COLLATERAL SPROUTING OF UNMYELINATED PRIMARY AFFERENTS LACKING RECEPTORS FOR NERVE GROWTH FACTOR

By

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SYMBOLS AND ABBREVIATIONS

192-sap	saporin-conjugated antibody 192 (against p75)
ABC	avidin-biotin-HRP-complex kit
CAP	compound action potential
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CTM	cutaneus trunci muscle
CS	collateral sprouting
DβH	dopamine-beta-hydroxylase
DβH-sap	saporin-conjugated antibody against DβH
DRG	dorsal root ganglion/ganglia
DCn(n)	dorsal cutaneous nerve (nerves)
FRAP	fluoride-resistant acid phosphatase
GDNF	glial cell line-derived neurotrophic factor
GS-I-B_4	isolectin B_4 from *Griffonia simplicifolia* type I
HRP	horseradish peroxidase
IR	immunoreactive/immunoreactivity
LCn(n)	lateral cutaneous nerve (nerves)
LTn(n)	lateral thoracic nerve (nerves)
NGF	nerve growth factor
trkA	high-affinity nerve growth factor receptor
P2X3	P2X family of ATP-gated ion channels - subunit 3
p75	low-affinity neurotrophin receptor
PACS	primary afferent collateral sprouting
PBS	phosphate buffered saline
PFA	paraformaldehyde
PGP 9.5	protein G product 9.5 (ubiquitin hydroxylase)
PNS	peripheral nervous system
PRV	pseudorabies virus
RET	GDNF-receptor - tyrosine kinase subunit
RF	receptive field
SOM	somatostatin
TH	tyrosine hydroxylase
TSA	tyramide signal amplification kit
VR-1	vanilloid receptor 1 (capsaicin-sensitive ion channel)

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COLLATERAL SPROUTING OF UNMYELINATED PRIMARY AFFERENTS LACKING RECEPTORS FOR NERVE GROWTH FACTOR

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Collateral sprouting occurs in both the peripheral and central nervous systems. It is a mechanism for rapid recovery of function after certain injuries, and also underlies such vital processes as the establishment of new anatomical connections during learning and memory formation.

Primary afferent collateral sprouting (PACS) is a process whereby uninjured sensory neurons respond to the denervation of adjacent tissue by extending branches into the denervated territory. It was previously determined that this process was limited to small diameter afferents with high thresholds to electrical stimulation. It was also determined that sensory neurons with receptors for nerve growth factor (NGF) were capable of PACS, and that NGF was paramount for the process. This study examined whether or not the population of small diameter neurons lacking NGF receptors (i.e., non-trkA) was capable of PACS.

The non-trkA small diameter afferents are those that become dependent on glial cell line-derived neurotrophic factor (GDNF) during early postnatal life. Since NGF has been shown to regulate PACS of trkA expressing neurons, it is possible that GDNF may have a role in PACS of the GDNF-dependent neurons. This would indicate that another neurotrophic system may be involved in plasticity of adult neurons.

It was determined, using multi-labelling immunohistochemistry, that axons with markers specific for non-trkA neurons were present in collaterally reinnervated skin. Further, contrary to the common understanding, non-trkA neurons expressed GAP-43 (a marker associated with axonal growth) in both normal ganglia and those undergoing PACS. These results indicate that the small diameter afferents lacking trkA are likely involved in PACS, but had been missed by previous experiments.

It was determined, using 1) selective destruction of neurotrophin receptor (i.e., trkA) bearing neurons and 2) transynaptic neuroanatomical tracing, that the small diameter neurons lacking trkA were not involved in the reflex pathway that had been the standard measure for the success and extent of PACS. This implies that the reflex is not suitable for assessing PACS of non-trkA neurons, and provides a partial explanation as to why the non-trkA neurons had been missed in previous PACS investigations.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Overview

Collateral sprouting (CS) is a process by which neurons extend newly-formed appendages out from their original axonal processes. This phenomenon can be part of a normal process such as the formation of new synaptic contacts during learning[193, 201, 221-223]. CS can also be induced in response to an injury where it can play a role in the restoration of numerous functions[17, 26, 46, 51, 52, 152, 240, 242]. However, this response to injury can sometimes lead to the development of pathologic conditions[35, 36, 124, 183]. Thus, experimentation to determine which neurons are capable of CS under both normal and pathologic conditions is important for the understanding of the mechanisms of collateral sprouting.

Collateral sprouting is a dynamic growth process that can be observed in certain neurons in the central nervous system (CNS) and in the peripheral nervous system (PNS). In the CNS, indicators of CS are routinely observed in neurons that are involved in the establishment of new anatomical connections in areas such as the hippocampus and cerebellum. These new connections are believed to provide a portion of the substrate for learning and memory. Collateral sprouting in the PNS has been observed in response to injury of neighboring axons or manipulation of the growth factor environment, and is displayed by motoneurons[17, 24, 25, 26, 41, 83, 152, 240, 242], sympathetic ganglion neurons[1, 70, 71, 91, 96, 100, 102, 103, 124, 136, 189], and primary afferent neurons, though limited to Aδ- and C-fibers[5, 47, 49-

[53, but see 58, 82, 84, 90, 93, 101/, 108, 216]. Studies focussing on primary afferent collateral sprouting (PACS) deal with the easily accessed and manipulated periphery, and may provide vital insights that can be applied to all forms of CS. The experiments detailed herein focus on PACS, which appears to be limited to the small diameter DRG neurons[47, 84, 93].

Most PACS experimentation thus far has primarily employed the cutaneous trunci muscle (CTM) reflex system as a monitor of its success and progress (see below). It has been determined that PACS, as it is currently understood, is a nerve growth factor (NGF) *dependent* process involving afferents that respond to directly NGF [49, 52]. **The methods used to test and monitor PACS, however, have not addressed the possible role of a subpopulation of unmyelinated primary afferents *lacking* receptors for NGF.** This dissertation recounts the investigation of the role of this subpopulation of primary sensory neurons in PACS.

The sensory neurons in question are a subpopulation of the small diameter, dark type-B dorsal root ganglion neurons. This subpopulation of small diameter DRG neurons is the group that does not express neurotrophin receptors, but binds the isolectin B_4 from *Griffonia* (or *Bandeireae*) *simplicifolia* type I (GS-I-B_4)[7, 146, 157, 158, 160]. The role of these neurons in signal transduction and recovery after injury is unknown. The entire small diameter, dark type-B population has unmyelinated, or thinly myelinated, processes [120, 122, 172] and is generally believed to be involved in transducing noxious stimuli, but may also subserve other functions which are currently unclear[203]. The anatomy and regulatory factors of these GS-I-B_4-reactive small diameter neurons have only recently begun to be elucidated[12, 64, 159, 176, 180, 181, 186, 187, 205, 207, 211, 212, 235, 236].

Primary Afferent Neurons

Non-cranial primary afferents are located in the intervertebral foramina in gross structures called dorsal root ganglia (DRG). DRG afferents are derived from the neural crest, and innervate all tissues including skin, muscle, and viscera.

Afferents of a given DRG have segmentally arranged central termination patterns as well as having a relatively well defined peripheral dermamyotome. The course of the peripheral axons from a DRG vary greatly from ganglion to ganglion. DRG from the cervical and lumbar plexus regions supply a great variety of nerves. Many DRG feed axons to a single nerve, and a single DRG has neurons that form many different nerves. The thoracic DRG, however, are more regular and segmentally restricted. The "cross-talk" between the thoracic cutaneous nerves and the DRG of adjacent segments is very small[247]. Therefore, the thoracic DRG are the most appropriate for PACS modeling.

Each DRG houses many different functional families of neurons. The particular groups and proportions vary with the tissues innervated at each segment. There are dozens of different functional types of primary afferents. General families include low-threshold mechanoreceptors, high-threshold mechanoreceptors, thermoreceptors, and chemoreceptors. These general functions are often mixed in single neurons, and the latter three are generally associated with nociceptor function (afferents transducing noxious and/or tissue damaging stimuli).

Certain correlations have been made between afferent structure and function. Unmyelinated axons are the smallest in diameter, and axon diameter increases with increasing thickness of myelination. Conduction velocity also increases with increasing axon diameter. The neurons that give rise to unmyelinated axons consistently have a small diameter soma (<35um), while the majority of neurons that give rise to myelinated

axons are medium to large diameter, with there being a trend of larger somal diameter giving rise to larger axon diameter[78, 79, 122]. However, some neurons with very small somal diameter have large myelinated axons[78, 79, 122].

Certain correlations have also been demonstrated between structure or function and various histochemical phenotypes. DRG neurons have been broken up into small-dark and large-light groups based on their somal size and appearance under light microscopic examination. These groups were shown to generally correspond to the myelination state of axons, with the small-dark being unmyelinated and the large-light being myelinated. Recent work has supported this relation between phenotype and myelination, but has offered a more accurate interpretation. One new development is the correction of the "large" in large-light. There are, in fact, some small neurons that display the "light" phenotype. These neurons have been shown to have other indicators of having myelinated axons [78, 79, 122]. For this reason, Lawson and colleagues have dropped the term "large-light" and simply distinguish light neurons from small-dark neurons. Lawson and colleagues have also generated highly convincing data that the light population (i.e., myelinated) expresses high levels of neurofilament triplet proteins (NF-H, NF-M, and NF-L), while the small-dark population expresses very low levels. The two populations can be reliably distinguished based on their immunoreactivity for neurofilaments[122, 172]. Thus, neurofilament (NF) immunoreactivity can be an indicator of myelination state.

The small-dark population can be further subdivided into two groups, those that express one of more of the major known sensory neuropeptides (peptidergic; eg., calcitonin gene-related peptide - CGRP, substance P - SP, somatostatin - SOM), and those that do not (non-peptidergic). CGRP is considered the prototypical sensory

neuropeptide, being expressed either alone, or co-expressed with other neuropeptides[69, 125, 126, 209, 239]. The majority of the non-peptidergic small-dark neurons are bound by the isolectin IB_4 from *Griffonia simplicifolia* type one (GS-I-B_4) which binds primarily to α-D-galactose residues[205, 207, 211, 212]. Thus, nearly every DRG neuron will demonstrate either NF-IR, CGRP-IR, or GS-I-B_4-binding, and many will display a combination.

It is the combinations of markers that can dramatically improve the utility and power of histochemical examinations of DRG neurons. Families can be more accurately defined by the presence or absence of multiple markers, as opposed to the presence or absence of a single marker. For example, NF-IR defines myelinated neurons, but does not distinguish between Aβ and Aδ afferents, and certainly can not indicate whether any of these may be nociceptors. CGRP-IR encompasses the vast majority of peptidergic nociceptors, but will not reveal whether any given CGRP-IR neuron may be myelinated. However, if used together, the markers will delineate nearly all myelinated non-nociceptive neurons ($NF^+/CGRP^-$), unmyelinated peptidergic nociceptors ($NF^-/CGRP^+$), and myelinated nociceptors ($NF^+/CGRP^+$), and non-peptidergic unmyelinated afferents ($NF^-/CGRP^-$)[144].

A variety of markers that define sensory neuron subgroups have been described. These include the expression of sensory neuropeptides (CGRP, SP, SOM), neurofilaments (NF), intermediate filaments (peripherin and α-internexin), enzymes (fluoride-resistant acid phosphatase - FRAP; carbonic anhydrase; neuron specific ubiquitin C-terminal hydroxylase - PGP 9.5; choline acetyltransferase - ChAT), calcium-binding proteins (calmodulin, calbindin, parvalbumin), growth factor receptors (neurotrophin receptor tyrosine kinases - trk family or p75; glial cell line-derived neurotrophic factor tyrosine kinases - RET and GFR family), chemical receptors (VR-1,

P2X family, AChR family), ion channels (PN1, SNS, Kv family), and binding of lectins. Figure 1 is a schematic representation of some of the overlaps of the markers in the DRG.

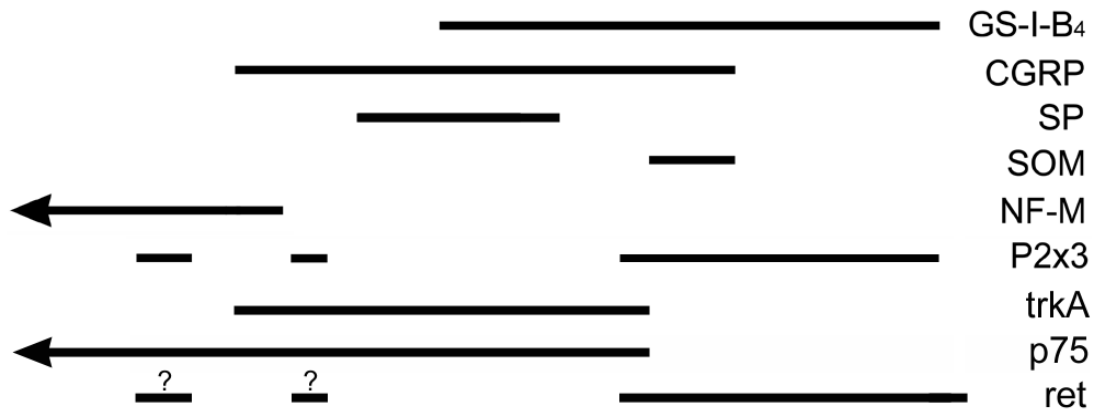

Figure 1. Schematic representation of the general overlaps of certain markers in small and medium diameter DRG neurons. Not all comparisons have been tested directly and therefore some overlaps, or lack thereof, may not be entirely correct. However, the general scheme is highly representative. Arrows indicate a continuation of the marker into the large diameter population. The distribution of RET outside of the GS-I-B_4-reactive population in medium and larger neurons has been described[159], but the overlaps with other markers are unclear, as indicated with the question marks.

Characteristics of Primary Afferent Collateral Sprouting (PACS)

Peripheral nerve injury results in the induction of two growth processes - regeneration of all fiber types in the injured nerve and CS of the adjacent uninjured nerves[47]. The second form of growth, CS, is unique in that the *uninjured* neurons somehow respond to the injury and begin to grow and restore function. The restoration of function is achieved by an apparent growth of peripheral axons into denervated areas of tissue. CS is known to occur in humans, but the extent of the process appears to be somewhat less than what occurs in animal models[5, 82, 90]. Neurons involved in this form of plasticity appear to maintain proper modality transduction and somatotopic

arrangement[47, 51, 52, 173], and are thus able to provide a more rapid restoration of function to denervated regions than would occur from regeneration alone[23, 47, 82, but see 102, 103].

The primary model for PACS investigations in the rodent has been the dorsal hairy skin of the back and the cutaneus trunci muscle (CTM) reflex system. Though others have been used[47, 108, 109, 164, 174, 191, 216, 238], such as denervating the plantar surface of the hindpaw by tibial nerve ligation and allowing reinnervation by the saphenous and caudal cutaneous sural nerves, these do not have the anatomical homogeneity present in the thoracic segments (the spared nerve/nerves is supplied by a single DRG). When an area of insensitivity is produced in the rat CTM reflex system by transection of multiple adjacent dorsal cutaneous nerves (DCnn), it is the high threshold stimulus transducing primary afferent axons that begin to sprout into the denervated areas of skin. Thus far, only examinations with pinch or heat have been reported[166, 173, 217]. Expansion of the low threshold peripheral fields has been tested using natural stimuli and electrical recording of the isolated nerve. Such work has shown no evidence of large diameter axon (low threshold / light touch transducing) collateral sprouting[84, 92, 93], though there may be evidence for this in other systems[58]. Certain lines of evidence strongly suggest that this expansion of high threshold sensitivity is due, at least in part, to an actual growth of axons through the skin into the denervated areas. Silver stains of behaviorally insensitive skin (skin areas where noxious stimuli are incapable of driving the CTM reflex) reveal no visible axons in dermal endoneurial tubes[51]. These same silver stains used on sections of skin regions to which high threshold (but not low threshold) sensitivity has returned after collateral sprouting revealed that axons were present in dermal bundles in areas of skin that had regained high threshold sensation. Such staining was not present after pharmacological treatments to prevent sprouting[51].

The speed of recovery and expansion of the sensitive field into the denervated area is very similar to what would be expected based on other neural growth systems[51, 109, 225]. It can first be reliably detected at 10-12 days post DCn transection and its maximum extent (regardless of the size of the denervated field) is reached at approximately 4 weeks post-transection. In models sparing a single DCn inside of a denervated field, the high threshold receptive field (RF) can approximately double its size over the course of PACS. Intravenous administration of Evan's Blue dye in conjunction with antidromic stimulation of spared sensory nerves at different times after denervation of surrounding areas revealed a gradual expansion of the area of skin into which the dye was extravasated. These extravasation fields matched extremely well with the behaviorally responsive areas of skin at all time points[51, 59]. Further, mRNA for the protein GAP-43, shown to be a marker of neuronal growth and axonal extension[13, 16, 31, 199], increases in neurons of the DRG supplying the spared DCn[151].

Molecular Signalling of PACS

The expansion of the high threshold receptive field into denervated areas of skin has been shown to be a nerve growth factor (NGF) dependent process[49, 52]. Administration of anti-NGF antibodies during PACS prevented any expansion of the receptive field for the duration of the treatment[49, 52, 58]. The expansion resumed once the treatment had been terminated. Additionally, exogenous NGF could induce PACS *de novo*, as revealed by the ratio of high threshold : low threshold receptive field sizes[52, 148]. Other systems where NGF concentration has been increased have also lead to collateral innervation by unmyelinated sensory afferents[101].

Message (mRNA) for both the high affinity (trkA) and the low affinity (p75) NGF receptors has been shown to be upregulated in DRGs that housed neurons involved in PACS[149, 151], as well as in other neurons undergoing CS[115]. These studies did not directly examine whether the increases were due to a new population of neurons beginning to express the receptors or whether those already expressing them simply increased their production, the latter is more likely. It has been shown that the proportion of neurons expressing NGF-receptor mRNA was similar between normal and sprouting groups[149, 151]. It has also been shown that the supply of target-derived NGF can influence the expression of NGF receptors[112]. Therefore, it is more likely that the increases in trkA and p75 in PACS DRG were due to the increases in expression levels by neurons already expressing the receptors, as opposed to the recruitment of a new group of neurons to express trkA and/or p75.

It has also been shown that NGF mRNA was increased in denervated skin[150]. These findings support the hypothesis that PACS, and perhaps CS in general, is induced by a buildup of NGF (or other factors) in a denervated area that is detected by intact neighboring axons. The buildup is likely due to the increased production of NGF by the target tissue and the reduced uptake and transport by damaged neurons. These axons may interpret the change in the growth factor environment as a cue to sprout toward the source of NGF. It has been shown that NGF-sensitive axons will alter their direction of growth in response to gradients of NGF [66, 86, 106]. It appears that this buildup of endogenous NGF is local and perhaps even compartmentalized[48]. Endogenous increases of NGF as a result of focal denervation is unlikely to cause widespread collateral sprouting of NGF responsive neurons[173].

Further evidence for PACS being an active growth process and involving, at very least, trkA-bearing DRG neurons, is found with examinations of the expression of growth-associated protein 43 (GAP-43). GAP-43 has been definitively shown to be involved in the growth of neural processes in both the normal and regenerating CNS and PNS [2, 3, 14, 31, 32, 153, 170, 200, 241]. GAP-43 has also been shown to be expressed by neurons involved in PACS and other forms of CS [17, 98, 148, 149, 151, 152], and found only in neurons also expressing NGF receptors[148, 149, 230], though this observation is questioned by work presented herein (see below - Limitations in testing PACS; Specific questions raised by previous PACS data).

As mentioned above, PACS has been shown to be limited to the medium-small diameter DRG neurons, to the exclusion of larger low-threshold neurons, and involves those with NGF receptors. As indicated in Figure 1 (above), trkA is normally found in about half of DRG neurons, and overlaps almost entirely with the peptidergic (i.e., CGRP-IR) medium and small diameter neurons[157]. The low-affinity receptor, p75, has a more extensive distribution, but is limited to the population of neurons expressing one or more of the high affinity neurotrophin receptors (trk family)[243]. Quite importantly, neither of these NGF receptors (nor other trk receptors) are expressed by about 30% of the small diameter neurons[157, 243]. These neurons instead express the components of the glial cell line-derived neurotrophic factor (GDNF) complex (RET, GFRα) and are included in the population that shows GS-I-B_4-binding[12, 157, 159]. Therefore, the small diameter population consists of two general subpopulations - those expressing NGF receptors, and those expressing GDNF receptors. There is clear evidence for the role of NGF and the trkA-bearing neurons in PACS. However, there is little evidence regarding the possible role of GDNF and/or the RET-bearing neurons in PACS. Some of the NGF-

related data could be taken to indicate that there may not be a role in PACS of non-trkA neurons, but such conclusions would be premature as their role was not directly tested (see below). Most examinations did not address, or even acknowledge, the non-trkA-bearing small diameter afferent population[23, 49, 50, 52, 149, 151, 173, 216].

Methodological Limitations in Previous PACS Testing

Examinations of collateral sprouting thus far have focussed, either by commission or omission, on the trkA receptor-expressing DRG neurons. TrkA-expressing neurons are primarily small-medium diameter *peptidergic* neurons[157, 227](Figure 1). This focus has ignored another subpopulation of small diameter DRG neurons which may be involved in PACS. This other population of neurons has unmyelinated axons, includes the *non-peptidergic* neurons[43, 205, 207, 235] as well as those peptidergic neurons that express somatostatin-IR [227, 228, 230]. All of the neurons in this other population *lack* expression of any of the known neurotrophin receptors, including trkA and p75 [146, 160, 227, 228, 243]. Examination of normal rat hairy and glabrous skin has revealed the presence of this population in the shallow dermis and certain areas of epidermis[64, 176, 186]. A detailed description of this unique population is provided below (see below - Unmyelinated Primary Afferents Lacking NGF Receptors).

