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Retrograde NGF Signaling in Pain

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Abstracts of the Dissertation

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Nerve growth factor (NGF) is a member of the nerurotrophin family that plays a crucial role during development. Although NGF was discovered because of its actions during development, it is now known to function throughout the life of the animal. NGF plays a profound role in nociception because its high affinity receptor, TrkA, is expressed in nociceptors. In mammals, NGF causes thermal hyperalgesia that develops within minutes (acute hyperalgesia) and lasts for several days (chronic hyperalgesia). While NGF-induced acute peripheral sensitization/hyperalgesia occurs via TRPV1 receptor activation, chronic hyperalgesia is thought to take place with a delay, caused by upregulation of genes. This delay would involve

1) retrograde transport of the NGF-TrkA complex from the axon terminals (DAs) to the cell bodies (CBs) and 2) modulation of gene expression at CBs. If altered gene expression can sensitize nociceptors, chronic hyperalgesia could thus develop. To test this idea, I looked at the expression of two genes, VGF, a neuropeptide, and Nav1.7, a sodium channel. Both proteins are widely expressed in primary sensory neurons including nociceptors, have been functionally linked to pain perception, and can be upregulated by NGF in some peripheral neurons. I first asked whether NGF can upregulate these proteins in sensory dorsal root ganglion (DRG) neurons and whether upregulation can be mediated by retrograde signaling. Here I show that NGF treatment increased Nav1.7 and VGF protein levels, and by using microfluidic devices to biochemically separate CBs from DAs, I show NGF stimulation at the DA retrogradely upregulated both mRNA and protein levels of these genes. Since Pincher, a member of the EHD protein family, is required for NGF/TrkA internalization and retrograde transport of NGF-TrkA endosomes in cultured sympathetic neurons, I investigated the role of Pincher in mediating this upregulation. NGF-induced retrograde upregulation in DRG neurons, indeed, was abolished in the neurons expressing a dominant negative form of Pincher, PincherG68E. In mice, NGF injection into the paw upregulated both VGF and Nav1.7 expression in DRG cell bodies through retrograde signaling. However, NGF failed to retrogradely upregulate these proteins in PincherG68E expressing DRG neurons. Finally, I show that mice injected with virus to express PincherG68E didn't develop NGF-induced chronic thermal hyperalgesia (tested 24 hours after NGF injection). Thus, Pincher-mediated retrograde NGF signaling may be a means by which NGF mediates a transition from acute to chronic hyperalgesia through changes in gene expression.

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Chapter I

General Introduction

Neurotrophins (NTs) are a family of target derived peptide growth factors that promote neuronal phenotype and survival, both in the central and peripheral nervous system. While NGF, the first discovered member of NT family, was originally discovered as a survival factor for dorsal root ganglion (DRG) neurons, NTs have since been implicated in multiple functions including axonal and dendritic growth, axonal guidance, synapse formation, cell proliferation, cell migration, and synaptic plasticity (reviewed by Zweifel et al, 2005). Other members of NT family are: Brain Derived Neurotrophic Factor (BDNF), Neurotrophin 3 (NT3) and Neurotrophin 4/5 (NT4/5). NTs bind to two different types of membrane receptors, tropomyosin receptor kinase (Trk) and p75NTR, to mediate signaling. The latter, p75NTR, can bind to all NTs, albeit with low affinity; furthermore, proNTs—the precursors of matures NTs—preferentially to P75 receptors (NyKjaer et al, 2004; Teng et al, 2005). Trk receptors, on the other hand, exhibit selectivity, but bind to NTs with high affinity. NGF, for example, binds to TrkA, BDNF and NT4 to TrkB, and NT3 to TrkC, though NT3 can bind to TrkA as well, with lower affinity (Ryden, and Ibanez, 1996). p75NTR is often associated with cell death for its pro-apoptoic effect in the absence of Trk receptors (reviewed in Miller and Kaplan, 2001).

Role of NGF in mediating pain:

NTs play a crucial role in the development of the nervous system. During prenatal development, for example, most peripheral neurons depend on NGF for survival. In NGF or TrkA knock-out

mice, ~70% of DRG sensory neurons die before birth (Crowley et al, 1994, Smeyne et al, 1994). These neurons lose their dependency on NGF for survival within two weeks after birth; however, in this early postnatal period, NGF is also required for expression of the appropriate nociceptor phenotype. Lewin and Mendell (1994) showed that in rats, deprivation of NGF during postnatal day 4 to 11 lead to reduced number of neurons from Aδ high threshold mechanoreceptors and a corresponding increase in the non-nociceptive Aδ D-hair population. Later studies on Bax and TrkA double knockout mice also revealed the importance of NGF in appropriate nociceptor development: unlike TrkA knockout mice, DRG neurons in Bax-/-, TrkA-/- mice do survive, however, due to the absence of TrkA signaling, these neurons fail to express distinct biochemical markers of nociceptive, Trk-expressing DRG neurons such as Calcitonin Gene-related Peptide (CGRP) and substance P (SP) (Patel et al, 2000).

Interestingly, in rats, between postnatal days 7 and 14, TrkA expressing sensory neurons lose their dependency on NGF for survival and simultaneously, about half of these cells downregulate TrkA and upregulate Glial cell-derived Neurotrophic Factor (GDNF) receptor components (Bennett et al 1998). However, about 40-45% of lumbar dorsal root ganglia (DRG) neurons continue to express TrkA throughout adulthood, and respond to NGF (Molliver et al, 1997). Since these neurons are not dependent on NGF for survival, what is the function of NGF in these neurons in adult animals?

Research done over the last two decades illustrate that NGF profoundly modulates the functions of nociceptors in adult animals (discussed later), and thus plays a key role in the generation and potentiation of pain. Local or systemic administration of NGF, for example, elicits both thermal hyperalgesia and mechanical allodynia in rats (Lewin et al., 1994). Similar results were also seen in humans where local NGF administration in healthy subjects caused

hyperalgesia and allodynia at the injection site (Petty et al., 1994). Moreover, the NGF level is increased in several animal models of inflammation caused by agents such as complete Freund's adjuvant (Donnerer et al. 1992; Safieh-Garabedian et al. 1995) and subcutaneous carrageenin (Westkamp and Otten 1987; Otten 1991; Aloe et al. 1992); anti-NGF antibody (Woolf et al, 1994) or sequestration of endogenous NGF by TrkA-IgG (McMahon et al, 1995) prevents this increase and diminishes inflammatory hyperalgesia. Taken together, these studies indicate that NGF is not only sufficient for the production of hyperalgesia, but also is necessary for the production of inflammatory hyperalgesia. Indeed, NGF-neutralizing antibodies are currently being monitored for their potential use as an analgesic in clinical trials: Tanezumab, for example, is an anti-NGF antibody that has reached Phase III clinical trials for osteoarthritis.

Two human genetic disorders also highlight the pivotal role played by NGF in pain perception. Human sensory and autonomic neuropathies (HSAN V), a rare disorder characterized by impaired temperature sensitivity, ulcers, and in some cases self-mutilation, is caused by a mutation in the coding region of the nerve growth factor beta (NGFB) gene (Einarsdottir et al, 2004). Similarly, HSAN IV an autosomal recessive hereditary disorder characterized by recurrent episodic fever, inability to sweat, self-mutilating behavior, mental retardation, and absence of reaction to noxious stimuli, is caused by the mutations, including frameshift, nonsense or missense variation found in either the extracellular binding domain or intracellular signal transduction domain of TrkA (Indo et al, 2001). While the developmental versus post-natal impact of NGF/TrkA mutation in these two disorders remain unclear, these disorders highlight the importance of proper NGF/TrkA function. NGF signaling, discussed below, provide insights into how it can modulate pain perception.

NGF Signaling & Pathways

p75NTR, the low affinity receptor for NTs, is a member of the tumor necrosis superfamily, and is often described as a cell-death receptor for its pro-apoptoic effect in the absence of Trk receptors (reviewed in Miller and Kaplan, 2001). p75NTR induced apoptosis is mediated by the activation of JNK pathway, ceramide production and association with cell death inducing proteins. However, there is a twist: in the presence of Trk receptors, p75NTR can interact with Trk receptors and cooperatively promote cell survival (Hamanoue et al, 1999). The p75NTR-Trk interaction can not only result in a high affinity binding receptor complex (Hempstead et al, 1991), but also can alter Trk ligand specificity (Davies et al 1993, Brennan et al, 1999). This complex interaction between p75NTR and Trk receptors is the reason why the function of p75NTR, despite being the first NT receptor to be discovered, remains unclear. However, several Trk-independent actions of p75NTR have been demonstrated. For example, in the absence of NTs, p75NTR interacts with RhoA, a member of Rho family proteins that control organization of actin cytoskeleton in many cell types. p75NTR-RhoA interaction leads to RhoA activation and inhibition of axonal growth. On the other hand, interaction of P75 with NTs causes p75NTR to dissociate from RhoA, and leads to RhoA inactivation and axonal growth (Yamashita et al, 1999). Preferential binding of proNTs to p75NTR adds another layer of complexity: although mature NTs stimulate neuronal survival, long term potentiation and synaptic strengthening, pro-Neurotrophins (proNTs)—the precursors of mature NTs—have been shown to induce apoptosis and long term depression (Pang et al, 2004, Woo et al, 2005).

Trk receptors, on the other hand, are prototypical receptor tyrosine kinases, and are thought to mediate the majority of NT classical actions. Activation of Trk-dependent pathways during early development blocks apoptosis and promotes cell survival and differentiation.

Furthermore, activation of Trk receptors can affect the sensitivity of nociceptor neurons; indeed, NGF-induced hyperalgesia seems to be mediated by the TrkA receptor (reviewed by Pezet and McMahon, 2006). For example, while p75NTR null mice have increased mechanical and thermal withdrawal thresholds, they do develop both heat and mechanical hyperalgesia after systemic injection of NGF. This suggests that NGF-induced hyperalgesia can occur in the absence of the p75NTR and that the TrkA receptor is sufficient to mediate the noxious action of NGF (Bergmann et al, 1998). The importance of NGF/TrkA signaling also has been demonstrated in an animal model of pancreatic pain (Winston et al, 2003). The authors showed that treatment with K252A, a TrkA inhibitor, reversed both the behavioral changes and the increase in CGRP and SP expression associated with pancreatitis. While these studies strongly indicate involvement of TrkA receptor, but not of p75NTR, in NGF-induced hyperalgesia, there is evidence that p75NTR-antibody prevents NGF-induced excitability in nociceptor culture (Zhang and Nicol, 2004), and p75NTR might have a role in some pain states (Peterson et al, 1998).

Trk receptors have an extracellular domain which contain two immunoglobulin-like domains and bind to NTs, a single transmembrane domain and a cytoplasmic region with a kinase domain (Kaplan et al, 1991). NTs, upon binding to Trk receptors, homodimerize, and stimulate autophosphorylation the receptors at multiple tyrosine residues. Two major autophophorylation sites of TrkA receptors are Tyr490 and Tyr785. The binding of specific effectors to these sites activates signaling cascades including Shc (Ras), FRS2 (Rap), phospholipase Cγ1 (PLCγ1) and phosphatidyl inositol-3 kinase (PI3K) pathways (reviewed by Sofroniew et al 2001, Huang and Reichardt, 2003; Figure 1).

Phosphorylation of Tyr490 initiates recruitment and phosphorylation of Shc, an adapter protein (Stephens et al, 1994). Shc recruitment and phosphorylation leads to recruitment of

adapter Grb-2, and Ras exchange factor, SOS. This leads to transient activation of Ras, and activates c-Raf/ERK, P38MAP Kinase and the PI3K pathway (discussed later). These signaling pathways mediate classical signaling actions of NGF. C-Raf/ERK pathway leads to sequential phosphorylation of MEK1/MEK2, and phosphorylation of Erk1 and Erk2 by MEK1, or MEK2. MEK1/MEK2 pathway can also be activated by Tyr490 phosphorylation through the binding and activation of FRS-2, and sequential activation of CrK, C3G, Rap-1, and B-raf (Kao et al, 2000). Activation of MEK results in phosphorylation of CRE-binding protein (CREB) and other transcription factors in the nucleus. It has been shown that CREB regulates genes that are essential for survival of sympathetic neurons *in vitro* (Riccio et al, 1999).Furthermore, MEK activation is essential for differentiation in PC12 cells since pharmacological inhibition of MEK has been shown to abolish neurite outgrowth in response to NGF (Pang et al, 1995).

As stated above, NGF can stimulate the PI3K pathway, which in many neurons is the most important pathway for NGF-induced cell survival. PI3K can be activated by both Ras-dependent and Ras-independent signaling after phosphorylation at Tyr490. The Ras-independent pathway involves three adapter proteins: Shc, Grb2, and Gab-1. Phosphorylated Grb-2 provides a docking site for Gab-1, which in turn is bound by PI3K (Holgado-Madruga et al, 1997). Activated PI3K generates phosphatidylinositides and activates PDK-1, which then activates Akt. Activated Akt phosphorylates many proteins important for survival, such as BAD, FKHR, YAP, MDM2, IKK, and GSK 3-β (reviewed in Downward, 2004).

When TrkA is phosphorylated on Tyr785, PLCγ1 is phosphorylated and activated. Activated PLCγ1 then hydrolyses PIP_2 to generate inositol tris-phosphate (IP_3) and diacylglycerol (DAG). IP_3 causes the release of Ca^{2+} from internal stores (Streb et al, 1983), and activates various enzymes such as Ca^{2+}-regulated isoforms of protein kinase C (PKC) and

calcium-calmodulin regulated protein kinases. DAG, on the other hand, stimulates DAG-regulated protein kinase C isoforms. In PC12 cells, PKC-δ, activated by NGF, can induce neurite outgrowth (Corbit et al, 1999).

In PC12 cells, phosphorylation on Tyr785, and subsequent binding of PLCγ1 is necessary for NGF-stimulated expression of genes including a sodium channel gene, PN1/Nav1.7 (Choi et al, 2001), implicated in pain transmission (see below). Interestingly, 2-minute stimulation of NGF was enough to cause PLC phosphorylation that remained at a high level even after an hour It is possible that sustained PLCγ1 signaling is responsible for this long term NGF-induced PN1/Nav1.7 expression. NGF can also increase expression of a number of genes, including VGF, in PC12 cells, through a transcriptional activation pathway mediated by a Ras→MAPK signaling cascade (D'Arcangelo and Halegoua, 1993, D'Arcangelo et al, 1996 Figure 2).

Retrograde Signaling By Neurotrophins

Although the NGF pathways emanating from the plasma membrane and leading to the nucleus are well delineated in cultured cells, *in vivo*, most NGF signals are initiated at the axon terminals. How do NGF signals then get transmitted to the cell body of a neuron that can be as far as a meter away? How, specifically, can NGF upregulate various genes from TrkA signaling generated at the nerve terminal? Elucidating the mechanism by which the NGF signal is transmitted retrogradely from the nerve terminal to the cell body has been an important focus of research in the neurotrophin field. Several models have been proposed to explain this mechanism including the retrograde effector model, the wave model, and the signaling endosome hypothesis (reviewed by Ginty and Segal, 2002).

The wave model suggests that binding and activation of TrkA by NGF at the distal axonal terminals leads to a wave of signaling that is propagated to the cell body. The retrograde effector model, on the other hand, suggests that binding and activation of TrkA by NGF activates signaling effectors that are then transported to the cell body. One key feature of both the wave model and the retrograde effector model is that retrograde signals transported to the cell body from the axon terminal are independent of NGF-TrkA transport. Support for both of these models is based on the evidence that covalently linked NGF, which renders NGF incapable of internalization, can support neuronal survival from the axonal terminal in compartmentalized chamber where neuronal cell body was biochemically separated from the axon terminals (MacInnis and Campenot, 2002). Furthermore, support for signaling effector model came from the demonstration that in compartmentalized chambers, inhibiting Trk kinase activity at the cell body by TrkA-antagonist K252A doesn't block retrograde survival signaling (Macinnis et al, 2003). However, in sympathetic neurons, Trk kinase activity in the cell body was found to be required for retrograde survival and blocking Trk endocytosis with a dominant negative dynamin (Ye et al, 2003) or dominant negative Pincher mutant (Valdez et al, 2005, discussed below) lead to cell death. These and other studies argue against both the wave model, and the retrograde effector model, and strongly supports the third model: the signaling endosome hypothesis (Ginty and Segal, 2002).

According to the signaling endosome model (Halegoua et al 1991, Mobley et al, 1996), NGF binds to its high affinity receptor TrkA at the nerve terminal, and activates it. The activated TrkA-NGF complex is then internalized and physically transported along the axonal microtubule network to the cell body where it can mediate its effects. Several lines of evidence suggest the signaling endosome model as being the dominant mechanism for retrograde signaling by NTs.

NGF itself has been shown to be retrogradely transported in sympathetic and sensory neurons, both *in vivo* and *in vitro*. Hendry et al., for example, had shown that injection of NGF, labeled with radioisotope Iodine (^{125}I), into the anterior chamber led to accumulation of radioactivity in the cell bodies of postganglionic superior cervical ganglion (Hendry et al, 1974). Later, other NTs, for example, BDNF and NT3, were also shown to be transported retrogradely along neuronal axons. ^{125}I-BDNF and ^{125}I-NT3 were found in neuronal cell bodies when the NTs were injected in the target fields (DiStefano et al, 1992). Similarly, retrograde transport of Trks has also been demonstrated. Phosphorylated TrkA, for example, accumulated in the rat sciatic nerve when the nerve was ligated, and this accumulation was enhanced, and abolished by NGF or anti-NGF administration, respectively (Ehlers et al, 1995). Downstream effectors such as PI3K, ERK, MEK were also shown to accumulate distal to the ligation site (Johanson et al, 1995). Co-precipitation studies have shown that TrkA and TrkB receptors are co-transported with NGF, or BDNF, respectively (Tsui-Pierchala and Ginty, 1999, Watson et al, 1999). Furthermore, after 18-24 hours of NGF injection into the footpad (the target field of lumbar 4-6 DRG neurons) of adult rats, Delcroix et al (2003) detected NGF, TrkA, and activated forms of Erk1/2 and p38 in endosomes in both axons and cell bodies of DRG neurons. Blocking endosomes with dominant negative Dynamin (Watson et al, 2001) and dominant negative Pincher (Valdez et al, 2005) prevented retrograde activation of Erk5. These findings indicate that NGF signaling is transmitted retrogradely through axonal transport of endosomes that contain NGF, TrkA, and activated signaling proteins.

Evidence for the signaling endosome model also came from the experiments that linked microtubule transport machinery to NT/Trk retrograde transport and signaling. Blocking microtubule based transport by colchicine treatment, Conner and Varon (1992) showed that NGF

immune-reactivity in the basal forebrain cholinergic neurons was greatly reduced. Light chain of Dynein was shown to bind Trk receptors (Yano et al, 2001), and inhibition of Dynein based transport blocked NT dependent retrograde survival signal (Heerssen et al, 2004). Though these experiments demonstrate the signaling endosome model, alternative mechanisms that don't require internalization and NT retrograde transport have been proposed (MacInnis and Campenot, 2002), and it is possible that multiple mechanisms can contribute to the transmission of retrograde NT signal.

Receptor Endocytosis Mechanisms:

Cells internalize materials by a variety of mechanisms that are collectively called endocytosis. Three major modes of endocytosis have been implicated in NT/Trk endocytosis: clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis.

Clathrin-mediated endocytosis involves formation of clathrin coated pits to internalize ligand-receptor complexes at the plasma membrane. This classic mode of receptor tyrosine kinase endocytosis has been demonstrated for epidermal growth factor receptor (EGFR) uptake (Beguinot et al, 1984). Binding of epidermal growth factor (EGF) to its receptor, EGFR leads to coordinated nucleation, and polymerization of clathrin by adapter protein like AP-2 and AP-180 into curved lattices at the plasma membrane. Dynamin, a large GTPase, mediates the fission of clathrin coated pints and release clathrin coated vesicles (CCVs) inside the cell. CCVs are then rapidly uncoated by auxilin and hsc70, and subsequently trafficked and processed through early endosomes for either degradation or recycling. The early endosomal protein Rab5 and its effector

EEA1 and Rabenosyn, the late endosomal protein Rab7, or the recycling Protein Rab11 are involved in the processing of CCVs (reviewed by Takei and Haucke, 2001).

Since internalization of EGFR is mediated by clathrin-dependent endocytosis, internalization of NT/Trk was thought to be mediated by this mechanism. Support for this suggestion came from experiments done by Grimes et al (1996): the authors showed that clathrin-coated vesicles, isolated by subcellular fractionation, contained NGF/TrkA complexes. In PC12 cells, Beattie et al (2000) showed that NGF treatment increased clathrin at the plasma membrane and enhanced clathrin-mediated membrane trafficking. Furthermore, treatment of DRG axon terminals with monodansylcadaverine (MDC), a transglutaminase inhibitor thought to specifically block clathrin-mediated endocytosis, prevented BDNF/TrkB internalization (Watson et al, 2001, Zheng et al, 2008).

However, there are caveats in the experiments described above. Later studies done by Grimes et al (1997) indicated that despite being derived from CCVs, NGF/TrkA endosomes could be small vesicles that are distinct from CCVs. Similarly, MDC is hardly a specific inhibitor for clathrin mediated endocytosis since MDC has been shown to interact with autophagic vacuole membrane lipids, (Niemann et al, 2000); also, the transglutaminases carry out several functions in the cell including cell adhesion (Menter et al, 1991) and hence they are not specific to clathrin-mediated endocytosis. Since the effect of specifically blocking clathrin on NT/Trk endocytosis has not been studied yet, these experiments need to be carried out to determine the role of clathrin on NT/Trk endocytosis. The observation that a dominant negative form of Dynamin blocks Trk endocytosis—often used to prove clathrin-mediated internalization of NT/Trk complexes—does not exclude other modes of internalization for NT/Trk endocytosis since dynamin is involved in clathrin-independent endocytosis as well (discussed below).

Furthermore, unlike clathrin-mediated endocytosis of EGFR which undergoes rapid degradation or recycling, retrogradely transported NT/Trk endosomes can signal for a prolonged period, and the temporal difference suggests that some means of subversion of the classical clathrin-mediated endocytosis pathway is likely to take place in NT/Trk internalization.

Caveolae-mediated endocytosis involves caveolin, a family of integral membrane proteins which acts as the principal components of caveolae membrane. Caveolae are flask-shaped invaginations that occur at cholesterol- and sphingolipids- rich plasma membrane microdomains (Reeves et al, 2012). The GTPase activity of dynamin is required for the budding of caveolae from purified endothelial plasma membranes (Oh et al, 1998), and internalization of caveolae is facilitated by disruption of the actin cytoskeleton (Reviewed by Nabi and Le, 2003). This mode of endocytosis is involved in cholesterol transport and homeostasis; endocytosis of some glycosylphosphatidylinositol-anchored proteins also seems to be mediated via this mechanism (Kurchalia and Parton, 1999).

Are NT/Trk complexes internalized by this mechanism? Support for this idea comes from several lines of evidence. TrkA and caveolin, for example, were shown to colocalize in caveolae microdomains (Huang et al, 1999). Isolated caveolae fractions contained active TrkA associated with Shc and PLCγ (Peiro et al, 2000), and caveolin coimmunoprecipitated with TrkA (Bilderback et al, 1999).

The studies described above were done in PC12 cells, and as some studies failed to detect caveolin mRNA in the brain (Oka et al, 1997), the role of caveolin in mediating NT/Trk endocytosis remains unclear. However, several studies did show that caveolin is expressed in the nervous system: caveolin expression was found in isolated hippocampal nerve terminals (Braun

and Madison, 2000), brain astrocytes (Cameron et al, 1997), and in DRG neurons (Galbiati et al, 1998). These conflicting results need to be resolved in order to link NT/Trk internalization with caveolin-mediated endocytosis. Moreover, experiments done by Sandvig et al (2008) suggest that the vast majority of caveolae remain surface-bound and tethered to the membrane and this argues against caveolin-mediated signaling endosomes since NT/Trk signaling endosomes need to be transported. While it's possible that caveolae could serve as a platform to initiate signaling from cell-surface or could act as a nucleating domain to concentrate cargo for other endocytic processes, the role of caveolin in mediating NT/Trk internalization remains controversial.

Macropinocytosis involves formation of macropinosomes, large heterogeneous vesicular structures that are originated by massive membrane ruffling and actin reorganization at the plasma membrane. Activity of the small GTPase, Rac, is required for this process. This mode of endocytosis is associated with not only nonspecific uptake of extracellular solutes and plasma membrane (Sawnson and Watts, 1995), but also in growth factor internalization. EGFR in fibroblasts, for example, has been shown to internalize via clathrin-independent, actin- and dynamin-dependent membrane ruffles. Interestingly, endocytosis of EGFR in these ruffles seems to be ligand-specific as other receptors weren't internalized in these ruffles (Orth et al, 2006). This suggests that macropinocytosis or a similar mechanism could mediate specific receptor endocytosis.

Indeed, several lines of evidence suggest that Trk internalization is mediated by a macropinocytic process. NGF treatment induces membrane ruffling both in PC12 cells and sympathetic neurons (Connolly et al, 1979, Connolly et al, 1981). In the axons of DRG neurons, Nakata et al (1998) found large, retrogradely moving, globular vesicles carrying TrkA-GFP together with dextran, a marker for macropinosomes. Furthermore, studies from our lab suggest

that endocytosis and retrograde transport of NT/Trk complexes is mediated by a novel, pinocytic mechanism called macroendocytosis (Shao et al, 2002, Valdez et al 2005, Valdez et al, 2007). Like macropinocytosis, this mechanism uses circular, dorsal membrane ruffling and actin reorganization, but is specifically receptor-mediated. Pincher, a member of ESP15 homology domain (EHD) protein family, and Rac, play crucial roles in this endocytosis mechanism. Moreover, endosomes formed by pincher-mediated endocytosis, unlike EGFR, are refractory to lysosomal degradation, and this could explain the prolonged signaling mediated by NT/Trk complexes (Philippidou et al, 2011).

EHD Proteins:

C-terminal Esp15 homology domain (EHD) proteins are members of the dynamin superfamily involved in endocytosis and endosomal trafficking (reviewed by Daumke et al, 2007). Like other members of the dynamin superfamily, these proteins have low affinity to nucleotides (low micromolar range), bind to negatively charged membranes, show lipid-induced hydrolysis of a nucleoside triphosphate (discussed below) on membrane binding, and oligomerize around lipid tubules in ring like structures.

Mammalian cells express four EHD isoforms, EHD1-4, and these isoforms are highly homologous. As expected from their homology, EHD proteins have a common motif: they have a nucleotide-binding region at the N-terminus, the EH domain at the C-terminus, and a coil-coiled region in-between (Naslavsky and Caplan, 2005; figure 3). The nucleotide binding-region binds to adenine nucleotides, and hydrolyses ATP, even though the region resembles the G-domain of dynamin that normally binds to GTP (Lee et al, 2005, Daumke et al, 2007).

Overexpression of hydrolysis-deficient mutant EHD proteins, like other members of dynamin superfamily, creates tube-like structure at the membrane. EHD proteins can oligomerize through their G domain and bind to other proteins through their EH domains.

These four members, despite their similarity, have distinct functions and subcellular distributions (George et al, 2007). EHD1 and EHD3 share the highest degree of homology, and are involved in the recycling of several receptors such as transferrin, major histocompatibility complex class I, and GLUT4. Moreover, these two members have been shown to interact with each other (Galperin, 2002). The second member of the family, EHD2, has the least structural similarity with the other members, and is involved in clathrin-mediated endocytosis of GLUT4 and transferrin (Grant and Caplan, 2008). EHD4/Pincher, on the other hand, seems to be involved in NT-induced Trk endocytosis (discussed later). Pincher has also been implicated in endocytosis of cell adhesion molecule L1/NgCAM where it forms hetero-oligomer with EHD1 (Yap et al, 2010). Moreover, Nogo-A, myelin-associated inhibitors for axonal growth, has been shown to be internalized by a Pincher- and rac-dependent, but clathrin-, and dynamin-independent mechanism (Joset et al, 2010).

