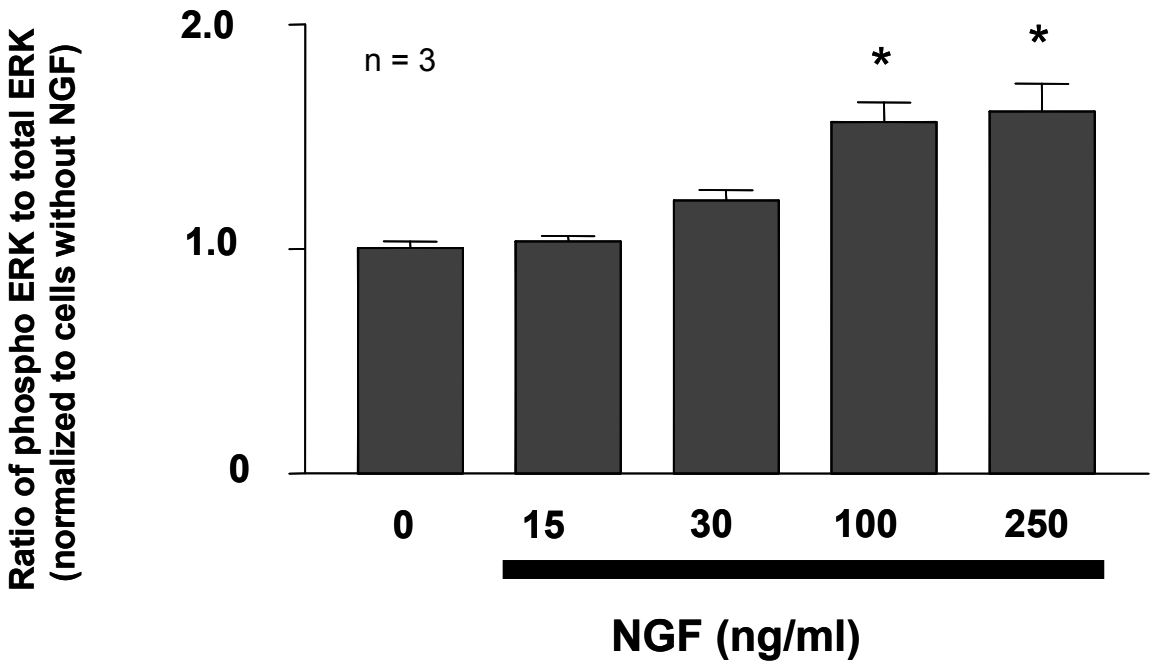


Figure 11. Seven-day treatment with NGF (100 and 250ng/ml) increases the level of pERK relative to total ERK in sensory neurons in culture. The ratio of pERK/total ERK is normalized to levels measured with no added NGF (0 ng/ml NGF). An (*) indicates a significant increase in the ratio of pERK/total ERK compared to cells treated with media alone ($p<0.05$, n=3 from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

Expression of dominant negative Ras (dnRas) in sensory neurons attenuates NGF-induced increases in iCGRP expression

To understand whether the Ras pathway mediates the NGF-induced increases in iCGRP content, Ras was manipulated directly. Neurons were infected with 150pfu of lentivirus expressing either a dominant negative isoform of Ras (17N dnRas) and EGFP, or EGFP alone (control virus). Neurons began expressing EGFP after 5 days of infection as determined by visualization of green fluorescence, but neurons were allowed to incubate for 2 more days to mimic time courses used in previous experiments and then photographs were obtained. As shown in Figure 12, neurons expressed EGFP at 7 days of infection with lentivirus. Approximately 70-90% of neurons in each of the fields expressed EGFP, and approximately 5 fields were viewed for each condition in each experiment, indicating that the majority of neurons were infected with lentivirus. EGFP expression was visible in the cell bodies and in the processes of some neurons.

Neurons were placed in culture with media containing no added NGF. Then cells were infected with lentivirus. Two days after infection fresh media was added to cells containing various amounts of added NGF. Cells were incubated with NGF for a total of 7 days. After treatment of neurons with lentivirus and NGF, cells were collected and lysed. Western blot analysis of cell lysates as described in Methods demonstrated an increase in Ras expression in neurons infected with the lentivirus containing the dnRas construct (Fig. 13). Values are reported as a ratio of Ras expression to actin expression, and are normalized to

control cells. Controls were uninfected sensory neuronal cultures (1.0 ± 0.0). Cells infected with the control virus containing only EGFP had a ratio of 0.7 ± 0.3, which was not significantly different than control. However, Ras expression was approximately 3 times the amount in lysates from cells that were uninfected or infected with lentivirus containing only EGFP, with a ratio of 3.0 ± 0.5 (Fig. 13). These data, showing high expression of EGFP in cells in culture and increased expression Ras in cell lysates from neuronal cultures, indicated there was an overexpression of the dnRas protein, including in the sensory neuronal population of these mixed cultures.

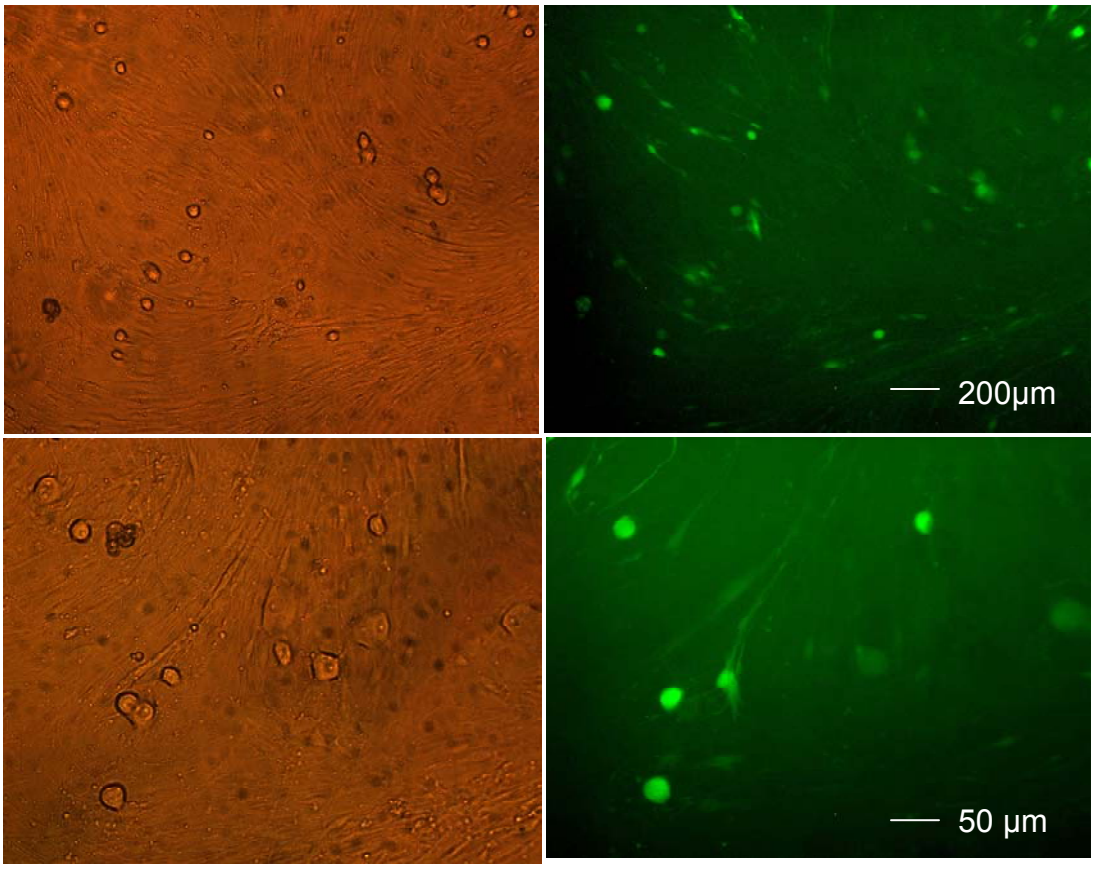


Figure 12. Neurons after 7d infection with 150pfu of lentivirus expressing EGFP with dnRas insert. Neurons were visualized using an inverted microscope with filter to visualize EGFP. The left top and left bottom panels show a brightfield view of neurons at 10X and 40X, respectively. The right top and right bottom panels are the same fields viewed with a filter to visualize EGFP. Neurons in brightfield are identified by their round cell bodies, while fibroblasts have a long spindle shape. Both support cells, such as fibroblasts, and neurons express EGFP.

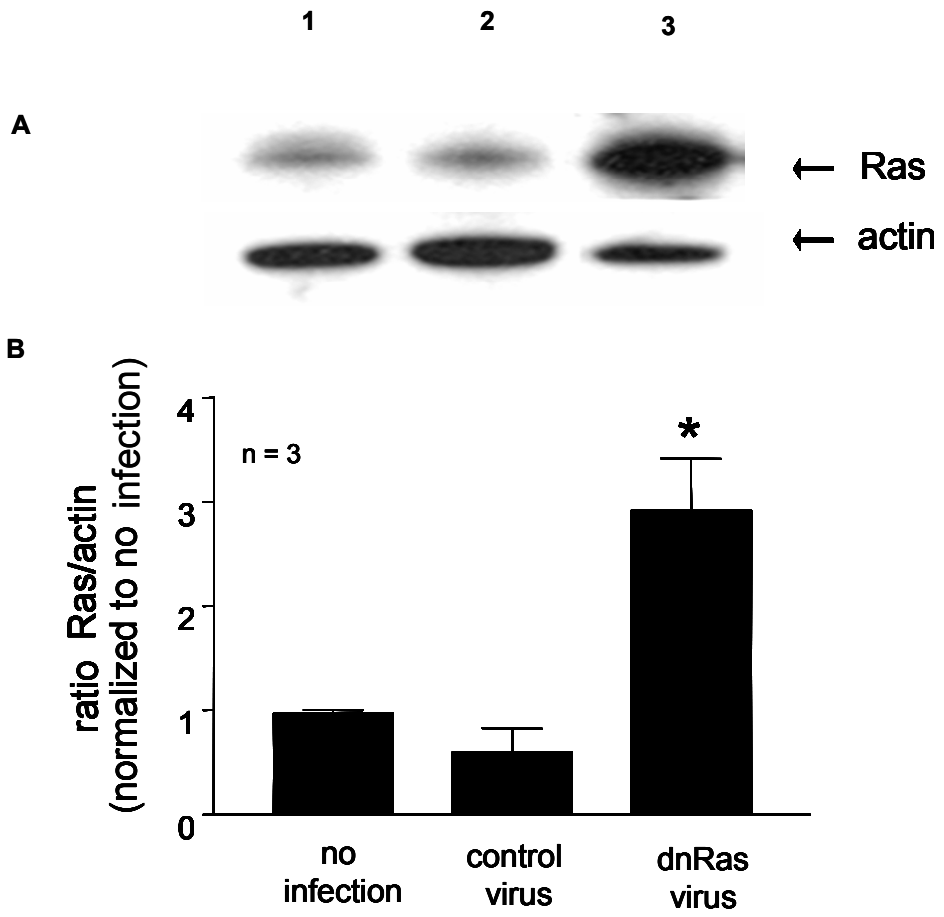


Figure 13. Cells from neuronal cultures infected with lentivirus expressing dnRas have increased expression of Ras protein. (A) Representative Western blot of protein from cells with (1) no infection, (2) infection with control lentivirus expressing EGFP only, or (3) infection with lentivirus expressing dnRas with EGFP. Immunoblotting was performed with a pan-Ras monoclonal antibody and anti-actin monoclonal antibody. (B) Summary data from Western blots demonstrating an increase in Ras expression in neuronal cultures infected with lentivirus expressing dnRas. Cells from these cultures have an approximate 3-fold increase in Ras expression. Infection with control virus does not affect the expression of Ras in neurons (n=3, from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

The overexpression of Ras in these experiments was dramatic. Ras is known to regulate the activity of downstream transcription factors. Therefore, the protein content of samples was measured to determine if overexpression of dnRas altered overall protein expression relative to infection with a virus containing empty vector but still expressing EGFP (control virus). Protein was measured in cell lysates using a Bradford assay as described in Methods. In these experiments, infection with the control virus or virus expressing dnRas did not alter protein levels in cell lysates (Fig. 14).

To determine dnRas altered the expression of iCGRP in neurons in response to NGF, iCGRP content was measured and normalized to the total amount of protein in the sample (Fig. 15). As shown previously, exposing neurons to 30 and 100 ng/ml NGF for 7 days increased the level of iCGRP expressed in sensory neurons in culture (Fig. 15). Control neurons expressed 2.0 ± 0.4 fmol iCGRP per µg protein, while neurons grown in 30 and 100 ng/ml NGF expressed 7.9 ± 0.7 and 7.5 ± 0.7 fmol iCGRP per µg protein, respectively, which was a significant increase from control. When neurons were exposed to the control virus, results were similar. Neurons grown in no added NGF expressed 3.0 ± 0.3 fmol iCGRP per µg protein, while neurons grown in 30 and 100 ng/ml NGF expressed 9.1 ± 0.9 and 7.5 ± 0.8 fmol iCGRP per µg protein, respectively. However, when neurons were infected with lentivirus expressing dnRas, there was a significant decrease in iCGRP content in neurons exposed to 30 or 100 ng/ml NGF. Neurons grown in no added NGF in the presence of dnRas expressed 2.0 ± 0.3 fmol iCGRP per µg protein. In neurons grown in 30 and 100

ng/ml NGF, they expressed 5.3 ± 0.6 and 4.0 ± 0.4 fmol iCGRP per μg protein, respectively. This was significantly lower expression than neurons exposed to control virus or no virus. However, it is important to note that the effect of NGF was not completely blocked by 150pfu of lentivirus. There remained a significant increase in the level of iCGRP, albeit smaller, in neurons expressing dnRas after long-term exposure to NGF. These data indicated that expression of the dnRas in neurons attenuates the NGF-induced increase in iCGRP expression.

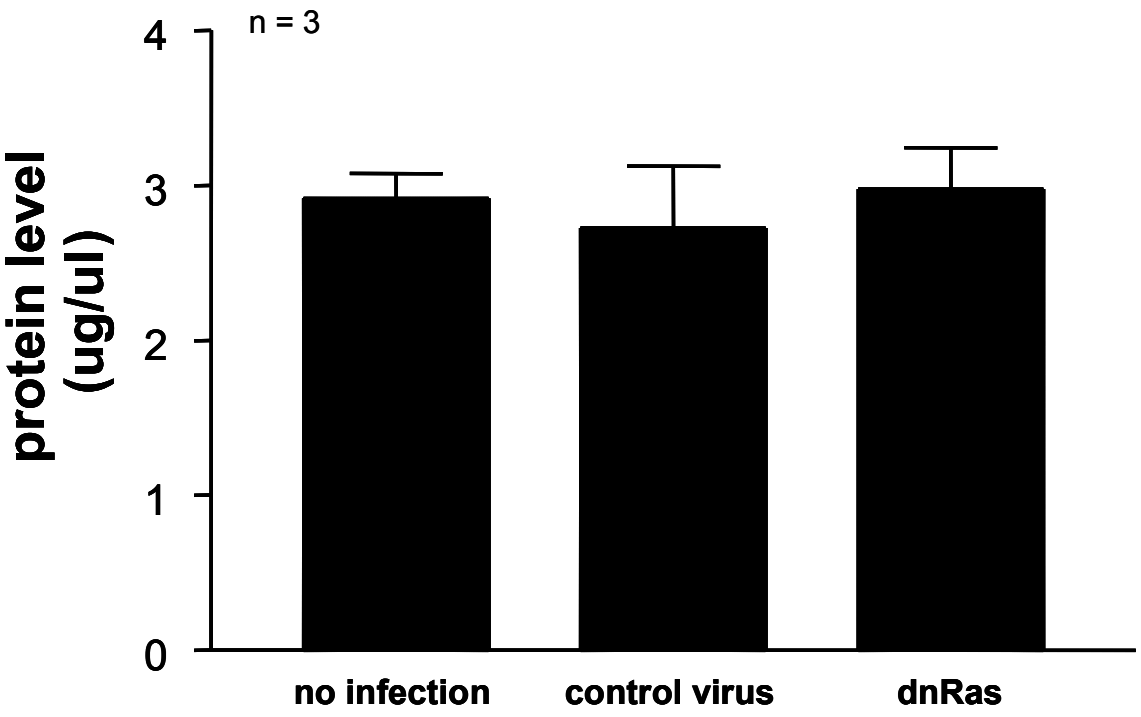


Figure 14. Infection with lentivirus expressing EGFP alone or EGFP with 17N dnRas does not alter protein levels measured in cell lysates. In these experiments, cells were exposed to media alone, control lentivirus, or virus expressing 17N dnRas. They were subsequently exposed to NGF ranging from 0, 30 or 100ng/ml. Neurons were collected and the protein levels assayed. There was no difference under any condition (n=3, from 3 separate harvests. Data was analyzed using one-way ANOVA).

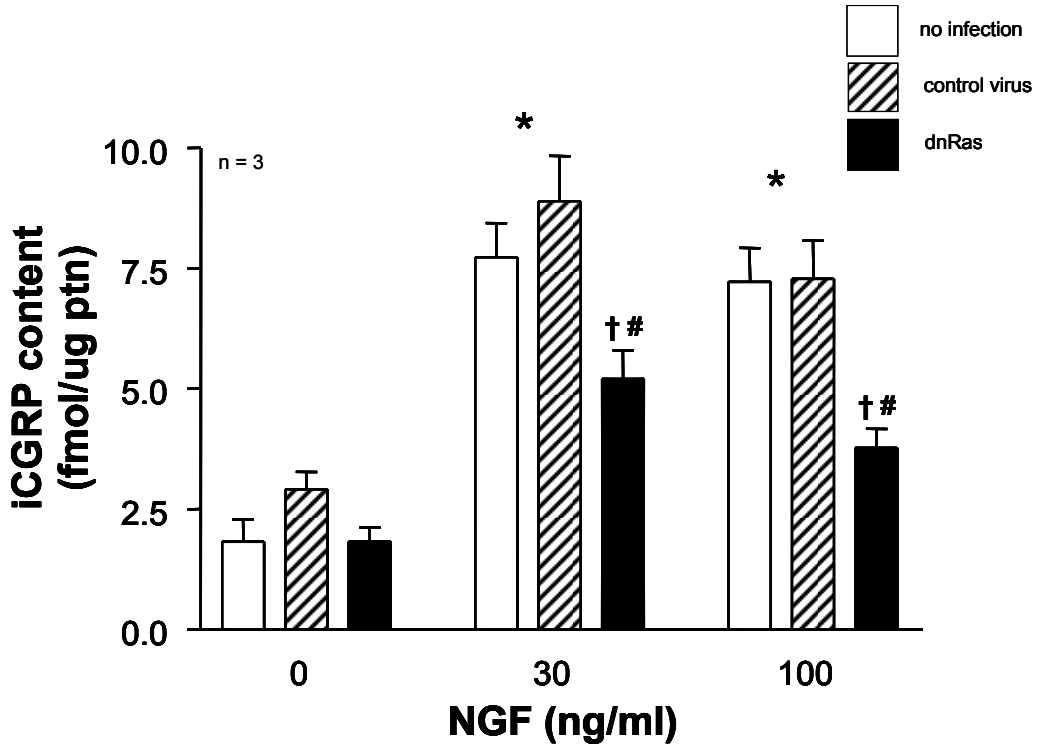


Figure 15. Expression of dnRas inhibits the NGF-induced increase of iCGRP expression in sensory neurons in culture. Neurons were exposed to media (white bars), virus expressing EGFP (hatched bars), or virus expressing dnRas and EGFP (black bars). After infection, they were exposed to increasing concentrations of NGF as indicated, and then cells were collected to measure iCGRP content. An (*) or (†) indicates a significant increase in content when neurons were exposed to 30 or 100ng/ml NGF compared to 0 NGF for each of the three conditions (media, control virus, or dnRas virus treatment). An ($^{\#}$) indicates a significant decrease in the NGF-induced augmentation of iCGRP at 30 and 100ng/ml NGF compared to uninfected cells or cells treated with control virus ($p<0.05$, n=3, from 3 separate harvests. Data were analyzed using two-way ANOVA with Bonferroni's post-hoc test).

Expression of constitutively active Ras (caRas) in sensory neurons increases in iCGRP expression

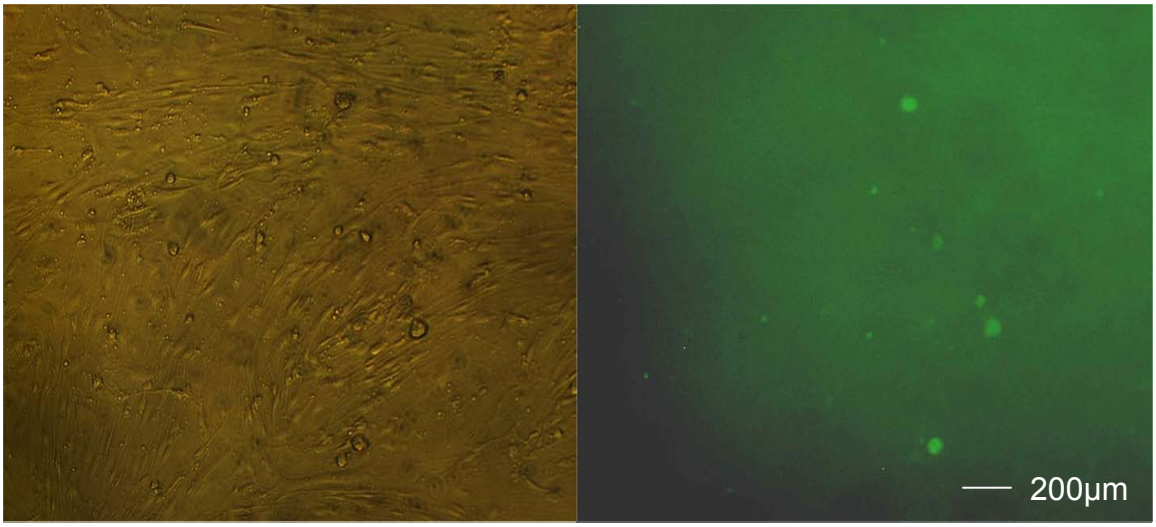
If Ras activation by NGF is sufficient for the increase of iCGRP expression in neurons in response to NGF, then infecting neurons with constitutively active Ras (caRas) also should increase the neuropeptide content even in the absence of NGF. Neurons were infected with 150 pfu of lentivirus expressing EGFP only or caRas with EGFP. Cells were incubated with virus in media for 48 hours, and then media was replaced with fresh F-12 containing no virus. On the seventh day, cells were collected and lysed to measure iCGRP content.

Figure 16A represents a brightfield view of dorsal root ganglia cells in culture and the same field viewed with a filter to visualize EGFP. Neurons expressed EGFP mostly in cell bodies. The number of cells infected with lentivirus as determined by EGFP expression was similar to the number of cells infected with virus containing dnRas (see above). As demonstrated by the Western blot, which is normalized to an actin loading control, lysates from neurons infected with virus expressing caRas with EGFP (lane 2) expressed higher levels of Ras than neurons infected with control virus alone.

The iCGRP content in lysates from cells infected with control virus or virus expressing caRas are shown in Figure 17. The average values from two different experiments are presented. The values were derived from duplicate measurements from each experiment. While this series of experiments is not complete, it appeared that caRas increased the iCGRP content in the absence of NGF. Experiments are currently being performed to complete these studies. If

caRas increases expression of iCGRP, then Ras activation is both necessary and sufficient for upregulation of iCGRP.

A



B

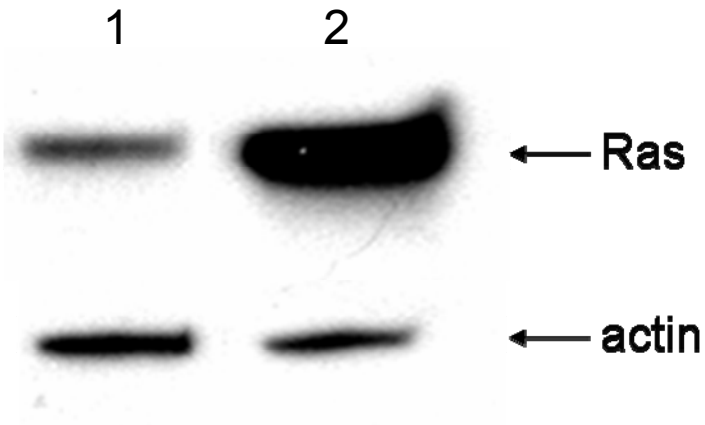


Figure 16. Characterization of neuronal cultures infected with lentivirus expressing EGFP or EGFP with caRas. (A) Neurons infected with virus were visualized using an inverted microscope with filter to visualize EGFP. The left panel shows a brightfield view of neurons at 10X, and left panel is the same field viewed with a filter to visualize EGFP. (B) Cell lysates from neuronal cultures infected with lentivirus expressing dnRas have increased expression of Ras protein. Representative Western blot of protein from cells with (1) infection with control lentivirus expressing EGFP only, or (2) infection with lentivirus expressing caRas with EGFP. Immunoblotting was performed with a pan-Ras monoclonal antibody and anti-actin monoclonal antibody.

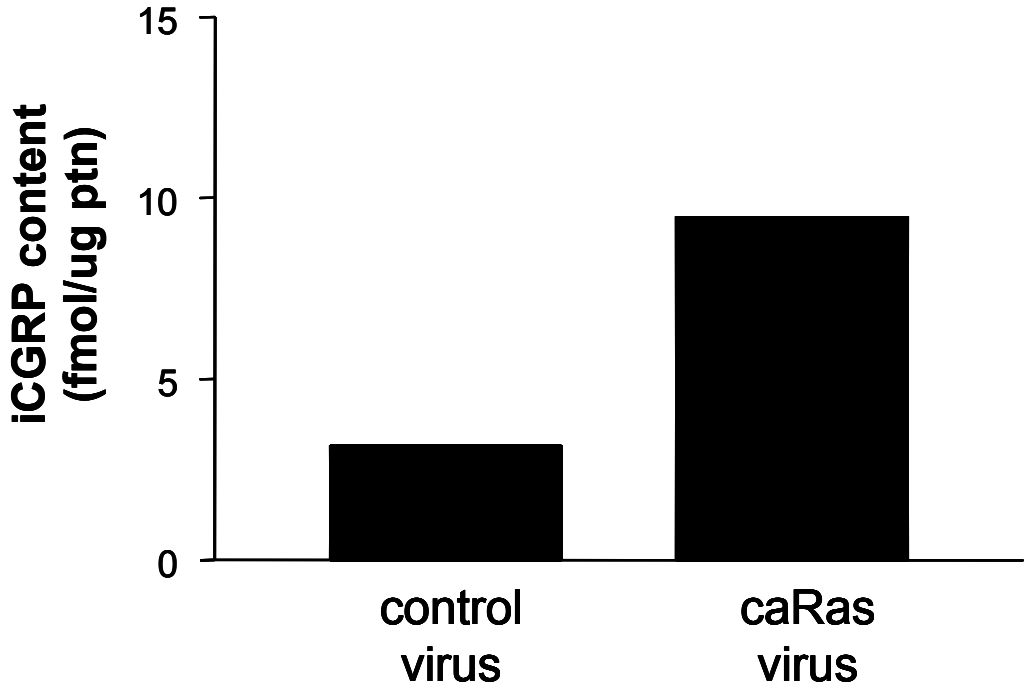


Figure 17. The iCGRP content in cell lysates from neurons infected with control virus or virus expressing caRas. Neurons were grown in culture in the absence of NGF, and were infected with virus for 7 days. After 7 days neurons were collected and lysed to measure iCGRP content. The means are derived from measurements from two different experiments. There is a trend for increased expression of iCGRP in the presence of the virus expressing caRas, but the experiment must be replicated to draw conclusions.

FTI-276 attenuates increases in iCGRP expression with chronic NGF exposure

While expression of dnRas and caRas are specific ways to inhibit or activate Ras signaling, respectively, it is possible that these manipulations could affect iCGRP expression through other mechanisms. For instance, simply overexpressing protein levels could alter signaling pathways and lead to incorrect conclusions about the native function of Ras in NGF signaling. Therefore, cells in culture were treated with a farnsyltransferase inhibitor, FTI-276, as another way to manipulate Ras-mediated signaling. As described in the Introduction, the Ras protein is post-translationally processed and a farnesyl lipid moiety is added to the carboxy terminal end of the molecule (Sebti and Hamilton, 1997). By blocking the enzyme that catalyzes the addition of farnesyl, using FTI-276, Ras cannot localize to the membrane and its activity is decreased.

The half-life of the Ras protein is approximately 24hr (Politi and Senderowicz, 2001). Therefore, treatment with FTI-276 for 4 days would theoretically reduce Ras by greater than 90% because the existing Ras would be degraded, and fanesylation of newly formed Ras would be inhibited. Consequently, cells were treated 4 days with 5μM FTI-276, and then cells were exposed to NGF in the presence or absence of inhibitor for 4-5 more days. This concentration was chosen because it is approximately 100x the IC_{50} for farnesylation of Ras in various cell lines (Sebti and Hamilton, 1997). Neurons were collected and iCGRP content was measured by radioimmunoassay, whereas inhibition of farnesylation was confirmed by Western blotting.

Figure 18A shows a representative Western blot from sensory neuronal cultures treated with FTI-276. The unfarnesylated Ras (top band) was resolved from the farnesylated Ras (bottom band). To quantify the effectiveness of FTI-276, the ratio of the density of the top band to bottom band was calculated.

In untreated neurons, there was no difference in the ratio at any concentration of NGF. Values are reported as a ratio of unprenylated to prenylated Ras, and are normalized to control values. At 0, 30, and 100 ng/ml NGF the ratios are 1.0 ± 0.0, 0.7 ± 0.2, and 1.0 ± 0.1, respectively. These values are not different. However, when cells were exposed to 5μM FTI-276 and NGF, the ratio increased from 1.6 ± 0.5 at 0 ng/ml added NGF to 2.7 ± 0.2 and 2.5 ± 0.7 in 30 and 100 ng/ml NGF, respectively. This indicates there was a significant increase in the ratio, or a decrease in farnesylated Ras, when neurons were treated with FTI-276 and NGF compared to FTI-276-untreated controls (Fig. 18B).

Because cells were incubated with FTI-276 for 7 days, it is possible that the level of Ras protein was altered. Altering levels of the Ras protein could change its activity, and could bias the interpretation of the data. Therefore, we measured total levels of Ras protein in these samples. When Ras (including farnesylated and unfarnesylated protein) was normalized to actin, the total amount of Ras expressed in sensory neurons does not change (Fig. 19). These data also indicated that there were not significant increases in total Ras expression in response to 30 or 100 ng/ml NGF after 7 days.

In sensory neurons treated with FTI-276 in the presence of NGF, there was a significant reduction in neurotrophin-induced upregulation of iCGRP (Fig. 20). Neurons with no added NGF expressed 3.0 ± 0.1 fmol iCGRP, and neurons with no added NGF in the presence of 5μM FTI-276 expressed 1.8 ± 0.1 fmol iCGRP per μg protein. These two values were not significantly different from one another. When 30 ng/ml NGF was added, cells expressed 6.3 ± 0.2 and 3.8 ± 0.5 fmol iCGRP per μg protein in the absence and presence of FTI-276, respectively. Again, these two measurements were not significantly different from one another. However, both measurements were significantly increased from neurons with no added NGF. At 100 ng/ml NGF, measurements were 9.9 ± 1.7 and 6.7 ± 1.3 fmol iCGRP per μg protein in the absence and presence of FTI-276, respectively. These values were significantly higher than those with no added NGF, and the values at 100 ng/ml NGF were significantly different than one another. These data indicate that NGF increases the expression of iCGRP in these neurons and FTI-276 inhibits the neurotrophin-induced increases at higher concentrations of NGF.

Because of the long duration of treatment with FTI-276, the cells in culture may contain less iCGRP because there are fewer cells present. The viability of cells was measured using an MTS assay. This assay requires living cells to break down a substrate to a colorimetric product through energy-dependent enzymes. Dead cells cannot catalyze the reaction, and would be reflected in lower absorbance at a designated wavelength. In Figure 21, cells were treated with a fixative to kill them. They were unable to convert the substrate and the

absorbance measured was not higher than background values. As shown in other treatment groups, FTI-276 incubation for 7-10 days did not affect viability up to 30μM compared to cells treated with media alone or vehicle (Fig. 21).

Together these data showed Ras expression did not change in cells treated chronically with FTI-276, and FTI-276 the NGF-induced increases in iCGRP expression. There were no changes in cell viability which also could be another uncontrolled variable in these experiments. Incubation with the drug reduced the increase in iCGRP expression in sensory neurons in response to 7 day treatment with NGF. These data indicate Ras is involved in regulation of iCGRP content in neurons.