Evan's Blue Dye Extravasation

Many of the experimental techniques that have been used thus far in testing PACS have provided a great deal of important and conclusive data, but are more limited in their scope of interpretation than was originally believed. Thus, some of the conclusions that have been made based on these data are perhaps incomplete, or

overstated. For example, revealing the functional terminations of axons in skin using the Evan's Blue dye/antidromic stimulation method actually reveals the terminal fields of only a *subset* of axons. The axons revealed are those expressing neuropeptides - in particular SP and CGRP - as these peptides are released during antidromic stimulation and cause vasodilation and extravasation of the dye[94, 134, 147, 195, 244]. This method revealed that the spared fields expanded[23, 139, 174, 191, 238], and that the CTM reflex receptive fields defined by behavioral testing matched extremely well with extravasation-revealed terminal innervation field [52, 174]. However, this method could not address the terminal distribution of non-peptidergic axons, such as much of the GS-I-B_4 reactive population[64, 176, 186], or those expressing SOM, which also bind GS-I-B_4 [235] (See Figure 1).

Histochemical Analysis of Peripheral Targets

Silver staining of axons in the collaterally reinnervated skin was also limited. This method revealed only axons in dermal endoneurial tubes[52], and could not address terminals or axons in the sub-epidermal plexus, a major path for growth of axons in skin[81], even though such structures were shown to exist in collaterally reinnervated skin[107]. Also, it could not discriminate between different histochemical subpopulations of axons, and was likely also to not label a significant portion of the smallest axons. It has been shown that silver stains specifically and preferentially label neurofilament proteins[42, 67, 138, 177], but not intermediate filament proteins [6]. It has also been shown that myelinated neurons are enriched with neurofilament, while unmyelinated neurons are neurofilament poor[120, 122, 172] (Figure 1), but are enriched with intermediate filament proteins (eg., peripherin, α-internexin)[73, 75] . Thus, silver stains likely revealed only the A δ axons involved in PACS, and potentially some C-fibers in dermal bundles.

Immunohistochemical analyses have the potential for greater sensitivity than silver stains and also to provide far better identification of the populations involved in PACS. However, very few histochemical analyses of the reinnervated skin have been reported. Those that have examined the innervation have used antibodies against either the neuropeptides CGRP and/or SP, or pan-neuronal markers such as PGP 9.5 [108, 109, see also 140, 216], or anterograde tracers that also could not distinguish between various populations[107]. No analyses were found that used markers specific for the non-trkA small diameter DRG neurons.

Electron microscopic examination has produced some promising indirect evidence for a role of non-trkA small diameter afferents in PACS. Unmyelinated axons lacking CGRP were identified in collaterally reinnervated root dentin in rat molars[216]. On the other hand, dermal bundles in denervated skin from animals treated with anti-NGF lacked any evidence of axons[49]. This particular finding should be regarded cautiously, however, when relating to non-trkA collateral sprouting. First, as mentioned above, the dermal bundles are not the primary route of growth for collaterally sprouting axons. They instead primarily use the subepidermal plexus[81]. Further, no study has addressed the possibility that anti-NGF treatments could, in fact, affect non-trkA afferent collateral sprouting via some intermediary (discussed below).

Histochemical Analysis of DRG

Other studies on PACS have examined the DRG housing neurons that were undergoing PACS. These examinations primarily focussed on mRNA of the NGF receptors (trkA and p75) and GAP-43 [115, 148, 149, 151]. While these studies furthered the understanding of the role of the NGF system in PACS, they did not address the

possibility that other growth factor systems might be involved. This is due in part to the lack of knowledge at that time about the growth factors regulating the non-trkA-bearing unmyelinated neurons.

Further, the expression of GAP-43 mRNA was not correlated to any other DRG family markers (i.e., trkA-IR, CGRP-IR, GS-I-B_4-binding, etc.) in those studies. It was assumed that the GAP-43 mRNA was expressed in, and increased in, the trkA-positive neurons. While it is very likely that the population of neurons that increased their production of GAP-43 mRNA included the trkA-positive neurons, it was possible that lower levels of GAP-43 were also produced by non-trkA-bearing neurons. In fact, assessment of GAP-43 mRNA in non-trkA-bearing neurons has proven difficult. The initial studies assessing which populations of normal DRG neurons expressed GAP-43 mRNA revealed two families of GAP-43 expressing neurons - those expressing high levels and those expressing very low levels[230]. The neurons expressing high levels of GAP-43 mRNA were consistently those that also expressed trkA, the highest GAP-43 levels being found in neurons that also expressed SP-IR. The neural population that expressed GAP-43 mRNA at low levels was consistently the small diameter neurons that lacked trkA (some of which expressed SOM-IR). An important finding was that the level of GAP-43 mRNA signal in neurons other than those with trkA was extremely low, very close to the level of background signal. Thus, assessments of GAP-43 mRNA in normal non-trkA-bearing DRG neurons has proven consistently difficult.

This difficulty has extended into GAP-43 detection via immunocytochemistry. Studies examining GAP-43-IR in DRG consistently report numbers of GAP-43-IR neurons that are far lower than would be expected based on mRNA studies[15, 199, 200, 210, 241]. While it is possible that some neurons simply produce GAP-43 mRNA but do not

translate detectible levels of protein, as may be the case with motor neurons[21, 133, 152, 231, versus 240], it is also possible that the immunocytochemical detection of GAP-43 in DRG neurons has been insufficient. Detection of GAP-43 in axons rarely suffers from such problems, as the protein is rapidly concentrated in axons, especially at terminals in muscle or skin. Evidence for insufficient detection of GAP-43-IR in DRG neurons in previous studies is presented as part of the results of this study.

Since GAP-43 is widely accepted, based on excellent demonstrations, to be intrinsically involved in neuronal anatomical plasticity, it may have been some of the seminal studies demonstrating which populations of DRG neurons expressed GAP-43 that prevented earlier examinations of the possibility that non-trkA small diameter primary afferent may be involved in PACS. For example, it was demonstrated that high levels of GAP-43 mRNA were selectively expressed by DRG neurons expressing trkA [230]. Other DRG populations expressed levels of GAP-43 mRNA that were very close to background. The combination of this demonstration with the lack of knowledge about the non-trkA small diameter population is likely to have played a role in the generation of the general focus on the trkA-positive population in sprouting studies. At the same time, however, Verge et al. (1990) also recognized that while the level of GAP-43 mRNA may be correlated with the capacity for anatomical plasticity in some neural populations, it was not necessarily indicative for all populations, such as motor neurons. They realized that "the possible contribution of GAP-43 to sprouting might be clarified by better histochemical definition of sensory axons that are capable of collateral sprouting" (Verge et al., 1990, p.933). While the current study does not focus on the role of GAP-43 per se, it does mirror the sentiment that a better definition of the populations that are capable of PACS is needed.

Electrophysiological Assessments of PACS

There have also been a limited number of electrophysiological assessments of the neurons involved in PACS. The most consistent finding was that the sprouted afferents were limited to high-threshold mechanoreceptors and thermoreceptors[5, 47, 59], to the exclusion of low-threshold A-fibers [but see 58, 84, 92, 93]. The electrophysiological data indicate quite strongly that PACS is indeed primarily, if not exclusively, limited to small diameter afferents (including at least a portion of the Aδ group). This fits well with what is known about which histochemical types of afferents participate. As stated above, afferents responsive to NGF (thus trkA-positive and peptidergic[157]) have a known role in PACS. Further, it has been shown that the vast majority of afferents expressing SP and/or CGRP displayed clear nociceptive capacities, or nociceptor-associated properties[54, 119, 121, 142-144, 171]. Therefore, the demonstrations of trkA expression and nociceptive roles for peptidergic neurons go hand-in-hand with the demonstration that afferents capable of PACS (an NGF-dependent phenomenon) are primarily nociceptors.

While these data fit together nicely and provide compelling evidence for the role of trkA-positive, peptidergic neurons in PACS, they have at no point actually addressed whether or not the trkA-*negative* population could participate in PACS. The same studies that provided direct evidence for the nociceptive capabilities of SP- and/or CGRP-expressing afferents gave evidence for nociceptive capabilities of *non-peptidergic* C-fibers [121, 142-144, 234]. This means that the trkA-*negative* small diameter afferents are included in the functional types of afferents capable of PACS. Therefore, unless a previous PACS study had been designed to specifically test for a role of non-trkA (mostly non-peptidergic) afferents in PACS, then any role they played could have been missed. No such design was found in any of the PACS studies to date. Further, none of

the PACS studies done to date provided evidence that could *eliminate* a possible role of the non-trkA afferents in PACS.

Assessments of PACS Using the CTM Reflex

Assessments of PACS using the CTM reflex are limited because it is unclear which particular types of afferents are involved in the reflex, and also what stimuli are adequate for the reflex. As previously stated, anti-NGF treatments have been shown to halt the expansion of an isolated CTM reflex-inducing sensory receptive field[49, 52]. Accordingly, if the trkA-negative, GS-I-B_4 reactive axons *do participate* in PACS, then it is likely that they *do not* contribute to the CTM reflex, unless they do so by transducing a sensory modality that has not yet been tested in relation to the CTM reflex. The function of the GS-I-B_4 reactive population is still unclear, and the only modalities tested to date and shown to be adequate for induction of the CTM reflex were noxious heat and pinch[52, 59, 166, 217]. It is possible that some types of A-delta and/or C-fibers may still participate in PACS, but may not be involved in the CTM reflex, and would therefore not have been observed with the behavioral tests. Given that the trkA-negative C-fiber terminations are anatomically distinct from those of the trkA-positive neurons, and that the two populations contain different neurotransmitters and/or neuromodulators, express different receptors, and also rely on different neurotrophic factors (see below), it is not at all unreasonable to believe that the two populations may be differentially involved in certain reflexes and/or afferent processes.

Unmyelinated Primary Afferents Lacking NGF Receptors

The focus of this dissertation was on the subset of unmyelinated primary afferents which are not directly responsive to NGF, lack trkA, and therefore were not examined in the previous work on PACS (described above). These neurons are almost entirely encompassed in the population that binds GS-I-B_4 [205, 207, 211, 212, 235, 236] and expresses the enzymes fluoride-resistant acid phosphatase (FRAP) and/or thiamine monophosphatase (TMP)[12, 40, 113, 145, 182, 204, 205, 207, 235].

Understanding of this population has lagged behind that regarding the trkA-bearing group, however, primarily due to the lack of suitable anatomic and functional markers for the population, as well as a lack of understanding of which factors regulate their function. FRAP histochemistry has been used to visualize the neuronal somata in the DRG, and the terminals in the dorsal horn of the spinal cord, but was not suitable for visualizing most peripheral processes[45, 62, but see 77, 113, 204, 207]. However, Streit and co-workers described the binding of GS-I-B_4 in a subpopulation of small diameter primary afferent somata and processes[211, 212] that was subsequently shown to include the FRAP expressing population of primary afferents[207, 235]. The GS-I-B_4-binding population includes the non-peptidergic unmyelinated DRG neurons, as well as the entirety of the population expressing the neuropeptide SOM [235]. Both the non-peptidergic and the SOM-expressing GS-I-B_4-binding neurons have been shown to lack NGF receptors[10, 12, 157, 202, 227]. The distribution of GS-I-B_4 reactive processes in peripheral tissues has since been described[64, 65, 175, 176, 186, 187, 205-207, 236], as well as their central terminations in the spinal cord[110, 111, 157, 181, 213].

Although the exact functional subclasses encompassed in this population remains unclear, certain functions are strongly suggested. Terminal distribution patterns of GS-I-

B_4 axons in the spinal cord and skin suggest a possible role as thermoreceptors[157, 176]. Recent evidence has demonstrated that a subtype of purine receptor (P2X3) is almost exclusively expressed in GS-I-B_4 reactive DRG neurons and likely in their cutaneous axons[233]. This lends credibility to the possibility that this population may demonstrate chemosensitivity, or at least have a sensitivity to tissue damage and/or inflammatory processes. Other recent work offers further evidence that at least a portion of the GS-I-B_4-positive, trkA-negative population is likely to contain nociceptors. The capsaicin-sensitive ligand-gated ion channel (VR-1) was shown to be primarily localized to this subpopulation of DRG neurons[75], and pain is the primary sensation elicited by administration of capsaicin[9, 117, 118, 208, 215, 218].

In vitro classification of acutely dissociated DRG neurons by current signature has provided some more direct evidence that the GS-I-B_4-binding neuron population that lacks peptides (except SOM) contain nociceptors. Classification of neurons based on their repertoire of voltage activated currents (current signature) has generated a large number of different subclassifications that maintain a high degree of internal consistency in regard to histochemistry, action potential shape, and pharmacological sensitivity to numerous agents[29, 30, 44]. This classification scheme is very powerful in that it allows the tracking and compilation of characteristics across experiments. Type 1 and type 2 neurons that had been recorded and then examined for their histochemical characteristics both consistently displayed GS-I-B_4-binding. Type 2 neurons consistently lacked CGRP and SP, while type 1 neurons expressed CGRP and SOM, but lacked SP, indicating that both types were part of the non-trkA small diameter afferent population (Petruska, J.C., J. Napaporn, R.D. Johnson, J.G. Gu, B.Y. Cooper; unpublished observations). Both types displayed numerous characteristics of nociceptive primary afferents (Petruska et al.,

unpublished observations)[29, 30, 44]. These included sensitivity to acidic solutions and capsaicin, as well as a wide action potential and a long-duration after-hyperpolarization (AHP), characteristics that correlate extremely well with a nociceptive function in normal animals[54, 144, 192]. Type 2 neurons also displayed ATP-induced currents with rapid kinetics that were likely mediated by homomeric P2X1 and P2X3 receptors (Petruska et al., unpublished observations), a characteristic associated with nociceptors[38, 39].

It has recently been shown that trkA-negative, GS-I-B_4-binding DRG neurons expressed the components of the glial cell line-derived neurotrophic factor (GDNF) receptor complex[12, 159]. They bound and transported GDNF and were supported during development and after injury by GDNF [123, 141]. Further, while both NGF and GDNF both regulated the expression of various proteins - including TTX-insensitive Na^+ channels, ATP-sensitive P2X3 receptors, and potentially the VR-1 receptor - they did so in mutually exclusive groups of neurons[12, 22, 63, 75, 99, 123, 141, 154]. The group of trkA-negative small diameter neurons regulated by GDNF also completely encompassed the SOM-expressing DRG neurons[12, 235]. SOM expression has been shown to be regulated by GDNF, and SOM-IR neurons have been shown to be insensitive to NGF, as they did not express either trkA or p75 [10, 12, 157, 202, 227]. Based on its expression of GS-I-B_4-binding, its regulation by GDNF, and its lack of NGF receptors, the SOM-IR population was included in the group under investigation in these experiments.

These recent discoveries should greatly enhance efforts to understand the role of these neurons in sensory systems. The findings described above are in line with the current proposal that these sensory neurons are capable of collateral sprouting since it is likely that the GDNF system is involved in at least one other form of collateral sprouting, namely that of motoneurons[132, 165].

Hypotheses and Plan for Testing

It was the basic hypothesis that the non-trkA small diameter DRG afferents were involved in PACS, but have been missed in previous studies because of methodological limitations. In order to address this hypothesis initially, collaterally reinnervated skin was histochemically analyzed for the presence of axons that displayed markers indicative of the non-trkA small diameter afferent population. As detailed in the results below, such axons were, in fact, observed. This was taken as a direct indication that the non-trkA C-fiber population was capable of collateral sprouting. While this finding supported the basic hypothesis, there were questions that arose from the previous work that needed to be addressed.

Since non-trkA neurons are likely capable of PACS, then 1) why did anti-NGF treatments block PACS as observed by microscopy of the skin?, and 2) why did anti-NGF treatments block PACS as observed by the CTM reflex? These questions can be addressed individually and are summarized below.

In addressing the first question, namely, the effect of anti-NGF treatments on PACS as assessed by microscopic examination of skin, two possibilities are raised. First, it is possible that silver staining of the skin may have missed some of the axons that were present, or was simply an insufficient stain to reveal the trkA primary afferent population. There are certain lines of evidence that support this possibility. First, it is known that silver stains primarily reveal neurofilament content[6, 67, 177]. In regards to DRG neurons, silver stains would reveal myelinated axons due to their high content of neurofilament proteins[120, 122, 172]. However, the majority of $trkA^+$ axons, and all of the $trkA^-$ axons (of interest) lack myelin. Second, the only myelinated axons that are involved in PACS are a subgroup of Aδ axons. Following that, the only Aδ subgroup

involved in PACS is NGF-sensitive, and therefore likely $trkA^+$ [128, 190]. This means that any myelinated axons that did participate in PACS as revealed by silver stain were those that were sensitive to NGF. Lastly, the only axons (of *any* type) revealed by the silver stain of skin were axons in dermal bundles[52, 166]. The highest density and occurrence of unmyelinated afferent axons is in the subepidermal plexus and in the epidermis. The silver stain did not reveal any such axons. This speaks directly to the likely insufficiency of the silver staining technique to reveal unmyelinated axons, the group of primary interest for these studies. The possibility that silver staining was an insufficient technique to reveal the full array of axons in collaterally reinnervated skin is addressed by experiments detailed in Chapter 3.

Second, it is possible that the anti-NGF treatment blocked PACS of both the $trkA^+$ and $trkA^-$ groups because NGF may play some role in PACS of the $trkA^-$ population. It is possible that $trkA^-$ DRG neurons may begin to express trkA (and/or p75) in response to the surgical isolation of their terminal fields. This possibility was indirectly addressed by experiments detailed in Chapter 3.

In addressing the second question, namely why anti-NGF appeared to block PACS as assessed by the CTM reflex, two possibilities are raised. First, it is possible that the anti-NGF treatment blocked only the PACS of the $trkA^+$ neurons and the PACS of the $trkA^-$ group was not observed. This would occur if the $trkA^-$ neurons do not participate in the CTM reflex, and therefore any collateral sprouting would have been missed since the CTM reflex was the primary method of assessment. This possibility was directly tested by experiments detailed in Chapter 4. The second possibility is the same as for the first question above, namely, that NGF could somehow affect PACS of this group. These arguments are summarized in Outline 1.

Since non-trkA neurons are capable of PACS, then why did anti-NGF block PACS:

1) as assessed by skin histochemistry?
    A) silver stains were insufficient (insensitive to the axons of interest):
        i) silver stains show myelinated, but the axons of interest are unmyelinated
        ii) the only involved myelinated are Aδ, and all are $trkA^+$
        iii) primary innervation fields of axons of interest not revealed
    B) NGF plays a role in $trkA^-$ PACS

2) as assessed by CTM reflex?
    A) $trkA^-$ PACS not observed because:
        i) $trkA^-$ not part of CTM reflex
    B) NGF plays a role in $trkA^-$ PACS

Outline 1. Summary of arguments addressing previous PACS data.

The experiments described herein were designed to address the possibility that non-trkA small diameter DRG afferents were capable of PACS, and to address some possible explanations as to why they had not been observed in previous examinations. In order to demonstrate the presence of non-trkA DRG axons in collaterally reinnervated skin, multi-labelling histochemistry for highly specific markers was performed on sections of normal and collaterally reinnervated skin. These experiments would also address the possibility that stains other than silver stains might be better suited to revealing the true array of innervation in collaterally reinnervated skin (Question 1A in Outline 1). In order to demonstrate that non-trkA DRG neurons expressed markers indicative of PACS, multi-labelling histochemistry for GAP-43 and markers highly specific for the non-trkA small diameter population was carried out on sections from the DRG involved in PACS. In order to address the possibility that NGF may play some role in $trkA^-$ PACS because the $trkA^-$ neurons begin to express trkA during PACS (Question 1B in Outline 1), multi-labelling histochemistry for trkA and markers highly specific for the non-trkA small diameter population was carried out on sections of skin. **The guiding hypothesis of this set of experiments was that the non-trkA small diameter DRG**

afferents were involved in PACS, but have been missed thus far. The specific hypotheses were: 1) axons displaying markers specific for the trkA-negative small diameter afferents would be observed in collaterally reinnervated skin, 2) neurons in the DRG involved in PACS that displayed markers specific for the trkA-negative small diameter afferents would begin to express GAP-43.

In order to address one of the possible reasons that non-trkA PACS was missed, namely, that the $trkA^-$ small diameter DRG neurons are not involved in the CTM reflex (Question 2A in Outline 1), experiments were undertaken to demonstrate which afferent populations were involved in the CTM reflex. **The guiding hypothesis of this set of experiments was that the non-trkA small diameter DRG afferents were not involved in the CTM reflex.** The primary means of examining this possibility was the selective destruction of p75-expressing DRG neurons (which included the $trkA^+$ neurons) with a directed neurotoxin. The toxin (saporin) gains access to the interior of only targeted cells based on the internalization of a transmembrane protein with an external antigen to which the antibody-neurotoxin complex has bound. The saporin is cleaved from the antibody and then inactivates ribosomes, which eventually kills the cells[178, 179]. The ability of the spared neurons to drive the CTM reflex was then examined, and the selective destruction of the targeted population was confirmed histologically. In addition, transneuronal tracing with PRV was employed to anatomically retrogradely trace the reflex circuit from the motoneurons to the primary afferents.