Pincher may have a distinct role among the EHD proteins. Unlike other members of EHD family, Pincher was not detected in recycling endosomes in NIH 3T3 cell lines (Blume et al, 2007). Pincher-mediated macroendocytosis requires Rab5, a protein localized at the plasma membrane and early endosomes and functions as a key regulator of vesicular trafficking during early endocytosis (Philippidou et al, 2011). Pincher has also been shown to bind to Rab-5-binding partners Rabenosyn and Rabankyrin. These binding partners could coordinate Pincher- and Rab5-dependent endocytosis and may also play a role in Pincher-dependent Trk endocytosis (see below).

Pincher-mediated Trk macroendocytosis and retrograde transport:

Pincher was originally identified by our lab as an NGF-inducible RNA and protein in PC12 cells. Subsequently, Pincher was shown to be involved in NT endocytosis and retrograde transport. Trk/Pincher endosomes were shown to recruit Erks1/2 and Erk5 and signaled persistently during a 24 hour period (Shao et al, 2002). Overexpression of a dominant negative form of Pincher (Pincher G68E, figure 1) led to accumulation of activated TrkA and Erks on distinctive membrane regions, blocked TrkA internalization (Shao et al, 2002), and eliminated retrograde NGF signal-induced neuronal survival (Valdez et al, 2005). Recently, our lab has shown that Pincher-generated endosomes are refractory to lysosomal degradation, and this allows sustained signaling and neuronal gene expression in the cell bodies (Valdez et al, 2007; Philippidou et al, 2011). The evidence for this observation comes from the observation that Trk and EGFR endosomes are differentially processed: unlike Trk endosomes, EGFR endosomes rapidly turn into Rab7-positive multi vesicular bodies (MVBs) that fuse with cathepsin-containing, electron-dense lysosomes. Retrograde Trk endosomes, on the other hand, are Rab5-positive MVBs, and the exchange of Rab5 for Rab7 seems to be the rate-limiting step for their lysomal processing (Valdez et al, 2007; Philppidou et al, 2011). Thus, Pincher seems to process not only NGF/TrkA internalization, but also subsequently transported NGF-TrkA endosomes in a distinctive, clathrin-independent manner that may be shared by other retrograde signaling receptors (see above).

Gene Upregulation by Retrograde NGF Signaling:

In compartmentalized chambers, where neuronal cell bodies are separated from the distal axons by an impermeable barrier, Ricco et al. showed that NGF treatment of distal axons failed

to induce CREB phosphorylation at the cell body when it was cross-linked to bead, thereby rendering NGF incapable of internalization (Riccio et al, 1997). In a similar experimental set up, a dominant negative form of Dynamin was shown to prevent Erk5 phosphorylation in the cell body in response to NGF treatment of distal axons (Watson et al, 2001). In DRG neurons, Pazrya-Murphy et al (2009) showed that the transcription factor MEF2D and the anti-apoptotic Bcl-2 family member Bcl-w are regulated by target-derived NGF and BDNF—these genes were preferentially induced by neurotrophin stimulation of distal axons compared with neurotrophin stimulation of cell bodies. Our lab has also shown that retrograde NGF signal from distal axons can upregulate Erk kinase activities and VGF expression in Superior Cervical Ganglion (SCG) neurons (Philippidou et al, 2011). Taken together, these studies suggest that after NGF binds to TrkA receptor and activates it, an endosome containing NGF-TrkA is formed; the endosome is then physically transported along the axonal microtubule network to the cell body to modulate gene expression. Furthermore, studies from our lab suggest that Pincher plays a crucial role not only in the internalization, and formation of NGF-TrkA complex, but also renders the endosome refractory to lysosomal degradation so that signal can persist in the cell body.

Mechanisms of NGF-induced Hyperalgesia:

NGF can induce both acute and chronic hyperalgesia in animals: systemic application of a single dose of NGF (1mg/kg i.p) in rats, for example, produces thermal hyperalgesia that develops within minutes (acute hyperalgesia) and lasts for 4 days (chronic hyperalgesia) (Lewin et al. 1993). Acute hyperalgesia is thought to be independent of gene upregulation while chronic hyperalgesia is generally believed to involve gene upregulation in nociceptors and central sensitization by activation of NMDA receptors (discussed below, figure 4).

Acute hyperalgesia involves indirect sensitization of nociceptors via mast cell activation, and acute sensitization of nociceptors via TRPV1 sensitization. NGF can activate mast cells under conditions such as tissue injury and inflammation when the local NGF level is increased. Activated mast cells release other pain mediators such as 5-hydroxytryptamine (5-HT), prostaglandins, bradykinin, histamine, ATP, and H^+ (Kawamoto et al, 2002); release of these inflammatory mediators from mast cells contributes to the sensitization of nociceptors. Interestingly, activated mast cells also release NGF (Leon et al, 1994), and this positive-feedback loop most likely lead to acute nociceptor sensitization as well as gene upregulation (discussed below).

NGF can sensitize the nociceptors directly with a very short latency (within 10 minutes of NGF application), and this leads to acute thermal hyperalgesia. In dissociated DRG neuronal culture, NGF acutely conditions the cellular response (enhancement of inward current) to capsaicin (Shu and Mendell 2001), and noxious heat (Galoyan et al, 2003); accordingly, in rats, thermal hyperalgesia can be observed within 15 minutes of subcutaneous NGF injection into the paw (Andreev et al, 1995). The mechanism underlying this NGF-induced sensitization is mediated by sensitization of transient receptor potential vanilliod receptor 1 (TRPV1) cation channels since both heat and capsaicin signal via TRPV1 (Caterina et al, 1997). However, the signaling pathways behind NGF-induced TRPV1 sensitization remain controversial. Different studies, for example, have suggested involvement of Protein Kinase A (Shu and Mendell, 2001; Bonnington and McNaughton 2003), protein Kinase C (Vellani et al, 1999), PI3K and Erk (Zhuang et al. 2004), p38 (Ji et al, 2002), and PIP_2 mediated inhibition of TRPV1 (NGF-mediated breakdown of PIP_2 releases the channels from the inhibition) (Chuang et al. 2001). Interestingly, intrathecal administration of NGF (for three days, twice per day) did not increase

TRPV1 mRNA levels in DRG neurons; instead, increased expression of TRPV1 protein was observed (Ji et al, 2002). Similar results were found when inflammatory agents such as CFA and carrageenan were injected into the paw: while TRPV1 mRNA levels showed no upregulation, the expression of protein levels was increased (Ji et al, 2002, Tohda et al, 2001). These studies suggest that increased NGF levels—caused by either direct injection of NGF or by injection of inflammatory compounds—can lead to increased TRPV1 protein levels in a transcription independent manner, and this increased TRPV1 expression can lead to hyperalgesia.

While activation of TRPV1 channels can lead to acute thermal hyperalgesia, increased levels of NGF can lead to chronic hyperalgesia. Lewin et al (1994) showed that activation of N-methyl-D-Aspartate (NMDA) receptors in the dorsal horn plays an important role in mediating this chronic hyperalgesia as the non-competitive NMDA antagonist MK-801 blocked the late phase of hyperalgesia in NGF treated rats (7 hours to 4 days after NGF injection). Gene upregulation by retrograde NGF transport may also play an important role in generating and maintaining chronic hyperalgesia (discussed by McMahon, 1996). Besides TRPV1 as discussed earlier, several functionally important proteins including neurotransmitters, receptors and ion channels are also upregulated by NGF. For example, NGF upregulates both mRNA and protein levels of two neuropeptides that are normally expressed in TrkA expressing neurons: SP and CGRP (Pezet et al, 2001, Christensen and Hulsebosch, 1997). Similarly, expression of BDNF—another modulator of inflammatory and neuropathic pain—can be modulated by NGF. BDNF, for example, is normally expressed in only 10% of total DRG neurons, and NGF stimulation leads to BDNF expression in virtually all TrkA expressing DRG neurons (Priestley et al, 2002).VGF, a neuroendocrine specific gene widely expressed in peripheral neurons, is also upregulated by NGF in both PC12 cells (Levi et al, 1985; Salton SR, 1991, D'Arcangelo and

Halegoua, 1993) and in superior cervical ganglion (SCG) neurons (Philippidou et al, 2011). A recent study has shown that a VGF-derived peptide may be involved in inflammatory pain. This study focused on the C-terminal internal VGF-derived peptide, TLQP-21, and demonstrated that in mice, peripheral injection of TLQP-21 increased pain-related licking response in the second inflammatory phase of formalin administration (Rizzi et al, 2008). Several ion channels are also upregulated by NGF. For example, Acid-sensing Ion channels 3 (ASIC 3)—a class of sodium channels that are activated by protons in cardiac ischemic and inflammatory pain—are expressed in nociceptors, and are upregulated by NGF (Mamet et al, 2003). Tetrodotoxin (TTX)-sensitive PN1 or Nav1.7, a sodium channel expressed in peripheral neurons, can also be upregulated by NGF. In PC12 cells, NGF transcriptionally upregulated mRNA levels of this channel (Toledo-Aral et al, 1995) and subcutaneous paw injection of NGF increased the expression of this channel in DRG neurons that lasted for over a week (Gould III et al, 2000). Interestingly, mutation in SCN9A—the gene that encodes PN1/Nav1.7—leads to complete lack of pain sensation in affected humans (Cox et al, 2006, Goldberg et al, 2007). In mice, application of CFA leads to Nav1.7 upregulation and development of long-lasting hyperalgesia; this hyperalgesia can be prevented by expressing Nav1.7 RNAi (Yeomans et al., 2005). Accordingly, Nav1.7 conditional knockout mice show reduced pain sensitivity in CFA induced inflammation (Nassar et al, 2004). Taken together, these studies not only indicate that NGF modulates gene expression in nociceptors, but also suggests how these changes might lead to hyperalgesia or altered pain perception in adult animals as many of them are vital for either normal pain perception or could potentially result in prolonged hyperalgesia at increased expression levels.

Unlike NGF-induced acute peripheral sensitization of nociceptors that occurs via TRPV1 receptors, or mast cell activation, hyperalgesia caused by NGF-induced upregulation of a

particular protein is thought to take place with a delay (from hours to days). This delay would be expected because 1) NGF-TrkA complex—as suggested by signaling endosome hypothesis—needs to be retrogradely transported from the axon terminals to the cell bodies and 2) modulation of gene expression by retrograde NGF-TrkA signal (at the cell body) need to take place. Furthermore, since altered gene expression can lead to changes in nociceptor function (i.e. more sensitized nociceptors), hyperalgesia can last for a prolonged period. Conversely, while peripheral sensitization can occur rapidly leading to hyperalgesia, pain is less likely to last long: once the stimulus is removed, hyperalgesia should decrease since cell physiology is not altered (i.e, no change in gene expression). Peripheral sensitization, hence, should lead to acute hyperalgesia while upregulation of genes and/or NMDA activation should lead to increased pain sensitivity with a longer duration. Since upregulation of proteins such as sodium channels could make the cells more excitable for a prolonged period, upregulation of genes by retrograde NGF signaling may be one mechanism via which chronic hyperalgesia develops. NGF-stimulated upregulation of genes could lead to peripheral sensitization of nociceptors while NGF-stimulated upregulation of genes such as BDNF and VGF could activate NMDA receptors at the dorsal horn and lead to central sensitization of the nociceptors.

Several interesting questions emerge from the model discussed above. For example, if the central component of chronic hyperalgesia mediated by retrograde NGF signaling, what are the genes (if any) that are upregulated via retrograde signaling in nociceptors? What happens when retrograde NGF signaling is blocked? Is it possible, for example, to attenuate chronic hyperalgesia by blocking retrograde NGF signaling (to block central component) while retaining acute hyperalgesia? In my dissertation, I focused on two genes, Nav1.7 and VGF, not only to address those questions, but also to understand the role played by Pincher in mediating

retrograde NGF signaling in DRG neurons. Here I show that 1) NGF increases mRNA and protein levels of both Nav1.7 channel and neuropeptide VGF in DRG neurons, 2) retrograde NGF signaling can induce this upregulation both in in vitro and in vivo systems, 3) Pincher is a critical component of this retrograde signaling since a dominant negative form of Pincher (PincherG68E) abolishes this upregulation, and 4) NGF injection causes both mechanical and thermal hyperalgesia in mice infected with virus expressing YFP, however, mice infected with virus expressing PincherG68E don't show NGF-induced thermal hyperalgesia.

Figure 1: Schematic representation of major signaling cascades activated by NGF-TrkA. Three important signaling pathways are activated by NGF binding to TrkA in neurons: the phosphatidylinositol 3 kinase /Akt pathway, the extra-cellular signal regulated protein kinase pathway, and the phospholipase C pathway (modified from Pollack and Harper, 2002).

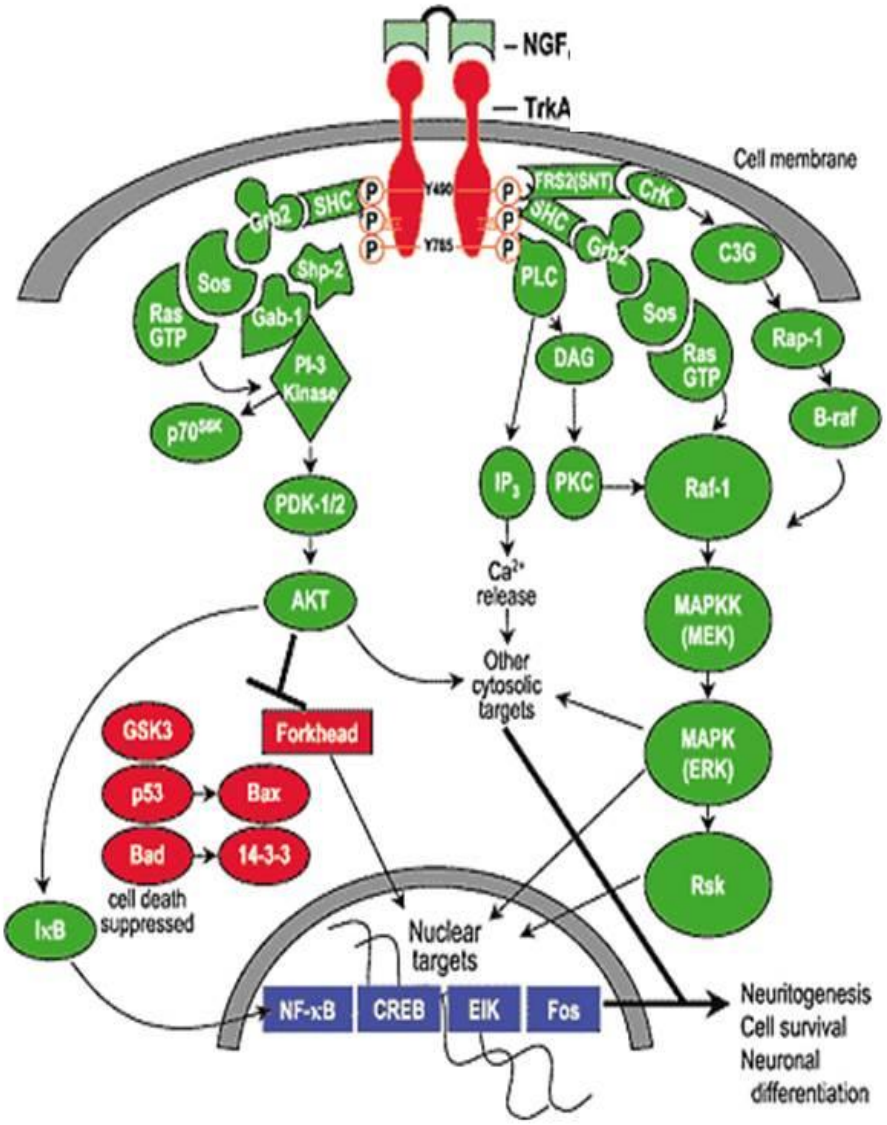

– NGF
— TrkA
Cell membrane
Grb2
SHC
Y490
Y785
P
FRS2(SNT)
CrK
C3G
Sos
Shp-2
Ras GTP
Gab-1
PI-3 Kinase
p70S6K
PLC
DAG
Rap-1
B-raf
PDK-1/2
IP3
PKC
Raf-1
AKT
Ca2+ release
MAPKK (MEK)
Other cytosolic targets
GSK3
Forkhead
p53
Bax
Bad
14-3-3
cell death suppressed
MAPK (ERK)
Rsk
IκB
Nuclear targets
NF-κB
CREB
EIK
Fos
Neuritogenesis
Cell survival
Neuronal differentiation

Figure 2: Model for NGF-TrkA gene induction in PC12 cells.

Model for NGF-induced gene induction of VGF and Nav1.7. Ras-Raf pathway is required for VGF mRNA upregulation upon NGF binding to TrkA. Induction of Nav1.7 mRNA, on the other hand, involves activation of PLCγ1 pathway (modified from D'Arcangelo and Halegoua, 1993)

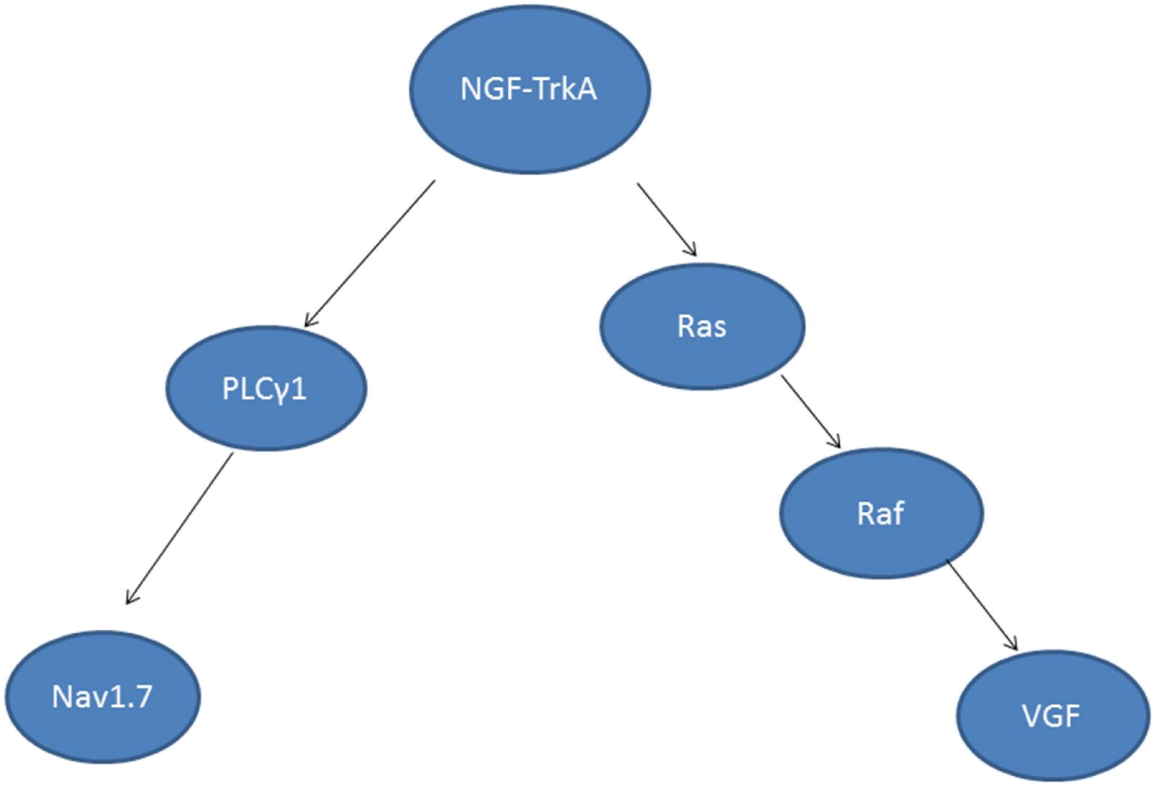

NGF-TrkA
PLCγ1
Ras
Raf
Nav1.7
VGF

Figure 3: Domain structure of Pincher protein

Pincher, similar to other EHD proteins, has an ATP-binding loop, coil-coil region and an EH domain. Mutation (depicted by an asterisc) at ATP binding loop creates a dominant negative form of Pincher, PincherG68E.

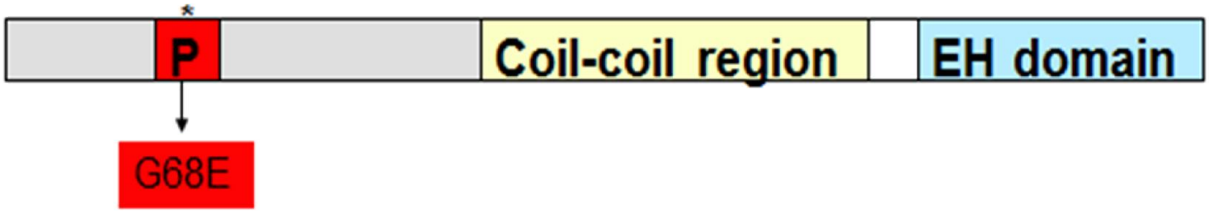
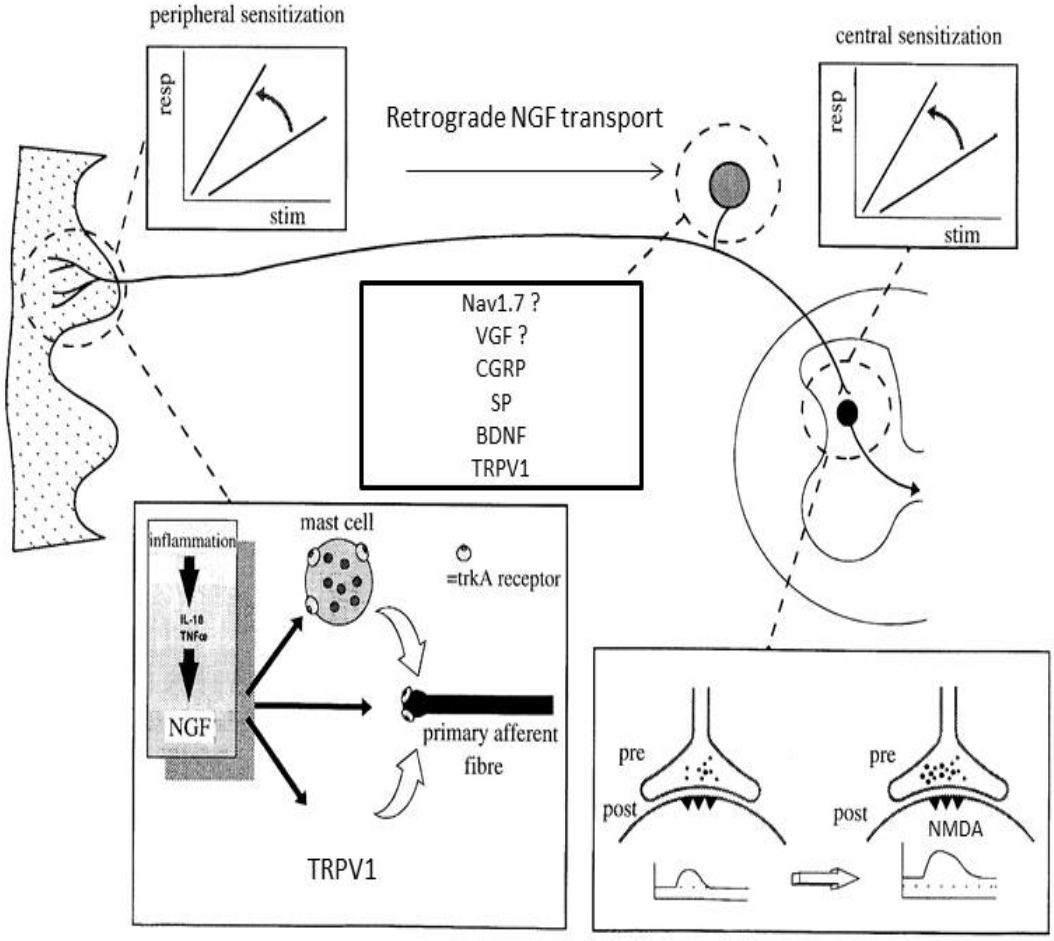

*
P
Coil-coil region
EH domain
G68E

Figure 4: Model for NGF-induced hyperalgesia

Pain can be mediated by increased expression and sensitization of TRPV1 channel while chronic hyperalgesia can be mediated by NGF-induced upregulation of several genes, as well as NMDA receptor activation at dorsal horn (modified from McMahon, 1996). Activation of NMDA receptor could facilitate synaptic transmission to the central nervous system whereas upregulation of ion channels could sensitize neurons to cause chronic hyperalgesia.


peripheral sensitization
resp
stim
Retrograde NGF transport
central sensitization
resp
stim
Nav1.7 ?
VGF ?
CGRP
SP
BDNF
TRPV1
inflammation
IL-1β
TNFα
NGF
mast cell
=trkA receptor
primary afferent
fibre
TRPV1
pre
post
pre
post
NMDA

Chapter II

Materials and Methods:

Animals

Pregnant (E15) Sprague Dawley rats and 4 week-old C57Bl/6 mice were ordered from Taconic. The animals were maintained in an air-conditioned environment on a 12-h light–dark schedule at 20–22°C and had free access to food and water until the experimental day. Pseudotyped (see below) lentiviral vectors with rabiesG coat protein carrying either GFP or Pincher-G68E were injected into the plantar surface of the right hind paw. After 12 days of infection, mice were injected with either NGF or PBS at the same spot, and behavioral experiments were done 24 hours after the NGF or PBS injection.

Cell Culture

Dorsal Root Ganglia (DRG) were collected from embryonic day 16 (E16) Sprague Dawley rats, or from eight week old adult mice. DRG neurons from E16 rats were dissociated and plated on Poly-L-Lysine-coated coverslips and maintained in Neurobasal Medium supplemented with B-27, L-Glutamine, Glucose, Fluorodeoxyuridine/Uridine and 100ng/ml NGF for 3 days before experiments. The cells were also grown in microfluidic devices coated with Poly-L-lysine (described below) to carry out retrograde assays.

Making Pseudotyped Lentiviral Vectors:

Pseudotyped lentiviral vector with RabiesG coat protein vector carrying PincherG68E was generated in the lab. HA-PicnherG68E DNA was subcloned into pHAGE-SYN-MVL under

synapsin promoter. 293T cells were transfected with HCMV.G (the RabiesG coat protein), gag/pol, rev, tat (kindly provided by Dr. Kevin Czaplinski), and pHASE-SYN-MVL-PincherG68E DNA. The cells then produced the desired pseudotyped virus and media from the culture was collected on day 2, day 3, day 4, and on day 5. On day 5, the collected media was filtered through .045µm filter and transferred to a sterile ultracentrifuge tube. Viral particles were pelleted by centrifugation (100,000g) for 1.5 hours at 4C. The supernatant was then poured off, and 100ul DMEM was added to the tube. The tube was incubated on ice for 2 hours, and viral particles were then carefully suspended in the media. The tube was then centrifuged for 10 minutes at 14000 rpm speed at 4C (to remove cell debris), and aliquot into 5ul samples. The viral particles were stored at -80C.

