THE REGULATION AND FUNCTION OF INTERLEUKIN-6 AFTER PERIPHERAL NERVE TRANSECTION

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ABSTRACT

Previous work has demonstrated that interleukin-6 (IL-6) is synthesized in approximately one-third of lumbar dorsal root ganglion neurons during the first week after sciatic nerve transection. The present study was undertaken to determine the signals influencing IL-6 synthesis in the peripheral nerve and the functional consequences of IL-6 production in response to nerve injury. The first sets of experiments demonstrate that IL-6 is induced by an injury factor arising from the nerve stump rather than by interruption of normal retrograde trophic support from the target tissues or distal nerve stump. Furthermore, the use of agents that stimulate or inhibit degranulation of mast cells suggest that they release a factor(s) that can induce IL-6 mRNA in the sciatic nerve after injury. Additional studies propose that endogenous IL-6 performs at least two important functions in sensory neurons after nerve injury. First, IL-6 supports neuronal survival in cooperation with brain-derived neurotrophic factor (BDNF). Specifically, IL-6 is required for *de novo* synthesis of BDNF mRNA in injured sensory neurons, and BDNF mediates the survival of DRG neurons in response to IL-6. Second, endogenous IL-6 mediates the development of behavioural changes associated with neuropathic pain. Following chronic constriction injury to the sciatic nerve, IL-6 deficient mice do not develop thermal hyperalgesia or mechanical allodynia and exhibit changes in expression of several neuropeptides. These findings suggest that IL-6 may contribute to neuropathic pain by altering neuropeptide levels. The last sets of experiments were aimed at determining if IL-6 contributes to neuropathic pain by influencing the complement of ion channels expressed by DRG neurons. Preliminary findings characterizing and comparing the array of voltage-gated potassium currents and their properties in injured versus uninjured nociceptive neurons, show that these currents are not consistently altered by nerve transection. The lack of injury-induced changes in K+ channel expression suggests that this is not the mechanism through which IL-6 contributes to neuropathic pain.

RÉSUMÉ

Par le passé, il a été démontré qu'interleukine 6 (IL-6) était synthétisé par approximativement un tiers des neurones résidant dans les ganglions lombaires à racine dorsale (GRD), une semaine après le sectionnement du nerf sciatique. La présente étude a été entreprise afin de déterminer la nature des signaux qui influencent la synthèse d'IL-6 dans le nerf périphérique, ainsi que les effets découlant de la production d'IL-6 à la suite d'une lésion nerveuse. La première série d'expériences démontrent que l'expression d'IL-6 n'est pas dûe à une interruption du support trophique émanent des tissus cibles et agissant via la partie distale du nerf mais est plutôt induite par des facteurs provenant du tronçon nerveux proximal. De plus, les résultat obtenus en utilisant des molécules stimulant ou empêchant la dégranulation des mastocytes suggèrent que celles-ci sécrètent un ou des facteurs pouvant induire la transcription de l'ARN messager d'IL-6 dans le nerf sciatique après une lésion. Nous croyons que l'IL-6 endogène accompli au moins deux functions importantes au sein des neurones sensorielles après le sectionnement. En premier lieu, IL-6 supporterait la survie des neurones en coopérant avec BDNF (Brain-Derived Neurotrophic Factor) de façon réciproque. En effet, IL-6 est requis pour l'induction de la synthèse de novo de l'ARN messager de BDNF et l'expression de BDNF est nécessaire pour qu'IL-6 favorise la survie neuronale. En second lieu, l'IL-6 endogène est important dans le développement de changements de comportements associés avec la douleur neuropathique. Il appert que les souris déficiente en IL-6 ne développent pas, après une constriction chronique du nerf sciatique, d'hyperalgésie thermale ou d'allodynie mécanique et exhibent des changements dans les niveaux d'expression de plusieurs neuropeptides. Ces résultats suggèrent qu'IL-6 pourrait contribuer au processus de douleur neuropathique en altérant les niveaux d'expression de neuropeptides. La dernière partie de cette étude avait pour but de déterminer si IL-6 contribue au processus de douleur neuropathique en influençant les niveaux d'expression des canaux ioniques des neurones du GRD. Nos résultats, encore préliminaires, ont permis la caractérisation de multiple courants potassiques dépendant du voltage (voltage-gated potassium currents) au sein de ces neurones. Par contre, aucune altération de ces courants ne fut déceler lorsque les

enregistrements de neurones provenant de RGD controle vs lésionnés furent comparés. Ainsi, les effets d'IL-6 durant le processus de douleur neuropathique ne semblent pas être liés à des altérations majeures des courants potassiques dépendant du voltage.

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Finally, I would like to thank my parents, my brothers and all my friends, especially Mathieu, who have guided and encouraged me along the way.

CONTRIBUTIONS OF AUTHORS

As required when a manuscript-based thesis includes co-authored papers, the following section is included from the "Guidelines for Thesis Preparation» of McGill University."

In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

The first two manuscripts included in this thesis were jointly written by Dr. Patricia Murphy and Dr. Peter Richardson.

In the first manuscript, entitled "Nature of the retrograde signal from injured nerves that induces Interleukin-6 mRNA in neurons," I contributed the quantifications of IL-6 mRNA labelling by *in situ* hybridization in Figure 3 and 5. Dr. Patricia Murphy contributed the *in situ* hybridizations (Figure 1, 2 and 4) and the neuronal counts (Table 1) along with Rob Johnson.

In the second manuscript, entitled "Interdependent actions of Interleukin-6 and Brain-derived Neurotrophic Factor on rat and mouse primary sensory neurons," I contributed the IL-6/BDNF colocalization data presented in Figure 3 and Figure 4, and the quantification of BDNF mRNA labelling by *in situ* hybridization in Figure 5. Dr. Patricia Murphy performed the Southern blot/RT-PCR (Figure 1), the immunocytochemistry for gp80 (Figure 1), and the survival assays of embryonic DRG neurons (Figure 2).

The third manuscript, entitled "Endogenous Interleukin-6 contributes to hypersensitivity to cutaneous stimuli and changes in neuropeptides associated with chronic nerve constriction in mice" was co-authored by Dr. Matt Ramer and Dr. Patricia Murphy. I contributed the quantification of galanin mRNA labelling in DRG from uninjured rats (Figure 7, *bottom*) and in DRG from wild-type and IL-6 deficient mice (see text), and the

neuronal counts of galanin mRNA-positive neurons (see text). Dr. Patricia Murphy performed the *in situ* hybridizations for IL-6 mRNA (Figure 1), substance P mRNA (Figure 3) and galanin mRNA (Figure 7A and B). Dr. Matt Ramer performed the behavioural testing in control and IL-6 deficient mice (Figure 2), the immunohistochemistry for substance P (Figure 4) and galanin (Figure 6) and the quantification of the immunohistochemical data for galanin (Figure 5).

I developed the cell culture protocol used in Chapter 5 and performed all the animal surgeries, cell cultures, patch clamp experiments and data analysis presented.

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ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
AMPA	alpha-amino-3-hydroxy-5-methyl-isoxazole
AP	action potential
AP-1	activation protein - 1
BDNF	brain-derived neurotrophic factor
BK	bradykinin
CAMK	calcium/calmodulin kinase
CAP	compound action potential
CCI	chronic constriction injury
CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
$CGRP_{1/2}$	calcitonin gene-related peptide receptor $_{1/2}$
CNTF	ciliary neurotrophic factor
CREB	cAMP response element binding protein
CT-1	cardiotrophin-1
DCN	dorsal column nuclei
DH	dorsal horn
DR	delayed-rectifier
DRG	dorsal root ganglia
E16	embryonic day 16
EAA	excitatory amino acids
EM	electron microscopy
EPSP	excitatory post-synaptic potential
aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
GABA	gamma-aminobutyric acid
GAL	galanin
GDNF	glial cell line-derived neurotrophic factor
GM-CSF	granulocyte/macrophage colony stimulating factor
H/R	hypoxia/reoxygenation
IFN	interferon
IB(4)	isolectin B(4)
IL-1	interleukin-1
IL-6	interleukin-6
IL-6R	interleukin-6 receptor (gp80)
IR	immunoreactivity
JAK	janus kinase
L4, L5, L6	lumbar 4^{th}, 5^{th} and 6^{th}
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
MAPK	mitogen-associated protein kinase
MEK	MAPK kinase
mGLUR	metabotropic glutamate receptor

MM	multiple myeloma
NF-κB	nuclear factor kappa B
NF-IL6	nuclear factor – interleukin-6
NGF	nerve growth factor
NK_1	neurokinin 1 receptor
NKA	neurokinin A
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NPY	neuropeptide tyrosine
NR2A/B	NMDA receptor 2A/B
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
OSM	oncostatin M
PC12	pheochromocytoma
PDGF	platelet-derived growth factor
PKC	protein kinase C
PG	prostaglandin
RBP-Jκ	recombinant signal sequence binding protein J kappa
SCN	sciatic cryoneurolysis
SNL	spinal nerve ligation
SOCS	suppressor of cytokine signalling
SOM	somatostatin
SP	substance P
STAT	signal transducer and activator of transcription
TGF-β	transforming growth factor beta
TNF-∝	tumor necrosis factor alpha
TTX-R	tetrodotoxin-resistent
TTX-S	tetrodotoxin-sensitive
VIP	vasoactive intestinal peptide

CHAPTER 1: GENERAL INTRODUCTION

1.0 Interleukin-6

Interleukin-6 (IL-6) is a member of the neuropoietic family of cytokines that includes ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin-11 (IL-11), cardiotrophin-1 (CT-1), and oncostatin M (OSM) (Taga and Kishimoto, 1997). This family of molecules has been grouped together based on similarities in tertiary structure and sharing of receptor subunits (Bazan, 1990; Ip and Yancopoulos, 1992; Patterson, 1992; Davis and Yancopoulos, 1993; Stahl et al., 1995). These cytokines are expressed in a wide range of tissues, including the nervous system, where they elicit similar and overlapping biological responses. IL-6 is a multifunctional cytokine that regulates many aspects of the immune and inflammatory response. Recently, IL-6 has been implicated in physiological and pathological events in the central and peripheral nervous system. This thesis will focus on efforts to define the regulation and function of IL-6 in the PNS, where it has been shown to enhance neuronal survival and regeneration and to participate in neuropathic pain. Three topics will be highlighted: the nature of the injury factor that leads to the induction of IL-6, the mechanism by which IL-6 supports the survival of sensory neurons, and the contribution of IL-6 to neuropathic pain.

1.1 IL-6 Receptor Complex

Neuropoietic cytokines use the common receptor subunit gp130 for signal transduction, which accounts for their overlapping biological activities (for review see Taga and Kishimoto, 1997). Specificity is achieved through different high-affinity cytokine receptors (the α-receptors), which bind their particular ligand before recruiting gp130. IL-6 initially binds to the IL-6R (gp80), and then the IL-6/IL-6R complex recruits and associates with gp130 (Yamasaki et al., 1988), leading to the homodimerization of gp130 and activation of signal transduction (Murakami et al., 1993). Recent evidence suggests that this functional signalling complex is likely a hexamer composed of two molecules each of IL-6, IL-6R, and gp130 (Ward et al., 1994).

The IL-6R is found as a transmembrane protein or as a soluble receptor (sIL-6R) (Honda et al.1992; Novick et al., 1989). The soluble form is generated either by alternative mRNA splicing or by proteolytic cleavage of the extracellular domain of the membrane-bound form (Rose-John and Heinrich, 1994; Mackiewicz et al., 1995). Both forms of the receptor have been shown to bind IL-6 with a similar affinity. Importantly, the IL-6/sIL-6R complex maintains its ability to associate with gp130 and to initiate a normal signal transduction cascade (Narazaki et al., 1993). This suggests that cells expressing gp130, but not the IL-6R, may still be able to respond to IL-6 in the presence of sIL-6R.

In the nervous system, the distribution of IL-6R is restricted to certain neuronal and glial populations in a regionally dependent manner (Gadient and Otten, 1994; Gadient and Otten, 1996; Thier et al., 1999). In contrast, gp130 is expressed ubiquitously. Thus, the presence of sIL-6R could significantly expand the population of cells that are IL-6 responsive. This effect has been demonstrated in several studies (Hirota et al., 1996; Hirota et al., 1995; Ikeda et al., 1996; Thier et al., 1999). For instance, endogenous IL-6 was able to promote the survival of newborn rat DRG neurons *in vitro,* if soluble IL-6R was supplied exogenously (Thier et al., 1999).

A soluble form of gp130 (sgp130) has also been identified (Narazaki et al., 1993; Mullberg et al., 1993). It is thought to antagonize the effects of IL-6 by binding the IL-6/sIL-6R complex in solution, thus inhibiting its ability to associate with gp130 at the membrane (Saito et al., 1992). Thus, sgp130 may play a role in dampening the effects of IL-6 *in vivo.*

1.2 IL-6 Receptor Signalling

IL-6 signals through the JAK/STAT pathway and the Ras-dependent mitogen activated kinase (Ras/MAPK) pathway (Kishimoto et al., 1994; Kishimoto et al., 1995) in neurons (see Figure 1). The JAK/STAT pathway is used by all neuropoietic cytokines, interferons, and many other cytokines and growth factors (Lutticken et al., 1994; Stahl et al., 1994; Darneli, Jr. et al., 1994). It consists of a family of non-receptor tyrosine kinases

of the Janus kinase family (Jak1-3 and Tyk2), and latent cytoplasmic transcription factors of the signal transducers and activators of transcription family (STAT1-5a,b and STAT6).

Jak1, Jak2, and Tyk2 are associated with the cytoplasmic domain of gp130. Homodimerization of the receptor results in reciprocal tyrosine phosphorylation, and consequent activation of the JAK's. Activated JAK's phosphorylate the cytoplasmic tail of gp130 on tyrosine residues that serve as docking sites for SH2 domain-containing proteins, in particular, the STAT's. IL-6 specifically recruits STAT1 and STAT3 to these docking sites, where they are phosphorylated by the JAK's. Serine phosphorylation is also required for full activation of STAT1 and 3 (Zhang et al., 1995; Boulton et al., 1995; Wen et al., 1995), which appears to be mediated by protein kinase C (Jain et al., 1999). Once activated, STAT1 and STAT3 form homo- or heterodimers and translocate to the nucleus where they regulate transcription of target genes directly (Stahl et al., 1995; Gerhartz et al., 1996). STAT's upregulate many IL-6 responsive genes, including multiple acute phase genes (Wegenka et al., 1993) and immediate-early genes (Lord et al.1991; Nakajima and Wall, 1991; Yuan et al., 1994).

There are several molecules that are involved in negative regulation of the JAK/STAT pathway. The protein PIAS3 has been shown to inhibit activated STAT3 as well as STAT3-mediated gene activation (Chung et al., 1997a). A similar protein has been identified for STAT1. Furthermore, suppressors-of-cytokine-signalling proteins (SOCS) comprise a family of negative-feedback inhibitors that act on this pathway. Specifically, IL-6 induces transcription of SOCS1 and once expressed, SOCS1 inhibits phosphorylation of gp130, STAT1 and STAT3 by interacting with the kinase domain of Jak2 (Endo et al., 1997). SOCS1 interacts with other members of the JAK family as well.

As stated previously, IL6 has also been shown to signal through the Ras/MAPK pathway (Nakafuku et al., 1992; Boulton et al., 1994; Daeipour et al., 1993). Activation of gp130 leads to the successive activation of Shc, Grb2, Sos1, Ras, Raf1, MEK and MAPK, by phosphorylation at serine or threonine residues. In this cascade, Shc and Grb2 function as adaptor proteins, while Sos1 (Son of Sevenless 1), a Ras GTP-GDP exchange factor, converts inactive GDP-bound Ras to the active GTP-bound form. Once activated, MAPK translocates to the nucleus where it phosphorylates the transcription factors NF-

IL-6 (nuclear factor IL-6) and AP-1 (activation protein-1) (Nakajima et al., 1993). These two transcription factors bind to their recognition sites in the promoter region of IL-6 responsive genes, thereby modulating transcription (Akira and Kishimoto, 1992; Nakajima et al., 1993).

Recently, it has been shown that the phosphotyrosine phosphatase SHP2 functions as an adaptor protein in the recruitment of the Grb2/Sos complex (Ali et al., 1997; Tauchi et al., 1996; Yin et al., 1997). This protein is also recruited to gp130 (via it's SH2 domain) and phosphorylated by JAK's following IL-6 stimulation, and may serve as a link between the JAK/STAT and Ras/MAPK pathways. A modulatory effect of SHP2 on IL-6 responsive genes has recently been demonstrated (Symes et al., 1997; Kim and Baumann, 1999).

Recently, IL-6 has been shown to activate the PI3K/Etk (Qiu et al., 1998), PI3K/Akt (Chen et al., 1999) and the SAPK (stress-activated protein kinase) (Zauberman et al., 1999) signal transduction pathways, in various non-neuronal cells. Specifically, the PI3K/Ekt pathway is activated by IL-6 in prostate cancer cells and functions to promote neuroendocrine-like differentiation of these cells (Qiu et al., 1998). The PI3K/Akt pathway is activated in response to IL-6 in the Hep3B hepatoma cell line and serves to protect these cells against TGFβ-induced apoptosis (Chen et al., 1999). Finally, p38 MAPK (a member of the SAPK family of proteins) and a downstream effector molecule, MAPKAP-K2, are phosphorylated and activated by IL-6 in HepG2 hepatoma cells, leading to secretion of acute phase proteins, and in B9 hybridoma cells, leading to IL-6 dependent proliferation (Zauberman et al., 1999).

These findings emphasize the complexity of events downstream of IL-6R/gp130 activation and demonstrate that cells stimulated by IL-6 respond in a variety of different ways. IL-6 may mediate cell survival, growth promotion, differentiation, growth arrest, and specific gene expression in a given cell type, though it is often unclear which of these signal transduction pathways control these different biological responses and to what degree they cooperate. A recent study suggests that IL-6 can trigger growth via the Ras/MAPK cascade in IL-6-dependent B9 and MM cell lines, as inhibition of the MAPK cascade inhibited proliferation of these cells (Ogata et al., 1997). On the other hand, IL-6

stimulates neurite outgrowth in PC12 cells, a cell line used to study neuronal differentiation, via activation of STAT3 (Wu and Bradshaw, 1996a; Wu and Bradshaw, 1996b; Ihara et al., 1997). STAT3 activity is also critical for growth arrest and terminal differentiation of macrophages, as well as gp130-mediated anti-apoptotic signals in some B cells (Nakajima et al., 1996; Yamanaka et al., 1996; Fukada et al., 1996). Thus, it remains to be determined which of these numerous signal transduction pathways mediate the effects of IL-6 in different cell types.

1.3 Regulation of IL-6 Expression

IL-6 gene expression is strongly induced by tissue injury and in many disease states. As a result, there is a growing body of work aimed at understanding how IL-6 is regulated. Many types of cells synthesize IL-6 in response to extracellular molecules such as cytokines, growth factors and other mediators of inflammation. These include IL-1β (Content et al., 1985; Zhang et al., 1990), TNFα (Kohase et al., 1986; Brach et al., 1990), PDGF (Kohase et al., 1987), LIF (Gruss et al.1992; Villiger et al.1993), OSM (Brown et al.1991), CT-1 (Robledo et al.1997), GM-CSF (Cicco et al., 1990), IFNγ (Faggioli et al., 1997a; Costanzo et al., 1999), stem cell factor (Gagari et al., 1997), histamine (Mor et al., 1995; Takamatsu and Nakano, 1998), substance P (Lieb et al., 1998), leukotriene B4 (Brach et al., 1992), prostaglandins (Leal-Berumen et al., 1995; Fiebich et al., 1997) and reactive oxygen species (Shibanuma et al., 1994). Bacterial products (Bauer et al., 1988; Fong et al., 1989), viral infection (Ray et al., 1988), and cyclohexamide (Faggioli et al., 1997b) also stimulate IL-6 synthesis. In contrast, IL-4 (Herrmann et al., 1991), IL-10 (de Waal et al., 1991), IL-13 (Minty et al., 1993), glucocorticoids (Ray et al., 1990), estrogen (Stein and Yang, 1995), retinoblastoma and p53 (Santhanam et al., 1991), and adenoviral E1A proteins (Janaswami et al., 1992) inhibit IL-6 production.

Generally, the effects of these activators and inhibitors are cell-type specific. The degree to which some of these mediators control IL-6 expression in the nervous system is yet to be determined. However, cytokines (IL-1β, TNFα, IFNγ, IL-13) (Ringheim et al., 1995; Benveniste et al., 1990; Chai et al., 1996; Sawada et al., 1992; Sebire et al., 1996), prostaglandins (PGE1 and 2) (Fiebich et al., 1997), bacterial and viral pathogens (Romero

et al., 1993; Gottschall et al., 1992), neurotransmitters (noradrenaline) (Maimone et al., 1993; Norris and Benveniste, 1993), and several neuropeptides (histamine, substance P, VIP) (Lieb et al., 1998; Gitter et al., 1994; Cadman et al., 1994; Palma et al., 1997; Grimaldi et al., 1994; Maimone et al., 1993) can upregulate IL-6 in glial cells and/or neurons.

The transcription factors NF-κB, NF-IL6, AP-1, Sp1 and the repressor RBP-Jκ all interact with the promoter of IL-6, thereby regulating transcription levels. Analysis of the IL-6 gene promoter revealed a region of homology to the c-fos serum response element, which encompasses an NF-IL6 binding site (Akira et al., 1990), a multiple response element (MRE) (Ray et al. 1989), and a potential recognition sequence for the *ets* family of transcription factors (Seth et al., 1992). Upstream of the c-fos homology region there is an AP-1 consensus sequence, as well as two glucocorticoid response homologies (GRE) (Tanabe et al., 1988), while the downstream region contains a κB site and a potential GATA-helix-loop-helix (HLH) site (Libermann and Baltimore, 1990; Shimizu et al., 1990; Zhang et al., 1990). Additive, cooperative, and competitive effects of these different transcription factors determine the spatial and temporal distribution of IL-6.

The NF-κB/Rel family of transcription factors consists of five members: NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel (for review see Ghosh et al., 1998). NF-κB is constitutively expressed in most cell-types, including neurons and glia. It is located primarily in the cytoplasm as an inactive homo- or heterodimer, associated with the inhibitory protein IκB (Baeuerle and Baltimore, 1988; Baeuerle et al., 1988). IκB inhibits NF-κB by masking its nuclear localization signal. In response to an activating signal, NF-κB is phosphorylated and IκB is cleaved, freeing it to translocate to the nucleus. Once in the nucleus, NF-κB binds the κB site in the promoter-region of various genes. κB sites have been identified in genes involved in the acute-phase response, inflammation, lymphocyte activation, and cell growth and differentiation. NF-κB is activated by a similar array of stimuli as IL-6, including cytokines, antigens, stress factors and viral and bacterial products. It has proven to be the dominant transcription factor regulating IL-6 expression in response to stimuli such as IL-1, TNFα, INFγ, LIF and leukotriene B4. It has also been shown to cooperate with other transcription factors, particularly NF-IL6, Sp1, and AP-1,

to control IL-6 synthesis (Stein and Baldwin, Jr., 1993; Stein et al., 1993; LeClair et al., 1992; Betts et al., 1993).

Transcriptional repressors act in concert with activators to determine IL-6 expression levels. The recombinant signal sequence binding protein Jκ (RBP-Jκ) is one of these. It is a ubiquitously expressed DNA binding protein that is an important gene regulatory factor involved in Notch signalling (for review see Honjo, 1996), expression of the Epstein-Barr virus nuclear Ag-2 protein (Henkel et al., 1994; Waltzer et al., 1994), and the adenovirus pIX gene (Dou et al., 1994). Studies have shown that RBP-Jκ is a constitutive silencer of the IL-6 gene in the absence of NF-κB (Plaisance et al., 1997; Kannabiran et al., 1997). It competitively binds the IL6-κB site thereby limiting the access of NF-κB. RBP-Jκ also functions as a modulator of NF-κB binding and transactivation after stimulation with TNFα (Plaisance et al., 1997).

1.4 Expression and Function of IL-6 Outside the Nervous System

IL-6 is expressed during development, but it is downregulated to undetectable levels in the adult. However, IL-6 synthesis can be induced in a variety of cell types, including B cells, T cells, monocytes/macrophages, mast cells, endothelial cells, fibroblasts, keratinocytes, chondrocytes, and mesangial cells (for review see Lotz, 1995). IL-6 was originally cloned as a factor that induced the differentiation of B cells into antibody-producing cells (Hirano et al., 1985; Hirano et al., 1986). Since then, IL-6 has been found to mediate the immune and inflammatory response (Hirano et al., 1986), the acute phase response (Gauldie et al., 1987), proliferation and differentiation of T cells and hematopoietic precursors (Ikebuchi et al., 1987; Shabo et al., 1988; Lotz et al., 1988), and the induction of fever (Helle et al., 1988). IL-6 deficient mice develop normally, but are compromised in their acute-phase response to tissue injury, resistant to the induction of fever, fail to control microbial infections and are defective in the production of T cell-dependent antibodies. (Kopf et al., 1994; Poli et al., 1994; Kozak et al., 1998). These findings suggest that IL-6 is required for an optimal protective response against trauma and infection. In addition to its classical functions, IL-6 is known to stimulate bone remodeling by osteoblasts (Kopf et al., 1994), protein breakdown in muscle (Goodman,

1994), and synthesis of metalloproteinase inhibitors by fibroblasts (Lotz and Guerne, 1991). Many of these changes have protective and restorative functions, suggesting that IL-6 may be part of a generalized response to tissue injury.

1.5 Expression and Function of IL-6 in the CNS

In the nervous system IL-6 is also present in development (Gadient and Otten, 1993; Gadient and Otten, 1994), undetectable in the adult, and rapidly induced in inflamed or traumatized tissue. Both IL-6 and the IL-6R are expressed in specific neuronal subpopulations (Yan et al., 1992; Schobitz et al., 1992; Gadient and Otten, 1993; Gadient and Otten, 1994). The highest levels of IL-6 mRNA are found in the hippocampus, neocortex and cerebellum (Gadient and Otten, 1994; Pousset, 1994). IL-6 mRNA has been detected in primary cultures of necocortical, striatal, and cerebellar neurons, demonstrating that IL-6 is produced by CNS neurons (Gadient and Otten, 1994; Ringheim et al., 1995). Microglia and astrocytes synthesize and release IL-6 as well (Aloisi et al., 1992; Benveniste et al., 1990; Lee et al.1993; Schobitz et al., 1992).

IL-6 performs a variety of beneficial functions in the CNS. It promotes the survival of certain neuronal populations and plays a protective role following insult to the brain. In particular, IL-6 promotes the survival of cholinergic neurons in the basal forebrain and septum, and catecholaminergic neurons of the mesencephalon (Hama et al., 1989; Hama et al., 1991; Kushima and Hatanaka, 1992; Kushima et al., 1992). Pretreatment of cultured hippocampal neurons with IL-6 protects them from glutamate-induced cell death (Yamada and Hatanaka, 1994). Furthermore, IL-6 is protective against other forms of neuronal insult such as MPP^+ toxicity to dopaminergic neurons (Akaneya et al., 1995), axonal injury due to head trauma (Hans et al., 1999), and ischemia (Matsuda et al., 1996).

Several studies have focused on IL-6 production in experimental models of ischemia (Maeda et al., 1994; Matsuda et al., 1996; Loddick et al., 1998; Suzuki et al., 1999). First, hypoxia followed by reoxygenation (H/R) triggers astrocytes to synthesize and release large quantities of IL-6 *in vitro* (Maeda et al., 1994). Second, H/R triggers an increase in bioactive IL-6 in the ischemic hemisphere *in vivo*, and an increase in IL-6

immunoreactivity in neurons and microglia (Loddick et al., 1998; Suzuki et al., 1999). Last, intracerebroventricular injection of IL-6 rescues neurons from lethal ischemia, prevents synaptic loss, and ischemia-induced learning disabilities (Matsuda et al., 1996; Loddick et al., 1998). Overall, these findings suggest that IL-6 plays a protective or restorative role in the CNS following ischemic injury.