CHAPTER 2
METHODS

General Methods

Histochemical Analysis of PACS

The intent of these experiments was to determine whether or not GS-I-B_4-binding neurons lacking NGF receptors were involved in PACS. This was assessed by examining which primary afferent subpopulations were present in collaterally reinnervated skin, and also by examining which primary afferent subpopulations in the spared DRG expressed the growth associated protein-43 (GAP-43) - a marker for axonal growth. Collateral sprouting was induced by surgical isolation of the T13 dermatomal cutaneous nerves. Animals were allowed to survive for either 14 days (for DRG cell counts and cutaneous innervation examinations; n=6) or 28 days (for cutaneous innervation examinations; n=4). They were then euthanized, perfused, and the tissue prepared for histochemical processing.

CTM Reflex Afferents

The intent of these experiments was to determine which subgroup or subgroups of primary afferents were involved in the induction of the nociceptive specific cutaneus trunci muscle (CTM) reflex. The first set of experiments involved the injection of a directed neurotoxin into the left T13 DRG. The neurotoxin was the ribosomal inactivating protein saporin conjugated to a monoclonal antibody against p75 (192-sap).

Animals (n=8) were allowed to survive for 7-23 days, and then underwent a terminal electrophysiological assessment of the ability of the DCn from the injected DRG to generate a CTM reflex. Animals were euthanized at the end of the experiment, perfused, and the tissue prepared for processing to examine the expression of NGF-related markers (trkA, p75, CGRP, SP) and markers associated with the trkA-negative population of small diameter afferents (GS-I-B_4-binding, SOM, P2X3).

A second set of experiments designed to address the same question involved injections of the transneuronal tracer pseudorabies virus into the CTM muscle or LTn (or control tissues) in order to characterize the afferents involved in the CTM reflex. Animals (n=24) were allowed to survive for 24-72 hours, and were then euthanized, perfused, and the tissue prepared for processing to localize the virus. The virus was localized with antibodies directed against the virus. This was combined with other markers specific for various subpopulations of primary afferents in order to determine if the virus was localized to particular subpopulations.

Specific Methods

Histochemical Analysis of PACS

Sprouting surgeries

Adult female Wistar rats were anesthetized with ketamine/xylazine. Fluids were usually administered at this time (1.5 - 3cc lactated Ringer's i.p.). They were maintained at 36°C (±1°C, monitored via a rectally placed thermistor) with an electric heating pad, and the heart rate was monitored with a stethoscope attached to an audio amplifier. For the surgical induction of collateral sprouting, an incision was made approximately 1cm to

the right of midline along the low thoracic and upper lumbar dorsal skin. This incision was designed to leave intact any cutaneous innervation from the left dorsal cutaneous nerves that may have crossed midline. The skin was pulled away from the body and the subcutaneous fascia freed from the underlying body wall musculature to reveal the DCnn emerging from the body wall musculature. The T11, T12, L1, and L2 DCnn were isolated, ligated with 7-0 monofilament nylon as they emerged from muscle and transected. For animals that were part of the DRG cell count studies, the corresponding lateral cutaneous nerves (LTnn) were also identified by approximate landmarks and ligated and transected. The LTnn were transected in these experiments in order to provide a greater area of neighboring denervation for the T13 dermatome. This would increase the probability of quantifying any changes in the PACS DRG, since a greater proportion of the T13 afferents would be undergoing PACS than if only the DCnn were transected. The incision was sutured in layers and closed with Michel clips. As the animals regained mobility during their recovery from anesthesia, the CTM reflex fields were mapped with a fine pinch stimulus. Fields were marked with a permanent marker on the skin. At the end of the survival period (28 or 14 days for skin studies, 14 days for DRG cell count studies), animals were anesthetized with 50-60 mg/kg sodium pentobarbital and the CTM reflex field mapped again. A survival time of 14 days was used for cell counting studies, where GAP-43-IR was a major focus, because a previous study had indicated that GAP-43 mRNA in PACS DRG peaked at 12 days[151]. Therefore, 14 days should offer an excellent indication of the GAP-43 protein signal in the PACS DRG. A survival time of 28 days was included for the skin histochemical analyses because a previous study had indicated that PACS reached its spatial extent at 28 days

post-denervation[52]. In all cases the formerly unresponsive fields had regained the ability to drive the CTM reflex in response to pinch. Animals were then overdosed with urethane and perfused (see below).

Control experiments were done in order to ensure that any IR or GS-I-B_4-binding observed in the collaterally reinnervated regions were actually axons that had grown into the area and were not simply residual profiles from axons that had originally innervated the tissue. These consisted of carrying out the surgical procedures as described above, but only allowing the animal to survive for 3 days before euthanizing and perfusing as described above (n=1).

This animal also served as a control for the accuracy of the innervation map produced by using pinch testing and the CTM reflex. The skin was tested prior to euthanizing the animal to ensure that the delineations had not changed. The skin sections were examined microscopically to determine how well the behavioral and the histochemical innervation patterns matched.

CTM reflex testing

The areas of skin that were capable of driving the CTM reflex were assessed as the animal recovered from the T13 isolation surgery as well as just prior to sacrifice. The reflex was sensitive to the ketamine/xylazine anesthesia used during surgery. Therefore, the testing was not done until the animal was able to move all four limbs and raise its head. This level of recovery was suitable to give an indication of the CTM reflex receptive field that did not differ from that revealed under pentobarbital anesthesia, to which the reflex was highly resistant. Assessment was done by lightly pinching the skin with fine forceps. The reflex could be induced with light pinch of very small areas of

skin, and was often induced by the prick of the forceps onto the skin surface. The forceps were closed perpendicularly to the body axis, so as to provide a very narrow application of force parallel to the borders of the receptive fields. The borders were drawn with permanent ink, which was re-applied every 2-3 days. The reflex was readily visible as a rapidly appearing and disappearing puckering of the skin just rostral to the pinch site.

Histochemical procedures for collaterally reinnervated skin

Samples of skin that included both the normal T13 dermatome and the collaterally reinnervated region were sectioned at 20-35μm and retrieved onto slides. Procedures for the detection of markers are summarized below. Antisera used for this and other procedures are listed in Table 1. Tissue underwent an initial blocking incubation in a solution of 1:30 goat serum in PBS with 0.4% Triton X-100 (GS-PBS-T) to prevent non-specific protein-protein binding of the subsequent antisera. The sections were then incubated overnight in a rabbit primary antisera This step, and all others, was followed by repeated rinses with 1% GS-PBS-T. The primary antisera were then detected with a 1:75 solution of Texas Red®-conjugated, or a 1:100 solution of AlexaFluor® 594-conjugated goat α-rabbit IgG. Detection of the trkA or P2X3 antisera was done with amplification, described below. This was followed by incubation with mouse primary antisera. The primary antisera were then detected with a 1:100 solution of FITC- (or AlexaFluor 488-) conjugated goat α-mouse IgG. The α-mouse antisera were preadsorbed against rat serum that was prepared in our lab to prevent non-specific binding of the secondary antisera to rat proteins. Controls for this uniformly showed that the preadsorbtion procedure eliminated non-specific binding. Following the rinse of the

secondary antisera, the tissue was incubated overnight in a solution of HRP-conjugated GS-I-B_4, or in some cases, α-PGP 9.5. PGP 9.5 was detected with Pacific Blue-conjugated α-rabbit antiserum, and the lectin was detected with the coumarin (blue) conjugate of the TSA amplification system (New England Nuclear, Inc.), similar to that used for the trkA or P2X3 antisera. This system utilizes the HRP molecule to catalyze the deposition of conjugated tyramide onto the tissue[18, 19, 20, 224]. Detection of the trkA or P2X3 antisera was done by incubation with biotinylated secondary antisera, followed by the avidin-biotin-HRP complex (ABC kit, Vector Labs). The ABC complex then catalyzed the deposition of FITC-conjugated TSA. If the TSA-detected lectin was to follow this, then the HRP present from detection of the trkA or P2X3 was quenched prior to application of the lectin with a 20 minute incubation in a solution of H_2O_2 and methanol diluted in PBS.

Markers used for skin histochemistry

The classification of a cutaneous axon into a particular histochemical family (and thus often a functional family) is more difficult than the histochemical classification of neurons in the DRG. This is primarily because the skin is not only innervated by sensory axons, but also by sympathetic axons. Sympathetic neurons share many markers found on families of DRG neurons, including trkA-IR. Morphological features of axons in normal skin can be a good indicator of whether an axon is sympathetic or sensory. However, since sympathetic axons also undergo collateral sprouting, and the only evidence that collaterally sprouted axons retain some morphological features is indirect and merely suggestive[107], morphology could not be used as the primary indicator of a sensory or sympathetic identity. Therefore, multi-labelling histochemistry was

employed. By labelling the skin with markers whose combinations would clearly identify sympathetic and sensory axons, as well as the various subfamilies of sensory axons, the types of axons innervating the reinnervated skin could be identified. TrkA provided an excellent indicator of both *sympathetic* and trkA-bearing *sensory* neurons. Therefore, any axons in collaterally reinnervated skin that bound GS-I-B_4, expressed GAP-43-IR, but lacked trkA-IR could be considered to be axons derived from the population of interest that had successfully sprouted into the denervated skin. P2X3, which is not present in sympathetic neurons of normal rats[131] or PACS rats (Petruska, unpublished observations), also provided an excellent marker for axons of interest since it is primarily expressed in GS-I-B_4-binding DRG neurons that lack CGRP [22], and are therefore unlikely to express trkA [157]. Axons binding GS-I-B_4 but lacking CGRP are indicative of successful non-trkA collateral sprouting by the same reasoning.

Multiple samples of skin were taken from the collaterally reinnervated regions and represented a span of 1.5 - 2.0 cm of skin. Generally, 2-3 samples from the reinnervated skin were sectioned, and 3-4 sections with 150-200μm separation were placed on slides for staining. This procedure generated a good sampling of the reinnervated skin regions.

Histological procedures for cell counts

Euthanized animals were transcardially perfused with exsanguination solution (heparinized phosphate buffered saline) followed by 4% paraformaldehyde in phosphate buffered saline (PBS). Tissue was removed and placed in a solution of 30% sucrose in PBS until they were sectioned. Tissue sections were made with a cryostat. Sections were cut at 10-12μm and collected in a serial series with 10-12 slides in the series. This

collection procedure generated slides holding sections with 90-132mm between each section. This separation guaranteed that no single neuron would be present in more than one section per slide and that one slide would contain a representative sample of the entire ganglion.

Immunohistochemical detection of markers for cell counts generally followed the procedures described above, with some modifications. Following the initial blocking step, the sections were incubated with a solution of mouse-α-GAP-43 overnight. This was followed by detection similar to that described for P2X3 or trkA in skin (bitotinylated secondary antiserum and ABC kit). However, instead of using TSA-FITC, TSA-biotin was used. This was then followed by an incubation with avidin-AlexaFluor 488 (green). The appropriate rabbit antisera were then applied (P2X3, trkA, SOM) and then detected with AlexaFluor 594-conjugated α-rabbit antiserum. Prior to application of the lectin, the HRP on the tissue sections from the amplification of the GAP-43 signal was quenched. The tissue was thoroughly rinsed prior to application of the lectin conjugate. The sections were incubated overnight in the lectin solution. The following day the GS-I-B_4-binding was detected with TSA-coumarin.

Cell counting

Black-and-white digital images of each section on the stained slides were taken in montage fashion and saved on computer disk. Images were captured either with a Zeiss Axiophot equipped with a Dage 72-S 10-bit integrating single-chip CCD camera, or a Zeiss Axiophot II equipped with a Spot II three-chip CCD camera. Images were opened in Adobe Photoshop 5.0 and positively-stained neurons with nuclei were circled. The circled cells and the marking layers were all compared and counted to generate the

percentages of neurons with various combinations of markers. A minimum of three sections per slide were used to generate the proportions.

The various staining combinations were assessed and the percentages generated for each individual animal. These percentages were then subjected to a two-tailed t-test to determine whether there was any significant difference between the normal control group and the PACS group.

CTM Reflex Afferents

Neurotoxin experiments

Adult female Wistar rats were anesthetized with ketamine/xylazine. Fluids were usually administered at this time (1.5 - 3cc lactated Ringer's ip). They were maintained at 36°C (±1°C, monitored via a rectally placed thermistor) with an electric heating pad, and the heart rate was monitored with a stethoscope attached to an audio amplifier. Under aseptic conditions, a midline incision was made in the dorsal back skin. The L6 spinal process was identified by subcutaneous palpation, and the T13 vertebra identified based on counting spinous processes. The left T13 DRG was then exposed. The capsule was pierced with a 28g needle, and 75-90ng (in 1.0-1.2µl) of the monoclonal antibody 192 (directed against the low-affinity NGF receptor p75) conjugated to saporin was then injected into the DRG with 10µl Hamilton syringe coupled to a glass micropipette with a tip outer diameter of 30-50µm. The injection was done in 0.2µl increments with a few minutes between each. The injected fluid was visualized to cause brief and minor swelling inside the DRG capsule and root compartment. There was no visible leakage of the fluid out of the injection hole either during the injection or during withdrawal of the

pipette. The muscle layers that had been retracted were sutured together again and the fascia and skin closed in layers. The wound was finally sealed with Michel clips. Animals (n=8) were housed separately and allowed to survive for 7-23 days.

At the end of the survival period they were prepared for a terminal physiological examination. They were anesthetized with sodium pentobarbital (50-60mg/kg) and placed on a circulating water heating pad to maintain their core temperature (monitored with a gastric thermistor). The trachea was intubated to monitor end-expired pCO_2. They were given 0.1cc of atropine (0.1mg/ml, s.c.) every 2 hours to counteract the fluid build-up in the lungs that accompanies pentobarbital anesthesia. A midline incision was made in the dorsal back skin and the T11, T12, T13, and L1 DCnn isolated bilaterally. In some cases, the middle branch of the LTn was dissected free from where it entered the CTM and was placed on recording electrodes. Each of the DCnn were placed sequentially on bipolar silver-silver chloride stimulating electrodes. The threshold to elicit a visible contraction of the CTM, or a recordable LTn response, was then established. The strength of the contraction at various stimulus intensities was also noted. In cases where the LTn recordings were done, the LTn response was recorded with a DCn stimulus of 1.5mA (Aδ strength) or 3-5mA (C-fiber strength). Finally, a laminectomy was performed in order to expose the dorsal roots. The dorsal root from the injected DRG was placed on bipolar recording electrodes and the compound action potential (CAP) elicited by C-fiber strength stimulation of the appropriate DCn was averaged and recorded. At the end of the physiological experiment the animals were overdosed with anesthetic and perfused as described above.

Tissue processing

Procedures for the detection of histochemical markers in sections of the injected and control DRG from toxin-injected animals were similar to those described above. Various combinations of markers were run in order to assess the efficacy and specificity of the toxin. The following markers (in various combinations) were assessed: mse-α-p75 (clone 192 - same as used for toxin conjugation), rbt-α-p75, trkA, SOM, P2X3, GS-I-B_4.

Pseudorabies virus injections

Adult female Wistar rats were anesthetized with ketamine/xylazine. Fluids were usually administered at this time (1.5 - 3cc lactated Ringer s ip). They were maintained at 36°C (±1°C, monitored via a rectally placed thermistor) with an electric heating pad, and the heart rate was monitored with a stethoscope attached to an audio amplifier. Under aseptic conditions, a midline incision was made in the dorsal back skin and the skin/CTM layer was separated from the underlying muscle in order to introduce the transneuronal tracer pseudorabies virus (PRV) into the cutaneus trunci muscle (CTM) reflex circuit (n=18). Care was taken to avoid damaging the dorsal cutaneous nerves (DCn). The skin/CTM layer was held away from the body with hemostats, and the thin fascia covering a small portion of the CTM was cleared away to facilitate injection of the muscle. The caudal-most portion of the cleared region was consistently at least 15mm rostral to the point where the T13 (the segment of focus in this study) DCn pierced the CTM to innervate the overlying skin. This ensured that the PRV injection was not placed into the region of CTM directly underlying the T13 dermatome to avoid any direct uptake of the virus by the DCn axons. Further, previous research determined by electromyography (EMG) that the zone of shortest latency response (SLR) of the CTM reflex elicited by electrical stimulation of a single DCn was 1cm rostral to the dermatome

of the stimulated nerve[217]. Focussing the injection within the T13 SLR would optimize the transport of the virus through the CTM reflex circuit to the T13 DRG. In another 6 cases, PRV was injected directly into the middle branch of the left LTn near the origin of the CTM.

The virus (1-5×10^8 plaque-forming units (PFU)/ml) was pulled into a 10µl Hamilton microsyringe equipped with a 33 gauge needle. The virus was injected directly into multiple sites within the CTM in 0.5-1.0µl increments. Care was taken to avoid injections near any penetrating DCn, but injections were often very close to the many branches of the lateral thoracic nerve (LTn) innervating the CTM. Any residual fluid was removed with a cotton-tipped applicator. The incision was then sutured closed with 7-0 nylon and secured with Michel clips.

Animals were allowed to recover to the point of regaining mobility of their trunk while still being monitored for rectal temperature and heart rate. Once they had regained this level of mobility, the stethoscope and thermistor were removed and the animal placed in a bedding-free cage which had one half over a heating pad. They were monitored until regaining mobility of their hindlimbs for weight-support and were then placed in a normal cage (full bedding and *ad libitum* water and food) which also was placed partially on a heating pad. Once they had fully recovered they were removed to a designated cubicle in the University of Florida Health Center Animal Resources Department (HCARD) infectious disease suite. The animals were observed at least three (3) times per day (usually at 8 hour intervals), with more frequent monitoring on the final day of the survival period. Animals were generally allowed to survive from 24-72 hours, depending on the particular experiment. Animals were allowed to survive for the entire

designated survival period unless they began to display signs of a more widespread neural infection. These signs included significant lethargy, head-bobbing, sporadic bouts of rapid motion, or ballistic movements. If these behaviors were observed, the animal was euthanized as soon as possible and perfused. Delays prior to euthanization were generally less than 30 minutes, as the supplies for this procedure were kept at the ready from 48 hours post-injection. No signs were ever observed prior to 60 hours post-injection.

Pseudorabies virus control experiments

In order to ensure that the virus localized to the lower thoracic and upper lumbar DRG neurons was indeed derived from retrograde infection of the various neurons of the CTM reflex, control experiments were performed. The injections of PRV made directly into the LTn branches near the origin of the CTM also served as a control to ensure that lower thoracic DRG infection could arise from LTn-derived primary infection (n=6). Injections of PRV were also made into the CTM as described above but were coupled with specific nerve transections (n= 1 each): 1) transection of the ipsilateral LTn branches, 2) transection of the ipsilateral T10-L2 DCnn, 3) transection of the ipsilateral LTn and T10-L2 DCnn. These transections were designed to dissect the routes of entry of the virus into the nervous system from the CTM. Animals were allowed to survive for up to 72 hours before being euthanized, perfused, and their tissue processed for the localization of PRV.

Previous studies had showed that the virus displayed differential tropism for certain subgroups of neurons[27, 28]. In order to assess whether or not there was any differential tropism of the virus for the central terminals of subtypes of DRG neurons,

injections of virus were made directly into the dorsal horn of the T12 or T13 spinal cord (n=3). Animals were allowed to survive for approximately 24 hours before being euthanized, perfused, and their tissue processed for the localization of PRV (see below).

Tissue processing and histological procedures

At the end of the designated survival period, or with the onset of symptoms of serious central viral infection, animals were euthanized with an overdose of urethane (0.5g/ml). Perfusion and cryostat sectioning of tissue was as described above.

All tissue was obtained from perfusion-fixed animals. Paraformaldehyde (4%) in PBS was used for all tissue with the exception of the first few PRV animals. These were perfusion-fixed with a lysine-periodate-paraformaldehyde (2%) fixative as previously used by other labs[28].

Procedures for the detection of PRV and its overlap with other markers is summarized below. The sections were then incubated overnight in a 1:1200 solution of rabbit anti-trkA [37]. This step, and all others, was followed by repeated rinses with 1% GS-PBS-T. The primary antisera were then detected with a 1:75 solution of Texas Red®-conjugated, or a 1:100 solution of AlexaFluor® 594-conjugated goat anti-rabbit IgG. In order to avoid cross-reactivity with the subsequent antisera also raised in rabbit and detected with anti-rabbit antisera, the sections were incubated briefly in a 1:50 solution of unconjugated goat anti-rabbit IgG. This was rinsed only briefly, and the sections were incubated for 5 minutes with 4% PFA, which was then thoroughly washed off. The sections were then incubated overnight in a 1:2000 solution of rabbit-anti-PRV [28]. The primary antiserum was then detected with a 1:100 solution of FITC- (or AlexaFluor 488-) conjugated goat anti-rabbit IgG. Control experiments were performed to establish the dilutions necessary to completely avoid the second set of rabbit and anti-rabbit antisera

from cross-reacting with the first. The dilutions used were highly reliable for clean, strong, and specific signals. Controls were run with each procedure, and all were negative.

Immunohistochemical localization of PRV in DRG was also compared to the pattern of GS-I-B_4-binding. This was accomplished by processing the tissue as described above for the localization of PRV. This was followed by an overnight incubation of the tissue in 1:200 GS-I-B_4-biotin. The tissue was rinsed the following morning and the lectin detected with avidin-FITC.

Table 1. List of antisera used and their sources and dilutions.