Western Blot Analysis

Proteins were extracted from DRG neurons using RIPA buffer (150mM Nacl, 10mM NaPhos, 2mM EDTA, 50mM NaF, 10mM Na pyrophosphate, 1mM Na3VO4 , 1% Triton X-100, .1% SDS, .5% Na deoxycholate), diluted in 2x sample buffer (2% SDS, 80mM Tris-HCl pH 6.8, 10% glycerol, 10% β-mercaptoethanl, 2ug/ml bromophenol blue) and analyzed by western blot. The following antibodies were used to probe western blots: rabbit anti-VGF (1:500, kind gift from Dr. Rock Levinson), mouse anti-Nav1.7 (1:100 LSBio), and GAPDH (1: 2000, Calbiochem). Anti-rabbit or anti-mouse Alexa680 secondary antibody was used (1:5000), and blots were developed using Odyssey Infrared Imaging System (Version 2.1, Licor). Quantitation of scanned images was done using ImageJ (NIH).

Immunocytochemistry:

Cells were fixed in 4% Paraformaldehyde solution for 15 minutes, and then washed three times with PBS (5 min, RT). After blockage of nonspecific binding sites (5.5% goat serum and .5% Triton-X) for an hour in room temperature, the cultures or tissue samples were probed with primary antibodies against VGF or Nav1.7, and GFP or HA-tag (the latter two antibodies were used in infected neuronal culture or DRG tissue samples from virus-injected mice) in the blocking solution overnight at 4C. After overnight incubation, the slides were washed 3 times in PBS at room temperature, and incubated with secondary antibodies in blocking solution for an hour at room temperature. After three 5 minute PBS final washes, the cultures were mounted with Vectashield H-1000 (Vector Labs) or Fluoromount-G containing DAPI (0.5 μg/ml) (for DRG tissues).

Microfluidic Devices:

Microfluidic devices were made by using soft lithography and replica molding (Taylor et al., 2005, Park et al, 2007). A master with positive relief patterns of cell culture compartments and microgrooves was made using photolithography to pattern two layers of negative photoresist, SU-8, on a silicon wafer. Each silicon wafer contained patterns for 9 microfluidic devices. Prepolymer mixture of Sylgard 184 (Dow Corning) was cast and cured against the positive relief master to create a negative replica molded piece. After curing, I punched out reservoirs using a biopsy punch and cut out individual devices. The devices were sterilized in 70% ethanol and then gently placed onto dried Poly-L-Lysine coated coverslips (100ug/ml). By maintaining a volume difference between the two compartments, a biochemical separation between cell body and distal axon terminals can be established in these devices.

Retrograde VGF and Nav1.7 Induction

After 3 days of plating DRG neurons in microfluidic devices, the cells were starved for 24 hours in the presence of anti-NGF (1:10000, Sigma) and MEK inhibitor U0126 (10uM), and then treated with 100ng/ml NGF with fluospheres 565/580 (1:1000, Invitrogen) for 16 hours at the distal axon compartment and stained for either VGF or Nav1.7.

Blocking Endocytosis

After 3 days of plating, pseudotyped lentiviral vectors carrying GFP or PincherG68E were added to the distal axon compartment and fluidic volume difference was maintained to create a biochemical barrier. 12 days after infection, the cells were starved for the cells were starved for 24 hours in the presence of anti-NGF (1:10000, Sigma) and MEK inhibitor U0126 (10uM), and then treated with 100ng/ml NGF for 16 hours at the distal axon compartment and stained for VGF or Nav1.7, and anti-HA.

In situ hybridization

3 days after plating the cells in microfluidic devices, the cells were starved for 24 hours in the presence of anti-NGF and MEK inhibitor U0126 and then treated with 100ng/ml for 16 hours at the distal axon compartment and then fixed for in situ hybridization. 1152bp Nav1.7 cDNA (Cooperman et al, 1987) and 2123bp VGF cDNA (D'Arcangelo and Halegoua, 1993) from SP64 vectors were cut with EcoRI and HindIII and the DNA fragments were inserted into pBluescript II SK vectors. The vectors were linearized by using SalI or HindIII restriction enzymes, and using T7 promoter forward primer TAATACGACTCACTATAGGG and T3 promoter forward

primer, GCAATTAACCCTCACTAAAGG sense or anti-sense probes were generated. Briefly, the cells were incubated in 0.1 mol/L triethanolamine-OH, pH 7.2, 0.15 mol/L NaCl (tetraethylammonium buffer) for 5 minutes at room temperature. The cells were then treated with acetic anhydride (0.25% in tetraethylammonium buffer) for 20 minutes at room temperature, and washed 3 times in DEPC containing PBS (5 minute for each wash). The cells were then incubated in 2× SSC (1× SSC contains 150 mmol/L NaCl and 15 mmol/L Na3 citrate, pH 7.0) for 10 minutes at room temperature, and then prehybridized with 50% deionized formamide, 4× SSC, 1× Denhardt's solution, 0.5 mg/mL salmon sperm DNA, 0.5 mg/mL yeast tRNA, and 10% dextran sulfate for an hour at 60C. Digoxigenin-labeled probe (100 ng/mL) was then used to probe mRNA at 60C overnight. The samples were then washed with 4× SSC for 10 minutes at room temperature, 1× SSC for 10 minutes at room temperature, 0.1× SSC at 60C for 30 minutes, and 0.1× SSC for 10 minutes at room temperature. Cells were then washed in 100 mmol/L Tris-HCl plus 150 mmol/L NaCl (pH 7.5) and incubated for 30 minutes in the same buffer containing 2% normal sheep serum and 0.3% Triton X-100. The cells were then incubated with Cy3 anti-digoxigenin antibody (1:500) for an hour at room temperature. After incubation, the reactions were stopped using 100 mmol/L Tris-HCl (pH 9.5), 100 mmol/L NaCl, and 50 mmol/L MgCl2. Immunohistochemistry was carried out on some of the cells after FISH.

Behavioral Analysis:

Mechanical withdrawal threshold in mice was measured with Von Frey hairs. The threshold was taken as the lowest force that elicited withdrawal response. Thermal paw withdrawal latency was measured using the Hargreaves heat apparatus and averaged over three trials.

Tissue Collection:

The mice were sacrificed after the behavioral experiment and L4-L6 DRG neurons from both ipsilateral (compared to injection side) and contralateral side were collected from them. A few thoracic DRG neurons were also collected. The neurons were incubated in 30% sucrose solution overnight, and then fixed in 4% paraformaldehyde for 2 hours. After fixation, the neurons were immerged in OCT freezing media and incubated at

Quantification and Statistics:

The images were captured with an Olympus confocal microscope, and fluorescence images were digitally processed first with Olympus Fluoview FV1000 software, and then using Adobe Photoshop. The quantification of signal intensity was done in ImageJ (NIH) software. Unless otherwise stated, a cell was counted as positive (a cell with elevated protein or mRNA (FISH) levels) if the cell's immunofluorescent signal intensity was above two standard deviations from the mean signal intensity from all the cells from a particular experiment. Unless otherwise indicated, statistical analyses on quantified experiments were carried out using Z Test for 2 Population Proportions and P value was calculated by Z value obtained from the Z score using GraphPad software.

Chapter III

Retrograde NGF signaling mediates upregulation of Nav1.7 and VGF in DRG neurons

Nerve growth factor (NGF) was originally discovered as a survival factor for DRG neurons during development (Levi-Montalcini, 1964). However, it is now clear that NGF has a wide repertoire of effects that span throughout the life of an animal. There is now, for example, considerable evidence that NGF acts as a peripheral pain mediator. Direct application of NGF elicits both thermal hyperalgesia and mechanical allodynia in rats (Lewin et al., 1994). Hyperalgesia and allodynia also develop in human subjects upon local NGF application (Petty et al., 1994). The NGF level also increases in several animal models such as complete Freund's adjuvant (Donnerer et al. 1992; Safieh-Garabedian et al. 1995) or subcutaneous carrageenin (Westkamp and Otten 1987; Otten 1991; Aloe et al. 1992) application. Administration of anti-NGF antibody (Woolf et al, 1994) or sequestration of endogenous NGF by TrkA-IgG (McMahon et al, 1995) prevents this increase and reduces inflammatory hyperalgesia. Taken together, these studies indicate that NGF plays a key role in the generation and potentiation of pain in adult animals.

Acute thermal hyperalgesia develops within 10 minutes of NGF application, and this acute pain is thought to be caused by sensitization of TRPV1 channels, a member of the transient potential family of receptors (Shu et al, 1999). In addition, NGF has been shown to upregulate several functionally important proteins including neurotransmitters, receptors and ion channels. For example, in PC12 cells, our lab has shown NGF can upregulate mRNA levels of the Nav1.7

sodium channel (Toledo-Aral et al, 1995). Phosphorylation on Tyr785 of TrkA, and subsequent binding and activation of PLCγ1 mediates NGF-induced Nav1.7 upregulation (Choi et al, 2001). The voltage-gated sodium channel, Nav1.7, is expressed in peripheral neurons and has been linked to pain pathways (Momin et al, 2008). Mutation in SCN9A, the gene that encodes this sodium channel PN1/Nav1.7, causes complete lack of pain sensation in humans (Cox et al, 2006; Goldberg et al, 2007). Knocking down Nav1.7 expression after CFA injection prevented development of hyperalgesia in C- and Aδ thermonociceptive tests (Yeomans et al, 2005). *In vivo*, subcutaneous paw injection of NGF led to hyperalgesia (lasting for up to 24 hours), and increased the expression of Nav1.7 in DRG neurons that lasted for over a week (Gould III et al, 2000).

Similarly, VGF, a neuroendocrine specific gene widely expressed in peripheral neurons, is also upregulated by NGF in both PC12 cells (Levi et al, 1985, Salton SR, 1999, D'Arcangelo and Halegoua, 1993) and in superior cervical ganglion (SCG) neurons (Philippidou et al, 2011). It has been shown that VGF or VGF-derived peptide may be involved in inflammatory pain (Reidl et al 2009; Rizzi et al, 2008). VGF gene induction is mediated via activation of Ras→MAPK signaling (D'Arcangelo and Halegoua, 1993, D'Arcangelo et al, 1996).

If NGF upregulates expression of genes such as SCN9A and vgf to cause hyperalgesia, how is the NGF signal conveyed to the cell body from the nerve terminal? According to the signaling endosome model, neurotrophin bound and activated Trk receptors are endocytosed and retrogradely transported to the cell body to convey the signal from the axon terminals. Retrograde NGF signaling has been suggested to upregulate VGF protein expression in SCG neurons ((Philippidou et al, 2011), however, whether or not NGF can retrogradely upregulate VGF expression in DRG neurons remains to be demonstrated. If NGF does indeed upregulate

mRNA/protein levels of VGF, does the mechanism involve retrograde NGF signaling? In rats, high dose of subcutaneous application of NGF has been shown to upregulate Nav1.7 protein expression in DRG neurons (Gould et al, 2000); however, it is unclear whether retrograde NGF signaling or indirect release of other factors from glial cells caused Nav1.7 upregulation. Since most non-neuronal cells were eliminated by FUDR treatment, cells grown in microfluidic devices enabled me to ask whether or not retrograde NGF signaling upregulates this gene.

Here I show that NGF application upregulates Nav1.7 and VGF expression in DRG neurons. Furthermore, by using microfluidic devices to biochemically separate cell bodies from the axon terminals, I show that retrograde NGF signal upregulates both of these genes: NGF stimulation at the axon terminals leads to upregulation of not only Nav1.7 and VGF mRNA levels, but also protein expression in DRG neurons.

Results:

NGF upregulates Nav1.7 and VGF protein levels in DRG neuron Culture.

To determine if application of NGF results in upregulation of Nav1.7 and VGF, I first performed western blot analysis of mass neuronal cultures, using antibodies directed against each protein. DRG neurons were collected from E16 rats and grown on Poly-L-Lysine coated coverslips in media containing NGF. FUDR/uridine was added to the media after 24 hours to kill the majority of the glial cells as 3-5 days of FUDR/uridine treatment eliminates 99% of the support cells (Lange et al, 2012). 3 days after plating the cells, the media was replaced with media containing anti-NGF, but no NGF. 24 hours later, the starvation media was removed, and the cells were treated with NGF (50ng/ml) for 16 hours. Artemin, a glial cell- derived neurotrophic factor (GDNF), which has been shown to sensitize nociceptors *in vitro* and

hyperalgesia *in vivo* (Malin et al, 2006), was also used to see if it could upregulate VGF and Nav1.7. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene, was used as loading control. As figure 1A shows, NGF, and Artemin each upregulates VGF protein levels, however, only NGF was able to upregulate both Nav1.7 and VGF proteins. Figure 1B shows the quantification of western blot analyses from 3 different experiments: NGF upregulates VGF expression by over 3 fold, whereas it upregulated Nav1.7 by almost 2 fold. Artemin also upregulated VGF expression (2.6 folds compared to control), but not Nav1.7 expression.

I then characterized the time-course of NGF-induced upregulation of VGF by immunohistochemistry. After 3 days of plating, cells were starved for NGF (NGF was removed from the media and anti-NGF antibody was added) for 24 hours. After 24 hours, the media containing anti-NGF was removed and the cells were treated with 50ng/ml NGF. As figure 1C shows, VGF protein level is upregulated after 6, 8, or 24 hours of NGF treatment, though the expression level decreases by 24 hour.

Developing Microfluidic Devices to assess the role of retrograde NGF signaling.

To determine whether the upregulation of Nav1.7 and VGF expression can be mediated by retrograde NGF signaling, I used microfluidic devices developed by Taylor et al (2005). These devices can be placed on Poly-L-Lysine coated coverslips with slight pressure, and no adhesive compound is required to attach the devices onto the coverslips. Compared to traditional Campenot chambers (Campenot, 1977) that use silicone grease to biochemically separate cell bodies from the axonal compartment, these microfluidic devices offer three advantages: 1) the neurons send axons faster through the microgrooves 2) they are less leaky, 3) since there is no grease to deal with, the system is less messy (Taylor et al, 2005, Park et al., 2006 and see below).

I developed these chambers in PDMS using light lithography and replica molding. First, I created two master masks on photosensitive Cr plates using Heidelberg Instruments DWL 2000 at Cornell Nano Facility: one mask contained the design of channels (figure 2A), and the other mask (figure 2B) contained the pattern of micro-grooves (300um) between two channels. Each mask contained replica for 9 microfluidic devices. Using these masks, I used photolithography to pattern two layers of negative photoresist, SU-8, on a silicon wafer. ABM Contact Aligner was used for this process. These two layers of photoresist resulted in a master replica with positive relief patterns of cell culture compartments and microgrooves (figure 2C). I then used prepolymer mixture of Sylguard 184 (Dow Corning) against the master replica to obtain a negative replica-molded piece (figure 2D). After curing, I peeled the PDMS away from the master and punched out reservoirs with 8mm biopsy punches (figure 2E), and cut into individual microfluidic chambers. Before using these chambers, I sterilized the PDMS pieces by immersing them in 70% ethanol for 30 minutes, and then washed them with sterilized water. Once dried, I could snap these devices on to coverslips coated with 100ug/ml Poly-L-lysine.

These devices consist of a molded elastomeric polymer device placed against glass coverslip. The device has four reservoirs and each two of them are connected by a channel (two channels in total). These two channels are connected by microgrooves (Figure 2F-G). Neurons are plated in one of these channels, and within a day of plating, the neurons send their axons to the other channel via these microgrooves. This platform allows the fluidic isolation of axonal microenvironments by maintaining a volume difference between the two compartments. The high fluidic resistance of the microgrooves produces a sustained flow between the compartments that counteracts diffusion between the two channels and thus, cell bodies can be biochemically separated from the axonal compartment.

I tested the biochemical separation between axonal and cell body compartment by adding dye in one compartment (lower liquid volume) and PBS in the other compartment (higher liquid volume). The volume difference was maintained for 24 hours. Figure 3C shows a photograph of such a microfluidic device. As the picture shows, maintaining volume difference between two compartments can lead to biochemical separation between the two since the dye did not enter the other compartment. Occasionally, a device would leak (about 3% of the number of devices I used), and this was evident by media coming out of the chambers. In all of those devices that leaked, the leak was due to either dislocation of the devices from coverslips, or improper placement of the devices onto coverslips, or debris. These devices weren't used for any retrograde assay.

Though the mechanism is not well understood, fluorescent latex microspheres (fluospheres) have been previously used as a retrograde tracer (Persson and Gatzinsky, 1993), and here, I used fluospheres 565/580 (Invitrogen) to mark the projection neurons: fluospheres were added to distal axon compartments and a biochemical barrier was established for 16 hours. Within 3 days of plating the cells, 70-80% of neurons would send their projections through the microgrooves as seen by the presence of the microspheres in the cell body. Since these numbers reflect the number of cells that took up fluospheres in the cells body, it's possible that even greater percentage of cells had sent out their projections but did not take up fluospheres.

In order to further test if the microfluidic devices create a biochemical barrier between the axonal compartment and cell bodies, I added modified Herpes Simplex virus expressing GFP to the axonal compartment (Halterman et al, 2006) while lowering the liquid volume in that compartment. This difference in volume between two compartments of the microfluidic produces a sustained flow between the compartments that counteracts diffusion between the two channels

and thus if virus (or other chemicals such as NGF or fluospheres) is added to the compartment with lower liquid volume, it cannot reach the other compartment simply via diffusion: the virus or reagents added to the axonal compartment can reach the cell body only via retrograde axonal transport in this system. Since HSV virus can retrogradely infect cells (Antinone and Smith, 2010), this allowed me to retrogradely label cells grown in these chambers.

After 5 days of infection, I added fluospheres to the same compartment (with lower volume) and maintained the volume difference between the compartments for 16 hours. After 16 hours, the elastomeric polymer device was removed, and the cells were stained for VGF (to visualize all the cells), and GFP (to visualize virus-infected, GFP expressing neurons). Figure 3A is a representative confocal image from the experiment; green arrow depicts a cells that shows GFP expression and had taken up fluospheres, red arrows, on the other hand, depict two cells that had taken up fluospheres, but did not express GFP. As figure 3B shows, out of 73 cells, only 15% of the cells expressed GFP after 5 days of HSV-GFP expression (n=11), however 70% of the cells had taken up fluospheres (n=51). In the absence of the biochemical barrier, all of the cells would have taken up fluospheres. Furthermore, all the cells that expressed GFP also had fluospheres in the cell body. This suggests that while not all the projecting cells expressed GFP, the ones that did express GFP definitely had projecting axons as confirmed by their uptake of fluospheres.

The number of infected cells were low here most likely due to properties of DRG neurons. The number of retrograde infected cells increased considerably when I let the cells express desired protein for 12 days. About 30% cells showed levels of expression when the cells were analyzed 12 days after the initial infection.

NGF retrogradely upregulate VGF protein levels.

To determine whether retrograde NGF signaling upregulates VGF expression, after 3 days of plating the DRG neurons in microfluidic devices, I replaced the media with media containing anti-NGF and MEK inhibitor U0216 in the absence of NGF. This modified paradigm from earlier experiments—that is treatment with both anti-NGF and MEK inhibitor U0216 instead of using only anti-NGF in the starvation media—was necessary because the basal VGF expression, even after starving the cells for 24 hours in anti-NGF media, was too high to faithfully detect NGF's influence on VGF expression (Figure 4A). It's most likely that even though NGF was removed from the media during starvation, the Trk-NGF signaling endosome located within the axons could continue signaling, Since NGF-induced VGF transcription is mediated by Ras-MEK pathway (D'Arcangelo and Halegoua, 1993), I used MEK inhibitor (U0126) to block the residual signaling that could lead to the high background.

Indeed, MEK inhibitor with anti-NGF reduced the basal VGF expression within 24 hours, and allowed me to determine the influence of NGF on VGF expression. After 24 hours, starvation media was washed off, and NGF was added to the axonal compartment. The volume of axonal compartment was reduced to maintain a biochemical barrier between axonal and cell body compartments: NGF added to the axonal compartment, hence, could modulate gene expression in the cell body only if it has been retrogradely transported from the axonal terminal to the cell body. To mark the projecting cells, I also added fluospheres to the axonal compartment. Only the cells that had taken up fluospheres were counted for analysis because presence of fluospheres in the cell body meant that the fluospheres were taken up from the nerve terminal and retrogradely transported to the cell body. In control devices, I only added fluospheres (no NGF) in the axonal compartment. After 16 hours of NGF treatment, the cells

were stained with VGF antibody for confocal imaging. Figure 4A shows a representative image of the experiments. In order to quantify the percentage of cells that showed elevated VGF levels, I first determined baseline VGF signal intensity by using ImageJ. Once more, only the cells that had taken up the fluospheres were used to determine the baseline. Elevated VGF level was defined by the following criteria: if a cell had taken up the fluospheres, and had the signal intensity two standard deviations above the baseline, the cell counted as showing elevated VGF expression. As figure 4B shows by this measure, compared to control, NGF stimulated VGF expression by about 3 fold (total control n=154; NGF n=159, from 3 separate experiments).

Retrograde NGF signaling upregulates both VGF and Nav1.7 mRNA levels.

I then determined whether mRNA levels of Nav1.7 and VGF levels are increased by retrograde NGF signaling. Cells were treated in media containing anti-NGF antibody, and MEK inhibitor (U0126) for 24 hours before NGF stimulation at the axon terminals. The distal axon terminals were treated with NGF (100ng/ml), and then fixed for in situ hybridization to detect mRNA levels, and immunocytochemistry to detect protein levels, respectively. Probes for in situ hybridization were generated by first isolating Nav1.7 (Cooperman, et al. 1987) and VGF cDNA (D'Arcangelo and Halegoua, 1993) and inserted into pBluescript II SK vectors. T7 and T3 dual promoter system of the vector allowed me generate both sense and antisense probes from the same DNA respectively. Sense probes were used as controls to test whether in situ hybridization was working or not. Figure 5A shows representative images of VGF and Nav1.7 in situ hybridization. Baseline signal intensity was established by measuring the signal intensity of either VGF or Nav1.7 from all cells, and then averaging the value. Cells with signal intensity higher than 2 standard deviation from the baseline were counted as cells with elevated VGF or Nav1.7 mRNA levels. As shown in figure 5B, compared to control, cells treated with NGF

showed elevated mRNA levels of VGF and Nav1.7 by almost four fold. For VGF in situ hybridization, a total of 343 cells were counted (control n=178, NGF treated n=167), and for Nav1.7 in situ hybridization, a total of 314 cells were counted (control n=174, NGF treated n=140)

Retrograde NGF signaling can stimulate VGF and Nav1.7 protein levels in the same cell.

Since DRG neurons are not homogeneous (some are positive for IB4 and some are SP positive, for example), does retrograde NGF signal translate into different outcomes in these cells? To answer this question, I determined whether the same cell that showed elevated VGF protein level also showed elevated Nav1.7 protein levels by immunocytochemistry. Figure 6A shows representative confocal images. Basal fluorescent level for both VGF and Nav1.7 signals were determined by averaging signal intensity from confocal images, and elevated VGF and Nav1.7 levels were defined by the following criterion: if a cell's signal intensity was two standard deviations above the baseline, the cell was counted as showing elevated response. The Van diagram in figure 6B shows the results: cells with increased VGF expression usually showed increased Nav1.7 expression levels levels: 50% cells with elevated VGF expression also showed elevated Nav1.7 mRNA and 88% cells with elevated Nav1.7 expression also showed increased VGF expression.

Retrograde NGF signaling can stimulate VGF protein expression and Nav1.7 mRNA levels in the same cell.

I also determined whether the same cells that showed elevated VGF protein levels also showed elevated Nav1.7 mRNA levels. I measured VGF expression using immunocytochemistry

and Nav1.7 mRNA levels using Fluorescent In Situ Hybridization (FISH). Figure 7A shows a representative image of the result. Basal fluorescent level for both VGF and Nav1.7 signals were determined by averaging signal intensity from confocal images, and elevated VGF and Nav1.7 levels were defined by the following criterion: if a cell's signal intensity was two standard deviations above the baseline, the cell was counted as showing elevated response. The result is shown in figure 7B (control n=46; NGF treated n=46). Cells with increased VGF expression usually showed increased Nav1.7 mRNA levels: 55% cells with elevated VGF expression also showed elevated Nav1.7 mRNA and 77% cells with elevated Nav1.7 mRNA also showed increased VGF expression.

Discussion:

Here I show that NGF can upregulate VGF and Nav1.7 expression in DRG neurons, and this upregulation can be mediated via retrograde NGF signaling. First, in mass culture, I treated DRG neurons with NGF in the media and then looked at protein levels by western blot. The upregulation of VGF expression in the presence of NGF, furthermore, was demonstrated by immunohistochemistry. Second, to mimic the physiological condition where DRG neuronal cell bodies are separated from their axonal terminals, I used microfluidic devices, and asked whether or not retrograde NGF signaling modulates VGF and Nav1.7 expression.

In compartmentalized chambers where cell bodies were biochemically separated from the axonal terminal, Pazrya-Murphy et al (2009) showed that transcription factor MEF2D and the anti-apoptotic Bcl-2 family member Bcl-w are regulated by target-derived NGF and BDNF. Interestingly, these genes were preferentially induced by neurotrophin stimulation of distal axons compared with neurotrophin stimulation of cell bodies. Our lab has also shown that retrograde

NGF signal from distal axons can upregulate VGF expression in Superior Cervical Ganglion (SCG) neurons (Philippidou et al, 2011). Here I show that retrograde NGF signaling can upregulate VGF expression in DRG neurons as well. Since internalization of NGF-TrkA receptor, and formation of NGF-TrkA signaling endosomes are necessary for conveying NGF signal from the nerve terminal to the cell body (Riccio et al, 1997, Watson et al, 2001), it is most likely that retrogradely trafficked NGF-TrkA endosomes caused VGF upregulation. Furthermore, my data show that VGF mRNA levels are increased by retrograde NGF signaling. This observation has physiological consequences since VGF has recently been linked to pain phenotype in mice. It is possible that increased VGF expression we demonstrated in vitro could have physical consequences i.e. increased hyperalgesia in vivo.

Another crucial aspect of our result relates to Nav1.7. Since sodium channels play a vital role in signal transmission in neurons, any change in the number of functional channels at the synapse can lead to direct physiological response. In PC12 cells, our lab had previously demonstrated that NGF upregulates the mRNA levels of Nav1.7. Here I show that NGF can upregulate Nav1.7 protein levels in DRG neuron mass cultures. Even though it has been shown that NGF injection into the paw led to upregulation of Nav1.7 in DRG neurons (Gould et al, 2000), the result was unclear as to if retrograde NGF signaling from the paw, or some other factors released by glial cells lead to the upregulation. Since my neuronal cultures were mostly free from non-neuronal cells, cells grown in microfluidic devices allowed me to address the question directly, and I show here that retrograde NGF signal can upregulate both Nav1.7 mRNA and protein levels.

I also show that retrograde NGF signal can upregulate not only mRNA or protein levels of VGF or Nav1.7, but can also upregulate both protein and mRNA levels of the two gene in the

same cells. The upregulation of mRNA suggests that retrograde NGF signal was capable of modulating transcription for both VGF and Nav1.7 mRNA in a heterogeneous DRG neuronal culture. My data also suggests that upregulation of mRNA/protein level of Nav1.7 and VGF aren't mutually exclusive: upregulation of Nav1.7, for example, doesn't exclude the upregulation of mRNA/protein level of VGF, and vice versa. However, there is a discrepancy: as described in the result section, 50% cells with increased VGF protein levels showed increased Nav1.7 expression, whereas 88% of cells with increased Nav1.7 levels showed elevated VGF expression. Similar results were observed when I looked at the VGF protein levels and Nav1.7 mRNA levels in the same cell: 55% cells with elevated VGF expression showed elevated Nav1.7 mRNA levels and 77% cells with elevated Nav1.7 mRNA showed increased VGF expression. Where does this discrepancy come from?

