

**NEUROPEPTIDES AND CYTOKINES IN REGENERATING AND  
DEGENERATING PERIPHERAL NERVE**

**by  
Michael. M.T. Khan**

**A thesis presented for the degree of  
Doctor of Philosophy  
in Neurobiology  
at London University**

**Department of Biology  
Medawar Building  
University College London**

ProQuest Number: 10016792

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 10016792

Published by ProQuest LLC(2016). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code.  
Microform Edition © ProQuest LLC.

ProQuest LLC  
789 East Eisenhower Parkway  
P.O. Box 1346  
Ann Arbor, MI 48106-1346

## ABSTRACT

This thesis concerns the role of neuropeptides and cytokines in regeneration of injured peripheral nerve.

A novel experimental technique is described for the rapid preparation of pure cell populations from rat sciatic nerve, involving immunoselection by "panning".

Forskolin elevates Schwann cell cAMPi *in vitro*, permitting growth factor-induced proliferation and expression of nerve growth factor (NGF) and of the transcription factor suppressed cAMP inducible POU (SCIP). The neuropeptide calcitonin gene-related peptide (CGRP) acts through cAMPi in other systems and is upregulated in regenerating motor axons following peripheral nerve injury. I show that CGRP and isoprenaline elevate cAMPi acutely in non-neuronal cells derived from rat sciatic nerve, but that this cAMPi elevation rapidly attenuates due, probably, to the desensitisation of receptors by cAMP-dependant protein kinase (PKA) and the  $\beta$ -adrenoceptor kinase ( $\beta$ ARK), respectively. CGRP fails to promote Schwann cell proliferation, *in vitro*, due to the time course of desensitisation rather than lack of signalling downstream of cAMPi, since CGRP elevates Schwann cell SCIP, and inhibits fibroblast proliferation. Degradation of CGRP by Schwann cell endopeptidase 24.11. may reduce desensitisation *in vivo*. Supraphysiological magnesium ( $Mg^{++}$ ) concentrations enhance receptor / G protein coupling, allowing maintenance of cAMPi elevation in response to CGRP. Consequently, previously desensitising levels of CGRP and isoprenaline are now mitogenic and increase NGF synthesis.

In the second section, I show that interleukin-1 $\beta$  (IL-1 $\beta$ ) promotes Schwann cell proliferation and survival in culture and, with tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), additively increases NGF production. These responses are lost with time and thus are not apparent in cells purified by conventional methods. IL-1 $\beta$  is active alone and stimulates several distinct intracellular signals: these probably include PKA, a pertussis toxin sensitive G protein, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and a tyrosine kinase or protein kinase C (PKC). Inhibiting any of these transduction signals reduces IL-1 $\beta$  action. Gamma-interferon ( $\gamma$ -IFN) dramatically enhances IL-1 $\beta$  stimulated NGF synthesis, yet inhibits proliferation. When PGE<sub>2</sub> synthesis or PKA are downregulated, IL-1 $\beta$  becomes weakly mitogenic for fibroblasts but no longer promotes NGF synthesis. Transforming growth factor type  $\beta$  (TGF $\beta$ ), a macrophage product, does not influence quiescent Schwann cells, but inhibits mitogen induced proliferation.

A model is proposed whereby the responses of non-neuronal cells after axotomy are regulated by a combination of axon and macrophage-derived signals, with a priming action by T cells. Clinical ramifications of the work are then discussed.



## **ACKNOWLEDGEMENTS**

I would like to thank my supervisor, Anne Mudge, for being not only a source of wisdom but also a friend. I thank Ellen Collarini and Bob Harris for their help with the Western blots. It is difficult to single out individual colleagues and friends, who have been of help and support during my studentship: I therefore thank all my friends in the Medawar building. A special thanks is due my wife, for proof reading the entire thesis, and also for putting up with me while I wrote it.

## CONTENTS

<b>ABSTRACT</b>	<b>2</b>
<b>ACKNOWLEDGEMENTS</b>	<b>4</b>
<b>CONTENTS</b>	<b>5</b>
<b>LIST OF FIGURES AND GRAPHS</b>	<b>8</b>
<b>LIST OF PLATES</b>	<b>11</b>
<b>SUMMARY</b>	<b>12</b>
<b>CHAPTER 1. INTRODUCTION</b>	<b>15</b>
<b>OVERVIEW</b>	<b>16</b>
<b>PERIPHERAL NERVE ULTRASTRUCTURE</b>	<b>17</b>
<b>PERIPHERAL NERVE REGENERATION</b>	<b>17</b>
<b>THE SCHWANN CELL IN NERVE REGENERATION</b>	<b>20</b>
<b>NERVE GROWTH FACTORS IN REGENERATION</b>	<b>21</b>
<b>SCHWANN CELL PROLIFERATION <i>IN VIVO</i></b>	<b>22</b>
SIGNAL TRANSDUCTION	25
MOLECULAR SIGNALLING BY GROWTH FACTORS	26
CELL CYCLE REGULATION	30
NEOPLASIA, ONCOGENES AND GROWTH FACTORS	33
NEUROFIBROMATOSIS	36
PLATELET DERIVED GROWTH FACTOR	37
<b>SCHWANN CELL MITOGENS</b>	<b>40</b>
<b>CHAPTER 2. NEUROPEPTIDES IN REGENERATING AND DEGENERATING PERIPHERAL NERVE</b>	<b>43</b>
<b>INTRODUCTION</b>	<b>44</b>
OVERVIEW	44
BIOCHEMICAL CHANGES FOLLOWING AXOTOMY	44
CALCITONIN GENE-RELATED PEPTIDE	45

RECEPTOR ADENYLATE CYCLASE SYSTEMS	47
G PROTEINS	48
DESENSITISATION OF $\beta$ -ADRENERGIC RECEPTORS	50
DESENSITISATION OF OTHER CYCLASE LINKED RECEPTORS	54
DESENSITISATION IN DISEASE	54
CYCLIC AMP AND GENE REGULATION	55
REGULATION OF THE NERVE GROWTH FACTOR GENE	56
REGULATION OF SCIP IN SCHWANN CELLS	57
CILIARY NEUROTROPHIC FACTOR	58
VASOACTIVE INTESTINAL POLYPEPTIDE	60
<b>MATERIALS AND METHODS</b>	<b>61</b>
MATERIALS	61
ANIMALS AND SURGICAL PROCEDURES	61
TISSUE PREPARATION FOR IMMUNOHISTOCHEMISTRY	61
CGRP IMMUNOHISTOCHEMISTRY	62
CELL DISSOCIATION	62
PANNING	63
MACROPHAGE SELECTION	63
FIBROBLAST SELECTION	63
SCHWANN CELL SELECTION	63
PANNING METHOD	68
CELL CULTURE	68
cAMPi ASSAYS	69
MITOGEN ASSAYS	69
NERVE GROWTH FACTOR ASSAY	70
SCIP ASSAYS	71
STIMULATIONS	71
EXTRACTION OF NUCLEAR PROTEINS	72
SCIP WESTERN BLOT	72
SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS	72
IMMUNOLABELLING OF BLOTS	73
<b>RESULTS</b>	<b>74</b>
SUMMARY	74
CGRP IN REGENERATING NERVE	74
CGRP AND cAMPi	82
SCHWANN CELL PROLIFERATION	86
DESENSITISATION OF RESPONSES TO CGRP	97
MAGNESIUM AND DESENSITISATION	105
NGF SYNTHESIS	113

CGRP AND INHIBITION OF FIBROBLAST PROLIFERATION	118
SIGNAL TRANSDUCTION IN FIBROBLAST PROLIFERATION	123
DISCUSSION	129
<b>CHAPTER 3. THE ROLE OF MACROPHAGE DERIVED CYTOKINES IN REGENERATING AND DEGENERATING PERIPHERAL NERVE</b>	<b>133</b>
<b>INTRODUCTION</b>	<b>134</b>
OVERVIEW	134
MACROPHAGES	135
IL-1 ACTION AND BREAKDOWN	137
SIGNAL TRANSDUCTION IN IL-1 STIMULATED CELLS	138
IL-1 AS A MITOGENIC FACTOR	138
IL-1 IN AXOTOMISED PERIPHERAL NERVE AND SCHWANN CELL CULTURE	139
REGULATION OF NGF SYNTHESIS BY CYTOKINES	140
<b>MATERIALS AND METHODS</b>	<b>141</b>
<b>RESULTS</b>	<b>142</b>
SUMMARY	142
IL-1 AND SCHWANN CELL PROLIFERATION	142
SIGNAL TRANSDUCTION IN IL-1 ACTION	148
DISCUSSION	171
<b>CHAPTER 4. DISCUSSION</b>	<b>172</b>
<b>REFERENCES</b>	<b>182</b>

## LIST OF FIGURES AND GRAPHS

FIG. 1. THE RAS SIGNALLING PATHWAY.	29
FIG.2A. LONGITUDINAL SECTIONS OF RAT SCIATIC NERVE, SHOWING PROXIMAL AND DISTAL ACCUMMULATION OF CGRP, 24 HOURS AFTER NERVE CRUSH.	76
FIG.2B. LONGITUDINAL SECTIONS OF RAT SCIATIC NERVE, SHOWING LINEAR STAINING FOR CGRP PROXIMAL TO THE LESION SITE.	77
FIG.2C. LONGITUDINAL SECTIONS OF RAT SCIATIC NERVE, SHOWING LOSS OF CGRP IMMUNOSTAINING DISTAL TO THE LESION, UNLESS REINNERVATION IS ALLOWED.	78
FIG.2D LONGITUDINAL SECTIONS OF RAT SCIATIC NERVE, SHOWING CONTINUED PRESENCE OF CGRP IMMUNOSTAINING AT THE NERVE FRONT, AT 7 AND 14 DAYS AFTER NERVE TRANSECTION..	79
FIG.2E. LONGITUDINAL SECTIONS OF RAT SCIATIC NERVE, SHOWING CONTINUED PRESENCE OF CGRP IMMUNOSTAINING AT THE REGENERATING NERVE FRONT, AT 7 AND 14 DAYS AFTER NERVE CRUSH, ALLOWING REGENERATION INTO THE DISTAL DEGENERATING NERVE.	80
FIG.3. DOSE RESPONSE CURVE FOR CGRP STIMULATION OF SCHWANN CELL CAMPI.	83
FIG.4A. SCHWANN CELL CAMPI DOSE RESPONSE TO CGRP + / - IBMX.	84
FIG.4B. FIBROBLAST CAMPI DOSE RESPONSE TO CGRP + / - IBMX.	85
FIG.5A. INCORPORATION OF <sup>125</sup> IDU BY PURE CULTURED SCHWANN CELLS, IN THE PRESENCE OR ABSENCE OF PMA.	88
FIG.5B. SCHWANN CELL INCORPORATION OF <sup>125</sup> IDU IN THE PRESENCE OF INHIBITORS OF PKA OR PKC.	89
FIG.6A. THE EFFECT OF TGFβ ON SCHWANN CELL INCORPORATION OF <sup>125</sup> IDU.	91
FIG.6B. THE EFFECT OF TGFβ ON SCHWANN CELL PROLIFERATION, AS ASSESSED BY MTT ASSAY.	92
FIG.7. TIMECOURSE OF SCHWANN CELL CAMPI ELEVATION AFTER CGRP OR FORSKOLIN ADDITION.	94
FIG.8. SCIP WESTERN BLOTS ON CGRP- AND FORSKOLIN- STIMULATED SCHWANN CELLS.	96
FIG.9. THE EFFECT OF SCHWANN CELL E.C.24.11 ON CGRP DEGRADATION.	98
FIG.10. THE ROLE OF G <sub>i</sub> AND PHOSPHODIESTERASE IN REGULATING ACUTE SCHWANN CELL CAMPI ELEVATION.	99
FIG.11. THE ROLE OF G <sub>i</sub> AND PHOSPHODIESTERASE IN REGULATING CAMPI IN SCHWANN CELLS OVER A 24 HOUR INCUBATION WITH CGRP.	101
FIG.12. THE EFFECT OF INHIBITING PKA ON CGRP MEDIATED SCHWANN CELL CAMPI ELEVATION.	103
FIG.13. THE EFFECT OF HEPARIN, AN INHIBITOR OF βARK, ON ISOPRENALINE- AND CGRP- INDUCED SCHWANN CELL CAMPI ELEVATION.	104

FIG.14A. THE EFFECT OF SUPRAPHYSIOLOGICAL MAGNESIUM CONCENTRATION ON ISOPRENALINE AND CGRP MEDIATED SCHWANN CELL DESENSITISATION.	106
FIG.14B. THE EFFECT OF MAGNESIUM ON HETEROLOGOUS AND HOMOLOGOUS DESENSITISATION.	107
FIG.15A. THE EFFECT OF MAGNESIUM ON THE PERMISSIVE ACTION OF ISOPRENALINE AND CGRP ON SERUM INDUCED SCHWANN CELL PROLIFERATION.	109
FIG.15B. THE EFFECT OF MAGNESIUM ON THE PERMISSIVE ACTION OF CGRP AND ISOPRENALINE ON PDGF INDUCED SCHWANN CELL PROLIFERATION IN DEFINED MEDIUM.	110
FIG.15C. THE EFFECT OF MAGNESIUM ON THE PERMISSIVE ACTION OF ISOPRENALINE AND PGE <sub>2</sub> ON SERUM INDUCED PROLIFERATION.	111
FIG.15D. THE EFFECT OF MAGNESIUM ON THE PERMISSIVE ACTION OF CGRP ON FGF INDUCED SCHWANN CELL PROLIFERATION.	112
FIG.16A. THE INDUCTION OF FIBROBLAST NGF SYNTHESIS BY CGRP IS POTENTIATED BY MAGNESIUM.	114
FIG.16B. NGF PRODUCTION IN CULTURED FIBROBLASTS IS STIMULATED BY CGRP AND POTENTIATED BY MAGNESIUM.	115
FIG.16C. MAGNESIUM POTENTIATION OF CGRP INDUCED FIBROBLAST NGF SYNTHESIS.	116
FIG.16D. MAGNESIUM AND ISOPRENALINE INDUCED SCHWANN CELL NGF SYNTHESIS.	117
FIG.17. THE EFFECT OF CGRP ON SERUM INDUCED FIBROBLAST PROLIFERATION.	119
FIG.18. THE EFFECT OF FORSKOLIN ON SERUM - INDUCED FIBROBLAST PROLIFERATION.	120
FIG.19A. THE EFFECT OF IBMX ON CGRP-MEDIATED INIBITION OF FIBROBLAST PROLIFERATION.	121
FIG.19B. THE EFFECT OF THEOPHYLLINE ON THE INHIBITION OF FIBROBLAST PROLIFERATION BY CGRP AND BY CNTF.	122
FIG.20A. THE ROLE OF G <sub>T</sub> IN FIBROBLAST PROLIFERATION.	124
FIG.20B. THE ROLE OF VARIOUS SECOND MESSENGERS IN MEDIATING INHIBITION OF FIBROBLAST PROLIFERATION BY CGRP, FORSKOLIN AND $\gamma$ -IFN.	125
FIG.21. DOSE RESPONSE CURVE FOR VIP INDUCED CAMPI ELEVATION IN SCHWANN CELLS.	127
FIG.22. SECOND MESSENGERS IN FIBROBLAST PROLIFERATION. THE ACTION OF CNTF ON FIBROBLAST PROLIFERATION.	128
FIG.23A. THE ACTION OF IL-1 AND PDGF ON SCHWANN CELL PROLIFERATION ASSESSED BY <sup>125</sup> IDU ASSAY.	143
FIG.23B. IL-1 INDUCED SCHWANN CELL PROLIFERATION IN DEFINED MEDIUM, AS ASSESSED BY MTT ASSAY.	144
FIG.23C. IL-1 $\beta$ INDUCED SCHWANN CELL PROLIFERATION IN DEFINED MEDIUM, COMPARED TO FORSKOLIN / PDGF SYNERGY, AS ASSESSED BY <sup>125</sup> IDU ASSAY.	145

FIG.23D. TNF $\alpha$ AND IL-1 $\beta$ INDUCED SCHWANN CELL PROLIFERATION IN SERUM CONTAINING MEDIUM, AS ASSESSED BY <sup>125</sup> IDU ASSAY.	146
FIG.23E. IL-1 $\beta$ INDUCED SCHWANN CELL PROLIFERATION IN SERUM CONTAINING MEDIUM, AS ASSESSED BY MTT ASSAY.	147
FIG.24. IL-1 SYNERGISES WITH PDGF AND FORSKOLIN.	149
FIG.25 THE EFFECT OF IL-1 AND PGE <sub>2</sub> ON SCHWANN CELL CAMPI.	150
FIG.26. THE COMPARATIVE EFFECTS OF PERTUSSIS TOXIN AND INDOMETHACIN PRE-TREATMENT ON IL-1-INDUCED SCHWANN CELL PROLIFERATION.	151
FIG.27A. H-89 IMPAIRS IL-1-INDUCED SCHWANN CELL PROLIFERATION.	152
FIG.27B. H-8, AN INHIBITOR OF PKA, IMPAIRS IL-1 MEDIATED SYNERGY WITH PDGF ON SCHWANN CELL PROLIFERATION.	153
FIG.28. PGE <sub>2</sub> ACTION ON PROLIFERATION OF SCHWANN CELLS CULTURED IN SUPRAPHYSIOLOGICAL MAGNESIUM .	154
FIG.29A. THE EFFECT OF PERTUSSIS TOXIN PRE-TREATMENT ON IL-1-INDUCED SCHWANN CELL PROLIFERATION.	155
FIG.29B. EFFECT OF PERTUSSIS TOXIN PRE-TREATMENT ON IL-1-INDUCED PROLIFERATION OF SCHWANN CELLS CULTURED IN SERUM-CONTAINING MEDIUM.	156
FIG.30A. THE EFFECT OF PERTUSSIS TOXIN AND H-89 ON IL-1 ACTION ON ENDONEURIAL FIBROBLAST PROLIFERATION.	158
FIG.30B. THE EFFECT OF PERTUSSIS TOXIN AND H-89 ON IL-1, $\gamma$ -IFN AND FORSKOLIN MEDIATED ACTIONS ON FIBROBLAST PROLIFERATION.	159
FIG.31. IL-1 IS A SCHWANN CELL SURVIVAL FACTOR IN INSULIN FREE MEDIUM.	161
FIG.32. TGF $\beta$ INHIBITS IL-1-INDUCED SCHWANN CELL PROLIFERATION.	162
FIG.33. PGE <sub>1</sub> INHIBITS SCHWANN CELL PROLIFERATION MEDIATED BY IL-1.	163
FIG.34. IL-1 AND TNF INDUCTION OF FIBROBLAST NGF PRODUCTION; SYNERGISTIC ACTION OF $\gamma$ -IFN.	165
FIG.35A. IL-1 INDUCTION OF SCHWANN CELL NGF PRODUCTION.	166
FIG.35B. POTENTIATION OF IL-1-INDUCED NGF SYNTHESIS BY $\gamma$ -IFN.	167
FIG.36. ADDITIVE ACTION OF IL-1 AND TNF ON SCHWANN CELL NGF PRODUCTION.	168
FIG.37. $\gamma$ -IFN POTENTIATES IL-1-INDUCED FIBROBLAST NGF SYNTHESIS AND INHIBITS PROLIFERATION.	170
FIG.38. SIGNAL TRANSDUCTION DURING CELL-CELL INTERACTIONS FOLLOWING PERIPHERAL NERVE INJURY	178

**LIST OF PLATES**

**Plate 1. S100 immunostaining of cultures of mixed Schwann cells and fibroblasts derived from rat sciatic nerve. 65.**

**Plate 2. Comparative morphology of Schwann cell and fibroblast cultures, 4 days after dissociation. 66.**

**Plate 3. Pure Schwann cell cultures, derived by fibroblast and macrophage depletion. 67.**



## SUMMARY

The studies presented in this thesis are concerned with the role of cytokines and neuropeptides in regeneration of peripheral nerve following injury. Specifically, we have investigated the action of these agents in controlling the proliferation and synthesis of trophic factors by non-neuronal cells during degeneration and in assisting regeneration of the injured peripheral nerve. The contribution of factors derived from lesioned axons and recruited macrophages will be discussed.

I developed a novel technique for generating pure populations of non-neuronal cells from rat sciatic nerve, which involves immunoselection by "panning". Homogeneous cells approaching 100% purity are obtained within a few hours of sciatic nerve dissociation by applying mixed dissociated cells to dishes coated with cell-type specific antibodies to surface antigens. Such cells are less than 24 hours old, in culture, and their behaviour is consistent with that demonstrated *in vivo*. Traditional techniques require a week or more and use cytotoxic agents to eliminate contaminating fibroblasts, followed by rounds of growth factor mediated expansion, to yield sufficient numbers for *in vitro* assays. Consequently, the biological responses differ from acutely isolated Schwann cells whose behaviour should more closely resemble what occurs after axotomy *in vivo*.

The first section of the thesis concerns the role of neuropeptides in the control of proliferation and gene expression in non-neuronal cells during regeneration of peripheral nerve. Agents that elevate cAMPi, such as forskolin and cholera toxin, permit Schwann cell proliferation when used in conjunction with growth factors such as PDGF, FGF or foetal calf serum (FCS) *in vitro*. To date, no endogenously occurring agents have been shown to elevate Schwann cell cAMPi and thereby allow proliferation. The neuropeptides CGRP and vasoactive intestinal polypeptide (VIP) are known to elevate cAMPi in other systems and, furthermore, following axotomy are upregulated in regenerating motor and sensory axons respectively. Therefore, these neuropeptides are ideally positioned to influence responses associated with axonal regeneration *in vivo*. We show that CGRP and the  $\beta$ -adrenergic agonist isoprenaline acutely elevate cAMPi in Schwann cells and fibroblasts, as does VIP to a lesser extent. CGRP and isoprenaline demonstrate synergy in cAMPi elevation, suggesting independent receptor mechanisms for adenylate cyclase activation. Ligand-induced cAMPi elevation is short lived in cultured cells, due to desensitisation, explaining why these agents do not reliably permit Schwann cell proliferation *in vitro*. Agents that elevate cAMPi also increase levels of the transcription factor SCIP in Schwann cells. CGRP also elevates SCIP levels, showing that intracellular signals are generated and reach the nucleus. The lack of mitogenic

action, therefore, is likely to be a result of receptor desensitisation and not due to failure of signalling downstream of cAMPi elevation. Experimental constraints, including cell losses during replacement of medium and loss of available peptide through adhesion and degradation, have led investigators to add agents to cultured cells at concentrations often in the micromolar range, exposing cells to continuous high concentrations of agents, such as CGRP. This is unlike the situation *in vivo* where continuous release of CGRP by axons followed by removal or degradation would prevent the accumulation of desensitising concentrations. To support this, we find that Schwann cells degrade CGRP via a surface endopeptidase e.c.24.11 which is inhibited by phosphoramidone *in vitro*. Evidence is presented that desensitisation is mediated by PKA and possibly by  $\beta$ ARK. Inhibitors of PKA, such as H-8 and H-89, reduce cAMPi attenuation to CGRP but also prevent proliferation. Heparin inhibits  $\beta$ ARK, in digitonin permeabilised cells, and also reduces cAMPi attenuation to isoprenaline, but under these conditions no proliferation occurs and cells eventually die. Inhibitors of phosphodiesterase or the inhibitory G protein,  $G_i$ , potentiate acute responses but do not prevent desensitisation, suggesting that neither cAMPi breakdown nor downregulation of adenylate cyclase are important for desensitisation. To prove our contention that, in the absence of desensitisation, both CGRP and isoprenaline would be permissive signals for growth factors as Schwann cell mitogens, we have utilised a novel technique. Theoretical concepts suggest a critical role for  $Mg^{++}$  in favouring the dissociated state of the heterotrimeric  $G_s$  complex following receptor ligand interaction. In this state the  $G_{s\alpha}$  subunit is free to activate adenylate cyclase. Increasing the concentration of  $Mg^{++}$  could therefore enhance receptor G protein coupling and thereby overcome the effects of receptor desensitisation. At concentrations between 5 and 10mM  $Mg^{++}$  the rapid loss of cAMPi elevation to both CGRP and isoprenaline is reduced and they are permissive in conjunction with serum, PDGF or FGF.  $Mg^{++}$  itself neither elevates cAMPi nor is mitogenic and is therefore potentiating a specific receptor response. Other neuropeptides such as somatostatin, met-enkephalin, ACTH, galanin and substance P, which do not elevate Schwann cell cAMPi, are not mitogenic even in high  $Mg^{++}$ . In the presence of high  $Mg^{++}$ , CGRP is able to increase NGF synthesis by fibroblasts, and isoprenaline stimulates NGF synthesis by Schwann cells. CGRP, even in the absence of  $Mg^{++}$ , inhibits fibroblast proliferation. In this case a brief elevation of cAMPi is sufficient as a similar time course of desensitisation is seen in these cells also.

The second section examines the role of the macrophage-derived cytokines, IL-1 and TNF, in regulating proliferation and NGF synthesis by non-neuronal cells. Data is presented which shows that IL-1 and TNF both increase NGF synthesis by cultured fibroblasts and weakly by Schwann cells, and that their action is additive. IL-1 is also

shown to be a Schwann cell mitogen. These Schwann cell responses are lost with time in culture and are not apparent, therefore, in cells prepared by older methods. This is the first *in vitro* demonstration that products of activated, recruited macrophages may regulate Schwann cell responses. IL-1 stimulates NGF production by endoneurial fibroblasts and stimulates proliferation weakly if PGE<sub>2</sub> synthesis is inhibited by indomethacin. PGE<sub>2</sub> inhibits fibroblast proliferation via activation of PKA as IL-1 is mitogenic if PKA is inhibited with H-89.  $\gamma$ -IFN potentiates the action of IL-1.  $\gamma$ -IFN is a product of activated T-cells and is upregulated in injured neurons. Co-treatment of cells with  $\gamma$ -IFN and IL-1 leads to marked synergistic stimulation of NGF synthesis, suggesting that it may sensitise non-neuronal cells to macrophage products.  $\gamma$ -IFN inhibits proliferation of fibroblasts and Schwann cells, even when IL-1 stimulated, suggesting that it may operate a switch from proliferation to synthesis of NGF.  $\gamma$ -IFN inhibits fibroblast proliferation independently of cAMPi and is not prevented by H-89. It may be of interest to examine the role of TGF- $\beta$  in this respect, as it also inhibits proliferation of fibroblasts and Schwann cells. IL-1 stimulates Schwann cell proliferation through more than one intracellular signalling pathway. Data is presented that a pertussis toxin sensitive G protein mediates part of IL-1 action; we have indirect evidence that IL-1 increases Schwann cell production of the E-series prostaglandin, PGE<sub>2</sub>. These pathways are both critical for synergy between IL-1 and PDGF, which is prevented by pertussis toxin or indomethacin pre-treatments. Part of intrinsic IL-1 mitogenic activity is unaffected by these agents but is by the tyrosine kinase inhibitor genistein. PKA activity must be intact for cell division as H-89 prevents the action of all mitogens. IL-1 acts in synergy with either forskolin or PDGF to induce proliferation. I propose that IL-1 is a mitogen by the activation of at least two intracellular signalling pathways; one of which is linked to cAMPi elevation and activation of PKA and one which is via a tyrosine kinase. TGF- $\beta$ , also a macrophage product, has previously been suggested as a Schwann cell mitogen. I show that TGF- $\beta$  does not influence acutely isolated Schwann cells, whilst quiescent, but inhibits mitogen induced proliferation.

Finally, a unifying model is suggested whereby the responses of non-neuronal cells following axotomy are regulated by a combination of both neuron-derived and macrophage-derived signals. Clinical aspects of the work are also discussed, including the therapeutic potential of supraphysiological serum Mg<sup>++</sup> in situations, such as heart failure, where down regulation of adenylate cyclase linked receptors is prominent.

## **CHAPTER 1**

### **INTRODUCTION**

## OVERVIEW

In mammals, neurons within the peripheral nervous system (PNS) regenerate whereas those in the central nervous system (CNS) do not. This fundamental difference has profound clinical implications. Although the proximal stumps of transected axons in the CNS begin to regenerate, this growth ceases by two weeks (Cajal, 1928) whereas, in the PNS, regrowth continues and functional reinnervation of target tissues may occur. A large body of evidence suggests that glial cells and other non-neuronal cells provide a permissive environment for neuronal regeneration to occur, within the PNS, and that failure of functional regeneration within the CNS may be due to differences in surrounding non-neuronal cells rather than in the neurons themselves. Regeneration in the optic nerve may be promoted by applying grafts of PNS tissue such as the sciatic nerve. Regeneration in the CNS can also be promoted by applying grafts of foetal CNS tissue, suggesting that the properties of neurons or glial cells differ between embryonic and adult stages. It is therefore of great interest to understand the biology of non-neuronal cells within the PNS. Identification of those factors influencing Schwann cell and fibroblast activity during PNS regeneration may not only lead to methods of improving functional outcome after peripheral nerve injury or in neuropathic disorders, but might also suggest therapeutic strategies for promoting regeneration within the CNS. Sufferers of motor neuron disease might benefit if trophic factor production by glial cells could be enhanced: understanding those factors which regulate this process could have great impact on the treatment of this and related disorders. Furthermore, many diseases involve primarily disordered function of the non-neuronal cells themselves, including Guillain-Barré syndrome in the PNS and multiple sclerosis within the CNS: again, identification of factors controlling proliferation and myelination by these cells could have therapeutic implications. Finally, the progression or genesis of nerve sheath tumours, occurring sporadically or in individuals with neurofibromatosis type 1 (NF-1) or type 2 (NF-2), could be manipulated if those factors governing proliferation were identified. Therapeutically, this might be of critical importance particularly where tumour location either precludes surgery or where surgical intervention is likely to result in damage to surrounding structures such as cranial nerves around the internal auditory meatus. It might also shed some light on the processes governing malignant transformation in subsets of glial cells bearing the NF-1 mutation.

In this thesis, we set out to identify factors regulating proliferation and trophic factor synthesis by Schwann cells and fibroblasts. Intracellular second messenger systems mediating these effects have also been studied and the relative contributions of axon and macrophage derived signals on these examined.

## **PERIPHERAL NERVE ULTRASTRUCTURE**

Peripheral nerves contain axons which are the distal extensions of sensory, motor and autonomic neurons whose cell bodies lie in dorsal root ganglia, the anterior horn of the spinal cord, and autonomic ganglia, respectively. A peripheral nerve, such as the sciatic, consists of numerous fascicles, or axon bundles, accompanied by Schwann cells that provide the axons with myelin or membranous/cytoplasmic ensheathment. A sleeve of basement membrane covers the Schwann cell and extends uninterrupted from one Schwann cell to the other along the same axon. Schwann cells form the basal lamina components on contact with axons (Bunge et al., 1986). The innermost part of the nerve, containing axons, is called the endoneurium and is surrounded by a flattened cellular layer, the perineurium, which surrounds each fascicle. Around the entire complement of fascicles is the epineurium. The connective tissue stroma, which lies between axon-Schwann cell units surrounding the nerve fibres, is formed by the endoneurial and specialised epineurial fibroblasts which produce the majority of endoneurial collagen (Salonen et al., 1988; Salonen et al., 1987; Roytta et al., 1987). The perineurial fibroblasts also contribute to the blood-nerve barrier (Bunge et al., 1989). Anastomotic networks of blood vessels, with numerous perforating feeders, along the entire length of the nerve supply intra fascicular networks. The peripheral nerve trunks contain substantial connective tissue; a third of protein in peripheral nerve trunks is collagen. The endoneurium also contains macrophages, particularly along the blood vessels (Oldfors, 1980), and mast cells in varying numbers depending on the species (Gamble and Goldby, 1961; Nennesmo and Reinholt, 1986) which contribute to post-injury oedema (Powell et al., 1979). The vasa nervora communicate with the extracellular fluid of the peripheral nervous system and play a critical role in composition of that fluid (blood-nerve barrier). The intrafascicular cells and axons themselves also add products which are released into the endoneurium (Skene and Shooter, 1983).

## **PERIPHERAL NERVE REGENERATION**

It is well established that peripheral nerves of most vertebrates have the capacity to regenerate after injury, to the extent that sensory and motor function can return. Peripheral nerve injuries fall into two broad categories: first, transection injuries where regeneration into the disconnected stump is prevented by spatial separation and, second, those such as crush injuries or non-displaced transection injuries where axonal regeneration into the distal stump can occur. Initially, the structural changes occurring in the distal stump are identical following both types of injury; axonal degeneration is accompanied by the proliferation of non-neuronal cells. Ischaemia, however, is not induced in peripheral nerve even after crush/transection. Both

proximal and distal stumps are supplied from perforating vessels, ensuring that the distal stump retains a good blood supply, even prior to regeneration and any new vessel formation or vascular repair. Crushed axons which retain their Schwann cell and basal lamina sheaths may regrow and reinnervate their targets; severed axons may grow out from their proximal tips to reconnect to their original targets, some remaining intact axons may sprout fine processes both at their terminals and at the nodes of Ranvier, some of which may succeed in innervating denervated muscle fibres. In the case of nerve section, the gap between the proximal and distal stumps is closed by Schwann cells and by perineurial and endoneurial cells which differentiate, proliferate, and move in from both sides of the lesion (Bijlsma et al., 1988). The entry of axons into the distal stump may be delayed or absent if the distance between severed ends is too great. Cut axons can grow over 1-2cm to reach the stump, but functional recovery will be poor (Lundborg et al., 1989). Transected nerves may traverse greater distances if a guidance channel or implanted chamber is employed (Aebischer et al., 1989). Using the Schwann cell as a guide, the regenerating axons reconnect with their targets. The quality of this reconnection depends on the ability of the fibres to enter the correct channels distal to the lesion, and on the survival of damaged neurons until they achieve this. In man, the best rate of growth is approximately 1mm per day, whereas in adult rat, the rate of regeneration of sciatic nerve neurons after crush is 2-3mm per day. There is a latent period of 5-6 days before appearance of regenerating axons beyond the crush site, for a lesion located 45mm from the target tissue (Guttman et al., 1942). Rates are faster following crush than following transection, and functional outcome is more complete because the basal lamina tubes have not been interrupted and can direct axons back to their correct targets. It has been estimated that only 6% of growing neurites do not reach their targets after crush injuries measuring 2mm in width.

Trauma to neurons results in significant biochemical rearrangements and readjustments. These changes have been described extensively: the soma undergoes what is collectively called chromatolysis, which eventually leads to either regeneration of the injured neuron or to its eventual degeneration; the axons, disconnected from their cell bodies, undergo Wallerian degeneration (named after A.V.Waller who first described these changes, in severed peripheral nerves in 1850). Similar changes occur after crush (Powell et al., 1979), cold injury (Myers et al., 1980), after damage by the immune system (Ridley and Rainbird, 1978), or when the blood supply is interrupted (Hess et al., 1979; Perry and Brown, 1981). Following nerve fibre injury, axons and myelin in the distal stump are degraded; this stage of Wallerian degeneration is largely executed by macrophages invading the distal stump, though Schwann cells may also play a part. There has been considerable debate as to the direction of degeneration; morphological changes proceed centripetally while

electrophysiological changes appear first at the nerve terminals. These differences may reflect the fact that axons are degenerating from the distal end, whereas myelin degradation occurs in the reverse direction. In baboon motor axons there is failure of neuromuscular transmission by 4-5 days, whereas conduction block appears later and develops centripetally.

The changes in the proximal stump are similar to those in the distal stump over the first 2 millimetres, representing about 3-4 internodes proximal to the injury, and include axonal degeneration and Schwann cell proliferation. These changes are locally confined, although a variable number of axons undergo degeneration and there is a small loss of cell-bodies through retrograde degeneration.

The question of which cells are responsible for clearing the myelin debris has been clarified. Recent evidence suggests that both macrophages and Schwann cells may be involved, but that the majority of clearance is by ED1-positive macrophages. Macrophages begin entering the nerve from blood vessels by the second day following injury, migrate to degenerating nerve fibres, penetrate the basal lamina and phagocytose myelin (Stoll et al., 1989). There are only rare resident macrophages present in mature adult peripheral nerve (Samuel et al., 1987b). Macrophage and polymorphonuclear leukocyte (PMNL) recruitment is required for normal progression of the degenerative response (Lunn et al., 1989; Beuche and Friede, 1984). In its absence, as is seen in the C57Bl/Ola mutant mouse strain, Wallerian degeneration proceeds extremely slowly and Schwann cell proliferation remains confined to the actual injury site (Brown et al., 1991). The lack of proliferation is paralleled by a failure of NGF production in the distal stump. The mutation seems to be a defect within the nerve, leading to absence of a recruitment stimulus rather than in circulating monocytes (Perry et al., 1990), though the nature of this is not known. Macrophages are prominent participants in inflammatory demyelinating neuropathies. In patients with chronic inflammatory neuropathy and Guillain-Barré syndrome (Griffin et al., 1990b), it was found that Ia-positive macrophages were adherent to both normal and demyelinating fibres. Foamy macrophages, judged to be postphagocytic, were Ia-negative. The actual recruitment stimulus for macrophages is not known but could be a T-cell product. T-lymphocytes are frequently present in nerves obtained from patients with various types of neuropathy (Cornblath et al., 1990). Monocytes can be demonstrated in 76% of biopsies taken from neuropathic nerves, with CD3<sup>+</sup> cells (T lymphocytes) at the greatest density. Factors that attract T lymphocytes into nerves may thus be important in pathogenesis.



## THE SCHWANN CELL IN NERVE REGENERATION

It has been known for more than forty years that injury to peripheral nerve causes multiplication of non-neuronal cells, especially Schwann cells. These cells may determine the success of axonal regeneration (Taniuchi et al., 1986) by secreting trophic factors, such as NGF (Heumann et al., 1987; Lindholm et al., 1987) and brain derived neurotrophic factor (BDNF) (Acheson et al., 1991). Numerous observations suggest that axons may grow over short distances on their own, but that over longer distances they require Schwann cells to do so. Although there are reports of basal lamina tubes deprived of living Schwann cells supporting regeneration (Ide et al., 1983), they do not appear to do so if Schwann cells are prevented from growing out from the proximal stump along with regenerating axons (Hall, 1986a). When Schwann cell mitosis is impaired by irradiation or anti-mitotic agents, axonal growth is retarded and myelin formation impaired (Hall, 1986b; Hall and Gregson, 1977; Love, 1983). Five days after axotomy of rat sciatic nerve proliferating Schwann cells have migrated within endoneurial spaces and within basal lamina to form longitudinal arrays with longitudinally overlapping processes just within the perineurium, referred to as the Bands of Bungner. It is generally believed that Schwann cells within distal bands provide trophic support for regenerating axons, this is most clear for sensory and sympathetic axons. Following axotomy, Schwann cells and fibroblasts produce NGF (Heumann et al., 1987c; Bandtlow et al., 1987), and Schwann cells start to produce receptors for NGF (Taniuchi et al., 1988; DiStefano and Johnson, 1988). In the C57Bl/Ola mutant mouse, normal NGF synthesis by non-neuronal cells does not occur, and the regeneration of sensory neurons is greatly impaired (Brown et al., 1991) although this may be coincidental. Schwann cells may secrete and bind NGF to their cell surface, providing a sink of NGF and thereby trophic support for regenerating axons. Schwann cells also make ciliary neurotrophic factor (CNTF) (Rende et al., 1992; Dobrea et al., 1992; Muir et al., 1989; Wewetzer et al., 1990), and BDNF. CNTF is a potential trophic factor for motoneurons (Sendtner et al., 1990); BDNF, neurotrophin-3 (NT-3) and neurotrophin-5 (NT-5) can prevent the death of cultured embryonic rat spinal motoneurons (Henderson et al., 1993), though it is not known if NT-3 and NT-5 are made by Schwann cells, or how they are regulated following injury. This dependence of neurons on exogenous trophic factors may be primarily a feature of developing or regenerating peripheral nerves. It is known that neurons express mRNA for high affinity NGF receptors (Trk A) and up to 35% of DRG neurons express both high affinity BDNF receptors (Trk B), and the preferred NT-3 receptor (Trk C) *in vivo* (Meakin and Shooter, 1992; McMahon et al., 1994); what is surprising however, is the finding that BDNF produced by dorsal root ganglion (DRG) neurons may act as an autocrine survival factor *in vitro*, for a subpopulation of sensory neurons at least (Acheson et al., 1995). It is therefore

possible that exogenous trophic support may not be required for some adult neurons, but equally supports the hypothesis that some form of survival signals are needed in adult as well as developing cells, which can be autocrine. The ability of nerves to successfully regenerate in the peripheral nervous system (PNS) is, therefore, at least in part due to the permissive environment provided by proliferating Schwann cells and secreted growth factors.

## **NERVE GROWTH FACTORS IN REGENERATION**

Nerve growth factor (NGF), was discovered in the 1950s by Levi-Montalcini and Hamburger (Levi-Montalcini and Hamburger, 1951). It was found to enhance the outgrowth of neurites selectively from neurons of sympathetic and embryonic dorsal root ganglia both *in vivo* during foetal development, and *in vitro* (Levi-Montalcini and Angeletti, 1963; Levi-Montalcini et al., 1954). NGF is produced by target tissues of NGF-sensitive fibres, taken up by presynaptic terminals and retrogradely transported to the perikaryon (Hendry et al., 1974; Stoeckel et al., 1975; Stoeckel et al., 1976). The importance of NGF for neuronal survival during development has been demonstrated conclusively by immunosympathectomy. Administration of NGF antiserum to new-born rats results in a significant reduction of neuronal number in the lumbar DRG (Yip et al., 1984). Addition of NGF during development reduces the extent of naturally occurring cell death (Hamburger et al., 1988), possibly by suppression of an active cell death program (Martin et al., 1988). Considerable cell death occurs in sympathetic and DRG neurons following peripheral axotomy in rats (Hendry, 1975; Yip and Johnson, 1984; Tessler et al., 1985; Arvidsson et al., 1986). Deprivation of NGF does not increase the degree of cell death in the lumbar DRG or affect the success of regeneration after sciatic nerve transection (Rich et al., 1984). NGF, however, partially reverses the central changes in the cell body following transection, when applied locally to lesioned nerve (Fitzgerald et al., 1985; Rich et al., 1987). NGF may be involved in collateral sprouting of sensory axons in the skin (Diamond et al., 1992). NGF mRNA increases distal to a sciatic nerve lesion (Heumann et al., 1987b), localised to Schwann cells and fibroblasts, with peaks at 6 hours and 3 days. This persists until regeneration occurs (Heumann et al., 1987c). This increased expression following axotomy may enable the distal stump to act as a surrogate target, providing trophic support to regenerating sensory and sympathetic fibres. However, although NGF receptors transiently accumulate around a nerve lesion by axonal transport (Raivich and Kreutzberg, 1987), both retrograde and anterograde transport decrease rapidly from one day after injury (Raivich et al., 1991). NGF receptors on Schwann cells conversely reach a peak at 6 days after injury

(Raivich et al., 1987), and this expression only decreases with the arrival of regenerating axons (Taniuchi et al., 1988). In contrast to NGF peptide, the receptor is not regulated by recruited macrophages (Brown et al., 1991; Heumann et al., 1987) but probably by axonal contact. It is, therefore, not clear what role NGF is playing in regeneration. It has been suggested that NGF produced by non-neuronal cells in the distal stump (Matsuoka et al., 1991) could bind to Schwann cell receptors and support regeneration of sensory axons by acting as a bridge between the two (Taniuchi et al., 1988). The disappearance of axonal expression and retrograde receptor transport after axotomy suggests that the regenerating axons may not be the target of NGF (Raivich et al., 1991). No local action on non-neuronal cells has been shown to date. Many related factors have now been described; BDNF has been isolated and sequenced (Barde et al., 1982; Leibrock et al., 1989) and may be a trophic factor for subpopulations of sensory neurons (Acheson et al., 1995); PCR cloning techniques have revealed the identity of the NGF-like neurotrophic factors, NT-3 (Maisonpierre et al., 1990) and NT-4/5 (Berkemeier et al., 1991; Ip et al., 1992). These neurotrophins exert motoneuron trophic activity *in vitro* (Henderson et al., 1993). All the neurotrophins including NGF bind to two classes of receptor; the tyrosine kinases encoded by the *trk* genes (*trkA*, *trkB*, and *trkC*) which mediate neurotrophin action (Cordon-Cardo et al., 1991), and the protein p75 which enhances sensitivity of cells bearing *trk* receptors to neurotrophins (Hempstead et al., 1991), and may be a functional element of the receptor. Interestingly, Schwann cells express both p75 (Schattman et al., 1990; Heuer et al., 1990) and *trkB* mRNA (Klein et al., 1990; Martin-Zanca et al., 1990), though biological responses of Schwann cells to neurotrophins have so far not been described.

### **SCHWANN CELL PROLIFERATION *IN VIVO***

In normal uninjured adult peripheral nerve non-neuronal cells consisting of Schwann cells and fibroblasts, and a smaller number of vascular endothelial cells and resident macrophage/ monocytes, are essentially quiescent and neither proliferate nor secrete detectable amounts of NGF. Knowledge of the signals involved in regulating cell proliferation and growth factor expression following nerve injury and during subsequent regeneration may lead to advances in the treatment of traumatic or neuropathic nerve damage, and may throw light on factors involved in the genesis or maintenance of the nerve sheath tumours of neurofibromatosis. Schwann cell division, meticulously regulated throughout development, occurs at an extremely low level in normal adult nerves. Loss of the myelin sheath in disease results in active proliferation of Schwann cells which, in the case of some demyelinating neuropathies, can be patchy with only parts of the nerve involved. The dividing cells are usually thought to be the Schwann cells of the demyelinated fibres and their daughters,

though it has been suggested that there is a 'surround' of Schwann cell proliferation around foci of demyelination; in this surround, multiple populations of Schwann cells are recruited to proliferate, including Schwann cells of intact unmyelinated fibres (Griffin et al., 1990a; Griffin et al., 1990). The release of diffusable mediators, possibly by recruited macrophages, at these sites of demyelination could be influencing all Schwann cells in range of the stimulus, which in the case of a completely transected nerve would involve most of the distal stump. In sciatic nerve chamber experiments extracellular fluid conditioned by nerve regeneration promotes Schwann cell proliferation (Le Beau et al., 1988). Fluid harvested at one day after transection was more effective in promoting Schwann cell mitosis than that collected at later days, the activity approaching control levels by day 5 and climbing from day 7 to peak again at day 14. These peaks coincide with the occurrence of Schwann cell mitosis *in vivo*, and with the recruitment of macrophages and arrival of regenerating axons, respectively.

Macrophages recruited to foci of demyelination, either by axonal or T-cell factors, may in turn release mitogens for Schwann cells.  $\gamma$ -IFN is known to be present in the nervous system (Kiefer and Kreutzberg, 1990) and is upregulated in sciatic nerve neurons following axotomy (Olsson et al., 1989; Schmidt et al., 1990). It inhibits Schwann cell proliferation (Eccleston et al., 1989) and regulates the expression of MHC class II antigens (Armati et al., 1990).  $\gamma$ -IFN can activate macrophages and is synthesised by activated T-cells. The exact role of neuronal  $\gamma$ -IFN is not known, although there is indirect evidence that it may be involved in regulation of MHC antigen induction and possibly the recruitment of T-lymphocytes into the nerve after axotomy. After axotomy, motor neurons transiently express  $\gamma$ -IFN, and T-cells invade such areas of the spinal cord, also MHC antigens are induced in and around motor neurons (Olsson et al., 1989; Maehlen et al., 1989).

$\gamma$ -IFN expression by sensory neurons may be important in the prevention or outcome of viral infection in sensory ganglia (Eneroth et al., 1992). It is tempting to speculate that  $\gamma$ -IFN may also play a role in the recruitment as well as the activation of macrophages in axotomized nerve, either directly or via T-lymphocyte recruitment.

Mast cell products may be important after nerve injury, and in certain types of neuropathy where these products may be elevated and mast cell numbers are increased. Mast cells differentiate from resident precursors under the influence of IL-3 and NGF, and interact closely with axons (Matsuda et al., 1991; Theoharides, 1990; Blennerhassett et al., 1991), though their role in regeneration is not known. Activated mast cells secrete a number of biologically active factors, including leukotrienes, histamine, heparin, eosinophil chemotactic factor, and serotonin, and are important in

the acute inflammatory response. Some of these mediators may play a role in peripheral nerve (Powell et al., 1980b). Repeated injuries can lead to progressively more severe reactions, as mast cells proliferate after each trauma.

In addition to factors produced by recruited or resident non-neuronal cells, the axons themselves are now known to upregulate the production of several neuropeptides, including CGRP, VIP, as well as galanin (Villar et al., 1989; Hokfelt et al., 1987); these neuropeptides are transported to the injury site and remain concentrated at the leading edge of regenerating axons, where they may be released. These peptides are therefore in a position to influence the behaviour of non-neuronal cells following axotomy and during regeneration.

Following axotomy, Schwann cells start to proliferate during the first day following injury, and within the distal stump this peaks at day 3 in the rodent and day 4 in the cat, as assessed by  $^3\text{H}$ -thymidine uptake. The timing of Schwann cell proliferation in the adult mouse has been reviewed previously (Brown and Asbury, 1981). At the peak proliferative rate, thymidine incorporation is some 40-fold above normal. The rate of proliferation falls to normal by day 11, and is already falling rapidly from day 5. This peak of Schwann cell division does not correlate with myelin breakdown which proceeds in a proximal-distal gradient. Schwann cell proliferation appears to occur simultaneously in all fibres and at several points measured along the length of the distal stump. There is a second peak of Schwann cell proliferation which coincides with regrowth (Pellegrino et al., 1986), closely paralleling re-establishment of axonal contact, and therefore does show a proximal-distal gradient. Concluding from this, one can say that after transection, when reinnervation is prevented, there is a monophasic proliferation by Schwann cells, whereas a biphasic response is seen if regeneration is permitted. The second wave of Schwann cell proliferation is paralleled by a much decreased proliferation of fibroblasts, which overgrow and populate the denervated distal stump if reinnervation is prevented (Haftek and Thomas, 1968; Miyamoto et al., 1986). Scar formation and fibroblast overgrowth in front of regenerating axons prevents reinnervation, so making regulation of fibroblast proliferation as important as that of Schwann cells if adequate recovery after injury is to occur.

In many respects, regeneration may be recapitulating peripheral nerve development. During development, Schwann cells proliferate along axons while they are growing into nerves. Axon numbers are adjusted by cell death (Reier and Hughes, 1972), and Schwann cell proliferation is curtailed (Peters and Muir, 1959) allowing differentiation into the myelin or non-myelin forming phenotypes. Myelin forming cells differentiate first; these Schwann cells attain a 1:1 relationship with large axons,

and produce the myelin sheath. Several smaller axons are ensheathed within the cytoplasm of non-myelin forming Schwann cells. Distances between myelin internodes increase with maturity, and redundant Schwann cells degenerate (Berthold, 1973), leading to the required ratio of Schwann cells to axons. This reduction in Schwann cell number may be due to apoptosis, but this is not known. Schwann cells undergo a phase of active proliferation *in vivo*, which is followed by a growth inhibition, a necessary step prior to differentiation (Morgan et al., 1991).

## SIGNAL TRANSDUCTION

External factors are able to regulate cell division, differentiation, and other responses from the cell surface by activating intracellular signalling pathways which can integrate a variety of extracellular stimuli. Signalling pathways within cells are formed by chains of intercommunicating proteins which integrate signals from upstream activators and pass them on to downstream effectors. This process is known as signal transduction, and allows signals to be amplified, dampened down, or processed, before being passed on to downstream targets. Because mammalian gene expression is largely regulated at the level of transcription, it is the activation of transcription factors that converts the stimulus into a new genetic program. Many neurotransmitters interact with membrane receptors to induce conformational changes that generate intracellular second messengers such as, cAMP,  $\text{Ca}^{++}$ , and diacylglycerol (DAG), via activation of G proteins. These, in turn, act via a group of serine/threonine kinases; cAMP dependent protein kinase (PKA), calcium-calmodulin kinase (CaM), and protein kinase C (PKC), respectively, which modulate cell responses by phosphorylating target proteins on serine or threonine residues. Growth factors, conversely, act via receptors that have intrinsic tyrosine kinase activity and do not require intermediary G proteins or second messengers to stimulate kinase activity. Receptor tyrosine kinases are autophosphorylated on ligand interaction and transmit mitogenic signals through the phosphorylation of key growth regulatory targets on tyrosine residues. In this manner, phosphorylation on tyrosine residues might activate the mitogenic potential of target proteins either by enhancing their enzymatic activity, or by altering their interactions with other cellular proteins.

Non-receptor tyrosine kinases include the protein product pp60<sup>c-src</sup> of the protooncogene *c-src*. These factors play a role in proliferation, and in chamber experiments increased expression of pp60<sup>c-src</sup> occurs in Schwann cells and neurons seven days after axotomy and peaking at thirty-five days, coinciding with the period of neurite outgrowth and myelination *in vivo*. In normal adult peripheral nerve there are generally undetectable levels of expression.

A number of proteins phosphorylated by tyrosine kinases have been identified. The level of tyrosine phosphorylation of some enzymes thought to play a role in signal transduction, such as phospholipase C, correlates well with their increased activity upon growth factor stimulation. Recently, there has been increased interest in the protein tyrosine phosphatases which may constitutively reverse the action of tyrosine kinases and probably have other receptor mediated functions. Downregulation of growth factor receptors occurs on prolonged exposure to agonist or by stimulation of other growth factor receptors, a process called transmodulation. EGF receptor downregulation occurs via PKC, with resultant serine/threonine phosphorylation of the receptor and decreased EGF binding; this process can be reversed by cAMPi elevation, which stimulates phosphatases that restore normal binding (Winston et al., 1991).

### MOLECULAR SIGNALLING BY GROWTH FACTORS

The role of growth factors, receptors, and tyrosine kinases, in the control of mammalian cell proliferation has been well established, as has the potential for viral subversion and oncogenic disturbance in each of these components. The mechanisms of signal transduction, amplification, and the biochemical pathways stimulated by the mitogenic growth factors and oncogenic tyrosine kinases, are being clarified. Several lines of evidence suggest that direct or indirect activation of cytosolic threonine/serine protein kinases by tyrosine kinase activity, may provide a crucial downstream function following membrane-mediated molecular signalling events. Functional links have been shown for activation of phospholipase C $\gamma$  (PLC $\gamma$ ) and thence PKC by growth factor receptor tyrosine kinases. A number of cytosolic serine/threonine kinases are directly activated by tyrosine phosphorylation. Mitogen-activated or microtubule-associated kinase (MAP kinase), a 42kD tyrosine kinase substrate which itself exhibits serine/threonine kinase activity, is phosphorylated in mammalian cells stimulated with EGF, PDGF, IGF-II or phorbol myristate acetate (PMA) (Rossomando et al., 1989). Cloning reveals that MAP kinase is identical to ERK-1 (extracellular signal-regulated kinase) (Boulton et al., 1990). Functioning as a key intermediary in the cascade of growth factor-mediated protein phosphorylation, the MAP kinase is only active when phosphorylated on both tyrosine and threonine residues (Anderson et al., 1990). In CCL39 fibroblasts, MAP kinase is synergistically phosphorylated by FGF and thrombin, which are also synergistic mitogens. Interestingly, the action of thrombin, but not FGF, is mediated via a pertussis toxin sensitive G protein, suggesting that MAP kinase may lie at a convergence point downstream of at least two distinct signalling pathways. Activated MAP kinase in turn phosphorylates and activates the 'growth associated' (40s ribosomal protein) S6 kinases (Sturgill et al., 1988), revealing a pathway of sequential protein

phosphorylation extending from the plasma membrane to the principal phosphoprotein of the eukaryotic ribosome.