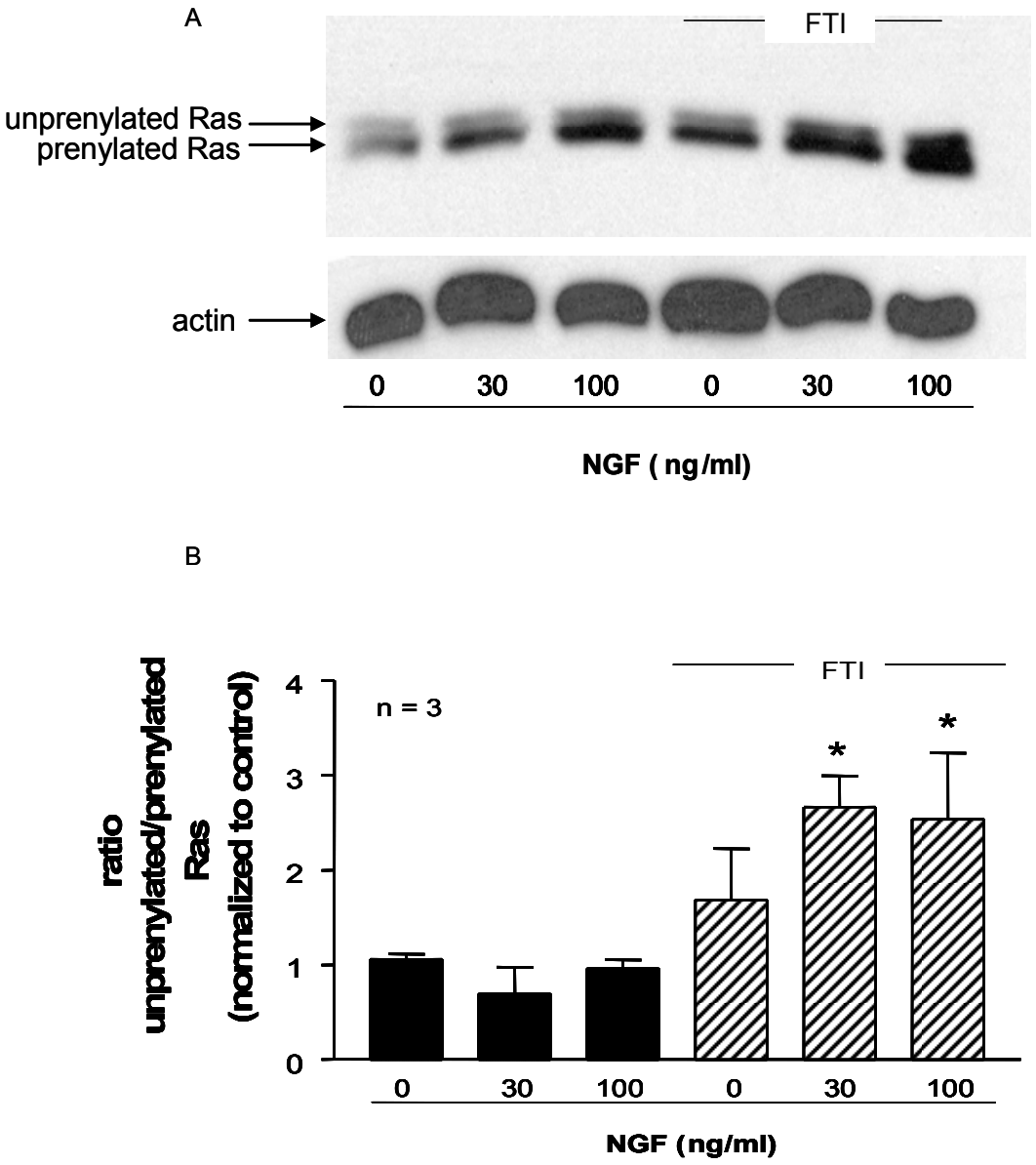


Figure 18. FTI-276 increases the ratio of unprenylated Ras to prenylated Ras in neuronal cultures. Cells were treated with vehicle or FTI-276 (5μM) as described in Methods. (A) Representative Western blot demonstrating the level of unprenylated Ras (top band) relative to prenylated Ras (bottom band), and the actin loading controls as indicated. (B) Summary densitometry data from 3 separate experiment using FTI-276. An (*) indicates a significant increase in the ratio of unprenylated to prenylated Ras compared to control (0 NGF only) ($p<0.05$, n=3 from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

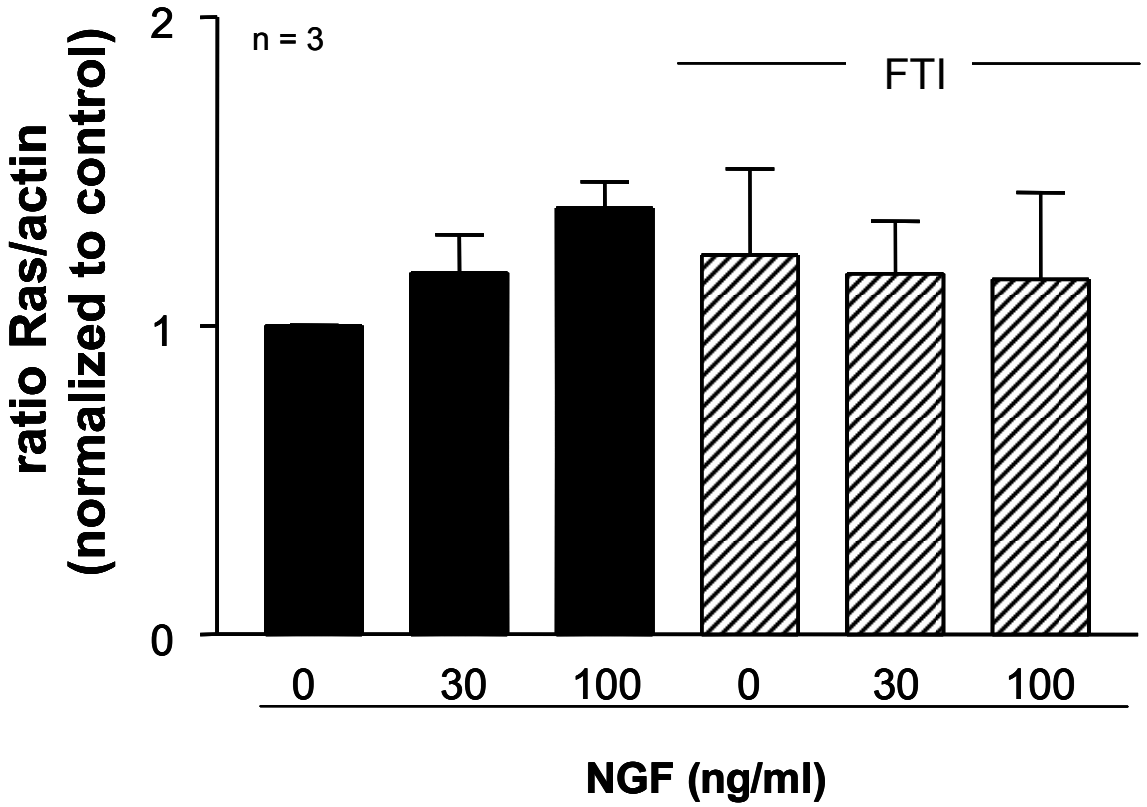


Figure 19. Expression of Ras in neuronal cultures is not altered by treatment with vehicle or FTI-276 (5μM) nor by treatment with 30 or 100 ng/ml NGF for 7 days. Neurons were treated with increasing amounts of NGF in the presence or absence of FTI-276. Summary data from densitometry measurements of Western blots for total Ras protein normalized to actin. There was no significant difference among any of the conditions in the relative amounts in sensory neurons in culture (n=3, from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

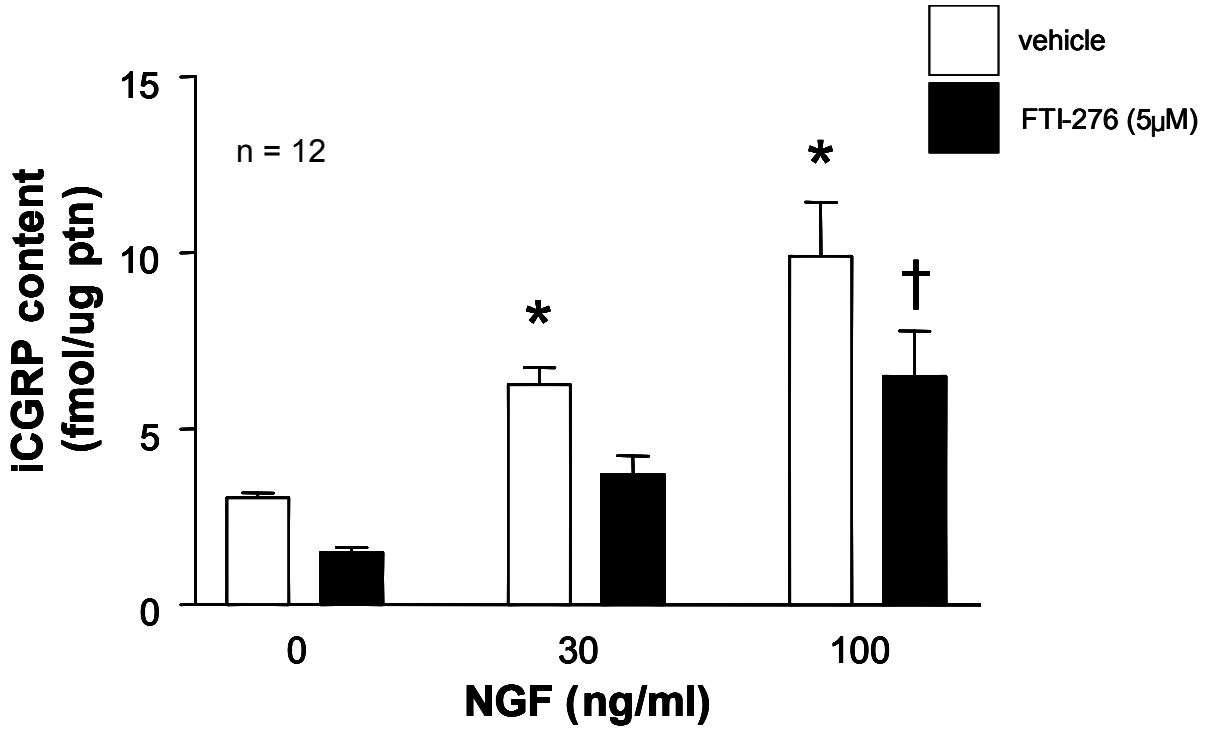


Figure 20. FTI-276 reduces the NGF-induced increase in iCGRP content in neurons exposed to 100 ng/ml NGF. Neurons were treated with vehicle (white bars) or FTI-276 (5μM, black bars) in the presence or absence of increasing concentrations of NGF as indicated. An (*) indicates an increase in iCGRP relative to treatment with no NGF. An (†) indicates significantly lower iCGRP content compared to vehicle control treated with 100 ng/ml NGF ($p<0.05$, n=12, 3 separate harvests, using two-way ANOVA with Bonferroni's post-hoc test).

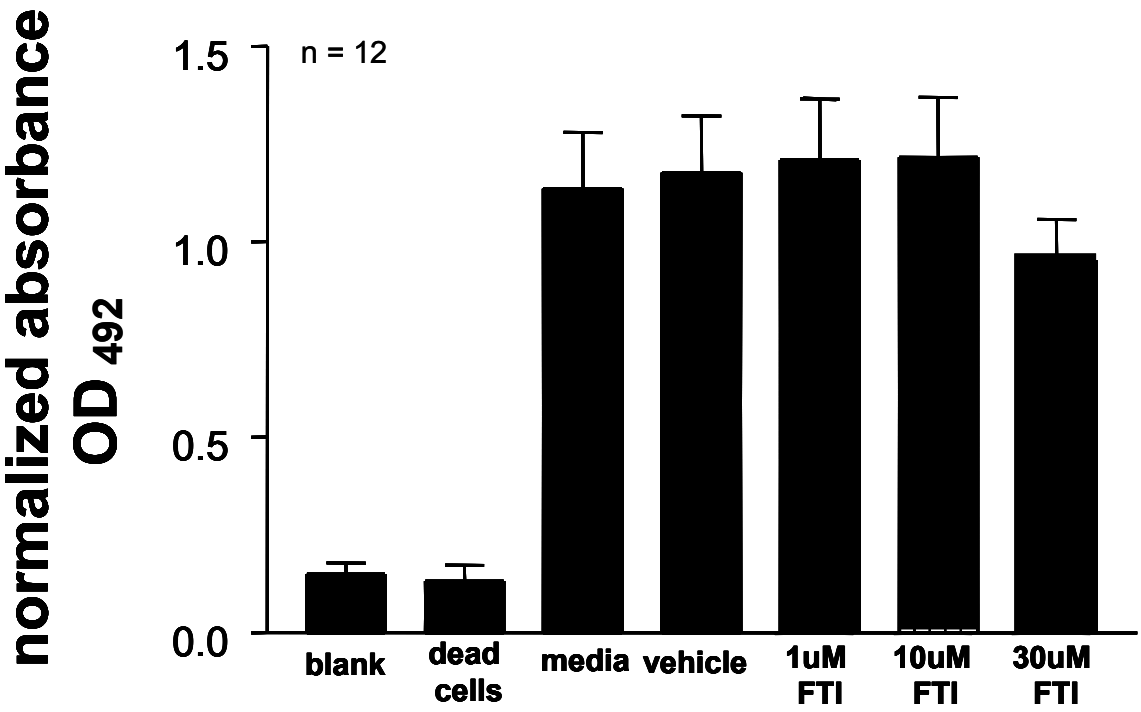


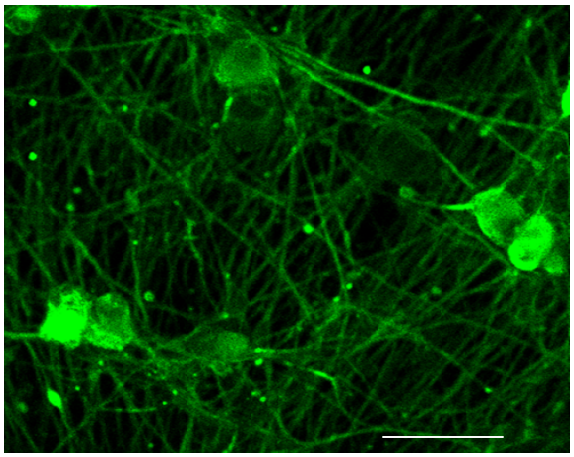
Figure 21. Cell viability of cultures from dorsal root ganglia is not affected by treating with FTI-276 for 7 days. Neurons were treated as indicated. Wells containing media but not cells were used as blanks. For controls, wells of cell were treated with media alone (media). After 7days, one group of cells was treated with paraformaldyhyde as a positive control for cell death. The neurons were then subjected to an MTS assay and absorbance was measured at 492 nm (see Methods). Data were normalized to control cells (media). The blank and dead cells had significantly lower absorbances compared to untreated cells. There were no differences among untreated cells and cells treated with FTI-276 ($p<0.05$, n=12 from three separate harvests, using one-way ANOVA with Tukey's post-hoc test).

SynGAP, a neuronal-specific RasGAP, is expressed in rat primary sensory neurons in culture

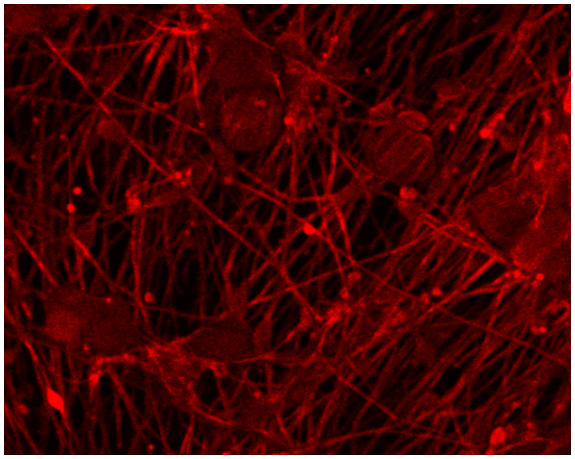
Ras activation is tightly regulated at the membrane by proteins that hydrolyze GTP to shut off signaling to MEK and ERK. The negative regulatory proteins are referred to as GTPase activating enzymes, or GAPs. SynGAP is a GTPase activating enzyme than has been demonstrated in neurons of the central nervous system, such as mouse hippocampal and cortical neurons. The enzyme accelerates the rate of reaction for hydrolysis of GTP, and inactivates RasGTP signaling to downstream cascades (Fig. 3).

Previously, the presence of synGAP in peripheral neurons had not been demonstrated. For these studies, dorsal root ganglion neurons derived from rats were grown for 7 days in the presence of 30ng/ml NGF. After fixing cells using paraformaldehyde, immunohistochemical studies were performed using antibodies for both synGAP and peripherin. Fluorescently labeled secondary antibodies were used against the primary antibodies. SynGAP (red) and peripherin (green) colocalized in sensory neurons (yellow) (Fig. 22). SynGAP was expressed in the processes of neurons in culture. There did not appear to be synGAP expression in non-neuronal support cells, which were present in these cultures.

Peripherin



synGAP



merge

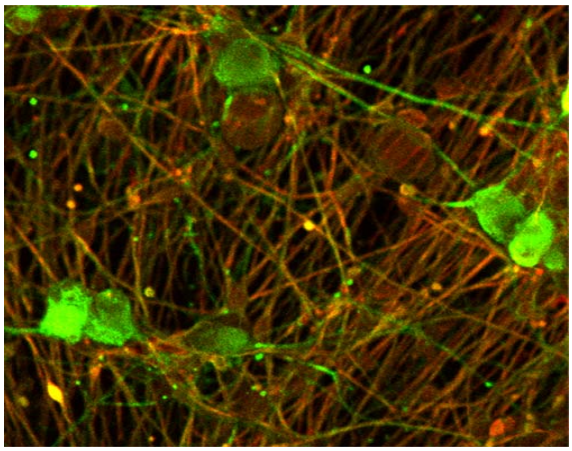


Figure 22. Dorsal root ganglion neurons in culture express the neuronal RasGAP, synGAP. Neurons in culture were fixed and stained using antibodies directed against peripherin and synGAP. Peripherin is localized in cell bodies and processes of neurons (top). SynGAP (middle) is localized heavily in processes with some staining in cells bodies. Colocalization shows synGAP is present in neurons that co-express peripherin. (—) represents 50 μm, fields are views from 40X lens.

After chronic exposure to 100 or 250 ng/ml NGF, neurons from mice with a heterozygous deletion of synGAP have increased iCGRP content compared to neurons from wildtype littermates

Mice that have a heterozygous deletion of the synGAP gene have been developed by the laboratory of RL Huganir (Kim et al., 2003). In neurons that have decreased expression of synGAP, Ras is more active. In previous studies using hippocampal neurons from synGAP heterozygous animals, downstream targets of Ras such as MEK and ERK show increased activation (Komiyama et al., 2002). Thus, these mice provide another means to manipulate the Ras signaling cascade in sensory neurons to examine the role of this small G-protein in NGF-induced expression of iCGRP.

Using dorsal root ganglion neurons from mice with a heterozygous deletion of synGAP, the question was asked whether the genetic alteration would alter the expression of iCGRP in cells exposed to NGF for 7 days. Neurons from synGAP heterozygous animals and their wildtype littermates were exposed to increasing amounts of NGF for 7 days, and the iCGRP content in these neurons was measured (Fig. 23). As can be seen in Figure 23, 100ng/ml NGF there was a significant increase in the expression of iCGRP compared to the littermate controls. These data suggested that neurons from mice with a heterozygous deletion of synGAP were more responsive to NGF compared to neurons from wildtype mice, suggesting that the Ras pathway is important in the regulation of iCGRP expression in sensory neurons in culture.

An interesting finding from these experiments is that neurons from mice appear to have more iCGRP per microgram of protein compared to cell neurons derived from dorsal root ganglia from rats. On average, neurons from rats express approximately 2.5 fmol iCGRP per microgram protein while neurons from mice express about 3 times that amount. More importantly, neurons from wildtype mice do not have a robust increase in iCGRP expression with NGF treatment.

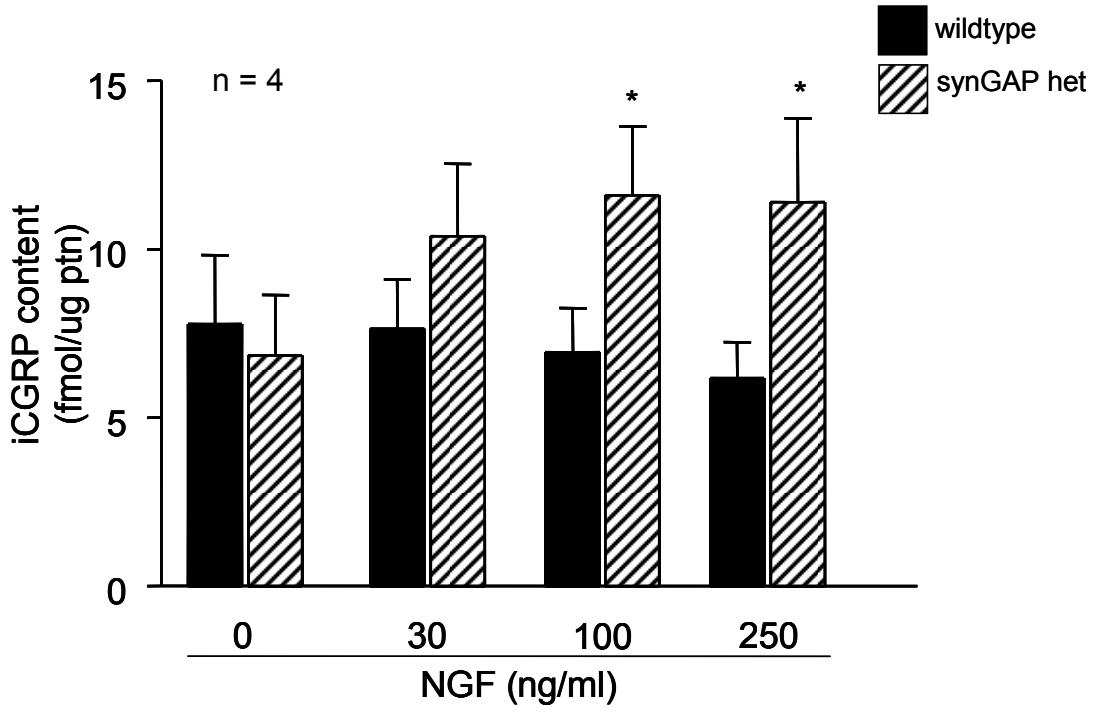


Figure 23. Compared to littermate controls, dorsal root ganglion neurons from synGAP heterozygous mice have increased levels of iCGRP expression in response to 100 and 250 ng/ml NGF. Neurons from wildtype animals (black bars) do not have increased expression of iCGRP when exposed to NGF as observed in neurons from rats. However, when neurons from synGAP mice (hatched bars) are exposed to NGF they demonstrate a significant increase in expression compared to their littermate controls. An (*) indicates a significant increase in iCGRP content compared to wildtype neurons ($p<0.05$, n=4, from 4 separate harvests, using two-way ANOVA with Bonferroni's post-hoc test).

Incubation of neuronal cultures with MEK inhibitor, U0126, attenuates the NGF-induced increases in iCGRP expression

RasGTP activates Raf kinase, which phosphorylates and activates MEK (see Fig. 2 and Introduction). As a different means to manipulate the downstream effectors of Ras in neurons, the MEK inhibitor U0126 was used. The drug inhibits the activation of MEK by blocking phosphorylation and activation by Raf-1 kinase at an IC_{50} of less than 100nM (Kohno and Pouyssegur, 2003). Thus, U0126 was used to determine if signaling molecules downstream from Ras were important in the regulation of NGF-induced changes in neuropeptide expression.

Rat neurons in culture were exposed to 0, 30, or 100 ng/ml NGF for 7 days in the absence or presence of (Fig. 24). Neurons were collected and the iCGRP content was measured in cell lysates. In neurons with no added NGF, cells expressed 3.1 ± 0.5 and 2.1 ± 0.7 fmol iCGRP per µg protein in the absence and presence of U0126, respectively. These measurements were not significantly different. In neurons exposed to 30 ng/ml NGF, cells expressed 5.6 ± 0.9 and 3.2 ± 0.4 fmol iCGRP per µg protein in the absence and presence of U0126, respectively. As described in previous experiments, the U0126-untreated cells at 30 ng/ml NGF demonstrated a significant increase in iCGRP expression compared to the cells with no added NGF. However, the neurons treated with U0126 and 30 ng/ml NGF did not demonstrate a significant increase in iCGRP expression relative to NGF-untreated neurons. In neurons exposed to 100 ng/ml NGF, cells expressed 7.0 ± 1.0 and 4.3 ± 0.6 fmol iCGRP per µg protein in the absence and presence of U0126, respectively. As in previous experiments, the

U0126-untreated neurons demonstrated a significant increase in iCGRP expression compared to cells with no added NGF. Furthermore, U0126 significantly decreased the expression of iCGRP in the presence of 100 ng/ml NGF (Fig. 24). Notably, 100ng/ml NGF significantly increased iCGRP content compared to 0ng/ml NGF even in the presence of U0126. There are at least two explanations for this finding. It is possible that at high levels of the neurotrophin, iCGRP transcription may involve MEK-independent processes. Alternatively, 1μM U0126 may not be a high enough concentration of the drug to block the increase of iCGRP expression by 100 ng/ml NGF.

As with FTI-276, the viability of rat neurons treated for 7 days with U0126 was measured to ensure long-term exposure did not harm cells in culture. Treatment with U0126 did not cause cell death in cultures as measured by MTS assay (Fig. 25).

Throughout the experiments using 1μM U0126, it was assumed the drug was inhibiting the activation of MEK in neurons from rats. Although U0126 is a relatively selective MEK inhibitor (English and Cobb, 2002), experiments also were performed to confirm that U0126 at the concentration utilized inhibited the phosphorylation of ERK. Because ERK is specifically activated by MEK, is a valid indicator of MEK inhibition. Neurons from rats were exposed to 100ng/ml NGF for 7 days in the presence or absence of U0126 (Fig. 26). NGF significantly increased pERK relative to total ERK compared to non-treated neurons, whereas U0126 significantly attenuated the effect of NGF. These data indicated U0126 partially attenuates the NGF- induced increases in pERK in rat sensory neurons

in culture, suggesting that this concentration of U0126 does not produce a complete inhibition of MEK.

In Figure 23, neurons from synGAP heterozygous mice showed an increase in iCGRP expression when they were exposed to 100ng/ml NGF compared to neurons from wildtype littermates. To determine whether this increase was dependent on MEK activity, neurons from synGAP heterozygous mice and their littermate controls were exposed to 100ng/ml NGF in the presence or absence of U0126 (1μM) for 7 days. The cells were collected, and iCGRP content was measured in lysates. As in the previous studies, there was no increase in the iCGRP expression in neurons from wild-type mice, whereas there was a significant increase in the amount of iCGRP from neurons exposed to 100ng/ml NGF in heterozygous mice. This increase was abolished by incubation with 1μM U0126 (Fig. 27), showing that the NGF-induced increase in iCGRP express is through MEK activation in mouse sensory neurons. Furthermore, these data were strikingly similar to those obtained by using FTI-276 in rat neurons (Fig. 20). In both cases there is a significant decrease in the NGF-induced upregulation of iCGRP content in sensory neurons. These experiments together strongly implicate the Ras/MEK/ERK pathway in the regulation of iCGRP expression by NGF in mouse and rat sensory neurons.

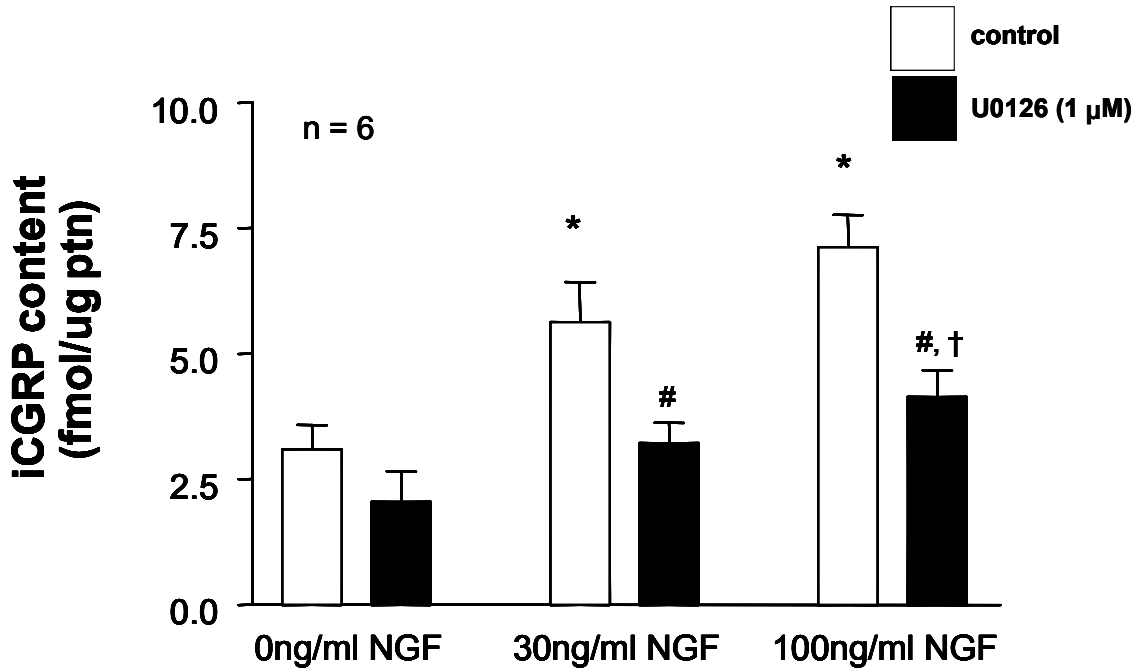


Figure 24. The MEK inhibitor, U0126 (1μM), significantly decreases NGF-induced upregulation of iCGRP content in sensory neurons in culture. An (*) indicates a significant increase in iCGRP content compared to 0ng/ml NGF. An (#) indicates a significant decrease in iCGRP content within an NGF-treatment groups compared to U0126-untreated ccells. An (†) indicates a significant increase in iCGRP content compared to neurons at 0ng/ml NGF treated with U0126 ($p<0.05$, n=6 from 6 separate harvests. Data were analyzed with two-way ANOVA with Bonferroni's post-hoc test).

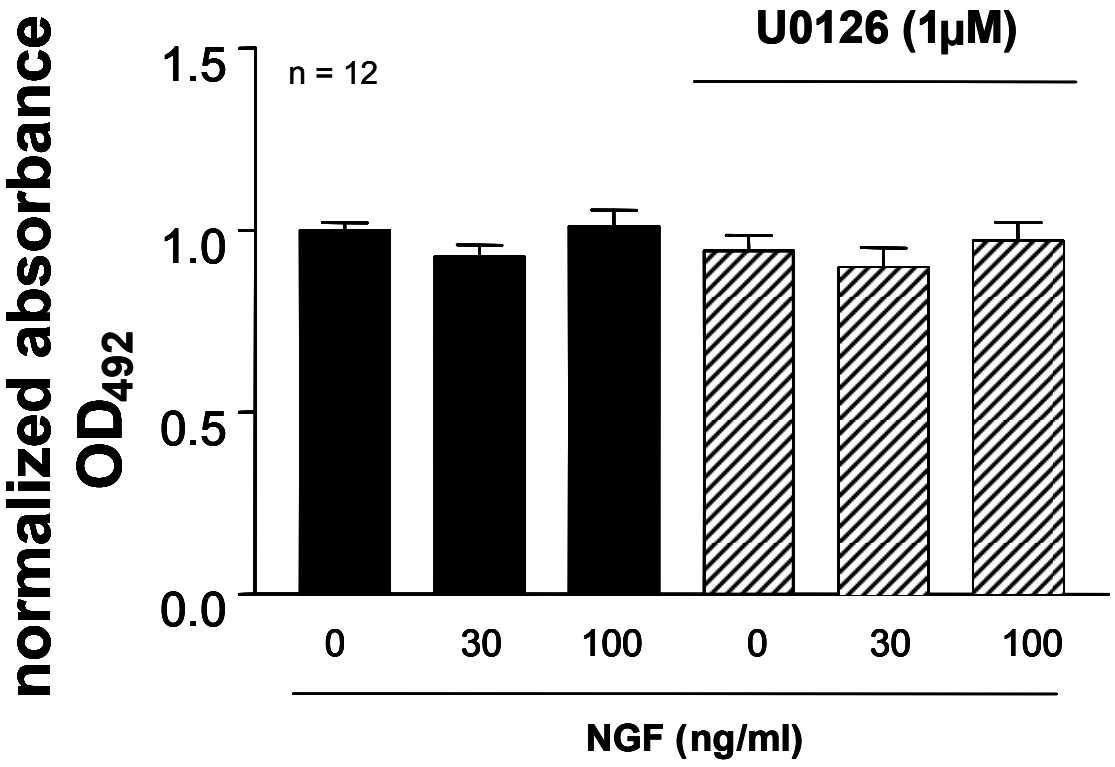


Figure 25. U0126 treatment for 7 days does not alter cell viability in cultures of dorsal root ganglion neurons. Cells were treated with the indicated amount of NGF in the absence (solid columns) or presence (hatched columns) of 1μM U0126. After 7 days, cultures were then subjected to an MTS assay per manufacturer's instructions. Absorbance was measured at 492 nm (see Methods). Data were normalized to untreated cells (0ng/ml NGF).