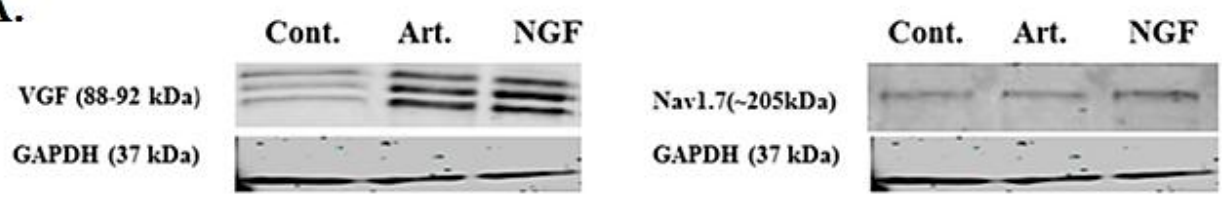
One possibility is that the discrepancy comes from the limitation of my detection system: it's possible that I failed to identify some cells because our detection system wasn't sensitive enough. It will be interesting to see in the future whether the same NGF can upregulate both Nav1.7 and VGF protein/mRNA levels in all neurons by using a different/more sensitive detection system. Another possibility is that the discrepancy results from two diverse signaling cascades that emanates from activated TrkA receptors. For example, in PC12 cells, our lab has shown that phosphorylation on Tyr785, and subsequent binding of PLCγ1 is necessary for NGF-induced Nav1.7 upregulation (Choi et al, 2001) and activation of Ras->MAPK signaling is involved in VGF upregulation (D'Arcangelo and Halegoua, 1993). Furthermore, it appears that NGF could regulate transcription of these genes via two different promoter systems. SCN9A promoter, located about 64,000 nucleotides upstream of the ATG translation start site, has a high degree of sequence conservation between human and mouse. The promoter has an extensive

CpG island around the transcription initiation sites as well as numerous GC boxes that could serve as binding transcription factor. Diss et al (2007) showed that NGF treatment not only upregulated Nav1.7 mRNA levels, but also upregulated the luciferase activity of promoter-luciferase construct. The authors also suggested Brn-3a, and egr1 as possible transcription factors for SCN9A, but their results did not address the question directly. Transcriptional control of VGF by NGF, on the other hand, is better understood. Our lab has shown that at least two promoter elements CRE, and CCAAT are required for VGF gene induction; furthermore, a third promoter element, G(S)G element, located between the TATA box and transcriptional start site that binds the NGF- and Ras-induced transcription factor, NGFI-A, has been shown to amplify transcriptional response (D'Arcangelo et al, 1996). It is possible that the discrepancy I observed here is due to two divergent signaling cascades that lead to two divergent transcriptional regulations.

Figure 1: NGF upregulates Nav1.7 and VGF protein expression in mass cultures.

1A(Left): Western blot image showing VGF protein expression in the presence or absence of NGF and Artemin. 1A(right): Western blot image showing Nav1.7 protein expression in the presence of absence of NGF and artemin. 1B: Quantification of the protein expression levels from 3 different western blot experiments.

1A.


Cont.
Art.
NGF
VGF (88-92 kDa)
GAPDH (37 kDa)
Cont.
Art.
NGF
Nav1.7(~205kDa)
GAPDH (37 kDa)

1B.

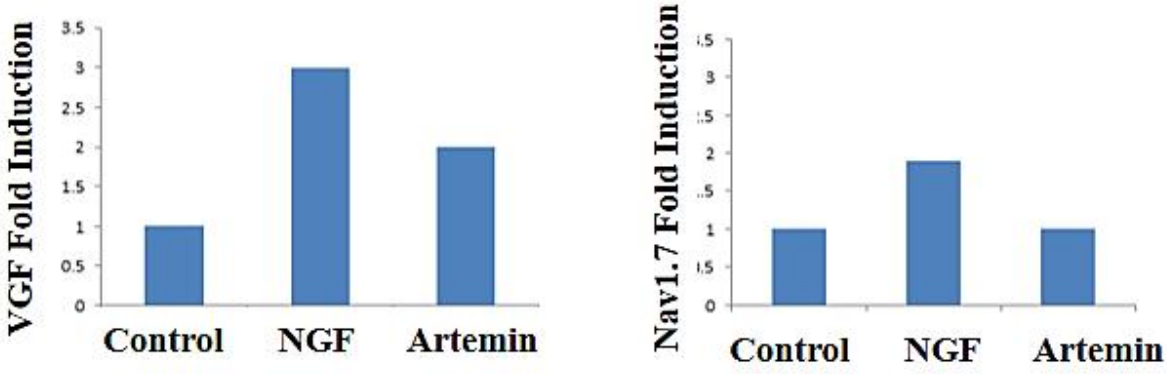

VGF Fold Induction
3.5
3
2.5
2
1.5
1
0.5
0
Control
NGF
Artemin
Nav1.7 Fold Induction
Control
NGF
Artemin

Figure 2: Developing microfluidic devices.

2A: Photo of master mask with microgrooves. 2B: Photo of second master mask containing chamber replica. 2C: 5-inch wafer (master mold) with showing molds for 9 microfluidic devices. 2D: Peeled PDMS containing replica of 9 microfluidic devices obtained from master mold. 2E. Photo of a single microfluidic device with punched reservoirs. 2F: Diagram of a single microfluidic device with two channels (black and red), and four reservoirs. Photo at the bottom is from the side view of the device (Taylor et al, 2005). 2G: Top-down view of a microfluidic device showing cell bodies in one channel and their projections through the microgrooves to the other channel.

2A.

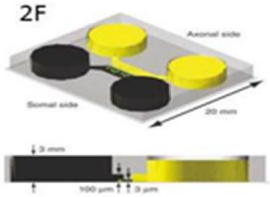
2B.

2C.

2D.

2E.

2F.



2G.

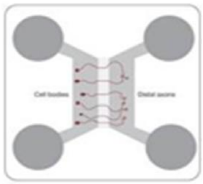
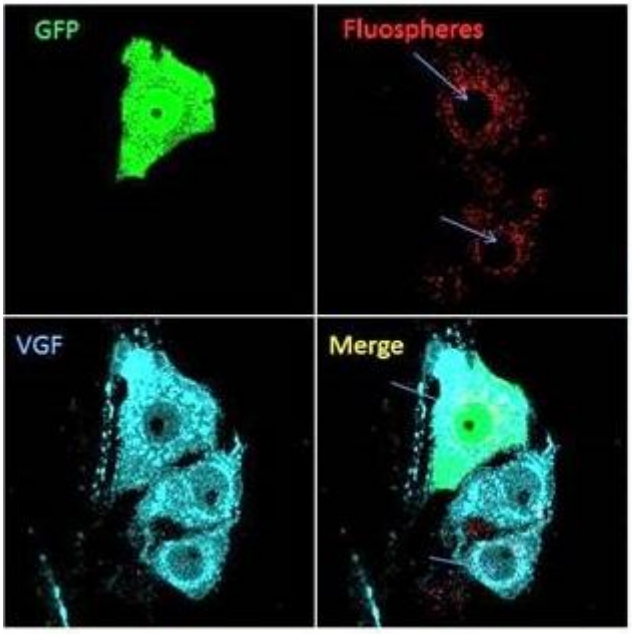


Figure 3: Microfluidic devices biochemically separate cell bodies from the axonal compartment.

3A: Loading dye was added to one channel and PBS was added to the other. Difference in volume was maintained to create a biochemical barrier—volume of dye was lower than the PBS channel. The picture, taken after 24 hours of maintaining the biochemical barrier, shows no diffusion of the dye to the PBS compartment. **3B:** Confocal images showing cell bodies expressing GFP, and fluospheres. The virus and the fluospheres were added to the axonal compartment and volume difference between two channels was maintained to biochemically isolate cell bodies from the axonal compartment. **3C:** Quantification of the cells expressing GFP, and/or containing fluospheres compared to total number of cells counted.

3A

3B


GFP
Fluospheres
VGF
Merge

3C

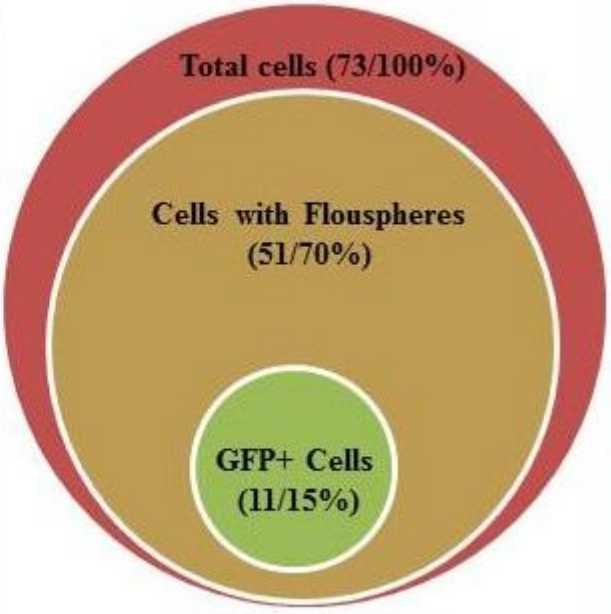
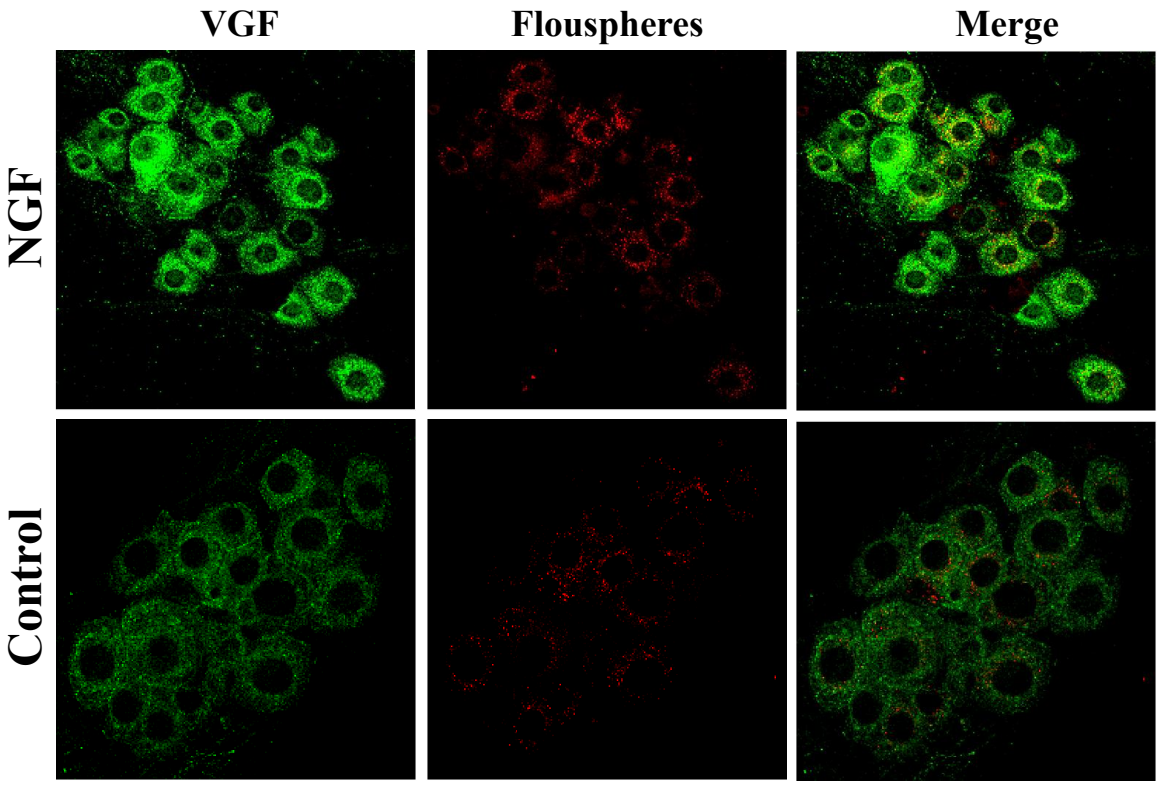

Total cells (73/100%)
Cells with Flouspheres
(51/70%)
GFP+ Cells
(11/15%)

Figure 4: NGF retrogradely upregulate VGF protein level.

4A (left): Confocal images of control cells that were not treated with NGF after starvation (red: fluospheres, green: VGF). Cells were starved in NGF-free media containing anti-NGF, and MEK-inhibitor U0126 for 24 hours. 4A (right): Confocal images of the cells that were treated with 100ng/ml (for 16 hours) NGF 24 hours after starvation protocol. NGF was added to the axonal compartment and biochemical separation was established. Flouspheres were added to the axonal compartments in both the control and experimental chambers to mark the projecting cells. Only cells with fluospheres were counted for the analysis. **4B:** Quantification of the cells showing elevated VGF expression from 3 different experiments.

4A:

:


VGF
Flouspheres
Merge
NGF
Control

4B:

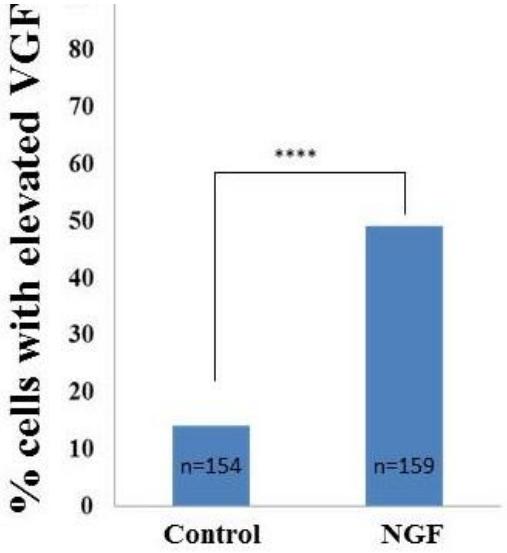
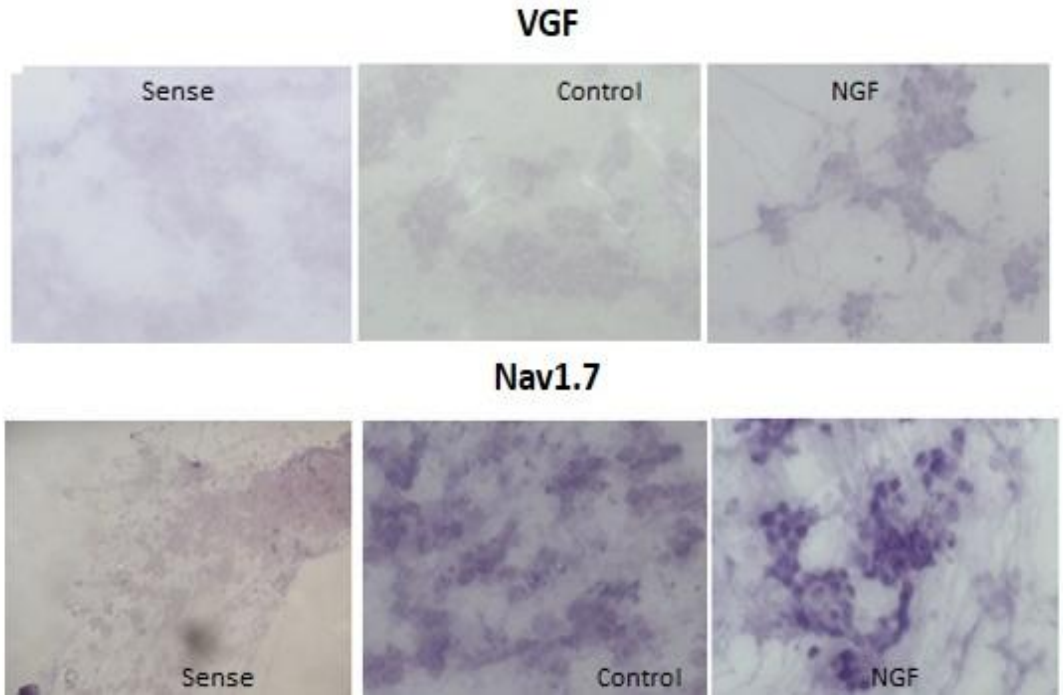

% cells with elevated VGF
80
70
60
50
40
30
20
10
0
****
n=154
n=159
Control
NGF

Figure 5: Retrograde NGF signal upregulates both VGF and Nav1.7 mRNA levels.

5A (Top left): Images of cells probed with VGF sense probe. Top middle: control cells that were starved for 24 hours in NGF-free medium with MEK inhibitor U0126, and anti-NGF antibody. and no NGF was added to the axonal compartment after the starvation. The cells were probed with VGF anti-sense probe. Top right: cells treated with NGF 24 hours after starvation, and probed with VGF anti-sense probed. NGF was added to the axonal compartment 16 hours and biochemical separation was maintained during the course of the experiment. Bottom left: Images of cells probed with Nav1.7 sense probe. Bottom middle: control cells that were starved for 24 hours and weren't treated with NGF after starvation. The cells were probed with Nav1.7 anti-sense probe. Bottom right: cells treated with NGF 24 hours after starvation, and probed with Nav1.7 anti-sense probe. NGF was added to the axonal compartment for 16 hours and biochemical separation was maintained during the course of the experiment. 5B:Quantification of cells showing elevated VGF mRNA levels compared to control (left), and quantification of cells showing elevated Nav1.7 mRNA levels compared to control (right)

5A:


VGF
Sense
Control
NGF
Nav1.7
Sense
Control
NGF

5B

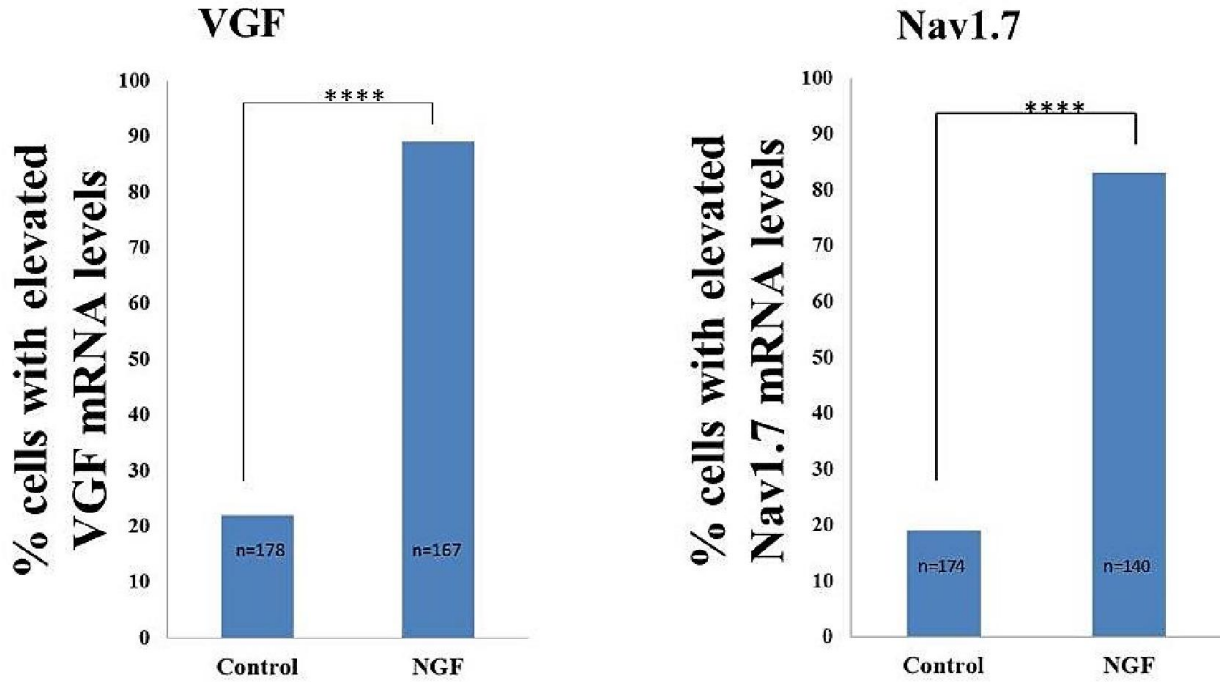
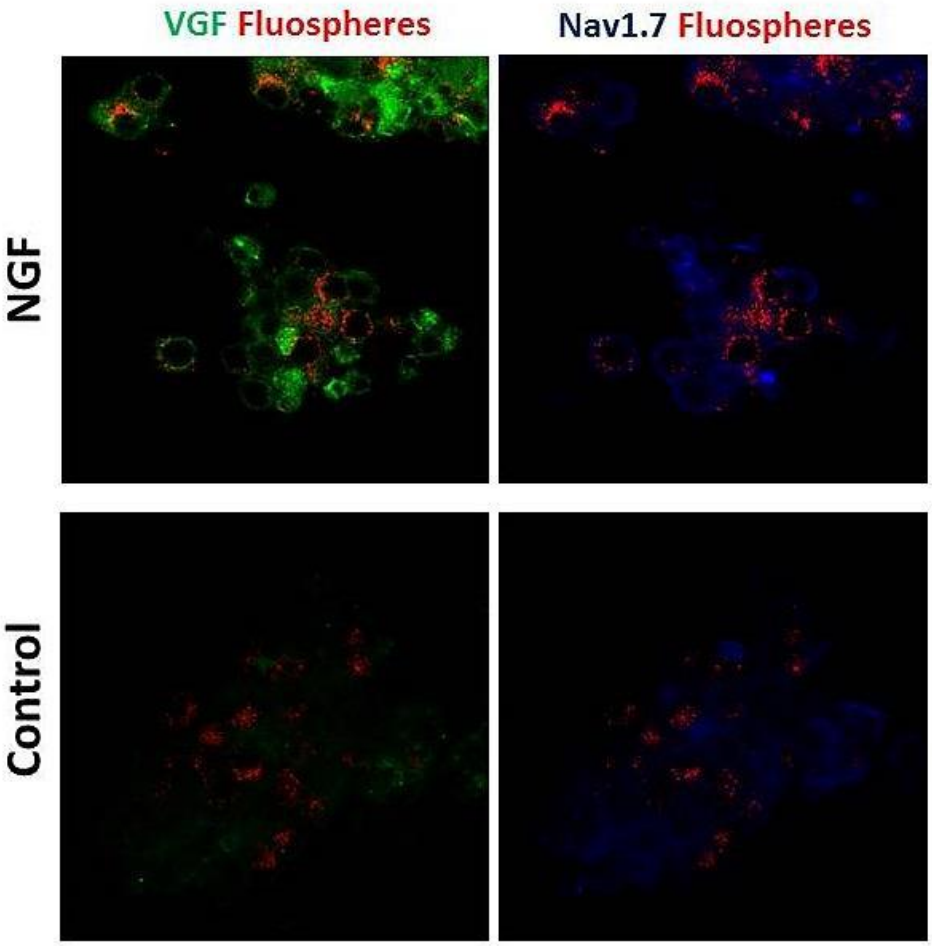

VGF
% cells with elevated VGF mRNA levels
****
n=178
n=167
Control
NGF
Nav1.7
% cells with elevated Nav1.7 mRNA levels
****
n=174
n=140
Control
NGF

Figure 6: Retrograde NGF signaling can stimulate VGF and Nav1.7 protein levels in the same cell.

6A top: Confocal images of NGF-treated cells stained for VGF (Green) and Nav1.7 (blue) antibody. Red dots are fluospheres that were added to mark the projecting cells. Cells were starved for 24 hours, and NGF and fluospheres were added to the distal axons after starvation. 6A bottom: confocal images of control cells 24 hours after starvation, only fluospheres were added to the axonal compartment for 16 hours and cells were stained for VGF and Nav1.7. 6B (left): Van diagram showing percentage of cells with elevated VGF levels showing elevated Nav1.7 protein levels. Right: Van diagram showing percentage of cells with elevated Nav1.7 protein levels also showing elevated VGF expression.

6A:


VGF Fluospheres
Nav1.7 Fluospheres
NGF
Control

6B:

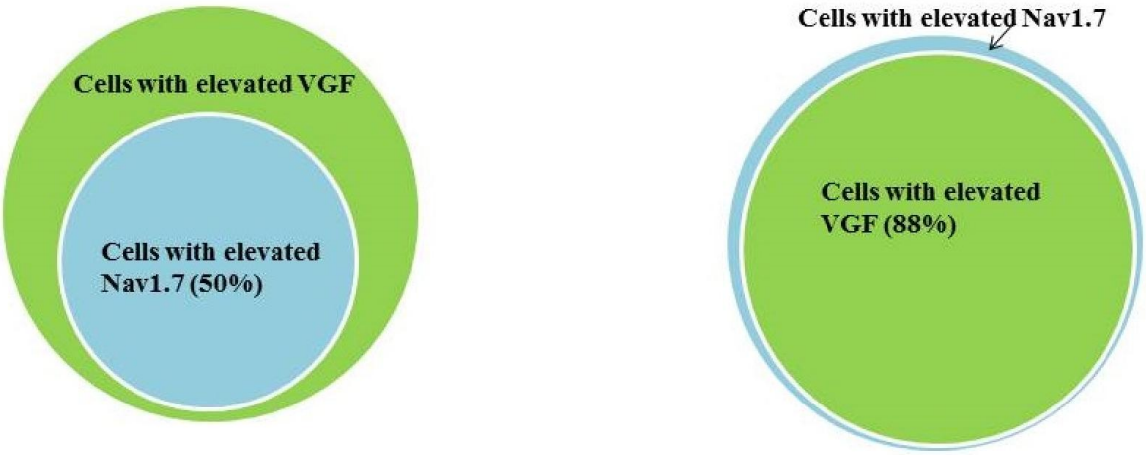
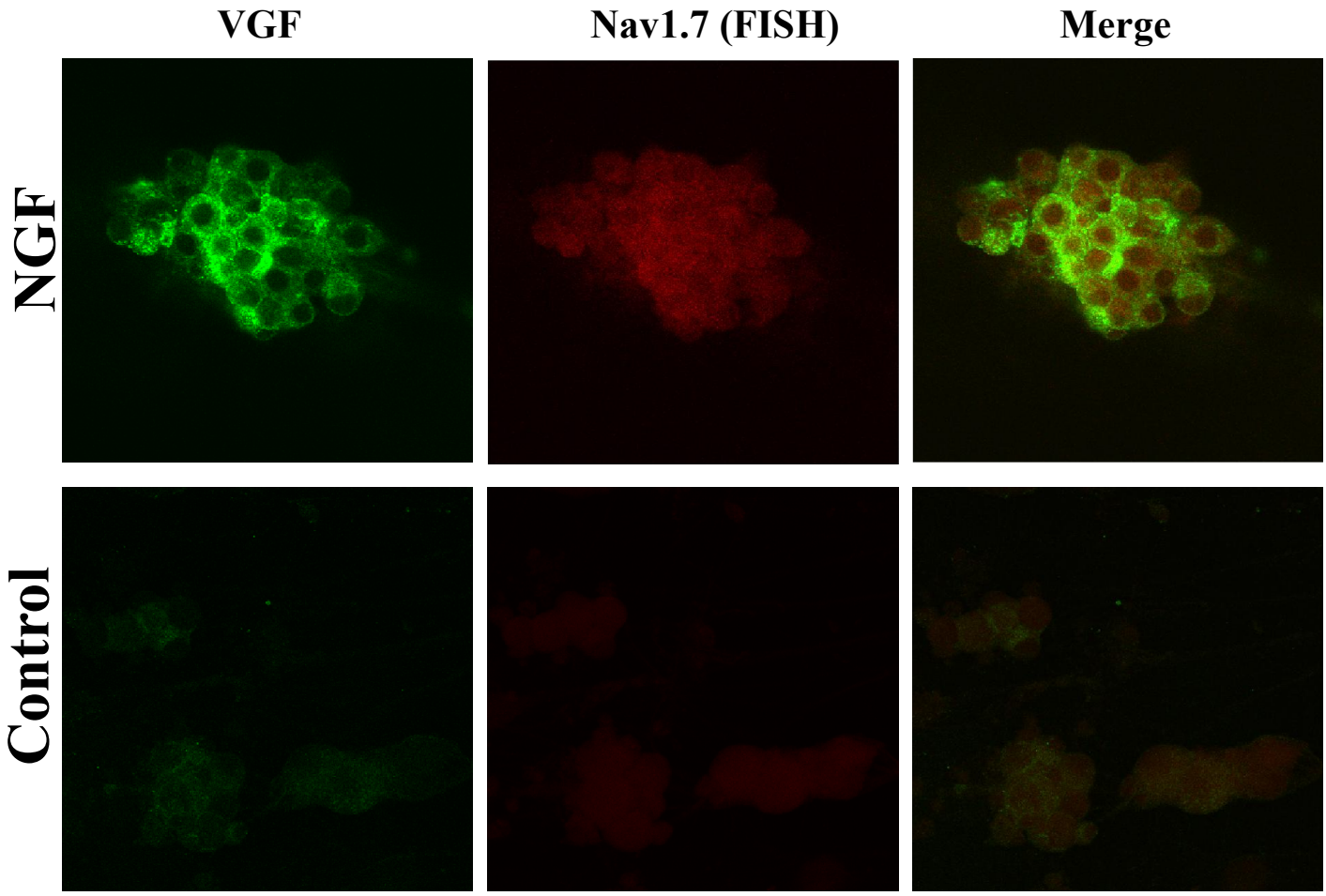

Cells with elevated VGF
Cells with elevated Nav1.7 (50%)
Cells with elevated Nav1.7
Cells with elevated VGF (88%)

Figure 7: Retrograde NGF signaling can stimulate VGF protein levels and Nav1.7 mRNA levels in the same cell.