The *ras* and *raf-1* proto-oncogenes are indispensable elements in the transduction of growth and differentiation signals initiated by receptor and non-receptor tyrosine kinases. The normal products of the *ras* gene are members of a large family of proteins known to bind guanine nucleotides. Like the structurally related G proteins, the Ras proteins function as a binary switch. In the inactive state, Ras binds GDP and is located at the inner cell membrane; upon activation it binds GTP and releases growth stimulating signals into the cell. Normally, this process is short-lived and terminated by the proteins intrinsic GTPase activity, which hydrolyses bound GTP back to GDP and restores the inactive state. Oncogenic Ras proteins have greatly reduced GTPase activity, allowing prolonged activation and growth stimulation. It is known that GTPase-activating proteins (GAPs) associate with Ras proteins and stimulate their intrinsic GTPase activity, thereby shutting them off (Trahey and McCormick, 1987). GAP proteins are defective in patients with type-1 Neurofibromatosis (NF-1) (Viskochil et al., 1990; Wallace et al., 1990; Basu et al., 1992), and consequently these patients have an increased risk of developing malignancy through defects in shutting off Ras. Interestingly, accelerated proliferation of Schwann cells occurs even in the absence of malignant change, and may reflect a simple imbalance in tonic Ras activity. The element regulated directly by yeast Ras proteins is adenylate cyclase, but in higher eukaryotes their direct target enzymes are only now being elucidated. Several cytoplasmic oncogenic serine/threonine kinases are rapidly and possibly directly regulated by Ras, including c-Raf-1 (Robbins et al., 1992), MEK, MAP kinase (Wood et al., 1992), and Rsk (Wood et al., 1992), stimulated by several mitogenic growth factors. Evidence suggests that these serine/threonine kinases are arranged in a linear cascade; Rsk is phosphorylated and activated by p42/p44 MAP kinases (Sturgill et al., 1988), which are in turn activated by MEK (Gomez and Cohen, 1992). c-Raf-1 phosphorylates and activates MEK *in vitro*, and a drosophila MEK-like kinase can act as a suppressor of a drosophila *raf-1* loss of function mutation (Tsuda et al., 1993). Of these serine/threonine kinases, c-raf-1 is situated most proximal to the signal generated by Ras. In NIH 3T3 cells, c-Raf-1 is essential in transducing proliferative signals initiated by receptor tyrosine kinases and transmitted through c-Ras. Antisense *c-raf-1* RNA, or dominant negative *c-raf-1* mutations, can block the DNA synthesis and growth stimulated by serum, TPA, or *ras* oncogenes (Kolch et al., 1991). Expression of *v-raf* can overcome the blockade to proliferation present in *ras* revertant lines (Rapp et al., 1988), or induced by microinjection of inhibitory anti-Ras antibodies. In most cells, growth factor induced Raf-1 activation is accompanied by multisite serine/threonine phosphorylation of Raf-1 through a PKC independent pathway and



can be deactivated *in vitro* by the serine/threonine-specific phosphatase-1 (Kovacina et al., 1990). Co-ordinated activity between protein kinases and phosphatases are probably a feature of most signalling pathways (Review by Hunter, 1995). Ras and Raf-1 may interact directly: Raf is, in fact, a direct effector of Ras (Zhang et al., 1993; Warne et al., 1993). The GTP bound, active form, of Ras has the highest affinity for Raf-1. An intact Ras effector region is required for interaction with Raf-1, and may exhibit competitive binding between Raf-1 and GAP polypeptides (which stimulate Ras GTPase activity). Raf may in turn phosphorylate and activate MAP kinase kinase, the homologue of mammalian MEK kinase.(Hughes et al., 1993). Raf-1 may, however, be activated by tyrosine phosphorylation as a direct result of the intrinsic kinase activity of the PDGF receptor (Morrison et al., 1989). Once activated, the Raf-1 kinase phosphorylates a factor/factors that then stimulate transcription of *c-fos* and the  $\beta$ -actin gene (Blackshear et al., 1990). The activity of Raf-1 is thus linked to induction of early response genes.

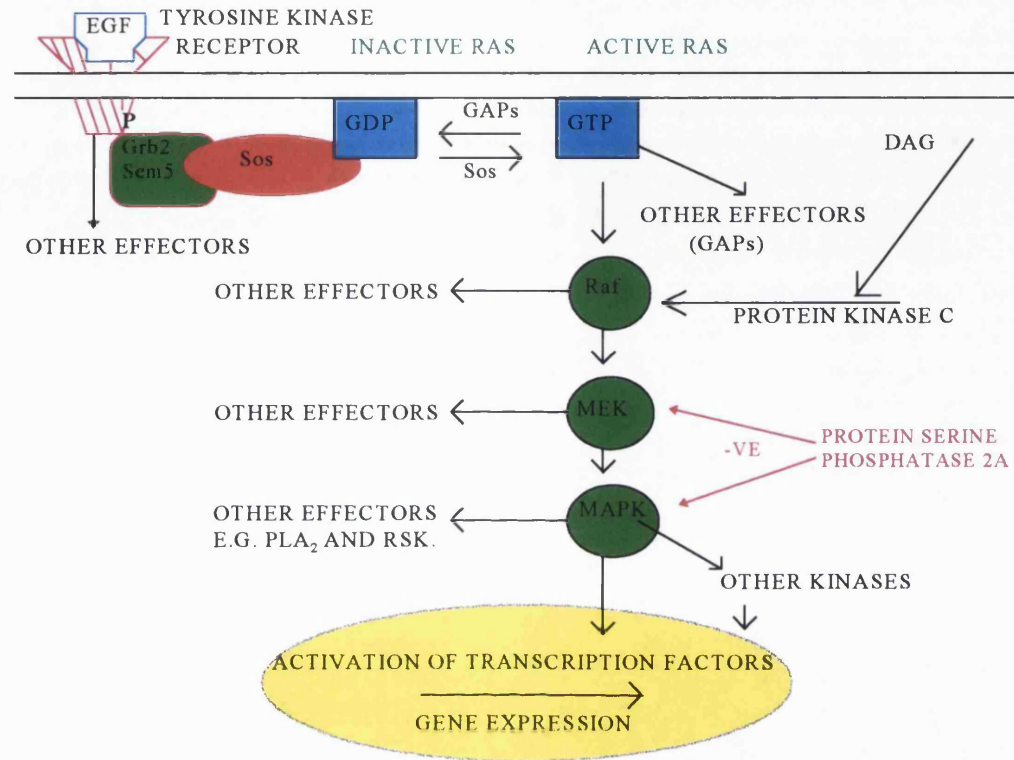


Fig. 1. The Ras signalling pathway.

Binding of growth factor, in this case EGF, leads to autophosphorylation of the receptor on tyrosine residues. Controllers of RAS exchange factors, such as Grb2 / Sem5, recruit exchange factors, like Sos, which act as guanine-nucleotide releasing factors. This converts Ras-GDP to active Ras-GTP. Several downstream targets are shown. Activated Raf phosphorylates MAP kinase kinase (or MEK) which via ERK1/2 (MAP kinase) can produce signals which enter the nucleus and phosphorylate transcription factors that regulate gene expression.

## CELL CYCLE REGULATION

The regulation of the cell cycle maintains the balance between cell proliferation, differentiation, survival and death. Positive regulation is provided by the cyclins and cyclin-dependent kinases (CDKs), and negative regulation by tumour suppressor proteins, notably those encoded by the tumour suppressor genes *p53* and *RBA*, and the SDK inhibitors. In the active state, cyclin/SDK complexes enable transition of the cell through distinct phases of the cell cycle: the checkpoint phases (G1 and G2), DNA synthesis (S) and mitosis (M).

A major control point in the mammalian cell cycle is the G1-S transition. It is during G1 that serum factors such as PDGF, EGF and IGF-1 act to stimulate transition to S phase (Gould and Nurse, 1989), and after which exogenous growth factors are no longer required to complete the cell division cycle. In the animal cell cycle this restriction point in mid-G1 phase, at which cells commit themselves to the mitotic cycle rather than differentiation or quiescence, is analogous to Start in yeast. One protein kinase stands out as the critical sentinel of the S phase, p34<sup>cdc2</sup>. This is the founding member of a family of protein serine/threonine kinases, CDKs, whose activity depends on their association with specific regulatory subunits known as cyclins. Studies using immunodepletion (Pardee, 1989), and antisense oligodeoxynucleotide strategies (Blow and Nurse, 1990), confirm that p34<sup>cdc2</sup> plays a pivotal role in the initiation of DNA replication in higher eukaryotes.

The cyclins are an ever growing family of proteins defined by an 150 amino acid region of structural homology, called the cyclin box, responsible for binding to the CDK subunit. The G1 or START cyclins act before S phase, whereas the G2 or mitotic cyclins act after S phase. The B cyclins, the first described cyclins isolated from marine invertebrates, regulate transition through mitosis. Cyclin A facilitates progression through S phase in mammalian cells. The G1 cyclins operate in the earliest phase of the cycle, including the G1/S transition. The G1 cyclins consist of at least three D cyclins and cyclin E. These G1 cyclins and their CDK partners are rate-limiting for progression through G1 into S phase (Dulic et al., 1992; Reznitsky et al., 1994). The D cyclins are uniquely modulated by exogenous growth factors (Matsushime et al., 1991). The D cyclins are induced by CSF-1 and downregulated by TGF- $\beta$ .

Different cyclins that bind the same CDK alter the specificity of the complex, and in fact CDKs in non-proliferating cells may serine/threonine phosphorylate targets unconnected with the cell cycle. The D type cyclins are short lived proteins, like the yeast CLNs, whose mRNA and protein synthesis are serum-dependent (Matsushime

et al., 1991). The D type cyclins associate with CDK2, CDK5 and CDK6, but primarily CDK4 (Matsushime et al., 1992). The cyclin D-CDK4 complexes form at the restriction point, and dissociate when the cell enters S phase (Baldin et al., 1993). Cyclins D1 and D2 are protooncogenes overexpressed in certain tumours; the PRAD1 and *bcl1* protooncogenes are both cyclin D1 (Motokura et al., 1991). The cyclin D-CDK4 complex may be rate limiting in G1; overexpression of cyclin D1 shortens the cell cycle and the G1 phase. The cyclin D-CDK4 complex may neutralise Rb-mediated growth inhibition (Kato et al., 1993), by phosphorylating the Rb protein. D cyclins may also bind directly to a critical pocket region of Rb, and inactivate it.

Cyclin E, whose expression is cell cycle-dependent, may be involved in initiation of DNA replication once cells have passed the restriction point (Reznitsky et al., 1994). Cyclin E primarily activates CDK2 and is raised in tumour cell lines. TGF $\beta$  inhibition of growth may be by inducing G1 arrest by production of an inhibitor which binds and inactivates the cyclin E-CDK2 complex (Koff et al., 1993). Other inhibitors include Cip1/pic1 (21kDa) which bind and inhibit numerous cyclin-CDK complexes *in vitro*, and are found associated with cyclin-CDK complexes in normal but not transformed cells. The Cip1/pic protein is upregulated in senescent cells and is induced by wild-type but not mutant p53 (El-Deiry et al., 1993), suggesting a mechanism whereby DNA damage could inhibit the cell cycle. Once cells begin to synthesise DNA, cyclin E is rapidly degraded and replaced by cyclin A which associates with CDK2; the resulting complex may share many substrates. The cyclin A-CDK2 complex is needed for continuing DNA replication; although the exact role is unknown the complex can interact with replication machinery including PCNA and helicases. Both cyclin E and A complexes with CDK interact with E2F and DP1 and could regulate their transcriptional activity. The cyclin B-CDK1 complex is the primary mitotic kinase, and is regulated by wee1/mik1 mediated phosphorylation of Y15 to prevent its premature activation. Once the cell is ready to enter the cell cycle then stockpiled cyclin B-CDK1 complexes are rapidly activated and translocated to the nucleus. Destruction of cyclin B and inactivation of the CDK complex are crucial for regulation of the metaphase-anaphase transition. Mutant indestructible cyclin B will block a cell in metaphase (Glotzer et al., 1991).

The retinoblastoma (Rb) susceptibility protein is underphosphorylated in G0, and in early G1 becomes highly phosphorylated on serine and threonine residues at the G1-S phase boundary and remains so during S and G2 (Furukawa et al., 1990; Ludlow et al., 1990). This has led to the suggestion that phosphorylation and hence inactivation of this protein may be an obligatory step in the transition from G1 to S phase. Interestingly, Rb phosphorylation can be inhibited by TGF- $\beta$  (Mihara et al., 1989), or other treatments leading to cytodifferentiation (Laiho et al., 1990). Quiescent, but not

senescent, fibroblasts are capable of phosphorylating Rb protein when stimulated with serum. Active p34<sup>cdc2</sup> kinase complexes are known to be capable of phosphorylating the Rb protein at multiple sites *in vitro* (Chen et al., 1989). The identity of the Rb kinase remains unresolved, but belongs to the CDK class, because coexpressing cyclins such as cyclin A and E can override Rb induced cell cycle arrest correlating with increased Rb phosphorylation (Zhu et al., 1993; Hinds et al., 1992). These cyclins can not override p107 induced cell cycle arrest. The most convincing *in vivo* evidence for p34<sup>cdc2</sup> kinase being linked with Rb comes from studies in human T-cells, in which p34 mRNA and protein were induced on mitogenic activation. In this study, p34 induction, Rb phosphorylation, and transition to S phase were blocked by incorporation of antisense oligonucleotides. The underphosphorylated Rb protein may interact negatively with members of the cell cycle specific transcription factor family, such as DRTF1/E2F or DP1, that are important for cell-cycle progression (Chellappan et al., 1991). Two other members of the Rb family have been described, p107 and p130; these so called "pocket proteins" may interact directly with cyclins and when upregulated will prevent cell cycle progression (Zhu et al., 1993). They may also interact with DRTF/E2F, but p130 complexes appear predominantly during G<sub>0</sub> and G<sub>1</sub>, and the p107 complex during G<sub>1</sub> and S. E2F mRNA increases towards the end of G<sub>1</sub>; they can overcome Rb-dependent cell cycle arrest (Zhu et al., 1993) and if artificially increased in quiescent fibroblasts causes them to progress into S phase (Johnson et al., 1993).

Speculation continues on the role of other G<sub>1</sub>-S phase specific cyclins in cell cycle control. A large number of studies have suggested the existence of a so called S-phase promoting factor (SPF) in somatic cells analogous to MPF of oocytes. It has been postulated that this SPF enzymatic activity may consist of p34 in complex with a G<sub>1</sub>-specific cyclin (Taya et al., 1989) or possibly cyclin A itself (Murray and Kirschner, 1989a). Recent studies suggest that not only is p34 kinase necessary, but that addition of cyclin A to a G<sub>1</sub> cell extract is sufficient to initiate DNA replication (Murray and Kirschner, 1989b). In rat PC12 cells, tyrosine hydroxylase is phosphorylated at multiple sites by proline directed protein kinase (PDPK) (Vulliet et al., 1989). Western blotting and use of specific antisera reveal the active form of this kinase consists of the p34<sup>cdc2</sup> catalytic subunit and p58<sup>cyclin A</sup> (Hall et al., 1990). A model predicts that the association of p34 and p58 and/or activation of the resulting heterodimer is under growth factor control. The cyclin A subunit is tyrosine phosphorylated in PDPK, suggesting differences in regulation compared to H1K (another p34 complex with cyclin B). The latter seems to be active later in the cycle, during G<sub>2</sub> just prior to M phase, whereas PDPK seems to peak during S phase. Both Rb and p53 tumour suppressor proteins are putative substrates for p34, and known to be phosphorylated during interphase. Complexes of cyclin A-cdc2 and cyclin B-cdc2

are required for the G2-M transition; activation of cyclin B-cdc2 triggers entry into M phase. and inactivation of both complexes is required for completion of mitosis (Review by King et al., 1994). Unlike regulation of protein phosphorylation by classic serine/threonine protein kinases, such as PKA and PKC, governed by minute to minute changes in steady state levels of respective second messengers, the p34<sup>cdc2</sup> containing protein kinase complexes, proposed to control both S phase (p34/p58 PDPK) and M phase (p34/p62<sup>cyclin B</sup>), are designed to remain active for more prolonged times. The activity of the p34 catalytic subunit is regulated by dephosphorylation and phosphorylation, and appears relatively independent of ionic fluctuations and second messenger molecules. Once triggered, the p34 containing PK complex may remain active for hours, and its phosphotransferase activity remain until cyclins are removed by proteolysis. This makes sense for their functional roles; control of onset and maintenance of S and M phase. Neither of these is considered reversible in somatic cell cycles, and failure to complete would be catastrophic. P34<sup>cdc2</sup>, is also phosphorylated on tyrosine residues, however in this case it is profoundly inactive until dephosphorylated for activation at the onset of M phase (Jamal and Ziff, 1990).

## NEOPLASIA, ONCOGENES AND GROWTH FACTORS

In 1911 Peyton Rous reported the transmission of sarcomas in chickens by injections of cell-free filterable extracts of tumours into healthy birds (Rous, 1911). This agent was subsequently shown to be an RNA virus, the retrovirus. The retroviruses capable of causing malignant tumours contain a gene that is not essential for viral replication, and may have been picked up from a host cell; these viral oncogenes (v-onc genes) have similar counterparts in normal cells (reviewed in Bishop, 1985), which are called protooncogenes. An oncogene may be defined as a gene whose aberrant activity is essential to the neoplastic state. Retroviruses may pick up and damage a normal protooncogene and hence become transforming viruses. In addition, mutagens or other mechanisms, may activate these genes which are then known as cellular oncogenes or c-oncs. Oncogenes are often classified by cellular location as either cytoplasmic or nuclear. A major class of cytoplasmic oncogenes are receptor tyrosine kinases. This group includes the products of the *v-src*, *v-yes*, *v-fps/fes*, *v-erb-B*, *v-fms* and *v-ros*. Some of these are now known to be growth factor receptors, the oncogene *v-erb-B* is homologous to the EGF receptor. The internal tyrosine kinase domain, is able to tyrosine phosphorylate substrate proteins and the receptor itself. The *v-fms* gene product is homologous to the receptor for CSF-1. Aberrant activation of these growth factor receptors is one mechanism for uncontrolled growth. The *v-sis* oncogene encodes a protein p28<sup>v-sis</sup>, that is virtually identical to the B chain of PDGF. Growth factors or their receptors may be protooncogenes, as indeed may

GTP-binding proteins, intracellular second messengers, protein kinases or transcription factors. Cells transformed by certain cytoplasmic oncogenes (*ras*, *src*, *mos*, *fes*, *abl*, *erb-B*, *yes* and *mil/raf*) which do not themselves code for growth factors, release growth-stimulating factors into culture medium. This can lead to autocrine growth if appropriate receptors are expressed. The *ras* oncogene family consists of at least 3 genes; Harvey(*Ha*), Kirsten(*Ki*) and *N-ras*. The protein products are 21kD peptides called p21. Ras proteins specifically bind GTP and GDP and resemble receptor-linked G proteins. A characteristic feature of mutant ras proteins is their reduced GTPase activity, leading to impaired inactivation and potential unregulated signalling. Several nuclear oncogenes show similarity to one another (*myc*, *N-myc*, *myb*). A common functional feature is their ability to immortalise cultured cells. Other members of this class are p53, *L-myc*, *ski*, *ets* and *fos*. Activation of protooncogenes is varied: *ras* genes may be activated by point mutations in amino acid positions 12 or 61; *Ha-ras* and *myc* may become oncogenic by overexpression, due to translocation away from regulatory elements. Loss of tumour-suppressors such as p53 or Rb may lead to excessive proliferation and reduction in apoptosis. Chromosomal rearrangement, as occurs for *abl* in chronic myeloid leukaemia (CML), and *myc* in Burkitts lymphoma, lead to activation by translocation of the proto-oncogene. In most cells it seems that two different oncogenes are needed for tumorigenesis. Combinations of nuclear and cytoplasmic genes are most common, suggesting that nuclear genes are important for immortalising the cell, and cytoplasmic ones for promotion of growth. Tumour initiation or progression is likely to arise from abnormalities in both tumour promoters and or tumour suppressors.

Excessive, aberrant or temporally inappropriate expression of various cyclins during the cell cycle could be involved in tumour pathogenesis. Gene amplification at the D1 locus is seen in up to 15% of breast cancers, 33% of oesophageal cancers and some non-small-cell lung cancers (Motokura et al., 1993). The cyclin D2 gene is located in a region often disturbed in germ-cell tumours, and abnormalities in cyclin D3 are found in many lymphomas and acute lymphocytic leukaemias. The vast majority of breast cancers demonstrate abnormalities in cyclin E (Keyomarsi et al., 1993). Alterations in cyclin E correlate with tumour aggressiveness and invasiveness, and may help discriminate malignancy from non-neoplastic states. Cyclin E abnormalities may be a general mechanism for tumour initiation and promotion.

Many CDK inhibitors linked to p53, exogenous growth inhibitors, and Rb are known. The p53 transcription factor, is lost or mutated in a large number of malignancies, including the familial Li-Fraumeni syndrome (Malkin et al., 1990) and the breast-ovarian cancer syndrome (Jolly et al., 1994). The normal role of p53 probably includes the induction of cell cycle-arrest in the presence of DNA damage, and

induction of apoptosis if subsequent repair is impossible (Kuerbitz et al., 1992). Part of this activity may be mediated via production of growth-arrest DNA damage-inducible (GADD) gene products (Kastan et al., 1992), which prolong G1 phase, and allow DNA repair. P53 also increases transcription of the genes for a CDK inhibitor known as p21WAF-1/CIP-1 (El-Deiry et al., 1993; Harper et al., 1993), which may prevent cyclin-CDK complexes from phosphorylating and inactivating Rb (Xiong et al., 1993; Dulic et al., 1994). The loss of p53 expression in fibroblasts is accompanied by a loss of p21 from G1 cyclin/CDK complexes (Dulic et al., 1993). Transfected p21, can inhibit the growth of numerous tumour cell cultures, and may be a key mediator of p53-induced cycle arrest, and link p53 and Rb tumour-suppressor action. It is not known what controls the outcome of p53 action in terms of growth-arrest or apoptosis. Proliferating cell nuclear antigen (PCNA), which interacts with both p21 and GADD has been suggested as a possible switch (Waga et al., 1994; Smith et al., 1994). Other CDK inhibitors: p27<sup>KIP-1</sup> (kinase inhibitory protein), P28<sup>ICK</sup> (inhibitor of cyclin-dependent kinase), are induced by different factors, namely TGF $\beta$  or cell-cell contact (Polyak et al., 1994). Another CDK inhibitor, p16<sup>INK4</sup> specifically inactivates cyclin D/CDK4 complexes (Serrano et al., 1993). The multiple tumour suppressor-1 (*MTS1*) gene, is structurally abnormal in many tumour cell-lines. The *MTS2* gene is adjacent to *MTS1* and encodes p15<sup>INK4B</sup>, which binds CDK4 and CDK6, in favor of D cyclins. Both p15 and p27, CDK inhibitors are increased by TGF $\beta$  and mediate TGF $\beta$ -induced cell cycle-arrest (Hannon et al., 1994).

The CDK inhibitors, suppress the cell cycle, in response to cell-cell contact, oxidative stress, and TGF $\beta$ , by preventing the phosphorylation, and thereby inactivation of Rb by cyclin/CDK complexes.



## NEUROFIBROMATOSIS

Von Recklinghausen neurofibromatosis, also known as Neurofibromatosis type-1 (NF-1), is an autosomal dominant inherited condition due to a mutation of a gene on chromosome 17 (Barker et al., 1987), and is characterised by the development of peripheral nerve sheath tumours, and some tumours in the CNS (Riccardi, 1981). The prevalence of the disease is 30-40 per 100,000, and occurs in one per 2500-3300 live births (Crowe et al., 1956). There is sequence homology between the NF-1 gene and the negative regulators of the ras cAMP pathway, *ira-1* and *ira-2* (Buchberg et al., 1990). The NF-1 gene product is also closely related to GTPase-activating protein (GAP) (Xu et al., 1990b), and can stimulate ras-GTPase and complement yeast *ira* mutants (Xu et al., 1990a; Ballester et al., 1990; Martin et al., 1990). The GAP-related domain of NF-1 can stimulate p21<sup>ras</sup> GTPase activity. Growth factors may activate p21<sup>ras</sup> by stimulating conversion of the GDP to the active GTP bound form. Activated p21<sup>ras</sup> may then mediate the activation of the MAP kinase, erk2, by growth factors such as PDGF and insulin, but not by phorbol esters (de Vries Smits et al., 1992). The NF-1 gene product is not exclusively expressed in cells of neural crest origin, such as Schwann cells, but is found mainly in nervous tissue (Daston et al., 1992).

Neurofibromatosis type-2 (NF-2) is characterised by autosomal dominant inheritance, the development of bilateral acoustic neuromas usually before the age of 21 years, and an increased susceptibility to gliomas and meningiomas. Many cases of acoustic neuroma contain deletions of part of chromosome 22 (Rouleau et al., 1987), which may contain a tumour suppressor gene (Comelli et al., 1992; Seizinger et al., 1987; Bijlsma et al., 1992) with homology to a cytoskeletal element.

Both types of tumour contain growth factor activity; elevated levels of FGF protein and mRNA have been described in acoustic neuromas (Murphy et al., 1989), and have also been isolated from neurofibromas in NF-1 (Ratner et al., 1990a; Ratner et al., 1990b). Glial growth factor (GGF) has also been isolated from sporadic acoustic neuromas not associated with NF-1 or NF-2 (Brockes et al., 1986). Other mitogenic activity has been isolated from Schwannoma cell lines, e.g. schwannoma-derived growth factor (Kimura et al., 1990). The *HER-2 / neu* oncogene encodes a receptor tyrosine kinase, p185<sup>erbB2</sup>, originally identified as a dominant transforming gene in neurogliomas and Schwannomas, induced by ethylnitrosurea in rat embryos. It is structurally related to the EGF receptor (Ulrich et al., 1984) and to erbB3, but does not bind EGF, although EGF can induce its phosphorylation (Stern and Kamps, 1988). Ethylnitrosurea-induced Schwannomas all carry a mutation in the *erbB2 (neu)* gene (Nikitin et al., 1991). Neu mRNA and immunoreactive p185<sup>erbB2</sup> are expressed

in rat sciatic nerve between postnatal days 1 and 7, but decrease in adulthood. After axotomy, however, *neu* mRNA and p185*erbB2* protein progressively increase. Schwann cells in culture express low levels of *neu*, but can be greatly increased on treatment with forskolin (Cohen et al., 1992), which also potentiates the mitogenic response to GGF (Davis and Stroobant, 1990). Several recombinant GGFs, and several putative p185*erbB2* receptor ligands, are derived from alternatively spliced mRNAs of the same GGF gene (Marchionni et al., 1993), and are expressed primarily in primary sensory neurons and motoneurons. These so called "neuregulins" (named after their interaction with the *neu* gene product) are all activators of the p185 *erbB2* receptor tyrosine kinase, and include heregulins (Holmes et al., 1992), *neu* differentiation factor (Wen et al., 1992), and probably acetylcholine receptor inducing activity (ARIA) (Falls et al., 1993). Thus, p185 *erbB2* is probably the receptor for GGF *in vivo*, and may play an important role in the neuronal regulation of Schwann cell proliferation and differentiation in development, and during nerve regeneration. Interestingly, the *neu* oncogene, but not the protooncogene, downregulate alpha and beta PDGF receptor (PDGFR) subtypes in transfected NIH 3T3 cells (Lehtola et al., 1991); this receptor transmodulation occurs by decreased mRNA for PDGFR.

It seems likely that in neurofibromas, particularly in NF-1, a genetic defect could lead to activation of the cAMP pathway. In other cases, transformation might involve synthesis or activation of growth factor receptors independently of cAMP. In either case, proliferation could then occur in concert with local growth factors, which are clearly present within these tumours. Many tumours of connective tissue origin, including dermatofibromas, contain PDGF beta receptor and PDGF BB peptide, but less clearly PDGF AA. Similarly, malignant gliomas in the CNS produce PDGF BB and receptor (Mauro et al., 1991), suggesting that autocrine stimulation by PDGF BB may be important, if not itself sufficient, for malignant growth (Smits et al., 1992). Interestingly, after injury increased levels of PDGF and receptor occur as part of the wound healing response. However, in this situation a glycoprotein, SPARC, is produced concurrently, which reduces PDGF receptor binding (Raines et al., 1992) and may thereby limit its activity. It remains to be shown whether SPARC plays a role in tumours.

## PLATELET DERIVED GROWTH FACTOR

PDGF, consists of a family of three homodimeric and heterodimeric combinations (AA, AB, BB) of the A and B chains of PDGF. The chains are linked by disulphide bonds; the B chain is 92% homologous with part of the transforming protein product p28<sup>*sis*</sup> of the simian sarcoma virus, which also shows 50% homology with the A chain. The *c-sis* proto-oncogene is expressed by activated human monocytes

(Martinet et al., 1986). PDGF A chain is expressed by mammalian neurons (Yeh et al., 1991) in the CNS and PNS, whereas, PDGF B chain has been demonstrated in neurons only within the CNS (Sasahara et al., 1991), although it is expressed within peripheral nerve, probably by glia. PDGF stimulates a number of physiological and morphological changes in cultured fibroblasts, including proliferation (Ross et al., 1986), enhanced phosphatidylinositol turnover (Habenicht et al., 1981), and increases in the transcription of certain genes (Bravo et al., 1987) (Greenberg and Ziff, 1984). The ability of PDGF to initiate these varied responses lies in the tyrosine kinase activity intrinsic to its receptor. Functionally distinct PDGFR subtypes are described (Gronwald et al., 1988; Matsui et al., 1989; Claesson-Welsh et al., 1988; Hart et al., 1988; Heldin et al., 1988), which bind different dimeric forms of PDGF. The alpha receptor binds all three isoforms via distinct binding sites (Heidaran et al., 1992), whereas, the beta receptor recognises the BB homodimer, and AB heterodimer (Drozdoff and Pledger, 1991). The B chain residues, arginine 27 and isoleucine 30, were identified as critical for receptor binding and activation, by site directed mutagenesis studies (Clements et al., 1991). PDGF receptor subtypes may phosphorylate unique or common target substrates (Eriksson et al., 1992b); and PDGF isomers induce unique responses (Roth et al., 1991; Kazlauskas et al., 1988; Nister et al., 1988). In BALB/c 3T3 cells, the alpha receptor activates the serum response element (SRE) only via a PKC dependent signalling mechanism, whereas, the beta receptor, which can also induce prolonged activation of *c-fos*, also acts via PKC independent signalling (Salhany et al., 1992). The binding of PDGF to its receptor results in a change in receptor conformation and dimerisation (Eriksson et al., 1992a) and induces receptor autophosphorylation (Ek and Heldin, 1982) and the phosphorylation of a number of cellular proteins on tyrosine residues (Cooper et al., 1982). Dimerisation of receptor by ligand binding is believed to be important in activation of kinase activity, autophosphorylation, and consequently signalling. Interestingly, even the monomeric form of PDGF seems able to induce receptor dimerisation (Andersson et al., 1992). Mutations abolishing the tyrosine kinase activity, eliminate the signalling capacity of the receptor (Escobedo and Williams, 1988). PDGF stimulation of inositol phospholipid hydrolysis is, in fact, dependent on phosphorylation of PLC1 on tyrosines 783 and 1254 (Kim et al., 1991). The PDGF alpha receptor may interact with a phosphatidylinositol 3-kinase (PI3-kinase) (Whitman et al., 1988) of unknown biological function, and whose interaction is prevented by mutations in the receptor at tyrosines 731 or 742 which do not prevent PDGF mitogenic signalling (Yu et al., 1991). The proteins identified that are phosphorylated on tyrosine residues following PDGF treatment, include the tyrosine kinase c-src, an 85 kd PI kinase (Whitman et al., 1987), and the serine/threonine kinase Raf-1 (Morrison et al., 1989). Many signal transduction enzymes associate with the PDGF receptor only when it is phosphorylated; two of these, GAP and PI3-

Kinase, bear *src* homology (SH) 2 domains which interact with specific phosphotyrosine containing sequence motifs in the kinase site of the receptor (Fantl et al., 1992). GAP can bind to activated PDGF receptors (Kazlauskas et al., 1990) and is rapidly tyrosine phosphorylated by PDGF. The stimulated beta receptor interacts with a signalling complex (Kaplan et al., 1990). In NIH 3T3 cells, there is an associated increase in p21ras bound to GTP which is sufficient to support proliferation (Molloy et al., 1992). PDGF-induced p21 ras activation may in turn lead to activation of ERK2 (extracellular signal-related kinase 2) (de Vries Smits et al., 1992). GAP and PI3-kinase bind to specific regions of the PDGF receptor beta subunit (Kazlauskas et al., 1992). Phosphorylation sites on the beta receptor have different binding specificities for GAP and PI3 kinase (Kashishian et al., 1992); Tyr 740 and 751 are involved in PDGF-stimulated binding of PI3-kinase, and Tyr 771 is required for efficient binding of GAP. Raf-1 which has been implicated in signal transduction mediated by membrane bound oncogene products and growth factor receptors, may be activated directly by phosphorylation on tyrosine residues by the PDGF  $\beta$  receptor (Morrison et al., 1989). However, phorbol esters which enhance the serine/threonine phosphorylation of Raf-1, also increase the kinase activity of Raf-1 (Morrison et al., 1989). It seems that for interaction with the PDGF receptor, certain *src* homology domains are required, notably SH2 (McGlade et al., 1992). The C-terminal SH2 domain of p85 is sufficient for directing the association of PI3-kinase with the PDGF beta receptor (Klippel et al., 1992). PDGF also activates PLC, thereby increasing diacylglycerol (DAG) levels (Habenicht et al., 1981) and activating PKC. It is possible that serine/threonine phosphorylation may also be important in activating Raf-1 kinase activity in response to PDGF. Raf-1 kinase activity and proliferation in response to PDGF are correlated, and treatment of serum-starved quiescent 3T3 cells with the amino-terminal truncated Raf-1 will induce DNA synthesis, suggesting a role for Raf-1 proteins in cellular proliferation.

Some of the actions of PDGF may be abolished by the use of the tyrosine-kinase inhibitors, genistein or the tyrphostins (Bryckaert et al., 1992), which prevent receptor autophosphorylation and tyrosine phosphorylation of many target proteins. Genistein inhibits PDGF and EGF mitogenesis in 10T1/2 fibroblasts and rat liver T51B cells with an ED50 of 40 $\mu$ M and 10 $\mu$ M respectively, and also inhibits IP<sub>3</sub> generation and calcium signalling in response to PDGF. Activation of PLC $\gamma$  by PDGF is not required for stimulation of DNA synthesis in some cells (Hill et al., 1990), although down-regulating PKC with TPA leads to a 50% reduction in PDGF-stimulated mouse fibroblast proliferation (Nishizawa et al., 1990). PKC seems to be required for transducing the mitogenic signals of PDGF from cytoplasm to nucleus in NIH/3T3 fibroblasts (Fields et al., 1990). Inhibition of PKC in malignant glioma cell lines abolishes PDGF induced proliferation (Pollack et al., 1990). However, in MG-63

human osteogenic sarcoma cells, PDGF can stimulate *c-myc* expression and this is not prevented by downregulating PKC (Frick et al., 1988), or by preventing elevation in  $\text{Ca}^{++}$ . Experiments using mutant PDGF receptors suggest that neither activation of tyrosine kinase activity nor PI turnover are sufficient for induction of proliferation (Escobedo and Williams, 1988). PDGF stimulated fibroblast proliferation can be inhibited by TGF- $\beta$ ; in human bone marrow fibroblasts TGF- $\beta$  inhibits PDGF BB receptor autophosphorylation and PI metabolism, through induction of a serine/threonine phosphatase (Fontenay et al., 1992). Okadaic acid, which inhibits phosphatases restores PDGF mitogenic activity and autophosphorylation. PDGF beta receptors on cultured human fibroblasts, are downregulated after 48 hour incubations with either PDGF BB, IL-1, or TNF- $\alpha$ . This is associated with a decreased mitogenic response to subsequent PDGF BB (Tingstrom et al., 1992) and suggests a modulatory role for macrophage derived cytokines in regulating fibroblast proliferation. PDGF BB can also transiently upregulate the expression of both alpha and beta PDGF receptors in human fibroblasts (Eriksson et al., 1991).

### SCHWANN CELL MITOGENS

The actual signals regulating Schwann cell proliferation *in vivo* are not known. A chemical signal was first proposed by Abercrombie and Johnson (1946). Since then, several mitogens have been described which are active *in vitro*. Salzer and Bunge (1980) suggested that the mitotic signal was a product of myelin breakdown, yet Schwann cells proliferate in unmyelinated nerves (Abercrombie et al., 1959; Clemence et al., 1989), and in the absence of myelin breakdown (Lunn et al., 1989). Other mitogens active *in vitro* are serum, PDGF, GGF (Brockes et al., 1980), basic-FGF (Ratner et al., 1988b), TGF- $\beta$  (Ridley et al., 1989), axolemmal fragments (Ratner et al., 1988a), and the matrix molecules, laminin and fibronectin (Baron-VanEvercooren et al., 1982; McGarvey et al., 1984). Agents that increase cAMPi, such as forskolin and cholera toxin, act as permissive agents for Schwann cell proliferation (Raff et al., 1978b) and synergise with GGF and TGF- $\beta$  in serum (Ridley et al., 1989). None of the growth factors, PDGF, TGF- $\beta$  or GGF, or the agents which elevate cAMPi, are mitogenic alone when cells are cultured in defined medium (Stewart et al., 1991). Elevating cAMPi may induce the synthesis of receptors for growth factors such as PDGF (Weinmaster and Lemke, 1990; Davis and Stroobant, 1990a), and GGF (Cohen et al., 1992), whose lack of mitogenic action alone may at least in part be explained by an absence of these receptors. At least two distinct signals are required for Schwann cell proliferation, firstly a growth factor such as PDGF or FGF, and secondly an absolute requirement for an agent able to elevate cAMPi (Stewart et al., 1991). Acidic-FGF (Eckenstein et al., 1991) and PDGF (Hardy et al., 1992) are present in peripheral nerve, and axons contain mRNA

for GGF's (Marchionni et al., 1993). Both FGF and PDGF are known to be released by activated macrophages at other sites of injury. Acidic FGF levels actually decline in the distal stump after axotomy (Eckenstein et al., 1991), and it remains to be shown that FGF or PDGF are available to non-neuronal cells after axotomy and what role they play *in vivo*. Supernatants from activated cultured monocytes are mitogenic for Schwann cells *in vitro* (Lisak et al., 1985), and in the absence of or exclusion of macrophage recruitment neither Schwann cell proliferation (Beuche and Friede, 1984; Brown et al., 1992), nor prolonged NGF expression occur (Brown et al., 1991). The time course of both these responses *in vivo* coincides with the recruitment of circulating macrophages / monocytes into the nerve (Clemence et al., 1989). The identity of the macrophage activity for Schwann cells is, however, still not known. Macrophages are known to synthesise numerous cytokines, including IL-1 (Auron et al., 1984) and the biologically similar TNF (Kohase et al., 1987), which have wide ranging effects on numerous different cell types, including astrocytes and fibroblasts (Gadient et al., 1990). IL-1 may be a factor regulating NGF production *in vivo*, and is able to influence NGF production by cultured endoneurial fibroblasts (Lindholm et al., 1988). However, this activity could not be demonstrated for Schwann cells *in vitro* (Matsuoka et al., 1991a). IL-1 receptor antagonists can impede peripheral nerve regeneration if introduced to guidance chambers (Guenard et al., 1991). In addition, others have failed to show any mitogenic activity for IL-1 or TNF on Schwann cells (Stewart et al., 1991), though antibodies to IL-1 can inhibit the mitogenic activity of cytokine-containing supernatants derived from cultured activated monocytes (Lisak and Bealmear, 1991) *in vitro*. VIP, secretin and noradrenaline, have previously been shown to elevate Schwann cell cAMPi (Yasuda et al., 1988b) but, to date, no endogenously occurring agents have been shown to elevate Schwann cell cAMPi and thereby induce proliferation, either in serum or in the presence of growth factors such as PDGF (Stewart et al., 1991; Raff et al., 1978b; Raff et al., 1978c; Davis and Stroobant, 1990b). The identification of endogenous cAMPi elevating factors would seem to be of key importance in understanding the regulation of Schwann cell behaviour post-axotomy. Axolemmal preparations may synergise with growth factors in a similar manner to forskolin, yet do not elevate cAMPi (Davis and Goodearl, 1991). It has been suggested that localisation of the cAMP signal to a small defined region of the cell might explain these anomalies, but this is not a feature of the numerous receptor-adenylate cyclase linked systems extensively studied *in vivo* or *in vitro*.

Cessation of Schwann cell growth can be brought about by growth inhibitory molecules (Muir et al., 1990; Eccleston et al., 1989). Cultured Schwann cells can secrete an autocrine growth inhibitory molecule(s), which may explain their low proliferation rate and unresponsiveness to many protein mitogens *in vitro* (Muir et al.,

1990). This factor resembles stromelysin and is able to cleave fibronectin to generate a fragment which inhibits Schwann cell proliferation (Muir and Manthorpe, 1992). These same Schwann cells are also thought to secrete PDGF B-chains (Eccleston et al., 1990), express PDGF receptors, and respond to exogenous PDGF by DNA synthesis. More recently, however, it has been shown that cultured Schwann cells do not produce PDGF B-chain, although they may produce some A-chain (Hardy et al., 1992). As Schwann cells do not respond to this form of PDGF, it is unlikely that they have an autocrine growth activity in short term culture, though immortalised cells which do produce B-chain may do so (Hardy et al., 1992). DNA synthesis in spontaneously immortalised Schwann cells, which have a high basal proliferation rate, was inhibited by conditioned medium from short-term cultures of Schwann cells, raising the possibility that the increased proliferation of immortalised Schwann cells is due to lack of secretion of an autocrine growth inhibitor. Loss of such a growth inhibitory factor or its receptors could have a role in the expression of the NF-1 or NF-2 mutant phenotype in Schwannomas and neurofibromas *in vivo*. As the PDGF B-chain is found in peripheral nerve during development *in vivo*, and probably also after injury, and to a lesser extent in normal adult nerve (Eccleston et al., 1990; Hardy et al., 1992), it is of interest to speculate as to the source of this peptide.

The aims of our work were therefore, to examine the role of endogenous agents on the regulation of cAMPi in Schwann cells, and to understand the signalling mechanisms of these key regulators of proliferation. We also set out to re-examine the role of macrophage-derived cytokines in mediating proliferation and NGF synthesis in cultured cells.

**CHAPTER TWO**

**NEUROPEPTIDES IN REGENERATING AND DEGENERATING  
PERIPHERAL NERVE**



## INTRODUCTION

### OVERVIEW

Injury to sciatic nerve results in proliferation of both fibroblasts and Schwann cells (Abercrombie and Johnson, 1946) which, in turn, may promote successful regeneration (Taniuchi et al., 1986) by producing trophic factors, such as NGF (Lindholm et al., 1987). There are two waves of Schwann cell proliferation following axotomy *in vivo*, one closely following nerve lesioning, and a second coinciding with the ingrowth of regenerating axons. The latter is also followed by a reduction in the proliferation of fibroblasts which otherwise overgrow and populate the distal stump when reinnervation is prevented (Haftck and Thomas, 1968; Miyamoto et al., 1986). Scar formation and fibroblast overgrowth in front of regenerating axons prevent reinnervation, making regulation of fibroblast proliferation as important as that of Schwann cells if adequate recovery after injury is to occur.

PDGF (Davis and Stroobant, 1990a; Davis and Stroobant, 1990b), GGF (Raff et al., 1978a; Brockes et al., 1980) and basic-FGF (Ratner et al., 1988a; Chen et al., 1991) are Schwann cell mitogens *in vitro*, but only in the presence of agents that increase cAMPi, such as forskolin and cholera toxin (Raff et al., 1978b; Stewart et al., 1991). Elevation of cAMPi induces the synthesis of receptors for growth factors, such as PDGF (Weinmaster and Lemke, 1990), thus allowing Schwann cells to respond to the growth factor. No endogenous cAMPi-elevating agent has been shown to allow growth factor stimulated Schwann cell proliferation *in vitro*. Schwann cells have previously been shown not to respond to  $\beta$ -adrenergic agonists or PGE<sub>1</sub> (Raff et al., 1978b; Raff et al., 1978c) and although VIP and noradrenaline elevate cAMPi (Yasuda et al., 1988a), they have not been shown to be mitogenic (Stewart et al., 1991; Davis and Stroobant, 1990b).

Fibroblast proliferation, in other systems, has variously been shown to be inhibited by forskolin and TGF- $\beta$ , and stimulated by PDGF.

### BIOCHEMICAL CHANGES FOLLOWING AXOTOMY

The processes of axonal transport and protein synthesis are essential for nerve regeneration. There is no change in the overall rate of axonal transport in injured nerve, but there is an increase in certain specific proteins reaching the nerve terminals. The preferential increase in transport includes polypeptides destined for the plasma membrane and cytoskeleton, e.g. GAP-43, one of a small family of related growth associated peptides (GAPs), is found in considerably higher concentrations in

developing and regenerating nerves (Verge et al., 1990). More recently, it has been shown that some neuropeptides are also preferentially transported, and are upregulated in specific populations of neurons after axotomy. Immunochemical studies have demonstrated the presence of a number of peptides in the dorsal and ventral horns of the spinal cord, as well as in the DRG. These neuropeptides are possible neurotransmitters or neuromodulators, and normally co-exist with classical neurotransmitters. Following sciatic nerve transection, there is a reduction in substance P and tachykinins in the L5 DRG (Noguchi et al., 1989; Nielsch and Keen, 1989); this is thought to be part of a diversion of metabolic priorities away from molecules required for normal nerve function towards those required for axonal regeneration. Peptides which are upregulated following injury might be expected to play a role in the regenerative process. Following sciatic nerve injury, CGRP is upregulated in motor neurons (Haas et al., 1990), whereas Galanin (Villar et al., 1991) and VIP (Nielsch and Keen, 1989; Shehab and Atkinson, 1986) are upregulated in sensory neurons. Both CGRP and VIP accumulate in increased amounts proximal, and transiently distal, to the site of nerve injury (Haas et al., 1990; Noguchi et al., 1990).

#### CALCITONIN GENE-RELATED PEPTIDE

CGRP is a 37- amino acid neuropeptide which exists in two forms,  $\alpha$ -CGRP and  $\beta$ -CGRP.  $\alpha$ -CGRP is generated by alternative splicing of the primary transcript of the calcitonin gene (Amara et al., 1982). However,  $\beta$ -CGRP, which was discovered in brain and thyroid (Amara et al., 1985), is generated from a different gene which does not encode calcitonin. Comparison of the peptide sequences of the two CGRP molecules reveals only a single amino acid substitution of a lysine in  $\beta$ -CGRP for a glutamate in  $\alpha$ -CGRP at position 35 in the rat, whereas there is a three amino acid difference between the human forms (Steenbergh et al., 1985). CGRP is widely distributed within the nervous system (Skofitsch and Jacobowitz, 1985; Yamamoto and Tohyama, 1989). Particularly, it is found in a large population of primary sensory neurons, and it is the first peptide to be localised in motoneurons (New and Mudge, 1986; Fontaine et al., 1986). When the distribution of mRNA for  $\alpha$ -CGRP and  $\beta$ -CGRP is analysed, it is  $\alpha$ -CGRP which is more abundant in sensory ganglia and motoneurons (Gibson et al., 1988). CGRP is found within synaptosomal vesicles (Gulbenkian et al., 1986; Freid et al., 1989), and is released from electrically or  $K^+$  stimulated neurons *in vitro* (Uchida et al., 1990). CGRP is highly concentrated in nerve terminals by axonal transport (Kashihara et al., 1989) from the cell body (New and Mudge, 1986; Fontaine et al., 1986); the rate of anterograde transport of CGRP being about 1mm/hr in both sensory and motor fibres (Kashihara et al., 1989). Peripheral axotomy induces an increased immunostaining for CGRP in the axons of

injured motoneurons, as well as up-regulation of mRNA encoding  $\alpha$ -CGRP, but not  $\beta$ -CGRP, in motoneuron cell bodies (Streit et al., 1989; Haas et al., 1990; Arvidsson et al., 1990; Noguchi et al., 1990). This contrasts with decreased  $\alpha$ -CGRP and  $\beta$ -CGRP mRNA in the DRG following peripheral nerve injury (Noguchi et al., 1990). Since levels of transmitter-related enzymes usually decrease in response to axotomy, it is surprising that CGRP in motoneurons increases after injury. This raises the possibility that CGRP plays a role in peripheral nerve regeneration. There is a clear differential effect of nerve injury on neuropeptide expression. Immunohistochemically detectable substance P-like-immunoreactivity (SPLI) rapidly disappears from sciatic nerve fibres trapped in nerve-end neuromas, but CGRP-LI and neuropeptide Y-like-immunoreactivity remain. Following axotomy, CGRP accumulates proximal, and transiently distal, to the lesion site within a few hours (N.Bindemann, pers.comm.; Kashiwara et al., 1989). Immunohistochemistry shows this to be axonal, presumably due to anterograde and retrograde pile-up of peptide, with greatest levels in the region extending from the lesion to 2mm proximal to it. CGRP rapidly disappears from the distal stump and is undetectable by 24 hours. However, during regeneration, CGRP can be shown to be present at the leading edge of regenerating axons. Following sciatic nerve ligation, proximally accumulating organelles (i.e., organelles which were in transport distally in the axons) contained CGRP-LI. Retrogradely transported organelles, however, contained virtually no CGRP-LI (Dahlstrom and Booj, 1988); early accumulation of CGRP distal to the lesion, prior to reinnervation, may be due to local synthesis or leakage from damaged axons, rather than through retrograde transport. Many neuropeptide receptors have been characterised, all belong to the family of GTP binding protein (G protein) coupled receptors with 7 membrane-spanning domains (Hokfelt, 1991).

The actions of CGRP in many systems seem to be mediated via elevation of cAMPi (Boudard and Bastide, 1991; Laufer and Changeux, 1987). The ability of CGRP to regulate acetylcholine receptor (AChR) expression at the motor end plate (Laufer and Changeux, 1987), and to stimulate proliferation of human umbilical vein endothelial cells (Haegerstrand et al., 1990), are mediated via cAMPi elevation and by interaction with specific high affinity receptors (Roa and Changeux, 1991). The effect of CGRP on HUVECs may be important for formation of new vessels during physiological and pathophysiological events such as, ischaemia, inflammation, and wound healing. CGRP elevates cAMPi in skeletal (New and Mudge, 1986; Laufer and Changeux, 1987) and cardiac muscle (Sigrist et al., 1986). cAMP levels are elevated below and above a peripheral nerve lesion within 6 hours distal and 24 hours proximal to the crush injury (Appenzeller, 1972).

Endopeptidase 24.11 or enkephalinase (E.C.24.11) has been shown to be present on the surface of both myelinating and non-myelinating Schwann cells in sciatic nerve, and is expressed by Schwann cells in culture (Kioussi and Matsas, 1991; Kioussi et al., 1992; Matsas et al., 1986; Barnes et al., 1988). CGRP is a substrate for EC 24.11, which is upregulated in peripheral nerve following injury (Kioussi et al., 1992). Inhibitors of this enzyme are available and include phosphoramidone. Interestingly, during metabolism of CGRP by EC 24.11, a homologous sequence to eosinophil chemotactic factor is liberated (Davies et al., 1992). This could influence the progression of inflammation by recruiting eosinophils to inactivate mast cell products.

## RECEPTOR-ADENYLATE CYCLASE SYSTEMS

The  $\beta$ -adrenergic receptors are the best studied examples of receptor systems using cAMP as a second messenger. Tissues contain a heterogeneous population of  $\beta$ -adrenergic receptor ( $\beta$ AR) subtypes (Carlsson et al., 1972; Minnemann et al., 1981; Stiles et al., 1983). In human heart,  $\beta_1$ AR (Kaumann and Lemoine, 1987; Lemoine et al., 1988) and  $\beta_2$ AR (Kaumann and Lobnig, 1986; Kaumann et al., 1989) subtypes have been described.

The many effects of the endogenous catecholamines, adrenaline and noradrenaline, follow from their interaction with high affinity receptors on the cell surface of specific target cells. This interaction links hormone-mediated chemical signals at the cell surface to intracellular effector proteins, via increases in cAMPi. Agonist receptor interaction leads to activation of a stimulatory GTP-binding protein ( $G_s$ ), with the consequent liberation of its active subunit  $G_{s\alpha}$ . This activation is dependent on GTP binding and  $Mg^{++}$  concentration.  $G_{s\alpha}$  activates adenylate cyclase to increase synthesis of cAMPi. A major feature of this system is its rapid attenuation on prolonged exposure to catecholamines. This desensitisation results in reduced cAMPi elevation in response to agonist.

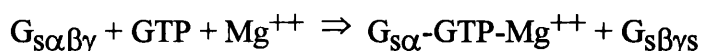
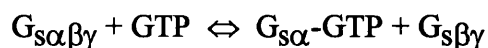
Prevention of desensitisation is an important therapeutic goal in the management of patients with heart disease, and many other clinical situations.

More than thirty GTP-binding protein (G protein) linked receptors have now been cloned (Dohlman et al., 1987); many of these are integral membrane glycoproteins, and in common with the  $\beta$ AR and the prototypic rhodopsin (Findlay and Pappin, 1986), consist of seven hydrophobic,  $\alpha$ -helical transmembrane spanning domains. This conserved structure has resulted in various pseudonyms for G protein linked receptors, including serpentine and magnificent seven. The intervening hydrophilic loops are arranged with the N-terminus and three short loops exposed extracellularly,

and two short and one long cationic loop and the C-terminus within the cytoplasm (Parker and Ross, 1989; Findlay and Pappin, 1986; Wang et al., 1990). Sequence homology is largely confined to the membrane spanning domains and to a lesser degree the shorter connecting loops. Individuality is conferred by the longer loop between spans five and six, and the carboxy terminal end of the third cytoplasmic loop which links to specific G proteins (Kubo et al., 1988; Kobilka et al., 1988; O'Dowd et al., 1988; Hausdorff et al., 1990b; Franke et al., 1990; Okamoto et al., 1991). The agonist binding site is contained within the hydrophobic core in a pocket formed by the bundle of membrane spanning helices (Strader et al., 1989b; Wong et al., 1988; Tota and Strader, 1990; Strader et al., 1988; Strader et al., 1989a).

### G PROTEINS

Many receptor systems activate signalling pathways which utilise G proteins to link surface signals to second messenger formation (Rodbell et al., 1971; Stryer and Bourne, 1986; Ross, 1989; Birnbaumer et al., 1990a). G proteins are designated either stimulatory ( $G_s$ ) or inhibitory ( $G_i$ ) according to their initially recognised regulatory activity on adenylate cyclase (Bourne et al., 1991). Each G protein is a heterotrimer of a GTP binding  $\alpha$  subunit (Bray et al., 1986; Kozasa et al., 1988) and regulatory  $\beta$  and  $\gamma$  subunits. Agonist binding to the  $\beta$ AR promotes exchange of GDP to GTP on the  $\alpha$  subunits of  $G_s$  (Gilman, 1987; Birnbaumer et al., 1987; Birnbaumer et al., 1985). The divalent metal ion  $Mg^{++}$  is required for the formation of the activated GTP-bound species,  $\alpha$ - $Mg^{++}$ -GTP (Gilman, 1987; Higashijima et al., 1987), which is then available for promoting cAMP formation through activation of adenylate cyclase. It seems a general rule that signal transducing G proteins, not just  $G_s$ , are activated by the binding energy of  $Mg^{++}$ -GTP. Agonist-receptor (AR) interaction accelerates dissociation of GDP, perhaps by lowering the  $Mg^{++}$  concentration required to facilitate this reaction (Freissmuth et al., 1989). There is negative cooperativity between the binding of GTP and  $\beta\gamma$  to G protein subunits; thus, GTP promotes G protein subunit dissociation and this reaction is shifted far to the right by  $Mg^{++}$  (Gilman, 1987).



The effect of  $Mg^{++}$  on binding of GTP is striking; the rate of dissociation of the nucleotide from  $G_{s\alpha}$  is reduced to near zero at mM concentrations (Higashijima et al., 1987). Negative control is provided in the system by  $\beta\gamma$ , through increased affinity of GDP for  $G_{s\alpha}$  and inhibition of GDP dissociation from  $G_{s\alpha}$ . However, in

the presence of 10 mM  $Mg^{++}$ , the effect of  $\beta\gamma$  on affinity of  $G_{s\alpha}$  is not great. The effect of  $Mg^{++}$  is thus to increase dissociation and decrease association of GDP, and enhance stability of  $G_{s\alpha}$ -GTP, the physiologically active form of the G protein.  $Mg^{++}$  has diverse actions on receptor- G protein interaction depending on concentration; in general, at supraphysiological concentrations, it enhances activation of  $G_s$  by AR, by stimulating GTP binding and GDP dissociation, and also GTP-induced subunit dissociation (Ross, 1989; Parker and Ross, 1989).