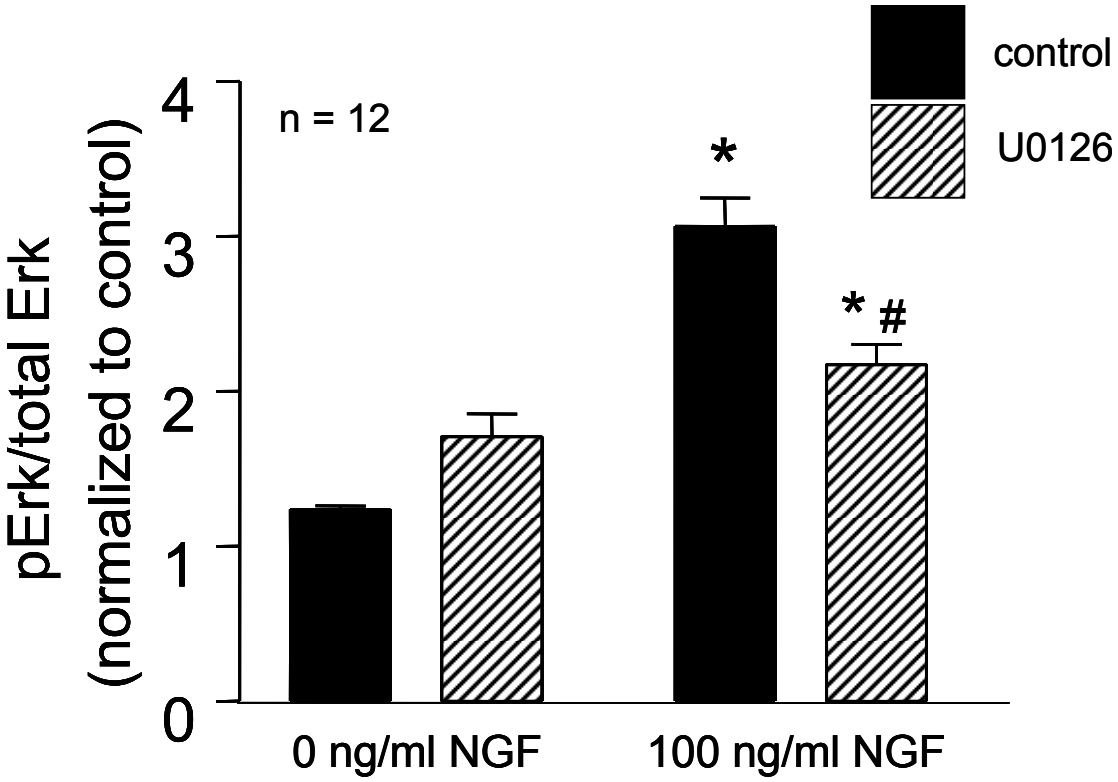


Figure 26. U0126 blocks the increase in pERK observed after 7 day treatment with 100ng/ml NGF. Rat sensory neurons were exposed for 7 days to 100ng/ml NGF in the presence (hatched bars) or absence (solid bars) of 1μM U0126. Neurons were then fixed and the levels of pERK relative to total ERK were measured (see Methods). The data are presented relative to control values (0 ng/ml NGF, U0126-untreated neurons). An (*) indicates a significant increase in the ratio of pERK/total ERK relative to control. An (#) indicates a significant decrease in the NGF-induced unregulated of iCGRP expression in neurons compared to U0126-untreated neurons ($p<0.05$, n=12 from 3 separate harvests, using two-way ANOVA with Bonferroni's post-hoc test).

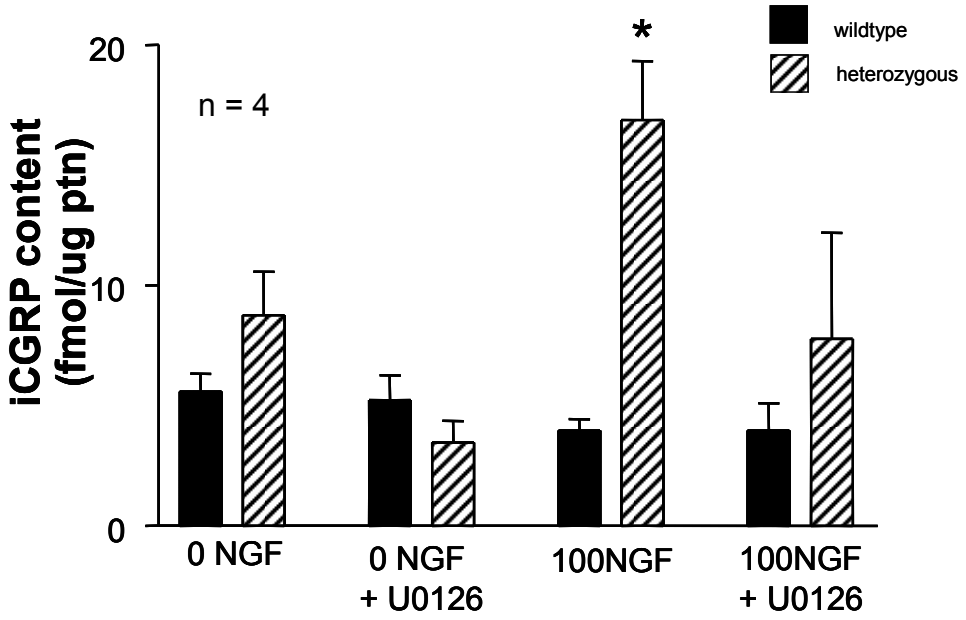


Figure 27. U0126 blocks the NGF-induced increase in iCGRP content in neurons from synGAP heterozygous mice. Neurons from synGAP heterozgyous mice (hatched bars) or their wildtype littermate (black bars) were exposed to either 0 or 100ng/ml NGF as indicated. Where indicated, groups of neurons were simultanesouly exposed to U0126 (1μM) for 7 days. The iCGRP content was measured in these neurons and normlaized to the amount of protein in the sample. An (*) indicates a significant increase in iCGRP content compared to neurons that were not treated with NGF ($p<0.05$, n=4 from 4 separate harvests, using two-way ANOVA with Bonferroni's post-hoc test).

Effects of Acute Exposure to NGF on the Capsaicin-evoked Release of iCGRP from Dorsal Root Ganglion Neurons in Culture

NGF increases the release of iCGRP from capsaicin-stimulated neurons

Acute exposure to NGF has been shown to increase the capsaicin-stimulated release of iCGRP independent of changes in content (Fehrenbacher, 2005). As shown in Figure 28, we were able to replicate this finding. The capsaicin-evoked release in the absence of NGF is 148.1 ± 16.42, while 10 min exposure to 100 ng/ml NGF prior to and throughout capsacin treatment causes more than a doubling in the release of iCGRP from sensory neurons to 323.3 ± 40.2 fmol/well/min (Fig. 28). This difference is not due to changes in content as the levels of iCGRP do not change with just 10 min treatment of NGF (Fehrenbacher 2005). These data suggest that acute exposure to NGF activates intracellular signaling cascades, and these post-translational events occur which alter neuronal sensitivity to capsaicin stimulation. These data compliment previous work that shows acute exposure to NGF increase the excitability of sensory neurons (Shu and Mendell, 1999; Bonnington and McNaughton, 2003; Bron et al., 2003; Zhang and Nicol, 2004; Zhuang et al., 2004). Controversy remains, however, as to the intracellular signaling that mediates the sensitivity of sensory neurons in response to treatment with NGF.

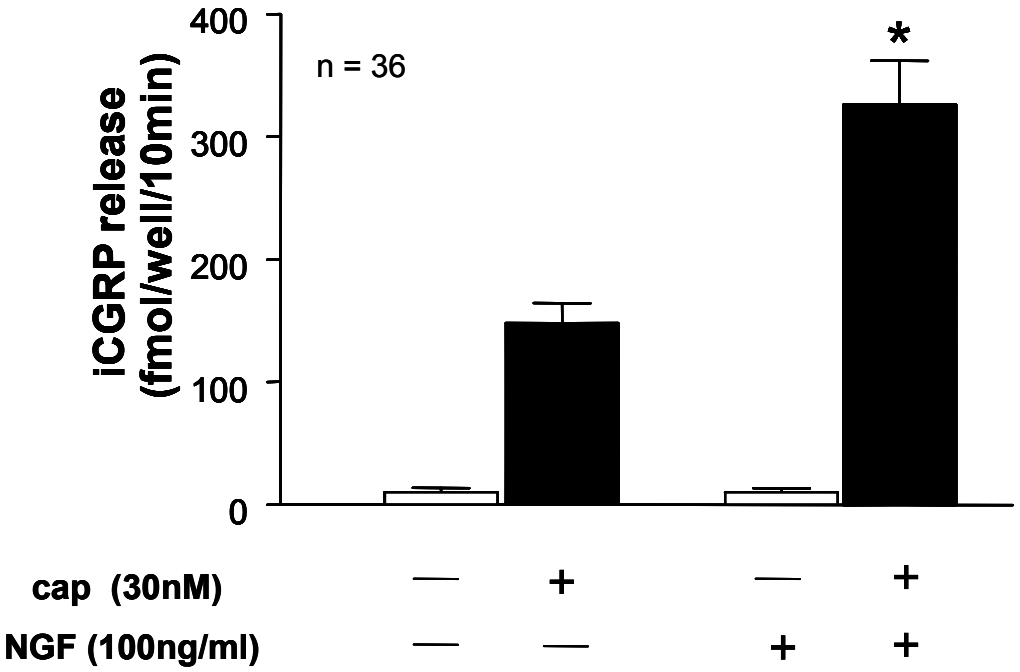


Figure 28. Brief exposure to NGF enhances capsaicin-evoked iCGRP release from neurons in culture. Resting release of iCGRP is indicated in the absence of capsaicin (cap) stimulation. Cells were exposed to HEPES buffer alone or to 100ng/ml NGF for 10 min. Next, cells were exposed to HEPES buffer containing capsaicin (30nM) with or without NGF for 10 min (stimulated release). NGF does not affect the amount of resting release from neurons in these experiments. An (*) indicates a significant increase in the stimulated release of iCGRP from neurons treated with NGF compared to non-treated controls ($p<0.05$, n=36, from 12 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

Expression of dnRas does not alter the degree of NGF-induced increases in capsaicin-evoked iCGRP release from sensory neurons

It has been shown that the Ras/MEK/ERK pathway is necessary for NGF-induced increases in cobalt uptake by neurons exposed to capsaicin (Bron et al., 2003). This and other studies suggest that the Ras/MEK/ERK pathway mediates acute sensitization of sensory neurons with brief exposure to NGF. Since we demonstrated that acute NGF increases capsaicin-evoked release of iCGRP, we examined whether the Ras/MEK/ERK pathway mediates this phenomenon. In the first series of experiments, we asked whether the NGF-induced increase in capsaicin-evoked release from neurons is altered when cells express dnRas. Because the expression of dnRas alters total content of iCGRP in neurons (Fig. 15), the capsaicin-evoked release of iCGRP was normalized to the total amount of neuropeptide measured to reflect changes in the sensitivity of neurons.

When neurons treated with media only were exposed to 30nM capsaicin, they released approximately 6.0 ± 1.5 percent of the total cellular iCGRP content When separate wells of neurons were exposed to NGF for 10 min prior to and throughout the capsaicin stimulation, the amount of iCGRP released doubled to 12.3 ± 2.2 percent of the total content (Fig. 29). This magnitude of change was observed in all treatment groups (media alone, control virus, or virus expressing dnRas). In cells expressing control virus, acute NGF exposure increased release from 7.5 ± 1.4 to 15.1 ± 1.0 percent. In cells expressing dnRas, release increased from 4.3 ± 0.6 to 7.8 ± 1.1 percent of the total content of iCGRP. Hence, dnRas did not affect the magnitude of sensitization by NGF. These data

suggest that acute NGF sensitization in neurons is not affected by Ras activity in these neurons.

However, in cells treated with virus expressing dnRas, the capsaicin-evoked release from neurons was lower from both NGF-treated and non-treated groups when compared to release from neurons treated with control virus. This indicated an overall decrease in capsaicin-evoked release when Ras activity was decreased. Because the effect was observed using the stimulating agent, capsaicin, it is possible that the decrease in evoked release was a result of decreased expression or sensitivity of TRPV1 in response to overexpression of dnRas (see Discussion).

Table 1 shows the resting release from sensory neurons treated with media alone, control virus, or virus expressing dnRas. Also reported in Table 1 are values for resting release from cells exposed to 100 ng/ml NGF for 10 minutes. In contrast to the capsaicin-evoked release, there were no significant changes in the resting release of iCGRP from sensory neurons in culture infected with lentivirus compared to resting release from cells treated with media alone (Tab. 1). These data indicate the neither infection with lentivirus nor brief exposure to NGF alters the resting release of iCGRP from sensory neurons in culture.

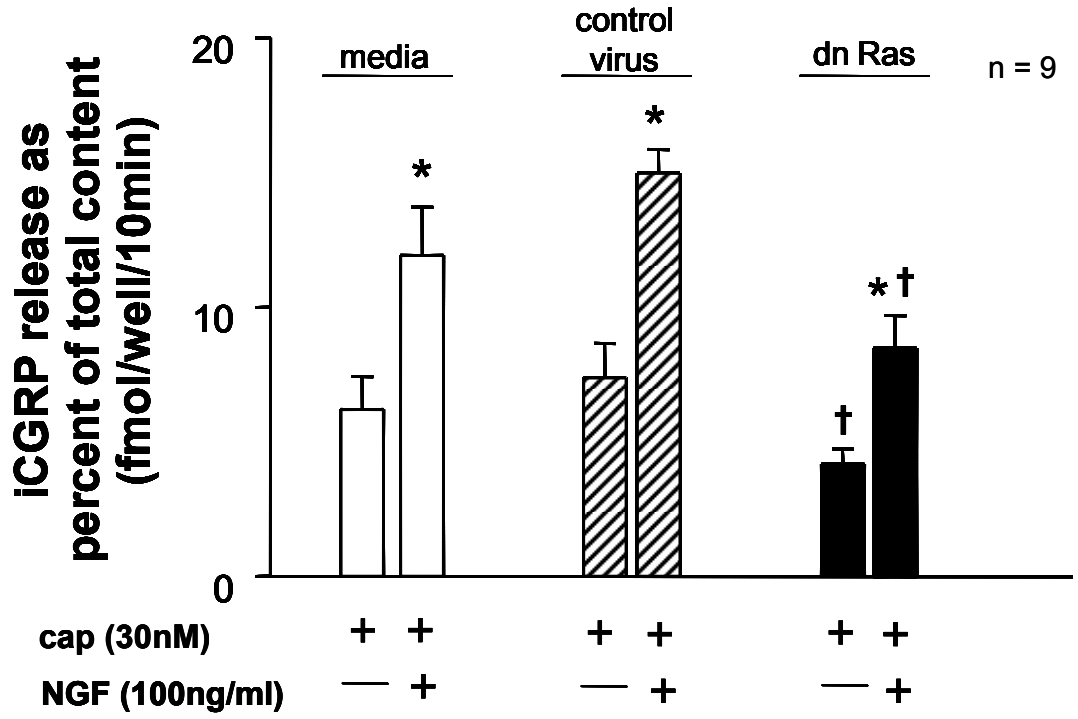


Figure 29. The increase in capsaicin-evoked iCGRP release by NGF is not altered by expression of dnRas. Neurons were treated with media alone (white bars), control virus (hatched bars), or virus expressing dnRas (black bars). After 7 days, a standard release assay was performed. Neurons were exposed to 30nM capsaicin with or without brief pretreament (10min) with 100 ng/ml NGF. An (*) indicates a significant increase in capsaicin-evoked release in the presence of NGF compared to its non-treated control. An (†) indicates a significant decrease in capsaicin-evoked release (with or without NGF) compared to neurons treated with control virus ($p<0.05$, n=9 from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

Resting iCGRP Release (Percentage of Total Content)

	no infection	control virus	dnRas
no NGF	0.16 ± 0.07	0.12 ± 0.03	0.15 ± 0.04
NGF	0.26 ± 0.09	0.09 ± 0.01	0.26 ± 0.06

Table 1. The resting release of iCGRP from sensory neurons in culture is not altered when neurons are infected with lentivirus expressing EGFP alone (control virus) or expressing EGFP with dnRas (dnRas). The values are reported as a percentage of the total iCGRP content expressed in neurons. Resting release in the presence of HEPES buffer alone (no NGF) or HEPES buffer with 100 ng/ml NGF for 10 min are presented. There were no differences among any of the values (mean ± SE, n = 9 from three separate harvests two-way ANOVA with Bonferroni's post-hoc test).

FTI-276 alters the capsaicin-evoked release of iCGRP from sensory neurons

In the experiments above, manipulation of the Ras pathway by incubation with FTI-276, a farnesyltransferase inhibitor, decreased the expression of iCGRP in response to NGF (Figs. 18 and 20). This suggested that the Ras pathway regulates the expression of iCGRP in response to NGF. Thus, we asked whether incubation with FTI-276 could alter the NGF-induced increase in capsaicin-evoked iCGRP release from sensory neurons to determine if the Ras pathway might be involved in regulating the increase in iCGRP release in response to brief exposure to the neurotrophin.

Neurons were treated with FTI-276 (5μM) for four days because the half-life of the Ras protein is approximately 24hr (Politi and Senderowicz, 2001). Control neurons were treated with vehicle. After 4d, greater than 90% of the existing Ras would be degraded, and fanesylation of newly formed Ras would be inhibited. After treatment, neurons were subjected to a standard release protocol. Neurons from each treatment group were exposed to 100ng/ml NGF for 10 min prior to and throughout the capsaicin-evoked release. As can be seen in Figure 30, vehicle-treated neurons showed the characteristic increase in capsaicin-evoked release when exposed to NGF acutely. Unexpectedly, the capsaicin-evoked release from FTI-276-treated neurons was significantly higher compared to capsaicin-evoked release from vehicle-treated neurons even in the absence of acute treatment with NGF. Because FTI-276 increased capsaicin-evoked release dramatically on its own, this agent could not be used to examine whether

inhibiting Ras alters the NGF-induced increase in transmitter release. In these experiments with FTI-276, the basal release was not altered (Table 2).

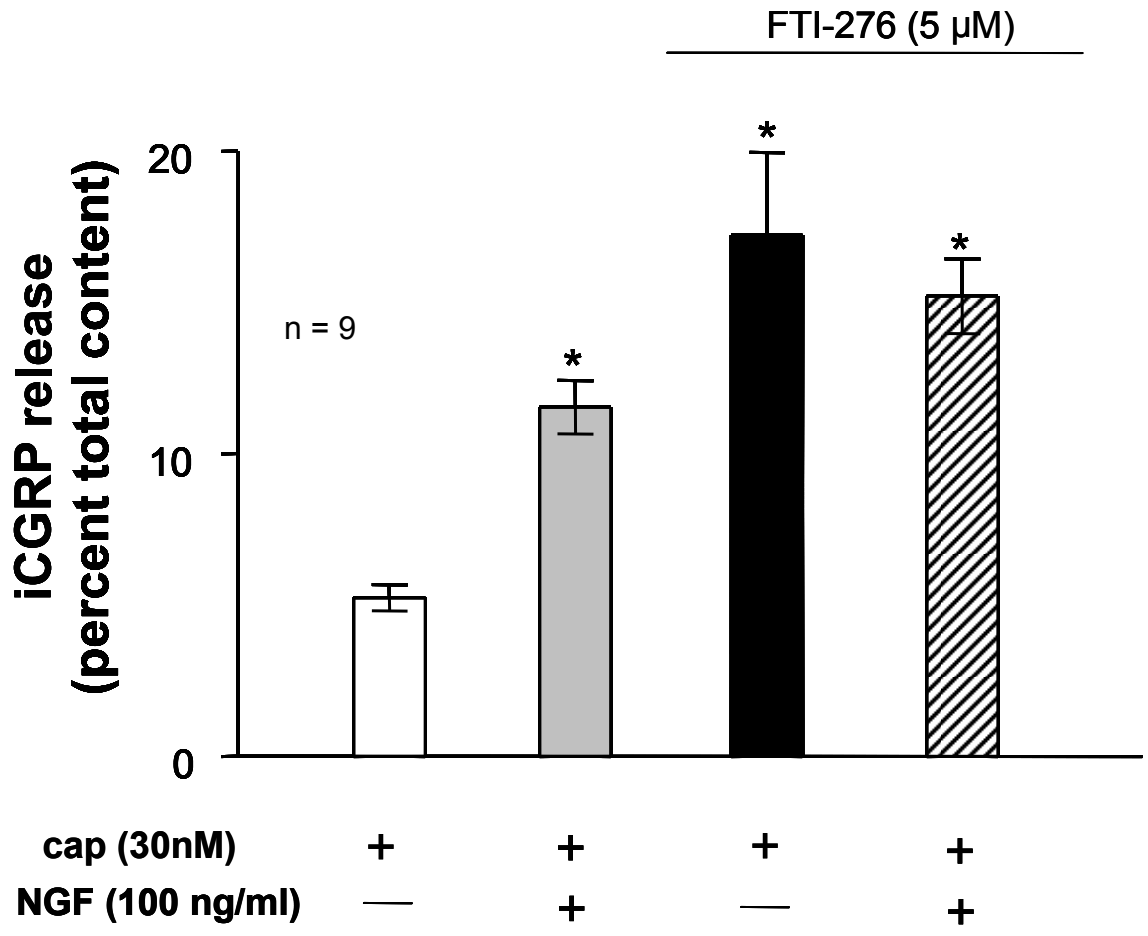


Figure 30. The inhibitor, FTI-276, increases the capsaicin-evoked release of iCGRP from sensory neurons in culture. Neurons were treated as previously described with vehicle or FTI-276 (5μM) as indicated. After treatment, the capsaicin-evoked release was measured in the presence or absence of 10 minute treatment with NGF (100ng/ml). The capsaicin-evoked release is shown from four conditions: vehicle treated (white bar), NGF treated (grey bar), FTI-treated (black bar), and FTI-treated neurons with acute NGF treatment (hatched bar). When vehicle-treated neurons were exposed to NGF acutely, the capsaicin-evoked release was increased. However, when neurons are treated with FTI-276, the capsaicin-evoked release is increased from control. An (*) indicates a significant increase in capsaicin-evoked release compared to control ($p<0.05$, n=9 from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

Resting iCGRP Release as a Percentage of Total Content

(fmol/well/10min)

	vehicle- treated	FTI-276 treated
no NGF	0.12 ± 0.04*	0.14 ± 0.02
NGF	0.13 ± 0.02	0.18 ± 0.03

Table 2. The resting release of iCGRP from sensory neurons in culture is not altered when neurons are treated with FTI-276 (5μM). The values are reported as a percentage of the total iCGRP content in neurons. Resting release in the presence of HEPES buffer alone (no NGF) or HEPES buffer with 100 ng/ml NGF for 10 min are presented. There were no differences among any of the values (*mean ± SE, n = 9 from three separate harvests, two-way ANOVA).

The NGF-induced increase in the capsaicin-evoked release of iCGRP from synGAP heterozygous neurons is not different compared to release from wildtype littermates

The capsaicin-evoked release from neurons isolated from synGAP heterozygous mice was tested to determine if neurons were sensitized by brief exposure to 100 ng/ml NGF. The rationale for testing the effects of NGF on capsaicin-evoked release was to test the hypothesis that neurons from heterozygous animals have an increased capsaicin-evoked release upon brief exposure to NGF secondary to increased Ras/MEK/ERK activation in cells with less expression of synGAP.

Neurons from wildtype and synGAP heterozygous mice were harvested and grown in culture with 30ng/ml NGF, and the resting and capsaicin-evoked release of iCGRP was measured from wildtype and synGAP heterozygous neurons. Groups of neurons from both genotypes were exposed to NGF for 10 minutes prior to and during capsaicin stimulation. When wildtype neurons were exposed to 100 ng/ml NGF for 10 minutes prior to and throughout capsaicin-evoked release, there was a significant increase in the iCGRP outflow from neurons from 5.6 ± 1.0 percent of total iCGRP content to 8.9 ± 1.9 percent (Fig. 31). The same trend was observed in neurons from synGAP heterozygous animals. In the presence of acute NGF, release increased from 6.5 ± 0.9 percent to 11.2 ± 1.3 percent. No difference was observed between the degree of NGF sensitization when comparing neurons from heterozygous neurons and their littermate controls. Furthermore, there was no alteration of resting release in

heterozygous neurons compared to wildtype controls. These data taken with the above experiments showing that expression of dnRas does not alter the NGF-induced increase in iCGRP release from sensory neurons suggest that the Ras pathway does not regulate the sensitivity of sensory neurons to capsaicin with brief exposure to NGF.

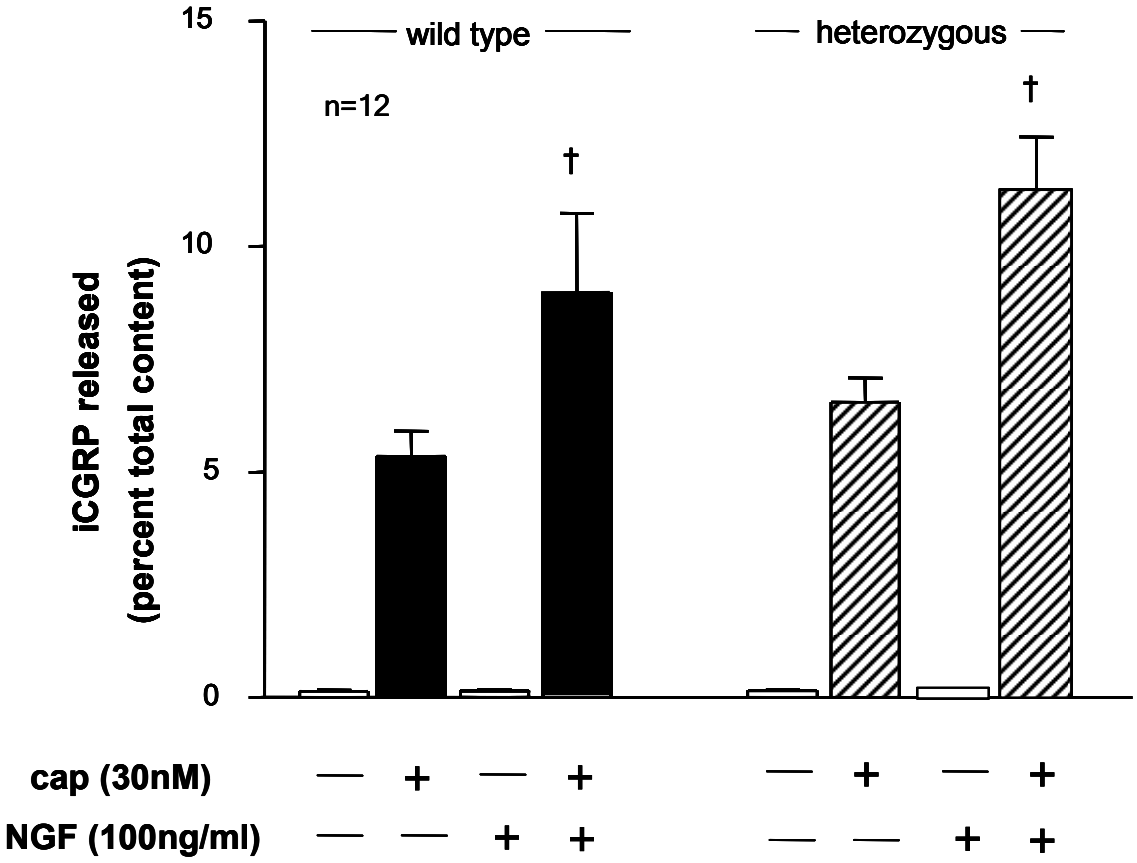


Figure 31. NGF augments capsaicin-evoked release of iCGRP from wildtype neurons and from neurons with a heterozygous mutation of synGAP. The resting- and 30nM capsaicin-evoked release for each condition is shown. NGF treatment (100ng/ml) for 10 min prior to and throughout capsaicin stimulation augments iCGRP release. Wildtype and heterozygous animals demonstrate the same degree of sensitization in response to NGF. (†) indicates a significant increase in capsaicin-evoked release relative release in the presence of acute NGF treatment. There is no difference among the resting releases under any condition ($p<0.05$ vs. release without NGF treatment, n=12 from 4 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

siRNA decreases the expression of synGAP in rat sensory neuronal cultures, and increases the level of RasGTP measured in cell lysates

With transgenic animals, congenital changes could be masked by compensation that can occur in cells. Thus, in the presence of decreased synGAP expression, it is possible that another GAP becomes overexpressed to correct aberrant Ras signaling in cells. To address this concern, siRNA against synGAP was designed and transfected into neurons from adult rats to transiently decrease the expression of synGAP in sensory neurons. Using this methodology, we examined whether decreasing the expression of synGAP in wildtype adult rat neurons could alter the NGF-induced sensitization of sensory neurons.

Neurons were grown in culture in the presence of 30 ng/ml NGF for two days. The neuronal cultures then were treated with serum-free media alone, serum-free media containing the transfection reagent, NeuroPorter®, media containing NeuroPorter® with 100nM of control siRNA (apurinergic/apyridinimic endonuclease, APE, see Methods), or media with NeuroPorter® containing 100nM synGAP siRNA. After 2 days, the serum-free media was replaced with fresh F-12 media, and media containing 30 ng/ml NGF was changed every other day for 6 days. APE siRNA was chosen as a control because the protein was under study in the laboratory, and because APE siRNA significantly depletes APE without affecting the release of iCGRP from neurons (data not shown).

Figure 32A shows a representative Western blot of cell lysates from the four different treatment groups, whereas Figure 32B summarizes densitometry results from Western blots from three separate experiments. Protein from the

hippocampus was used as a positive control for Western blotting as mouse neurons from this brain region express synGAP (Komiyama et al., 2002c). As can be seen in the representative blot in Figure 32A, an immunoreactive band appears near 135kDa, the size of synGAP. The levels of synGAP expression in NeuroPorter® or APE siRNA-treated neurons after three separate experiments were not different from control (Fig. 32B). However, the levels of synGAP in cells treated with synGAP-siRNA were decreased by 60% compared to control.

Once we confirmed that synGAP siRNA reduced synGAP expression in cell lysates, cells were treated with APE or synGAP siRNA, and the levels of RasGTP were measured to demonstrate a functional effect of the siRNA. As seen in Figure 33, there was a significant four-fold increase in the level of RasGTP in neurons treated with synGAP siRNA compared to neurons treated with NeuroPorter® alone. Therefore, these results demonstrate that a decrease in the level of synGAP increases the level of RasGTP present in cell lysates of sensory neuronal cultures, suggesting that Ras signaling is increased in these cells.

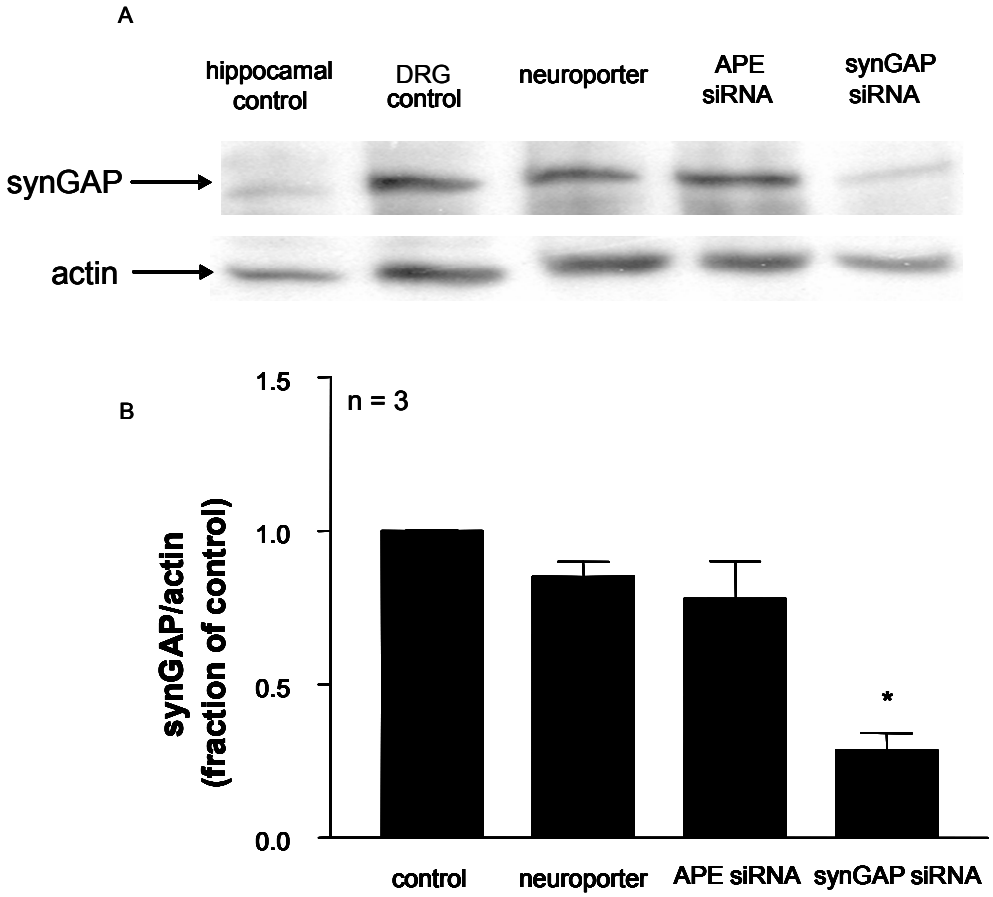


Figure 32. Rat sensory neurons in culture exposed to synGAP siRNA (100nM) have reduced expression of the synGAP protein. (A) Representative Western blot demonstrating the presence of synGAP protein in both hippocampal neurons and dorsal root ganglion neurons in culture. (B) Summary data from Western blots of neurons treated with siRNA. The levels of synGAP are normalized to actin, and the values are reported as a fraction of the control level. An (*) represents a significant reduction of the synGAP protein relative to control levels ($p<0.05$, n=3 from three separate harvests, using one-way ANOVA with Tukey's post-hoc test).