7A (top): Confocal images of NGF-treated cells stained for VGF (Green) and probed with Nav1.7 anti-sense probe (red). Cells were starved for 24 hours and NGF was added for 16 hours after starvation. 7A bottom: 24 hours after starvation, no NGF was added to the axonal compartment and cells were stained for VGF and probed with Nav1.7 probe. 7B (left): Van diagram showing percentage of cells with elevated VGF levels showing elevated Nav1.7 mRNA levels. Right: Van diagram showing percentage of cells with elevated Nav1.7 mRNA levels also showing elevated VGF expression.

7A:


VGF
Nav1.7 (FISH)
Merge
NGF
Control

7B:


Cells with elevated VGF
Cells with elevated Nav1.7 mRNA levels (55%)
Cells with elevated Nav1.7 mRNA levels
Cells with elevated VGF (77%)

Chapter IV

PincherG68E blocks NGF-stimulated upregulation of VGF and Nav1.7

How does the NGF initiated at the neuronal terminal mediate signaling at the cell body? Several models, such as retrograde effector model, the wave model, and the signaling endosome hypothesis, have been proposed to explain this retrograde NGF signaling mechanism (reviewed by Ginty and Segal, 2002). One model in particular, the signaling endosome hypothesis, has now gained considerable amount of support and is thought to be the dominant mechanism for conveying this retrograde NGF signal. According to this model, neurotrophin bound and activated Trk receptors are endocytosed at the axon terminals and then retrogradely transported via microtubule network to the cell body.

Support for this model comes from several lines of evidence. For example, following NGF application into the target fields of ganglion neurons, NGF can be detected in the cell bodies (DeStefano et al, 1992, Delcroix et al, 2003). Retrograde transport of Trks have also been demonstrated (Ehlers et al, 1995), and co-precipitation studies have shown that TrkA and TrkB receptors are co-transported with NGF, or BDNF, respectively (Tsui-Pierchala and Ginty, 1999, Watson et al, 1999). Furthermore, NGF stimulation at the axon terminals failed to induce CREB phosphorylation at the cell body when NGF internalization was prevented (Riccio et al, 1997). Data from the experiments where transport machinery was compromised also support that idea that NT/Trk endosomes are indeed retrogradely transported. For example, colchicine—an inhibitor of microtubule polymerization that blocks microtubule based transport—treatment

reduced NGF immuno-reactivity in the basal forebrain of cholinergic neurons (Conner and Varon 1992). Inhibition of Dynein based transport also blocked NT dependent retrograde survival signal (Heerssen et al, 2004). Similarly, overexpression of a dominant negative form of Dynamin has been shown to prevent Erk5 phosphorylation in response to NGF (Watson et al, 2001). While these results strongly argue in favor of the signaling endosome hypothesis, the identity of molecular entities/mechanisms responsible for the formation and processing of NGF/TrkA complex remained elusive until a pinocytic chaperone molecule, Pincher, was discovered (discussed below).

Studies from our lab suggest that endocytosis and retrograde transport of NT/Trk complexes is mediated by a novel, pinocytic mechanism called macroendocytosis (Shao et al, 2002, Valdez et al 2005, Valdez et al, 2007). Like macropinocytosis, this mechanism show membrane ruffling and actin reorganization, but happens to be specific, and receptor-mediated. Pincher, a member of ESP15 homology domain (EHD) protein family, plays a crucial role in this endocytosis mechanism. Pincher was initially identified in PC12 cells as an NGF-induced protein (Shao et al, 2002) and subsequently it was shown to be involved in NT endocytosis and retrograde transport. In PC12 cells, these Pincher derived NGF/TrkA endosomes were shown to cause Erk5 activation and Erk5 remained activated for 24 hours (Shao et al, 2002). Overexpression of a dominant negative form of Pincher, PincherG68E, blocked Trk internalization (Shao et al, 2002). This blockade led to accumulation of activated Trk and Erk5 on the distinctive membrane regions and eliminated retrograde signal-induced neuronal survival (Valdez et al, 2005). Recently, our lab has shown that Pincher-generated endosomes are refractory to lysosomal degradation, and this allows sustained signaling and neuronal gene expression in the cell bodies (Philippidou et al, 2011). The evidence for this observation comes

from the fact that Trk and EGFR endosomes are differentially processed: unlike Trk endosomes, EGFR endosomes rapidly turn into Rab7-positive multi vesicular bodies (MVBs) that fuse with cathepsin-containing, electron-dense lysosomes. Retrograde Trk endosomes, on the other hand, are Rab5-positive MVBs, and the exchange of Rab5 for Rab7 seems to be the rate-limiting step for their late endosome/lysome processing. Thus, Pincher seems to process not only NGF/TrkA internalization, but also subsequent retrograde transport of NGF-TrkA endosomes.

In chapter III, I have shown that retrograde NGF signaling upregulated both mRNA and protein levels of VGF and Nav1.7. Here I asked whether Pincher is necessary for this retrograde gene upregulation. Specifically, by using a pseudotyped lenitviral vector, I expressed PincherG68E in DRG neurons grown in microfluidic devices and asked whether NGF-induced upregulation of mRNA and protein levels of VGF and Nav1.7 can be abolished.

Results:

DRG neurons expressing a dominant negative form of pincher, PincherG68E, fail to upregulate VGF protein and Nav1.7 mRNA levels upon retrograde NGF stimulation.

To retrogradely mark the projecting neurons, our lab developed a lentiviral vector that carries a dominant negative form of Pincher, PincherG68E (tagged with HA) (Figure 1A-B). This pseudotyped virus has rabiesG coat protein which allows the virus to infect host cells retrogradely. The construction of the pseudotyped virus is shown in figure 1B. Since this pseudotyped virus can retrogradely infect DRG neurons in microfluidic devices, this viral construct allowed us to identify projecting neurons without the necessity of using fluospheres or Q-dots. As control, I used similar pseudotyped lenviral vector carrying GFP (Figure 1C).

Since my results in chapter III showed that retrograde NGF signaling can upregulate Nav1.7 and VGF expression in microfluidic devices, I proceeded to determine whether this retrograde signaling can be blocked by using a dominant negative form of pincher (PincherG68E). In microfluidic devices, virus vectors carrying PincherG68E construct was added to the distal axon chambers and biochemical separation was maintained for 3 hours. 12 days post-infection, NGF was removed from the media and replaced with media containing anti-NGF antibody and Erk inhibitor U0126. After 24 hours of NGF starvation, media was replaced with NGF-free media, and NGF was added to the distal axonal compartment for 16 hours. After 16 hours of NGF treatment, the cells were fixed and VGF protein and Nav1.7 mRNA levels were determined by using immunocytochemistry or Fluorescent in Situ Hybridization (FISH), respectively. Figure 2A shows the experimental paradigm. Figure 2B (top) shows confocal images of NGF treated cells that were stained for HA to detect HA-PincherG68E (cyan), VGF (Green), and FISH to detect Nav1.7 mRNA (red). Figure 2B (bottom) shows confocal images of control cells that did not receive NGF stimulation. In order to analyze the results, both control and NGF treated cells were divided into two groups: cells that expressed PincherG68E and cells that did not. Furthermore, a threshold for elevated expression was determined by averaging the signal intensity and cells that showed a value above mean+ 2 standard deviation were counted as the positive cells that had increased VGF expression or elevated Nav1.7 mRNA levels.

NGF treatment upregulated VGF mRNA levels in cells that didn't express PincherG68E: in these NGF-treated cells, 17.1% cells that expressed PincherG68E (n=54) and 40.1% cells that did not express PincherG68E (n=137) showed elevated VGF expression. The difference between these two groups was statistically significant (P=.002). Cells that weren't treated with NGF, only low levels of VGF expression was seen: 14.9% cells expressing PincherG68E, and 15.7% non-

PincherG68E expression cells showed elevated levels of VGF expression. The difference between the two groups wasn't statistically significant (P=.39). These results suggest that retrograde NGF signal fail to upregulate VGF expression levels in neurons expressing dominant negative Pincher. The bar graph in Figure 2C summarizes the data from 3 separate experiments.

Nav1.7 mRNA levels upon NGF-stimulation showed similar results. 33.9% cells that didn't express PincherG68E showed elevated Nav1.7 mRNA levels whereas only 16.1% PincherG68E-expressing cells showed increased Nav1.7 mRNA levels. The difference between these two groups was statistically significant (P=.009). Non-NGF treated cells showed only low levels of Nav1.7 mRNA: 14.8% PincherG68E expressing cells and 15.1% non-PincherG68E expressing cells showed elevated Nav1.7 mRNA levels. There was no significant difference between these two groups (P=.88). These results suggest that retrograde NGF signal fail to upregulate Nav1.7 mRNA levels in neurons expressing dominant negative Pincher. The bar graph in figure 2D summarizes the data from 3 different experiments.

NGF stimulates VGF and Nav1.7 expression in GFP expressing DRG neurons, however this upregulation is abolished in PincherG68E expressing neurons.

Since I had used lentiviral vectors to block retrograde NGF signaling, one concern was that the viral infection could have somehow altered NGF signaling. In order to address that concern, I used a similar lentiviral construct carrying GFP, instead of PincherG68E, and asked whether GFP-virus could also block NGF-stimulated upregulation of VGF and Nav1.7 protein levels. In microfluidic devices, I added viral vectors carrying either GFP or PincherG68E to the distal axon chambers and maintained a fluidic volume difference for 3 hours. After 12 days of infection, NGF was removed from the media and replaced with media containing anti-NGF

antibody and Erk inhibitor U0126. After 24 hours of NGF starvation, media was replaced with NGF-free media, and NGF was added to the distal axonal compartment for 16 hours. After 16 hours of NGF treatment, the cells were fixed and VGF and Nav1.7 protein levels were determined by using immunocytochemistry. Figure 3A (top row) shows confocal images of cells infected with GFP virus and treated with NGF and then stained for GFP (green) and VGF (red). Figure 3A (second row) shows confocal images of PincherG68E-HA expressing cells that were treated with NGF. Figure 3A (third row) shows confocal images of GFP infected cells that weren't stimulated with NGF. Figure 3A (last row) shows confocal images of control cells that were infected with PincherG68E, but did not receive NGF treatment. In order to analyze the results, only cells that showed either GFP or PincherG68E expression were counted. A threshold for elevated expression was determined by averaging the signal intensity and cells that showed a value above mean+ 2 standard deviation were counted as the positive cells that had increased VGF expression.

15.1% GFP expressing cells showed elevated VGF expression in the absence of NGF stimulation, but 38.1% GFP expressing neurons showed elevated levels of VGF upon NGF stimulation. The difference was statistically significant ($P<.001$). 12.8% PincherG68E expressing neurons showed elevated VGF level, and the number slightly increased after NGF stimulation. However, the difference was not statistically significant. The bar graph in figure 3B summarizes the data.

Figure 4A (top row) shows confocal images of cells infected with GFP virus and treated with NGF and then stained for GFP (green) and Nav1.7 (red). Figure 3A (second row) shows confocal images of PincherG68E-HA expressing cells that were treated with NGF. Figure 3A (third row) shows confocal images of GFP infected cells that weren't stimulated with NGF.

Figure 3A (fourth row) shows confocal images of control cells that were infected with PincherG68E, but did not receive NGF treatment. In order to analyze the results, only cells that showed either GFP or PincherG68E expression were counted. A threshold for elevated expression was determined by averaging the signal intensity and cells that showed a value above mean+ 2 standard deviation were counted as the positive cells that had increased Nav1.7 expression.

13.1% GFP expressing neurons showed elevated Nav1.7 level without NGF stimulation, and 32.1% GFP expressing neurons showed elevated Nav1.7 level upon NGF stimulation. GFP expressing neurons, hence, did respond to NGF. In PincherG68E infected cultures, 12.1% neurons showed elevated Nav1.7 expression in the absence of NGF, and did not respond to NGF stimulation. 14.2% PincherG68E expressing, NGF-treated cells showed Nav1.7 expression. The bar graph in Figure 4B summarizes the data for NGF-induced Nav1.7 expression.

Discussion:

Here I show that Pincher plays a crucial role in mediating NGF signaling. By using a pseudotyped lentiviral vector, I expressed a dominant negative form of Pincher, PincherG68E, in DRG neurons, and show that cells expressing PincherG68E don't respond to retrograde NGF stimulation. Since a similar pseudotyped virus expressing GFP did respond to NGF stimulation, PincherG68E virus didn't compromise the cells ability to respond to NGF. My results, hence, demonstrate that NGF-induced retrograde gene induction is Pincher-dependent.

NGF bound Trk receptors are endocytosed and retrogradely transported to the cell body leading to long-term changes in gene expression (Halegoua et al, 1991, Beattie et al, 1996). Prolonged Trk kinase mediated signaling is necessary for this long term changes in gene

expression. Interestingly, EGFR, a similar receptor tyrosine kinase, fails to mediate this long-term expression changes. Our lab has shown that short endosomal lifetime of EGFR results in transient signaling of Erk kinases that is insufficient to mediate long-term changes in gene expression (Valdez et al, 2005). Since classical clathrin-dependent, receptor-mediated endocytosis of EGFR generates endosomes that are rapidly targeted for recycling or lysosomal degradation, it's likely that an alternative mean of endocytosis, which could protect TrkA endosomes from recycling or lysosomal degradation, is necessary for nerurotrophin endocytosis. Indeed, results from our lab indicate that Trk receptors are internalized via macroendocytosis, and that this process is mediated by the Pincher/EHD4 (Valdez et al, 2005, Valdez et al, 2007, Philippidou et al, 2011). Unlike clathrin-dependent EGFR endosomes, Pincher-mediated endosomes can signal for a relatively longer timeframe due to delayed transition of Trk endosomes to Rab7-dependent lysosomal breakdown (Valdez et al, 2005, Philippidou et al, 2011). The importance of this protection of Trk endosomes from lysosomal degradation has also been demonstrated by our lab: retrograde infection of distal axons with PincherG68E-Herpes Simplex Virus (HSV)-derived vector blocked NGF-induced upregulation of VGF in SCG neurons (Philippidou et al, 2011). The data from my experiments also underscores the importance of Pincher in mediating Trk endocytosis as cells expressing PincherG68E failed to upregulate Nav1.7 and VGF expression upon NGF stimulation at the distal axons.

By using, Fluorescent In Situ Hybridization (FISH) to determine Nav1.7 mRNA levels, and immunocytochemistry to determine VGF expression in DRG neurons expressing PincherG68E, I show that NGF stimulation at the axon terminals failed to upregulate Nav1.7 mRNA and VGF protein levels. The cells that didn't express PincherG68E, however, responded to NGF stimulation by upregulation both Nav1.7 mRNA and VGF protein levels (figure 2).

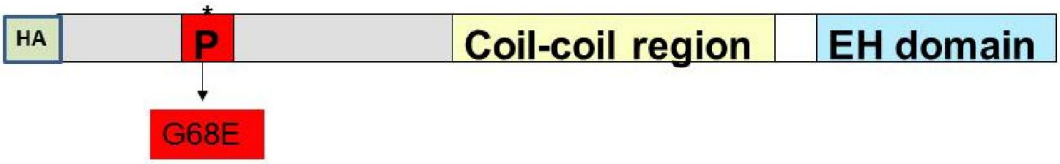
Furthermore, by using immunocytochemistry, I show that NGF stimulation failed to upregulate protein levels of both VGF and Nav1.7 (Figure 3 and figure 4, respectively). My results favors the following model: Pincher-mediated macroendocytosis generates Trk endosomes that are refractory to lysosomal processing, and this leads to a sustained endosomal signaling after retrograde transport to the cell body.

Since the Nav1.7 channel has gained considerable amount of attention in recent years due to its link with normal pain perception (Cox et al, 2006), and Pincher mediates retrograde NGF-induced upregulation of this channel in primary DRG neuronal cultures, it would be interesting to see if expression of PincherG68E can somehow mitigate inflammatory pain.

Figure 1: Generation of psuedotyped lentiviral vectors that can retrogradely infect DRG neurons to express either PincherG68E or GFP:

1A: Pincher, similar to other EHD proteins, has an ATP-binding loop, coil-coil region and an EH domain. Mutation (depicted by an asterisc) at ATP binding loop creates a dominant negative form of Pincher, PincherG68E. **1B:** Construction of pseudotyped lentiviral vector with rabiesG coating carrying a dominant negative form of Pincher, PincherG8E**. 1C:** Construction of pseudotyped lentiviral vector with rabiesG coating carrying GFP protein.

1A


HA
P
Coil-coil region
EH domain
G68E

1B


RSV
RRE
cPPT
Ubi
PincherG68E
WPRE
3´LTR
CMV
Rabies-G
pA

1C

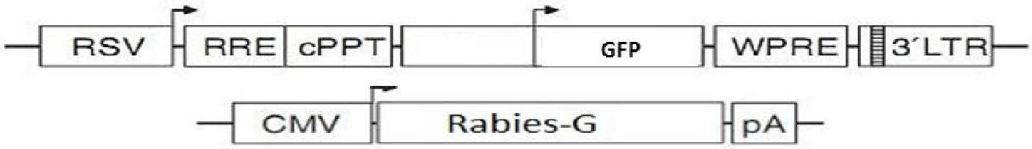
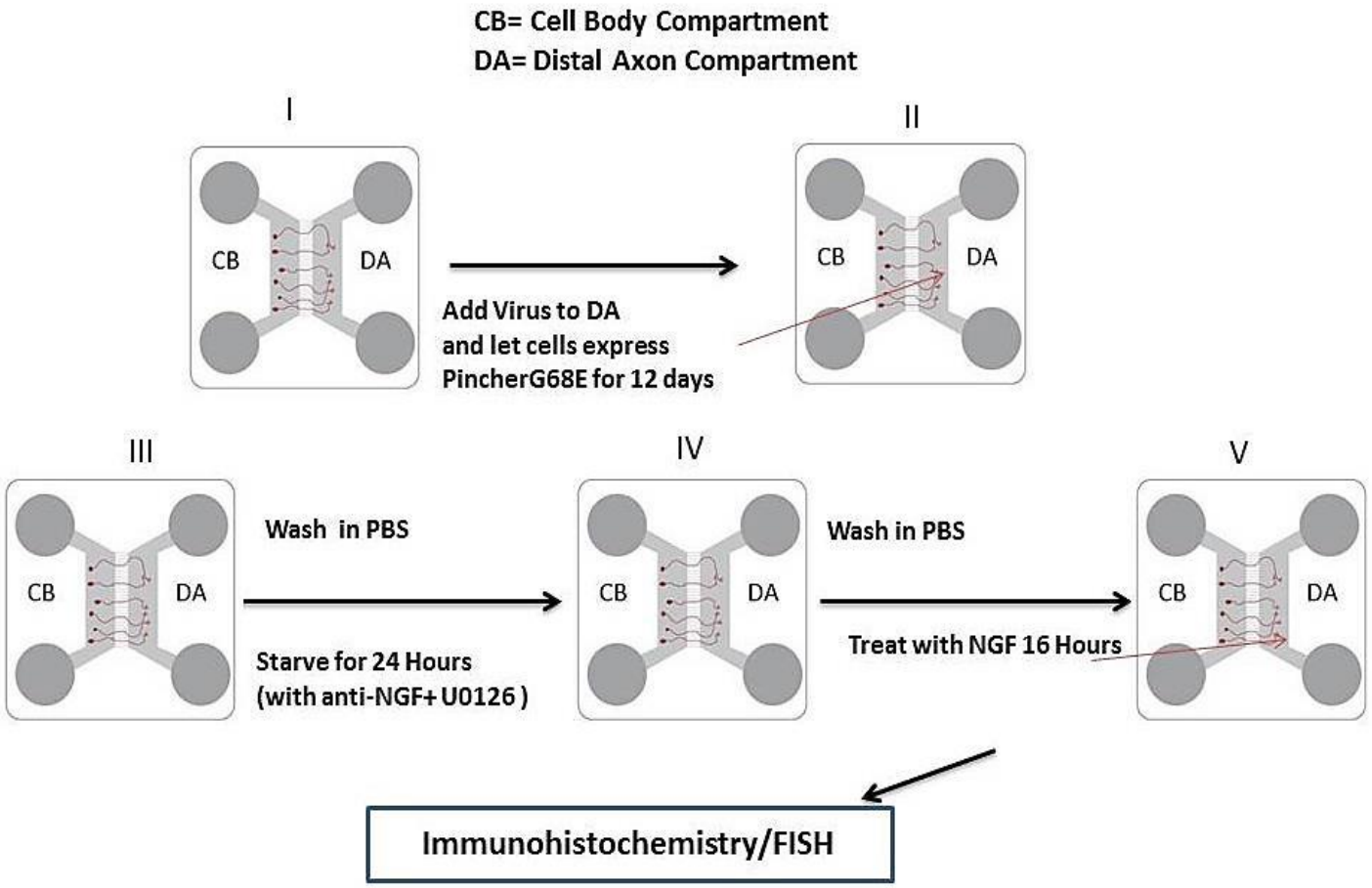
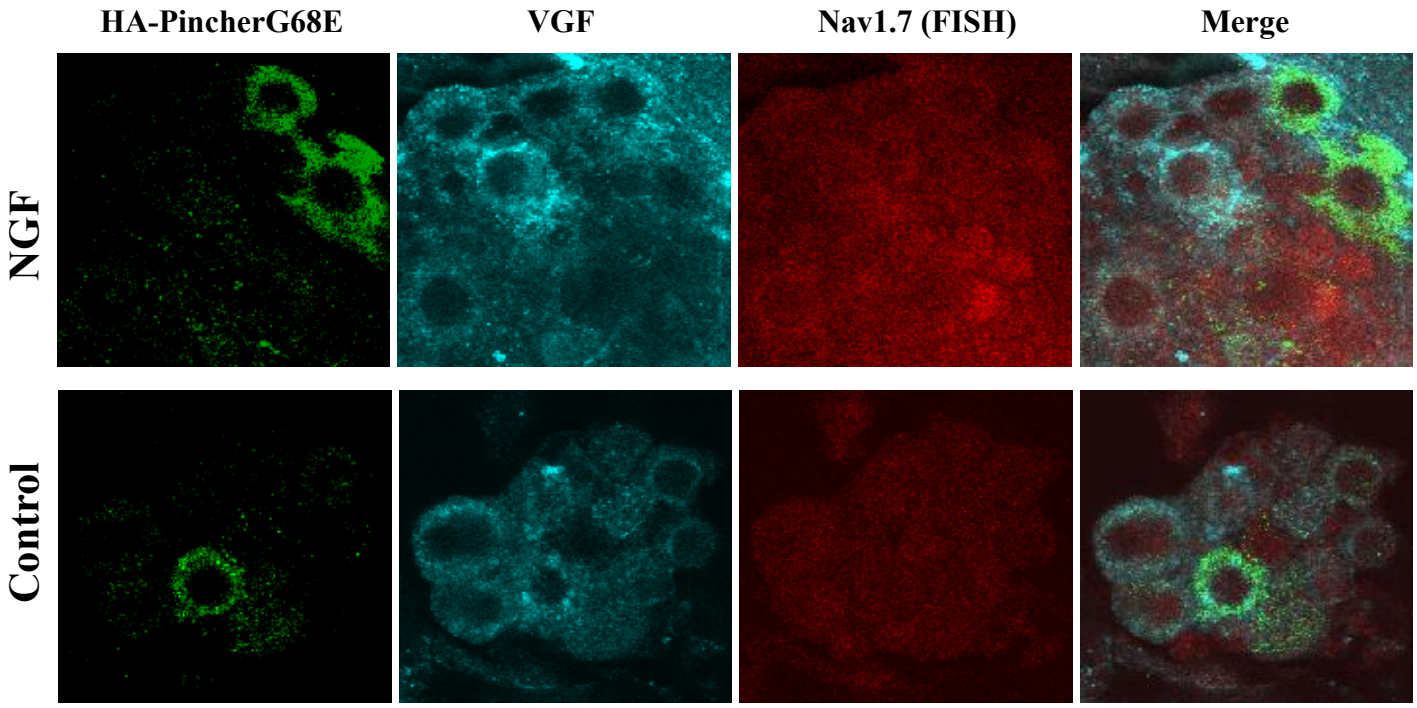

RSV
RRE
cPPT
GFP
WPRE
3´LTR
CMV
Rabies-G
pA

Figure 2: DRG neurons expressing a dominant negative form of pincher, PincherG68E, fail to upregulate VGF protein and Nav1.7 mRNA levels upon retrograde NGF stimulation: **2A:** Schematic diagram of viral infection and subsequent retrograde NGF stimulation. **2B:** Top: Confocal images of DRG neurons expressing PincherG68E after 16 hour NGF stimulation at the distal axon chamber. Cyan=PincherG68E-HA, green=VGF, and red=FISH for Nav1.7. Bottom: DRG neurons that were infected with lentivirus carrying PincherG68E, but were not treated with NGF. **2C:** Bar graph showing neurons that were infected with PincherG68E virus but did not express PincherG68E after 12 days of infection responded to NGF stimulation at the axonal terminals, whereas untreated neurons and PincherG68E expressing, NGF-treated neurons did not show increased VGF levels. **2D:** Bar graph showing neurons that were infected with PincherG68E virus but did not express PincherG68E after 12 days of infection responded to NGF stimulation at the axonal terminals, whereas untreated neurons and PincherG68E expressing, NGF-treated neurons did not show elevated Nav1.7 mRNA levels.

