AN IN VIVO STUDY OF GENE EXPRESSIONS DURING COLLATERAL SPROUTING ACCELERATED BY ELECTRICAL STIMULATION IN RAT DORSAL ROOT GANGLIA

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BY

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ABSTRACT

Previous studies have demonstrated that collateral sprouting in sensory neurons is an NGF dependent process, and that the onset of this sprouting can be accelerated by electrical stimulation (depolarization) of nerves, producing a precocious collateral sprouting. However, the mechanism underlying this phenomenon is not clear.

In the present study, the mechanisms underlying precocious collateral sprouting were studied in adult rats *in vivo*. Electrical stimulation was performed on the intact cutaneous nerves at 8V, 20 HZ, 1 min. These intact nerves were isolated from the adjacent nerves by dissecting the nearby nerves. The intact dorsal cutaneous nerves were treated under three paradigms: i) electrical stimulation (S); ii) isolation of intact nerves (I); iii) electrical stimulation plus isolation (S+I). After varying periods of time (1h, 4h, 8h, 1d, 2d, 4d, 8d, and 14d), the dorsal root ganglia (DRGs) connected with these nerves were dissected and the possible factors related to precocious sprouting were investigated in the DRG neurons using *in situ* hybridization (ISH), immunocytochemistry (ICC) and Western blot assays. The parameters examined included immediate early genes (IEGs), such as CREB, egr-1, c-fos, c-jun, and Oct-2; NGF receptors (Trk A and p75); and potential members of the NGF - Trk A signal transduction pathways inducing downstream signaling (PI3-kinase, SHC, PLC-γ, ERK1).

The results showed that, among IEGs, CREB mRNA was quickly induced after 1h

electrical stimulation, and this increase lasted to 4d. The effects of isolation started at 1d, and the combination of isolation plus stimulation resulted in this occurring sooner. At the protein level, the expression of pCREB was only significantly increased under stimulation at 8h ($p<0.05$). After 4h, electrical stimulation started to induce the elevation of egr-1 mRNA and this induction lasted until 2d, but the protein level was significant increased only at 8h. Isolation, which would result in increased NGF levels in the skin due to the adjacent cutaneous denervation, did not induce significant changes in Egr-1 protein during the experimental period. Isolation plus stimulation shortened the duration of Egr-1 increase (at 1d) and this increase lasted to 4d. Except for electrical stimulation alone and isolation alone, isolation and stimulation together induced significant increases of Fos in DRGs at 2d and 4d. Stimulation did not have significant effects on Jun protein, but after 8h, isolation plus stimulation, respectively, resulted in significant increases in Jun protein compared with control. Oct-2 was not affected by any of the treatments in these experiments.

Under the treatments of electrical stimulation and isolation, expression of Trk A receptor mRNA and protein showed different patterns in these experiments. The mRNA level of Trk A did not significantly increase after electrical stimulation; however, isolation alone resulted in a significant increase of TrkA mRNA and this increase reached a peak at 4d . Combined with electrical stimulation, isolation induced a large increase at a very early time point (1h), but this gradually declined at later time points (2d and 4d).

The protein level of Trk A was only increased at 1h and 4h stimulation time points.

There was, however, an increase induced by isolation plus stimulation at later time points (4d and 8d). The phosphorylated state of Trk A receptor did not appear to be increased except at the isolation treatment at 1h and longer time points of 4d and 8d. With respect to p75, mRNA levels were altered little by electrical stimulation. Isolation alone induced a peak change at 2d. The combination of electrical stimulation and isolation resulted in increased expression of p75 by 4h, peaking at 1d, and then gradually decreasing.

Among the proteins which propagate NGF signals, PLC-γ1 was slightly induced by stimulation and isolation at the short time periods (1h, 4h) and the very late time point (8d). PI-3 kinase was increased only at the latest time point (8d) following treatments. SHC and MAP kinase (ERK1) were not obviously affected by any of these treatments.

The results addressed the hypotheses that, during precocious collateral sprouting, electrical stimulation induces some alterations in IEG expression and elevated Trk A receptor expression and/or activation, and acts in concert with the increased availability of NGF to result in an accelerated terminal sprouting response. This study provided information about the potential mechanisms associated with precocious sprouting at the molecular level.

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ANOVA	analysis of variance
bp	base pair
BSA	bovine serum albumin
^{0}C	degree Celsius
cAMP	cyclic adenosine 5'- phosphate
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CRE	cAMP response element
CREB	cAMP response element binding protein
CTM	*cutaneus trunci* muscle
DCN	dorsal cutaneous nerve
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
DTT	dithiothreitol
ECL	enhanced chemiluminescence

egr	early growth response gene
ERK	early response kinase
Grb2	growth factor receptor-bound protein 2
ICC	immunocytochemistry
IEG	immediate early gene
ISH	*in situ* hybridization
Kda	kilodalton
MAPK	mitogen-activated protein kinase
MEK	MAP kinase or ERK kinase kinase
mRNA	messenger RNA
NGF	nerve growth factor
NGS	normal goat serum
pCREB	phosphorylated cAMP response element binding protein
PBS	phosphate buffered saline
PI-3 kinase	phosphatidylinositol-3 kinase
PLC-γ	phospholipase C-γ
PMSF	phenylmethylsulfonyl fluoride
PNS	peripheral nervous system

POU	named for its founder member, Pit-1, Oct-1 and 2 and *unc*-86
RNA	ribonucleic acid
Rsk	ribosomal-activated protein kinase
SD	small dark
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SNT	*suc*-associated neurotrophic factor-induced tyrosine-phosphorylated target
SOS	son of sevenless, a nucleotide exchange factor
TBS	tris buffered saline
TBST	tris buffered saline plus tween-20
TDT	terminal deoxynucleotidyl transferase
Trk	tyrosine response kinase
TTX	tetrodotoxin

Chapter I

INTRODUCTION

1.1 The collateral sprouting of sensory neurons in adult animals

Collateral sprouting is a phenomenon whereby intact axons produce many terminals in their target tissue to form a neuritic arbor. This process happens in the central nervous system (CNS) (Gilad *et al.*, 1979; Friedman *et al.*, 1986) and the peripheral nervous system (PNS) (Diamond *et al.*, 1976; Kinnman *et al.*, 1986) during the developmental (rev. in Crutcher, 1986) or adult stage of the animal (Diamond *et al.*, 1992c). However, in different stages, collateral sprouting appears to have different functions (rev. in Diamond *et al.*, 1992d). During development, collateral sprouting not only meets the need that the axon has enough neuritic connections with its target tissues, but also ensures the efficiency of an axon's function. In the adult animal, collateral sprouting in the PNS has a positive function; that is, after axonal damage in one innervation field, the axons in an adjacent field will sprout to re-innervate the denervated target area. However, in the highly organized CNS, the effect of collateral sprouting may not be advantageous due to the possibility of the formation of aberrant connections. In this introduction, I will discuss the collateral sprouting in the PNS, but not in the CNS, of adult mammals.

In adult animals, the peripheral sensory neurons experience two kinds of outgrowths which are different from those seen in their developmental stage: one is called regeneration,

and the other is called collateral sprouting. These two processes are different in several aspects. Regeneration occurs after injury to axons; that is, after cutting or crushing of the axon, the injured terminal close to the peripheral area will degenerate and then new axonal growth will occur back to the original target tissue resulting in recovery of sensory function. Collateral sprouting, on the other hand, is growth from intact axons. When axons are injured, the intact axons in an adjacent undamaged target area will elaborate multiple collateral fine branches to reinnervate the denervated target area. Another difference between the two processes is that collateral sprouting is nerve growth factor (NGF)-dependent (Diamond *et al.*, 1987; Diamond *et al.*, 1992a), while regeneration does not appear to be influenced by NGF (Diamond *et al.*, 1992b).

Collateral sprouting in the adult animal is important in sensory recovery in the PNS after damage. Since the first demonstration of collateral sprouting in sensory neurons (Weddell *et al.*, 1941), other workers have conducted further experiments to confirm this phenomenon in adult salamanders and rats (Aguilar *et al.*, 1973; Stirling, 1973; Cooper *et al.*, 1977 a, b; Devor *et al.*, 1979). It was found that while some sensory neurons in the skin indeed underwent collateral sprouting, not all peripheral sensory neurons had this ability.

Due to its easy dissection and large size, the dorsal root ganglion (DRG) is often used as a model to investigate various neuronal properties, from ion channel to neurotrophic factor dependence. According to the cell morphology and the staining of clumps of Nissl substance in the cytoplasm, the sensory neurons in DRGs have been classified into two types of cells:

large light (L) and small dark (SD) neurons (Lawson, 1979; rev. in Lawson, 1994). The size distributions of L and SD neurons overlap (Lawson, 1979). L neurons cover the entire size range of the ganglion, from small, medium to large cells; whereas SD neurons are normally small cells. Further classification can be made according to the type of nerve fibers arising from these neurons. Large neurons generally give rise to myelinated fibers which can be divided into two groups: Aδ and Aα/β fibres. In rats, Aα fibers are low threshold mechanoreceptors which have fast conduction velocities, and Aδ fibres are high threshold mechanonociceptors with slower conduction velocities. The SD neurons are generally associated with unmyelinated C-fibers, which are categorized as high threshold thermonociceptors (Harper and Lawson, 1985a, b; Lawson, 1994). In a study of collateral sprouting, it was found that the low threshold mechanoreceptive Aα nerve fibers had the ability of collateral sprouting only during a critical period during development, for example, when rats were younger than 20 days (Jackson and Diamond, 1981), while in the adult animal, Aα fibres failed to produce functional collateral spouts into denervated areas of mammalian skin (Diamond & Jackson, 1978; Horch, 1981; Jackson & Diamond, 1984). On the other hand, the high threshold myelinated mechanonociceptive Aδ fibers and unmyelinated thermonociceptive C fibers do undergo collateral sprouting into adjacent denervated area in the adult. By physiologically mapping the enlargement of the denervated area (due to invasion of collateral sprouts) using the *cutaneus trunci* muscle (CTM) reflex (Nixon *et al.*, 1984; Doucette & Diamond, 1987; Theriault and Diamond, 1988), it was found that only

nociceptive Aδ and C fibers contributed to the enlarged area. Earlier studies in Salamander skin indicated that collateral sprouting was regulated by an agent manufactured continually by the target skin (Diamond *et al.*, 1976). Subsequent investigation by daily administration of anti-NGF serum in adult rat suggested that it was NGF which regulated the collateral sprouting of sensory nerves in the skin (Diamond *et al.*, 1987). Further studies demonstrated that collateral sprouting was a NGF dependent process (Diamond *et al.*, 1992a; Mearow *et al.*, 1993; Mearow *et al.*, 1994).

1.2 The role of NGF in collateral sprouting

Nerve growth factor (NGF) was first found and purified from male mouse submaxillary gland (Levi- Montalcini and Hamburger, 1953; Cohen, 1959) and later purified from snake venom (Cohen, 1960). It is a complex of three subunits (α, β, γ), and among them, the β subunit acts as the biologically functional unit (Greene et al, 1971). Early studies indicated that NGF was an important trophic factor in the development and survival of sympathetic neurons and certain sensory neurons (rev. in Segal and Greenberg, 1996; Lewin and Barde, 1996). In adult animals, although NGF is not so critical to sensory neuron survival, it still exerts other effects on adult sensory or sympathetic neurons (rev. in Johnson *et al.*, 1986; Lewin and Barde, 1996). Experiments indicated that, for sensory neurons and sympathetic neurons, NGF is supplied exclusively from their peripheral targets and that there was a competition between these two types of neurons for limiting amounts of target-derived NGF

(Shelton & Reichardt, 1984; Korsching & Thoenen, 1985). Subsequent experiments indicated that NGF was manufactured in target skin (both dermis and epidermis) of sensory axons (Davies *et al.*, 1988), and also in Schwann cells along the degenerating peripheral nerves of adult animals (Taniuchi *et al.*, 1987). In addition, subsequent to skin denervation, NGF mRNA levels in the skin increased, suggesting that the production of NGF might be regulated by sensory axons (Mearow *et al.*, 1993). Sensory neurons are able to take up and retrogradely transport NGF in adult mammals (Stoeckel et al, 1975; Richardson and Riopelle, 1987). Further experiments showed that endogenous NGF regulated the sprouting of Aδ and C fibres (Diamond *et al.*, 1987, 1992a). In these experiments, sub-cutaneous injection of anti-NGF daily for 25-28 days blocked collateral sprouting, but not regeneration. Furthermore, systemic injections of NGF into skin could induce the collateral sprouting of intact DRG neurons in the absence of denervation (Diamond *et al.*, 1992c; Mearow, 1997). However, NGF did not affect regeneration of sensory axons in these experiments (Diamond *et al.*, 1992a). After the denervation of adult rat, the NGF mRNA expression was upregulated in the skin, suggesting that the increased NGF concentration was responsible for the collateral sprouting of the remaining intact sensory neurons (Diamond *et al.*, 1987; Mearow et al , 1993a). The available evidence supports the view that collateral sprouting of nociceptive afferents is an NGF-dependent process.

1.3 The phenomenon of precocious sprouting of sensory neurons

In the study of collateral sprouting of nociceptive nerves, Nixon *et al.* (1984) developed a method to measure collateral sprouting of high threshold nociceptors. When the back skin of anaesthetized rats was pinched, it produced a bilateral reflex excitation of the underlying *cutaneus trunci* muscle (CTM), resulting in a very visible wrinkling of the loose dorsal skin. This reflex could also be elicited when Aδ and C fibres were electrically stimulated. Therefore, by measuring the area of skin from which the reflex response could be elicited, they estimated the extent of the area innervated by Aδ and C fibres (Nixon *et al.*, 1984). If collateral sprouting happened, the area of enlargement could also be detected by this reflex. Using this method to study collateral sprouting, they found an interesting phenomenon, *i.e.*, periodic examination of the recovery of CTM reflex by pinching the skin resulted in an earlier recovery of innervation, due to the field expansion of the remaining intact Aδ and C fibres (Nixon *et al.*, 1984). Subsequently, it was observed that electrical excitation of intact Aδ fibres in the remaining skin also resulted in an earlier recovery of innervation. It was suggested that impulses shortened the latency to the onset of collateral sprouting of the activated Aδ fibres. The same phenomenon was also found for polymodal nociceptive C fibres; pinch, heat, or electrical stimulation respectively also caused the precocious sprouting of C fibres (Doucette & Diamond, 1987).

Normally, following skin denervation and isolation of one cutaneous nerve, the time required to first observe functional re-innervation of the denervated skin (by the sprouting axons of the remaining 'isolated' intact nerve) ranges from 10-12 days. However, electrically

stimulating Aδ and C fibres for several minutes prior to, or at the same time of, denervation, the sprouting of dorsal cutaneous nerves in adult rats appeared earlier than in the non-stimulated nerves. Further studies investigated the coinvolvement of electrical stimulation and NGF (Diamond *et al.*, 1992a; Mearow, 1997). It was found that electrical stimulation of dorsal cutaneous nerves at voltages sufficient to activate the high threshold Aδ and C fibres caused the collateral sprouting to be observed 5-6 days earlier than the normal sprouting. The intriguing feature of this phenomenon is the fact that NGF must be available at the time of stimulation. If, at the time of stimulation, the animals were treated with 1 or 2 injections of anti-NGF, the stimulation lost its effectiveness and the earlier onset of collateral sprouting did not appear (Diamond *et al.*, 1992a).

1.4 Molecular mechanisms of precocious sprouting

Electrical stimulation and increased availability of NGF together result in the accelerated sprouting of nociceptive neurons. However, the mechanism underlying this precocious sprouting is uncertain. From other unrelated studies, it was observed that electrical stimulation and/or membrane depolarization via KCl and NGF, respectively, induce some changes of certain gene expressions and protein synthesis through the activation of different transduction pathways (Sukhatme *et al.*, 1988; Bartel *et al.*, 1989; Sheng *et al.*, 1990; Sheng *et al.*, 1991; Birren *et al.*,1992; Taylor *et al.*, 1993; Kendall *et al.*, 1994; Ensor *et al.*, 1996). These experiments have given some information regarding genes that might be involved in

the precocious sprouting phenomenon after membrane depolarization and NGF treatment. Such candidates could involve immediate early genes (IEGs) such as c-fos, c-jun, egr/zif268, pCREB, which have been shown to be induced by noxious stimulation or NGF. In addition, since NGF appears to be central to the response, perhaps alterations in NGF receptor expression or activity might be of importance.

1.4.1 The changes of NGF receptor induced by NGF or electrical stimulation during sprouting

NGF, like other growth factors, exerts its effects by binding to cell membrane receptors, and subsequent activation of signaling pathways (e.g., Kaplan and Stephens, 1994; Chao 1994; Segal and Greenberg, 1996). NGF binds to two receptors: a low affinity neurotrophin receptor, known as p75 (Chao *et al.*, 1986; Radeke *et al.*, 1987) and a high affinity specific receptor tyrosine kinase, Trk A (Kaplan *et al.*, 1991a, b; Klein *et al.*, 1991 a).

Since collateral sprouting is an NGF-dependent process, the involvement of both Trk A and the low affinity p75 receptor in this process was examined. It was found that in DRG neurons undergoing collateral sprouting, the mRNA expression of TrkA and p75 were both elevated (Mearow et al, 1994). In experiments where collateral sprouting had been blocked by anti-NGF treatment, there was no upregulation of the mRNAs for the NGF receptors (Mearow and Kril, 1995). If NGF was given systemically, in the absence of any denervation, an upregulation of both p75 and Trk A mRNA was observed (Mearow, 1994; Miller *et al.*,

1994). Further analysis indicated that the NGF level in the skin was upregulated after two days denervation (Mearow *et al.*, 1993).

It is generally accepted that most of the effects of NGF are exerted via binding to the Trk A receptor; p75 appears to be neither necessary nor sufficient for many aspects of NGF signalling (eg., Chao, 1994; Segal and Greenberg, 1996). The role of the p75 receptor is not clearly defined although suggested roles have included modulation of the cellular response to NGF (Barker *et al.*, 1994; Hantzopoulos *et al.*, 1994; Verdi *et al.*, 1994) and involvement in neuronal apoptosis (Bartlett *et al.*, 1996; VanderZee *et al.*, 1996). For example, studies with p75-/- knockout mice indicate that in the absence of p75 receptors, sensory neurons exhibit a decreased sensitivity to NGF such that they require a higher concentration of NGF for survival (Davies *et al.*, 1993). In a recent study, it was observed that there was little or no collateral sprouting in p75-/- KO mice; however sprouting could be induced with injections of NGF (Diamond *et al.*, 1995).

Signalling events subsequent to the binding and concomitant activation of the Trk receptors have been the subject of intense investigation. Most of those studies (until fairly recently) have dealt with the effects of NGF on a variety of neural tumor cell lines, most notably the PC12 cell line. The binding of NGF to its cognate receptor results in autophosphorylation and activation. *In vivo* studies indicate that, in sciatic nerves, following ligation of the nerves at several centimetres away from DRG cell body, Trk A receptor was retrogradely transported in the axon, presumably after binding to NGF at the nerve terminal

(Ehlers *et al.*, 1995). It was also shown that Trk A was transported in a phosphorylated or active state, because the Trk A retrograde transport and phosphorylation were increased by NGF injection in footpad, and abolished by blocking the endogenous NGF with anti-NGF (Ehlers *et al.*, 1995).

On the basis of all these observations, it seems reasonable to suggest that denervation of the skin results in increased levels of NGF which subsequently bind and activate the Trk A receptor, followed by the initiation of a cascade of biochemical events which may ultimately result in, or at least influence the process of, collateral sprouting.

With respect to the role of electrical stimulation in the acceleration of the sprouting response, experiments have provided some evidence that depolarization may directly influence either NGF receptor activation states, or levels of expression. Previous studies indicated that membrane depolarization could cause the expression of Trk A receptor in MAH cells, an immortalized sympathoadrenal progenitor cell line, not initially responding to NGF (Birren *et al.*, 1992). Membrane depolarization also results in the phosphorylation of Trk A in NGF-sensitive PC12 cells and subsequent neurite formation (Solem *et al.*, 1995). However, the neurite growth induced by this depolarization only occurs if NGF receptors were partly activated by overexpression of Trk A or treatment with a low level of NGF. Therefore, the possible involvement of electrical stimulation in collateral sprouting may be that the depolarization induces the pathways which are also activated by NGF; for example, the neurite growth induction in PC12 cells by depolarization as a result of calcium influx was

due to the activation of Ras signal transduction pathway (Solem *et al.*, 1995; Rusanescu *et al.*, 1995) which also can be induced by NGF (eg., Kaplan and Stephens, 1994).

1.4.2 The changes of growth-related immediate early genes (IEGs) induced by NGF or electrical stimulation

The function of IEGs in the nervous system has been extensively studied in the PC12 cell system. Extracellular stimulation, such as NGF or electrical activation, or depolarization with KCl, caused the induction of certain genes whose transcription is activated rapidly and transiently within minutes of stimulation, and does not need *de novo* protein synthesis (Sukhatme et al, 1988;Bartel et al, 1989; Sheng *et al.*, 1990; Herdegen *et al.*, 1991 a; Sheng *et al.*, 1991). These kinds of genes are called immediate early genes (IEGs). IEGs are the earliest downstream nuclear targets for extracellular stimulations and their products act as transcription factors, somehow regulating the expression and activity of downstream genes and finally generating long term biological change of cells (Cole, *et al.*, 1989; Rusak, *et al.*, 1990; Abraham *et al.*, 1991). Therefore, IEGs function as mediators to couple the external stimulation to cellular biological change. c-fos and c-myc protooncogenes were the first two genes found to respond to growth factor treatment, and later many more IEGs were found (rev. in Sheng and Greenberg, 1990). Among them, c-fos, c-jun, and egr-1 (early growth response gene, also called zif/268, NGFI-A, or Krox-24) are well studied genes more directly related to growth response induced by NGF and membrane depolarization (Herdegen *et al.*,

1991 a; rev. in Stephens, 1993; Bartel *et al.*, 1989; Sukhatme *et al.*, 1988; Sheng *et al.*, 1990,1993; Changelian *et al.*, 1989; Robertson, 1992). c-fos protooncogene belongs to a leucine zipper gene family, and can form dimer complexes with c-jun which belongs to another IEG family (Jalava and Mai, 1994). The dimer, referred to as AP-1, can bind to a relatively specific sequence in the promoter region of other genes and activate their transcription(Curran and Franza, 1988; Kerppola and Curran, 1992). It was found that the transcription of c-fos was mediated by the cAMP response element binding protein (CREB) (Konradi et al, 1995). CREB is a member of a gene family whose protein product has a similar structure as Fos and Jun, and forms functional homodimers via a leucine zipper interaction (Dwarki *et al.*, 1990). The increased transcription of c-fos induced by membrane depolarization, Ca2+ influx, cAMP elevation in cells is mediated through a cAMP response element (CRE)-like sequence that binds CREB (Fisch *et al.*, 1989; Sheng *et al.*, 1988, 1990). Subsequently, CREB is phosphorylated at a specific residue (Ser-133) known to be recognized by cAMP-dependent protein kinase and this process is important for activation of CREB as a transcription factor in vivo and in vitro (Yamamoto et al, 1988; Gonzales and Montminy, 1989; Sheng et al, 1990). Furthermore, the activation of c-fos by NGF also requires intact CREs and involves the activation of CREB through a Ras-dependent protein kinase.

In DRG neurons, NGF induced a rapid c-fos gene expression after 30 min treatment in cultured cells (Lindsay *et al.*, 1990). In PC12 cells, NGF stimulation resulted in an increase

within 30 min in c-jun, similar to that seen for c-fos (Bartel *et al.*, 1989). Membrane depolarization also elevated c-jun mRNA expression, but the change was relatively small (Bartel *et al.*, 1989). egr-1 has the same motif as c-fos and c-jun, and can be activated by both NGF and membrane depolarization (Sukhatme *et al.*, 1988; Bartel *et al.*, 1989), but it was activated more strongly by NGF than by membrane depolarization, like c-jun (Bartel *et al.*, 1989). Treatment of adult DRG neurons with NGF increased egr-1 mRNA expression after 60 minutes (Kendell *et al.*, 1994). In the 5' upstream sequence of egr-1 gene, a CRE-like sequence is present (Christy *et al.*, 1989), indicating that this IEG might be also activated by CREB through the binding to CRE site in DNA sequence.

In addition to the above IEGs, sensory neuron octamer-binding protein Oct-2 is another interesting IEG. Oct-2 was firstly identified as a B cell immunoglobulin-specific transcription factor (He *et al.*, 1989; Lilycrop *et al.*, 1991 a). Subsequently, Oct-2 and other POU (named for its founder member, Pit-1, Oct-1 and 2 and *unc*-86) proteins, Oct-1 and Brn-3, were found specifically expressed in adult rat sensory neurons (Latchman *et al.*, 1992). Among them, Oct-2 was the only gene coding for a transcription factor whose mRNA level and functional protein level were elevated in the presence of NGF in isolated DRG neurons (Wood *et al.*, 1992; Ensor *et al.*, 1996). An *in vivo* study showed that Oct-2 was induced in a situation where target NGF levels were increased following an inflammatory response induced after injection of complete Freunds adjuvant; anti-NGF treatment abolished this increase (Ensor *et al.*, 1996). In the upstream sequences of the neuropeptide, calcitonin gene-related peptide

(CGRP), whose expression is regulated by NGF, there are also octamer binding sites (Stolarsky-Freedman et al, 1990). It was suggested that Oct-2 might play a similar role as a transcription factor in regulating gene expression in DRG neurons that respond to NGF. Further studies indicated that Oct-2 presented the kinetics of an IEG when it was induced by NGF (Kendall *et al.*, 1995). Therefore, Oct-2 is a potential candidate in mediating NGF signal transduction during precocious sprouting.

Most of experimental results for depolarization have come from the cell culture model (Bartel *et al.*, 1989; Lindsay *et al.*, 1990; Sheng *et al.*, 1990; Kendall *et al.*, 1994), but *in vivo* studies would give more direct evidence of the involvement of electrical stimulation in regulation of IEGs. In spinal cord, noxious electrical stimulation of rat sciatic nerves at intensity activating Aδ/C fibres strongly induced the expression of Fos, Jun and Egr-1 protein in the dorsal horn of the spinal cord (Herdegen *et al.*, 1991 a). Further, in DRG neurons, basal expression of c-fos, c-jun, egr-1, CREB, Oct-2 has been shown (Herdegen *et al.*, 1991a, b, 1992; Jenkins *et al.*, 1993; Kendall *et al.*, 1995). Even though there is evidence that electrical stimulation caused little or no change in the expression of these IEGs in DRG neurons themselves (eg. Hunt *et al.*, 1987; Herdegen *et al.*, 1991), the time period after electrical stimulation and the spinal level of DRGs are different than those used in the experiments described in this thesis. Therefore, under electrical stimulation, investigating the changes in expression of these IEGs in DRG neurons will give us some information about the involvement of electrical stimulation in regulating the gene expressions of IEGs and help us

to understand the mechanism of precocious sprouting.

1.4.3 Related pathways through NGF receptors

As indicated above, NGF exerts its effects primarily via a high affinity receptor. while the contribution of the low affinity receptor p75 is not fully understood. It was found that the protooncogene Trk A tyrosine kinase was the high affinity receptor for NGF and the binding of NGF to Trk A receptor causes rapid autophosphorylation of tyrosine kinase of Trk A (Kaplan *et al.*,1991). The phosphorylated tyrosine kinase activates other proteins through the Ras transduction pathway and related pathways (PI3-kinase, PLC-γ) (rev. in Kaplan and Stephens, 1994). It has been suggested that the Ras MAP kinase is necessary for the effects of NGF on neuronal differentiation and neurite outgrowth as opposed to survival (*e.g.*, Kaplan and Stephens, 1994; Segal and Greenberg, 1996).