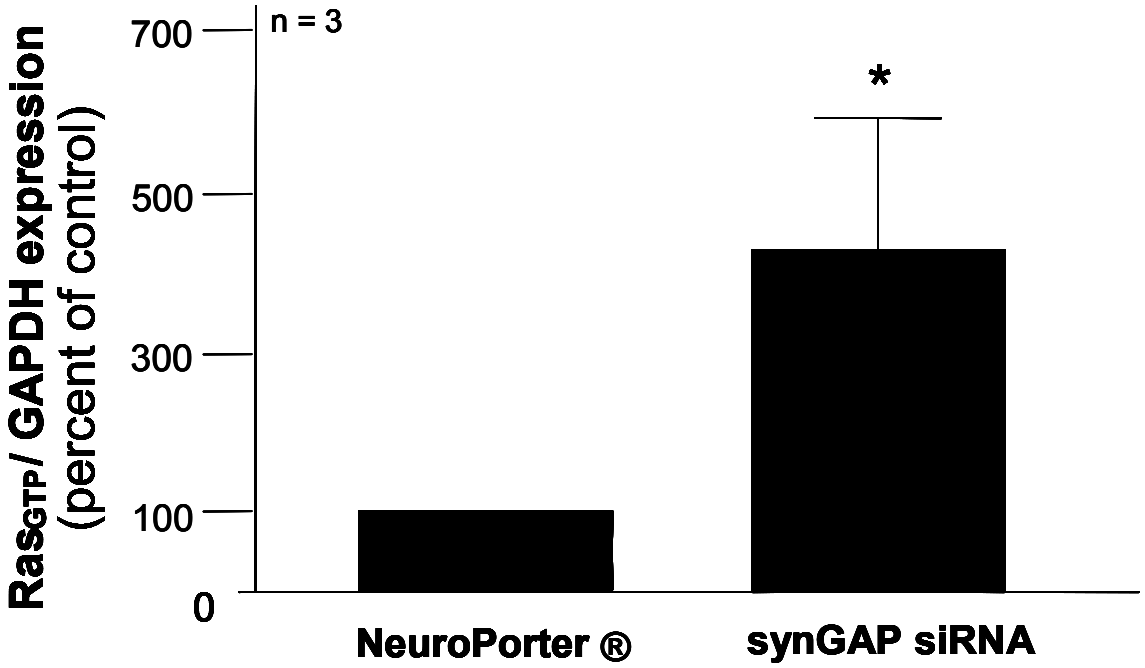


Figure 33. The level of RasGTP in neurons treated with synGAP siRNA in increased compared to neurons treated with NeuroPorter® alone. After treatment with either NeuroPorter® or 100nM synGAP siRNA, cells were collected and the amount of RasGTP was measured. The RasGTP present in the cells was normalized to the loading control GAPDH, and the results were reported as a percentage of the control value. An (*) indicates a significant increase in the RasGTP level in synGAP siRNA-treated neurons relative to its NeuroPorter® control ($p<0.05$, n=3 from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

Decreased synGAP expression augments NGF-induced increase in iCGRP release from neurons

In these experiments, we examined whether siRNA knockdown of synGAP in neurons derived from wildtype rats would alter the NGF-induced increase in capsaicin-evoked iCGRP release. Neurons were treated with synGAP siRNA or control siRNA as previously described. After treatment, we measured iCGRP release following our standard protocol. Neurons were treated for 10 minutes with either HEPES buffer alone or buffer containing 100 ng/ml NGF. Then, neurons were exposed for 10 minutes to 30nM capsaicin in the presence or absence of NGF.

Treatment with siRNA did not change the resting release of iCGRP from neurons as there were no significant differences among the resting release measured from the treatment groups (Table 3). However, the capsaicin-evoked release is presented in Figure 34. There was a significant increase in the capsaicin-evoked release of iCGRP when neurons were exposed acutely to 100ng/ml NGF. Release from neurons treated with APE siRNA increased from 100 ± 23 fmol iCGRP/well/10min to 260 ± 30 fmol iCGRP when treated briefly with NGF. In neurons treated with synGAP siRNA, NGF treatment increased capsaicin-evoked iCGRP release from 130 ± 17 to 360 ± 45 fmol iCGRP/well/10min. The increased release in response to brief exposure to NGF was observed in both the APE siRNA-treated neurons and synGAP siRNA–treated neurons. However, the capsaicin-evoked release from synGAP siRNA–treated cells in the presence of NGF was slightly, but significantly greater than

the NGF-induced increase in release for APE siRNA–treated neurons. The NGF-induced increase in capsaicin-evoked release was 2.60-fold greater for neurons treated with APE siRNA, while the increase was 2.75-fold in neurons treated with synGAP siRNA.

While NGF causes an increase in the iCGRP release in these experiments, these data appear to be inconsistent with data in experiments measuring NGF-induced sensitization in dnRas-treated and in synGAP heterozygous neurons. With overexpression of dnRas in rat neurons or in neurons from synGAP heterozygous mice, the degree of NGF sensitization was not different from controls (Figs. 16 and 31). One explanation for the data in neurons derived from synGAP heterozygous mice is that the animals adapt to the increased Ras activity so that changes in iCGRP release in the presence of NGF are masked by the cells ability to offset changes in Ras activity through unknown mechanisms (Fig. 31). Another possible explanation is that release values obtained in experiments using siRNA were not normalized to cellular iCGRP content (Fig. 32), therefore the increase in release is a result of increased expression of iCGRP. Thus, further experiments are needed to eliminate the impact of potential content changes on release.

Resting iCGRP Release

(fmol/well/10min)

	APE siRNA-treated	synGAP siRNA-treated
no NGF	16.9 ± 2.9*	13.3 ± 1.1
NGF	18.9 ± 3.1	11.6 ± 1.3

* mean ± SE, n = 16 from four separate harvests

Table 3. The resting release of iCGRP from sensory neurons in culture is not altered when neurons are treated with synGAP siRNA. The release values are reported fmol release per well of cells over 10 min. Resting release in the presence of HEPES buffer alone (no NGF) or HEPES buffer with 100 ng/ml NGF for 10 min are presented. There were no differences among any of the values (two-way ANOVA).

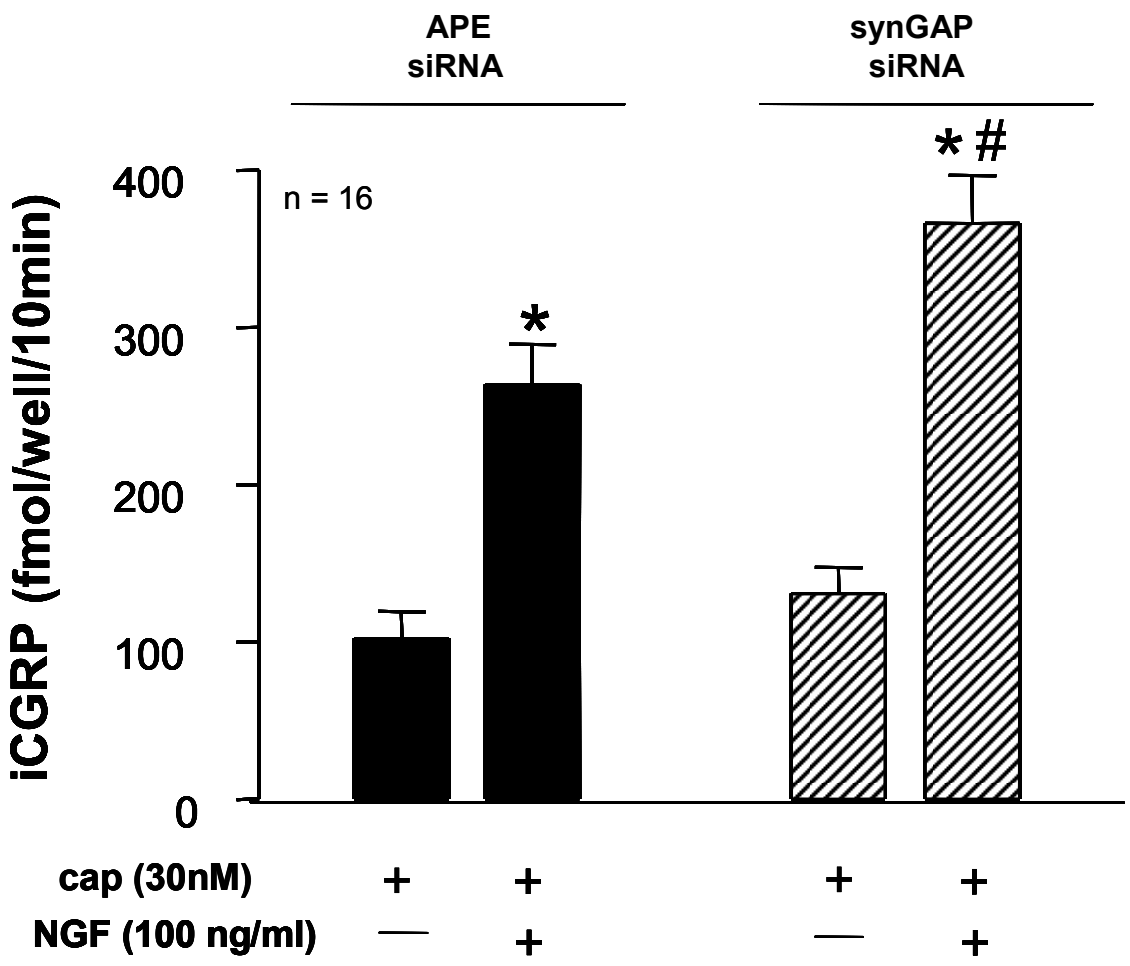


Figure 34. The NGF-induced sensitization of capsaicin-evoked iCGRP release (not normalized to content) is greater in neurons treated with synGAP siRNA. The 30nM capsaicin-evoked release from neurons treated with either siRNA to APE (black bars) or synGAP (hatched bars) is shown. Where indicated, groups of cells are exposed to 100ng/ml NGF for 10 min prior to and throughout the 10 min stimulation with capsaicin. An (*) indicates a significant increase in release of iCGRP from neurons treated with NGF acutely compared to non-treated controls. An (#) indicates a significant difference between iCGRP released in the presence of NGF when comparing APE siRNA-treated and synGAP siRNA-treated neurons ($p<0.05$, n=16, from 4 separate harvests, using two-way ANOVA with Bonferroni's post-hoc test).

The MEK inhibitors, U0126 and PD98059, do not alter the NGF-induced increase in capsaicin-evoked iCGRP release from sensory neurons in culture

To further explore whether the Ras/MEK/ERK pathway regulates the NGF-induced increases stimulated release of iCGRP from sensory neurons, the activation of ERK was measured after acute exposure to the trophic factor. Neurons were grown in 30 ng/ml NGF for 7 days, and then the cultures were washed once with HEPES. Each well of cells was then exposed for a total of 20 minutes to 15, 30, 100, or 250 ng/ml NGF. Immediately after treatment with NGF, cells were exposed to fixative, 4% paraformaldehyde, for 20 minutes. Then cells were permeabilized as described in Methods, and immunocytochemistry was used to measure the levels of pERK and total ERK in fixed cells. As shown in Figure 35, neurons exposed acutely to NGF had no changes in the levels of pERK relative to total ERK expression (Fig. 35).

However, to ensure changes in the level of pERK could be detected under the experimental conditions using immunocytochemistry, we included a positive control in experiments. We examined whether a 10 minute exposure to 100nM of the phorbol ester, phorbol 12,13-dibutyrate (PDBu), would increase pERK relative to total ERK expression. We used PDBu since activation of the PKC pathway has been shown in other cell systems to phosphorylate ERK through direct activation of Raf-1 kinase (Buscher et al., 1995; Ambrosini et al., 2000). As can be seen in Figure 35, 100nM PDBu increased the level of pERK 3-fold in dorsal root ganglion cultures. Thus, the lack of an increase in levels of pERK in

neurons treated with NGF for 10 minutes was not secondary to limitations of detection.

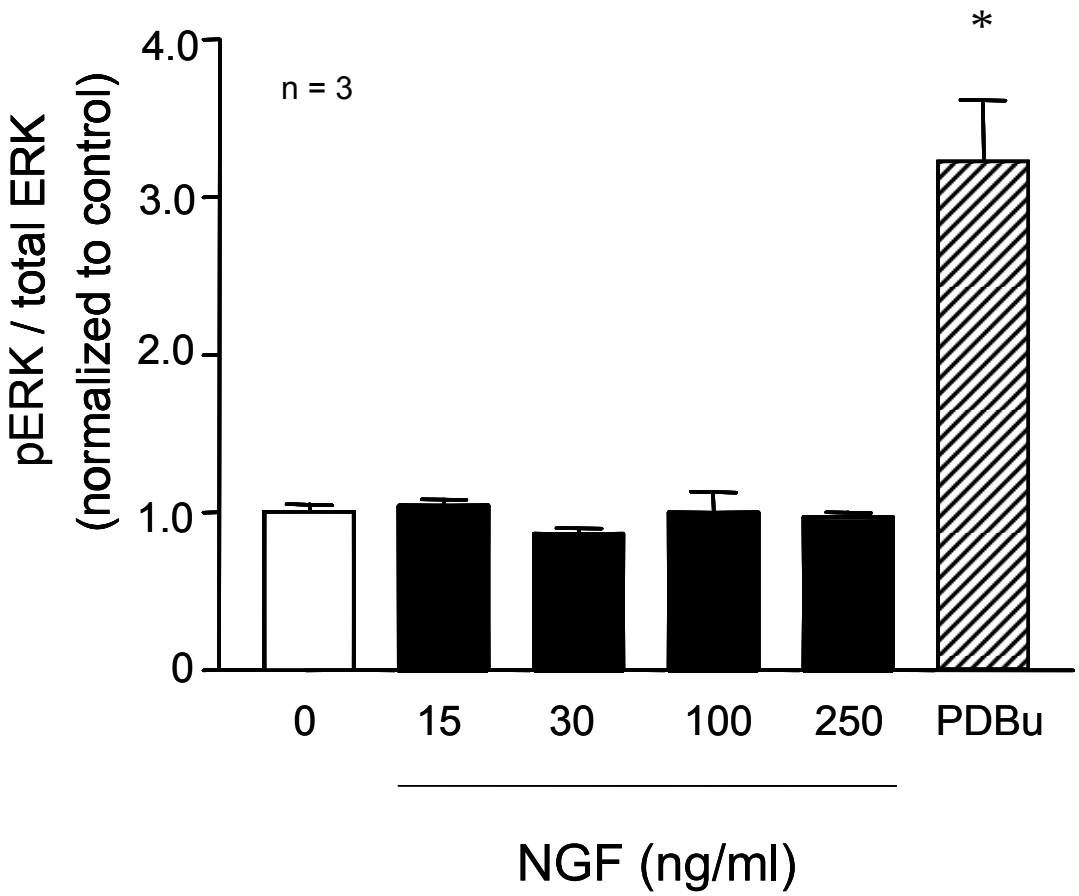


Figure 35. Ten-minute treatment with NGF does not alter the level of pERK relative to total ERK in sensory neurons in culture. Neurons were grown for 7 days in 30ng/ml NGF. On the 7th day, neurons were washed with HEPES buffer, and then were treated for 20 min with either buffer alone (0 NGF), the indicated concentration of NGF, or 100nM PDBu. After the brief treatment, neurons were fixed immediately and levels of pERK and total ERK were measured. The ratio of pERK/total ERK is normalized to control levels. An (*) indicates a significant increase in the ratio compared to cells treated with media alone ($p<0.05$, n=3 from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

As described in the Introduction, MEK and ERK have been implicated in the regulation of the effects of NGF on cells *in vitro*. Although changes in pERK levels were not observed in response to brief NGF exposure (Fig. 35), it is possible that small changes may be sufficient to cause an increase in NGF-induced augmentation of iCGRP release from neurons. Therefore, we examined whether U0126 could block the NGF-induced increase in capsaicin-evoked iCGRP release from rat sensory neurons in culture. As shown in previous experiments, NGF treatment for 10 minutes prior to and throughout capsaicin stimulation increases the amount of iCGRP released from sensory neurons by approximately two-fold from 143 ± 10 to 375 ± 53 fmol iCGRP/well/10min (Fig. 36). When cells were pretreated for 10 minutes prior to and throughout capsaicin-evoked release with U0126 (1μM) in HEPES, and through release with the MEK inhibitor did not alter capsaicin-evoked release. Furthermore, when cells were pretreated with U0126 and then treated with U0126 and 100ng/ml NGF simultaneously, the MEK inhibitor did not alter the sensitizing effect of the neurotrophin. These results indicated MEK is not involved in the mechanism of NGF-induced increases in iCGRP released from sensory neurons.

In the above experiments, we demonstrated that PDBu increased pERK in neuronal cultures (Fig. 35), and it is known that PDBu augments capsaicin-evoked iCGRP release from sensory neurons (Barber and Vasko, 1996b). These two events, the increase in pERK and the increase in evoked release of iCGRP in response to PDBu, may be related. Consequently, we tested whether the ability of PDBu to augment basal or capsaicin-evoked release of iCGRP release

could be blocked by the MEK inhibitor, U0126 (Fig. 37). For these studies, wells of cells were exposed to PDBu (10nM) for 10 minutes prior to and throughout capsaicin-evoked release, and the PDBu treatment increased the capsaicin-evoked iCGRP release from 225 ± 15 to 548 ± 25 fmol iCGRP/well/10min. PDBu also increased resting release of iCGRP from sensory neurons in culture from 15 ± 2 to 75 ± 24 fmol iCGRP/well/10min. These results agreed with previous experiments performed in our laboratory using embryonic dorsal root ganglion neurons (Barber and Vasko, 1996). Treatment with U0126 did not block the ability of PDBu-induced increase in iCGRP release from sensory neurons (Fig. 37). These data indicated that phorbol ester induced increases in iCGRP release are not mediated by MEK activation.

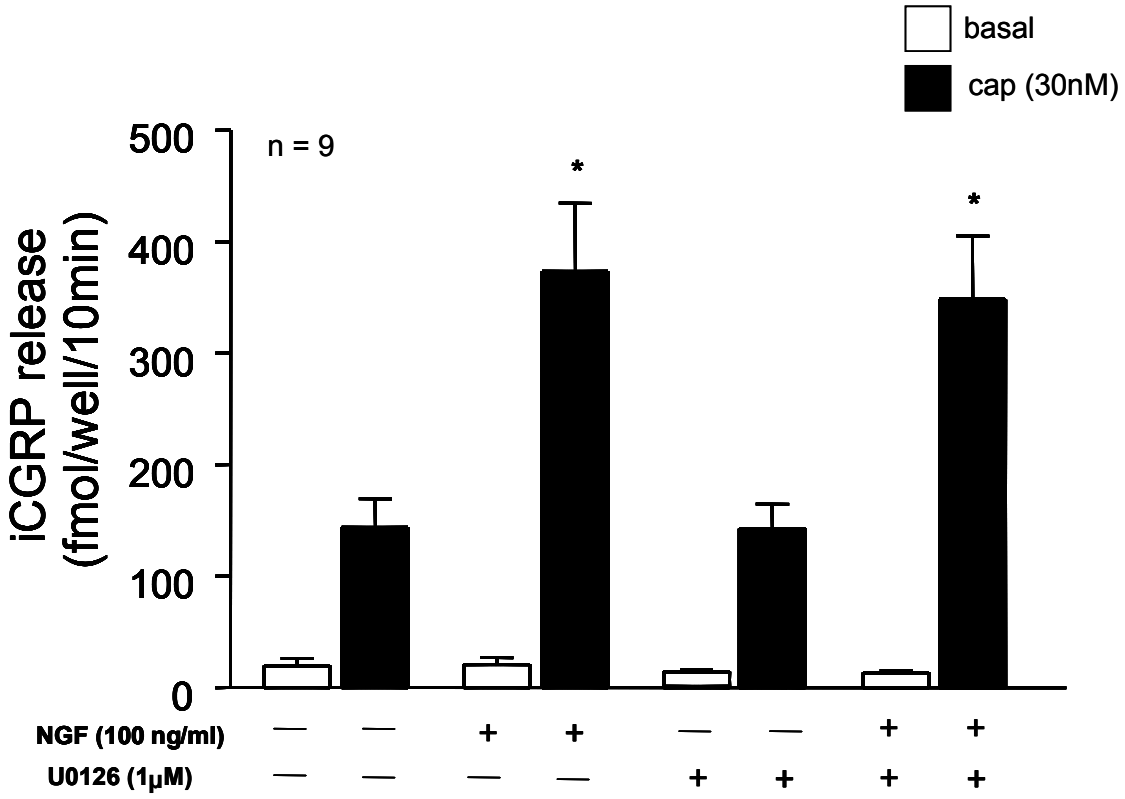


Figure 36. U0126 does not block the NGF-induced increase in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture. The resting (white bars) and capsaicin-evoked (black bars) iCGRP release from neurons are shown. Neurons were exposed to 100ng/ml NGF for 10 min prior to and throughout capsaicin-evoked release as indicated. Some groups of neurons were pretreated for 10 min with U0126 (not shown), and then exposed to 1μM U0126 for 10 min prior to and throughout capsaicin-evoked release as indicated. An (*) indicates a significant increase in iCGRP release from sensory neurons compared to non-treated controls ($p<0.05$, n=9 from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

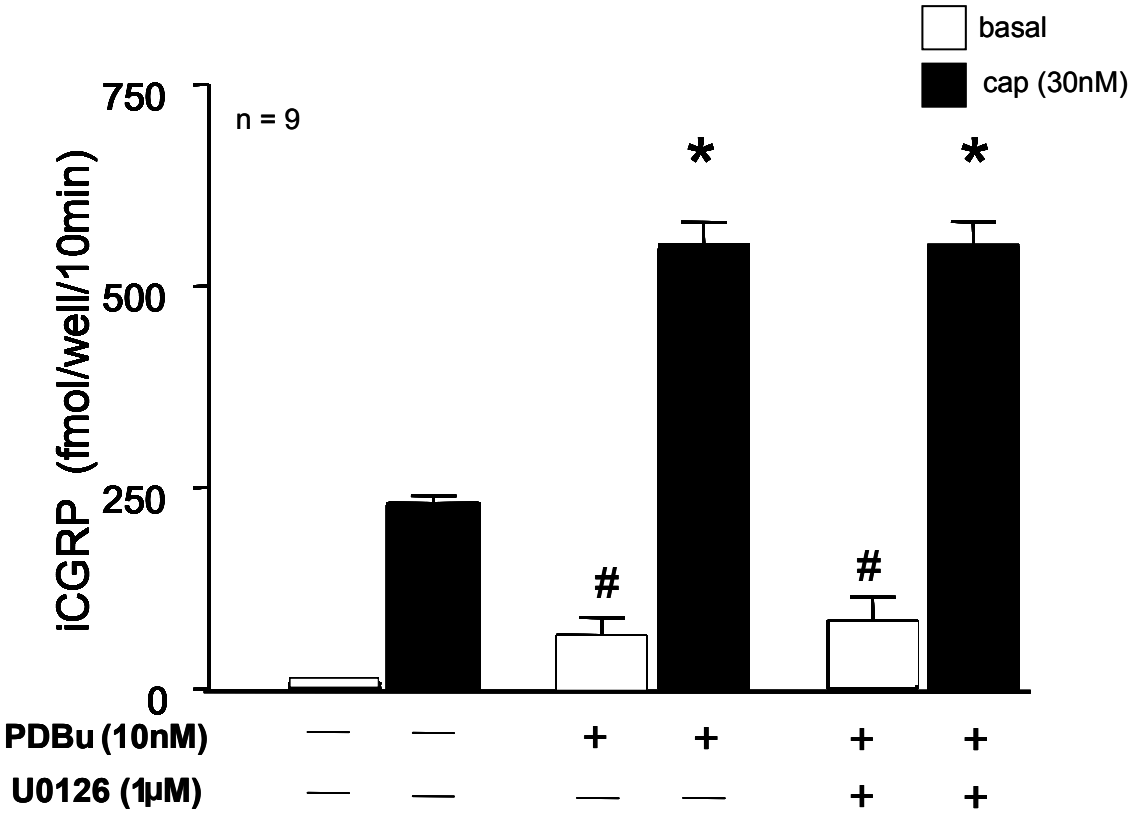


Figure 37. U0126 does not block the PDBu-induced increase in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture. The resting (white bars) and capsaicin-evoked (black bars) iCGRP release from neurons are shown. Neurons were exposed to 10nM PDBu for 10 min prior to and throughout capsaicin-evoked release as indicated. Some groups of neurons were pretreated for 10 min with U0126 (not shown), and then exposed to 1μM U0126 for 10 min prior to and throughout capsaicin-evoked release as indicated. An (*) indicates a significant increase in iCGRP release from sensory neurons compared to non-treated controls. An (#) indicates a significant increase in the resting release compared to untreated controls ($p<0.05$, n=9 from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

To confirm that 1μM U0126 is inhibiting the activation of MEK under our experimental conditions, studies were designed to show that this drug inhibited the PDBu-induced increase in phosphorylation of ERK. Because ERK is selectively activated by MEK, a blockade of pERK would indicate that U0126 inhibited MEK activity.

When neuronal cultures are exposed to 10nM PDBu for 10 minutes there is a 2-fold increase in the ratio of pERK to total ERK (Fig. 38). This increase was partially attenuated by treating cultures with 100nM U0126 and completely blocked by U0126. These data indicated that the MEK inhibitor at this concentration blocks activation of MEK and activation and phosphorylation of ERK. Thus the lack of effect of U0126 on NGF-induced increases in iCGRP release from neurons is not due to a lack of MEK inhibition, but likely because NGF-sensitivity is not mediated by ERK activation. This data in combination with data from Figure 36 showing no inhibition of NGF-induced increases in iCGRP release from neurons using the same concentration of U0126 (Fig. 36) suggest that pERK is not a mediator of NGF-induced sensitization.

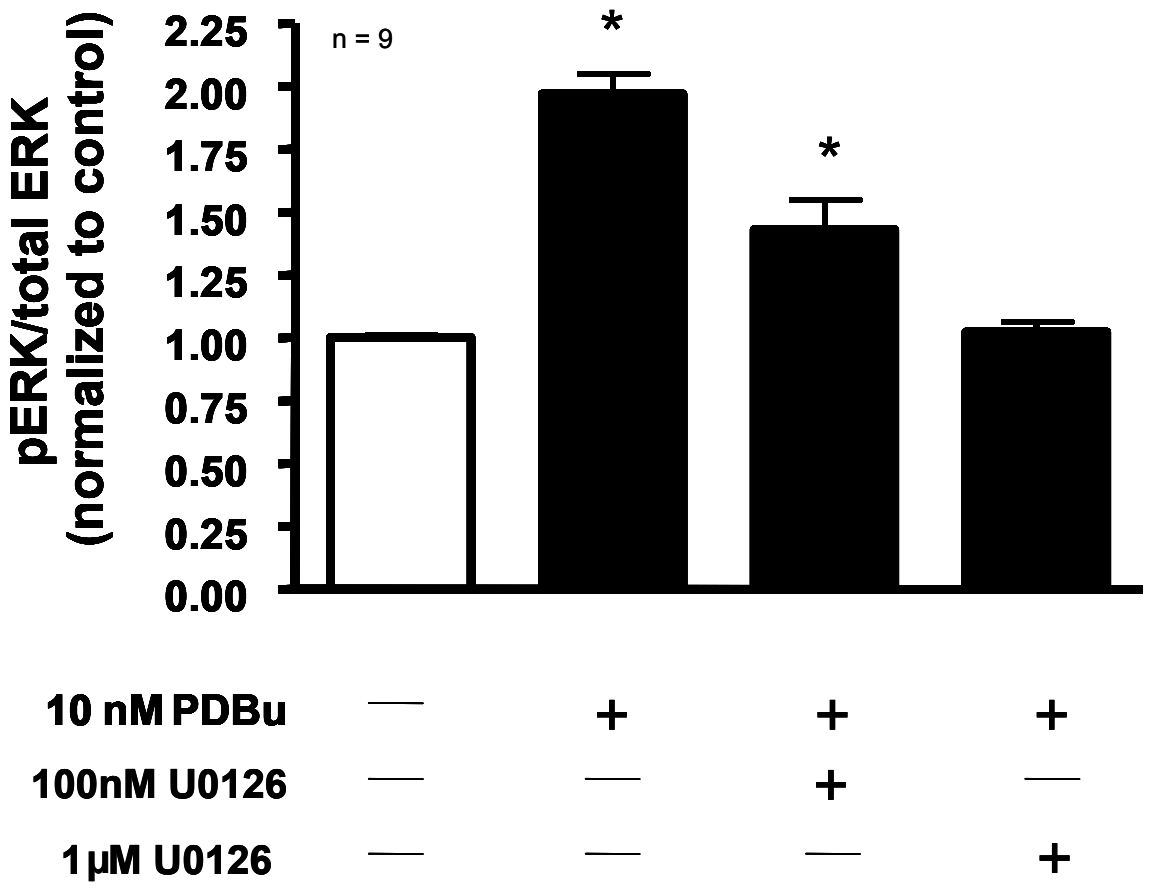


Figure 38. U0126 (1µM) blocks PDBu-induced upregulation of pERK in sensory neurons in culture. Neurons were exposed to the phorbol ester, PDBu (10nM) for 10 min. Where indicated, groups of cells were treated throughout PDBu stimulation with 100nM or 1µM U0126. An (*) indicates a significant increase of the ratio of pERK to total ERK compared to control (no PDBu treatment) ($p<0.05$, n=9 from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

We also examined whether PD 98059, another MEK inhibitor, could block the ability of NGF to augment capsaicin-evoked iCGRP release from sensory neurons (Fig. 39). We used this alternative inhibitor as a second means to determine whether MEK regulates the increase in NGF-induced increases in capsaicin-evoked release.

When cells were pretreated with 20μM PD 98059, 10 times the IC_{50} (Pang et al., 1995), the drug did not attenuate the increase in capsaicin-evoked release with acute exposure to 100 ng/ml NGF. When the concentration of the drug was increased, it stimulated iCGRP release directly to a level similar in magnitude to stimulation with 30nM capsaicin (data not shown). Because of the lack of effect at the lower concentration of the inhibitor and the alteration of basal release in the presence of the higher concentration of drug, the experiments were not repeated. However, these initial data combined with data using the MEK inhibitor, U0126, are evidence that MEK and ERK do not regulate the NGF-induced increase in capsaicin-evoked iCGRP release from sensory neurons in culture.

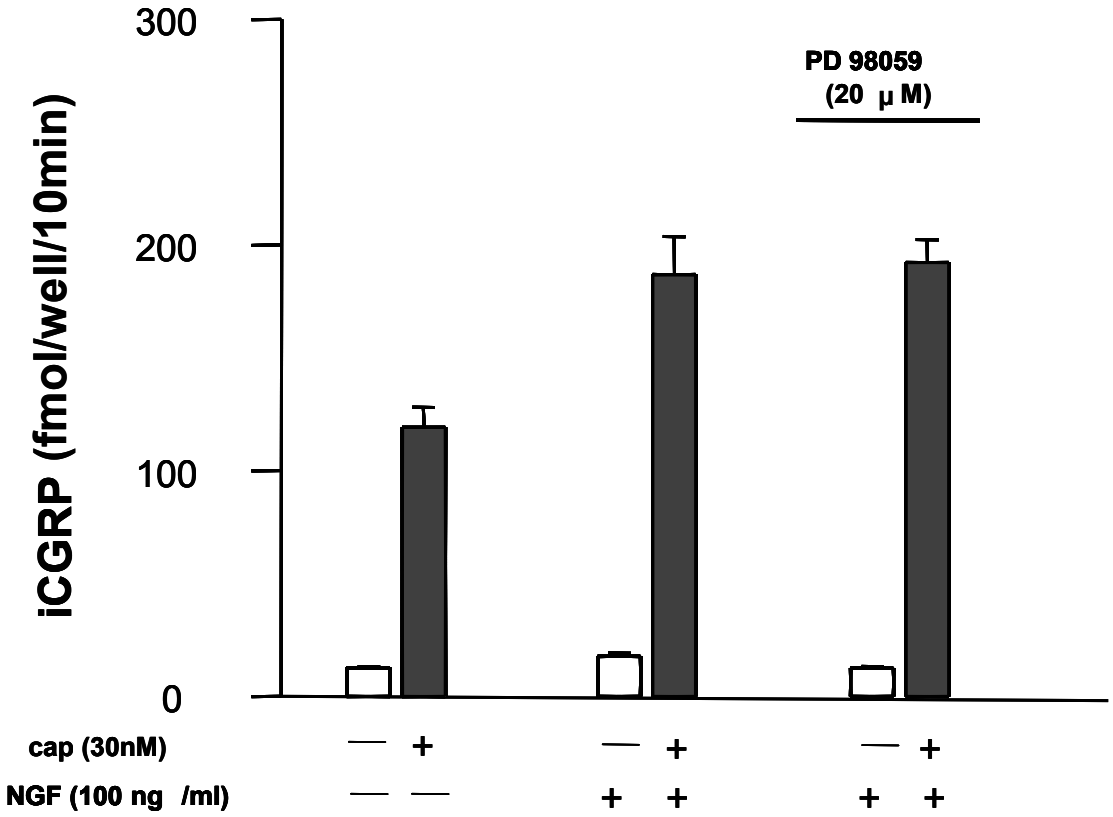


Figure 39. PD 98059 does not affect NGF-induced increases in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture. The resting and capsaicin-evoked iCGRP release from neurons are shown for each of the five conditions. Neurons were exposed to100 ng/ml NGF for 10 min prior to and throughout capsaicin-evoked release as indicated below the x-axis. One group of cells were pretreated for 10 min with 20µM PD 98056, and then throughout the release protocol as indicated (n=3, from a single harvest of neurons).