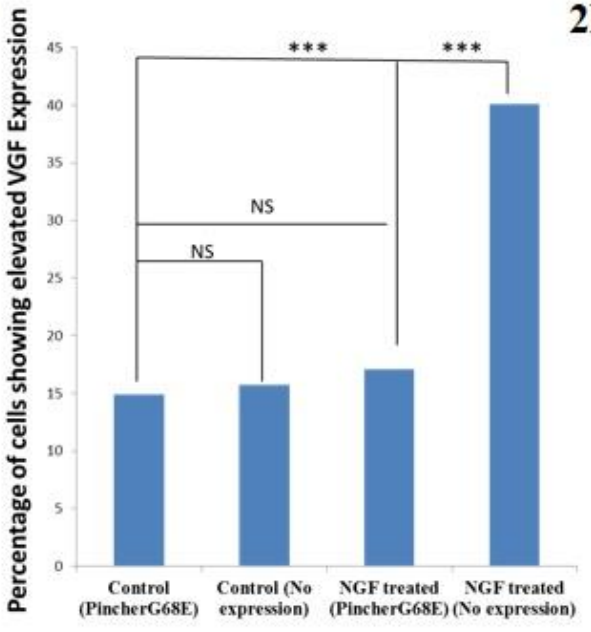
2A:


CB= Cell Body Compartment
DA= Distal Axon Compartment
I
II
CB
DA
CB
DA
Add Virus to DA
and let cells express
PincherG68E for 12 days
III
IV
V
CB
DA
Wash in PBS
Starve for 24 Hours
(with anti-NGF+ U0126)
CB
DA
Wash in PBS
Treat with NGF 16 Hours
CB
DA
Immunohistochemistry/FISH

2B:


HA-PincherG68E
VGF
Nav1.7 (FISH)
Merge
NGF
Control

2C.


Percentage of cells showing elevated VGF Expression
45
40
35
30
25
20
15
10
5
0
***
***
NS
NS
Control (PincherG68E)
Control (No expression)
NGF treated (PincherG68E)
NGF treated (No expression)

2D.

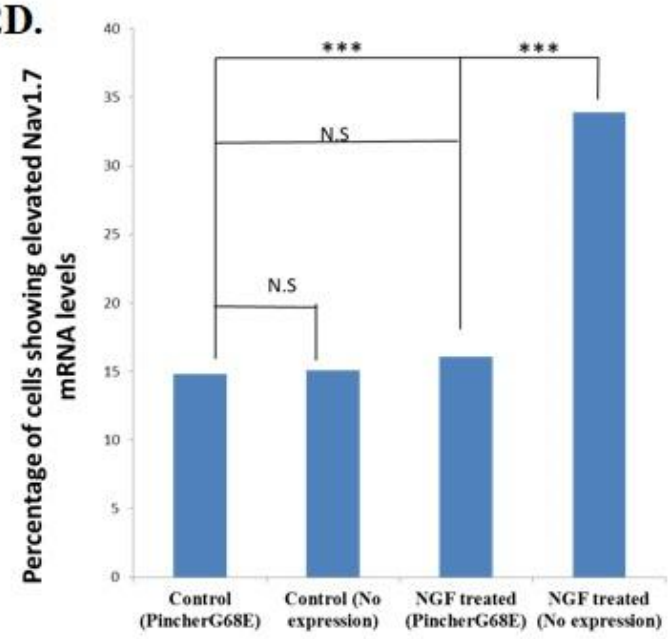
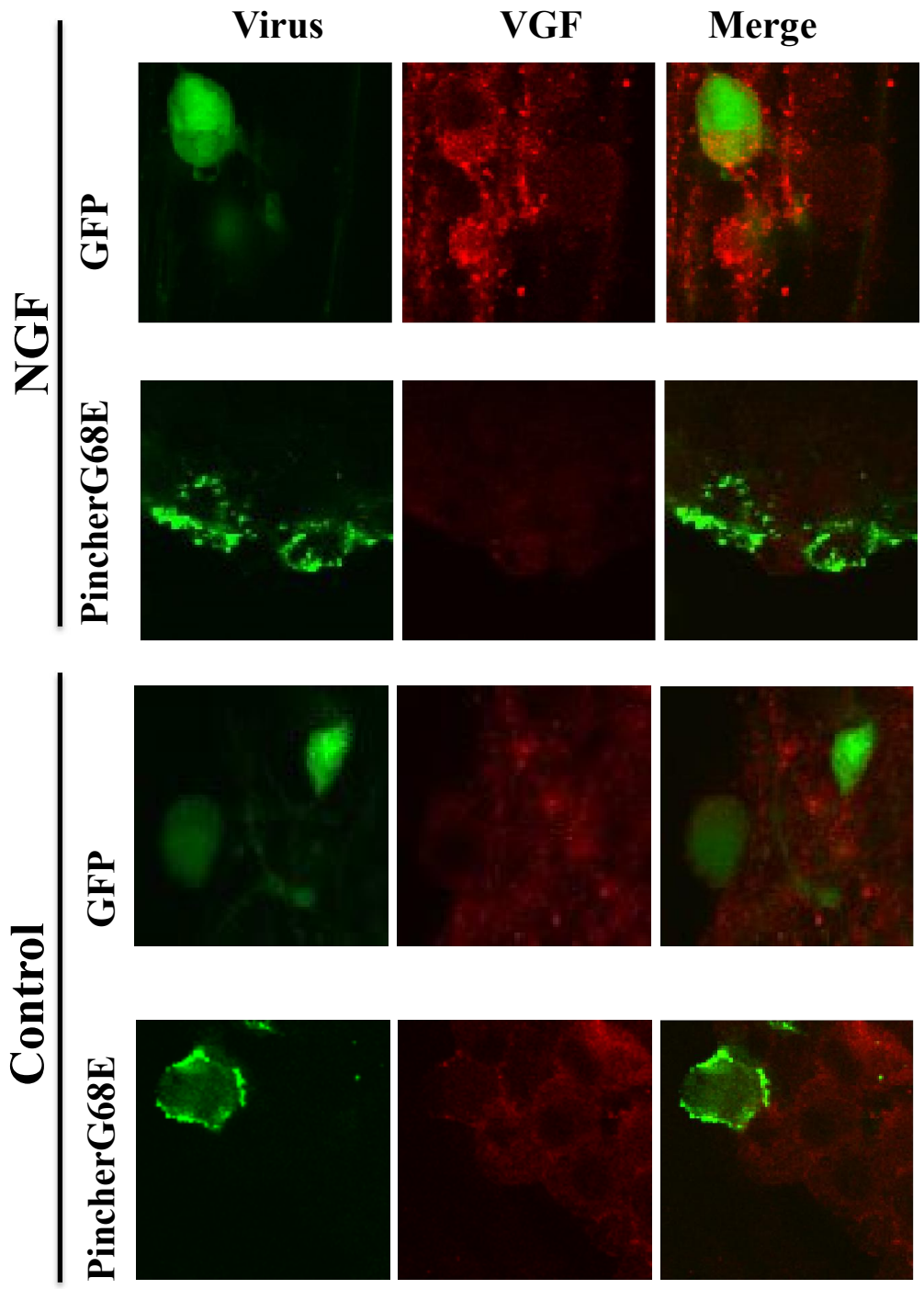

Percentage of cells showing elevated Nav1.7 mRNA levels
40
35
30
25
20
15
10
5
0
***
***
N.S
N.S
Control (PincherG68E)
Control (No expression)
NGF treated (PincherG68E)
NGF treated (No expression)

Figure 3: NGF stimulates VGF expression in GFP expressing DRG neurons, however this upregulation is abolished in PincherG68E expressing neurons. 3A: (top row) confocal images of cells infected with GFP virus and treated with NGF and then stained for GFP (green) and VGF (red). Second row: confocal images of PincherG68E-HA expressing cells that were treated with NGF. Third row: confocal images of GFP infected cells that weren't stimulated with NGF. Last row: confocal images of control cells that were infected with PincherG68E, but did not receive NGF treatment. **3B:** Data from 3 different experiments are summarized in the bar graph.

3A:


Virus
VGF
Merge
NGF
GFP
PincherG68E
Control
GFP
PincherG68E

3B:

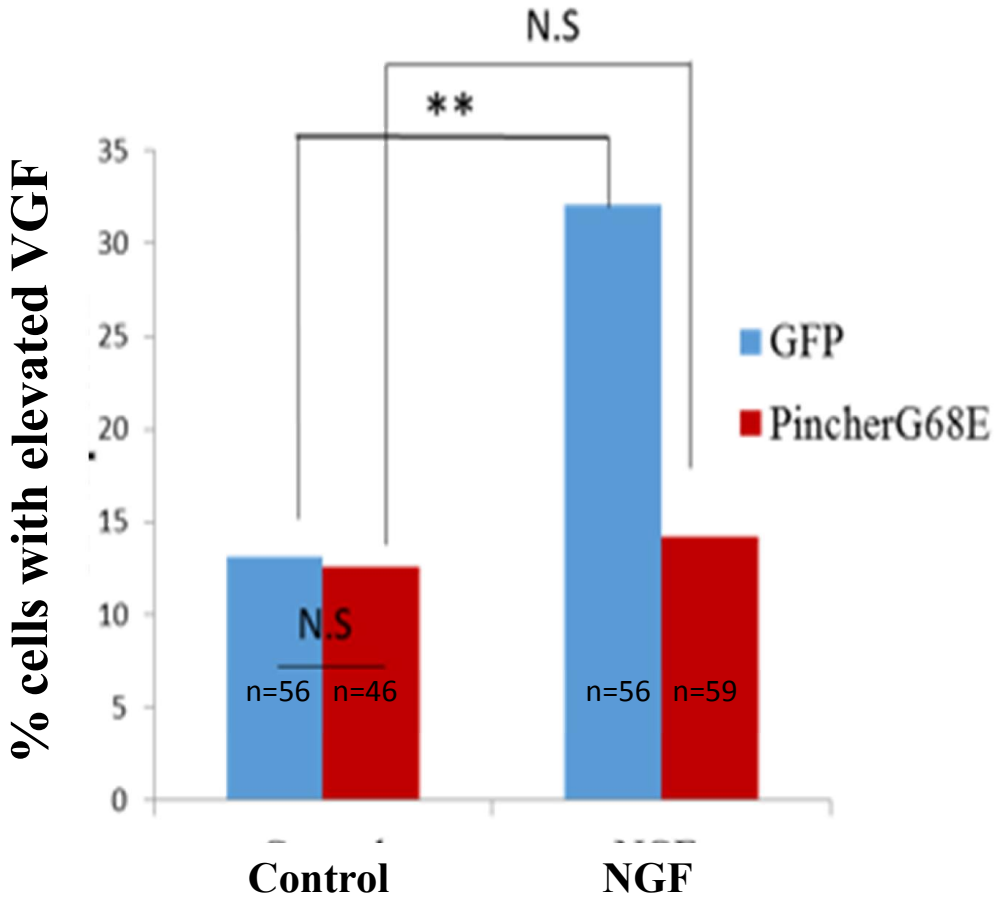
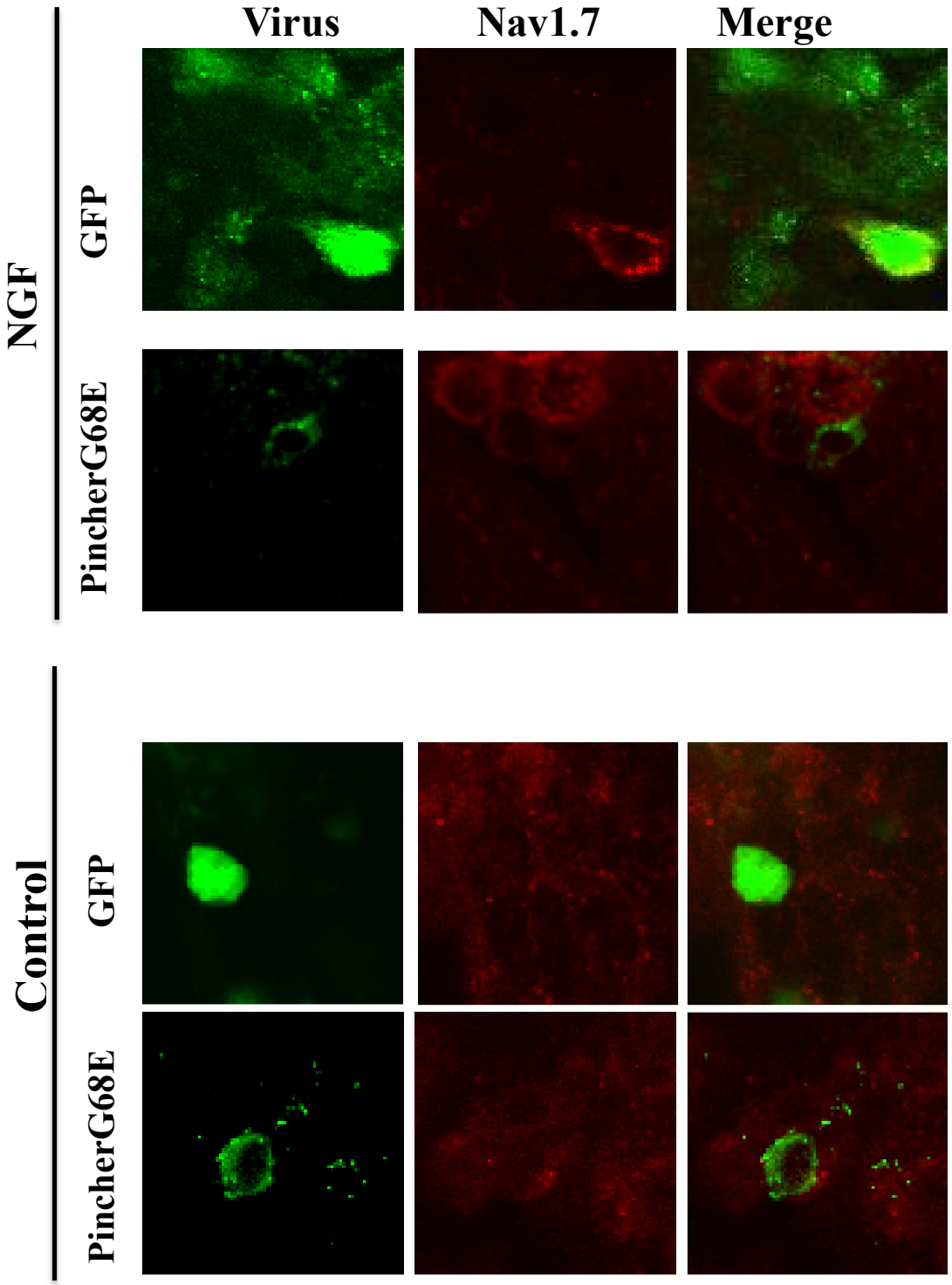
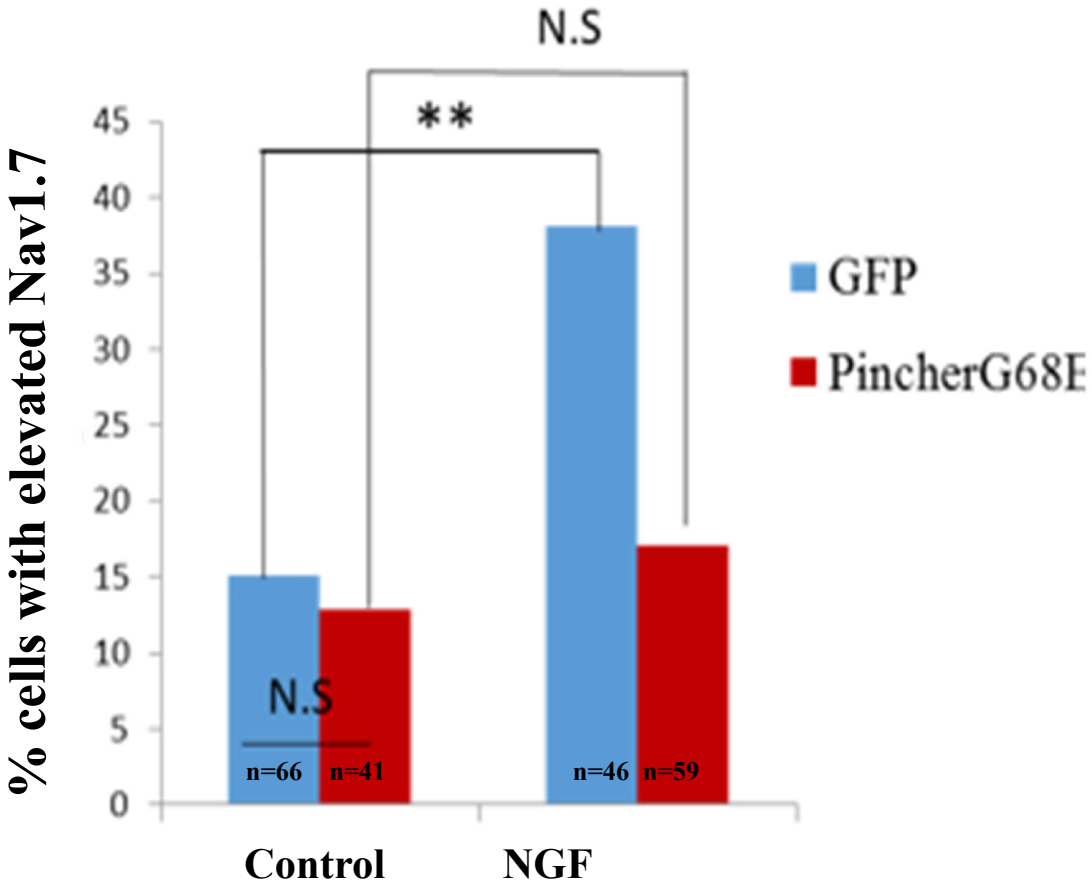

N.S
**
% cells with elevated VGF
35
30
25
20
15
10
5
0
GFP
PincherG68E
N.S
n=56
n=46
n=56
n=59
Control
NGF

Figure 4: NGF stimulates Nav1.7 expression in GFP expressing DRG neurons, however this upregulation is abolished in PincherG68E expressing neurons. 4A: (top row) confocal images of cells infected with GFP virus and treated with NGF and then stained for GFP (green) and Nav1.7 (red). Second row: confocal images of PincherG68E-HA expressing cells that were treated with NGF. Third row: confocal images of GFP infected cells that weren't stimulated with NGF. Last row: confocal images of control cells that were infected with PincherG68E, but did not receive NGF treatment. **4B:** Data from 3 different experiments are summarized in the bar graph.

4A:


Virus
Nav1.7
Merge
NGF
GFP
PincherG68E
Control
GFP
PincherG68E

4B


N.S
**
% cells with elevated Nav1.7
45
40
35
30
25
20
15
10
5
0
N.S
n=66
n=41
n=46
n=59
Control
NGF
GFP
PincherG68E

Chapter V:

Pincher-mediated retrograde NGF signaling upregulates VGF and Nav1.7, and causes thermal hyperalgesia

One striking aspect of NGF signaling is that in adult animals, it can profoundly modulate both thermal and mechanical pain sensitivity. NGF can not only sensitize nociceptors via mast cell and TRPV1 receptor activation in a transcription independent manner, but also it can change gene expression which might alter nociceptor physiology. The former transcription independent mechanism is thought to be responsible for NGF-induced acute hyperalgesia while the change is gene expression is thought to cause chronic hyperalgesia (Discussed by McMahon 1996, discussed below).

Several functionally important proteins including neurotransmitters, receptors and ion channels are upregulated by NGF. NGF, for example, upregulates both mRNA and protein levels of two neuropeptides that are normally expressed in TrkA expressing neurons: SP and CGRP (Pezet et al, 2001, Christensen and Hulsebosch, 1997). Similarly, expression of BDNF can be modulated by NGF (Priestley et al, 2002).VGF, a neuroendocrine specific gene that expressed in peripheral neurons, is also upregulated by NGF in both PC12 cells (Levi et al, 1985; Salton et al, 1991, D'Arcangelo and Halegoua, 1993) and in cultured superior cervical ganglion (SCG) neurons (Philippidou et al, 2011). Ion channels, such as PN1/Nav1.7, a Tetrodotoxin (TTX)-sensitive sodium channel expressed in peripheral neurons, can also be upregulated by NGF. In PC12 cells, NGF upregulated mRNA levels of this channel (Toledo-Aral et al, 1995) and subcutaneous paw injection of NGF increased the expression of this channel in DRG neurons that lasted for over a week (Gould III et al, 2000). Nonfunctional mutations in PN1/Nav1.7

channels lead to complete lack of pain sensation in affected humans (Cox et al, 2006, Goldberg et al, 2007). In mice, application of CFA leads to Nav1.7 upregulation and development of hyperalgesia; this hyperalgesia can be prevented by expression of shRNA for Nav1.7 (Yeomans et al., 2005). Accordingly, Nav1.7 conditional knockout mice show reduced pain sensitivity in CFA induced inflammation compared to littermate control animals (Nassar et al, 2004). VGF-derived peptide also seems to be involved in inflammatory pain: peripheral injection of TLQP-21 C-terminal internal VGF-derived peptide, increased pain-related licking response in the second inflammatory phase of formalin administration (Rizzi et al, 2008).

While NGF-mediated sensitization of the nociceptors occurs with a very short latency (within 10 minutes of NGF application) to cause acute thermal hyperalgesia, NGF-induced changes in gene expression would take place with a delay (from hours to days). This delay would be expected because 1) NGF-TrkA complex—as suggested by signaling endosome hypothesis—needs to be retrogradely transported from the axon terminals to the cell bodies and 2) modulation of gene expression by retrograde NGF-TrkA signal (at the cell body) needs to take place. Furthermore, since altered gene expression can lead to changes in nociceptor function (i.e. more sensitized nociceptors), hyperalgesia might be expected to last for a prolonged period. Conversely, while peripheral sensitization can occur rapidly leading to hyperalgesia, pain is less likely to last long: once the stimulus is removed, hyperalgesia should decrease since cell physiology is not altered (i.e, no change in gene expression).

Pincher, a pinocytic chaperone molecule, is required for NGF/TrkA internalization, and subsequent retrograde transport of NGF-TrkA endosomes. Recently, our lab has shown that Pincher-generated endosomes are refractory to lysosomal degradation, and this allows sustained signaling and neuronal gene expression in the cell bodies (Valdez et al, 2007; Philippidou et al,

2011). Furthermore, overexpression of a dominant negative form of Pincher (Pincher G68E) in cultured PC12 cells and neurons blocked Trk internalization, led to accumulation of activated TrkA and Erk5 on the distinctive membrane regions (Shao et al, 2002), and eliminated retrograde signal-induced neuronal survival (Valdez et al, 2005). While a crucial contribution of Pincher in mediating retrograde NGF signaling has been documented in vitro, several questions remained unanswered. For example, is Pincher required for retrograde NGF signaling in vivo? If so, is it possible to attenuate chronic hyperalgesia by blocking retrograde NGF signaling while retaining acute hyperalgesia?

In order to address those questions in vivo, I focused on two genes encoding VGF and Nav1.7. These genes—as described earlier—not only are upregulated by NGF, but are also involved in mediating pain. By using virus expressing PincherG68E, I abolished retrograde NGF signaling, and determined whether expression of dominant negative pincher eliminates NGF-induced upregulation of VGF and Nav1.7 in mice. Finally, I used Hargreaves Test to determine how PincherG68E expression affects thermal hyperalgesia in mice.

Results:

Retrograde NGF signaling upregulates VGF, and Nav1.7 expression in vivo.

To determine whether retrograde NGF signaling can upregulate VGF and Nav1.7 expression in vivo, I injected 200ng of NGF (Malin et al. 2006) into the right hind paw of mice. NGF aliquots were mixed with quantum dots (QD-605) to mark the cells that would likely take up NGF. 24 hours after injection, the mice were euthanized and L4-6 DRG neurons were collected both from ipsilateral and contralateral sides. The DRGs were then cryo-sectioned and the tissue sections were histologically stained with anti-VGF and anti-Nav1.7 antibodies. The

samples were then visualized by confocal microscopy and images were analyzed in ImageJ. 370 cells were analyzed for VGF expression, and 307 cells were analyzed for Nav1.7 expression. A threshold for elevated expression was determined by averaging the signal intensity and cells that showed a value above mean+ 2 standard deviation were counted as the positive cells that had increased Nav1.7 or VGF expression.

Figure 1A shows confocal images for VGF protein levels. 39% of the ipsilateral cells that had taken up Qdots showed increased VGF expression: NGF increased VGF expression by 4.3 fold compared to contralateral side, and 2.6 fold compared to ipsilateral cells that did not take up Q-dots (P=.001, and P=.005 respectively). Compared to the contralateral side (in which no cells took up Q-dots), neurons on the ipsilateral side that had not taken up Q-dots, NGF injection did not increase VGF expression to a statistically significant level (1.67 fold : 9% contralateral cells versus 15% ipsilateral cells without Q-dots; P=0.07). In contrast, Bar graph 1D (left) summarizes the data.

Figure 1B shows confocal images for Nav1.7 expression. 33% of the ipsilateral cells that had taken up Qdots showed increased VGF expression: NGF increased Nav1.7 expression by 4.7 fold compared to contralateral side, and 2.75 fold compared to ipsilateral cells that did not take up Q-dots (P=.001, and P=.006, respectively). Relative to the contralateral side, neurons on the ipsilateral side that had not taken up Q-dots, NGF again did not increase Nav1.7 expression in a statistically significant manner (1.7 fold, 7% contralateral cells versus 12% cells without Q-dots; P=0.06). However, Bar graph in Figure 1D (right) shows the quantification of the data. One interesting observation was that the cells here seem to expel Q-dots via exocytosis. It's possible that the Q-dots were toxic to the cells and unless it went to the nucleus, the cytoplasmic Q-dots were expelled.

DRG neurons expressing a dominant negative form of pincher, PincherG68E, fail to upregulate VGF upon retrograde NGF stimulation.

Since my results suggested that retrograde NGF signaling can upregulate Nav1.7 and VGF expression in vivo, I proceeded to determine whether this retrograde signaling can be blocked by using a dominant negative form of Pincher (PincherG68E). To mark the cell bodies that send projections to the paw, our lab developed a lentiviral vector expressing HA-PincherG68E. This pseudotyped virus has rabiesG coat protein which allows the virus to infect host cells retrogradely. By using microfluidic devices, I have shown that this chimeric virus can retrogradely infect cells in vitro. However, before using the virus for my experiments, I needed to ascertain that the virus can retrogradely infect host cells in vivo. I injected 4ul of viral particles and 1ul cholera toxin (beta subunit) in the right hind paw of mice. Beta-subunit of cholera toxin binds to ganglioside GM1, a plasma membrane (PM) glycolipid, that carries the toxin from the nerve terminal to the cell body, and thus can be used as retrograde tracer (Fujinaga et al, 2003). After 14 days of infection, I sacrificed the mice to analyze the efficacy of the retrograde infection/transport. I collected L4-6 DRG neurons, and after cryosectioning, I stained the samples with anti-HA antibody (for HA-PincherG68E). Figure 2A (top panel) shows cells expressing HA-PincherG68E; there is a considerable overlap between cells expressing the dominant negative form of Pincher and the cells that had taken up cholera toxin. Lower levels of dominant negative Pincher expression can be seen from the cells from contralateral side (figure 2A, bottom panel). Since high volume of virus was used for this experiment, for subsequent experiments, I used lower volume (1ul), but high titer virus. This allowed me to label only cells from ipsilateral side.

Since the virus we created could retrogradely infect cells to express PincherG68E in DRG neurons, we then used similar pseudotyped lentivirus expressing GFP as a control for effects of viral infection (a kind gift from Dr. Kevin Czaplinski). Figure 2B shows DRG neurons expressing GFP (stained with anti-GFP antibody) after 14 days of injection with GFP virus (top). The bottom figure shows the contralateral DRG neurons which don't show any GFP expression. Figure 2B illustrates that there are most likely "hot spots" of neurons that where most cells could express GFP (top), and there are other spots where very little GFP expression can be seen (bottom)

Having established the tools I needed to manipulate retrograde signaling, I then proceeded to determine whether dominant negative Pincher could block retrograde NGF signaling in vivo. To address the question, I injected GFP or HA-PincherG68E virus in one hindpaw. 12 days post-infection, I injected the GFP virus-infected or the PincherG68E virus-infected mouse hindpaw with NGF (200ng).