Antigen	**Host**	**Source**	**Dilution**
PRV	Rbt	J.P. Card [28]	1:2000
trkA	Rbt	L.F. Reichardt or Chemicon, Inc.[37]	1:1200
p75	Rbt	M.V. Chao[87, 88]	1:5000
p75	Mse	Oncogene, Inc.	5µg/ml
CGRP	Rbt	Peninsula Labs, Inc.	1:15k
CGRP	Mse	RBI, Inc.	1:2000
PGP 9.5	Rbt	Biogenesis, Inc.	1:1500
TH	Mse	Sigma, Inc.	1:1000
P2X3	Rbt	E.J. Kidd, Glaxo-Wellcome[104]	1:1500
P2X3	GP	Neuromics, Inc.[232]	1:3000
SP	Rbt	Peninsula Labs, Inc.	1:3000
SOM	Rbt	Peninsula Labs, Inc.	1:1000
SOM	Mse	Biomeda, Inc.	1:10
GAP-43	Mse	Boerhinger-Mannheim, Inc.	5µg/ml
GAP-43	Rbt	P. Caroni[2]	1:1000
GS-I-B_4		Sigma, Inc.	5-10µg/ml

CHAPTER 3
RESULTS - HISTOCHEMICAL ANALYSIS OF PACS

Histochemical Analysis of Collaterally Reinnervated Skin

Skin regions that had been denervated and subsequently reinnervated by collateral sprouting of the spared T13 cutaneous axons were examined in order to determine the characteristics of the axons present in the reinnervated skin.

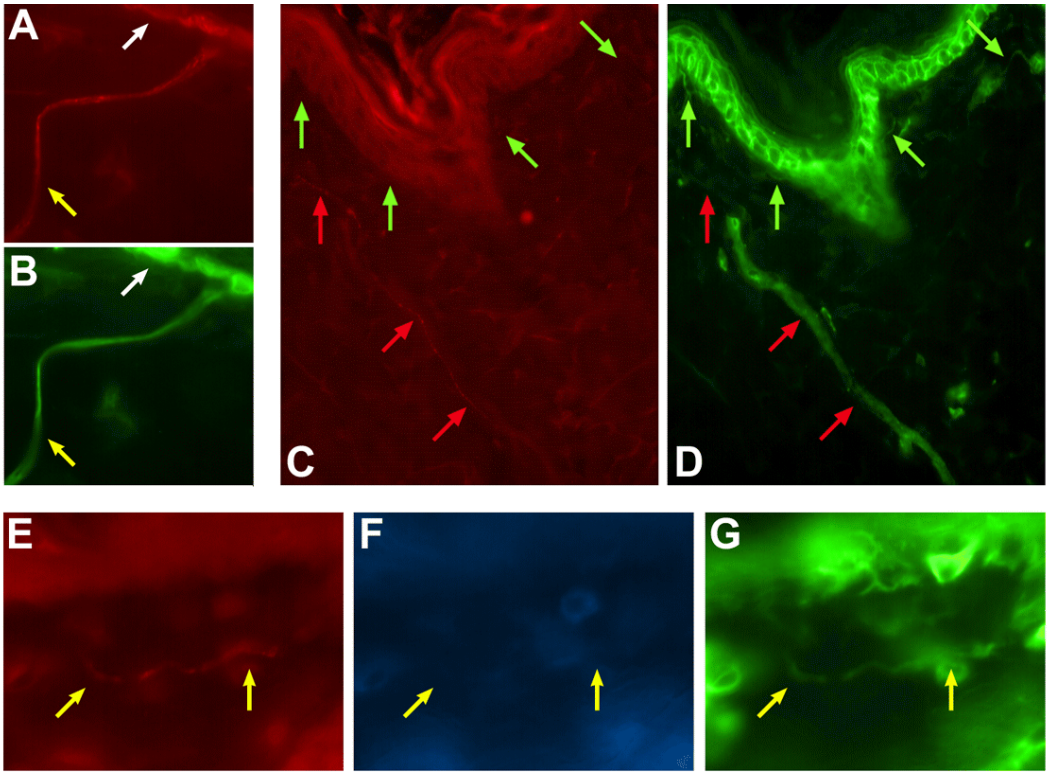

Figure 2. Examples of multi-labelling histochemistry of collaterally reinnervated skin. Skin was stained for GAP-43 (A) and GS-I-B_4 (B), CGRP (C) and GS-I-B_4 (D), or GAP-43 (E), trkA (F), and GS-I-B_4 (G). The yellow arrow in panels A and B reveals a GAP-43-IR axon(s) that also has lectin-binding approaching epidermis (white arrow). The red arrows in C and D indicate a CGRP-IR axon that lacks GS-I-B_4-binding, and the green arrows indicate subepidermal GS-I-B_4 axons that lack CGRP-IR. The yellow arrows in E-G reveal a GAP-43-IR axon (E) that also has GS-I-B_4-binding (G), but completely lacks trkA-IR (F). Epidermis is at the top of all images.

Skin samples from animals undergoing PACS for 2 weeks (n=6) or 4 weeks (n=4) were examined with markers specific for axons undergoing active growth (GAP-43), as well as markers associated with trkA-IR neurons (trkA, CGRP, SP). GAP-43-IR axonal profiles that were GS-I-B_4^+ but lacked either CGRP or trkA were consistently observed in the collaterally reinnervated regions of skin (Figure 2). These regions had been unresponsive to noxious stimulation (as assessed by attempting to elicit the CTM reflex)

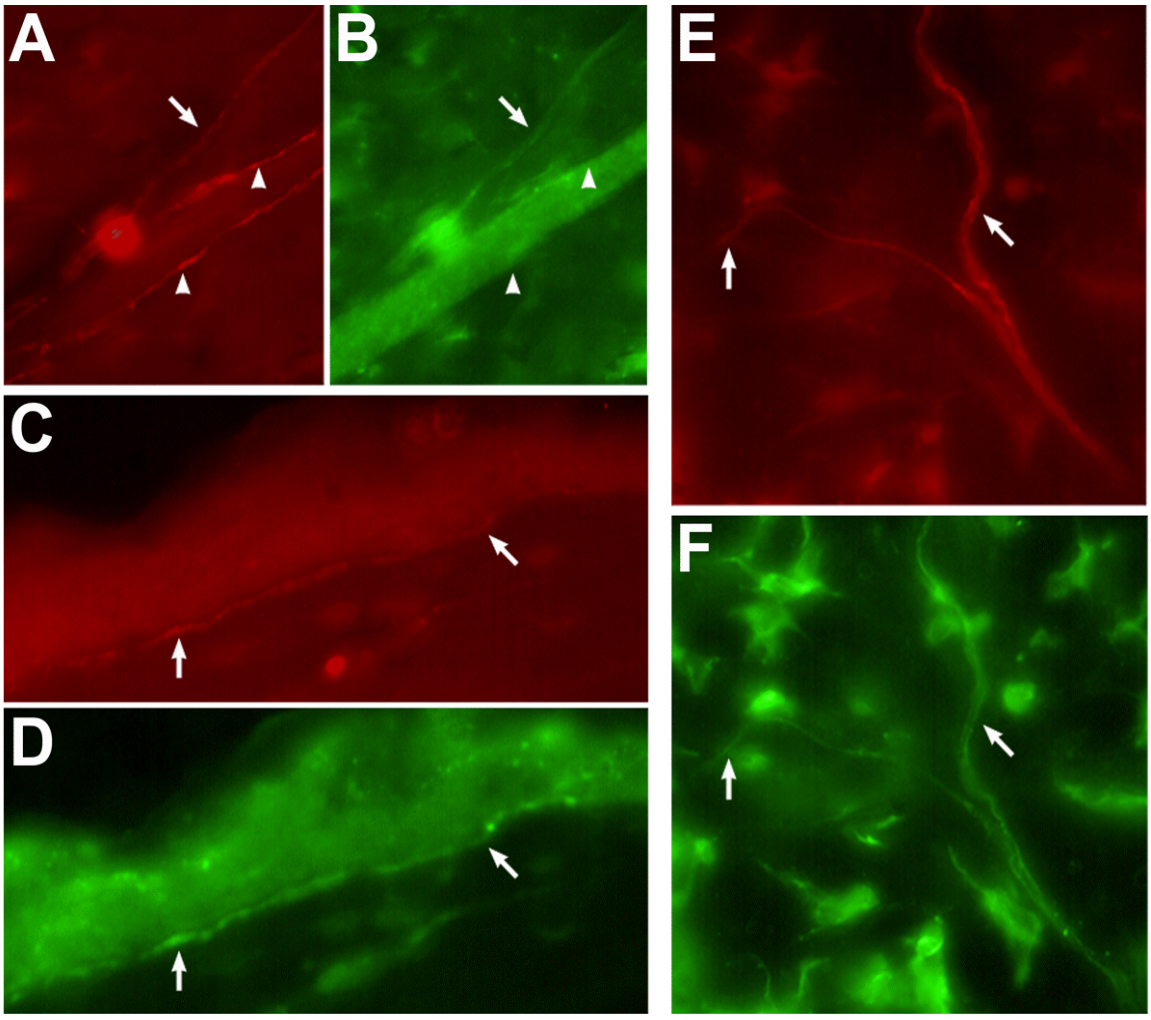

Figure 3. Examples of multi-labelling histochemistry of collaterally reinnervated skin. Skin was stained for GAP-43 (A, C) and P2X3 (B, D), or GAP-43 (E), and SOM (F). White arrows (A-D) indicate GAP-43-positive axons that also displayed P2X3-IR. Arrowheads (A, B) indicate GAP-43-positive axons that lack P2X3-IR. White arrows (E, F) indicate GAP-43-positive axons that also displayed SOM-IR.

after the acute transections, but had regained responsiveness by the end of the survival period. Further, these regions also displayed axonal profiles that were GAP-43^{+}/GS-I-B_4^{+}/TH , indicating that they were likely not sympathetic in origin. This conclusion was further reinforced by the demonstration of GAP-43-IR and GS-I-B_4-binding in axons that *lacked trkA* since trkA is also present in sympathetic axons. These profiles were not observed as frequently as GAP-43-IR axons that displayed trkA-IR or associated markers. However, their presence was consistent. No difference was observed in the reinnervation patterns of skin from the two week or four week survival groups.

Skin samples were also examined for markers associated with the non-trkA small diameter DRG neurons (SOM, P2X3). SOM-IR was found in a few axonal profiles as revealed by co-labelling with PGP 9.5 or GAP-43 (Figure 3). Further, many P2X3-IR axon profiles were consistently observed in the collaterally reinnervated regions of skin (Figure 3). These profiles could sometimes be observed entering the epidermis.

Interestingly, certain staining patterns were observed on axonal profiles in the underlying CTM in samples from experimental animals that were not observed in those from control animals. In particular, many CTM motor axons, morphologically identified based on axon caliber and the direct observation of motor end-plates, were GAP-43-IR and CGRP-IR (Figure 4). Motor axons with GAP-43-IR and CGRP-IR were found in the CTM underlying the normal spared T13 dermatome as well as the denervated field.

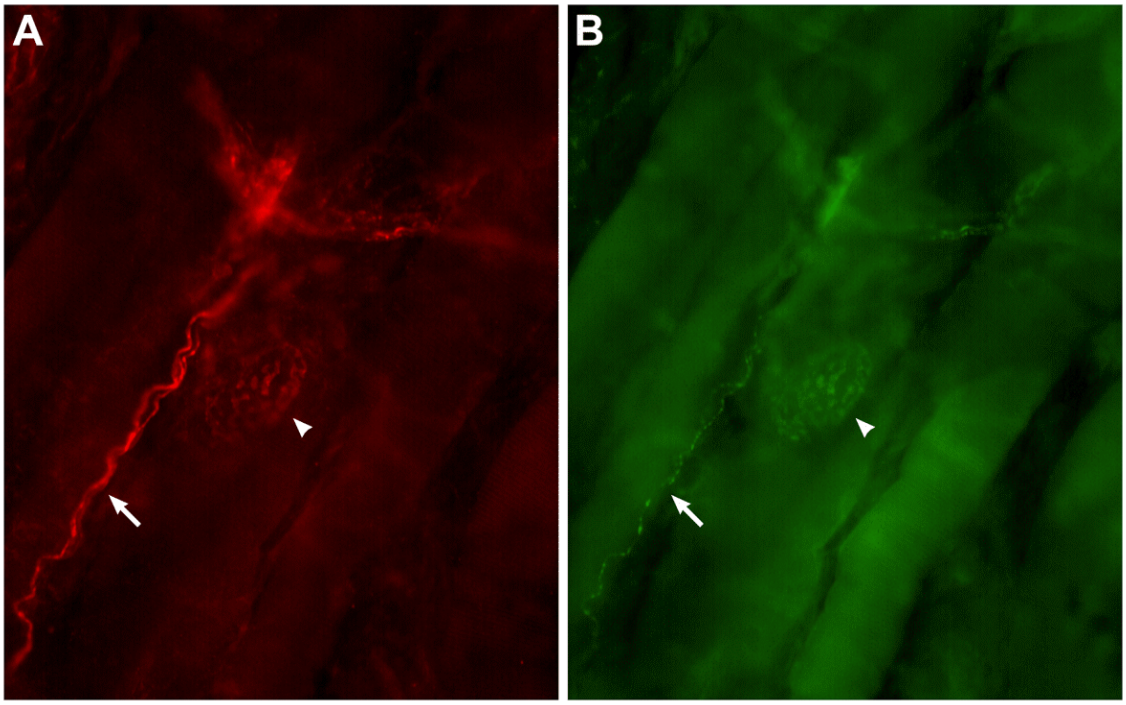

Figure 4. Example of CTM from below collaterally reinnervated skin stained for GAP-43 (A) and CGRP (B). The arrow indicates labelled axons, and the arrowhead indicates a labelled end-plate.

Skin Histochemistry Controls

Control experiments were run in order to ensure that any IR or GS-I-B_4-binding observed in the collaterally reinnervated regions was actually due to axons that had grown into the area and was not simply residual signal from axons that had originally innervated the tissue. One animal underwent the same surgical procedures as the others used for examination of skin reinnervation, but was allowed to survive for only 3 days. The regions that had been defined by pinch-induced CTM reflex activity to be denervated showed a dramatically decreased innervation density that was clear upon histological examination. The rare profiles that could be observed displayed extremely weak IR for PGP 9.5, CGRP, SP, trkA, or GAP-43. GS-I-B_4-binding could still be observed, but it was very weak. GS-I-B_4^+ profiles also lacked the normally accompanying PGP 9.5-IR.

The same skin samples were examined in order to determine how well the behaviorally defined innervation fields matched with the histochemically revealed innervation fields. The pinch-induced CTM reflex provided an excellent behavioral indicator of the extent of innervation as revealed by immunohistochemistry for axons. Only the extremely rare axon profile (approximately one axon profile per ten sections examined) was found more than 500µm beyond the behaviorally-defined border.

Other control tissue was also examined to assess the rate of denervation-induced marker loss. This tissue included the distal segment of a transected T12 DCn and sciatic nerve that had been crushed either 7 or 14 days prior. All nerve segments revealed rapid and nearly complete loss of all markers.

Histochemical Analysis of DRG Sections

Cell counts were done to assess the proportion of trkA-negative neurons expressing GAP-43-IR in both control (n=7) and PACS (n=4) DRGs. Quantitation of the proportion of trkA-negative/GAP-43-positive neurons that displayed GS-I-B_4-binding was also done in order to directly assess the GAP-43-IR of the population in question. The guiding hypothesis was that PACS DRGs would contain more trkA-negative/GS-I-B_4-binding neurons with GAP-43-IR than would controls. This was based on the ideas that there was a general lack of GAP-43 in non-trkA neurons in *normal* animals[15, 230], but that GAP-43-IR axons displaying markers of non-trkA afferents were found in *collaterally reinnervated* skin (present data).

Qualitatively, in control DRG, there were three clear levels of GAP-43 staining, namely negative, low-intensity, and high-intensity (Figure 5B). These three levels were

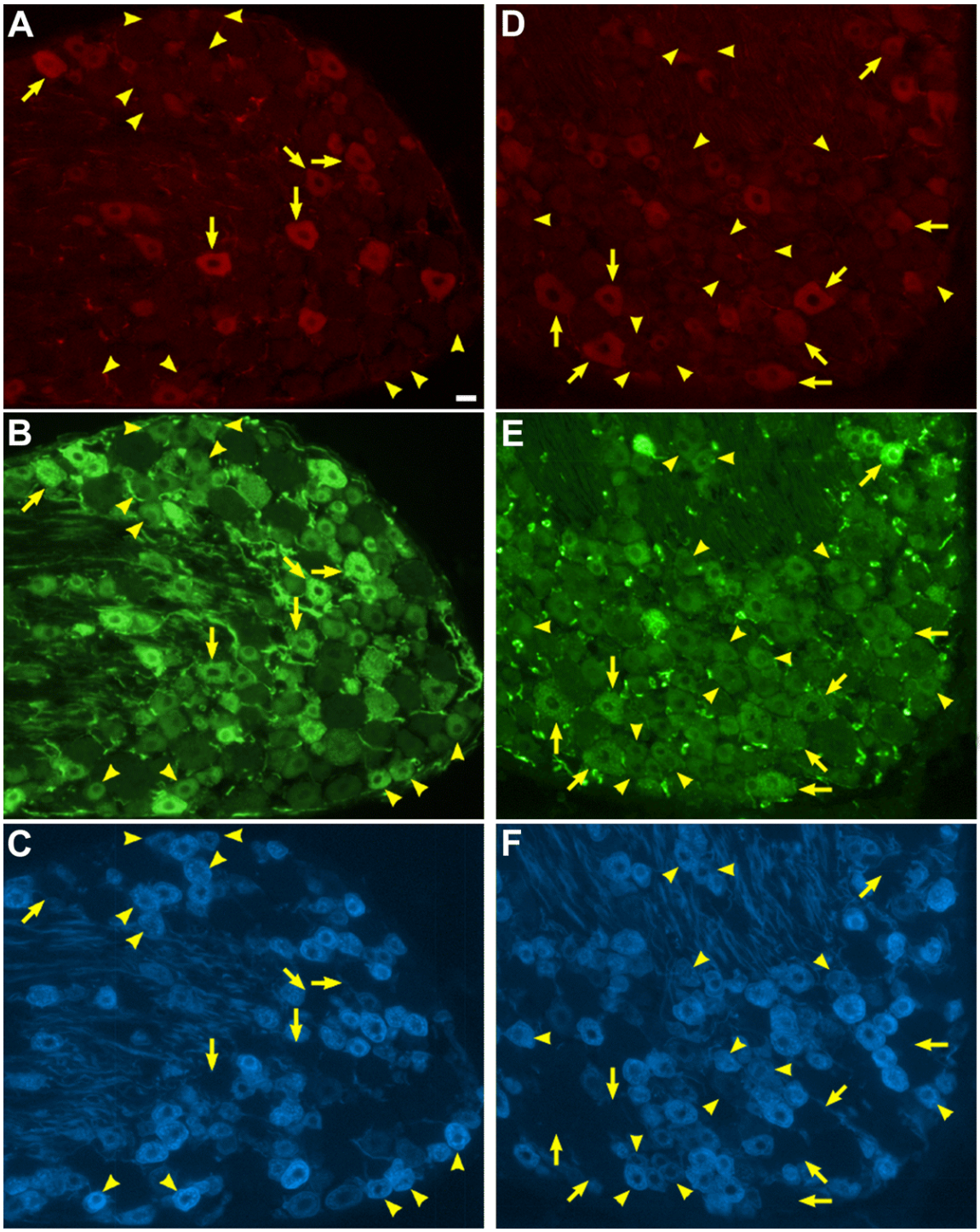

Figure 5. Histochemistry of both normal (A-C) and PACS (D-F) DRG. Sections were stained for trkA (red; A, D), GAP-43 (green; B, E), and GS-I-B4-binding (blue; C, F). Arrows indicate GAP-43-positive neurons that also displayed trkA-IR, while arrowheads indicate GAP-43-positive neurons that lacked trkA-IR, but expressed GS-I-B4-binding.

also visible in the experimental DRG. Interestingly, there appeared to be a general reduction in signal intensity, though certain neurons had even higher intensity GAP-43-IR than was present in the controls.

As expected from previous reports[230], many GAP-43-IR neurons in the control DRGs also expressed trkA-IR (55.3±9.8%). Qualitatively, it was clear that those neurons with high levels of GAP-43-IR were most often trkA-IR (Figure 5). Somewhat surprisingly in light of previous reports[15, 230], there was a large proportion of the GAP-43-IR population that lacked trkA-IR (44.7±9.8%). These neurons generally displayed low levels of GAP-43-IR, but were clearly positive. Most of these trkA-negative/GAP-43-IR neurons also expressed GS-I-B_4-binding (94.2±2.9%), indicating that they were part of the population of interest.

The distribution of GAP-43-IR in DRG neurons was not changed in the DRGs involved in PACS (Table 2). Contrary to the hypothesis, the percentage of neurons expressing GAP-43-IR but lacking trkA-IR was not found to be significantly different between control and experimental DRGs by two-tailed t-test. Qualitatively, though the general GAP-43-IR signal intensity was reduced, GAP-43-IR could still be observed in trkA-negative DRG neurons (Figure 5).

Table 2. Percentages of neurons in control and PACS ganglia.

	$trkA^-$ of $GAP\text{-}43^+$	**GS-I-B_4^+ of $GAP\text{-}43^+/trkA^-$**
Control	46.0±7.5	94.2±2.9
PACS	41.5±5.1	77.3±7.5**

Values represent the mean of the group ± SD. ** indicates significance at $p<0.01$

The proportions of the GAP-43-positive/trkA-negative group that expressed GS-I-B_4-binding was also examined. Most of the GAP-43-positive/trkA-negative neurons

did bind GS-I-B_4 (Figure 5). There was, however, a significant difference between groups for GAP-43^+/trkA^-/GS-I-B_4^+ neurons by two-tailed t-test ($p=0.01$)(Table 2).

In order to focus on the trkA-negative small diameter population, the distribution of GAP-43-IR was also assessed in relation to SOM-IR or P2X3-IR. Quantitatively, nearly all SOM-IR neurons in control DRG (n=7) expressed GAP-43-IR (Table 3).