The autophosphorylation of Trk A tyrosine kinase provides recognition or docking sites for components of cellular signalling pathways. For example, NGF was shown to induce the complex formation of Trk A with PLC-γ1 (Ohmichi *et al.*, 1991) and with SHC (Obermeier *et al.*, 1993b; Stephens *et al.*, 1994). Besides these two proteins, another protein molecule activated by NGF is PI-3 kinase. PI-3 kinase also binds to the Trk A receptor (Ohmichi *et al.*,1992; Obermeier *et al.*, 1993b) and the binding of PI-3 to Trk A results in the phosphorylation of PI-3 kinase. In addition to the above three protein molecules which are directly associated with Trk A receptor, another molecule, SNT is also phosphorylated after

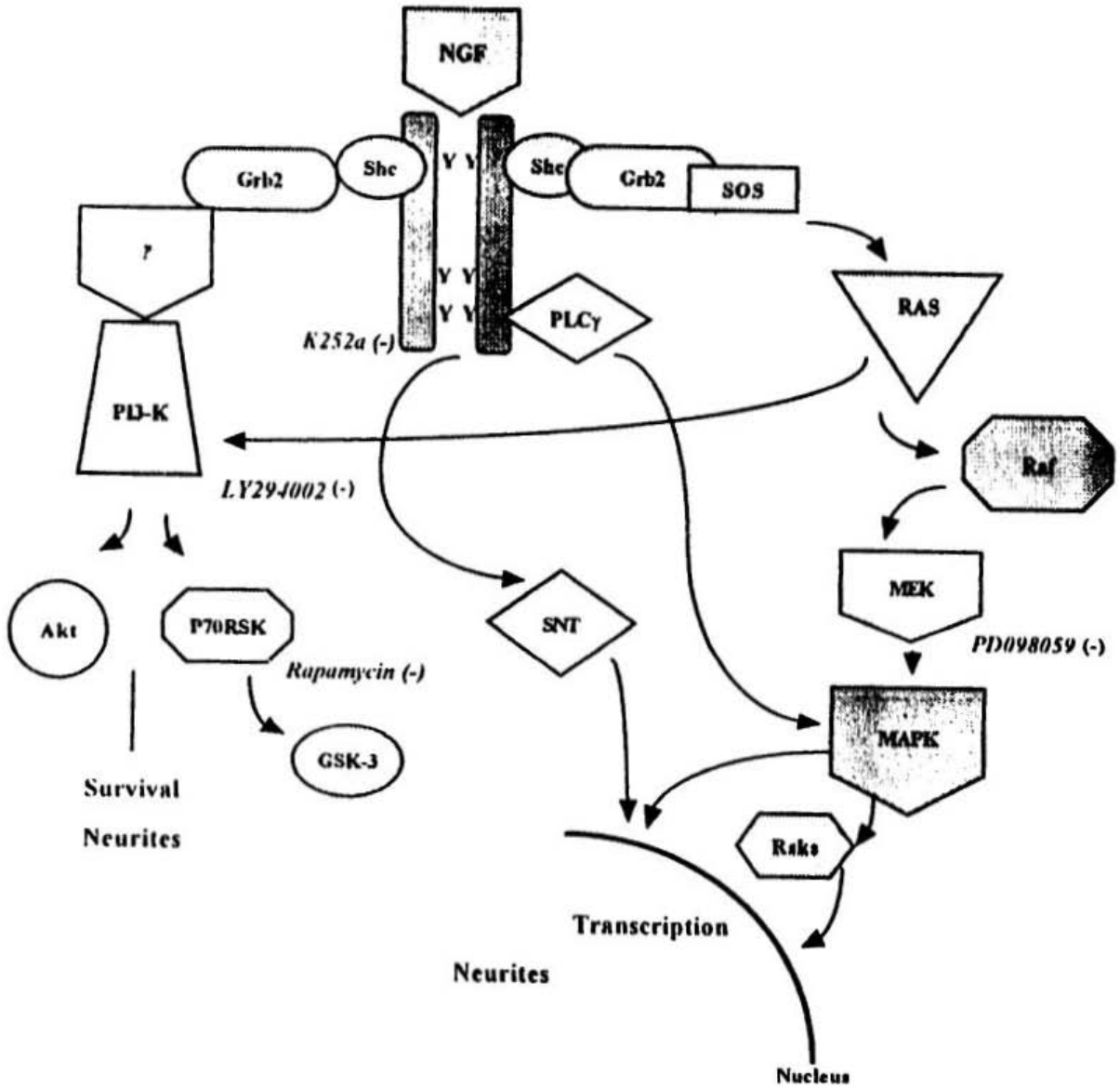
NGF binding to Trk A (Rabin *et al.*, 1993). PLC-γ, SHC, and PI-3 kinase form complexes with the activated Trk receptors relatively rapidly after NGF treatment (Obermeier et al, 1993b; Loeb et al, 1992; Stephens et al, 1994). Each of these molecules is thought to activate a distinct pathway that may function in differentiation (SHC, PLC-γ) or survival (PI-3 kinase) (Kaplan and Stephens, 1994; Rasouly *et al.*, 1995; Kaplan, 1996; Kaplan and Miller, 1997; Tolkovsky, 1997) (Fig.1).

SHC binding and activation links Trk activation to the p21ras-MAP kinase signalling pathway, which has been shown to be essential for NGF-induced neurite outgrowth in PC12 cells (Hagag et al, 1986). Stimulation of Ras results in the sequential activation of Raf-B, MEK (MAPK kinase), MAPK (also ERKs) and the Rsk kinases; the latter two are responsible for activation gene transcriptional events (Kaplan and Stephens, 1994; Segal and Greenberg, 1996).

PLC-γ 1 activation results in increased activity of this enzyme, and resulting downstream cellular events may include alterations in the activity of protein kinase C (PKC), ion fluxes (especially calcium), cytoskeletal reorganization, and gene expression (Vetter et al, 1991; Obermeier et al, 1993a). PLC-γ 1 activation may also provide another mechanism for activating the Ras cascade , since mutated Trk receptors no longer able to bind PLC-γ 1 and SHC are not able to induce Ras-MAPK signalling (Stephens et al, 1994).

PI-3 kinase stimulates the formation of phosphoinostides that appear to act as second messenger molecules stimulating other serine/threonine kinases; these effects may function

Figure 1. Trk A signal transduction pathways. Adapted from Kaplan and Stephens, 1994; Rasouly et al., 1995; Kaplan, 1996; Kaplan and Miller, 1997; Tolkovsky, 1997.


NGF
Grb2
Shc
Y Y
Shc
Grb2
SOS
Y Y
Y Y
PLCγ
K252a (-)
RAS
PI3-K
LY294002 (-)
Raf
MEK
PD098059 (-)
Akt
P70RSK
Rapamycin (-)
SNT
GSK-3
MAPK
Survival
Neurites
Rsk
Transcription
Neurites
Nucleus

to regulate aspects of protein synthesis and cellular morphology. It may also play a role in both survival and neurite formation, although the data in this regard is somewhat confusing (Yao and Cooper, 1995; Peng *et al.*, 1995; Creedon *et al.*, 1996).

Another protein which appears to be specifically activated by NGF in PC12 cells (the former signalling proteins can also be activated by EGF in PC12 cells) is SNT (Rabin et al, 1993). This SNT protein appears to act via a pathway distinct from the PLC-γ 1 or SHC pathways, and may be important in the differentiation and NGF-induced neurite formation in PC12 cells (Peng *et al.*, 1995).

Based on the available information, it seems likely that multiple NGF-regulated pathways are involved in survival, differentiation and neurite promotion activities (Kaplan and Stephens, 1994; Greene and Kaplan, 1995; Obermeier *et al.*, 1995; Segal and Greenberg, 1996). While most of this information has come from studies of PC12 cells, it is likely that similar pathways may be activated in primary neurons, although there may be individual differences (Klinz and Heumann 1993; Klinz *et al.*, 1996; Segal *et al.*, 1996) depending upon the age and type of neuron. Thus, both ras-dependent and ras-independent cascades are likely to be involved, although the data provide conflicting results suggesting that in some cases MEK, Ras and PI-3 kinase are required for neurite growth, while in other cases they are not required or not sufficient (Borasio *et al.*, 1993; Peng *et al.*, 1994; Obermeier *et al.*, 1994; Klinz *et al.*, 1996).

A further complication arises from studies suggesting that the duration of the activation

may play a role in the specificity of different growth factors and that certain pathways are or are not contributing to the observed cellular responses (rev. in Marshall, 1995). Thus, NGF results in the sustained activation of the Ras-MAP kinase pathway that lasts for several hours (Traverse *et al.*, 1992). It is apparent that the phosphorylation of the various tyrosine residues on the Trk receptors does not occur simultaneously, but rather in some coordinated fashion, and this could also be a method of determining the outcome of the activation (Segal *et al*, 1996).

1.5 Objective of this study

Based on the above information, it was hypothesized that prior to the precocious collateral sprouting, electrical stimulation might activate certain IEGs or elevate Trk A receptor activity to act in concert with the increased availability of NGF to result in an accelerated terminal sprouting response. Therefore, the purpose of this study was to investigate the gene expressions and possible transduction pathways induced by these extracellular factors.

1. *In vivo*, precocious collateral sprouting was generated in partially denervated rat skin by electrical stimulation of the remaining intact nerves. In these experiments, IEGs, including CREB, egr-1,c-fos, c-jun, and Oct-2 which can be induced by membrane depolarization or elevated NGF levels or both) were assayed using primarily immunocytochemisty (ICC).

2. Because NGF exerts its function via NGF high affinity receptor Trk A, potential changes

in Trk A receptor expression and its phosphorylation (activation) state were also investigated under denervation and electrical stimulation status using *in situ* hybridization and Western (immunoblot) analyses. The potential downstream signalling components activated by such manipulations were also assayed using Western analysis.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Animals, female Sprague-Dawley rats (130-180 g, at least 6 weeks old), were supplied by the Memorial University of Newfoundland Animal Care Facility (96 rats in total). Tissue freezing medium was purchased from Triangle Biomedical Sciences. Sodium Pentobarbital (Somnotol) was obtained from MTC Pharmaceuticals. Sigma Chemical Company is the supplier of aprotinin, phenyl methyl sulphonyl fluoride (PMSF), leupeptin and vanadate. Protein-A agarose or sepharose beads were obtained from Santa Cruz Biotechnology, Inc.. GIBCO-BRL supplied normal goat serum and bovine serum albumin. Aqua perm mounting media was purchased from Fisher. Eastman Kodak company was the producer of Kodak autoradiography NTB2 emulsion.

The rainbow colour protein ladders were supplied by Amersham Corp. and Bio-Rad Laboratories.

Dc protein assay kit was purchased from Bio-Rad Laboratories. Amersham Corp./Life Sciences division supplied Hybond ECL membrane, Enhanced Chemiluminescence reagents (ECL reagents) and Hyperfilm.

Vectastain Elite ABC kit was obtained from Vector laboratories.

The monoclonal RTA antibody (Trk A antibody) was a kind gift from Dr. L. Reichardt;

a 203 pan-Trk antibody was kindly provided by Dr. David Kaplan, Montreal Neurological Institute. Santa Cruz was the supplier of the rabbit polyclonal antibodies Egr-1, Trk, ERK1, and PLC-γ1. The mouse monoclonal phosphotyrosine antibody 4G10, rabbit polyclonal antibodies against pCREB, PI-3 kinase and SHC were obtained from Upstate Biotechnology Inc. c-Fos antibody was purchased from Oncogene Science, Inc.

[^{35}S]dATP and [^{32}P]dATP were supplied by Dupont.

Terminal Deoxynucleotidyl Transferase (TDT) was purchased from GIBCO-BRL.

The Northern Exposure software for computer-based Image analysis system was the product of EMPIX Imaging. MCID image analysis system was from Imaging Research Inc.. Pharmacia LKB Biotechnology was the supplier of Ultraspectrophotometer II and Ultroscan XL Laser Densitometer.

2.2 Surgery and Stimulation/isolation paradigms

The procedures for rat surgery were similar to those described in Diamond et al. (1992a,b) and Mearow *et al.* (1994). Briefly, female Sprague Dawley rats were injected intraperitoneally with sodium pentobarbital (4.5 mg/100g rat weight). Five to ten min later, the reflex reaction was checked by squeezing the paws. When the rats were anaesthetized, the fur was clipped from the skin of thoracolumbar region of the back, and the skin was sterilized by wiping with 75% (v/v) ethanol. A 4 - 5 cm midline incision was made and the skin was gently separated from underlying tissue and the dorsal cutaneous nerves (DCNs)

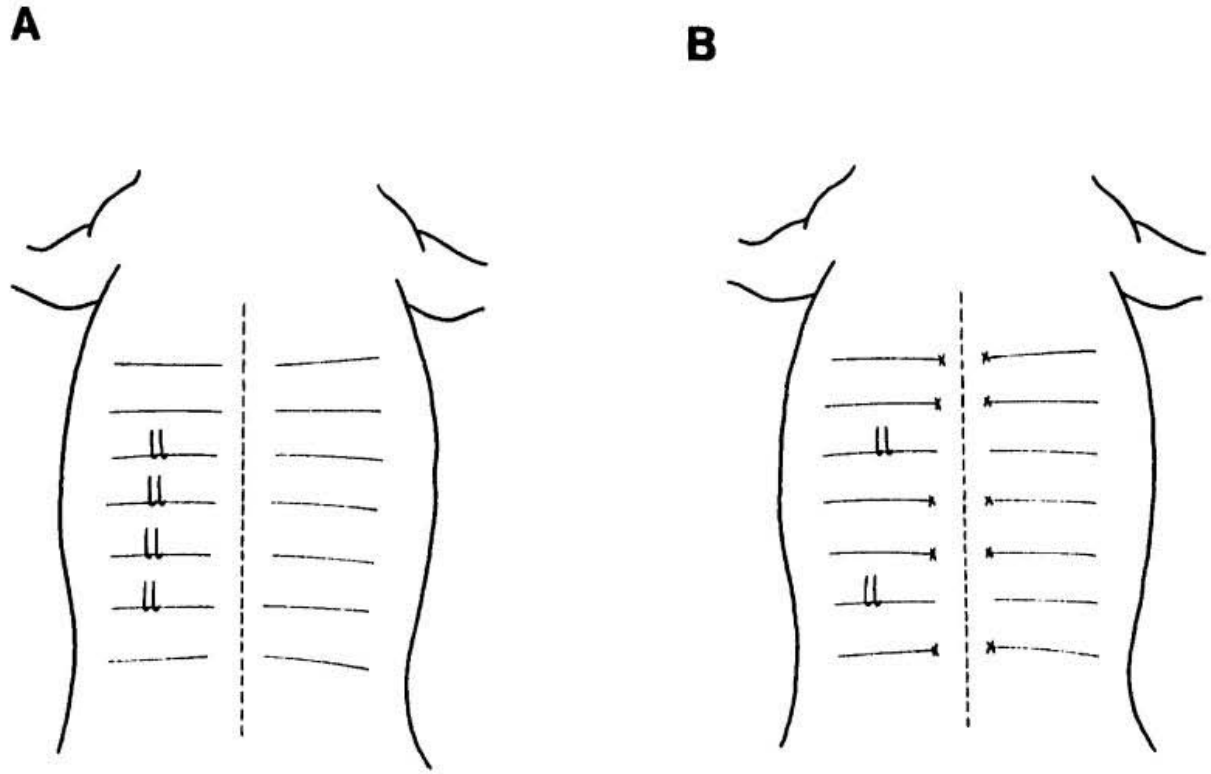
on both sides were exposed. For the stimulation paradigm, the DCNs from T13 to T10 (Fig. 2A) were separated from body wall, and a string of silk thread was inserted underneath the nerves. The left side DCNs from T13 to T10 were stimulated by bipolar stimulating electrodes at 8 V, 20 HZ for 1 min which excited both Aδ and C fibres and elicited the CTM (*cutaneus trunci* muscle) reflex (Nixon et al, 1984), whereas the right side T13 to T10 nerves, as internal controls, were not stimulated. For the isolation plus stimulation paradigm (Fig. 2B), on both sides L1, T12, T11, T9 and T8 nerves were cut just distal to their exits from the body wall. This treatment gave the target areas of T13 and T10 nerves intact sensory fields, and isolated their fields from those of denervated skin. The right side T13 and T10 nerves were separated from the body wall (producing the normal sprouting); the left side T13 and T10 nerves were respectively both separated and stimulated (producing the accelerated sprouting paradigm). After these treatments, the skin was sutured with clips (for short term, less than 1 day) or silk thread (for long term, more than 1 day), and the animals were permitted to recover.

2.3 DRG dissection

At different time points following surgery, the rats were deeply reanaesthetized with sodium pentobarbital (4.5 mg/100 g rat weight). The skin was quickly opened again at previous suture site. Along the spine, the muscles around it were cut open with scissors. At 2-3 cm posterior to the last rib, the spine was transected and exposed dorsally and

Figure 2. Schematic diagrams of stimulation and isolation plus stimulation paradigms. These diagrams show the back view of a rat. The back skin was opened after anaesthetization. **A.** The stimulation paradigm. The left side DCNs, T10 to T13 were separated from the body wall and stimulated with electrical bipolar electrodes at 8 V for 1 min. The right side DCNs were just separated from body wall and not treated as a sham control. **B.** The isolation plus stimulation paradigm. On both sides of the rat, T8, T9, T11, T12, and L1 DCNs were cut at the exit point from body wall. The left side T10 and T13 were separated from body wall and electrically stimulated with electrodes at 8 V for 1 min --- isolation plus stimulation treatment. The right side T10 and T13 were only separated from body wall --- isolation treatment. The back skin was sutured and rats were allowed to recover. After different time periods, the DRGs connected with the DCNs were dissected from the rats.

√√- electrical stimulation **X** - nerve cutting


A
B

bilaterally. After pulling up the spinal cord and cutting the rest of bones on both sides, DRGs were exposed. T13 DRG is located in the first intervertebral space posterior to the last rib. Forward from this point are DRGs T12, T11 and T10 in order; caudally is DRG lumbar 1. DRGs were removed quickly. For protein (western blot and immunoprecipitation assays) and RNA (RT-PCR assay) extraction, DRGs were put directly into a microcentrifuge tube and immediately frozen in liquid nitrogen. Normally, 4 DRGs were taken for each time point. For frozen-sectioning (immunocytochemistry and *in situ* hybridization assays), DRGs were embedded in tissue freezing medium and quickly frozen in liquid nitrogen. Tissues were stored at -70^{0}C.

2.4 Protein extraction and quantitation

The DRGs were taken out from the -70^{0}C freezer and placed on ice. Cold lysis buffer, containing 0.1% (v/v) NP-40, 10% glycerol, 1 mM phenylmethyl sulfomyl fluoride (PMSF), 10 mg/ml aprotinin, 1 mg/ml leupeptin, and 0.5 mM sodium orthovanadate in TBS (Tris Buffered Saline) (Kaplan and Knusel, 1997) was then mixed with the frozen tissues. The DRGs were homogenized on ice with an electrical homogenizer for several seconds and then kept on ice for 20 minutes. The insoluble material was removed by centrifugation at 13,000 rpm, 4^{0}C for 15 min and the supernatant was transferred into another fresh microcentrifuge tube.

Total protein concentration was measured by a Bio-Rad Dc protein assay kit. Briefly,

a standard curve was made each time when this assay was performed. Five dilutions of protein standard I (Bio-Rad) from 0.1 mg/ml to 1.43 mg/ml protein were prepared in lysis buffer. 25 µl of standards and samples were mixed with 125 µl of reagent A'(reagent A + S) and 1 ml of reagent B then incubated at room temperature for 15 min. The absorbances were measured at 750 nm using a spectrophotometer. Finally, the concentration of each sample was determined according to the standard working curve. DRG samples were duplicated to ensure the accuracy of determination. The lysates were stored at -70°C or used directly for Western blot or immunoprecipitation.

To extract enough protein for western blot or immunoprecipitation, at least 4 DRGs were used. In certain conditions, such as isolation, only 2 DRGs were obtained from one animal. Normally, one DRG was used for ICC, the other one was used for protein extraction or both were used for protein. Therefore, the DRGs for protein extraction came from at least 2-4 animals. On the other hand, since western blot were repeated several times, more animal were used to get enough protein to repeat the experiments.

2.5 Western blot assay

Normally, 10 µg of protein was used for the Western blot assay. Sample buffer [10% glycerol, 2% sodium dodecyl sulfate(SDS), 0.1 M dithiothreitol (DTT), 0.005% bromophenol blue] was added into the protein lysate, boiled for 3 minutes, and then loaded onto a 8%-12% SDS polyacrylamide gel (29:1 acrylamide:bisacrylamide). Electrophoresis

was run in electrophoresis buffer [25 mM Tris.base, 250 mM glycine (pH 8.3), 0.1% SDS] at 200 V (constant voltage) for 45 min in a Bio-Rad mini-electrophoresis apparatus. The nitrocellulose membranes were soaked in transfer buffer (25 mM Tris.base, 192 mM glycine, 20% methanol) for at least 15 min. After electrophoresis, the gel was put into transfer buffer for 10 min. Subsequently, the proteins were transferred onto nitrocellulose membranes in a transfer tank at 100 V, 200 mA, 4°C for 90 min. After transfer, the blot was allowed to air dry and used directly for probing or sealed with Saran Wrap at 4°C prior to use. To eliminate nonspecific binding of antibodies, the blot was blocked with 3% BSA in TBST (TBS with 0.1% Tween-20) or 5% skim milk in TBST at room temperature for 1 hour. Subsequently, the blot was incubated with primary antibody in 5% milk in TBST or 3% BSA in TBST overnight with shaking. The dilutions of primary antibodies are RTA, 1:10.000; TrkA, 1:100; pCREB, 1:250; anti-phosphotyrosine, 1:1000; Egr-1, 1:400; ERK-1, 1:5000; PLC-g1, 1:5000; PI-3 kinase, 1:800; SHC, 1:1000. After washing with TBST for 10 min three times, the blot was incubated in secondary antibody (goat anti-rabbit or goat anti-mouse antibody) at 1:3000 dilution in Blotto for 1 hour. After rinsing three times with TBST, the blot was placed between a transparent plastic folder, incubated with Enhanced chemiluminescence reagents (ECL, Amersham) for exactly one minute, and exposed to a sheet of Hyperfilm® from 30 seconds to 10 minutes, depending on the strength of signals.

2.6 Immunoprecipitation Assay

This assay follows Kaplan and Knusel's protocol (1997). Briefly, 50 μg of protein lysate was incubated with 20 μl of anti-Trk A or Pan-Trk antibody at 4°C overnight with rotation. Then 30 μl of protein-A sepharose beads was added and incubated with rotation at 4°C for 2 hours. Beads were pelleted by spinning 35 seconds at 3000 rpm, washed with cold lysis buffer three times, and boiled in 1 x sample buffer for 3 min. The supernatant was loaded on 10 or 12 % SDS-PAGE gels and run in electrophoresis buffer at 100 V, 120 mA for 45 min. Subsequently, the proteins were transferred to Hybond nitrocellulose membrane. The blot was probed by anti-trkA or anti-phosphotyrosine antibody as described in section 2.5.

The signals were quantitated by Ultrascan XL Laser Densitometer(Pharmacia LKB Biotechnology). Statistical analysis was performed with the Prism program.

2.7 DRG sectioning

DRGs stored in tissue freezing medium at -70°C were removed and immediately put into liquid nitrogen. Next, the tissue was mounted on the cryostat chuck with tissue freezing medium and again quickly frozen in liquid nitrogen. Frozen sections were made using a cryostat microtome at 14 μm, then thaw-mounted onto gelatin-coated slides for several time points and dried at room temperature. Each slide contained control and experimental sections and was stored at -20°C until used in immunocytochemistry (iCC) or *in situ* hybridization (ISH) assay.

2.8 Immunocytochemistry

The protocol used for immunocytochemisty utilized a peroxidase detection method (Mearow et al., 1994). The procedure is as follows: sections removed from the freezer were allowed to air dry for 20 minutes at room temperature and demarcated by a PAP pen to form compartments around them. Subsequently, the sections were fixed in 4% freshly-made formaldehyde in PBS (Phosphate Buffered Saline) for 20 min. Endogenous peroxidase was removed with 0.3% H_2O_2 in 100% methanol for 30 min, followed by three washes with 1 x PBS. Sections were incubated in 10% normal goat serum (NGS) containing 0.1% Triton-X 100 in PBS to block nonspecific protein binding at room temperature for 1 h, then directly incubated with primary antibody in 3% NGS plus 0.1% Triton-X 100 at 4^0C overnight. For rabbit polyclonal antibodies Fos and Egr-1, the dilution factors were 1:200 and 1:100, respectively. For rabbit polyclonal antibodies pCREB, Jun and Oct-2, the dilutions were 1:2000, 1:7500, and 1:2000. After incubation in primary antibody, slides were washed with PBS three times for 10 minutes each, and subsequently, incubated with biotinylated secondary antibody (goat anti-rabbit or goat anti-mouse IgG) for 1 h. Visualization of the antibody pattern was carried out in avidin/biotin/peroxidase reagent for 1 hour. Finally, sections were incubated with 0.05% 3,3'- diaminobenzidine (DAB) in 100 mM Tris buffer (PH 7.0) and 0.012% H_2O_2. The slides were firstly dehydrated with Aqua perm mounting media, dried and secondarily mounted with Permount or D.B.X. neutral mounting medium for permanent protection. The proteins of pCREB, Egr-1, Fos, Jun and Oct-2 genes were

primarily located in nuclei. Therefore, the different sizes of cells (diameter: small, <30 μm; medium, between 30 μm to 40 μm; large, > 40 μm) containing dark nuclear staining and total cells in each DRG section were counted under the light microscope using the computer based image analysis system (Northern Exposure) and the data were stored in the Excel® program.

The ICC experiments for each gene were performed on a series of slides. Each slide contained the sections from control and three treatments: stimulation, isolation and stimulation plus isolation at one of the following time points (1h, 4h, 8h, 1d, 2d, or 4d). Normally, at one time point, one slide had 3-5 sections for each treatment (from one DRG). So, for one single experiment, one DRG was used for one treatment at each time point. For each gene, 2-6 experiments were run (2-6 DRGs were used for each treatment at each time point (n=2-6), and some of these DRGs were from the same or different animals since one or two DRGs could be obtained for certain treatment). After ICC experiments, nuclear stained neurons and total neurons from one DRG section were counted under light microscope and the percentage of nuclear stained neurons in total neurons was calculated. One to five sections could be sampled on one slide for each treatment and each time point and these data were averaged as one value for one experiment at that treatment/time point. Subsequently, the values from repeated experiments (DRGs) at each treatment/time point were analysed and the mean values and standard errors of the means were obtained. Here, the n value was the times of experiments run for this gene and also equal to the numbers of

DRGs used for this gene at each treatment/time point. To statistically analyse the effects of treatments and times on the gene expression, two types of tests were performed on Minitab statistics software. Firstly, multiple analysis of variance by general linear model (MANOV by glm) was used to analyse the significant difference ($p<0.05$) between control group and treatment groups (stimulation, isolation, isolation plus stimulation) during experimental time period, and also the significant difference ($p<0.05$) within each treatment with the change of the time. Then, if group significant differences existed, oneway ANOVA with Dunnett's test was further used to test the significant differences ($p<0.05$) between control and each treatment (stimulation, isolation, stimulation plus isolation) at each time point. The data for each gene were plotted using the SigmaPlot® program and each figure is accompanied by a summary data table. The n values in the table represent the number of DRGs used for each gene. In these experiments, individual DRGs were subjected to the different treatments and an acceptable way to analyze the data is to consider each DRG as an n = 1 (G. Skanes, J. Evans personal communications)

2.9 *In situ* hybridization (ISH) assay

Oligonucleotide probes were used for ISH. The anti-sense sequences of oligos for the probes are: Trk A (Merlio *et al.*, 1992), 5'AAG GTT GAA CTC AAA AGG GTT GTC CAT GAA GGC AGC CAT GAT GGA GGC3'; Trk A (Meakin et al, 1992), 5'GC AAG AAA GAC CTT TCC AAA GGC TCC CTC CCC TAG CTC CCA CTT GAG3'; P75 (Radeke

et al., 1987), extracellular domain - 5'CAC GAG TCC CGA GCC CAC CTC GCA CAC GCT GCA AGC CTC ACA GTG GCC3', membrane spanning element - 5'GAA AGC AAT ATAGGC CAC AAG GCC CAC GAC CAC AGC AGC CAA GAT GGA3'. CREB (Gonzalez *et al.*, 1989), 5'GTC TGC TCC AGA GTC CAT GGT CAT CTA GTC ACC GGT GGT3'; NGFI (EGR-1, Wisden *et al.*, 1990), 5'GCG TTG CTC AGC AGC ATC ATC TCC TCC AGT TTG GGG TAG TTG TCC3';

On ice, 5 pmol of oligos, 50 mCi of ^{35}S dATP, 1/10 of reaction volume of 5 x Tailing buffer were mixed with 15-30 units of TDT (Terminal Deoxynucleotidyl Transferase) in 50 µl and incubated at 37^{0}C for 30 min. 350 µl of TE8, 20 µl of 5 M NaCl, 2.5 µl of tRNA (10 mg/ml), and 425 µl of Phenol/chloroform mix were then added to the above mixture. After vortexing and spinning down, the aqueous phase was precipitated with ethanol. The pellet was air-dried and resuspended in 100 µl of H_2O with 100 mM DTT. Finally, 1 µl of sample was taken to count incorporation efficiencies.

In situ hybridization (ISH) was carried out on frozen DRG sections (14 µm thick) according to established procedure (Mearow *et al.*, 1989 and 1994). Briefly, sections were removed from the freezer, air-dried and fixed with 4% formaldehyde in PBS-DEPC for 5 min at room temperature. After three 5 min washes with PBS-DEPC, slides were placed into 0.25% acetic anhydride in 0.1 M triethanolamine HCl for 10 min, subsequently dehydrated with 70%, 90% and 100% ethanol and air-dried. The ^{35}S-labelled oligonucleotide probes were mixed with buffer A (4 x SSC, 50% formamide, 250 mg/ml yeast tRNA, 500 mg/ml

sonicated salmon sperm DNA, 1 x Denhardts solution, 5% dextran sulfate, and 100 mM DTT). Normally, 100 µl of the above mix (containing 1 x 10^6 dpm of ^{35}S) were needed per slide. Slides were coverslipped and incubated at 42°C in a humidified chamber overnight. Coverslips were removed in 4 x SSC containing 1 mM β-mercaptoethanol, and then the sections were washed at 40°C according to the following sequences: two times in 2 X SSC for 30 min each; two times in 1 x SSC for 30 min each. After brief rinsing in cold tap water, the slides were allowed to air dry.