After cryo-sectioning, the tissue samples were stained with antibody against either GFP (for GFP-infected mice) or HA (for HA-Pincher-G68 infected mice) and VGF for confocal imaging. Figure 3A-B shows confocal images of DRG neurons from ipsilateral side of GFP+NGF, and PincherG68E+NGF injected mice, respectively. Once more, cells that had signal intensity above mean+two standard deviations were counted as cells with increased VGF expression. 10% neurons from the contralateral (n=69) side showed elevated VGF expression. Cells from the ipsilateral side were divided in two categories: GFP-expressing neurons and not GFP-expressing neurons. 11% non-GFP expressing neurons (n=146) showed elevated VGF expression, whereas 36% GFP-expressing neurons showed elevated VGF expression. This value

was statistically significant from the other two groups. This suggests that NGF upregulated VGF levels in GFP expressing neurons only.

Neurons collected from PincherG68E-infected mice showed a different patterns. 15% neurons from contralateral neurons (n=45), 13% neurons that did not express PincherG68E (n=68), and 12% neurons that expressed PincherG68E (n=54) showed elevated VGF expression. This suggests that NGF-induced VGF upregulation that was seen in GFP expressing mice was abolished in PincherG68E-infected mice. There was no statistically significant difference among the groups. The Bar graph in figure 3D summarizes the data.

DRG neurons expressing a dominant negative form of pincher, PincherG68E, fail to upregulate Nav1.7 upon retrograde NGF stimulation.

Having shown that Pincher plays a critical role in NGF-induced VGF upregulation in mice, I then proceeded to see if dominant negative Pincher could also block NGF-induced Nav1.7 upregulation (as seen in vitro). To address the question, I injected viruses expressing either GFP or HA-PincherG68E into one hindpaw. 12 days post-infection, I injected the GFP and the HA-PincherG68E virus-infected mice with NGF (200ng). After 24 hours, the mice were euthanized and DRGs collected to determine Nav1.7 expression.

After cryo-sectioning, the tissue samples were stained for either GFP (for GFP-infected mice) or HA (for Pincher-G68 infected mice) and Nav1.7 for confocal imaging. Figure 4A-B shows confocal images of DRG neurons from ipsilateral side of GFP+NGF, and PincherG68E+NGF injected mice, respectively. Cells that had signal intensity above mean+two standard deviations were counted as cells with increased Nav1.7 expression. In GFP-infected mice, 9% cells (n=69) showed elevated Nav1.7 expression, and from non-GFP expressing neurons from the ipsilateral

side, 10% cells (139) showed elevated Nav1.7 expression. However, in GFP-expressing neurons, 28% cells (78) showed elevated Nav1.7 levels. This value was significantly different from other two groups.

In PincherG68E infected mice, there was no difference between contralateral (14%, n=54), PincherG68E-expressing ipsilateral neurons (15%, n=45), and non-PincherG68E expressing neurons (13%, n=84). This suggests that dominant negative form of pincher could block NGF-induced upregulation of Nav1.7. Bar graph in figure 4D summarizes the data.

Mice injected with PincherG68E fail to develop thermal hyperalgesia after 24 hours of NGF administration.

Since both VGF and Nav1.7 have been implicated in pain perception, and as blocking retrograde NGF signaling blocks NGF-induced upregulation of VGF and Nav1.7, I then asked whether this could lead to altered pain perception in mice. Four groups of mice (6 mice per group) were used for two different nociceptive tests: Hargreaves Test which measures thermal hyperalgesia from an intense heat source and Von Frey Test which measures mechanical pain threshold. Six-week old mice were used for this experiment. At the beginning of the experiment, for each mouse, one hindpaw was injected with virus expressing either PincherG68E or GFP. Twelve days after the injection, either 200ng NGF or PBS (control) was injected at the site of viral injection. The mice were then tested for thermal (Hargreaves Test) and mechanical allodynia (Von Frey Test). There were no statistical differences among the four groups when the uninjected hind paw was tested on either Hargreaves or Von Frey Test (data not shown). Figure 5A shows the results from the injected hind paws using the Hargreaves Test. Surprisingly, only mice infected with GFP-Virus and NGF showed a lower latency (they withdrew their paws faster

than any other groups when heat was applied). The other groups, including mice injected with both PincherG68E and NGF did not show heightened pain sensitivity. The average paw withdrawal latency for GFP+NGF, GFP+PBS, PincherG68E+NGF, and PincherG68E+PBS injected mice was 2.64s, 6.49s, 6.53s, and 5.54s. There was no statistically significant difference among the latter 3 groups, however, the difference between these three groups and GFP-virus+NGF injected group was statistically significant (P=.02, P=.021, and P=.017 for GFP+PBS, PincherG68E+NGF, and PincherG68E+PBS injected mice respectively).

The result from Von Frey Test (Figure 5B), however, was different: NGF seemed to have failed to induce mechanical allodynia in GFP/NGF treated mice; though the value was lower for these mice (threshold was 2.65 grams) compared to PBS treated mice (threshold was 3.56), it was not statistically significant. Behavioral experiments on more animals need to be carried out in order to figure out why this has happened. NGF, however, did cause mechanical allodynia in PincherG68E-expressing mice as NGF treated mice showed significantly lower mechanical threshold compared to PBS-treated mice (3.02 grams versus 4.51 grams respectively). The data suggests that NGF-induced mechanical allodynia develops normally in PincherG68E-expressing mice.

Discussion

Here I show that NGF injections in the mouse hindpaw can retrogradely upregulate two proteins that had been implicated in pain perception: VGF and Nav1.7. By using Q-dots to mark the recipient DRG neurons, I was able to show that the cells that take up Q-dots (and most likely, NGF) from the axon terminals to the cell body show marked elevation in VGF and Nav1.7

expression; this was demonstrated by counting the number of cells that had high fluorescence signal intensity (Figure 1). Since Q-dots were injected with NGF, and they were absent in contralateral side, it's clear that NGF did not upregulate these genes by systemically entering into the bloodstream. Hence, the NGF from injected site at the paw was signaling retrogradely from the nerve terminals to the cell body of DRG neurons to upregulate both VGF and Nav1.7 expression. The NGF-stimulated level of these proteins was consistently higher in ipsilateral NGF-injected neurons that did not have Qdots than in contralateral neurons, although this was not statistically significant. This tendency could however, be due to a higher efficiency of NGF vs. Qdots being retrogradely transported by specific TrkA receptors.

Furthermore, I have used pseudotyped lentiviral constructs to retrogradely mark DRG neurons locally innervating the mouse hindpaw. Between 5-20% DRG neurons from stained tissue samples expressed the gene (either GFP or HA-PincherG68E) carried by these pseudotyped viruses. The virus particles did not significanly enter the bloodstream since DRG neurons collected from either the thoracic (ipsilateral) or contralateral sides did not show NGF-stimulated gene expression. Since GFP expressing cells did show increased VGF and Nav1.7 levels upon NGF stimulation, infection by GFP-expressing virus doesn't seem to affect retrograde gene induction by NGF.

However, the response to NGF was attenuated when neurons were expressing a dominant negative form of Pincher. By using virus expressing PincherG68E, I have shown that retrograde NGF signaling can be blocked in vivo, and this lead to a dramatically decreased neuronal response to NGF stimulation. Specifically, I used this virus to express dominant negative form of Pincher to show that Pincher not only plays an important role in this retrograde signaling, but also leads to defects in retrogradely stimulated gene expression mediated by NGF. As shown in

Figures 3 and 4, the cells expressing PincherG68E fail to upregulate both Nav1.7 and VGF expression upon NGF stimulation of nerve terminal fields.

Since the Nav1.7 channel has gained considerable attention in recent years due to its link to normal pain perception (Cox et al, 2006), I was also curious to see if an increase in expression level of Nav1.7 could lead to hyperalgesia. Mice injected with GFP-virus showed heightened thermal hyperalgesia 24 hours after NGF injection (Figure 5). However, mice infected with Pincher-G68E virus showed significant deviation in this paradigm: these mice did not withdraw their paws from the heat source as quickly as the former group; in fact, their response was similar to mice that had been injected with PBS only. This result may be due to the necessity for increased expression of Nav1.7, VGF and other NGF stimulated genes, for mediating the chronic NGF hyperalgesic effect.

In mice, about 80% of small diameter DRG neurons express TrkA at E15, however, by P7, about half of them stops responding to NGF, and becomes responsive to GDNF (Molliver et al, 1999). Hence, if VGF and Nav1.7 levels are increased by NGF stimulation, one would expect less than 40% of the neurons to show increased levels of these protein upon NGF injection. My finding here is indeed consistent with this idea as about 35% neurons showed elevated levels of VGF, and about 30% neurons showed elevated levels of Nav1.7 upon NGF injection.

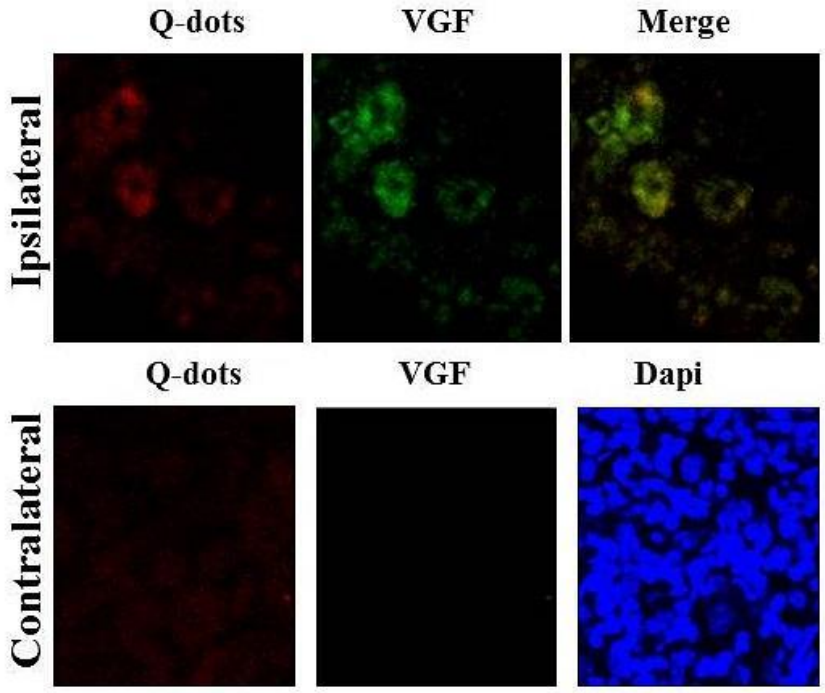
One interesting aspect is that these mice had similar response on Von Frey Test: NGF did not cause mechanical allodynia in either GFP-expressing mice, however, NGF did induce mechanical hyperalgesia in PincherG68E mice. It is possible NGF-induced mechanical pain transmission was not altered by the expression of dominant negative form of Pincher. The observation that NGF failed to induce mechanical hyperalgesia in GFP-expressing mice is also perplexing as one would expect these mice to develop NGF-induced hyperalgesia. Since for each

group, I tested 6 animals, more sample size would be required to get a better understanding of the action of PincherG68E-infected mice.

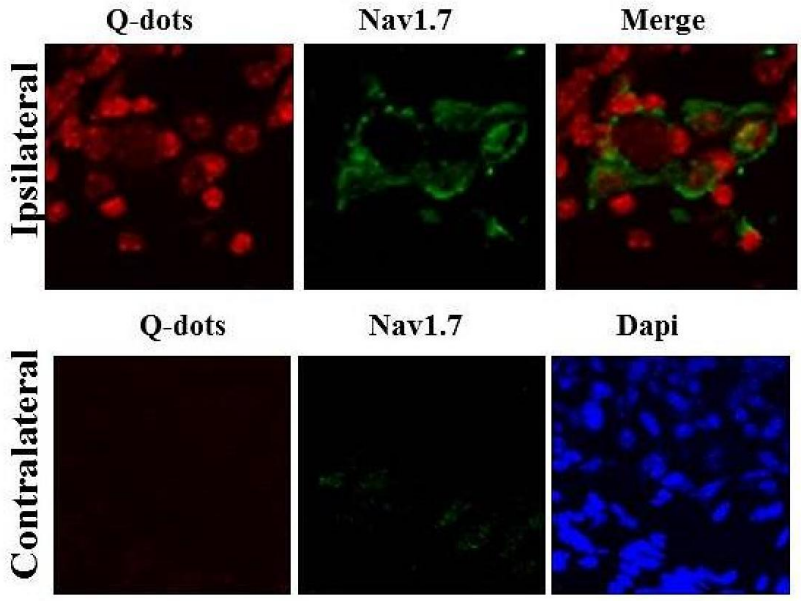
Figure 1: Retrograde NGF signal upregulates VGF, and Nav1.7 expression in vivo:

1A: VGF expression in L4-L6 DRG neuron with or without NGF treatment in the paw. Top panels shows VGF expression from ipsilateral (injection site) and bottom panel shows VGF expression from contralateral side. **1B:** Nav1.7 expression in L4-L6 DRG neuron with or without NGF treatment in the paw. Top panels shows Nav1.7 expression from ipsilateral (injection site) and bottom panel shows Nav1.7 expression from contralateral side. **1C:** High magnification image showing Q-dot labeled neurons have elevated Nav1.7 expression when treated with NGF. **1D:** Bar Graph depicting percentage of cells showing either elevated VGF (left) or Nav1.7 (right) expression after 24 hours of NGF+Q-dot treatment.

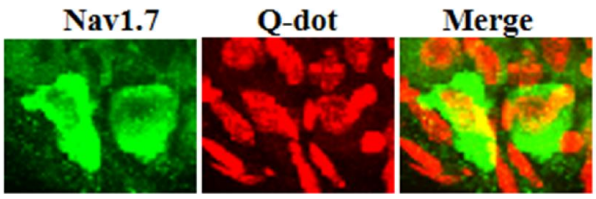
1A:


Q-dots
VGF
Merge
Ipsilateral
Q-dots
VGF
Dapi
Contralateral

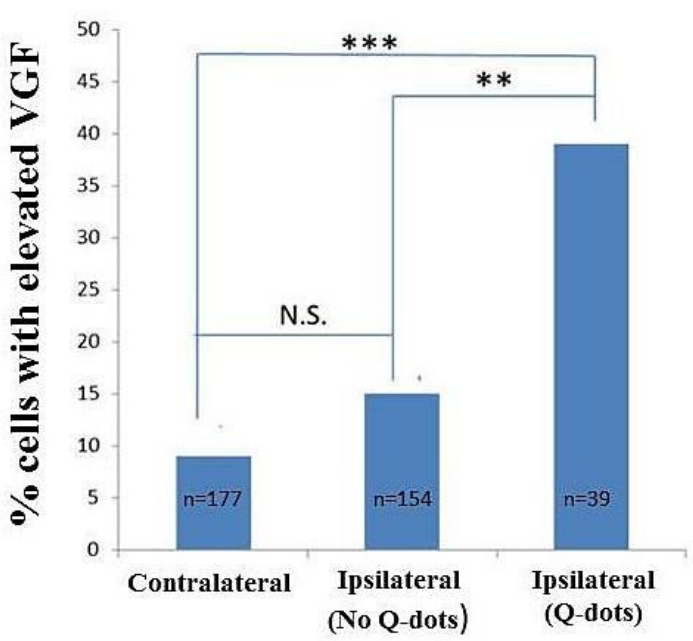
1B:


Q-dots
Nav1.7
Merge
Ipsilateral
Q-dots
Nav1.7
Dapi
Contralateral

1C:


Nav1.7
Q-dot
Merge

1D:


% cells with elevated VGF
50
45
40
35
30
25
20
15
10
5
0
***
**
N.S.
n=177
n=154
n=39
Contralateral
Ipsilateral (No Q-dots)
Ipsilateral (Q-dots)

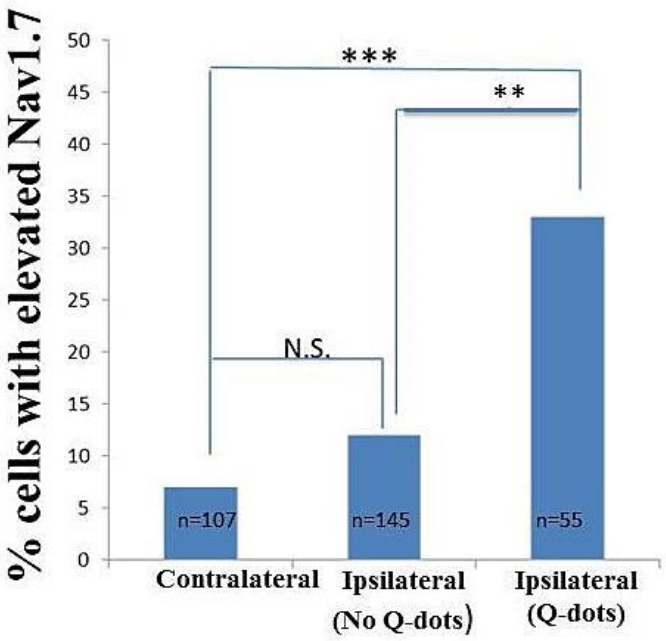

% cells with elevated Nav1.7
50
45
40
35
30
25
20
15
10
5
0
***
**
N.S.
n=107
n=145
n=55
Contralateral
Ipsilateral (No Q-dots)
Ipsilateral (Q-dots)

Figure 2: DRG neurons express GFP, or dominant negative pincher upon retrograde injection of pseudotyped lentivirus:

Pseudotyped lenivirus has rabiesG coat protein which allows the virus to infect host cells retrogradely. **2A:** GFP is expressed in L4-L6 DRG neurons from ipsilateral side after 14 days of infection (top panel). No GFP signal was detected in the neurons from contralateral side (bottom panel). Anti-GFP antibody was used to detect GFP signal. **2B**: Dominant negative form of pincher, PincherG68E, is expressed in L4-L6 DRG neurons from ipsilateral side after 14 days of infection (top panel). Some HA signal was detected (due to high virus concentration used) in the neurons from contralateral side (bottom panel). Anti-HA antibody was used to detect PincherG68E since its linked to HA tag. Choleratoxin conjugated to fluospheres (red channel) can also be used to retrogradely label cells. **2C** shows high-level (upper panel) and low-level (lower panel) GFP expression pockets.

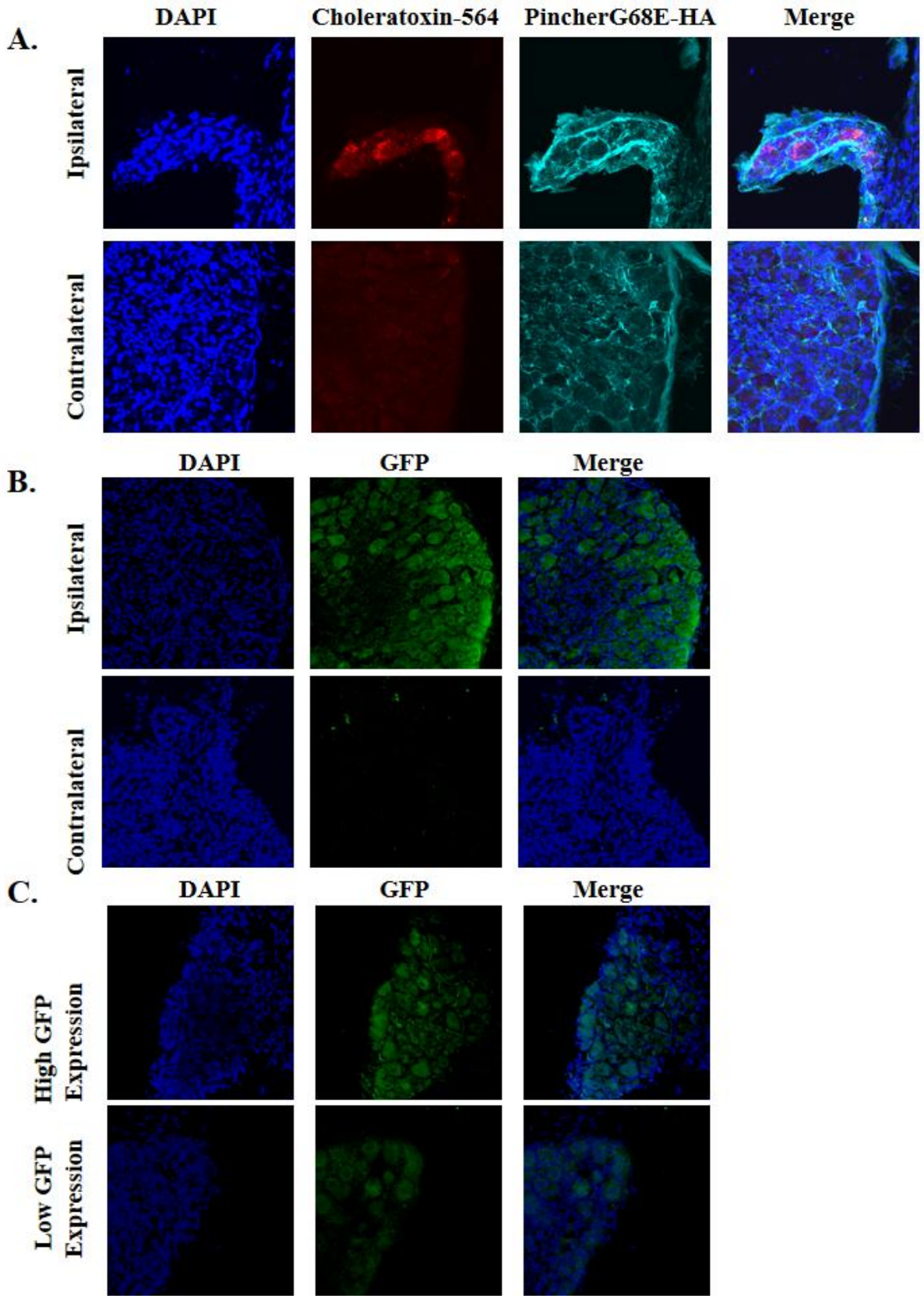
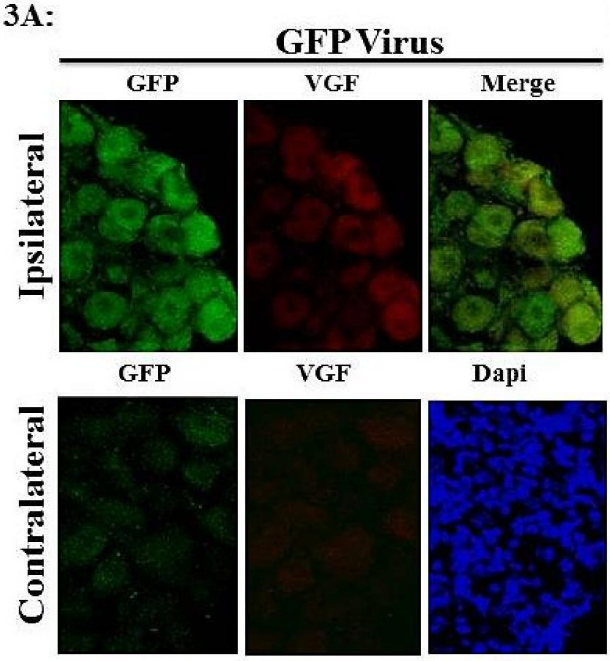

A.
DAPI
Choleratoxin-564
PincherG68E-HA
Merge
Ipsilateral
Contralateral
B.
DAPI
GFP
Merge
Ipsilateral
Contralateral
C.
DAPI
GFP
Merge
High GFP Expression
Low GFP Expression

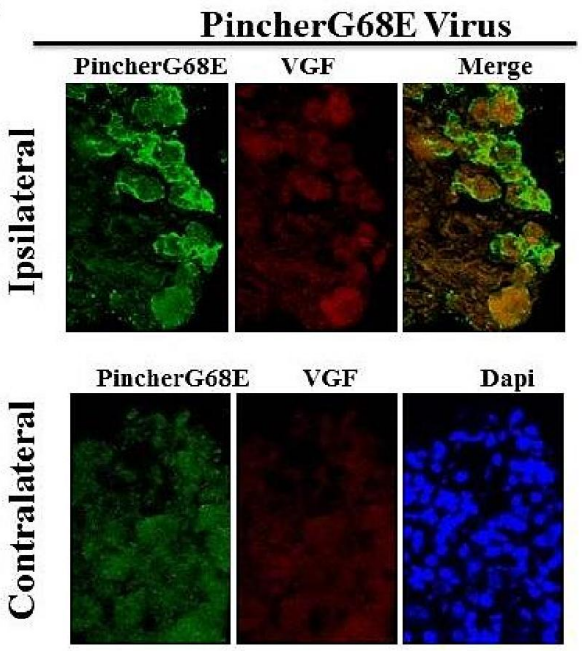
Figure 3: NGF stimulation fails to upregulate VGF expression in DRG neurons expressing PincherG68E:

3A: Ipsilateral cells were infected with GFP-virus and stained with VGF (top panel) after 24 hours of NGF treatment; contralateral cells (bottom panel) don't show any GFP expression, and very low levels of VGF expression. **3B:** Ipsilateral cells were infected with PincherG68E-virus and stained with VGF (top panel) after 24 hours of PBS treatment. Only these cells show PincherG68E expression. Neither contralateral cells (bottom panel) nor ipsilateral neurons give strong VGF signal. **3C:** Bar graph depicts percentage of cells showing elevated VGF expression from different treatment groups. While cells expressing GFP-virus responds to NGF and upregulates VGF, cells expressing dominant negative pincher show no such response.