$G_{s\alpha}$  (Birnbaumer et al., 1990b; Birnbaumer et al., 1990a), dissociated from the receptor and from the  $\beta\gamma$  subunit, activates adenylate cyclase (Kaumann and Birnbaumer, 1974; Kaumann et al., 1983), thereby increasing cAMPi formation. The response is terminated by the intrinsic GTPase activity of the  $\alpha$  subunit that stimulates hydrolysis of bound GTP to GDP, thereby promoting reassociation of the G protein  $\alpha\beta\gamma$  trimer. In mutants where this GTPase activity is lost, the  $\alpha$  subunits remain constitutively active and may therefore behave as oncogenes (Climenti et al., 1990; Landis et al., 1989) in cell types in which cAMPi elevation is mitogenic (Hen et al., 1989; Dumont et al., 1989). Basal GTPase activity for  $G_s$  is extremely low (Brandt and Ross, 1985). The  $\alpha$  subunits, although they have unique regulatory regions, exhibit highly conserved sequences including the nucleotide binding sites, which share structural homology with other GTP binding proteins such as the oncogene product p21<sup>ras</sup> (de Vos et al., 1988; Holbrook and Kim, 1989). The  $\alpha$  subunit of G proteins contains at least one high affinity binding site for divalent metal ions. The more variable carboxy-terminal regions are responsible for receptor binding (Sullivan et al., 1987), and are targets for pertussis, or cholera toxin induced ADP-ribosylation (West et al., 1985). The  $\beta$  and  $\gamma$  subunits, in contrast, are relatively interchangeable among G proteins, although some heterogeneity of  $\gamma$  subunits is seen. They act as a  $\beta\gamma$  dimer (Whiteway et al., 1989) to regulate signalling by inhibiting  $\alpha$  subunit activation by GTP (Northup et al., 1983; Brandt and Ross, 1985), and may promote the intrinsic GTPase activity of the G protein. Recently it has become clear that the  $\beta\gamma$  subunit may potentiate the stimulatory action of  $G_{s\alpha}$  on the type II and IV adenylate cyclases (Tang and Gilman, 1991), whereas it inhibits  $G_{s\alpha}$  stimulation of adenylate cyclase type I. Insufficient  $\beta\gamma$  subunits are released from  $G_s$  *in vivo* to mediate this effect due to the relatively low abundance of  $G_s$ . Far greater quantities are liberated by dissociation of  $G_i$  or  $G_o$ . This could in part explain the ability of  $G_i$  to inhibit adenylate cyclase type I and to synergise with  $G_s$  in activating type II (Tang and Gilman, 1992), and provides a further mechanism for cross-talk between signalling pathways.

DESENSITISATION OF  $\beta$ -ADRENERGIC RECEPTORS

A remarkable feature of the  $\beta$ AR / adenylyl cyclase system is that, even in the continuous presence of agonist, cAMPi responses generally plateau and wane within a few minutes; subsequently administered  $\beta$ -agonists are less effective at elevating cAMPi if receptors have previously been exposed to maximally effective  $\beta$ -agonists (micromolar concentrations) (Johnson et al., 1978; Shear et al., 1976). As with many other receptor-effector systems it undergoes desensitisation upon agonist exposure. This property is not confined to G protein linked systems but has been shown for the tyrosine kinase receptors for EGF and insulin (Schlessinger, 1988). Receptor desensitisation has profound physiological implications. Clinically manifested heart failure can be precipitated by loss of myocardial contractility. Reduction in maximum myocardial contractility occurs in the presence of supranormal  $\beta$ -agonist levels, either endogenous or exogenous, as there are no functional spare  $\beta$ -receptors in human myocardium (Bristow et al., 1982; Colucci, 1990). This desensitisation of cardiac  $\beta$ -receptors can be measured as a decrease in isoprenaline induced cAMPi production, following prior  $\beta$ -agonist exposure (Bristow et al., 1985), and is termed homologous desensitisation (Clark, 1986; Reithmann et al., 1989; Reithmann and Werdan, 1988; Reithmann and Werdan, 1989; Sibley and Lefkowitz, 1985). Desensitisation has separate components, short-term (Su et al., 1979) and long-term; recovery from the former is rapid whereas, prolonged agonist exposure results in a more profound desensitisation, requiring new protein synthesis and thus days for recovery (Perkins et al., 1990). Desensitisation can also be classified according to its specificity, as homologous or heterologous; the former does not impair responsiveness to a different receptor ligand whereas, the latter will reduce the second messenger responses of numerous different agents. Rapid agonist induced desensitisation of mammalian cells involves receptor phosphorylation by two kinases, PKA and  $\beta$ ARK. Homologous desensitisation is mediated primarily by  $\beta$ ARK whereas, heterologous desensitisation may require PKA mediated phosphorylation of receptors (Sibley et al., 1987). Longer exposures, hours rather than minutes, are generally required to induce heterologous desensitisation in addition to homologous desensitisation, but both will reverse within hours upon withdrawal of agonist. Prolonged exposure, usually days, may induce downregulation of receptor mRNA by decreased stability and increased receptor degradation following phosphorylation. Once receptor numbers are decreased, reversal of desensitisation, on withdrawing agonist, requires several days and new protein synthesis. This system is less well studied, and mechanisms discussed below refer to "short-term" desensitisation occurring within 24 hours of agonist exposure. Recent work suggests that PKA mediated desensitisation occurs even at nanomolar concentrations, and may be important in regulating response to low levels of

circulating catecholamines, whereas  $\beta$ ARK (as well as PKA) mediated desensitisation occurs at micromolar concentrations and may be more important at synaptic sites.

Intracellular cAMP levels are the product of at least three processes, the generation of cAMP from ATP by adenylate cyclase, the degradation of cAMP by phosphodiesterase (PDE), and the export of cAMP out of the cell. Desensitisation leads to the decreased generation of cAMP. Export is not critical in mediating desensitisation, as the total cAMP levels (both extracellular and intracellular) plateau under desensitising conditions. PDE gene expression can be upregulated by cAMPi within hours (Swinnen et al., 1989). However, both the rapid time course of desensitisation, and the inability of PDE inhibitors to prevent it, suggest that PDE is not critical in mediating desensitisation. PDE inhibitors, such as milrinone and amrinone, may in fact further promote desensitisation of the  $\beta$ -adrenergic receptor and other cyclase linked receptors, by potentiating and maintaining acute cAMPi elevation (Bobik and Little, 1984; Benovic et al., 1985; Brown et al., 1986; Hadcock and Malbon, 1988; Maisel et al., 1989b). These agents probably depend on receptor induced cAMPi generation to be effective (Feldman et al., 1987). Rolipram, a specific PDE type III inhibitor, is less prone to cause this in some *in vitro* systems (Gordeladze, J.O., 1990). Prolonged exposure of cells to cAMPi elevating agents, including iloprost, a stable prostacyclin analogue, and cholera toxin can down regulate  $G_s$ . Chronic exposure to agonist over several days, leads to a decrease in the density of ventricular  $\beta$ AR (Neve and Molinoff, 1986; Hadcock and Malbon, 1988; Hadcock et al., 1989), and reduced activation of adenylate cyclase (Harden, 1983; Fowler et al., 1986; Ginsburg et al., 1983; Bristow et al., 1982; Karliner and Scheinman, 1988; Colucci et al., 1989; Murphree and Saffitz, 1989; Otto-Erich et al., 1989; Tse et al., 1979), whereas acute exposure over a matter of hours, does not. Acute exposure, however, still results in attenuation of catecholamine action (Vatner et al., 1988), cAMPi elevation, and contractility. This is due to phosphorylation of the receptor (Benovic et al., 1986; Strasser, 1989; Lohse et al., 1990; Benovic et al., 1985; Lefkowitz et al., 1984) and consequently, an uncoupling of the receptor from  $G_s$  (Strasser et al., 1984; Vatner et al., 1985; Kaumann et al., 1989; Kaumann and Birnbaumer, 1976; Kaumann et al., 1982; Bristow et al., 1989; Kassis and Fischman, 1984; Vatner et al., 1989). Acute, homologous desensitisation is mediated primarily by the  $\beta$ ARK (Benovic et al., 1986; Lohse et al., 1990; Strasser et al., 1986b; Strasser et al., 1986a; Hausdorff et al., 1990a; Lohse et al., 1989) in co-operation with an arrestin-like molecule which binds to the phosphorylated receptor (Benovic et al., 1987; Lohse et al., 1992; Rapoport et al., 1992). Extracellular cysteine residues on the  $\beta$ AR may be important for action (Liggett et al., 1989), but phosphorylation sites are believed to occupy the serine/threonine rich C-terminus (Bouvier et al., 1988). PKA contributes little to acute desensitisation (Lohse et al., 1989), but is important in



mediating heterologous desensitisation on more prolonged agonist exposure (Benovic et al., 1985; Hausdorff et al., 1990a; Clark et al., 1988). PKA may also act by phosphorylating part of the C-terminal end of the third cytoplasmic loop and thereby prevent activation of  $G_s$  (Okamoto et al., 1991). This process has been extensively studied using permeabilised cells or membrane preparations. Desensitisation of the  $\beta_2$ AR can be induced by incubation of a purified plasma membrane fraction with the catalytic subunit of PKA (Clark et al., 1987), or by treatment of cells with PMA to elevate PKC (Johnson et al., 1990). In these "broken cell" studies, heterologous desensitisation is most marked at  $Mg^{++}$  concentrations of 1mM or less (Clark et al., 1987), and can be partly overcome by increasing the  $Mg^{++}$  concentration to between 5 and 10mM (Kunkel et al., 1989), which improves receptor-G-protein coupling (Gilman, 1987). In fact, at 10 mM  $Mg^{++}$  the coupling efficiency of phosphorylated receptors (Pitcher et al., 1992) is only 15% less than the unphosphorylated one. A similar effect has been demonstrated for cell membranes at 10mM  $Mg^{++}$  (Johnson et al., 1990; Hausdorff et al., 1989). Under conditions where desensitisation is believed to be mainly PKA mediated, sub-millimolar  $Mg^{++}$  levels allow desensitisation to occur, but at higher concentrations this is largely abolished. In addition, at supraphysiological  $Mg^{++}$  concentrations (i.e.  $> 0.9mM$ ), the usual inhibitory action of  $\beta\gamma$  on GTP binding to the  $\alpha$  subunit is reversed and GDP release is facilitated. These results suggest that  $Mg^{++}$  can facilitate receptor/ $G_s$  coupling by interacting with a lower affinity binding site. PKA mediated heterologous desensitisation may be most sensitive to inhibition by supraphysiological levels of  $Mg^{++}$ .  $\beta$ ARK mediated homologous desensitisation, which requires the cofactor arrestin for maximum efficiency, is not impaired at a wide range of  $Mg^{++}$  concentrations (0.5-5mM) *in vitro*. In this chapter, I provide the first evidence for an effect of supraphysiological  $Mg^{++}$  on desensitisation in intact, living cells.

Cross talk between separate signalling pathways can occur. Receptor phosphorylation can be induced by PKC (Bouvier et al., 1987), which might allow regulation of the  $\beta$ AR by  $\alpha$ -agonists, or cholinergic inputs (review by Pitcher et al., 1992). In some systems,  $G_i$  activation may induce desensitisation through liberation of excess  $\beta\gamma$ . Pertussis toxin pretreatment partially blocks desensitisation to parathormone in ROS 17/2.8 cells, as well as potentiating acute cAMPi elevation; inhibiting tonic  $G_i$  activity or concurrent activation of  $G_i$  by agonist may explain this (Abdul-Badi Abou-Samra et al., 1989). In S49 lymphoma cells, treatment with isoprenaline or forskolin increases levels of  $G_i$ , thereby increasing the inhibitory control of cAMP by somatostatin; this effect is also pertussis toxin sensitive (Haddock et al., 1990). Somatostatin can act via  $G_i$ , and in many systems exerts negative control over cAMPi elevation. Pertussis toxin can prevent homologous desensitisation of adenylate cyclase (Wilson et al., 1986), which can also be inhibited via  $G_i$ . Melatonin antagonises the

ability of forskolin to activate adenylate cyclase via a pertussis toxin sensitive G protein, and thereby reduces cAMPi accumulation (Carlson et al., 1989). Prolonged treatment of adipocytes with PGE<sub>1</sub> leads to loss of G<sub>i</sub> activity, and subsequent PGE<sub>1</sub> or other agents such as nicotinic acid, are less effective at suppressing cAMPi elevation. Through the action of G<sub>i</sub>, cAMPi production may be reduced by choline esters, somatostatin, epinephrine (via  $\alpha$ -receptors), dopamine, IL-1, serotonin, and in the presence of an adenylate cyclase activator, vasopressin.

Treatment with noradrenaline may also cause heterologous, or agonist independent desensitisation of other cyclase linked receptors, such as the PGE<sub>1</sub> and histamine receptors (Reithmann and Werdan, 1988; Bohm et al., 1988), and also those for CGRP, (this thesis).

In several cell types, activation of cell responses results from receptor-mediated hydrolysis of inositol phospholipids, generating two second messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP<sub>3</sub>), which activate PKC and elevate intracellular Ca<sup>++</sup> concentration, respectively (Nishizuka, 1988; Berridge and Irvine, 1989). PKC activation has variously been shown to inhibit, stimulate, or not change, cAMPi concentrations in different systems. The phorbol ester PMA may induce desensitisation via receptor phosphorylation. This has been shown for the  $\alpha$ -adrenergic receptor in C6 rat glioma cells (Kassis et al., 1985). PMA or phospholipase C, which activate PKC directly or via elevation of intracellular DAG levels, were able to antagonise both isoprenaline and forskolin mediated elevation of cAMPi in C6 rat glioma cells, via an increase in phosphodiesterase activity (Bressler and Tinsely, 1990). A calmodulin-dependent phosphodiesterase is upregulated in human glioma cells by muscarinic cholinergic agonists (Tanner et al., 1986) within minutes of cholinergic stimulation. PMA treatment reduces receptor binding of calcitonin to T47D human breast cancer cells and BEN human lung cancer cells via phosphorylation of the receptor, and consequently decreases receptor mediated adenylate cyclase activation and cAMP production. This is dependent on PKC activation and is inhibited by H7. Conversely, in some systems PKC may promote adenylate cyclase activation, ie. S49 lymphoma cells and 3T3 fibroblasts (Rozengurt et al). In prolactin-secreting rat adenoma (GH4C1) pituicytes, PKC can stimulate adenylate cyclase activity by inhibiting G<sub>i</sub> and thereby may potentiate the activity of agents such as VIP (Gordeladze et al., 1989). In swine luteal cells TPA markedly potentiates the cAMPi elevation obtained with leuteinizing hormone, forskolin, or cholera toxin, but has no effect on basal cAMPi levels. TPA also augments the stimulatory effect of pertussis toxin when combined with maximally effective doses of forskolin or cholera toxin (Wheeler and Veldhuis, 1989), but there was no effect on basal cAMPi. This potentiation of maximal levels of cAMPi had little effect on the ED<sub>50</sub> and was seen within 15 minutes and was still seen after 48 hours. This effect

was seen in the presence and absence of IBMX which potentiated cAMPi levels of all combinations.

#### DESENSITISATION OF OTHER CYCLASE LINKED RECEPTORS

Receptors for CGRP on cultured cardiac myocytes (Chatterjee et al., 1991) and on mesenteric artery (Han et al., 1990), elicit markedly attenuated cAMPi and vasodilatation responses, respectively, following prior exposure to CGRP (Davies et al., 1992). Short-term exposure of mesenteric artery to CGRP leads to homologous desensitisation of CGRP responses, leaving those to isoprenaline unaffected. CGRP may also mediate heterologous desensitisation (Miles et al., 1989). CGRP regulates phosphorylation of the AChR in primary rat myotube cultures, which is increased in the presence of the phosphodiesterase inhibitor Ro 20-1724. Phosphorylation of the AChR in the presence of CGRP, appears to be mediated by CGRP-stimulated increases in cAMP levels leading to activation of PKA. These results suggest that CGRP may play a physiological role as a regulator of AChR desensitisation by modulating AChR phosphorylation at the neuromuscular junction. CGRP is a potent vasodilator (Kawasaki et al., 1988; Greenberg et al., 1987) and enhances myocardial contractility via a cAMPi dependant mechanism (Sigrist et al., 1986), and via stimulation of inward calcium current (Ono et al., 1989).

#### DESENSITISATION IN DISEASE

Heart failure is an extremely common condition associated with great morbidity and a considerable shortening of life expectancy. This syndrome is associated with a loss of responsiveness to endogenous catecholamines and rapid loss of response to exogenously administered inotropes, due to receptor desensitisation. The limitation or prevention of desensitisation by preventing receptor phosphorylation, or by augmenting receptor effector coupling, is an important therapeutic goal.

One difficulty in assessing receptor desensitisation in living patients with congestive cardiac failure (CCF), is accessibility of myocytes. Most studies have relied on transplant specimens, animal models, or haemodynamic measures of contractility and heart rate. Another approach is to study the regulation of  $\beta$ -AR found on more readily available tissue. Several studies confirm that  $\beta$ -AR on circulating lymphocytes closely resemble those on myocardium with regards to their regulation by catecholamines and in heart failure (Brodde et al., 1986; Aarons and Molinoff, 1982; Maisel et al., 1989a; Colucci et al., 1981; Mancini et al., 1989; Horn and Bilezikian, 1990).

#### CYCLIC AMP AND GENE REGULATION

## CYCLIC AMP AND GENE REGULATION

Regulation of gene expression is modulated by the interactions of positive and negative factors with specific DNA motifs. The hormonal stimulation of a variety of eukaryotic genes is mediated by cAMPi, through a conserved cAMP response element (CRE)(Montminy et al., 1986). Transcriptional induction by cAMP is rapid, peaking at 30 minutes, and gradually declining over 24 hours. This burst in transcription is resistant to inhibitors of protein synthesis, suggesting that cAMP may stimulate gene expression by inducing the covalent modification rather than *de novo* synthesis of specific nuclear factors. Since the majority of known cellular effects of cAMP occur via the catalytic subunit (C-subunit) of PKA, with the notable exception of direct cAMP gating of channels in the olfactory system, it is likely that this enzyme mediates the phosphorylation of factors that are critical for the transcriptional response. PKA-deficient cell lines are unable to stimulate somatostatin gene expression in response to forskolin (Montminy et al., 1986). Furthermore, micro-injection of the C-subunit into cells can directly activate CRE-dependent transcription, of genes for VIP and also *c-fos*, even without simultaneous addition of cAMP (Riabowol et al., 1988). Treatment of cells with cAMP induces the transport of the active C-subunit of PKA to the nucleus. The CRE binding protein (CREB), which stimulates the transcription of the somatostatin gene has been described. CREB is constitutively bound to the CRE, even in non-stimulated cells, but acts as a repressor, and can block the action of upstream enhancer binding transcription factors. Once phosphorylated at Ser-133 by the C-subunit, however, activation of genes with CRE promoters progresses (Gonzalez and Montminy, 1989). *De novo* protein synthesis is not required for activation of genes by phosphorylated CREB, although a CREB binding protein may be involved in the action of phosphorylated CREB. This contrasts with some effects mediated via the AP-1 site which require the *de novo* synthesis of the fos and jun proteins, which in turn, then activate gene transcription via an AP-1 site. When cAMPi levels decline, the catalytic subunit of PKA is inactivated, resulting in an increase in phosphatase activity, particularly protein phosphatase 1 (PP1), which dephosphorylates and inactivates CREB (Hagiwara et al., 1992).

Jun protein, the product of protooncogene *jun* and a member of the AP-1 family of transcription factors, can upregulate the transcription of its own gene, whereas a related JunB protein can suppress its transcription. Similarly the product of the nuclear oncogene *fos* can suppress the transcription of its own promoter, but activates the transcription of promoters containing AP-1 binding sites. The AP-1 site present in the human and mouse *c-jun* promoter are similar. Also, the core consensus sequence of CRE required for induction by increases in cAMPi, is very similar to the AP-1 binding site (Montminy et al., 1986). CREB can bind to the AP-1 site present in the promoter of *c-jun*, and when phosphorylated by the C- subunit of PKA, will activate

this promoter. CREB, unphosphorylated in the absence of PKA activity, is actually a repressor of the *c-jun* promoter (Lamph et al., 1990). This CREB repression extends to both serum and PMA induction. The *c-jun* promoter can be activated by Jun or Fos-Jun proteins, but unphosphorylated CREB can still interfere with this transactivation, suggesting that CREB is a potent repressor of transcription but reverses its role after phosphorylation. CREB acts to inhibit binding of the Fos-Jun heterodimer, but when phosphorylated, either allows its transactivation domain to interact with other transcription factors, or that the phosphorylated CREB has a greater binding affinity for its cognate CRE than to the AP-1 site. Recent work suggests a similar effect on the *c-fos* promoter. PDGF mediated proliferation and stimulation of *c-myc* mRNA levels are inhibited by elevating cAMPi in human foreskin fibroblasts (Heldin et al., 1989). Elevation of cAMPi also inhibits IL-2 stimulated *c-myc* levels in T-lymphocytes. However, although cAMPi inhibits proliferation in macrophages, it has no effect on growth factor related *c-myc* expression, though it does increase *c-fos* mRNA (Vairo et al., 1990). cAMPi elevation in macrophages may also reduce IL-1 production (Knudsen et al., 1986).

#### REGULATION OF THE NERVE GROWTH FACTOR GENE

Mechanisms for the transcriptional activation of the NGF gene have been studied in fibroblasts. IL-1 can increase the stability of NGF mRNA as well as activating transcription (Lindholm et al., 1988). Following axotomy, the increase in NGF mRNA levels is preceded by an increase in mRNAs for *c-fos* and *c-jun*. Heterodimers of Fos and Jun protein are known to regulate activity of certain genes after binding to a consensus sequence (AP-1 site) in the promoter of these genes. In fibroblasts, regulation of NGF transcription can be achieved by interaction of transacting factors with a specific AP-1 site. Interestingly, the transcriptional activation of the NGF gene can be prevented by pre-treatment with dexamethasone, both after nerve lesion *in vivo*, and after stimulation of fibroblasts with serum or IL-1 *in vitro*. This transcriptional regulation is prevented by deletion of a 162 bp segment of the NGF promoter. Forskolin and activated PKA increase NGF mRNA transcription in the absence of protein synthesis, possibly by phosphorylation of CREB, whereas, TPA and other inducers of Fos protein are ineffective. If CREB is involved, then synergy would be expected with TPA or PDGF once CREB had been phosphorylated by the C-subunit of PKA. Schwann cell NGF-mRNA transcription is activated synergistically by forskolin and TPA, independently of protein synthesis (Matsuoka et al., 1991b). This activation of NGF mRNA transcription requires PKA, as it can be prevented with H-8, whereas, H-7 only blocks the synergistic action of TPA, without affecting forskolin induced elevation. Interestingly, TPA alone reduces basal NGF-mRNA levels slightly. Growth factors such as PDGF, FGF, EGF, IL-1, IGF-I, or

IGF-II did not increase Schwann cell NGF mRNA even in the presence of serum or forskolin. In fact, TGF- $\beta$  abolishes forskolin-stimulated increases, and reduces basal levels of NGF mRNA. Forskolin-mediated increases in NGF mRNA are potentiated by pre-treatment with ionomycin, which stimulates  $\text{Ca}^{++}$  influx by an EGTA inhibitable pathway. This could be physiologically relevant, as  $G_s$  activates a dihydropyridine sensitive  $\text{Ca}^{++}$  channel, in addition to adenylate cyclase (Mattera et al., 1989; Brown and Birnbaumer, 1988). Agents such as noradrenaline and CGRP which act via  $G_s$ , could also activate these  $\text{Ca}^{++}$  channels. Further experiments are needed to examine whether this could lead to enhanced ability to promote NGF synthesis. Forskolin, which directly activates adenylate cyclase bypassing the G protein, would thus be expected to be less effective. However, upregulation of NGF-mRNA by isoprenaline is also enhanced by ionomycin. It would be interesting to determine the effect of EGTA or of a selective calcium-calmodulin protein kinase inhibitor on isoprenaline induced NGF-mRNA elevation.

Elevation of cAMPi increases levels of NGF mRNA but reduces the amount of BDNF mRNA in Schwann cell cultures (Acheson et al., 1991). In nerve explants, IL-1 elevates NGF mRNA but does not effect BDNF mRNA. As for the NGF receptor, mRNA increases concomitant with axonal degeneration. During regeneration, axonal contact down regulates the NGFr mRNA. Administration of TGF- $\beta$ 1 down regulates NGFr mRNA in cultured Schwann cells and this is potentiated by forskolin.

#### REGULATION OF SUPPRESSED cAMP-INDUCIBLE POU IN SCHWANN CELLS

The POU proteins constitute a large family of putative transcription factors, named from the first 3 members to be described (pit, oct and unc). They are most closely related in their DNA binding homeobox domains (a helix-turn-helix motif) and in a contiguous POU-specific domain immediately upstream of this domain (Herr et al., 1988; He et al., 1989). In these regions, the degree of amino acid sequence conservation between SCIP and the other class III POU proteins, Brn-1 and -2, is striking. Sequence analysis suggests that mRNA for SCIP and the previously described *Tst-1*, encode the same protein (Monuki et al., 1989; Monuki et al., 1990; Kuhn et al., 1991). POU proteins have been shown to transcriptionally activate cell-specific genes and to participate in the determination of cell fate. It is therefore thought that these proteins function in development through the stable activation of genes that define specific developmental pathways. SCIP is expressed by developing Schwann cells in the nervous system (Treacy et al., 1991; Monuki et al., 1990; Monuki et al., 1989). SCIP expression in cultured Schwann cells may be stimulated by agents that elevate cAMPi (Monuki et al., 1989). Both in normal development and

in response to nerve transection, SCIP expression is transiently activated only during the period of rapid cell division that separates the premyelinating and myelinating phases of Schwann cell differentiation. Following axotomy, SCIP mRNA (normally at very low levels) is transiently elevated with a peak between 2 and 5 days post-lesion, coinciding with the first wave of Schwann cell proliferation. In cotransfection assays, SCIP acts as a transcriptional repressor of myelin-specific genes (Monuki et al., 1990) and was shown to repress the P<sub>O</sub> promoter (He et al., 1991). It has thus been suggested that SCIP expression is linked to proliferation, and acts to repress myelin gene expression. The association with proliferation may have functional significance, as the POU transcription factors Oct-1 and Oct-2 have been shown to stimulate DNA replication *in vitro* (Verijzer et al., 1990). Oct-3 is expressed by the embryonic stem cell line p19 only under conditions that permit cell proliferation but prevent differentiation (Okamoto et al., 1990). Nearly all the POU proteins analysed to date are expressed at high levels in regions of the early embryo, such as the ventricular zone of the neural tube, that are populated by rapidly proliferating progenitor cells (He et al., 1989). POU proteins may therefore act as transiently expressed regulators in proliferating progenitors.

#### CILIARY NEUROTROPHIC FACTOR

Ciliary neurotrophic factor (CNTF) was originally described as a survival factor for chick ciliary ganglion neurons. It has been cloned and expressed (Lin et al., 1989; Negro et al., 1991), and demonstrates no significant amino acid homology to any other known protein. It is a cytoplasmic protein, as shown by the absence of a hydrophobic signal sequence and its lack of release from HeLa cells transfected with a CNTF expression vector (Stockli et al., 1989). The receptor has also been cloned and found to resemble that for IL-6 (Davis et al., 1991). CNTF has been immunolocalised to peripheral nerve (Rende et al., 1992), particularly to myelinating Schwann cells (Dobrea et al., 1992). It may function as a motor neuron trophic factor following axotomy (Sendtner et al., 1990), though others have shown it to act only *in vitro* (Wewetzer et al., 1990). A survival promoting action has also been demonstrated for hippocampal neurons (Ip et al., 1991) and chromaffin cells (Unsicker et al., 1985). Expression in peripheral nerve in the rat, starts at postnatal day 4 and increases to high adult levels by three weeks. Interestingly, developmental expression of CNTF occurs after the time period of physiological cell death of CNTF-responsive neurons. It is unlikely, in rodents at least, to act as a target derived trophic factor in development. CNTF can be released from Schwann cells after injury even though it lacks a signal sequence. Very low levels are seen in cultured Schwann cells, although they are a rich source *in vivo*, suggesting that this property may be lost with time in culture.

## CGRP RECEPTORS

Different biological effects of CGRP analogs have suggested the existence of receptor subtypes. Alpha-human CGRP binding sites exist in rat cerebellum, liver, and spleen exhibiting the same rank order of analog displacement. However, CGRP activates adenylate cyclase in the liver and spleen but not in the cerebellum, suggesting existence of CGRP receptor subtypes (Bushfield et al., 1993; Stangl et al., 1993). CGRP8-37 is believed to be a selective antagonist of the CGRP1 receptor, which has now been cloned (Aiyar et al., 1996). In situ studies show specific localization of the CGRP1 receptor mRNA to alveolar cells in the lung and to cardiac myocytes in the heart. Other putative subtypes have not been cloned. The distribution of CGRP receptors in the peripheral nervous system has not previously been examined.

## LOCALISATION OF CGRP IN THE NERVOUS SYSTEM

In the sensory nervous system CGRP distribution correlates with expression of NGF receptors. In dorsal root ganglia virtually all trkA-immunoreactive cells express CGRP immunoreactivity (Averill et al., 1995). In the dorsal horn TrkA immunoreactivity is heaviest in laminae I and II outer and has a similar distribution to CGRP. CGRP is depleted by dorsal rhizotomy.

CGRP-LI is not found in all motor terminals of the sciatic nerve. Endplates of IIB fibres located in the superficial portion of tibialis anterior (TA) display CGRP-LI, whereas in the deep portion of TA some endplates lack CGRP-LI. Thirty per cent of the IIB fibres in extensor digitorum longus (EDL) show CGRP-LI at the endplates. All endplates of IIA fibres in TA and EDL lack CGRP-LI. CGRP-LI is rarely detected at endplates in the soleus muscle. CGRP-LI levels in general are lower in motoneurons of small and slow-twitch motor units (eg. innervating soleus) than those of large and fast-twitch motor units (eg. innervating TA and EDL), but that there are also differences between the different types of fibres in TA and EDL (Forsgren et al., 1995). After axotomy, although all motoneuron pools display stronger CGRP labeling than corresponding unlesioned pools, in a fraction of the axotomized motoneurons the increase seems to be discrete or even absent (Piehl et al., 1993).

It is interesting that CGRP-LI appears in normally non-reactive motor endplates during axon regeneration after nerve injury (Csillik et al., 1993). The number of CGRP-IR motoneurons is also increased after injury to the hypoglossal nerve (Grothe, 1993). CGRP immunoreactivity is absent from preganglionic parasympathetic motoneurons, but may be induced if these nerves are surgically implanted into striated muscles normally innervated by CGRP immunoreactive neurons (McWilliam et al., 1995), suggesting the existence of a muscle-derived factor able to induce CGRP expression. Members of the FGF family including bFGF can abolish the upregulation of CGRP that follows axotomy of motor neurons *in vivo* (Piehl et al., 1995); deprivation of such muscle-derived factors could account for CGRP upregulation in injured motor neurons.

During development CGRP-LI is uniformly high in lateral horn motoneurons (the reticulospinal nucleus) of males and females at all ages examined. The majority of motoneurons of the dorsolateral nucleus are also positive throughout postnatal development although the percentage of positive motoneurons is slightly higher in males than in females (Forger et al., 1993).



## VASOACTIVE INTESTINAL POLYPEPTIDE

VIP is a major regulatory peptide in the mammalian brain and a member of the glucagon-secretin family of gastrointestinal peptide hormones. It is 28 amino acids long and was originally isolated from porcine duodenum (Said and Mutt, 1970). It has been identified in neurons of the CNS and in primary sensory neurons in the PNS (Fuji et al., 1983; Hokfelt et al., 1980). VIP is not normally detectable in sensory neurons in lumbar 4/5 DRG, but VIP mRNA and peptide appear after sciatic nerve lesion (Nielsch and Keen, 1988). VIP mRNA appears 3 days following injury and also in the dorsal horn (Shehab and Atkinson, 1986). The VIP gene was recently isolated and found to contain 7 exons. cAMP induces a substantial increase in VIP immunoreactivity regulated at the transcriptional level (Hayakawa et al., 1984). Increased protein kinase activity by phorbol ester may increase VIP synthesis in adrenal chromaffin cells (Pruss et al., 1985). Levels of VIP mRNA in rat are low at birth, peak at 14 days, and decrease in aged rats.

## MATERIALS AND METHODS

### MATERIALS

Anti rat O4 and Thy 1.1 hybridomas were a kind gift from Prof. R. Mirsky (Dept. of Anatomy); Ox42 was purchased from Seralab; anti mouse immunoglobulins were from Dako. Anti-CGRP antibody was from Peninsula. Antibodies to NGF were from Boeringer. CGRP was from Bachem; VIP and Somatostatin were from Peninsula, PDGF-BB and basic FGF were from Promega, Isoprenaline, galanin, theophylline, IBMX, staurosporine, H-7, PGE<sub>2</sub>, laminin, PMA, forskolin, Pertussis toxin, Cholera toxin, Poly-D-lysine, type 1a trypsin, type III collagenase and general lab chemicals were from Sigma. H-8, H-89, Genistein, and chlorophenol red  $\beta$ D-galactopyranoside, were from Calbiochem. The dual range cAMP radioimmunoassay kit and <sup>125</sup>IIdU were from Amersham. The CellTitre non-radioactive cell proliferation assay kit was from Promega. Foetal calf serum was from Gibco. Culture media were from Flow. Tissue culture plastics were from Falcon. Tissue culture 24 and 96-well plates were Falcon Primaria grade except where stated.

### ANIMALS AND SURGICAL PROCEDURES

Young adult male Sprague-Dawley rats (approximately 200g) were operated under deep halothane anaesthesia. For nerve lesion experiments, the sciatic nerve crush or transection was performed in the mid-femoral position. Following transection the distal stump was displaced to prevent reinnervation. Nerve crush was performed with watchmaker forceps ( No.5) for 1 minute, followed by application of a tight silk suture for 1 minute ( 2/0 Mersilk). The animals were killed by CO<sub>2</sub> narcosis, between 2 hours and 14 days post lesioning. Nerve segments, measuring 2cm proximal and 5cm distal to the lesion were removed. Contralateral nerve segments were removed as controls.

For cell cultures, p4-6 rat pups were killed by decapitation, and sciatic nerves collected into cold calcium-magnesium free dissociation buffer.

### TISSUE PREPARATION FOR IMMUNOHISTOCHEMISTRY

After dissection, the nerves were immediately immersed in 4% (w/v) paraformaldehyde in phosphate buffered saline, pH 7.4 (PBS) and fixed overnight at 4°C. The following day they were placed in 30% (w/v) sucrose in PBS for 2 hours for cryoprotection. The tissue was embedded in OCT embedding medium ( Tissue-tek, Miles) and then stored at -70°C until sectioned on a freezing microtome (Bright). Longitudinal sections of nerve, 10-15  $\mu$  thick were cut and thawed onto gelatin, and

pottassium permanganate, coated glass microscope slides. For crushed nerve, sections traversed the lesion site and included 5mm proximal and distal to it. Further distal segments were also examined. For cut nerves, proximal and distal segments were embedded and sectioned separately.

### CGRP IMMUNOHISTOCHEMISTRY

Sections were washed for 15 minutes in 5% (v/v) horse serum (Gibco), 0.1% (v/v) Triton-x100 (Sigma), 0.01% (w/v) sodium azide and 20mM L-lysine(Sigma) in PBS ("immunohistochemistry buffer" (IHB)). Slides were labelled overnight at 4°C with a rabbit anti-human CGRP polyclonal antibody (Peninsula) at 1/700 dilution in IHB. Control sections also received 1µM human CGRP or IHB only. Following incubations, slides were washed 3 times, for 5 minutes each, in IHB. The second antibody, a goat anti-rabbit flourescein-conjugate (Amersham), preabsorbed with 20% rat serum and microfuged to remove precipitates, was applied to sections at 1/100 in IHB for 45 minutes at room temperature.

Triplicate washes were repeated and slides mounted in Citiflour ( City University, U.K), prior to viewing with a fluorescence microscope (Zeiss Universal).

### CELL DISSOCIATION

Sciatic nerves were dissected from up to 14 p4-6 rats per preparation and collected into cold dissociation buffer ( 136 mM NaCl, 5mM KCl, 5mM Na phosphate, pH 7.4 with 33mM glucose). Sterile plastic was used throughout. Sciatic nerves were finely chopped with a scalpel and forceps in a 100mm Petri dish and transferred to a 15ml conical tube. Nerves were incubated for 35 minutes at 37°C in 2ml of 0.025% type 1a trypsin and 0.15% type III collagenase, in dissociation buffer. Final dissociation was achieved by trituration with a 1ml Pipetteman. Enzymes were inactivated by addition of an equal volume of culture medium (1:1 DMEM/ HAM-F12 with 10% foetal calf serum) or with trypsin inhibitor (for defined medium experiments) and cells collected by centrifugation at 1600 rpm for 10 minutes. The cell pellet was resuspended in culture medium, filtered through nylon mesh and then applied to precoated and washed panning dishes.

## PANNING

Schwann cell cultures were prepared by positive immunoselection by panning. Cells newly dissociated from sciatic nerve are panned on 150 mm Petri dishes pre-coated with cell-type specific antibodies. Panning dishes were prepared by coating Petri dishes for 2 hours at 37°C, or overnight at 4°C with a non-specific rabbit anti-mouse immunoglobulins (Dakopatts) diluted 1 in 400 in 15mM Trisma base buffered to pH 9.6 with HCl, to a final concentration of 10µg/ml. Following aspiration of buffer, the dishes were washed three times in normal saline before coating with cell specific antibodies, for at least 1 hour in pH 7.4 medium, with 0.2% (w/v) BSA, at 37°C. Triplicate washes were repeated before application of cell suspensions.

## MACROPHAGE SELECTION

Macrophages are removed from cell suspensions by panning on OX 42 (Seralab) coated Petri dishes. Cell suspensions are serially transferred from one OX 42 coated dish to another, until no further cell binding can be observed under microscopic control. Resultant cell suspensions are, consequently, free of macrophages, which remain adherent to the OX 42 coated Petri dishes, when cell suspensions are transferred to further dishes.

## FIBROBLAST SELECTION

Following removal of macrophages, suspensions are enriched for Schwann cells by depletion of fibroblasts through serial pannings on Thy 1.1 coated dishes. As described for macrophage depletion, suspensions are transferred serially from one Thy 1.1 coated Petri dish to another until no further binding could be observed microscopically. If resulting pure fibroblasts cultures were to be used for parallel experiments, then Petri dishes with bound fibroblasts were washed several times, with buffer, under microscopic control, until all loosely bound cells have been washed off. This step is included to increase the purity of resultant cultures by excluding contaminating non-antibody bound cells.

## SCHWANN CELL SELECTION

Pure Schwann cells were derived either by positive immunoselection involving panning on O4, or by total depletion of macrophages and fibroblasts. Pure O4 bound Schwann cells were harvested by trypsinisation. To avoid this extra trypsinisation step, and to increase the eventual Schwann cell yield per preparation, Schwann cell cultures of at least 99% purity were derived by total depletion of macrophages and

fibroblasts. The resulting cell suspension containing almost exclusively Schwann cells could then be counted and plated. Purity was assessed by trypan staining of ethanol-extracted cultures in all cAMP assays, fibroblasts and Schwann cells were apparent by morphology (and comparable results were obtained with immuno-stained preparations using Thy 1.1 and S100).

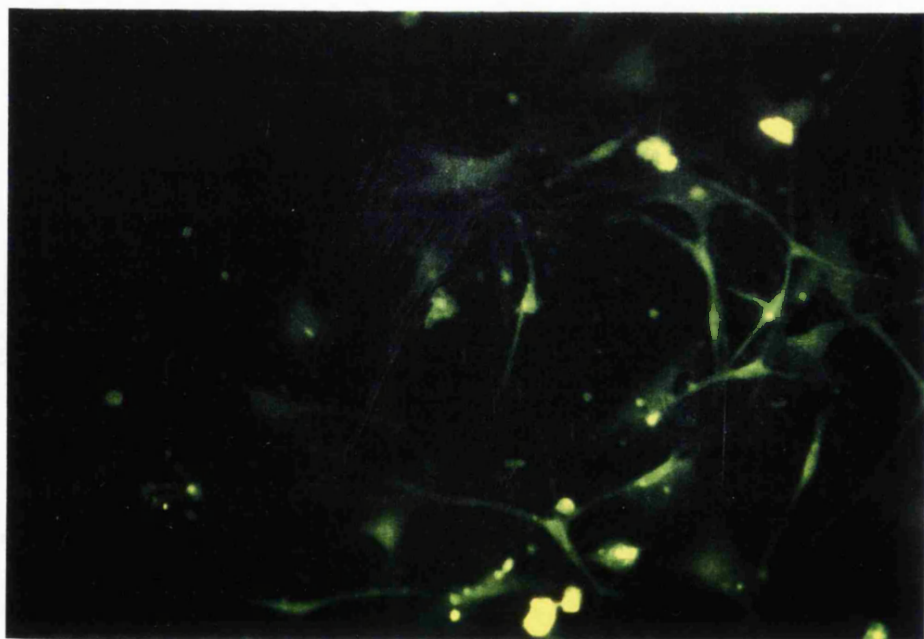


Plate 1. S100 immunostaining of cultures of mixed Schwann cells and fibroblasts derived from rat sciatic nerve.

Upper plate taken at higher power.

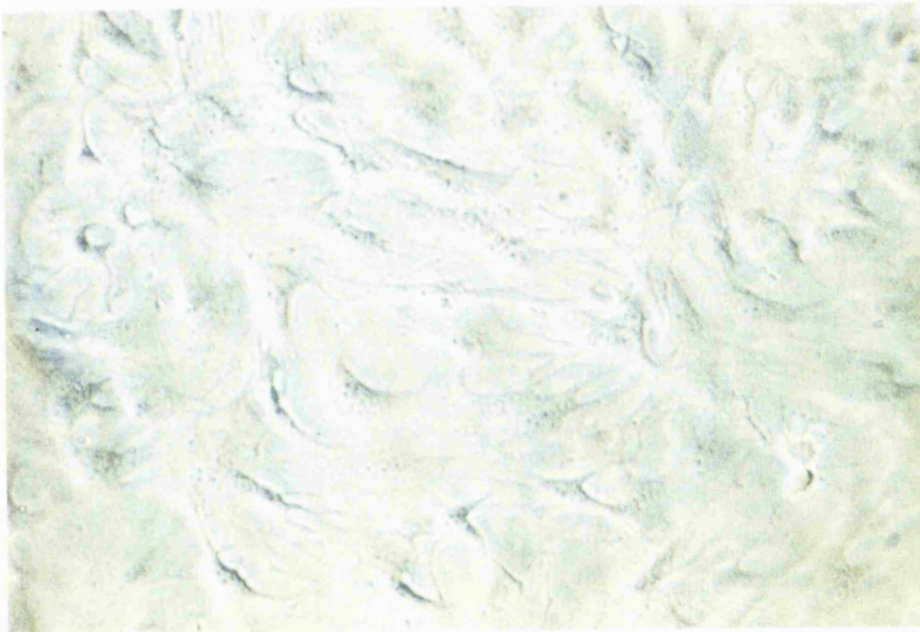
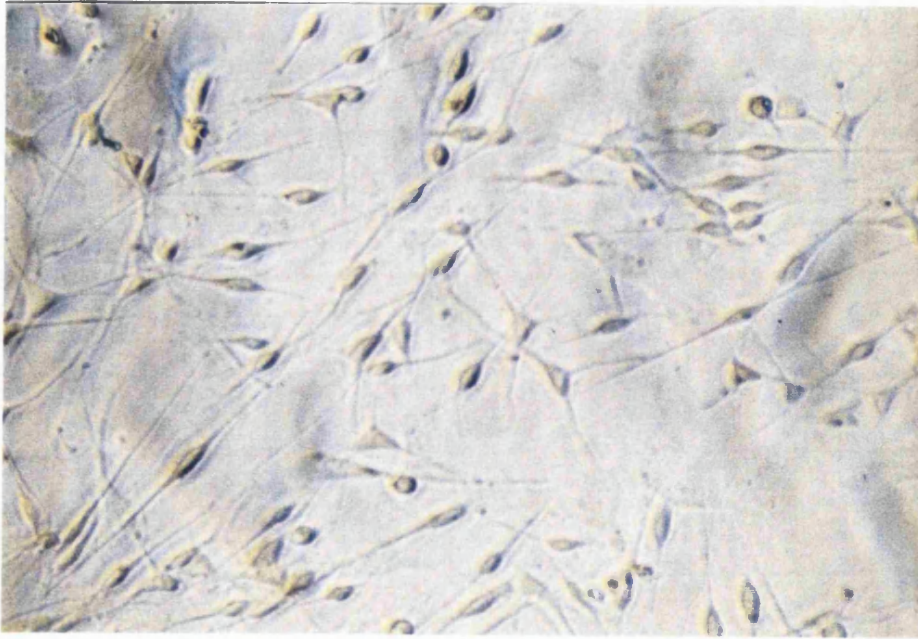


Plate 2. Comparative morphology of Schwann cell and fibroblast cultures, 4 days after dissociation.

Upper plate, Schwann cells and lower plate fibroblasts.



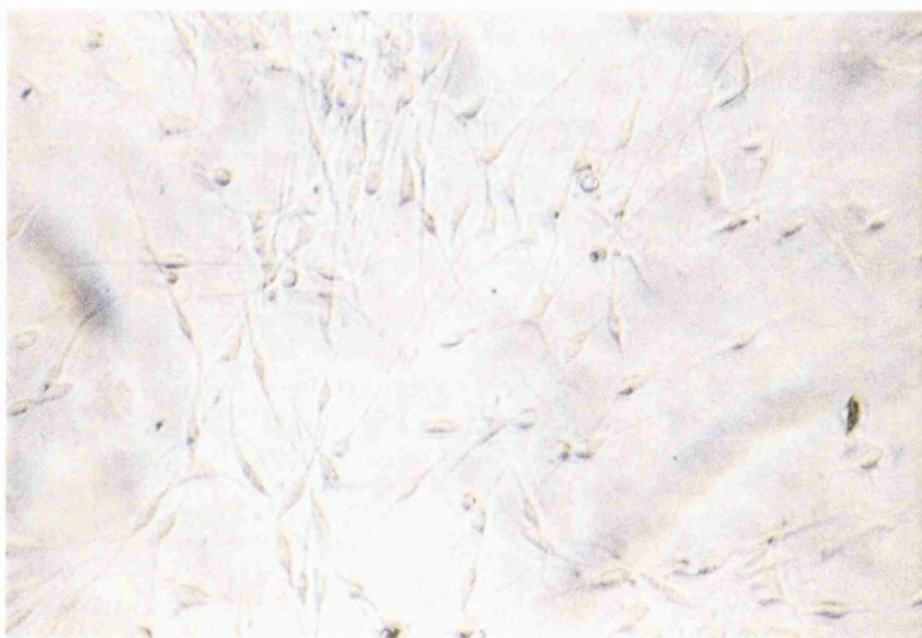


Plate 3. Pure Schwann cell cultures, derived by fibroblast and macrophage depletion.  
Upper plate taken at lower power.



## **.Error! Bookmark not defined.PANNING METHOD**

Panning is performed by applying cell suspensions serially to coated dishes. Cells are applied to individual dishes for 15-30 minutes in culture medium at 37°C in a CO<sub>2</sub> gassed incubator and agitated vigorously at 10 minute intervals to prevent non-specific binding. After a final vigorous agitation, the medium containing non-adherent cells is transferred to the next dish. This is monitored microscopically and cells transferred to the next series of dishes when no further adhesion of cells is seen for a particular antibody and that cell type may be regarded as maximally depleted. This is most critical where Schwann cells are being purified solely by depletion of other cell types. Dishes with adherent cells are washed three or more times, under microscopic control, until all unbound and loosely attached cells are removed. Adherent pure cells are harvested immediately by trypsinisation (0.125 % trypsin in dissociation buffer for 10 minutes at 37°C) and squirting cells off the dish with a 1ml Pipetteman. Anti-trypsin or 10% FCS is added and cell suspension transferred to a 15ml conical tube. Cells are collected by centrifugation at 1600rpm for 10 minutes, resuspended in culture medium and following counting are plated into 96 or 24-well primaria tissue culture plates (falcon) for use. To avoid contamination of cultures with exogenous growth factors, cells are washed several times in defined medium when adherent and assays performed without added serum, except where stated. This technique allows assays of cell function to be performed within 24 hours of dissociation from the nerve.

## **CELL CULTURE**

For mitogen and cAMP assays cells were grown in Falcon Primaria 96- or 24-well plates at a density of 5-10,000 cells per 96-well or 50-100,000 per 24-well, respectively. For NGF assays cells were plated at 250-500,000 per well in 24-well plates.

In all cases cells were grown in 1:1 Dulbeccos modified Eagles medium (DMEM) and HAM F12 (Flow) with either 10% FCS or modified rat Sato's defined medium (transferrin 50µg/ml; progesterone 60µg/ml; putrescine 1.6µg/ml; insulin 5µg/ml; thyroxine 0.4µg/ml; selenium 100µg/ml; triiodothyronine 0.1µg/ml; BSA 0.3mg/ml) and supplemented with penicillin 100 IU/ml; glutamine 2mM; streptomycin 100µg/ml and amphotericin except where stated. Schwann cell cultures were variously grown on uncoated, laminin (2µg/cm<sup>2</sup>) (Sigma) or PDL (1mg/ml) (Sigma) coated wells as stated. Cells were allowed to settle overnight following plating prior to experimentation. Medium was changed regularly and always prior to commencement of experiments.

### cAMPi ASSAYS

Cells were grown in 24-well dishes for no longer than 24 hours in defined medium before assays. Preincubations with IBMX, 2mM (Sigma); theophylline, 0.5mM (Sigma); rolipram, 10 $\mu$ M; phosphoramidone, 100nM (Sigma); where used, were commenced at least 30 minutes before addition of CGRP (Bachem) or Isoprenaline (Sigma). Preincubations with pertussis toxin, 1 $\mu$ g/ml (Sigma); H-7, 10mM (Calbiochem); staurosporine, 10nM (Sigma) or the protein kinase A inhibitor H-89, 30 $\mu$ M (calbiochem) ,where used, were commenced at least three hours before addition of CGRP or Isoprenaline.

For acute dose-response studies, CGRP or isoprenaline were added to wells for 10 minutes, except where stated. Reactions were stopped by rapid simultaneous replacement of culture medium with ice-cold 80% ethanol and cAMP extracted by a subsequent incubation for a minimum of 2 hours at -20°C. This extraction buffer was then aspirated, transferred to clearly labelled Eppendorfs and dried by vacuum spinning using a Speed Vac SC100 (Savant). Recovery of cAMP of greater than 90% was achieved by this method. The resultant cAMP containing pellet was resuspended in assay buffer and cAMP content for each sample measured in duplicate using the, more sensitive, acetylation method of the dual range cAMP competitive radioimmunoassay kit (Amersham). Samples of cAMP were incubated with a <sup>125</sup>I radiolabelled cAMP standard and could thus compete for added anti-cAMP antibody. Antibody bound cAMP was then precipitated as a pellet using a conjugate antibody linked to a magnetic bead. The amount of bound labelled cAMP tracer, inversely related to the amount of sample cAMP present, was determined using a  $\gamma$ -counter (Nuclear enterprises NE1600), and could be compared to known cAMP standards.

### MITOGEN ASSAYS

Schwann cell division was measured from the incorporation of [5 -<sup>125</sup>I] 2-deoxy-uridine (<sup>125</sup>IdU) (Amersham) into newly synthesised DNA in pure Schwann cells. Schwann cell division could thus be measured in microwells, speeding up assay times and allowing a greater number of conditions to be simultaneously investigated, compared to methods requiring microscopic cell counting, such as BrdU labelling. Cells were cultured in 96-well Primaria plates, in defined or serum-containing medium. Fibroblasts were grown on uncoated dishes, Schwann cells on laminin (2 $\mu$ g/cm<sup>2</sup>), except where stated, at densities of 5000 and 10,000 per well respectively. After plating, cells were left overnight, before gentle washing and replacement of medium. Drug incubations were performed over 48 hours with addition of agents at 0

and 24 hours. Enzyme inhibitors were preincubated for at least 3 hours prior to the start of stimulations.  $^{125}\text{I}$ IdU at  $2\mu\text{Ci/ml}$  was added for the final 24 hours of the assay. Following washes, cells were harvested onto glass fibre filter mats and DNA synthesis determined by measuring incorporated radioactivity by  $\gamma$ -counting.

To confirm that increased DNA synthesis was actually followed by cell division, mitogen experiments were repeated using a non-radioactive CellTitre cell proliferation kit (Promega). Cells were plated and handled exactly as previously described. MTT, a tetrazolium salt, was added for the final 4 hours in culture, to allow conversion to a blue formazan reaction product by cell mitochondrial enzymes. Cells were then solubilised and viable cell number determined by measuring absorbance at  $570\text{-}630\text{\AA}$  in an ELISA plate reader. The reaction product, and hence O.D., is directly proportional to the number of viable cells present, and in control experiments there was a linear correlation with the number of cells plated as measured by haemocytometer.

#### NERVE GROWTH FACTOR ASSAY

Cells were prepared as previously described, by panning, and were immediately plated in Falcon Primaria 24 well plates. For fibroblast cultures, plates were not coated with substrate. For Schwann cell cultures, all plates were coated with laminin at  $100\mu\text{l}$  of a  $10\mu\text{g/ml}$  solution per well for 30 minutes at  $37^\circ\text{C}$ . Cells were plated at  $4\times 10^5$  per well for Schwann cells and  $5\times 10^4$  for fibroblasts. Medium in all cases consisted of a 1:1 mixture of DMEM and HAM F-12 supplemented with penicillin, streptomycin and glutamine, and either 10% FCS or the rat defined medium and insulin mixture as previously described. Prior to the start of experiments, the volume of medium per well was kept at 0.5 ml. Cells were left overnight, before the medium was changed. For the experiment, serum free medium was added at 0.2ml per well, and final volume prior to assay kept at 0.225ml, to allow determination of basal unstimulated NGF levels in the assay without using a concentration step. Cells were kept in humidified boxes, in a gassed incubator at  $37^\circ\text{C}$ . Additions were made to cultures at 0 and 24 hours, after a further 24 hours medium was collected for assays. All additions were made in serum free medium and volumes were kept constant by addition of medium with carrier, if used, to control wells. Cell free wells receiving additions were used as further assay controls to exclude any immunoreactivity of various additions.

NGF was assayed using a modified two-site sandwich immunoassay (Korsching and Thoenen, 1983). Linbro 96-well rigid EIA plates (ICN) were coated with an anti-mouse- $\beta$ -(2.5S)nerve growth factor antibody (Boehringer Mannheim). This was

added at 0.5µg/ml in coating buffer at pH 9.6 at 120µl per well overnight at 4°C. 100µl of coating buffer containing 2% albumen was then added for 2 hours at room temperature, to reduce subsequent non-specific binding. The plates were then washed three times, following aspiration of coating solution, in albumin free wash buffer containing 0.2% Triton X-100 at pH 7.0 immediately prior to the addition of samples and standards.  $\beta$ -(2.5S) nerve growth factor standards (Boehringer Mannheim) were made up in serum free medium at doubling concentrations from 1pM- 0.5nM. Samples and standards were added at 100µl per well, and prior to addition received 11µl of 10x extraction buffer, consisting of 2% albumin, 70g/ml aprotinin, 40mmol/l EDTA, and 0.5% Na-azide in Tris-HCl, 50mmol/l; NaCl, 200mmol/l at pH 7.0. Samples and standards were then incubated overnight at 4°C. Washes were repeated three times following aspiration of solutions. Anti-mouse- $\beta$ -(2.5S)nerve growth factor- $\beta$ -gal (Boehringer Mannheim) was then added to the wells at 0.1U/ml in 100µl of conjugate buffer for 4 hours at 37°C. Conjugate buffer consisted of Tris HCl, 50mmol/l; NaCl, 200mmol/l; CaCl<sub>2</sub>, 10mmol/l; albumin, 1%(w/v); triton X-100, 0.1%(w/v) and Na-azide, 0.01%(w/v); pH7.0. Triplicate washes were again performed and then substrate added. The substrate used was chlorophenol red- $\beta$ -D-galactopyranoside (Calbiochem) at 40mg in 20 ml of substrate buffer consisting of Hepes, 100mmol/l; NaCl, 150mmol/l; MgCl<sub>2</sub>, 2mmol/l; Na-azide, 0.1%; albumin, 1%; pH 7.0. This was added at 120µl/well. Absorbance was then measured at 2 hours and at subsequent 1 hour intervals at 570-630λ against substrate solution until all standard solutions were clearly readable against blanks.