For autoradiographic visulization, slides were initially exposed to Kodak X-OMAT film for 1-2 days, and then dipped in Kodak NTB2 emulsion, dried, placed in foil-wrapped black boxes, and exposed at 4°C for 2-4 weeks depending on the kind of probes used. Finally, the slides were developed with Dektol, coverslipped and analyzed using an computer-based image analysis system (MCID.St. Catharines. Ont.).

One experiment was run for each gene on a series of slides. The slides carried at least 5 sections representative of areas throughout the same DRG; control and experimental sections were processed on the same slides. A grain counting software option was used to randomly count grains from neurons in which the nucleus was visible under phase contrast. Over 100 neurons on three sections from one DRG at each treatment / each time point were analyzed. The grain numbers and area of each neuron were computed and grain density (number of grains per μm^2 neuronal area) was calculated for each neuron. Subsequently, the data from each treatment / time point were statistically analyzed, first, as a function of the

total cell population sampled, and, second , following classification of the samples into three groups based upon neuronal size. Neurons were classified as small (<30 μm diam.), medium(30-40μm diam.), and large (>40μm diam.). Then, the changes of grain density at each treatment / time point were statistically analyzed by oneway ANOVA plus student t-test using Minitab software to investigate the significant difference ($p<0.05$) caused by treatments or times.

The figures were drawn with Prism® program and the bar of SEM were put onto the figures. The n value for each bar was 100 neurons, representing 1 or 2 DRGs per each time point. These experiments were carried out to further confirm results from previous experiments (Mearow, 1998).

Chapter III

RESULTS

3.1 The expression of mRNAs and proteins of immediate early genes (IEGs)

3.1.1 mRNA expression detected by *In Situ* Hybridization

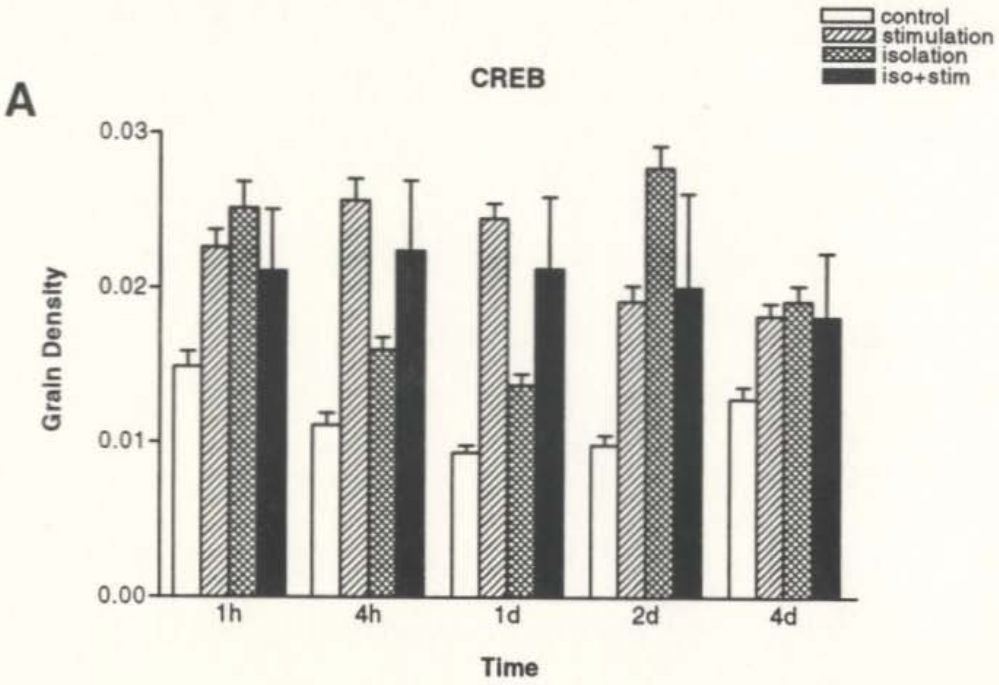
In order to examine the response of IEGs during precocious sprouting, the mRNA expression of several IEGs was detected by *in situ* hybridization (ISH) on 14 μm frozen sections from DRGs prepared under situations which would cause the depolarization of the DRG neurons and/or the elevation of NGF levels in the target area: i) electrical stimulation of DCNs (depolarization), ii) nerve isolation (NGF level increase), and iii) stimulation plus isolation (the combination of these two factors). As described in the methods, the nerves on the left side of the animals were stimulated, while the contralateral nerves were not stimulated and used as controls for effects produced by surgery. After 1h, 4h, 1d, 2d, and 4d, the appropriate DRGs were dissected and frozen sections were cut using a cryostat. Each slide contained the control (C), stimulated (S), isolated (I) and stimulated plus isolated (S+I) conditions for a given time point.

The raw data were subsequently analyzed in two steps. First, the grain density (number of grains per neuron area, μm^2) at each time point was calculated for each treatment. Subsequently, in order to learn more about the response of the different populations of DRG

neurons, the grain density was further analyzed as a function of neuron size (diameter). The neuronal population was grouped into three sizes: small (<30 μM diam.), medium (30-40 μM diam.) and large (>40 μM diam.) neurons.

CREB expression in DRG neurons The grain density of CREB under stimulation (S), isolation (I), and isolation plus stimulation (I+S) treatments at 1h, 4h, 1d, 2d, 4d is presented in Fig. 3A. The rats which were treated only by surgery were used as controls at each time point. Statistical analysis indicated that all three treatments induced significant increase ($p<0.05$) of CREB expression during the experimental period, but the amplitude presented different characters depending on the treatment. Electrical stimulation alone resulted in 1.5X increase of CREB expression as early as 1h compared with control, and this increase was further elevated at 4h , reached a peak 1d after stimulation, and subsequently declined. Isolation alone induced an unexpected large increase of CREB after 1h treatment, and later it resulted in a peak increase (2.5X compared with control) after 2d. Isolation plus stimulation induced a consistent increase of CREB expression from 1h to 4d, with little change in amplitude. Further analysis of mRNA expression in the small neuronal population is shown in Fig. 3B. The expression of CREB in the small sized neurons was very similar to that in the total population, suggesting that CREB was expressed primarily in the small neurons.

Figure 3. Expression of CREB mRNA in DRG neurons. *In situ* hybridization and analysis were performed on frozen sections of DRGs from animals at 1h, 4h, 1d, 2d, and 4d after stimulation and isolation plus stimulation paradigms as described in methods. **A.** The grain density of CREB mRNA in the total neuronal population at control and each treatment per time point. **B.** The grain density of CREB mRNA in small neurons at each control and treatment per time point. Significant differences between control and each treatment at different time points were analyzed by Oneway ANOVA plus student's t-test on Minitab program ($p<0.05$). The different treatments are represented as: control (empty bar), stimulation (hatched bar), isolation (cross-hatched bar) and isolation plus stimulation (solid bar). Each bar represents the analysis of 100 neurons from 1 DRG per treatment.


A
CREB
control
stimulation
isolation
iso+stim
Grain Density
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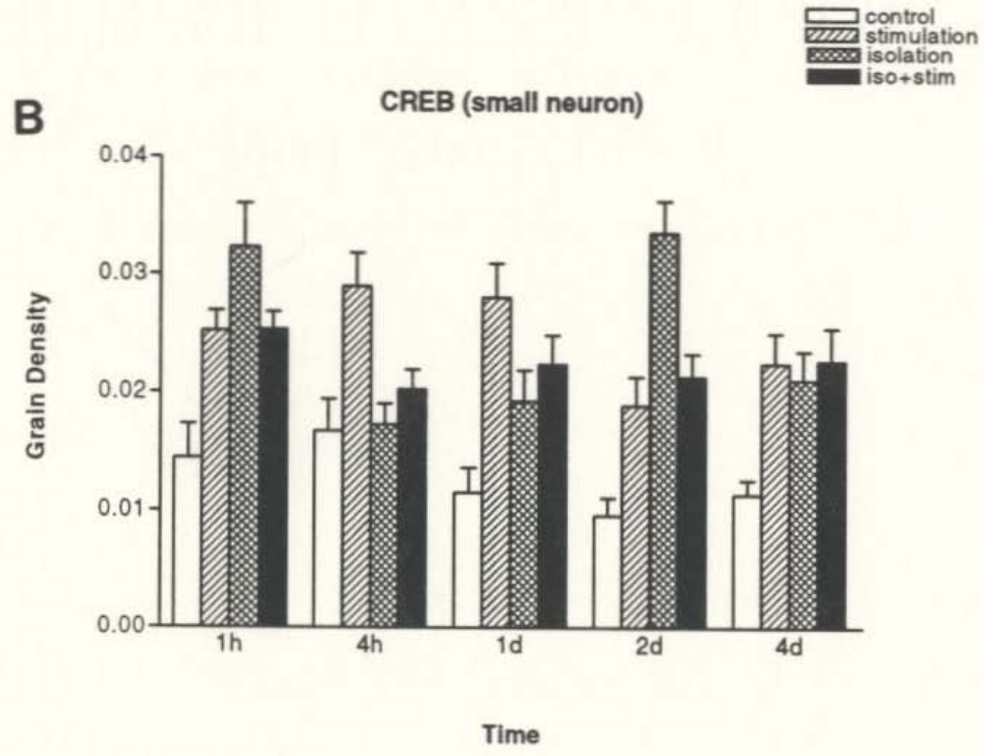
0.02
0.01
0.00
1h
4h
1d
2d
4d
Time


B
CREB (small neuron)
control
stimulation
isolation
iso+stim
Grain Density
0.04
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0.02
0.01
0.00
1h
4h
1d
2d
4d
Time

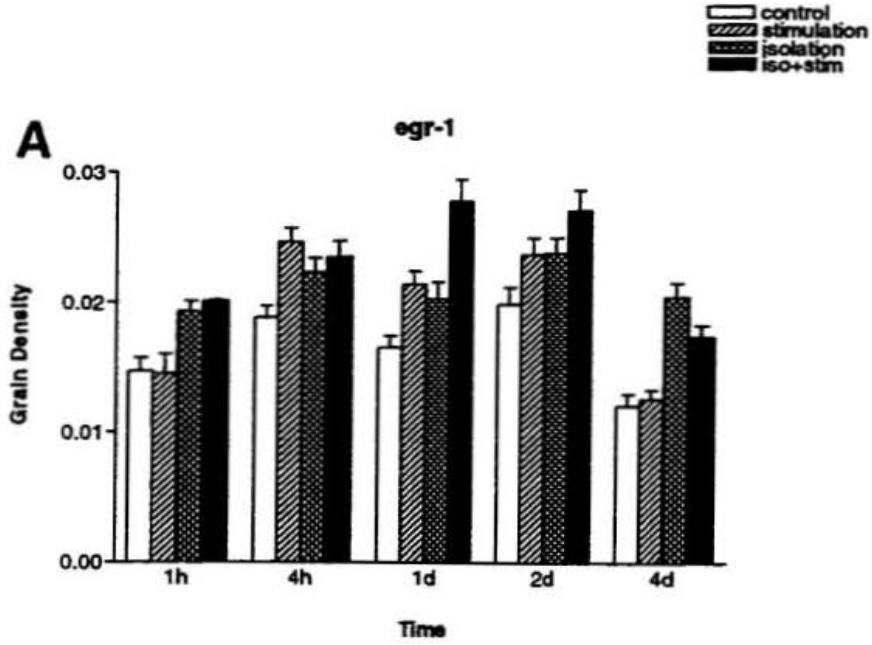
egr-1 mRNA expression in DRG neurons The results of mRNA expression of egr-1 are shown in Fig. 4. Although there were no changes in egr-1 mRNA expression in DRG neurons after 1h post-stimulation, there was an significant increase ($p < 0.05$) at the 4h and 1d time points, but was not significant at 2d. In the isolation paradigm, there was an unexpected increase of egr-1 expression after 1h. Even though there was some increased egr-1 expression at 4h, 1d and 2d, these changes were not significant ($p<0.05$). However, after 4d, a significant increase of the egr-1 expression was detected again after isolation alone, and this increase was 1.7 X relative to control. The combination of the stimulation and isolation treatment resulted in significantly increased expression of egr-1 during the whole period of experiments. This increase peaked by 1d, and remained somewhat elevated by 4d.

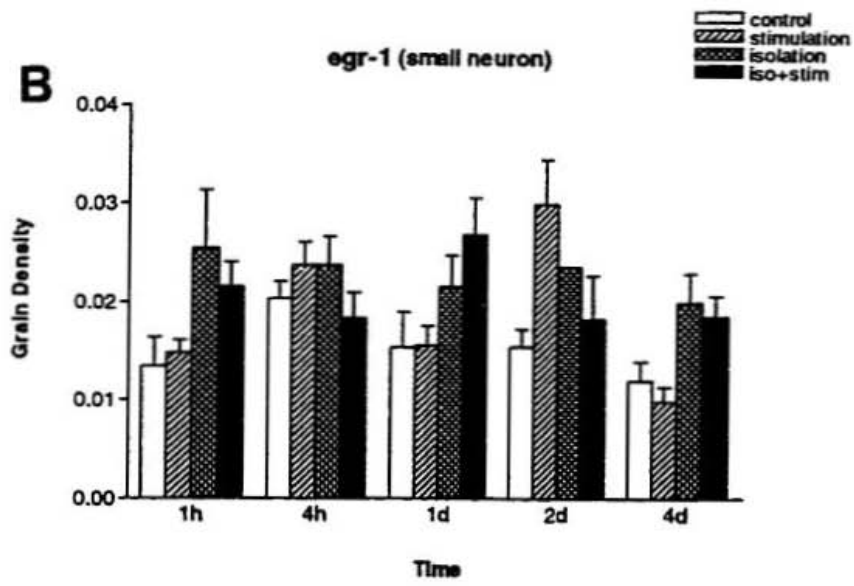
The egr-1 expression as a function of neuronal size was further analyzed (Fig. 4B). In small size neurons (which are the neurons expected to be initiating the sprouting response) egr-1 expression did not show a similar expression pattern to that in the total population of neurons, suggesting that there was no neuronal specificity to the expression of egr-1 gene.

3.1.2 Protein expression in DRG neurons

To investigate the functional products of certain IEGs, protein expression in DRG neurons was investigated by immunocytochemistry, immunoprecipitation and Western-blot assays in the situation of stimulation, isolation and stimulation plus isolation at several time

Figure 4. Expression of egr-1 mRNA in DRG neurons. *In situ* hybridization was performed on frozen sections of DRGs from animals at 1h, 4h, 1d, 2d, and 4d after stimulation and isolation plus stimulation paradigms as described in methods. **A.** The grain density of egr-1 mRNA on the total neuronal population at control and each treatment per time point. **B.** The grain density of egr-1 mRNA in small neurons at each control and treatment per time point. Significant differences between control and each treatment at different time points were analyzed by Oneway ANOVA plus student's t-test on Minitab program ($p<0.05$). The different treatments are represented as: control (empty bar), stimulation (hatched bar), isolation (cross-hatched bar) and isolation plus stimulation (solid bar). Each bar represents the analysis of 100 neurons from 1 DRG per treatment.


control
stimulation
isolation
iso+stim
A
egr-1
Grain Density
0.03
0.02
0.01
0.00
1h
4h
1d
2d
4d
Time


control
stimulation
isolation
iso+stim
B
egr-1 (small neuron)
Grain Density
0.04
0.03
0.02
0.01
0.00
1h
4h
1d
2d
4d
Time

periods after three treatments.

3.1.2.1 Immunocytochemistry (ICC) analysis

In order to identify the location of certain IEG proteins, ICC was performed on frozen sections of DRG neurons; use of DAB and HRP linked secondary antibody resulted in an insoluble brown product at the sites of positive antibody staining. For each primary antibody used for ICC, the optimal primary antibody dilution was determined by incubating the slides in 5 dilutions of primary antibody, and the one which showed clear nuclear staining with the lowest background was selected as the optimal antibody dilution for future use. The specificity of the antibody was checked by two steps. The first one was to omit the primary antibody and determine the status of nuclear staining. These experiments showed that in the absence of primary antibody, the nuclei of DRGs were not stained. Next, the same series of slides were treated with the best dilutions of primary antibody plus ten times amount of control peptide which blocked the epitope of this antibody. The results indicated that, in the presence of control peptide, the nuclear staining by this antibody was totally blocked, indicating that the staining was specific to this antibody. All the antibodies used in these experiments were specifically tested. Former studies indicated that the influence of stimulation occurs at very early time points, less than 2 days (Nixon *et al.*,1984; Doucette and Diamond, 1987; Kril *et al.*, 1993); on the other hand, the increase of NGF level induced by isolation occurs at least 2 days later (Mearow et al., 1993). Here, what is interesting is the separate and combined effects of electrical stimulation and isolation on gene expression.

My preliminary experiments indicated that electrical stimulation resulted in an increase of certain gene expressions shortly after treatment (0.5h, 1h, 4h, 8h and 1d) and isolation increased these gene expression after longer period of treatment (1d, 2d, 4d, 8d, and 14d) (data not shown). Therefore, based on the preliminary experiments, the time points chosen for further study were designated as 1h, 4h, 8h, 1d, 2d and 4d for the later experiments.

pCREB protein expression As a cAMP binding protein, CREB exerts its function when it is phosphorylated at ^{133}Ser. Therefore, using a phospho-specific CREB antibody, the phosphorylated protein, pCREB, was examined in DRG neurons at different time points under three types of treatments (stimulation, isolation, and isolation plus stimulation). ICC results showed that pCREB was specifically localized in the nucleus of DRG neurons. The pCREB staining in DRG neurons under control and isolation plus stimulation at 2d is representatively given in Fig.5. The immunostaining of pCREB was distributed in different sized neurons, but occurred predominantly in small neurons. The percentages of pCREB stained cells in total counted cells from different treatments at different time points were presented in Fig. 6 and all the data for the figure were listed in the attached table. The basal expression of pCREB in total neurons was 20.63 % to 30.47%. Except the mean value at 4h stimulation was less than control (might be due to difference of individul DRGs), the rest of mean values were all larger than control. Statistical analysis by MANOVA by glm indicated that, within each treatment group (stimulation, isolation, and isolation plus

Figure 5. Immunostaining of pCREB protein in DRG neurons. The micrographs show the immunostaining of pCREB protein in DRG neurons. ICC was performed on frozen section as described in the text. The phospho-specific pCREB monoclonal antibody was from Upstate Biotechnology Inc. It can be seen that the nuclei of DRG neurons are darkly stained by pCREB antibody. **A.** Nuclear staining of pCREB under control treatment, and **B.** Nuclear staining of pCREB at 2d under isolation plus stimulation treatment. The arrow heads point out the dark staining of the nuclei. Scale bar = 100 μm.

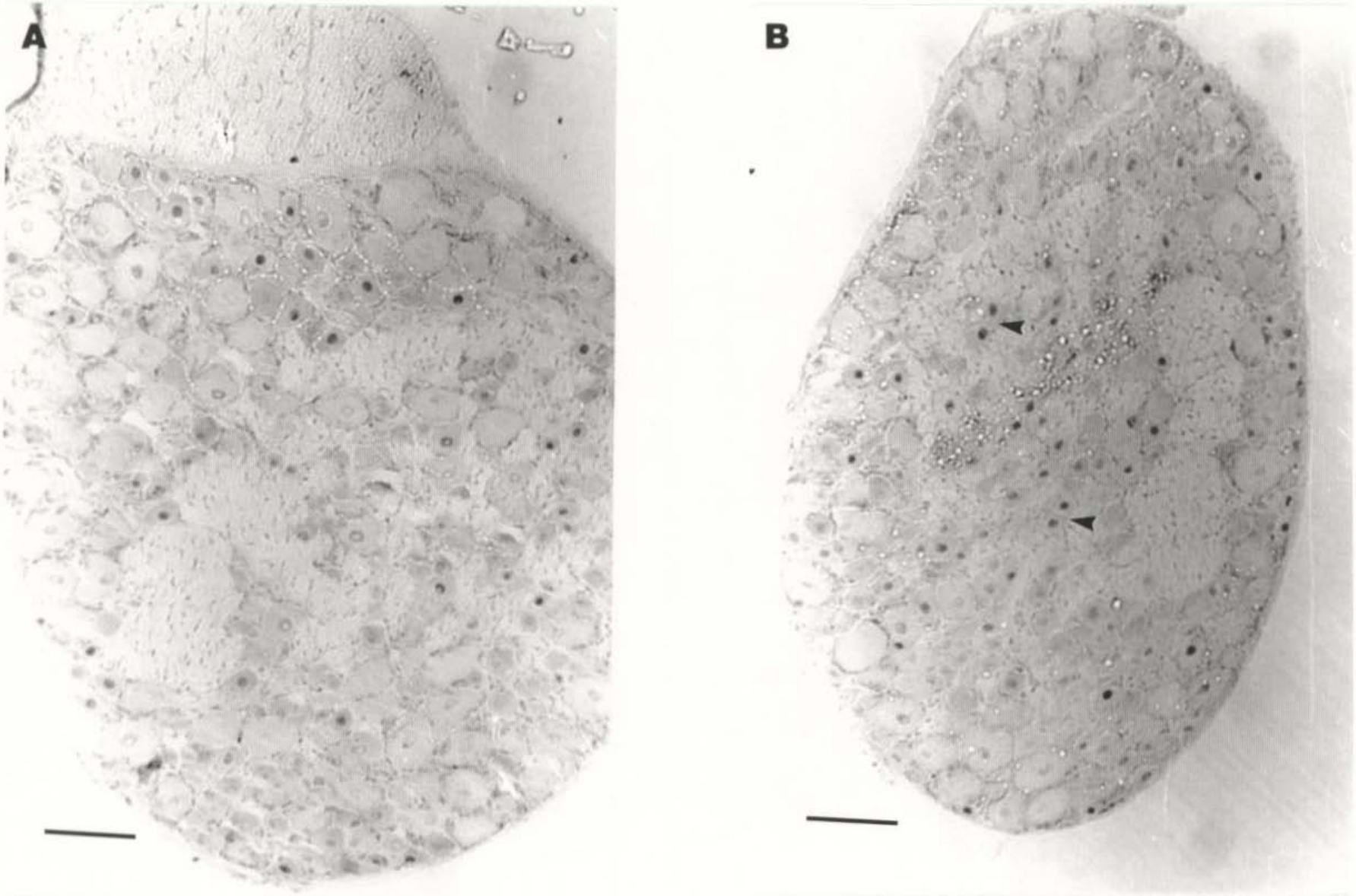

A
B

Figure 6. Analysis of the percentage of pCREB immunostained neurons in total DRG neurons. The immunostained cells and total DRG neurons per section were counted under the light microscope. On each slide, the 3-5 sections for control and each experimental treatment (stimulation, isolation and isolation plus stimulation) at different time points (1h, 4h, 8h, 1d, 2d and 4d) were analyzed. For each control or treatment per time point, one DRG was used per experiment. The cells with stained nuclei and total cells in one DRG section were counted and the percentage of stained cells in total (%) was calculated. Thus, a given experimental series is the data from 1 DRG per treatment per time point, ie, a total of 24 DRGs per experiment from at least 12 rats. The plotted data represent the Mean of 2-6 experiments; the table presents the Mean , SEM, n value and statistical analysis. Statistical analysis was carried out using the following tests. MANOVA by glm was used to test group difference ($p<0.05$, significant difference) caused by treatments across the time points. Significant differences ($p<0.05$) between control and treatments at each time point were tested by oneway ANOVA with Dunnett's test (represented by * in the figure and table). The different treatments are presented as follows: control, solid dot; stimulation, open dot; isolation, solid triangle; isolation plus stimulation, open triangle.

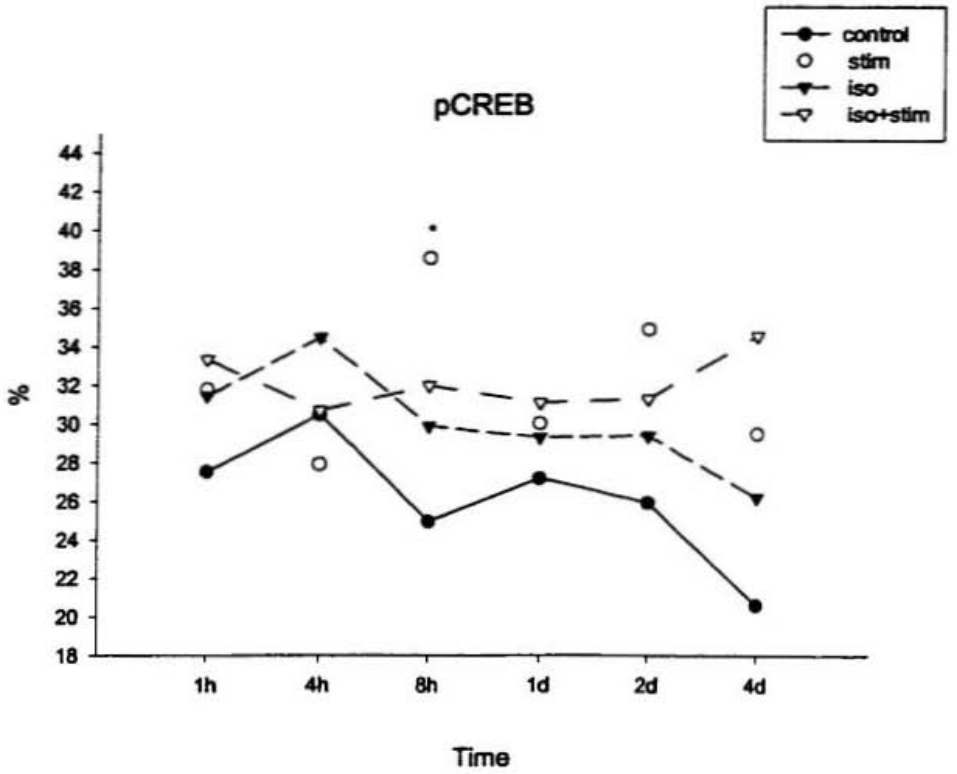


Table Immunocytochemistrical data for pCREB (total stained cells in total counted cells, %)

Time	1h				4h				8h			
Treatment	Mean	SEM	n	Signif.	Mean	SEM	n	Signif.	Mean	SEM	n	Signif
Control	27.54	0.83	3	No	30.47	1.87	3	No	24.96	2.79	3	No
Stim	31.80	2.90	3	No	27.91	4.58	3	No	38.59	1.42	2	*
Iso	31.45	6.70	3	No	34.47	4.53	3	No	29.89	2.31	3	No
Iso+Stim	33.34	8.52	2	No	30.69	4.02	3	No	32.00	2.33	2	No
Time	1d				2d				4d			
Treatment	Mean	SEM	n	Signif.	Mean	SEM	n	Signif.	Mean	SEM	n	Signif
Control	27.21	0.66	3	No	25.95	3.64	3	No	20.63	2.26	3	No
Stim	30.03	6.10	3	No	34.93	4.74	2	No	29.53	9.65	2	No
Iso	29.31	4.87	3	No	29.41	3.82	3	No	26.23	3.57	3	No
Iso+Stim	31.10	5.61	3	No	31.33	7.79	2	No	34.61	4.70	3	No

stimulation), treatment did not induce significant increase of the gene expression with the change of tested time period (1h, 4h, 8h, 1d, 2d, and 4d) ($p<0.05$). However, with the change of time, all the treatment groups showed significant differences from the control group ($p<0.05$). Based on these results, Oneway ANOVA plus Dunnett's test was used to investigate that at which time points the significant increases for each treatment occurred. The results indicated that, except at 8h stimulation induced significant increase of pCREB expression ($p<0.05$), at other time points (1h, 4h, 8h, 1d, 2d, and 4d), stimulation, isolation and isolation plus stimulation did not induce significant increase of pCREB expression in DRG neurons ($p<0.05$).

Egr-1 protein expression Egr-1 protein was also localized to the nucleus. The nuclear staining of Egr-1 protein under control and isolation plus stimulation treatment in DRG neurons is shown in Fig. 7. The percentage of nuclear stained cells in total DRG neurons was analyzed statistically and the data are shown in Fig. 8 and the attached table. Under control situation, the basal expression of Egr-1 protein in total DRG neurons was 32.29% to 34.37% at different time points. MANOVA by glm indicated that all treatment groups (stimulation, isolation, isolation plus stimualtion) were significant differences from control group along with the time periods (1h, 4h, 8h, 1d, 2d, and 4d) ($p<0.05$). Based on these results, onewayANOVA with Dunnett's test were used to further detect at which time point, the significant difference induced by each treatment occurred ($p<0.05$). It was found that

Figure 7. Immunostaining of Egr-1 protein in DRG neurons. ICC was performed on frozen sections as described in the text. Egr-1 polyclonal antibody was purchased from Santa Cruz Biotech. The nuclei of DRG neurons are darkly stained by Egr-1 antibody. **A.** Nuclear staining of Egr-1 under control treatment, and **B.** Nuclear staining of Egr-1 at 2d under isolation plus stimulation treatment. The arrow heads pointed out the dark staining of the nucleus. Scale bar = 100 μm.