The PI3 kinase inhibitors, LY294002 and wortmannin, do not alter the NGF-induced increase in capsaicin-evoked iCGRP release from sensory neurons

Because the Ras/MEK/ERK cascade is not involved mediating the ability of NGF to augment capsaicin-evoked release of iCGRP, we examined whether another signaling pathway implicated in signaling downstream from NGF could mediate this action. The PI3 kinase pathway is thought to control a number of NGF-mediated actions in sensory neurons (Bonnington and McNaughton, 2003c; Zhuang et al., 2004a). Zhuang *et. al*. found that Akt activation was increased with 2 minute exposure to NGF, and this increase was blocked by the PI3 kinase inhibitor, LY294002. Bonnington and McNaughton found just 1-2 minute exposure to NGF induced an increase in intracellular Ca^{2+} upon exposure to capsaicin. This effect was attenuated by a PI3 kinase inhibitor, wortmannin. Based on these experiments, studies were designed to test whether the PI3 kinase inhibitors, LY294002 and wortmannin, could block the NGF-induced increase in capsaicin-evoked iCGRP release from sensory neurons in culture.

In these experiments, 10 minute treatment with 100 ng/ml NGF caused a two-fold increase in 30nM capsaicin-evoked release, from 185 ± 45 fmol iCGRP/well/10min to 392 ± 57 iCGRP/well/10min. When neurons were incubated with the PI3 kinase inhibitors, LY294002 (3μM) or wortmaninnin (3nM), neither of the drugs caused a changed capsaicin-evoked release alone. In addition, at the concentrations used these inhibitors did not cause changes in resting release from neurons. Drug concentrations higher than 3μM LY294002 or 3nM

wortmannin caused a significant increase in the resting release of iCGRP from sensory neurons (data not shown).

Unlike previous studies using these inhibitors, the effects of NGF on sensory neurons were not inhibited. The drugs did not alter the NGF-induced increase in iCGRP release from sensory neurons. These data suggest that the PI3 kinase pathway does not regulate the increase in capsaicin-evoked iCGRP release induced by brief exposure to 100ng/ml NGF.

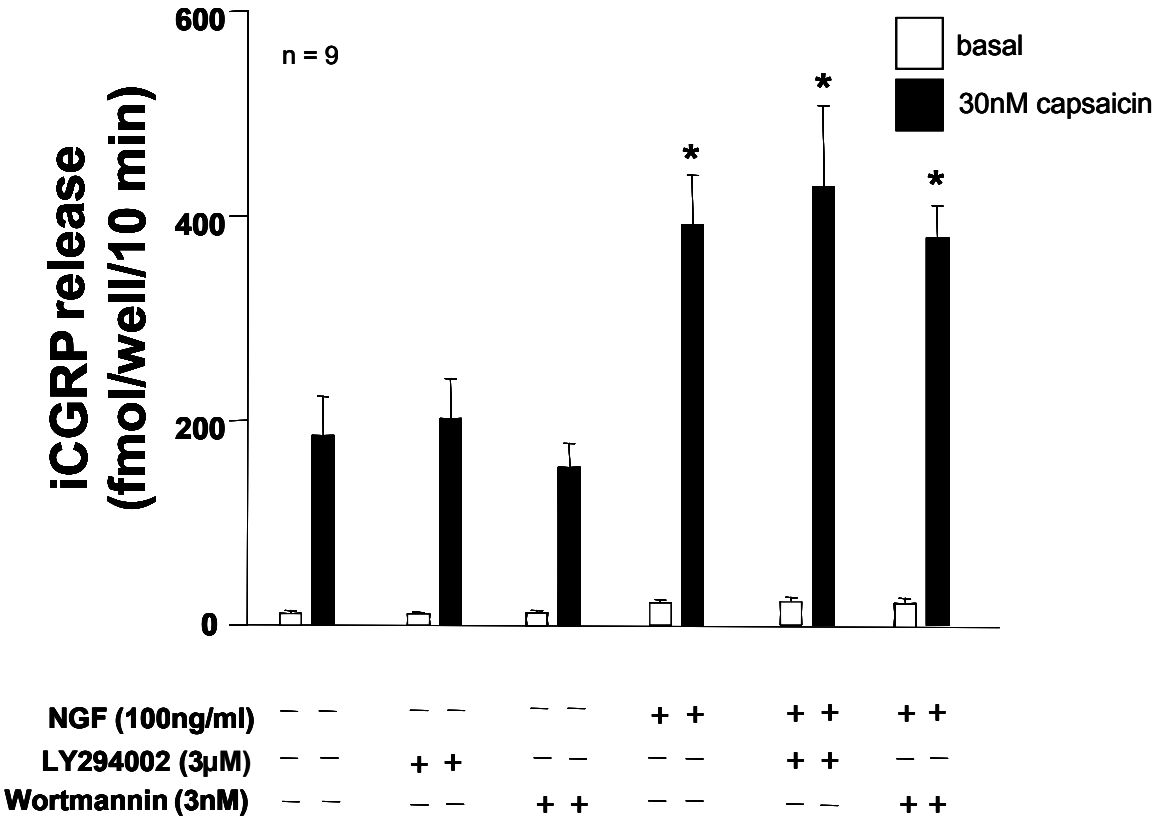


Figure 40. Neither LY294002 (3μM) nor wortmannin (3nM) inhibits the NGF-induced increase in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture. The resting and capsaicin-evoked iCGRP release from neurons is shown for each of the six conditions. Neurons were exposed to 100ng/ml NGF for 10 min prior to and throughout capsaicin-evoked release as indicated. Groups of neurons were pretreated for 10 min with the indicated inhibitor, and then exposed again to the same concentration of inhibitor for 10 min prior to and throughout capsaicin-evoked release as indicated. An (*) indicates a significant increase in iCGRP release from sensory neurons compared to NGF non-treated controls ($p<0.05$, n=9 from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

The PKC inhibitor, BIM, blocks the increase in capsaicin-evoked iCGRP release cause by brief exposure to NGF

Multiple studies demonstrate that activation of PKC is involved in the regulation of the sensitivity of sensory neurons (Barber and Vasko, 1996; Frayer et al., 1999; Khasar et al., 1999; Richardson and Vasko, 2002). In addition, NGF exposure has been shown to activate PLCγ, which in turn liberates DAG and activates PKCs (Chuang et al., 2001). Interestingly, many of these studies showing PKC activation by NGF have been performed in cell lines or heterologous expression systems, and brief NGF exposure caused an increase in the activation of atypical PKC isoforms (Wooten et al., 1991; Wooten et al., 2004).

Bonnington and McNaughton showed that the PKC inhibitor, bisindolylmalemide I (BIM), abolished the NGF-induced sensitization of mouse sensory neurons (Bonnington and McNaughton, 2003). However, Shu and Mendell found that BIM did not alter NGF's ability to block capsaicin-induced tachyphylaxis in sensory neurons in culture (Shu and Mendell, 2001). Thus, controversy exists whether PKC activation is involved in the acute sensitization of sensory neurons by NGF. Furthermore, the involvement of PKC in the regulation of evoked release of iCGRP in neurons has not been tested. Therefore, we examined whether PKC inhibition could block the NGF-induced increases in capsaicin-evoked iCGRP release from neurons in culture.

For these studies, cells in culture were exposed to 100 ng/ml NGF in the absence or presence of various concentrations of BIM and basal and capsaicin-

evoked release was measured (Fig. 41). As in previous studies, acute exposure to NGF increased in the capsaicin-evoked release of iCGRP from neurons from 140 ± 15 fmol iCGRP/well/10min to 244 ± 28 fmol iCGRP. In neurons treated with 100nM BIM for 10 minutes prior to NGF treatment and throughout capsaicin-evoked release, the inhibitor abolished the the NGF-induced augmentation of capsaicin-evoked iCGRP release from neurons (149 ± 21 fmol iCGRP/well/10min). These data implicate PKC in the regulation of the acute effects of NGF on sensory neurons.

The capsaicin-evoked release of iCGRP in the presence of BIM alone appeared to trend downward (73 ± 10 fmol iCGRP/well/10min), although not significantly different from control. Premkumar and Ahern have shown that PKC activates the TRPV1 channel (Premkumar and Ahern, 2000). Therefore, it is logical to expect that inhibition of PKC with BIM may reduce the capsaicin-induced release of iCGRP from neurons. If this were the case, there would be no difference in the degree of sensitization by NGF between the BIM-treated and non-treated groups. Furthermore, the baseline for capsaicin-evoked release would shift making comparisons in the presence of drug difficult to interpret. Therefore, we repeated these studies using 30nM BIM (Fig. 42).

In these experiments, 30nM BIM did not alter the capsaicin-evoked release of iCGRP alone (Fig. 42). Capsaicin-evoked release was 209 ± 25 fmol iCGRP/well/10min. In the presence of 30nM BIM, capsacin-evoked release was 205 ± 27 fmol iCGRP/well/10min. NGF-induced sensitization was reduced from

320 ± 41 fmol iCGRP/well/10min to 247 ± 27 fmol iCGRP. In these experiments, BIM did not affect resting iCGRP release.

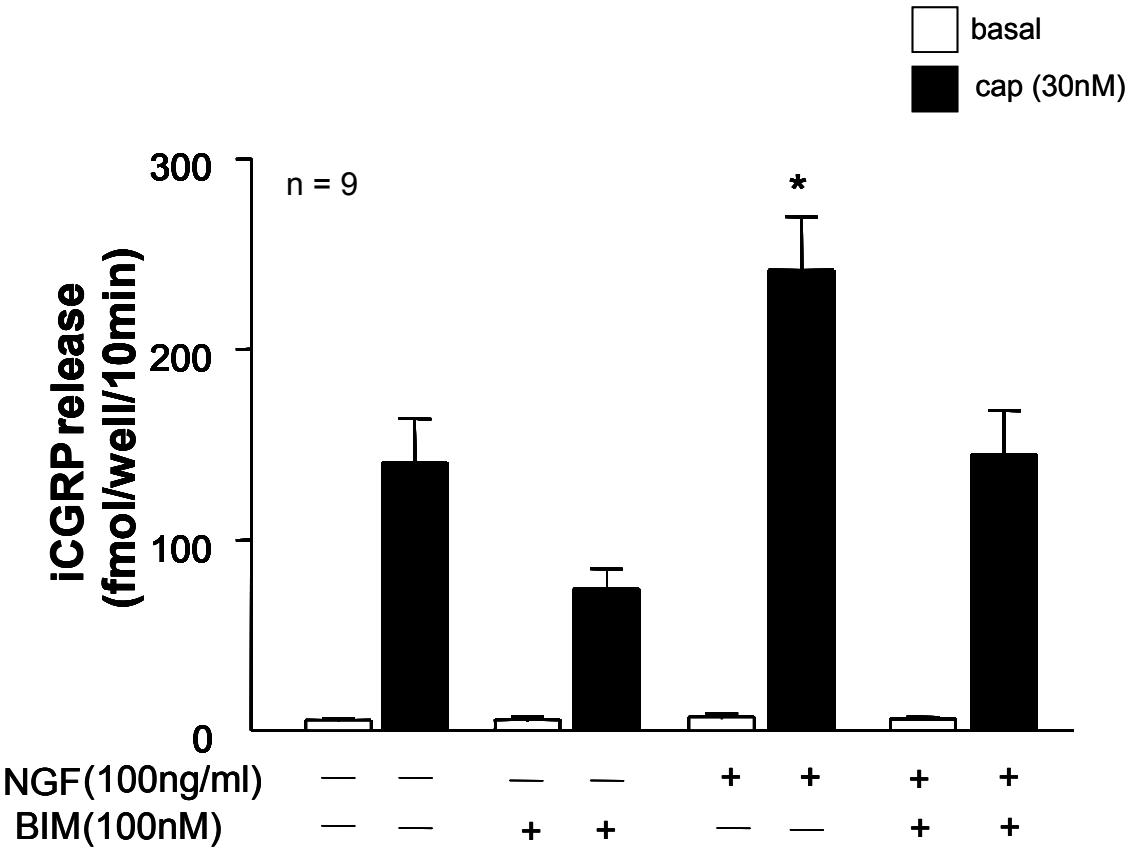


Figure 41. The effect of BIM (100nM) on the NGF-induced increases in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture. The resting and capsaicin-evoked iCGRP release from neurons is shown for each of the four conditions. Neurons were exposed to 100ng/ml NGF for 10 min prior to and throughout capsaicin-evoked release as indicated below the x-axis. Some groups of neurons were pretreated for 10 min with the 100nM BIM, and then exposed again to the same concentration of inhibitor for 10 min prior to and throughout capsaicin-evoked release as indicated. An (*) indicates a significant increase in iCGRP release from sensory neurons compared to NGF non-treated controls ($p<0.05$, n=9 from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

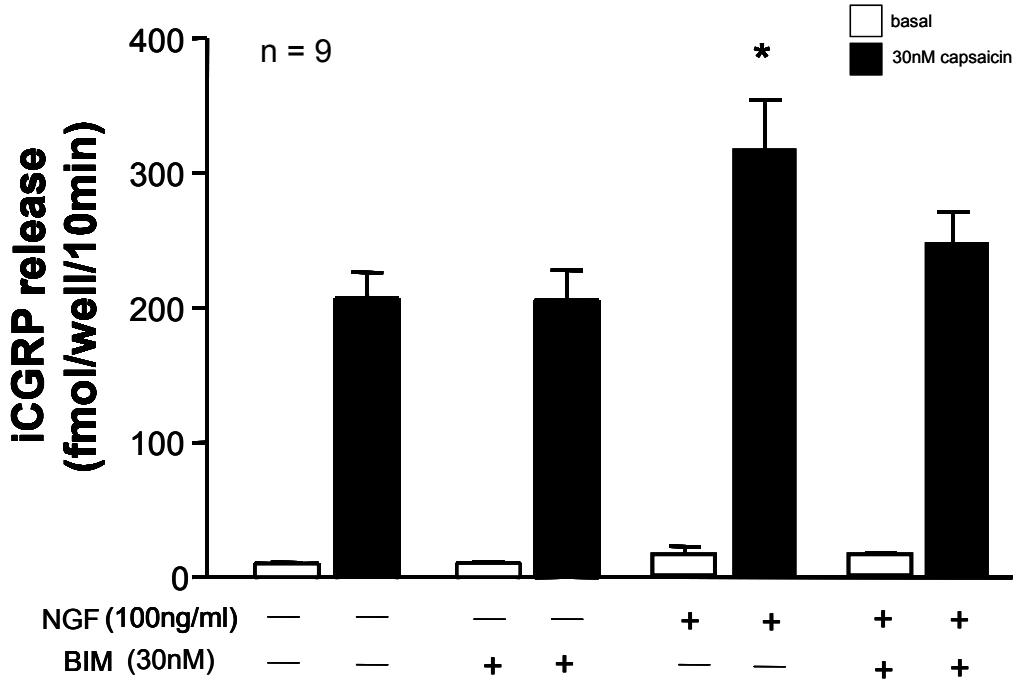


Figure 42. The effect of BIM (30nM) on the NGF-induced increases in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture. The resting and capsaicin-evoked iCGRP release from neurons is shown for each of the four conditions. Neurons were exposed to 100ng/ml NGF for 10 min prior to and throughout capsaicin-evoked release as indicated below the x-axis. Some groups of neurons were pretreated for 10 min with 30nM BIM, and then exposed again to the same concentration of inhibitor for 10 min prior to and throughout capsaicin-evoked release as indicated. An (*) indicates a significant increase in iCGRP release from sensory neurons compared to NGF non-treated controls ($p<0.05$, n=9 from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

Because inhibition of PKC attenuated the NGF-induced sensitization of sensory neurons using BIM, we began studies to ascertain which isozymes of PKC are involved in NGF signaling. If NGF were signaling through a specific PKC isoform to augment neurotransmitter release, then reducing the expression of that specific PKC isoform in neurons would reduce the ability of NGF to increase the capsaicin-evoked iCGRP release from sensory neurons.

To date, siRNA-knockdown has been attempted for PKCα, PKCζ, and PKCε in the Vasko laboratory. However, the only molecule that has been successful in reducing the expression of its respective isoform was siRNA developed against PKCα. Consequently, we examined whether reducing expression of PKCα would alter NGF-induced augmentation of capsaicin-evoked release of iCGRP. In these experiments, cells in culture were treated with 200nM siRNA targeting APE (control siRNA) or PKCα. After treatment, cells were subjected to a standard iCGRP release protocol in the presence or absence of 100ng/ml NGF 10 minutes prior to and throughout capsaicin stimulation. Then cells were lysed and immunoblotting was performed to confirm knockdown.

The representative Western blot (Fig. 43A) shows that the signal for PKCα was reduced by approximately 50%. Despite this reduction there was no inhibition of the sensitizing action of NGF on peptide release (Fig. 43B). The data represent the mean and standard deviation from triplicate measurements from a single harvest. This could indicate PCKα has no effect on sensitization. Alternatively, a 50% decrease in the protein expression may not be sufficient to affect NGF-induced augmentation of evoked release.

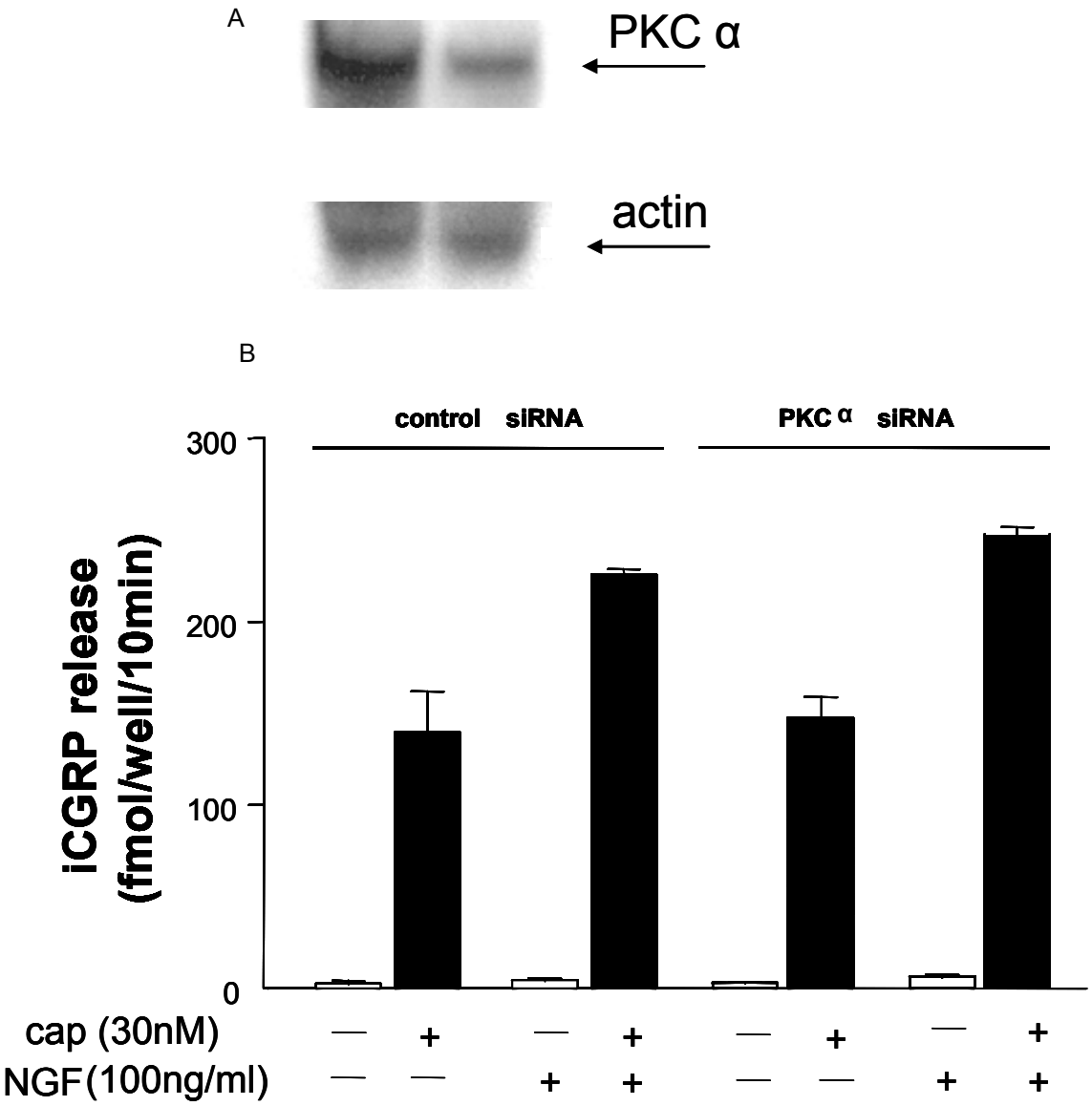


Figure 43. The NGF-induced sensitization of capsaicin-evoked iCGRP release (not normalized to content) does not appear to be different in neurons treated with control or PKCα siRNA. (A) Western blot demonstrating the expression of PKCα and actin in neurons treated with control siRNA (left lane) or PKCα siRNA (right lane). (B) The 30nM capsaicin-evoked release from neurons treated with control siRNA or PKCα siRNA as indicated. Where indicated, groups of cells are exposed to 100ng/ml NGF for 10min prior to and throughout the 10 min stimulation with capsaicin (n=3, from a single harvest of neurons).

Inhibition of Src kinase signaling in neurons blocks the increase in capsaicin-evoked release of iCGRP in response to brief exposure to NGF

Evidence in PC12 cells shows that brief exposure to NGF activates Src kinase (Wooten et al., 2001). Furthermore, Src kinase may activate various PKC isoforms (Wooten et al., 1994; Seibenhener et al., 1999). Finally, Zhang *et. al.* showed that the NGF-induced increase in Ca^{2+} into mouse sensory neurons was blocked by a Src kinase inhibitor (Zhang et al., 2005).

Consequently, it is possible that activation of Src kinases is the mechanism through which NGF could alter transmitter release from sensory neurons in culture. To test this notion, a Src kinase inhibitor, PP2, was used to determine if this signaling pathway regulates the augmented release of iCGRP in the presence of acute treatment with NGF. Neurons were cultured as previously described. Cells were subjected to a release protocol in the presence or absence of NGF. In addition, cells were simultaneously exposed to PP2 (10μM) or PP3 (10μM), which is the inactive analogue of PP2 that was used for control for the non-specific effects of the inhibitor.

When neurons were exposed to 100 ng/ml NGF for 10 minutes prior to and throughout capsaicin-evoked release, there was a significant increase in capsaicin-evoked iCGRP release, from 107 ± 8 fmol iCGRP/well/10min to 169 ± 6 fmol iCGRP (Fig. 44). Some groups were pretreated with PP2 (10μM) or PP3 (10μM) for 10 minutes and then treated with the inhibitors prior to and throughout capsaicin-evoked release. In neurons exposed to PP2 or PP3, there was no effect on capsaicin-evoked release alone which was 99 ± 6 and 95 ± 5 fmol

iCGRP/well/10min, respectively. However, the NGF-induced increase in capsaicin-evoked iCGRP release was significantly reduced by PP2 from 169 ± 6 fmol iCGRP/well/10min to 109 ± 7 fmol iCGRP. PP3 had no significant effect on NGF-induced sensitization of release from neurons (166 ± 6 fmol iCGRP/well/10min). These data indicated Src kinase regulates sensitization of sensory neurons by NGF.

In these experiments, there was a small, but significant increase in the resting release of iCGRP from neurons treated with either PP2 or PP3 in NGF-treated neurons. Resting release increased from 17 ± 2 fmol iCGRP/well/10min to 35 ± 10 fmol iCGRP in the presence of NGF and PP2 or PP3. Considering the increase occurred in both the PP2- and PP3-treated neurons, it is likely that the effect is non-specific, and perhaps an interaction between the compounds and NGF.

These experiments strongly implicate Src kinase signaling in the effects of NGF on release from sensory neurons. Presently, it is not known what PKC isoforms signal downstream of Src kinases in sensory neurons. Nor is it known which Src kinases may be regulating NGF sensitization. Further studies are needed to distinguish these elements involved in regulation of evoked iCGRP release from sensory neurons in the presence of NGF.

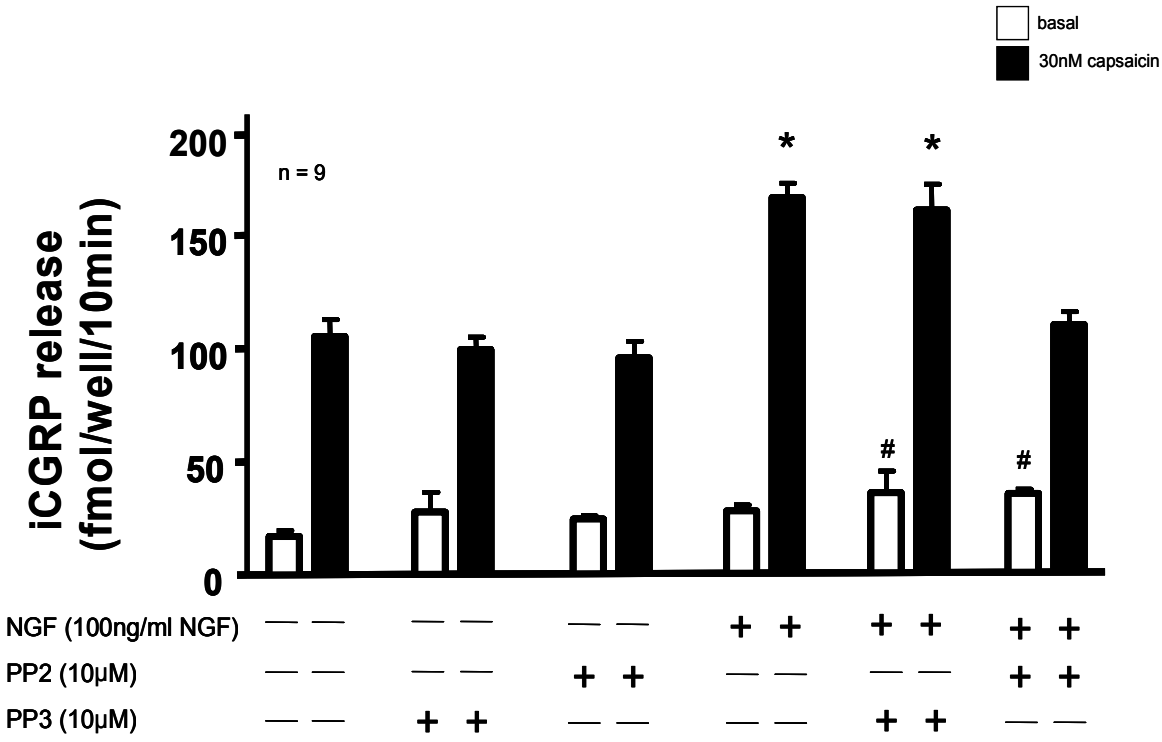


Figure 44. The effect of PP2 (10μM) and PP3 (10μM) on the NGF-induced increases in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture. The resting (white bars) and capsaicin-evoked (black bars) iCGRP release from neurons are shown for each of the six conditions. Neurons were exposed to 100ng/ml NGF for 10 min prior to and throughout capsaicin-evoked release as indicated below the x-axis. Some groups of neurons were pretreated for 10 min with 10μM PP2 or PP3, and then exposed again to the same concentration of inhibitor for 10 min prior to and throughout capsaicin-evoked release as indicated. An (*) indicates a significant increase in iCGRP release from sensory neurons compared to NGF non-treated controls. A (#) indicates a significant increase in resting release compared to resting release from control neurons ($p<0.05$, n=9 from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

The effects of manipulating the sphingomyelinase pathway on capsaicin-evoked iCGRP release from sensory neurons

Ceramide (10μM) does not affect capsaicin-evoked iCGRP release, while S1P (1 and 10 μM) increase stimulated release

The experiments described above test the involvement of pathways that are commonly associated with the TrkA receptor activation in response to acute treatment with NGF. However, there are studies that show the neurotrophin receptor, $p75^{NTR}$ regulates the sensitivity of sensory neurons both *in vivo* and *in vitro.* When $p75^{NTR}$ binds NGF, it activates the enzyme sphingomyelinase. This enzyme cleaves sphingomyelin to produce ceramide. Zhang *et. al.* demonstrated that ceramide, which increases with activation of the $p75^{NTR}$, increases the sensitivity of sensory neurons as measured by changes in the number of action potentials fired in response to a ramp of depolarizing current (Zhang et al., 2002). Furthermore, the ability of acute NGF to augment the number of APs is attenuated by blocking the p75 receptor (Zhang and Nicol, 2004).

Based on these data, we designed studies to examine whether the resting and 30nM capsaicin-evoked iCGRP release from neurons was augmented by treating neurons for 10 min with ceramide (100nM, 1μM and 10μM). Capsaicin-evoked release from control neurons was 188 ± 27 fmol iCGRP/well/10min (Fig. 45). Capsaicin-evoked release in the presence of 1nM, 1μM, or 10uM ceramide was 182 ± 22, 178 ± 14, and 229 ± 46 fmol iCGRP/well/10min, respectively. There was no significant change in the capsaicin-evoked release using up to 10μM ceramide, suggesting ceramide does not play a role in the regulation of sensitivity to capsaicin.

Ceramide is converted to sphingosine, by ceramidase (Roux et al., 2001). Sphingosine is converted to sphingosine-1-phosphate (S1P) by sphingosine kinase (Toman et al., 2001). Like ceramide, S1P increases the excitability of sensory neurons in culture (Zhang et al., 2006). Therefore, a second group of experiments was performed to test whether S1P increases the capsaicin-evoked release from sensory neurons in culture. As with studies using ceramide, the resting and 30nM capsaicin-evoked release from sensory neurons in culture was tested in the presence or absence of increasing concentrations of S1P (treatment 10min prior to and through stimulation). Cells treated with 30nM capsaicin released 255 ± 15 fmol iCGRP/well/10min (Fig. 46). Vehicle, which was 0.1% MPL, did not alter capsaicin-evoked release (295 ± 18 fmol iCGRP/well/10min). However, both 1 and 10μM S1P caused a significant increase in the capsaicin-evoked iCGRP release from neurons (391 ± 23 and 402 ± 38 fmol iCGRP/well/10min). There were no alterations in the resting release from neurons exposed to these same concentrations of S1P. These data implicated S1P in the regulation of sensitivity of sensory neurons and support the notion that $p75^{NTR}$ signaling and the sphingomyelinase pathway regulate sensitization of sensory neurons.

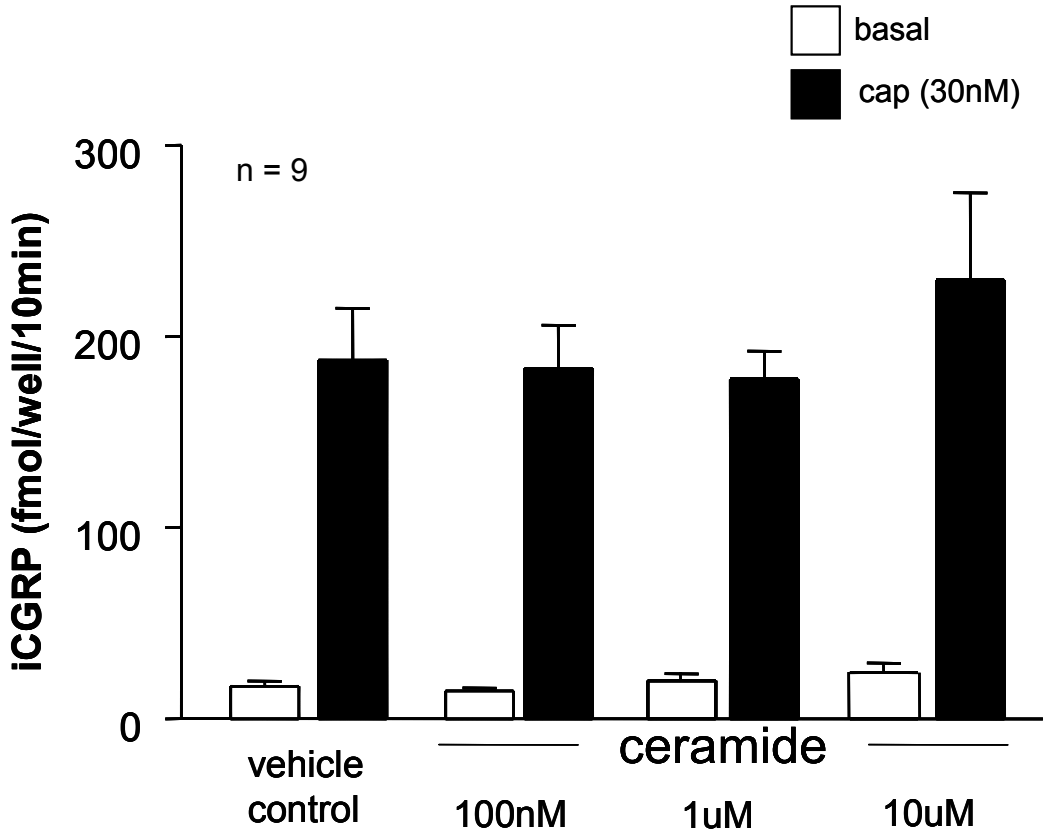


Figure 45. The effect of ceramide on 30nM capsaicin-evoked iCGRP release from sensory neurons in culture. The resting and capsaicin-evoked iCGRP release from neurons is shown for each of four conditions. Neurons were exposed to the indicated concentration of ceramide for 10 min prior to and throughout capsaicin-evoked release as indicated below the x-axis. There were no differences among any of the measurements and there were no changes in resting release of iCGRP from neurons ($p<0.05$, n=9 from 3 separate harvests, using one-way ANOVA).