## SCIP ASSAYS

### STIMULATIONS

For SCIP assays, cultures of pure Schwann cells, approximately 500,000 cells per dish, were prepared by O4 +ve immunoselection by panning, as previously described. Having an excess of Schwann cells in the supernatant, dissociating 30 sciatic nerves for 6 dishes, and applying equal aliquots to 100mm panning plates in parallel, ensured there was less than 5% variability in cell numbers per plate, as determined by both MTT assays (conducted in dishes) and haemocytometer counting following trypsinisation.. Cells were allowed to incubate on panning dishes overnight in defined medium before commencing stimulations. For stimulations, medium was replaced and CGRP or forskolin added for 24 hours. To minimise desensitisation, medium and additions were exchanged 4 times at 4 hour intervals, with a 30 minute addition free period between each, during the day and a final 8 hour incubation overnight prior to extraction of SCIP. Each condition was duplicated, and samples pooled for assay. These experiments were repeated on separate occasions to validate results

## EXTRACTION OF NUCLEAR PROTEINS

For extraction of SCIP, dishes were first washed in 10 ml of ice-cold PBS. Cells were next scraped in 1ml of ice-cold PBS, using a glass rod and rubber policeman, and transferred to an Eppendorf tube. Cells were pelleted for 15 seconds in a microfuge at 4°C, the supernatant aspirated, and the pellet resuspended, by gentle pipetting, in 400µl of cold buffer A (10mM HEPES pH 7.9; 10mM KCl; 0.1mM EDTA; 0.1mM EGTA; 1mM DTT and protease inhibitors 0.5mM PMSF; 0.5 µg/ml leupeptin; 0.7 µg/ml pepstatin; 40µg/ml bestatin and 1µg/ml aprotinin). These were then incubated on ice for 15 minutes before addition of 25µl of 10% NP-40 solution and vigorous vortexing for 10 seconds. Eppendorfs were microfuged for 30 seconds at 4°C, and the resultant nuclear pellet resuspended in 50µl of ice-cold buffer C (20mM HEPES pH 7.9, 0.4 M NaCl; 1mM EDTA; 1mM EGTA and 1mM DTT with protease inhibitors). Resuspension of this pellet required a combination of vigorous pipetting and vortexing for 15 seconds. The mixture was then vigorously rocked for 30 minutes on a shaking platform at 4°C. Eppendorfs were well sealed and taped securely to the platform with masking tape. Nuclear debris was then removed by spinning in a microfuge for 5 minutes at 4°C. Supernatants were stored at -70°C until assayed.

## SCIP WESTERN BLOT

### SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Stacking and resolving gels were cast. The resolving gel (20 ml at 12%) consisted of 7.9 ml of 30% protogel acrylamide mix; 2.5 ml of resolving mix; 0.2 ml of 10% SDS; 9.2 ml H<sub>2</sub>O; 0.2 ml of 10% APS and 20 µl of TEMED. The stacking gel, which was warmed, (10ml at 5%) consisted of 1.7 ml of protogel; 2.5 ml of stacking mix; 0.1 ml of 10% SDS; 5.6 ml of H<sub>2</sub>O; 0.1 ml of APS and 10 µl of TEMED. In both cases the APS and TEMED were added just before pouring to run the polymerisation. The resolving gel was poured first and butanol layered on top 1:1 with resolving buffer. This was rinsed off, after the gel set, the stacking gel poured and combs added to create wells.

Nuclear extracts were run on the SDS gels. Before loading, samples were boiled for 3 minutes in 1x sample buffer (50mM Tris.Cl (pH 6.8); 100mM dithiothreitol; 2% SDS; 0.1% bromphenol blue and 10% glycerol). 50µl of sample was then added to each well in the gel. Markers were also added and an extract from a BBB Schwannoma line as a positive control. Gels were run at 15V/cm in electrophoresis buffer (25mM Tris; 250mM glycine (pH 8.3); 0.1% SDS), until the dye front reached the bottom. Samples were transferred to Immobilon P, nitro-cellulose paper, membranes by semi-dry electrophoretic transfer. Membranes were wet in MeOH, immersed in water for 2 minutes and transfers performed with 0.8mA/cm<sup>2</sup> for 60-90

minutes, in transfer buffer (48mM Tris; 39mM glycine; 0.037% (v/v) and 20% MeOH).

#### IMMUNOLABELLING OF BLOTS

Blots were then incubated in blocking buffer (5%(w/v) non-fat dry milk containing; 0.2%(v/v) TWEEN 20; 0.02% Na-azide and 1xPBS). Blots were washed 7 times for 15 minutes each in wash buffer (1xPBS and 0.2%(v/v) TWEEN 20). Next, to conserve antibody stocks, blots were sealed in plastic bags with affinity purified rabbit anti-SCIP antibody (Gift from G.Lemke) at 1:100 in 3ml of blocking buffer overnight at room-temperature on a slow rotating wheel. Blots were then washed in wash buffer 7 times for 15 minutes each. Peroxidase conjugated swine anti-rabbit antibody (Dako p217) was next added at 1:1000, in blocking buffer, for 1 hour at room temperature. Washes were repeated as previously described. Blots were then developed using an Enhanced chemi-luminescence kit (Amersham). Equal amounts of solutions A and B were mixed and added to the blot in a dark room for 1 minute. Blots were drained, wrapped in saran wrap and exposed to film (Amersham x-ray film) in a sealed cassette. In general reactions were so strong that a few seconds of exposure to film was sufficient and remained detectable by brief film exposure, inspite of fading, for several hours.

## RESULTS

### SUMMARY

In this chapter, I show that CGRP is concentrated at the injury site and at the regenerating nerve front, by axonal transport *in vivo*. CGRP elevates Schwann cell and fibroblast cAMPi *in vitro*, and increases fibroblast NGF synthesis. CGRP inhibits fibroblast proliferation, via elevation of cAMPi. The action of CGRP on Schwann cells is limited by the rapid attenuation of cAMP elevation. This desensitisation does not involve  $G_i$  or PDE, but is mediated by PKA. Desensitisation to  $\beta$ -adrenergic agonists is mediated by  $\beta$ ARK-like activity. Improving receptor-effector coupling by increasing extracellular  $Mg^{++}$ , limits cAMPi attenuation, and unmasks a permissive action of both CGRP and isoprenaline on Schwann cell proliferation. Schwann cells metabolise CGRP via a phosphoramidone sensitive enzyme, which may limit desensitisation *in vivo*. This degrading enzyme is likely to be the surface endopeptidase, enkephalinase (EC.24.11). CGRP may mediate axonal influences on non-neuronal cells after axotomy *in vivo*, particularly at the regenerating nerve front. By promoting Schwann cell proliferation and inhibiting fibroblast proliferation, as well as by stimulating NGF production, CGRP may play an essential role in regeneration.

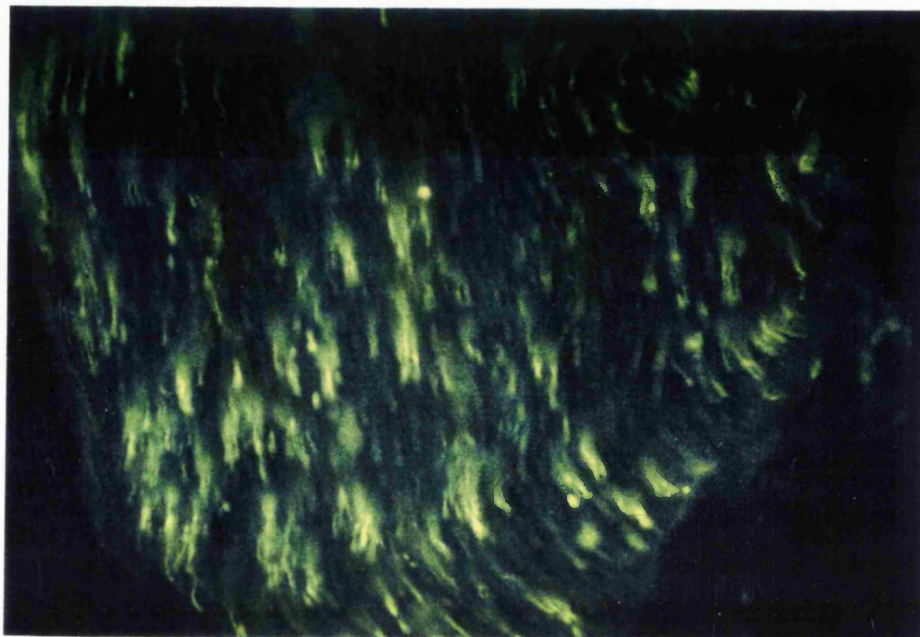
### CGRP IN REGENERATING NERVE

The distribution of CGRP in adult rat sciatic nerve following a cut or crush injury, was examined immunohistochemically and by radioimmunoassay. As shown in Fig.2a, there is a marked accumulation of CGRP proximal to and to a lesser extent distal to the nerve lesion, which occurs within 2 hours. Initially this is similar for both crushed and transected nerves. The linear, fibre-like staining pattern, suggests that this CGRP immunoreactivity is within axons (Fig.2b). A transient distal accumulation of CGRP occurs within hours of axotomy, and may be due to leakage of CGRP from damaged axons as well as anterograde transport. This distal accumulation declines over 24-48 hours unless reinnervation of the distal stump occurs (Fig.2c). The appearances of some CGRP-stained sections, suggested there might also be a more cellular distribution of CGRP-like immunoreactivity in the distal stump. However, in follow up experiments, where sections were examined under electron microscopy, no immunostaining was localised to Schwann cells or other resident non-neuronal cells (Fig.2f). Following transection, the proximal accumulation of CGRP is still present at 2 weeks (Fig.2d), but there is a loss of staining in the degenerating distal nerve by 48 hours. Following a crush injury, where reinnervation is permitted, CGRP immunostaining is increased both proximal and distal to the crush for 3 weeks post

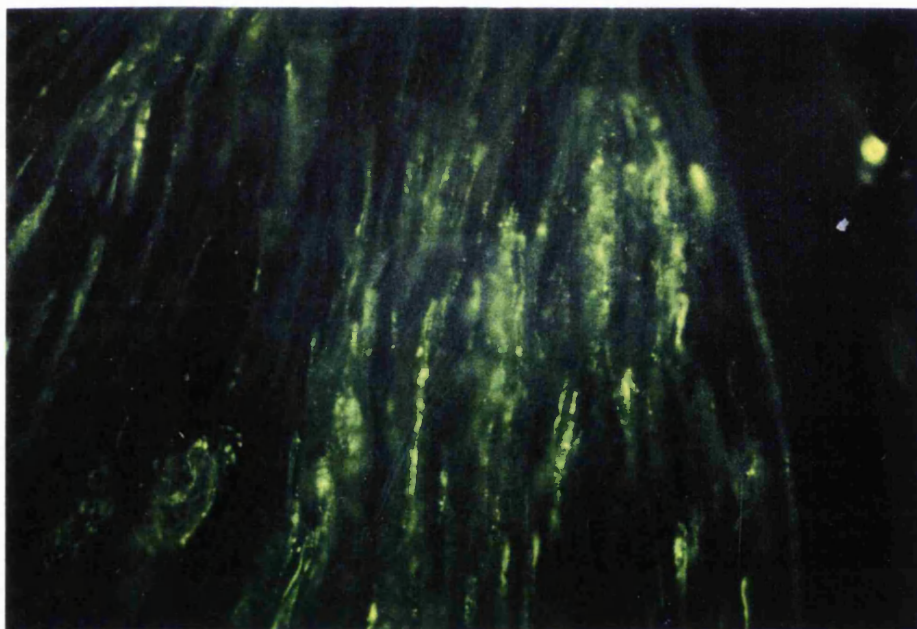
injury (the longest time course examined) (Fig.2e). It is likely that CGRP accumulation is initially due to antero- and retro-grade transport, and that thereafter CGRP seen distal to a crush injury is due to CGRP accumulation at the leading edge of regenerating axons, and not through any significant local synthesis within the distal nerve. CGRP present in crushed and control nerves has been quantified by radioimmunoassay, confirming the immunohistochemical findings (Bindemann and Mudge, unpublished results). The continued presence of high levels of CGRP at the regenerating nerve front, suggested that it might have a role in the regenerative process. Most neurotransmitters are downregulated during regeneration, with synthesis in the cell body largely confined to substances of value to axonal growth.



## PROXIMAL



## SITE OF LESION



## DISTAL

Fig.2a. Longitudinal sections of rat sciatic nerve, showing proximal and distal accumulation of CGRP, 24 hours after nerve crush. Orientation of sections is shown in red type.

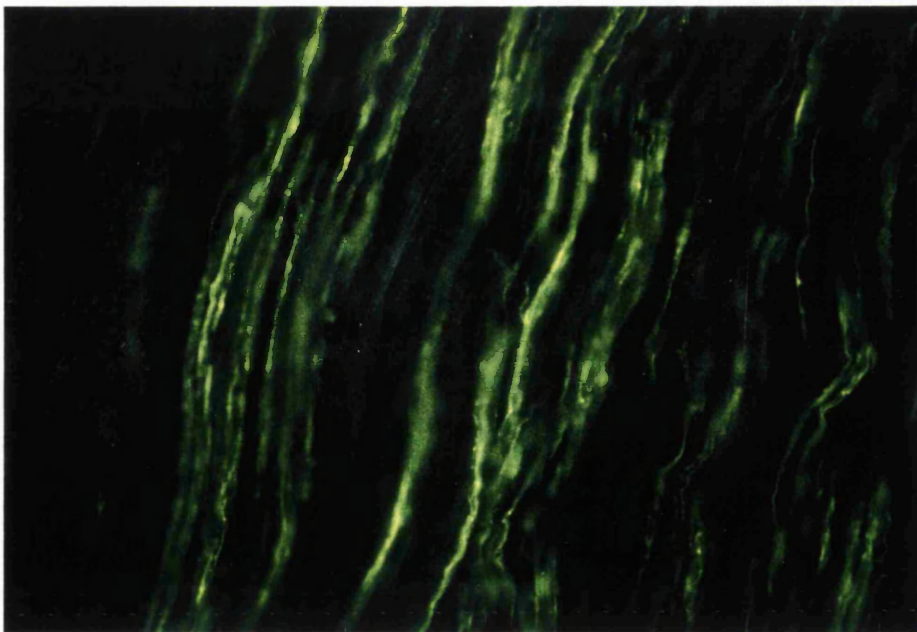
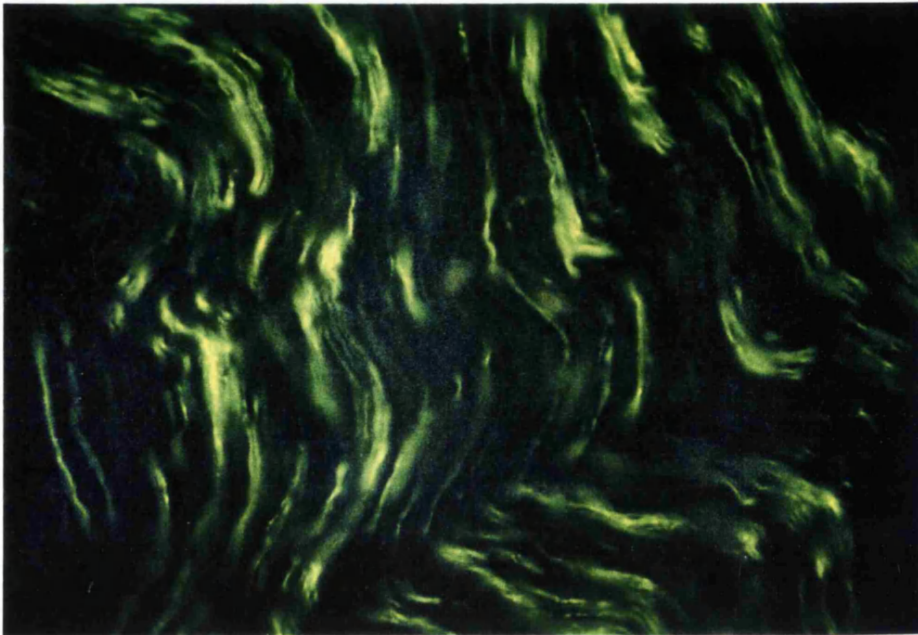
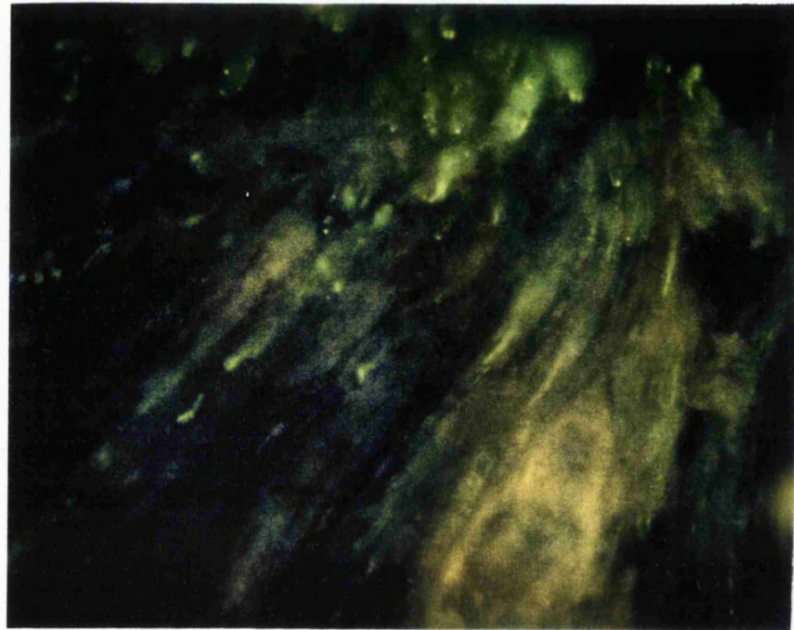


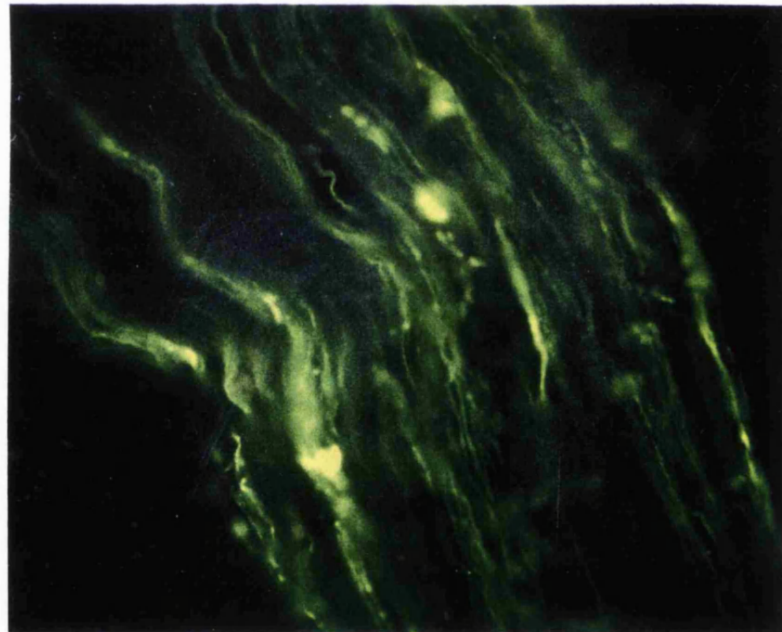
Fig.2b. Longitudinal sections of rat sciatic nerve, showing linear staining for CGRP proximal to the lesion site.

## SITE OF TRANSECTION



## DISTAL

## PROXIMAL



## SITE OF INJURY

Fig.2c. Longitudinal sections of rat sciatic nerve, showing loss of CGRP immunostaining distal to the lesion, unless reinnervation is allowed.

The top section shows the distal stump 48 hours after transection, the bottom section shows the proximal and distal sides around the crush lesion, 7 days after injury.



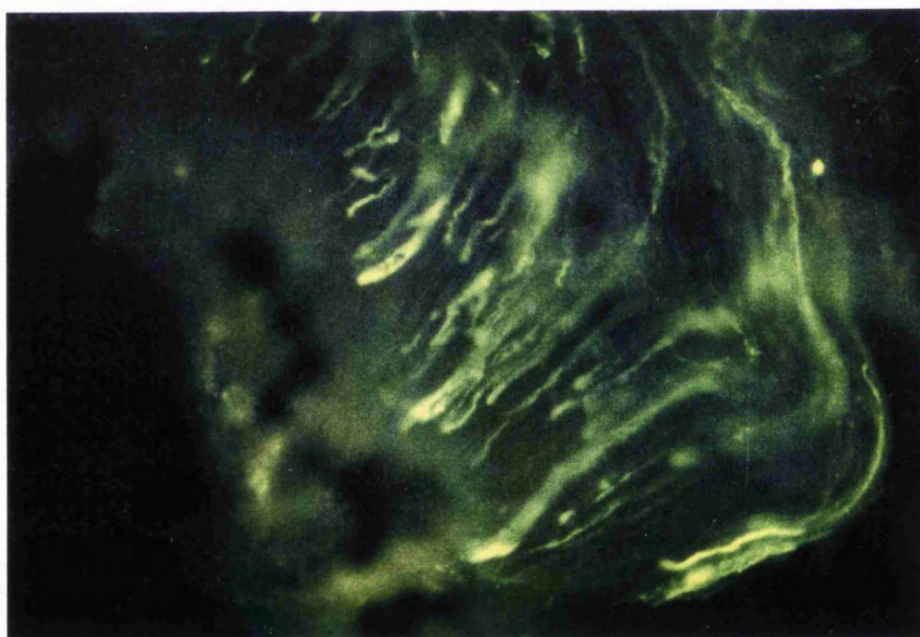
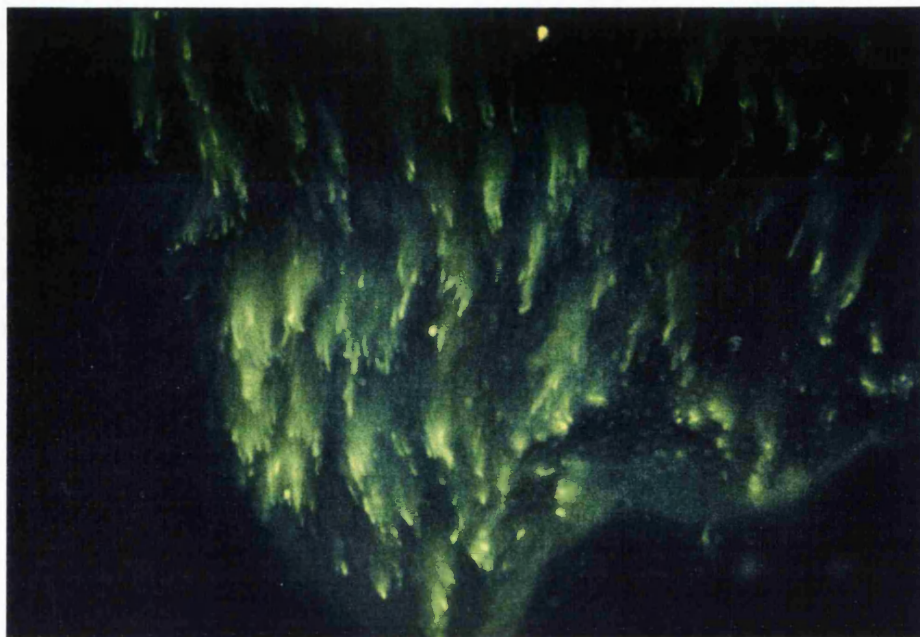


Fig.2d. Longitudinal sections of rat sciatic nerve, showing continued presence of CGRP immunostaining at the proximal stump nerve front, at 7 and 14 days after nerve transection.

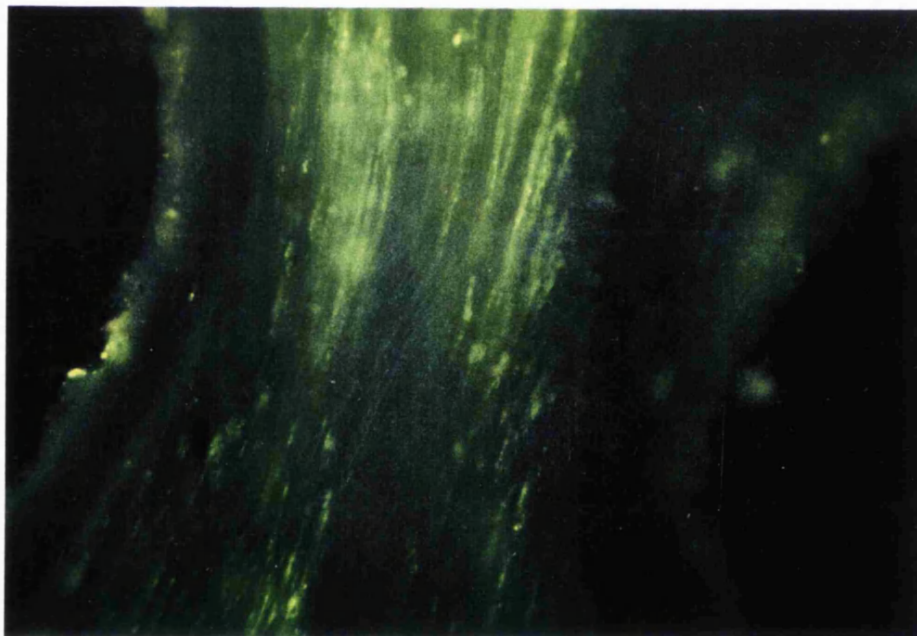
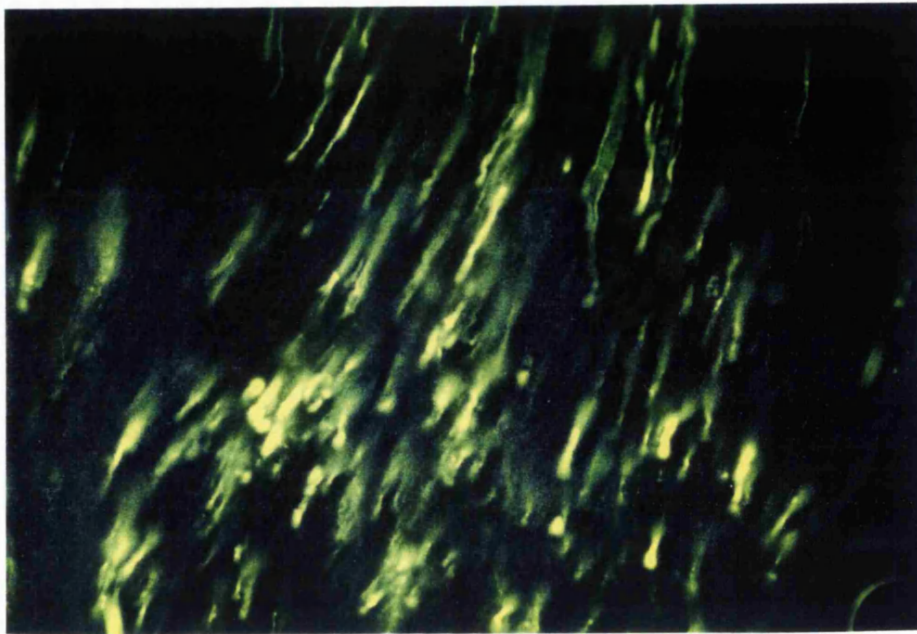


Fig.2e. Longitudinal sections of rat sciatic nerve, showing continued presence of CGRP immunostaining at the regenerating nerve front, at 7 and 14 days after nerve crush, allowing regeneration into the distal degenerating nerve.

## CGRP AND cAMPI

The effect of CGRP on cAMPI elevation and DNA synthesis of cultured non-neuronal cells derived from rat sciatic nerve, was examined. For these studies we prepared cultures of pure Schwann cells or fibroblasts using a novel adaptation of immunoselection by panning (Barres et al., 1988).

CGRP was added to cultures for a 10 minute period following which cAMPI levels were determined. CGRP stimulates a dose-dependent elevation of cAMPI in both Schwann cells (Fig. 3) and fibroblasts (Fig. 4b) *in vitro*, which is potentiated by the phosphodiesterase inhibitor isobutyl methyl xanthine (IBMX) (Fig. 4a,b).

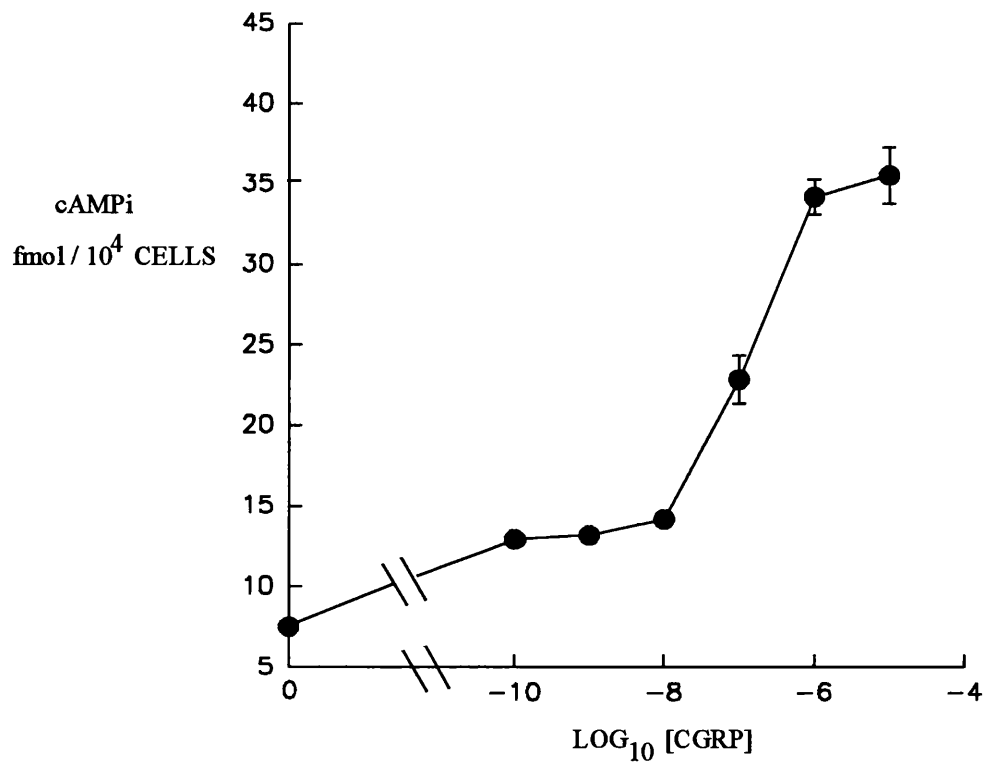


Fig.3. Dose response curve for CGRP stimulation of Schwann cell cAMPi.

Schwann cells were plated at 10,000 cells per well of a 24-well plate in serum-free defined medium, and were 24 hours old in culture at the time of assay. Cells were exposed to CGRP for 10 minutes, at varying doses, and cAMPi assayed. Values are mean  $\pm$  s.e. of triplicate wells. Where no error bars are shown, in this and subsequent figures, these fall within the confines of the point symbol.

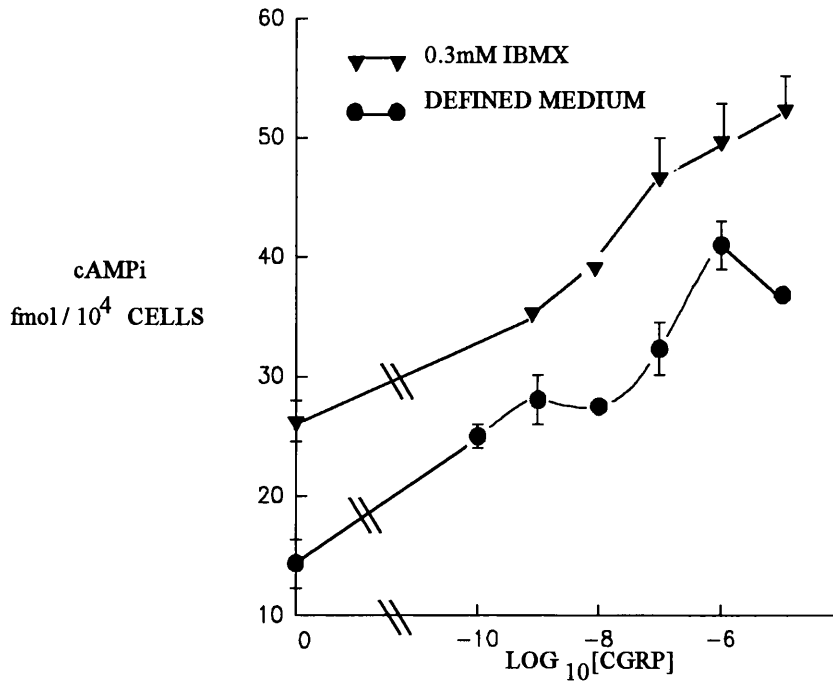


Fig.4a. Schwann cell cAMPi dose response to CGRP + / - IBMX

Schwann cells were plated at 10,000 cells per well of a 24-well plate in serum-free defined medium. Cells were exposed to CGRP for 10 minutes, at varying doses, and cAMPi assayed. IBMX, where used, was added 30 mins. before CGRP. Values are mean  $\pm$  s.e. of triplicate wells. Where error bars are shown with only positive or negative deflection this is to facilitate graphical representation.



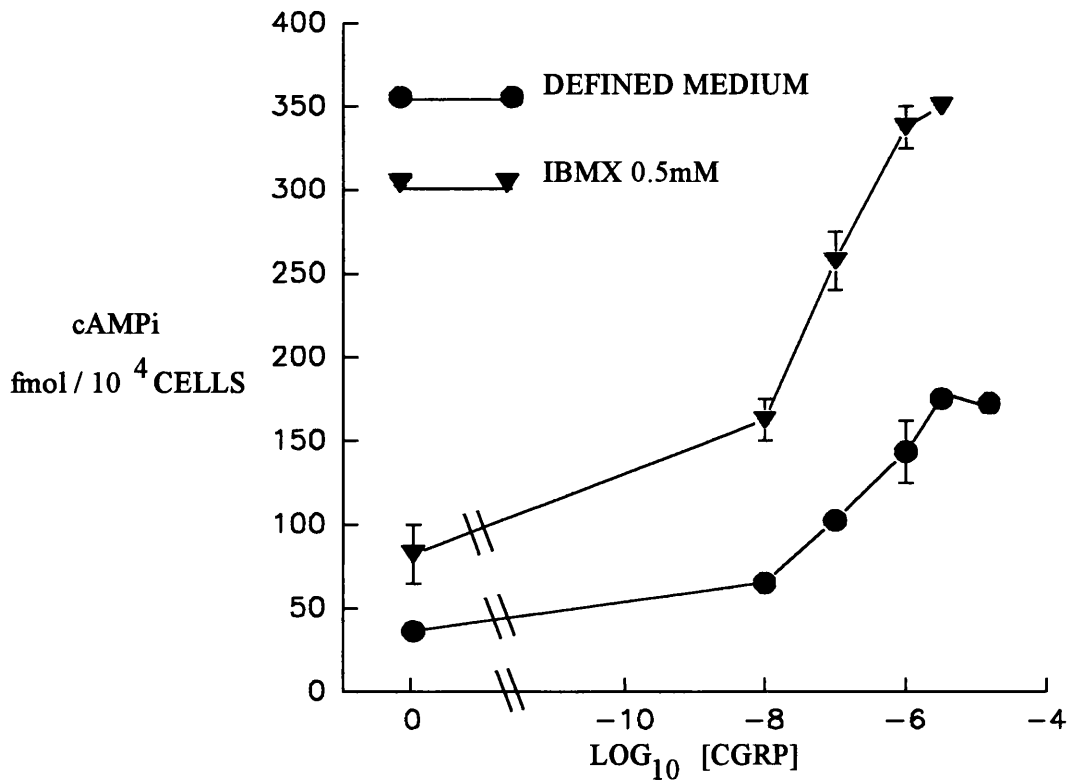


Fig.4b. Fibroblast cAMPi dose response to CGRP + / - IBMX

Endoneurial fibroblasts were plated at 10,000 cells per well of a 24-well plate in serum-free defined medium. Cells were exposed to CGRP for 10 minutes, at varying doses, and cAMPi assayed. IBMX, where used, was added 30 mins. before CGRP. Values are mean  $\pm$  s.e. of triplicate wells.

## SCHWANN CELL PROLIFERATION

Two methods were used to assess Schwann cell proliferation: the incorporation of  $^{125}\text{IdU}$  into newly synthesised DNA as a marker of replication, and a non-radioactive cell viability / proliferation assay based on the conversion of the tetrazolium salt MTT to a formazan-containing dye by the mitochondrial enzyme succinyl dehydrogenase. The MTT assay was used because criticisms have been levelled at techniques that measure only DNA synthesis as a marker of proliferation (Oliver et al., 1989); errors have arisen due to arrest of cells in S or G<sub>2</sub> phases of the cell cycle, whereby DNA synthesis progresses without subsequent cell division and proliferation. The MTT assay has been shown to correlate closely with cell number in many systems, but must also be interpreted with caution as it is dependent on the metabolic activity / viability of cells; a decrease in mitochondrial activity is an early sign of cell death by apoptosis.

Two distinct signals are required for Schwann cell proliferation. Firstly, a growth factor such as PDGF, FGF or IGF-I, and secondly, a permissive signal that elevates cAMPi. Forskolin, which elevates cAMPi via activation of adenylate cyclase, is widely used in experiments to elevate cAMPi.

Growth factor activity is mostly dependent on the activation of receptor tyrosine kinases, but may be mediated indirectly via phospholipase C $\gamma$  (PLC $\gamma$ ) followed by activation of PKC; in fact, activators of PKC can substitute for growth factors in many mitogen assays. The phorbol ester PMA is mitogenic for Schwann cells in defined medium but, as is the case with growth factors, only if cAMPi is elevated (Fig.5a). PDGF added to cultures already stimulated with PMA, in the presence of cAMPi elevation, promotes some additional activity (Fig.5a), but this is barely significant, unless PMA is used at submaximal doses (Fig.5b). Inhibiting PKC with either H-7 or staurosporine, prevents Schwann cell proliferation to mitogenic combinations such as PDGF and forskolin (Fig.5b). In cultures where basal proliferation was high, this was also inhibited. Schwann cell growth factor mitogens, such as PDGF, are known to act via receptor tyrosine kinases in other systems. Genistein, which inhibits tyrosine kinases, abolishes all Schwann cell mitogenic activity and leads to the rapid onset of cell death, probably by apoptosis. The activation of tyrosine kinase would therefore seem of prime importance in mediating growth factor action on Schwann cell proliferation and survival. Tyrosine kinases are not involved in the generation of cAMPi, and growth factors such as PDGF, do not elevate Schwann cell cAMPi. I suggest that PKC activation is at least one important downstream mediator of growth factor tyrosine kinase activation, which in itself is sufficient for promoting DNA replication if cAMPi is also elevated. It is not yet

possible to conclude that downstream PKC activation is sufficient to mediate the growth promoting activity of tyrosine kinases; where tyrosine kinases were inhibited with genistein, Schwann cells failed to proliferate and died, and no survival action / increase in cell number could be shown with phorbol esters, but DNA incorporation was increased above controls in the presence of both PMA and forskolin. Further experiments are required to distinguish whether tyrosine kinases promote cell survival and proliferation by distinct signalling systems. PKC seems sufficient to stimulate proliferation in forskolin-treated cultures, but may not be sufficient to prevent apoptosis. Inhibition of PKA prevents the permissive action of forskolin (Fig.5b), suggesting that PKA is important for enabling growth factor activity, and probably mediates the action of cAMPi on proliferation. Pertussis toxin which inhibits  $G_i$ , thereby increasing basal cAMPi levels, also enables the growth factor activity of PMA. Eventually, cultures incubated with inhibitory doses of any of these three classes of protein kinase inhibitors, die, probably via apoptosis; interestingly, only with tyrosine kinase inhibitors does this occur within 24-48 hours, whereas a longer time is required for other inhibitors.

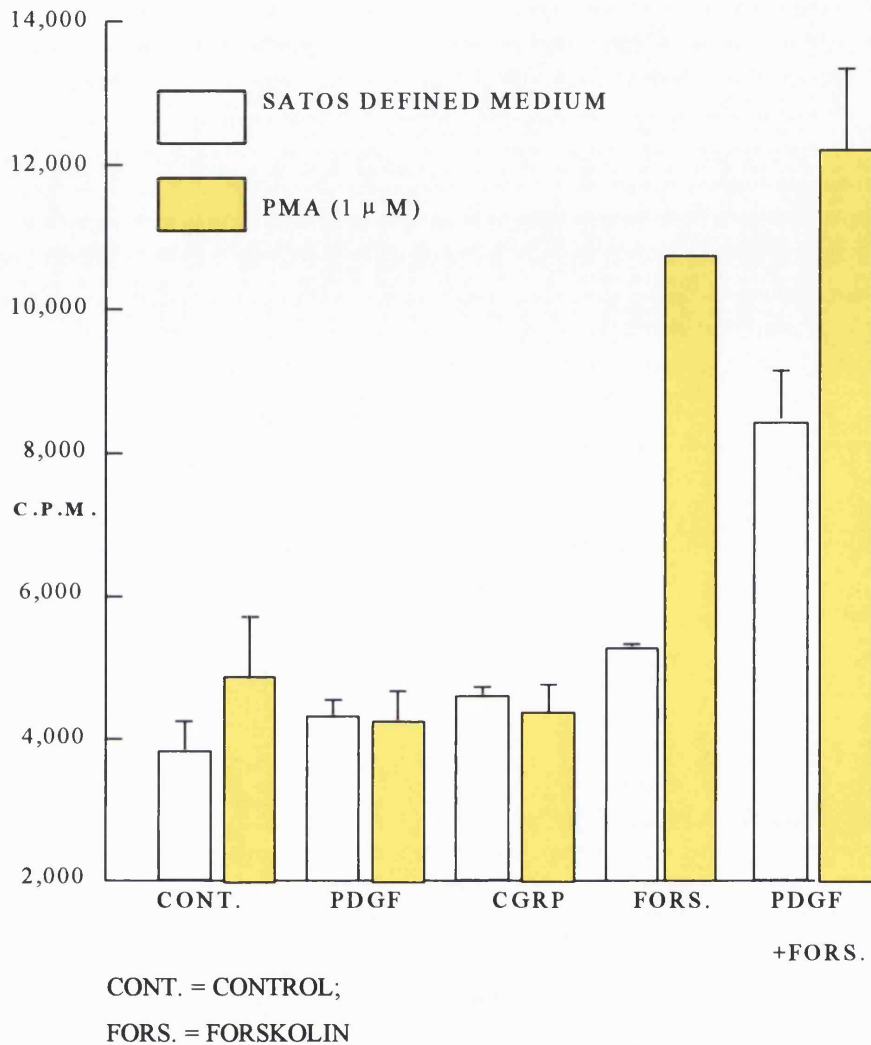


Fig.5a. Incorporation of  $^{125}$ IdU by pure cultured Schwann cells, in the presence or absence of PMA.

Cells were plated in serum-free defined medium, at 10,000 cells per well of laminin coated 96-well plates. Cells were 24 hours post-nerve dissociation at the start of incubations. Mitogens were added to cultures for a total of 48 hours, with additions repeated at 24 hours. Enzyme inhibitors and PMA were added 4 hours prior to mitogens. Values are mean  $\pm$  s.e. of triplicate cultures.

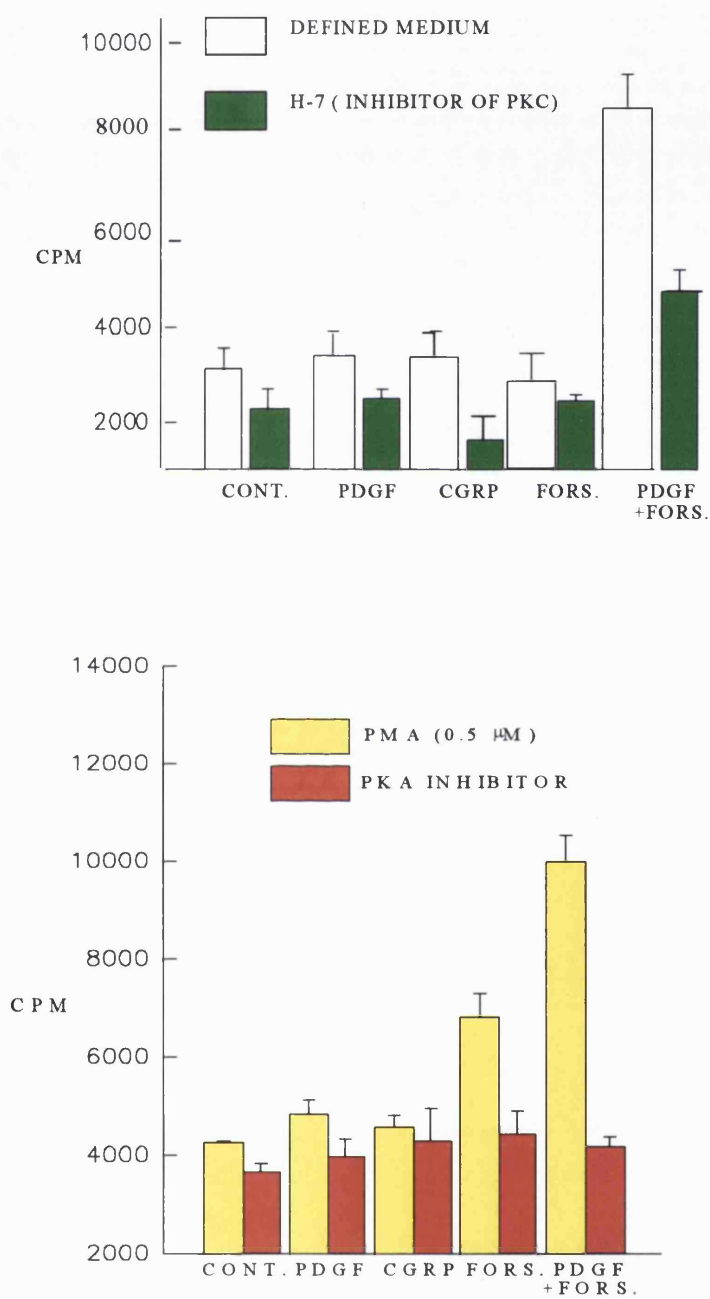


Fig.5b. Schwann cell incorporation of  $^{125}\text{IdU}$  in the presence of inhibitors of PKA or PKC.

Experiments were performed as in 5a.

It seems likely, therefore, that activation of PKA and both tyrosine kinases and PKC, are required for Schwann cell proliferation. Growth factor stimulation of DNA replication in Schwann cells, may require only PKC activation. It seems likely that PKC is activated via the tyrosine kinase activity of the growth factor receptor.

Other growth factors were investigated, and FGF was found to be mitogenic, whereas in our experiments TGF- $\beta$  was inhibitory on Schwann cell division, antagonising the proliferative effects of forskolin and PDGF in both MTT and  $^{125}\text{IdU}$  assays (Fig. 6a,b). Inhibition of proliferation was seen both in serum and in defined medium. TGF- $\beta$  is a weak mitogen and has synergistic action with forskolin in 10% FCS if cells are prepared by more traditional means (more than 7 days old in culture) (Davis and Stroobant, 1990).

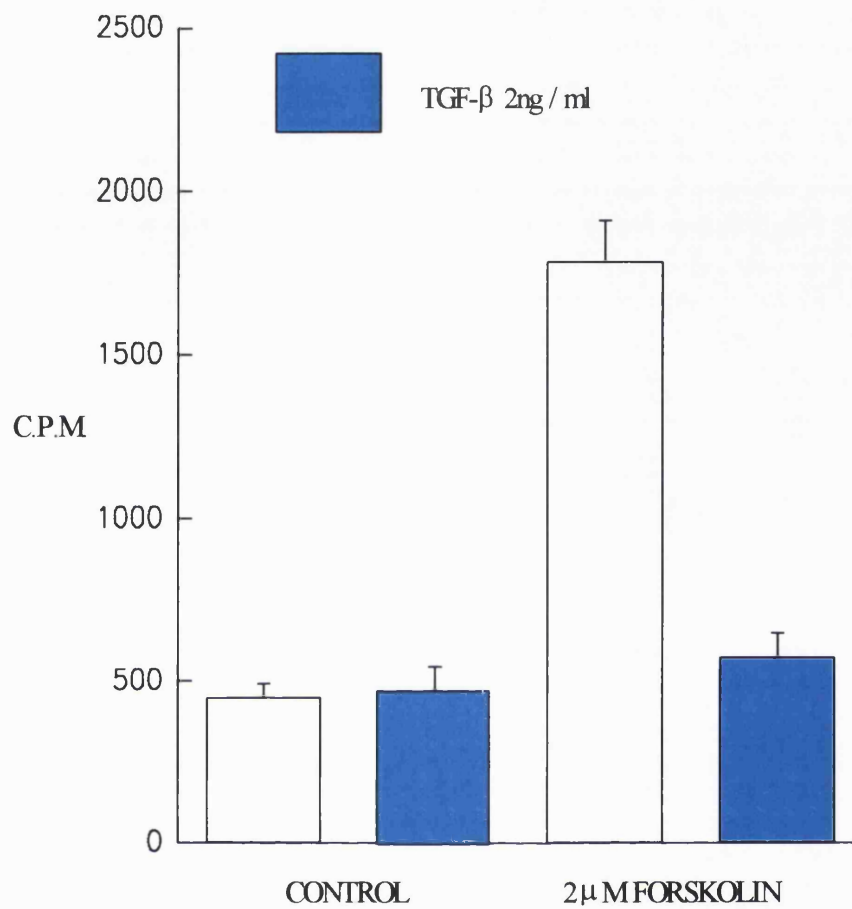


Fig.6a. The effect of TGFβ on Schwann cell incorporation of <sup>125</sup>IIdU.

Schwann cells were grown in DMEM and 10% FCS, at 10,000 cells per well of a PDL coated 96-well plate, and were 24 hours post-nerve dissociation at the start of incubations. Forskolin and TGFβ were added for 48 hours, and additions were repeated at 24 hours. Values are mean  $\pm$  s.e. of triplicate cultures.

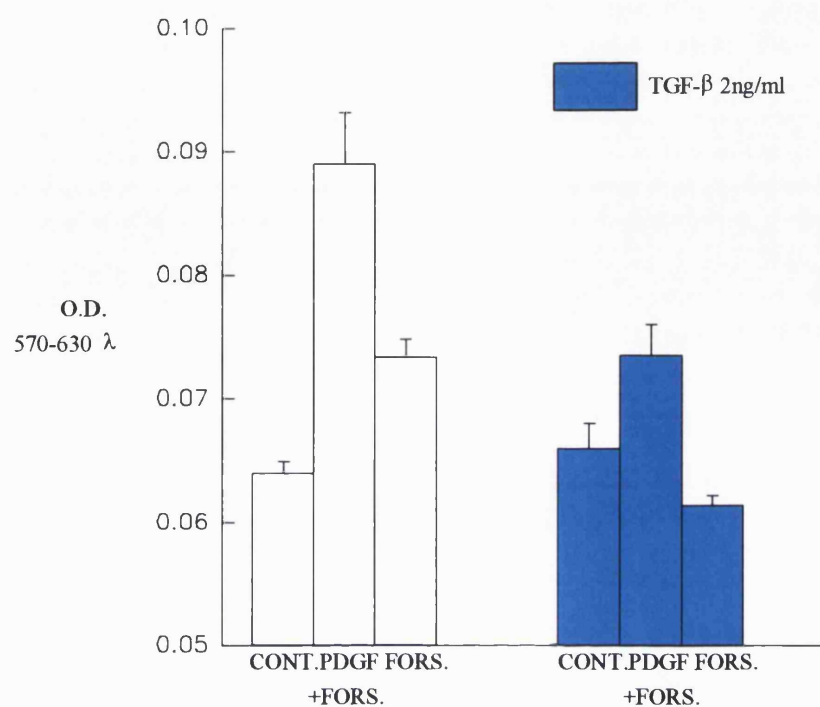


Fig.6b. The effect of TGF $\beta$  on Schwann cell proliferation, as assessed by MTT assay.

Schwann cells were plated at 10,000 cells per well of a laminin coated 96-well plate, and cultured in DMEM and 10% FCS. Cells were 24 hours old at the start of assays, and additions were made over 48 hours, following which cell number was determined by MTT assay. Values are mean  $\pm$  s.e. of triplicate cultures.



Experiments were now conducted to test the hypothesis that CGRP might be an endogenously occurring permissive factor for growth factor activity on Schwann cells. Although on some occasions CGRP was permissive for Schwann cell proliferation in 10% FCS and with PDGF in defined medium, on a number of occasions this was not seen. Forskolin was consistently permissive for proliferation in response to serum or PDGF. Plating efficiency was improved by using laminin as a culture substrate, but did not alter results obtained with CGRP. To further examine the relatively labile response of Schwann cells to CGRP as compared with forskolin, we plotted the time courses of cAMPi elevation for the two agents. As shown in Fig. 7, addition of  $10^{-6}$ M CGRP caused a marked 4-fold increase in cAMPi after 10 minutes, which diminished after 60 minutes, and had returned to baseline levels by 24 hours, even in the continuous presence of peptide. This contrasts with the results obtained over 24 hour incubations with  $10^{-5}$ M forskolin, where cAMPi levels declined gradually from an initial 10 minute peak to 50% of peak levels by 24 hours but were still elevated.

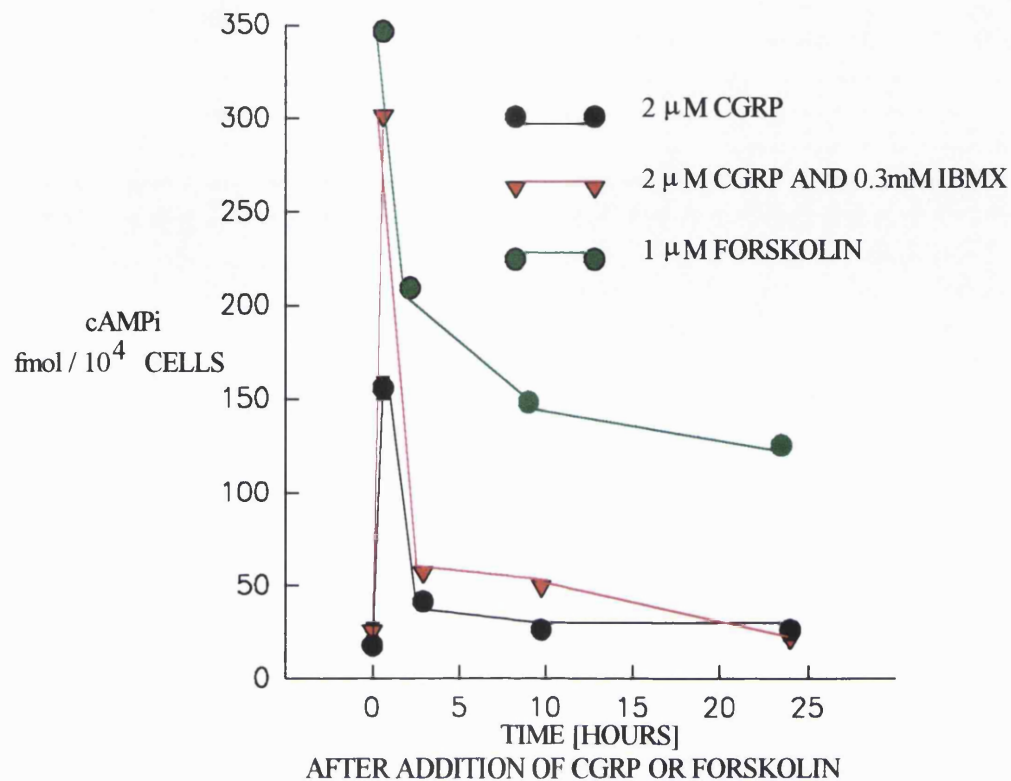


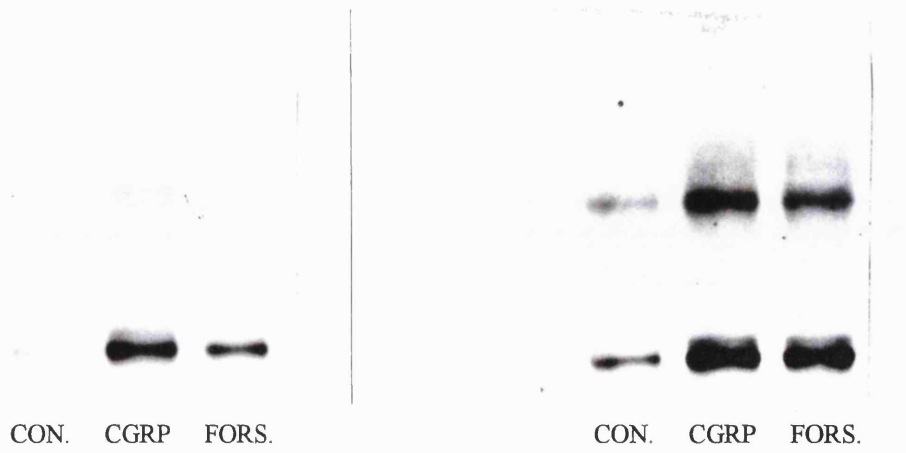
Fig.7. Timecourse of Schwann cell cAMPi elevation after CGRP or forskolin addition.