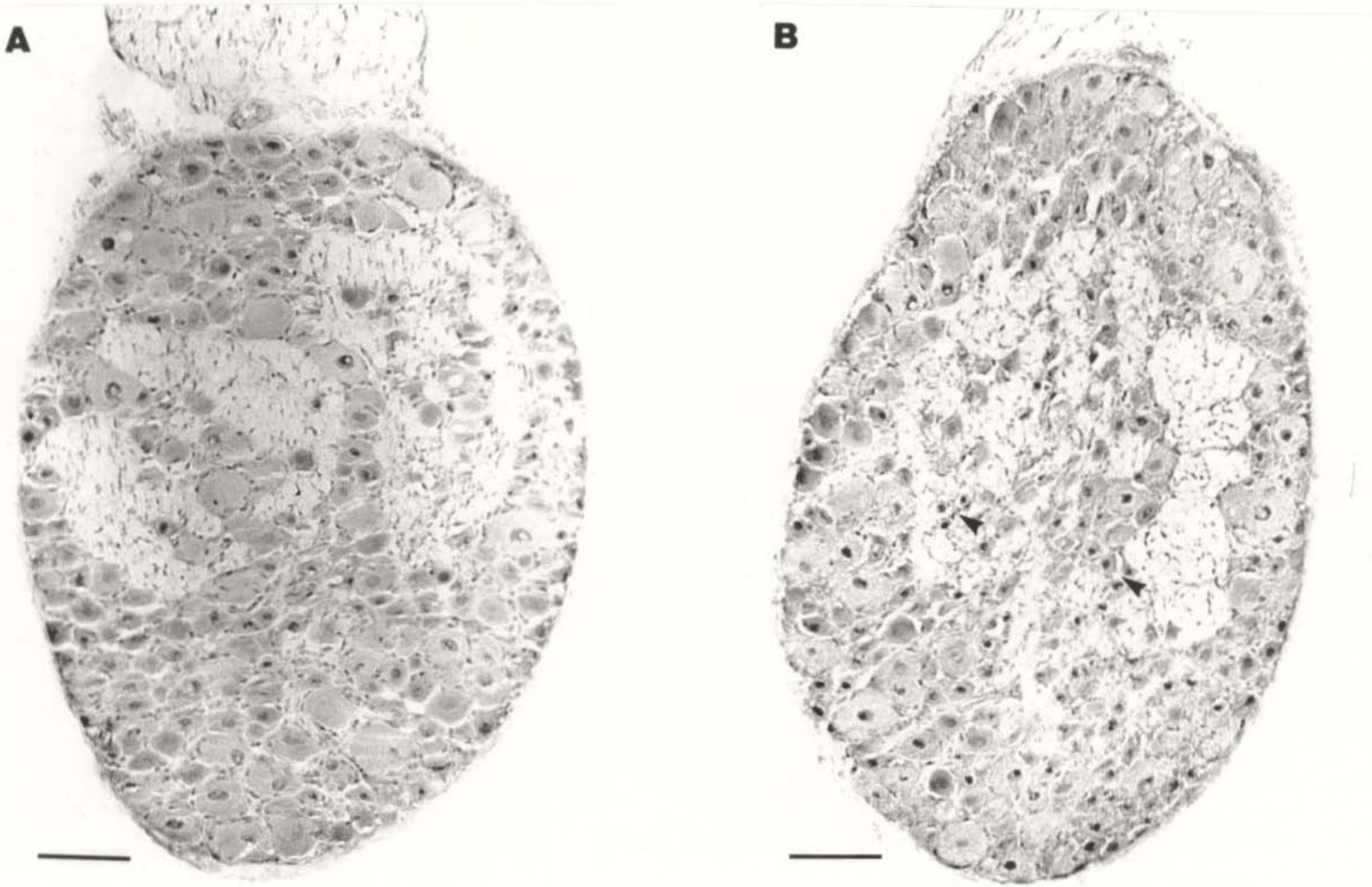

A
B

Figure 8. Analysis of the percentage of Egr-1 immunostained neurons in total DRG neurons. The immunostained cells and total DRG cells per section were counted under the light microscope. On each slide, the 3-5 sections for control and each experimental treatment (stimulation, isolation and isolation plus stimulation) at different time points (1h, 4h, 8h, 1d, 2d and 4d) were analyzed. For each control or treatment per time point, one DRG was used per experiment. The cells containing stained nuclei and total cells in one DRG section were counted and the percentage of stained cells in total (%) was calculated. Thus, a given experimental series is the data from 1 DRG per treatment per time point, ie, a total of 24 DRGs per experiment from at least 12 rats. The plotted data represent the Mean of 2-6 experiments; the table presents the Mean , SEM, n value and statistical analysis. Statistical analysis was carried out using the following tests. MANOVA by glm was used to test group difference ($p<0.05$, significant difference) caused by treatments across the time points. Significant differences ($p<0.05$) between control and treatments at each time point were tested by oneway ANOVA with Dunnett's test (represented by * in the figure and table). The different treatments are presented as follows: control, solid dot; stimulation, open dot; isolation, solid triangle; isolation plus stimulation, open triangle.

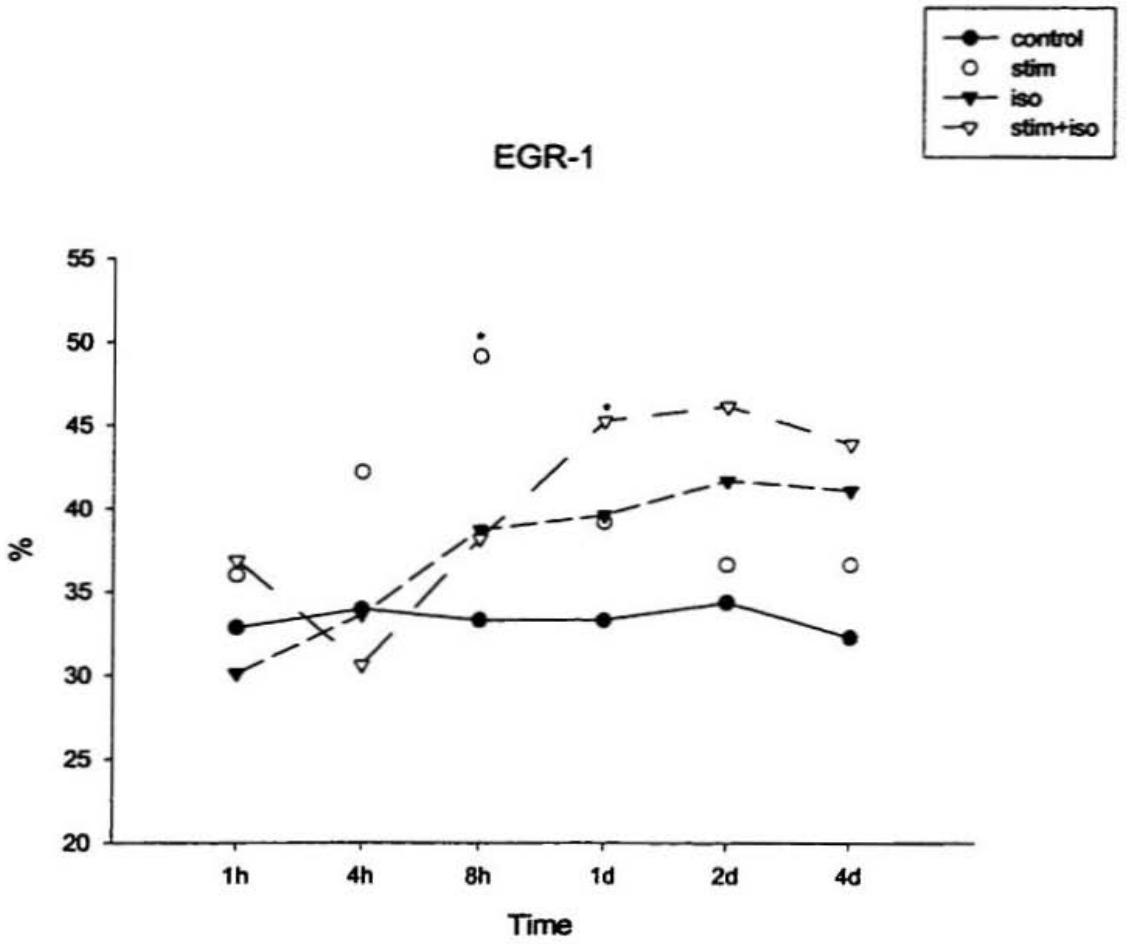


Table Immunocytochemistrical data for Egr-1 (total stained cells in total counted cells, %)

Time	1h				4h				8h			
Treatment	Mean	SEM	n	Signif.	Mean	SEM	n	Signif	Mean	SEM	n	Signif.
Control	32.68	1.95	6	No	33.94	2.18	5	No	33.30	1.68	4	No
Stim	36.00	1.05	3	No	42.18	1.67	3	No	49.13	5.48	2	*
Iso	30.12	4.19	4	No	33.61	3.99	3	No	38.72	1.13	3	No
Iso+Stim	36.85	3.41	3	No	30.60	0.30	2	No	38.16	4.82	2	No
Time	1d				2d				4d			
Treatment	Mean	SEM	n	Signif.	Mean	SEM	n	Signif	Mean	SEM	n	Signif.
Control	33.29	2.03	6	No	34.37	2.56	4	No	32.29	2.66	4	No
Stim	39.19	3.00	4	No	36.64	6.51	3	No	36.64	1.64	2	No
Iso	39.60	5.41	2	No	41.70	2.07	3	No	41.13	3.48	2	No
Iso+Stim	45.26	2.54	3	*	46.16	3.20	4	No	43.91	4.75	3	No

stimulation alone induced some increases of Egr-1 expression at 1h and 4h. However only at 8h, it induced a high value as 49.13% and resulted in a significant difference compared with control ($p<0.05$). At later time points, even though stimulation still cause some increase of Egr-1 expression, the value became decreased gradually. Isolation alone did not cause a significant increase of Egr-1 at each time point compared with control ($p<0.05$). However, interestingly, when isolation was combined with stimulation, the significant increase of Egr-1 protein expression appeared at 1d ($p<0.05$). Subsequently, it started to decrease a bit at 2d and 4d compared with control.

The distribution of Egr-1 expression in different sized neuronal populations showed that, as the percentage of immunostained small neurons in total immunostained neurons, Egr-1 protein was mainly expressed in small neurons and basal expression in small neurons was about 50% (data not shown).

Fos protein expression As shown in Fig. 9, Fos protein was predominantly localized in the nuclei of DRG neurons. The quantitative analysis of Fos expression in DRG neurons as the percentage of stained cells in total counted cells was done and the data were described in Fig. 10 and the attached table. Statistical analysis of stained neurons showed the basal expression of stained cells in total DRG cells was 22.92% to 32.06% (see table). Compared with control group, treatment groups were significantly different from it with the tested time periods (1h, 4h, 8h, 1d, 2d, and 4d) (MANOVA by glm, $p<0.05$). Based on the above

Figure 9. Immunostaining of Fos protein in DRG neurons. ICC was performed on frozen section as described in the text. Fos polyclonal antibody was from Oncogene Science. The nuclei of DRG neurons are darkly stained by Fos antibody. **A.**. Nuclear staining of Fos under control treatment, and **B.** Nuclear staining of Fos at 2d under isolation plus stimulation treatment. The arrow head point out the dark staining of the nuclei. Scale bar = 100 μm.

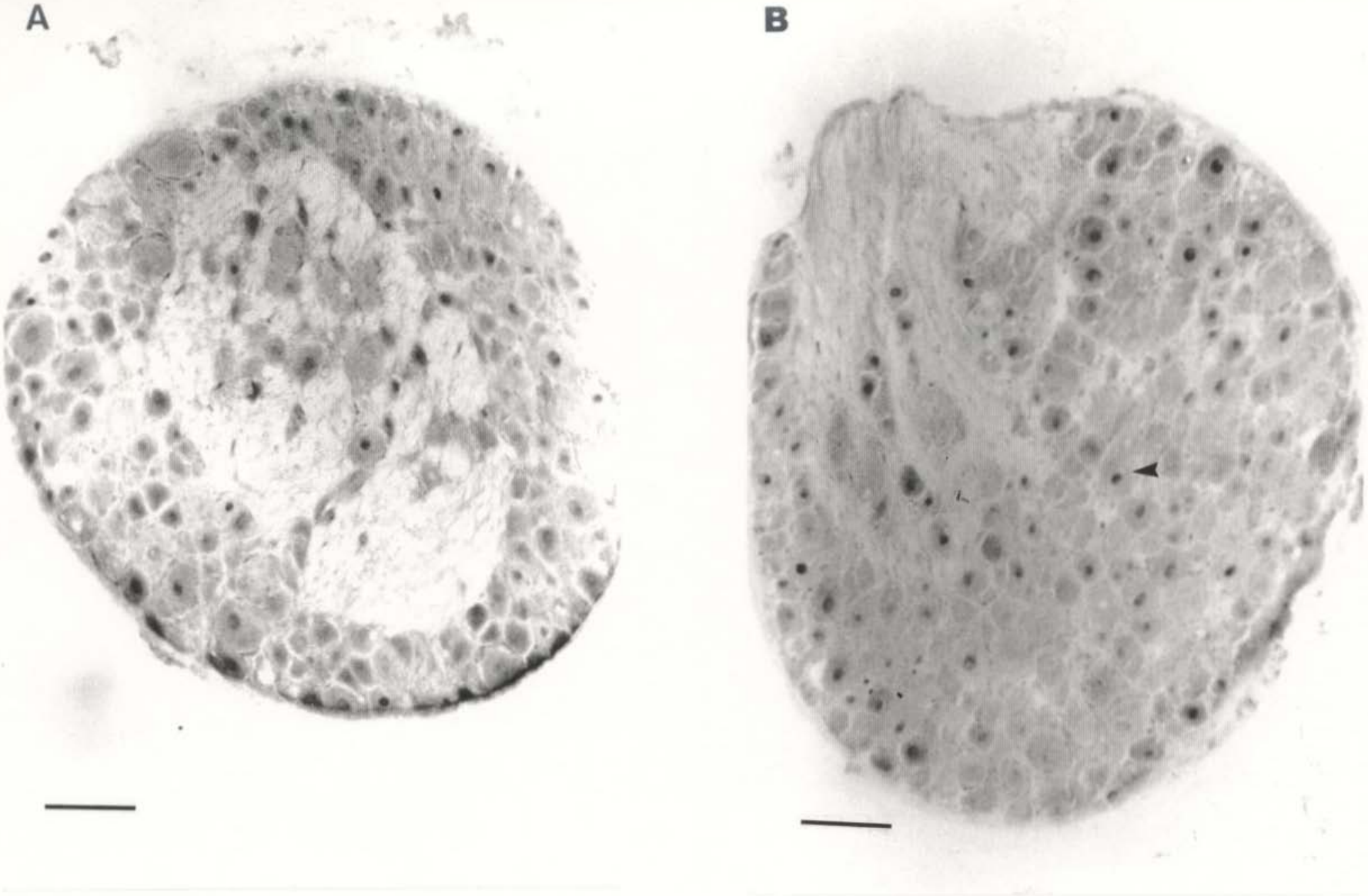

A
B

Figure 10. Analysis of the percentage of Fos immunostained neurons in total DRG neurons. The immunostained cells and total DRG cells per section were counted under the light microscope. On each slide, the 3-5 sections for control and each experimental treatment (stimulation, isolation and isolation plus stimulation) at different time points (1h, 4h, 8h, 1d, 2d and 4d) were analyzed. For each control or treatment per time point, one DRG was used per experiment. The cells containing stained nuclei and total cells in one DRG section were counted and the percentage of stained cells in total (%) was calculated. Thus, a given experimental series is the data from 1 DRG per treatment per time point, ie, a total of 24 DRGs per experiment from at least 12 rats. The plotted data represent the Mean of 2-4 experiments; the table presents the Mean , SEM, n value and statistical analysis. Statistical analysis was carried out using the following tests. MANOVA by glm was used to test group difference ($p<0.05$, significant difference) caused by treatments across the time points. Significant differences ($p<0.05$) between control and treatments at each time point were tested by oneway ANOVA with Dunnett's test (represented by * in the figure and table). The different treatments are presented as follows: control, solid dot; stimulation, open dot; isolation, solid triangle; isolation plus stimulation, open triangle.

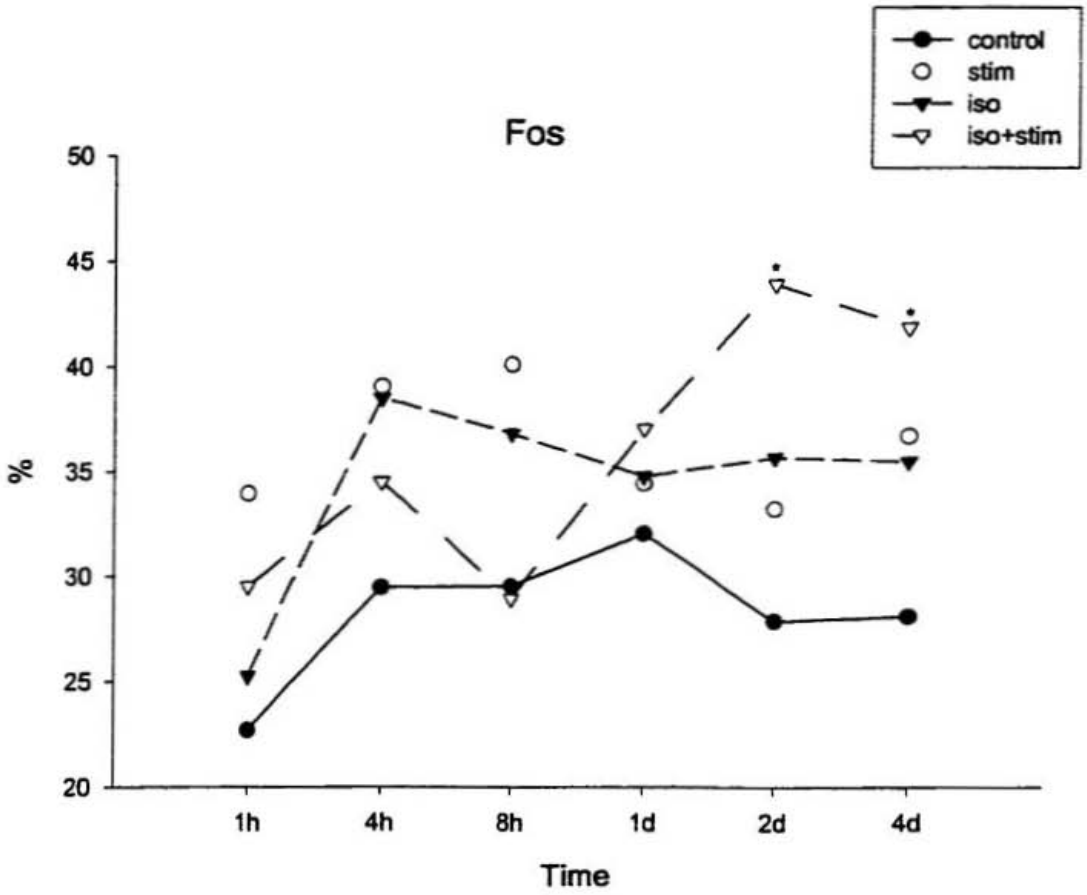


Table Immunocytochemistrical data for Fos (total stained cells in total counted cells, %)

Time	1h				4h				8h			
Treatment	Mean	SEM	n	Signif.	Mean	SEM	n	Signif.	Mean	SEM	n	Signif.
Control	22.77	1.64	3	No	29.48	3.81	3	No	29.53	5.35	3	No
Stim	33.94	4.93	3	No	39.06	5.68	4	No	40.13	1.83	2	No
Iso	25.25	0.25	2	No	38.50	1.50	2	No	36.82	3.19	2	No
Iso+Stim	29.50	1.00	2	No	34.50	0.50	2	No	28.94	7.07	2	No
Time	1d				2d				4d			
Treatment	Mean	SEM	n	Signif.	Mean	SEM	n	Signif.	Mean	SEM	n	Signif.
Control	32.06	4.08	3	No	27.87	3.62	3	No	28.11	1.86	3	No
Stim	34.44	5.44	2	No	33.25	1.25	2	No	36.75	0.25	2	No
Iso	34.77	1.91	2	No	35.70	2.83	2	No	35.52	2.52	2	No
Iso+Stim	37.05	1.45	2	No	43.95	0.70	2	*	41.90	3.40	2	*

results, onewayANOVA with Dunnett's t-test were further used to test the significant change of Fos at each single time point ($p<0.05$). The results showed that stimulation and isolation alone did not induce significant increase of Fos protein expression at all the tested time points ($p<0.05$). However, the combination of isolation and stimulation treatment resulted in the number of positive neurons reaching as high as 43.95% at 2d, and 41.90% at 4d respectively, significantly increased compared with control ($p<0.05$). In this case, the combination of stimulation and isolation induced a larger increase than individual stimulation and isolation treatments, indicating that the electrical stimulation and NGF had some overlapping effects on Fos protein expression.

Jun protein expression Like other IEGs, Jun protein was also present in the nucleus in DRG neurons (Fig.11). The expression of Jun was examined only in the later experiments, so at some time points, the data was not obtained (8h: stimulation, 1d: all the treatments). According to the statistical analysis, 28.00% to 32.50%of the total DRG neurons had a basal expression of Jun according to the data from control at 1h, 4h, 8h, 2d, and 4d (Fig. 12 and attached table). MANOVA by glm were performed to investigate the group differences. It was shown that all treatment groups (stimulation, isolation, isolation plus stimulation) were significantly different from control group with the tested time points ($p<0.05$). Based on the above results, oneway ANOVA with Dunnett's test were then used to test the significant differences between control and each treatment at each time point ($p<0.05$). It was shown

Figure 11. Immunostaining of Jun protein in DRG neurons. ICC was performed on frozen sections as described as the text. Jun polyclonal antibody was from Santa Cruz Biotech. The nuclei of DRG neurons are darkly stained by Jun antibody. **A.** Nuclear staining of Jun under control treatment, and **B.** Nuclear staining of Jun at 4d under isolation plus stimulation treatment. The arrow head point out the dark staining of the nuclei. Scale bar = 100 μm.

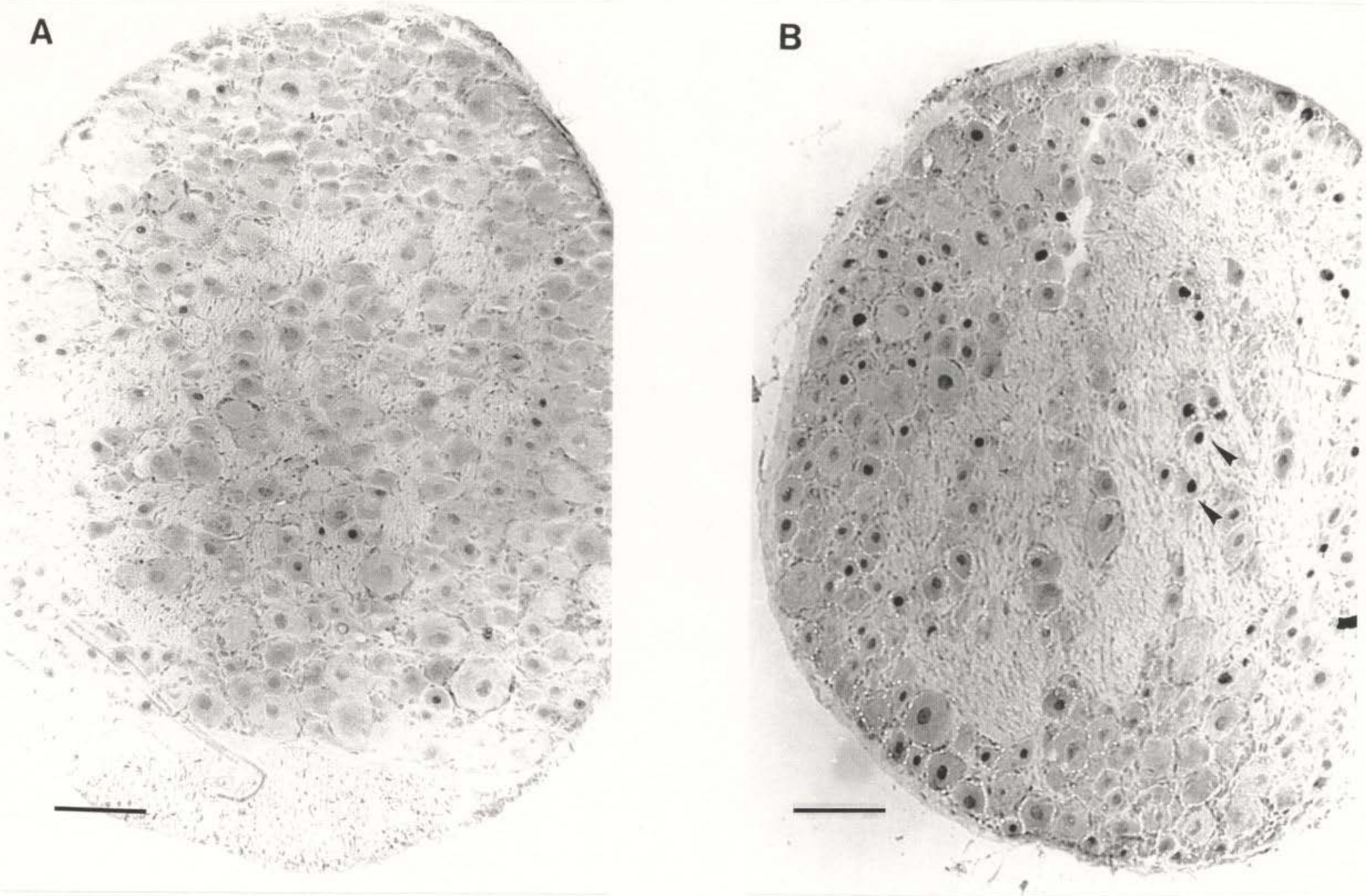

A
B

Figure 12. Analysis of the percentage of Jun immunostained neurons in total DRG neurons. The immunostained cells and total DRG cells per section were counted under the light microscope. On each slide, the 3-5 sections for control and each experimental treatment (stimulation, isolation and isolation plus stimulation) at different time points (1h, 4h, 8h, 2d and 4d) were analyzed. For each control or treatment per time point, one DRG was used per experiment. The cells containing stained nuclei and total cells in one DRG section were counted and the percentage of stained cells in total (%) was calculated. Thus, a given experimental series is the data from 1 DRG per treatment per time point, ie, a total of 20 DRGs per experiment from at least 10 rats. The plotted data represent the Mean of 2 experiments; the table presents the Mean , SEM, n value and statistical analysis. Statistical analysis was carried out using the following tests. MANOVA by glm was used to test group difference ($p<0.05$, significant difference) caused by treatments across the time points. Significant differences ($p<0.05$) between control and treatments at each time point were tested by oneway ANOVA with Dunnett's test (represented by * in the figure and table). The different treatments are presented as follows: control, solid dot; stimulation, open dot; isolation, solid triangle; isolation plus stimulation, open triangle.