Table 3. Percentages of neurons expressing GAP-43-IR.

	SOM	**P2X3**
Control	95.3±7.9	94.4±7.3
PACS	90.5±4.7	88.3±6.2

Qualitatively, the GAP-43-IR signal intensity was mixed. Rarely did SOM-IR neurons display the highest levels of GAP-43-IR, but they did often display moderate to strong GAP-43-IR, and rarely displayed the lowest levels of GAP-43-IR (Figure 6).

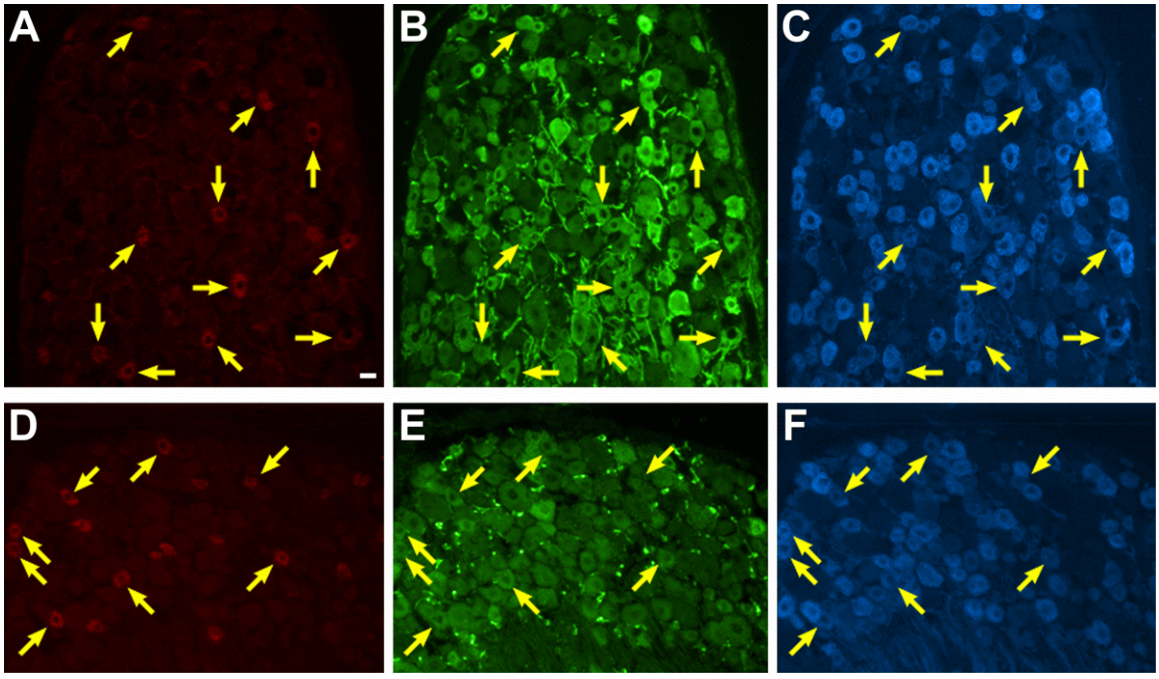

Figure 6. Examples of multi-labelling histochemistry of both normal (A-C) and PACS (D-F) DRG sections. Sections were stained for SOM (red; A, D), GAP-43 (green; B, E), and GS-I-B4-binding (blue; C, F). Arrows indicate SOM-IR neurons that also displayed GAP-43-IR. All SOM-IR neurons were GS-I-B4-reactive.

The P2X3-IR population also consistently displayed GAP-43-IR. In control DRG (n=5), 94% of P2X3-IR neurons displayed GAP-43-IR. Qualitatively, the majority of these neurons displayed moderate to weak GAP-43-IR (Figure 7).

These distributions were not different in the PACS group. The proportion of SOM-IR neurons displaying GAP-43-IR in the PACS DRG was not significantly different from controls. The same was true for the P2X3-IR population. The proportion of P2X3-IR neurons displaying GAP-43-IR in the PACS DRG was not significantly different from controls. Qualitatively, GAP-43-IR was appeared to have a very similar distribution pattern to control DRG (Figures 6, 7).

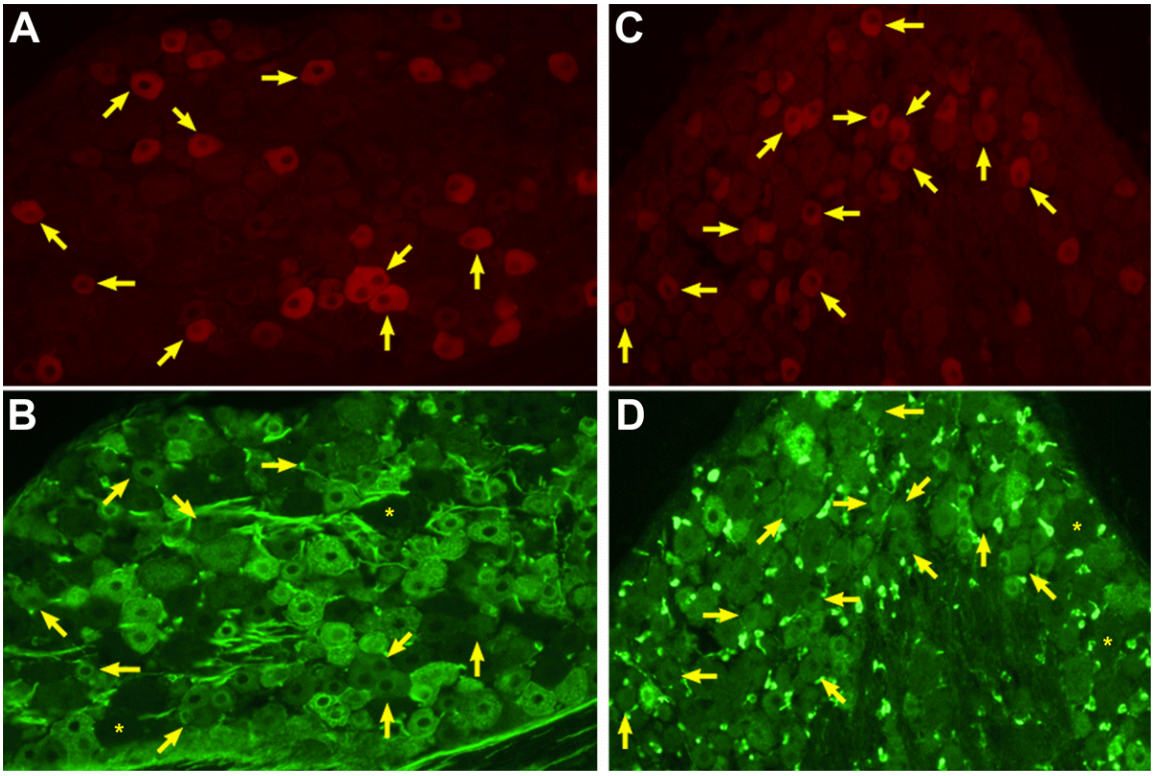

Figure 7. Examples of multi-labelling histochemistry of both normal (A, B) and PACS (C, D) DRG sections. Sections were stained for P2X3 (red; A, C) and GAP-43 (green; B, D). Arrows indicate P2X3-IR neurons that also displayed GAP-43-IR.

Detection of GAP-43 Protein

It was clear during the development of the GAP-43 immunohistochemical protocols that the method of detection employed could significantly affect the quantitative outcome. This was confirmed with the unexpected discovery of large proportions of non-trkA DRG neurons that displayed GAP-43-IR. A brief assessment was made of a variety detection methods. This provided an illustration of the likely cause of the discrepancies between previous reports of GAP-43 mRNA and protein, and also between the current report and previous ones.

The direct comparison of the detection methods made it clear that strong signal amplification was a requirement for the accurate assessment of GAP-43-IR in the DRG soma. Indirect fluorescence revealed very few trkA-negative neurons with GAP-43-IR (Figure 8A, B), and this was not significantly improved with the use of an avidin-biotin amplification step (Figure 8C, D). Reliable and reproducible detection of the trkA-negative GAP-43-IR population required the use of enzymatic amplification. The current results were obtained using the avidin-biotin-HRP complex (ABC kit - Vector Labs, Inc.) to catalyze the deposition of the tyramide signal amplification system (TSA - NEN, Inc.; Figure 8E, F).

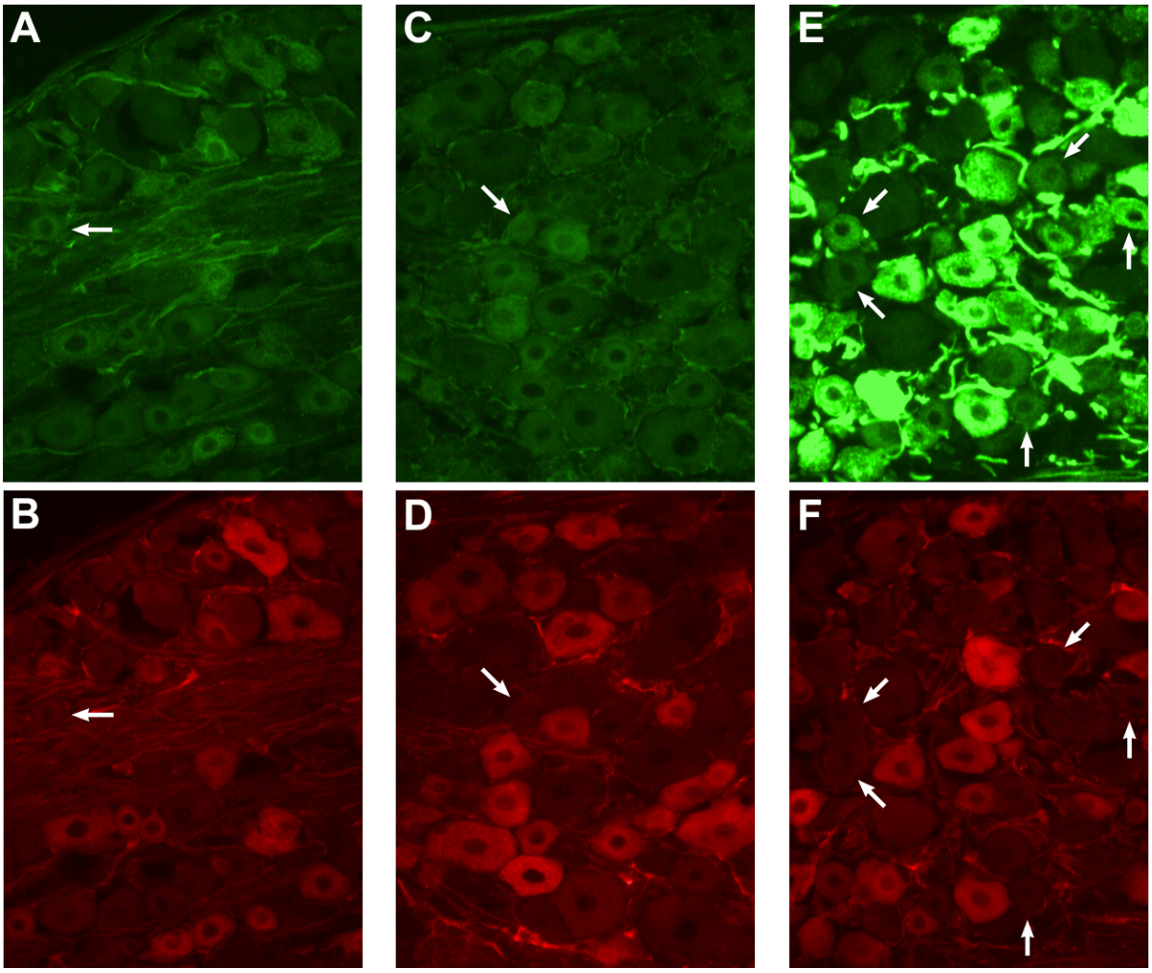

Figure 8. Examples of multi-labelling histochemistry of normal DRG sections with different methods of visualizing GAP-43-IR. Sections were stained for GAP-43 (green; A, C, E) and trkA (B, D, F). Arrows indicate GAP-43-IR neurons that lacked trkA-IR. GAP-43-IR was visualized with fluorophore-conjugated secondary antiserum (A), biotinylated secondary with fluorophore-conjugated avidin (C), or with enzymatic amplification via a biotinylated secondary followed by the avidin-biotin-HRP complex (ABC kit) which deposited FITC-conjugated tyramide (E). Note the dramatic increase in the number of clearly GAP-43-IR neurons in E. Note also that there were still many clearly GAP-43-negative neurons.

CHAPTER 4
RESULTS - CTM REFLEX AFFERENTS

192-Saporin Injections

In order to directly examine the contribution of the trkA-IR and non-trkA-IR DRG neurons to the CTM reflex, a neurotoxin conjugate directed against the p75 neurotrophin receptor was injected into the left T13 DRG. It was previously demonstrated that p75 mRNA was expressed in nearly all trkA-positive DRG neurons, but was not expressed in neurons lacking mRNA for any of the trk receptors[243]. Brief examinations of p75-IR, trkA-IR, SOM-IR, and GS-I-B_4-binding performed as part of this work demonstrated that the conclusions made by Wright and Snider (1995) at the mRNA level were likely to be true at the protein level as well. Nearly all trkA-IR neurons also expressed p75-IR. Further, even though many non-trkA-IR neurons displayed p75-IR, none of the SOM-IR neurons expressed trkA-IR or p75-IR, and only those GS-I-B_4-binding neurons that also had trkA-IR showed p75-IR. Thus, since the desired end was the destruction of trkA-expressing neurons, a toxin directed at p75-bearing neurons should have been sufficient.

Electrophysiology

Terminal electrophysiological experiments were performed 7 to 23 days following injection of 192-sap (75ng in 1.0-1.2µl) into the T13 DRG (n=7) or T12 DRG (n=1; animal had only 5 lumbar vertebrae which caused a mis-identification of T13

during the injection surgery). When the CTM reflex was elicited by bipolar electrical stimulation of DCnn from *uninjected* DRG, it appeared normal in threshold, latency, duration, and magnitude. In contrast, the CTM elicited from stimulation of the DCn from the *injected* DRG was absent, or extremely weak, in comparison with the reflex elicited from other nerves in the same animal and uninjected animals in 5 of 8 cases (Figure 9).

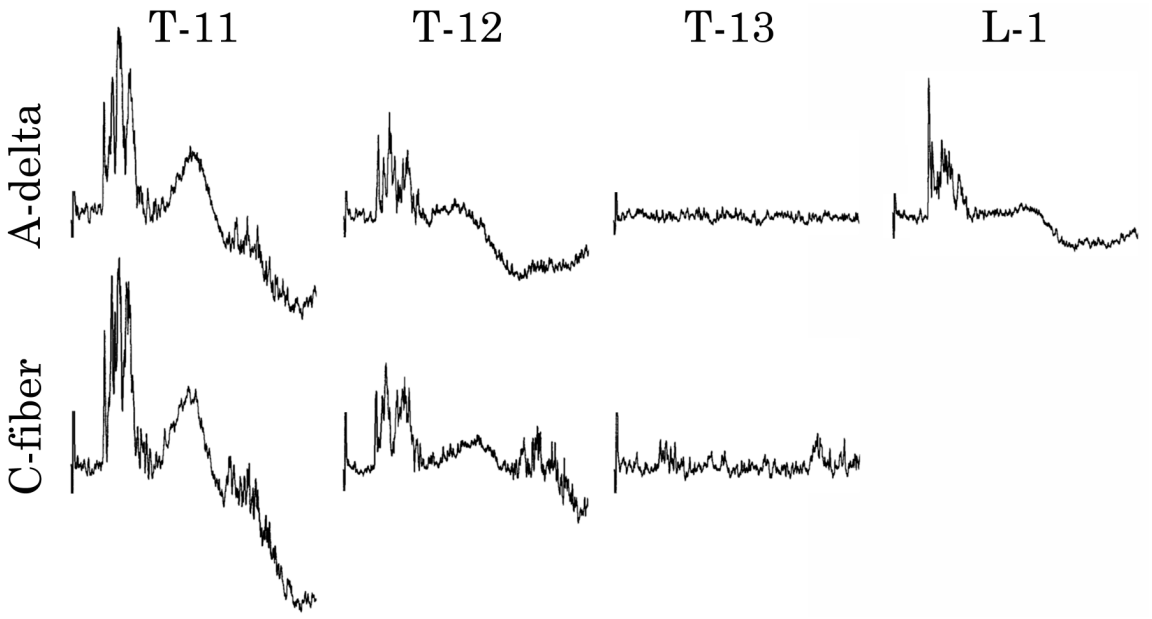

Figure 9. Recorded responses of the Lateral Thoracic nerve (LTn) ipsilateral to the injected DRG to stimulation of ipsilateral Dorsal Cutaneous nerves (DCnn). The DCn stimulated is indicated above the columns of traces, and the stimulus intensity is indicated to the left of the rows of traces. The T13 DRG was injected in this case. The large waves present in some of the traces is motion artifact due to contraction of the CTM lying under the recorded LTn.

Further, the threshold for generating a visible response (if possible at all) was greater for the injected nerve than for the other nerves. Averaged recordings from filaments of the dorsal root from the injected DRG in response to stimulation of the DCn revealed the presence of functional A- and C-fibers in the DCn (Figure 10). The A-fiber compound wave was dramatically reduced compared with both ipsilateral and contralateral control nerves, as was the C-fiber wave, though to a lesser degree (Figures 10 , 11).

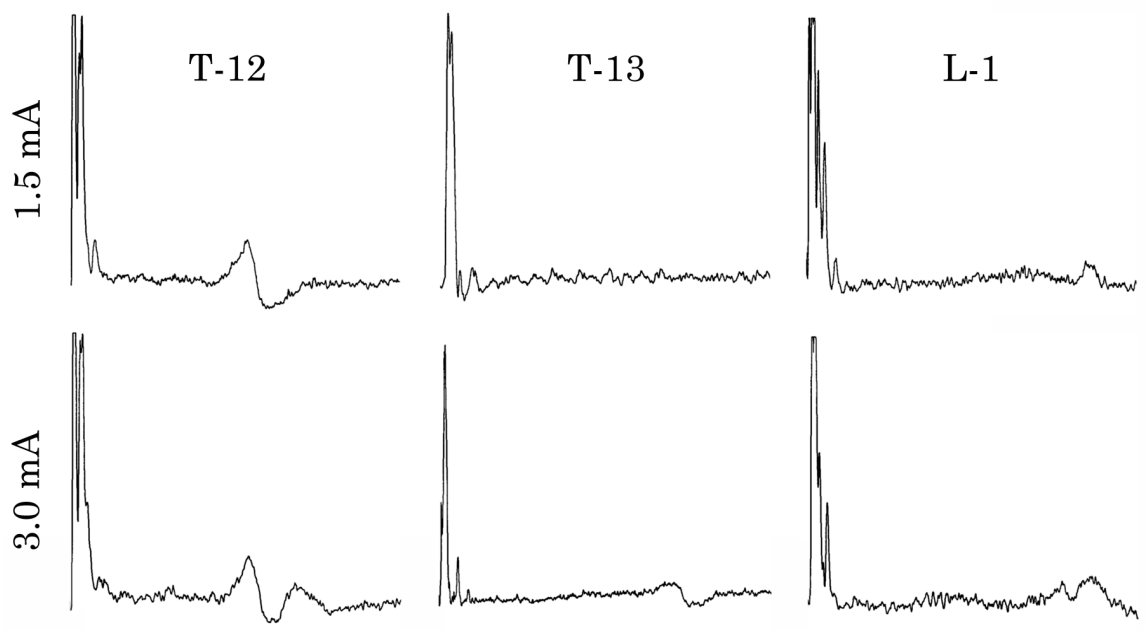

Figure 10. Recorded responses of dorsal roots (level indicated above columns of traces), in response to stimulation of the appropriate DCn. Stimulus intensity is indicated to the left of the rows of traces. The T13 DRG was injected in this case. Note that the T13 response indicates that there are still functional A- and C-fibers present. Also note that the A-fiber responses from the uninjected segments have saturated the amplifier, capping the trace, whereas the responses from the T13 segment have not since they are much smaller.

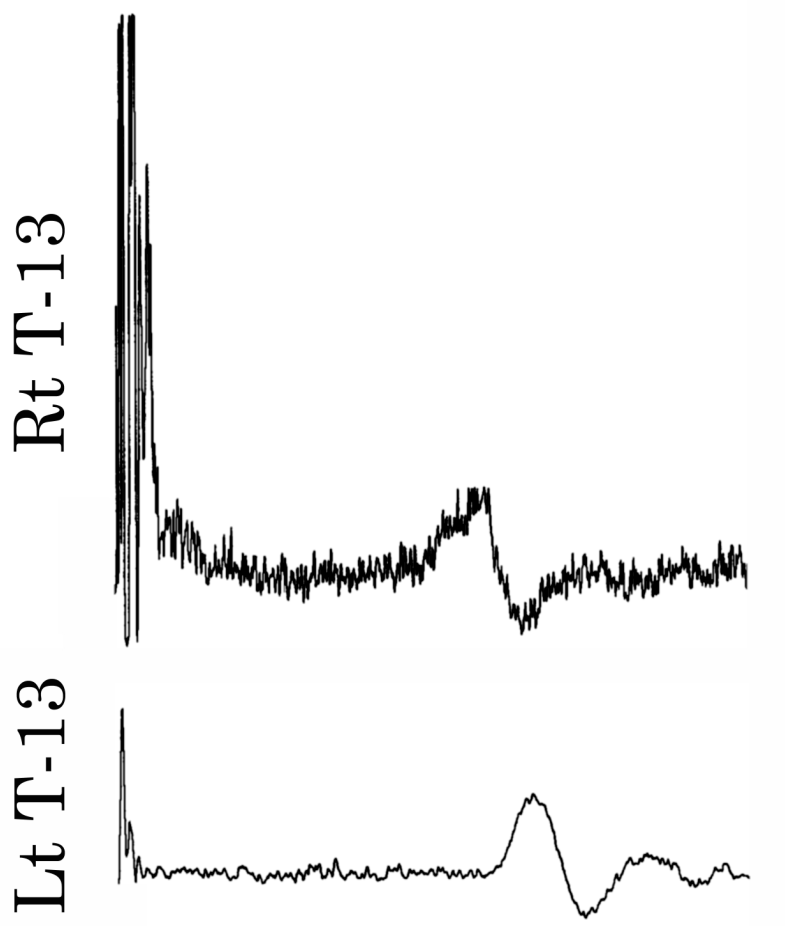

Figure 11. Recorded responses of the dorsal roots of both the injected (Lt T13) and uninjected (Rt T13) DRG in response to stimulation of their DCn. The higher level of noise from the Rt T13 recording is a result of the stimulated DCn being intact, as opposed to the Lt T13 DCn, which had been transected. The delay in the Lt T13 C-fiber wave could be accounted for by differences in conduction distance.