There is increasing evidence to suggest that though acute IL-6 exposure can be beneficial to the nervous system, chronic exposure may be harmful. For example, astrocyte proliferation is a well-recognized CNS response to injury, which is thought to play an important role in the activation, survival, and regeneration of adjacent neurons, microglia, and oligodendrocytes (for review see Eddleston and Mucke, 1993; Norenberg, 1994). IL-6 has been shown to promote differentiation and proliferation of astrocytes *in vivo* and *in vitro* (Selmaj et al., 1990; Campbell et al., 1993; Chiang et al., 1994; Fattori et al., 1994), and to increase the synthesis and release of NGF from cultured astrocytes (Frei et al., 1989). Neuroglial activation in response to axotomy of the facial nerve or focal cryo injury to the cortex is impaired in IL-6 deficient mice, as is synthesis of GM-CSF and metallothionein (Klein et al., 1997; Penkowa et al., 1999). These findings suggest that null mice have a reduced posttraumatic astrocytic response, and that IL-6-induced proliferation appears to be protective. In contrast, transgenic mice overexpressing IL-6 in the brain exhibit neuropathological abnormalities, including astrogliosis and microglial reactivity, damage and loss of neurons, breakdown of the blood-brain-barrier, and monocyte infiltration (Campbell et al., 1993; Chiang et al., 1994; Brett et al.,1995). Furthermore, the firing properties of cerebellar Purkinje neurons are altered, accounting for the observed ataxia in transgenic mice (Nelson et al., 1999). These results show that chronic IL-6 exposure can be harmful, suggesting that dysregulation of IL-6 in the nervous system could have pathological consequences.

The involvement of IL-6 in pathophysiological events is supported by a variety of clinical observations. Increased expression of IL-6 is associated with several neurodegenerative diseases, including Alzheimer's (Bauer et al., 1991; Strauss et al., 1992; Wood et al., 1993; Brugg et al., 1995) and Parkinson's Disease (Mogi et al., 1994; Blum-Degen et al., 1995), inflammatory disorders such as bacterial and viral meningitis (Frei et

al., 1989; Waage et al., 1989), HIV-related encephalitis (Laurenzi et al., 1990; Perrella et al., 1992), and the autoimmune diseases multiple sclerosis (Woodroofe and Cuzner, 1993; Okuda et al., 1998) and systemic lupus erythematosus (Hirohata and Miyamoto, 1990).

Despite these findings, very little is known about the consequences of IL-6 elevation in diseased states. Several studies have assessed the pathophysiological effects of IL-6 on developing CNS neurons *in vitro*. Chronic exposure to IL-6 enhanced the intracellular calcium response to NMDA and altered resting calcium levels in cultured cerebellar granule neurons (Holliday et al., 1995; Qiu et al., 1995). Furthermore, treatment with IL-6 enhanced membrane and current responses to NMDA (in parallel with the increases in intracellular calcium) and NMDA receptor-mediated neurotoxicity (Qiu et al., 1998). These studies suggest that elevated levels of IL-6 in the CNS can alter neuronal physiology and lead to neurotoxicity. They also imply that IL-6 may be a participant in disease processes, not merely an associate.

1.6 Expression and Function of IL-6 in the PNS

IL-6 is rapidly upregulated in the sciatic nerve after nerve injury. Specifically, IL-6 mRNA levels increase for approximately one day in the proximal nerve stump and throughout the distal nerve segment, following nerve crush or transection (Zhong et al., 1999; Reichert et al., 1996; Bourde et al., 1996). Bolin et al. (1995) implicated Schwann cells as the major source of IL-6 in the injured peripheral nerve. They demonstrated that an immortal Schwann cell line produces IL-6 when stimulated with IL-1, TNFα, or LPS. However, Reichert et al. (1996) cultured primary Schwann cells isolated from degenerating sciatic nerves and were unable to detect IL-6 in conditioned media from these cells. In contrast, cultured fibroblasts and macrophages from the same degenerating nerves produced high levels of IL-6. Thus, the main cellular source of IL-6 in the periphery remains unclear.

Over the past decade, work in our lab has shown that inflammatory events are elicited in DRG after nerve injury and can contribute to axonal regeneration (Lu and Richardson, 1991; Lu and Richardson, 1993). In particular, the presence of macrophages in DRG is beneficial to the regeneration of dorsal root axons (Lu and Richardson, 1991)

and macrophages accumulate in appropriate DRG after nerve injury (Lu and Richardson, 1993). Macrophages are known to synthesize several cytokines associated with inflammation and repair of non-neuronal tissue, specifically IL-6, IL-1 and TNFα. Therefore, mRNA levels of these three cytokines were determined in lumbar DRG neurons associate with normal and cut sciatic nerves. The surprising result of this work was that IL-6 mRNA is induced for approximately one week in a subpopulation of medium and large lumbar dorsal root ganglia (DRG) neurons, following sciatic nerve transection (Murphy et al., 1995). *In situ* hybridization was used to confirm that IL-6 mRNA was localized in neurons, though non-neuronal cells in the DRG may serve as an additional source of IL-6. Mast cells initiate many inflammatory processes and are known to produce and release IL-6 from preformed granules in response to certain stimuli. Therefore, endoneurial mast cells may represent an additional source of IL-6 in the peripheral nerve after injury.

Upregulation of IL-6 in injured neurons suggests that it may promote neuronal survival in the PNS as well as in the CNS. Hirota et al. (1996) demonstrated that ligation of the hypoglossal nerve increased IL-6 and IL-6R immunoreactivity in the hypoglossal nucleus and in Schwann cells at the lesion site. Furthermore, regeneration of the nerve was retarded by administration of an anti-IL-6R antibody and accelerated in transgenic mice overexpressing human IL-6 and human IL-6R. Similarly, Ikeda et al. (1996) showed that *in vivo* administration of IL-6 and sIL-6R delayed motor neuron degeneration in the wobbler mouse, resulting in increased neuronal survival, decreased muscle atrophy and potentiated grip strength. These results support the idea that IL-6 contributes to the survival and regeneration of motor neurons.

Zhong et al. (1999) addressed the contribution of IL-6 to regeneration of sensory nerves using an IL-6 deficient mouse (Kopf et al., 1994). They demonstrated that the sensory system of IL-6 deficient mice is developmentally altered. In particular, null mice had smaller compound action potentials (CAP) in the sensory branch of the uninjured sciatic nerve, suggesting a reduced number of functional myelinated nerve fibres. This finding was corroborated by neuronal counts, which revealed that a population of large DRG neurons (> 700 μm^2) was reduced in number by approximately one-third. They also

demonstrated that regeneration was impaired in IL-6 deficient mice following sciatic nerve crush. First, functional recovery was delayed in null mice compared to wild-type mice, as analyzed by a behavioural footprint assay. Second, recovery of the CAP was selectively impaired in the sensory branch of the sciatic nerve, indicated that fewer peripheral fibres were conducted action potentials after nerve lesion. These findings suggest that IL-6 is essential to the development and maintenance of sensory neurons *in vivo*.

2.0 Neuropathic Pain

Neuropathic pain is associated with damage to nervous tissue, which may be caused by partial nerve injury or a variety of peripheral neuropathies. It is characterized by several syndromes including hyperalgesia (increased sensitivity to noxious stimuli), allodynia (a painful response to normally innocuous stimuli), and spontaneous pain. Furthermore, it is chronic and does not respond well to conventional analgesic therapies. Recently, several animal models have been developed that are useful tools with which to study the mechanisms underlying neuropathic pain. These include chronic constriction injury of the sciatic nerve (Bennett and Xie, 1988), partial nerve transection (Seltzer et al., 1990), and L5/L6 spinal nerve ligation (Kim and Chung, 1992). The use of these animal models has helped distinguish the pathological mechanisms that underlie neuropathic pain from the physiological mechanisms of acute nociception. The critical feature of pathological pain is hypersensitivity, such that painful responses are exaggerated, amplified and prolonged. This section will focus on peripheral and central mechanisms of hypersensitivity, sources of sensitization, and the role of interleukin-6 in neuropathic pain.

2.1 Acute versus Chronic Pain

Acute pain is an adaptive response that serves to protect an organism from dangerous, tissue-damaging stimuli. It is associated with motor withdrawal (mediated via a reflex arc in the dorsal horn) elicited by acute exposure to noxious stimuli. In contrast, chronic pain is considered a pathological or maladaptive response to tissue injury. The key differences between acute and chronic pain are summarized in Table 1.

2.2 Transmission of Nociceptive Information

A noxious stimulus is encoded as a nociceptive signal and transmitted from the periphery to the dorsal horn, then on to higher centres in the nervous system. Primary afferent fibres synapse in the dorsal horn and form either monosynaptic or polysynaptic tracts that project rostrally. A key pathway in sensory transmission is the post-synaptic dorsal column pathway (or lemniscal system). The dorsal column nuclei (gracile nucleus and cuneate nucleus) are relay points in the caudal medulla. Sensory information passes through these relay stations on its way to the thalamus and other corticolimbic structures. The dorsal column nuclei also receive direct, ascending innervation from a population of primary afferent fibres that carry proprioceptive, normally innocuous information. This population of neurons has been implicated in the development of neuropathic pain.

The organization of primary afferent input to the dorsal horn is complex (for review see Willis and Coggeshall, 1991). Essentially, there are three classes of primary afferent fibres (C, Aδ and Aβ) that are categorized on the basis of size, structure, conduction velocity and differential sensitivity to noxious and innocuous stimuli. C fibres are thin, unmyelinated, slow-conductors that are activated by high threshold noxious thermal or mechanical stimuli. In contrast, Aβ fibres are large, myelinated, fast-conductors of non-noxious stimuli such as touch, vibration and pressure. The properties of Aδ fibres are intermediate to those of C and Aβ fibres. It is important to note that there are multiple subtypes of C and Aδ fibres that are just beginning to be characterized. For instance, some fibres have vanniloid receptors that allow them to respond to noxious chemicals such as capsaicin, while others do not. C, Aδ and Aβ fibres can also be distinguished on the basis of their neurotransmitter content, target neurons, and pattern of innervation in the dorsal horn. These properties are compared in Table 2.

2.3 Peripheral Mechanisms of Neuropathic Pain

Nerve damage may cause sensitization of C and Aδ fibres (peripheral sensitization), an event that is thought to underlie hypersensitivity to mechanical and thermal stimuli. Nociceptive fibres release neuropeptides such as SP, CGRP, and BK, and a local inflammatory response is associated with the release of neuroactive agents, such as ions

TABLE 1: Distinguishing Features of Acute and Chronic Pain*

Type	Duration	Temporal Features	Characteristics	Class	Sources of Pain	Adaptive Value
Acute	Seconds	• Instantaneous Onset • Concurrent with stimulus	• Proportional to cause	• Nociceptive	Transient nociceptor activation	• High • Preventative
Chronic	Months to Years	• Persistent • Long-term disease, may exceed resolution of tissue damage	• 1° and 2° hyperalgesia • Allodynia • Spontaneous Pain	• Nociceptive • Neuropathic	Complex central and peripheral mechanisms (see text)	• None • Maladaptive

* Adapted from Millan, M.J. (1999) The induction of pain: an integrative review. *Progress in Neurobiology, 57: 1-164.*

TABLE 2: Distinguishing Features of C and Aβ Fibres*

Type	Activation Threshold	Principal Transmitters	Principal Neurotrophin Receptors	Activated Postsynaptic Receptors	Innervated DH Laminae	Postsynaptic Neuronal Types Targeted	Sensation	
							Physiological	Pathological
C Fibre	High	SP/NKA CGRP EAA	TrkA, (TrkB)	$NK_{1/2}$ $CGRP_{1/2}$ NMDA/AMPA/mGLU	I//II_o IV/V, X	Nociceptor-specific Wide Dynamic Range§	Noxious	Hyperalgesia, Cold Allodynia
Aβ Fibre	Low	EAA	TrkC, (TrkB)	AMPA	III-VI	Non-nociceptive Wide Dynamic Range	Innocuous	Mechanical Allodynia

* Adapted from Millan, M.J. (1999) The induction of pain: an integrative review. *Progress in Neurobiology, 57: 1-164.*

§ Wide Dynamic Range neurons receive noxious and innocuous input

(K^+ and H^+), prostanoids (PGE_2, PGI_2), amines (5-HT, histamine), purines (ATP), nitric oxide (NO), cytokines (TNFα, IL-1β, IL-6, LIF), and growth factors (NGF) from infiltrating immune cells and proliferating Schwann cells (for review see Millan, 1999). These inflammatory mediators act directly or indirectly on C fibre terminals, causing decreases in terminal thresholds such that lower intensity stimuli can now initiate activity in the nociceptor. This effect is due to immediate posttranslational changes in transducing receptor molecules (for example, the vanniloid receptor VR1) (Caterina et al., 1997; Tominaga et al., 1998) or sodium channels located at the nerve terminal (England et al., 1996; Gold et al., 1998), and is independent of transcriptional alterations occurring in the DRG. Peripheral sensitization is sufficient to account for primary hyperalgesia (increased sensitivity to noxious stimuli at the site of injury), however it is insufficient to explain secondary hyperalgesia or allodynia (see below) (LaMotte et al., 1982).

2.4 Central Mechanisms of Neuropathic Pain

Dorsal horn (DH) neurons can become hyperexcitable due to increased excitatory input from the periphery, or reduced inhibitory input from within the spinal cord and higher centres. Central sensitization is an increase in the excitability of DH neurons as a consequence of C fibre-mediated afferent barrage from sensitized nociceptors. It is associated with several functional changes in the spinal cord, including: increased responsiveness to suprathreshold inputs, expansion of receptive field size, and reduction in action potential threshold (Woolf, 1983; Woolf and Wall, 1986; Cook et al., 1987; Dickenson and Sullivan, 1987; Simone et al., 1989; Coderre and Melzack, 1992). Behaviourally, it manifests as pain that spreads to regions beyond the site of tissue injury (secondary hyperalgesia), and includes Aβ fibre-mediated hyperalgesia (allodynia) (Torebjork et al., 1992). Unlike peripheral sensitization, central sensitization is the result of activity-dependent posttranslational mechanisms in the DH, which arise several hours after noxious stimulation.

The mechanisms underlying central sensitization are complex, though several salient events stand out. C fibre stimulation normally results in the release of excitatory amino acids (EAAs) from presynaptic terminals in the DH, resulting in fast excitatory

postsynaptic potentials (EPSPs) at AMPA receptors (AMPARs). AMPARs are the predominant mediators of nociceptive transmission under baseline conditions and at low frequency rates (Hunter and Singh, 1994; Dickenson et al., 1997). Higher frequency stimulation evokes the release of EAAs, SP, CGRP, and BDNF from C fibre terminals. These neurotransmitters/neuromodulators bind to their postsynaptic receptors (AMPAR, NMDAR, mGluR, NK_1, $CGRP_{1/2}$, and trkB, respectively) and generate a larger EPSP that initiates calcium-entry through voltage-gated calcium channels. Calcium entry causes further depolarization, leading to removal of the Mg^{++} block from NMDARs, enabling NMDAR activation by glutamate or aspartate. NMDARs are highly permeable to calcium, therefore NMDAR activation results in a build up of intracellular Ca^{++} that in turn activates a number of calcium-dependent second messenger pathways, including protein kinase C (PKC), calcium-calmodulin kinase (CAMK), and NO (Bliss and Collingridge, 1993; Mao et al., 1995; Mayer and Westbrook, 1987). PKC has been shown to phosphorylate the NMDAR, which dramatically changes the channel kinetics and reduces the voltage-dependent Mg^{++} block, leading to an enhanced responsiveness to glutamate (Chen and Huang, 1992; Wang and Salter, 1992). These posttranslational modifications increase synaptic efficacy, thereby enabling previously subthreshod inputs to drive neuronal output (Woolf and King, 1990).

There is growing evidence that BDNF is a critical mediator of central sensitization. In particular, treatment with exogenous BDNF caused a sustained increase in nociceptive spinal reflex activity and enhanced NMDA-induced depolarization in a rat spinal cord slice preparation (Kerr et al., 1999). Furthermore, trkB-IgG, which sequesters endogenous BDNF, reduced spinal nociceptive reflex activity when endogenous BDNF levels were enhanced by NGF-treatment or peripheral inflammation (Kerr et al., 1999). These findings are supported by the well-defined role of BDNF as a neuromodulator in the CNS. BDNF rapidly enhances synaptic transmission in hippocampal neurons through trkB receptor stimulation and postsynaptic phosphorylation mechanisms (Levine et al., 1995). In particular, it has been shown to cause rapid phosphorylation of the NMDAR (Suen et al., 1997) and to potentiate NMDAR responsiveness by increasing the channel open time (Levine et al., 1998). It also induces expression of NR2A and NR2B, two NMDAR

subunits (Small et al., 1998). Thus, BDNF may function to enhance excitatory input from the periphery, thereby contributing to hypersensitivity in neuropathic pain.

Studies on BDNF illustrate another important concept in the development of persistent pain states. In addition to initiating many posttranslational changes in DRG and DH neurons, increased C fibre-mediated afferent input can also initiate long-term, activity-dependent changes in gene transcription. BDNF mRNA in DRG neurons and trkB mRNA in DH neurons are increased soon after intense C fibre activity. Both of these changes are likely due to an activity-dependent calcium influx that causes activation of calcium-dependent signalling pathways, which converge on the cAMP responsive element-binding protein (CREB) (Tao et al., 1998; Shieh et al., 1998). An increase in the amount of presynaptic neuromodulator and postsynaptic receptor will result in synaptic potentiation between primary afferent C-fibres and second-order DH neurons.

As mentioned earlier, another cause of increased excitability in the DH is loss of inhibitory tone. First, inhibitory interneurons in lamina I-II degenerate after peripheral nerve injury, thereby reducing the amount of inhibitory control in the DH (Sugimoto et al., 1990). This process may be initiated by EAA-induced PKC activation/translocation and NO production (Kitto et al., 1992). Second, changes in neuropeptide and neurotransmitter levels may cause transient functional alterations in DH neurons. For instance, GABA is reduced in the DH and GABA receptors are reduced presynaptically (Sugimoto et al., 1990; Sivilotti and Woolf, 1994). Furthermore, CCK, an endogenous inhibitor of opioid receptors, is increased in injured sensory neurons. All of these changes dampen endogenous spinal inhibitory mechanisms.

2.5 Sources of Sensitization

There are three possible sources of sensitizing signals from injured neurons: changes in neurotransmitter/neuromodulator content, increased synaptic connectivity in the dorsal horn, and increased spontaneous activity.

2.51 Changes in Neurotransmitter/Neuromodulator Content

Peripheral nerve injury initiates long-lasting transcriptional changes in neurons of the DRG, DH and DCN, which manifest several hours after injury. The expression levels of various neuropeptides, prostanoids, nitric oxide, opioids, cytokines, growth factors and ion channels are modified after nerve injury in DRG. Some of these alterations may be adaptive responses aimed at reducing chemical transmission and promoting survival of sensory neurons, while others may lead to enhanced or reduced nociception, with either protective or pathological consequences.

The numerous transcriptional changes in DRG neurons after injury can be accounted for by two differing mechanisms. First, interruption of the retrograde supply of trophic factors from the periphery leads to phenotoypic changes in axotomized neurons. For instance, loss of NGF-dependent trophic support to small, trkA-positive neurons alters the array of neuropeptides expressed by these neurons, including substance P (SP), calcitonin-gene related peptide (CGRP), galanin (GAL) and neuropeptide tyrosine (NPY). Administration of NGF after axotomy prevents decreases in SP and CGRP (Fitzgerald et al., 1985; Wong and Oblinger, 1991) and partially prevents increases in GAL and NPY (Verge et al., 1995; Corness et al., 1998). In contrast, NGF does not regulate the expression of vasoactive intestinal peptide (VIP) or somatostatin (SOM) in trkA-positive neurons. Loss of neurotrophic support is the main mechanism influencing neuronal phenotype after total nerve transection, an event where communication between the cell body and the periphery is completely disrupted.

Second, retrograde transport of specific signalling molecules, produced as a result of peripheral inflammation, may also lead to phenotypic changes in the DRG. These signalling molecules include many of the same inflammatory mediators that initiate peripheral sensitization, such as cytokines, purines, amines, ions, prostanoids, and nitric oxide. This mechanism is only valid after partial nerve injury (where many nerve fibres remain intact) with an inflammatory component. Chronic constriction injury is an animal model of neuropathic pain with both of these features; sciatic nerve connection to the periphery is partially spared and uninjured axons reside in a peripheral nerve environment where injured axons are undergoing Wallerian degeneration. One of the most potent

changes in neuropeptide expression after CCI, is the upregulation of GAL. LIF, a neuropoietic cytokine induced at the site of nerve injury (Kurek et al., 1996), is retrogradely transported to small DRG neurons (Curtis et al.1993; Thompson et al., 1997) where it stimulates GAL synthesis (Corness et al., 1996; Kerekes et al., 1999). The effects of GAL on spinal cord transmission and nociception are contradictory, therefore the functional correlates of plasticity in galanin expression following nerve injury are unclear. However, several lines of evidence indicate that GAL can potentiate nociceptive transmission, including 1) low doses of intrathecally applied galanin generate hyperalgesia in rats (Wiesenfeld-Hallin et al., 1988), 2) the spontaneous release of GAL in the superficial DH following CCI is associated with ectopic activity within sensory neurons and hence with the CCI-induced pain (Colvin et al., 1997), and 3) GAL knockout mice do not develop hyperalgesia and allodynia after nerve injury (Kerr et al., 1998). Therefore, signal-dependent changes in transcription, in addition to activity-dependent changes, can result in a potentiated nociceptive system.

Signal-dependent phenotypic changes also occur in large DRG neurons, a population that normally transmits innocuous, proprioceptive information. Following nerve injury, SP is downregulated in small DRG neurons and upregulated in large DRG neurons. Furthermore, the de novo synthesis of SP is accompanied by an increase in NK_1 receptors in the DH (Krause et al., 1995). Recently, it has been demonstrated that newly synthesized SP is released from Aβ fibre terminals in the dorsal horn following nerve injury (Malcangio et al., 1999). These changes result in a potentiated system, and one in which the specific type of stimulus that can evoke central sensitization has changed. Now, hypersensitivity can be invoked by low-intensity Aβ fibre inputs, in addition to high-intensity C fibre inputs, as a result of a signal-dependent transcriptional changes in large DRG neurons. This phenomenon is known as progressive tactile hypersensitivity, and is thought to underlie the development of mechanical allodynia, a prominent feature of neuropathic pain. This form of central sensitization in response to Aβ fibre inputs can be mimicked by direct Aβ fibre stimulation (Neumann et al., 1996; Ma and Woolf, 1996). It remains to be determined which growth factors and/or inflammatory mediators influence gene expression in large DRG neurons, but likely candidates include BDNF, NT-3, FGF,

and IL-6 (Murphy et al., 1995; Murphy et al., 1999; Fu and Gordon, 1997; Sterne et al., 1998; Kerekes et al., 1997).

2.52 Altered Synaptic Connectivity

The second potential cause of sensitization in neuropathic pain is changes in synaptic connectivity. First, peripheral nerve injury induces sprouting of both injured and intact myelinated fibres into denervated areas in the DH (Woolf et al., 1992; Mannion et al., 1996; Doubell et al., 1997). In particular, Aβ fibre terminals have been shown to sprout into lamina II of the DH, a region which normally contains only C fibre terminals. Direct communication between Aβ fibres and neurons in lamina II may lead to the interpretation of innocuous stimuli as noxious (Woolf et al., 1992; Woolf et al., 1995). Second, postganglionic adrenergic sympathetic neurons sprout into dorsal root ganglia following SNL (Chung et al., 1993), CCI (Ramer and Bisby, 1997) or complete sciatic nerve axotomy (McLachlan et al., 1993). The sympathetics form basket-like structures around neuronal cell bodies (particularly large DRG neurons), and EM studies have shown close contacts between sympathetic and sensory neurons (Chung et al., 1997b). The formation of functional synapses could provoke aberrant firing of large DRG neurons and alter sensory processing. In fact, adrenergic antagonists and surgical sympathectomy alleviate allodynia and other symptoms of neuropathic pain (Kim et al., 1993; Raja et al., 1996). Several signals have been implicated in sympathetic sprouting, including NGF (Davis et al., 1998; Ramer and Bisby, 1999), LIF (Thompson and Majithia, 1998), and IL-6 (Ramer et al., 1998a). Specifically, anti-NGF treatment can reduce injury-induced basket formation, infusion of LIF induces noradrenergic sprouting and basket formation in intact dorsal root ganglia (Thompson and Majithia, 1998), and sprouting and basket formation are both largely reduced in IL-6 deficient mice after SNL (Ramer et al., 1998a).

2.53 Spontaneous Activity

Studies in both animals and humans support the idea that spontaneous activity plays a key role in the development of neuropathic pain (Nystrom and Hagbarth, 1981; Seltzer et al., 1991; Gracely et al., 1992; Devor, 1991). There are three sources of

spontaneous action potential activity in injured primary sensory afferents: the neuroma (Wall and Gutnick, 1974; Calvin et al., 1982), demyelinated plaques (Calvin et al., 1982), and dorsal root ganglia (Wall and Devor, 1983). Most of the spontaneous activity recorded in primary afferent fibres after chronic constriction injury arises in the DRG (Kajander et al., 1992; Xie et al., 1995; Study and Kral, 1996). Spontaneous activity has been reported in Aβ, Aδ and C fibres after CCI (Xie and Xiao, 1990; Kajander et al., 1992) and in acutely isolated DRG neurons of all sizes (Study and Kral, 1996; Zhang et al., 1997). The latter finding suggests that an intrinsic change in these neurons is sufficient to trigger spontaneous discharge after nerve injury. Resting potential and input resistance are unchanged after injury, however several groups have demonstrated a decreased action potential threshold (Zhang et al., 1997). These findings suggest that enhanced excitability in DRG neurons is a function of changes in active ionic currents, in particular an increase in sodium currents (I_{Na}) and/or a decrease in potassium currents (I_K).

DRG contain numerous sodium currents that can be divided into two main types: tetrodotoxin-sensitive (TTX-S) and TTX-resistant (TTX-R) (Kostyuk et al., 1981; Caffrey et al., 1992; Elliott and Elliott, 1993; Rush et al., 1998). TTX-S, fast-inactivating currents are observed in all DRG neurons and are mediated by a variety of α-subunits, namely, brain types α-I, -IIA, -III, PN1, and NaCh6 (Black et al., 1996; Toledo-Aral et al., 1997; Sangameswaran et al., 1997; Souslova et al., 1997). In contrast, TTX-R currents are only seen in a subpopulation of capsaicin-sensitive, small diameter DRG neurons and have a high threshold for activation and slow inactivation kinetics (Cummins and Waxman, 1997). The properties and distribution of the ∝-subunits PN3 (SNS) (Akopian et al., 1996; Sangameswaran et al., 1996; Souslova et al., 1997) and NaN (Dib-Hajj et al., 1998a; Tate et al., 1998) suggest that they are TTX-R channels that give rise to the TTX-R currents in small DRG neurons.

In addition to causing excessive production and redistribution of sodium channels, nerve injury changes the levels of sodium channel α-subunit expression in DRG neurons (Rizzo et al., 1995). Alterations in the balance of TTX-R I_{Na} and TTX-S I_{Na}, reflecting changes in sodium channel expression, would be expected to have profound changes on neuronal excitability. Sciatic nerve transection causes a decrease in TTX-R currents,

which correlates with a downregulation of the PN3 and NaN genes (Dib-Hajj et al., 1996; Cummins and Waxman, 1997). Furthermore, the properties of the TTX-S current are altered (they begin to reprime rapidly), which correlates with a strong upregulation of the α-III gene (Cummins and Waxman, 1997). These changes may enable DRG neurons to fire spontaneously or to sustain unusually high rates of firing.

Alterations in channel distribution within a neuron are complex and injury dependent. Unlike transection of the sciatic nerve, CCI does not change the amplitude or I-V relationship of either TTX-S or TTX-R currents in small sensory neurons. Instead, PN3 levels in the cell body are initially decreased, then subsequently redistributed to the peripheral nerve, just proximal to the site of injury. SNL results in a similar redistribution of PN3 in L5 and L6 ganglia, but PN3 expression actually increases in the uninjured L4 ganglion. These complex changes in sodium channel redistribution may reflect differential availability of trophic factors (NGF, GDNF) after various types of nerve injury (Dib-Hajj et al., 1998b; Hilborn et al., 1998; Fjell et al., 1999).