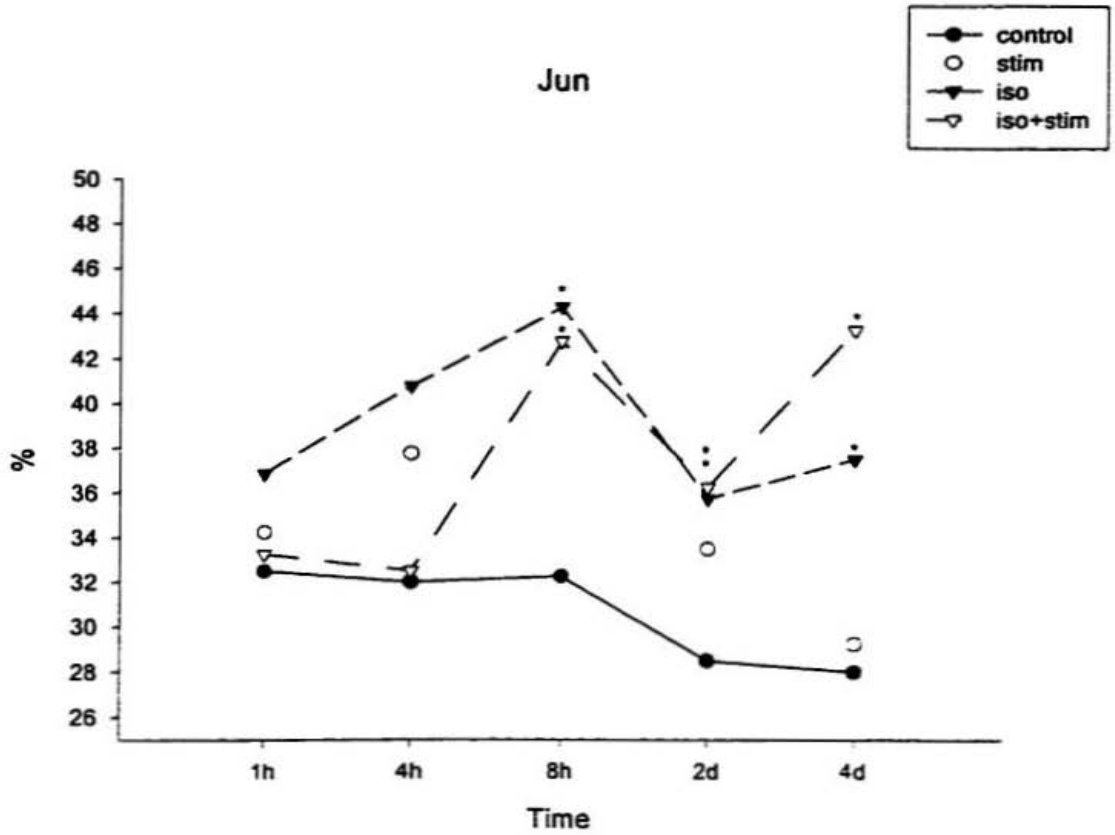


Table Immunocytochemistrical data for Jun (total stained cells in total counted cells, %)

Time	1h				4h				8h			
Treatment	Mea	SEM	n	Signif.	Mean	SEM	n	Signif.	Mean	SEM	n	Signif.
Control	32.5	3.00	2	No	32.00	0.00	2	No	35.25	1.75	2	No
Stim	34.2	1.25	2	No	37.75	0.25	2	No	—	—	2	No
Iso	36.8	0.85	2	No	40.75	1.25	2	No	44.25	0.25	2	*
Iso+Stim	33.2	0.75	2	No	32.50	4.50	2	No	42.75	1.75	2	*
Time	1d				2d				4d			
Treatment	Mea	SEM	n	Signif.	Mean	SEM	n	Signif.	Mean	SEM	n	Signif.
Control	—	—	—	—	28.50	0.50	2	No	28.00	1.00	2	No
Stim	—	—	—	—	33.50	1.00	2	No	29.25	0.75	2	No
Iso	—	—	—	—	35.75	1.75	2	*	37.50	1.00	2	*
Iso+Stim	—	—	—	—	36.25	2.25	2	*	43.25	1.25	2	*

in Fig.12 that the percentage of stained cells increased more or less at different time points compared with control. Stimulation alone did not cause large changes at the tested time points. Isolation alone caused significant increase of Jun protein compared with control as early as 8h ($p<0.05$), and this situation lasted until 4d. As the same as isolation alone, isolation plus stimulation induced significant increase of Jun expression as early as 8h ($p<0.05$), and this effect existed till 4d.

Oct-2 protein expression Oct-2 protein was localized in the nucleus and the representative example of nuclear staining at control and isolation plus stimulation treatment is provided in Fig.13. Unfortunately, from the data of Oct-2 (Fig.14), it seems that the results did not show any consistent pattern under control and the three treatments at the time points which were checked (1h, 4h, 8h, 1d, 2d and 4d) (data not shown). Therefore, further analysis was not performed.

3.1.2.2 Western blot analysis - Egr and pCREB

Western blot analysis was used to further examine the protein expression of Egr-1 and pCREB. HeLa cell and NIH3T3 cell lysates were used as positive controls, and Egr-1 protein was observed on the blots at the size of 87 KD in these samples. However, at different time points (1h, 4h, 8h, 1d, 2d, 4d), there were no obvious changes among control and other treatments for Egr-1 (data not shown).

Figure 13. Immunostaining of Oct-2 protein in DRG neurons. ICC was performed on frozen section as described in the text. Oct-2 polyclonal antibody was from Santa Cruz. The nuclei of DRG neurons are darkly stained by Oct-2 antibody. **A.** Nuclear staining of Oct-2 under control treatment, and **B.** Nuclear staining of Oct-2 at 2d under isolation plus stimulation treatment. The arrow head pointed out the dark staining of the nuclei. Scale bar = 100 μm.

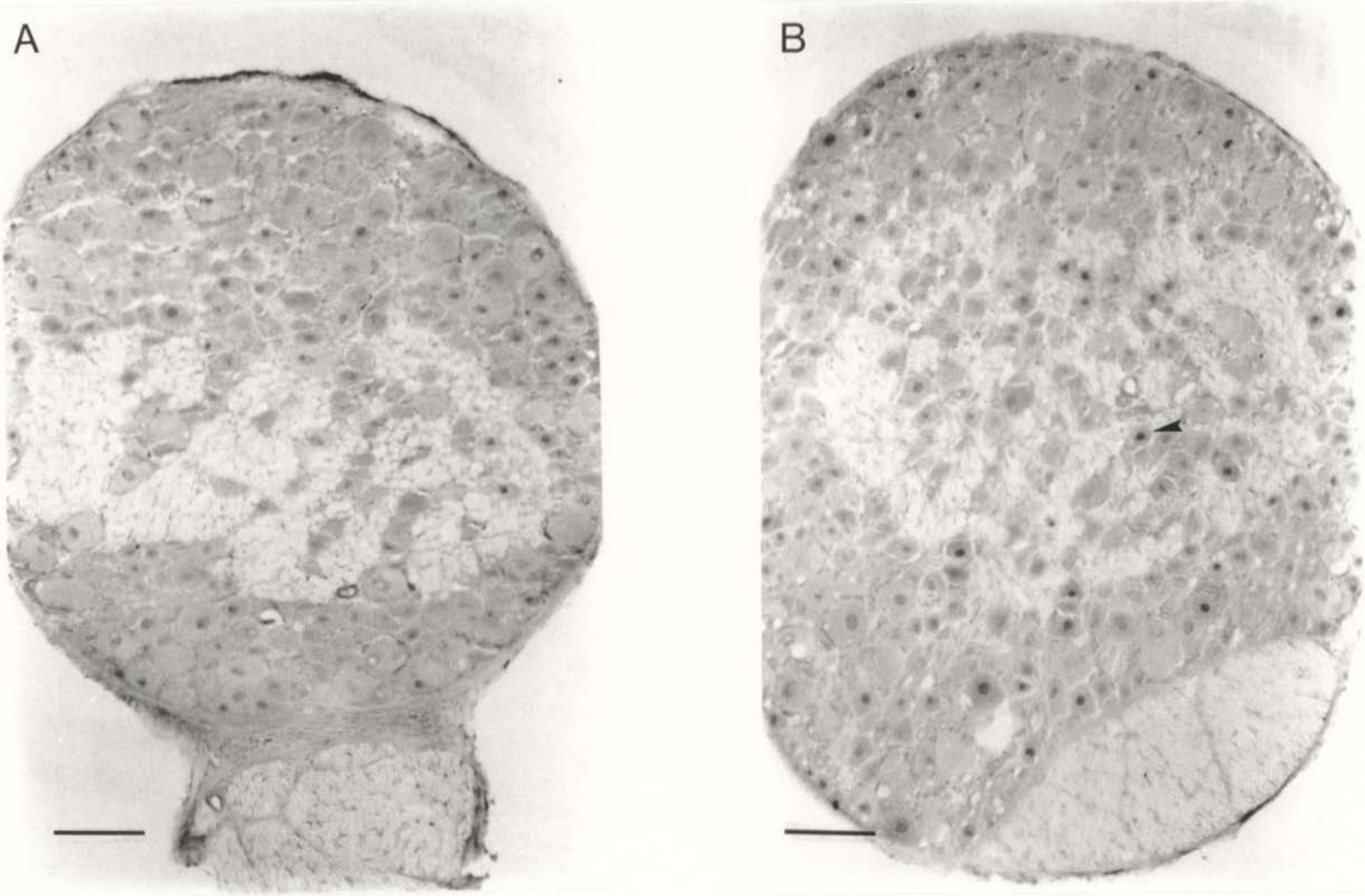

A
B

Figure 14. Analysis of the percentage of Oct-2 immunostained neurons in total DRG neurons. The immunostained cells and total DRG cells per section were counted under the light microscope. On each slide, the 3-5 sections for control and each experimental treatment (stimulation, isolation and isolation plus stimulation) at different time points (1h, 4h, 8h, 1d, 2d and 4d) were analyzed. For each control or treatment per time point, one DRG was used per experiment. The cells containing stained nuclei and total cells in one DRG section were counted and the percentage of stained cells in total (%) was calculated. Thus, a given experimental series is the data from 1 DRG per treatment per time point, ie, a total of 20 DRGs per experiment from at least 10 rats. The plotted data represent the Mean of 2 experiments; the table presents the Mean , SEM, n value and statistical analysis.

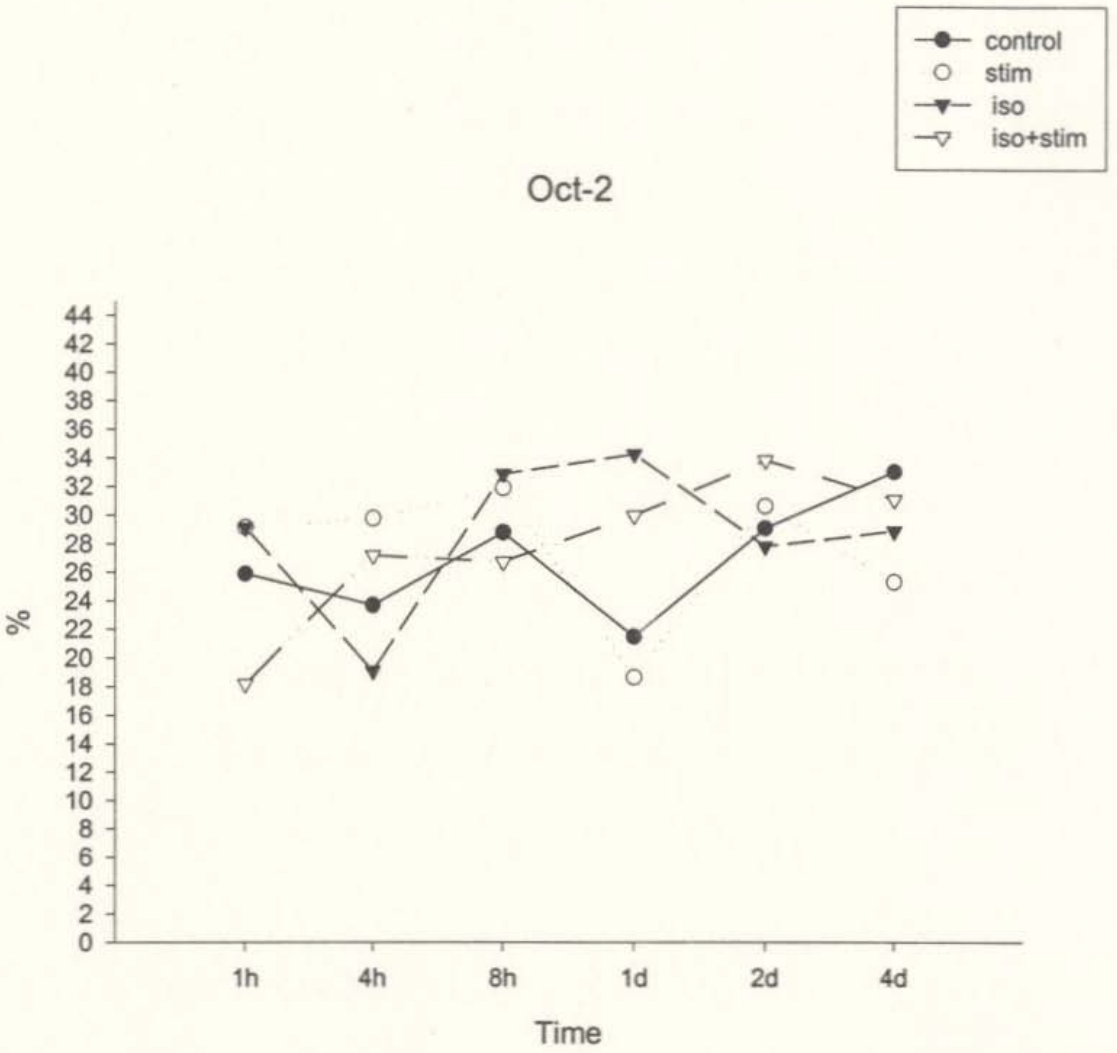


Table Immunocytochemistrical data for Oct-2 (total stained cells in total counted cells, %)

Time	1h				4h				8h			
Treatment	Mea	SEM	n	Signif.	Mean	SEM	n	Signif.	Mea	SEM	n	Signif.
Control	25.8	0.52	2	---	23.67	1.34	2	---	28.7	0.83	2	---
Stim	29.1	0.44	2	---	29.73	0.84	2	---	31.8	0.00	2	---
Iso	29.0	4.24	2	---	19.07	3.47	2	---	32.8	7.27	2	---
Iso+Stim	18.7	0.83	2	---	27.13	1.69	2	---	26.6	0.59	2	---
Time	1d				2d				4d			
Treatment	Mea	SEM	n	Signif.	Mean	SEM	n	Signif.	Mea	SEM	n	Signif.
Control	21.4	0.70	2	---	29.04	2.83	2	---	32.9	2.03	2	---
Stim	18.6	1.18	2	---	30.58	1.24	2	---	25.2	1.76	2	---
Iso	34.2	2.90	2	---	27.76	0.30	2	---	28.8	1.64	2	---
Iso+Stim	29.9	0.84	2	---	33.79	0.86	2	---	31.0	1.33	2	---

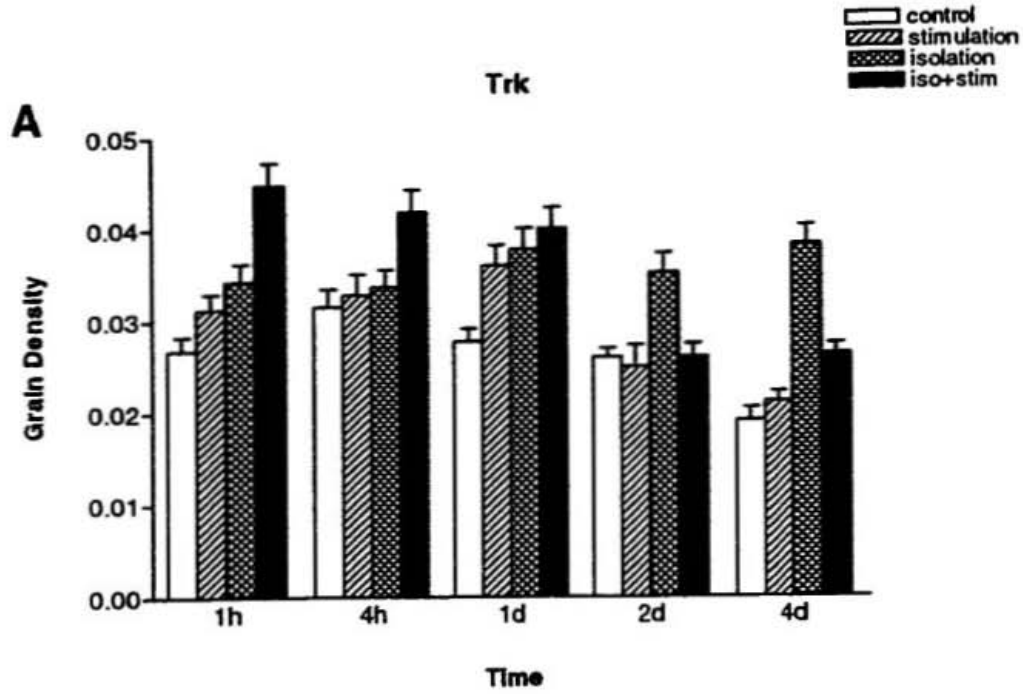
The size of pCREB was 43 KD. Hela cell lysates showed a strong band at 86 KD and a band at 43 KD for pCREB-probed blots. But for the DRG lysates only faint 86 KD bands appeared and the 43 KD band was not detectable. This is probably due to the relatively low amounts of phosphorylated CREB in the samples, and the possibility is that most of the pCREB was present in a dimerized form.

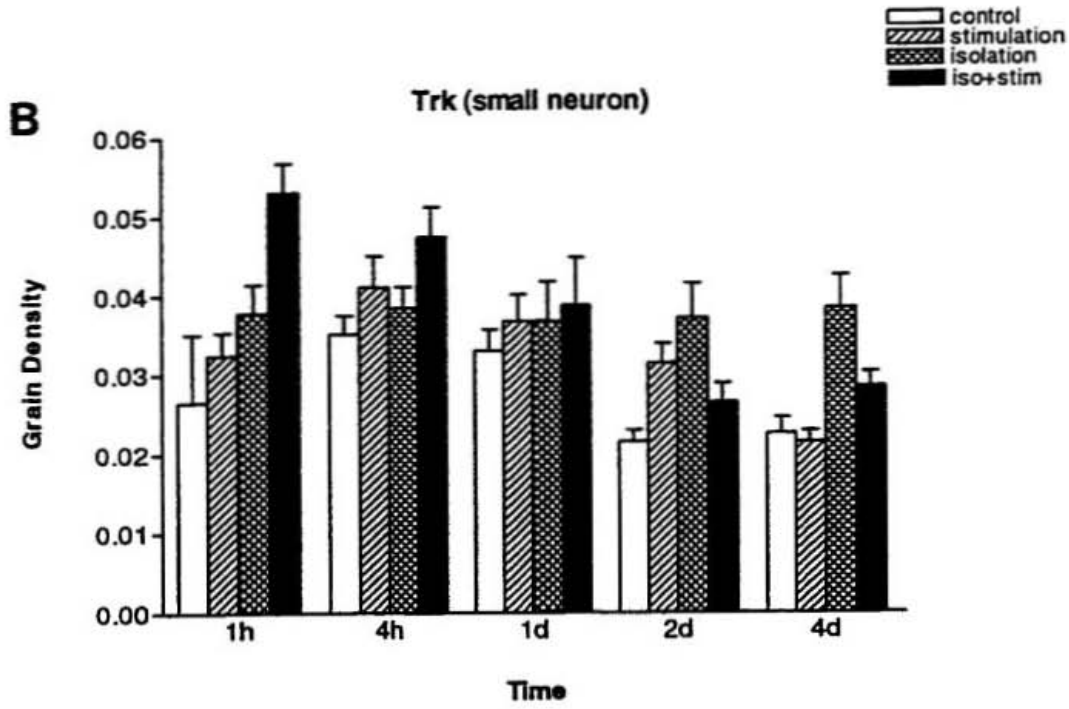
3.2 The expression of mRNAs and proteins of NGF receptors

The mRNA expression of the NGF receptors, Trk A and p75, was analyzed on frozen sections by ISH after stimulation, isolation, and isolation plus stimulation treatments. In addition, the Trk A receptor protein and its phosphorylation state were detected by immunoprecipitation and western blot analysis.

3.2.1 Trk A and p75 mRNA expression detected by *In Situ* hybridization The grain densities of Trk A receptor mRNA at 1h, 4h, 1d, 2d and 4d under stimulation, isolation, isolation plus stimulation treatments are shown in Fig. 15A. While stimulation alone appears to cause little change in Trk A receptor mRNA, both isolation and isolation plus stimulation produced large changes. With isolation alone the grain density significantly increased (ANOVA, $p< 0.05$) after 1d and lasted to 4d. Isolation plus stimulation induced a significant increase in the grain density of Trk A as early as 1h ($p < 0.05$), but then decreased to control level at 2d. Further analysis of grain density with respect to neuronal size classification

Figure 15. Expression of Trk A receptor mRNA in DRG neurons. *In situ* hybridization was performed on frozen sections of DRGs from animals at 1h, 4h, 1d, 2d, and 4d after stimulation and isolation plus stimulation paradigms as described in methods. **A.** The grain density of Trk A mRNA on the total neuronal population at control and each treatment /time point. **B.** The grain density of TrkA mRNA in small neurons at each control and treatment/time point. Significant differences between control and each treatment at different time points were analyzed by Oneway ANOVA plus student's t-test on Minitab program ($p<0.05$). The different treatments are represented as: control (empty bar), stimulation (hatched bar), isolation (cross-hatched bar) and isolation plus stimulation (solid bar). Each bar represents the analysis of 100 neurons from 1 DRG per treatment.


control
stimulation
isolation
iso+stim
Trk
A
Grain Density
0.05
0.04
0.03
0.02
0.01
0.00
1h
4h
1d
2d
4d
Time


control
stimulation
isolation
iso+stim
Trk (small neuron)
B
Grain Density
0.06
0.05
0.04
0.03
0.02
0.01
0.00
1h
4h
1d
2d
4d
Time

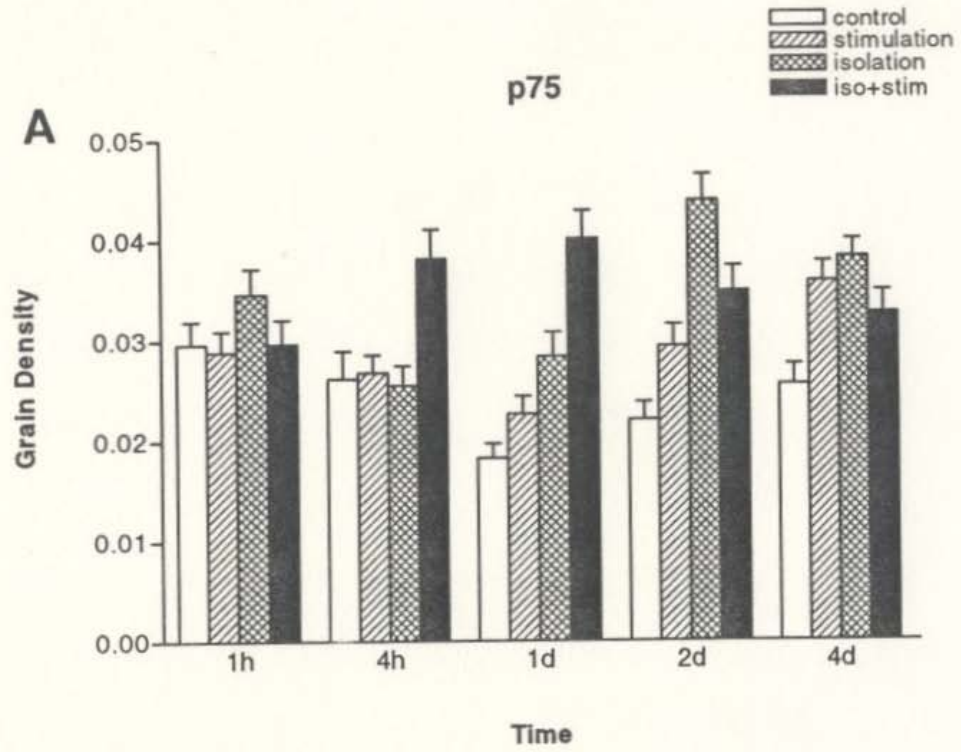
indicated that the expression of Trk A in small neurons presented a similar pattern with that seen in the total neuron population, suggesting that the changes were taking place mainly in small neurons (Fig.15B).

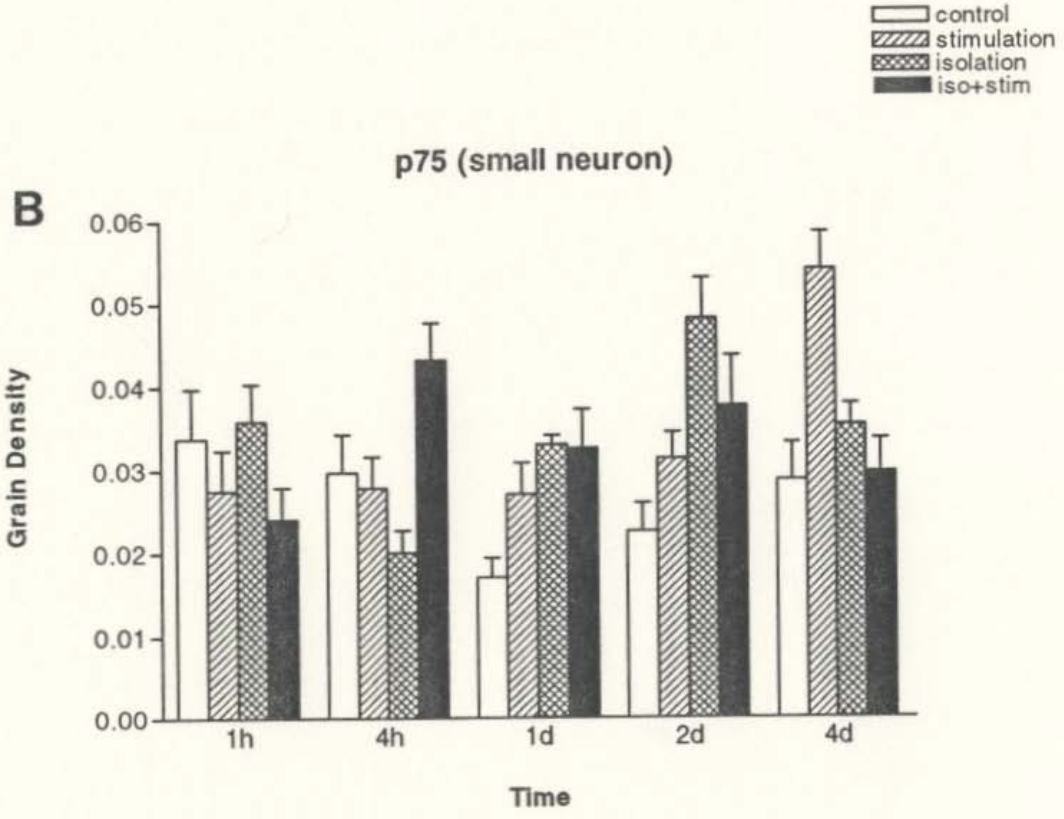
As the low affinity of NGF receptor, the mRNA expression of p75 was also investigated. The grain density of this receptor at 1h, 4h, 1d, 2d and 4d is shown in Fig.16. Stimulation alone did not cause a significant elevation of p75 mRNA over time. With isolation alone, the significant change in grain density was observed at the 2d and 4d time points. The increase of p75 expression with isolation plus stimulation was significant from 4h to 2d. In small size DRG neurons, the expression was similar, although there was more variability in the results. This might reflect the fact that the intensity of the ISH signal was much greater over the small neurons, and was more difficult to assess accurately with the image analysis program used.

3.2.2 Immunoprecipitation and Western analysis of Trk A expression and phosphorylation state

To visualize the Trk A receptor and its phosphorylated form, immunoprecipitation was performed firstly by using a Trk A antibody, and then the Western blots were probed sequentially with Trk A and anti-phosphotyrosine. Lysates from spinal cord treated *in vitro* with NGF were used as a positive control. The Trk A receptor has a molecular weight of 140 KD, as seen in the Trk A blot.. The blot probed with anti-phosphotyrosine also showed

Figure 16. Expression of p75 receptor mRNA in DRG neurons. *In situ* hybridization was performed on frozen sections of DRGs from animals at 1h, 4h, 1d, 2d, and 4d after stimulation and isolation plus stimulation paradigms as described in methods. **A.** The grain density of p75 mRNA on the total neuronal population at control and each treatment /time point. **B**. The grain density of p75 mRNA in small neurons at each control and treatment/time point. Significant differences between control and each treatment at different time points were analyzed by Oneway ANOVA plus student's t-test on Minitab program ($p<0.05$). The different treatments are represented as: control (empty bar), stimulation (hatched bar), isolation (cross-hatched bar) and isolation plus stimulation (solid bar). Each bar represents the analysis of 100 neurons from 1 DRG per treatment.


control
stimulation
isolation
iso+stim
p75
A
0.05
0.04
0.03
0.02
0.01
0.00
Grain Density
1h
4h
1d
2d
4d
Time


control
stimulation
isolation
iso+stim
p75 (small neuron)
B
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Grain Density
1h
4h
1d
2d
4d
Time

a 140 KD band, indicating that this was phosphorylated Trk A (data not shown).

To test the potential change in Trk A receptor expression and activity, lysates were extracted from DRG neurons from control, stimulation, isolation, isolation plus stimulation at 1h, 4h, 2d, 4d and 8d. Protein concentrations were determined and aliquots of equivalent amounts of protein of the lysates were electrophoresed and transferred to nitrocellulose filters. Western blots were probed with Trk A antibody (RTA) or an anti-phosphotyrosine antibody. Experiments were conducted in triplicate. The results of a representative Western blot of Trk A are shown in Fig. 17. In panel 1, compared with the Trk A band in control lane corresponding to 140 KD, there appeared to be a slight increase in the density of bands, especially at 1h and 4h stimulation time points. In panel 2, there seems to be increased density of Trk A bands (relative to control) were presented at 4d and 8d isolation and isolation plus stimulation time points. The samples in panel 1 were run on the same gel; samples in panel 2 were electrophoresed on 2 separate gels - although in the same electrophoretic run. This representative blot was the final one run on the last of the available samples; while the blots of the 2d and 4d samples are not optimal, they were included as representative of the series.

The representative results of phosphotyrosine expression from Western blot are shown in Fig.18. There were no major differences in the patterns of Trk A proteins phosphorylated on tyrosine under any of the conditions, except perhaps at the 1h time point and for the isolation condition.