Histochemistry

Qualitative histological examinations of both the injected and contralateral uninjected DRG for trkA and related markers revealed that the neurotoxin had severely disrupted, or destroyed, nearly all trkA-IR neurons. TrkA-IR was clearly diminished in terms of the number of IR neurons, and nearly completely abolished in terms of IR intensity in the injected DRG. The same was true for p75-IR (detected with two separate antisera), although the extracellular p75-IR remained nearly unchanged between the DRG (Figure 12). Substance P-IR (SP-IR) neurons were clearly fewer in number and weaker in intensity in the injected DRG than the contralateral DRG, although there were many SP-IR axons that appeared normal. CGRP-IR appeared in many neurons in the injected DRG. However, the pattern of CGRP-IR in many of the neurons was clearly different from normal DRG. The CGRP-IR was diffuse throughout the cytoplasm, as opposed to the usual strong and granular morphology of vesicular neuropeptides. In such neurons, GS-I-B_4-binding was either absent or extremely weak.

Qualitative histological examinations of the DRG with markers specific for non-trkA-IR small diameter neurons revealed that while the trkA-IR population had been severely reduced, the non-trkA-IR, GS-I-B_4-binding neurons appeared unaffected. Large numbers of GS-I-B_4-binding neurons remained in the injected DRG and had a completely normal appearance. SOM-IR was intact and appeared normal in morphology and number in the injected DRG (Figure 13). P2X3-IR also appeared completely normal in the injected DRG (Figure 13). A summary of the data is presented in Table 4.

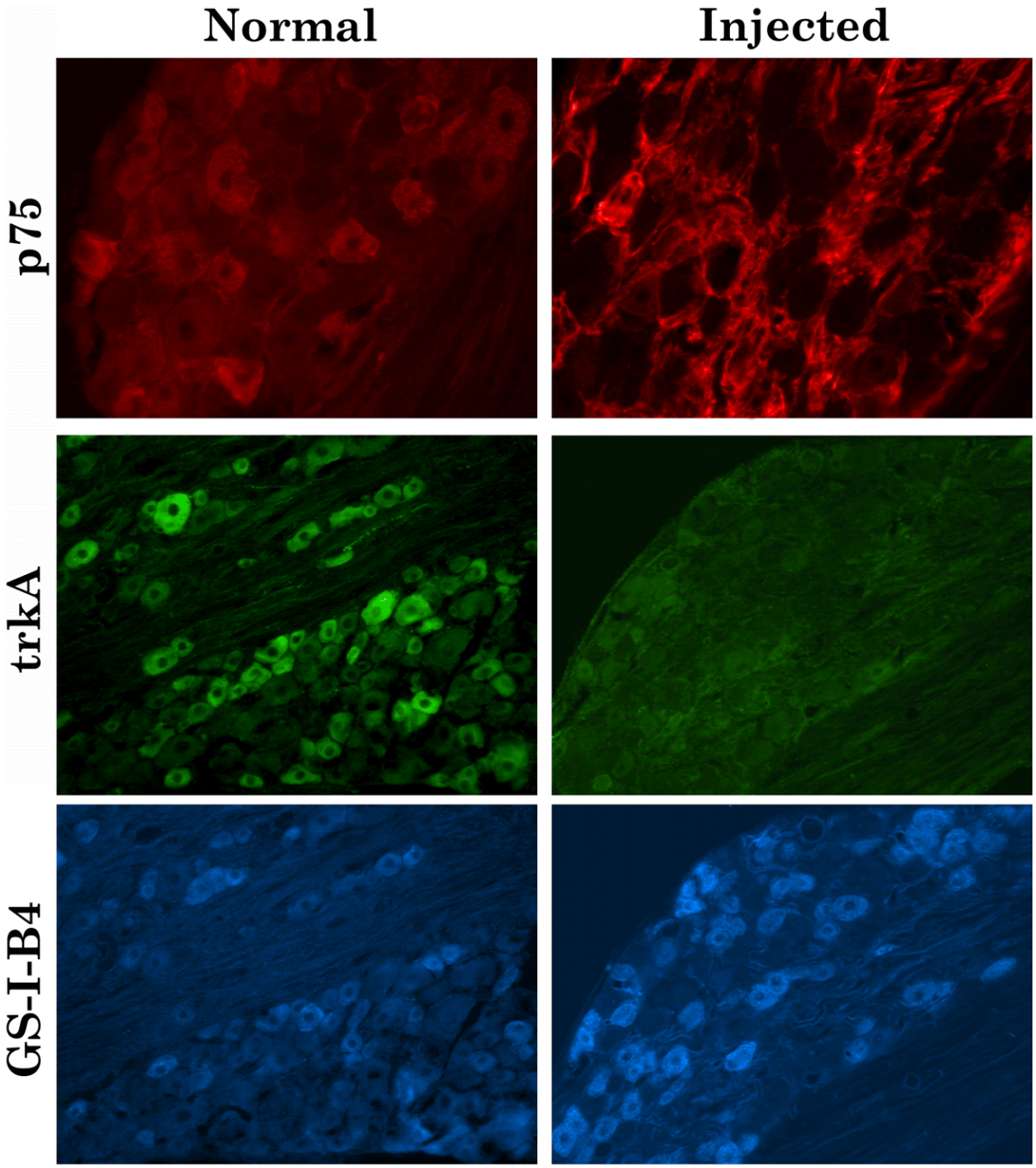

Figure 12. Histochemical stains of both injected and contralateral uninjected DRG. For each group, the trkA and GS-I-B4 stains are from the same sections, and the p75 stains are from a different section. Note the dramatic loss of both p75 and trkA from neurons in the injected DRG, while GS-I-B4-binding appears unaffected.

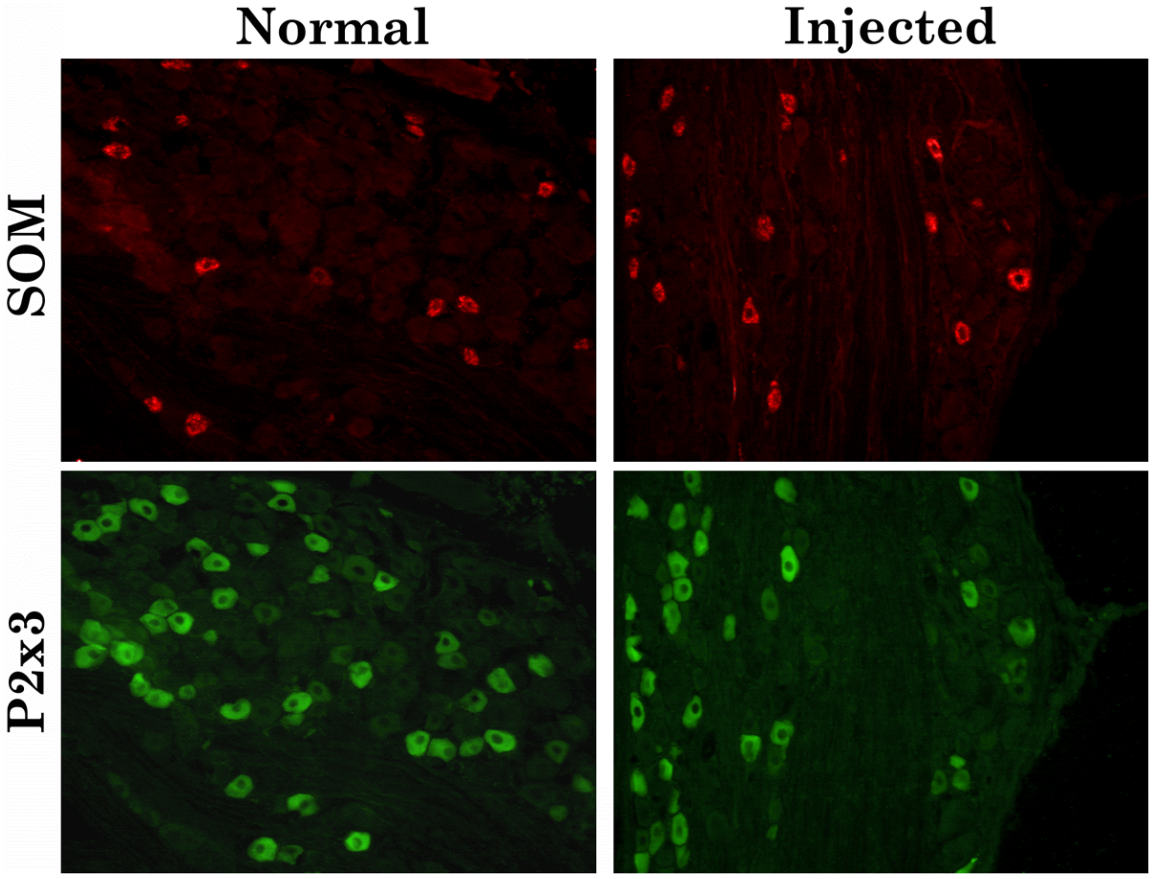

Figure 13. Histochemical stains of both injected and contralateral uninjected DRG. For each group, the SOM and P2X3 stains are from the same section. Note that the staining for both markers in the injected DRG appears unaffected.

Table 4. Summary of 192-saporin injection experiments.

Injected DRG (left)	Case	Dose (ng)	Vol. (µl)	Time (days)	Reflex	Threshold* Ratio (L:R)	Histochemistry	
							trkA	non-trkA
T13	7	90	1.2	16	absent	n/a	depleted	normal
T13	4	75	1.0	23	absent	n/a	depleted	normal
T12	6	90	1.2	22	absent	n/a	depleted	normal
T13	2	75	1.0	13	weak	16.2	depleted	normal
T13	8	90	1.2	21	weak	10.5	depleted	normal
T13	3	75	1.0	17	strong	1.3	normal	normal
T13	1	50-90	1.0	7	strong	1.0	normal	normal
T13	5	90	1.2	21	strong	1.0	normal	normal

* Threshold was determined by electrical stimulation of the DCnn of the injected DRG and the contralateral uninjected DRG.

Pseudorabies Virus Circuit Tracing

Forty-eight to 76 hours following injections of PRV into either the left CTM or the left LTn, the C7, C8, T12, and T13 spinal cord segments and the bilateral T12, T13, and L1 DRG were retrieved and processed to detect the presence of PRV. Many of the injections did not result in successful infections. An injection was considered to result in a successful infection if PRV was detected in the CTM motoneuron pool. Of the 17 injections designed to trace the CTM circuit, 4 resulted in clear infection. PRV was reliably detected in the DRG in experiments where the injections lead to successful infections. The PRV was present almost exclusively in a subpopulation of small diameter neurons (Figure 14). Multi-labelling fluorescence experiments were carried out to determine the relationship of PRV-IR to trkA-IR and GS-I-B_4-binding. PRV-IR was consistently demonstrated to co-exist primarily with trkA-IR (Figure 14). PRV-IR was observed in GS-I-B_4-binding neurons, but the majority of these also expressed trkA-IR. Therefore, most neurons infected with PRV up to 76 hours post-injection were those that expressed the NGF receptor trkA.

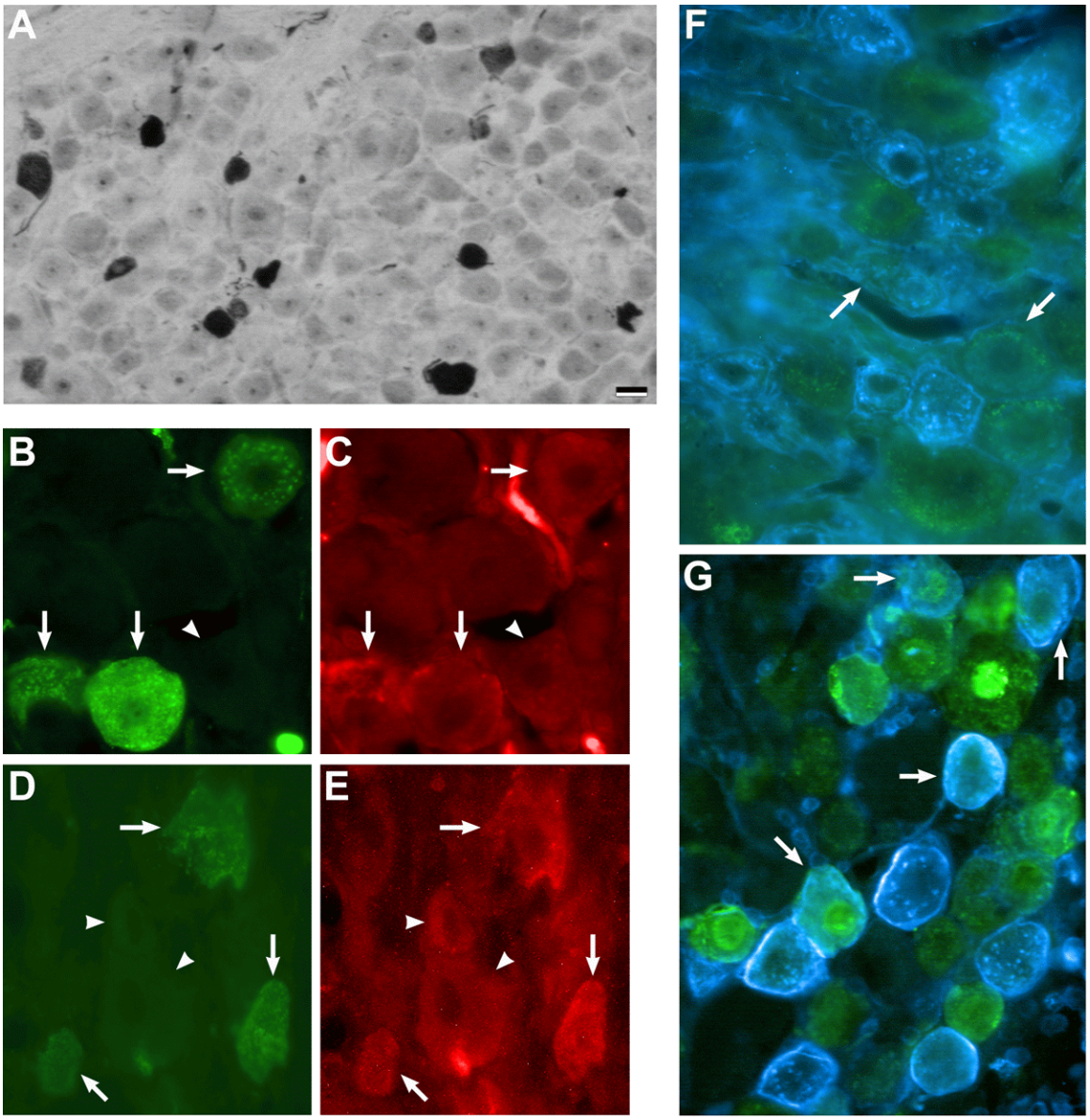

Figure 14. Examples of DRG from PRV injected animals. Panel A demonstrates that PRV primarily infected small diameter DRG neurons (scale bar indicates 25µm). PRV (green; B, D, F, G) was also localized primarily to neurons that expressed trkA (red; C, E), and those that lacked GS-I-B4-binding (blue; F, G). Arrows indicate neurons that are infected with PRV and co-expressed the other marker. Arrowheads (B-E) indicate trkA-positive neurons that were not infected with PRV.

Control Experiments

Control experiments to examine whether or not there was any differential tropism of dorsal root processes for the Becker PRV were performed by injecting PRV directly

into the dorsal horn of the T12/T13 spinal cord. PRV-IR was detectable in the vast majority of T12 and T13 DRG neurons 24 hours after injection, indicating that nearly all neurons were capable of taking up and transporting the virus from their central terminals (Figure 15).

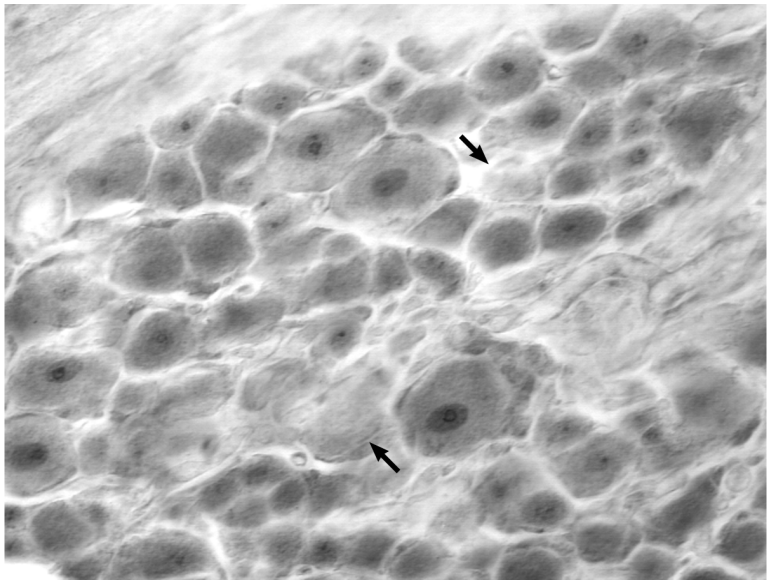

Figure 15. Example of a T12 DRG section stained for PRV 24 hours after injection of PRV into the T12-T13 dorsal horn. Nearly every neuron showed evidence of infection, regardless of diameter. Arrows indicate neurons that appear to lack any sign of infection.

Control experiments were also done to assess whether PRV injected into the CTM muscle might be gaining access to the CNS from some route other than the CTM motor axons. Injections of PRV were made into the CTM in the same fashion as circuit tracing animals. In addition, certain nerve transections were also made. In order to investigate the possibility that the PRV was infecting afferents innervating the overlying skin, a PRV injection was coupled with transection of all ipsilateral LTn branches near the origin of the CTM (n=1). This resulted in no sign of infection. In order to investigate if the PRV was gaining access via some route other than axons innervating the injected

tissue, a PRV injection was coupled with transection of the ipsilateral LTn (as above), and also with transection of nearby DCnn (n=1). This resulted in no sign of infection. In order to investigate whether the central infection was due to infection of the CTM motor axons, a PRV injection was coupled with transection of the ipsilateral neighboring DCnn (n=1). This animal showed signs of infection in both the CTM motor neuron pool and the low thoracic DRG. While the controls that showed no infection certainly provide evidence in support of the route of infection being limited to the CTM motor axons, negative results must be interpreted cautiously due to the low rate of infection intrinsic in this method. The latter control, however, provided positive evidence that the CTM motor axons did provide a route of infection that could give rise to subsequent infection of the low thoracic DRG.

CHAPTER 5
DISCUSSION

Unmyelinated Afferents Lacking trkA Participate in PACS

Skin Histochemistry

Histochemical analysis of the collaterally reinnervated skin regions revealed the presence of axons that appeared to have been derived from the small diameter DRG neurons lacking trkA and expressing GS-I-B_4-binding. While these axons were regularly and consistently observed, they were not present as frequently as those expressing NGF-related markers (sensory or sympathetic). This could indicate that while the population in question was likely capable of PACS, it may not have exhibited quite as robust a response as the trkA-expressing afferents and sympathetic neurons. This finding supported one of the guiding hypotheses of this work, namely, that **the non-trkA small diameter DRG afferents were involved in PACS, but have been missed thus far.**

In order to be certain that the hypothesis has truly been supported, the identification of the axons in collaterally reinnervated skin must be highly reliable. The areas of greatest concern were 1) the possible mis-identification of trkA-positive axons as trkA-negative axons; 2) the possible mis-identification of sympathetic axons as sensory axons; and 3) the possible mis-identification of axon remnants as collaterally sprouted axons. This latter possibility was of least concern. Previous studies have shown that axons in target tissues deteriorate rapidly following transection[61, 116, 168, 169, 185, 135, 155, 85]. No remnants of transected axons should have remained in the denervated skin into the

time that the collaterally sprouting axons reinnervated the tissue, let alone into the time when the animal was euthanized and the skin examined for innervation. Nonetheless, controls were also examined. The skin examined after a 3 day survival following T13 isolation surgery (the same as used for induction of PACS) revealed that the skin was essentially completely devoid of axons as revealed by stains for PGP 9.5, SP, CGRP, and GAP-43. Some GS-I-B_4-binding did remain on structures that appeared to be axons, but the intensity was very weak, and was not accompanied by PGP 9.5 as it was in normal or collaterally reinnervated skin. These same stains were used to examine the distal segments of transected or crushed nerve (with survival times of 7 to 14 days). None of the stains, including the lectin, revealed any signal. Therefore, it was concluded that the axons revealed in the collaterally reinnervated skin were in fact derived from collaterally sprouted axons from the spared segment, and were not remnants of transected axons.