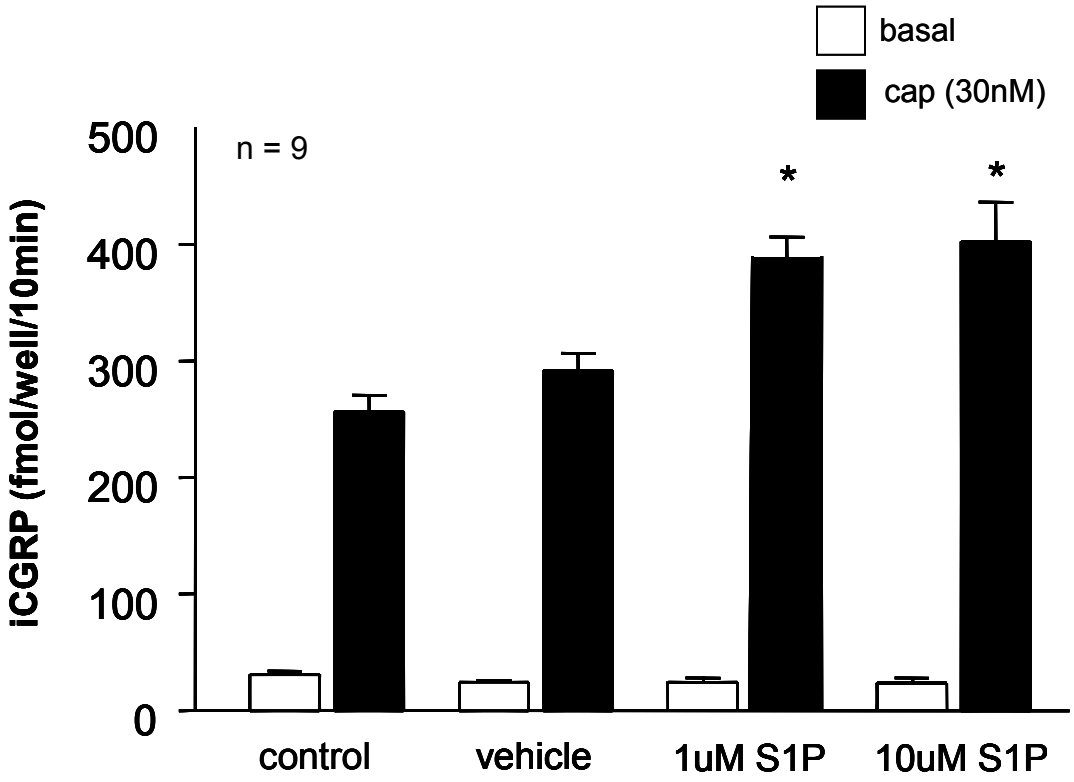


Figure 46. The effect of sphingosine-1-phosphate on the NGF-induced increases in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture. The resting and capsaicin-evoked iCGRP release from neurons are shown for each of four conditions. Neurons were exposed to the indicated amount of S1P or vehicle (0.1% MPL) prior to and throughout capsaicin-evoked release as indicated below the x-axis. An (*) indicates a significant increase in iCGRP release from sensory neurons compared to NGF non-treated controls ($p<0.05$, $n=9$ from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

Effects of sphingosine kinase inhibitor (DMS) or a neutral sphingomyelinase inhibitor on sensory neurons in culture

Since S1P augments capsaicin-evoked transmitter release, further experiments were designed to determine whether inhibition of enzymes upstream in the metabolic pathways of S1P could alter NGF-induced increases in capsaicin-evoked release of iCGRP from sensory neurons. For these studies, we used *N,N*-dimethyl sphingosine (DMS), which is a sphingosine kinase inhibitor (Natarajan et al., 1994; Zhang et al., 2006). In a similar trend to those experiments shown above, NGF appears to increase the 30nM capsaicin-evoked release from neurons from 77 ± 5 fmol iCGRP/well/10min to 105 ± 19 fmol iCGRP (Fig. 47). Surprisingly, when neurons were treated with DMS (10μM) for 20 minutes prior to and throughout capsaicin-treatment, there were alterations in both the resting and capsaicin-stimulation release (Fig. 47). Resting release increased 3-fold in the presence of DMS, while 30nM capsaicin-evoked release was reduced to 29 ± 2 fmol iCGRP/well/10min. The capsaicin-evoked release was nearly abolished. Because the drug altered both the resting and the capsaicin-evoked release, it was difficult to interpret the results and further experiments using this drug were not performed.

We also attempted to determine whether a sphingomyelinase inhibitor would block NGF-induced sensitization. A neutral sphingomyelinase inhibitor (N-Smase) (15μM) was used in the presence or absence of NGF and capsaicin stimulation. As with DMS, the inhibitor increased the resting release of iCGRP by 2 to 5 times, whereas capsaicin-evoked release was not affected (Fig. 48).

Because this concentration of the sphingomyelinase inhibitor had a dramatic effect on resting release, interpretation of experiments using capsaicin and/or NGF is not practical.

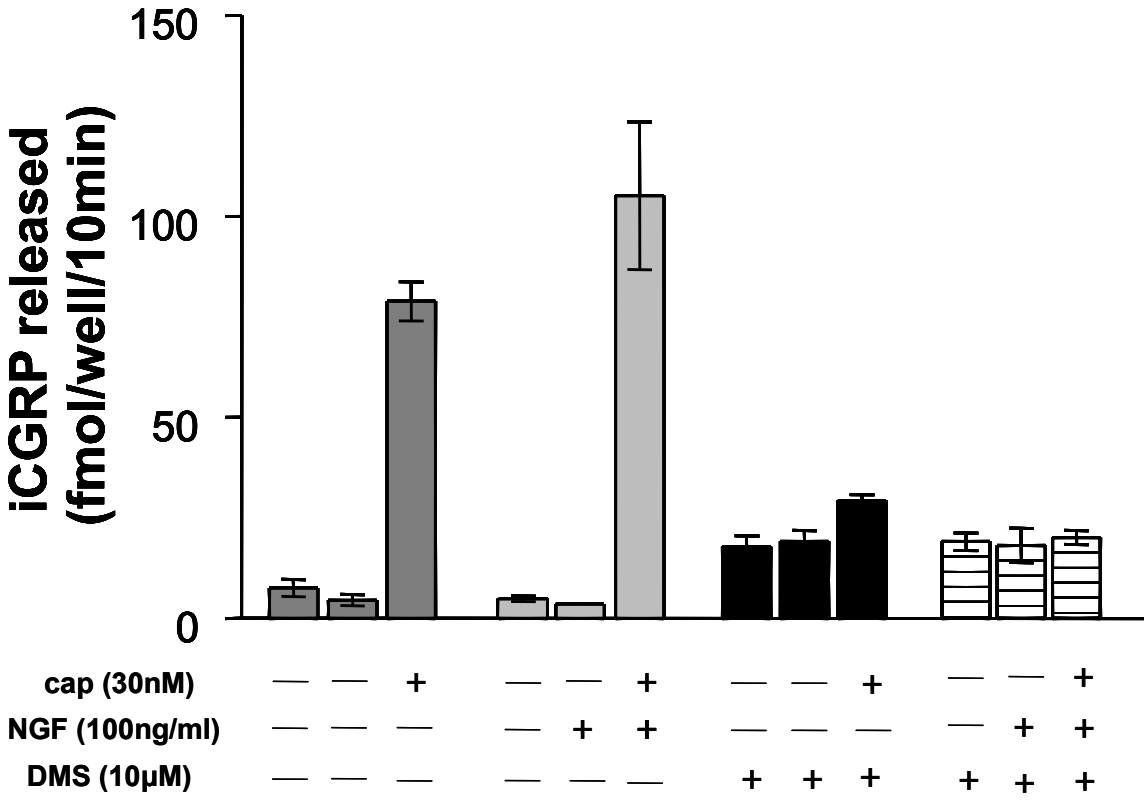


Figure 47. The effect of DMS (10µM) on NGF-induced increases in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture. The resting release from two consecutive 10 min incubations and the capsaicin-evoked iCGRP release from neurons are shown for each of the four conditions. Neurons were exposed to 100ng/ml NGF for 10 min prior to and throughout capsaicin-evoked release as indicated below the x-axis. Neurons were treated with DMS during the two resting incubation (20 min) and throughout capsaicin-evoked release as indicated (n=3 from a single harvest, mean ± SD).

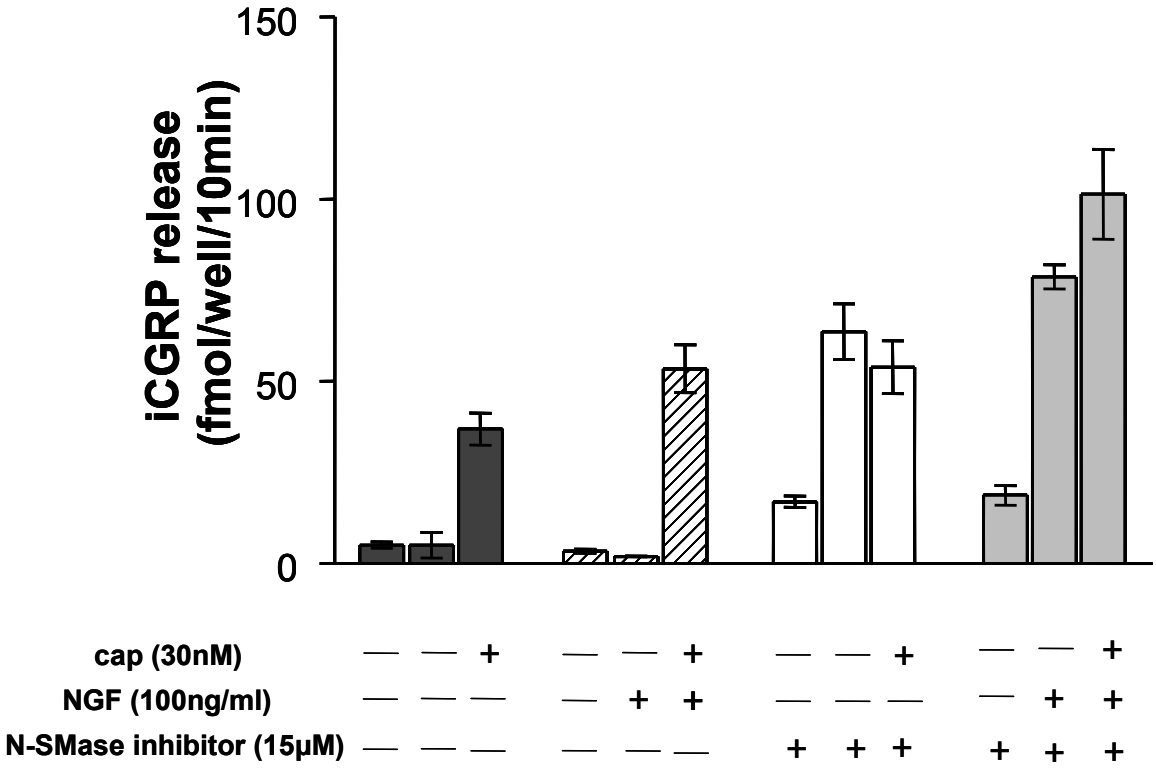


Figure 48. The effect of neutral sphingomyelinase inhibitor (N-SMase inhibitor, 15μM) on NGF-induced increases in 30nM capsaicin-evoked iCGRP release from sensory neurons in culture. The resting release from two consecutive 10 min incubations and the capsaicin-evoked iCGRP release from neurons are shown for each of the four conditions. Neurons were exposed to 100ng/ml NGF for 10 min prior to and throughout capsaicin-evoked release as indicated below the x-axis. Neurons were treated with the N-SMase inhibitor during the two resting incubation (20 min) and throughout capsaicin-evoked release as indicated (n=3 from a single harvest, mean ± SD).

DISCUSSION

In this thesis, the cell signaling cascades that regulate the NGF-induced increases in iCGRP content and stimulated release from sensory neurons were examined. NGF is an important inflammatory mediator and its signaling cascades in neurons are important to understand. First, it is well-established that NGF expression increases with inflammation (Lewin and Mendell, 1994; Woolf et al., 1994; Safieh-Garabedian et al., 1995; Falcini et al., 1996; Nonogaki et al., 1996). Second, injection of NGF causes an inflammatory response in the paw and increases pain and hypersensitivity in animals and humans, respectively (Lewin et al., 1993; Petty et al., 1994). Finally, inhibiting NGF signaling by a blocking antibody reduces thermal hyperalgesia that develops with CFA-induced inflammation of the rat paw (Woolf et al., 1994). Therefore, NGF regulates the sensitivity of sensory neurons, and is a neurotrophin that should be studied to determine the possible intracellular signaling cascades that regulate its effects in sensory neurons. Understanding the intercellular mechanisms that regulate sensitivity of sensory neurons will lead to a better understanding of hypersensitivity of neurons and of acute and chronic pain.

As described above, a number of studies performed previous to this work have shown that NGF increases the expression of neuropeptides both *in vivo* and *in vitro* (Lindsay and Harmar, 1989; Verge et al., 1995; Malcangio et al., 1997; Fehrenbacher 2005). However, the intracellular signaling pathways that regulate this alteration in expression in response to NGF have not been characterized in primary sensory neurons. Therefore, experiments described in this work were designed to determine

what regulatory pathways increase expression of neuropeptides in response to NGF in primary sensory neurons.

While multiple studies show that exposure of neurons to NGF for a number of days increases the expression of both SP and CGRP, it should be mentioned that one study by Bowles *et. al.* did not see a difference in content of CGRP or SP in dorsal horn slices after 7 or 14 day treatment of animals with NGF (Bowles et al., 2004). They found only an increase in the capsaicin-stimulated release of CGRP after NGF treatment. It is not clear why Bowles *et. al.* observed no changes in peptide expression with NGF treatment while many other studies have. For instance, Malcangio *et. al*. used the same doses of NGF (1mg/kg) *in vivo*, and observed increases in peptide content in rat spinal cord tissues (Malcangio et al., 1997). Verge *et. al.* demonstrated that intrathecal administration of NGF (125ng/ul/hr) reversed the loss of neuropeptide expression in response to nerve injury (Verge et al., 1995). Furthermore, there is evidence *in vitro* that NGF regulates the expression of iCGRP in primary sensory neurons (Lindsay et al., 1989; Fehrenbacher, 2005, and Fig. 2).

In addition to studies on signaling cascades regulating neuropeptide expression, experiments were performed to determine the signaling pathways important in regulating the increase in evoked release of iCGRP from neurons in response to brief exposure to NGF (Fehrenbacher, 2005). However, a more subtle finding emerged from the studies by Fehrenbacher. There are at least two mechanisms that result in increased outflow of iCGRP from neurons in response to NGF. Fehrenbacher demonstrated that the stimulated release of peptide from sensory neurons increased if the cell content of iCGRP was increased after chronic exposure to NGF. Whether the

stimulus was capsaicin or high extracellular potassium, both elicited the increase in iCGRP release if content was increased. This suggested that there was an increase in expression of iCGRP, which was confirmed by our experiments (Fig. 8). However, if neurons were exposed briefly (10 minutes) to NGF, and then stimulated with capsaicin, there was an increase in capsaicin-evoked iCGRP release. This increase is not dependent on alterations in expression of the neuropeptide because changes in neuropeptide expression do not occur within 10 minutes of NGF exposure. Furthermore, if the data are normalized to the total cellular iCGRP content, then the percentage of iCGRP released from neurons is higher in neurons exposed to NGF for 10 minutes. These data indicate that there is both an acute and chronic effect of NGF on sensory neurons, and determining the regulatory molecules that mediate these changes is the focus of this thesis.

In this thesis, it is purported that the increase in release of neurotransmitters from sensory neurons is important in the development of hyperalgesia, allodynia, neurogenic inflammation, and pain. This idea is supported by studies that show hyperalgesia and allodynia develop when there is an increase in neurotransmitter release into the spinal cord. Malmberg *et. al.* delivered capsaicin and PGE_2 simultaneously to the thecal space, and observed increases in the EEAs released into the extracellular space (Malmberg et al., 1995). This increase in EEAs induced allodynia in animals. In a similar group of studies, Warsame *et. al.* measured capsaicin-evoked SP release into the thecal space after CFA-induced inflammation of the paw (Warsame et al., 2004). There was a significant increase in SP release compared to control animals. These data suggest that increases in release of EEAs and

neuropeptides from primary sensory neurons into the spinal cord contribute to the development of hyperalgesia and allodynia in animals.

The studies outlined in this thesis show that Ras is necessary for the increased expression of iCGRP in response to NGF in sensory neurons in culture. A significant increase in RasGTP levels was measured in response to chronic exposure to NGF in dorsal root ganglion cultures (Fig. 11). These results confirm previous studies in PC12 cells showing incubation with NGF increases Ras activation as measured by increases in RasGTP levels (Ng and Shooter, 1993; Basu et al., 1994; Ganju et al., 1998; Egea et al., 2000) . However, these data were correlative and to test the direct involvement of the Ras/MEK/ERK pathway in regulation of iCGRP expression, cells were infected with lentivirus expressing dominant negative Ras. Results show that cells expressing dominant negative Ras have a diminished response to chronic exposure to NGF. Neurons expressing dnRas do not express as much iCGRP when grown in the presence of 30 or 100 ng/ml NGF as those infected with control virus (Fig. 15). When cells express caRas, there appears to be an increase in iCGRP content even in the absence of neurotrophic factors. However, the data are derived from multiple measurements in a single experiment. Therefore, further studies are needed to determine if this result will be reproducible. If the increase occurs in the absence of NGF, one could conclude Ras activation is sufficient for the expression of iCGRP in sensory neurons.

As shown in Figure 15, there is still an increase in iCGRP in neurons exposed to 30 and 100 ng/ml NGF in the presence of dnRas. There are at least two possibilities to explain why expression is still upregulated in neurons expressing dnRas. First, the

activity of Ras may not be sufficiently blocked to completely inhibit iCGRP expression in response to NGF. This possibility could be resolved by increasing the number of pfu used to infect the cells, and then measure whether iCGRP expression is blocked to a greater extent in the presence of NGF. However, an alternate explanation is that there may be redundant signaling pathways to increase iCGRP expression, such that NGF signaling to Ras may not be the only pathway involved in upregulation of transcription. A recent report by Bellamy *et. al.* revealed that iCGRP expression in trigeminal neurons could be increased by treatment of cells with nitric oxide (Bellamy et al., 2006). In their studies, they showed that PI3 kinase and cGMP were not required for the increase in peptide expression, which suggests that in some types of sensory neurons, there are other signaling cascades that regulate iCGRP expression. Whether this regulatory pathway is similar in dorsal root ganglion neurons has not been explored.

As another means to inhibit Ras, cells were exposed to the farnesyltransferase inhibitor, FTI-276 (Fig. 18). This drug blocks the addition of a farnesyl group to the carboxy terminus of Ras. The data in Figure 18 show there is an increase in the level of unfarnysylated Ras with exposure to FTI-276. Presumably, this results in improper localization and decreased activity. The data in Figure 20 show that FTI-276 attenuates the NGF-induced increase in iCGRP expression at 100 ng/ml NGF. These data suggest that proper localization of Ras, which affects its ability to signal in neurons, is necessary for NGF to signal to this protein and increase expression of CGRP. Interestingly, at 30 ng/ml NGF or with no added NGF there seems to be a downward trend in the expression of iCGRP, but the difference was not siginificant. This may

suggest that even without added NGF, the Ras pathway is active and the addition of FTI-276 causes some level of inhibition of iCGRP expression in our experiments.

As another means to manipulate the Ras pathway, neurons from synGAP heterozygous mice were used. Neurons from these mice have a reduced expression of synGAP, a RasGAP that decreases activity of the Ras protein. Komiyama *et. al.* showed that decreased expression of synGAP led to activation of ERK in hippocampal neurons from mice (Komiyama et al., 2002). In experiments in this thesis, 100 or 250 ng/ml NGF caused a significant increase in the iCGRP expression in dorsal root ganglion neurons derived from mice with a heterozygous deletion of the synGAP gene compared to expression of iCGRP from wildtype littermate controls (Fig. 25). These data suggest upregulating activity of the Ras pathway controls expression of iCGRP in neurons derived from mice. However, what is interesting is that Komiyama *et. al.* showed that the Ras/MEK/ERK pathway is upregulated in tissues derived from heterozygous mice which were not treated with NGF (Komiyama et al., 2002). This would imply with no added NGF, if Ras is constitutively active, there should be a difference in iCGRP expression when comparing synGAP heterozygous and wildtype neurons. Yet, no differences were observed in content in neurons from synGAP heterozygous mice versus neurons from wildtype animals in the absence of NGF (Fig. 25). The neurons must be stimulated with higher concentrations of NGF (100 or 250 ng/ml NGF) before differences from their littermate controls are observed. One explanation for the discrepancy between the data in sensory neurons and hippocampal neurons is that the level of activity of the Ras/MEK/ERK pathway in peripheral neurons versus neurons from the hippocampus might be different. It also is possible that higher

levels of the pERK are needed compared to those in unstimulated tissues to drive the expression of iCGRP in dorsal root ganglion neurons. Finally, it is possible that pathways other than Ras/MEK/ERK promote the expression of iCGRP. In fact, other studies show that synGAP may also regulate another small G-protein, Rap (Knuesel et al., 2005). This G-protein is not well-studied in neurons, but could play also play a role in the regulation of iCGRP expression in neurons.

The most surprising result from studies using synGAP heterozygous mice is that the neurons from the wildtype mice did not show an increase in iCGRP content with increasing concentrations of NGF after 7 days. In the literature, transgenic mice on a C57Bl background were used to assess the effects chronic NGF exposure to SP or CGRP expression. These reports show increases in neuropeptide immunoreactivity in response to chronic exposure to the neurotrophin (Schmidt et al., 1995; Ma and Bisby, 1998; Dinh et al., 2004). In these cases, the concentrations of NGF were similar to those used in studies in this thesis. No clear explanation to date can explain this discrepancy in findings. The wildtype mice for all practical purposes should have an intact promoter and enhancer elements of the CGRP gene. It could be that this particular strain of mice has very efficient GAPs, and the concentrations of NGF used in these experiments did not cause changes in iCGRP levels in adult animals. Further studies could be performed to determine the reasons why neurons from these animals do not respond to NGF in a similar manner to C57Bl transgenic mice demonstrated in other studies.

Based on data from this thesis using dnRas and FTI-276, which both reduce Ras activity, it is the small GTPase is functioning to control the expression of iCGRP

in sensory neurons in culture. Whether the sphingomyelinase pathway regulates peptide expression was not tested in experiments in this thesis. Evidence exists that the $p75^{NTR}$ is involved in the regulation of SP expression in sensory neurons in culture (Skoff and Adler, 2006). When neurons in culture are exposed to NGF (10ng/ml) for 7 days in the presence of a blocking antibody to either the TrkA receptor or to $p75^{NTR}$, there was a significant decrease in the amount of SP expressed as measured by radioimmunoassay in a manner similar to those described in this thesis (Skoff and Adler, 2006). This finding demonstrates a need for specific inhibitors of downstream signaling cascades of $p75^{NTR}$ that do not affect the resting release of neuropeptides from sensory neurons.

In the studies in this thesis, the signaling cascades regulating an increase in evoked release of iCGRP with brief exposure to NGF were examined. The Ras cascade was tested first. There was no difference in the degree of sensitization by NGF when neurons expressed dnRas compared to neurons infected with a control virus (Fig. 29). All treatment groups showed an approximate two-fold increase in capsaicin-stimulated iCGRP release brief exposure to NGF (Fig. 29). These results provide evidence that Ras does not mediate the NGF sensitization of release. However, it was observed that in neurons expressing dnRas there was a significant decrease in capsaicin-evoked release from both the control and NGF-treated neurons. A likely explanation is that there is an overall decrease in the expression of the neuropeptide due to decreased Ras activity. It is probable because we showed that dnRas decreases iCGRP expression in response to NGF to a similar degree (Fig. 15). However, another possible explanation for these findings is the dnRas expression induces a decrease in

the expression of TRPV1. Although TRPV1 expression was not measured in these studies, it has been shown that capsaicin-sensitivity and TRPV1 expression in sensory neurons is increased by activation of the Ras/MEK/ERK pathway (Winter et al., 1988, Bron et al., 2003). Furthermore, there are multiple studies that show that the upregulation of TRPV1 is an underlying cause of changes in capsaicin-sensitivity in response to NGF (Bonnington and McNaughton, 2003; Bowles et al., 2004).

MEK and ERK are not mediators of the increased evoked-release of iCGRP in response to 20 minute stimulation with NGF (Fig. 33). There was no increase in the levels of pERK in response to 10 minute incubation with NGF (Fig. 31). These results conflict with other studies in the literature which use comparable concentrations of NGF to stimulate sensory neurons. First, Zhuang *et. al.* showed that 2 and 10 minute stimulations with NGF-induced a significant upregulation of pERK levels in sensory neuronal cultures (Zhuang et al., 2004). Bron *et. al.* also found a significant increase in pERK with brief NGF exposure (Bron et al., 2003). The major differences between their studies and those presented in Figure 31 are culture conditions. Neurons used in the studies by Zhuang *et. al*. and Bron *et. al.* are grown in culture for 24 hours or 4-5 days, respectively. The neurons used in their experiments are kept in serum free media for at least 24 hours prior to their experiments. In contrast, neurons in the studies presented in the current work are grown for 7 days in 30 ng/ml NGF in media with serum. In experiments in this thesis, we chose to use serum because physiologically neurons would likely be exposed to serum continuously. Furthermore, we chose to grow neurons in 30 ng/ml NGF for 7 days because we were still able to observe NGF-induced sensitization while simultaneously increasing peptide content enough to

measure capsaicin-evoked release. Neurons still can be sensitized by acute NGF exposure after just 2 days in 30 ng/ml NGF in media with serum (data not shown). Therefore, the differences observed between the data presented here and the data presented by the groups above are most likely derived from differences in exposure to serum with or without NGF.

What is important to note, is even without significant increases in pERK in neurons grown in serum with 30ng/ml NGF in our studies, there is still a significant increase in the levels of capsaicin-evoked iCGRP release when neurons are exposed to NGF for 10 minutes (Fig. 35). This indicates that sensitization is occurring even in the absence of an increase in ERK activity, or there is no correlation between NGF-induced increases in capsaicin-evoked iCGRP release and changes in pERK levels. Furthermore, this NGF-induced increase in iCGRP expression is not blocked by exposing neurons to 1μM of the MEK inhibitor, U0126, showing that pERK is not a mediator of NGF-induced increases in capsaicin-evoked release of iCGRP (Fig. 36). It is unlikely that the concentrations of U0126 used in these experiments (1μM) are not effective in blocking MEK activity because this concentration blocked the PDBu-induced increase in pERK in neuronal culture (Fig. 38).

In experiments where neurons were treated with FTI-276, and then capsaicin-evoked release was measured, FTI-276 increased capsaicin-evoked release (Fig. 30). Because FTI-276 increased capsaicin-evoked release to levels greater than the amount of release observed under control conditions, the effect of this drug on NGF-induced sensitization could not be interpreted. These results suggest that FTI-276 may act non-specifically to alter stimulated release from sensory neurons in culture. It is known that

FTI-276 may target a variety of proteins that are prenylated by the enzyme, farnesyltransferase (Sebti and Der, 2003). The majority of the studies using FTI-276 were performed in cell lines. Therefore, it is not known what proteins are targeted by this enzyme in primary sensory neurons besides Ras. Possible targets of FTI-276 that may cause the increase in capsaicin-stimulated iCGRP release from sensory neurons are protein tyrosine phosphatases. Some phosphatases, specifically PTP(CAAX) or PRL-1, 2, and 3, are prenylated by farnesyltransferase, and are localized to the membrane (Cates et al., 1996). These proteins are expressed in neurons of the central nervous system, but their presence in dorsal root ganglion neurons has not been studied (Takano et al., 1996). In many cases, these phosphatases inhibit the actions of receptor tyrosine kinases by dephosphorylating them. A loss of inhibition of receptor tyrosine kinases after incubation with FTI-276 could lead to a gain of function in proteins that are negatively regulated by dephosphorylation. As proof of concept that phosphatases are critical in regulating sensitivity of sensory neurons, the Vasko laboratory has previously shown that exposing sensory neurons in culture to the phosphatase inhibitor, okadaic acid, causes increased evoked release of iCGRP (Hingtgen et al., 1994). As in our experiments using FTI-276, there was no change in the resting release of neurons exposed to okadaic acid.

It also has been shown that FTI-276 blocks the addition of a farnesyl group, but other lipid groups are still added. Therefore, the lipid composition of the molecule is changed (Sebti and Der, 2003). An example is the Rho-b protein, which is in the family of small GTPases. Two lipids, a farnesyl group and geranylgeranyl group, can be added to this protein. When the protein contains only the geranylgeranyl group, it

localizes to another location in the cell and can signal to a different complement of molecules (Lebowitz et al., 1997; Lebowitz and Prendergast, 1998). While there is too little data in neurons to draw any conclusions about what is occurring, one could speculate that this differential localization of proteins also occurs in neurons. These findings may help to explain why there is an increase in capsaicin-evoked iCGRP release in the presence of FTI-276. For instance, the TRPV1 receptor may be inhibited constitutively by phosphatases that require the addition of lipid groups post-translationally (Chuang et al., 2001). If these phosphatases are not properly localized, TRPV1 could be more active. Upon capsaicin-stimulation, a more active TRPV1 channel could result in the increase in capsaicin-evoked release.

In addition to the expression of mutant isoforms of Ras and the use of farnesyltransferase inhibitors, the Ras pathway was manipulated using mice with a heterozygous deletion of the RasGAP, synGAP. One limitation in using these genetically altered mice is the neurons with a heterozygous deletion of synGAP may exhibit some level of compensation so that the cell develops the ability to overcome increased Ras activation in order to function normally. We also developed siRNA to decrease synGAP expression in neurons derived from normal adult animals, and measured release of iCGRP in response to capsaicin in the presence or absence of acute stimulation with NGF. In cells treated with synGAP siRNA, there was a slight increase in the magnitude of sensitization compared to neurons treated with a control siRNA (Fig. 34). The data from these experiments could be interpreted two ways. First, it is clear that a decrease in synGAP may lead to the increased expression of iCGRP especially when the neurons are grown in NGF, as these neurons were. This slight

increase could be secondary to an increase in the level of CGRP expressed. In this group of experiments release was not measured as a percentage of total content. However, these data also could indicate that indeed decreased synGAP leads to greater sensitization, and indicate that Ras plays a role in sensitization. However, these data taken into account with the other experiments that discount Ras and ERK as mediators of sensitization, leads this author to believe that this small increase in the degree of sensitization is likely due to slight changes in content. To confirm this, experiments should be repeated and measure release as a percentage of total content.

Inhibitors of the PI3 kinase pathway, another cascade activated by Ras, did not affect the NGF-induced increase in iCGRP (Fig. 40). The two drugs used to inhibit PI3 kinase in these studies were wortmannin (3nM) and LY294002 (3μM). The IC_{50} for wortmannin in inhibiting PI3 kinase is 6nM , and for LY294002 it is approximately 1.4μM (Yano et al., 1995). While the concentrations used for these experiments were near the IC_{50}, using greater concentrations caused an increase in the resting iCGRP from neurons when they were exposed to the drug alone without any stimulating agent. For instance, using LY294002 at 10 or 20μM increased resting release from neurons in culture by 2-3 fold (data not shown). Other studies demonstrated that brief exposure to NGF increased the phosphorylation of Akt using immunohistochemistry (Zhuang et al., 2004). Prior to their brief exposure to NGF, the neurons were not exposed to added NGF or serum in media in these experiments. However, in experiments in this thesis, neurons grown in serum at 30 ng/ml NGF for 7 days did not appear to have increased pAkt after a 10 minute exposure to NGF (personal communication, Dr. Judith Richter). The differences in the results are likely due to different culture conditions and NGF

exposure times. Because there was no change in NGF-induced sensitization in the presence of LY294002 or wortmannin, and there was no change in the levels of pAkt in neurons exposed for 10 minutes to the neurotrophin, this pathway is not a likely mediator of the NGF-induced increase in capsaicin-evoked release in our experiments.

In contrast to the classic pathways described above, activation of Src kinase does appear necessary for NGF-induced sensitization of sensory neurons (Fig. 44). In the current studies, it was determined that a protein that does regulate the increase in capsaicin-evoked release of iCGRP with 10 minute exposure to NGF is Src kinase (Fig. 44). The Src kinase inhibitor, PP2 (10μM), was used to block activity of this protein in neurons. The NGF-induced increase in capsaicin-evoked release was blocked completely at this concentration of PP2, whereas the inhibitor had no affect on capsaicin-evoked release in the absence of NGF or on basal release from the neuorns (Fig. 44). In contrast to these findings, PP3, the inactive analogue to PP2, did not affect the NGF-induced increase in capsaicin-evoked release and did not affect basal release. These results suggest that indeed Src kinase is playing a role in NGF-induced sensitization of sensory neurons. The findings in these current studies substantiate published results using sensory neurons from adult mice and heterologous expression systems. Zhang *et. al.* found that the NGF-induced increase in Ca^{2+} influx in HEK293 cells expressing the TRPv1 receptor could be blocked by the Src kinase inhibitor (Zhang et al., 2005). The mechanism by which this increased Ca^{2+} occurs is not known. Zhang *et. al.* suggest that Src kinase phosphorylates the TRPV1 receptor and causes translocation of TRPV1 to the membrane of the HEK293 cells. This may be the case,

but these results must be duplicated in specifically in sensory neurons to substantiate their findings in heterologous expression systems.