Figure 17. Western blot of Trk A receptor expression in DRG neurons. Treated (stim, iso, iso+stim) and control DRGs were removed at 1h, 4h, 2d, 4d, and 8d. DRGs were homogenized in lysis buffer. Lysate was extracted with lysis buffer and protein concentration was measured by protein assay kit (Bio-Rad). 10 μg of protein was subjected to 10 % SDS PAGE, and transferred to ECL nitrocellulose membrane (Amersham). After blocking with 5 % skim milk, the blot was probed with Trk antibody (RTA). The binding of this antibody was detected with ECL kit (Amersham). The size of Trk A receptor is 140 KD. Panel 1 were samples from 1h and 4h. Panel 2 were samples from 2d, 4d and 8d. **C** - control; **S** - stimulation; **I** - isolation; **I +S** - isolation plus stimulation.

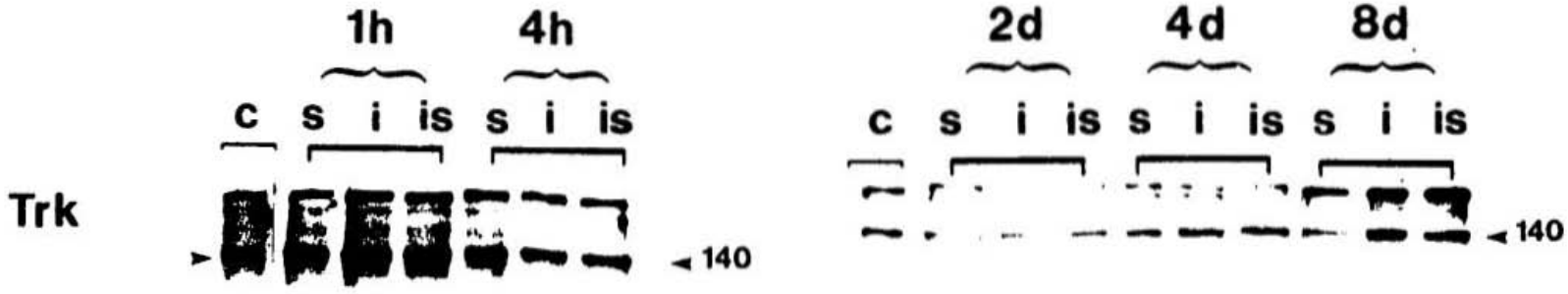
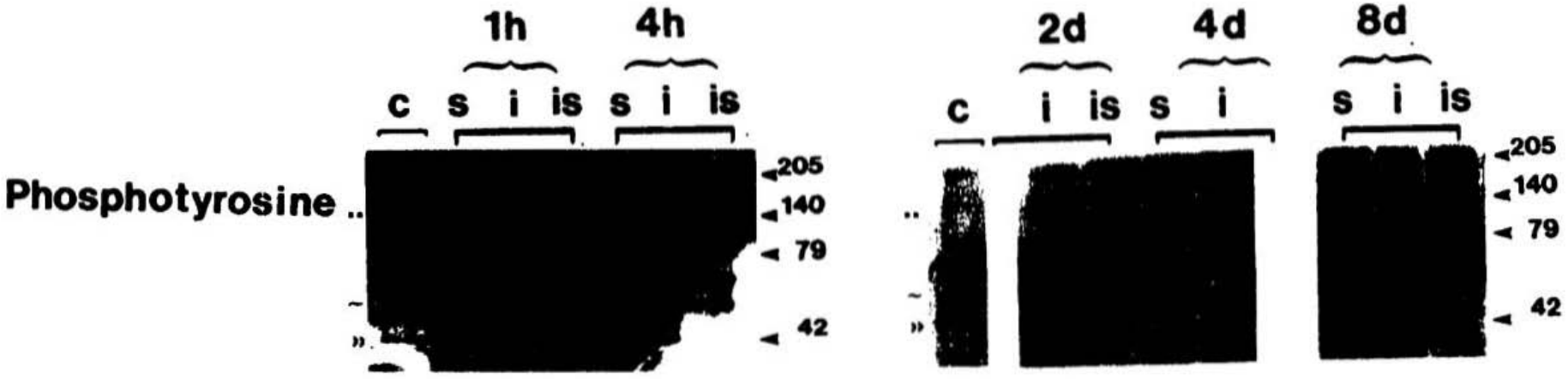

1h
4h
2d
4d
8d
c s i is s i is
c s i is s i is s i is
Trk
140
140

Figure 18. Western blot of phosphotyrosine of Trk A in DRG neurons. Experimental procedure was as described in Fig.17. Phosphotyrosine antibody was from Upstate Biotechnology Incorp. Panel 1 shows samples from 1h and 4h. Panel 2 shows samples from 2d, 4d and 8d. **C** - control; **S** - stimulation; **I** - isolation; **I +S** - isolation plus stimulation. There was a band around 140 KD and might be the phosphorylated Trk A tyrosine kinase. The markers on the left side indicate the possible location of several protein molecules: .. (phosphorylated Trk A); ~ (phosphorylated SHC); » (phosphorylated ERK)


Phosphotyrosine
1h
4h
2d
4d
8d
c
s
i
is
205
140
79
42

3.3 Expression of proteins related to NGF receptor mediated pathways

The binding of NGF to Trk A receptor stimulates the intrinsic tyrosine kinase activity of this receptor, initiating the autophosphorylation of this receptor on several sites (tyrosine residues). There are several cytoplasmic proteins which associate with the Trk A receptor after activation, and subsequently activate downstream signaling cascades (eg. Kaplan and Stephens, 1994). As part of the experiment to investigate the potential contribution of Trk activation and subsequent cellular signaling to collateral sprouting, preliminary experiments to examine the presence of these protein were carried out. The same Western blots were probed sequentially with antibodies to PLC-γ1 and, PI-3 kinase (p85), SHC, and MAP kinase (ERK1).

PLC-γ1 The molecular weight of the PLC-γ1 protein is 145 KD. The expression of this protein at 1h, 4h, 2d, 4d and 8d under four conditions were shown in Fig. 19. There were some small increases in expression of PLC-γ1 (compared to control) detected at the early time points, although at 8d there seems to be greater increase in PLC-γ1.

PI-3 The antibody used recognizes the 85 KD subunit of PI-3 kinase (Fig. 20). Compared to the control sample, there was an increased expression of PI-3 kinase in the experimental samples, particularly at 8d. There was little difference between the different experimental conditions.

Figure 19. Western blot of PLC-γ1 in DRG neurons. Experimental procedure was as described in Fig.17. PLC-γ1 was from Santa Cruz Biotech. The size of PLC-γ1 on the blot is 145 KD. Panel 1 shows samples from 1h and 4h. Panel 2 shows samples from 2d, 4d and 8d. **C** - control; **S** - stimulation; **I** - isolation; **I +S** - isolation plus stimulation.

PLC-γ1

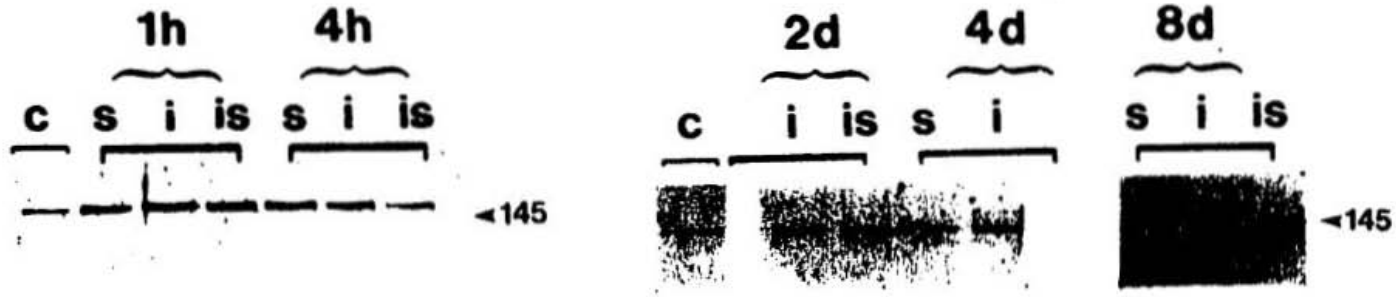
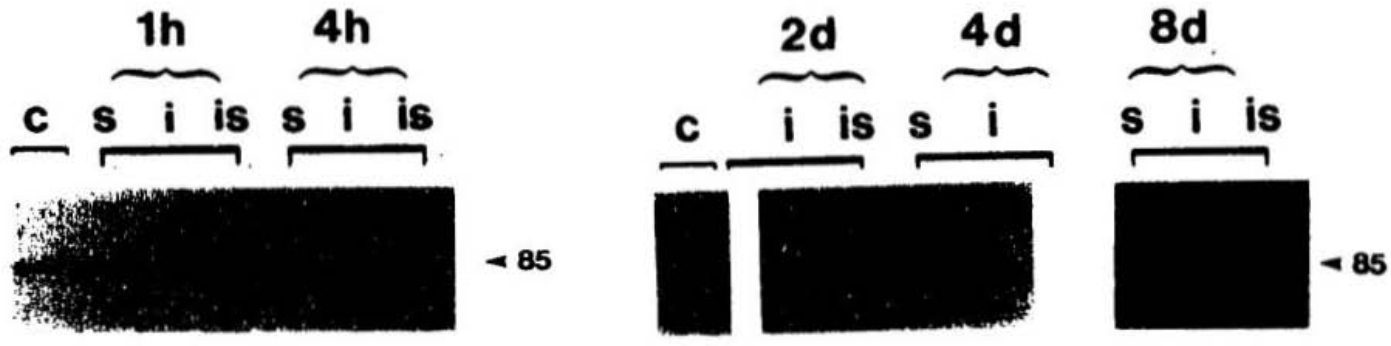


Figure 20. Western blot of PI-3 kinase in DRG neurons. Experimental procedure was as described in Fig.17. PI-3 kinase antibody was from Upstate Biotechnology Incorp. The size of PI-3 kinase on the blot is 85 KD. Panel 1 shows samples from 1h and 4h. Panel 2 shows samples from 2d, 4d and 8d. **C** - control; **S** - stimulation; **I** - isolation; **I +S** - isolation plus stimulation.



PI.3-kinase

SHC When the blot was probed with the SHC antibody, three bands appeared on the blot, 66 KD, 52 KD and 46 KD (as expected) (Fig.21). However, the 52 KD bands was always more strongly expressed in DRG lysates. Under the four conditions of treatments, there were no obvious differences in SHC signals at the time points examined.

MAP kinase (ERK) The antibody recognizes two isoforms of MAP Kinase (ERK), ERK1 (44 KD) and ERK2 (42 KD). The Western blot results with ERK are shown in Fig. 22. Under all conditions investigated and at all time points, there was a strong expression of both isoforms, but there appeared to be only slight difference among the different treatments and time points. There does seem to be an increase in the expression of the p44 isform at later time points.

It is possible that the above results may be due to large differences in the amount of protein loaded per lane, however, this is not likely to be the case, since the PI-3 kinase and PLC-γ1 results are from the same blot probed sequentially, and the SHC and MAPK results are from another blot probed sequentially. If, for example, the large differences in the 8d expression of MAPK compared to the 2d experiment were due to loading differences, we would expect to see similar magnitudes of differences with the anti-SHC blot.

These proteins are also phosphorylated as part of their activation by NGF binding to Trk A. The Fig. 18 shows a series of bands identified by the phosphotyrosine antibody. The bands corresponding in size to those discussed above can be seen. However, there appears to be little change with the different times and treatments.

Figure 21. Western blot of SHC in DRG neurons. Experimental procedure was as described in Fig.17. SHC antibody was purchased from Upstate Biotechnology Incorp. There were three SHC bands showed up on the blot: 66 KD, 52 KD and 46 KD. Panel 1 shows samples from 1h and 4h. Panel 2 shows samples from 2d, 4d and 8d. **C** - control; **S** - stimulation; **I** - isolation; **I +S** - isolation plus stimulation.

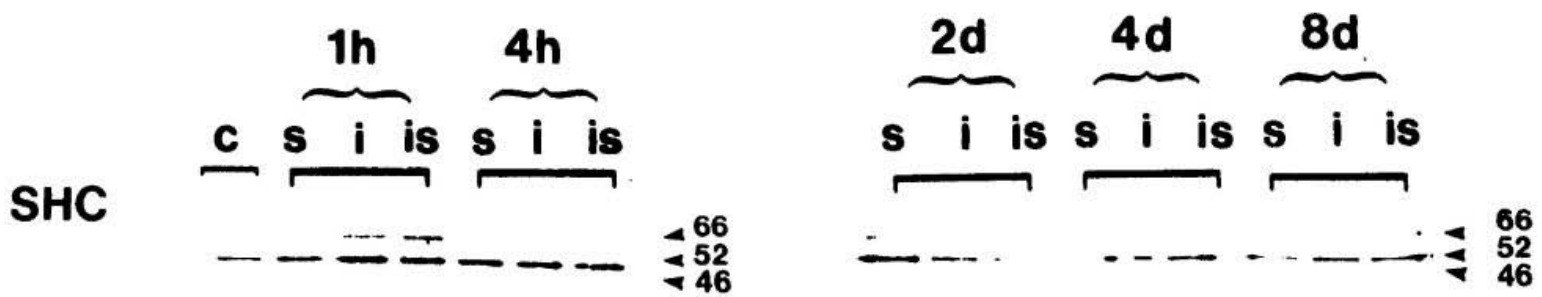
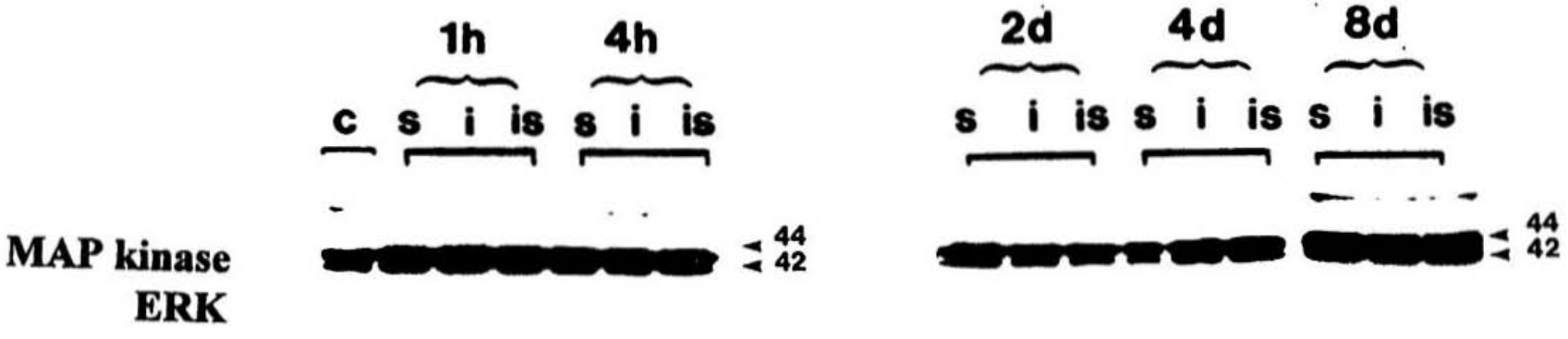

SHC
1h
4h
c
s
i
is
s
i
is
66
52
46
2d
4d
8d
s
i
is
s
i
is
s
i
is
66
52
46

Figure 22. Western blot of ERK-1 in DRG neurons. Experimental procedure was as described in Fig.17. ERK-1 antibody was from Santa Cruz Biotech. The sizes of ERK-1 on the blot were 44 KD and 42 KD. Panel 1 shows samples from 1h and 4h. Panel 2 shows samples from 2d, 4d and 8d. **C** - control; **S** - stimulation; **I** - isolation; **I +S** - isolation plus stimulation.


1h
4h
2d
4d
8d
c
s
i
is
s
i
is
s
i
is
s
i
is
s
i
is
MAP kinase
ERK
44
42
44
42

Attempts to carry out experiments where specific proteins were immunoprecipitated and then the immunoprecipitates probed with anti-phosphotyrosine were not entirely successful due to the limited amount of both sample and antibodies, as well as a problem with the particular anti-phosphotyrosine antibody used. This is also the reason why the blots in Fig.19-22 were not re-probed with anti-phosphotyrosine.

3.4 Summary

The results of this study can be summarized as follows. CREB mRNA was quickly induced after 1h electrical stimulation, and this increase lasted to 4d. The effect of isolation started at 1d, and the combination of isolation plus stimulation resulted in this occurring sooner. At the protein level, compared with control, the expression of pCREB protein was only significantly increased under stimulation at 8h ($p<0.05$), not under other treatments at other time points.

Electrical stimulation started to induce the elevation of egr-1 mRNA levels after 4h and this induction lasted until 2d for mRNA; for protein, compared with control, only at 8h, the significant elevation were seen. Isolation, which would cause the increase of NGF level in skin (Mearow *et al.*, 1993) did not induce significant increase of Egr-1 protein expression in the nucleus during the experimental time period. However, isolation plus stimulation induced a significant increase of Egr-1 protein after 1d and this increase lasted to 4d. Therefore, isolation plus stimulation not only shortened the duration of egr-1 increase, but

also caused a long time increase of this gene.

Compared with control, stimulation or isolation alone did not affect the expression of c-Fos, However, at 2d and 4d, isolation plus stimulation respectively induced significant increase of Fos.

Stimulation alone did not have significant affects on Jun protein level, however, from 8h, isolation and isolation plus stimulation respectively caused significant increases in Jun protein level compared with control.

Under the treatments of electrical stimulation and isolation, the change of Trk A receptor mRNA and protein showed different patterns in these experiments. The mRNA level of Trk A did not significantly increase after electrical stimulation; however, isolation alone resulted in a significant increase of TrkA mRNA and this increase reached a peak at 4d (2.0X relative to control). Combined with electrical stimulation, isolation induced a large increase at very early time point (1h), but the increase gradually decreased at late time points (2d and 4d).

The protein level of Trk A was only increased at 1h and 4h stimulation time points. The increase induced by isolation plus stimulation only showed up at the late time points (4d and 8d). The phosphorylation state of Trk A receptor did not appear to be increased except after the isolation treatment at 1h and at the longer time points of 4d and 8d.

p75 mRNA level was altered little by electrical stimulation. Isolation alone induced a peak change at 2d. The combination of these two factors resulted in increased expression of p75 by 4h, and their effects was largest at 1d, and then gradually decreased.

Among the proteins which propagate NGF signals, PLC-γ1 was slightly induced by stimulation and isolation at the earlier time period (1h, 4h) and at the very late time point (8d). PI-3 kinase was increased only at the late time point (8d) after these three treatments. SHC and MAP kinase (ERK1) were not obviously affected by any of these treatments.

Chapter IV

DISCUSSION AND FUTURE DIRECTIONS

4.1 The significance of this study

The phenomenon of precocious sprouting in sensory neurons was first recognized by Nixon *et al.*, (1984) and Doucette and Diamond (1987). In order to test the recovery of responsiveness in rat skin after denervation, they periodically pinched or hot probed the skin (i.e, testing the skin with a warm (60^0C) metal probe) and found that this testing resulted in an earlier development of the recovery. Electrical stimulation in their experiments mimicked the effects induced by pinching or hot probing. However, when TTX was used to block the propagation of electrical impulse from the peripheral processes to the cell bodies, this precocious sprouting did not occur. This suggested that impulses produced by electrical stimulation were transmitted to the DRG neurons first, and somehow induced some changes in the responsive neurons and produced the accelerated sprouting of the nerve terminals (Nixon *et al.*, 1984; Doucette and Diamond, 1987). Further studies showed that this precocious sprouting is an NGF-dependent process (Diamond *et al.*, 1987; Diamond *et al.*, 1992a) because this process did not occur in the absence of NGF. Studies in adult rats indicated that when electrical stimulation was performed prior to or just at the same time of denervation of the dorsal cutaneus nerves, the onset of collateral sprouting was earlier than

in untreated animals (Doucette and Diamond; 1987; Diamond *et al.*, 1992 a, b). Therefore, it seems that the effects of electrical stimulation act to reduce the latency of sensory terminal collateral sprouting which is physiologically detectable in the skin. However, what sorts of factors regulate this interesting phenomenon is still a mystery. My study firstly investigated the possible elements, related to this process at the molecular level, in particular, whether electrical stimulation might activate certain IEGs or elevate Trk A receptor activity to act in concert with the increased availability of NGF to result in an accelerated collateral sprouting response. This study will help to understand the mechanism of this collateral sprouting.

4.2 The role of immediate early genes (IEGs) in precocious sprouting in DRGs

Some IEGs, as transcription factors, regulate downstream target genes by largely unknown mechanisms, and cause long lasting biological effects. For example, IEGs have been shown to be activated by a variety of extracellular stimuli such as depolarization, neurotrophins, light and so on (e.g., Bartel *et al.*, 1989; Herdegen *et al.*, 1991; Taylor *et al.*, 1993; Ginty *et al.*, 1993; Kendall *et al.*, 1995). Electrical stimulation, which depolarizes the nerve membrane and is similar to the depolarization by KCl, can also regulate certain gene expression (e.g. Sukhatme *et al.*, 1988; Bartel *et al.*, 1989; Sheng *et al.*, 1990; Lu *et al.*, 1991; Sheng *et al.*, 1993; Yoon and Lau, 1994) and control growth, differentiation and sprouting of many cell types (Saffen *et al.*, 1988; Bartel *et al.*, 1989; Manivannan and

Terakawa, 1994; Solem *et al.*, 1995). One possible common pathway activated by these different stimuli may be regulated by depolarization influenced cyclic AMP levels (rev. in Ghosh and Greenberg, 1995). For example, elevation of cAMP levels induces the translocation of the cAMP response element binding protein (CREB) from the cytoplasm to the nucleus, where the phosphorylated form of CREB (pCREB) binds to a cAMP response element (CRE)-like sequence in DNA (Fisch *et al.*, 1989; Sheng *et al.*, 1988, 1990). In the promoter regions of certain immediate early genes, such as c-fos, c-jun and egr-1, CRE elements have been described (eg., Angel *et al.*, 1988; Christy *et al.*, 1988; Changelian *et al.*, 1989; Fisch *et al.*, 1989). Thus, the expression of these genes may be regulated by CREB after depolarization of membranes. Therefore, if electrical stimulation causes some changes in IEG expression or induction, CREB might be the one mediating the effects of electrical stimulation, and the first gene to be activated during this process. Besides depolarization, NGF is another factor regulating IEG expression. Among IEGs, c-fos (e.g. Greenberg *et al.*, 1985; Kruijer *et al.*, 1985; Bartel *et al.*, 1989; Buckmaster *et al.*, 1991), egr-1 (Milbrandt, 1987; Bartel *et al.*, 1989; Cao *et al.*, 1990; Kendall *et al.*, 1994), c-jun (Bartel *et al.*, 1989) and Oct-2 (Kendall *et al.*, 1995) are the examples of genes regulated by NGF in neurons. Since electrical stimulation and NGF are the factors related to precocious sprouting in my study, the IEGs which can be activated by these two factors will be discussed in the following sections.

CREB CREB, as one of the members of a Ca^{2+}-mediated transduction pathway, is

expressed in many cell types (eg. Sheng *et al.*, 1990, 1991; Ruppert *et al.*, 1992; Imaki *et al.*, 1994; Konradi and Heckers, 1995; Shiromani *et al.*, 1995) and activated by many factors, such as depolarization (Sheng *et al.*, 1990), NGF (Ginty *et al.*, 1994), and light (Ginty *et al.*, 1993). Previous studies showed that CREB-immunolabeled neurons were found in lumbar DRGs of untreated rats; this basal expression exhibited a high variability which ranged from absence to weak labeling of around 8% of all counted cells (Herdegen *et al.*, 1992).

In my study, since CREB exerts its function subsequent to phosphorylation on Ser^{133} (eg. Sheng *et al.*, 1990), an antibody against phosphorylated CREB, pCREB (Ginty *et al.*, 1993) was used to reflect the activity of CREB in DRG neurons. My results found that pCREB presented a basal expression in the nuclei of DRG neurons (Fig. 5), and that the percentage of total immuno-stained cells at the basal level was from 20.63 % to 30.47% depending on different DRGs (Fig.6), higher than that in the previous studies. The possible reasons which might account for the difference between my study and the former studies may be as follows: firstly, it may be related to the localization of DRG neurons. The DRGs examined in my study were from thoracic (T10-T13) DRGs, but the DRGs in the former studies were from lumbar levels. The lumbar DRGs have a greater total number of neurons than thoracic DRGs (10 - 12,000 vs 6 - 8, 000), and also have a higher representation of low threshold and limb propriceptive afferents, which may influence the proportion of labeled cells; secondly, it may be possibly due to the difference in antibodies. The titer of the antibody might be a factor to influence the binding of antibody to the epitope. In my study,

the antibody specificity was tested by adding pCREB control peptide into the optimal concentration of pCREB antibody to block its function. In these control situations, staining was abolished, therefore, my results should not be due to the non-specific staining of the neurons.

The change of CREB mRNA and protein under noxious stimulation in *in vivo* DRG neurons has not been previously investigated. In my study, the effects of electrical stimulation on this gene in DRG neurons were studied *in vivo* for the first time. Interestingly, it was found that electrical stimulation induced the increase of mRNA for CREB expression as early as 1h (see Fig. 3A) and the mRNA level of CREB expression reached a peak at 1d. However, for the pCREB protein, compared with control at each time point, electrical stimulation only induced significant increase of its expression at 8h ($p<0.05$), later and shorter than the increase of mRNA level (Fig. 6). However, this apparent discrepancy is reasonable since the mRNA levels reflect the amount of CREB mRNA in each neuron, while the protein level reflects the number of neurons that were PCREB positive. Furthermore, protein expression was that of the activated form of CREB, while the ISH examined total CREB message.

Isolation alone did not have effects on the expression of pCREB protein in my study. Previous studies indicated that an increase in NGF mRNA level was detected in skin 2d after denervation (Mearow *et al.*, 1993). According to my results, isolation alone did not result in significant changes of pCREB protein at later time point ($p<0.05$) (Fig.6). The reason for

this might be that since these neurons are normally expressed to low levels of NGF, an increase in cutaneous NGF did not furher induce the change of pCREB.

The change of pCREB was further detected by the cooperative effects of electrical stimulation and isolation treatments. Under the influence of electrical stimulation and isolation, while pCREB expression was elevated compared to control, the increases were not significant ($p<0.05$).

In DRG neurons, although CREB mRNA and protein were observed in all sizes of neurons, pCREB expression was most marked in small-size neurons (65.6%) (data not shown); the CREB mRNA expression profile in small neurons was similar to that in the total population of neurons (Fig. 3B). Since collateral sprouting occurs primarily in small neurons, these results provided further evidence that CREB may be involved in mediating signals during precocious sprouting.

However, an interesting result for these experiments is that, as early as 8h, stimulation induced a significant increase of pCREB expression ($p<0.05$). It suggested that electrical stimulation might be an important factor in the induction of pCREB expression. Since pCREB was upregulated by electrical stimulation at early time point, the possible role of pCREB may be that it is involved in the regulation of downstream genes expression important for priming the neurons to respond more rapidly to the sprouting stimulus, NGF.

egr-1 egr-1 is a gene activated in different neuronal tissues by many factors (eg. Milbrandt,

1987; Cole *et al.*, 1989; Hughes *et al.*, 1992; Bartel *et al.*, 1989; Felipe *et al.*, 1993; Kendall, 1994; Mundschau *et al.*, 1994). In cultured PC12 cells and adult DRG neurons, depolarization and NGF resulted in upregulation of this gene (Bartel *et al.*, 1989; Kendall *et al.*, 1994). However, in the *in vivo* situation, a change in the expression of this gene induced by electrical stimulation and NGF in DGR neurons has not been well studied. Nevertheless, previous studies have shown an increase in the expression of this gene in dorsal horns after sciatic nerve section (Herdegen *et al.*, 1992) . The results from an *in vivo* study in rat showed that Egr-1 protein was either absent or gave a weak basal immunostaining in up to 10% of large and small lumbar DRG neurons (Herdegen *et al.*, 1992). On the other hand, it was highly expressed in superior cervical ganglion of the adult rat (Milbrandt, 1987), indicating that it has some cell-specific expression. In my study, electrical stimulation alone or in cooperation with NGF was examined in the *in vivo* situation in thoracic (T10-T13) DRG neurons. Unlike the former study where Egr-1 presented a variable immunostaining in lumbar DRGs (Herdegen *et al.*, 1992), Egr-1 basal expression was detected consistently in three sizes of DRG neurons, and the protein was detectable in as high as 32.29% to 34.37% of total labeled cells (Fig. 8). An increase of Egr-1 expression started at 4h (42.18%), but the significant increase of Egr-1 protein was first observed at 8h after electrical stimulation ($p<0.05$), later than that found in PC12 cells (Bartel *et al.*, 1989). The expression of protein of Egr-1 in the isolation alone experimental condition did not show any significant increases compared with control ($p<0.05$). This suggests that the change of

NGF alone might be not strong enough to induce the change of Egr-1 protein expression. This was further supported by another group of experiments where the isolation plus stimulation acted to induce a significant increase of Egr-1 protein ($p<0.05$) as early as 1d and which lasted until 4d.