In order to address the other possible mis-identifications, a number of combinations of stains and detection methods were used to identify axons in collaterally reinnervated skin. Since it had been shown previously (by electrophysiological recording, not CTM reflex) that the only sensory axons present in collaterally reinnervated skin were small diameter high threshold axons (to the exclusion of larger myelinated axons)[84, 92, 93], identifications did not have to be concerned with eliminating the larger trkA-negative sensory afferent population. The task was to clearly identify trkA-negative unmyelinated sensory afferents. To do this, GAP-43-IR or PGP 9.5-IR distinguished axonal structures in the collaterally reinnervated skin, and GAP-43-IR also identified growing axons. In combination with these, stains for trkA or CGRP were used (see overlap of trkA and CGRP - Figure 1). GS-I-B_4-binding, P2X3-IR, and SOM-IR were also used to identify the trkA-negative unmyelinated sensory afferents.

GAP-43/trkA/GS-I-B_4: The combination of trkA-IR and lectin-binding revealed trkA-positive axons (sensory or all sympathetic) as $trkA^+/GS\text{-}I\text{-}B4^{+/-}$, or trkA-negative unmyelinated sensory axons as $trkA^-/GS\text{-}I\text{-}B_4^+$ (Figure 2). Since trkA is expressed by all sympathetic axons, and the detection method employed very strong enzymatic signal amplification, axons that lacked trkA-IR could definitively be considered trkA-negative unmyelinated sensory afferents.

GAP-43/CGRP/GS-I-B_4: The combination of CGRP-IR and lectin-binding revealed trkA-positive sensory axons (CGRP is not found in sympathetic axons but has a strong degree of overlap with trkA in sensory neurons[157]) as $CGRP^+/GS\text{-}I\text{-}B4^{+/-}$. TrkA-negative unmyelinated axons (sensory or sympathetic) were revealed as $CGRP^-/GS\text{-}I\text{-}B_4^+$ (Figure 2). Since both trkA-negative unmyelinated sensory axons and nearly all sympathetic axons display GS-I-B_4-binding, morphology of the innervation was used to determine whether the CGRP-negative axon was sensory or sympathetic. Such axons on vasculature were considered sympathetic, although some axons on vasculature were trkA-negative. Such axons that were located in the subepidermal area or extended into the epidermis were considered trkA-negative sensory axons.

GAP-43/P2X3: P2X3-IR was taken to indicate a trkA-negative small diameter unmyelinated sensory axon (Figure 3). The overlap of P2X3 with CGRP and/or trkA is very small, but P2X3-expression in small diameter sensory neurons is almost completely encompassed inside of the GS-I-B_4-binding population[22, 232, 233]. Further, P2X3 is not expressed by sympathetic neurons[34].

GAP-43/SOM: SOM-IR, when not found on/near vasculature, was taken to indicate trkA-negative unmyelinated sensory afferents (Figure 3). Sensory neurons expressing SOM are entirely encompassed inside of the trkA-negative, GS-I-B_4-binding

population[235]. However, since SOM-IR is found in some sympathetic neurons, morphology was considered when assessing a sensory versus sympathetic identity for SOM-IR axons.

Numerous combinations of stains conclusively revealed the presence of trkA-negative unmyelinated sensory axons in collaterally reinnervated skin. The mis-identification of sympathetic axons as sensory axons, as well as the mis-identification of trkA-positive axons as trkA-negative was eliminated by staining the skin with antibodies against trkA and detecting trkA-IR with strong signal amplification. Further, stains specific for the trkA-negative population were used, and also revealed many axons in collaterally reinnervated skin. The demonstration of trkA-negative axons in reinnervated skin also indicated that at least some portion of the unmyelinated sensory axons that previously lacked trkA-IR remained trkA-negative.

DRG Histochemistry

Histochemical analysis of DRG housing neurons undergoing PACS (PACS DRG) was undertaken to determine whether or not trkA-negative unmyelinated afferents expressed GAP-43, an indicator of ongoing plasticity. It had been previously shown that GAP-43 was preferentially expressed in small diameter trkA-positive neurons of the DRG [15, 230]. It was hypothesized, based on the distribution of GAP-43 in normal DRG and the novel findings of trkA-negative axons in collaterally reinnervated skin, that **the trkA-negative unmyelinated neurons of the PACS DRG would begin to express GAP-43 in response to the surgical isolation of the dermatome.** It was expected that there would be an increase in the proportion of GAP-43^{+}/trkA^{-} neurons in PACS DRG.

Contrary to the hypothesis, it was found that there was no significant difference in the proportion of GAP-43^{+}/trkA^{-} neurons between the normal and PACS DRG. There was, however, a unique finding. It was clear that the reason that there was no difference between the control and PACS DRG was *not* because there were very few trkA-negative neurons that expressed GAP-43 in PACS DRG. Instead, the normal DRG housed a large group of trkA-negative unmyelinated neurons that expressed GAP-43, which was unexpected based on the literature[15, 230]. The difference between the present results and what was expected based on the literature is likely due to a much improved means for visualizing the GAP-43-IR (Figure 8). The current results were obtained using a degree of signal amplification much greater than what has been previously reported for a fluorescence immunohistochemical study of GAP-43 [15, 200]. The novel GAP-43-IR distribution is demonstrated in the context of other markers in Figure 16.

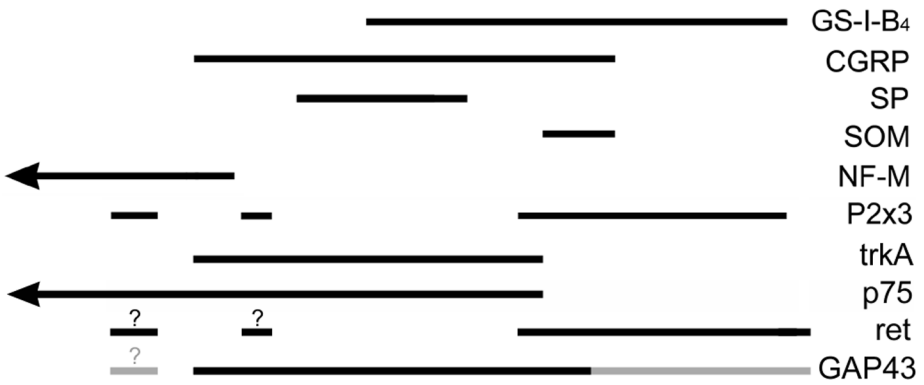

Figure 16. Schematic representation of the general overlaps of certain markers in small and medium diameter DRG neurons. The gray portions of the GAP-43 distribution represent the new findings reported in this dissertation. The question mark indicates a portion of the GAP-43 distribution that lacks trkA-IR and GS-I-B4-binding. It is possible that it may overlap with the larger RET-positive neurons, but this is not yet known.

The vast majority of the GAP-43^{+}/trkA^{-} neurons also displayed GS-I-B_4-binding. There was also no significant difference between normal and PACS DRG in the proportion of neurons displaying SOM-IR and GAP-43-IR. A significant difference was found, however, between normal and PACS DRG for the proportion of GAP-43^{+}/trkA^{-} neurons that displayed GS-I-B_4-binding. In normal DRG, the vast majority displayed GS-I-B_4-binding. In PACS DRG however, this proportion was significantly lower (94% vs. 77%). There are multiple possible interpretations for these results.

Since there was not a significant decrease in the proportion of GAP-43^{+}/trkA^{-} neurons between normal and PACS DRG, it is possible that there was a loss of GS-I-B_4-binding neurons, or more likely, a loss of GS-I-B_4-reactivity by some neurons. It has been proposed that lectin-binding elements (in the case of GS-I-B_4 it is α-D-galactose) play a role in axonal outgrowth, pathfinding, and fasciculation[4, 55-57, 89, 95, 184]. As such, it seems unlikely that GS-I-B_4-binding should be lost during PACS. On the other hand, GS-I-B_4-binding is lost after transection of peripheral axons[156, 237]. It is possible that the difference between normal and PACS DRG is due to the transection of axons from the PACS DRG that were traveling in one of the neighboring DCnn. While the number of axons that project via a DCn other than their own is very low[247], they do exist. If this were the case, then transected axons would lose GS-I-B_4-binding, but would increase their levels of trkA-IR. The increase in trkA-IR intensity could change neurons that would been deemed negative during cell counts of normal DRG to being considered positive. These two changes together could create a significant difference in the proportion of GAP-43^{+}/trkA^{-} neurons that displayed GS-I-B_4-binding between control and PACS DRG.

It is also possible that there was an increase in the number of neurons expressing trkA in the PACS DRG. If this occurred primarily in GS-I-B_4-binding neurons, then the *proportion* of GAP-43^+/$trkA^-$ neurons that displayed GS-I-B_4-binding could have changed significantly. It has been shown that NGF levels can regulate the expression levels of its receptors[149, 220, 226]. It has also been shown that trkA and p75 levels were increased in PACS DRG [149, 151], though it was not clear if any neurons began to express trkA and/or p75 in PACS DRG that did not express them previously. Preliminary evidence was produced as part of this work that suggested that there was little, if any, increase in the number of SOM-IR (thus also GS-I-B_4-binding) neurons that expressed trkA. That did not, however, address the remainder of the normally $trkA^-$/GS-I-B_4^+ population. Both this possibility and the previous could be addressed by quantitatively measuring the proportion of trkA-expressing and GS-I-B_4-binding neurons in normal and PACS DRG in relation to the entire DRG population. It must also be considered, however, that although there was not a significant difference between normal and PACS DRG in the proportion of GAP-43-IR neurons that displayed trkA-IR, the raw number of neurons being considered in the GAP-43^+/$trkA^-$ group is much smaller than the GAP-43-IR group as a whole. Neurons lacking trkA made up only 46% and 41% of the GAP-43-IR population in normal and PACS DRG, respectively. Therefore, while differences may not have reached significance when considered in relation to the entire GAP-43-IR population, they may have become significant when compared to the much smaller group of GAP-43-IR neurons that lacked trkA.

As a summary for the possible explanations for the finding of a significant difference:

1) Not *all* GS-I-B_4-binding neurons expressed SOM. Therefore, there is still a group of $trkA^-/GS\text{-}I^+$ without SOM-IR that could lose GAP-43-IR without there being a change to the SOM^+/GAP^+ population;

2) There could have been a decrease in the number of GS-I-B_4-binding neurons or a downregulation of the GS-I-B_4-binding elements in some neurons;

3) There could have been an increase in the number of neurons expressing trkA, which could have occurred together with, or independently from, possibility #2;

4) There could have been an increase in the number of $trkA^-$/GS-I-B_4-negative very small, or larger, DRG neurons, that expressed GAP-43. This would have to be a small enough group that it would not be significant as part of the full GAP-43 population, but it could become significant when viewed inside of the smaller $GAP\text{-}43^+/trkA^-$ population.

The Role of Unmyelinated Afferents Lacking trkA in PACS Has Gone Unrecognized

PACS of the unmyelinated afferents lacking trkA has been clearly demonstrated. However, their role in PACS has gone unrecognized until this work. Some of the possible reasons for this have been presented in Chapter 1, and addressed as part of this dissertation.

Previous Histochemical Examinations Could Have Been Insufficient

It was possible that the histochemical stains (silver stains) employed to visualize axons that had reinnervated skin by PACS were not sufficient to reveal all axons that may have actually been present. If that were the case, then studies where PACS was prevented by administration of anti-NGF antiserum[52, 166] and subsequently found no axons (by silver stain) in the denervated skin may have simply missed axons that were actually present, but were not revealed by the silver stains.

The insufficiency of stains also extends to previous work which used immunohistochemistry. Previous studies used stains for markers (e.g., PGP 9.5) which were not specific for the various subpopulations of afferents (or sympathetic efferents, for that matter)[108, 109, see also 140, 216]. Moreover, most studies focussed on the trkA-positive, peptidergic population, and did not take the trkA-negative population into account at all. As a result, such studies could not address whether the trkA-negative neurons were indeed involved in PACS.

Other studies attempted to reveal the terminal distribution patterns of axons that had undergone PACS, or were in the midst of PACS, by inducing the extravasation of Evan's Blue dye into the skin[23, 52, 139, 174, 191, 238]. This method was excellent for revealing the distribution of axons expressing SP and/or CGRP. However, it was completely insufficient to address the distribution of any non-peptidergic axons (therefore most trkA-negative unmyelinated afferents), since SP and/or CGRP are required for the extravasation of the dye[94, 134, 147, 195, 244].

Studies examining mRNA may have also been insufficient to reveal any role of the non-trkA afferents in PACS. Studies of the distribution of GAP-43 mRNA in normal

DRG revealed that neurons with high levels of GAP-43 mRNA almost invariably co-expressed trkA [230]. Neurons lacking trkA, however, expressed levels of GAP-43 mRNA that were very low, and very close to the background signal levels. Examination of GAP-43 mRNA in PACS DRG did not examine *which* neurons expressed GAP-43 mRNA [151]. The low levels of GAP-43 mRNA in non-trkA DRG neurons, and the lack of correlation of GAP-43 mRNA in PACS DRG with trkA, have made examination of the distribution of GAP-43 in normal and PACS DRG incomplete.

The histochemical examinations presented here have been designed to address the insufficiencies present in previous studies. Examinations of axons in skin were performed with markers specific for the trkA-bearing axons (trkA, CGRP) as well as the non-trkA bearing unmyelinated afferents (GS-I-B_4, P2X3, SOM). Further, the examinations of the distribution of GAP-43 in normal and PACS DRG presented here had two significant differences from previous relevant examinations. First, the present examinations looked at GAP-43 protein, not mRNA. This had the advantage of examining the actual functional unit of GAP-43, but the disadvantage that if the low levels of GAP-43 mRNA in non-trkA afferents actually translated to very low levels of GAP-43 protein, then the protein would be difficult to detect. This was a problem with previous studies as well[15]. Second, this very disadvantage was addressed. The current results were produced with a very strong and clean signal amplification system. This allowed the clear identification of even very low levels of GAP-43 protein. Thus, the current examinations have overcome the insufficiencies of the previous studies in order to directly examine the role of the non-trkA unmyelinated afferents in PACS.

NGF Insensitive Afferents Could Become Sensitive

Previous studies determined that NGF was paramount for PACS [49, 52, 70, 71, 173]. These studies detailed excellent data to support this conclusion, but could have suffered from insufficiencies that could have caused the overstating of conclusions. Some of these insufficiencies were addressed above. Of particular importance was the data demonstrating that anti-NGF treatments could halt PACS. This conclusion was reached after histochemical examinations of skin and behavioral examinations of the CTM reflex both indicated that there were no axons that had undergone PACS during anti-NGF treatments[49, 52, 70, 71, 173]. Possible insufficiencies in the histochemical examinations were discussed above, and possible insufficiencies in the CTM reflex examinations are presented below. If these examinations truly suffered from such insufficiencies, then the conclusion reached in those studies - that NGF is paramount for PACS - could be an overstatement. *It is possible that NGF is paramount only for PACS of trkA-positive afferents (and CS of sympathetic efferents)*, and that the PACS of trkA-negative afferents was missed in those studies for any number of the reasons discussed above.

However, it is also possible that NGF is in fact paramount for *all* PACS. If this is the case, then the possible insufficiencies of the previous studies may have been irrelevant. This possibility could be brought about in a number of ways. First, it is possible that the afferents that lack trkA and/or p75 in normal DRG began to express these receptors in response to isolation of their RF. It has been shown that the population of unmyelinated afferents that lack trkA in the adult did express trkA during development, but then ceased expressing the receptor[158, 159, 202]. If this expression occurred, then their participation in PACS may have been be entirely dependent on their

newly acquired responsiveness to NGF via the trkA and/or p75 receptors. If this were the case, then anti-NGF treatments would have prevent PACS of this population as well. Second, it is possible that the neurons that lacked NGF receptors in normal DRG still did not express them in PACS DRG, but their PACS mechanism was still somehow dependent on NGF in another way. This possibility is addressed in detail below.

The current experiments did not test these possibilities directly, though some data was generated that offers some indication that the possible *de novo* expression of NGF receptors was *unlikely* to have occurred. First, if the neurons that lacked trkA expression in normal DRG began to express trkA in PACS DRG and were involved in PACS, then it would be expected that there would be a significant decrease in the proportion of GAP-43^{+}/$trkA^{-}$ neurons in PACS DRG. This was not the case (Table 2). It is still possible, however, that a group of neurons did express trkA *de novo*, but was too small to become significant. It is also possible that the neurons expressed p75 *de novo*, since this was not examined. There is preliminary evidence that p75 knockout mice have a reduced capacity for PACS. However, they were still capable of PACS with administration of exogenous NGF, and all examinations were again focussed on only the trkA-bearing populations. Preliminary evidence was produced as part of this work that suggested that there was little, if any, increase in the number of SOM-IR (thus also GS-I-B_4-binding) neurons that expressed trkA. That did not, however, address the remainder of the normally $trkA^{-}$/GS-I-B_4^{+} population. These possibilities could be addressed by quantitatively measuring the proportion of trkA-expressing and p75-expressing neurons in normal and PACS DRG in relation to the entire DRG population.

Further evidence against the possibility of *de novo* synthesis of trkA in formerly non-trkA afferents is found in the present histochemical examinations of collaterally reinnervated skin. Clear evidence of the presence of numerous GAP-43^{+}/trkA^{-} axons was found in reinnervated skin regions (Figure 2). This provides evidence that at least a portion of the axons involved in PACS do not express trkA.

CTM Reflex Testing Could Be Insufficient For Non-trkA Afferents

A number of previous studies used induction of the CTM reflex as a test for the progress of PACS and to reveal its spatial extent[49, 50, 52, 59, 151, 166, 173]. It was also shown that anti-NGF could halt the expansion of the isolated RF capable of inducing the CTM reflex[49, 52, 70, 71, 173]. If non-trkA afferents did participate in PACS, then why did anti-NGF treatments prevent the expansion of the RF capable of inducing the CTM? This was possible if 1) the anti-NGF actually prevented PACS of the non-trkA afferents (discussed above and further below), 2) the non-trkA afferents did sprout and could have evoked the CTM reflex, but the adequate stimuli were not applied, or 3) the non-trkA afferents were not part of the CTM reflex circuit.

Certain possibilities regarding a role of NGF in any PACS mechanisms of the non-trkA afferents do exist. Some of these have been discussed, and others are discussed below. The possibility that the non-trkA afferents did sprout and could have evoked the CTM reflex had the adequate stimuli been applied is unlikely. It has been shown that the CTM reflex in rats can be evoked by noxious pinch and heat[217]. It has also been shown that the non-trkA unmyelinated afferent population has a number of properties that indicate that it encompasses families of neurons that are not only nociceptive, but

specifically have the capability to transduce noxious heat and/or mechanical stimuli. The capsaicin receptor VR-1 has been shown to be primarily localized to the non-peptidergic (thus non-trkA), GS-I-B_4-binding afferent population[75]. Further, the VR-1 receptor has been shown to be regulated by GDNF[154], which also regulates the non-trkA unmyelinated population[12, 159]. Capsaicin application has been shown to induce the sensation of noxious heat[117, 196, 215, 218], and in vitro experiments have shown that DRG neurons that respond to capsaicin also respond to noxious levels of heat[9, 33, 60, 161, 162, 246]. Type 2 neurons[29, 30, 44] also display strong capsaicin sensitivity and have been shown directly to lack SP-IR, CGRP-IR (and thus are non-trkA), but to express GS-I-B_4-binding (Petruska et al, unpublished observations). It has also been shown that the ATP receptor P2X3 was primarily localized to the non-peptidergic (thus non-trkA), GS-I-B_4-binding afferent population. This receptor confers rapid ATP-induced currents which have been associated with mechano-nociceptors[39]. P2X3 has also been shown to be present on type 2 neurons, which display rapid ATP-induced currents (Petruska et al, unpublished observations). Type 2 neurons have also been shown to be sensitized by PGE_2 [29]. This array of characteristics is consistent with non-trkA GS-I-B_4-binding afferents containing families with the capability to transduce noxious heat and/or mechanical stimuli. Therefore, the non-peptidergic, non-trkA unmyelinated afferents contain at least some neurons that have the capacity to transduce stimuli which are also capable of inducing the CTM reflex in rats, making it *unlikely that non-trkA PACS was missed because the proper stimulus for these afferents to evoke the CTM reflex was not applied.*

As an aside, there is another possible mechanism that could account for the applied heat stimuli in previous reports[59, 166, 173, 217] **not** activating non-trkA afferents that

may have sprouted into denervated skin during anti-NGF treatments. There is good evidence that different groups of thermo-nociceptors are responsible for transducing different cutaneous heating rates. Specifically, it appears that noxious heat applied at a low rate is transduced by capsaicin-sensitive C-fiber afferents, while that applied at a high rate is transduced by other nociceptors, likely Aδ-fiber afferents[245, 246]. The previous reports do not provide sufficient detail to determine whether the heating rates fell into one or the other category. However, the heating rates could very well have been rapid, as could be expected with the application of heat being through the advancement of a strong heat source toward the skin. If this were truly the case, then this stimulus *may not have activated* any non-trkA afferents that did sprout into the denervated territory, and thus the CTM reflex would not have been activated, leading to the possible mistaken conclusion that no sprouting had occurred.

A final possibility was that the non-trkA afferents were simply not involved in the CTM reflex circuit, and therefore would have been consistently missed in examinations of PACS that relied on the CTM reflex. This possibility was directly examined in the present experiments.