Pure Schwann cells, 24 hours following nerve dissociation, were cultured in defined medium at 30,000 cells per well of a 24-well plate, and IBMX added 1 hour prior to start of assays. Reactions were stopped by simultaneous replacement of medium with 70% ice cold methanol, and extracted cAMPi WAS measured by competitive radioimmunoassay. Values are mean  $\pm$  s.d. of duplicate cultures, and all assays were performed in duplicate.

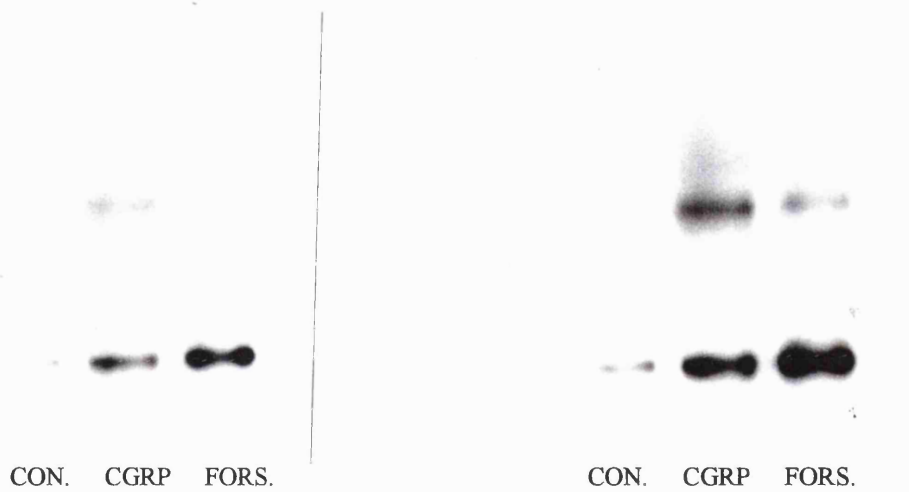
Forskolin (which directly activates adenylate cyclase) may bypass those intracellular regulatory mechanisms that CGRP (which acts via a membrane receptor) would be subject to. Loss of cAMPi elevation during the first few hours of an extended incubation, is typical of other receptor-linked adenylate cyclase systems, and reflects the development of receptor desensitisation. CGRP *in vivo* is likely to be released continuously at low concentration, and presented to Schwann cells at non-desensitising doses. In addition, mechanisms for removal of peptide may help maintain this. Variable responses in culture, could arise from experimental constraints that necessitate the use of relatively high doses of CGRP, which unfortunately promote rapid desensitisation. Incubating cultures with low concentrations of CGRP (less likely to induce desensitisation), unfortunately results in a relatively rapid loss of cAMPi inducing activity in the medium, as determined by transfer of medium to fresh cultures, suggesting that CGRP is being actively degraded, or lost by adsorption or sticking to plastics or substrates. Only at initial micromolar concentrations was there still significant cAMPi inducing activity left in the medium after 24 hours. Attempting to avoid desensitisation to CGRP as well as losses of peptide activity from the medium, by delivering intermittent pulses of CGRP at low concentration, with replacement of medium to avoid incremental rises in peptide concentration, inevitably led to cell losses. These cell losses resulted in unacceptably high levels of variability between replicate cultures, even for PDGF and forskolin combinations, making analysis unreliable.

Cells firmly bound to panning dishes remain adherent during washes. Administering pulses of 100nM CGRP (with washes and replacement of medium) to Schwann cells on 100mm panning dishes, enabled us to investigate whether, under these conditions, some cell responses downstream of cAMPi elevation could be promoted. In fact, CGRP is able to increase levels of the POU-domain protein, suppressed cAMP-inducible POU (SCIP), in cultured Schwann cells when administered in pulses. This action is mediated by elevated cAMPi, and shared by forskolin (Fig 8). As previously shown in Western blots immunostained with a SCIP antiserum (Collarini et al., 1991), a second specific band is visible.

These results suggested that a more prolonged elevation of cAMPi is required for Schwann cell responses, particularly a permissive action on growth factor-mediated proliferation. Further experiments were therefore performed to investigate the contribution of peptide degradation and desensitisation in regulating CGRP activity.



Two exposures of the same Western blot. The longer exposure on the right shows a second band.



Two exposures of the same Western blot. The longer exposure on the right shows a second band.

Fig.8. SCIP Western blots on CGRP- and forskolin- stimulated Schwann cells.

## DESENSITISATION OF RESPONSES TO CGRP

We determined whether biologically-active CGRP remained in medium incubated for 24 hours with Schwann cells. Following incubation, medium with or without added 100nM CGRP or forskolin, was transferred to fresh test cells and cAMPi measured after 10 minutes. Elevation of cAMPi by this incubated medium in test cells, was compared with fresh stock peptide. CGRP-containing medium was less effective in increasing cAMPi following a 24 hour preincubation with Schwann cells, demonstrating that CGRP may be significantly degraded in this period (Fig 9). Forskolin was as effective as fresh equimolar stock. Part of the loss of biologically active CGRP could be prevented by including an inhibitor of endopeptidase 24.11 during the 24 hour incubation (Fig.9). There was no inhibitory activity in the medium, as transferred medium alone had no effect on cAMPi levels and, furthermore, supplementation of transferred medium with fresh peptide restored cAMPi elevating ability. CGRP degradation alone is not sufficient to account for the attenuation of cAMPi. Repeated addition of fresh peptide to cultured cells does not alter the time course of desensitisation, and Schwann cells remain refractory to subsequent peptide for 24 hours or more. Cells are down regulating their responsiveness over a 24 hour period, even in the presence of biologically active forskolin and CGRP. Forskolin acts directly on adenylate cyclase, thereby bypassing receptor mediated regulation, suggesting that the fall in cAMPi during a 24 hour incubation with forskolin involves downregulation of adenylate cyclase or increased breakdown of cAMPi via phosphodiesterase. Addition of IBMX largely inhibits the fall in cAMPi seen with forskolin over a 24 hour incubation. Phosphodiesterase does not significantly contribute to desensitisation to CGRP. Time courses to  $10^{-6}$ M CGRP in the presence and absence of the phosphodiesterase inhibitor IBMX are similar, but basal cAMPi levels are increased and acute CGRP responses potentiated (Fig.10). After 24 hours there is still a marked decline in cAMPi levels, although levels are slightly greater than with IBMX alone (Fig.11). Although breakdown of cAMPi is prevented, synthesis of cAMPi in the presence of CGRP is still curtailed over a short time course, and acutely generated cAMPi lost via other mechanisms over the 24 hour period. In mitogen assays, no effect of CGRP beyond that achieved by IBMX alone could be detected. IBMX alone exhibited synergy with PDGF, but potentiated neither the actions of forskolin or CGRP in mitogen assays.

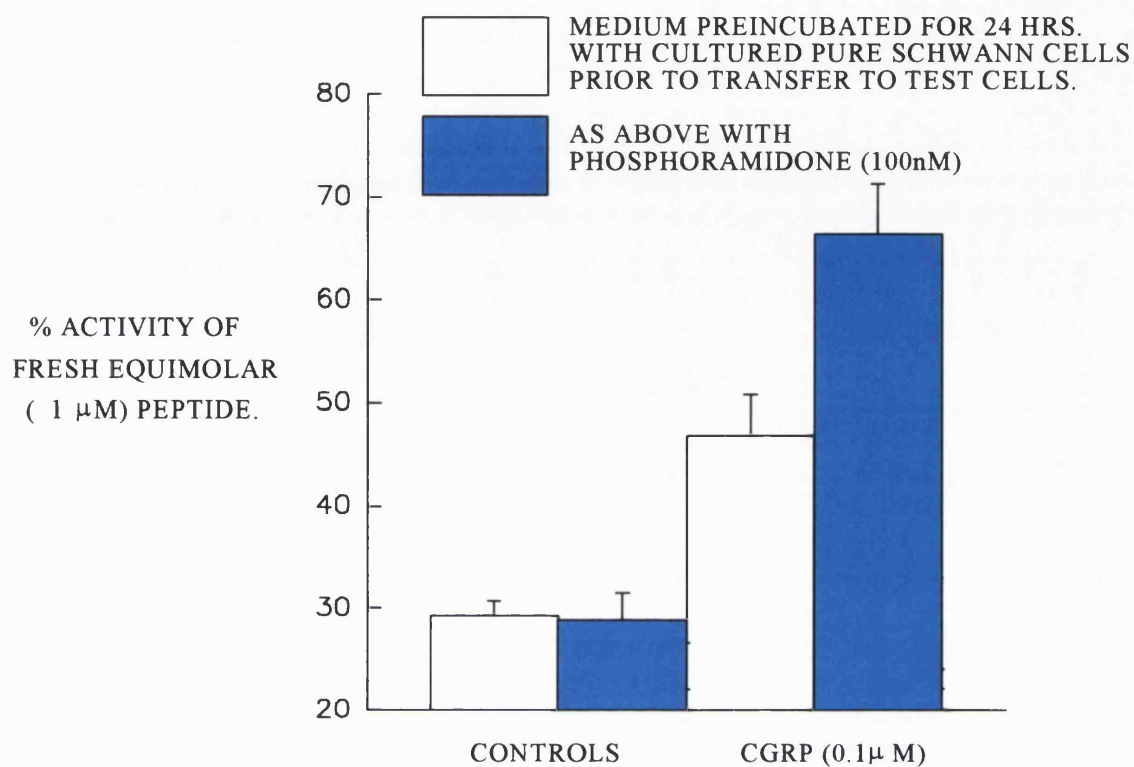


Fig.9. The effect of Schwann cell E.C.24.11 on CGRP degradation.

Pure Schwann cells, cultured in defined serum-free medium at 15,000 cells per well of a 96-well plate, were preincubated with 0.1µM CGRP for 24 hours in the presence or absence of phosphoramidone. Resultant preincubated medium was then transferred to fresh cells, and CGRP bioactivity was assessed by determining cAMPi elevation in test cells following a 10 minute incubation. cAMPi elevation is expressed as a percentage of that obtained with fresh 0.1µM CGRP, representing proportion of CGRP bioactivity retained following a 24 hour preincubation. Values are mean  $\pm$  s.e. of triplicate cultures.

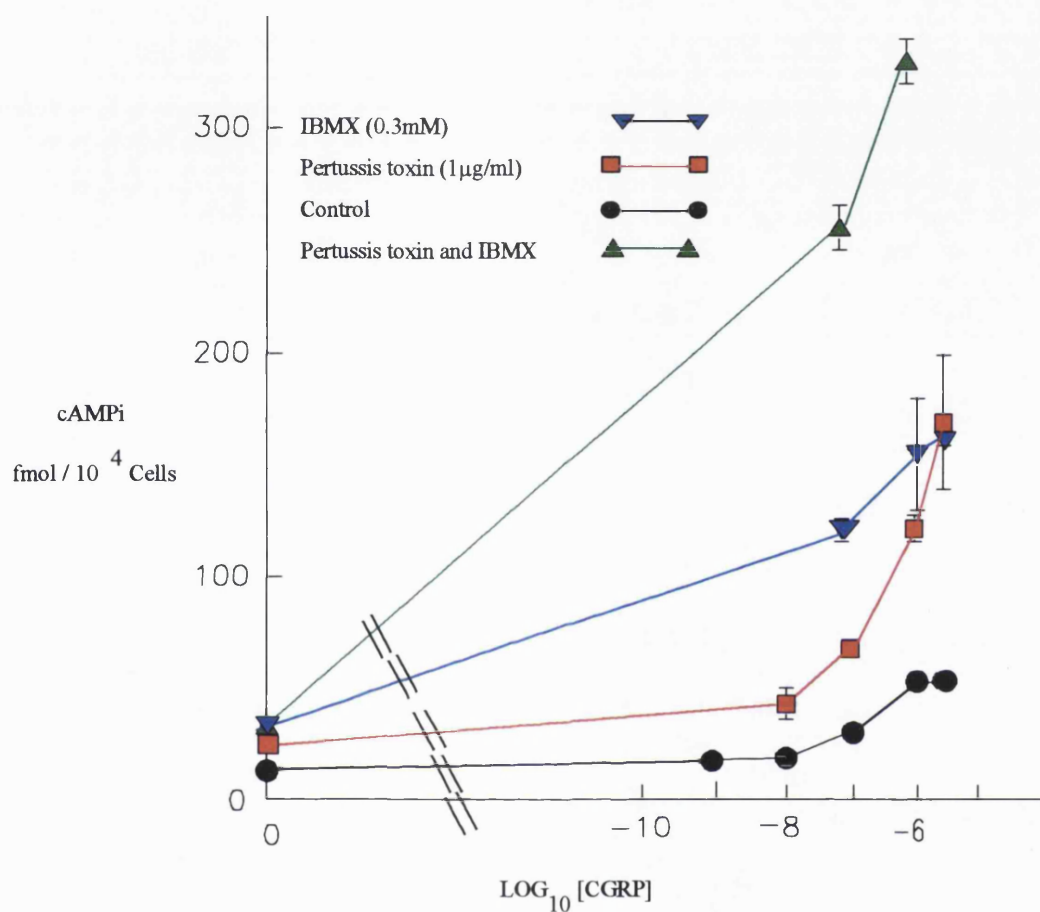


Fig. 10. The role of G<sub>i</sub> and phosphodiesterase in regulating acute Schwann cell cAMPi elevation.

Dose response curves are shown for pure cultured Schwann cells, following a 10 minute exposure to CGRP. Cells were plated at 10,000 cells per well of a laminin coated 96-well plate. Pertussis toxin and IBMX were added 2 hours prior to CGRP stimulations. FCS-containing medium was replaced by serum-free defined medium, four hours prior to start of assays. Values are mean  $\pm$  s.e. of triplicate cultures.

The complete loss of cAMPi elevation and rapid time course of desensitisation, suggest that receptor downregulation, or reduced activation of adenylate cyclase, possibly via  $G_i$ , are important. This is characteristic of most agonists using cAMP as an intracellular second messenger, and has been demonstrated for the  $\beta$ -adrenergic receptor, and also for VIP. Pertussis toxin, which can inhibit sensitive G proteins,  $G_i$  and  $G_o$ , markedly potentiates acute cAMPi elevation to CGRP but has little effect on basal cAMPi. This action is not therefore due to inhibition of tonic  $G_i$  activity. Further acute potentiation is seen in the presence of IBMX, suggesting that  $G_i$  inhibition leads to increased synthesis of cAMPi, and not reduced breakdown (Fig.10). Pertussis toxin does not, however, prevent the rapid time course of desensitisation. Over 24 hours, cAMPi still attenuates (Fig.11) and, in mitogen experiments, although pertussis toxin accentuates the permissive action of forskolin, it does not enable the mitogenic action of isoprenaline or CGRP. Isoprenaline (10 $\mu$ M) weakly elevates cAMPi and demonstrates potent synergy with CGRP, but in combination desensitisation was unchanged and they were not mitogenic. Isoprenaline also induces desensitisation to CGRP after a 24 hour preincubation, whereas after 4 hours this is far less prominent, suggesting that heterologous desensitisation requires a more prolonged time course (Fig.14b). The importance of receptor phosphorylation on desensitisation was assessed by inhibiting the kinases  $\beta$  ARK and PKA. These enzymes are known to mediate homologous and heterologous desensitisation of  $\beta$ -adrenergic receptors, respectively.



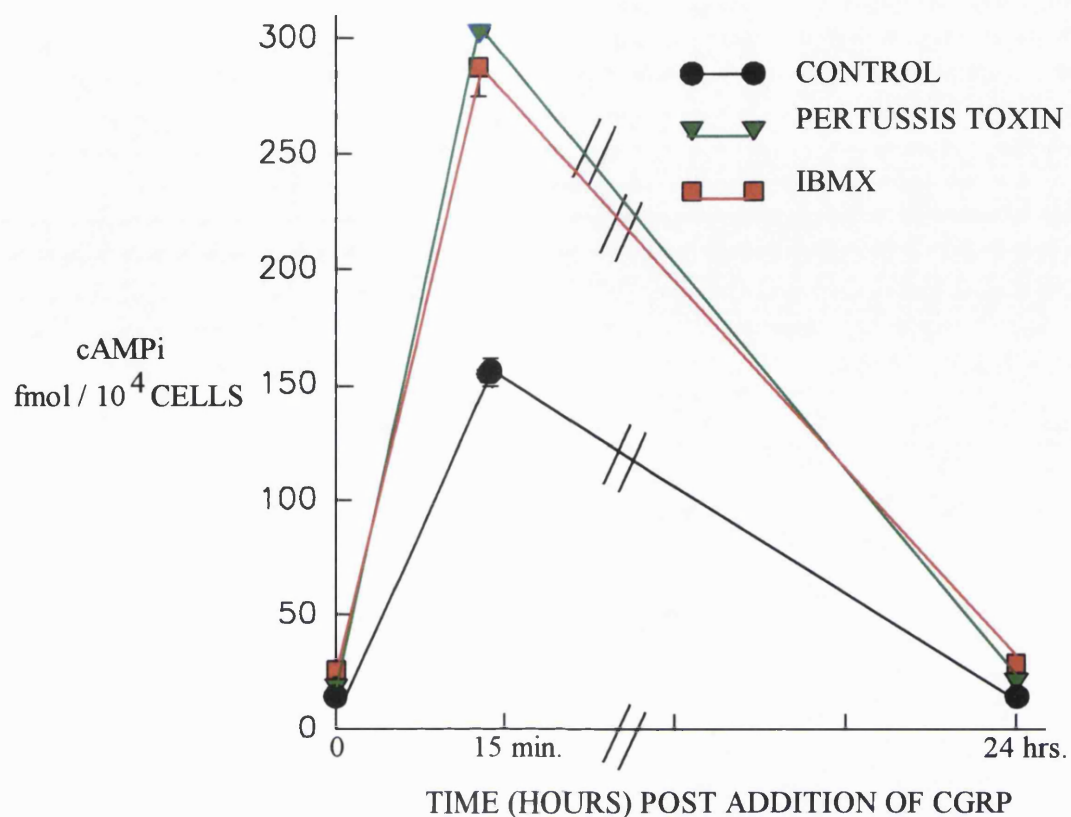


Fig. 11. The role of  $G_i$  and phosphodiesterase in regulating cAMPi in Schwann cells over a 24 hour incubation with CGRP.

Elevation of cAMPi in Schwann cell cultures, was measured following stimulation with  $2\mu\text{M}$  CGRP in the presence or absence of enzyme inhibitors. Schwann cells were plated at 10,000 cells per well of laminin coated 96-well plates in serum-free defined medium. cAMPi at 15 minutes and 24 hours after CGRP addition are compared. Enzyme inhibitors were added 4 hours prior to assays. Values are mean  $\pm$  s.e. of triplicate cultures.

The PKA inhibitors, H-8 and H-89, dramatically increase basal and stimulated cAMPi production in acute assays. These high cAMPi levels were maintained over 24 hours, and loss of cAMPi response to CGRP was greatly reduced (Fig.12). To inhibit  $\beta$ ARK, heparin (100nM) was added to cells permeabilised with digitonin (0.15%); permeabilisation was necessary to allow heparin to enter the cell. Heparin reduces desensitisation to isoprenaline, but not CGRP, suggesting that  $\beta$ ARK is important in mediating the loss of cAMPi response during prolonged exposure to  $\beta$ -adrenergic agonists, but not to CGRP (Fig.13). These results suggest that the regulation of adenylate cyclase-linked systems differ for individual classes of agonists. In contrast to the results seen with inhibition of PKA, inhibiting  $\beta$ ARK had no effect on basal cAMPi levels. This suggests that PKA mediated phosphorylation is providing a degree of negative feedback inhibition on cAMPi turnover, even in the resting state. In order to study the role of receptor desensitisation in regulating a potential mitogenic activity in Schwann cells, proliferation assays with isoprenaline were conducted in the presence or absence of heparin (100nM), which was added either with digitonin or during cell dissociation and trituration, to aid cell entry. Unfortunately, cells did not survive long enough for proliferation to be measured. Proliferation assays were also conducted in the presence of a PKA inhibitor. In the presence of H-89, despite massive elevation of cAMPi and prevention of desensitisation, no Schwann cell proliferation was seen, with either CGRP or isoprenaline. Even the action of forskolin and PDGF was prevented, confirming the importance of PKA mediated phosphorylation in mediating the permissive actions of cAMPi on growth factor activity. These results suggest that PKA mediated desensitisation, possibly by inactivation of the CGRP receptor, is preventing the permissive action of CGRP on growth factor activity under these assay conditions. Avoiding desensitisation, by repeated addition of low doses of CGRP and exchange of medium might thus be expected to avoid desensitisation and allow a growth promoting activity. However, attempting this in mitogen assays led to inconsistent results within assays due to washing off of cells. Further optimisation of this is required, though preliminary experiments suggest that CGRP is a mitogen under these conditions.

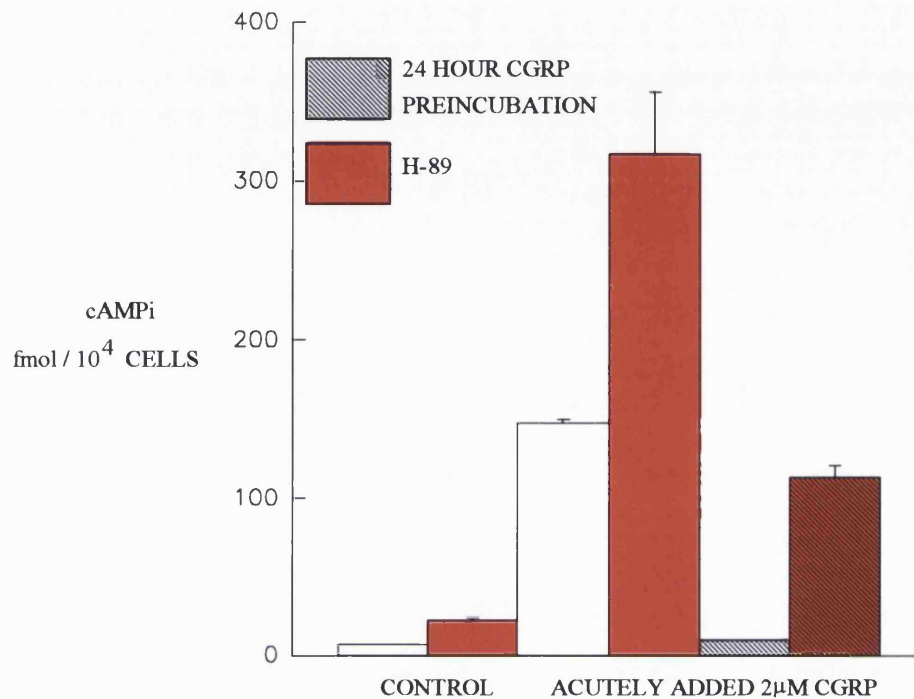


Fig. 12. The effect of inhibiting PKA on CGRP mediated Schwann cell cAMPi elevation.

Schwann cells were plated at 10,000 cells per well of laminin coated 96-well plates in serum-free defined medium. Schwann cells were preincubated for 24 hours with medium alone or with 2μM CGRP +/- the PKA inhibitor H-89. Following the preincubation, medium was replaced, and after 30 minutes cAMPi elevation in response to a 10 minute incubation with 2μM CGRP, determined. Results are mean +/- s.e. of triplicate cultures.

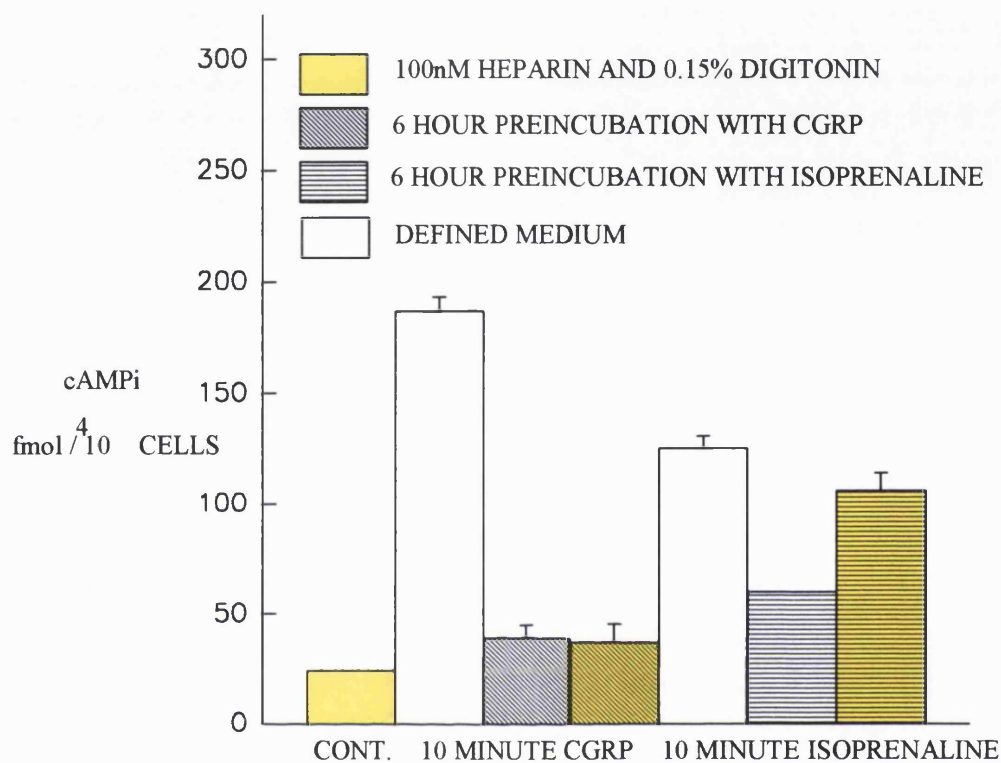


Fig. 13. The effect of heparin, an inhibitor of  $\beta$ ARK, on isoprenaline- and CGRP-induced Schwann cell cAMPi elevation.

Schwann cells were plated at 100,000 cells per well of laminin coated 24-well plates in serum-free defined medium. Schwann cell cultures were preincubated for 6 hours with isoprenaline or CGRP in the presence or absence of heparin and digitonin. Medium was then replaced and, after 30 minutes, acute 10 minute stimulations with CGRP and isoprenaline were performed and cAMPi assayed. Values are mean  $\pm$  s.d. of duplicate cultures.

## MAGNESIUM AND DESENSITISATION

It has long been known that  $Mg^{++}$  is critical for G protein receptor coupling. We therefore set out to determine the effects of increasing extracellular  $Mg^{++}$  concentration on isoprenaline and CGRP-mediated cAMPi elevation. We show that by increasing  $Mg^{++}$  in the medium to 4mM, we greatly reduced the loss of cAMPi elevation to both isoprenaline and CGRP, suggesting that coupling was enhanced and the effect of desensitisation reduced (Fig.14a). Following a 4 hour exposure to isoprenaline, there was only minimal reduction of subsequent cAMPi elevation in response to CGRP.  $Mg^{++}$  had no effect on this low level of heterologous desensitisation induced by preincubation of Schwann cells with isoprenaline before CGRP stimulation (Fig. 14b).

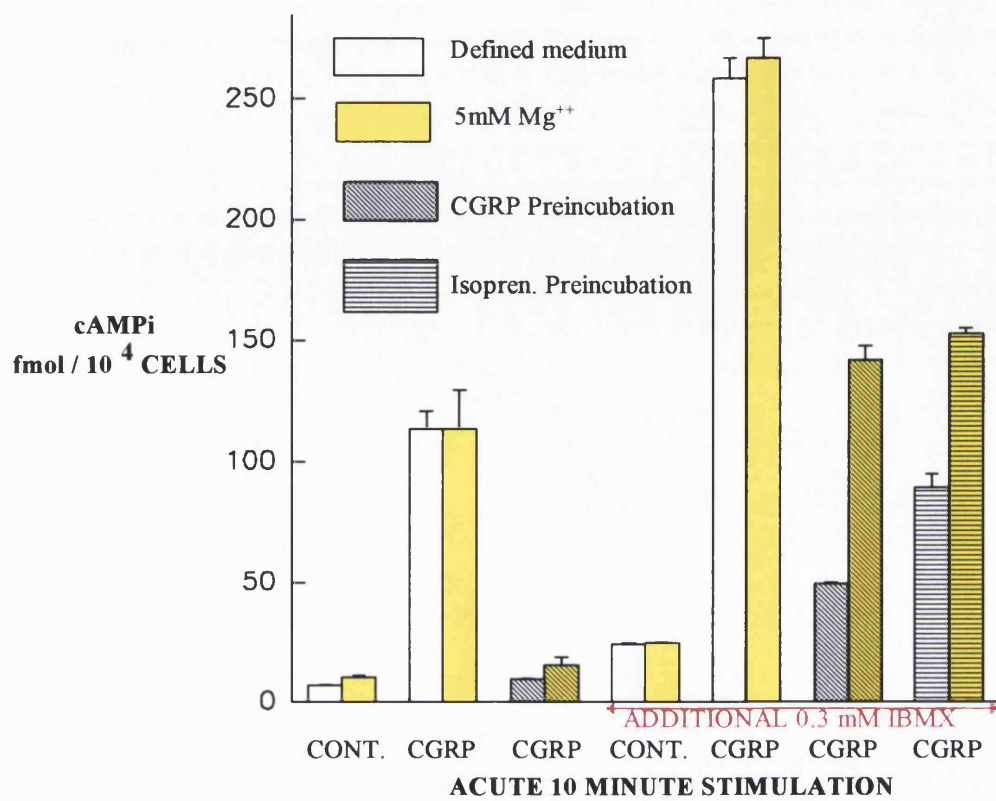


Fig. 14a. The effect of supraphysiological magnesium concentration on isoprenaline and CGRP mediated Schwann cell desensitisation.

Schwann cells were plated at 10,000 cells per well of laminin coated 96-well plates in serum-free defined medium. Cultures were preincubated for 4 hours with 2 $\mu$ M CGRP or 5 $\mu$ M isoprenaline in the presence or absence of 5mM magnesium. Cells were then washed and after 45 minutes, acute 10 minute stimulations with CGRP performed. Magnesium and IBMX were added to relevant wells directly following washes. Values are mean  $\pm$  s.e. of triplicate cultures.

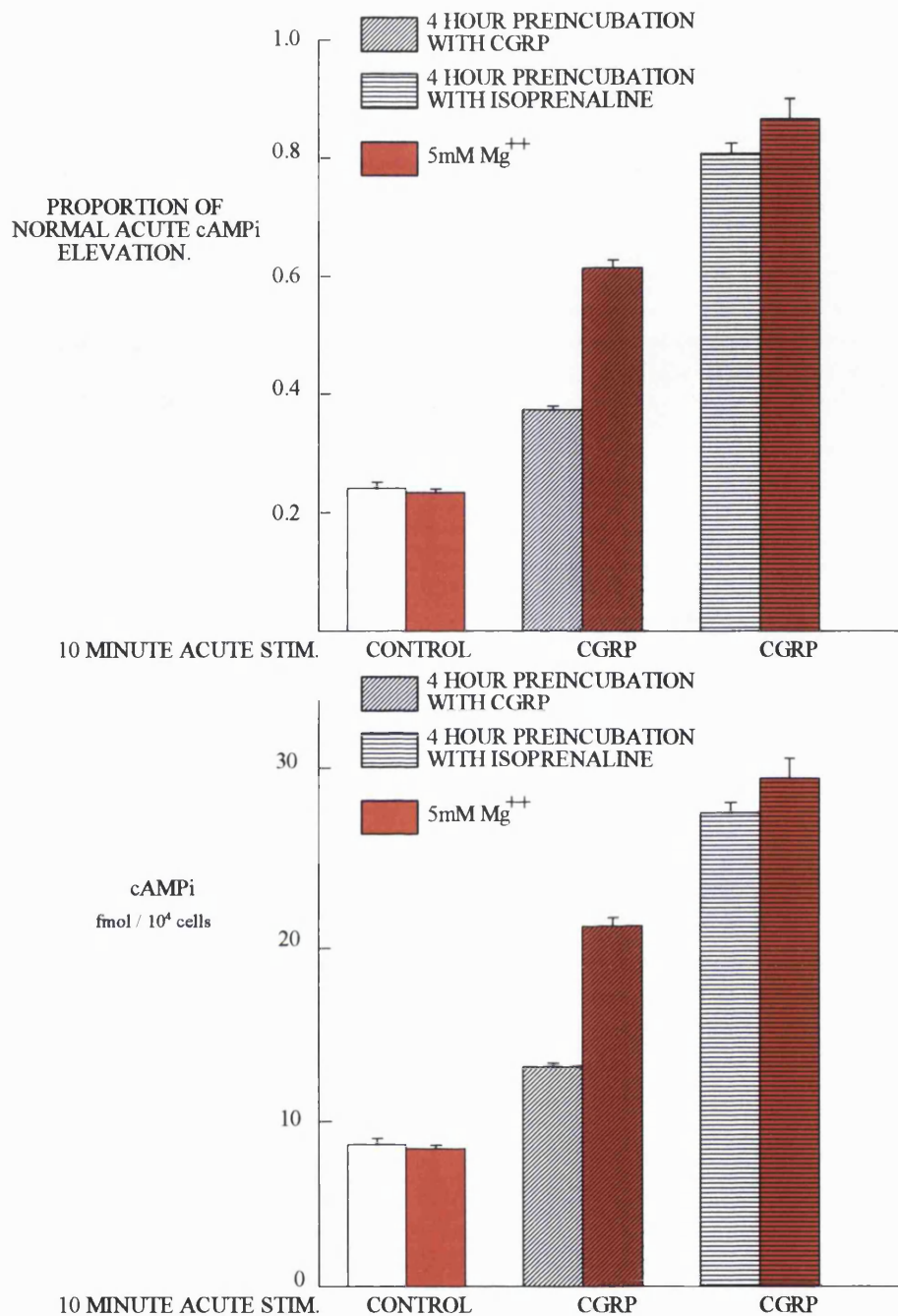


Fig. 14b. The effect of magnesium on heterologous and homologous desensitisation. The lower graph shows actual cAMPi values whereas, the upper graph represents this as a proportion of normal acute cAMPi elevation.

Schwann cells were grown in defined serum-free medium at 10,000 cells per well of a 96-well plate. Cells were preincubated for 4 hours with medium only, 5 $\mu$ M isoprenaline, or 2 $\mu$ M CGRP. Following preincubations, cells were washed, IBMX added, and then after 1 hour acute 10 minute exposures to 2 $\mu$ M CGRP performed. Values are mean  $\pm$  s.e. of triplicate cultures.

Of great interest, in the presence of 4mM  $Mg^{++}$ , both these agents are permissive for growth factor induced Schwann cell proliferation, enabling serum (Fig.15a,c.), PDGF (Fig.15c.), and also FGF induced proliferation (Fig.15d.). In the latter experiment, CGRP alone was not analysed as some of these wells had become infected. However, the increase in O.D. seen with CGRP and FGF combined is greater than would have been anticipated for CGRP alone from other assays. PDGF has little additional activity above that seen with 10% FCS (Fig.15c.).  $Mg^{++}$  alone neither elevates cAMPi nor is mitogenic. It therefore seems likely that  $Mg^{++}$  is merely augmenting a direct and specific action of these agents that act via cAMPi.



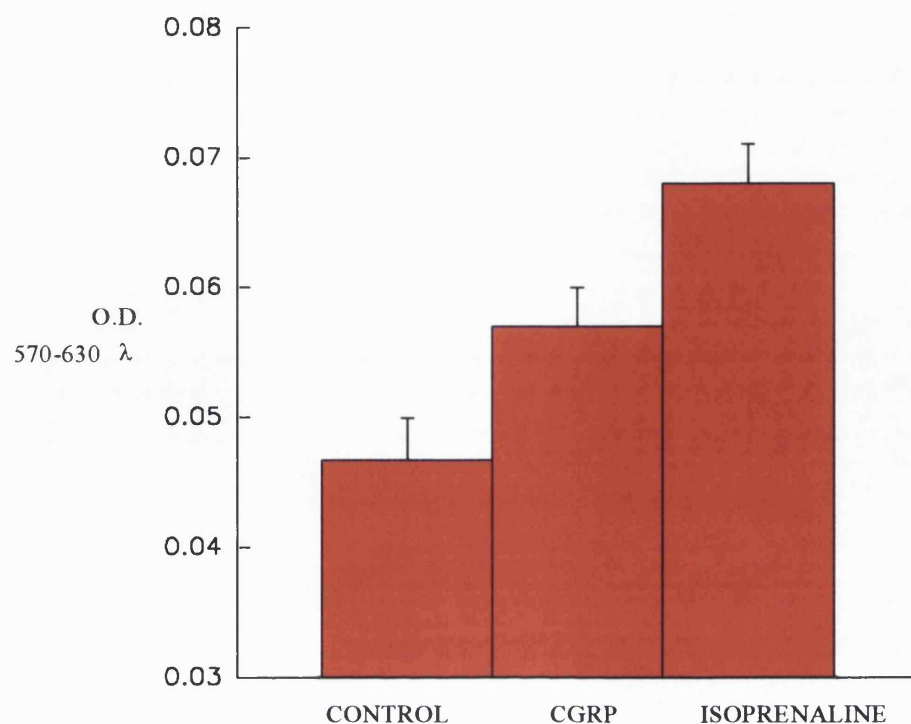


Fig. 15a. The effect of magnesium on the permissive action of Isoprenaline and CGRP on serum induced Schwann cell proliferation.

Schwann cells were plated at 10,000 cells per well of a laminin coated 96-well plate in DMEM with 10% FCS and 8mM  $Mg^{++}$ . Additions were repeated at 24 hour intervals over a total of 48 hours, and cell number assessed by MTT assay. Values are mean  $\pm$  s.e. of O.D. readings of triplicate cultures.

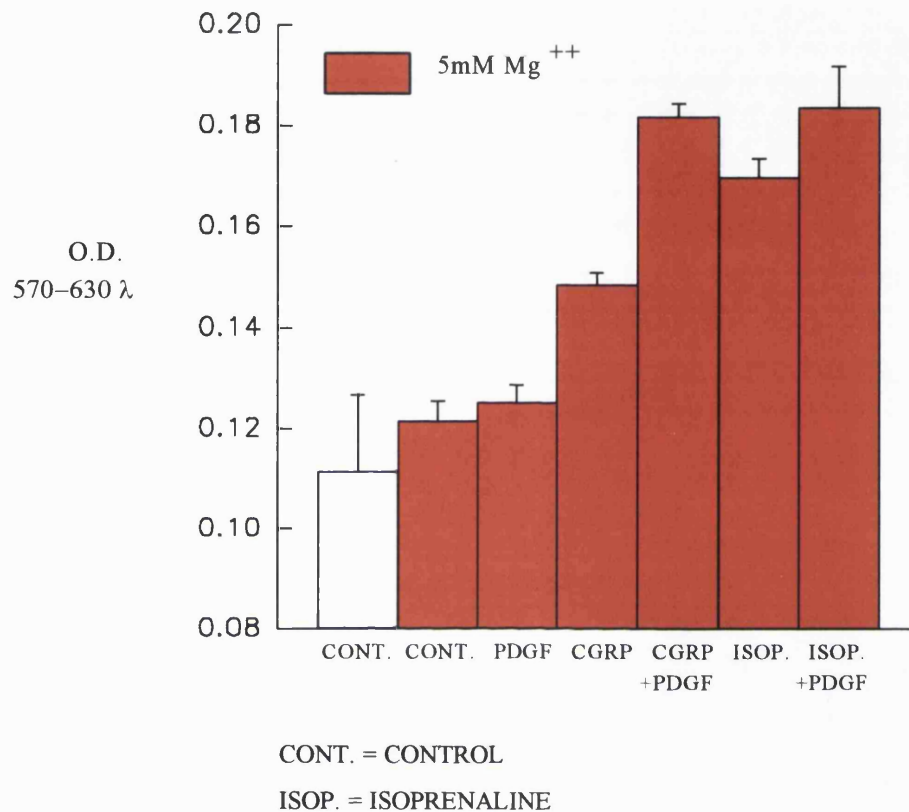


Fig. 15b. The effect of magnesium on the permissive action of CGRP and Isoprenaline on PDGF induced Schwann cell proliferation in defined medium.

Schwann cells were plated at 10,000 cells per well of a laminin coated 96-well plate in serum-free defined medium, with or without 5mM magnesium. Cells were incubated for 48 hours with additions. Cell number was determined by optical density following a 4 hour incubation with MTT. Values are mean  $\pm$  s.e. of triplicate cultures.

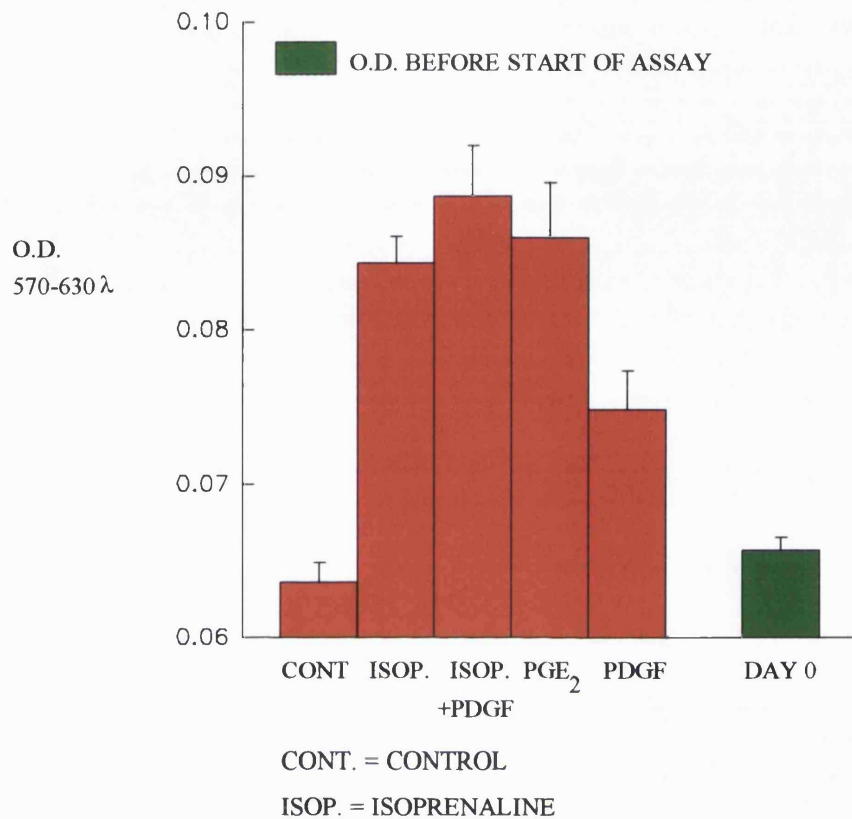


Fig.15c. The effect of magnesium on the permissive action of Isoprenaline and PGE<sub>2</sub> on serum induced proliferation.

Schwann cells were plated at 10,000 cells per well of a laminin coated 96-well plate in DMEM with 10% FCS and 4mM magnesium. Cells were incubated with additions for 48 hours before assessing viable cell number by optical density. Values are mean  $\pm$  s.e. of triplicate cultures.

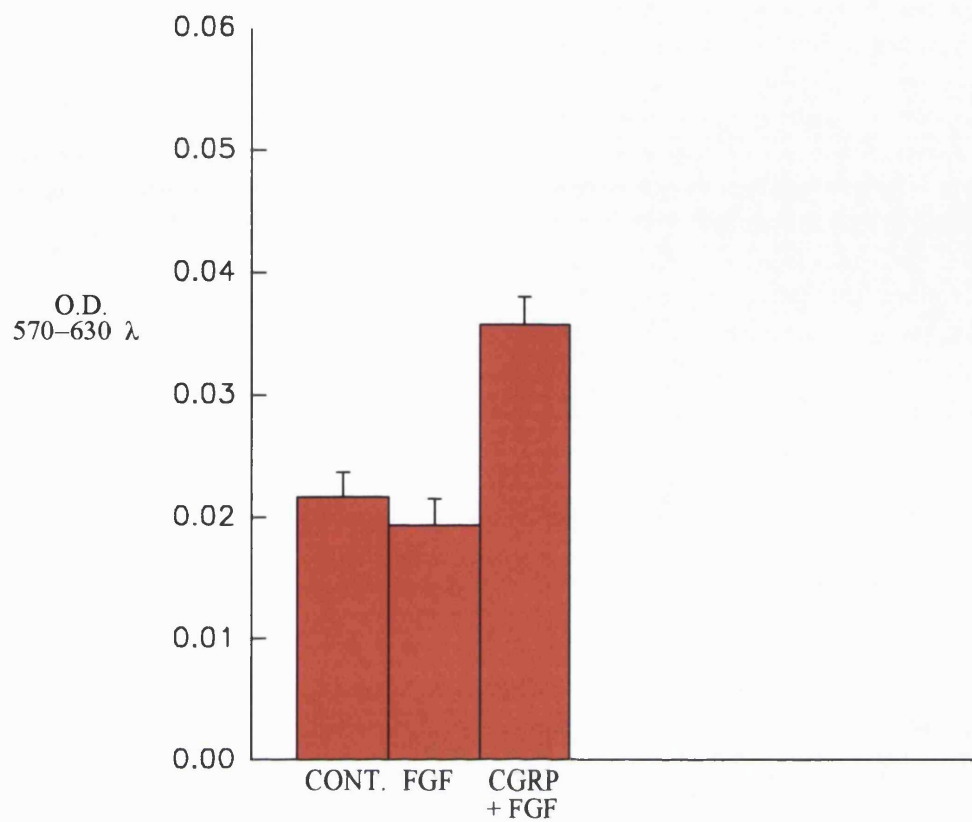


Fig. 15d. The effect of magnesium on the permissive action of CGRP on FGF induced Schwann cell proliferation.

Schwann cells were plated at 10,000 cells per well of a laminin coated 96-well plate in serum-free defined medium, with or without 5mM magnesium. Cells were incubated for 48 hours with additions. Cell number was determined by optical density following a 4 hour incubation with MTT. Values are mean  $\pm$  s.e. of triplicate cultures.

## NGF SYNTHESIS

CGRP and isoprenaline increase fibroblast NGF production. In fact, CGRP is only weakly active in the absence of  $Mg^{++}$ , but this is potentiated strongly in its presence (Fig.16a,b). Isoprenaline is active in the absence of increased extracellular  $Mg^{++}$  (Fig.16c). NGF production by Schwann cells is stimulated by isoprenaline in the presence of  $Mg^{++}$  (Fig.16d), but to date no action of CGRP on Schwann cells has been confirmed. These results suggest that  $Mg^{++}$  is able to increase receptor signalling thereby minimising desensitisation in the presence of receptor agonists.

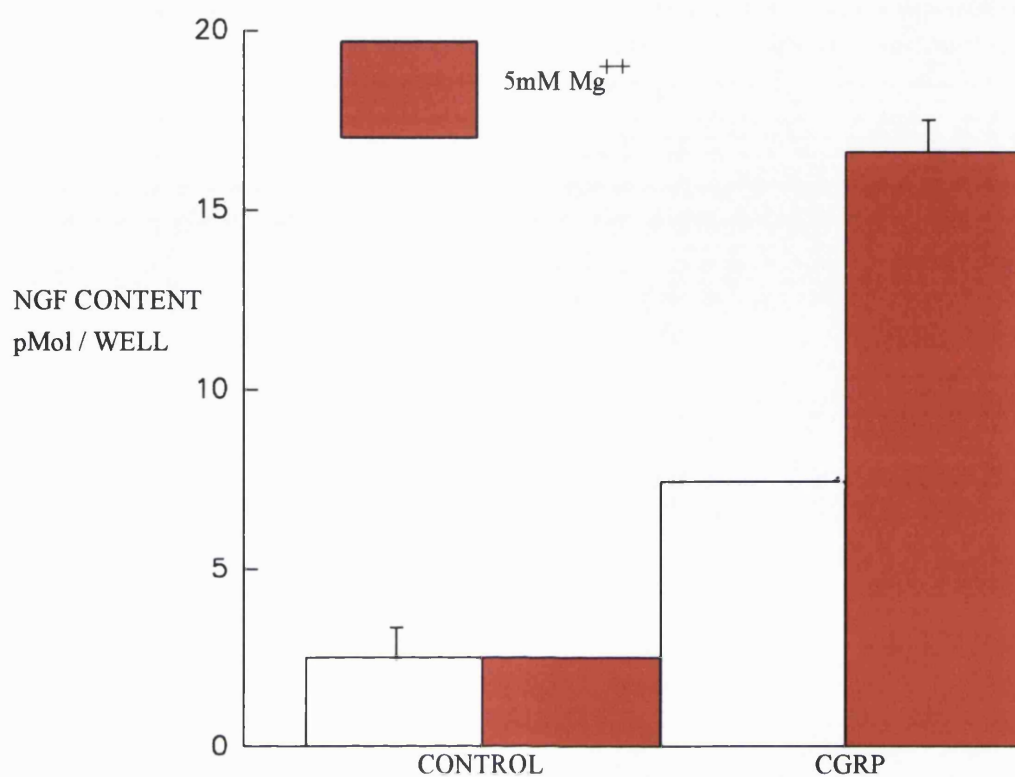


Fig. 16a. The induction of fibroblast NGF synthesis by CGRP is potentiated by magnesium.

NGF immunoassay of endoneurial fibroblast supernatants, following a 72 hour incubation of pure cultured fibroblasts with 2 $\mu$ M CGRP with or without added magnesium. Cells were cultured in defined serum-free medium at 200,000 cells per well of a 24-well plate, in a final volume of 200 $\mu$ l/well of medium. Values are mean  $\pm$  s.d. of duplicate cultures assayed in duplicate.

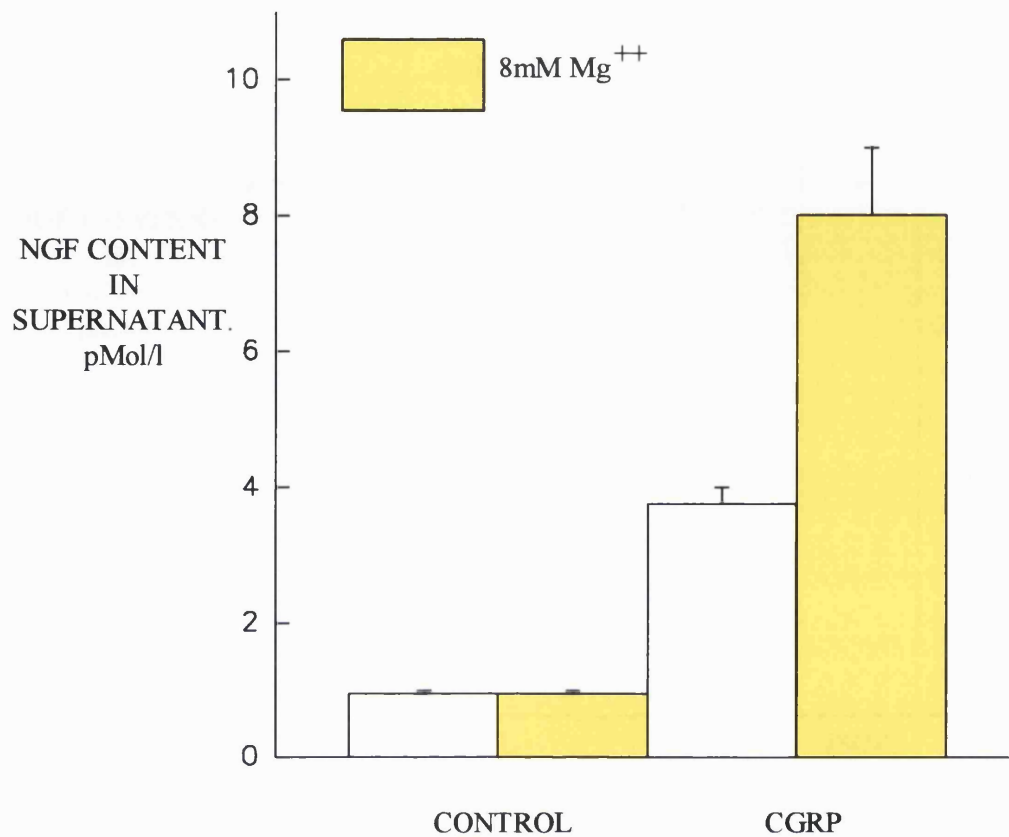


Fig. 16b. NGF production in cultured fibroblasts is stimulated by CGRP, and potentiated by magnesium.

NGF immunoassay of endoneurial fibroblast supernatants, following a 72 hour incubation of pure cultured fibroblasts with 2 $\mu$ M CGRP with or without added magnesium. Cells were cultured in defined serum-free medium at 200,000 cells per well of a 24-well plate, in a final volume of 200 $\mu$ l/well of medium. Values are mean  $\pm$  s.d. of duplicate cultures assayed in duplicate.

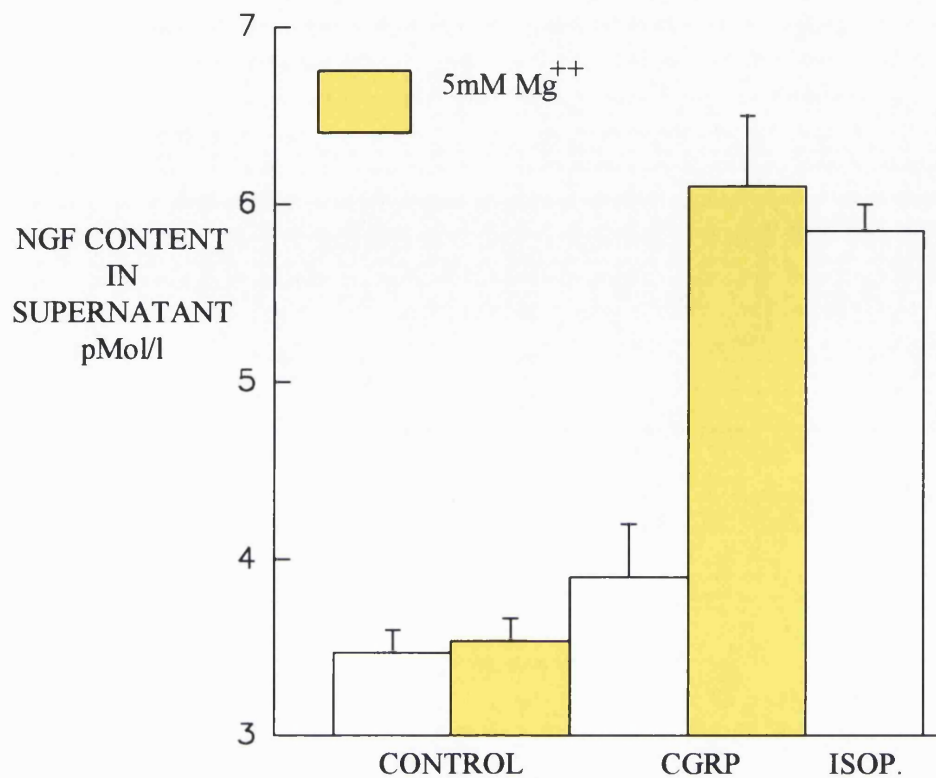


Fig.16c. Magnesium potentiation of CGRP induced fibroblast NGF synthesis.

NGF immunoassay of endoneurial fibroblast supernatants, following a 72 hour incubation of pure cultured fibroblasts with isoprenaline or CGRP with or without added magnesium. Additions were repeated at 24 hour intervals. Cells were cultured in DMEM with 2% FCS at 200,000 cells per well of a 24-well plate, in a final volume of 200 $\mu$ l/well of medium. Values are mean  $\pm$  s.d. of duplicate cultures assayed in duplicate.