Since there are CRE elements in the promoter region of egr-1 (Changelian et al., 1989), it might be a potential downstream target of the pCREB. In my study, the change of Egr-1 protein did show some relationship with that of pCREB protein (Fig.8). From the results of pCREB (Fig. 6), electrical stimulation induced significant increase of pCREB protein at 8h ($p<0.05$); similarly, electrical stimulation also resulted in a significant increase of Egr-1 at the same time point. These results suggest that the increase of pCREB might induce the increase of Egr-1 protein level and regulate its expression during precocious sprouting.

Of interest is that electrical stimulation and isolation appeared to have certain interactive effects in inducing the expression of Egr-1 protein. As shown in Fig. 8, when the nerves were treated by electrical stimulation and isolation together, the expression of Egr-1 protein was not a simple accumulation of these two effects. At early time points (4h and 8h), electrical stimulation induced a large increase of Egr-1 protein expression, but the combination of electrical stimulation and isolation did not show a significant increase. However, at 1d, even though the separate effects of electrical stimulation or isolation did not show significant changes ($p<0.05$), their cooperative effects induced significant increase of Egr-1 protein.

According to the results from ICC (data not shown), although Egr-1 was expressed in all sizes of neurons, most of the labeled cells were the small neurons. The increasing trend after stimulation or/and isolation treatment for the small neurons was similar to that of total cell population. From previous knowledge, it is known that these small to medium size neurons would be expected to undergo collateral sprouting (Diamond *et al.*, 1992a; Mearow *et al.*, 1994). Therefore, these results further suggested that, although the mechanism of their interaction is not clear, electrical stimulation and isolation contribute to the activation of egr-1 and might be involved in collateral sprouting process.

Previous studies showed that electrical stimulation of sciatic nerves at Aδ/C intensities induced the expression of egr-1 in the central postsynaptic targets of the noxious afferents in spinal cord (Herdegen *et al.*, 1991), which may be an important mechanism in the physiology of sensing noxious/painful stimulation. Little change was observed in the DRG neurons themselves, although the time points examined were somewhat different than those used in the present experiments. A further point that might account for differences in expression could be the anaesthetic used. In my experiments, the animals were relatively lightly anaesthetized, so that the effects of the stimulation on the CTM reflex could be observed. More complete anaesthesia (as used in the previous studies) might depress some potential responses.

c-fos c-fos is a well studied gene that can be activated by many factors during different

biologic processes (eg. rev. in Angel and Karin, *et al.*, 1991; Sheng and Greenberg, 1991). In different tissues and under different treatments, c-fos expression presents a variety of patterns (eg. Bartel *et al.*, 1989). While there are a number of studies examining the influence of noxious stimulation in c-fos activation in DRG neurons (Hunt *et al.*, 1987; Jenkins and Hunt, 1991), *in vivo* studies on the involvement of c-fos in DRG neurons under both electrical and NGF stimulation are rarely documented. For example, it was shown that basal expression of c-Fos was not detected in lumbar DRG neurons in untreated animal (Herdegen *et al.*, 1992), and its expression in DRGs was neither activated by noxious stimulation of sciatic nerve or hind paw (Hunt *et al.*, 1987; Jenkins and Hunt; 1991), even though c-fos was upregulated in dorsal horn after noxious stimulation (Herdegen *et al.*, 1991). NGF does induce a relatively rapid c-fos expression in cultured DRGs cells (Lindsay *et al.*, 1990), and an *in vivo* study also showed that NGF induces Fos protein in Trk A immunoreactive lumbar DRG neurons (Michael *et al.*, 1996). In my study, the basal expression of c-fos in thoracic DRGs and the involvement of electrical stimulation and NGF was initially found during the collateral sprouting accelerated by electrical stimulation (Fig.10). The expression of c-Fos was not significantly increased ($p<0.05$) after electrical stimulation alone and isolation alone but it was significantly increased after co-treatment of stimulation and isolation at 2d and 4d ($p<0.05$). The results of some studies have indicated that CREs exist in the promoter region of c-fos (Sheng *et al.*, 1988; Sheng *et al.*, 1990). Since the response of c-fos was later than CREB, and NGF cooperated with stimulation to

result in some changes on c-Fos, it seems that CREs might be directly or indirectly involved in the regulation of c-Fos response.

Since under stimulation conditions the basal expression of c-fos was not detected in the previous studies (Hunt *et al.*, 1987; Herdegen *et al.*, 1991a), in order to clarify that the immunostaining in my experiment was real, the experiments were carefully controlled. A control peptide which blocks c-fos epitopes was added into primary antibody incubation solution in some experiments. Further, it is known that electrical stimulation of the sciatic nerves results in the c-fos expression in the dorsal horn of the spinal cord (Herdegen *et al.*, 1991). Therefore, spinal cord sections, from cord segments whose related DCNs were electrically stimulated, were used as a positive control to test the signal of c-fos. In these experiments, the peptide blocked the Fos expression, but the signal was also observed in the appropriate spinal cord sections (data not shown). Thus, my observation should reflect a real staining for c-Fos protein. Compared with my experiments, the observation that c-fos expression was not detected in DRGs in other experiments might be due to the following reasons. Firstly, the DRGs were from different sources. The DRGs tested in former experiments were from the lumbar area, while in my experiments, the DRGs were from thoracic area. Secondly, the electrical stimulation was conducted on different nerves derived from these DRGs, and it is conceivable that they might have different responses to the electrical stimulation. Thirdly, the condition of electrical stimulation used in these treatments were different in terms of frequency and duration. Fourthly, the antibodies used in different

experiments were different, as were the time points investigated; the effects of anaesthesia as discussed above may also be relevant.

Recent results supported our observations of c-Fos expression in DRG neurons, and its upregulation by NGF (Michael *et al.*, 1996). This preliminary report indicated that 3 hr after an intrathecal injection of NGF there was a significant increase (about 10%) in the number of DRG neurons expressing c-Fos. In cultured DRGs, NGF upregulated the expression of c-Fos rapidly; by adding NGF to the culture medium, c-Fos expression was elevated within 30 min (Lindsay *et al.*, 1990). In my study, c-fos appeared to increase under the treatment of isolation, however, this was not significant ($p<0.05$) (Fig. 10). A potential explanation is that c-fos is not very sensitive to small changes in NGF levels in the skin. Unlike stimulation and isolation alone, electrical stimulation and isolation together induced significant increases ($p<0.05$) in c-fos protein after 2d, suggesting that depolarization and NGF might work in coordination with each other through the same or different pathways in inducing this protein expression.

c-Jun As a component of the AP-1 DNA-binding protein, c-Jun has been well studied. *In vivo* studies showed that c-Jun expression is more related to the transection/injury of nerves. Transection of the sciatic nerves resulted in a rapid induction of c-Jun expression in its lumbar DRG neurons (Herdegen *et al.*, 1992). However, in L4/5 DRG, small and large neurons showed low basal c-Jun expression which was restricted to the cell nucleus (Leah

et al., 1991; Herdegen *et al.*, 1991; Herdegen *et al.*, 1992; Jenkins *et al.*, 1993). In my study, the basal expression of c-Jun in DRGs was stable and high in all sizes of neurons. Noxious electrical stimulation, which activated Aδ and C- fibers, produced a quick increase of c-Jun expression in ipsilateral dorsal horn of lumbar spinal cord, but did not provoke additional c-Jun immunoactivity in dorsal root ganglia within 2 h after electrical stimulation (Herdegen *et al.*, 1991). Similar results were obtained in my study (Fig.12). During my experimental period (1h, 4h, 8h, 1d, 2d, and 4d), electrical stimulation did not induce any change of c-Jun protein in DRGs after electrical stimulation of DCNs. The involvement of c-Jun in DRG during collateral sprouting was previously investigated (Jenkins *et al.*, 1993). It was shown that after denervation of L4/L5 nerves, the saphenous nerve, which was derived from L3 DRG, could collaterally sprout into the L4/L5 area. During this process, an elevation in c-Jun protein expression was visualized in the intact L3 DRG nucleus even though the results were equivocal due to the high variation of immunopositive cells (Jerkins *et al.*, 1993). Like the above results, in my study, isolation alone significantly upregulated expression of c-Jun protein as early as 8h after isolation and this increase lasted to 4d ($p<0.05$). An interesting result in my study is that, under the treatments of stimulation and isolation, c-Jun protein expression also significantly increased ($p<0.05$) even though that the increases were not significantly different from isolation alone treatment at the same time point ($p<0.05$). Again, these results support the idea of cooperativity between depolarization and the provision of the sprouting stimulus (i.e., nerve isolation).

Oct-2 Oct-2, as a member of the POU (named for its founder members, Pit-1, Oct-1 and 2, *uns*-86) family , is specifically expressed in sensory neurons (Latchman et al 1992). In cultured DRG neurons, it is expressed at high levels and regulated by NGF (Wood *et al.*, 1992; Latchman *et al.*, 1992; Kendall *et al.*, 1995). An *in vivo* study also showed that NGF increased Oct-2 mRNA level in DRG neurons innervating inflamed tissue during inflammation, and that anti-NGF blocked this increase, indicating that the expression of Oct-2 was NGF-dependent (Ensor *et al.*, 1996). However, the effects of electrical stimulation on the expression of this gene have not been studied. I carried out preliminary experiments and found that although Oct-2 protein had a basal expression in DRG neurons (Fig.14), as previously reported (Wood *et al.*, 1992; Latchman *et al.*, 1992), the results of Oct-2 under different treatments were not consistent. Since the sample number used here was small, further experiments need to be performed in order to find out the involvement of Oct-2 in precocious sprouting.

While it is appreciated that larger sample sizes would provide greater accuracy to the results obtained, this was not always possible to achieve due to time constraints, experimental design, and availability of reagents. For example, with respect to the ICC analyses, loss of sections from slides resulted in smaller sample sizes than original expected. However, although for some samples, the n value is small, the results from my study still suggested that stimulation and isolation indeed caused some change of these IEGs. In order to further confirm these results, in the future, more samples can be used and further studies

can be done based on my experiments.

The protein expression of these above IEGs was also examined by Western blot assay. Since IEG proteins of interest were likely present in the lysates at relatively small amounts and the Western technique may not have been sensitive enough to specifically detect the band of interest, the Western blot analysis did not yield satisfactory results to show the change of protein level on blots. Because only limited amounts of lysate were available, immunoprecipitation, which is a more sensitive technique to check protein expression, was not performed to investigate the changes on the protein of interest under the present situations.

4.3 The role of NGF receptor in precocious sprouting

The effects of electrical stimulation or depolarization on NGF receptor expression have not been studied extensively *in vivo*. In *in vitro* experiments, membrane depolarization has been shown to induce the expression of the Trk A receptor in MAH cells, an immortalized sympathoadrenal progenitor cell line which did not initially respond to NGF due to the absence of Trk A receptor (Birren *et al.*, 1992), suggesting the relationship between depolarization and NGF receptor. In my study, the effects of electrical stimulation on the expression of Trk receptor were for the first time investigated *in vivo*. At the mRNA level (Fig.15) and protein level (Fig.17), Trk A expression was increased very little after electrical stimulation compared with control. Although p75 receptor presented an elevation over time,

the elevation was also not significant. These results are consistent with those from other studies in adult DRGs (Mearow, 1998), suggesting that electrical stimulation had some effects on NGF receptors, but that it was not sufficient in itself to significantly alter the receptor mRNA expression.

Previous *in vitro* studies showed that NGF receptor was upregulated by NGF in adult sensory neurons (Lindsay *et al.*, 1990). *In vivo* studies have also indicated that NGF mRNA level increased in the skin during collateral sprouting (Mearow *et al.*, 1993). In addition, in DRG neurons both p75 and Trk A mRNAs were upregulated prior to and during collateral sprouting (Mearow *et al.*, 1994; Mearow, 1994). Similar results were obtained in my study but in my experiments earlier time points were examined. So, as early as 2d, increases in these two NGF receptor mRNAs could be seen.

Interestingly, in my experiments, although electrical stimulation alone did not induce significant upregulation of Trk A and p75 receptor expression, electrical stimulation combined with isolation resulted in significant increase of these two receptor starting at 1h and 4h respectively.

Since the phosphorylation state of the Trk A receptor is an indication of its activation, any change on this phosphorylation is a useful parameter to evaluate the involvement of Trk A in mediating biological phenomena. The phosphorylation of Trk A is a rapid process; for example, phosphorylated Trk A was detectable 5 min following the addition of NGF to PC12 cell culture (Kaplan *et al.*, 1991b). Membrane depolarization by KCl resulted in the phos-

phorylation of Trk A in NGF sensitive PC12 cells and subsequent neurite formation (Solem *et al.*, 1995).

Based on the possible involvement of NGF and membrane depolarization in the phosphorylation of Trk A in other tissues, phosphorylated TrkA was detected in DRG neurons *in vivo* in my study. The basal expression of phosphorylated Trk A was detected in DRG neurons using Western analysis (Fig. 18). However, electrical stimulation did not increase the activity of Trk A. Even in the presence of increased NGF (isolation) at later time points (2d, 4d, 8d), electrical stimulation did not induce an obvious increase in the phosphorylation state of Trk A.

According to the results, there might be two possibilities to explain the function of electrical stimulation. One is that the change of Trk A activity induced by electrical stimulation and NGF might be very fast and transient, and the time duration selected in my study might not be narrow enough to cover the time points where the phosphorylated Trk A was increased. The other one is that depolarization (electrical stimulation) might not be strong enough to induce the change of phosphorylation of Trk A, but it could be transiently "priming" the cells to response to the real sprouting stimulus, *i.e.*, NGF.

4.4 The involvement of NGF receptor down-stream proteins in precocious sprouting

As the high affinity receptor, Trk A is thought to mediate the effects of NGF, and result

in the activation of intracellular proteins (rev. Kaplan and Stephens, 1994; Segal and Greenberg, 1996k; Kaplan and Miller, 1997). In PC 12 cells, the proteins which propagate the information from Trk A receptor have been of intensive study (e.g. Loeb *et al.*, 1994; Stephens *et al.* 1994; Soltoff *et al.*, 1994; Obermeier *et al.*, 1993; Ohmichi *et al.*, 1991; Vetter et al, 1991). It is thought that PLC-γ, PI-3 kinase and SHC mediate different pathways to produce different biological effects on the cells. In addition to the above proteins, MAPK is an important protein recognized to mediate the signals. Based on information obtained by studying PC12 cells, potential changes in the above proteins under electrical stimulation or/and isolation were investigated in DRG neurons in my study. However, while the results showed that these protein were basally expressed in adult DRG neurons, electrical stimulation or/and isolation did not result in any obvious influence on these proteins during collateral sprouting process. Again this may be because basal levels are already relatively high, and as such our stimuli may not influence changes detectable with the methods employed. Furthermore, at the time I carried out my experiments, phospho-specific antibodies against these signalling components were not available. Use of such reagents would allow for the investigation of whether the various treatments resulted in activation of these proteins, rather than changes in their levels of expression.

4.5 The probable pathways mediating electrical stimulation and NGF signals in precocious sprouting

Evidence from PC12 cells show that depolarization induced neurite outgrowth occurs only when NGF receptor was partially activated, either by overexpression of TrkA or by treatment with a low concentration of NGF; depolarization alone was insufficient to stimulate neurite formation (Solem *et al.*, 1995). Further studies indicated that the inhibitors of Ca^{2+} channels and Ca^{2+} /Calmodulin-dependent protein kinase II and IV reduced this depolarization-induced outgrowth, suggesting that depolarization stimuli act together with subthreshold activation of NGF receptors to induce neurite growth through a Ca^{2+} and CaM kinase-dependent signal transduction pathway (Solem *et al.*, 1995). From the results in my study, it seems that electrical stimulation of the DCN nerves and the likely NGF increase after denervation (isolation) could have similar effects. In my study, electrical stimulation induced increased expression of certain IEGs (egr-1), and NGF also induced the expression of this gene separately, even though there was a time delay on this induction. The mRNA levels and protein expression of CREB, and egr-1 significantly ($p<0.05$) increased at very early time points under the treatment of electrical stimulation and the appearance of the increases in these two genes showed a time order (CREB appeared firstly, then egr-1 appeared). Isolation, which activated NGF receptor expression in my experiment at the later time points (after 1d), also stimulated egr-1 expression. Interestingly, under the situation of electrical stimulation and isolation, the increase in the expression of this gene appeared earlier than isolation alone. In addition, the amplitude of increase was not the simple

addition of these two treatments. For example, Egr-1 protein was increased by electrical stimulation as early as 8h. However, it was increased by isolation plus stimulation at 1d, later than stimulation alone.

From former evidence it is known that electrical stimulation or depolarization can increase the influx of Ca^{2+} into the cytoplasm of electrically excitable cells and subsequently, the increased Ca^{2+} can upregulate the expression of CREB or pCREB in the nucleus (Sheng *et al.*, 1991). Since the existence of CRE binding site in the promoter regions of egr-1, c-fos, c-jun, these genes were thought to be the downstream genes of CREB (e.g., Angel *et al.*, 1988; Christy *et al.*, 1988; Changelian *et al.*, 1989; Fisch *et al.*, 1989). Previous studies suggest that calcium influx induces neurite growth through a *src*-ras signaling cassette in PC12 cells and egr-1 was also activated in this process (Rusanescu *et al.*, 1995). My results suggest a similar order of regulation among these genes: the expression of egr-1 and c-fos were partially regulated by the increase of CREB under electrical stimulation alone. However, since under the co-treatment of electrical stimulation and isolation, the expression of the IEGs were different, other mechanisms might be involved in this process. The results suggested that isolation and stimulation might induce an increase in NGF receptors, and this increase in expression and potential for increased activation might consequently activate ras-mediated transduction pathways (Ginty *et al.*, 1994). Downstream genes regulated by CREB, egr-1, c-fos and c-jun might also be controlled by this transduction pathway. Therefore, the effects of electrical stimulation on precocious sprouting could be such that, electrical

stimulation directly activated IEGs, such as CREB, egr-1 expression. In addition, denervation induced increases in skin levels of NGF might lead to activation of the Trk A receptor at certain time points, which could enhance the transduction through the ras-pathway and subsequently upregulate expression of these IEGs. When these two effects are combined together, it could produce a particular pattern of IEGs expression. In this way, it is possible that target genes which subsequently regulate collateral sprouting could be expressed earlier with the combination of stimulation plus isolation than isolation alone, thus shortening the latency of the onset of collateral sprouting. For example, with electrical stimulation and systemic NGF injection (rather than nerve isolation), the expression of mRNAs for GAP43 and Tα1 (proteins known to be involved in collateral sprouting), are upregulated earlier than with just NGF alone; stimulation alone has little influence on their expression (Kril *et al.*, 1993; Mearow, 1998).

4.6 Future directions

The results of the present series of experiments have addressed the hypothesis that, during the precocious collateral sprouting, electrical stimulation activates the expression of certain IEGs and elevates Trk A receptor expression; electrical stimulation acts in concert with the increased availability of NGF to result in an accelerated terminal sprouting response. With respect to the role of particular IEG expression in the phenomenon of accelerated sprouting, the results are not fully conclusive. However, as the results from the different

methodological approaches (*i.e.*, ICC and ISH) are at least consistent, it would seem that the trends seen and the seemingly inconsistent increases and /or decrease in expression may be of importance in the neuronal response to changes in the external environment. The alterations seen in p75 and Trk A mRNA expression are essentially the same as those observed in previous studies of this system (Kril *et al.*, 1993; Mearow, 1994; Mearow *et al.*, 1994; Mearow, 1998). The present results add further information by examining earlier time points. In addition, to examining alterations in mRNA expression of the NGF receptors, which might be assumed to be a downstream event, it was of interest to investigate the possibility that electrical stimulation (i.e., depolarization) might have a much earlier effect on at least the Trk A receptor.

Thus, the influence of stimulation on the activity of the Trk receptor (as reflected by its phosphorylation state) was examined, as were the potential effects on downstream signaling interaction that could eventually lead to the sorts of alterations expected in gene expression. The results obtained provide for the first time information from in vivo experiments about phosphorylation states of Trk and potential downstream interactions. While a more involved study was beyond the scope of the present thesis, these data provide a basis upon which to continue further studies.

Such studies could include:

i) The duration between each sampling could be narrowed in the future experiments and the change of phosphorylation state of Trk A would be detected under these conditions.

ii) Since the NGF- mediated activation of the ras pathway is most likely involved in the process, the activation of ras or inhibition of ras after isolation and isolation plus stimulation treatments could be tested by Western blot analysis in the future.

iii) In addition to examining the DRG neurons, ligation studies of stimulated nerves could be included to assess changes in retrograde signals. It is possible that any alterations in signaling components may have been attenuated by the time the cell body is reached. The NGF receptor complex or other signaling intermediates must travel several centimeters from the nerve terminals to the cell bodies. Alternatively, the signaling events may be purely local events, occurring at the site of NGF interaction with its receptors at the terminal site (eg., Campenot, 1994; Kimpinski *et al.*, 1997; Senger and Campenot, 1997).

iv) Studies in which endogenous NGF is blocked using anti-NGF are useful. It is possible that in these in vivo experiments the prolonged exposure of the axons to target-derived NGF may act to either 1) result in relatively high basal levels of activation, where small changes (as might be induced by our stimuli) would be difficult to detect (Knusel *et al.*, 1996). 2) Alternatively, the increased exposure could act to depress Trk activation (Knusel *et al.*, 1997); the timing of the exposure compared to when samples are taken for analyses would be an important variable. ICC may be another approach which might show small changes better; with the Western analysis, where lysates of total DRGs were analyzed, small changes in Trk A activation in the NGF responsive population (50% of the neurons, at least) may be diluted out by the total DRG signal.

v) The use of phospho-specific antibodies (i.e., p-trk and MAPK and others) that are currently becoming available may prove to be more sensitive and allow for detection of small and more specific changes.

REFERENCE

Abraham, W. C., Dragunow, M., Tate, W. P. (1991) The role of immediate early genes in the stabiiization of long term potentiation. **Mol. Neurobiol.** 5: 297-314.

Aguilar, C. E., Bisby, M. A., Cooper, E., and Diaomon, J. (1973) Evidence that axoplasmic transport of trophic factors is involved in the regulation of peripheral nerve fields in salamanders. **J. Physiol. Lond** 234:449-464.

Angel, P. and Karin, M. (1991) The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. **Biochim Biophys Acta** 1072 (2-3): 129-157.

Barker, P. A., Barbee, G., Misko, T.P., Shooter, E.M. (1994) The low affinity neurotrophin receptor, p75LNTR, is palmitoylated by thioester formation through. **J Biol Chem** Dec 2, 269:48, 30645-50.

Bartel, D. P., Sheng, M., Lau, L.F., and Greenberg, M. E. (1989) Growth factors and membrane depolarization activate distinct programs of early response gene expression: dissociation of *fos* and *jun* induction. **Genes Dev.** 3:3.4-313.

Birren, S. J., Verdi, J. M. and Anderson, D. J.(1992) Membrane depolarization induces $p140^{trk}$ and NGF responsiveness, but not $p75^{lngfr}$. **Science** 257:395-397.

Borasio, G.D. Markus. A., Wittinghofer, A., Barde, Y.A., Heumann, R. (1993) Involvement of ras p21 in neurotrophin-induced response of sensory, but not sympathetic neurons. **J Cell Biol** 121(3): 665-672.

Buckmaster, A., Nobes, C. D., Edwards, S. N., and Tolkovsky, A. M. (1991) Nerve growth factor is required for induction of c-fos immunoreactivity by serum, depolarization, cyclic AMP or trauma in cultured rat sympathetic neurons. **Euro. J. Neurosci.** 3: 698-707.

Campenot, R. B. (1994) NGF and the local control of nerve terminal growth. **J. Neurobiology** 25 (6): 599-611.

Cao, X., Koski, R. A., Gashler, A., Mckiernan, M., Morris, C. F., Gaffney, R., Hay, R. V., and Sukhatme, V. P. (1990) Identification and characterization of the egr-1 gene product, a DNA-binding zinc finger protein induced by differentiation and growth signals. **Mol. Cel. Biol.** May: 1931-1939.

Changelian, P.S.,Feng, P., King, T.C., Milbrandt, J. (1989) Structure of the NGFI-A gene and detection of upstream sequences responsible for its transcriptional induction by nerve growth factor. **Proc Natl Acad Sci U S A** 86(1): 377-381.

Chao, M.V., Bothwell, M.A., Ross, A.H., Koprowski, H., Lanahan, A.A., Buck, C.R., Sehgal, A. (1986) Gene transfer and molecular cloning of the human NGF receptor. **Science** 232 (4749): 518-521 (1986)

Chao, M.V. (1994) The p75 neurotrophin receptor. **J Neurobiol** 25(11): 1373-1385

Christy, B., Nathans, D. (1989) Functional serum response elements upstream of the growth factor-inducible gene zif268.. **Mol Cell Biol** 9(11): 4889-4895.

Cohen, S. (1959) Purification and metabolic effects of a nerve growth-promoting protein from snake venom. **J. Biol. Chem.** 234:1129-1137.

Cohen, S. (1960) Purification of a nerve-growth promoting protein from the mouse salivary gland and its neurocytotoxic antiserum. **Proc. Natl. Acad. Sci. USA** 46:302-311.

Cole, A. J., Saffen, D. W., Baraban, J. M., Worley, P. F. (1989) Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. **Nature** 340 (6233): 474-476.

Cooper, E., Diamond, J., and Turner, C. (1977a) The effects of nerve section and of colchicine treatment on the density of mechanosensory nerve endings in salamander skin. **J. Physiol. Lond** 264: 725-749.

Cooper, E., Scott, S. A., and Diamond, J. (1977b) Control of mechanosnsory nerve sprouting in salamander skin. **Neurosci. Symp.** 2:120-138.

Creedon, D. J., Johnson, E. M., Lawrence, J. C. (1996) Mitogen-activated protein kinase-independent pathways mediate the effects of nerve growth factor and cAMP on neuronal survival. **J Biol Chem** 271(34): 20713-20718.

Crutcher, K.A. (1986) The role of growth factors in neuronal development and plasticity. **CRC Crit Rev Clin Neurobiol** 2(3): 297-333.

Curran, T., Franza, B. R. Jr. (1988) Fos and Jun: the AP-1 connection. **Cell** 55: 395-397.

Davies, A. M., Bandtlow, C., Heumann, R., Korsching, S., Rohrer, H., and Thoenen, H. (1987) Timing and site of nerve growth factor synthesis in developing skin in relation to

innervation and expression of the receptor. **Nature** 326: 353-358.

Davies, A. M., Lee, K. F., Jaenisch, R. (1993) p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. **Neuron** 11(4): 565-574.

Devor, M., Schonfield, D., Seltzer, Z., and Wall, P.D. (1979) Two modes of cutaneous reinnervation following peripheral nerve injury. **J. Comp. Neurol.** 185: 211-220.

Diamond, J., Cooper, E., Turner, C., and Macintyre, L., (1976) Trophic regulation of nerver sprouting. **Science** 193:371-377.

Diamond, J., Coughlin, M., Macintyre, L., Holmes, M., Visheau, B. (1987) Evidence that endogenous beta nerve growth factor is responsible for the collateral sprouting, but not the regeneration, of nociceptive axons in adult rats. **Proc Natl Acad Sci U S A** 84(18): 6596-6600.

Diamond, J., Jackson, P. (1987) Plasticity and stability in the sensory innervation of skin. In Effects of Injury on Trigeminal and Spinal Somatosensory Systems. Pubols, L.M., and Sessle, B. J. (eds.) Alan R. Liss, New York 43-48.

Diamond J., Holmes, M., and Coughlin, M. (1992a) Endogenous NGF and nerve impulses regulate the collateral sprouting sensory axons in the skin of the adult rat. **J. Neurosci.** 12: 1454-1466.

Diamond, J., Foerster, A., Holmes, M., and Coughlin, M. (1992b) Sensory nerves in adult rats regenerate and restore sensory function to the skin independently endogenous NGF and nerve impulses. **J. Neurosci.** 12: 1467-1476.

Diamond, J., Foerster, A. (1992c) Recovery of sensory function in skin deprived of its innervation by lesion of the peripheral nerve. **Exp Neurol** 115(1): 100-103.

Diamond, J., Gloster, A., Kitchener, P. (1992d) Regulation of the sensory innervation of skin: trophic control of collateral sprouting. In **Sensory Neurons - Density, Development, and Plasticity.** p. 309-331. S. A. Scott (eds). Oxford University Press, New York.

Diamond, J., Lourenssen, S., Pertens, E., and Urschel, B. (1995) Lack of collateral sprouting of nociceptive nerves in adult p75 knock-out mice. **Soc. Neurosci Abstr** 21:1539.

Doucette, D., Diamond, J. (1987) Normal precocious sprouting of heat nociceptors in the skin of adult rats. **J. Comp. Neurol.** 261:592-603.

Dwarki, V. J., Montminy, M., Verma, I. M. (1990) Both the basic region and the 'leucine zipper' domain of the cyclic AMP response element binding (CREB) protein are essential for transcriptional activation. **EMBO J** 9(1): 225-232.