The importance of this sensory neuron-specific (SNS/PN3) sodium channel is highlighted in studies using antisense oligonucleotides to PN3, which reduced thermal hyperalgesia and tactile allodynia after SNL (Tate et al., 1998). Similar studies on sensory neuron-specific NaN did not alter pain behaviours, suggesting that this TTX-R channel does not have a prominent role in the development of neuropathic pain (Porreca et al., 1999). In fact, generation of a PN3 (SNS) null mouse has shown that the PN3 α-subunit is responsible for all TTX-R I_{Na} in sensory neurons (Akopian et al., 1999). These mice also show deficits in mechano- and thermoreception after nerve transection. Thus, modulation of sodium channel expression can contribute to pain behaviours.

Potassium (K^+) channels also play an important role in regulating the level of neuronal excitability. In general, K^+ currents act to hyperpolarize a cell, thereby limiting neuronal excitability. It follows that a reduction in the hyperpolarizing or inhibitory influence of K^+ currents would tend to lead to a hyperexcitable state. This mechanism has been proposed to underlie the development of hyperexcitability in DRG neurons following axonal injury, and therefore may contribute to the development of neuropathic pain. In fact, several studies have suggested that a facilitation of K^+ currents may be involved in

attenuation of neuropathic pain by the anaesthetic agent, mexilitine (Sato et al., 1995; Khandwala et al., 1997; Kingery, 1997).

There are two broad categories of voltage-gated K^+ currents: fast, transient outward currents termed A currents; and slower, sustained outward currents termed delayed rectifiers (DR). These two currents regulate many aspects of neuronal excitability, including the resting membrane potential, AP repolarization, AP afterhyperpolarization, spike threshold and spike frequency adaptation (Rudy, 1988). Recently, several groups have studied the distribution and types of voltage-gated K^+ currents in adult rat DRG neurons. Gold et al. (1996) identified three A-type currents and three DR-type currents, four of which were differentially distributed within cells classified as nociceptors or non-nociceptors. Safronov et al. (1996) specifically studied small DRG neurons and identified one A-type and four DR-type currents. Everill et al. (1997) studied cutaneous afferent neurons, which give rise to myelinated fibres (including Aβ fibres), and identified two transient and one sustained current. These three studies demonstrated that: 1) multiple K^+ current components exist in each cell, 2) DR-type currents predominate in DRG neurons of all sizes, 3) A-type currents are present but masked by DR-type currents, 4) K^+ current components manifest in different ratios in cells that are morphologically identical, and 5) individual K^+ current components do not correlate with distinct neuronal populations. Overall, these studies emphasized that DRG neurons are extremely heterogeneous with respect to the variety and distribution of voltage-gated potassium currents.

These and other studies of K^+ currents in DRG neurons have provided insights into the functional role played by A-type and DR-type currents. One important finding is that DRG neurons show very rapid accommodation to an applied sustained depolarization, whereby only a single or a small group of AP's is generated (Birch et al., 1991; Kocsis et al., 1982). DR-type currents mediate this effect; therefore, a decrease in the amount of DR would be expected to increase the number of AP generated in response to a tonic depolarizing stimulus. Safronov et al. (1996) found that DR's are involved in setting the resting membrane potential of DRG neurons. If the resting membrane potential is kept close to E_K (the equilibrium potential for K^+), a large depolarization is required to reach AP threshold. Thus, modulating the amount of DR will influence the level of excitability

and vary the time to reach AP threshold in these neurons. They also found that DR's participate in AP repolarization, thereby determining the duration of individual AP's. A decrease in the amount of DR current would be expected to broaden the AP, leading to increased release of neurotransmitter in the DH. Lastly, DR's also participate in the AP afterhyperpolarization, where they tend to slow down the return of membrane potential to threshold, thereby lengthening the interspike interval and limiting repetitive firing. It follows that a decrease in DR-type currents could diminish the afterhyperpolarization and increase the rate of repetitive firing. Transient A-type currents also contribute to the repolarizing phase of the AP and tend to slow the recovery from afterhyperpolarization, thereby limiting the ability of a neurons to fire rapid trains of AP's (Safronov et al., 1996; Everill et al., 1998). These currents are instrumental in transducing graded stimulating currents into graded firing rates (Connor and Stevens, 1971). The mechanisms outlined above illustrate the importance of voltage-gated potassium currents in determining neuronal excitability and illustrate how a decrease in outward potassium currents would lead to a hyperexcitable state.

A point of key interest is whether or not potassium currents are altered by neuronal injury. Several electrophysiological studies have shown plasticity of potassium currents after axotomy (Kelly et al., 1986; Everill and Kocsis, 1999) and following seizure activity (Tsaur et al., 1992). For instance, Everill et al. (1999) found that the total mean peak current density was reduced (~50%) in cutaneous afferent neurons following sciatic nerve axotomy. Recently, immunostaining has revealed that Kv1.1, 1.2, 1.3, 1.4, 1.6 and 2.1 are expressed in DRG neurons, however there was no clear relationship to cell size (Ishikawa et al., 1999). After axotomy, Kv1.1, 1.2 and 2.1 were downregulated in small neurons and Kv1.1 and 2.1 were reduced in large neurons. The Kv1.2 channel was the only one to be reduced exclusively in small sensory neurons. This subunit usually forms heterodimers with other members of its family, therefore it is impossible to predict what type of current it underlies *in vivo*, without further studies. Unfortunately, little is known about the distribution of the Kv2, Kv3, or Kv4 families of voltage-gated potassium channel subunits in DRG.

It is clear that the expression of K^+ channels is altered in response to neuronal injury, however it is unclear which factors are regulating these changes *in vivo*. Many factors have been shown to contribute to the developmental expression of voltage-gated and Ca^{++}-dependent potassium currents, including NGF (Sharma et al., 1993; Raucher and Dryer, 1995), aFGF (Dourado and Dryer, 1992), TGFβ (Cameron et al., 1998), PDGF (Timpe and Fantl, 1994), bFGF (Timpe and Fantl, 1994), and CNTF (McFarlane and Cooper, 1993). Specifically, CNTF was able to prevent the loss of A-type currents and to prevent the increase in a DR-type current in cultured rat sympathetic neurons, suggesting a developmental role for CNTF in determining neuronal phenotype. Whether or not any of the other members of the neuropoietic cytokine family influence potassium currents is unknown. In addition to these developmental studies, certain factors have been shown to modify neuronal excitability by suppressing potassium currents (Wu and Barish, 1994; England et al., 1996; Nicol et al., 1997). In particular, prostaglandins alter membrane excitability by inhibiting a DR-like current in cultured embryonic rat sensory neurons (Nicol et al., 1997). Therefore, inflammatory mediators are capable of modulating the expression of K^+ currents in sensory neurons, and could potentially alter neuronal excitability in persistent pain states.

2.6 Role of IL-6 in Neuropathic Pain

IL-6 has been implicated in pain by reports that injection of IL-6 into the cerebral ventricle or lumbar subarachnoid space induces thermal hyperalgesia and/or mechanical allodynia (Oka et al., 1995; DeLeo et al., 1996). IL-6 immunoreactivity is increased in the spinal cord following sciatic cryoneurolysis (SCN), another technique used to induce neuropathic pain in rodents (DeLeo et al., 1996). SCN upregulated IL-6 in the dorsal and ventral horns ipsilateral to the side of injury. Interestingly, only allodynic rats displayed an increase in IL-6, which was coincident with the development of pain-related behaviours. A later study looked at changes in IL-6 mRNA levels after SCN, and showed that increases in IL-6 mRNA levels paralleled the changes in IL-6-IR, and that the cellular source of IL-6 was predominantly neuronal (Arruda et al., 1998). These studies

demonstrate the spinal production of IL-6 in response to peripheral nerve injury, where it may play a role in central mechanisms of neuropathic pain.

As mentioned earlier, IL-6 deficient mice exhibit reduced sympathetic sprouting and basket formation following SNL (Ramer et al., 1998a). This was accompanied by delayed development of mechanical allodynia relative to wild-type mice. Sympathetic neurons express both the IL-6R and gp130, suggesting that IL-6 may act directly on these neurons to induce sprouting. However, a second possibility is that IL-6 influences sympathetic sprouting through interactions with nerve growth factor (NGF). Both IL-6 and NGF are synthesized in DRG neurons after injury (Murphy et al., 1995; Sebert and Shooter, 1993), and IL-1 can induce expression of both IL-6 and NGF (Lindholm et al., 1987; Ringheim et al., 1995). Furthermore, NGF can induce sprouting of sympathetic axons in the CNS (Isaacson et al., 1992) and may be responsible for injury-induced sympathetic sprouting in DRG neurons (Ramer et al., 1998b). Synergistic effects of IL-6 and NGF have also been reported in the nervous system. In particular, IL-6 and NGF can promote neurite outgrowth in PC12 cells (Wu and Bradshaw, 1996a; Ihara et al., 1996), possibly through NGF-induced upregulation of the IL-6R (Sterneck et al., 1996), and elevated levels of IL-6 in cerebrospinal fluid may induce NGF synthesis by cultured astrocytes (Kossmann et al., 1996). Thus, the accumulation of IL-6 in DRG may affect neuronal excitability either directly or indirectly.

OBJECTIVES

The general aim of these studies is to understand the regulation and function of neuronal IL-6 after peripheral nerve injury. The first set of objectives is addressed by quantifying changes in mRNA labelling by *in situ* hybridization. The objectives are as follows:

1. To identify the injury signal driving the induction of IL-6 mRNA in DRG neurons after nerve transection,
2. To identify the cellular origin of the signal driving the upregulation of IL-6 mRNA,
3. To elucidate the mechanism through which IL-6 mitigates the death of DRG neurons,
4. To elucidate the mechanisms through which IL-6 contributes to behavioural changes associated with neuropathic pain after nerve injury.

The second set of objectives is addressed by whole-cell voltage clamp recordings of acutely isolated DRG neurons. The main objectives is as follows:

1. To determine if IL-6 is influencing spontaneous firing of DRG neurons after nerve injury.

In order to address this question, the following two objectives have to be met.

2. To develop a protocol for culturing primary sensory neurons from adult mouse dorsal root ganglia,
3. To determine if voltage-gated potassium currents are altered in response to peripheral nerve transection.

CHAPTER 2

NATURE OF THE RETROGRADE SIGNAL FROM INJURED NERVES THAT INDUCES INTERLEUKIN-6 mRNA IN NEURONS

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PURPOSE OF THE STUDY

Numerous factors activate second messenger systems and signal transduction cascades that converge on the IL-6 promoter, regulating transcription of the IL-6 gene. Over the last decade, the second messengers, transducing molecules and transcription factors influencing IL-6 expression have been uncovered. What has remained unclear, however, is which extracellular molecules are responsible for activating these systems in particular distinct cell types.

Our laboratory demonstrated that IL-6 is upregulated following sciatic nerve transection in medium to large DRG neurons, as soon as 1 day post-injury (Murphy et al., 1995). The temporal expression of IL-6 suggests that it is one of the earliest signals generated in response to nerve injury. This finding has prompted us to investigate 1) the identity of the injury signal driving IL-6 expression, 2) the cellular origin of this signal, and 3) the function of neuronal IL-6.

The early induction of IL-6 could reflect a direct intraneuronal mechanism initiated by interruption of retrograde axonal transport or an indirect mechanism involving perineuronal cells in the DRG. Adult sensory neurons are known to be highly dependent upon the retrograde transport of target derived trophic factors for maintenance of their differentiated phenotype. Inhibition of retrograde axonal transport with microtubule-binding proteins, such as colchicine, mimics many of the neuronal responses of DRG neurons to nerve transection (Gold and Austin, 1991; Leah et al., 1991). Therefore, we have employed colchicine to investigate if a loss of target-derived trophic support is inducing IL-6 expression.

Perineuronal cells, such as fibroblasts, macrophage, and mast cells, are present in the DRG and axon of the sciatic nerve and are well situated to respond immediately to nerve injury. Mast cells, in particular, are found in direct contact with peripheral nerves. They rapidly degranulate in response to nerve injury (Olsson, 1967), releasing a variety of molecules that can act back on neighbouring neurons (for review see Metcalfe et al., 1997). We have employed several agents that either stabilize or degranulate mast cells to investigate the possibility that a mast cell-derived factor is responsible for initiating rapid IL-6 expression in DRG neurons.

Finally, the neuroprotective role of IL-6 in the CNS and the growth-promoting effect of IL-6 on PC12 cells suggest that IL-6 might promote neuronal survival and regeneration in the PNS. The last part of this study uses IL-6 deficient mice to explore the possibility that endogenous IL-6 supports neuronal survival after nerve transection.

ABSTRACT

In previous studies, interleukin-6 was shown to be synthesized in approximately one third of lumbar dorsal root ganglion neurons during the first week after nerve transection. In present studies, interleukin-6 mRNA was found to be induced also in axotomized facial motor neurons and sympathetic neurons. The nature of the signal that induces interleukin-6 mRNA in neurons after nerve injury was analyzed. Blocking of retrograde axonal transport by injection of colchicine into an otherwise normal nerve did not induce interleukin-6 mRNA in primary sensory neurons but injection of colchicine into the nerve stump prevented induction of interleukin-6 mRNA by nerve transection. Therefore, it was concluded that interleukin-6 is induced by an injury factor arising from the nerve stump rather than by interruption of normal retrograde trophic support from target tissues or distal nerve segments. Next, injection into the nerve of a mast cell degranulating agent was shown to stimulate interleukin-6 mRNA in sensory neurons and systemic administration of mast cell stabilizing agents to mitigate the induction of interleukin-6 mRNA in sensory neurons following nerve injury. These data implicate mast cells as one possible source of the factors that lead to induction of interleukin-6 mRNA after nerve injury.

In search of a possible function of inducible interelukin-6, neuronal death after nerve transection was assessed in mice with null deletion of the intereukin-6 gene. Retrograde death of neurons in the fifth lumbar dorsal root ganglion was 45% greater in knockout than in wild type mice. Thus, endogenous IL-6 contributes to the survival of axotomized neurons.

INTRODUCTION

IL-6 (interleukin-6) is virtually absent in the PNS (peripheral nervous system) of normal mature animals but after sciatic nerve transection is induced for approximately one day in the nerve (Zhong, Heumann, 1995; Bourde et al., 1999) and approximately one week in a subpopulation of medium and large lumbar DRG (dorsal root ganglion) neurons (Murphy et al., 1995). IL-6 mRNA persists in axotomized neurons much more briefly than GAP-43 mRNA and other induced mRNAs. This relative brevity suggests that the inductive signal from the injured nerve might be unusual.

Following nerve transection, nerve cell bodies undergo many retrograde reactions (Lieberman, 1971) including alteration of neuropeptides and ion channels (Hokfelt et al., 1994; Zhang et al., 1997; Cummins, Waxman, 1998; Verge et al., 1995), and synthesis of molecules that promote regeneration (McQuarrie, Grafstein, 1973; Richardson, Issa, 1984; Skene, 1989). Most of these responses can be attributed to interruption of normal retrograde trophic support from target tissues and/or distal nerve segments (Lieberman, 1974; Gordon et al., 1991). Pharmacological inhibition of retrograde axonal transport with microtubule-binding proteins such as colchicine or vinblastine mimics many of the neuronal and perineuronal responses of DRG neurons to nerve transection (Landmesser, Pilar, 1974; Aldskogius, Svensson, 1988; Woolf et al., 1990; Leah et al., 1991). Some of the changes in DRG neurons can be attributed to loss of retrograde influence of specific molecules, for example NGF (Fitzgerald et al., 1985; Verge et al., 1995; Verge et al., 1995) and GDNF (Bennett et al., 1998). Other changes are due to interruption of retrograde transport of unknown molecules. (Verge et al., 1990)

In response to sufficient stimuli, mast cells quickly release many products from preformed granules and initiate several inflammatory processes in addition to acute hypersensitivity (Echtenacher et al., 1996; Malaviya et al., 1996; Wershil et al., 1988; Kubes, Granger, 1996; Galli, 1993). Following peripheral nerve crush, mast cells in the immediate vicinity are rapidly degranulated (Olsson, 1967). Degranulation of mast cells contributes to the pain elicited by NGF (Woolf et al., 1996; Lewin, Mendell, 1994), and

to other pathological processes in peripheral nerves (Dines, Powell, 1997; Zochodne et al., 1994; Brosman et al., 1985).

An extreme neuronal response to axonal interruption is death at least sometimes due to apoptosis (Berkelaar et al., 1994). Neuronal death is thought to be due to loss of retrograde trophic support from target tissues and/or glial cells (Lieberman, 1974; Pettmann, Henderson, 1998) and can be reduced by exogenous trophic agents (Li et al., 1994; Li et al., 1994).

Data presented here indicate that the induction of IL-6 mRNA in DRG neurons is initiated by an injury factor from the nerve stump to which mast cells may contribute and that endogenous inducible IL-6 attenuates the death of axotomized neurons.

METHODS

SURGERY FOR NERVE MANIPULATIONS IN RATS

Adult female Sprague-Dawley rats weighing approximately 200 gm. were anaesthetized with Pentothal (50 mg/kg intraperitoneally) and submitted to a variety of microsurgical procedures. i) The right sciatic nerve was exposed in midthigh and either transected (3 rats) or crushed (3 rats) with jeweler's forceps for 10 seconds while the left sciatic nerve was uninjured. Rats were sacrificed 4-7 days later. ii) In 3 rats, the L5 dorsal root was sectioned with microscissors 2mm from its DRG. Rats were sacrificed 4-7 days later. iii) In 3 rats, the right facial nerve was exposed near the stylomastoid foramen and transected with removal of a 2mm segment to impede regeneration. The completeness of the transection was confirmed by observations of whisker paralysis and failure of eye closing. Rats were sacrificed 2-7 days later. iv) In 4 rats, the external and internal post-ganglionic nerves were transected several mm from the superior cervical ganglion or the pre-ganglionic cervical sympathetic trunk was severed proximal to the ganglion. Rats were sacrificed 4 days later.

NERVE INJECTIONS

2-5 μl of 5 mM colchicine, 0.25 - 1.0 μg of the mast cell degranulating compound 48/80 (Sigma), normal rat serum, or saline were injected slowly into the right sciatic nerve in midthigh through a glass micropipette with tip diameter approximately 50 μm attached to a manual pressure injection system filled with mineral oil (Beitz, King, 1976). Colchicine was injected in uninjured nerves (3 rats), in the stump or distal segment of transected nerves (3 rats), or in contralateral nerves (3 rats). Colchicine, injected intraneurally at this dose, has been shown to block axonal transport for at least 5 days (Richardson, Verge, 1986). A total of 14 rats were injected with 48/80. In 3 rats, recombinant TNF-α (tumor necrosis factor) was injected into the sciatic nerve and, in 3 rats, IL-1β.

INTRAPERITONEAL INJECTIONS

Two mast cell stabilizing agents were injected individually and intraperitoneally in combination with sciatic nerve transection at midthigh in an attempt to influence the induction of IL-6 mRNA in DRG neurons. Cromolyn sodium (1 mg/ml of a 1% solution) (Sigma) or ketotifen (1 ml/kg of a 1% solution) were injected twice a day for 5 days before surgery and for a further 5 days until sacrifice. Three rats were injected in each of the two groups and 3 control rats underwent nerve transection alone.

IN SITU HYBRIDIZATION

In most experiments, L4 and L5 DRG were removed, frozen immediately in N-methyl butane at -55°C, embedded in Tissue-Tek (Miles Laboratory), and stored at -70° C. DRG to be compared were embedded in the same mold. Where appropriate, superior cervical ganglia or the brainstem were removed, in the latter case, after perfusion of the rats per aorta with phosphate-buffered saline. Antisense oligonucleotides for IL-6 (Murphy et al., 1995) or GAP-43 (Verge et al., 1990) approximately 50 nucleotides in length were labeled with ^{33}P by the terminal transferase reaction. Frozen sections cut on a cryostat set at 5-10 μm, were thaw mounted on Probe-On slides (Fisher) and hybridized 16-18 hours at 42°C with a solution containing 500,000 cpm oligonucleotide, 50% formamide, 4x SSC, 100 mg/ml dextran sulfate, 1% sarcosyl, 500 μg/ml salmon sperm DNA, and 200 mM DTT. Following hybridization, the sections were washed four times in 1X SSC at 55°C for 15 minutes, fixed briefly in 65% and 95% ethanol, dried, dipped in radiosensitive emulsion (Kodak NTB2), exposed in the dark at 4°C for 4-6 weeks, developed, fixed, and stained with 0.002% Toluidine blue.

Neuronal labeling for IL-6 mRNA was quantified with a computerized image analysis system (Richardson et al., 1989) for groups of 2-4 sections on the same slide. Only cells with a visible nucleolus were quantified. The percentage of cross-sectional area covered by silver grains was measured and a correction factor applied to yield a parameter linearly related to grain number. Labeling index refers to the ratio of grain density over neurons to grain density over non-neuronal regions of the DRG.

NEURONAL CELL COUNTS AFTER NERVE TRANSECTION IN MICE

Nine male C57BL6/129 mice aged 9 weeks and 9 mice of the same strain with null mutation of the IL-6 gene (Kopf et al., 1994) were anesthetized by intramuscular injection of 0.75 mg/g ketamine and 0.01 mg/g xylazine. Through a midline dorsal incision, the right sciatic nerve was transected at its origin from the L4 and L5 spinal nerves while the left sciatic nerve was uninjured.

Fourteen days after nerve transection, ipsilateral and contralateral L5 DRG were removed from deeply anesthetized mice. The DRG were fixed overnight in 4% formaldehyde, washed 3 times with phosphate buffered saline, protected overnight in 18% sucrose, and frozen in cryomolds at -55°C in 2-methyl butane. Serial frozen sections were cut on a cryostat set at 5 μm, thaw mounted onto gelatin-coated slides, and stained with 0.002% Toluidine blue. Neurons with clearly visible nucleoli were counted under oil immersion light microscopy in every fifth section by an observer blinded to the mouse genotype. No correction was made for split nucleoli. Percentage survival was calculated as the ratio of ipsilateral to contralateral counts.

RESULTS

MOTOR AND SYMPATHETIC NEURONS SYNTHESIZE IL-6 mRNA AFTER NERVE INJURY

Experiments were performed to determine whether nerve injury induces IL-6 mRNA in PNS neurons that were not DRG neurons.

In sections of the brainstem from rats sacrificed 2-4 days after facial nerve transection, IL-6 mRNA was detected by in situ hybridization in neurons of the ipsilateral but not contralateral facial motor nucleus. (Figure 1). In semi-quantitative analysis of two nuclei, 64% (89/141) and 65% (90/138) of neurons were deemed to be labeled including few or no small motoneurons. With this technique of in situ hybridization, IL-6 mRNA was not detected in non-neuronal cells. The data support a prediction (Klein et al., 1997) that at least some of the IL-6 mRNA detected in the facial motor nucleus after nerve transection (Kiefer et al., 1993) is in neurons.

After transection of the post-ganglionic sympathetic nerves but not after transection of the preganglionic trunk, IL-6 mRNA was found in many sympathetic neurons in the superior cervical ganglion (Figure 1). Again, no hybridization signal was detected in non-neuronal cells of the superior cervical ganglion. In contrast to a previous report (Marz et al., 1996), we did not detect IL-6 mRNA in sympathetic neurons of uninjured mature rats or contralateral superior cervical ganglia.

The results of these experiments indicate that, following nerve injury, IL-6 mRNA is induced in many neurons in the corresponding motor nucleus or sympathetic ganglion.

IL-6 mRNA IS INDUCED IN SOME DRG NEURONS BY NERVE CRUSH OR DORSAL SPINAL NERVE ROOT TRANSECTION

After sciatic nerve crush, IL-6 mRNA was detected in L5 DRG neurons albeit in fewer neurons than after nerve transection (data not shown). Transection of the L5 dorsal spinal root elicited clear IL-6 mRNA labeling in a few (2-5%) L5 DRG neurons. After section of a dorsal spinal nerve root, most neurons in the corresponding DRG are not visibly perturbed (Carmel, Stein, 1969) but(Hare, Hinsey, 1940) GAP-43 (Chong et

al., 1994) and c-jun (Jenkins et al., 1993) are found in a few neurons as is IL-6 mRNA. IL-6 mRNA is induced in DRG neurons after nerve crush or dorsal spinal nerve root transection but in fewer neurons than after nerve transection.

A SIGNAL FROM INJURED NERVES STIMULATES IL-6 SYNTHESIS IN DRG NEURONS

Colchicine was injected intraneurally to investigate whether interruption of retrograde axonal transport mimics or blocks the induction of induction of nerve transection. Injections were either into a previously uninjured nerve or immediately proximal to the site of nerve transection. After sacrifice 3 days later, GAP-43 and IL-6 mRNAs were analyzed in L5 DRG by in situ hybridization. As anticipated, GAP-43 mRNA was induced in DRG neurons by simple intraneural injection of colchicine and its induction by nerve transection was not blocked by proximal injection of colchicine (Figure 2). This is the expected pattern of responses for a molecule that is induced (directly or indirectly) by interruption of retrograde transport to the nerve cell body of normal trophic influence from the distal nerve or target tissues. For IL-6 mRNA, very different responses were obtained (Figures 2 and 3). Injection of colchicine into the uninjured sciatic nerve did not induce IL-6 mRNA and injection of colchicine into the stump of a transected nerve blocked induction of IL-6 mRNA in L5 DRG neurons. In other control experiments, injection of colchicine into the distal segment of the transected nerve or into the contralateral nerve did not interfere with the induction of IL-6 in axotomized DRG neurons (data not shown). Therefore, the blockage by colchicine of induction of IL-6 after nerve injury is not a non-specific toxic effect. In previous experiments (Richardson, Verge, 1986), intraneural injection of 5 mM colchicine was shown to cause considerable axonal degeneration. This action of colchicine does not invalidate the conclusions of these experiments since IL-6 is not induced despite axonal degeneration. The results of these experiments suggest that the induction of IL-6 in injured DRG neurons is triggered by a positive signal from the injury site rather than from loss of retrograde inhibition by molecule(s) arising from the distal nerve or target tissues.

DEGRANULATION OF MAST CELLS INFLUENCES IL-6 mRNA IN NEURONS

Injection of the mast cell degranulating agent 48/80 but not saline into the uninjured sciatic nerve induced IL-6 mRNA in medium and large neurons in the ipsilateral DRG (Figure 4, Figure 5). IL-6 mRNA was seen in L5 DRG removed 24 or 48 but not 6 hours after injection of 48/80. Approximately one fifth of neurons were seen to contain IL-6 mRNA after 48/80 injection. Given that 48/80 disrupts the blood-nerve barrier (Harvey et al., 1994), we investigated whether intraneural injection of serum or surgical disruption of the blood-nerve barrier (Gentili et al., 1981) was a sufficient stimulus for IL-6 induction in neurons. Neither neurolysis nor injection of up to 5µl serum into the nerve induced IL-6 in DRG neurons (data not shown). These observations suggest that degranulation of endoneurial mast cells induces IL-6 mRNA in neurons by a mechanism more complicated than simple increase in vascular permeability.

Not only does degranulation of endoneurial mast cells induce IL-6 mRNA in DRG neurons, but agents that stabilize mast cells attenuate the induction of IL-6 mRNA. Injected for 5 days before and after nerve transection, cromolyn sodium (Figure 4, or ketotifen (data not shown) substantially reduced the induction of IL-6 mRNA (Figure 4, Figure 5).

EFFECTS OF CYTOKINES ON IL-6 INDUCTION

TNF-α is present in the granules of resident mast cells, (Gordon, Galli, 1990), mediates the initiation of some inflammatory reactions by mast cells (Echtenacher et al., 1996; Malaviya et al., 1996), and stimulates IL-6 synthesis in many cell types (Brach et al., 1990) including cortical neurons in vitro (Ringheim et al., 1997). Therefore, TNF-α was deemed to be one candidate signaling molecule from injured nerves to stimulate IL-6 synthesis in neurons. However, a single intraneural injection of TNF-α (100ng) did not induce enough IL-6 mRNA in DRG neurons to be detected by in situ hybridization. In similar manner, intraneural injection of IL-1β (100 ng) failed to stimulate IL-6 mRNA in DRG neurons. While not supportive of the hypothesis that endogenous IL-1β and/or TNF-α are responsible for IL-6 mRNA induction after nerve injury, these negative results with a single technique do not exclude the possibility.