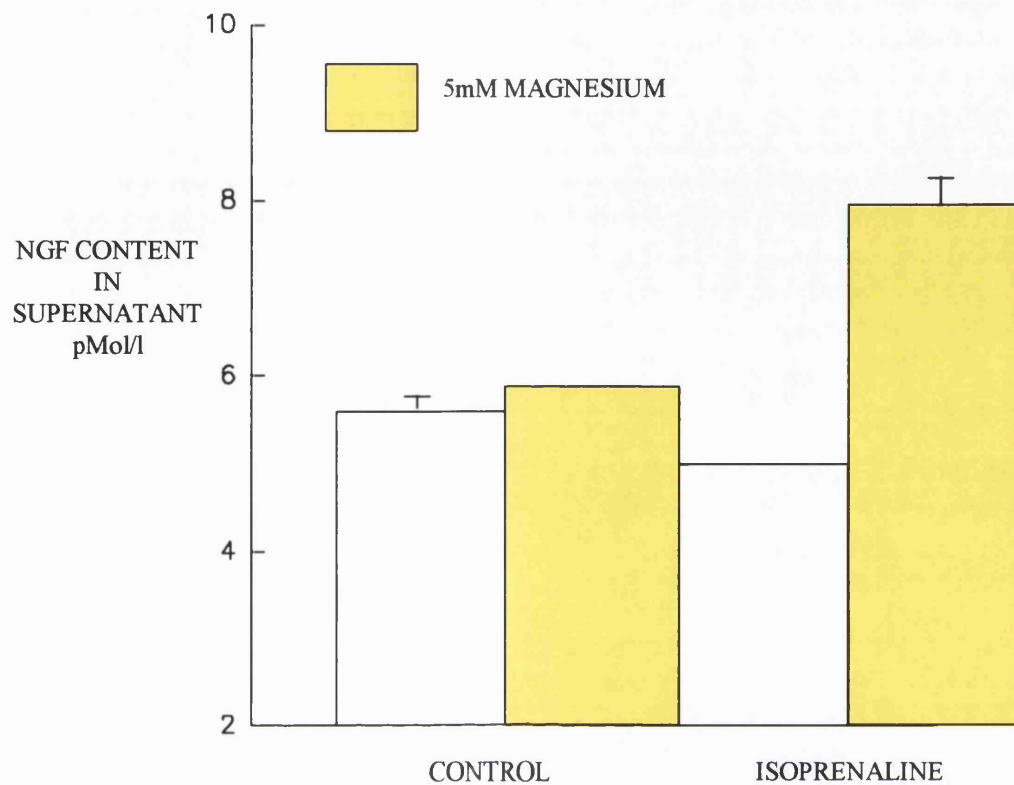


Fig. 16d. Magnesium and isoprenaline induced Schwann cell NGF synthesis.

NGF immunoassay on supernatants of pure cultured rat Schwann cells, following a 72 hour incubation with 5 $\mu$ M Isoprenaline, with or without added magnesium. Cells were grown in 5 % FCS at a density of 500,000 cells per well of a 24-well plate, and restricted to a 150 $\mu$ l total volume of supernatant by the end of assays. Values are mean  $\pm$  s.d. of duplicate cultures assayed in duplicate.

## CGRP AND INHIBITION OF FIBROBLAST PROLIFERATION

As already shown, CGRP also elevates fibroblast cAMPi (Fig.4b). CGRP causes a consistent, dose dependent reduction in fibroblast proliferation, whether induced by serum (Fig.17) or PDGF (Fig.19a), and this is potentiated by IBMX. This action is shared by forskolin (Fig.18), IBMX (Fig 19a), and theophylline (Fig.19b), confirming that this effect is mediated by cAMPi.

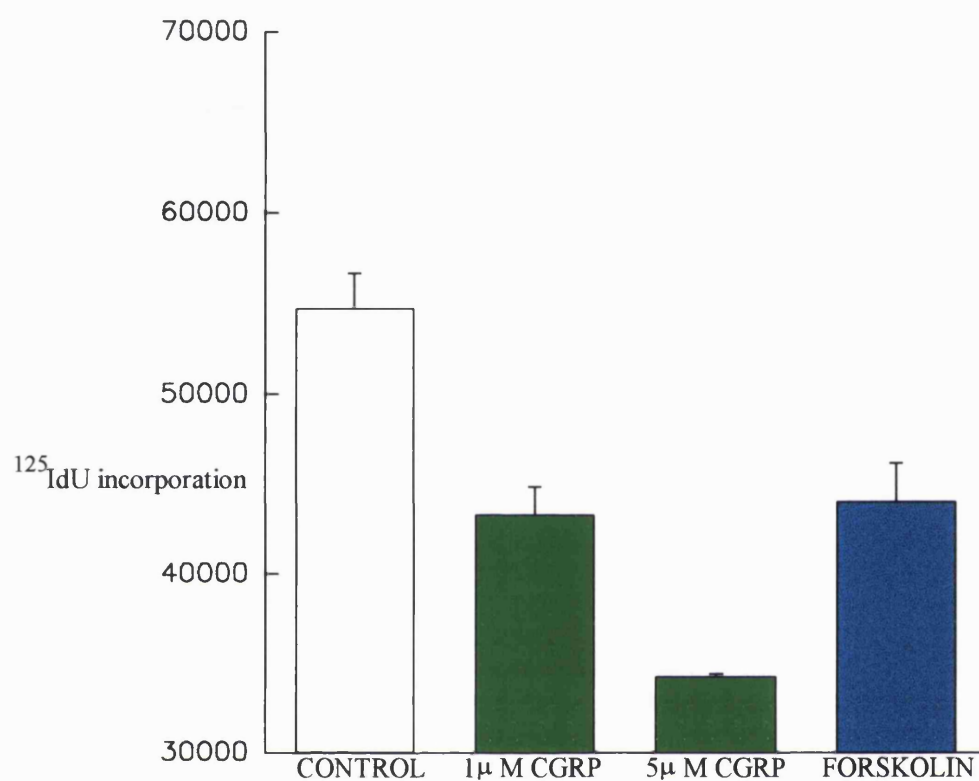


Fig.17. The effect of CGRP on serum induced fibroblast proliferation.

<sup>125</sup>IIdU proliferation assay on cultured endoneurial fibroblasts. Fibroblasts were 24 hours old in culture at the start of assays and were plated at 5,000 cells per well of a 96-well plate in DMEM with 10% FCS. Additions were made over 48 hours, with additions repeated at 24 hours. Values are mean  $\pm$  s.e. of triplicate cultures.

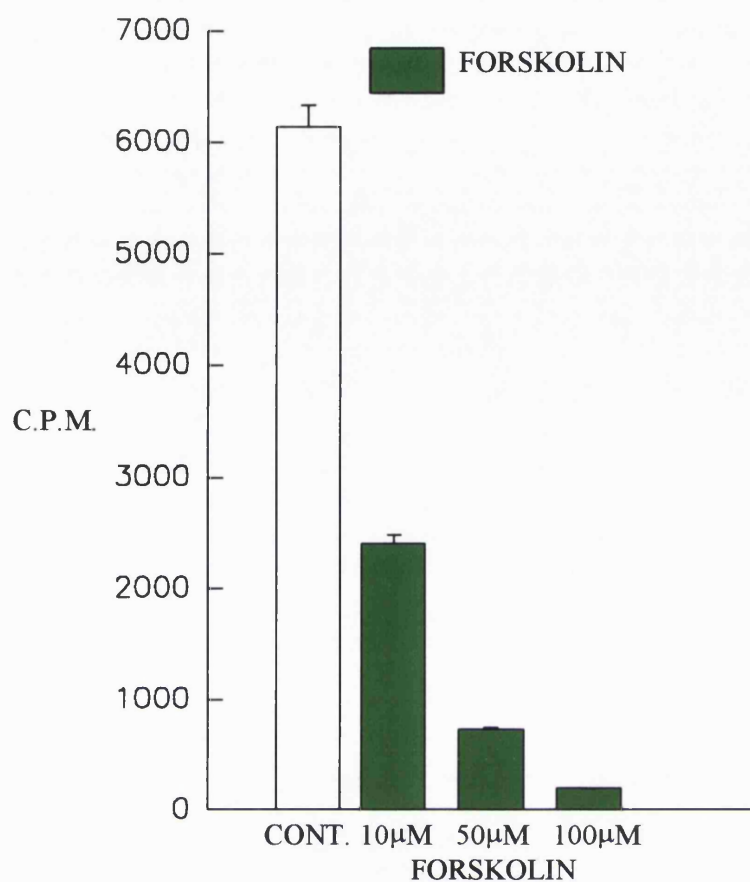


Fig.18. The effect of forskolin on serum - induced fibroblast proliferation.

<sup>125</sup>IIdU proliferation assay on cultured endoneurial fibroblasts. Fibroblasts were 24 hours old in culture at the start of assays and were plated at 5,000 cells per well of a 96-well plate in DMEM with 10% FCS. Additions were made over 48 hours with additions repeated after 24 hours. Values are mean  $\pm$  s.e. of triplicate cultures.

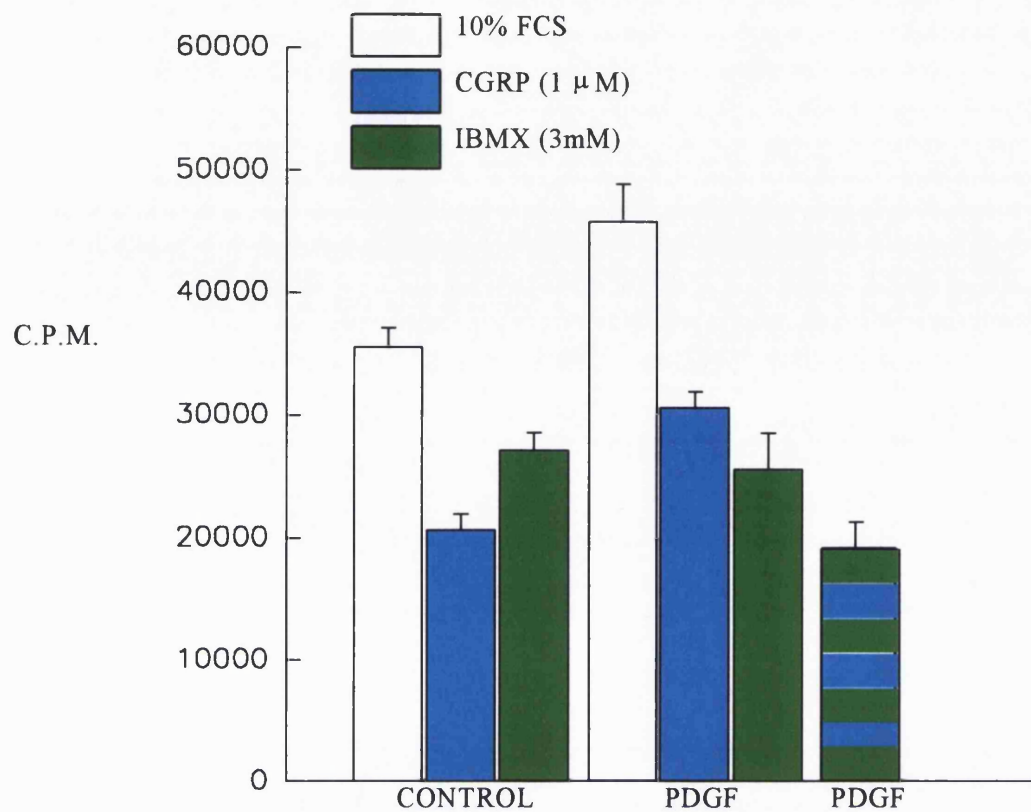


Fig. 19a. The effect of IBMX on CGRP-mediated inhibition of fibroblast proliferation.

<sup>125</sup>IIdU proliferation assay on cultured endoneurial fibroblasts. Fibroblasts were 24 hours old in culture at the start of assays and were plated at 5,000 cells per well of an uncoated Primaria 96-well plate in DMEM with 10% FCS. Additions were made over 48 hours with additions repeated after 24 hours. Values are mean  $\pm$  s.e. of triplicate cultures.

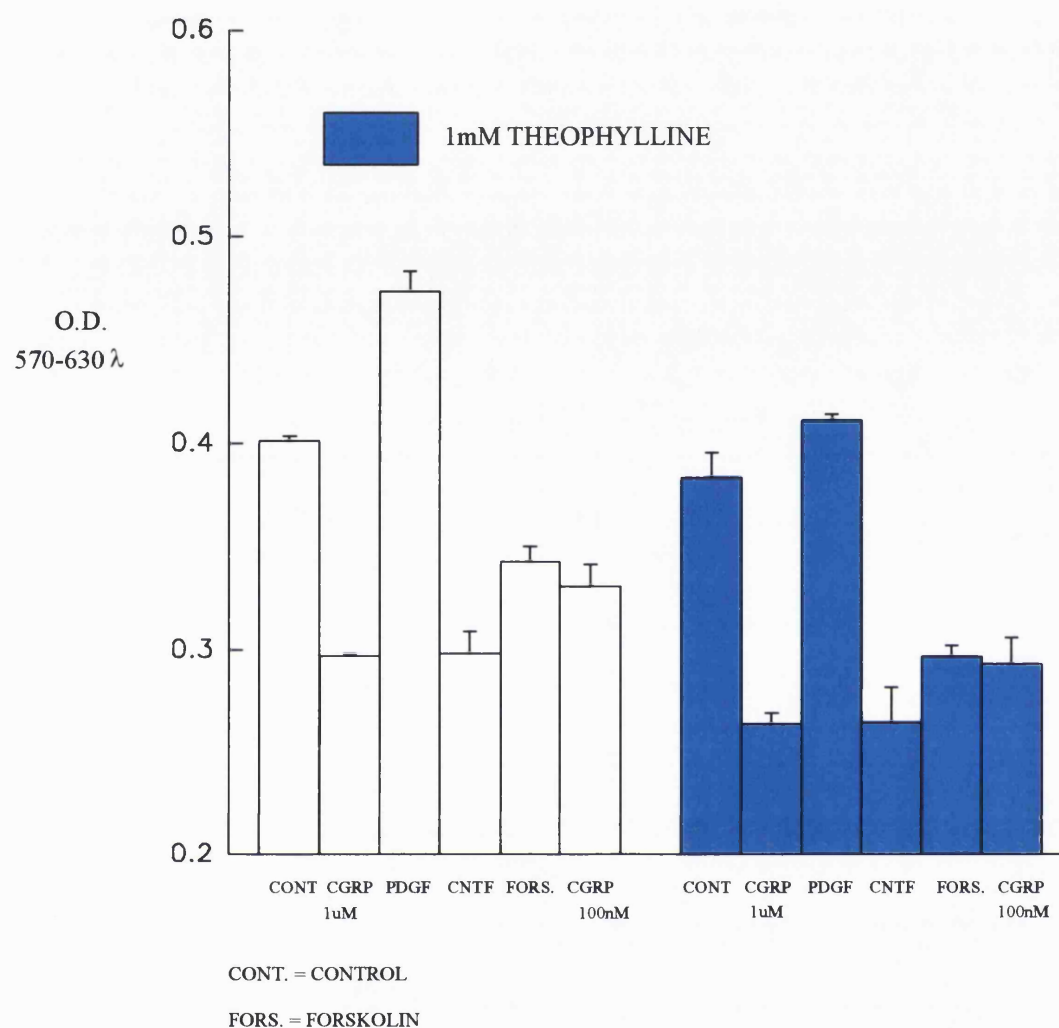


Fig. 19b. The effect of theophylline on the inhibition of fibroblast proliferation by CGRP and by CNTF.

MTT assay on cultured endoneurial fibroblasts. Fibroblasts were 24 hours old in culture at the start of assays and were plated at 5,000 cells per well of a 96-well plate in DMEM with 10% FCS. Additions were made over 48 hours with additions repeated after 24 hours. Values are mean  $\pm$  s.e. of triplicate cultures.

## SIGNAL TRANSDUCTION IN FIBROBLAST PROLIFERATION

In fibroblasts, proliferation can be inhibited by pertussis toxin, which reduces the activity of the inhibitory G protein,  $G_i$ , suggesting that tonic inhibition of cAMPi formation by  $G_i$  may play a role in enabling fibroblast proliferation. Pertussis toxin inhibits fibroblast proliferation, and no additional inhibition is seen with agents that act via cAMPi (Fig.20a), suggesting that cAMPi formation may be constitutive in this situation. This result, however, does not exclude the possibility that another pertussis toxin sensitive G protein is involved in the transduction of growth promoting signals. The fact, however, that  $\gamma$ -IFN, which does not act via cAMPi, is still able to cause further inhibition suggests that mitogenic signals are still being transduced even in the presence of pertussis toxin (Fig.20b). Basal cAMPi levels have little inhibitory action, as H-89, an inhibitor of PKA, does not increase proliferation, though it prevents the inhibitory action of CGRP (Fig.20b). It may be that inhibitory levels of cAMPi are contained by the action of  $G_i$ . PDGF probably acts, at least in part, via PKC, as little added mitogenic action of PDGF is seen beyond that of phorbol ester. The action of phorbol is antagonised by CGRP via elevation of cAMPi.

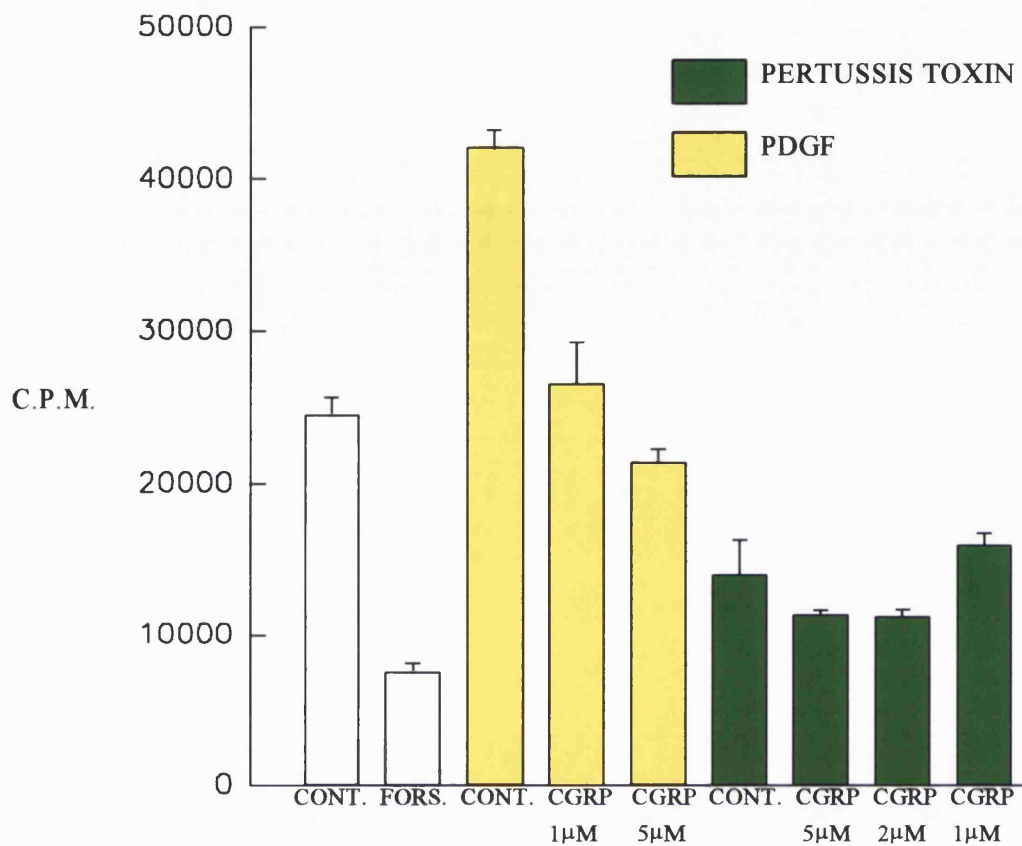


Fig.20a. The role of  $G_i$  in fibroblast proliferation.

$^{125}$ IIdU proliferation assay on cultured endoneurial fibroblasts. Fibroblasts were 24 hours old in culture at the start of assays and were plated at 5,000 cells per well of a 96-well plate in DMEM with 10% FCS. Additions were made over 48 hours with additions repeated after 24 hours. Values are mean  $\pm$  s.e. of triplicate cultures.



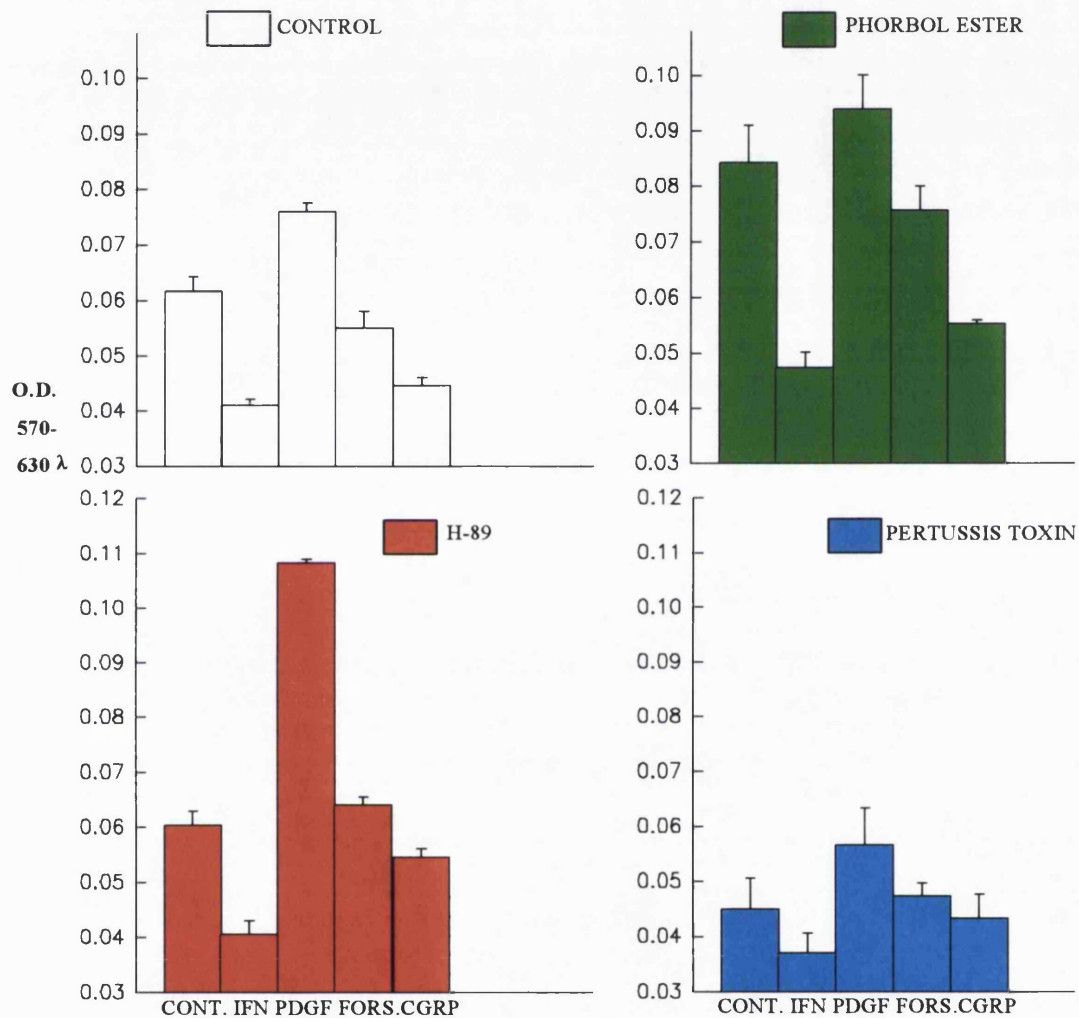


Fig.20b. The role of various second messengers in mediating inhibition of fibroblast proliferation by CGRP, forskolin and  $\gamma$ -IFN.

<sup>125</sup>IIdU proliferation assay on cultured endoneurial fibroblasts. Fibroblasts were 24 hours old in culture at the start of assays and were plated at 5,000 cells per well of a 96-well plate in DMEM with 10% FCS. Additions were made over 48 hours with additions repeated after 24 hours. Values are mean  $\pm$  s.e. of triplicate cultures.

The inhibition of fibroblast proliferation by CGRP is not as sensitive to desensitisation as Schwann cell proliferation seems to be. Sustained elevation of cAMPi does not appear necessary for this action. CGRP affects cAMPi with a similar time course and magnitude to that seen in Schwann cells, yet still inhibits fibroblast proliferation. This may reflect the more rapid turnover of fibroblasts in culture.

Other agents that are known to elevate Schwann cell cAMPi were also examined. VIP elevates Schwann cell cAMPi, though this effect is less potent than that of CGRP (Fig.21). VIP had no demonstrable mitogenic activity for Schwann cells in standard culture conditions. Further experiments are planned to examine the potential role of VIP as a mitogen in the presence of supraphysiological magnesium.

Other growth factors were examined for a potential role in controlling non-neuronal cell proliferation. CNTF, which is made by Schwann cells, may inhibit fibroblast proliferation (Fig.22). No consistent action on Schwann cells themselves was shown for CNTF, suggesting that, at least as regards proliferation, there is no autocrine function for this factor. It may however provide a means for Schwann cell inhibition of fibroblast proliferation.

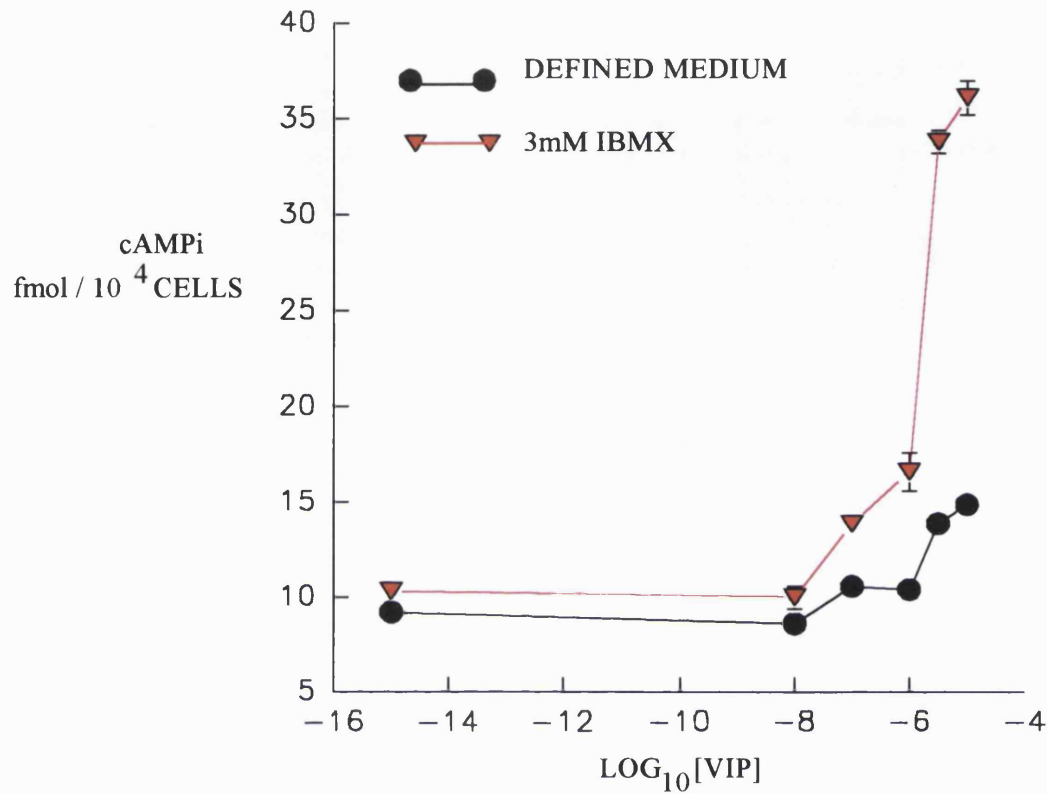


Fig.21. Dose response curve for VIP induced cAMPi elevation in Schwann cells.

Schwann cells plated at 100,000 cells per well of a 24-well plate were grown in serum-free defined medium, and IBMX added 1 hour prior to stimulations. Cultures were exposed to VIP for 10 minutes at different concentrations of peptide, and cAMPi assayed. Values are mean  $\pm$  s.d. of duplicate cultures.

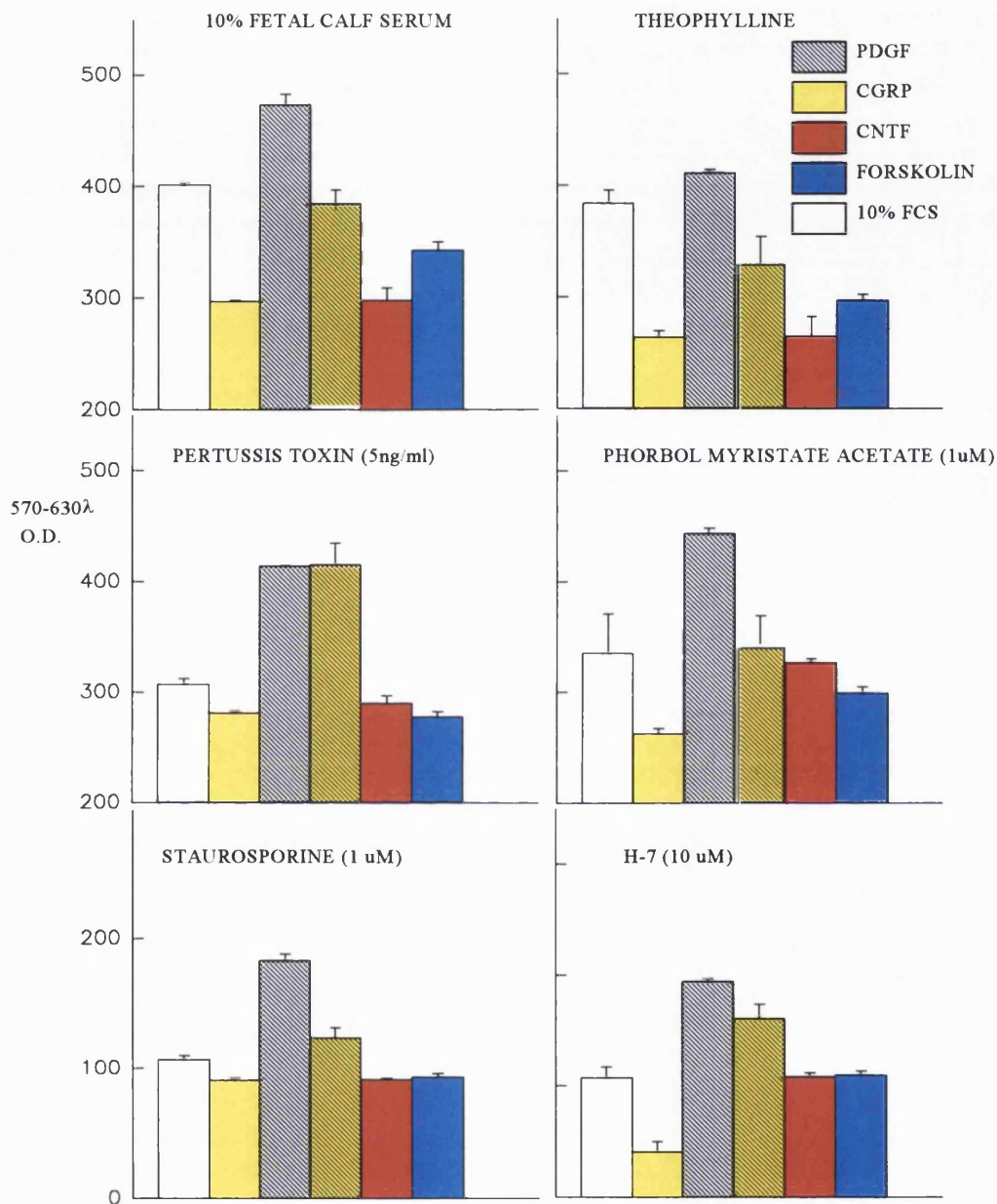


Fig.22. Second messengers in fibroblast proliferation. The action of CNTF on fibroblast proliferation.

MTT cell proliferation/viability assay on pure fibroblasts following a 48 hour incubation with agents. Cells were plated at 5,000 cells per well of a 96 well plate, and grown in DMEM with 10% FCS. Additions were made at 24 hour intervals, and cell number determined by formation of a coloured formazan product detectable by O.D. after 48 hours. Values are mean  $\pm$  s.e. of triplicate cultures.

## DISCUSSION

A model may be proposed in which axotomy results in increased amounts of CGRP at the site of injury by anterograde and retrograde transport of peptide, and possibly also by increased synthesis by motoneurons. CGRP is present *in vivo* when and where both waves of Schwann cell proliferation are occurring, and is therefore in a position to trigger Schwann cell proliferation and to prevent fibroblast proliferation at the leading edge of regenerating or damaged axons. CGRP can elevate fibroblast cAMPi and causes a dose dependent inhibition of fibroblast proliferation *in vitro*. CGRP when added to Schwann cells *in vitro*, produces an elevation in cAMPi and a variable increase in the rate of cell division when added in the presence of 10% FCS. The action of cAMPi elevation on Schwann cells may involve augmenting or enabling the action of other growth factors such as PDGF. We suggest that Schwann cell cAMPi elevation may in part be mediated by neuronal CGRP and by circulating or neuronal catecholamines acting on Schwann cell CGRP and  $\beta$ -adrenergic receptors, respectively.

Formation of cAMPi by Schwann cells in response to CGRP and  $\beta$ -agonists is regulated by many factors, but of prime importance, is the onset of receptor desensitisation by phosphorylation of the receptor. Investigation of the various second messenger systems and regulatory enzymes involved in cAMPi responses, has enabled us to outline their contributions in regulating Schwann cell cAMPi formation. The inhibitory G-protein,  $G_i$  (which can downregulate adenylate cyclase and thereby inhibit agonist induced cAMPi elevation), and the enzyme phosphodiesterase (involved in the breakdown of cAMPi), function primarily to limit the level of acute cAMPi elevation in response to CGRP, and do not play an important role in desensitisation on prolonged (hours or more) exposure to agonist. It seems that phosphorylation of receptors for CGRP and  $\beta$ -agonists is responsible for desensitisation, and can be blocked by inhibitors of PKA and  $\beta$ ARK, respectively. These agents are incompatible with proliferation; PKA activation is required to promote downstream responses essential for proliferation, and currently available techniques for inhibiting  $\beta$ ARK, such as heparin and digitonin, are too toxic for anything other than very brief assays, such as acute cAMPi dose responses. Schwann cells are able to metabolise CGRP via endopeptidase 24.11, and this may represent a mechanism for preventing desensitisation *in vivo*. Both homologous and heterologous desensitisation can be demonstrated, with a more prolonged time course required for the latter. We have developed a novel strategy for overcoming desensitisation in living cell culture. In the presence of supraphysiological  $Mg^{++}$  in the culture medium, desensitisation is greatly attenuated, cAMPi responses are maintained, and CGRP and isoprenaline seem to be permissive for proliferation in serum and with

growth factors, such as PDGF and FGF. CGRP is metabolised by Schwann cells in culture, and this is inhibitable by phosphoramidone, an inhibitor of enkephalinase (endopeptidase E.C.3.4.24.11). This is known to exist on the surface of Schwann cells (Kioussi and Matsas, 1991), and CGRP may be a substrate (Davies et al., 1992).

The source of mitogenic growth factors, such as PDGF and FGF, *in vivo* is not clear. PDGF and TGF- $\beta$  are both known to be released from macrophages at sites of injury (Rappolee et al., 1988), and this may also prove to be so for peripheral nerve. Macrophages are known to invade injured nerves and may indeed be a necessary requirement for Schwann cell proliferation *in vivo* (Lunn et al., 1989). Recent work has confirmed the presence of PDGF A-chain mRNA in mouse peripheral nerve, both in sensory and motor cell bodies; our own preliminary results confirm the presence of A-chain mRNA in rat anterior horn. It remains to be shown, however, if these neurons contain B-chain, the form most active on Schwann cells. Schwann cells themselves may produce a number of growth factors *in vitro*, including PDGF and CNTF, and may also produce TGF- $\beta$  (Unsicker et al., 1991). However, it is not known whether Schwann cells synthesise PDGF following axotomy or whether the form of PDGF produced would exhibit autocrine action. The exclusion of macrophages from our cultures is of great importance when investigating the activity of potential mitogens; traditional purification methods do not specifically exclude macrophages.

CGRP, in addition to the inhibitory effects on fibroblast proliferation that we have found, has previously been shown to inhibit both macrophage and also T-cell proliferation (Umeda et al., 1988) via elevation of cAMPi (Boudard and Bastide, 1991; Umeda and Arisawa, 1989). It is possible that the main function of CGRP *in vivo*, is to downregulate the immune and inflammatory response through a negative action on T-cells and macrophages, and to prevent excessive scar formation by a negative action on endoneurial fibroblasts. Following injury *in vivo*, circulating catecholamine levels are high and could contribute to the local effects of neuropeptides, such as CGRP and possibly VIP. VIP is present in sensory neurons and has been shown to inhibit human monocyte function via elevation of cAMPi (Wiik, 1989).

In conclusion, therefore, we suggest that CGRP and possibly also VIP in concert with  $\beta$ -adrenergic agonists, contribute to cAMPi elevation in Schwann cells following axotomy, and that by doing so they permit proliferation of these cells in response to growth factors, such as PDGF. The failure, to date, in demonstrating a mitogenic effect of these agents *in vitro*, is due to a rapid desensitisation of the response. This is not surprising, and is a feature of cAMP-linked systems studied to date.

Desensitisation is less likely to be a problem *in vivo*, where cells would be exposed to continuously released low concentrations of peptide and these removed by uptake or breakdown. Heterologous desensitisation for CGRP may have other implications. Endopeptidase 24.11 (enkephalinase) is a cell surface enzyme involved in the degradation of atrial natriuretic peptide and also of CGRP (Davies et al., 1992). Experiments using inhibitors of endopeptidase 24.11 are underway to assess the potential benefit of inhibiting breakdown of atrial natriuretic peptide in diuresis and heart failure; one potential problem would be coincidental inhibition of CGRP breakdown. Although this may lead to beneficial vasodilatation, it could lead to desensitisation of adrenergic receptors and loss of inotropic action, this has been shown for isolated myocyte calcium influx (Ono et al., 1989).

In addition, CGRP is permissive for the mitogenic response to the growth factor PDGF. This response *in vitro* is, however, limited by the rapid development of homologous desensitisation to CGRP and also to isoprenaline. Furthermore, on prolonged stimulation (>8 hour) a degree of heterologous desensitisation is also induced. We show that this desensitisation can not be prevented by inhibitors of PKA, PKC, or phosphodiesterase, and is not dependent on pertussis toxin sensitive G protein inhibition. We provide the first evidence for the role of  $Mg^{++}$  in limiting the effect of desensitisation both to CGRP and isoprenaline. In the presence of 5mM  $Mg^{++}$ , CGRP and isoprenaline-mediated elevation of cAMPi is potentiated and maintained, indicating that the effect of desensitisation is being neutralised. Not surprisingly, in the presence of 5mM  $Mg^{++}$ , both CGRP and isoprenaline are permissive for Schwann cell proliferation;  $Mg^{++}$  on its own neither elevates cAMPi nor is mitogenic. CGRP and isoprenaline increase NGF synthesis by fibroblasts, and isoprenaline increases Schwann cell NGF synthesis only in the presence of 5mM  $Mg^{++}$ . We also show that Schwann cells are able to metabolise CGRP, and that this is probably via a phosphoramidone inhibitable surface endopeptidase, 24.11. We suggest that CGRP released from injured peripheral nerves may play an important role in nerve regeneration by regulating the responsiveness of Schwann cells to other growth factors via an elevation in cAMPi. Additionally, CGRP may be important in preventing fibroblast proliferation at the regenerating nerve front which could otherwise impair reinnervation. Desensitisation *in vivo* could be limited by the continuous release of low levels of CGRP from neurons, and the prevention of accumulation of desensitising levels of peptide by continuous degradation of CGRP by 24.11 on Schwann cells.

In some systems, such as S49 lymphoma cells and 3T3 fibroblasts, PKC may promote adenylate cyclase activation (Rozengurt et al). In prolactin-secreting rat adenoma (GH4C1) pituicytes, PKC can stimulate adenylate cyclase activity by inhibiting  $G_i$

and thereby may potentiate the activity of agents such as VIP (Gordeladze et al., 1989). In swine luteal cells, TPA markedly potentiates the cAMPi elevation obtained with leuteinizing hormone, forskolin, or cholera toxin. TPA also augments the stimulatory effect of pertussis toxin when combined with maximally effective doses of forskolin in the presence and absence of IBMX. This is of great interest as we have seen a similar action of these agents on forskolin stimulated proliferation of cultured Schwann cells. The combination of pertussis toxin and PMA resulted in a greatly amplified proliferation of Schwann cells. Pertussis toxin in this system also potentiates cAMPi elevation, as does PMA. One may infer from this that stimulatory coupling exists between PKC and cAMP generating systems in Schwann cells, and that this may be at the level of G-proteins or the catalytic units of adenylate cyclase. PMA, which is not mitogenic alone in defined medium, potentiated proliferative responses and synergised with forskolin but not PDGF. Inhibitors of PKC, such as H-7 and staurosporine, prevent the mitogenic action of PDGF, as does the tyrosine kinase inhibitor, genistein. This suggests that PDGF action on Schwann cell proliferation is wholly or partly mediated by protein kinase C, and less by tyrosine kinase activity. There is an additive effect when PMA is combined with pertussis toxin; the latter is not able to substitute for PDGF, but enhances the action of forskolin, suggesting that inhibition of  $G_i$  is able to augment the effects of cAMPi elevating agents. From cAMP assays, this is due to enhancing elevation of cAMPi. PKC activation does not seem to be at the level of  $G_i$ , as pertussis toxin potentiates the effects of PMA. One possibility is that it acts at the level of the catalytic subunit of PKA (C-subunit). PKC mediated phosphorylation of the C-subunit has been demonstrated in erythrocytes and has been suggested by studies in pituitary cells. The effect occurs both with and without added IBMX, suggesting that the effects are not mediated by decreased breakdown of cAMPi via an inhibitory effect of PKC on phosphodiesterase.

My description of an inhibitory effect of TGF $\beta$  on Schwann cell proliferation raises several possibilities. TGF- $\beta$ 3 is expressed in sciatic nerve, as is TGF $\beta$ 1 in connective tissue, and they have been localised to Schwann cells (Unsicker et al., 1991). The possibility exists, therefore, that TGF $\beta$  may play a negative autocrine role in control of proliferation. TGF $\beta$  is also made by macrophages, which may therefore provide both stimulatory and inhibitory signals for Schwann cell proliferation. CNTF may provide a mechanism for the Schwann cell mediated inhibition of fibroblast proliferation during regeneration and development, when synthesis of this factor are known to be high.



### **CHAPTER THREE**

#### **THE ROLE OF MACROPHAGE DERIVED CYTOKINES IN REGENERATING AND DEGENERATING PERIPHERAL NERVE**

## INTRODUCTION

### OVERVIEW

Peripheral nerves can regenerate after a crush or cut injury. Various cellular events in the distal stump seem crucial for successful axon regrowth: (i) Macrophages invade the damage site (Perry et al., 1987), then spread into the distal endoneurium (Lunn et al., 1990). (ii) There are two waves of Schwann cell division within the first week after injury, the first around the injury site and the second throughout the endoneurium with the same distribution and timing as the spread of macrophages (Clemence et al., 1989; Brown et al., 1991). (iii) NGF synthesis is increased in both Schwann cells and epineurial fibroblasts, with the timing also coinciding with macrophage invasion (Brown et al., 1991; Heumann, 1987a; Heumann et al., 1987b). Nerve growth factor receptor (NGFr) is upregulated in Schwann cells (Taniuchi et al., 1987; Taniuchi et al., 1988) independently of macrophages, and may be mediated by loss of axonal contact (Taniuchi et al., 1988; Lemke et al., 1988). (iv) Axons in the distal stump degenerate and their debris is phagocytosed (Bray et al., 1981).

Macrophages recruited from the circulation are crucial for Schwann cell division, NGF synthesis, and axonal degeneration, as shown by the following experiments: Exclusion of invading cells from chambers containing explanted nerve prevents Schwann cell division and myelin phagocytosis, but both events are restored by allowing their entry (Beuche and Friede, 1984). The mouse strain C57/Bl/Ola, does not recruit macrophages into the endoneurium; in these mice, nerve degeneration is greatly delayed, and both division of endoneurial Schwann cells and expression of NGF away from the crush site are absent, and the expression of NGFr lowered (Brown et al., 1991). Blocking macrophage recruitment into the distal endoneurium of normal mice with antibodies to the complement receptor, mimics the situation in C57/Bl/Ola mice (Bruck and Friede, 1990). Regulation of the two waves of Schwann cell proliferation may be different; macrophage recruitment and the second wave of proliferation are interdependent, whereas macrophages may not be critical for the first wave at the injury site. A further period of Schwann cell proliferation occurs during axonal regrowth and may be mediated by axonal signals. Macrophages secrete many cytokines, including Il-1 (Auron et al., 1984), and TNF (Kohase et al., 1987). Growth factors, such as FGF and PDGF are mitogenic for Schwann cells *in vitro*, but only in the presence of agents which increase cAMPi (Stewart et al., 1991). Several reported experiments show that Il-1 and TNF have no effect on Schwann cell proliferation (Stewart et al., 1991; Davis and Stroobant, 1990b). Il-1 increases NGF synthesis by epineurial fibroblasts in nerve explants and by cultured fibroblasts *in*

*vitro* , but is reported to have no effect on Schwann cell NGF synthesis *in vitro* (Matsuoka et al., 1991a).

## MACROPHAGES

Macrophages recruited into peripheral nerve after injury are of critical importance for normal degeneration and regeneration, NGF production, and Schwann cell proliferation to occur. It is not, however, clear how their actions on Schwann cells and fibroblasts are mediated.

There is considerable evidence that macrophage or leukocyte entry into peripheral nerve is required for myelin destruction and removal, and for Schwann cell mitosis (Perry et al., 1987; Lunn et al., 1989; Brown et al., 1991; Beuche and Friede, 1984). Peripheral nerve injury is slightly different to other sites of inflammation in that there is only a very slight, or no, early recruitment of neutrophils to the lesion. The major cellular response is the recruitment of macrophage/monocytes to the lesion and distal stump at a few days post injury. Normal uninjured nerve contains only a small population of resident macrophages, as defined by immunological criteria. Macrophages, as defined by the expression of ED1 marker, begin to enter injured peripheral nerve from blood vessels by day 2 post axotomy, and numbers are approaching maximum by 3 days post lesion. Macrophages thus begin to accumulate in the distal stump just prior to the period of Schwann cell proliferation (Thomas, 1966; Haftek and Thomas, 1968; Perry et al., 1987). A recently described mutant mouse strain, in which axons degenerate very slowly, demonstrates a much reduced macrophage recruitment following axotomy than a normal mouse strain (Lunn et al., 1989). These C57BL/Ola mice also demonstrate a much lower increase in NGF mRNA and NGFr mRNA (Brown et al., 1991) associated with impaired regeneration of sensory but not motor axons. Interestingly, there is almost no proliferation of Schwann cells following injury, except in the first few millimetres surrounding the injury site, possibly due to the absence of IL-1 or other growth factor production, by recruited monocytes / macrophages. Proliferation adjacent to the lesion might be mediated by axonal signals, such as CGRP released from damaged neurons, which act as permissive agents for proliferation in response to PDGF, FGF, or other growth factors. These growth factors could originate from the Schwann cells themselves or even the axons. Macrophages could also be involved in this, as even in the C57BL/Ola mouse, macrophages are seen adjacent to the lesion site.

Macrophages in culture secrete Schwann cell mitogens when presented with myelin membrane fractions (Baichwal et al., 1988). Macrophages secrete TGF- $\beta$  (Assoian et al., 1987) and also PDGF after injury (Shimokado et al., 1985a; Mornex et al., 1986;

Martinet et al., 1986; Rappolee et al., 1988). Macrophages produce the PDGF-B protein (Ross et al., 1990; Shimokado et al., 1985b), but they stop making PDGF about 12 hours after stimulation. Lipid loading of macrophages, as occurs in Wallerian degeneration, leads to the production of TGF- $\beta$  and Il-1 (Rappolee et al., 1988). These macrophages then attract more macrophages by secreting TGF- $\beta$ , which may also be produced by Schwann cells in peripheral nerve (Unsicker et al., 1991). Platelet TGF- $\beta$  is a chemoattractant for monocytes and induces monocyte Il-1 production, an action shared by the complement fragment, C5a (Wahl et al., 1987).

Ia antigen is involved in antigen recognition by T-cells and can be induced on cultured macrophages and Schwann cells by gamma-interferon (Samuel et al., 1987a). During Wallerian degeneration, a sub population of ED1-positive monocytes/macrophages express Ia antigen; Schwann cells are Ia-negative (Stoll et al., 1989). Ia expression by monocytes/macrophages appears to be a transient event and is not seen in post-phagocytic macrophages, as indicated by the fact that ED1-positive phagocytes with large vacuoles are Ia-negative. The expression of Ia antigen during Wallerian degeneration indicates that Ia expression need not necessarily reflect specific immune events, but in some instances can represent a non-specific response to peripheral nervous system damage. Apolipoprotein E (apoE), a lipid-binding glycoprotein involved in transport and metabolism of phospholipids and cholesterol, is synthesised and secreted at elevated rates following transection of mature rat peripheral and central nerves. In the peripheral nervous system (PNS) infiltrating macrophages express apoE during Wallerian degeneration (Stoll et al., 1989b).

Colony stimulating factor-1 (CSF-1), granulocyte/macrophage colony stimulating factor (GM-CSF), IL-3, and PMA, are mitogens for cultured murine bone marrow derived macrophages. Agents that elevate macrophage cAMPi, such as 8-BrcAMP, IBMX, PGE<sub>2</sub> and cholera toxin, inhibit this proliferative response (Vairo et al., 1990). C1-inhibitor (C1-inh) is synthesised and secreted by mononuclear phagocytes. Amongst the most potent stimulators of monocyte C1-inh synthesis are the interferons (IFNs) IFN- $\alpha$ , IFN- $\beta$ , and  $\gamma$ -IFN. A number of agents which inhibit monocyte C1-inh secretion and reduce C1-inh mRNA levels (such as histamine, PGE<sub>2</sub>, C5a des arg, and serum treated immune complexes), elevate cAMPi and activate PKA (Lappin and Whaley, 1989). Interferons bind to specific receptors (IFN- $\alpha$  and - $\beta$  binding to Type I IFN receptors, and  $\gamma$ -IFN binding to type II IFN receptors). At least two mechanisms by which IFN receptor-ligand interaction elicit their effects exist. These are: 1) binding of an activated receptor transducer/regulatory component to specific DNA sequences on IFN sensitive genes. 2) the activation of protein kinase C and binding of its regulatory components to specific DNA sequences. Unlike, the well described Ras signalling pathway, the end result of which is the translocation of activated protein

kinases to the nucleus (where they activate nuclear transcription factors by phosphorylation), IFN's induce tyrosine phosphorylation and activation of transcription factor subunits in the cytoplasm, which then translocate into the nucleus and induce transcription.  $\gamma$ -IFN causes a dose related increase in monocyte C1-inh mRNA abundance and protein synthesis, maximal within 1-2 hours of treatment. This rapid stimulation of monocyte C1-inh synthesis, suggests that this increases the transcription of the C1-inh gene.  $\gamma$ -IFN alters the stability of C1-inh mRNA.

## IL-1 ACTION AND BREAKDOWN

Interleukin-1 (IL-1) includes two polypeptides, IL-1 $\alpha$  and IL-1 $\beta$ , both of which recognise the same receptor and share biological properties, although they are distinct gene products. They are synthesised by both leukocytic as well as non-leukocytic cells, and their effects are manifested in nearly every tissue. Although they are biologically similar to TNF, IL6, FGF, PDGF and TGF- $\beta$ , they are structurally distinct, with the exception of acidic-FGF which is related to IL-1 $\beta$ . IL-1 $\beta$  contains several cleavage sites for serine proteases, and recently, an endopeptidase that is membrane bound and is involved in the degradation of neuropeptides, has been shown to destroy the biological activity of IL-1 (Pierart et al., 1988). Enkephalinase, 24.11, a phosphoramidone-sensitive endopeptidase found in the nervous system, is associated with both myelinating and non-myelinating Schwann cell membranes in peripheral nerve, before and following axotomy (Kenny and Bourne, 1991).

In human macrophage lines, endotoxin-stimulated IL-1 transcription can be observed within 15 minutes (Fenton et al., 1987), and there appears to be some sort of negative regulation, as inhibitors of protein synthesis amplify and prolong this effect. IL-1 is under both transcriptional and translational control. When macrophages adhere to glass, IL-1 mRNA expression is stimulated, but no peptide is produced unless another signal (such as endotoxin) is also given. Endotoxin, however, stimulates both transcription and translation (Libby et al., 1986a). PGE<sub>2</sub>, often produced in response to IL-1 stimulation, can suppress IL-1 translation via induction of cAMPi (Knudsen et al., 1986), and this action is potentiated by dibutyryl cAMP (dbcAMP) or theophylline. No effect is seen at the transcriptional level. IL-1 stimulates human endothelial cells *in vitro*, to release PGI<sub>2</sub>, PGE<sub>2</sub>, and platelet-activating factor. Co-stimulation with PMA and IL-1, results in synergistic PGE<sub>2</sub> synthesis (Rollins et al., 1991). IL-1 has been shown to elevate cAMPi in a number of cell types, including lymphocytes and fibroblasts (Chedid et al., 1989). It is thought that this can be mediated directly by interaction of IL-1 with its receptor and associated G protein, as well as by an indirect action through increased PGE<sub>2</sub> production. Atypically, the G protein mediating IL-1-induced cAMPi elevation, appears to be pertussis toxin-

sensitive, as pre-treatment with pertussis toxin abolishes the cAMPi elevation seen with IL-1 (Chedid et al., 1989). Pertussis toxin, but not cholera toxin, also inhibits PGE<sub>2</sub> production in murine fibroblasts. In addition, the IL-1-induced GTPase activity of G-protein  $\alpha$  subunits, is impaired.

## SIGNAL TRANSDUCTION IN IL-1 STIMULATED CELLS

Addition of IL-1 to human synovial fibroblasts, results in increased arachidonic acid release and a transient rise in DAG. PMA, an activator of PKC, also increases arachidonic acid release, but with a different time course. This action of IL-1 is blocked by staurosporine and H-7 (both fairly non-specific inhibitors of PKC), suggesting that a protein kinase activity mediates IL-1 induced arachidonic acid release. However, as neither desensitising PKC with prolonged PMA pre-treatment, nor inhibiting PKA with H-8, prevents arachidonic acid release, this PK activity may not be PKC or PKA (Cisar et al., 1991), but another kinase inhibited by staurosporine or H-7. IL-1 stimulates PGE<sub>2</sub> production by osteoblasts, and in this case it is abolished by verapamil (100 $\mu$  M) and the calmodulin inhibitor W-7 (50 $\mu$  M), whilst potentiated by A23187 (0.1 $\mu$  M). PGE<sub>2</sub> production is also potentiated by IBMX, suggesting that both Ca<sup>++</sup> and cAMP second messenger systems are involved. This is of interest as it has recently become clear that G<sub>s</sub> can, in addition to activating adenylate cyclase, activate dihydropyridine-sensitive Ca<sup>++</sup> channels. In renal mesangial cells, genistein blocks IL-1-mediated PGE<sub>2</sub> production in serum-containing medium (Coyne and Morrison, 1990), suggesting a role for tyrosine phosphorylation. In the EL4 murine thymoma cell line, IL-1 stimulation leads to secretion of IL-2; cAMPi blocks or partially inhibits this response, whereas PKC activation leads to synergistic enhancement. This is also inhibited by pertussis toxin (Rollins et al., 1991). The regulation of the AP-1 transcription complex in these cells has been studied, and found to be regulated by several signalling pathways (Chedid et al., 1991). IL-1 and cAMP do not themselves induce AP-1 activation but synergise with phorbol esters. No effect of IL-1 on cAMP production could be demonstrated in these cells, suggesting different sites of activity for forskolin and IL-1. PKC activity, however, was critical as neither IL-1 or forskolin up-regulated AP-1 in the presence of a transfected PKC inhibitor. This inhibitory effect was reversed in the presence of added protein phosphatase inhibitor (okadaic acid). AP-1 may thus be regulated by the balance of several serine/threonine protein kinases and phosphatases.

## IL-1 AS A MITOGENIC FACTOR

*In vitro* IL-1-induced proliferation occurs in fibroblasts and smooth muscle cells, only if a cyclooxygenase inhibitor is included (Libby et al., 1986a; Libby et al., 1986b).

This may reflect an anti-proliferative action of IL-1-induced PGE<sub>2</sub> elevation on fibroblasts, though a weak mitogenic response has been demonstrated in endoneurial fibroblasts (Lisak and Bealmear, 1991; Lisak et al., 1985). Part of IL-1 action may be through increased production of receptors for other growth factors, such as EGF. IL-1 and TNF, mitogenic for fibroblasts even in the absence of insulin, act synergistically to promote fibroblast proliferation (Elias et al., 1987). This seems to occur at a second messenger level, and is not the result of up-regulation of receptors.