Primary Afferents and the CTM Reflex

Experiments were undertaken in order to investigate whether or not non-trkA small diameter DRG neurons played any role in the CTM reflex. This investigation was of vital importance in determining whether or not the CTM reflex was a suitable test to reveal the spatial extent of cutaneous innervation by C-fibers lacking NGF receptors. Induction of the CTM reflex by application of noxious stimuli to the skin has been the standard behavioral test for measuring the success and/or extent of PACS in rats[49, 52, 59, 93,

[166, 173]. Previous work had indicated that PACS, as measured by the CTM reflex, was entirely dependent on NGF [49, 52, 59, 150, 151, 173]. This being the case, the NGF-insensitive C-fibers have essentially been regarded either as incapable of PACS, or disregarded altogether in the context of PACS. The previous work left open the possibility that non-trkA C-fibers may in fact be capable of PACS, but only if these neurons were either not involved in the CTM reflex, or if they transduced some stimulus that was never applied as part of previous investigations. Therefore, experiments were undertaken to determine whether or not non-trkA C-fibers were involved in the CTM reflex.

Selective Destruction of Neurotrophin Receptor-Expressing Afferents

One approach taken to address the question of whether or not non-trkA C-fibers were involved in the CTM reflex was to acutely destroy the trkA-IR population and test the CTM reflex. This was accomplished through the use of a ribosome-inactivating toxin (saporin) that had been conjugated to a monoclonal antibody raised against an extracellular epitope of the rat low-affinity neurotrophin receptor (p75). No such toxin-conjugate directed against the trkA receptor itself was available at the time. However, detailed studies from other labs[243], as well as our own observations, had indicated that the p75 receptor was restricted in its DRG distribution to neurons expressing at least one of the high affinity neurotrophin receptors (trkA, trkB, trkC). Further, nearly all trkA-IR neurons co-expressed p75, and none of the non-trkA, GS-I-B_4-binding neurons expressed p75. As a result, while many more neurons than just the desired target neurons (trkA-IR) would be destroyed, the neurons in question (non-trkA, GS-I-B_4-binding) should have been immune to the toxin.

The results of these experiments demonstrated that the toxin did, in fact, severely affect the target neurons within the survival period. This was indicated histochemically by the clear loss of p75-IR (examined with two different antisera) and trkA-IR, as well as the morphological changes found in CGRP-IR and SP-IR from the injected DRG as compared to intact control and contralateral control DRGs (Figure 12).

The effects of the toxin also appeared to have been selective based on histochemical evidence. GS-I-B_4-binding was morphologically no different from controls. There were somewhat fewer neurons displaying GS-I-B_4-binding, but this was expected since many trkA-IR/CGRP-IR neurons also have GS-I-B_4-binding[157-159, 235]. More specifically, cell markers specific to the non-trkA, GS-I-B_4-binding neurons (P2X3 and SOM) showed no differences between controls and injected DRGs (Figure 13). This suggested that the synthesis and trafficking of neurotransmitters (SOM), membrane bound receptors/channels (P2X3), and Golgi apparatus and membrane markers (GS-I-B_4-binding) were intact. This in turn indicated that this subpopulation of neurons was likely functioning as normal.

Physiological examination of the primary afferent population in the DCn of the injected DRG indicated that a large complement of C-fibers remained (Figures 10, 11). This established that any differences between control and injected animals was not due to a general loss of C-fibers as a result of the injection. These results indicated that the 192-sap had altered the function of the target population, but had spared the non-trkA, GS-I-B_4-binding population. Further, this population had axons that were still functional.

The ability of electrical stimulation of the DCn from the injected DRG to induce the CTM reflex was also examined. This was a direct test of the involvement of the non-

trkA, GS-I-B_4-binding DRG neurons in the CTM reflex. Stimulation of the adjacent and contralateral DCnn revealed similar stimulus-intensity thresholds to CTM induction, as well as generating a normal-appearing CTM reflex. Electrical stimulation of the DCn from the injected DRG, however, revealed a higher threshold than the other nerves (if the reflex could be induced at all), as well as a severely weakened CTM reflex, even at stimulus intensities greater than those eliciting maximal responses when delivered to other nerves. *This was true only in animals where the histological examination of the injected DRG showed that the p75-bearing population had been affected.* Those that had a normal CTM response elicited from the injected dermatome were also histochemically revealed to have had normal appearing NGF-related markers. As an example, histochemical examination of the injected DRG from Case 2 (Table 4) revealed that a very small group of neurons with normal trkA-IR neurons was present in the injected DRG, as well as a group of axons with normal CGRP-IR and SP-IR morphology. This may indicate that the CTM-induction capacities that remained, weakened though they were, could have been due to those neurons and/or axons. Even if the remaining CTM-induction ability of the DCn from the injected DRG was due to the non-trkA, GS-I-B_4-binding neurons, it is clear that these neurons must play a very small role in the CTM reflex. It is most likely, however, that the weakened remaining capacities were due to residual functionality of the axons of moribund neurons, since the CTM reflex induction was completely absent, not simply weakened, in injected DRG from other animals.

Eight animals were involved in the toxin experiments. All underwent the same surgical procedures, and all received similar amounts of toxins in similar volumes of fluid. In spite of this, 3 of 8 cases showed no signs of destruction of the p75-bearing

neurons. They also showed normal CTM reflex induction from the injected dermatome. These animals therefore served as excellent controls for any possible effects of surgery or injection on the CTM reflex. It was likely that the Case 1 failed because of a short survival time (7 days). The other 2 cases, however, had 17 and 21 day survival periods, and received different doses of toxin. There appeared to be no correlation between failure of the toxin and dosage (within the range used here).

Transynaptic Neuronal Tracing Reveals Afferents Involved in the CTM Reflex

A neuroanatomical tracing approach was also taken to determine if the non-trkA C-fibers were synaptically connected to the CTM reflex circuitry. This technique was designed to retrogradely trace the elements of the CTM reflex circuit. While there may have been numerous other elements labelled, the only ones examined in this investigation were the CTM motoneurons and the low thoracic primary afferents. The CTM motoneuron pool was examined in all cases to ensure that there was infection of the motoneurons. In all cases where infection was successful, the extent of PRV infection of primary afferents from the level of injection was limited to a subgroup of small diameter afferents. Multiple-labelling histochemistry revealed that the vast majority of these neurons expressed trkA. This result indicated that the majority of the afferents that supplied input to the CTM reflex were limited to those expressing trkA. This conclusion supported the hypothesis that non-trkA C-fibers are not involved in the CTM reflex, making the CTM reflex an unsuitable measure to indicate the spatial extent of non-trkA C-fiber cutaneous innervation.

Control experiments were performed to examine the possibility that some route other than the CTM motoneurons had lead to the infection of the primary afferents. There were a number of possible routes of entry of the PRV tracer into the nervous system from the site of injection (CTM or LTn). These were the motoneurons, sympathetic neurons, and sensory neurons in the muscle and/or overlying skin. It was highly improbable that the specific infections that were observed were due to viral spread through the vasculature to some remote site[28]. Further, direct infection of the DCn cutaneous afferents from the overlying skin did not occur, which suggests that infection of the LTn afferents was also unlikely. Infection of the sympathetic axons in the injected CTM or overlying skin was another possible route of entry. This route of entry proved extremely difficult to control. Since there was no elimination of this route of infection in this study, the results of the PRV tracing studies must be interpreted with caution. As stated, the majority of DRG neurons that became infected by the PRV were trkA-positive, many were non-trkA, and often expressed GS-I-B_4-binding. One possible interpretation of this is that non-trkA afferents were synaptically connected to the CTM reflex circuit (this is discussed below). Another interpretation is that the populations of afferents infected with PRV represent both CTM reflex afferents and those that influence local sympathetic outflow.

Another set of control experiments were performed to assess whether or not all types, particularly the non-trkA C-fibers, were capable of being infected from their central terminals. It was unlikely that all neurons would be infected, since the terminations of neurons in a single DRG spread over a larger rostro-caudal area than was actually injected, and the injections also likely did not reach all lamina where the

afferents terminate. Since the PRV was rapidly taken up and transported, using the presence of the virus as an indicator of the injection sites was not possible. The results showed that the majority of DRG neurons were infected after PRV injections into the dorsal horn. More importantly, PRV was found in many non-trkA small diameter neurons, and in far more GS-I-B_4-binding neurons than when PRV was injected into the CTM or LTn. These results indicated that the both trkA-IR and non-trkA primary afferents were capable of being infected by exposure of their central terminals to PRV. This indicated that the reduced infection in non-trkA DRG neurons compared to trkA-positive neurons was not due to an inability of their central terminals to become infected by the virus.

The initial indication of the lack of non-trkA involvement as assessed by PRV-tracing must be accompanied by the possibility that the non-trkA, GS-I-B_4-binding primary afferents could have been involved in the CTM reflex, but were synaptically connected via a more complex path than the trkA-IR neurons. If this were the case, then the 72 hour PRV transport time may not have been sufficient for the virus to have reached them.

TrkA-Negative Unmyelinated Afferents and the CTM Reflex

The results of the two sets of experiments described above, when taken together, strongly support the hypothesis that *the non-trkA, GS-I-B_4-binding DRG neurons do not significantly participate in the induction of the CTM reflex.* Further, any ability that they *may* have to induce the CTM reflex is likely to be transmitted via a pathway that is distinct from that of the trkA-IR DRG neurons. Therefore, the CTM reflex is an insufficient test/monitor for PACS of the non-trkA afferent population.

It is certainly interesting to find that two different groups of unmyelinated afferents, each of which contains mechano-nociceptors and thermo-nociceptors, are differentially connected to a reflex circuit that is activated by noxious heat or mechanical stimui. There are a number of possible reasons for this, but it must still be considered that the non-trkA unmyelinated afferent may be capable of inducing the CTM reflex.

The present data and discussion make it clear that the non-trkA afferents are unlikely to drive the CTM reflex under the conditions studied, but this may not hold true for other conditions. It was clear from behavioral assessments that a strong CTM reflex could be induced in normal animals with very brief natural stimuli that was spatially constrained (and thus would not activate a large number of afferent fibers). Further, electrophysiological experiments showed that a full CTM reflex could be evoked with just a single electrical pulse. In light of the revelation that destruction of the trkA-bearing neurons eliminated these capacities, one must believe that these capacities were conferred by the trkA-bearing neurons.

It is unlikely that the elimination of the ability of a nerve to induce the CTM reflex was due to the destruction of a large population of C-fibers in a simple population type of effect. As mentioned above, a strong CTM could easily be evoked with a spatially constrained natural stimulus. Further, the dorsal root recordings from the toxin-injected animals revealed that a large group of C-fibers still existed and functioned in the injected segment, though these axons clearly were not capable of evoking a CTM reflex. Instead a general population type effect, the elimination of the ability to evoke a CTM reflex is most likely a specific type of effect. It indicates that the non-trkA afferents do not have the same capacity to rapidly and reliably induce a CTM reflex as do the trkA afferents.

It must be considered, however, that the non-trkA afferents might be capable of driving the CTM reflex under other circumstances or conditions. The PRV tracing studies did reveal that some non-trkA afferents became infected. This could have been a result of the possible sympathetic route of entry, or the result of some circuit path that connected the CTM motoneurons and non-trkA afferents. Further, a wide variety of electrical stimuli were not tested in the toxin experiments. Perhaps trains or bursts of stimuli would have driven the reflex. Perhaps the non-trkA afferents gain access to the CTM reflex when the target tissue is injured or inflamed. It is also possible that the non-trkA afferents might be capable of driving the CTM reflex as a result of PACS, but this was not investigated as part of the present experiments. If this were the case, the effects of anti-NGF treatments on expansion of the isolated CTM RF would have to be considered.

Regardless of whether the non-trkA afferents can drive the CTM reflex under some condition, it is clear that the trkA and non-trkA afferents are differentially connected to the reflex circuitry. This differential connectivity could be related to the development of the two populations. It has been shown that all unmyelinated afferents initially express trkA, and that a subgroup downregulates their expression of trkA by postnatal day 21 [158, 202]. This subgroup of small diameter afferents that ceases to express trkA begins to express the components of the GDNF receptor complex and becomes responsive to GDNF in late embryonic and early postnatal life[12, 159]. These two populations also have different central termination patterns in the spinal cord. Unmyelinated afferents that express trkA terminate primarily in lamina IIo, whereas those that lack trkA primarily terminate in lamina IIi [157]. The differences in development and central termination could directly underlie the differences in input to the CTM reflex.

PACS of trkA-Positive Versus trkA-Negative Afferents

Previous work has clearly demonstrated that trkA-positive afferents are capable of robust PACS, and that this PACS is a robust and rapid growth response to an acute denervation that is dependent on NGF [49, 52, 59, 150, 151, 173]. The current work demonstrates that the non-trkA unmyelinated afferents are also capable of PACS. There may be many, or few, differences between the two processes. It was noted that there were fewer trkA-negative axons present in collaterally reinnervated skin than trkA-positive axons. This could be due to a number of reasons. First, trkA-positive axons would represent not only trkA positive sensory afferents that had sprouted, but also sympathetic efferents that had sprouted[70, 71, 103, 163, 164]. Second, there are more trkA-positive than trkA-negative unmyelinated afferents in DRG [157]. In response to that argument, however, it should be noted that a greater percentage of cutaneous afferents express GS-I-B_4-binding and lack trkA than express trkA or CGRP [11]. Finally, it is possible that fewer trkA-negative afferents successfully sprouted into the denervated territory.

A recent study that examined PACS in great detail provided strong indications that NGF is paramount for PACS [173]. This study included examinations of the intraspinal changes that may occur in conjunction with the peripheral innervation changes during PACS. They used a retrograde tracer to show that the termination area of an isolated DCn that had undergone PACS was significantly larger than in normal animals. Further, they demonstrated that the number of second-order neurons that displayed IR for the inducible protein c-Fos increased significantly in response to noxious pinch of the RF of the sprouted DCn. All of these changes were shown to be due to PACS, as opposed to the sensitization that is known to occur in response to acute administration of exogenous NGF [127, 129, 130], and also to be prevented by administration of anti-NGF antibodies. While

the data would appear to strongly suggest that NGF is the primary, if not the sole, controlling factor in PACS, some other possibilities need to be addressed.

First, the tracer used to visualize the central terminals of the isolated DCn was HRP-conjugated wheat germ agglutinin (WGA-HRP). While this tracer has been widely used, it has intrinsic technical limitations. It was applied to the DCn by transecting the nerve and suturing it into a sealed tube containing the WGA-HRP. It has been shown that transection of the peripheral axons of a nerve prevent the transganglionic transport of GS-I-B_4-HRP by GS-I-B_4-binding afferents[156]. The lectin is still transported to the DRG cell bodies, but will not go centrally as it would if the axons had not been transected. It is possible that this same phenomenon could occur for WGA-HRP, since WGA is also a lectin and was applied to a transected nerve. If this were the case, then trkA-negative PACS would have been missed, and the effects of anti-NGF treatments overstated. Further, it is currently unclear whether WGA-HRP may have a selective affinity for particular afferent types when applied to a transected nerve[74], but this could have obvious implications for interpretation of the data. The data presented by Pertens et al. (1999) do not demonstrate whether or not the WGA-HRP in normal cord or on the contralateral control side of PACS animals is present in lamina IIi.

The fact that the increase in the number of c-Fos-expressing dorsal horn neurons could also be prevented by anti-NGF treatments brings us back to the possibility that NGF is the sole controlling factor in PACS. The arguments in favor of this data supporting the idea that NGF insensitive afferents do not sprout is clear, the changes are prevented by anti-NGF. However, this suggestion must be reconciled with the current data demonstrating that trkA-negative afferents are involved in PACS. One approach is to address the induction of c-Fos. It was not detailed by Pertens et al. (1999), nor was it found elsewhere, which specific lamina (in particular IIo or IIi) housed second order neurons that could be induced to express c-Fos in *normal* animals. The data presented by Pertens et al. (1999) do not demonstrate whether or not lamina IIi has any c-Fos-IR neurons in these control groups. If stimulation of the afferents that terminate in lamina IIi (non-trkA unmyelinated) do not induce significant c-Fos expression in lamina IIi neurons in normal animals, then it is reasonable to expect that they may not induce c-Fos expression after PACS, either, regardless of the presence of NGF. If this were the case, then it would be possible that the non-trkA afferents did in fact undergo PACS, with or without anti-NGF treatments, but would have been missed by the c-Fos studies.

A Role for NGF in PACS of Afferents Lacking trkA? A Likely Role for Other Factors.

While there may be many reasons that non-trkA PACS was missed by previous studies, it must be considered that the major line of evidence *against* a role for non-trkA afferent PACS may have been correct in spite of technical challenges in demonstrating the participation of these afferents. This evidence is the consistent and dramatic effects that anti-NGF treatments had on PACS-induced changes. How can the clear effects of

anti-NGF on numerous measures of PACS, and on PACS-related/induced changes, be reconciled with the clear demonstration of PACS by the non-trkA unmyelinated afferents provided by the current data? Possibly the non-trkA afferents express some unknown receptor for NGF. This is highly unlikely since it has been shown that trkA and p75 account very well for all NGF binding in DRG neurons[226]. The possibility that non-trkA unmyelinated afferents become sensitive to NGF during PACS has been discussed above. In short, this is also unlikely, at least for the majority of non-trkA unmyelinated afferents, based on 1) the demonstration of non-trkA axons in collaterally reinnervated skin, 2) the lack of any difference in the GAP-43^{+}/trkA^{-} population between normal and PACS DRG, and 3) preliminary evidence from direct examination of trkA-IR with markers specific for the non-trkA population.

Contrary to possibilities based on the direct sensitivity/sensitization of the non-trkA unmyelinated afferents to NGF, if there is a role for NGF in PACS of these neurons, then it is far more likely to be via an intermediary. Such an intermediary would have to itself be sensitive to NGF, and in turn produce some factor that would influence the non-trkA unmyelinated afferent population. The most likely candidate for a controlling factor at this point must be considered to be GDNF. The non-trkA unmyelinated afferents seem to display requirements of, and sensitivities to, GDNF that are very similar to those of the trkA-expressing afferents with NGF. These include survival dependency during development and regulation of cellular constituents in adulthood[12, 22, 63, 72, 141, 157, 159, 188, 219, 228, 229].

Directly related to PACS, both GDNF and NGF are capable of affecting the neurite outgrowth of adult DRG neurons, but each affects mutually exclusive populations (those with the appropriate receptor)[68, 123]. In addition, the current data has revealed that the GDNF-sensitive population of DRG neurons (non-trkA, GS-I-B_4-binding neurons) expresses GAP-43 protein in the normal adult, contrary to previous understanding. This expression is clearly present, but much weaker than that exhibited by trkA-IR neurons. Coincidentally, the current data also demonstrated that PACS of the non-trkA unmyelinated afferents was not as robust as that of the trkA-bearing neurons (sensory or sympathetic), and it was recently shown that although GDNF significantly enhanced neurite outgrowth of adult DRG neurons *in vitro*, its effect on total neurite length was far less than that of NGF [68, 123].

GDNF therefore appears to be a suitable and logical candidate for one possible factor that may influence PACS of the non-trkA unmyelinated afferents. The logical cellular intermediary is likely to be (at least) Schwann cells. It is known that denervated Schwann cells begin to express p75 [137, 248]. Further, it has been shown that denervated Schwann cells begin to express significant levels of GDNF [8, 76].

It is likely that the GDNF signal transduction system participates in at least one other form of peripheral plasticity, namely CS of motoneurons, and also some central CS [193]. It is known that GDNF levels play a role in the innervation of muscle[165, 167, 214]. It has recently been shown that GDNF is upregulated in denervated muscle[132]. It has also been shown that GAP-43 is upregulated in motoneurons and their axons innervating partially paralyzed or denervated muscle[17, 80, 152, 231]. These pieces of information are quite similar to those demonstrating the process of NGF-dependent PACS and sympathetic efferent CS.

The current data have demonstrated that non-trkA unmyelinated afferents are indeed capable of PACS. Their capacity for PACS, however, was clearly less than that for trkA-positive neurons. This could be related to their intrinsic low level of GAP-43 expression, or, if the role of GDNF is accepted, the differences between the NGF and GDNF systems on neurite outgrowth mechanisms[68]. It is possible that non-trkA PACS could be more robust under different conditions. Perhaps the PACS of this population would be stronger if the trkA-positive axons were not present. Perhaps PACS of this population would be more robust into inflamed or injured skin where the production of TGF-β, a necessary co-factor for full GDNF signalling[114, 198], would be increased[97, 105, 194, 197].

Summary

The current data demonstrate that the trkA-negative unmyelinated DRG afferents are capable of PACS. The factor(s) controlling this process are still unclear. If NGF plays no role, then it is most likely that PACS of this population has been overlooked in previous studies for numerous reasons. One major reason could be that, as demonstrated by the current data, this population of neurons does not participate in the CTM reflex, a primary monitor for PACS. It is quite possible, however, that NGF does play a role in PACS of this population, in spite of their lack of NGF receptors. If this is the case, then the influence of NGF on non-trkA PACS is likely via an intermediary. As a corollary to that, PACS of this population may *not* have been overlooked in previous studies. Instead, PACS of both the trkA-positive and trkA-negative populations would not have occurred.

It is now clear that a new population of afferents has been demonstrated to participate in PACS. With that comes the likelihood that some factor other then NGF could be capable of controlling PACS. As a result, any future research regarding attempts to manipulate PACS should include considerations for this new factor, which is likely to be GDNF. Of particular note, work attempting to "encourage" PACS as a means for recovery or improvement of function in response to peripheral or central injuries, or attempting to suppress PACS in an attempt to alleviate any number of pathological sensory processes in which PACS, or CS in general, may have played a role, should be concerned with these findings.

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