In other studies on Src kinase, Jin *et. al.* found that PP2 decreased capsaicin-evoked currents in dorsal root ganglion neurons, and they showed TRPV1 and Src kinase co-precipitated in lysates from a heterologous expression system. These data suggest that Src kinase may directly phosphorylate TRPV1 (Jin et al., 2004). Other data show that constitutively active Src can phosphorylate and activate the TrkA receptor in SK-N-MC neuroblastoma cell line (Tsuruda et al., 2004). This may represent a feed forward mechanism where NGF activates Src possibly through binding to phosphotyrosines on the cytoplasmic tail of TrkA (Fig. 2). In turn, active Src may phosphorylates the TrkA receptor to enhance its signaling.

Another study demonstrated Src could augment release of neuropeptides (Igwe, 2003). This study showed that Src regulates the increased expression and release of SP from dorsal root ganglion neurons in culture in response six hours of exposure to the cytokine, interleukin 1-β (IL-1β). The author used PP2 (100nM) to show that both release of neuropeptide and Src kinase activity in neurons were decreased in the presence of the inhibitor. What is not clear from this study using IL-1β is whether alterations in release of SP are a result of increased expression of the peptide alone or whether there is a component of SP release affected by altered excitability. It is more likely that IL-1β increases production of SP because it has been previously shown that this interleukin does not sensitize sensory neurons in culture (Nicol et al., 1997).

NGF-induced sensitization of iCGRP release also was blocked by the PKC inhibitor, BIM. These findings provide evidence that this kinase is involved in the

upregulation of stimulated outflow of neuropeptides caused by NGF (Figs. 41 and 42). BIM is an inhibitor of the classic and novel PKCs, but not the atypical isoforms. The mechanism by which PKC controls NGF-induced sensitivity in sensory neurons is not known, but experiments suggest that PKC phosphorylates and sensitizes the TRPV1 receptor (Bhave and Gereau, 2004). In light of the data showing Src kinase as a regulator of sensitivity, it may be that PKC is downstream from Src (Fig. 2). It has been demonstrated in cell lines that Src kinase can phosphorylate and active PKCδ, a novel PKC isoform which is inhibited by BIM (Joseloff et al., 2002).

Whether or not Src kinase directly modulates the release of iCGRP in response to brief stimulation with NGF, or indirectly through activation of PKC isoforms in sensory neurons is not known. Many studies have been performed on the Src kinase pathway in PC12 cells or in HEK heterologous expression systems (Igwe, 2003; Seibenhener et al., 1999; Wooten et al., 2001). The results from these experiments are mixed. As described above, Src kinase may directly bind to and phosphorylate the TRPV1 receptor to increase its activity (Jin et al., 2004). However, the atypical PKCs, such as PKCζ and PKCι (also called PKCλ in the rat), are activated by 15 minute stimulation of PC-12 cells with 100 ng/ml NGF (Wooten et al., 1994; Wooten et al., 2001). While these studies are compelling, more studies must be completed to understand how Src kinases may regulate the responses to NGF in sensory neurons both *in vitro* and *in vivo*.

The literature on PKC signaling downstream of NGF is extensive. The isoforms of PKC implicated in NGF-induced sensitivity and hyperalgesia include PKC-ε, PKC-δ, and PKC-α (O'Driscoll et al., 1995; Khasar et al., 1999; Ferreira et al., 2005). The

atypical isoforms, PKC-ι/λ and PKCζ, are activated by Src kinase in response to NGF. Futhermore, BIM, which inhibits many isoforms of PKC, blocks the NGF-induced increase in capsaicin-evoked iCGRP release (Figs. 41 and 42). Based on these data, it is likely that the classic and/or novel isoforms of PKC are involved. An attempt was made to selectively decrease isoforms of PKC in sensory neurons, and test the NGF-induced sensitization of neurons (Fig. 43). PKCα expression was reduced by 50% using siRNA, but there not appear to be a difference in the capsaicin-evoked release of iCGRP in the presence of NGF. While the experiment using PKCα siRNA is a single harvest of neurons and needs to be replicated, the results are compelling. They show that PKCα is not a likely mediator of NGF sensitization of capsaicin-evoked release from sensory neurons in culture. Other isoforms of PKC need to be tested to determine if any specific one can selectively block NGF-induced sensitization of sensory neurons.

In addition to testing signaling pathways that are classically linked to the TrkA neurotrophin receptor, such as MEK/ERK, data from these experiments demonstrate that S1P is involved in the regulation of evoked release (Fig. 46). While S1P could be produced from the activation of other pathways, it is interesting to speculate that the $p75^{NTR}$ could be involved. As shown above in Figure 2, NGF binds to the $p75^{NTR}$, which activates sphingomyelinase. Sphingomyelinase cleaves sphingomyelin to produce ceramide. Ceramide is metabolized to sphingosine, which can be phosphorylated by sphingosine kinase to yield S1P in the cell. S1P increased the capsaicin-induced release of iCGRP from sensory neurons in culture (Fig. 46). While our studies did not determine whether or not the $p75^{NTR}$

was involved in NGF-induced sensitization, further studies could be developed to determine if NGF does in fact alter capsaicin-evoked iCGRP release through this pathway.

In contrast to published findings, our data indicate that ceramide, the precursor to S1P, did not change the capsaicin-evoked release of iCGRP from neurons (Fig. 45). It is unclear why S1P, but not ceramide, sensitizes cells. Zhang and Nicol found the NGF enhances neuronal excitability through the $p75^{NTR}$ because using a p75 blocking antibody inhibits the increases in the number of action potentials in response to a ramp of depolarizing current (Zhang and Nicol, 2004). Also, the authors link NGF signaling specifically to downstream mediators of the $p75^{NTR}$. They show sensitization of sensory neurons by the neurotrophin could be blocked by inhibiting the conversion of sphingosine to S1P using DMS, an inhibitor of sphingosine kinase (Zhang et al., 2006). However, these authors also have determined that ceramide sensitizes sensory neurons in a similar manner to S1P (Nicol et al., 1997). One possible cause for the differences in results from our data and data from the Nicol laboratory, namely that ceramide does or does not sensitize neurons, is that the endpoints of our experiments, capsaicin-evoked iCGRP release, and the endpoints of studies by Zhang and Nicol, numbers of action potentials evoked by a ramp of current, are not equal and cannot be compared directly.

It is well established that there are interactions between the $p75^{NTR}$ and the TrkA receptor. The two receptors co-precipitate in spinal cord and brain tissues (Huber and Chao, 1995). It is purported that the interaction between the

two receptors confers specificity for NGF binding to TrkA, and increases the activity of TrkA when the neurotrophin is bound (Barker and Murphy, 1992; Benedetti et al., 1993). In the experiments described in this thesis, the receptor through which NGF was acting was not defined. However, both of these receptors, the TrkA and $p75^{NTR}$, are present on sensory neurons in culture, and it is likely that they are interacting as shown in cell lines. Although not the focus of the thesis, the contribution of these receptors to the NGF-induced increase in capsaicin evoked release could be determined by using antibodies that either block or activate TrkA and $p75^{NTR}$ and test the ability of NGF to increase capsaicin-evoked release. The laboratory of LF Reichardt has published the development of a TrkA activating antibody (Weskamp and Reichardt, 1991), which could be used to determine if the increases in NGF content and evoked release were mediated by TrkA. Furthermore, siRNA to specifically target either receptor could be used, and then the ability to NGF to sensitize neurons could be tested.

Overall, the experiments presented in this thesis show that NGF activates the Ras signaling pathway(s) to regulate the expression of iCGRP in neurons and Src kinase/PKC regulate the amount transmitter released from a neurons. This differential signaling through neurotrophin receptors is a concept well-established in the literature. Mutants of the TrkA receptor have been generated that lack the ability to signal to one or more of the downstream pathways activated by NGF (Loeb et al., 1994; Kaplan and Miller, 1997; Eggert et al., 2000). These mutants, which can then be expressed in cell lines, have given insight into which regions of the TrkA receptor are important for

activation of the Ras, PI3 kinase, and PLC pathways, and others. Eggert *et. al.* expressed wild-typeTrkA or one of five TrkA mutants into the SH-SY5Y cell line. In cells transfected with the wild-type TrkA, exposure to NGF slowed their proliferation and differentiated. This was also the case in cells transfected with TrkA mutants that no longer signaled to PI3 kinase. These results suggest that the PI3 kinase pathway was not involved in signaling cells to stop dividing and start differentiating. Conversely, when TrkA mutants with the inability to activate the PLC pathway were transfected into neurons, differentiation was blocked, which implies this pathway is involved in the regulation of differentiation in response to NGF. However, there may be a more complex story to TrkA receptor signaling that just individual pathways activated by specific locations on the TrkA receptor. Meakin *et. al.* have shown there is competition for binding a single location on the TrkA receptor (Meakin et al., 1999). The complement of proteins within the cell and the ability of those proteins to bind at the receptor at the time of NGF binding may dictate what signals are further propagated.

Based on current knowledge, NGF can activate multiple downstream signaling cascades. The data from experiments in this thesis indicate iCGRP expression after chronic exposure to NGF is regulated by a different pathway than alterations in capsaicin-stimulated release with brief exposure to NGF. It is interesting to speculate how these findings might explain what has been demonstrated by studies using NGF *in vivo*. For instance, Lewin *et. al.* showed that administration of NGF to rats resulted in a hyperalgesia with two observed components (Lewin et al., 1993). First there was a thermal hyperalgesia that developed within 15 min after NGF injection. Then a mechanical hyperalgesia

developed within 24 hours of NGF. The thermal hyperalgesia that develops immediately could be the result of post-translation changes in the TRPV1 receptor in response to NGF. Perhaps Src kinase is activated by NGF, which in turn phosphorylates and activates TRPV1. When the animal is stimulated with noxious heat, the primary sensory neurons release more neurotransmitter in a similar manner to neurons stimulated with capsaicin in the presence of NGF. Because the changes could be TRPV1 specific, only the thermal hyperalgesia would be apparent.

A global question that arises from these studies on the effects of NGF on sensory neurons is whether the neurotrophin and its signaling cascades are good targets for therapeutics for inflammation and chronic pain. Recent reviews suggest inhibition of NGF is a good strategy to decrease chronic pain (Hefti et al., 2005; Pezet and McMahon, 2006). In these two articles, the authors argue that current work both *in vitro* and *in vivo* suggests NGF augments the sensitivity of sensory neurons to and contributes to the development of chronic pain secondary to injury or inflammation. During inflammation, NGF upregulates multiple proteins that control the sensitivity of sensory neurons, such as ion channels, including TRPV1 and Nav 1.8 and 1.9, and neuropeptides (see above). Blocking NGF signaling reduces pain associated with a number of clinical problems including arthritis, pain associated with cancers invading bones, and post-operative pain (Voilley et al., 2001; Mamet et al., 2002; Mamet et al., 2003). Conversely, phase II trials are complete for recombinant human NGF therapy for improvement of sensory neuropathy associated with HIV infection (McArthur et

al., 2000). There was a significant improvement in the intensity of pain experienced by individuals receiving the NGF versus individuals receiving placebo.

While the molecular mechanisms responsible for physical findings and symptoms associated with acute and chronic pain are still being determined, it is clear NGF contributes to neuronal plasticity. Because the development of pain appears to be from various causes, such a peripheral neuropathy caused by diabetes, reflex sympathetic dystrophy after bone fracture, or chronic low back pain after repetitive use, this author no longer believes chronic pain is one type of disease, but it is specific to a disease process. Subsequently, it may be more fruitful to study sensory neurons in the context of each disease. NGF will likely be a central inflammatory mediator in many of these processes, and in some cases it will be necessary to block its effects, while in others it will be beneficial to treat with NGF. Using the key findings in this thesis, (1) Ras regulation of iCGRP expression in response to chronic NGF, and (2) Src and PKC regulation of NGF-induced increases in evoked release of iCGRP, may be good starting points for further research in determining the mechanisms of development of pain in one or more of these disease processes.

REFERENCES

Adler JE, Kessler JA, Black IB (1984) Development and regulation of substance P in sensory neurons in vitro. Dev Biol 102:417-425.

Adwanikar H, Karim F, Gereau RW (2004) Inflammation persistently enhances nocifensive behaviors mediated by spinal group I mGluRs through sustained ERK activation. Pain 111:125-135.

Ahlgren SC, Levine JD (1993) Mechanical hyperalgesia in streptozotocin-diabetic rats. Neuroscience 52:1049-1055.

Ahlgren SC, Wang JF, Levine JD (1997) C-fiber mechanical stimulus-response functions are different in inflammatory versus neuropathic hyperalgesia in the rat. Neuroscience 76:285-290.

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol 7:261-269.

Aley KO, Martin A, McMahon T, Mok J, Levine JD, Messing RO (2001) Nociceptor sensitization by extracellular signal-regulated kinases. J Neurosci 21:6933-6939.

Aloe L (2004) Rita Levi-Montalcini: the discovery of nerve growth factor and modern neurobiology. Trends Cell Biol 14:395-399.

Aloe L, Tuveri MA, Carcassi U, Levi-Montalcini R (1992) Nerve growth factor in the synovial fluid of patients with chronic arthritis. Arthritis Rheum 35:351-355.

Ambrosini A, Tininini S, Barassi A, Racagni G, Sturani E, Zippel R (2000) cAMP cascade leads to Ras activation in cortical neurons. Brain Res Mol Brain Res 75:54-60.

Averill S, McMahon SB, Clary DO, Reichardt LF, Priestley JV (1995) Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. Eur J Neurosci 7:1484-1494.

Barber LA, Vasko MR (1996) Activation of protein kinase C augments peptide release from rat sensory neurons. J Neurochem 67:72-80.

Barker PA, Murphy RA (1992) The nerve growth factor receptor: a multicomponent system that mediates the actions of the neurotrophin family of proteins. Mol Cell Biochem 110:1-15.

Bartlett SE, Reynolds AJ, Tan T, Heydon K, Hendry IA (1999) Differential mRNA expression and subcellular locations of PI3-kinase isoforms in sympathetic and sensory neurons. J Neurosci Res 56:44-53.

Basbaum AI (1999) Distinct neurochemical features of acute and persistent pain. Proc Natl Acad Sci U S A 96:7739-7743.

Basu T, Warne PH, Downward J (1994) Role of Shc in the activation of Ras in response to epidermal growth factor and nerve growth factor. Oncogene 9:3483-3491.

Baumann TK, Simone DA, Shain CN, LaMotte RH (1991) Neurogenic hyperalgesia: the search for the primary cutaneous afferent fibers that contribute to capsaicin-induced pain and hyperalgesia. J Neurophysiol 66:212-227.

Belanger S, Ma W, Chabot JG, Quirion R (2002) Expression of calcitonin gene-related peptide, substance P and protein kinase C in cultured dorsal root ganglion neurons following chronic exposure to mu, delta and kappa opiates. Neuroscience 115:441-453.

Bellamy J, Bowen EJ, Russo AF, Durham PL (2006) Nitric oxide regulation of calcitonin gene-related peptide gene expression in rat trigeminal ganglia neurons. Eur J Neurosci 23:2057-2066.

Benedetti M, Levi A, Chao MV (1993) Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. Proc Natl Acad Sci U S A 90:7859-7863.

Bernards A (2003) GAPs galore! A survey of putative Ras superfamily GTPase activating proteins in man and Drosophila. Biochim Biophys Acta 1603:47-82.

Besson JM, Chaouch A (1987) Peripheral and spinal mechanisms of nociception. Physiol Rev 67:67-186.

Bhave G, Gereau RW (2004) Posttranslational mechanisms of peripheral sensitization. J Neurobiol 61:88-106.

Bollag G, McCormick F (1991a) Differential regulation of rasGAP and neurofibromatosis gene product activities. Nature 351:576-579.

Bollag G, McCormick F (1991b) Regulators and effectors of ras proteins. Annu Rev Cell Biol 7:601-632.

Bonica JJ, Loeser JD (1990) Medical Evaluation of the Patient with Pain. In: The Management of Pain (Bonica JJ, ed), Philadelphia: Lea and Febiger.

Bonini S, Lambiase A, Bonini S, Angelucci F, Magrini L, Manni L, Aloe L (1996) Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. Proc Natl Acad Sci U S A 93:10955-10960.

Bonnington JK, McNaughton PA (2003) Signalling pathways involved in the sensitisation of mouse nociceptive neurones by nerve growth factor. J Physiol 551:433-446.

Bothwell M (1995) Functional interactions of neurotrophins and neurotrophin receptors. Annu Rev Neurosci 18:223-253.

Bowles WR, Sabino M, Harding-Rose C, Hargreaves KM (2004) Nerve growth factor treatment enhances release of immunoreactive calcitonin gene-related peptide but not substance P from spinal dorsal horn slices in rats. Neurosci Lett 363:239-242.

Bron R, Klesse LJ, Shah K, Parada LF, Winter J (2003) Activation of Ras is necessary and sufficient for upregulation of vanilloid receptor type 1 in sensory neurons by neurotrophic factors. Mol Cell Neurosci 22:118-132.

Brownlee S, Schrof JM (1997) The quality of mercy. Effective pain treatments already exist. Why aren't doctors using them? US News World Rep 122:54-7, 60, 62.

Budai D (2000) Neurotransmitters and receptors in the dorsal horn of the spinal cord. Acta Biologica Szegediensis 44:21-38.

Budai D, Larson AA (1998) The involvement of metabotropic glutamate receptors in sensory transmission in dorsal horn of the rat spinal cord. Neuroscience 83:571-580.

Burkey TH, Hingtgen CM, Vasko MR (2004) Isolation and culture of sensory neurons from the dorsal-root ganglia of embryonic or adult rats. Methods Mol Med 99:189-202.

Buscher D, Hipskind RA, Krautwald S, Reimann T, Baccarini M (1995) Ras-dependent and -independent pathways target the mitogen-activated protein kinase network in macrophages. Mol Cell Biol 15:466-475.

Castellucci V, Kandel ER (1976) Presynaptic facilitation as a mechanism for behavioral sensitization in Aplysia. Science 194:1176-1178.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816-824.

Cates CA, Michael RL, Stayrook KR, Harvey KA, Burke YD, Randall SK, Crowell PL, Crowell DN (1996) Prenylation of oncogenic human PTP(CAAX) protein tyrosine phosphatases. Cancer Lett 110:49-55.

Cesare P, Dekker LV, Sardini A, Parker PJ, McNaughton PA (1999) Specific involvement of PKC-epsilon in sensitization of the neuronal response to painful heat. Neuron 23:617-624.

Cesare P, McNaughton P (1996) A novel heat-activated current in nociceptive neurons and its sensitization by bradykinin. Proc Natl Acad Sci U S A 93:15435-15439.

Chen JJ, Dymshitz J, Vasko MR (1997) Regulation of opioid receptors in rat sensory neurons in culture. Mol Pharmacol 51:666-673.

Chen Y, Bacon G, Sher E, Clark BP, Kallman MJ, Wright RA, Johnson BG, Schoepp DD, Kingston AE (2000) Evaluation of the activity of a novel metabotropic glutamate receptor antagonist (+/-)-2-amino-2-(3-cis and trans-carboxycyclobutyl-3-(9-thioxanthyl)propionic acid) in the in vitro neonatal spinal cord and in an in vivo pain model. Neuroscience 95:787-793.

Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Chao MV, Julius D (2001) Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. Nature 411:957-962.

Coggeshall RE, Hong KA, Langford LA, Schaible HG, Schmidt RF (1983) Discharge characteristics of fine medial articular afferents at rest and during passive movements of inflamed knee joints. Brain Res 272:185-188.

Costigan M, Woolf CJ (2000) Pain: molecular mechanisms. J Pain 1:35-44.

Cullen PJ, Lockyer PJ (2002) Integration of calcium and Ras signalling. Nat Rev Mol Cell Biol 3:339-348.

Cunningham ME, Stephens RM, Kaplan DR, Greene LA (1997) Autophosphorylation of activation loop tyrosines regulates signaling by the TRK nerve growth factor receptor. J Biol Chem 272:10957-10967.

Dai Y, Iwata K, Fukuoka T, Kondo E, Tokunaga A, Yamanaka H, Tachibana T, Liu Y, Noguchi K (2002) Phosphorylation of extracellular signal-regulated kinase in primary afferent neurons by noxious stimuli and its involvement in peripheral sensitization. J Neurosci 22:7737-7745.

Devor M (2006) Sodium channels and mechanisms of neuropathic pain. J Pain 7:S3-S12.

Dinh QT, Groneberg DA, Peiser C, Springer J, Joachim RA, Arck PC, Klapp BF, Fischer A (2004) Nerve growth factor-induced substance P in capsaicin-insensitive vagal neurons innervating the lower mouse airway. Clin Exp Allergy 34:1474-1479.

Dobrowsky RT, Jenkins GM, Hannun YA (1995) Neurotrophins induce sphingomyelin hydrolysis. Modulation by co-expression of p75NTR with Trk receptors. J Biol Chem 270:22135-22142.

Dobrowsky RT, Werner MH, Castellino AM, Chao MV, Hannun YA (1994) Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. Science 265:1596-1599.

Donaldson LF, McQueen DS, Seckl JR (1995) Neuropeptide gene expression and capsaicin-sensitive primary afferents: maintenance and spread of adjuvant arthritis in the rat. J Physiol 486 (Pt 2):473-482.

Donnerer J, Schuligoi R, Stein C (1992) Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. Neuroscience 49:693-698.

Donnerer J, Schuligoi R, Stein C, Amann R (1993) Upregulation, release and axonal transport of substance P and calcitonin gene-related peptide in adjuvant inflammation and regulatory function of nerve growth factor. Regul Pept 46:150-154.

Donovan S, Shannon KM, Bollag G (2002) GTPase activating proteins: critical regulators of intracellular signaling. Biochim Biophys Acta 1602:23-45.

Durham PL, Russo AF (2003c) Stimulation of the calcitonin gene-related peptide enhancer by mitogen-activated protein kinases and repression by an antimigraine drug in trigeminal ganglia neurons. J Neurosci 23:807-815.

Durham PL, Russo AF (2000) Differential regulation of mitogen-activated protein kinase-responsive genes by the duration of a calcium signal. Mol Endocrinol 14:1570-1582.

Durham PL, Russo AF (2003) Stimulation of the calcitonin gene-related peptide enhancer by mitogen-activated protein kinases and repression by an antimigraine drug in trigeminal ganglia neurons. J Neurosci 23:807-815.

Egea J, Espinet C, Soler RM, Peiro S, Rocamora N, Comella JX (2000) Nerve growth factor activation of the extracellular signal-regulated kinase pathway is modulated by Ca(2+) and calmodulin. Mol Cell Biol 20:1931-1946.

Eggert A, Ikegaki N, Liu X, Chou TT, Lee VM, Trojanowski JQ, Brodeur GM (2000) Molecular dissection of TrkA signal transduction pathways mediating differentiation in human neuroblastoma cells. Oncogene 19:2043-2051.

English JM, Cobb MH (2002) Pharmacological inhibitors of MAPK pathways. Trends Pharmacol Sci 23:40-45.

Evans AR, Nicol GD, Vasko MR (1996) Differential regulation of evoked peptide release by voltage-sensitive calcium channels in rat sensory neurons. Brain Res 712:265-273.

Falcini F, Matucci CM, Lombardi A, Generini S, Pignone A, Tirassa P, Ermini M, Lepore L, Partsch G, Aloe L (1996) Increased circulating nerve growth factor is directly correlated with disease activity in juvenile chronic arthritis. Ann Rheum Dis 55:745-748.

Fehrenbacher JC (2005) The Contribution of Inflammation and Inflammatory Mediators To Sensitization of Sensory Neurons. Indiana University.

Feig LA, Cooper GM (1988) Relationship among guanine nucleotide exchange, GTP hydrolysis, and transforming potential of mutated ras proteins. Mol Cell Biol 8:2472-2478.

Ferreira J, Triches KM, Medeiros R, Calixto JB (2005) Mechanisms involved in the nociception produced by peripheral protein kinase c activation in mice. Pain 117:171-181.

Ferreira SH, Nakamura M, de Abreu Castro MS (1978) The hyperalgesic effects of prostacyclin and prostaglandin E2. Prostaglandins 16:31-37.

Ferri GL, Sabani A, Abelli L, Polak JM, Dahl D, Portier MM (1990) Neuronal intermediate filaments in rat dorsal root ganglia: differential distribution of peripherin and neurofilament protein immunoreactivity and effect of capsaicin. Brain Res 515:331-335.

Fitzgerald M, Wall PD, Goedert M, Emson PC (1985) Nerve growth factor counteracts the neurophysiological and neurochemical effects of chronic sciatic nerve section. Brain Res 332:131-141.

Frayer SM, Barber LA, Vasko MR (1999) Activation of protein kinase C enhances peptide release from rat spinal cord slices. Neurosci Lett 265:17-20.

Freeland K, Liu YZ, Latchman DS (2000) Distinct signalling pathways mediate the cAMP response element (CRE)-dependent activation of the calcitonin gene-related peptide gene promoter by cAMP and nerve growth factor. Biochem J 345 Pt 2:233-238.

Ganju P, O'Bryan JP, Der C, Winter J, James IF (1998) Differential regulation of SHC proteins by nerve growth factor in sensory neurons and PC12 cells. Eur J Neurosci 10:1995-2008.

Goedert M, Stoeckel K, Otten U (1981) Biological importance of the retrograde axonal transport of nerve growth factor in sensory neurons. Proc Natl Acad Sci U S A 78:5895-5898.

Gold MS, Levine JD, Correa AM (1998) Modulation of TTX-R INa by PKC and PKA and their role in PGE2-induced sensitization of rat sensory neurons in vitro. J Neurosci 18:10345-10355.

Goodman GR (2003) Outcomes measurement in pain management: issues of disease complexity and uncertain outcomes. J Nurs Care Qual 18:105-111.

Gould HJ, III, England JD, Liu ZP, Levinson SR (1998) Rapid sodium channel augmentation in response to inflammation induced by complete Freund's adjuvant. Brain Res 802:69-74.

Guard S, Watson SP (1987) Evidence for neurokinin-3 receptor-mediated tachykinin release in the guinea-pig ileum. Eur J Pharmacol 144:409-412.

Gureje O, Von KM, Simon GE, Gater R (1998) Persistent pain and well-being: a World Health Organization Study in Primary Care. JAMA 280:147-151.

Hall KE, Browning MD, Dudek EM, Macdonald RL (1995) Enhancement of high threshold calcium currents in rat primary afferent neurons by constitutively active protein kinase C. J Neurosci 15:6069-6076.

Hannun YA (1994) The sphingomyelin cycle and the second messenger function of ceramide. J Biol Chem 269:3125-3128.

Hefti FF, Rosenthal A, Walicke PA, Wyatt S, Vergara G, Shelton DL, Davies AM (2005) Novel class of pain drugs based on antagonism of NGF. Trends Pharmacol Sci.

Helke CJ, Charlton CG, Wiley RG (1986) Studies on the cellular localization of spinal cord substance P receptors. Neuroscience 19:523-533.

Helke CJ, Krause JE, Mantyh PW, Couture R, Bannon MJ (1990) Diversity in mammalian tachykinin peptidergic neurons: multiple peptides, receptors, and regulatory mechanisms. FASEB J 4:1606-1615.

Hingtgen CM, Vasko MR (1994) Prostacyclin enhances the evoked-release of substance P and calcitonin gene-related peptide from rat sensory neurons. Brain Res 655:51-60.

Hingtgen CM, Waite KJ, Vasko MR (1995) Prostaglandins facilitate peptide release from rat sensory neurons by activating the adenosine 3',5'-cyclic monophosphate transduction cascade. J Neurosci 15:5411-5419.

Hiruma H, Saito A, Ichikawa T, Kiriyama Y, Hoka S, Kusakabe T, Kobayashi H, Kawakami T (2000) Effects of substance P and calcitonin gene-related peptide on axonal transport in isolated and cultured adult mouse dorsal root ganglion neurons. Brain Res 883:184-191.

Holzer P (1988) Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. Neuroscience 24:739-768.

Hong Y, Abbott FV (1994) Behavioural effects of intraplantar injection of inflammatory mediators in the rat. Neuroscience 63:827-836.

Honore P, Rogers SD, Schwei MJ, Salak-Johnson JL, Luger NM, Sabino MC, Clohisy DR, Mantyh PW (2000) Murine models of inflammatory, neuropathic and cancer pain each generates a unique set of neurochemical changes in the spinal cord and sensory neurons. Neuroscience 98:585-598.

Huber LJ, Chao MV (1995) A potential interaction of p75 and trkA NGF receptors revealed by affinity crosslinking and immunoprecipitation. J Neurosci Res 40:557-563.

IASP Task Force on Taxonomy (1994) Classification of Chronic Pain. In: IASP Task Force on Taxonomy (Merskey H, Bogduk N, eds), pp 209-214. Seattle: IASP Press.

Igwe OJ (2003) c-Src kinase activation regulates preprotachykinin gene expression and substance P secretion in rat sensory ganglia. Eur J Neurosci 18:1719-1730.

Inaishi Y, Kashihara Y, Sakaguchi M, Nawa H, Kuno M (1992) Cooperative regulation of calcitonin gene-related peptide levels in rat sensory neurons via their central and peripheral processes. J Neurosci 12:518-524.

Ji RR, Kohno T, Moore KA, Woolf CJ (2003) Central sensitization and LTP: do pain and memory share similar mechanisms? Trends Neurosci 26:696-705.

Ji RR, Samad TA, Jin SX, Schmoll R, Woolf CJ (2002) p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. Neuron 36:57-68.

Jiang X, Zhang YH, Clark JD, Tempel BL, Nicol GD (2003) Prostaglandin E2 inhibits the potassium current in sensory neurons from hyperalgesic Kv1.1 knockout mice. Neuroscience 119:65-72.

Jin X, Morsy N, Winston J, Pasricha PJ, Garrett K, Akbarali HI (2004) Modulation of TRPV1 by nonreceptor tyrosine kinase, c-Src kinase. Am J Physiol Cell Physiol 287:C558-C563.

Joseloff E, Cataisson C, Aamodt H, Ocheni H, Blumberg P, Kraker AJ, Yuspa SH (2002) Src family kinases phosphorylate protein kinase C delta on tyrosine residues and modify the neoplastic phenotype of skin keratinocytes. J Biol Chem 277:12318-12323.

Julius D, Basbaum AI (2001) Molecular mechanisms of nociception. Nature 413:203-210.

Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF (1991a) The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science 252:554-558.

Kaplan DR, Martin-Zanca D, Parada LF (1991b) Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. Nature 350:158-160.

Kaplan DR, Miller FD (1997) Signal transduction by the neurotrophin receptors. Curr Opin Cell Biol 9:213-221.

Katz ME, McCormick F (1997) Signal transduction from multiple Ras effectors. Curr Opin Genet Dev 7:75-79.

Khasar SG, Lin YH, Martin A, Dadgar J, McMahon T, Wang D, Hundle B, Aley KO, Isenberg W, McCarter G, Green PG, Hodge CW, Levine JD, Messing RO (1999) A novel nociceptor signaling pathway revealed in protein kinase C epsilon mutant mice. Neuron 24:253-260.

Kim JH, Lee HK, Takamiya K, Huganir RL (2003) The role of synaptic GTPase-activating protein in neuronal development and synaptic plasticity. J Neurosci 23:1119-1124.

Knuesel I, Elliott A, Chen HJ, Mansuy IM, Kennedy MB (2005) A role for synGAP in regulating neuronal apoptosis. Eur J Neurosci 21:611-621.

Kohno M, Pouyssegur J (2003) Pharmacological inhibitors of the ERK signaling pathway: application as anticancer drugs. Prog Cell Cycle Res 5:219-224.

Komiyama NH, Watabe AM, Carlisle HJ, Porter K, Charlesworth P, Monti J, Strathdee DJ, O'Carroll CM, Martin SJ, Morris RG, O'Dell TJ, Grant SG (2002) SynGAP regulates ERK/MAPK signaling, synaptic plasticity, and learning in the complex with postsynaptic density 95 and NMDA receptor. J Neurosci 22:9721-9732.

Kuraishi Y, Hirota N, Sato Y, Kaneko S, Satoh M, Takagi H (1985) Noradrenergic inhibition of the release of substance P from the primary afferents in the rabbit spinal dorsal horn. Brain Res 359:177-182.

Lambiase A, Bonini S, Bonini S, Micera A, Magrini L, Bracci-Laudiero L, Aloe L (1995) Increased plasma levels of nerve growth factor in vernal keratoconjunctivitis and relationship to conjunctival mast cells. Invest Ophthalmol Vis Sci 36:2127-2132.

Lambiase A, Manni L, Rama P, Bonini S (2003) Clinical application of nerve growth factor on human corneal ulcer. Arch Ital Biol 141:141-148.

Lang E, Novak A, Reeh PW, Handwerker HO (1990) Chemosensitivity of fine afferents from rat skin in vitro. J Neurophysiol 63:887-901.

Lebowitz PF, Casey PJ, Prendergast GC, Thissen JA (1997) Farnesyltransferase inhibitors alter the prenylation and growth-stimulating function of RhoB. J Biol Chem 272:15591-15594.

Lebowitz PF, Prendergast GC (1998) Non-Ras targets of farnesyltransferase inhibitors: focus on Rho. Oncogene 17:1439-1445.

Leon A, Buriani A, Dal TR, Fabris M, Romanello S, Aloe L, Levi-Montalcini R (1994) Mast cells synthesize, store, and release nerve growth factor. Proc Natl Acad Sci U S A 91:3739-3743.

Leslie TA, Emson PC, Dowd PM, Woolf CJ (1995) Nerve growth factor contributes to the up-regulation of growth-associated protein 43 and preprotachykinin A messenger RNAs in primary sensory neurons following peripheral inflammation. Neuroscience 67:753-761.