#### IL-1 IN AXOTOMISED PERIPHERAL NERVE AND SCHWANN CELL CULTURE

IL-1 is released from activated macrophages, and is one of the first cytokines whose mRNA is expressed after injury (Fenton et al., 1987). Recently, using a polymeric guidance channel to bridge a transected mouse peripheral nerve, it was found that inclusion in the regeneration chamber of a receptor antagonist to IL-1, decreased the number of regenerating myelinated and unmyelinated axons (Guenard et al., 1991). Supernatants from activated macrophages are mitogenic for Schwann cells and endoneurial fibroblasts cultured in 10% new-born calf serum (Lisak et al., 1985), leading to a 2-3 fold increase in cell number, and increased <sup>3</sup>H-thymidine incorporation, after 48 hours. This action of unfractionated macrophage-derived cytokines on Schwann cells can be partially blocked by mixed antibodies to IL-1 $\alpha$  and IL-1 $\beta$  (Lisak and Bealmear, 1991). In this study, Schwann cells were cultured for 48 hours in 10% FCS, and contained no detectable monocytes as defined by Fc receptor expression, latex bead phagocytosis, or Ox42 expression. It was concluded that part of the activity of unfractionated cytokines was due to IL-1, and that this action was direct, as indomethacin (which blocks prostaglandin synthesis) did not impair IL-1-induced proliferation. However, in this study as well as others, neither recombinant nor purified IL-1 $\alpha$  or IL-1 $\beta$ , have been shown to promote Schwann cell proliferation (Davis and Stroobant, 1990a; Stewart et al., 1991). Equally, submaximal GGF-induced Schwann cell proliferation was not potentiated by IL-1.

Following axotomy, the amount of NGF mRNA present in rat sciatic nerve increases dramatically from nearly undetectable levels in the normal adult nerve, to a more than 10-fold elevation after nerve lesion. This response is biphasic: a first, rapid transient increase peaking 6 hours after nerve lesion, is followed by a second, long-lasting elevation, reaching maximum levels 3-7 days after injury. The induction of the second peak requires macrophage invasion and secretion of IL-1. This is suggested by the fact that IL-1 induces expression of NGF-mRNA in sciatic nerve explants, and that in mutant mice lacking a macrophage response, the second mRNA peak is not seen. Moreover, in explants devoid of a blood supply, the second peak is not seen but

may be restored by IL-1 $\beta$ . Other cytokines, including TNF, have not been studied in this context to date.

### REGULATION OF NGF SYNTHESIS BY CYTOKINES

Neonatal rat cortical astrocytes in primary culture, synthesise and secrete NGF. Levels of NGF mRNA may be increased 2-fold by stimulation of cultures with FGF or IL-1 (Vige et al., 1991). The IL-1-induced response is maximal after a 3 hour incubation period, and persists for 36 hours. Unlike FGF, IL-1 also increases the stability of NGF mRNA. The actions of FGF and IL-1 are additive. Primary cultures of rat cortical astrocytes contain low cellular levels of NGF (2 pg/mg of protein), but secrete NGF into the culture medium (540 pg of NGF/mg of cell protein/38 hours). A maximal 3-fold increase in NGF can be elicited by IL-1 (10U/ml) after a 38 hour incubation period (Carman Krzan et al., 1991). IL-1 elevates NGF mRNA and protein in cultures of rat hippocampus, and this is partially blocked by indomethacin. In keeping with this, PGE<sub>2</sub> also elevates NGF (Friedman et al., 1990).

IL-1 acts synergistically with TNF to elevate NGF content in supernatants of cultured rat neocortical astroglia. Purified astrocytes in defined medium, respond poorly to either added individually, but when IL-1 and TNF are combined, they increase NGF in culture supernatants 3-6-fold over 48 hours (Friedman et al., 1990). Both IL-1 and TNF can further enhance the maximal stimulation of NGF secretion induced by acidic or basic FGF in astrocyte cultures. FGFs also enhance secretion of NGF from fibroblasts (Yoshida and Gage, 1991); in both cases, PDGF is ineffective. In a fibroblast cell line, L929, NGF production may be increased by phorbol esters, implicating PKC. This seems to be cell-specific, as this is not seen in dispersed salivary cells.



## MATERIALS AND METHODS

Experimental and culture techniques have been described previously in Chapter 2. Cells were prepared by panning, and cultured as previously outlined. In all cases, MTT and  $^{125}\text{IdU}$  mitogen experiments, as well as cAMPi and NGF assays, were performed as previously described. Enzyme inhibitors were from the same sources as described in Chapter 2.

IL-1 $\beta$  (Promega) was used at concentrations between 1-100U/ml, IL-1 $\alpha$  (Promega) at 10ng/ml,  $\gamma$ -IFN at 100-300U/ml, and TNF $\alpha$  (Promega) at 10ng/ml.

## RESULTS

### SUMMARY

In this chapter I have used Schwann cells and fibroblasts dissociated from neonatal sciatic nerve and rapidly purified by immuno-panning, to re-investigate the action of the cytokines: IL-1, TNF and  $\gamma$ -IFN. In contrast to previous reports, I show that IL-1 by itself is a mitogen for Schwann cells, and IL-1 can synergize with PDGF to give a mitogenic signal without the need for exogenous signals to raise Schwann cell cAMPi. In addition, both IL-1 and TNF increase fibroblast NGF synthesis, and these effects are additive; IL-1 also increases NGF synthesis in freshly prepared Schwann cells. Moreover, the effects of IL-1 on NGF synthesis are greatly potentiated by  $\gamma$ -IFN which has no effect by itself and thus seems to act as a priming signal for IL-1 action.

### IL-1 AND SCHWANN CELL PROLIFERATION

Because Schwann cell properties change with time in culture ( Mirsky et al, 1990; Scarpini et al, 1990; Seilheimer et al, 1989), we developed a fast immuno-panning procedure for purification of cells. In all experiments, pure Schwann cells were grown *in vitro* for 24 hours before the start of the experiment, except where stated. As shown in Figure 23(a-b), treatment of such Schwann cells with recombinant IL-1 $\beta$  (10-30 U/ml) for 48 hours in defined serum-free medium, led to an increase in DNA synthesis and in viable cell number, as measured by the incorporation of <sup>125</sup>-iododeoxyuridine (<sup>125</sup>IdU) and the MTT assay, respectively. The synergistic stimulation of Schwann cell proliferation by IL-1 and PDGF, is comparable with that of forskolin and PDGF (Fig. 23c.). IL-1 is mitogenic in serum, as is TNF $\alpha$  (Fig.23d).

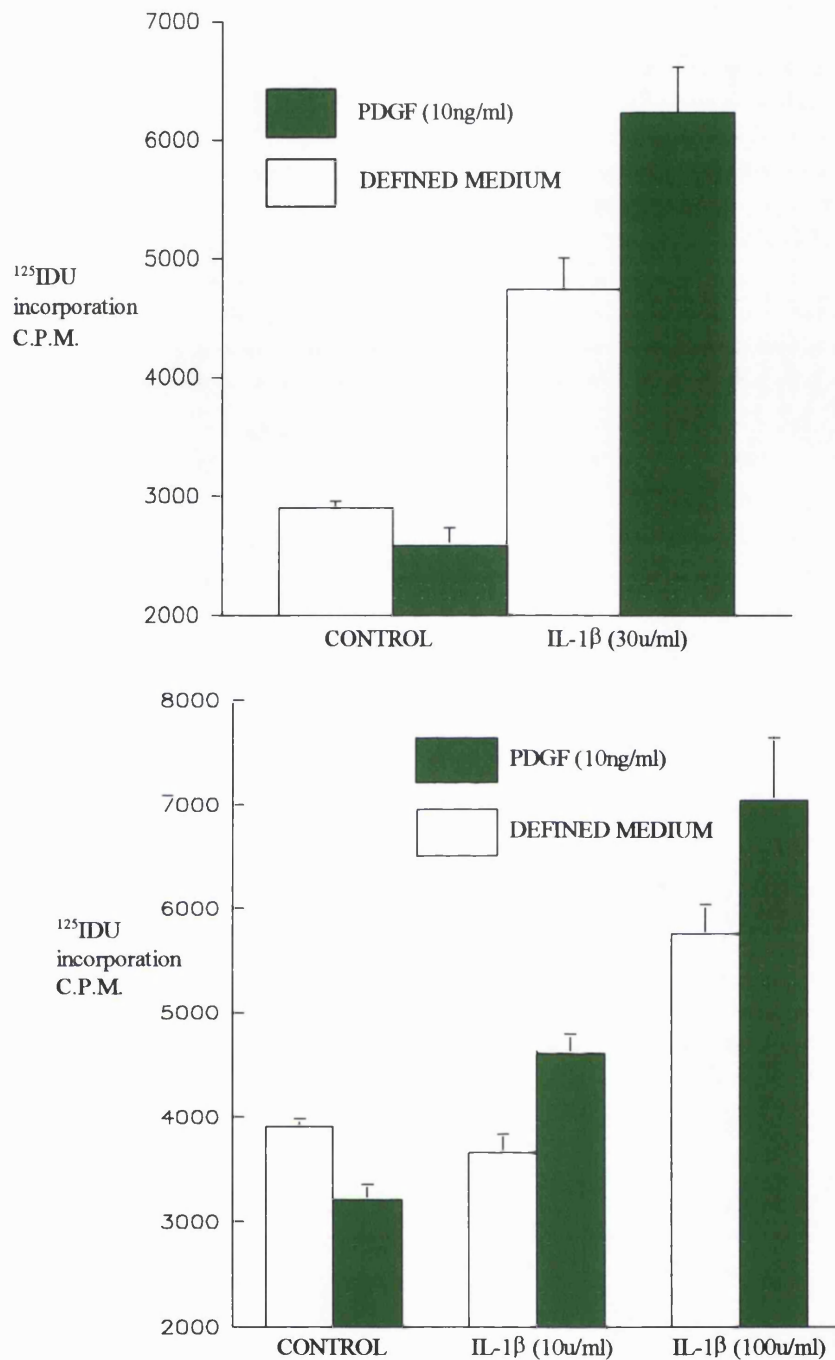


Fig.23a. The action of IL-1 and PDGF on Schwann cell proliferation assessed by  $^{125}\text{IdU}$  assay.

Schwann cells, plated at 10,000 cells per well of a laminin coated 96-well plate in serum-free defined medium, were 24 hours post nerve-dissociation at the start of assays. Agents were added over 48 hours, and repeated at 24 hour intervals.  $^{125}\text{IdU}$  was added for the final 24 hours of culture. Cells were then harvested and incorporated radioactivity determined. Values are mean  $\pm$  s.e. of triplicate cultures. Two separate experiments are shown.

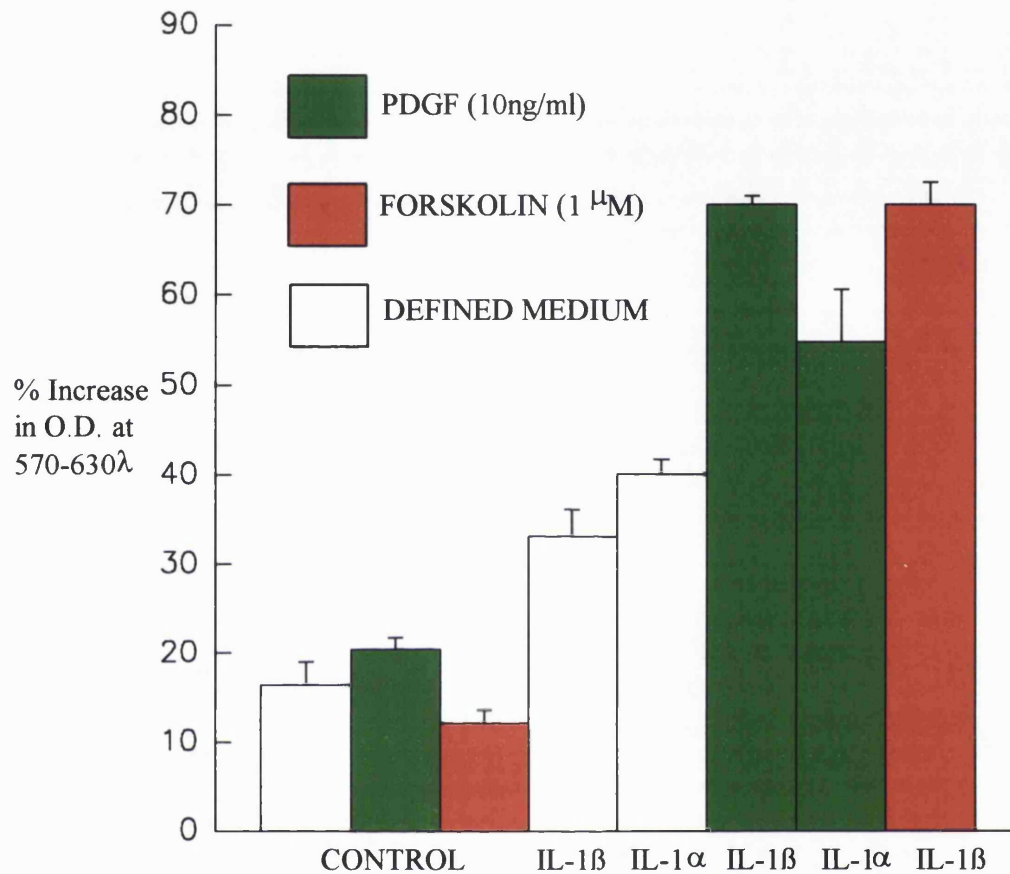


Fig.23b. IL-1 induced Schwann cell proliferation in defined medium, as assessed by MTT assay.

Cells were plated at 10,000 per well of a laminin coated 96-well plate in defined medium, and were 24 hours old at the start of assays. Cytokines were added over 48 hours with additions repeated at 24 hour intervals. IL-1 was used at 30u/ml. Values are mean  $\pm$  s.e. of % increase in O.D. from day 0 of triplicate cultures.

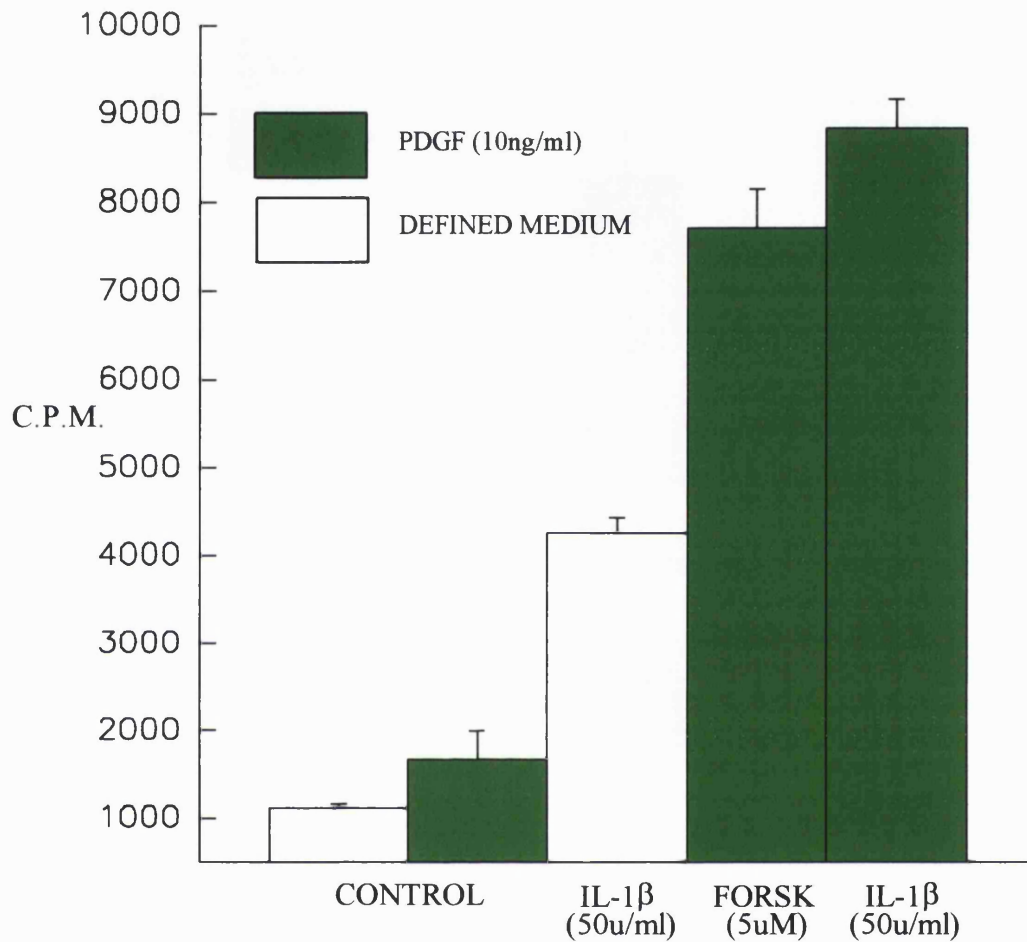


Fig23c. IL-1 $\beta$  induced Schwann cell proliferation in defined medium, compared to forskolin / PDGF synergy, as assessed by  $^{125}$ IdU assay.

Schwann cells, plated at 10,000 cells per well of a 96-well plate in serum-free defined medium, were 24 hours post nerve-dissociation at the start of assays. Agents were added over 48 hours and repeated at 24 hour intervals.  $^{125}$ IdU was added for the final 24 hours of culture. Cells were then harvested and incorporated radioactivity determined. Values are mean  $\pm$  s.e. of triplicate cultures.

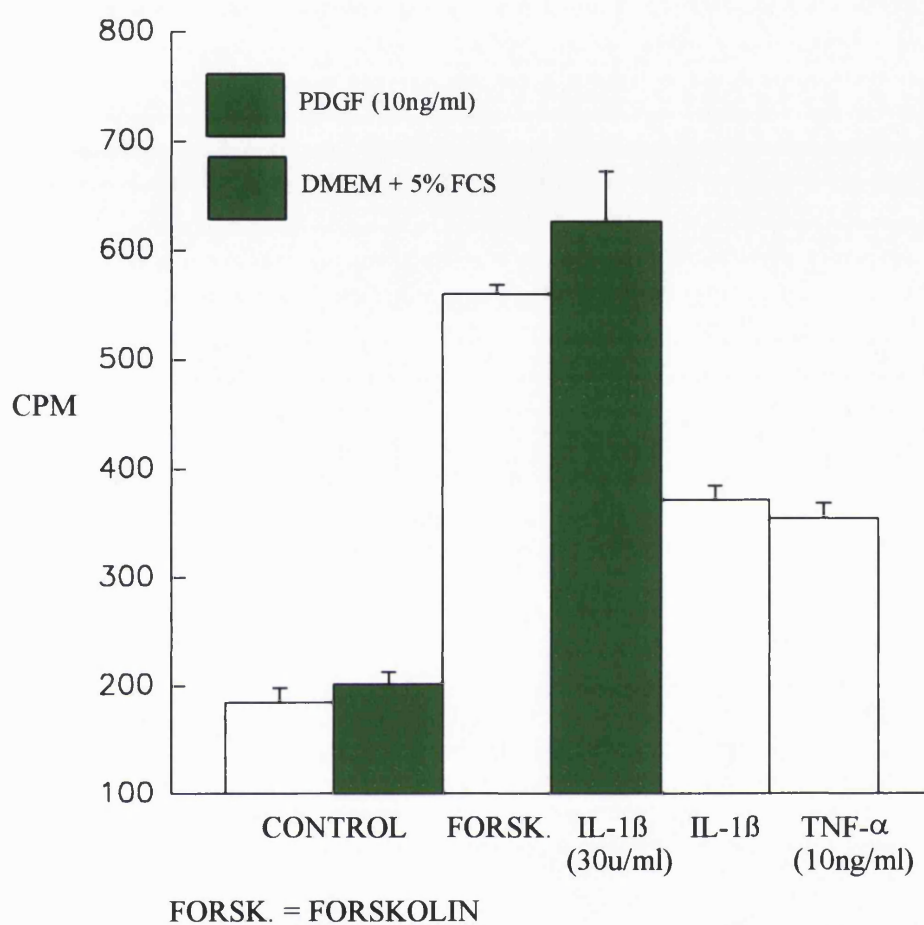


Fig.23d.  $\text{TNF}\alpha$  and  $\text{IL-1}\beta$  induced Schwann cell proliferation in serum containing medium, as assessed by  $^{125}\text{IdU}$  assay.

Schwann cells, plated at 10,000 cells per well of a 96-well plate in DMEM and 5% FCS, were 24 hours post nerve-dissociation at the start of assays. Agents were added over 48 hours and repeated at 24 hour intervals.  $^{125}\text{IdU}$  was added for the final 24 hours of culture. Cells were then harvested and incorporated radioactivity determined. Values are mean  $\pm$  s.e. of triplicate cultures.

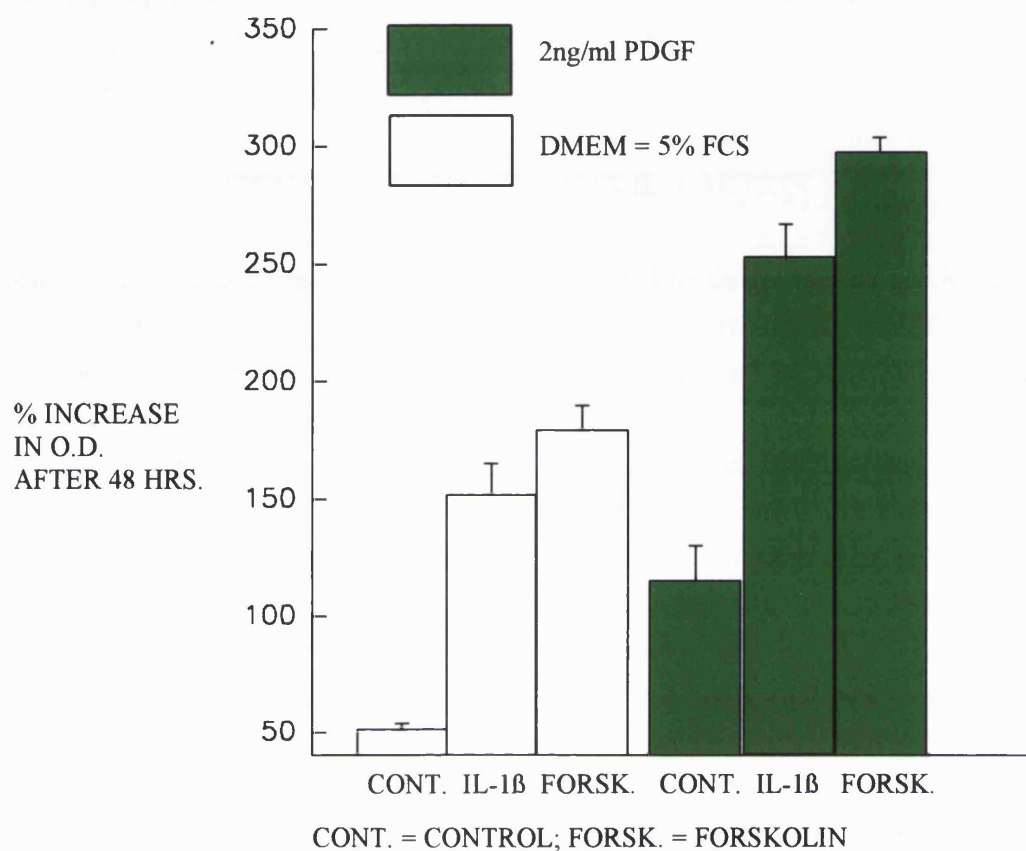


Fig.23e. IL-1 $\beta$  induced Schwann cell proliferation in serum containing medium, as assessed by MTT assay.

Schwann cells were plated at 10,000 per well of a 96-well plate in DMEM and 5% FCS, and were 24 hours old at the start of assays. Cytokines were added over 48 hours with additions repeated at 24 hour intervals. IL-1 $\beta$  was used at 30u/ml. Values are mean  $\pm$  s.e. of % increase in O.D. from day 0 of triplicate cultures.

Cells were generally cultured on laminin as this improved plating efficiency, but similar results were obtained using poly-D-lysine as a substrate. IL-1 $\beta$  was also able to synergize, in proliferation assays, with PDGF, as well as forskolin (Fig. 24). These Schwann cell responses to IL-1 were however, inconsistent if the cells were grown *in vitro* for a week before starting the experiment, and in fact, were not seen in cells prepared by traditional methods. (data not shown).

### SIGNAL TRANSDUCTION IN IL-1 ACTION

Various second messenger systems were investigated to determine the mechanism/s of IL-1 $\beta$  action. IL-1 $\beta$  produced a small elevation in Schwann cell cAMPi after 4 hours or more (Fig.25), though this could not be detected in acute assays. This relatively prolonged time-course, suggests that synthesis of an intermediate is required for cAMPi elevation, and argues against a direct link between the IL-1 receptor and adenylate cyclase. We do not have data confirming that indomethacin pre-treatment prevents IL-1 induced cAMPi elevation in Schwann cells, as in fact, it has proved difficult to consistently measure cAMPi elevation, in response to IL-1. Indomethacin pre-treatment does reduce the mitogenic activity of IL-1 (Fig.26), suggesting that prostaglandin synthesis is involved in IL-1-induced proliferation. The prostaglandin PGE<sub>2</sub> is able to acutely elevate cAMPi when added to Schwann cells *in vitro* (Fig.25), and is mitogenic if desensitisation is prevented with high magnesium (Fig.28). It seems likely, therefore, that IL-1 elevates cAMPi via a prostaglandin-mediated pathway. Pre-treatment of Schwann cells with either H-8 or H-89, both selective inhibitors of PKA, only attenuate IL-1 mitogenic activity although they virtually abolish the synergistic action of forskolin and PDGF, and of IL-1 and PDGF (Fig.27a, b).

Pertussis toxin, which in some systems potentiates the action of agents acting through cAMPi, partly inhibits IL-1 induced proliferation, in defined medium (Fig.26 and 29a), and in serum containing medium (Fig.29b), suggesting the involvement of a sensitive G protein.



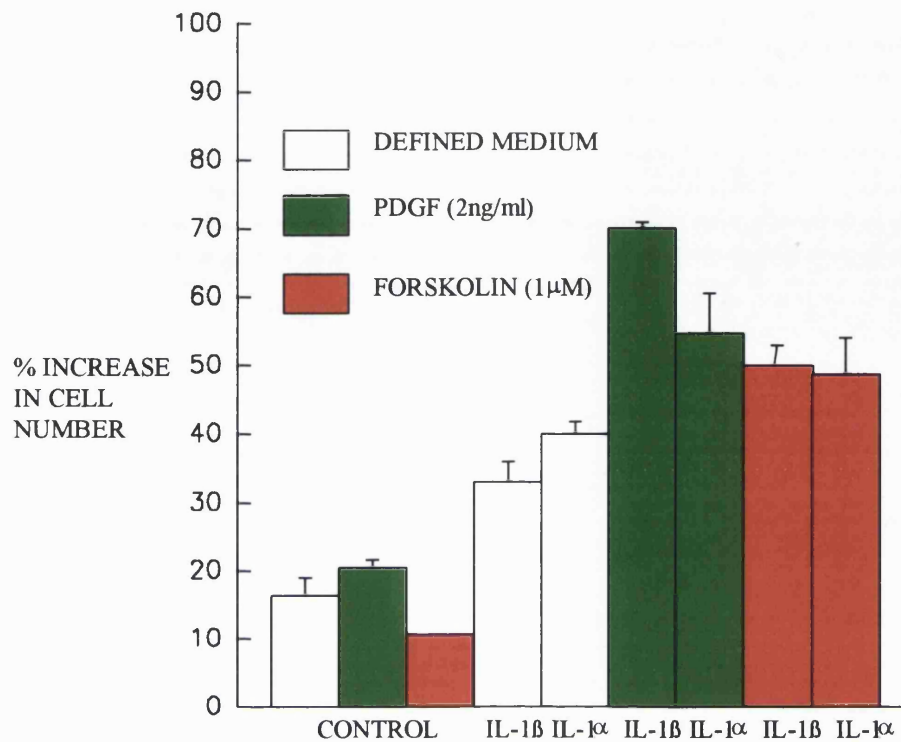


Fig.24. IL-1 synergises with PDGF and forskolin.

MTT cell proliferation/viability assay on pure cultured Schwann cells, following a 48 hour incubation with cytokines. Cells were plated at 10,000 per well of a 96-well plate in defined medium, and were 24 hours old at the start of assays. Cytokines were added over 48 hours with additions repeated at 24 hour intervals. IL-1 $\beta$  was used at 30u/ml and IL-1 $\alpha$  at 10ng/ml. Values are mean  $\pm$  s.e. of % increase in O.D. from day 0 of triplicate cultures

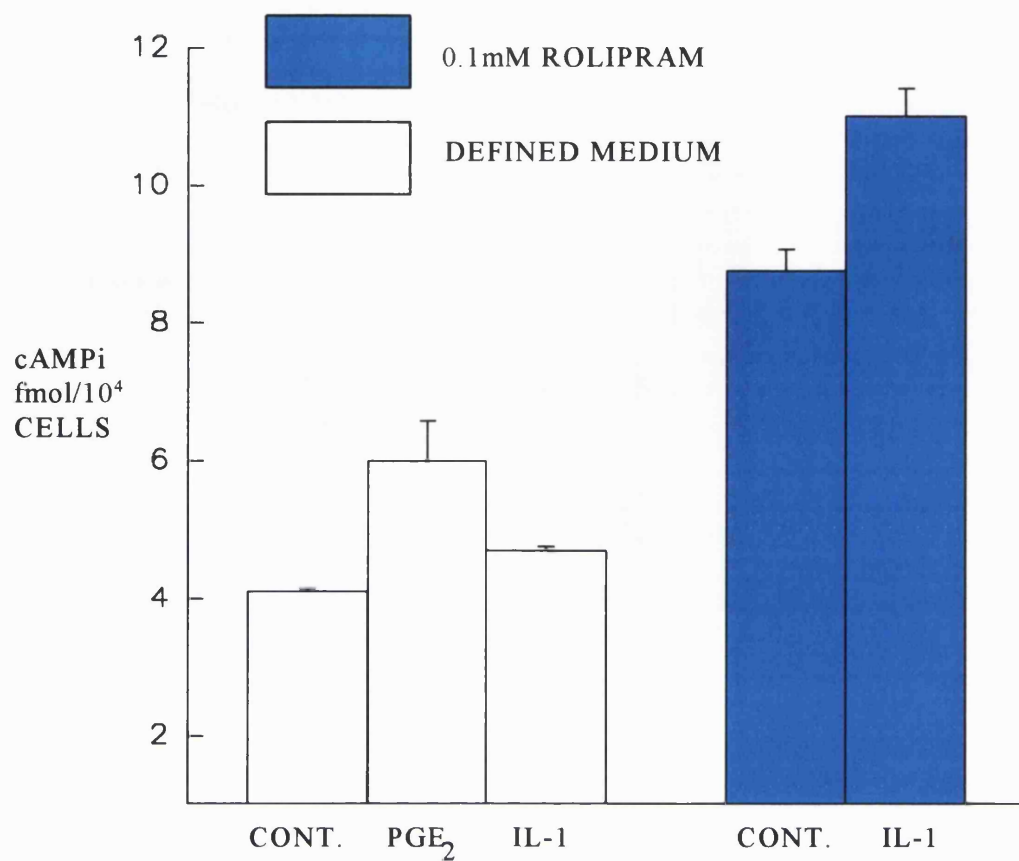


Fig.25. The effect of IL-1 and PGE<sub>2</sub> on Schwann cell cAMPi.

Schwann cells were plated at 10,000 cells per well of a 96-well plate in serum-free defined medium. cAMPi was determined, following 4 hour stimulations with IL-1, or after 10 minutes with PGE<sub>2</sub>. Rolipram was added for 4 hour stimulations only. Values are mean  $\pm$  s.e. of triplicate cultures.

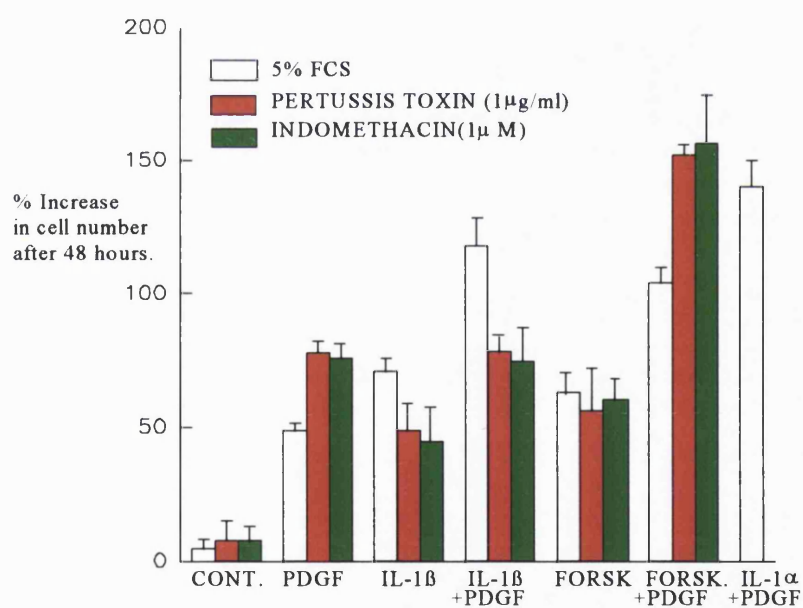
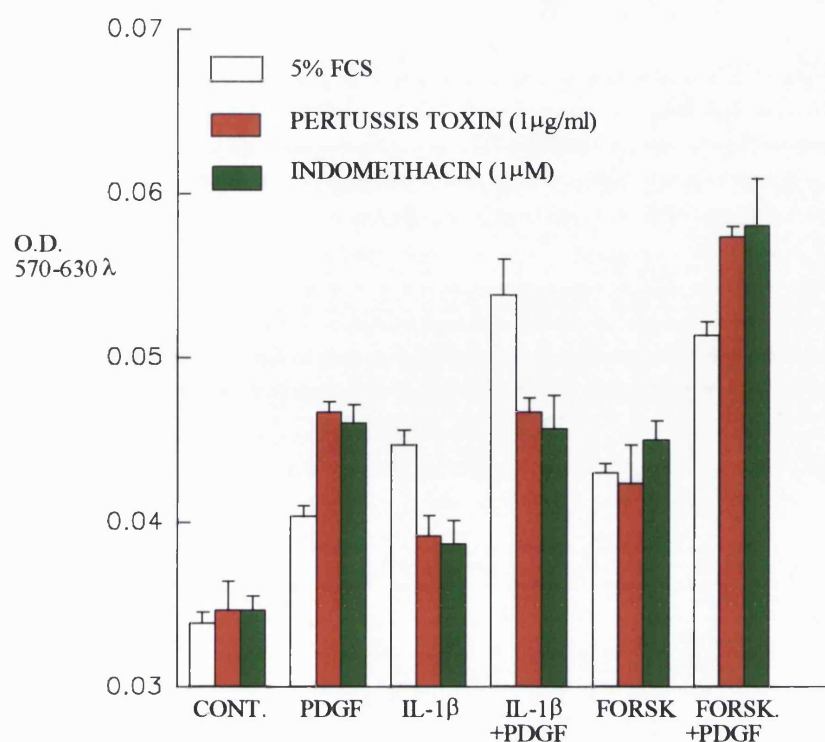


Fig.26. The comparative effects of pertussis toxin and indomethacin pre-treatment on IL-1-induced Schwann cell proliferation.

MTT assay on Schwann cells after 48 hour incubation with agents. Cells were plated at 10,000 cells per well in 5% FCS. IL-1β was used at 50u/ml; IL-1α at 10ng/ml; PDGF at 2ng/ml and forskolin at 2μM. Values are mean +/- s.e. of triplicate cultures.

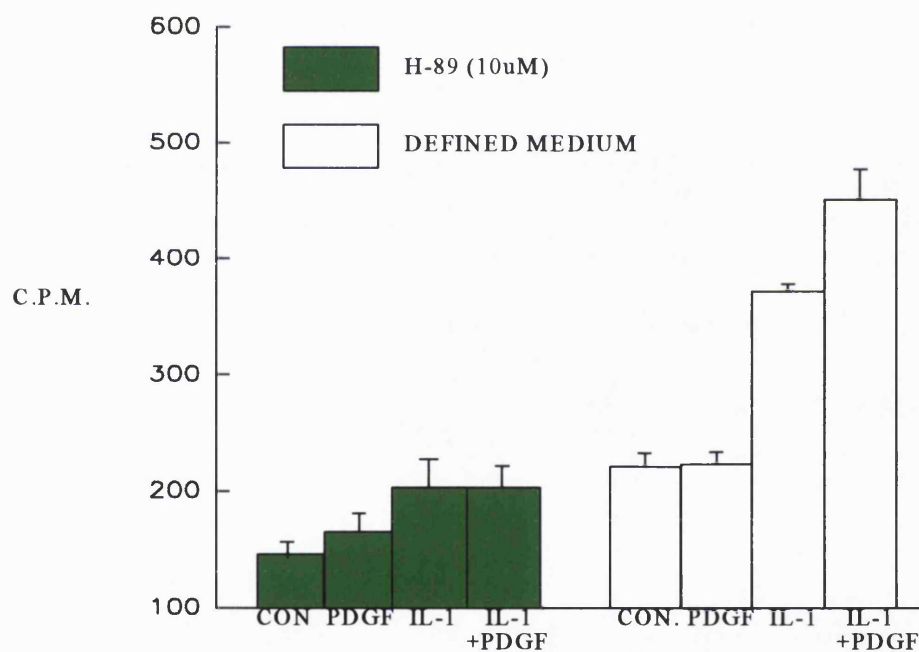


Fig.27a. H-89 impairs IL-1-induced Schwann cell proliferation.. H-89 impairs IL-1-

<sup>125</sup>IIdU cell proliferation assay on pure cultured Schwann cells. Cells were plated at 10,000 per well of a 96-well plate in defined medium, and were 24 hours old at the start of assays. Cytokines were added over 48 hours with additions repeated at 24 hour intervals. IL-1 $\beta$  was used at 30u/ml and PDGF at 2ng/ml. Values are mean  $\pm$  s.e. of triplicate cultures

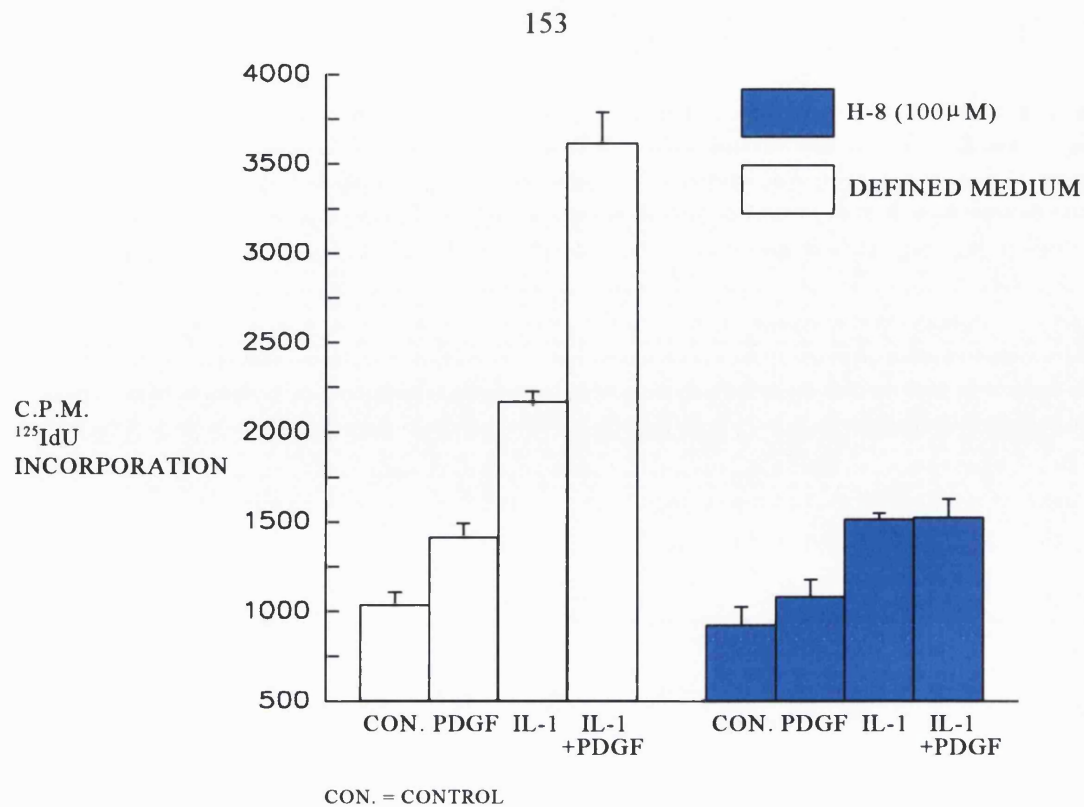


Fig.27b. H-8, an inhibitor of PKA, impairs IL-1 mediated synergy with PDGF on Schwann cell proliferation.

<sup>125</sup>IdU cell proliferation assay on pure cultured Schwann cells. Cells were plated at 10,000 per well of a 96-well plate in defined medium, and were 24 hours old at the start of assays. Cytokines were added over 48 hours with additions repeated at 24 hour intervals. IL-1 $\beta$  was used at 30u/ml and PDGF at 2ng/ml. Values are mean  $\pm$  s.e. of triplicate cultures

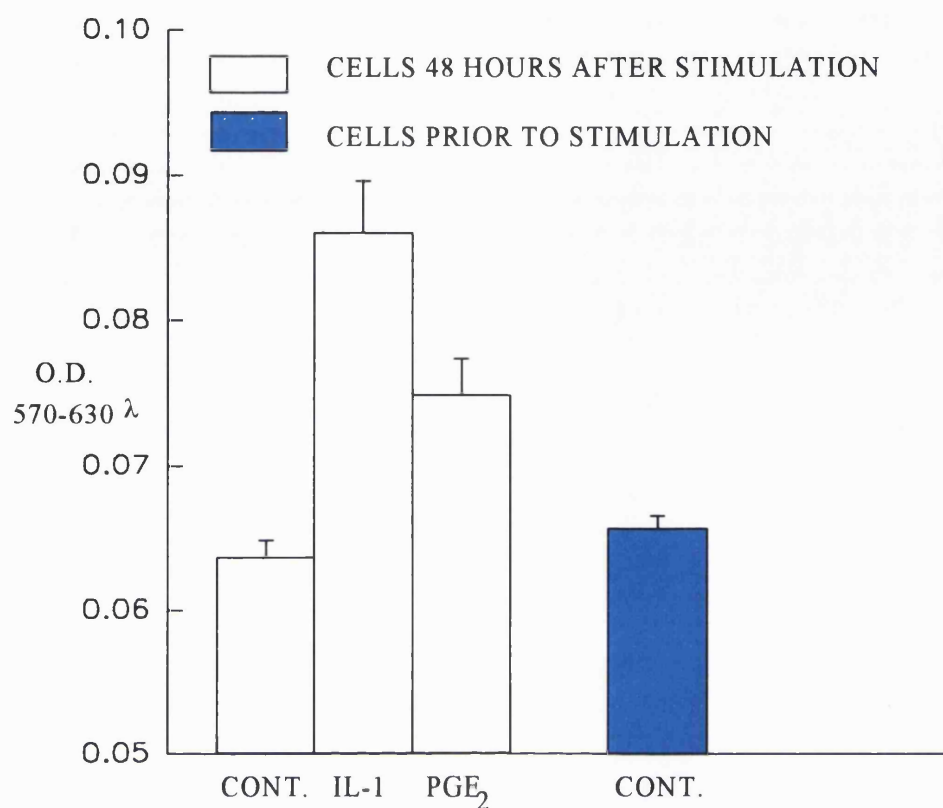


Fig.28. PGE<sub>2</sub> action on proliferation of Schwann cells cultured in supraphysiological magnesium .

MTT cell proliferation/viability assay on pure cultured Schwann cells. Cells were plated at 10,000 per well of a 96-well plate in defined medium, and were 24 hours old at the start of assays. Cytokines were added over 48 hours with additions repeated at 24 hour intervals. IL-1 $\beta$  was used at 30u/ml and PGE<sub>2</sub> at 1 $\mu$ M. Values are mean  $\pm$  s.e. of % increase in O.D. from day 0 of triplicate cultures.

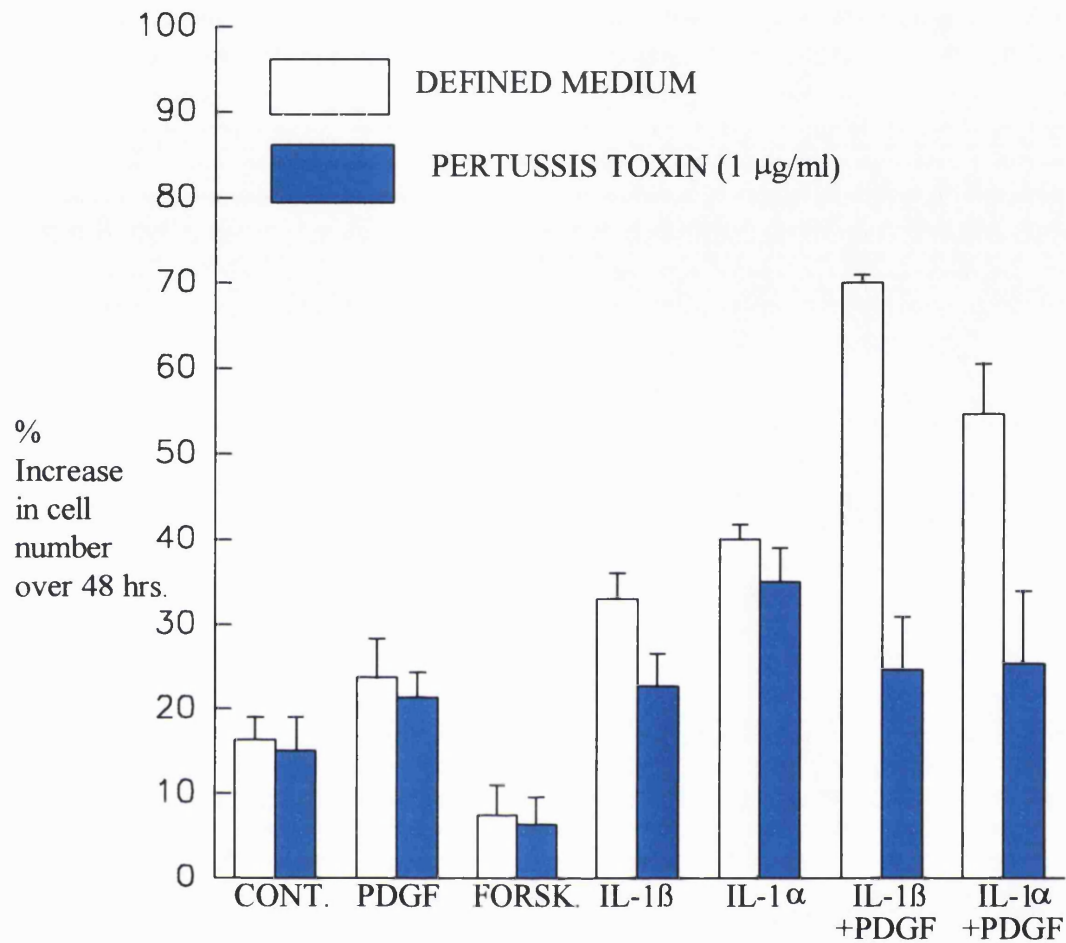


Fig.29a. The effect of pertussis toxin pre-treatment on IL-1-induced Schwann cell proliferation.

MTT assay on pure cultured Schwann cells, plated at 10,000 cells per well of a 96-well plate in defined medium. Cells were 24 hours old at the start of assays.

Cytokines were added over 48 hours with additions repeated at 24 hour intervals.

MTT assay was then performed. IL-1 $\beta$  was used at 30u/ml; IL-1 $\alpha$  at 10ng/ml; PDGF at 2ng/ml and forskolin at 2 $\mu$ M. Values are mean  $\pm$  s.e. of % increase in O.D. from day 0 of triplicate cultures

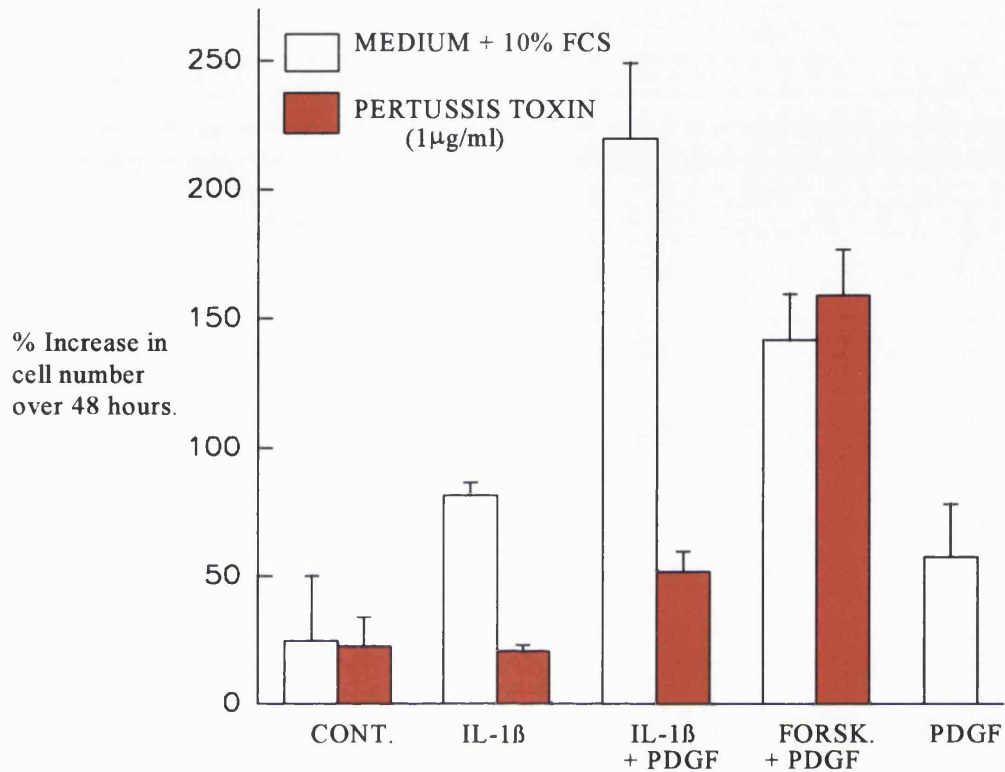


Fig.29b. Effect of pertussis toxin pre-treatment on IL-1-induced proliferation of Schwann cells cultured in serum-containing medium.

MTT cell proliferation/viability assay on pure cultured Schwann cells. Cells were plated at 10,000 per well of a 96-well plate in 10% FCS, and were 24 hours old at the start of assays. Cytokines were added over 48 hours with additions repeated at 24 hour intervals. IL-1 $\beta$  was used at 30u/ml and PDGF at 2ng/ml. Values are mean  $\pm$  s.e. of % increase in O.D. from day 0 of triplicate cultures



IL-1 is not mitogenic for endoneurial fibroblasts unless pertussis toxin or indomethacin are added, suggesting that any intrinsic mitogenic activity is neutralised by prostaglandin synthesis via a pertussis toxin sensitive G protein (Fig.30a, b). This probably involves cAMPi, as PKA inhibition also prevents inhibition of fibroblast proliferation. Production of PGE<sub>2</sub>, via a pertussis toxin-sensitive G protein, is probably also important for Schwann cells as pertussis toxin attenuates IL-1-induced proliferation, and in particular, prevents the synergy between IL-1 and growth factors, such as PDGF or serum (Fig.26; 29a,b). It is likely, therefore, that the pertussis toxin-sensitive G protein is important for activation of the cAMPi related pathway in some manner.

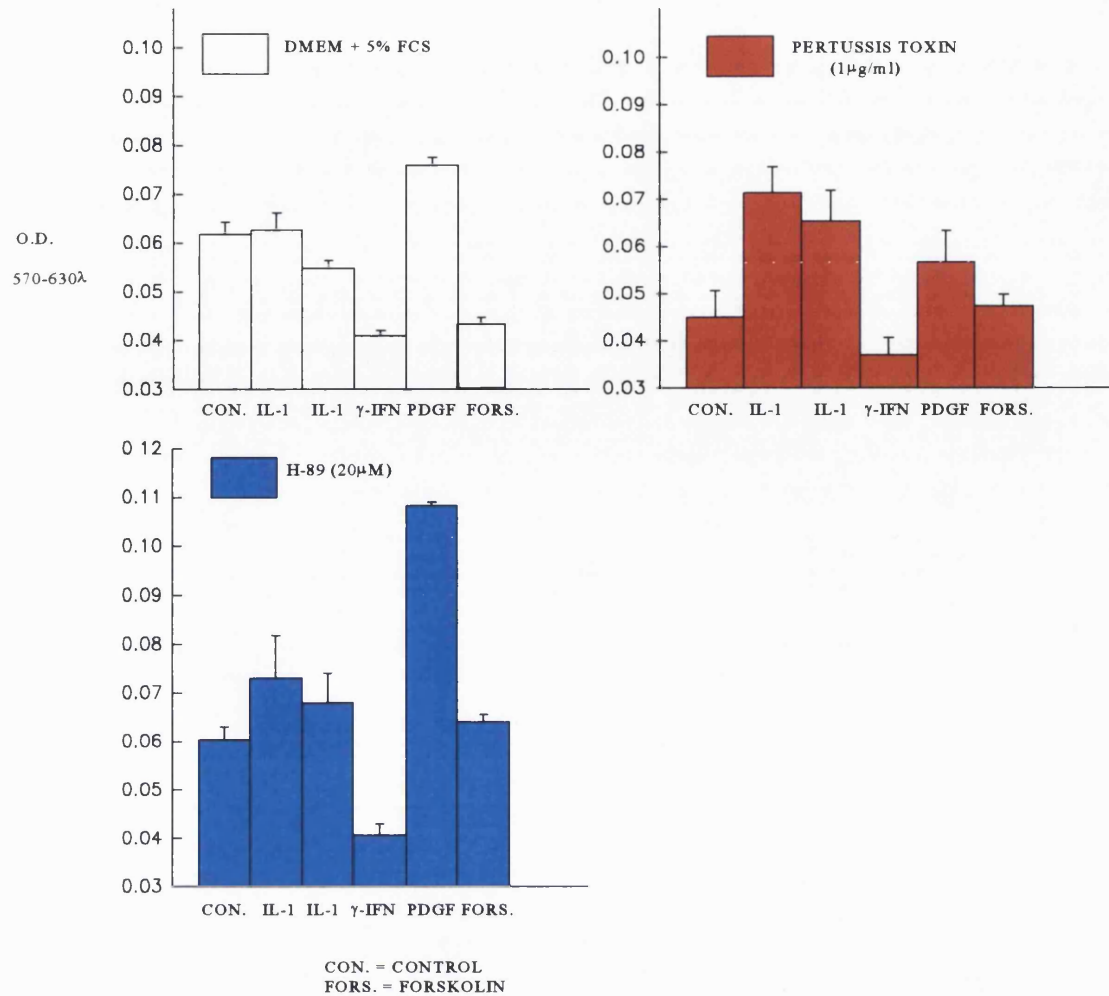


Fig. 30a. The effect of pertussis toxin and H-89 on IL-1 action on endoneurial fibroblast proliferation.

MTT cell proliferation assay on endoneurial fibroblasts. Cells were grown in 5% FCS, at 5,000 cells per well of a 96-well plate. Additions were made at 24 and 48 hours, and cell number determined at 72 hours by MTT assay. Where used, enzyme inhibitors were added 4 hours before cytokines. Values are mean  $\pm$  s.e. of triplicate cultures. IL-1 was used at 30u/ml; IFN at 300u/ml; PDGF at 2ng/ml and forskolin at 1μM.