INCREASED DEATH OF AXOTOMIZED DRG NEURONS IN IL-6 -/- MICE

Survival of neurons in L5 DRG after sciatic nerve transection was compared in IL-6 -/- and wildtype mice. IL-6 -/- mice breed well and appear normal but react abnormally to traumatic or infectious challenge (Kopf et al., 1994; Ramsay et al., 1994; Chai et al., 1996; Fattori et al., 1994; Cressman et al., 1996).

Two weeks following transection of the sciatic nerve at its origin in nine wildtype mice, the mean ratio of neuron counts in ipsilateral versus contralateral L5 DRG decreased by 30% (Table 1). In previous studies in rats, death of L5 DRG was estimated by cell counting, as 14% or 23%, 10 or 30 days after sciatic nerve transection at midthigh (Himes and Tessler, 1989; Arvidsson et al., 1986) and, by stereology, as 22% 15 days after spinal nerve transection (Vestergaard et al., 1997). The level of transection in rats in the latter study is similar to that in mice in the present study. These data indicate that, in C57BL6/129 mice, death of axotomized DRG neurons is comparable to that in rats.

The mean decrease in ratio of counts in ipsilateral versus contralateral L5 DRG following sciatic nerve transection in nine IL-6 -/- mice was 43% (Table 1), translating to 45% more neuronal loss than in wildtype mice. We interpret the excessive decrease in neuronal numbers in L5 DRG after sciatic nerve transection in IL-6 -/- mice to indicate that endogenous IL-6 induced by nerve injury contributes to the survival of injured neurons.

DISCUSSION

IL-6 IS INDUCED IN NEURONS BY A POSITIVE INJURY SIGNAL

The effects of intraneural injection of colchicine indicate that IL-6 induction is triggered by a positive rather than negative signal from injured nerves. Three other examples establish a precedent for this mechanism. The induction of p75 mRNA in motoneurons also appears to depend upon a positive retrograde axonal signal (Moix et al., 1991; Greeson et al., 1992) although for these neurons, it is not possible to exclude an influence from degenerating terminals of the central projections of sensory neurons. The induction of galanin in DRG neurons depends in part upon release of LIF from the nerve stump (Corness et al., 1996; Sun and Zigmond, 1996) but also upon release from a chronic inhibition by NGF (Verge et al., 1995; Corness et al., 1998). Perhaps the best documentation of a positive inductive signal from injured axons is for long-term hyperexcitability in Aplysia neurons (Gunstream et al., 1995).

The positive nature of the initial signal and the brief duration of IL-6 induction are unusual among the consequences of nerve injury on nerve cell bodies. Perhaps other growth factors with relatively brief induction in neurons after nerve injury for example, BDNF (Tonra et al., 1998; Averill et al., 1997; Kobayashi et al., 1996; Verge et al., 1996) and bFGF (Ji et al., 1995), also are induced by a positive signal from the nerve and are involved in neuronal survival after axotomy.

The present studies have emphasized cellular rather than molecular signals that induce IL-6 mRNA in DRG neurons. In a variety of cell types, IL-6 synthesis is stimulated strongly by several extracellular molecules including lipopolysaccharide (Zhang et al., 1994), IL-1 (Zhang et al., 1990), TNF-α (Brach et al., 1990; Ringheim et al., 1997), LIF (Villiger et al., 1993), cardiotrophin-1 (Robledo et al., 1997), oncostatin-1 (Brown et al., 1991), interferon-γ (Faggioli et al., 1997), granulocyte-macrophage colony-stimulating factor (Cicco et al., 1990), stem cell factor, (Gagari et al., 1997), histamine (Mor et al., 1995; Takamatsu, Nakao, 1998), leukotriene B4 (Brach et al., 1992) et al, 1992), prostaglandins (Leal-Berumen et al., 1995; Fiebich et al., 1997), and reactive oxidative species (Shibanuma et al., 1994). With respect to intracellular

signaling, IL-6 gene expression is dominated by NF-κB (Zhang et al., 1990; Lord et al., 1991; Sha et al., 1995) but is influenced by other transcription factors, for example NF-IL6 (Matsusaka et al., 1993; Zhang et al., 1994), Sp1 (Kang et al., 1996), and AP-1 (Dendorfer et al., 1994) plus the transcription repressor RBP (Kannabiran et al., 1997). NF-κB is constitutively expressed in neurons (Kaltschmidt et al., 1994) where it is perturbed by nerve injury (Doyle, Hunt, 1997; Ma, Bisby, 1998). Candidate molecules for the initiation of the signaling that induces IL-6 in neurons must be present in injured nerves, are likely to be among those that are known to stimulate IL-6 synthesis in non-neuronal cells, probably activate the NF-κB signaling pathway, and should have receptors on some but not all axons.

POSSIBLE IMPLICATION OF MAST CELLS IN INDUCTION OF IL-6 mRNA IN NEURONS

Intraneural injection of 48/80 was sufficient stimulus to induce IL-6 mRNA in neurons. It seems probable that this effect of 48/80 was due to mast cell degranulation rather than non-specific tissue damage or inflammation. Doses of 48/80 slightly higher than used here do not induce gross axonal damage or Wallerian degeneration and axonal interruption alone is not sufficient to induce IL-6 synthesis in neurons. One possible mechanism of action of 48/80 is breakdown of the blood-nerve barrier (Harvey et al., 1994). However, neither increasing of the permeability of nerve vasculature by surgical manipulation nor injection of serum sufficed to induce IL-6 mRNA in DRG neurons. We conclude that one or more of the molecules released from degranulated mast cells triggers a retrograde axonal signal that induces IL-6 mRNA.

Two agents that interfere with mast cell degranulation also mitigate the induction of IL-6 in neurons after nerve injury. Although the actions of these pharmacological agents may not be restricted to mast cells, the observations again are consistent with the hypothesis that mast cells in the stump of a transected nerve are a source of retrograde signals that induce IL-6 synthesis in DRG neurons.

Mast cells are known best for IgE-dependent responses to parasites and detrimental allergic reactions. However, mast cells have been shown recently to

counteract bacterial infection and stimulate neutrophil extravasation, both actions mediated through the release of TNF-α (Echtenacher et al., 1996; Malaviya et al., 1996). Results of present experiments raise the possibility that mast cells have a beneficial action in the PNS, stimulation of synthesis of IL-6 to support axotomized neurons.

ENDOGENOUS IL-6 CONTRIBUTES TO NEURONAL SURVIVAL AFTER NERVE INJURY

Endogenous IL-6 mitigates the death of sensory neurons after axotomy. This statement is justified by evidence that the decrease in neuronal numbers in L5 DRG after nerve transection is 45% greater in IL-6 -/- mice than in control mice.

Whereas the absence of endogenous IL-6 in mutant mice results in the death of sensory neurons after axotomy, infusion of exogenous IL-6 was not found to counteract death of facial motor neurons after injury in newborn rats (Li et al., 1994). One possible explanation for this paradox is that IL-6 is induced to biologically effective concentrations in the immediate vicinity of axotomized neurons (Murphy et al., 1995) so that exogenous IL-6 is superfluous in this circumstance.

The lack of IL-6 in mutant mice is not compensated by CNTF or LIF, which are active on DRG neurons and use gp130 as their signaling receptor. In contrast, null mutation of the LIF or CNTF gene alone does not impair neuronal survival after axotomy, although mutation of both genes does lead to increased neuronal death (Sendtner et al., 1996). Also, the absence of LIF in mutant mice has non-compensable consequences on neuropeptide synthesis in axotomized sympathetic and sensory neurons (Rao et al., 1993; Corness et al., 1996; Sun, Zigmond, 1996). The lack of compensation in the present experiments may reflect different sites of synthesis for the 3 cytokines: only IL-6 is synthesized in DRG neurons after nerve injury (Murphy et al., 1995; Curtis et al., 1994; Banner, Patterson, 1994; Sendtner et al., 1992; Seniuk et al., 1992).

Although IL-6 is neurotoxic under some circumstances (Campbell et al., 1993), endogenous IL-6 supports axotomized neurons just as another cytokine with toxic properties, TNF-α, supports ischemic neurons (Bruce et al., 1997).

A major negative signal, interruption of retrograde trophic support, leads to the death of many DRG neurons after nerve injury. Results of our experiments indicate that an additional positive signal leads to induction of IL-6, which counteracts the tendency to neuronal death.

FIGURE 1. Dark- and light-field photomicrographs of IL-6 in situ hybridization preparations from sections through the ipsilateral (A, E) or contralateral (C) facial motor nucleus of a rat sacrificed 4 days after unilateral facial nerve transection and of sections through the ipsilateral (B, F) and contralateral (D) superior cervical ganglion of rats sacrificed 4 days after external and internal carotid nerve transection. Note that many neurons in the ipsilateral facial motor nucleus and some neurons in the superior cervical ganglion contain IL-6 mRNA. Magnification x 230 (A), x290 (B, C, D), x1120 (E, F)

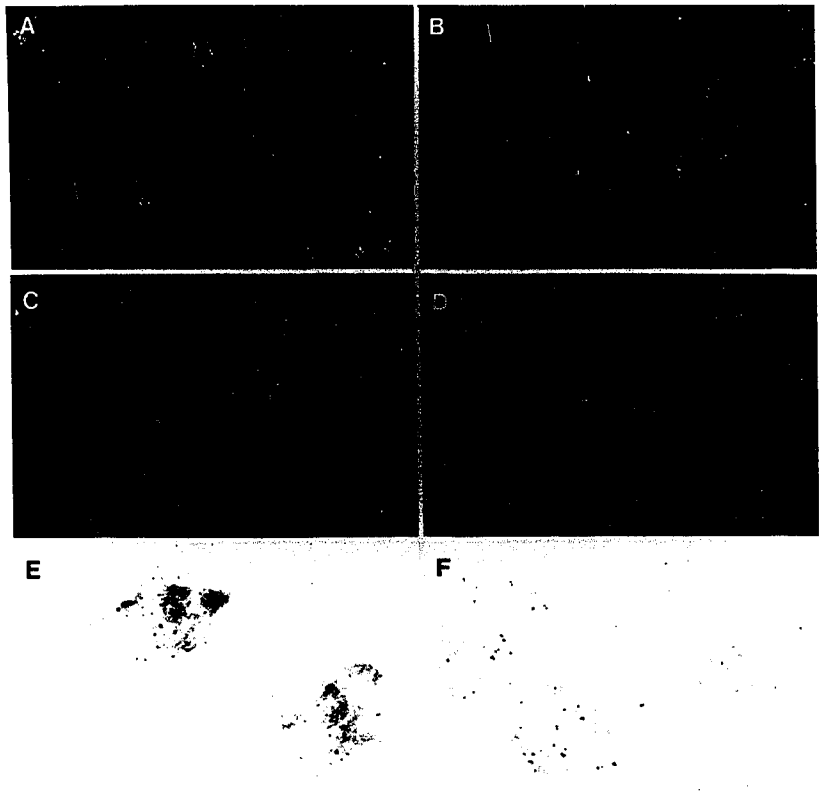
A
B
C
D
E
F

FIGURE 2. Dark field photomicrographs of in situ hybridization preparations for IL-6 mRNA (A, C, E, G) or GAP-43 mRNA (B, D, F, H) all of sections of L5 DRG. DRG are contralateral (A, B) or ipsilateral (C, D) to sciatic nerve transection, ipsilateral to intraneural injection of colchicine (E, F), or ipsilateral to nerve transection plus injection of colchicine into the nerve stump (G, H). Note that IL-6 mRNA is induced by nerve transection but not by intraneural injection of colchicine and that the effect of nerve transection on IL-6 mRNA is blocked by more proximal injection of colchicine. GAP-43 mRNA is also induced by nerve transection. However, in contrast to IL-6 mRNA, GAP-43 mRNA is induced by intraneural injection of colchicine and its induction by nerve transection is not blocked by more proximal injection of colchicine. Despite longer exposure times, the signal for IL-6 mRNA is consistently weaker than that for GAP-43 mRNA, presumably because the latter is much more abundant. Magnification x180.

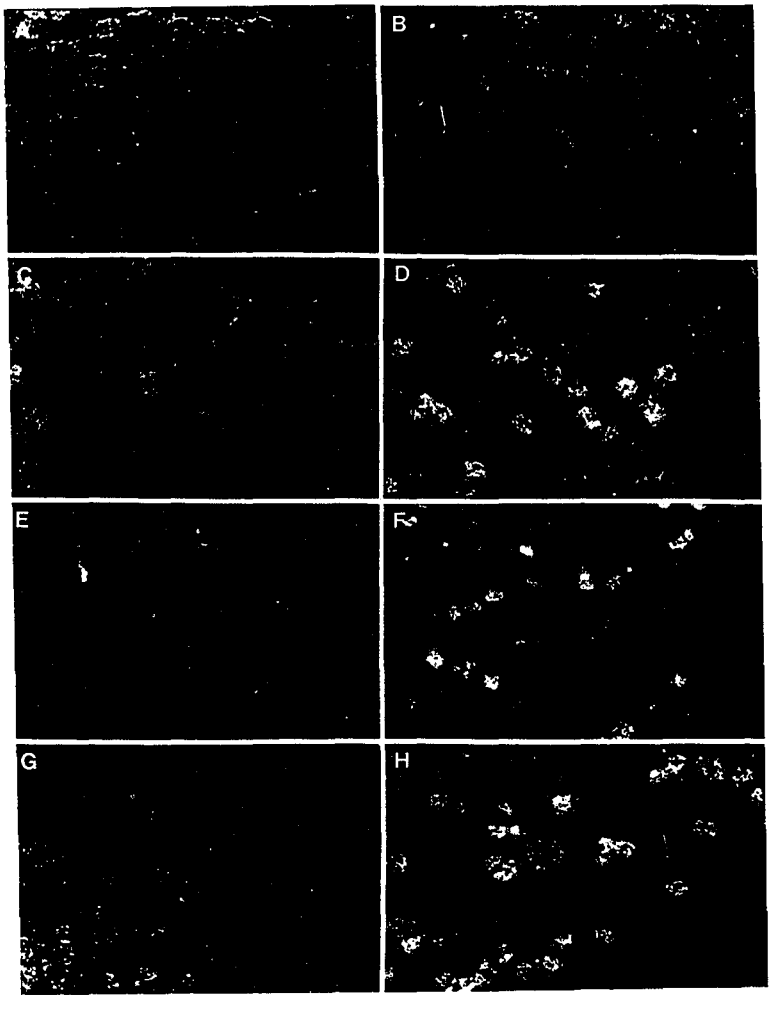
A
B
C
D
E
F
G
H

FIGURE 3. Quantification of IL-6 mRNA labeling by in situ hybridization. Each point represents a single DRG neuron with labeling index as the y-axis and volume as the x-axis (log-log scale). (A) normal DRG, (B) DRG removed 3 days after nerve transection, (C) DRG removed 3 days after injection of colchicine, (D) DRG removed 3 days after nerve transection plus injection of colchicine into the nerve stump. Note that many neurons with clear presence of IL-6 mRNA (> threefold background) are found after simple nerve transection but few or none in either of the experiments involving colchicine injection.

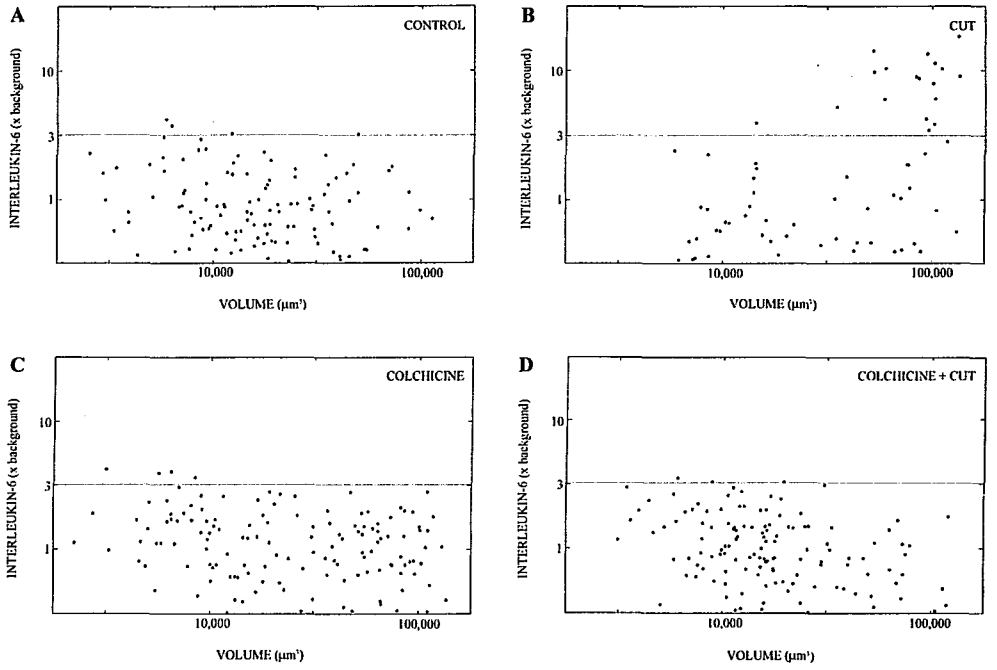
A
CONTROL
B
CUT
C
COLCHICINE
D
COLCHICINE + CUT
INTERLEUKIN-6 (x background)
10
3
1
10,000
100,000
VOLUME (μm³)

FIGURE 4. (A) Light-field photomicrograph of IL-6 in situ hybridization preparation of L5 DRG ipsilateral to a sciatic nerve that had been injected with 48/80, a mast cell degranulating agent. One heavily labeled neuron is seen. (B) Dark-field photomicrograph of IL-6 in situ hybridization preparation of DRG ipsilateral to transected sciatic nerve. (C) Dark-field photomicrograph of IL-6 in situ hybridization of DRG ipsilateral to sciatic nerve transection in a rat that had also been injected intraperitoneally with a mast cell stabilizing agent, cromolyn sodium. Note that the labeling evident after nerve transection is reduced by injection of cromolyn sodium. Magnification x1120 (A), x180 (B, C).

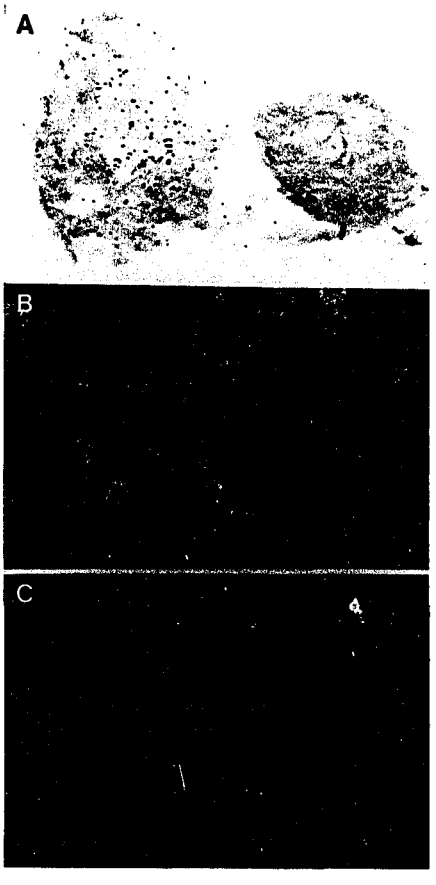
A
B
C

FIGURE 5. Quantification of IL-6 mRNA labeling to show the effects of 48/80 and cromolyn sodium. Again each point represents a single DRG neuron with labeling index as the y-axis and volume as the y-axis (log-log scale). (A) DRG associated with nerve injected with 48/80, (B) DRG associated with nerve injected with saline, (C) DRG associated with nerve transected 5 days previously, (D) DRG associated with nerve transected 5 days previously with intraperitoneal injection of cromolyn for 5 days before and after nerve transection. Note that 48/80 mimics and cromolyn sodium blocks the induction of IL-6 mRNA by nerve transection.

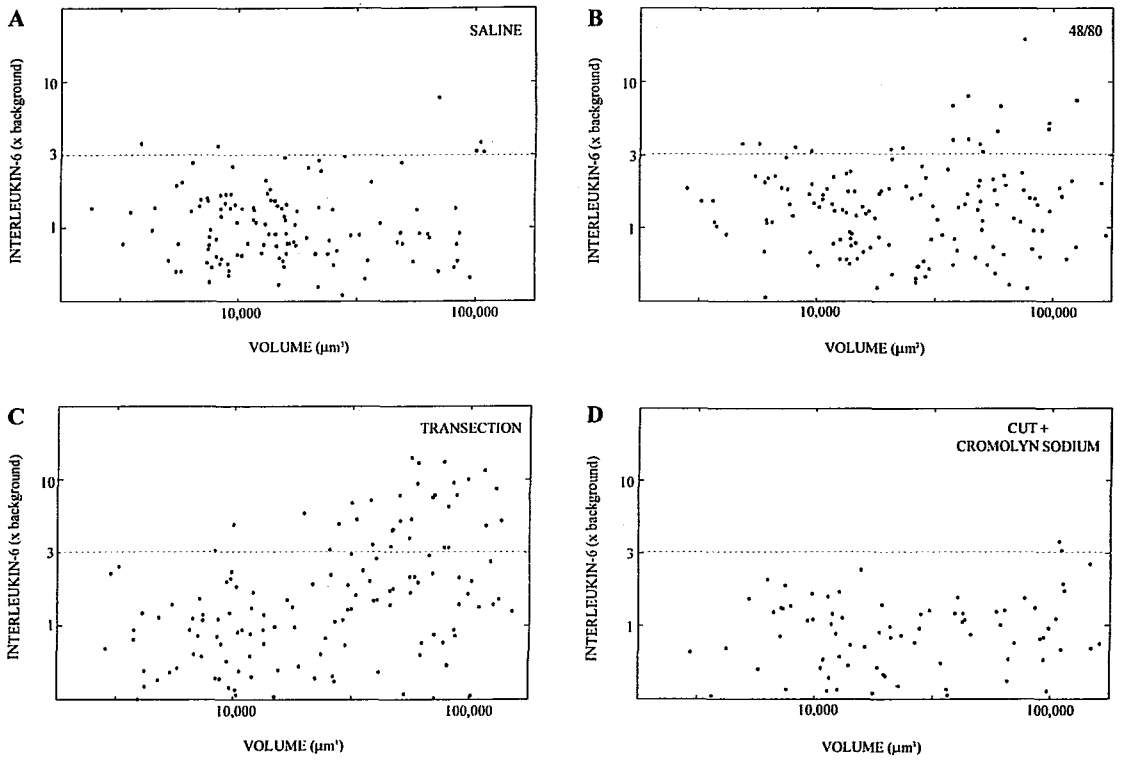
A
SALINE
B
48/80
C
TRANSECTION
D
CUT +
CROMOLYN SODIUM
INTERLEUKIN-6 (x background)
10
3
1
10,000
100,000
VOLUME (μm³)
VOLUME (μm²)

TABLE 1: NEURONAL COUNTS IN L5 DRG OF WILDTYPE (WT) AND IL-6 -/- (KO) MICE 14 DAYS AFTER SCIATIC NERVE TRANSECTION

	Contralateral	Ipsilateral	Ratio (I/C)
WT	3555 ± 201	2470 ± 134	70 ± 2
KO	3268 ± 261	1840 ± 157	57 ± 4*

Neurons with distinct nucleoli were counted by a blinded observer in every fifth section from contralateral and ipsilateral DRG removed 2 weeks after sciatic nerve transection (mean ± s.e.m.; n= 9 mice in each group). Note that the mean of the ratios is not the same as the ratio of the means. * $p < 0.05$ by Student's t-test or Mann-Whitney rank sum test. Neuronal counts in the contralateral DRG tended to be lower in IL-6 -/- mice than wildtype mice but this trend did not reach statistical significance.

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CHAPTER 3

INTERDEPENDENT ACTIONS OF INTERLEUKIN-6 AND BRAIN-DERIVED NEUROTROPHIC FACTOR ON RAT AND MOUSE PRIMARY SENSORY NEURONS

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PURPOSE OF THE STUDY

The previous chapter presented evidence that endogenous IL-6 mitigates the death of DRG neurons after nerve transection. However, evidence that IL-6 supports neurons directly is less compelling than for the neuropoietic cytokines CNTF and LIF. For instance, exogenous IL-6 has been reported to have little action on DRG neurons (Horton et al., 1998; Kurek et al., 1998) unless it is administered along with soluble gp80, the high-affinity IL-6 α-receptor (Thier et al., 1999). This suggests that the trophic influence of IL-6 on DRG neurons is limited by the distribution of gp80. This study investigates the responsiveness of DRG neurons to IL-6 and the distribution of gp80 mRNA and protein among DRG cell types.

Another factor that may influence the effects of IL-6 on DRG neurons is the availability of a cofactor. Several findings suggest that BDNF could act as a cofactor for IL-6. First, IL-6 can support the survival of enteroceptive neurons of the nodose ganglion that are also dependent on BDNF (Horton et al., 1998). Second, BDNF is synthesized by DRG neurons in culture and supports the survival of this subpopulation via an autocrine loop (Acheson et al., 1995). Third, BDNF is upregulated in response to nerve transection in medium to large DRG neurons (Michael et al., 1997). This raises the possibility that the same population of axotomized DRG neurons synthesize IL-6 and BDNF.

This study evaluates if IL-6 and BDNF cooperate to support the survival of DRG in culture. Furthermore, it seeks to determine if IL-6 and BDNF are colocalized in axotomized DRG neurons and if IL-6 contributes to the induction of BDNF in these neurons.

ABSTRACT

We describe here reciprocity in the actions of interleukin-6 and brain-derived neurotrophic factor on primary sensory neurons. In low-density, neuron-enriched cultures of neurons from fetal rat dorsal root ganglia, interleukin-6 supports the survival of approximately one third of the neurons yet virtually all of them bear interleukin-6 α-receptors. One possible explanation for this selectivity is that the actions of interleukin-6 on sensory neurons are mediated through brain-derived neurotrophic factor. Brain-derived neurotrophic factor mRNA is detected in the neuronal cultures and agents that block the biological activity of endogenous brain-derived neurotrophic factor also block the survival-promoting actions of interleukin-6. In adult rats, interleukin-6 mRNA and brain-derived neurotrophic factor mRNA are induced in a similar population of dorsal root ganglion neurons after nerve transection. In interleukin-6 knockout mice, the induction of brain-derived neurotrophic factor after nerve transection is severely attenuated.

In brief, the ability of interleukin-6 to support the survival of embryonic neurons depends upon the presence of endogenous brain-derived neurotrophic factor and the induction of brain-derived neurotrophic factor in injured sensory neurons depends upon the presence of endogenous interleukin-6.

INTRODUCTION

IL-6 (interleukin-6) is produced in the adult peripheral nervous system after nerve injury (Kiefer et al., 1993; Murphy et al., 1995; Zhong and Heumann, 1995; Bourde et al., 1996) and mitigates the death of DRG (dorsal root ganglion) neurons (Murphy et al., 1999a). Another demonstrated action of IL-6 on adult DRG neurons is to stimulate the synthesis of galanin in some of them (Thompson et al., 1998; Murphy et al., 1999b). In vitro, IL-6 supports the survival of fewer embryonic primary sensory neurons (Horton et al., 1998; Thier et al., 1999) than ciliary neurotrophic factor or leukemia inhibitory factor even though all three molecules act through the same signalling receptor gp130 (Taga and Kishimoto, 1997).

One putative limiting factor in the responsiveness of DRG neurons to IL-6 could be restriction in the distribution of gp80, the non-signalling ligand-binding α-component of the IL-6 receptor complex (Simpson et al., 1997; Yawata et al., 1993; Gadient and Otten, 1996; Schöbitz et al., 1993). Support for this hypothesis comes from the observation that addition of soluble IL-6 receptor enhances the ability of IL-6 to maintain DRG neurons (Thier et al., 1999).

Selectivity in the actions of IL-6 on DRG neurons might arise also from the need for a second cell-specific agent. Several pairs of growth factors including IL-6 and NGF (nerve growth factor) (Wu and Bradshaw, 1996; Sterneck et al., 1996) co-operate to promote neuronal survival and differentiation (Maina and Klein, 1999; Carnahan et al., 1994; Arce et al., 1998; Kriegelstein et al., 1998; Zinman et al., 1998). The neurotrophin, BDNF (brain-derived neurotrophic factor), is synthesized in a population of small and medium-sized trkA-containing neurons in normal rat DRG (Verge et al., 1996; Ernfors et al., 1990). Like IL-6, BDNF is induced in a population of large and medium sized neurons after nerve transection (Averill et al., 1997). We have discovered co-operation between IL-6 and BDNF in their actions on DRG neurons.