Levi-Montalcini R (1964) GROWTH CONTROL OF NERVE CELLS BY A PROTEIN FACTOR AND ITS ANTISERUM: DISCOVERY OF THIS FACTOR MAY PROVIDE NEW LEADS TO UNDERSTANDING OF SOME NEUROGENETIC PROCESSES. Science 143:105-110.

Levi-Montalcini R, Skaper SD, Dal TR, Petrelli L, Leon A (1996) Nerve growth factor: from neurotrophin to neurokine. Trends Neurosci 19:514-520.

Levine JD, Reichling DB (1999) Peripheral mechanisms of inflammatory pain. In: Textbook of Pain (Melzack R, Wall PD, eds), pp 59-84. Edinburgh: Churchill Livingstone.

Lewin GR, Mendell LM (1993) Nerve growth factor and nociception. Trends Neurosci 16:353-359.

Lewin GR, Mendell LM (1994) Regulation of cutaneous C-fiber heat nociceptors by nerve growth factor in the developing rat. J Neurophysiol 71:941-949.

Li JY, Jahn R, Hou XE, Kling-Petersen A, Dahlstrom A (1996) Distribution of Rab3a in rat nervous system: comparison with other synaptic vesicle proteins and neuropeptides. Brain Res 706:103-112.

Lindholm D, Heumann R, Meyer M, Thoenen H (1987) Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of rat sciatic nerve. Nature 330:658-659.

Lindsay RM, Harmar AJ (1989) Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 337:362-364.

Lindsay RM, Lockett C, Sternberg J, Winter J (1989) Neuropeptide expression in cultures of adult sensory neurons: modulation of substance P and calcitonin gene-related peptide levels by nerve growth factor. Neuroscience 33:53-65.

Liu YZ, Chrivia JC, Latchman DS (1998) Nerve growth factor up-regulates the transcriptional activity of CBP through activation of the p42/p44(MAPK) cascade. J Biol Chem 273:32400-32407.

Loeb DM, Stephens RM, Copeland T, Kaplan DR, Greene LA (1994) A Trk nerve growth factor (NGF) receptor point mutation affecting interaction with phospholipase C-gamma 1 abolishes NGF-promoted peripherin induction but not neurite outgrowth. J Biol Chem 269:8901-8910.

Ma W, Bisby MA (1998) Increase of preprotachykinin mRNA and substance P immunoreactivity in spared dorsal root ganglion neurons following partial sciatic nerve injury. Eur J Neurosci 10:2388-2399.

Ma W, Zheng WH, Powell K, Jhamandas K, Quirion R (2001) Chronic morphine exposure increases the phosphorylation of MAP kinases and the transcription factor CREB in dorsal root ganglion neurons: an in vitro and in vivo study. Eur J Neurosci 14:1091-1104.

Malcangio M, Garrett NE, Tomlinson DR (1997) Nerve growth factor treatment increases stimulus-evoked release of sensory neuropeptides in the rat spinal cord. Eur J Neurosci 9:1101-1104.

Malmberg AB, Hamberger A, Hedner T (1995) Effects of prostaglandin E2 and capsaicin on behavior and cerebrospinal fluid amino acid concentrations of unanesthetized rats: a microdialysis study. J Neurochem 65:2185-2193.

Mamet J, Baron A, Lazdunski M, Voilley N (2002) Proinflammatory mediators, stimulators of sensory neuron excitability via the expression of acid-sensing ion channels. J Neurosci 22:10662-10670.

Mamet J, Lazdunski M, Voilley N (2003) How nerve growth factor drives physiological and inflammatory expressions of acid-sensing ion channel 3 in sensory neurons. J Biol Chem 278:48907-48913.

Martin-Zanca D, Mitra G, Long LK, Barbacid M (1986) Molecular characterization of the human trk oncogene. Cold Spring Harb Symp Quant Biol 51 Pt 2:983-992.

Matallanas D, Arozarena I, Berciano MT, Aaronson DS, Pellicer A, Lafarga M, Crespo P (2003) Differences on the inhibitory specificities of H-Ras, K-Ras, and N-Ras (N17) dominant negative mutants are related to their membrane microlocalization. J Biol Chem 278:4572-4581.

Matsumura H, Sakurada T, Hara A, Sakurada S, Kisara K (1985) Characterization of the hyperalgesic effect induced by intrathecal injection of substance P. Neuropharmacology 24:421-426.

McArthur JC, Yiannoutsos C, Simpson DM, Adornato BT, Singer EJ, Hollander H, Marra C, Rubin M, Cohen BA, Tucker T, Navia BA, Schifitto G, Katzenstein D, Rask C, Zaborski L, Smith ME, Shriver S, Millar L, Clifford DB, Karalnik IJ (2000) A phase II trial of nerve growth factor for sensory neuropathy associated with HIV infection. AIDS Clinical Trials Group Team 291. Neurology 54:1080-1088.

McDonald NQ, Blundell TL (1991) Crystallization and characterization of the high molecular weight form of nerve growth factor (7 S NGF). J Mol Biol 219:595-601.

McDonald NQ, Lapatto R, Murray-Rust J, Gunning J, Wlodawer A, Blundell TL (1991) New protein fold revealed by a 2.3-A resolution crystal structure of nerve growth factor. Nature 354:411-414.

Meacci E, Vasta V, Donati C, Farnararo M, Bruni P (1999) Receptor-mediated activation of phospholipase D by sphingosine 1-phosphate in skeletal muscle C2C12 cells. A role for protein kinase C. FEBS Lett 457:184-188.

Meakin SO, Macdonald JI, Gryz EA, Kubu CJ, Verdi JM (1999) The signaling adapter FRS-2 competes with Shc for binding to the nerve growth factor receptor TrkA. A model for discriminating proliferation and differentiation. J Biol Chem 274:9861-9870.

Micera A, Lambiase A, Puxeddu I, Aloe L, Stampachiacchiere B, Levi-Schaffer F, Bonini S, Bonini S (2006) Nerve growth factor effect on human primary fibroblastic-keratocytes: Possible mechanism during corneal healing. Exp Eye Res.

Miki K, Fukuoka T, Tokunaga A, Noguchi K (1998) Calcitonin gene-related peptide increase in the rat spinal dorsal horn and dorsal column nucleus following peripheral nerve injury: up-regulation in a subpopulation of primary afferent sensory neurons. Neuroscience 82:1243-1252.

Naldini L (1998) Lentiviruses as gene transfer agents for delivery to non-dividing cells. Curr Opin Biotechnol 9:457-463.

Natarajan V, Jayaram HN, Scribner WM, Garcia JG (1994) Activation of endothelial cell phospholipase D by sphingosine and sphingosine-1-phosphate. Am J Respir Cell Mol Biol 11:221-229.

Natura G, von Banchet GS, Schaible HG (2005) Calcitonin gene-related peptide enhances TTX-resistant sodium currents in cultured dorsal root ganglion neurons from adult rats. Pain 116:194-204.

Ng NF, Shooter EM (1993) Activation of p21ras by nerve growth factor in embryonic sensory neurons and PC12 cells. J Biol Chem 268:25329-25333.

Nicol GD, Vasko MR, Evans AR (1997) Prostaglandins suppress an outward potassium current in embryonic rat sensory neurons. J Neurophysiol 77:167-176.

Noguchi K, Kawai Y, Fukuoka T, Senba E, Miki K (1995) Substance P induced by peripheral nerve injury in primary afferent sensory neurons and its effect on dorsal column nucleus neurons. J Neurosci 15:7633-7643.

Nonogaki K, Moser AH, Shigenaga J, Feingold KR, Grunfeld C (1996) Beta-nerve growth factor as a mediator of the acute phase response in vivo. Biochem Biophys Res Commun 219:956-961.

O'Driscoll KR, Teng KK, Fabbro D, Greene LA, Weinstein IB (1995) Selective translocation of protein kinase C-delta in PC12 cells during nerve growth factor-induced neuritogenesis. Mol Biol Cell 6:449-458.

Obata K, Tsujino H, Yamanaka H, Yi D, Fukuoka T, Hashimoto N, Yonenobu K, Yoshikawa H, Noguchi K (2002) Expression of neurotrophic factors in the dorsal root ganglion in a rat model of lumbar disc herniation. Pain 99:121-132.

Ohmichi M, Matuoka K, Takenawa T, Saltiel AR (1994) Growth factors differentially stimulate the phosphorylation of Shc proteins and their association with Grb2 in PC-12 pheochromocytoma cells. J Biol Chem 269:1143-1148.

Okuse K, Chaplan SR, McMahon SB, Luo ZD, Calcutt NA, Scott BP, Akopian AN, Wood JN (1997) Regulation of expression of the sensory neuron-specific sodium channel SNS in inflammatory and neuropathic pain. Mol Cell Neurosci 10:196-207.

Packer RJ, Gutmann DH, Rubenstein A, Viskochil D, Zimmerman RA, Vezina G, Small J, Korf B (2002) Plexiform neurofibromas in NF1: toward biologic-based therapy. Neurology 58:1461-1470.

Pang L, Sawada T, Decker SJ, Saltiel AR (1995) Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. J Biol Chem 270:13585-13588.

Parsons AM, Seybold VS (1997) Calcitonin gene-related peptide induces the formation of second messengers in primary cultures of neonatal rat spinal cord. Synapse 26:235-242.

Petty BG, Cornblath DR, Adornato BT, Chaudhry V, Flexner C, Wachsman M, Sinicropi D, Burton LE, Peroutka SJ (1994) The effect of systemically administered recombinant human nerve growth factor in healthy human subjects. Ann Neurol 36:244-246.

Pezet S, McMahon SB (2006) NEUROTROPHINS: Mediators and Modulators of Pain. Annu Rev Neurosci 29:507-538.

Planells-Cases R, Garcia-Sanz N, Morenilla-Palao C, Ferrer-Montiel A (2005) Functional aspects and mechanisms of TRPV1 involvement in neurogenic inflammation that leads to thermal hyperalgesia. Pflugers Arch 451:151-159.

Pohl M, Benoliel JJ, Bourgoin S, Lombard MC, Mauborgne A, Taquet H, Carayon A, Besson JM, Cesselin F, Hamon M (1990) Regional distribution of calcitonin gene-related peptide-, substance P-, cholecystokinin-, Met5-enkephalin-, and dynorphin A (1-8)-like materials in the spinal cord and dorsal root ganglia of adult rats: effects of dorsal rhizotomy and neonatal capsaicin. J Neurochem 55:1122-1130.

Politi PM, Senderowicz AM (2001) First symposium of novel molecular targets for cancer therapy. Oncologist 6:207-212.

Portenoy RK, Kanner RM (1996) Definition and assessment of pain. In: Pain Management: Theory and Practice (Portenoy RK, Kanner RM, eds), Philadelphia: F A Davis.

Premkumar LS, Ahern GP (2000) Induction of vanilloid receptor channel activity by protein kinase C. Nature 408:985-990.

Quirion R, van RD, Dumont Y, St-Pierre S, Fournier A (1992) Characterization of CGRP1 and CGRP2 receptor subtypes. Ann N Y Acad Sci 657:88-105.

Reeh PW, Kocher L, Jung S (1986) Does neurogenic inflammation alter the sensitivity of unmyelinated nociceptors in the rat? Brain Res 384:42-50.

Reynolds AJ, Bartlett SE, Hendry IA (1998) Signalling events regulating the retrograde axonal transport of 125I-beta nerve growth factor in vivo. Brain Res 798:67-74.

Rhee SG (2001) Regulation of phosphoinositide-specific phospholipase C. Annu Rev Biochem 70:281-312.

Richardson JD, Vasko MR (2002) Cellular mechanisms of neurogenic inflammation. J Pharmacol Exp Ther 302:839-845.

Rong R, Ahn JY, Chen P, Suh PG, Ye K (2003) Phospholipase activity of phospholipase C-gamma1 is required for nerve growth factor-regulated MAP kinase signaling cascade in PC12 cells. J Biol Chem 278:52497-52503.

Roux PP, Bhakar AL, Kennedy TE, Barker PA (2001) The p75 neurotrophin receptor activates Akt (protein kinase B) through a phosphatidylinositol 3-kinase-dependent pathway. J Biol Chem 276:23097-23104.

Ruiz G, Banos JE (2005) The effect of endoneurial nerve growth factor on calcitonin gene-related peptide expression in primary sensory neurons. Brain Res 1042:44-52.

Safieh-Garabedian B, Poole S, Allchorne A, Winter J, Woolf CJ (1995) Contribution of interleukin-1 beta to the inflammation-induced increase in nerve growth factor levels and inflammatory hyperalgesia. Br J Pharmacol 115:1265-1275.

Safieh-Garabedian B, Poole S, Haddad JJ, Massaad CA, Jabbur SJ, Saade NE (2002) The role of the sympathetic efferents in endotoxin-induced localized inflammatory hyperalgesia and cytokine upregulation. Neuropharmacology 42:864-872.

Sango K, Verdes JM, Hikawa N, Horie H, Tanaka S, Inoue S, Sotelo JR, Takenaka T (1994) Nerve growth factor (NGF) restores depletions of calcitonin gene-related peptide and substance P in sensory neurons from diabetic mice in vitro. J Neurol Sci 126:1-5.

Schaible HG, Jarrott B, Hope PJ, Duggan AW (1990) Release of immunoreactive substance P in the spinal cord during development of acute arthritis in the knee joint of the cat: a study with antibody microprobes. Brain Res 529:214-223.

Schaible HG, Schmidt RF, Willis WD (1987) Enhancement of the responses of ascending tract cells in the cat spinal cord by acute inflammation of the knee joint. Exp Brain Res 66:489-499.

Scheffzek K, Ahmadian MR, Kabsch W, Wiesmuller L, Lautwein A, Schmitz F, Wittinghofer A (1997) The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. Science 277:333-338.

Schicho R, Donnerer J (1999) Nerve growth factor stimulates synthesis of calcitonin gene-related peptide in dorsal root ganglion cells during sensory regeneration in capsaicin-treated rats. Neurosci Res 35:183-187.

Schmidt Y, Unger JW, Bartke I, Reiter R (1995) Effect of nerve growth factor on peptide neurons in dorsal root ganglia after taxol or cisplatin treatment and in diabetic (db/db) mice. Exp Neurol 132:16-23.

Schuligoi R, Amann R (1998) Differential effects of treatment with nerve growth factor on thermal nociception and on calcitonin gene-related peptide content of primary afferent neurons in the rat. Neurosci Lett 252:147-149.

Sebti SM, Der CJ (2003) Opinion: Searching for the elusive targets of farnesyltransferase inhibitors. Nat Rev Cancer 3:945-951.

Sebti SM, Hamilton AD (1997) Inhibition of Ras prenylation: a novel approach to cancer chemotherapy. Pharmacol Ther 74:103-114.

Segal M, Willumsen BM, Levitzki A (1993) Residues crucial for Ras interaction with GDP-GTP exchangers. Proc Natl Acad Sci U S A 90:5564-5568.

Seibenhener ML, Roehm J, White WO, Neidigh KB, Vandenplas ML, Wooten MW (1999) Identification of Src as a novel atypical protein kinase C-interacting protein. Mol Cell Biol Res Commun 2:28-31.

Severini C, Improta G, Falconieri-Erspamer G, Salvadori S, Erspamer V (2002) The tachykinin peptide family. Pharmacol Rev 54:285-322.

Shepherd G (1994) Neurobiology. Oxford University Press.

Shu X, Mendell LM (1999) Nerve growth factor acutely sensitizes the response of adult rat sensory neurons to capsaicin. Neurosci Lett 274:159-162.

Shu X, Mendell LM (2001) Acute sensitization by NGF of the response of small-diameter sensory neurons to capsaicin. J Neurophysiol 86:2931-2938.

Simone DA, Baumann TK, Collins JG, LaMotte RH (1989) Sensitization of cat dorsal horn neurons to innocuous mechanical stimulation after intradermal injection of capsaicin. Brain Res 486:185-189.

Skoff AM, Adler JE (2006) Nerve growth factor regulates substance P in adult sensory neurons through both TrkA and p75 receptors. Exp Neurol 197:430-436.

Sofroniew MV, Howe CL, Mobley WC (2001) Nerve growth factor signaling, neuroprotection, and neural repair. Annu Rev Neurosci 24:1217-1281.

Southall MD, Bolyard LA, Vasko MR (2002) Twenty-four hour exposure to prostaglandin downregulates prostanoid receptor binding but does not alter PGE(2)-mediated sensitization of rat sensory neurons. Pain 96:285-296.

Southall MD, Vasko MR (2001) Prostaglandin receptor subtypes, EP3C and EP4, mediate the prostaglandin E2-induced cAMP production and sensitization of sensory neurons. J Biol Chem 276:16083-16091.

Springer J, Geppetti P, Fischer A, Groneberg DA (2003) Calcitonin gene-related peptide as inflammatory mediator. Pulm Pharmacol Ther 16:121-130.

Stacey DW, Feig LA, Gibbs JB (1991) Dominant inhibitory Ras mutants selectively inhibit the activity of either cellular or oncogenic Ras. Mol Cell Biol 11:4053-4064.

Stephens L, Anderson K, Stokoe D, Erdjument-Bromage H, Painter GF, Holmes AB, Gaffney PR, Reese CB, McCormick F, Tempst P, Coadwell J, Hawkins PT (1998) Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. Science 279:710-714.

Stucky CL, Abrahams LG, Seybold VS (1998) Bradykinin increases the proportion of neonatal rat dorsal root ganglion neurons that respond to capsaicin and protons. Neuroscience 84:1257-1265.

Sun RQ, Lawand NB, Willis WD (2003) The role of calcitonin gene-related peptide (CGRP) in the generation and maintenance of mechanical allodynia and hyperalgesia in rats after intradermal injection of capsaicin. Pain 104:201-208.

Sun RQ, Tu YJ, Lawand NB, Yan JY, Lin Q, Willis WD (2004) Calcitonin gene-related peptide receptor activation produces PKA- and PKC-dependent mechanical hyperalgesia and central sensitization. J Neurophysiol 92:2859-2866.

Taha TA, Argraves KM, Obeid LM (2004) Sphingosine-1-phosphate receptors: receptor specificity versus functional redundancy. Biochim Biophys Acta 1682:48-55.

Takai Y, Sasaki T, Matozaki T (2001) Small GTP-binding proteins. Physiol Rev 81:153-208.

Takano S, Fukuyama H, Fukumoto M, Kimura J, Xue JH, Ohashi H, Fujita J (1996) PRL-1, a protein tyrosine phosphatase, is expressed in neurons and oligodendrocytes in the brain and induced in the cerebral cortex following transient forebrain ischemia. Brain Res Mol Brain Res 40:105-115.

Toman RE, Milstien S, Spiegel S (2001) Sphingosine-1-phosphate: an emerging therapeutic target. Expert Opin Ther Targets 5:109-123.

Toman RE, Payne SG, Watterson KR, Maceyka M, Lee NH, Milstien S, Bigbee JW, Spiegel S (2004) Differential transactivation of sphingosine-1-phosphate receptors modulates NGF-induced neurite extension. J Cell Biol 166:381-392.

Tominaga M, Caterina MJ (2004) Thermosensation and pain. J Neurobiol 61:3-12.

Tsuruda A, Suzuki S, Maekawa T, Oka S (2004) Constitutively active Src facilitates NGF-induced phosphorylation of TrkA and causes enhancement of the MAPK signaling in SK-N-MC cells. FEBS Lett 560:215-220.

Tverberg LA, Russo AF (1992) Cell-specific glucocorticoid repression of calcitonin/calcitonin gene-related peptide transcription. Localization to an 18-base pair basal enhancer element. J Biol Chem 267:17567-17573.

Tverberg LA, Russo AF (1993) Regulation of the calcitonin/calcitonin gene-related peptide gene by cell-specific synergy between helix-loop-helix and octamer-binding transcription factors. J Biol Chem 268:15965-15973.

van RD, Hanisch UK, Quirion R (1997) Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors. Neurosci Biobehav Rev 21:649-678.

Vasko MR, Campbell WB, Waite KJ (1994) Prostaglandin E2 enhances bradykinin-stimulated release of neuropeptides from rat sensory neurons in culture. J Neurosci 14:4987-4997.

Vellani V, Zachrisson O, McNaughton PA (2004) Functional bradykinin B1 receptors are expressed in nociceptive neurones and are upregulated by the neurotrophin GDNF. J Physiol 560:391-401.

Verge VM, Gratto KA, Karchewski LA, Richardson PM (1996) Neurotrophins and nerve injury in the adult. Philos Trans R Soc Lond B Biol Sci 351:423-430.

Verge VM, Richardson PM, Wiesenfeld-Hallin Z, Hokfelt T (1995) Differential influence of nerve growth factor on neuropeptide expression in vivo: a novel role in peptide suppression in adult sensory neurons. J Neurosci 15:2081-2096.

Verge VM, Riopelle RJ, Richardson PM (1989) Nerve growth factor receptors on normal and injured sensory neurons. J Neurosci 9:914-922.

Vetter ML, Martin-Zanca D, Parada LF, Bishop JM, Kaplan DR (1991) Nerve growth factor rapidly stimulates tyrosine phosphorylation of phospholipase C-gamma 1 by a kinase activity associated with the product of the trk protooncogene. Proc Natl Acad Sci U S A 88:5650-5654.

Voilley N, de WJ, Mamet J, Lazdunski M (2001) Nonsteroid anti-inflammatory drugs inhibit both the activity and the inflammation-induced expression of acid-sensing ion channels in nociceptors. J Neurosci 21:8026-8033.

Warsame AA, Gustafsson H, Olgart L, Brodin E, Stiller CO, Taylor BK (2004) Capsaicin-evoked substance P release in rat dorsal horn increases after peripheral inflammation: a microdialysis study. Neurosci Lett 368:226-230.

Watson A, Ensor E, Symes A, Winter J, Kendall G, Latchman D (1995) A minimal CGRP gene promoter is inducible by nerve growth factor in adult rat dorsal root ganglion neurons but not in PC12 phaeochromocytoma cells. Eur J Neurosci 7:394-400.

Watson A, Latchman D (1995) The cyclic AMP response element in the calcitonin/calcitonin gene-related peptide gene promoter is necessary but not sufficient for its activation by nerve growth factor. J Biol Chem 270:9655-9660.

Waxman SG, Dib-Hajj S, Cummins TR, Black JA (2000) Sodium channels and their genes: dynamic expression in the normal nervous system, dysregulation in disease states(1). Brain Res 886:5-14.

Waxman SG, Dib-Hajj S, Cummins TR, Black JA (1999) Sodium channels and pain. Proc Natl Acad Sci U S A 96:7635-7639.

Weskamp G, Reichardt LF (1991) Evidence that biological activity of NGF is mediated through a novel subclass of high affinity receptors. Neuron 6:649-663.

Wiley JW, Gross RA, MacDonald RL (1992) The peptide CGRP increases a high-threshold Ca2+ current in rat nodose neurones via a pertussis toxin-sensitive pathway. J Physiol 455:367-381.

Winter J, Bevan S, Campbell EA (1995) Capsaicin and pain mechanisms. Br J Anaesth 75:157-168.

Winter J, Forbes CA, Sternberg J, Lindsay RM (1988) Nerve growth factor (NGF) regulates adult rat cultured dorsal root ganglion neuron responses to the excitotoxin capsaicin. Neuron 1:973-981.

Wood KW, Sarnecki C, Roberts TM, Blenis J (1992) ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. Cell 68:1041-1050.

Woolf CJ (1983) Evidence for a central component of post-injury pain hypersensitivity. Nature 306:686-688.

Woolf CJ (2004) Pain: moving from symptom control toward mechanism-specific pharmacologic management. Ann Intern Med 140:441-451.

Woolf CJ, Decosterd I (1999) Implications of recent advances in the understanding of pain pathophysiology for the assessment of pain in patients. Pain Suppl 6:S141-S147.

Woolf CJ, Safieh-Garabedian B, Ma QP, Crilly P, Winter J (1994) Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. Neuroscience 62:327-331.

Woolf CJ, Safieh-Garabedian B, Ma QP, Crilly P, Winter J (1994b) Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. Neuroscience 62:327-331.

Woolf CJ, Salter MW (2000) Neuronal plasticity: increasing the gain in pain. Science 288:1765-1769.

Wooten MW, Vandenplas ML, Seibenhener ML, Geetha T, az-Meco MT (2001) Nerve growth factor stimulates multisite tyrosine phosphorylation and activation of the atypical protein kinase C's via a src kinase pathway. Mol Cell Biol 21:8414-8427.

Wooten MW, Zhou G, Seibenhener ML, Coleman ES (1994) A role for zeta protein kinase C in nerve growth factor-induced differentiation of PC12 cells. Cell Growth Differ 5:395-403.

Yang C, Kazanietz MG (2003) Divergence and complexities in DAG signaling: looking beyond PKC. Trends Pharmacol Sci 24:602-608.

Yano H, Agatsuma T, Nakanishi S, Saitoh Y, Fukui Y, Nonomura Y, Matsuda Y (1995) Biochemical and pharmacological studies with KT7692 and LY294002 on the role of phosphatidylinositol 3-kinase in Fc epsilon RI-mediated signal transduction. Biochem J 312 (Pt 1):145-150.

York RD, Yao H, Dillon T, Ellig CL, Eckert SP, McCleskey EW, Stork PJ (1998) Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature 392:622-626.

Zhang X, Aman K, Hokfelt T (1995) Secretory pathways of neuropeptides in rat lumbar dorsal root ganglion neurons and effects of peripheral axotomy. J Comp Neurol 352:481-500.

Zhang X, Huang J, McNaughton PA (2005) NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. EMBO J 24:4211-4223.

Zhang Y, Fehrenbacher JC, Vasko MR, Nicol GD (2006) Sphingosine-1-phosphate via activation of a G protein-coupled receptor(s) enhances the excitability of rat sensory neurons. J Neurophysiol.

Zhang Y, Vasko MR, Nicol GD (2006) Intracellular sphingosine 1-phosphate mediates the increased excitability produced by nerve growth factor in rat sensory neurons. J Physiol.

Zhang YH, Nicol GD (2004) NGF-mediated sensitization of the excitability of rat sensory neurons is prevented by a blocking antibody to the p75 neurotrophin receptor. Neurosci Lett 366:187-192.

Zhang YH, Vasko MR, Nicol GD (2002) Ceramide, a putative second messenger for nerve growth factor, modulates the TTX-resistant Na(+) current and delayed rectifier K(+) current in rat sensory neurons. J Physiol 544:385-402.

Zhuang ZY, Xu H, Clapham DE, Ji RR (2004) Phosphatidylinositol 3-kinase activates ERK in primary sensory neurons and mediates inflammatory heat hyperalgesia through TRPV1 sensitization. J Neurosci 24:8300-8309.

CURRICULUM VITAE

Education

Indiana University, Indianapolis, Indiana — 2001-2007
MD/PhD program
Doctor of Philosophy

Indiana University School of Medicine — 2001-present
MD/PhD program
Doctor of Medicine, expected May 2008

Hanover College, Hanover, Indiana — 1997-2001
Bachelor of Arts in Biology, Magna cum Laude
Minor in Biochemistry
Minor in Foreign Language, Spanish

Professional Experience

Indiana University School of Medicine, MD/PhD program — June 2003-2007
Graduate research in mechanisms
of sensory neuron sensitization
Graduate Training: Michael Vasko, PhD,
Chair, Department of Pharmacology and Toxicology

St. Francis Hospital of Indianapolis — Jan 2003-present
Extern, Emergency Department

Indiana University School of Medicine
Medical Scientist Training Program, Research rotations

Hal Broxmeyer, PhD, — Summer 2001
Chair, Depatment of Microbiology and Immunology
Cdc4 role in cell cycle checkpoints

Randy Brutkiewicz, PhD, — Summer 2002
Department of Microbiology and Immunology
Liver NK-T cell cytokine production

Harvard Medical School and
Brigham and Women's Hospital, Boston, MA Summer 1999 and 2000
Undergraduate research
Sean P. Colgan, PhD,
Department of Anesthesia and Reperfusion Injury
Undergraduate advisor, Walter J Bruyninckx, PhD, DVM,
Hanover College
Neutrophil's role in endothelial permeability

Hanover College 1998-1999
Undergraduate Research
Research Training, Jeff Hughes, PhD,
SAMase expression and bacterial phenotype

Hanover College 1998
Plant Taxonomy Training, advisor, Paul MacMillan, PhD,
Herbarium Preservation

Dow Corning Corperation Summer 1998
Undergraduate research
Research training, Anne-Marie Blanquert, DVM
Microbiology in silicones and bioremediation

Teaching Experience

Hanover College 2001
Teaching Assistant to Walter J. Bruyninckx, PhD, DVM
Introduction to Biology, Laboratory

Accomplishment

Sigma Xi Scientific Research Society, IU Medical Center Chapter
Third place winner, senior student presentations June 2006

Days in Molecular Medicine, MD/PhD Scholarship Award May 2006
Travel award for DMM Conference, Stockholm, Sweden

Chancellor's Scholar Award, IUPUI, Indianapolis April 2006
Top GPA for graduate student, School of Medicine

National Research Service Award April 2006
(NINDS 1 F30 NS055448-01): $107,000, 3 year training grant

INGen Scholar, IU School of Medicine 2001-2005
Tuition, fees, and living expenses for MD/PhD program

Education Enhancement Grant, IUPUI 2005
Award for Travel to participate in Abstract Presentation

Honorable Mention, Abstract Competition
American College of Physicians, Indiana Chapter 2005

Rhodes Scholar Finalist, Region V 2001

Mortar Board, Hanover College 2001

Goldwater Scholar 2000

J. Dan Webster Outstanding Student in Biology, Hanover College 2001

Richter Grant, Hanover College 1998-1999
Provides funding for student-designed projects
Project Name: HIV, AIDS, and the Response of the
Centers for Disease Control and Prevention, Atlanta

Horner Scholar, Hanover College 1997-2001
Tuition and fees for four years

Lowe's Building Foundations Scholar 1997-2001
Living expenses for four years

Affiliation

Society for Neuroscience 2005-present
Student member

American Medical Association, 2006-2007
Council on Scientific Affairs and Public Health
Council member

Medical Student Section-American Medical Association	2001-present
Committee on Scientific Issues, Chair	2005-2006
Alternate Trustee, State Chapter	2005-2006
American Medical Political Action Committee	
Student Advisory Board Member	2004-2006
Chair, Lobby Day Planning Committee	2005-2006
Secretary, State Chapter	2003-2004
Indiana University School of Medicine Delegate	2002-2003
Indiana Medical Political Action Committee Member	2002-2005
American College of Physicians	2005
Medical Student Member	
Tri-Beta Biology Club, Hanover College	
President	2000-2001
Secretary	1999-2000
General Member	1997-1999

Publications

Park KA and Vasko MR. Lipid Mediators of Sensitivity in Sensory Neurons. *Trends in Pharmacological Sciences*, 2005 Nov; 26(11):571-7.

Collard CD, Park KA, Montalto MC, Alapati S, Buras JA, Stahl GL, Colgan SP. Neutrophil-derived glutamate regulates vascular endothelial barrier function. *J Biol Chem*. 2002 Apr 26; 277(17):14801-11

Abstracts

2006 Society for Neuroscience Annual Meeting:
Park KA, Thompson EL, Richter JD, Vasko MR.
The Ras/MEK/ERK cascade mediates nerve growth factor-induced increases in expression of calcitonin-gene related peptide in sensory neurons.

2005 Annual MD/PhD Student Conference:
Park KA and Vasko MR. *Ras Contributes to NGF-Induced Increases in CGRP Content in Sensory Neurons*.

2005 Society for Neuroscience Annual Meeting:
Fehrenbacher JC, Park KA, Richter JA, Thompson EL, David MC, Vasko MR. *Exposure to nerve growth factor increases the capsaicin-evoked release of iCGRP by directed sensitization and by increasing peptide content in sensory neurons.*

2004 Society for Neuroscience Annual Meeting:
Park KA, Bohnstedt BN, Thompson EL, Fehrenbacher JC, and Vasko MR. *Reduced expression of synGAP sensitizes sensory neurons.*

Conference Participation

Days in Molecular Medicine Conference, Stockholm, Sweden May 2006
Abstract presentation

American Pain Society, Annual Meeting, San Antonio, Texas May 2006
Abstract presentation

20th Annual MD/PhD Student Conference, Keystone, Colorado July 2005
Abstract presentation

Society for Neuroscience, Annual Conference
Abstract presentation
San Diego, California 2004
Washington, D.C. 2005

Indiana University School of Medicine MD/PhD Annual Retreat
Oral Presentation August 2005
Abstract Presentation August 2004

Lobby Day Participant, MSS-AMA, Washington, DC 2005 and 2006

Interim Meeting MSS-AMA 2003 and 2004
Reference Committee Member, I-04
Abstract Presentation, I-04
Primary author of resolution passed through MSS-AMA General Assembly, I-04
Community Service Committee, I-03

Annual MSS-AMA Meetings, Chicago, Illinois 2003, 2004, 2005

Indiana State Medical Association, Annual Meeting 2003 and 2004
IMPAC Student Representative

Community Activity

Spring into Shape, 5K Run/Walk and Health Fair, MSS-AMA 2005 and 2006
Organizer

Community Service Committee, Interim Meeting MSS-AMA Dec 4-7, 2003

Coordinator, Forest Manor Health Fair — Sept 6 2003
Funded partly with 2003 AMA Community Service Grant
Organizer

Spring House Calls, Participant — April 2003

Indianapolis Life 500 Festival Mini-Marathon — 2002-2007

Hanover College Student Advisory Committee — 2000-2001

International Experience

Himalayan Health Exchange, Ancient Guge Valley (Tibet), Northern India
June, July 2002
Medical student participation in health care provision to remote countryside.

Yucatán Peninsula, Mérida, México
May 2001
Exchange Student, Hanover College.