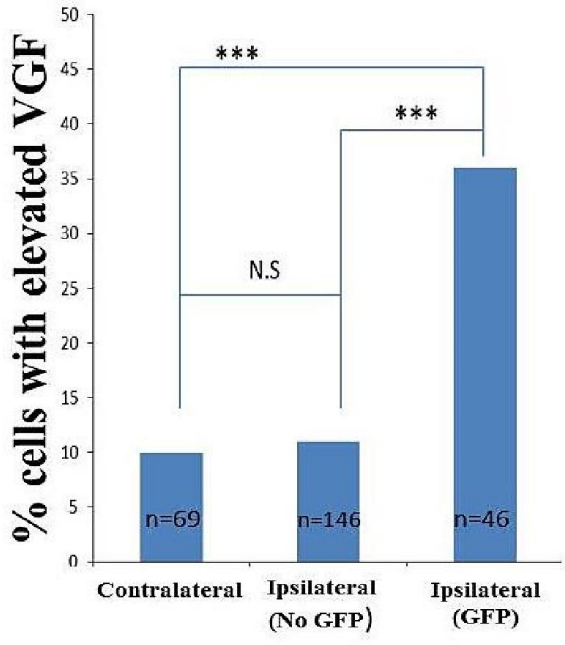
3A:


GFP Virus
GFP
VGF
Merge
Ipsilateral
GFP
VGF
Dapi
Contralateral

3B:


PincherG68E Virus
PincherG68E
VGF
Merge
Ipsilateral
PincherG68E
VGF
Dapi
Contralateral

3C


% cells with elevated VGF
***
***
N.S
n=69
n=146
n=46
Contralateral
Ipsilateral (No GFP)
Ipsilateral (GFP)

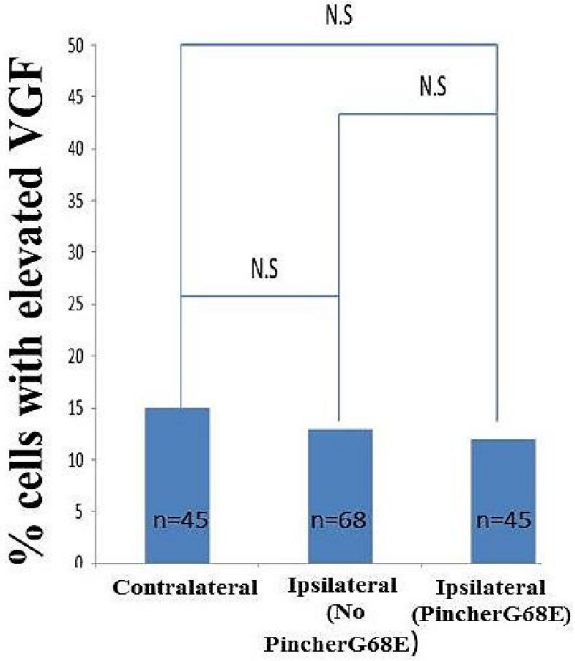
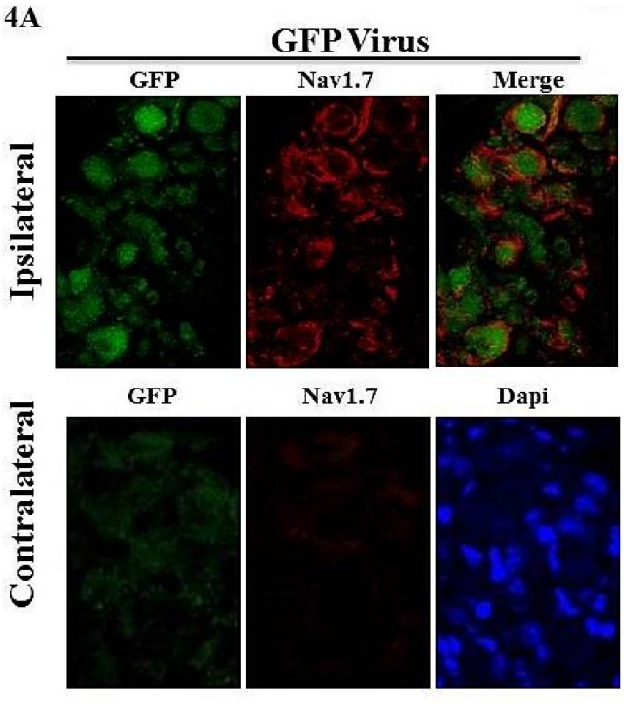

% cells with elevated VGF
N.S
N.S
N.S
n=45
n=68
n=45
Contralateral
Ipsilateral (No PincherG68E)
Ipsilateral (PincherG68E)

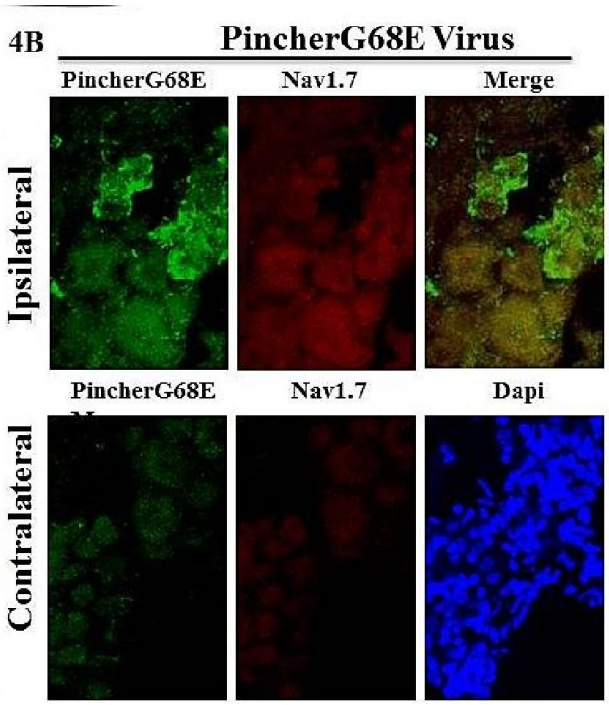
Figure 4: NGF stimulation fails to upregulate Nav1.7 expression in DRG neurons expressing PincherG68E:

4A: Ipsilateral cells were infected with GFP-virus and stained with Nav1.7 antibody (top panel) after 24 hours of NGF treatment; contralateral cells (bottom panel) don't show any GFP expression, and very low levels of Nav1.7 expression. **4B:** Ipsilateral cells were infected with PincherG68E-virus and stained with Nav1.7 (top panel) after 24 hours of PBS treatment. Only these cells show PincherG68E expression. Neither contralateral cells (bottom panel) nor ipsilateral neurons give strong Nav1.7 signal. **4C:** Bar graph depicts percentage of cells showing elevated Nav1.7 expression from different treatment groups. While cells expressing GFP-virus responds to NGF and upregulates Nav1.7 protein levels, cells expressing dominant negative pincher don't respond in similar fashion.

4A


GFP Virus
GFP
Nav1.7
Merge
Ipsilateral
GFP
Nav1.7
Dapi
Contralateral

4B


PincherG68E Virus
PincherG68E
Nav1.7
Merge
Ipsilateral
PincherG68E
Nav1.7
Dapi
Contralateral

4C:

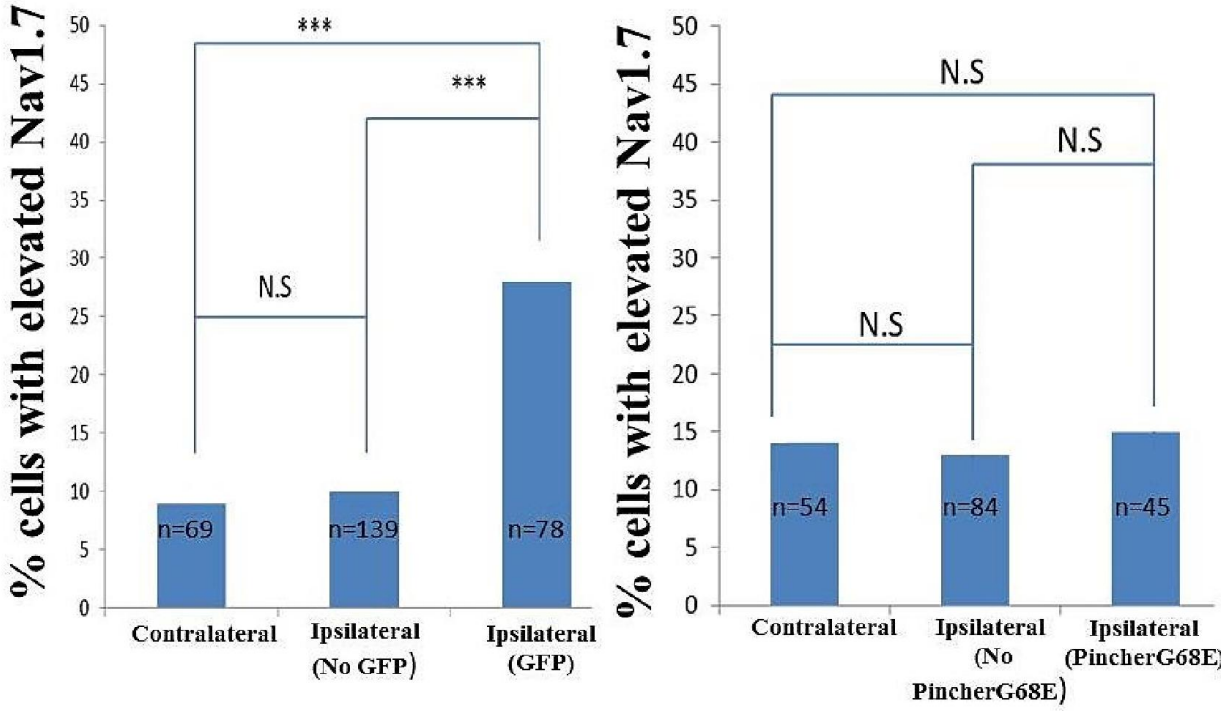
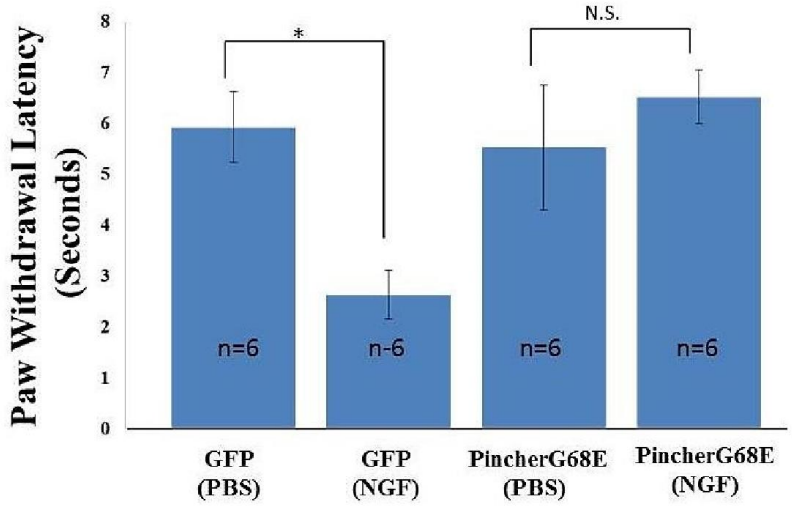

% cells with elevated Nav1.7
***
***
N.S
n=69
n=139
n=78
Contralateral
Ipsilateral (No GFP)
Ipsilateral (GFP)
N.S
N.S
N.S
n=54
n=84
n=45
Contralateral
Ipsilateral (No PincherG68E)
Ipsilateral (PincherG68E)

Figure 5: Mice injected with PincherG68E have attenuated thermal hyperalgesia after 24 hours of NGF administration:

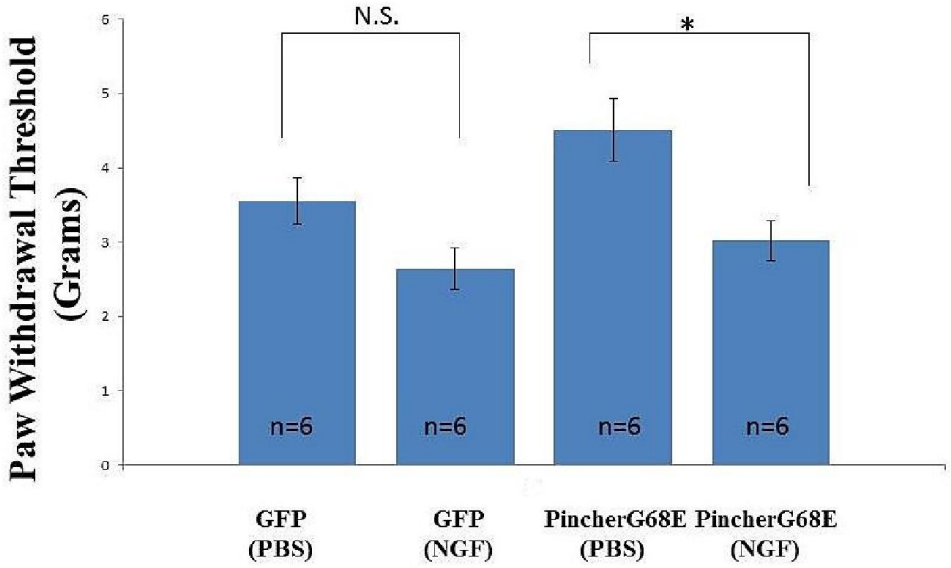
5A. Bar graph shows results from right hind paw withdrawal on Hargreaves Test. Mice were injected with either PincherG68E or GFP lentiviral vectors with rabiesG coat protein. After 12 days, the mice were injected with either 100ng of NGF or PBS for 24 hours before the test. Only mice infected with GFP-Virus and NGF showed lower latency (they withdrew their paws faster than any other groups when heat was applied), and other three groups, including mice injected with both PincherG68E and NGF did not show heightened pain sensitivity.

5B: Bar graph shows results from von Frey test. The minimum amount of force that elicited paw withdrawal was measured. GFP/NGF treated mice showed no significant mechanical hyperalgesia compared GFP/PBS treated mice. However, there was significant difference between PincherG68E/NGF treated mice and PicnherG68E/PBS treated mice suggesting that expression of PincherG68E did not compromise NGF-induced mechanical allodynia.

5A:


Paw Withdrawal Latency (Seconds)
8
7
6
5
4
3
2
1
0
*
N.S.
n=6
n-6
n=6
n=6
GFP (PBS)
GFP (NGF)
PincherG68E (PBS)
PincherG68E (NGF)

5B:


Paw Withdrawal Threshold (Grams)
6
5
4
3
2
1
0
N.S.
*
n=6
n=6
n=6
n=6
GFP (PBS)
GFP (NGF)
PincherG68E (PBS)
PincherG68E (NGF)

Chapter VI:

General Discussion

The results presented show that retrograde NGF signaling leads to not only the upregulation of VGF and Nav1.7, but also chronic hyperalgesia (hyperalgesia lasting for 24 hours). The results also underscore the key role played by the pinocytic chaperone molecule, Pincher, is mediating not only this chronic hyperalgesia, but also the upregulation of these proteins.

Retrograde axonal signaling by NGF has been shown to upregulate gene expression in neurons. In compartmentalized chambers, where neuronal cell bodies are separated from the axons by an impermeable barrier, NGF failed to induce phosphorylation of the transcription factor, CREB, at the cell body when NGF internalization was blocked (Riccio et al, 1997). In a similar experimental set up, a dominant negative form of Dynamin, which blocks TrkA endocytosis, has been shown to prevent Erk5 phosphorylation in response to NGF (Watson et al, 2001). In DRG neurons, Pazrya-Murphy et al (2009) had shown that transcription factor MEF2D and the anti-apoptotic Bcl-2 family member Bcl-w are regulated by target-derived NGF and BDNF—these genes were preferentially induced by neurotrophin stimulation of distal axons compared with neurotrophin stimulation of cell bodies. Also in compartmentalized chambers, our lab has shown that retrograde NGF signals from distal axons can upregulate VGF expression in Superior Cervical Ganglion (SCG) neurons (Philippidou et al, 2011).

Here, I used microfluidic devices to determine whether retrograde NGF signaling from the axon terminals can upregulate VGF and Nav1.7 protein and mRNA levels in DRG neurons. In these devices, by maintaining a volume difference between the axonal and cell body

compartment, a biochemical barrier can be established between the two compartments. My results show that DRG neurons grown in these devices do respond to NGF stimulation at the axonal terminal by upregulating both mRNA and protein levels of VGF and Nav1.7. While VGF transcriptional upregulation by NGF has been previously demonstrated in PC12 cells (Levi et al, 1985, Salton SR, 1991, D'Arcnagelo and Halegoua, 1993) and increased VGF expression in SCG neurons (Philppidou et al, 2011), to the best my knowledge, this is the first time retrograde NGF signaling has been shown to upregulate VGF mRNA and protein levels in DRG neurons.

In PC12 cells, NGF has also been shown to transcriptionally upregulate mRNA levels of Nav1.7channel (Toledo-Aral et al, 1995). Subcutaneous paw injection of NGF has been shown to increase Nav1.7 protein levels in DRG neurons (Gould III et al, 2000), however it's not clear if the upregulation of Nav1.7 was caused by retrograde NGF signaling or by other factors released by non-neuronal cells. Since my cells were mostly of pure neuronal population, cells grown in microfluidic devices allowed me to test whether NGF, added to the axon terminals, could retrogradely upregulate Nav1.7 mRNA and protein levels. My result show that retrograde NGF signaling is capable of upregulating Nav1.7 mRNA and protein levels: NGF stimulation at the axon terminals led to this upregulation.

I injected NGF into the mouse hindpaw to determine whether retrograde NGF signaling could upregulate VGF and Nav1.7 protein levels in vivo. NGF injection contained Q-dots to mark the cells that innervated the injection site, and for my analysis, I only counted the cells that had taken up the Q-dots. My results show that NGF injection at the paw could retrogradely upregulate both Nav1.7 and VGF protein levels in L4-L6 DRG neurons collected from the injected mice 24 hours after the injection. The number of cells showing elevated levels of VGF and Nav1.7 expression was significantly different from the number of neurons collected from the

contralateral side: Q-dot containing ipsilateral neurons had increased expression of VGF and Nav1.7.

The Signaling Endosome Hypothesis suggests that internalization of NGF bound TrkA, formation of endosomes containing NGF/TrkA complex, and subsequent retrograde transport of these signaling endosomes are crucial for retrograde NGF signaling. However, the mechanism of how these events take place and the identity of molecular players that carry out these tasks remained elusive until recently. Studies from our lab suggest that endocytosis and retrograde transport of NT/Trk complexes is mediated by a novel, pinocytic mechanism called macroendocytosis (Shao et al, 2002, Valdez et al 2005, Valdez et al, 2007), and Pincher, a member of ESP15 homology domain (EHD) protein family, plays crucial roles in this endocytosis mechanism. Overexpression of a dominant negative form of Pincher (PincherG68E), led to accumulation of activated TrkA and Erks on distinctive membrane regions, blocked TrkA internalization (Shao et al, 2002), and eliminated retrograde NGF signal-induced neuronal survival (Valdez et al, 2005). Having shown that retrograde NGF signal could upregulate VGF and Nav1.7 mRNA and protein levels in vivo and in vitro, I then asked whether this upregulation could be abolished by blocking endocytosis.

By using a pseudotyped lentiviral vector, I expressed a dominant negative form of Pincher, PincherG68E, in DRG neurons, and show that cells expressing PincherG68E do not respond to retrograde NGF stimulation. These pseudotyped lentiviral vector contained RabiesG coat protein that enable them to infect cells retrogradely and thus by adding the viral particles to the axonal compartment, I could mark the projecting cells, and determine their response to retrograde NGF stimulation. Here I show even though the cells that did not express PincherG68E upregulate VGF when NGF was added to axonal chambers, PincherG68E expressing neurons

don't show increased VGF expression. Nav1.7 mRNA and protein levels were also upregulated in cells that did not express PincherG68E, but the cells that expressed PincherG68E failed to show elevated Nav1.7 levels.

To address the concern that infection had compromised cells' ability to upregulate these two genes, I used a similar lentiviral vector to express GFP and show that the cells expressing GFP virus do respond to retrograde NGF stimulation to upregulate VGF and Nav1.7 protein levels. Furthermore, by infecting DRG neurons in microfluidic devices by GFP or PincherG68E virus, I show that compared to cells infected with GFP-virus, PincherG68E infected cells show markedly reduced expression of VGF and Nav1.7 upon NGF stimulation at the axon terminals. My results are similar to previous finding from our lab where PincherG68E-Herpes Simplex Virus (HSV)-derived vector was shown to block NGF-induced upregulation of VGF expression in SCG neurons (Philippidou et al, 2001).

I then proceeded to determine whether PincherG68E expression could lead to attenuation of NGF-induced upregulation of VGF and Nav1.7 expression in vivo. I injected the same pseudotyped lentiviral vector carrying PincherG68E into the paw to not only mark the cells innervating the injection site, but also do block retrograde signaling. As control, I also injected another group of mice with GFP-virus. After 12 days of infection, I injected the mice with NGF into the same injection site, and collected L4-L6 DRG neurons to determine VGF and Nav1.7 protein levels in these cells. My results show that while GFP expressing neurons do respond to NGF injection by upregulating VGF and Nav1.7 protein levels, PincherG68E expressing cells don't respond to NGF injection and the level of VGF and Nav1.7 expression is comparable to cells from contralateral side of the injection. Thus, data from both in vivo and in vitro experiments highlight the importance of Pincher in mediating retrograde NGF signal since cells

expressing the dominant negative form of Pincher did not respond to retrograde NGF stimulation.

One striking aspect of NGF is that the growth factor has been shown to cause both acute and chronic hyperalgesia in animals: systemic application of a single dose of NGF (1mg/kg i.p) in rats, for example, produces thermal hyperalgesia that develops within minutes (acute hyperalgesia) and lasts for 4 days (chronic hyperalgesia) (Lewin et al. 1993). Acute hyperalgesia involves indirect sensitization of nociceptors via mast cell activation, and acute sensitization of nociceptors via TRPV1 sensitization. While activation of TRPV1 channels can mediate the acute thermal hyperalgesia, increased levels of NGF also leads to chronic hyperalgesia. Lewin et al. (1994) showed that activation of NMDA centrally in the dorsal horn play an important role in mediating this chronic hyperalgesia as non-competitive NMDA antagonist MK-801 blocked the late phase of hyperalgesia in NGF treated rats (7 hours to 4 days after NGF injection). Activation of NMDA receptor leads to Ca^{2+} entry inside spinal cord neurons located at the dorsal horn and initiate calcium-sensitive intracellular signal cascades. The signaling cascade could then lead to the phosphorylation of the NMDA and other receptor/ion channels and the neurons would then remain excitable for a prolonged period (Woolf and Salter, 2000). This process, known as central sensitization, thus, could lead to chronic hyperalgesia. It is thought that primary afferent neurons can also be sensitized for prolonged period to cause chronic hyperalgesia and this sensitization may involve gene upregulation.

As discussed previously, hyperalgesia caused by NGF-induced upregulation of gene expression is generally thought to take place with a delay (from hours to days). This delay would be expected because 1) NGF-TrkA complex needs to be retrogradely transported from the axon terminals to the cell bodies and 2) modulation of gene expression by retrograde NGF-TrkA

signal (at the cell body) need to take place. Since protein upregulation could alter biochemical properties of a nociceptor, it's possible that increased expression of a particular gene could lower the cell's threshold for an action potential. Innocuous or low level of stimulation could lead the cell to fire, and elicit hyperalgesic response such as decreased paw withdrawal latency. Peripheral sensitization can occur rapidly leading to hyperalgesia, pain is less likely to last long: once the stimulus is removed, hyperalgesia should decrease since long term cell physiology is not altered (i.e, no change in gene expression). Peripheral sensitization, hence, should lead to acute hyperalgesia while upregulation of genes should lead to increased pain sensitivity with a longer duration. However, these ideas have never been tested. Since I have shown that retrograde NGF signaling upregulate VGF and Nav1.7 expression and these two genes have been implicated in normal pain perception/ hyperalgesia, I then asked whether blocking of retrograde NGF signaling would lead to attenuation of chronic hyperalgesia in mice.

Since the pseudotyped lentivirus expressing PincherG68E blocked retrograde NGF signaling, I injected mice with the same virus and determined paw withdrawal latency (to an intense heat source) of these mice upon subsequent NGF injection. I tested these mice on Hargreaves apparatus after 24 hours of NGF injection to specifically ask whether the chronic hyperalgesia could be attenuated by blocking retrograde NGF signaling, and show that PincherG68E infected mice don't develop chronic thermal hyperalgesia upon NGF injection: there was no statistical difference between the group of mice that had received NGF injection and the group of mice that had received only PBS injection. GFP-expressing mice, however, did respond to NGF injection and the paw withdrawal latency for these mice decreased significantly.

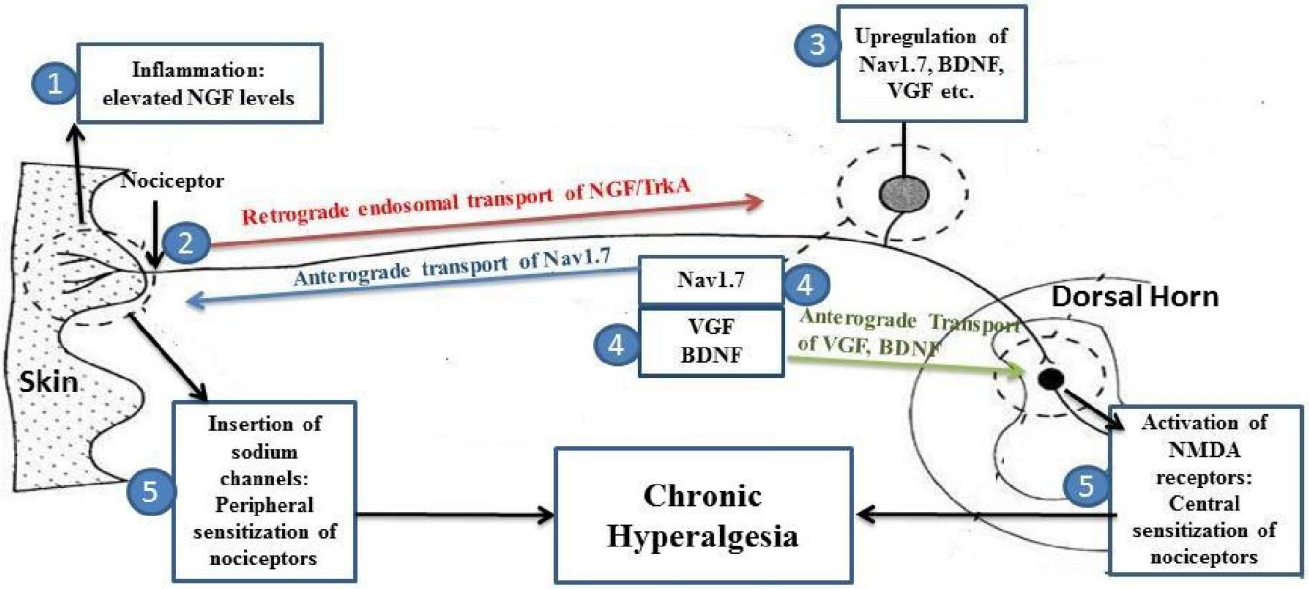
Based on this finding, I propose the following model: NGF-induced acute hyperalgesia could be mediated by TRPV1 sensitization and doesn't require gene upregulation, however,

NGF-induced chronic hyperalgesia involves gene upregulation. Gene upregulation could sensitize nociceptors by at least two ways: upregulation of genes that mediate signal transmission from the skin to the spinal cord, and upregulation of genes at the dorsal horn that can modulate signal transmission from the peripheral to the central nervous system. According to this model, upregulation of genes such as Nav1.7 could lead to chronic peripheral sensitization (by lowering the threshold for action potential in the nociceptors) and cause chronic hyperalgesia. On the other hand, upregulation of genes such as BDNF and VGF at the dorsal horn, which are capable of activating receptors like NMDA, would lead to increased signal transmission and increased pain perception/hyperalgesia (Figure 1). Retrograde NGF signaling is essential for this process as expression of PincherG68E not only blocked NGF-induced upregulation of Nav1.7 and VGF protein levels, but also attenuated chronic hyperalgesic response to NGF treatment. Since proper expression of sodium channel is critical for conveying pain signal from the paw to the spinal cord, it's most likely that NGF treatment upregulate Nav1.7 expression in neurons, and increased levels of this sodium channel make these neurons hyperactive and mice hyperalgesic. Since blocking retrograde NGF signal prevents this upregulation, these mice don't become hyperalgesic. In order to validate the model, however, a critical experiment needs to be done. It needs to be determined, for example, whether PincehrG68E infected mice develop acute hyperalgesia upon NGF injection. A future time course study on the development and progression of NGF-induced hyperalgesia in PincherG68E expressing mice would shed more light into the role played by retrograde endosomal signaling.

Figure 1: Model for the development of chronic hyperalgesia by retrograde endosomal transport.

Inflammation can increase the levels of NGF in the skin. NGF, with its high affinity receptor, TrkA can then be retrogradely transported in endosomes to the cell body where it can modulate gene expression. Upregulation of genes can lead to either peripheral or central nociceptor sensitization.. Peripheral sensitization: upregulation of Nav1.7, and subsequent transport of the channel to the skin can increase number of functional channels there. This could lower action potential threshold of the nociceptors, and lead to chronic hyperalgesia. Central sensitization: Upregulation of BDNF, or VGF, and subsequent transport and release of these proteins at the dorsal horn could activate NMDA receptors. Once activated, NMDA receptors can lead to central sensitization of nociceptors.

Figure 1:



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