Ehlers, M. D., Kaplan, D. R., Price, D. L., Koliatsos, V. E. (1995) NGF-stimulated retrograde transport of trkA in the mammalian nervous system. **J Cell Biol** 130(1):149-156.

Ensor, E., Kendall, G., Allchorne, A., Woolf, C. J., Latchman, D. S. (1996) Induction of the Oct-2 transcription factor in primary sensory neurons during inflammation is nerve growth factor-dependent. **Neurosci Lett** 204(1-2): 29-32.

Felipe, C. D., Jenkins, R., O'shea, R., Williams, T. S. C., Hunt, S. P. (1993) The role of immediate early genes in the regeneration of the central nervous system. **Advances in Neurology** 59:263-271.

Fisch, T. M., Prywes, R., Simon, M. C., Roeder, R. G. (1989) Multiple sequence elements in the c-fos promoter mediate induction by cAMP. **Genes Dev** 3(2): 198-211.

Friedman, B., Aguayo, A. J. (1985) Injured neurons in the olfactory bulb of the adult rat grow axons along grafts of peripheral nerve. **J Neurosci** 5(6): 1616-1625.

Ghosh, A. and Greenberg, M. E. (1995) Calcium signaling in neurons: molecular mechanisms and cellular consequences. **Science** 268(5208): 239-247.

Gilad, GM., Reis, D. J. (1979) Collateral sprouting in central mesolimbic dopamine neurons: biochemical and immunocytochemical evidence of changes in the activity and distrubution of tyrosine hydroxylase in terminal fields and in cell bodies of A10 neurons. **Brain Res** Jan 5160(1):17-26.

Ginty, D. D., Glowacka, D., Bader, D. S., Hidaka, H., Wagner, J. A. (1991) Induction of immediate early genes by Ca2+ influx requires cAMP-dependent protein kinase in PC12 cells. **J. Biol Chem** 266 (26): 17454-17458.

Ginty, D. D., Kornhauser, J. M., Thompson, M. A., Bading, H., Mayo, K. E., Takahashi, J. S., Greenberg, M. E. (1993) Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. **Science** 260(5105): 238-241.

Ginty, D. D., Bonni, A., Greenberg, M. E. (1994) Nerve growth factor activates a ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. **Cell** 77: 713-725.

Gonzalez, G. A., Montminy, M. R. (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. **Cell** 59(4): 675-680.

Greene, L., Varon, A. S., Piltch, A., and Shooter, E. M. (1971) Substructure of the β-subunit of mouse 7S nerve growth factor. **Neurobiology** 1:37-48.

Greene, L. A., Kaplan, D. R. (1995) Early events in neurotrophin signalling via Trk and p75 receptors. **Curr Opin Neurobiol** 5(5): 579-587.

Hagag, N., Halegoua, S., Viola, M. (1986) Inhibition of growth factor-induced differentiation of PC12 cells by microinjection of antibody to ras p21. **Nature** 319(6055): 680-682.

Hantzopoulos, P. A., Suri, C., Glass, D. J., Goldfarb, M. P., Yancopoulos, G. D. (1994) The low affinity NGF receptor, p75, can collaborate with each of the Trks to potentiate functional responses to the neurotrophins. **Neuron** 13(1): 187-201.

Harper, A. A., Lawson, S. N. (1985a) Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurones. **J Physiol (Lond)** 359: 31-46.

Harper, A. A., Lawson, S. N. (1985b) Electrical properties of rat dorsal root ganglion neurones with different peripheral nerve conduction velocities. **J Physiol (Lond)** 359: 47-63.

He, X., Treacy, M. N., Simmons, D. M., Ingraham, H. A., Swanson, L. W., Rosenfeld, M. G. (1989) Expression of a large family of POU-domain regulatory genes in mammalian brain development. **Nature** 340(6228):35-41.

Herdegen, T., Kovary, K., Leah, J., Bravo, R. (1991a) Specific temporal and spatial distribution of JUN, FOS, and KROX-24 proteins in spinal neurons following noxious transsynaptic stimulation. **J Comp Neurol** 313(1): 178-191.

Herdegen, T., Leah, J. D., Manisali, A., Bravo, R., and Zimmermann, M. (1991b) c-jun-like immunoreactivity in the CNS of the adult rat: basal and transynaptically induced expression of an immediate-early gene. **Neurosci.** 41 (2/3): 643-654.

Herdegen, T., Carlos, E., Fiallos-Estrada, Schmid, W., Bravo, R., and Zimmermann, M. (1992) The transcription factors c-Jun, Jun-D and CREB, but Fos and Krox-24, are differentially regulated in axotomized neurons following transection of rat sciatic nerve. **Mol. Brain Res.** 14: 155-165.

Horch,K. (1981) Absence of functional collateral sprouting of mechanoreceptor axons into

denervated areas of mammalian skin. **Exp. Neurol.** 74:313-317.

Hughes, P., Lawlor, P., Dragunow, M. (1992) Basal expression of Fos, Fos-related, Jun, and Krox 24 proteins in rat hippocampus. **Mol Brain Res** 13: 355-357.

Hunt, S. P., Pini, A., Evan, G. (1987) Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. **Nature** 28 (6131): 632-634.

Imaki, J., Yoshida, K., Yamashita, K. (1994) A developmental study of cyclic AMP-response element binding protein (CREB) by in situ hybridization histochemistry and immunocytochemistry in the rat neocortex. **Brain Research** 651: 269-274.

Jackson, P., and Diamond, J. (1981) Regenerating axons reclaim sensory targets from collateral nerve sprouts. **Science** 214:926-928.

Jackson, P. C., Diamond, J. (1984) Temporal and spatial constraints on the collateral sprouting of low-threshold mechanosensory nerves in the skin of rats. **J Comp Neurol** 226(3): 336-345.

Jalava, A., Mai. S. (1994) Fos and Jun form cell specific protein complexes at the neuropeptide tyrosine promoter. **Oncogene** 9: 2369-2375.

Jenkins, R., Hunt, S. P. (1991) Long-term increase in the levels of c-jun mRNA and jun protein-like immunoreactivity in motor and sensory neurons following axon damage. **Neurosci Lett** 129(1): 107-110.

Jenkins, R., Mcmahon, S. B., Bond, A. B., Hunt, S. P. (1993) Expression of c-Jun as a response to dorsal root and peripheral nerve section in damaged and adjacent intact primary sensory neurons in the rat. **Eur J Neurosci** 5(6): 751-759.

Jing, S., Tapley, P., Barbacid, M. (1992) Nerve growth factor mediates signal transduction through trk homodimer receptors. **Neuron** 9(6): 1067-1079.

Johnson, E. M. Jr., Koike, T., Franklin, J. (1986) A "calcium set-point hypothesis" of neuronal dependence on neurotrophic factor. **Exp Neurol** 115(1), 163-166.

Kaplan, D. R., Hempstead, B. L., Martin-Zanca, D., Chao, M. V., Parada, L. F. (1991a) The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. **Science** 252(5005): 554-558.

Kaplan, D. R., Martin-Zanca, D., Parada, L. F. (1991b) Tyrosine phosphorylation and

tyrosine kinase activity of the trk proto-oncogene product induced by NGF. **Nature** 350 (6314): 158-160.

Kaplan, D. R. and Knusel, B. (1997) Analysis of Trk/Neurotrophin Receptor Activity. In press.

Kaplan, D. R., Miller, F. D. (1997) Signal transduction by the neurotrophin receptors. **Current Opinion in Cell Biology** 9: 213-221.

Kaplan, D. R., Stephens, R. M. (1994) Neurotrophin signal transduction by the Trk receptor. **J Neurobiol** 25(11):1404-1417.

Kaplan, D. R. (1996) Studying signal transduction in neuronal cells: the Trk/NGF system. In **signal transduction: tools and techniques** for society for neuroscience Washington, D. C. 15-27.

Kendall, G., Ensor, E., Brar-Rai, A., Winter, J., Latchman, D. S. (1994) Nerve growth factor induces expression of immediate-early genes NGFI-A (Egr-1) and NGFI-B (nur 77) in adult rat dorsal root ganglion neurons. **Brain Res Mol Brain Res** 25(1-2): 73-79.

Kendall, G., Brar-Rai, A., Ensor, E., Winter, J., Wood, J. N., Latchman, D. S. (1995) Nerve growth factor induces the Oct-2 transcription factor in sensory neurons with the kinetics of an immediate-early gene. **J Neurosci Res** 40(2): 169-176.

Kerppola, T., Curran, T. (1992) Fos-Jun heterodimers and Jun homodimers bend DNA in opposite orientations: implications for transcription factor cooperativity. **Cell** 66: 317-326.

Kinnman, E., Aldskogius, H. (1986) Collateral sprouting of sensory axons in the glabrous skin of the hindpaw after chronic sciatic nerve lesion in adult and neonatal rats: a morphological study. **Brain Res** 377(1): 73-82.

Kimpinski, K., Compenot, ., Mearow, K. M. (1997) Effects of the neurotrophins nerve growth factor, neurotrophin-3 and brain-derived neurotrophic factor on neurite growth from adult sensory neurons in compartmented cultures. **Neurobiol.** 864: 1-16.

Klein, R., Jing, S. Q., Nanduri, V., O'Rourke, E., Barbacid, M. (1991a) The trk proto-oncogene encodes a receptor for nerve growth factor. **Cell** 65(1):189-197.

Klinz, F.J., Heumann, R. (1995) Time-resolved signaling pathways of nerve growth factor diverge downstream of the p140trk receptor activation between chick sympathetic and dorsal root ganglion sensory neurons. **J Neurochem** 65(3): 1046-1053.

Klinz, F. J., Wolff, P., Heumann, R.(1996) Nerve growth factor-stimulated mitogen-activated protein kinase activity is not necessary for neurite outgrowth of chick dorsal root ganglion sensory and sympathetic neurons. **J Neurosci Res** 46(6): 720-726.

Knusel, B., Kaplan, D. R., Hefti, F. (1996) Intraparenchymal NGF and BDNF injections in adult and aged rats induce long-lasting Trk tyrosine phosphorylation. **Expl Neurol.** 139: 121-130.

Knusel, B., Gao, H., Okazaki, T., Yoshida, T., Mori, N., Hefti, F., Kaplan, D. R (1997) Ligand-induced down-regulation of Trk messenger RNA, protein and tyrosine phosphorylation in rat bcortical neurons. **Neuroscience** 78(3): 851-862.

Konradi, C., Heckers, S. (1995) Haloperidol-induced Fos expression in striatum is dependent upon transcription factor cyclic AMP response element binding protein. **Neuroscience** 65(4): 1051-1061.

Korsching, S., and Thoenen, H. (1985) Nerve growth factor supply for sensory neurons: Site of origin and competition with the sympathetic nervous system. **Neurosci. Lett.** 54:201-205.

Kril, Y., Diamond, J., Mearow, K. M. (1993) NGF receptor mRNA expression in collaterally sprouting sensory neurons - the influence of stimulation. Soc. Neurosci. Abstr. 19: 1712.

Kruijer, W., Schubert, D., Verma, I. M. (1985) Induction of the proto-oncogene fos by nerve growth factor. **Proc Natl Acad Sci U S A** 82 (21): 7330-7334.

Latchman, D.S., Dent, C.L., Lillycrop, K.A., Wood, J. N. (1992) POU family transcription factors in sensory neurons. **Biochem Soc Trans** 20(3): 627-631.

Lawson, S. N. (1979) The postnatal development of large light and small dark neurons in mouse dorsal root ganglia: a statistical analysis of cell numbers and size. **J Neurocytol** 8(3): 275-294.

Lawson, S. N (1994) Morphological and biochemical cell types of sensory neurons. 27-59.

Lawson, S. N., Waddell, P. J. (1991) Soma neurofilament immunoreactivity is related to cell size and fibre conduction velocity in rat primary sensory neurons. **J Physiol (Lond)** 435: 41-63.

Leah, J. D., Herdegen, T., Bravo, R. (1991) Selective expression of Jun proteins following axotomy and axonal transport block in peripheral nerves in the rat: evidence for a role in the regeneration process. **Brain Res** 566 (1-2): 198-207.

Lewin, G. R., Barde, Y. A. (1996) Physiology of the neurotrophins. **Annu Rev Neurosci** 19: 289-317.

Loeb, D. M., Tsao, H., Cobb, M. H., Greene, L. A. (1992) NGF and other growth factors induce an association between ERK1 and the NGF receptor, gp140prototrk. **Neuron** 9(6): 1053-1065.

Loeb, D. M., Stephens, R. M., Copeland, T., Kaplan, D. R., Greene, L. A. (1994) A Trk nerve growth factor (NGF) receptor point mutation affecting interaction with phospholipase C-Gamma labolishes NGF-promoted peripherin induction but not neurite outgrowth. **J Biol chem** 269 (12): 8901-8910.

Levi-Montalcini, R., and Hamburger, V. (1953) Selective growth-stimulation effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. **J. Exp. Zoo.** 116:321-362.

Lillycrop, K. A., Latchman, D. S. (1991a) Cloning and sequencing of the rat Oct-1 POU box. **Nucleic Acids Res** 19(13): 3744.

Lillycrop, K.A., Dent, C. L., Wheatley, S. C., Beech, M. N., Ninkina, N. N., Wood, J. N., Latchman, D. S. (1991b) The octamer-binding protein Oct-2 represses HSV immediate-early genes in cell lines derived from latently infectable sensory neurons. **Neuron** 7(3): 381-390.

Lillycrop, K.A., Budrahan, V. S., Lakin, N. D., Terrenghi, G., Wood, J. N., Polak, J. M., Latchman, D. S. (1992) A novel POU family transcription factor is closely related to Brn-3 but has a distinct expression pattern in neuronal cells. **Nucleic Acids Res** 20(19): 5093-5096.

Lillycrop, K. A., Estridge, J. K., Latchman, D. S. (1994) Functional interaction between different isoforms of the Oct-2 transcription factor expressed in neuronal cells. **Biochem J** 298(Pt 1): 245-248.

Lindsay, R. M., Shooter, E. M., Radeke, M. J., Misko, T. P., Dechant, G., Thoenen, H., and Lindholm, D. (1990) Nerve growth factor regulates expression of the nerve growth receptor gene in adult neurons. **Eur. J. Neurosci.** 2: 389-396.

Lindsay, R.M. (1994) Neurotrophins and receptors. **Prog Brain Res** 103: 3-14.

Loeb, D. M., Tsao, H., Cobb, M. H., Greene, L. A. (1992) NGF and other growth factors induce an association between ERK1 and the NGF receptor, gp140prototrk. **Neuron** 9(6): 1053-1065.

Manivannan, S., Terakawa, S. (1994) Rapid sprouting of filopodia in nerve terminals of chromaffin cells, PC12 cells, and dorsal root neurons induced by electrical stimulation. **J. Neurosci.** 14(10): 5917-5928.

Marshall, M. (1995) Interactions between Ras and Raf: key regulatory proteins in cellular transformation. **Mol Reprod Dev** 42(4): 493-499.

Meakin, S. O., Suter, U., Drinkwater, C. C., Welcher, A. A., Shooter, E. M. (1992) The rat trk protooncogene product exhibits properties characteristic of the slow nerve growth factor receptor. **Proc Natl Acad Sci U S A** 89(6): 2374-2378.

Mearow, K. M., Kril, Y., Diamond, J. (1993) Increased NGF mRNA expression in denervated rat skin. **Neuroreport** 4(4): 351-354.

Mearow, K. M. Kril, Y., Gloster, A., Diamond, J. (1994) Expression of NGF receptor and GAP-43 mRNA in DRG neurons during collateral sprouting and regeneration of dorsal cutaneous nerves. **J Neurobiol** 25(2): 127-142.

Mearow, K. M., Kril, Y. (1995) Anti-NGF treatment blocks the upregulation of NGF receptor mRNA expression associated with collateral sprouting of rat dorsal root ganglion neurons. **Neurosci Lett** 184(1): 55-58.

Mearow, K. M. (1998) The effects of NGF and sensory nerve stimulation on collateral sprouting and gene expression in adult sensory neurons. Exp. Neurol (in press).

Merlio, J. P., Ernfors, P., Jaber, M., Persson, H. (1992) Molecular cloning of rat trkC and distribution of cells expressing messenger RNAs for members of the trk family in the rat central nervous system. **Neuroscience** 51(3): 513-532.

Michael, G. J., Priestley, J. V. (1996) Expression of trkA and p75 nerve growth factor receptors in the adrenal gland. **Neuroreport** 7(10): 1617-1622.

Michel, J. B., Ordway, G. A., Richardson, J. A., Williams, R. S. (1994) Biphasic induction of immediate early gene expression accompanies activity-dependent angiogenesis and myofiber remodeling of rabbit skeletal muscle. **J. Clin. Invest.** 94: 277-285.

Michael, G. J., Joshi, R. K., Bennett, D. L. H., McMahon, S. B., Priestley, J. V. (1996) Nerve growth factor (NGF) induces c-Fos protein in Trk A immunoreative dorsal root ganglion cells *in vivo*. Soc. Neurosci. Abstr 22:108.

Milbrandt, J. (1987) A nerve growth factor-induced gene encodes a possible transcriptional

regulatory factor. **Science** 238 (4828): 797-799.

Miller, F. D., Speelman, A., Mathew, T. C., Fabian, J., Chang, E., Pozniak, C., Toma, J. G. (1994) Nerve growth factor derived from terminals selectively increases the ratio of p75 to trkA NGF receptors on mature sympathetic neurons. **Dev Biol** 161(1): 206-217.

Mundschau, L. J., Forman, L. W., Weng, H., Faller, D. V. (1994) Platelet-derived growth factor (PDGF) induction of egr-1 is independent of PDGF receptro autophosphorylation on tyrosine. **J Bio Chem** 269 (23): 16137-16142.

Ninkina, N. N., Buchman, V. L., Akopian, A. N., Lawson, S. N., Yamamoto, M., Campell, E., Corcoran, L., Wood, J. N. (1995) Nerve growth factor-regulated properties of sensory neurons in Oct-2 null mutant mice. **Brain Res Mol Brain Res** 33 (2): 233-244.

Nixon, B.J., Doucette, R., Jackson, P.C., Diamond, J. (1984) Impulse activity evokes precocious sprouting of nociceptive nerves into denervated skin. **Somatosens Res** 2(2): 97-126.

Obermeier, A., Lammers, R., Wiesmuller, K. H., Jung, G., Schlessinger, J., Ullrich, A.(1993a) Identification of Trk binding sites for SHC and phosphatidylinositol 3'-kinase and formation of a multimeric signaling complex. **J Biol Chem** 268(31): 22963-22966.

Obermeier, A.,, Halfter, H., Wiesmuller, K. H., Jung, G., Schlessinger, J., Ullrich, A. (1993b) Tyrosine 785 is a major determinant of Trk--substrate interaction. **EMBO J** 12(3): 933-94..

Obermeier, A., Bradshaw, R. A., Seedorf, K., Choidas, A., Schlessinger, J., Ullrich, A. (1994) Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLC gamma. **EMBO J** 13(7): 1585-1590.

Obermeier, A., Bradshaw, R. A., Seedorf, K., Choidas, A., Schlessinger, J., Ullrich, A. (1995) Definition of signals for neuronal differentiation. **Ann N Y Acad Sci** 766: 1-17.

Ohmichi, M., Decker, S. J., Pang, L., Saltiel, A. R. (1991a) Nerve growth factor binds to the 140 kd trk proto-oncogene product and stimulates its association with the src homology domain of phospholipase C gamma 1. **Biochem Biophys Res Commun** 179(1): 217-223 .

Ohmichi, M., Decker, S. J., Pang, L., Saltiel, A. R. (1991) Phospholipase C-gamma 1 directly associates with the p70 trk oncogene product through its src homology domains. **J Biol Chem** 266(23): 14858-14861.

Ohmichi, M., Decker, S. J., Saltiel, A. R. (1992) Activation of phosphatidylinositol-3 kinase

by nerve growth factor involves indirect coupling of the trk proto-oncogene with src homology 2 domains. **Neuron** 9(4): 769-777.

Peng, Z. C., Chen, S., Bertini, G., Schmidt, H. H., Bentivoglio, M. (1994) Co-localization of nitric oxide synthase and NGF receptor in neurons in the medial septal and diagonal band nuclei of the rat. **Neurosci Lett** 166(2): 153-156.

Peng, X., Greene, L. A., Kaplan, D. R., Stephens, R. M. (1995) Deletion of a conserved juxtamembrane sequence in Trk abolishes NGF-promoted neuritogenesis. **Neuron** 15(2): 395-406.

Rabin, S. J., Cleghon, V., Kaplan, D. R. (1993) SNT, a differentiation-specific target of neurotrophic factor-induced tyrosine kinase activity in neurons and PC12 cells. **Mol Cell Biol** 13(4): 2203-2213.

Radeke, M. J., Misko, T. P., Hsu, C., Herzenberg, L. A., Shooter, E. M. (1987) Gene transfer and molecular cloning of the rat nerve growth factor receptor. **Nature** 325(6105): 593-597.

Rasouly, D., Lazarovici, P. (1995) Neurotrophin receptors: signal transduction of neurotrophin receptors: basic concepts and available pharmacological tools. Neural Note Spring from Promega. p3.

Richardson, P. M., Issa, V. M., Riopelle, R. J. (1986) Distribution of neuronal receptors for nerve growth factor in the rat. **J Neurosci** 6(8): 2312-2321.

Riopelle, R. J., Verge, V. M., Richardson, P. M. (1987) Properties of receptors for nerve growth factor in the mature rat nervous system. **Brain Res** 427(1): 45-53.

Ritter, A. M., Lewin, G. R., Mendell, L. M.(1993) Regulation of myelinated nociceptor function by nerve growth factor in neonatal and adult rats. **Brain Res Bull** 30(3-4): 245-249.

Robertson, H. A. (1992) Immediate-early genes, neuronal plasticity, and memory. **Biochem. Cell Biol.** 70: 729-737.

Ruppert, S., Cole, T. J., Boshart, M., Schmid, E., Schutz, G. (1992) Multiple mRNA isoforms of the transformation activator protein CREB: generation by alternative splicing and specific expression in primary spematocytes. **EMBO J** 11(4), 1503-1512.

Rusak, B., Robertson, H. A., Wisden, W, Hunt, S. P. (1990) Light pulses that shift rythms induce gene expression in the suprachiasmatic nucleus. **Science** 248: 1237-1240.

Rusanescu, G., Qi, H., Thomas, S. M., Brugge, J. S., Halegoua, S. (1995) Calcium influx induces neurite growth through a Src-Ras signaling cassette. **Neuron** 15(6): 1415-1425.

Saffen, D. W., Cole, A, J., Worley, P. F., Christy, B. A., Ryder, K., Baraban, J. M. (1988) Convulsant-induced increase in transcription factor messenger RNAs in rat brain. **Proc Natl Acad Sci U S A** 85(20): 7795-7799.

Sagar, S. M., Sharp, F. R., Curran, T. (1988) Expression of c-fos protein in brain: metabolic mapping at the cellular level. **Science** 240(4857): 1328-1331.

Sagar, S. M., Sharp, F. R. (1993) Early response genes as Markers of Neuronal activity and growth factor action. **Advances in Neurology** 59: 273-284.

Segal, R. A., Bhattacharyya, A., Rua, L. A., Alberta, J. A., Stephens, R. M., Kaplan, D. R., Stiles, C. D. (1996) Differential utilization of Trk autophosphorylation sites. **J Biol Chem** 271(33): 20175-20181.

Segal, R. A., Greenberg, M. E. (1996) Intracellular signaling pathways activated by neurotrophic factors. **Annu Rev Neurosci** 19: 463-489.

Shelton, D. L., Reichardt, L. F. (1984) Expression of the beta-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. **Proc Natl Acad Sci U S A** 81(24): 7951-7955.

Sheng, M., Dougan, S. T., McFadden, G., Greenberg, M. E. (1988) Calcium and growth factor pathways of c-fos transcriptional activation require distinct upstream regulatory sequences. **Mol Cell Biol** 8(7): 2787-2796.

Sheng, M., Greenberg, M. E. (1990) The regulation and function of c-fos and other immediate early genes in the nervous system. **Neuron** 4(4): 477-485.

Sheng, M., McFadden, G., Greenberg,, M. E. (1990) Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. **Neuron** 4(4): 571-582.

Sheng, M., Thompson, M. A., Greenberg, M. E. (1991) CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. **Science** 252(5011): 1427-1430.

Sheng, M., Fields, R. D., Nelson, P. G. (1993) Specific regulation of immediate early genes by patterned neuronal activity. **J. Neurosci. Res.** 35: 459-467.

Shiromani, P. J., Magner, M., Winston, S., Charness, M. E. (1995) ime course of phosphorylated CREB and Fos-like immunoreactivity in the hypothalamic supraoptic nucleus after salt loading. **Brain Res Mol Brain Res** 29(1): 163-171.

Solem, M., McMahon, T., Messing, R. O. (1995) Depolarization-induced neurite outgrowth in PC12 cells requires permissive, low level NGF receptor stimulation and activation of calcium/calmodulin-dependent protein kinase. **J Neurosci** 15(9): 5966-5975.

Soltoff, S. P., Rabin, S. L., Cantley, L. C., Kaplan, D. R. (1994) Nerve growth factor promotes the activation of phosphatidylinositol 3-kinase and its association with the trk tyrosine kinase. **J Biol Chem** 267 (24): 17472-17477.

Stephens, R. M., Loeb, D. M., Copeland, T. D., Pawson, T., Greene, L. A., Kaplan, D. R. (1994) Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma 1 to mediate NGF responses. **Neuron** 12(3): 691-705 (1994)

Stirling, R. V. (1973) The effect of increasing the innervation field sizes of nerves on their reflex response time in salamanders. **J Physiol (Lond)** 229(3), 657-679.

Stoeckel, K., Schwab, M., Thoenen, H. (1975) Specificity of retrograde transport of nerve growth factor (NGF) in sensory neurons: a biochemical and morphological study. **Brain Res 89(1): 1-14.**

Sukhatme, V. P., Cao, X. M., Chang, L. C., Tsai-Morris, C. H., Stamenkovich, D., Ferreira, P. C., Cohen, D. R., Edwards, S. A., Shows, T. B., Curran, T. (1988) A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. **Cell** 53(1): 37-43.

Taniuchi, M., Clark, H. B., Schweitzer, J. B., Johnson, E. M. Jr. (1988) Expression of nerve growth factor receptors by Schwann cells of axotomized peripheral nerves: ultrastructural location, suppression by axonal contact, and binding properties. **J Neurosci** 8(2): 664-681.

Taylor, L. K., Marshak, D.R., Landreth, G. E. (1993) Identification of a nerve growth factor- and epidermal growth factor-regulated protein kinase that phosphorylates the protooncogene product c-Fos. **Proc Natl Acad Sci U S A** 90(2): 368-372.

Theriault, E., Diamond, J. (1988) Nociceptive cutaneous stimuli evoke licalized contraction in a skeletal muscle. **J. Neurophysiol.** 60:446-462

Tolkovsky, A. (1997) Neurotrophic factors in action - new dogs and new tricks. **TINS** 20 (1): 1-3.

Verdi, J. M., Birren, S. J., Ibanez, C. F, Persson, H., Kaplan, D. R., Benedetti, M., Chao, M. V., Anderson, D. J. (1994) p75LNGFR regulates Trk signal transduction and NGF-induced neuronal differentiation in MAH cells. **Neuron** 12(4): 733-745

Vetter, M. L., Martin-Zanca, D, Parada, L. F., Bishop, J.M., Kaplan, D. R. (1991) Nerve growth factor rapidly stimulates tyrosine phosphorylation of phospholipase C-gamma 1 by a kinase activity associated with the product of the trk protooncogene. **Proc Natl Acad Sci U S A** 88(13): 5650-5654.

Weddell, G., Guttmann, L., and Gutman, E (1941) The local extension of nerve fibers into denervated areas of skin. **J. Neurol. Neurosurg. Psychiat.** 4:206-255.

Wisden, W., Errington, M. L., Williams, S., Dunnett, S. B., Waters, C., Hitchcock, D., Evan, G., Bliss, T. V., Hunt, S. P. (1990) Differential expression of immediate early genes in the hippocampus and spinal cord. **Neuron** 4(4): 603-614.

Wood, J. N., Lillycrop, K. A., Dent, C. L., Ninkina, N. N., Beech, M. M., Willoughby, J. J., Winter, J., Latchman, D. S. (1992) Regulation of expression of the neuronal POU protein Oct-2 by nerve growth factor. **J Biol Chem** 267(25):17787-17791.

Yamamoto, K. K., Gonzalez, G. A., Biggs, W. H. 3d., Montminy, M. R. (1988) Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. **Nature** 334(6182): 494-498.

Yao, R., Cooper, G. M. (1995) Regulation of the Ras signaling pathway by GTPase-activating protein in PC12 cells. **Oncogene** 11(8): 1607-1614.

Yoon, J. K., Lau, L. F. (1994) Involvement of JunD in transcriptional activation of the orphan receptor gene nur 77 by nerve growth factor and membrane depolarization in PC12 cells. **Mol Cell Biol** 14 (12): 7731-7743.