METHODS

CELL CULTURE

DRG were dissected from E16 (embryonic day 16) rat embryos removed under sterile conditions from pregnant Sprague-Dawley rats and dissociated with 0.005% trypsin and 0.05 mg/ml DNase 1. To enrich for neurons (Lindsay, 1988), cells were centrifuged through 15% bovine serum albumin, applied to a one-step Percoll gradient and preplated for 2 hours at 37°C on Falcon 3001 plastic. Non-adherent neurons or adherent non-neuronal cells were resuspended in modified neurobasal medium. In low-density cultures, 200-400 neurons from E16 rat DRG were plated on laminin-coated 16 mm plates in 200 µl modified neurobasal medium (GIBCO, MD, USA), with or without rat IL-6 (Braciak et al., 1993) or murine rIL-6 (kindly supplied by Gerald Fuller, University of Alabama at Birmingham) (Grenett et al., 1991). Two days later, putative neurons with processes at least twice the diameter of the cell body were counted and the counts compared to counts of neurons in the presence of 20 ng/ml βNGF. In other experiments, neurons were cultured in the presence of 40 ng/ml IL-6 together with an anti-NGF antibody (Cedarlane Laboratories, Canada) that cross-reacts with all neurotrophins, an anti-BDNF neutralizing antibody (Promega Corp, WI, USA) at 10 mg/ml, or trk-IgG fusion proteins (kindly supplied by David Shelton, Genentech).

In high-density cultures, 2000-3000 neurons were plated in laminin-coated wells in the absence of any survival factors and incubated with one of two anti-IL-6 blocking antibodies, a murine monoclonal antibody kindly supplied by Dr. Gerald Fuller (Grenett et al., 1991) and a rat polyclonal antibody (Thibault et al., 1996). Surviving neurons were counted 48 hours later.

The B9 lymphoma cell line was used to assay for IL-6 bioactivity (Aarden et al., 1987).

RT-PCR AND SOUTHERN BLOTTING FOR IL-6 and GP80

RNA was isolated from embryonic cultures as previously described (Murphy et al., 1995) or with a RNeasy kit (Qiagen GmbH, Germany) and 1 µg aliquots were reverse

transcribed with random hexanucleotide primers and Moloney murine leukemia virus reverse transcriptase (Murphy et al., 1995).

The polymerase chain reaction (PCR) and Southern blotting for IL-6 were performed as previously described (Murphy et al., 1995). PCR for gp80 was performed with DNA obtained from 50 ng RNA, primers corresponding to nucleotides 800-818 and 1422-1440 spanning the transmembrane domain of gp80 (Baumann et al., 1990) and 22 cycles of 1 minute at 95°C, 1 minute at 55°C, and 2 minutes at 72°C. For Southern blotting after electrophoresis of the PCR product and transfer to nylon membranes, a ^{32}P-labelled oligonucleotide corresponding to bp 1179-1227 was used.

IMMUNOCYTOCHEMISTRY FOR GP80 AND CELL MARKERS

Cells were plated onto 12 mm glass circular discs pre-coated with poly-orinithine and laminin and the discs were placed in 16 mm wells. After overnight incubation, cultures were fixed for 5 minutes with 4% paraformaldehyde, and washed. Cells were incubated sequentially in 0.6% H_2O_2, in a blocking solution containing 3% normal goat serum, 0.5% BSA, and 0.01% Triton X-100, overnight at 4°C with primary antibodies diluted in the same solution, for 45 minutes at room temperature with a biotinylated secondary antibody (Vector Laboratories, CA, USA), and with avidin-biotin complex containing 0.05% diaminobenzidine and 0.01% H_20_2. The reaction was terminated with water and the cells were lightly stained with Toluidine blue. Antibodies used to assess the composition of the cultures were rabbit anti-rat S100 (Sigma, MO, USA) at 1:2000, rabbit anti-rat Thy 1.1 (Cedarlane Laboratories, Canada) at 1:1000, and rabbit anti-human neurofilament (kindly supplied by Jean-Pierre Julien, Montreal General Hospital, 1:500). More than 95% of cells in neuron-enriched cultures were neurons. Three separate antibodies to gp80 were used at a 1:200 dilution, a rabbit polyclonal raised against the extracellular domain (Thibault et al., 1996), a monoclonal blocking antibody (kindly supplied by J. Van Snick, Ludwig Institute for Cancer Research, Brussels, Belgium) (Vink et al., 1990), and a rabbit antibody against a peptide corresponding to amino acids 441-460 at the carboxy terminus of mouse IL-6 (Santa Cruz Biotechnology, CA, USA).

RNASE PROTECTION ASSAYS FOR BDNF

For RNase protection assays (Seniuk et al., 1992), a BDNF cDNA product was obtained by RT-PCR from rat brain RNA, subcloned into the pGEM-7Zf(-) plasmid vector, and sequenced. The BDNF cDNA was linearized and transcribed into a ^{32}P-labelled antisense RNA probe by incubation with SP6 RNA polymerase. RNA extracted from cell samples was incubated overnight at 50°C with BDNF and cyclophilin riboprobes. Following digestion with RNase A and RNase T1, the protected fragments were separated by electrophoresis through a urea-polyacrylamide gel and visualized by radioautography.

SURGERY

Experiments were performed on adult Sprague-Dawley rats and on IL-6 -/- mice (Kopf et al., 1994) plus wild-type C57BL6/129 mice. Rats were anesthetized by intraperitoneal injection of phenobarbital (50 mg/kg) and mice by intramuscular injection of a mixture of ketamine (0.75 mg/g) and xylazine (0.01 mg/g). The right sciatic nerve was transected at the hip in mice and at its origin from the L4 and L5 spinal nerves in rats. Mice and rats were sacrificed 4 days after nerve transection.

Other rats were infused intrathecally with recombinant mouse IL-6 (250 ng/h) in a solution of phosphate-buffered saline containing penicillin and streptomycin or with control solution without IL-6. The infusion system consisted of an osmotic pump connected to silicon tubing that was inserted into the subarachnoid space through a laminectomy at the lumbosacral junction (Verge et al., 1995). Rats were sacrificed after 4 days of infusion.

IN SITU HYBRIDIZATION FOR IL-6 AND BDNF

DRG for in situ hybridization were frozen at -55°C in Tissutek (Miles Laboratories, IN, USA) and stored at -70°C. Cryomolds containing DRG from pairs of mice were joined before sectioning so that DRG to be compared could be processed and analyzed on the same slide. Oligonucleotides corresponding to bp 645-694 of the rat BDNF cDNA (Maisonpierre et al., 1991), or bp 137-184 and 602-649 of the rat IL-6

cDNA (Northemann et al., 1989) were ^{33}P-labelled by the terminal transferase reaction and purified (Murphy et al., 1995). Sections, cut on a cryostat set at 20 μm, were hybridized overnight at 42 °C in a solution containing 50% formamide, washed in 1X SSC at 55°C for 15 minutes, fixed briefly in ethanol, dried, dipped in radiosensitive emulsion, exposed at -20°C for approximately 10 days, developed, fixed, and stained with Toluidine Blue (Murphy et al., 1995).

For studies involving co-localization of IL-6 and BDNF mRNAs, labelling was quantified (Richardson et al., 1989) for individual neurons in adjacent sections that were identified in photomontages of the pairs of sections. Labelling index refers to the ratio of grain density over neurons to grain density over non-neuronal areas of the same section.

RESULTS

IN VITRO, MOST DRG NEURONS HAVE IL-6 α-RECEPTORS AND SOME RESPOND TO EXOGENOUS OR ENDOGENOUS IL-6

Analysis of the RNA from embryonic rat DRG cultures by RT-PCR and Southern blotting demonstrated a PCR product that co-migrated with gp80 RT-PCR products from spleen and liver (n=10). Upon segregation of cell types, the PCR product generated from neuronal cell mRNA was consistently more abundant than that generated from RNA from non-neuronal cells (Figure 1A). In DRG cultures, most cells with neuronal morphology but no non-neuronal cells were immunoreactive for gp80 (Figure 1B). Similar patterns of immunostaining were observed with all three antibodies to gp80. Immunoreactivity was not detected with omission of the primary antibody, incubation with anti-isotypic antibodies, or pre-incubation of the anti-peptide antibody with 100X fold excess of the receptor peptide. The observations indicate that most or all DRG neurons in culture have IL-6 α-receptors at least some of which are not truncated (Rose-John and Heinrich, 1994) since they are recognized by an antibody to a peptide at the carboxy terminus. The additional possibility that some non-neuronal cells, for example macrophages (Munck Petersen et al., 1990), have IL-6 α-receptors has been neither confirmed nor excluded.

Forty-eight hours after plating at low density in neurobasal medium without serum or exogenous neurotrophic support, no surviving DRG neurons were observed. IL-6 from each of the 3 sources caused a dose-dependent increase in survival of sensory neurons detectable at a concentration of 0.2 ng/ml and maximal at 40 ng/ml (Figure 2A). IL-6 supported approximately 60% as many neurons as NGF or one third of the plated population. Under these experimental conditions, IL-6 suffices to keep alive a subpopulation of DRG neurons that would otherwise die.

In high-density cultures, many DRG neurons survived for at least 72 hours without serum or exogenous trophic agents. Addition of polyclonal or monoclonal anti-IL-6 blocking antibodies, but not rabbit, mouse or anti-isotypic serum, decreased this spontaneous survival (Figure 2B). The inhibitory activity of the antibodies withstood pre-heating for 30 minutes at 56°C but was abolished by pre-incubation of the antibody with

recombinant IL-6. In analysis of RNA from neuron-enriched cultures by RT-PCR and Southern blotting for IL-6 mRNA, a PCR product was detected that co-migrated with the product from spleen: the band was not seen after degradation of RNA by RNase before PCR. In addition, neuron-conditioned medium was shown by assay with B9 cells to have IL-6 bioactivity (data not shown). The data indicate that cells in high-density DRG cultures synthesize IL-6 that promotes the survival of neurons by autocrine or paracrine mechanism.

BDNF IS PRESENT IN DRG CULTURES AND REQUIRED FOR SUPPORT OF NEURONS BY IL-6

Analysis of RNA from purified embryonic rat DRG neurons by RNase protection assays with a BDNF probe consistently revealed a protected fragment of appropriate size (approximately 700 bp) that co-migrated with a fragment from rat brain mRNA (data not shown). These observations confirm previous evidence that BDNF is synthesized by DRG neurons in in vitro (Acheson et al., 1995).

In further experiments, exogenous BDNF supported the survival of approximately one third of E16 DRG neurons (data not shown), although under other culture conditions, it supported only a few E15 mouse DRG neurons (Davies et al., 1993). BDNF maintains the viability in vitro of a subpopulation of adult DRG neurons through an autocrine mechanism (Acheson et al., 1995). Accordingly, we investigated the possible participation of endogenous BDNF in the maintainence of embryonic DRG neurons by IL-6.

Addition of an anti-pan neurotrophin antibody to low-density cultures of DRG neurons maintained with 40 ng/ml IL-6 almost completely blocked neuronal survival (Figure 1C). Normal rabbit or anti-isotypic serum did not interfere with survival. The inhibitory effects of the anti-neurotrophin antibody persisted after heating for 30 minutes at 56°C. They were abolished by pre-incubation with NGF but not IL-6. Maintenance of DRG neurons by IL-6 was also blocked by a neutralizing antibody to BDNF (data not shown) or by 40 ng/ml trkB-IgG but not by trkA-IgG (Figure 2D). The blocking effect of trkB-IgG was neutralized by pre-incubation with BDNF. Although NT-3 and NT-4/5 as well as BDNF might be blocked by trkB-IgG, the former two are not blocked by this

antibody. The combination of observations with the anti-BDNF antibody and trkB-IgG fusion protein implicates BDNF as the neurotrophin in neuronal cultures that mediates the actions of IL-6.

IL-6 IS INDUCED IN THE SAME AXOTOMIZED DRG NEURONS AS BDNF AND IS REQUIRED FOR INDUCTION OF BDNF IN THESE NEURONS

In L5 DRG associated with uninjured sciatic nerves taken from rats, wildtype mice, or IL-6 -/- mice, IL-6 mRNA was not detected by in situ hybridization and BDNF mRNA was present in approximately one third of the neurons, most of them small. Following nerve transection in rats, IL-6 mRNA was present in one third of DRG neurons, most of them large or medium in size, and BDNF mRNA was present in approximately two thirds of neurons including many that were large or medium in size. In co-localization studies of L5 DRG after nerve transection (Figure 3 and 4), virtually all neurons that contained IL-6 mRNA also contained BDNF mRNA. On the other hand, many small neurons containing BDNF did not contain IL-6 mRNA. The most parsimonious explanation of these results is that IL-6 mRNA is present in the population of DRG neurons that synthesize BDNF mRNA de novo after nerve injury (Averill et al., 1997).

In wild-type mice as in rats, the proportion of L5 DRG neurons with BDNF increased from approximately one third to approximately two thirds 4 days after nerve transection. In uninjured IL-6 -/- mice the proportion of neurons with BDNF was not significantly different than in wild-type mice. However, after nerve transection in IL-6 -/- mice, the proportion of neurons with BDNF mRNA did not increase above basal values (Figure 5). The results show that induction of BDNF mRNA in DRG neurons after nerve transection is severely attenuated or abolished in IL-6 -/- mice.

DISCUSSION

IL-6 DEPENDS UPON ENDOGENOUS BDNF TO MAINTAIN DRG NEURONS

IL-6 maintains a greater percentage of embryonic rat DRG neurons under these conditions than of newborn rat DRG (Thier et al., 1999) or embryonic mouse trigeminal neurons (Horton et al., 1998) under other conditions. The efficacy of IL-6 on embryonic rat DRG neurons is similar to that on embryonic mouse neurons (Horton et al., 1998). Age and species of animal, anatomical source of sensory neurons, and culture conditions may all contribute to the differences in responses to IL-6.

IL-6 supports the survival of a subpopulation of DRG neurons, depending upon the actions of endogenous BDNF synthesized by the neurons. The nature of the surviving subpopulation and the mechanism of the dependence of IL-6 on BDNF in vitro have not been defined.

At optimal concentrations, either IL-6 or BDNF supports approximately one third of E16 rat DRG neurons. The trkB population in adult rat DRG (McMahon et al., 1994), the reduction in numbers of DRG neurons in BDNF –/– mice (Jones et al., 1994) or trkB –/– mice (Klein et al., 1993) mice, and the killing of adult rat DRG neurons by BDNF antisense oligonucleotides (Acheson et al., 1995) are all in the order of 30-40% of the total population. The simplest explanation of these observations is that IL-6, through BDNF, supports only cells that bear trkB. However, the alternative possibility that BNDF has indirect actions on trkB-negative cells has not been excluded.

Three possible explanations for the dependence of IL-6 activity on endogenous BDNF are that IL-6 induces BDNF, that IL-6 induces trkB, or that IL-6 and BDNF have synergistic actions on convergent intraneuronal signalling pathways. IL-6 does participate in the induction of BDNF in DRG neurons, at least in vivo. Proof that IL-6 induces BDNF in vitro is difficult to obtain because these embryonic DRG neurons do not survive under basal conditions without IL-6 support. The ability of IL-6 to induce BDNF in DRG neurons provides sufficient but not necessarily exclusive explanation for its ability to support the survival of these neurons.

IL-6 CONTRIBUTES TO THE INDUCTION OF BDNF mRNA IN INJURED DRG NEURONS

After nerve injury in rats, BDNF is induced in the same or almost the same population of neurons as IL-6. After nerve injury in IL-6 -/- mice, BDNF is not demonstrably induced in DRG neurons. These observations indicate that IL-6 is necessary for the induction of BDNF and are consistent with an autocrine mechanism of action for endogenous IL-6.

Axotomy, NGF, or neuronal activity each can stimulate expression of the BDNF gene in neurons. The present results together with previous analysis of the BDNF promoter (Timmusk et al., 1995) suggest that the induction of BDNF in axotomized DRG neurons is mediated by IL-6 response element(s) active in the promoter region upstream of exon 4.

After nerve injury, IL-6 mitigates the death of DRG neurons (Murphy et al., 1999a) and promotes behavioural changes associated with neuropathic pain (Murphy et al., 1999b). It is plausible that both of these actions of IL-6 on sensory neurons are mediated through stimulation of synthesis of BDNF.

FIGURE 1: A: Southern blot following RT-PCR to analyze gp80 mRNA in RNA (50 ng) extracted from purified neuronal and non-neuronal preparations of E16 DRG. Cultures enriched for neurons or, to a lesser extent, those enriched for non-neuronal cells yield a PCR product of approximately 600 bp co-migrating with a product from liver. B: Immunocytochemistry of purified cultures from E16 DRG demonstrating immunoreactivity for gp80 in sensory neurons with an antibody raised against a peptide at the carboxy terminus. C: Loss of immunoreactive product following incubation of the antibody with 100X excess of peptide prior to immunocytochemistry.

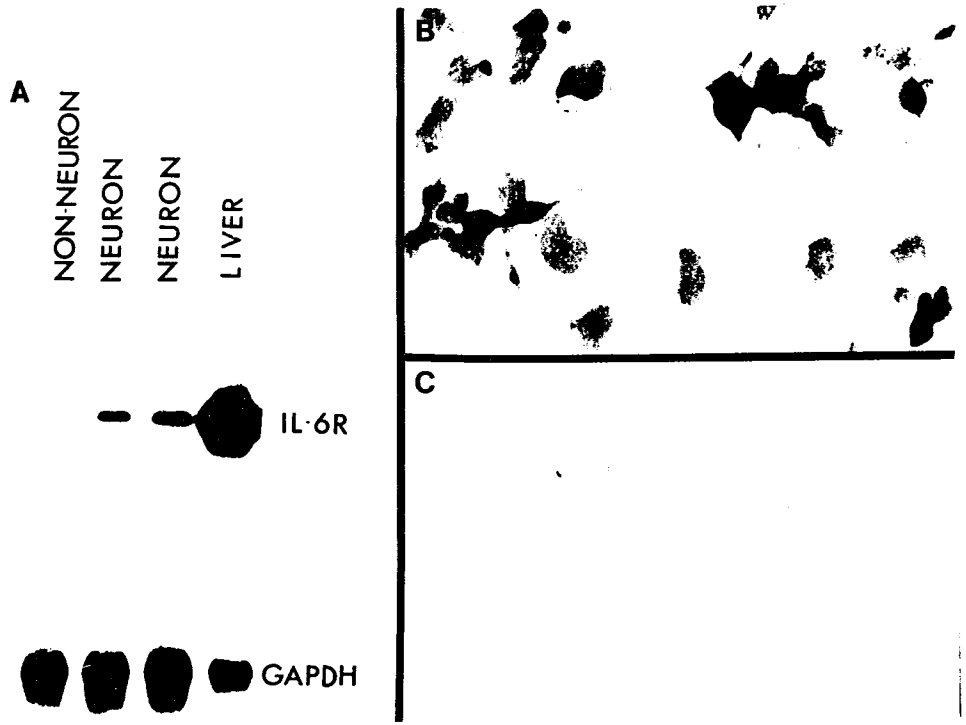

A
NON-NEURON
NEURON
NEURON
LIVER
IL-6R
GAPDH
B
C

FIGURE 2: A: Influence of exogenous IL-6 on survival of E16 rat DRG neurons (mean ± S.E.M., n=10). Survival is expressed as a percentage of survival of neurons supported by NGF. B: Inhibition by polyclonal anti-IL-6 antiserum of spontaneous survival of neurons cultured at high density. The number of neurons surviving in the presence of antibody is expressed as a percentage of the number surviving in duplicate cultures without antibody (mean ± S.E.M, n=10 for each antibody). The antibody is neutralized by pre-incubation with 10 ng (square), 25 ng (triangle), or 50 ng (inverted triangle) of IL-6. C: Inhibition by a pan anti-neurotrophin antibody of the survival-promoting activity of IL-6 (40 ng/ml) on DRG neurons cultured at low density (mean ± S.E.M, n=10). Antibody pre-incubated with NGF (square) does not inhibit IL-6 activity. D: A trkB-IgG fusion protein (0.5 - 2.0 μg/ml) (circles) but not trkA-IgG fusion protein (squares) blocks survival of DRG neurons cultured at low density in the presence of 40 ng/ml IL-6 (mean ± S.E.M, n=6). Pre-incubation of the trkB-IgG with BDNF (triangles) overcomes the inhibition of survival by the fusion protein.

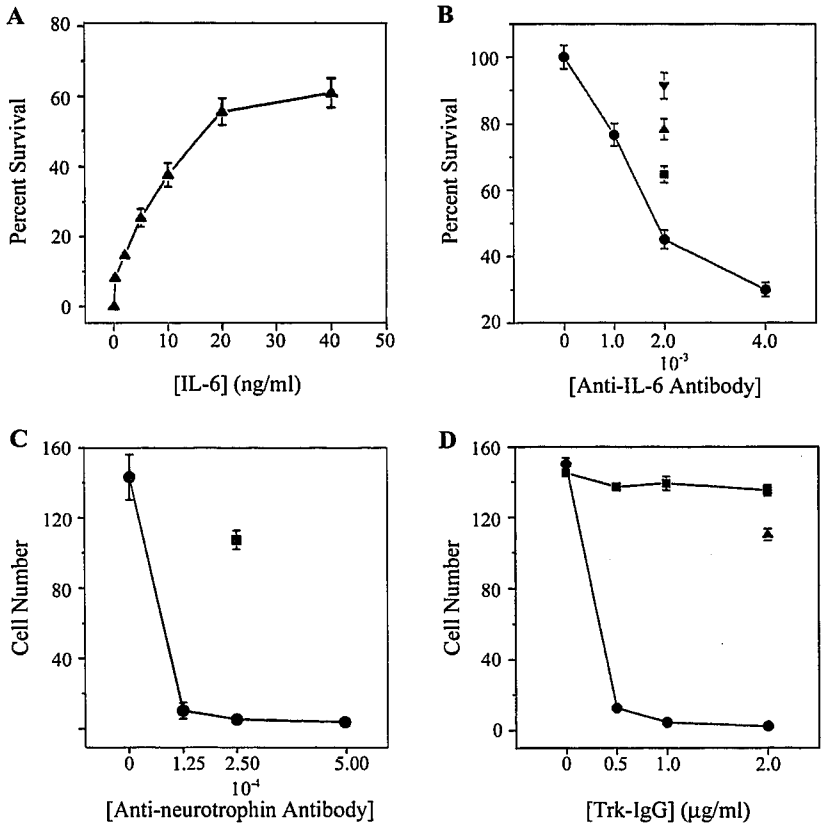

A
Percent Survival
80
60
40
20
0
0
10
20
30
40
50
[IL-6] (ng/ml)
B
Percent Survival
100
80
60
40
20
0
1.0
2.0
4.0
10^{-3}
[Anti-IL-6 Antibody]
C
Cell Number
160
120
80
40
0
1.25
2.50
5.00
10^{-4}
[Anti-neurotrophin Antibody]
D
Cell Number
160
120
80
40
0
0.5
1.0
2.0
[Trk-IgG] (μg/ml)

FIGURE 3: A: Photomicrographs of adjacent sections of a L5 DRG from an adult rat processed for in situ hybridization with oligonucleotide probes for IL-6 (A) or BDNF (B). Note two large neurons one of which is positive for both IL-6 and BDNF mRNAs and the other positive for BDNF mRNA but negative for IL-6 mRNA. (Magnification x 1200).

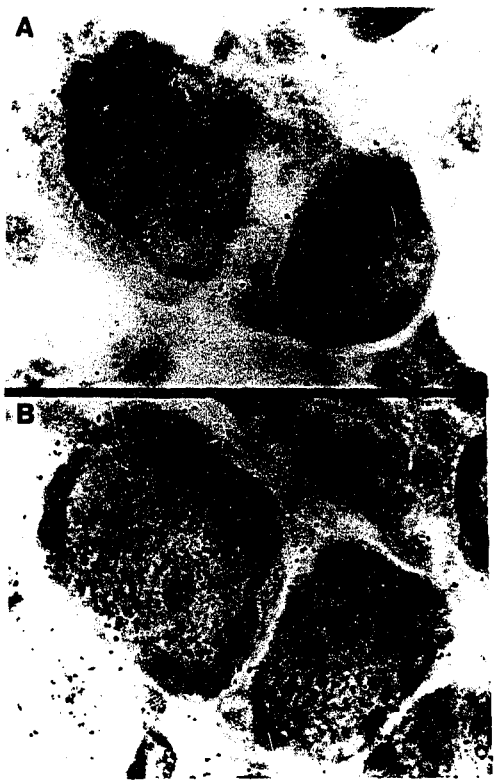

A
B

FIGURE 4: Scatter diagram in which labelling indices for BDNF mRNA (y axis, log scale) and IL-6 mRNA (x axis, log scale) are presented for individual neurons in a rat L5 DRG. Note that all but two of the neurons that are heavily labelled with IL-6 are also labelled with BDNF whereas many neurons are heavily labelled with BDNF but not IL-6.

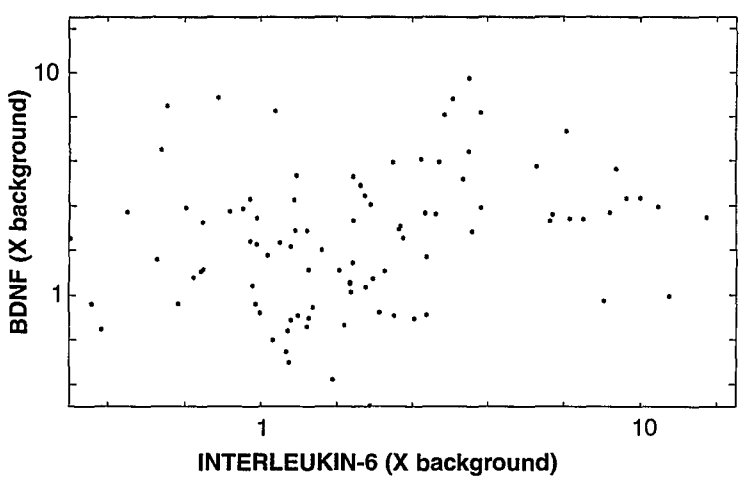
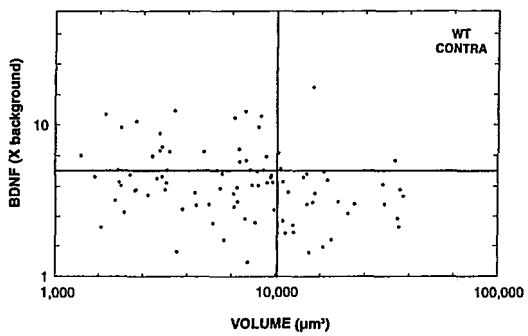
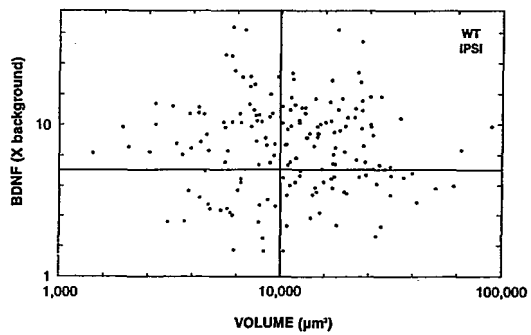
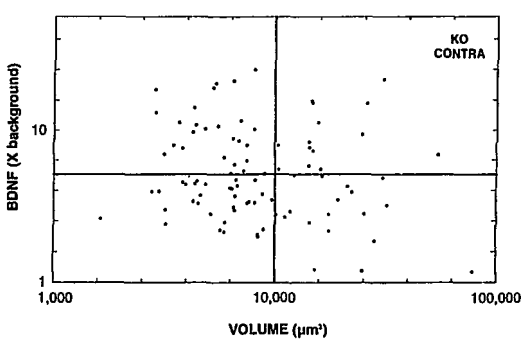
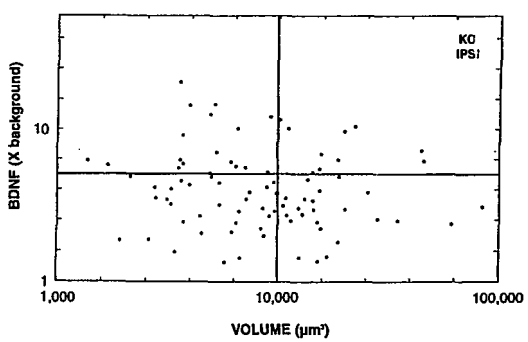

10
1
BDNF (X background)
1
10
INTERLEUKIN-6 (X background)

FIGURE 5: Scatter diagrams (log-log scale) to show BDNF labelling densities (y axes) and volume (x axes) of individual neurons in four L5 mouse DRG processed for BDNF in situ hybridization. DRG contralateral (A) and ipsilateral (B) to the side of sciatic nerve transection 4 days before sacrifice in a wild-type mouse. DRG contralateral (C) and ipsilateral (D) to the side of transection in a IL-6 -/- mouse. Note in all DRG the presence of a moderate number of small and medium sized neurons with high BDNF labelling indices. In wild-type but not in IL-6 -/- mice, BDNF mRNA is induced in many large neurons (upper right quadrant) after sciatic nerve transection.


WT
CONTRA
BDNF (X background)
10
1
1,000
10,000
100,000
VOLUME (µm³)


WT
IPSI
BDNF (X background)
10
1
1,000
10,000
100,000
VOLUME (µm³)


KO
CONTRA
BDNF (X background)
10
1
1,000
10,000
100,000
VOLUME (µm³)


KO
IPSI
BDNF (X background)
10
1
1,000
10,000
100,000
VOLUME (µm³)

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CHAPTER 4

ENDOGENOUS INTERLEUKIN-6 CONTRIBUTES TO HYPERSENSITIVITY TO CUTANEOUS STIMULI AND CHANGES IN NEUROPEPTIDES ASSOCIATED WITH PERIPHERAL NEUROPATHIC PAIN

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PURPOSE OF THE STUDY

The neuropoietic cytokines perform similar and often overlapping functions, due to sharing of the common receptor subunit, gp130. In addition to the well-documented role of LIF, CNTF and IL-6 in supporting neuronal survival, LIF and IL-6 have also been implicated in neuropathic pain. It has been shown, for instance, that injection of exogenous IL-6 into the cerebral ventricle or lumbar subarachnoid space induces thermal hyperalgesia and/or tactile allodynia in rats (Oka et al., 1995; DeLeo et al., 1996). The primary objective of this study is to examine the role of endogenous IL-6 in neuropathic pain, using the chronic constriction nerve injury model in IL-6 deficient mice.

LIF and CNTF have also been shown to regulate the expression of neuropeptides in sympathetic and sensory neurons (Kerekes et al., 1999; Corness et al., 1996; Thompson et al., 1998; Fann and Patterson, 1993; Symes et al., 1993; Zinman et al., 1998). The injury-induced upregulation of LIF mRNA leads to some striking changes in neuropeptide gene expression; the most recognized being the upregulation of galanin in small DRG neurons. The neuropeptides galanin and substance P are known to modulate nociceptive transmission and changes in the expression levels of the two molecules could potentially underlie behavioural changes associated with neuropathic pain. The second objective of this study is to examine whether IL-6 influences the expression of the pain-related peptides substance P and galanin.

ABSTRACT

Partial nerve injury can cause distressing chronic pain for which conventional analgesic treatment with opiates or anti-inflammatory agents is not very effective. Constriction nerve injury, widely used to study neuropathic pain, was shown to induce interleukin-6 (IL-6) mRNA in a subset of rat primary sensory neurons. The hypersensitivity to tactile and thermal stimuli that are caused by chronic nerve constriction in wild-type mice were not evident in mice with null deletion of the interleukin-6 gene. Also, after constriction nerve injury in mice without interleukin-6, the loss of substance P in sensory neurons was excessive and the induction of galanin in central sensory projections was reduced. In additional experiments, intrathecal infusion of interleukin-6 in rats was shown to stimulate synthesis of galanin in approximately one third of lumbar dorsal root ganglion neurons. The results of these experiments indicate that endogenous IL-6 mediates some of the hypersensitive responses that characterize peripheral neuropathic pain, and influences two neuropeptides that have been implicated in pain transmission.

INTRODUCTION

Despite recent advances in understanding molecular mechanisms of neuropathic pain, treatment of these symptoms is still unsatisfactory. One experimental model to study peripheral neuropathic pain is chronic constriction injury to the rodent sciatic nerve (Bennett and Xie, 1988), which produces allodynia (painful responses to innocuous stimuli), hyperalgesia (excessive responses to noxious stimuli), Wallerian degeneration particularly of large fibres, changes in peptides in primary sensory neurons, sprouting of sympathetic axons in the DRG (dorsal root ganglion), and sprouting of primary afferent fibres in the dorsal horn of the spinal cord (Basbaum et al., 1991; Kajander and Bennett, 1992; Nahin et al., 1994; Study and Kral, 1996; Shortland et al., 1997). Thermal hyperalgesia and tactile allodynia that reflect peripheral sensitization of primary sensory neurons and central sensitization of dorsal horn neurons (Bessou and Perl, 1969; LaMotte et al., 1983; Woolf and King, 1987; Campbell et al., 1988; Kuraishi et al., 1991; Koltzenburg et al., 1994; Woolf et al., 1994) that have been implicated in the development of such hypersensitivity include NGF (nerve growth factor), substance P, glutamate receptors, nitric oxide synthase and protein kinase C (McMahon et al., 1993; Neugebauer et al., 1993; Lewin and Mendell, 1994; Mantyh et al., 1997).

The pleiotrophic cytokine IL-6 (interleukin-6), undetectable by conventional methods in the normal peripheral nervous system, is induced following nerve transection in some motor and sensory neurons and in non-neuronal cells in the nerve (Kiefer et al., 1993; Murphy et al., 1995; Bolin et al., 1995; Kurek et al., 1996; Reichert et al., 1996). Little is known about the functions of IL-6 induced in the peripheral nervous system. IL-6 receptor mRNA is present in the DRG (Gadient and Otten, 1996), and the presence of IL-6 in the facial motor nucleus contributes to the glial responses found for axotomized neurons (Klein et al., 1997). The possibility that IL-6 might influence neuronal peptide synthesis is suggested by the actions on sympathetic and sensory neurons of LIF (leukemia inhibitory factor) and CNTF (ciliary neurotrophic factor) (Rao et al., 1993; Corness et al., 1996; Sun and Zigmond, 1996), both of which act through the same signalling receptor as IL-6 (Kishimoto et al., 1994; Taga and Kishimoto, 1997). IL-6 and LIF have been

implicated in pain by reports that injection of IL-6 into the cerebral ventricle or lumbar subarachnoid space induces thermal hyperalgesia and/or tactile allodynia (Oka et al., 1995; DeLeo et al., 1996), local injection of LIF induces tactile allodynia (Thompson et al., 1996), and IL-6 and LIF are involved in sympathetic sprouting about axotomized DRG neurons (Ramer et al., 1998; Thompson and Majithia, 1998). On the other hand, evidence has be published to suggest that IL-6 (Xu et al., 1997) and LIF (Banner et al., 1998) are analgesic.

Using mice with a null mutation of the IL-6 gene, we have examined possible contributions of endogenous IL-6 to the cutaneous hypersensitivity and changes in neuronal peptides that are associated with constriction nerve injury.

METHODS

ANIMAL SURGERY

Thirty adult female Sprague-Dawley rats weighing approximately 200 g were anaesthetized with pentobarbital (50mg/kg, intraperitoneally). The right sciatic nerve was exposed in the thigh and 4 ligatures (4-0 chromic gut suture, softened in saline) were loosely tied around the nerve at intervals of several millimetres (Bennett and Xie, 1988; Ramer et al., 1997). Adult male wild-type and IL-6 -/- mice (Kopf et al., 1994) weighing approximately 25 g were anaesthetized either intramuscularly with a mixture containing 0.75 mg/g ketamine and 0.01 mg/g xylazine or with methoxyflurane anaesthesia. Chronic constriction injuries in mice were performed with 3 5-0 chromic gut ligatures (Ramer et al., 1997).

Animal care was according to the guidelines the guidelines of the Canadian Council on Animal Care as monitored by animal care committees at McGill and Queen's Universities.

BEHAVIORAL TESTING

Latency of withdrawal to heat stimulus and threshold to withdrawal from mechanical stimulus were assessed preoperatively and one and two weeks following chronic constriction injury (Ramer et al., 1997) in wild type and IL-6 -/- mice. After acclimatization for 2-5 minutes, thermal stimulus was applied by a radiant heat source of variable intensity shone underneath the plantar hindpaws of mice (Hargreaves et al., 1988). Withdrawal was detected by photosensors, and the time between light onset and withdrawal (withdrawal latency) was automatically recorded by a computer. Intensity was established pre-operatively in control C57Bl/126 mice. Mechanic stimulus was applied with von Frey hairs (0.5g, 0.9g, 1.4g, 2.2g, 3.1g) applied ten times 0.25 Hz to each hindpaw beginning with the weakest hair and finishing with the hair eliciting a nocifensive response (defined as one that lasted for at least 2 seconds with shaking and/or licking the hindpaw or vocalization). For both thermal and mechanical stimuli, each hindpaw was tested alternatively three times, averaged, and differences in latency (seconds) or threshold

(grams) of wild-type and IL-6 mice were analyzed with the Student's t-test. Post-operative withdrawal latency difference scores were compared to pre-operative measurements using a one-way ANOVA. In all behavioral studies, the observer was blinded to the genotype of the mice.

INTRATHECAL INFUSION OF IL-6

Recombinant IL-6 from one of 2 different sources (Grenett et al., 1991; Braciak et al., 1993) or vehicle solution was infused into the rat lumbar subarachnoid space (Verge et al., 1990a). A mini-osmotic pump (Alza 2001) filled with IL-6 at 100 ng/μl in PBS with 0.1%BSA and penicillin/streptomycin was connected to silicone tubing (outer diameter 0.012 inches) that was thread into the lumbar subarachnoid space through a small laminectomy. Following infusion at 1.0 μl/h of either IL-6 or control solution for 3-4 days, L4 and L5 DRG were removed and processed for *in situ* hybridization.

IN SITU HYBRIDIZATION

For *in situ* hybridization (Schalling et al., 1988; Verge et al., 1995), mouse or rat DRG were removed, frozen immediately at -55°C in 2 methyl butane, embedded in Tissue-Tek, (Miles Laboratory), and stored at -80°C. Cryomolds containing contralateral and ipsilateral DRGs from wild-type and mutant mice were fused together before sectioning so that DRG from different mice were processed on the same slide. Sections cut on a cryostat set at 5 μm were mounted on Probe-on slides and hybridized 16-18 hours at 42°C with a solution containing 500,000 cpm of ^{35}S-or ^{33}P-labeled oligonucleotides, 50% formamide, 4X SSC, dextran sulphate (100 mg/ml), sarcosyl (1%), 500 μg/ml salmon sperm DNA, and 200 mM DTT. Oligonucleotides for IL-6, substance P, and galanin (Murphy et al., 1995; Verge et al., 1995) were labeled by the terminal transferase reaction (Sambrook et al., 1989) and purified (Murphy et al., 1995). Following hybridization, the slides were washed four times in 1X SSC at 55°C for 15 minutes, were fixed briefly in 65% and 95% ethanol, dried, dipped in emulsion (NTB2 Kodak), exposed in the dark at -20°C for 1-6 weeks, developed, fixed, and stained with 0.002% toluidine blue.

Neuronal labeling *in situ* hybridization was quantified with a computerized image analysis system (Verge et al., 1990b) for pairs of sections on the same slide. Only cells with a visible nucleolus were quantified. Labeling index refers to the ratio of grain density over neurons to grain density of non-neuronal portions of the DRG.

IMMUNOHISTOCHEMISTRY

Following nerve constriction for 2 weeks, mice were deeply anesthetized and perfused sequentially with ice-cold 1% sodium nitrite and 4% paraformaldehyde in phosphate buffer. Spinal cords (L3-L5) and brainstems were removed, post-fixed overnight in 4% paraformaldehyde, and cryoprotected in 30% sucrose for 2-4 days. Contralateral and ipsilateral tissues were embedded in Tissue-tek in the same mold. Transverse sections cut on a cryostat set at 40 µm were processed for immunocytochemistry for galanin (1:4000, Peninsula Laboratories Inc. Belmont CA) and substance P (1:6000, Chemicon Inc, Temecula CA) with horseradish peroxidase and diaminobenzidine (Ma and Bisby, 1997). Average intensity of the dorsal horn was measured with an image analysis software (SigmaScan, Jandel Scientific Inc., San Rafael CA) in five randomly chosen sections from each animal and the mean intensity was plotted as a function of depth in the dorsal horn. Galanin-immunoreactive fibre density in the gracile nucleus was expressed as the area of galanin-positive axons and terminals divided by the area occupied by the entire nucleus gracilis. A one-way ANOVA followed by Student-Newman-Keuls test for pairwise differences was used to compare the fibre densities of ipsilateral and contralateral gracile nuclei from wild-type and IL-6 -/- mice.

RESULTS

INDUCTION OF IL-6 mRNA BY CONSTRICTION NERVE INJURY

The presence of IL-6 mRNA in ipsilateral L4 and L5 DRG after chronic constriction injury was established by *in situ* hybridization and RNase protection assay. In ipsilateral DRG removed 5 or 14 days after chronic constriction injury, IL-6 mRNA was detected in some medium and large neurons (Figure 1), albeit with weaker signal and in fewer neurons than after nerve transection. 14 days after chronic constrictive injury, IL-6 mRNA was detected in ipsilateral L4 and L5 DRG by *in situ* hybridization and RNase protection assay. In contrast, IL-6 persists for less than 8 days after nerve transection (Murphy et al., 1995). These results indicate that IL-6 mRNA is present in L4 and L5 DRG at times after nerve constriction when tactile allodynia and thermal hyperalgesia are evident.

ATTENUATED BEHAVIORAL RESPONSES FOLLOWING CONSTRICTION NERVE INJURY IN IL-6 -/- MICE

Studies were performed on wild-type and IL-6 -/- mice (Kopf et al., 1994) which breed well, appear normal, and have no known abnormalities of neural development, but have deficiencies in protective and restorative responses to tissue injury (Fattori et al., 1994; Ramsay et al., 1994; Chai et al., 1996; Cressman et al., 1996). In the absence of nerve injury, withdrawal responses in IL-6 -/- mice and wild-type mice were not significantly different. As anticipated (Bennett and Xie, 1988; Ramer et al., 1997; Ramer and Bisby, 1997), constriction nerve injury to wild-type mice induced enhanced withdrawal responses to thermal and mechanical stimuli with statistically significant differences in sensitivity between the ipsilateral and contralateral limb evident at 7 and 14 days (Figure 2). After 14 days of nerve constriction injury in IL-6 -/- mice, the side-to-side difference in IL-6 -/- mice was significantly less than in wild-type mice ($p < 0.001$). Indeed, the threshold to withdrawal from thermal and latency to withdrawal from mechanical stimuli seemed to increase rather than decrease ipsilateral to nerve constriction in IL-6 -/- mice, but the side-to-side differences were not significantly different. We conclude that endogenous IL-6 is

required to produce the neuronal changes that underlie thermal hyperalgesia and mechanical allodynia.

NEUROPEPTIDE RESPONSES TO NERVE CONSTRICTION INJURY AND IL-6 INFUSION

In lumbar DRG contralateral to nerve injury in both IL-6 and wild type mice, substance P mRNA was found in many small neurons, presumably trkA positive, nociceptive neurons that project to superficial laminae of the spinal cord (Verge et al., 1989; Averill et al., 1995). In ipsilateral L4 and L5 DRG removed 5 or 14 days after chronic constrictive injury, substance P mRNA was not altered appreciably in the ipsilateral L4 or L5 DRG of wild-type mice but consistently was diminished in the ipsilateral DRG of IL-6 -/- mice (Figure 3) (n= 5). In the ipsilateral dorsal horn of the lumbar spinal cord, substance P immunoreactivity was unchanged by nerve transection in wild-type mice but reduced by approximately 20% in densitometric assay of layers I and II of IL-6 -/- mice (Figures 4 and 5).

As previously demonstrated in rats (Ma and Bisby, 1997), nerve constriction injury in wild-type mice induced galanin mRNA in many DRG neurons of differing sizes, galanin immunoreactivity in fibres projecting to layers III and IV of the dorsal horn, and galanin immunoreactivity in the ipsilateral nucleus gracilis. Following the same injury in IL-6 -/- mice, no increase in galanin immunoreactivity in deeper layers of the dorsal horn of the spinal cord was detected (Figure 6), and the increase in the nucleus gracilis was 50% of that in wild-type mice (Figure 5 and 6). Nerve constriction injury induced galanin mRNA in approximately one-third of DRG neurons in both wild-type and IL-6 -/- mice. In quantitative analysis of two pairs of DRG, the size and mean labelling index of the third of neurons most heavily labelled was not significantly different in the two strains.

In additional experiments in rats, infusion of IL-6 but not saline into the lumbar spinal subarachnoid space induced a strongly positive signal for galanin mRNA (> 5 x background) in 32 % of DRG neurons, small and medium in size (Figure 7). In control rats infused with saline, 13 % of neurons were positive for galanin mRNA by this criterion. These observations are consistent with an independent report that intraneural injection of

LIF or IL-6 induces galanin immunoreactivity in corresponding DRG neurons (Thompson et al., 1998). Thus, in normal rats, exogenous IL-6 stimulates expression of the galanin gene and, following constriction nerve injury in mice, endogenous IL-6 is partially responsible for an increase of galanin immunoreactivity in central projections of sensory neurons.

DISCUSSION

IL-6 IS INDUCED BY CHRONIC CONSTRICTION INJURY

Chronic constriction injury like nerve transection induces IL-6 in a subpopulation of medium and large sensory neurons, some of which presumably project to the dorsal column nuclei. In comparison to nerve transection, constriction nerve injury induces IL-6 in DRG neurons less intensively but more chronically. IL-6 induction in DRG persists for at least 14 days during constriction injury, as compared to less than 8 days after nerve transection. The results of these RNase protection assays and *in situ* hybridization preparations indicate that IL-6 is present in rat DRG neurons when hypersensitivity following chronic constriction injury is apparent. IL-6 also may be synthesized in non-neuronal cells of the constricted nerve.

POSSIBLE MECHANISMS OF ACTION OF IL-6 ON NEUROPEPTIDES IN DRG NEURONS

Exogenous IL-6 increases galanin mRNA in small- and medium-sized DRG neurons, and endogenous IL-6 contributes to the increase of galanin immunoreactivity in the dorsal horn and dorsal column nuclei after nerve injury. To the sensitivity of our quantitative analysis of *in situ* hybridization, the induction of galanin mRNA in DRG after nerve constriction injury was not shown to differ significantly in IL-6 -/- and wild-type mice. The consequences of lack of IL-6 on galanin gene expression in axotomized DRG neurons are probably counteracted by the effects of LIF and withdrawal of NGF (Verge et al., 1995; Corness et al., 1998; Sun and Zigmond, 1996; Kerekes et al., 1999). Nonetheless, full induction of galanin in the central projections of DRG neurons is dependent upon the presence of endogenous IL-6.

The factors that might influence substance P mRNA in DRG neurons after nerve constriction include axotomy which would tend to cause death of some neurons, and induce substance P *de novo* in some large neurons (Noguchi et al., 1995), plus inflammation with induction of NGF in the nerve which would tend to stimulate substance P synthesis in both small and large neurons (Fitzgerald et al., 1985; Neumann et al., 1996).

Although using *in situ* hybridization we detected no changes in substance P mRNA in DRG neurons of wild-type mice after chronic nerve constriction, others using RNA blotting (Nahin et al., 1994) have reported a modest reduction. The extent of loss of substance P mRNA after nerve constriction in IL-6 -/- mice suggests both that events leading to loss of substance P are accentuated and events leading to induction of substance P are impaired. We do not know whether IL-6 influences substance P synthesis in DRG neurons directly or indirectly, e.g. through the release of NGF from non-neuronal cells (Frei et al., 1989).

The abnormal responses of galanin and substance P, and the reduced propensity for hypersensitivity after nerve injury in IL-6 -/- mice could reflect abnormal development of DRG neurons in the absence of IL-6 after nerve injury.

FUNCTION OF IL-6 IN PERIPHERAL NEUROPATHIC PAIN

In contrast to one previous report (Xu et al., 1997), we observed no differences between IL-6 -/- and wild-type mice in withdrawal responses to thermal or mechanical stimuli in uninjured animals. The authors themselves raise the possibility that differences in cutaneous sensitivity in uninjured wild-type and IL-6 -/- mice might result from differences in the background genotype of the IL-6 -/- mice. In our experiments, IL-6 -/- mice did not develop the thermal hyperalgesia or mechanical allodynia that are consistently manifest within 14 days after nerve constriction in wild-type mice. The behavioural consequences of partial nerve injury were not assessed in the previously mentioned study of pain in IL-6 -/- mice (Xu et al., 1997).

The extent to which abnormal responses of galanin and substance P contribute to the attenuation of hypersensitivity in nerve-injured IL-6 -/- mice is unclear. Galanin is generally regarded as antinociceptive (Hokfelt et al., 1994), but several observations indicate a nociceptive action (Cridland and Henry, 1988; Wiesenfeld-Hallin et al., 1988) and very recently, galanin -/- mice have been shown to resemble IL-6 -/- mice in their pain phenotype (Kerr et al., 1998). Sustance P has been implicated in the rapid induction of hypersensitivity by C-fibre stimulation or formalin injection (De Felipe and Hunt, 1994; Ma and Woolf, 1995; Mantyh et al., 1997), but not in the chronic hyperalgesia associated with

partial nerve injury (Cao et al., 1998). Abnormal responses of galanin and/or substance P may contribute to the attenuation of symptoms in IL-6 -/- mice, but probably do not provide the entire explanation.

LIF (Thompson et al., 1996; Banner et al., 1998), NGF (McMahon et al., 1995), brain-derived neurotrophic factor (BDNF) (Dassan et al., 1998) and interferon-γ (Robertson et al., 1997)all contribute potentially to neuropathic and/or inflammatory pain. The possibility that the actions of IL-6 in neuropathic pain involve interaction with one or more of these neurotrophic factors or cytokines deserves consideration.

FIGURE1: Darkfield (A-C) and brightfield (D) photomicrographs of sections of rat L5 DRG processed for *in situ* hybridization with ^{33}P-labelled IL-6 oligonucleotide probes. Sections are from ipsilateral (A,D) and contralateral (C) L5 DRG removed 5 days after constriction nerve injury (A and C) or 4 days after nerve transection (B). Note that IL-6 mRNA is induced in some neurons (arrows) ipsilateral but not contralateral RG after constriction nerve injury, although in fewer neurons and at less intensity than after nerve transection. Magnification x 480 (A-C); x1200 (D).

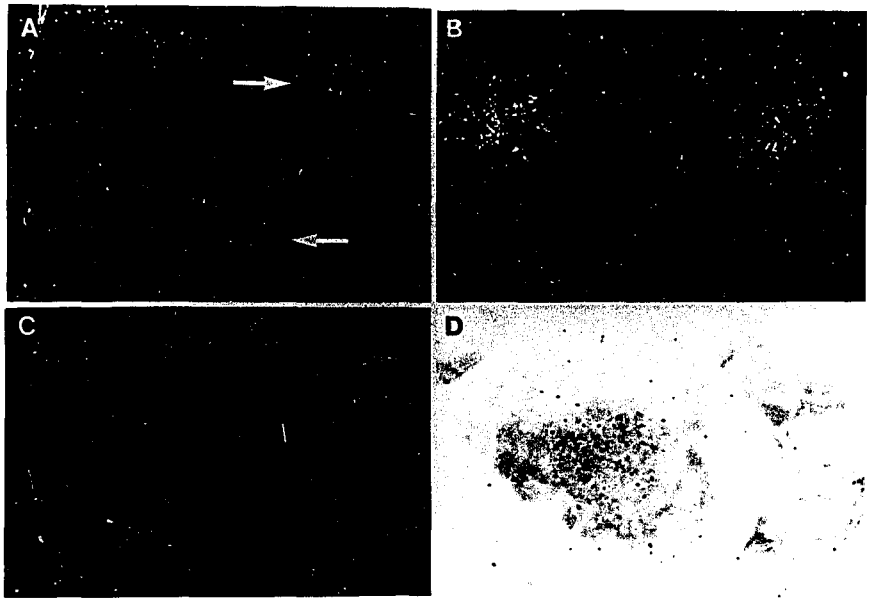

A
B
C
D

FIGURE 2. Thermal hypersensitivity (A) was assessed as the right-to-left difference in latency (s) to withdrawal from a radiant heat source. Mechanical hypersensitivity (B) was assessed as the right-to-left difference in threshold (g) to withdrawal from von Frei hairs. Testing was performed 0, 1, and 2 weeks after constriction nerve injury (mean ± SEM, n = 7 mice per group for each test). Note that the thermal and mechanical hypersensitivity that are evident in wild-type mice are not seen in IL-6 -/- mice. Although the sensitivity of IL-6 -/- mice to temperature and pressure appears to decrease after chronic nerve constriction, this trend was not significant.

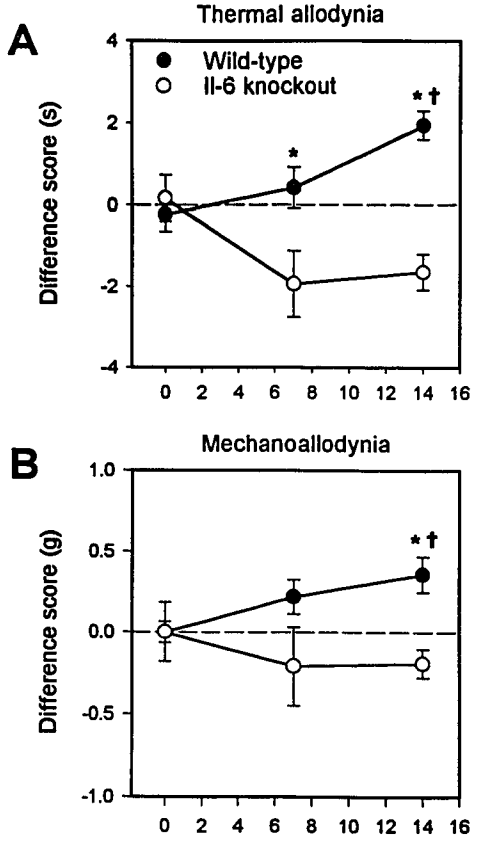

A
Thermal allodynia
Wild-type
Il-6 knockout
Difference score (s)
4
2
0
-2
-4
0 2 4 6 8 10 12 14 16
* †
*
B
Mechanoallodynia
Difference score (g)
1.0
0.5
0.0
-0.5
-1.0
0 2 4 6 8 10 12 14 16
* †

FIGURE 3. Darkfield photomicrographs of sections from L4 DRG removed from wild type (A and B) or IL-6 -/- (C and D) mice 14 days after constriction injury to the sciatic nerve and processed for *in situ* hybridization with an ^{35}S-labelled substance P oligonucleotide probe. Substance P is present in many small neurons in contralateral DRG from wild type (A) and IL-6 -/- mice (C). In ipsilateral DRG, substance P mRNA is not changed appreciably in wild-type mice (B) but is decreased markedly in IL-6 -/- mice (D). Magnification: X 120.

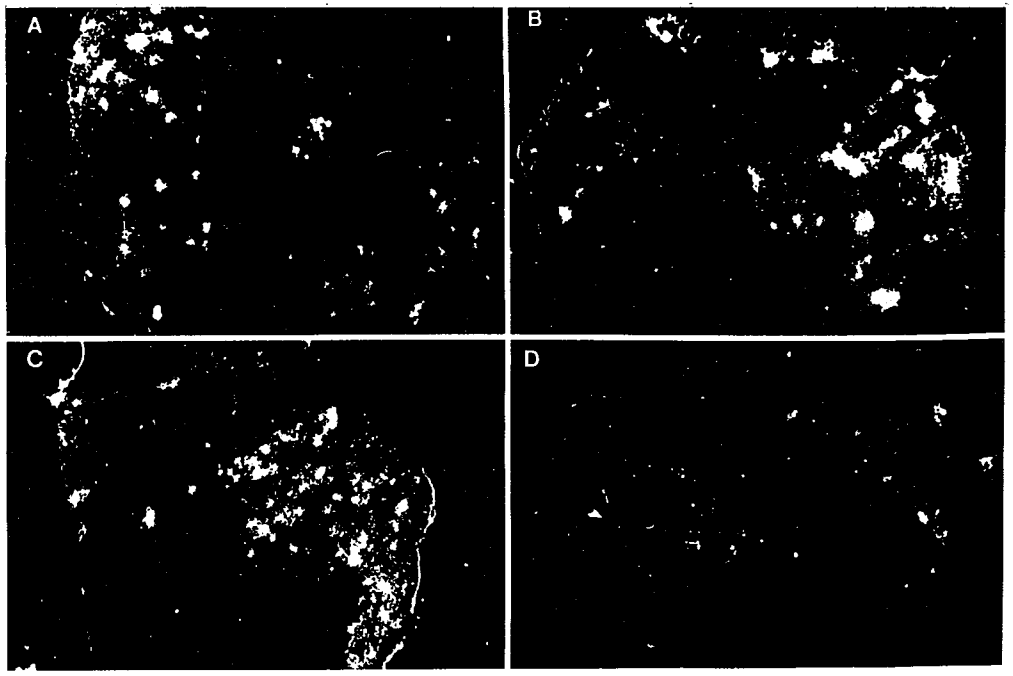

A
B
C
D

FIGURE 4. Substance P immunoreactivity in the dorsal horn of the spinal cord of wild-type (A and B) or IL-6 -/- (C and D) mice submitted to nerve constriction 2 weeks before being killed. (A and C) contralateral to the lesion, (B and D) ipsilateral. Note the diminution of immunoreactivity particularly in laminae I and II ipsilateral to the nerve injury in IL-6 -/- mice (D), but not in wild-ype mice (B). Magnification: X 100.

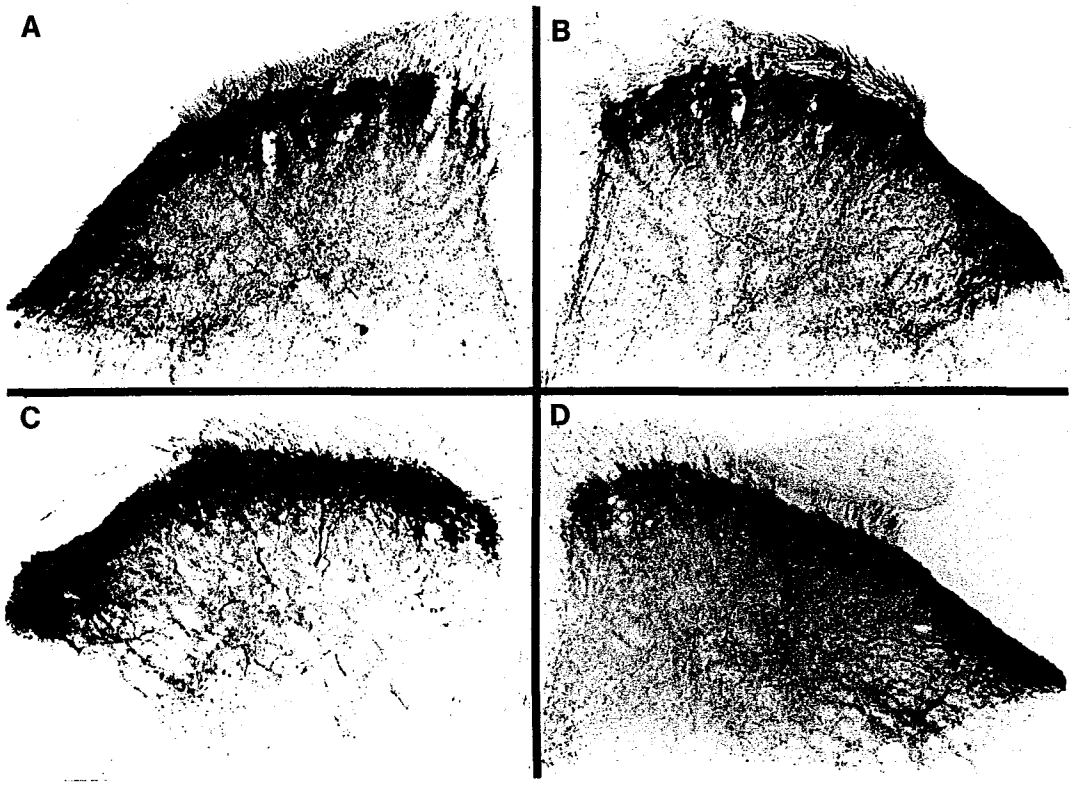

A
B
C
D

FIGURE 5. Quantification of immunohistochemical data for galanin in the spinal cord and brainstem (A and B), and substance P in the spinal cord (C and D) after nerve constriction for 2 weeks in wild-type (WT) and IL-6 -/- (IL-6 ko) mice. The values obtained in the spinal cord were obtained by averaging three sets of density measurements in each section along dorsal-ventral lines from lamina I-IV and then subtracting from this average the minimum greyscale value within that average. The average corrected measurements from the whole of laminae I-II and III-IV in ipsilateral aand contralateral dorsal horns were compared with the use of paired *t*-tests. *P*-values are indicated on the figures. For immunohistochemical data in the nucleus gracilis (B, inset), average values of the percentage of area of nucleus gracilis covered by immunopositive axons and terminals were compared in wild-type and IL-6 -/- mice, and significance estimated by *t*-testing. *N*=5 mice in each diagram.

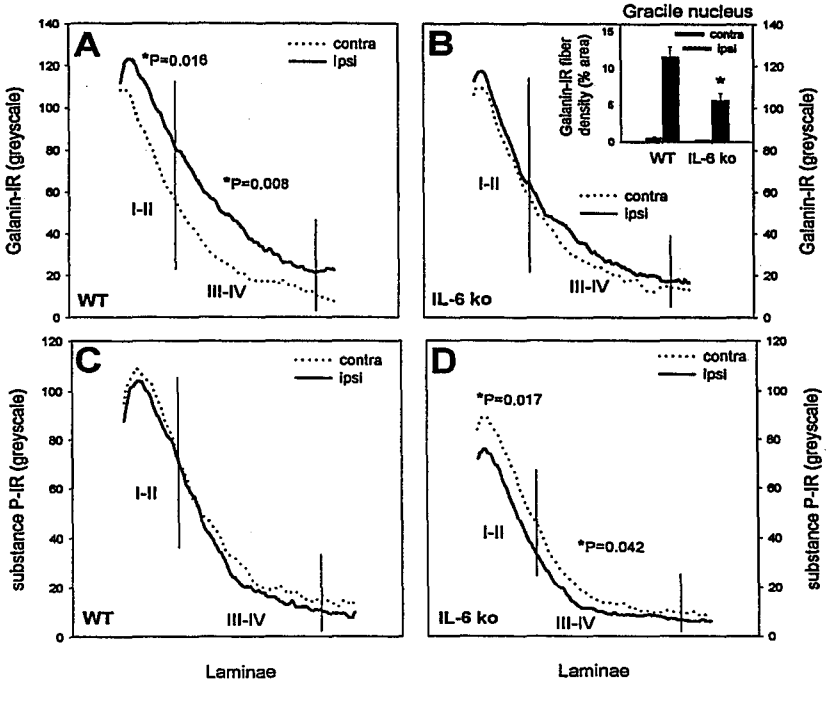

Gracile nucleus
A
B
C
D
contra
ipsi
*P=0.016
*P=0.008
*P=0.017
*P=0.042
I-II
III-IV
WT
IL-6 ko
Galanin-IR (greyscale)
substance P-IR (greyscale)
Galanin-IR fiber density (% area)
Laminae

FIGURE 6. Galanin immunoreactivity in the dorsal horn in transverse sections through the lumbar spinal cord and nucleus gracilis of wild type (A, B, E) and IL-6 -/- mice (C, D, F) sacrificed after two weeks of unilateral sciaitc nerve constriction. In wild type mice, immunoreactivity in layers III and IV of the dorsal horn (arrow) is increased ipsilateral (B) but not contralateral (A) to chronic constriction of the sciatic nerve. This augmentation of galanin immunoreactivity in primary sesnsory projections to deeper layers of the dorsal horn is not seen ipsilateral to a nerve constriction injury in IL-6 -/- mice (D). In the nucleus gracilis, galanin immunoreactivity is negligible in uninjured mice, induced ipsilaterally (arrow) by nerve constriction injury strongly in wild-type mice (E), and weakly in IL-6 -/- mice (F). (The dark area of galanin immunoreactivity ventral and medial to the nucleus gracilis is not derived from sensory neurons and is not influenced by nerve injury). Magnification: X 100.

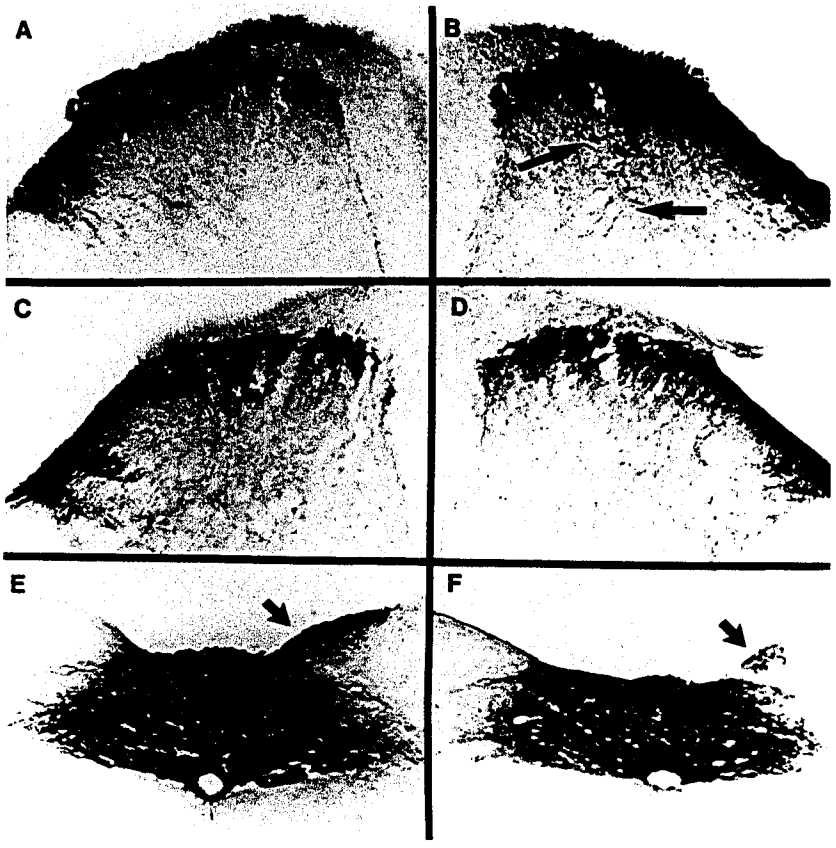
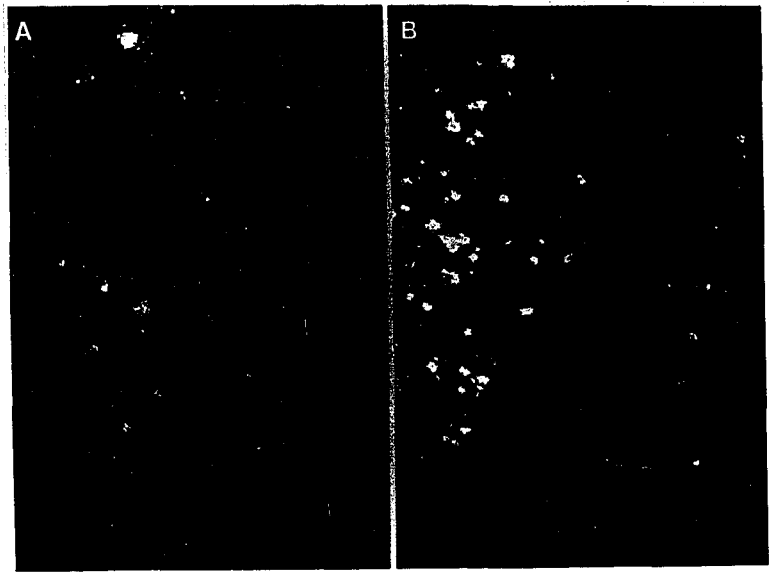
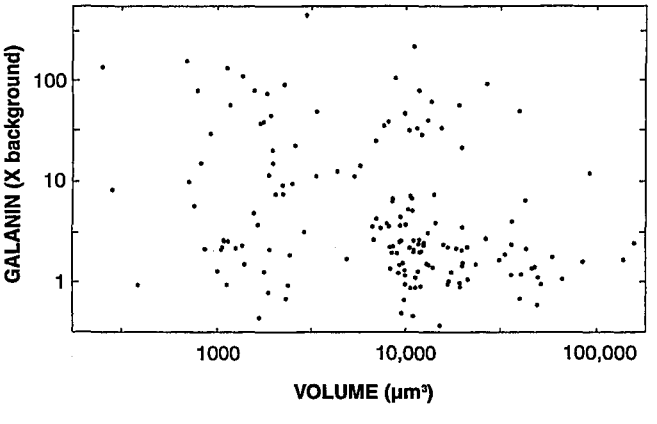

A
B
C
D
E
F

FIGURE 7. Dark field photomicrographs of sections from rat L5 DRG removed after 3 days of intrathecal infusion with saline (A) or IL-6 (B) processed for *in situ* hybridization with an ^{35}S-labelled galanin oligonucleotide. Magnification: X 100. (C) Scatter diagram on a log-log scale in which each point represents one neuron, the *x*- axis being cell volume and the y-axis percentage of neuronal area covered by silver grains as a multiple of background labelling . Note that induction of galanin is not limited to small neurons but does not include the largest neurons.


A
B


GALANIN (X background)
100
10
1
1000
10,000
100,000
VOLUME (μm³)

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CHAPTER 5:

VOLTAGE-GATED POTASSIUM CURRENTS IN SMALL DORSAL ROOT GANGLION NEURONS ARE NOT ALTERED BY SCIATIC NERVE TRANSECTION

PURPOSE OF THE STUDY

There are three possible changes in the sensory system that could drive the development of sensitization in dorsal horn neurons. These include changes in neurotransmitter/neuromodulator content (for review see Woolf, 1999), changes in synaptic connectivity (Woolf et al., 1992; Woof et al., 1995) and increased spontaneous firing in primary afferent neurons (Wall and Devor, 1983; Kajander and Bennett, 1992).

The previous study provided evidence to suggest that endogenous IL-6 plays an important role in the development of neuropathic pain and that it regulates synthesis of the neuromodulators substance P and galanin. However, it is unclear if the subnormal induction of substance P and galanin in IL-6 deficient mice accounts for the attenuation of hypersensitivity in these mice. Another study that looked at neuropathic pain in IL-6 deficient mice showed that sympathetic sprouting is greatly diminished, suggesting that IL-6 can also influence changes in synaptic connectivity (Ramer et al., 1998). As yet, no one has looked at the effect of IL-6 on spontaneous electrical activity in DRG neurons.

The long-term aim of this study is to evaluate whether IL-6 influences neuronal hyperexcitability by altering the expression levels or properties of voltage-gated ion channels. In particular, we want to test the possibility that IL-6 depresses voltage-gated potassium currents. Since voltage-gated potassium currents generally act to hyperpolarize neurons, thereby limiting neuronal excitability, a depression of these currents could lead to aberrant neuronal hyperexcitability. However, it is essential to determine if these currents are altered by axotomy before proceeding to evaluate the influence of interleukin-6. Therefore, the immediate aim of this study is to determine if voltage-gated potassium currents are altered in small DRG neurons in response to axotomy.

INTRODUCTION

DRG neurons convey information on stimulus events that occur at discreet loci in the periphery to the CNS. The nociceptors, one of several classes of DRG neurons, integrate noxious thermal, mechanical and chemical stimuli into a single output in the form of a train of patterned action potentials. This pattern of activity ultimately determines the sensation of pain. Thus nociceptors can be viewed as sensory gates that dynamically control the pattern of excitability being transmitted centrally.

Recently, there has been a great deal of interest surrounding the complement of ion channels expressed by dorsal root ganglion (DRG) neurons. First, the complement of ion channels on the DRG cell body is thought to be representative of channels that are functionally important on the axon (Everill et al. 1997). For this reason dissociated nociceptive DRG neurons *in vitro* are used as a model with which to study the properties of nociceptive terminals *in vivo*. *In vivo* data suggests that a number of the electrophysiological properties of the neuronal cell body are similar to those present in the terminal, including those that regulate firing frequency and response-adaptation (Harper and Lawson, 1985). Furthermore, *in vitro* data has shown that many physiological properties of nociceptors are well conserved in culture, including the shape of the action potential waveform, capsaicin-responsiveness and substance P-like immunoreactivity (Gold et al., 1996b). Second, peripheral nerve injury induces phenotypic changes in the cell body of DRG neurons such that these neurons begin to fire spontaneously. This novel source of aberrant electrical activity is thought to contribute to the array of sensitizing signals from the periphery, which lead to central changes underlying the development of neuropathic pain.

Modulation of voltage-gated ion channels on DRG neurons can alter the electrophysiological properties of the membrane, controlling how nociceptive input is translated into an output pattern of action potentials. Spontaneous action potential firing, which represents a novel pattern of activity, could reflect changes in the array of voltage-gated ion channels expressed on the soma of DRG neurons. Recently, several changes in sodium (Na^+) channel expression have been demonstrated following sciatic nerve injury (Akopian et al., 1996; Black et al., 1996; Sangameswaran et al., 1997). These changes in

channel expression correlate with the decrease in TTX-R and increase in TTX-S Na^+ currents that is seen in axotomized small DRG neurons (Zhang et al., 1997; Cummins and Waxman, 1997). The idea that these changes in Na^+ channel expression lead to neuronal hyperexcitability is supported by the observation that abnormal increases in Na^+ conductance can lead to inappropriate, repetitive firing, and that Na^+ channel blocking agents efficiently reduce neuropathic pain.

In addition to Na^+ channels, sensory neurons contain a variety of different voltage-gated potassium (K^+) channels. Voltage-gated K^+ currents are classified according to their kinetic and inactivation properties as either transient A-type currents, or delayed rectifiers (Rudy, 1988). Based on the fact that K^+ currents generally function to limit neuronal excitability, a suppression of K^+ currents in the soma could lead to increased neuronal membrane excitability in DRG neurons. For example, selective blockade of a slow A-type current in sensory neurons decreases both action-potential threshold and accommodation, suggesting that this current normally functions to limit excitability (Stansfeld et al., 1986). Also, blockade of 4-AP-sensitive K^+ currents (transient currents) on primary afferent axons unmasks a slow Na^+ current and gives rise to burst firing (Honmou et al., 1994; Kocsis et al., 1983). Delayed rectifiers are generally responsible for repolarization of the action potential; therefore, depression of this type of current would prolong the action potential, leading to increased transmitter release (Kocsis et al., 1987; Eng et al., 1988). In fact, prostaglandins have been shown to increase membrane excitability and the release of neuropeptides from sensory neurons by suppressing a sustained or delayed rectifier type K^+ current (Nicol et al., 1997). These findings suggest that suppression of K^+ currents can lead to hyperexcitability, and that changes in the relative number and spatial distribution of K^+ channels within a single sensory neuron will affect the excitability and firing properties of that neuron.

A recent report by Ishikawa et al. (1999) showed that the expression of voltage-gated K^+ channels Kv1.1, 1.2 and 2.1 are downregulated after axotomy in small DRG neurons. Another recent report showed that the total mean peak K^+ current density is decreased (~50%) in cutaneous afferent neurons after axotomy (Everill and Kocsis, 1999).

Therefore, the purpose of this study was to further characterize the effects of

axotomy on voltage-gated potassium currents in DRG neurons. DRG were obtained from adult mice that had undergone right sciatic nerve transection. Whole-cell recording techniques were used to identify potassium current components within the population of small-diameter nociceptive neurons.

METHODS

CELL CULTURE

All experiments were conducted on dissociated adult mouse dorsal root ganglion (DRG) neurons and great efforts were made to maximize neuronal health and survival. Dissociated adult DRG neurons have been well characterized *in vitro* and express many phenotypic markers seen *in vivo*. The use of DRG explants would have been less disruptive to the neurons and would have enabled us to classify neurons according to their conduction velocity, however, the number of neurons recorded per sitting would have been limited (Gold et al., 1996). DRG slices, another possible neuronal preparation, have proven problematic because cell bodies (particularly of medium to large-sized neurons) are difficult to access and rarely clear of cellular debris and satellite cells (Safronov et al., 1997).

Mice were anaesthetized intramuscularly with a mixture of ketamine (0.75 mg/g) and xylazine (0.01mg/g). The right sciatic nerve was exposed in midthigh and transected, while the left sciatic nerve was left intact. Ipsilateral (injured) and contralateral (control) L4 and L5 DRG were removed from mice anaesthetized 7 days after transection. A 7 d time-point was chosen because neuropathic pain-related behaviours are evident by 7 d and because changes in several other types of ion channels have been reported by this time (Dib-Hajj et al., 1996; Cummins et al., 1997; Dib-Hajj et al., 1998)

Ipsilateral and contralateral DRG were kept separate and placed in HBSS (Ca^{++} and Mg^{++} free) containing collagenase A (1 mg/ml), collagenase D (1 mg/ml) (Roche) and papain (20 U/ml) (Worthington) for 25 minutes at 37°C. Cells were dissociated by gentle trituration with fire-polished pipettes of different sizes. The dissociated neurons were centrifuged at 800 rpm, rinsed twice with HBSS, and resuspended in growth media composed of DMEM:F12 (1:1) (Sigma), 10% FBS (Gibco BRL), 20 mM L-glutamine (Gibco BRL), (100U/ml) penicillin, and (0.1 mg/ml) streptomycin (Gibco BRL). The neurons were plated on Aclar (Allied Chemicals) coverslips coated with poly-ornithine (1 mg/ml) (Gibco BRL) followed by laminin (0.1 mg/ml); these coverslips formed the bottom of modified 35 mm culture dishes, which also served as recording chambers for the

electrophysiological experiments. The cultures were maintained at 37°C in 95% air-5% CO_2 environment.

Neurons were examined 12-24 hours after plating. This time point was chosen in order to: 1) limit variations in expressed types and amounts of current as a function of time, and 2) circumvent space clamp problems, as neurite outgrowth was minimal at the time of recording.

DATA RECORDING AND ANALYSIS

DRG neurons were voltage clamped using whole-cell recording techniques. All experiments were done at room temperature (21-24 °C) with a List EPC-7 amplifier. Though elevated recording temperatures (32-38 °C) would have been more representative of core body temperature, higher ambient temperatures tend to increase current run-down and cause the cell membrane at the tip of the electrode tends to reseal (Atkins and McCleskey, 1993). Furthermore, recording at room temperature facilitates comparisons with other studies on dissociated DRG neurons.

Pipette resistances were 2-5 MΩ and were filled with intracellular media (described below). The current signal was balanced to zero with the pipette immersed in the bathing solution. The seal resistances were usually 5-20 GΩ, and the series resistances (6-10 MΩ) were partially compensated (20-30%). A pentium-based PC computer was used to deliver the voltage-clamp steps and acquire the membrane currents; the software for stimulation, data acquisition, and analysis was written by Mr. A. Shermann (Alembic Inc., Montreal). Membrane currents and voltages were filtered at 3 kHz with an eight-pole Bessel filter (Frequency Devices, Inc.), sampled, displayed, and stored on line.

The duration of the voltage steps was either 125 ms or 10 s: for the 125 ms steps the data was sampled at 5 kHz, whereas for the 10 s steps, the data was filtered at 100 Hz and sampled at 200 Hz. The 125 ms steps were sufficiently long to see complete inactivation of fast inactivating currents, but short enough to visualize the activation phase of all current-types at a high resolution. The 10 s steps allowed us to clearly and rapidly identify which current components were present in each cell, and to differentiate between slowly inactivating current components and non-inactivating ones.

To study the voltage-gated K currents, inward Na and Ca-dependent currents were blocked pharmacologically: Na currents were blocked with TTX (1 μM), and the external Na concentration was reduced to 2 mM and replaced by 140 mM choline chloride; Ca currents were blocked by 2 mM cobalt chloride and by lowering the external Ca concentration to 0.5 mM. Furthermore, any neuron in which there was a suggestion of inadequate space clamp, such as notches or oscillations, was excluded from this study.

As described in the RESULTS, the total outward current on small DRG neurons is made up of three different currents: I_K, I_{Af}, and I_{As}. These currents differ in their voltage dependence, therefore they could be isolated by subtraction procedures. To measure I_K, we delivered depolarizing voltage steps from -10mV; at this potential, I_K can be activated, whereas both I_{Af} and I_{As} are inactivated. I_K was corrected for leakage and capacity currents by digitally adding the current evoked by an equivalent hyperpolarizing step.

To isolate I_{As}, I_K evoked by deplarizing steps from -10 mV was subtracted from the outward currents (I_{As} and I_K) evoked from depolarizing steps to the same potentials from a holding potential of -40 mV, and the current evoked by a hyperpolarizing step from -10 to -40 mV was added to each record to remove the leakage current. To isolate I_{Af}, currents evoked by depolarizing voltage steps from -40 mV (I_{As} and I_K) were subtracted from the corresponding currents evoked from -90 mV (I_{Af}, I_{As}, and I_K). The current evoked by a hyperpolarizing step form -40 mV to -90 mV was added to each trace.

In order to measure the voltage dependence for activation, we plotted the steady-state conductance (for I_K or I_{As}) or the peak conductance (for I_{Af}) relative to the maximum conductance at +40 mV, against the step potential (mV). These curves were then fit with Boltzman distributions (Equ. 1)

$$G/G_{max} = 1/[1 + \exp((V_{½} - V_m)/k)] \quad (1)$$

where G is the conductance, V_m is the step potential, $V_{½}$ is the voltage at half maximal activation and k is RT/Kz where R is the gas contant, T is the absolute temperature, K is the Boltzman's constant, and z is the valence of the equivalent gating charge.

To quantify the expression of each current, current amplitudes were measured and normalized to membrane capacitance. The amplitudes were determined from the current evoked by a voltage step to +40 mV after each current was isolated from the others as

described above. For I_{Af}, measurements were made at the peak current, which occurred within the first 10 ms. For I_{As}, measurements were made at the plateau, 125 ms after the beginning of the step. Measurements of I_K were made at the end of a 10 s voltage step to +40 mV, at which time I_{As} had completely decayed. The membrane capacitance (pF) was obtained by integrating the capacitative current evoked by a 10 mV hyperpolarizing voltage step. Standard *t* tests were used to compare differences between mean current densities, and between the voltage dependence of activation in uninjured versus injured neurons.

SOLUTIONS

The ionic composition of the extracellular sollution was 140 mM choline Cl, 2 mM NaCl, 5.4 mM KCl, 0.5 mM $CaCl_2$, 0.18 mM $MgCl_2$, 10mM HEPES (pH 7.4, adjusted with NaOH), 5.6 mM glucose, 1 μM TTX (Sigma), and 2 mM $CoCl_2$. The pH was 7.3-7.4. The intracellular pipette solution contained 5 mM NaCl, 140 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4, adjusted with KOH), 10 mM EGTA, and 0.2 mM $CaCl_2$ (final free Ca concentration < 10^{-8} M). The pH was 7.3-7.4.

RESULTS

VOLTAGE-GATED K^+ CURRENTS IN DRG NEURONS

Figure 1A shows the total outward potassium current on adult mouse DRG neurons evoked by 125 ms (*left*) and 10 s (*right*) voltage-clamp steps from a holding potential of -90 mV to +40 mV, after blockade of inward currents and Ca^{2+}-activated currents (see METHODS). The outward current is composed of a non-inactivating component, a rapidly inactivating component, and a slowly inactivating component that activates at slightly more depolarized voltages than the fast transient component. These three components can be distinguished most clearly in response to a 10 s voltage step (*Fig. 1A, right*).

Figure 1B-D, demonstrates that these three current components can be separated on the basis of their voltage dependence. Specifically, the outward current evoked from a holding potential of -10 mV activates slowly (Fig. 1B, *left*) and exhibits very little inactivation over a 10 s period (Fig. 1B, *right*). This current is referred to as the delayed rectifier, I_K. Figure 1C shows the slowly inactivating current that is evoked from a holding potential of -40 mV, in isolation of I_K, by subtracting the current evoked from -10 mV for the total current evoked at -40 mV. This current displays faster activating kinetics than I_K (Fig. 1C, *left*) and slow inactivation over the 10 s period of the voltage step (Fig. 1C, *right*); it is referred to as the slow A-current (I_{As}). Depolarizing voltage steps from a holding potential of -90 mV evoke a third component (Fig. 1D) that activates and inactivates more rapidly than I_{As}, and is referred to as the fast A-current (I_{Af}). I_{Af} was separated from the other currents by subtracting the total current evoked at -40 mV from the total current evoked at -90 mV. I_K, I_{As}, and I_{Af} were difficult to separate completely due to some overlap between the voltages for activation and removal of inactivation.

Table 1 demonstrates that small DRG neurons are highly heterogeneous with respect to the contribution of I_K, I_{As}, and I_{Af} to the total current. The mean diameter of DRG neurons in this

minimally to the total current. A high degree of heterogeneity was observed for both control neurons and injured neurons. This made it difficult to distinguish qualitative differences between the distribution of I_K, I_{As}, and I_{Af} in injured versus uninjured neurons.

ACTIVATION PROFILES OF K^+ CURRENTS ON DRG NEURONS

Figure 2 shows the voltage dependence of activation for I_K, I_{As}, and I_{Af} in control and injured neurons. Activation of I_K was determined from the current evoked in response to a series of depolarizing voltage steps from a holding potential of –10 mV. In figure 2A, the ratio of the steady-state conductance to the maximum conductance (G/G_{max}) for I_K is plotted against the membrane potential (V_m). The *open circles* represent activation of I_K in uninjured neurons, while the *closed circles* represent injured neurons. The Boltzman distribution parameters of I_K were $V_{½}$ = 20.91 mV ± 0.41, k = 6.36 ± 0.37 (n = 6) and $V_{½}$ = 20.63 ± 0.38, k = 5.25 ± 0.35 (n = 6), for uninjured and injured neurons, respectively (see equation 1, METHODS). This data demonstrates that there is no significant change in the voltage dependence of activation of I_K after injury.

Figure 2B demonstrates the voltage dependence of activation of I_{As} in control (*open circles*) and injured (*closed circles*) neurons. The data from uninjured neurons (*dotted line*) are well fit by a Boltzman distribution with a $V_{½}$ = -8.31 ± mV, k = 8.68 ± 1.20. The data from injured cells (*solid line*) was much more variable, having a Boltzman fit of $V_{½}$ = -2.28 ± 1.75 mV, k = 15.84 ± 1.65. These values reflect a slight rightward shift and a decrease in the slope of the activation curve for injured neurons, however these changes did not reach significance.

The voltage dependence of activation of I_{Af} is shown in Fig. 2C. This figure shows the peak conductance (G) of I_{Af} as a ratio of the maximum conductance (G_{max}), plotted against the step potential (V_m). Once again, the activation curves for injured and uninjured neurons are represented on the same plot. I_{Af} activates over a wide range of voltages, beginning at more hyperpolarized potentials than I_{As}. Both sets of data are well described by a Boltzman distribution, with parameters of $V_{½}$ = -20.35 ± 0.61 mV, k = 11.11 ± 0.54 and $V_{½}$ = –19. 13 ± 1.22 mV, k = 15.72 ± 1.09, for uninjured and injured neurons, respectively. Clearly, the voltage dependence of activation of I_{Af} is not altered by neuronal

injury. Altogether, these data support the qualitative observation that the properties of the three current components are unchanged by nerve transection.

K^+ CURRENT DENSITIES BEFORE AND AFTER NERVE INJURY

The current densities for I_K, I_{As}, and I_{Af} were measured in ~25 injured and uninjured neurons in order to quantify changes in the expression of these current components in response to nerve transection. Figure 3A shows the distribution of I_K current densities on small DRG neurons in uninjured (*open bars)* and injured (*closed bars*) neurons. The distribution of current densities for I_K is skewed slightly to the left, indicating that the contribution of the delayed rectifier current to the total current density is small. Fifteen percent more injured neurons had current densities in the < 20 pA/pF range than uninjured neurons, suggesting a slight shift in I_K current densities toward smaller values after nerve injury. The distribution of current densities for I_{As} (Fig. 3B) is also skewed to the left. No significant difference was observed in the overall distribution of this current after nerve injury. Figure 3C shows the current density distributions for I_{Af}. In contrast to I_K and I_{As}, the distribution of I_{Af} is skewed to the right, illustrating that I_{Af} is the major current in most small DRG neurons. The percentages of uninjured versus injured neurons, which have current densities in the 60-99 pA/pF range, appear to differ. In fact, this difference is mainly due to arbitrary binning of the data, such that uninjured and injured neurons expressing I_{Af} in the range of 80 pA/pF are split unequally into two bins. Thus, there is no consistent change in the current density distribution for I_{Af} between injured and uninjured neurons. Overall, there was very little quantitative change in the expression of I_K, I_{As}, and I_{Af} after injury. Though a small trend towards decreased current densities was noted for I_K, the range of current densities between neurons was quite large, making it difficult to determine if this shift was significant.

Finally, Figure 4 compares the mean current densities obtained from the distributions for I_K, I_{As}, and I_{Af}, in injured versus uninjured neurons. No significant changes between injured and uninjured neurons were observed for any of the three current components. This figure also highlights the fact that I_{Af} was the major current in most small DRG neurons, followed by I_K and I_{As}.

DISCUSSION

These preliminary results demonstrate that small adult DRG neurons display at least three potassium currents *in vitro* that can be separated on the basis of their voltage dependence. Furthermore, these neurons represent a heterogeneous population with respect to the expression of I_K, I_{As}, and I_{Af}. Lastly, no qualitative or quantitative differences between I_K, I_{As}, and I_{Af} were found in injured neurons compared to uninjured neurons.

The I_K, I_{As}, and I_{Af} currents reported here are similar to those seen in neonatal nodose neurons (McFarlane and Cooper, 1991) and in small neonatal and adult DRG neurons (Safronov et al., 1996; Gold et al., 1996a). However, two additional delayed rectifier-type currents have been isolated in small DRG neurons on the basis of their sensitivity to the blocking agent TEA and permeability to Cs^+ ions (Safronov et al., 1996; Gold et al., 1996a). These findings suggest that the delayed rectifier isolated in this study may contain one or more sustained components that are subject to steady-state inactivation. Our failure to distinguish between various delayed rectifiers may have had an impact on the findings in this study. However, if an injury-induced change in any one of these currents had occurred, it should have affected the mean current density for I_K, though no significant change was observed.

Surprisingly, our preliminary results did not show any changes in I_K, I_{As}, or I_{Af} after injury. A stated earlier, Ishikawa et al. (1999) showed that the expression of voltage-gated K^+ channels Kv1.1, 1.2 and 2.1 are downregulated after axotomy in small DRG neurons and Everill and Kocsis (1999) showed that the total mean peak K^+ current density is decreased (~50%) in cutaneous afferent neurons after axotomy. There are several possible explanations for these contrasting results. First, the neurons in this study may not represent a pure population of nociceptors. One can determine if a small DRG neuron is a nociceptor by recording an AP waveform under current clamp or by testing for sensitivity to capsaicin. Using these two methods, Gold et al. (1996) found that nociceptive DRG neurons preferentially express two transient currents, termed I_{Aht} and I_{As}, which are very similar to the transient currents noted in this study. These findings make us reasonably confident that the majority of neurons in this study were indeed nociceptors. Second, our separation of I_K, I_{As}, and I_{Af} may not have been sufficient to pick up injury-induced changes

in a particular component. The use of pharmacological agents that block specific voltage-gated potassium channels could help in clarifying any differences that may exist after injury. Third, we did not study the voltage dependence of inactivation or the kinetics of activation and inactivation. Determination of these three additional parameters may have revealed injury-induced changes in small DRG neurons. Fourth, the downregulation of Kv1.1, 1.2 and 2.1 noted in the study by Ishikawa et al. could have been accompanied by upregulation of other potassium channel subtypes that were not examined. Last, Everill and Kocsis (1999) recorded from a different population of DRG neurons than those studied here. It is reasonable to suggest that changes within different functional populations of DRG neurons could be distinct. This idea is supported by the differential expression of voltage-gated Na^+ channels in small and large DRG neurons, after peripheral nerve injury. Also, Everill and Kocsis recorded from their neurons 2-3 weeks post-injury, which was 1-2 weeks later than the time point used in this study.

The high degree of heterogeneity between neurons in this studied is typical of small DRG neurons. Nociceptors can be distinguished on the basis of several properties including capsaicin-responsiveness and growth factor dependence. For instance, trkA bearing nociceptors that are supported by NGF, also synthesize and release a variety of neuropeptides in response to noxious stimuli and are termed peptidergic nonciceptors (Verge et al., 1989; Averill et al., 1995; Michael et al., 1997). A second, non-peptidergic populations of nociceptors bear the c-Ret receptor on their membranes, respond to GDNF (Molliver et al., 1997; Bennett et al., 1998), and are marked by the lectin IB(4) (Nagy and Hunt, 1982; Silverman and Kruger, 1990). Finally, about half of the peptidergic and non-peptidergic nociceptors respond to capsaicin (Tominaga et al., 1998). Recently, much work has focused on determining whether or not these distinct populations of nociceptors serve different physiological functions. Though small DRG neurons all give rise to thin, unmyelinated C fibres (nociceptive fibres), several functional classes of C fibres exist. For instance, some C fibres are chemospecific, while others respond to thermal or mechanical stimuli. Polymodal C fibres respond to all three types of noxious stimuli and play a central role in the development of neuropathic pain. Recently, IB(4)-positive neurons have been found to have longer-duration action potentials, higher densities of TTX-resistant sodium

currents, and smaller noxious heat-activated currents than IB(4)-negative neurons (Stucky and Lewin, 1999).

DRG neurons are also heterogeneous with respect to the expression of ion channels. PN3 and NaN are two sodium channel subtypes that are expressed exclusively in small DRG neurons (Dib-Hajj et al., 1996; Cummins and Waxman, 1997). NaN has an even more restricted distribution than PN3, suggesting that it may be expressed by a particular subpopulation of nociceptors. Similarly, the ATP-responsive $P2X_3$ channel is only expressed on c-Ret- and IB4-positive neurons and is therefore unique to the non-peptidergic nociceptors. This suggests that the differential distribution of ion channels among small DRG neurons may have direct functional implications. It remains to be determined whether or not any of the voltage-gated potassium channel genes are exclusively expressed in nociceptors.

These preliminary findings suggest that transection of the sciatic nerve does not alter the properties or array of voltage-gated K^+ currents in small DRG neurons and that voltage-gated K^+ currents are not critical mediators of hyperexcitability in these neurons. However, further studies need to be performed to support this idea. An additional approach to this study would have been to record from DRG neurons under current clamp. This method would have allowed us to compare the frequency and shape of action potentials in injured and uninjured neurons. This would have given us the ability to quickly distinguish nociceptors from non-nociceptors and to screen for neurons exhibiting altered patterns of activity. Once these neurons had been identified, we could have switched to whole-cell voltage clamp in order to characterize any underlying changes in the properties of K^+ currents.

FIGURE 1: Voltage-dependent K^+ currents on an adult DRG neuron.

(A) total outward current evoked by depolarizing voltage steps from a holding potential (V_h) of -90 mV. (B) I_K – depolarizing voltage steps from a V_h of -10 mV activate only a slowly activating, noninactivating current (I_K). (C) $I_{As} - I_{As}$ is isolated by subtracting the currents evoked at a V_h of -10 mV (I_K), from the corresponding currents evoked at a V_h of -40 mV ($I_K + I_{As}$). (D) $I_{Af} - I_{Af}$ is isolated by subtracting the currents evoked at a V_h of -40 mV ($I_{As} + I_K$) from the corresponding currents evoked at a V_h of -90 mV ($I_{Af} + I_{As} + I_K$). In (A), currents on the *left* were evoked by 125 ms depolarizing voltage steps in 20 mV increments to +40 mV, and on the *right* were evoked by 10 s voltage steps to +40 mV from a V_h of -90, -40, and -10 mV. In (B-D), currents were evoked by depolarizing voltage steps to +40 mV for either 125 ms (*left*) or 10 s (*right*). The leakage and capacity currents have been subtracted from all the traces. For the 125 ms steps, the currents were filtered at 3 kHz and sampled at 5 kHz, and for the 10 s steps, the currents were filtered at 100 Hz and sampled at 200 Hz.

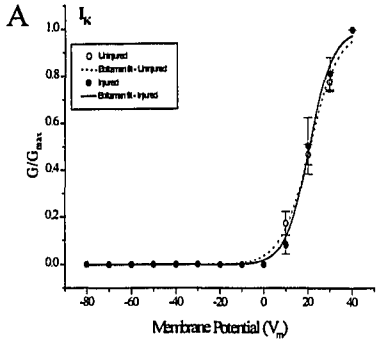
TABLE 1: Expression of voltage-gated K^+ currents in small diameter DRG neurons

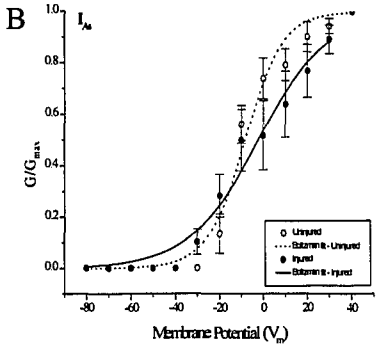
	I_K (pA/pF)	I_{As} (pA/pF)	I_{Af} (pA/pF)	I_{TOTAL} (pA/pF)
Uninjured	0	16	116	132
	35	24	88	147
	64	36	94	194
	36	14	158	208
	78	56	75	209
Injured	60	35	59	154
	0	46	122	168
	13	28	131	172
	53	102	51	206
	104	55	74	233

This table shows the variation in the expression of K-currents on small diameter DRG neurons. Isolate I_K, I_{As}, and I_{Af} currents evoked by a depolarizing step to +40 mV were measured in 5 representative uninjured and 5 representative injured neurons. Current densities were obtained by dividing the currents by cell capacitance (pF). Each line is the data from a different neuron. I_{TOTAL} is the total K-current density for each neuron and shows considerable variation. I_{Af}, rapidly inactivating current; I_{As}, slowly inactivating current; I_K, noninactivating current.

FIGURE 2: Voltage dependence of activation for I_K, I_{As}, and I_{Af} in uninjured or injured neurons.

(A-C) The steady-state activation curves for I_K, I_{As}, and I_{Af}, respectively. *Open circles* (mean ± SE, n = 6) represent the activation profiles in uninjured neurons and *closed circles* (mean ± SE, n = 6) represent the activation profiles in injured neurons. I_K, I_{As}, and I_{Af} were isolated from the total current, and corrected for leakage and capacity currents. The activation values were determined from the plateau current evoked from holding potentials of –10 mV (I_K) and –40 mV (I_{As}), or from the peak current evoked from a holding potential of –90 mV (I_{Af}). The conductance was determined by dividing the current by the driving force ($V_m - E_k$), and expressed as a proportion of G_{max} (where G_{max} = 1). The *dotted lines* represent Boltzman fits of the data from uninjured neurons, and the *solid lines* represent Boltzman fits for injured neurons. For I_K, the coefficients of activation before and after injury were $V_{½}$ = 20.91 ± 0.41 mV, k = 6.36 ± 0.37 and $V_{½}$ = 20.63 ± 0.38 mV, k = 5.25 ± 0.35, respectively. The coefficients for I_{As}, were $V_{½}$ = -8.31 ± 1.37 mV, k = 8.68 ± 1.20 and $V_{½}$ = -2.28 ± 1.74 mV, k = 15.84 ± 1.65, in uninjured and injured neurons respectively. For I_{Af}, the values are $V_{½}$ = -20.35 ± 0.61 mV, k = 11.11 ± 0.54 and $V_{½}$ = -19.13 ± 1.22 mV, k = 15.72 ± 1.09. There is no significant difference between the voltage dependence of activation for I_K, I_{As} or I_{Af}, in uninjured versus injured neurons.


A
I_K
Uninjured
Boltzmann fit - Uninjured
Injured
Boltzmann fit - Injured
G/G_{max}
Membrane Potential (V_m)


B
I_{As}
Uninjured
Boltzmann fit - Uninjured
Injured
Boltzmann fit - Injured
G/G_{max}
Membrane Potential (V_m)

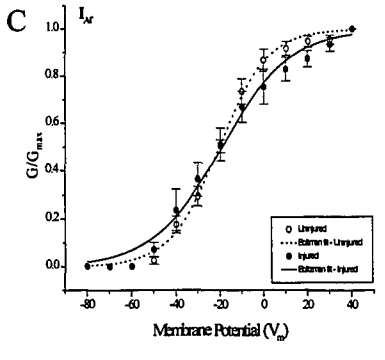
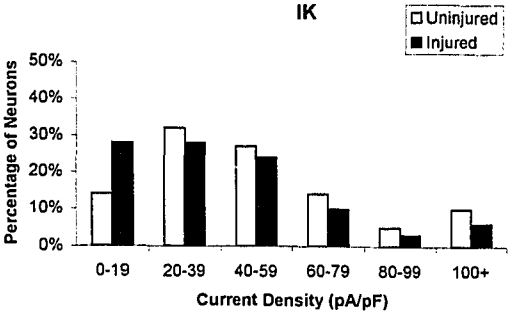
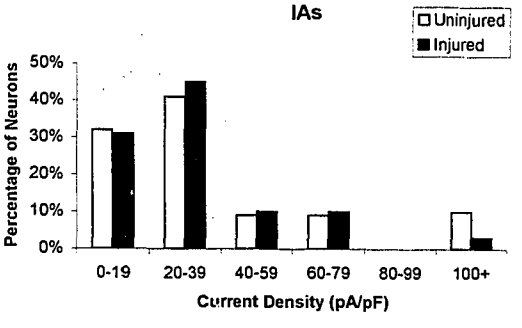

C
I_{Af}
Uninjured
Boltzmann fit - Uninjured
Injured
Boltzmann fit - Injured
G/G_{max}
Membrane Potential (V_m)

FIGURE 3: Current densities for I_K, I_{As}, and I_{Af} in uninjured and injured neurons.
(A-C) Current density distributions for I_K, I_{As} and I_{Af}, respectively. Current density (pA/pF) was measured as the peak isolated K^+ current (pA) at +40 mV divided by the membrane capacitance (pF), and plotted in bins of 20 (pA/pF) (x-axis) against the percentage of total neurons in each group (y-axis). The *open bars* represent the current densities in uninjured neurons (n = 29) and the *closed bars* represent the current densities in injured neurons (n = 22). The current density distributions are similar for I_K, I_{As} and I_{Af}, in injured and uninjured neurons.


IK
Uninjured
Injured
Percentage of Neurons
50%
40%
30%
20%
10%
0%
0-19
20-39
40-59
60-79
80-99
100+
Current Density (pA/pF)


IAs
Uninjured
Injured
Percentage of Neurons
50%
40%
30%
20%
10%
0%
0-19
20-39
40-59
60-79
80-99
100+
Current Density (pA/pF)

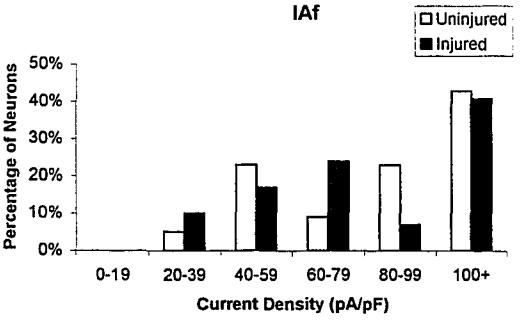
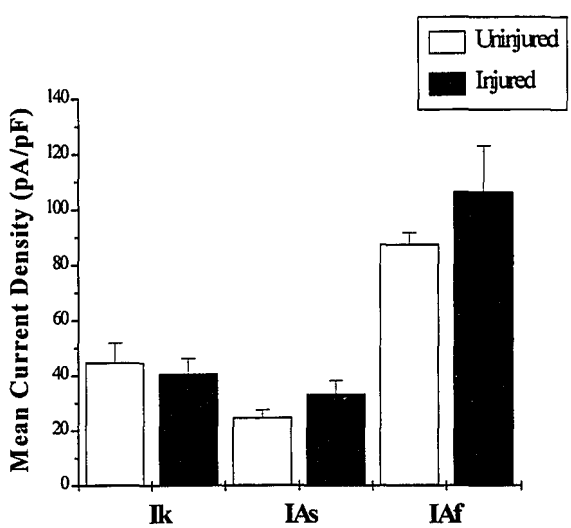

IAf
Uninjured
Injured
Percentage of Neurons
50%
40%
30%
20%
10%
0%
0-19
20-39
40-59
60-79
80-99
100+
Current Density (pA/pF)

FIGURE 4: Mean current densities for I_K, I_{As}, and I_{Af} in uninjured and injured neurons. Mean current densities (pA/pF) (mean ± SE) (y-axis) were calculated from the peak current densities in Figure 3. As in Figure 3, the *open bars* represent the current densities in uninjured neurons (n = 29) and *closed bars* represent the current densities in injured neurons (n = 22). There were no significant differences between I_K, I_{As} and I_{Af} before and after injury.


Uninjured
Injured
Mean Current Density (pA/pF)
140
120
100
80
60
40
20
0
Ik
IAs
IAf

SUMMARY AND CONCLUSIONS

I have contributed several findings to the larger body of work presented in this thesis. First, my series of quantifications of mRNA labelling by *in situ* hybridization have confirmed many qualitative observations. Second, the cell culture preparation for adult DRG neurons, developed for my whole-cell patch clamp recordings, will have many future applications. Third, my electrophysiological recording have addressed a potential mechanism underlying the generation of neuropathic pain following peripheral nerve injury.

Chapter two investigated the regulation of IL-6 after peripheral nerve transection and showed that a positive signal originating in the proximal nerve stump is inducing IL-6 mRNA in DRG neurons. I confirmed that blocking retrograde transport with the microtubule-binding protein colchicine does not induce IL-6 mRNA, suggesting that IL-6 is not upregulated by a loss of retrograde transport of a trophic molecule from the periphery. In contrast, nerve transection plus colchicine injection into the proximal nerve stump, a manipulation that wili block a positive signal originating in the proximal nerve stump, completely inhibited the induction of IL-6 mRNA normally seen after nerve transection.

These findings prompted us to investigate the source of the positive signal regulated IL-6 synthesis. The second part of this study explored the possibility that perineuronal mast cells were releasing a factor in response to nerve injury that was upregulating IL-6 mRNA in DRG neurons. I confirmed that the mast cell degranulating agent, compound 48/80, can upregulate IL-6 mRNA in some medium to large sized DRG neurons and that the mast cell stabilizing agent, cromolyn sodium, blocks the induction of IL-6 normally seen after nerve transection. These findings implicate a mast cell-derived factor in the regulation of IL-6 synthesis, though my quantifications suggest that this is not the sole factor upregulating IL-6 mRNA, as the number of IL-6 mRNA positive neurons was reduced after mast cell degranulation relative to nerve transection.

Chapter three investigated the mechanism through which endogenous IL-6 mitigates the death of DRG neurons. In particular, the relationship between IL-6 and the

neurotrophin BDNF was examined. My quantification of serial sections of DRG neurons hybridized for BDNF mRNA and IL-6 mRNA demonstrated that IL-6 and BDNF colocalize in a subpopulation of DRG neurons after nerve injury. Examination of the original sections showed that the neurons which colocalized these two molecules were medium to large in size. Furthermore, I demonstrated that the observed attenuation of BDNF mRNA levels after injury in the IL-6 KO mouse was specific to medium to large-sized neurons, which normally upregulate BDNF after injury.

These findings suggest that endogenous IL-6 is required for the induction of BDNF in medium to large DRG neurons. Axonal tracing studies would be necessary to confirm for certain that this effect is specific to axotomized neurons. The colocalization studies raise the possibility that IL-6 has autocrine actions on medium to large DRG neurons, as it does in other cell populations (Akira and Kishimoto, 1992; Screpanti et al., 1992), though this possibility has not been examined further.

The other studies presented in this chapter suggest that IL-6 supports neuronal survival in a BDNF-dependent manner. Recently, a synergistic effect of BDNF and CNTF on motor neuron survival has been reported (Hashimoto et al., 1999), suggesting that BDNF and the neuropoietic cytokines may cooperate to support survival in several neuronal populations. Though this effect of IL-6 on sensory neurons is mediated by BDNF, BDNF may also mediate the contribution of IL-6 to pain-related behaviours, as there is convincing evidence implicating BDNF in the development of neuropathic pain (Dassan et al., 1998; Kerr et al., 1999).

Chapter four focused on the role of interleukin-6 in neuropathic pain. Substance P and galanin levels were examined in an effort to understand the mechanism through which IL-6 is contributing to the behavioural changes associated with neuropathic pain. A striking change in galanin levels in the dorsal horn and gracile nucleus were observed in IL-6 deficient mice, prompting us to examine if exogenous IL-6 could regulate galanin mRNA levels in DRG neurons. I demonstrated that IL-6 infusion induces galanin mRNA in 37% percent of uninjured rat DRG neurons, compared to only 13% percent galanin-positive neurons following saline infusion. Furthermore, I showed that galanin positive neurons were small, medium and large in size, suggesting that a broad category of DRG

neurons were able to respond to exogenous IL-6. The ability of exogenous IL-6 to induce galanin and the attenuation of galanin induction in IL-6 deficient mice suggest that endogenous IL-6 may regulate galanin synthesis *in vivo*. As mentioned earlier, there are two main lines of evidence regarding the role of galanin in noociceptive transmission; one suggests that galanin is a pro-nociceptive peptide while the other suggests that it is anti-nociceptive. Therefore, it is unclear if these changes in galanin synthesis account for the pain-related behavioural changes seen in IL-6 deficient mice.

The results presented in chapter five are from a series of preliminary experiments, which were ultimately designed to test if IL-6 was influencing spontaneous firing of DRG neurons after nerve injury. I developed a cell culture procedure for acutely dissociated adult mouse DRG neurons. This population of neurons proved to be very fragile, hence, precise culture conditions had to be worked out to optimize the health of these neurons for electrophysiological recording. Next, I studied the voltage-dependent potassium currents in injured versus uninjured DRG neurons, to the address the hypothesis that voltage-gated potassium currents are reduced in response to axotomy, thereby contributing to neuronal hyperexcitability. I have presented findings to suggest that sciatic nerve transection does not alter the voltage-dependence of activation or current densities of the three potassium current components IK, IAs and IAf.

FUTURE DIRECTIONS

The studies presented here have contributed to the breadth of knowledge on neuronal interleukin-6 in the peripheral nervous system. They also point towards several lines of investigation that could be pursued in the future. I have listed several questions that are raised by these studies below:

1. Which mast cell-derived factor induces IL-6 in neurons? Histamine, substance P and the proinflammatory cytokines IL-1β and TNF-α are produced by mast cells and are known to influence IL-6 synthesis in a number of cell types.
2. Mast cell factors such as histamine, prostaglandins and mast cell degranulating peptide are known to influence ionic currents. Do any of these mast cell-derived factors alter neuronal currents indirectly, via upregulation of neuronal IL-6?
3. What is the exact phenotype of IL-6 mRNA positive neurons? Do these neurons express the high-affinity BDNF receptor trkB? These questions can be approached using immunohistochemistry double-labelling for protein expression or *in situ* hybridization of serial sections for mRNA levels.
4. What is the phenotype of the surviving population of DRG neurons? Do they express IL-6? trkB?
5. What is the mechanism by which IL-6 and BDNF promote neuronal survival? Does IL-6 induce BDNF or trkB directly? RNA protection assays and *in situ* hybridization studies of BDNF mRNA levels before and after IL-6 infusion have shown that IL-6 increases BDNF mRNA levels in DRG neurons (Murphy PG, unpublished data). Do IL-6 and BDNF have synergistic actions, which converge on intraneuronal signalling pathways?
6. Does IL-6 influence spontaneous activity in DRG neurons? This question could be approached by comparing the expression levels of sodium and potassium channels in IL-6 deficient mice and wild-type mice. Peptide or mRNA levels could also be examined in response to IL-6 infusion in uninjured animals or in response to the application of exogenous IL-6 to DRG neurons in culture. If a change in potassium or

sodium currents is uncovered using one of these approaches, one could proceed to investigate changes in ionic currents electrophysiologically.

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