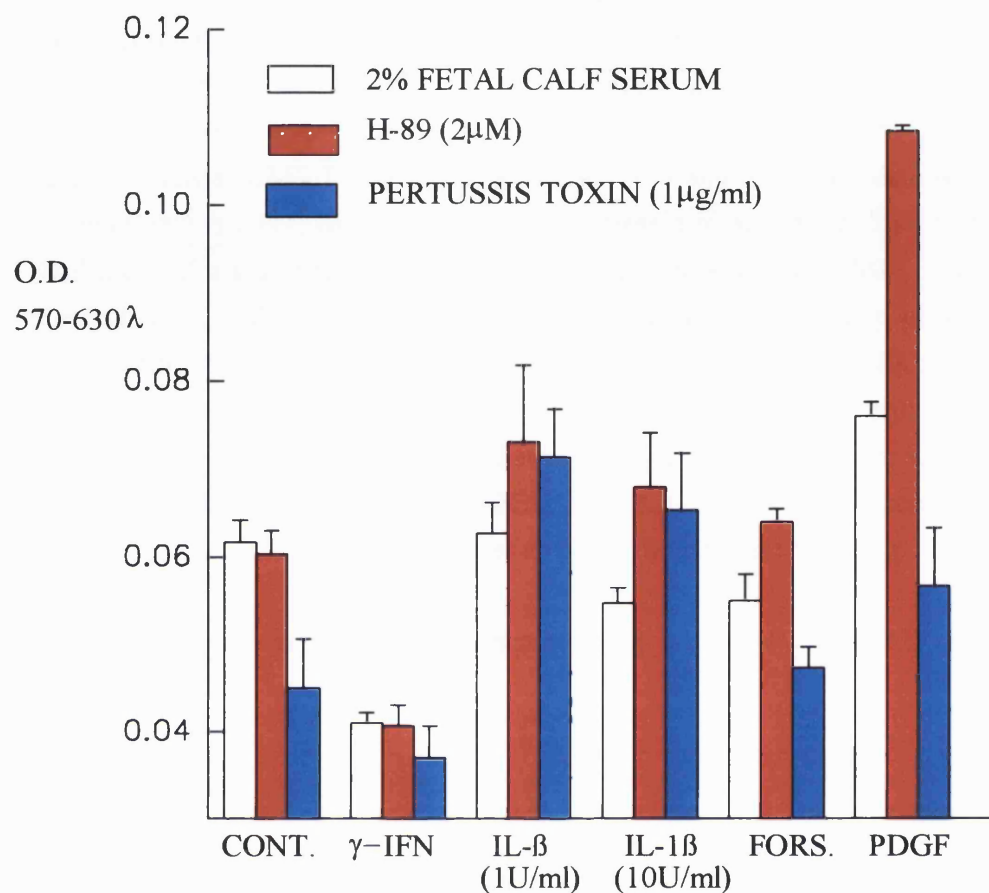


Fig.30b. The effect of pertussis toxin and H-89 on IL-1,  $\gamma$ -IFN and forskolin mediated actions on fibroblast proliferation.

MTT cell proliferation assay on endoneurial fibroblasts. Cells were grown in 5% FCS, at 5,000 cells per well of a 96-well plate. Additions were made at 24 and 48 hours, and cell number determined at 72 hours by MTT assay. Where used, enzyme inhibitors were added 4 hours before cytokines. Values are mean  $\pm$  s.e. of triplicate cultures. IL-1 was used at 30u/ml; IFN at 300u/ml; PDGF at 2ng/ml and forskolin at 1μM.

Inhibitors of PKC, such as staurosporine and H-7, prevent the mitogenic action of PDGF in the presence of IL-1 or forskolin, but do not affect the action of IL-1 itself (Fig. 30a,b). These results suggest that neither PKA or PKC are essential for IL-1-induced proliferation but activation of these kinases may potentiate IL-1 actions. IL-1-induced Schwann cell proliferation is also attenuated, but not abolished, in the presence of the tyrosine kinase inhibitor genistein; genistein also prevents the mitogenic activity of FGF and PDGF with both IL-1 and forskolin (data not shown). IL-1 may act via a pertussis toxin-sensitive G-protein to activate at least two distinct signalling pathways: one via a PGE<sub>2</sub> dependent increase in cAMP<sub>i</sub>, and another independent of both PKA and PKC. IL-1 can augment the activity of agents that activate both PKA and PKC, but can act independently of PKA and PKC.

Interestingly, when Schwann cells are cultured in the absence of insulin in defined medium, approximately 50% of cells have died within 48 hours, but survive in the presence of growth factors. Either PDGF, forskolin or IL-1 can rescue cells from death, and in fact IL-1 still demonstrates a weak mitogenic activity (Fig.31).

IL-1 $\beta$  induced proliferation can be antagonised by both TGF- $\beta$  (Fig.32) and also by PGE<sub>1</sub> (Fig.33).

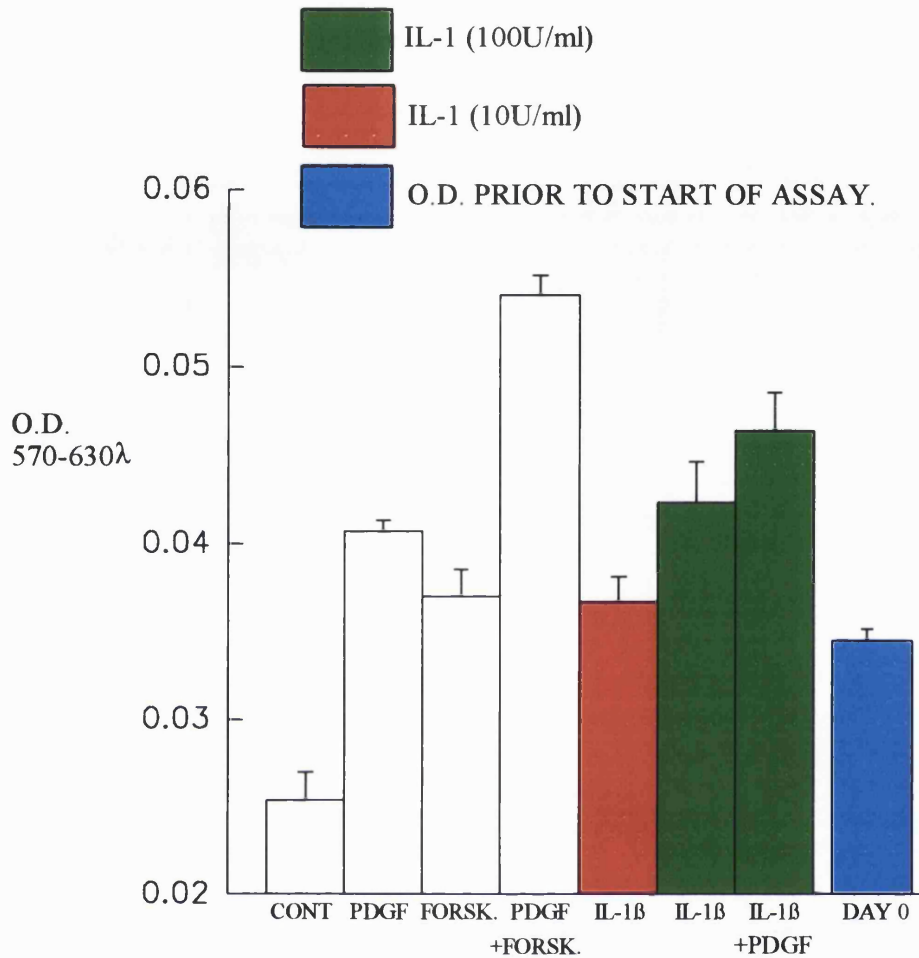


Fig.31. IL-1 is a Schwann cell survival factor in insulin free medium.

Schwann cells were grown in serum-free defined medium without insulin, at 10,000 cells per well of a laminin coated 96-well plate. Additions were made at 24 and 48 hours, and cell number was determined at 72 hours by MTT assay. Where used, enzyme inhibitors were added 4 hours before cytokines. Values are mean  $\pm$  s.e. of O.D. measurements of triplicate cultures. IL-1 was used at 30u/ml, IFN at 300u/ml, PDGF at 2ng/ml, and forskolin at 1 $\mu$ M.

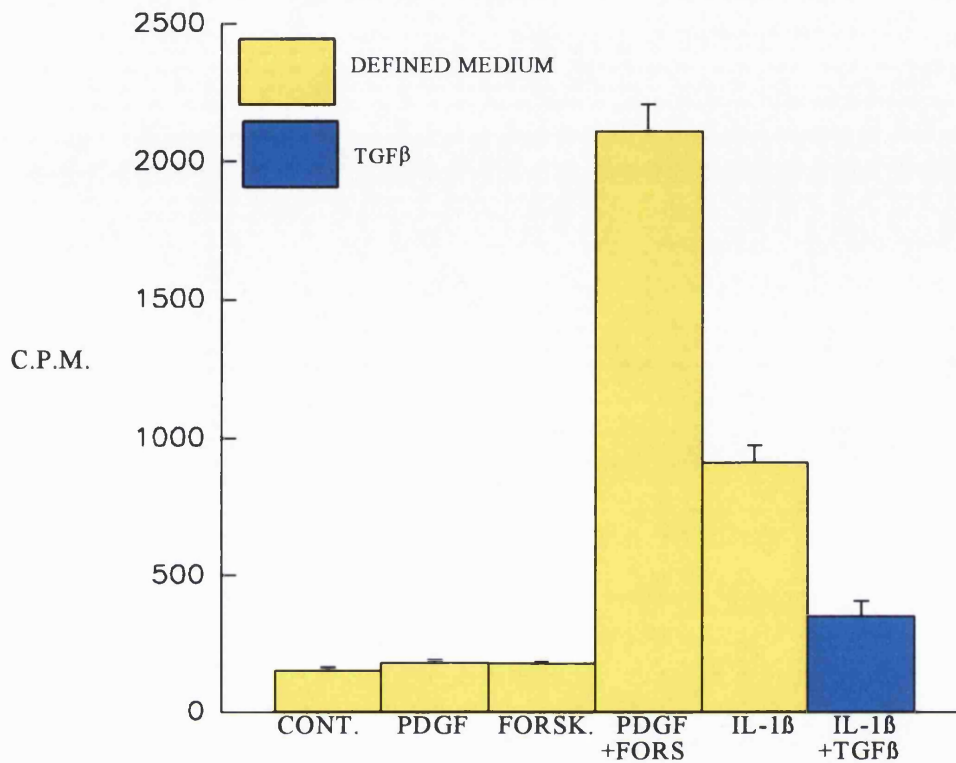


Fig.32. TGFβ inhibits IL-1-induced Schwann cell proliferation.

<sup>125</sup>IdU cell proliferation assay on pure cultured Schwann cells. Cells were plated on laminin at 10,000 per well of a 96-well plate in defined medium, and were 24 hours old at the start of assays. Cytokines were added over 48 hours with additions repeated at 24 hour intervals. IL-1β was used at 30u/ml and PDGF at 2ng/ml. Values are mean +/- s.e. of triplicate cultures

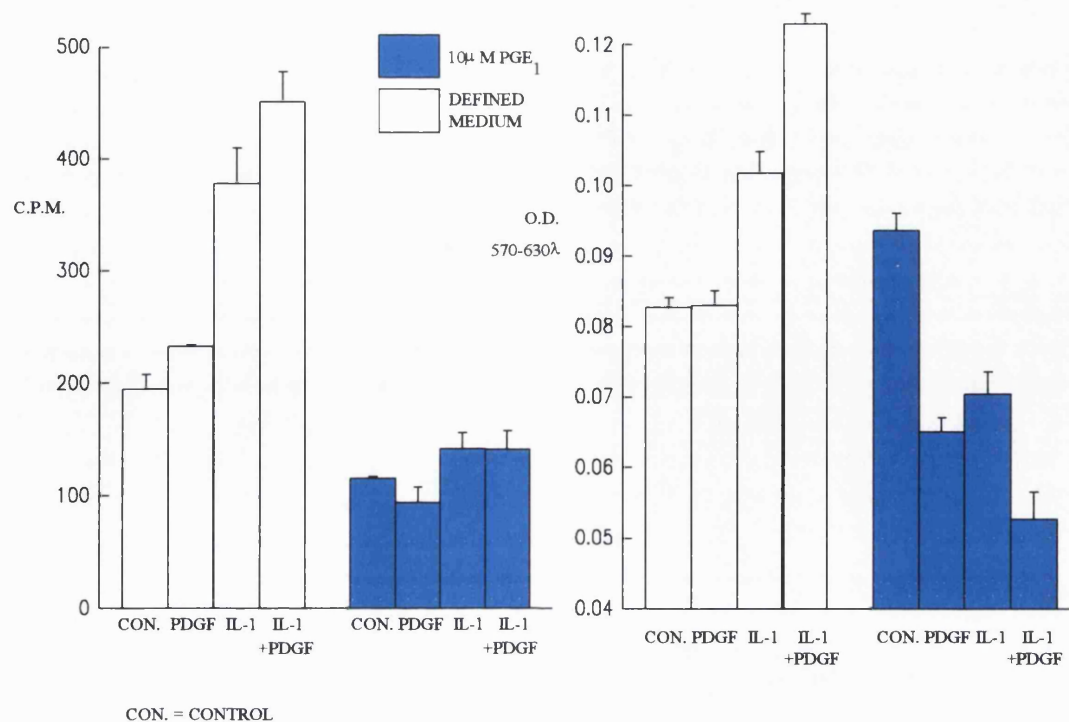


Fig.33.  $PGE_1$  inhibits Schwann cell proliferation mediated by IL-1.

$^{125}$ IIdU cell proliferation assay on pure cultured Schwann cells. Cells were plated on laminin at 10,000 per well of a 96-well plate in defined medium, and were 24 hours old at the start of assays. Cytokines were added over 48 hours with additions repeated at 24 hour intervals. IL-1 $\beta$  was used at 30u/ml and PDGF at 2ng/ml. Values are mean  $\pm$  s.e. of triplicate cultures

The mitogenic action of IL-1 prompted us to re-investigate its ability to increase NGF synthesis in non-neuronal cells. Using the sensitive two-site immunoassay previously described, we found that IL-1 produced a 2-3 fold elevation in NGF levels of sciatic nerve fibroblasts (Fig.34), and also a 50% increase in NGF production in freshly purified Schwann cells, in the absence of other added growth factors (Fig.35a,b). A similar magnitude of response was seen with TNF alone, and an additive effect with IL-1 was seen for Schwann cell (Fig.36) and for fibroblast NGF synthesis (Fig.37).



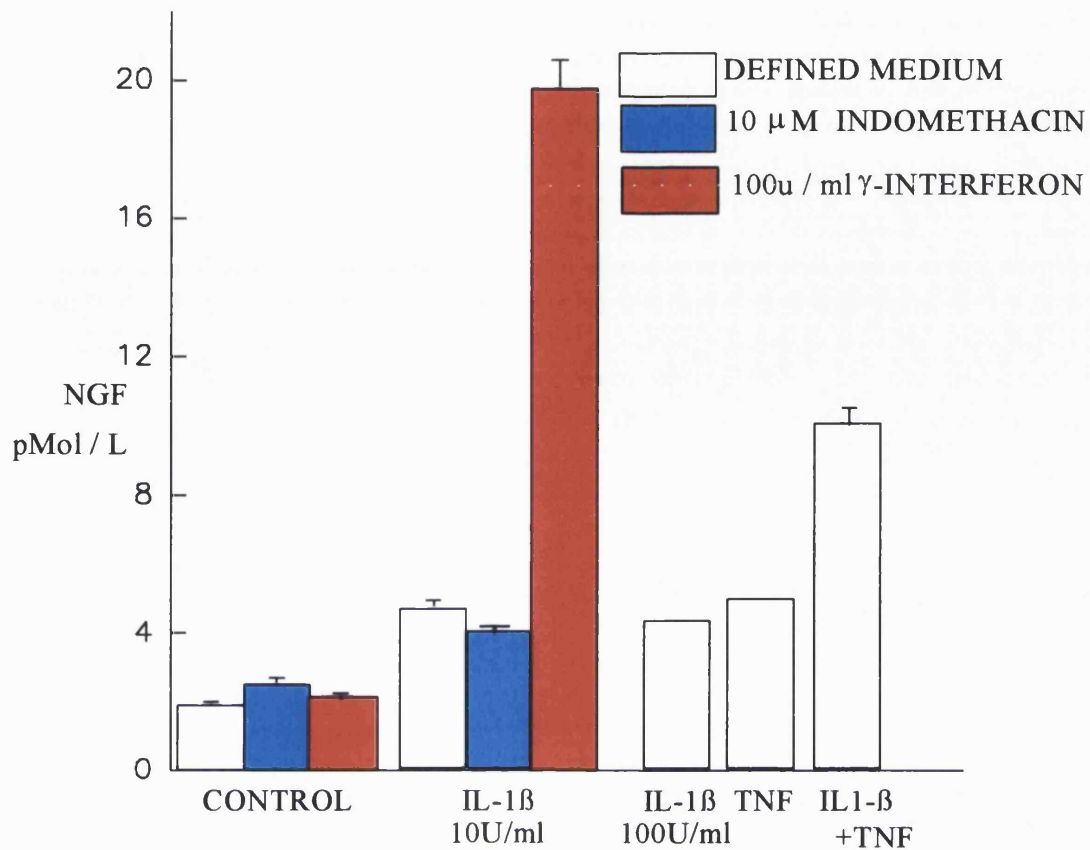


Fig.34. IL-1 and TNF induction of fibroblast NGF production; synergistic action of  $\gamma$ -IFN.

NGF immunoassay of endoneurial fibroblast supernatants, following a 72 hour incubation of pure cultured fibroblasts with IL-1 or TNF. Cells were cultured in defined serum-free medium at 500,000 cells per well of a 24-well plate in a final volume of 200 $\mu$ L/well of medium. Values are mean  $\pm$  s.d. of duplicate cultures.

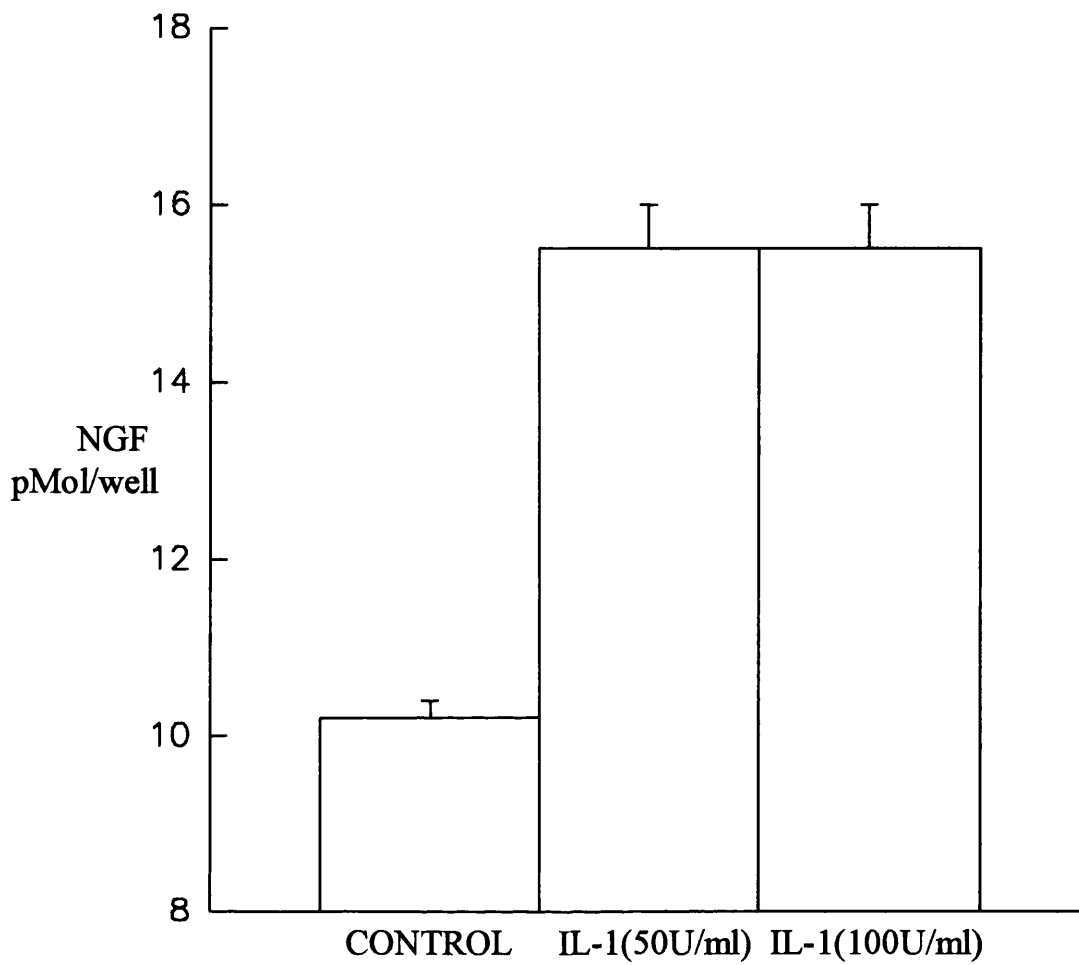


Fig.35a. IL-1 induction of Schwann cell NGF production.

NGF immunoassay of Schwann cell supernatants, following a 72 hour incubation of pure cultured Schwann cells with IL-1. Cells were cultured in defined serum-free medium at 500,000 cells per well of a 24-well plate in a final volume of 150 $\mu$ L/well of medium. Values are mean  $\pm$  s.d. of duplicate cultures.

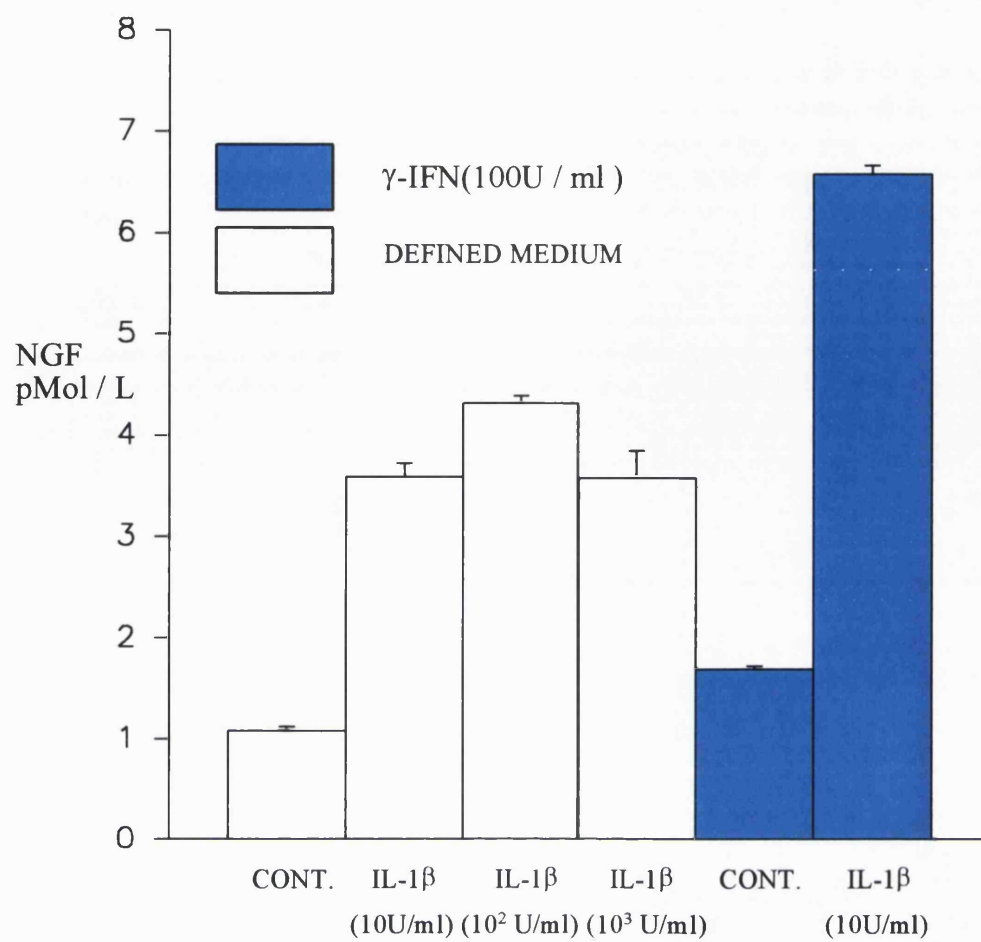


Fig.35b. Potentiation of IL-1-induced NGF synthesis by  $\gamma$ -IFN.

NGF immunoassay of Schwann cell supernatants, following a 72 hour incubation of pure cultured Schwann cells with IL-1. Cells were cultured in defined serum-free medium at 500,000 cells per well of a 24-well plate in a final volume of 150 $\mu$ L/well of medium. Values are mean  $\pm$  s.d. of duplicate cultures.

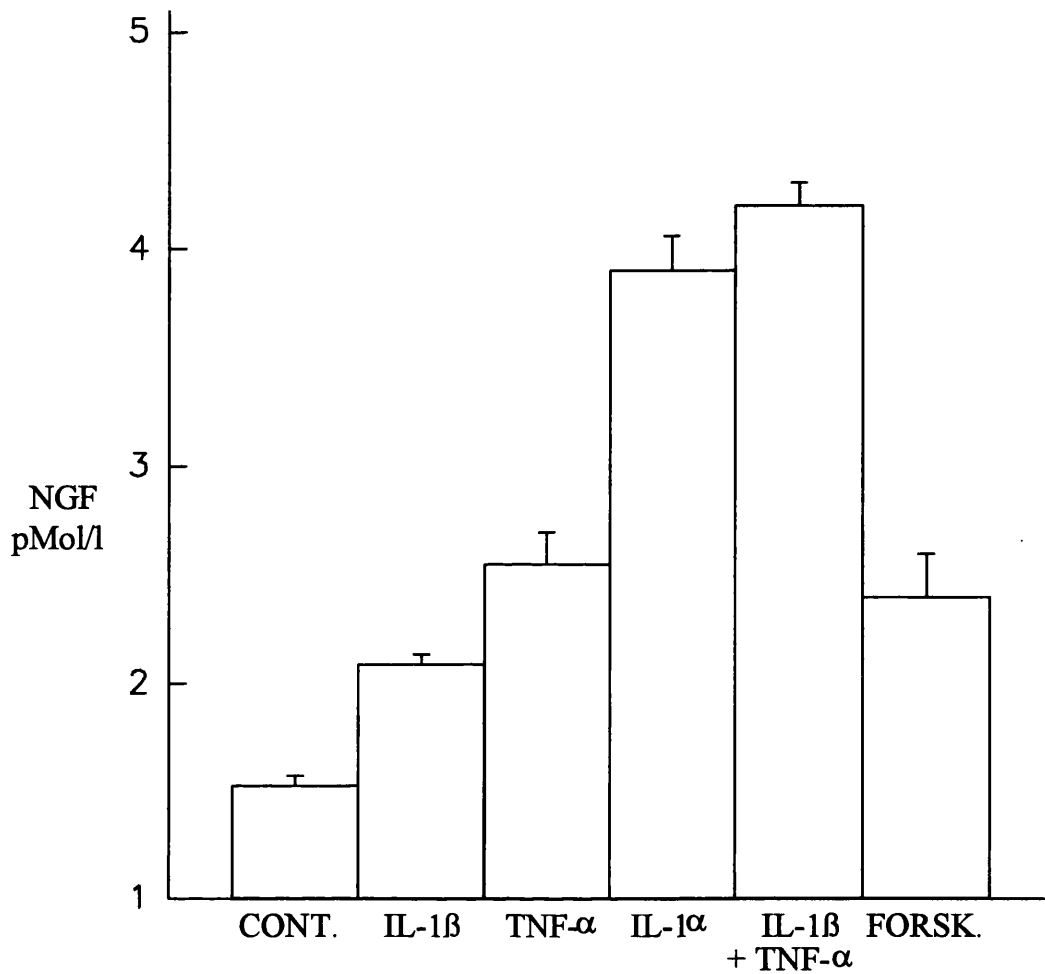


Fig.36. Additive action of IL-1 and TNF on Schwann cell NGF production.

NGF immunoassay of Schwann cell supernatants, following a 72 hour incubation of pure cultured Schwann cells with IL-1. Cells were cultured in defined serum-free medium at 500,000 cells per well of a 24-well plate in a final volume of 150 $\mu$ L/well of medium. Values are mean  $\pm$  s.d. of duplicate cultures.

Under these same conditions, forskolin treatment induced a similar increase in NGF production by Schwann cells.

In response to IL-1, NGF synthesis by Schwann cells and fibroblasts, increases by 50% and 200-300%, respectively. In the presence of  $\gamma$ -IFN, however, IL-1-induced NGF synthesis by both fibroblasts and Schwann cells is dramatically potentiated. Although  $\gamma$ -IFN has no effect by itself, IL-1-induced NGF synthesis in fibroblasts and Schwann cells is increased to 16-fold and 4-fold, respectively. This suggests a key role for  $\gamma$ -IFN in priming non-neuronal cells to respond to IL-1. It is known that  $\gamma$ -IFN, a potent macrophage activator, promotes release of IL-1 from recruited macrophages. The actual source of  $\gamma$ -IFN is not known, but may be from activated T-cells or from the axons themselves.

$\gamma$ -IFN inhibits proliferation of Schwann cells and fibroblasts, and in fibroblast cultures, particularly, there was a clear inverse relationship between proliferation and NGF synthesis (Fig.37). In these assays neither pertussis toxin nor indomethacin inhibited NGF synthesis in response to IL-1.

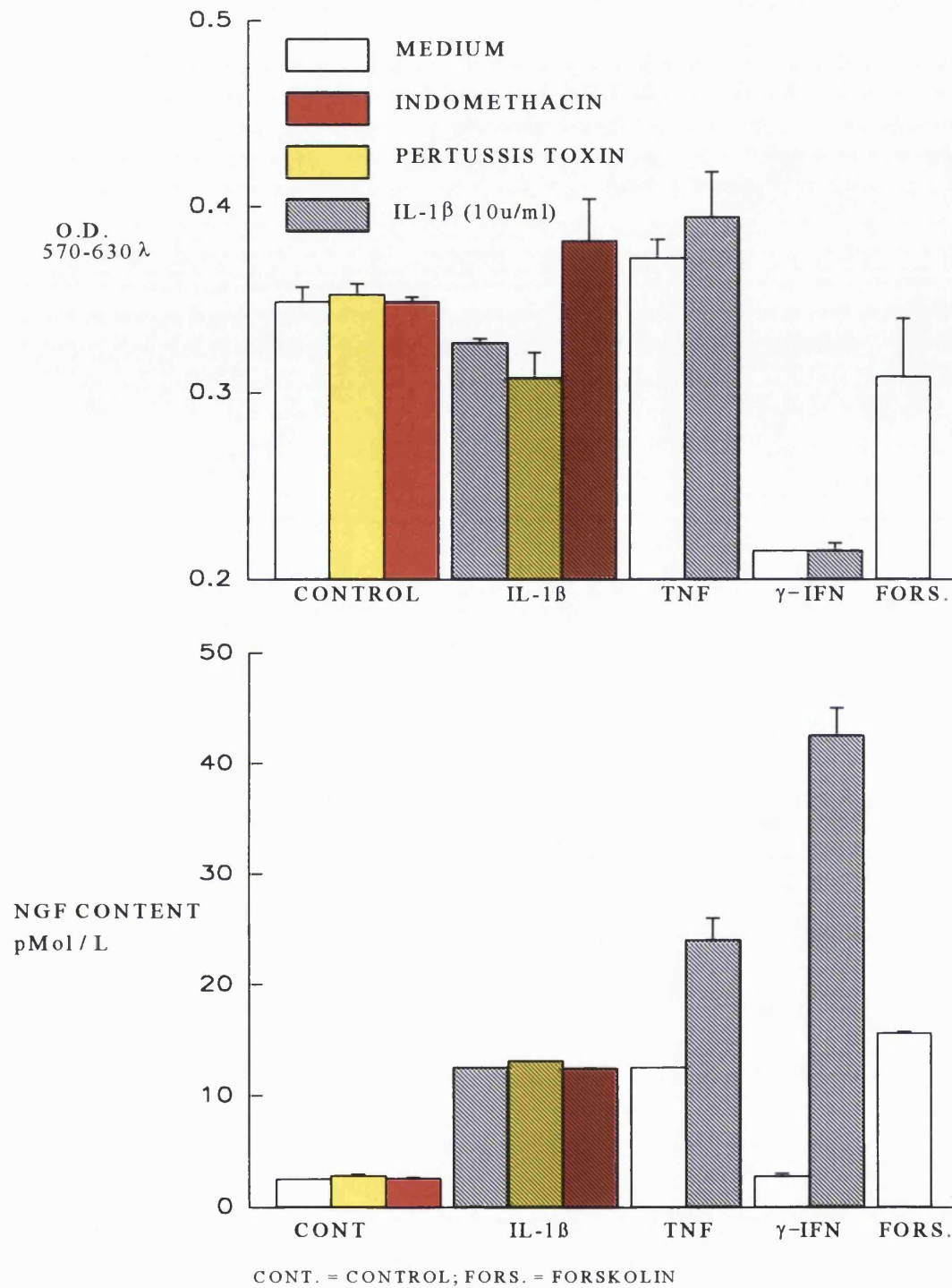


Fig.37.  $\gamma$ -IFN potentiates IL-1-induced fibroblast NGF synthesis and inhibits proliferation.

NGF immunoassay of supernatants, following a 72 hour incubation of pure cultured fibroblasts with IL-1 or TNF. Cells were cultured in defined serum-free medium at 200,000 cells per well of a 24-well plate in a final volume of 200 $\mu$ L/well of medium. Enzyme inhibitors were added 4 hours prior to cytokines. Parallel MTT assays were performed. Values are mean  $\pm$  s.d. of duplicate cultures.

## DISCUSSION

Macrophages are known to invade injured nerves and may indeed be a necessary requirement for Schwann cell proliferation *in vivo*. My demonstration of IL-1 as a Schwann cell mitogen and survival factor, provides further evidence that macrophages may have an important role in the control of Schwann cell proliferation after axotomy. Macrophages may influence regeneration primarily via IL-1 production, but may also do so via production of other cytokines such as TNF $\alpha$ . Additionally, they may also produce peptide growth factors; it is known that PDGF and TGFs are both released by macrophages at sites of injury, and this may also prove to be so for peripheral nerve. These growth factors however, unlike IL-1, are not mitogenic alone but require a permissive factor in order to stimulate proliferation. As PDGF is a mitogen in the presence of IL-1, it is possible that macrophage signals might be sufficient to stimulate Schwann cell proliferation, without requiring other cell products. The action of IL-1, in this respect, may be mediated via the production of PGE<sub>2</sub>, and consequent activation of the cAMPi / PKA pathway. The ability of IL-1 to act as a sole mitogen may be due to the activation of several distinct signalling pathways, including activation of PKA through elevation of cAMPi, and also PKC/tyrosine kinase. In fact, it is the only mitogen tested which was active even in the absence of insulin, and in this situation also improved survival/metabolic activity as assessed by an MTT assay. It is not possible from our data, to determine for certain whether tyrosine kinase activity in addition to activation of PKC via PLC $\gamma$ -induced DAG formation, plays a role in proliferation. The ability of IL-1 and TNF to elevate NGF synthesis in Schwann cells and fibroblasts, suggests that macrophage cytokines may also be vital for the success of axonal regeneration.  $\gamma$ -IFN dramatically potentiates IL-1 stimulated NGF synthesis thereby supporting an additional role for T cells other than activation or recruitment of macrophages. T cell factors, such as  $\gamma$ -IFN, may also prime non-neuronal cells to greatly enhance their responsiveness to macrophage products. It is tempting to speculate that the role played by  $\gamma$ -IFN may be to switch cell activity from a proliferative phase to one of synthesis of trophic factors such as NGF.  $\gamma$ -IFN inhibits both Schwann cell and fibroblast proliferation in response to various growth factor combinations, as well as potentiating IL-1-induced NGF synthesis. The cessation of Schwann cell proliferation, which occurs during regeneration of axons, may be due to  $\gamma$ -IFN or through down-regulation of cell responses to IL-1 by Schwann cells, as clearly occurs during prolonged time in culture. It is of considerable importance to examine what role cytokines, such as IL-1, may play in the regulation of abnormal Schwann cell proliferation, as in the nerve sheath tumours of neurofibromatosis. Blocking monocyte function / recruitment could be a viable therapeutic strategy as might the use of selective receptor antagonists for IL-1 and even TNF, which are now available.

## CHAPTER FOUR

## DISCUSSION

In this thesis I have attempted to describe the contributions of axon- and monocyte-derived signals in regulating events believed to underlie successful regeneration within the injured peripheral nervous system. To further understand how extracellular signals may mediate these responses, I have also described those second messenger systems involved in their action and regulatory events involved in limiting these responses.

Schwann cells are critical in the development, function, and regeneration of peripheral nerves. During embryogenesis, Schwann cell progenitors migrate from the neural crest (LeDouarin, 1982), and proliferate along tracts to be occupied by peripheral axons, where they support axon extension. The development of peripheral axons seems to rely on Schwann cells, particularly for the formation of the basal lamina (Bunge et al., 1986) and as a major source of trophic factors (Muir et al., 1989; Acheson et al., 1989), which regulate neurite outgrowth and neuronal survival. During later stages of development, Schwann cells produce the myelin sheath. Adult Schwann cells are normally quiescent, but retain the capacity for cell division and can de-differentiate to a proliferative state after injury (Salzer et al., 1980). Two early bursts of Schwann cell proliferation follow nerve injury, and could be influenced by inflammatory mediators (Baichwal et al., 1988), myelin breakdown products (Salzer et al., 1980), axonal signals, loss of axonal contact (Salzer and Bunge, 1980) or secreted factors. Later, during axon regrowth, a second wave of Schwann cell proliferation occurs and precedes remyelination (Pellegrino et al., 1986).

Schwann cell proliferation and production of trophic factors are essential for normal regeneration of sensory, sympathetic, and probably motor neurons, following axotomy of peripheral nerve. It is of great importance, therefore, to understand the signals which control these processes *in vivo*. Our work, and that of others, suggests that Schwann cell proliferation *in vitro* requires a combination of two distinct signals: one a growth factor such as PDGF, FGF, or GGF, and the second an elevation of cAMPi. Current evidence suggests that the stimulation of proliferation through the cAMPi pathway involves increased synthesis of receptor tyrosine kinases for growth factors such as PDGF and the GGFs, and probably others. Numerous growth factors might be present at the right time and place *in vivo*; neurons, themselves, can produce mRNA for GGFs during development (Marchionni et al., 1993), and macrophages have been shown to produce PDGF in other injury models. It remains to be seen whether growth factors are indeed transported within axons and presented to Schwann



cells, or whether macrophages provide growth factors within peripheral nerve. It is also not known to what extent circulating growth factors may gain access to and influence Schwann cell responses, particularly after disruption of the blood / nerve barrier following axotomy. Further experiments must follow to clarify this.

Growth factor stimulation of Schwann cell proliferation requires PKC. Agents such as PMA, which activate this kinase, are mitogenic in the presence of cAMPi elevation, and, furthermore, agents such as H-7 and staurosporine which inhibit PKC, block growth factor action. Tyrosine kinase activity may largely be involved upstream of PKC, probably linked to the activation of PLC $\gamma$  by growth factor receptor tyrosine kinase activity. Inhibitors of tyrosine kinase, such as genistein, impair growth factor action, but also rapidly lead to cell death. The development of more selective and less toxic inhibitors may allow these pathways to be more readily pharmacologically dissected. At least *in vitro*, many known growth factors, including PDGF, GGF, FGF, EGF and probably IGF-1, are capable of stimulating Schwann cell proliferation under permissive conditions.

The identity of endogenous agents capable of elevating Schwann cell cAMPi, and thereby enabling growth factor-induced proliferation has, however, remained much more elusive. Agents such as forskolin and cholera toxin enable growth factor action *in vitro*, by increasing the expression of Schwann cell growth factor receptors, for GGF (Cohen et al., 1992), and PDGF (Weinmaster and Lemke, 1990). However, endogenous agents, including  $\beta$ -adrenoceptor agonists and numerous peptides known to act via adenylate cyclase, have previously been shown not to have this activity (Eccleston et al., 1992). Increased synthesis of growth factor receptors can not be the only role of cAMPi, as even PMA, which acts down-stream of growth factor receptors, is mitogenic only if cAMPi is concurrently elevated. Part of the requirement for cAMPi elevation might be the lifting of transcriptional repression by CREB on genes activated via PKC after stimulation by growth factors (Lamph et al., 1990). The reversal of CREB action following cAMPi elevation could then result in synergistic stimulation of gene transcription. Another possibility is that PKA-mediated phosphorylation and inactivation of a cell-cycle inhibitor such as the retinoblastoma gene product, Rb, or a Schwann cell homologue, is required to allow progression through the important G1-S transition in the cell cycle. The cAMPi pathway may also be involved in promoting NGF synthesis by Schwann cells, and this seems not to require a growth factor stimulus (Matsuoka et al., 1992).

As experiments, to date, had failed to reveal a mitogenic activity for all known endogenously occurring cAMPi elevating agents, we set out to re-examine their activity. CGRP was chosen for an in depth study as firstly, it is expressed in and

transported by peripheral axons and is actually up-regulated following axotomy; and secondly, because it is a known elevator of cAMPi in most systems where it has been studied. As shown in this thesis, CGRP is a potent elevator of Schwann cell cAMPi, but in common with most studied systems of this type, readily undergoes desensitisation such that in standard tissue culture conditions CGRP responsiveness is completely lost within a few hours. Consequently, rather than searching for an unknown endogenous ligand, which might elevate cAMPi and enable growth factor activity *in vivo*, we undertook to reappraise the role of known factors, such as CGRP. CGRP was felt to be of particular interest as firstly it was actually upregulated following injury and concentrated at the edge of regenerating axons, and secondly it was a potent elevator of Schwann cell cAMPi. Earlier experiments have used levels of agonist that might rapidly desensitise Schwann cell responses/receptors, thereby preventing the demonstration of mitogenic activity *in vitro*. We thus investigated the mechanism of desensitisation of Schwann cells to CGRP, and the  $\beta$ -adrenoceptor agonists, which elevate cAMPi *in vitro*. Inhibition of cAMPi degradation with phosphodiesterase inhibitors, or prevention of  $G_i$ -mediated down-regulation of adenylate cyclase with pertussis toxin, potentiate the acute elevation of cAMPi, but do not prevent rapid agonist induced desensitisation. Phosphodiesterase and inhibitory G proteins may act primarily to regulate resting cAMPi levels, and the extent of acutely stimulated cAMPi elevation. PKA and  $\beta$ ARK mediate a large part of the desensitisation seen after a 6 hour exposure to CGRP and the  $\beta$ -agonist, isoprenaline, respectively. Inhibition of these kinases, however, is incompatible with proliferation; inhibiting PKA with H-8 or H-89 also blocks the downstream activation of signals required for entry to the cell cycle, and using heparin to inhibit  $\beta$ ARK requires digitonin to permeabilise cells, proving rapidly cytotoxic. When CGRP was administered to Schwann cells in a pulsatile manner, allowing periods of recovery without peptide present, CGRP was able to increase expression of Schwann cell SCIP. This activity is shared by forskolin, suggesting that cAMPi elevation, as has previously been described, is mediating this action. Early attempts at performing mitogen experiments under similar conditions were unsuccessful due to loss of cells during washes and mitogen experiments were generally unsuccessful when performed on antibody bound Schwann cells. I describe a novel method of circumventing desensitisation, involving the use of supraphysiological  $Mg^{++}$  concentrations to promote  $G_s$  dissociation, and liberation of the active  $G_{s\alpha}$  subunit. At 5mM  $Mg^{++}$ , down-regulation of cAMPi elevation is reduced and both CGRP and  $\beta$ -adrenergic agonists enable growth factor stimulated proliferation of Schwann cells.  $Mg^{++}$  alone is without activity, suggesting that a specific receptor response is being potentiated. It is known that CGRP is up-regulated after peripheral nerve injury and becomes concentrated at the regenerating nerve front. It is therefore in place to influence Schwann cell responses *in vivo*. This contention is further supported by my

demonstration that Schwann cell cultures *in vitro*, are able to metabolise CGRP via a phosphoramidone-sensitive endopeptidase, 24.11. This may be an important mechanism for preventing desensitisation *in vivo*. It has previously been shown that this enzyme is present on the Schwann cell surface and is up-regulated in peripheral nerve after axotomy. Regulation of enzyme levels may thereby represent a further control step by moderating desensitisation at the time when levels of peptide are also up-regulated. Circulating catecholamine levels are high following injury, but to what extent this may contribute to Schwann cell responses *in vivo* is not known. The regulation of Schwann cell SCIP by CGRP may play a role in the regulation of switches between myelination and proliferation, such as are known to occur during development and during nerve regeneration

In addition to stimulation of Schwann cell responses, fibroblast proliferation must be restricted to the nerve sheath and connective tissue, whilst preventing scar tissue formation from impeding the outgrowth of regenerating axons. We show that CGRP is able to elevate fibroblast cAMPi and thereby prevent growth factor mediated proliferation *in vitro*. Release of CGRP from regenerating axons could prevent fibroblast proliferation at the regenerating nerve front; if axonal regrowth is prevented, fibroblast proliferation goes unchecked and eventually the distal stump is entirely replaced by connective tissue. Axonal CGRP may play an important inhibitory role *in vivo*, further to fibroblast inhibition, by limiting both macrophage and T cell activity. CGRP can inhibit both T cells and macrophages in other *in vitro* systems, through cAMPi elevation.

It has long been known that macrophages, recruited following axotomy, are critical for successful nerve regeneration, Schwann cell proliferation, and the synthesis of trophic factors within the nerve. The identity of the macrophage products mediating these responses *in vivo*, has remained obscure. Others have shown no mitogenic activity for IL-1 or TNF, *in vitro*, although antibodies to IL-1 reduce the mitogenic activity of macrophage conditioned medium. Although, in isolated nerve segments, IL-1 results in increased expression of NGF by both fibroblasts and Schwann cells, neither IL-1 nor TNF have been shown to regulate NGF production by cultured Schwann cells, *in vitro*. We show that IL-1 is a mitogen and survival factor for Schwann cells, *in vitro*, but this response is lost if cells are cultured for more than a few days. Previous failure to reveal an action of this cytokine probably reflect, therefore, the method of cell isolation/purification previously used. It seems that IL-1 is uniquely able to act via both the growth factor and cAMPi pathways, and is both a mitogen and survival factor for Schwann cells as a sole agent. In keeping with this, we show that part of IL-1 action is via a PKC / tyrosine kinase pathway which shows synergy with forskolin, and part through an activation of PKA which shows synergy

with PDGF. Activation of PKA may require IL-1-induced PGE<sub>2</sub> synthesis as an intermediary, as it is inhibited by blocking prostaglandin synthesis with indomethacin. It also seems that a pertussis toxin sensitive G protein is involved, as pertussis toxin also inhibits part of IL-1 action. IL-1 also increases NGF production by cultured Schwann cells, as well as fibroblasts, and is additive in this with TNF $\alpha$ . Importantly, IL-1-induced NGF synthesis is greatly potentiated by  $\gamma$ -IFN.

Schwann cell proliferation can be influenced by both macrophage products, such as IL-1, which is a survival factor in the absence of insulin, and also by axon-derived signals such as CGRP, which require concurrent growth factor both for mitogenic action and for survival promotion in the absence of insulin. IL-1 synergises with PDGF and also with forskolin, suggesting that it stimulates both pathways submaximally.

The activity of CGRP and the  $\beta$ -adrenergic agonists are curtailed by the rapid development of receptor desensitisation, which prevents their exerting a permissive action on growth factor-driven Schwann cell proliferation at high dose *in vitro*. This may be less prominent *in vivo*, where continuous release of peptide at low level, and breakdown of CGRP by 24.11, may prevent the accumulation of desensitising levels of peptide. The action of IL-1, certainly *in vitro*, is limited by a loss of Schwann cell responsiveness to this cytokine after even a relatively short time in culture. The signals which regulate this expression of IL-1 responsiveness are of great interest and deserve further investigation. One possible candidate is  $\gamma$ -IFN; we show that this cytokine greatly potentiates the activity of IL-1 on Schwann cell and fibroblast NGF expression.  $\gamma$ -IFN is inhibitory to both Schwann cell and fibroblast proliferation, and may be important in mediating a switch from proliferation to differentiation. This switch may be important in myelination, and has been associated with the expression of the transcription factor SCIP. It is not known if  $\gamma$ -IFN can stimulate SCIP expression, but this would seem an interesting experiment.  $\gamma$ -IFN is likely to have a major role in promoting axonal regeneration through its profound augmentation of NGF synthesis by non-neuronal cells and as a macrophage-activating factor. It would certainly be important to examine the effect of “knocking out”  $\gamma$ -IFN, on NGF synthesis and nerve regeneration *in vivo* (these experiments are currently underway in our laboratory).  $\gamma$ -IFN may be originating from either T cells or possibly from the axons themselves. As summarised by Fig.38, considerable work is still required to define how these factors interact in development and after axotomy *in vivo*. However, work by ourselves and others has clarified to an extent how macrophage, T cell and axon derived signals may interact to regulate Schwann cell function after peripheral nerve injury.

It has been shown that macrophage products have a role in regulating both NGF production by non neuronal cells and also Schwann cell proliferation. Our results, which show that  $\gamma$ -IFN potentiates the action of IL-1 in promoting NGF synthesis, suggest that T cells (or possibly the axons themselves), as sources of  $\gamma$ -IFN, may also play a critical part in this response.  $\gamma$ -IFN also inhibits proliferation of both Schwann cells and fibroblasts, and conceivably could be operating a switch from a proliferative state to one of trophic factor synthesis. The contribution of axonal signals to nerve regeneration may also include the neuropeptide CGRP, which is a permissive signal for growth factor mediated Schwann cell proliferation if desensitisation is circumvented *in vitro*.

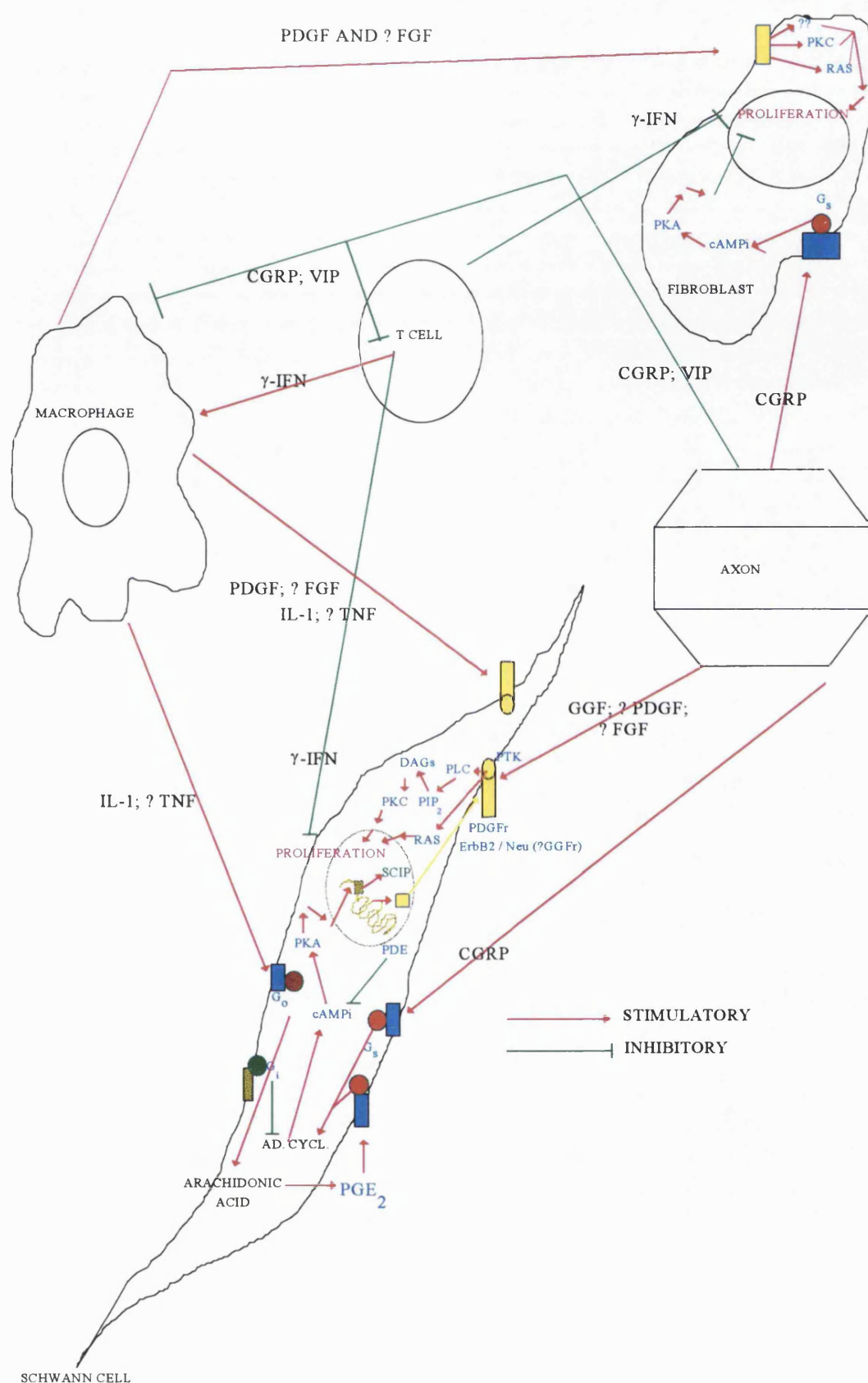


Fig.38. Signal transduction during cell-cell interactions following peripheral nerve injury.

The demonstration of CGRP degradation by cultured Schwann cells, via a phosphoramidate inhibitable enzyme, suggests that Schwann cells, *in vivo*, may avoid desensitisation by limiting local concentrations of CGRP. Axonal signals are probably of great importance. GGF is made in sensory and motoneurons and is mitogenic for Schwann cells. Receptors for this are up-regulated following nerve injury, during development, and by treatment with forskolin *in vitro*. It is tempting to speculate that macrophage products may be required for up-regulating ErbB2 expression. It is possible that the axons themselves may contribute to this via release of cAMPi elevating signals, such as CGRP. It is thus probable that the regulation of growth factor receptors is the critical control step, rather than the availability of growth factor. The actual time course of GGF expression in peripheral nerve is not known, although our own work shows that CGRP is up-regulated and concentrated in the areas of Schwann cell proliferation following axotomy. Therefore, CGRP could be influencing Schwann cell expression of growth factor receptors such as ErbB2 and PDGFr. In mice, at least, it would seem that macrophage factors as well as neuronal products are required for NGF expression and proliferation; in the C57Bl mutant, where macrophage recruitment into injured nerve fails to occur, neither proliferation nor NGF expression occur. It would be interesting to determine whether Schwann cell PDGF and GGF receptor expression are up-regulated normally in the C57Bl mutant, and if, in fact, neuronal expression of GGF occurs. Equally, it is not known whether CGRP upregulation occurs normally in the mutant, and it may be interesting to see if expression patterns of neuropeptides are different. Possibilities would include a further decrease in levels of neuronal peptides, particularly in sensory neurons, which are regulated by NGF; these include CGRP and VIP which are downregulated to some extent in subpopulations of neurons. Equally one might see an absence of the expected upregulation of CGRP in motoneurons, particularly if a similar regulatory mechanism exists between Schwann cell and macrophage for motoneurons. In this case one should examine the presence of other trophic factors including CNTF, BDNF, NT3 etc.

If neurons are, in fact, capable of providing the necessary complement of mitogens for Schwann cells, then the macrophage role may be to prevent apoptosis. In our experiments, IL-1 treated Schwann cell cultures, in contrast to non-treated, looked healthier on microscopic examination, produced greater responses in MTT assays (whereas IdU assays differed less), and in insulin free medium IL-1 exerted a survival-promoting action on Schwann cells. More experiments are clearly needed to delineate the relative contributions of all these factors on Schwann cell regulation.

Knowledge of the detailed cell-cell interactions present during nerve injury, and in regulating proliferation in non-neuronal cells may have clinical implications.

Strategies could be developed to improve the extent of nerve regeneration, and possible treatments developed for abnormally increased Schwann cell growth in the nerve sheath tumours of neurofibromatosis. In these tumours, abnormal upregulation of Ras protein activity occurs by inactivation of GTPase activating proteins (GAP's), through an inheritable gene mutation. Increased amounts of growth factors including GGF and PDGF have been isolated from these tumours. It is possible, that in these tumours there is activation of growth factor activity through Ras, which might bypass the requirement of cAMPi elevation, and increased receptor synthesis due to direct activation of signalling, downstream of the receptor tyrosine kinase. Immortalised Schwann cell lines, derived by repeated passaging of cultures, have increased levels of growth factor receptors, and basal cAMPi is raised. In fact, such cultures may exhibit autocrine growth by also secreting PDGF. It remains unexplained why neurofibromata only arise in restricted locations, even when the NF-1 mutation has occurred in the germ line, rather than as a somatic mutation. If increased Ras activity alone is not sufficient for abnormally increased growth, a further signal / signals may be required in those sites at which neurofibromata develop. These added signals might either be growth factors or cAMPi elevating factors; it would be of great interest to determine whether neuropeptides such as CGRP are present in these tumours, as well as the macrophage products, IL-1 and TNF. The PKC pathway would seem a potentially important control step, based on our own findings in normal cultured Schwann cells. Future experiments of interest could involve blocking IL-1 or CGRP activity with antibody or receptor antagonists, in human tumours in culture or implanted in nude-mice. Success in this situation could relatively easily be followed by clinical trials. Our panning technique would allow the rapid purification of these cells for use in mitogen and second messenger assays. Studying the growth factor requirements of Schwann cells purified and cultured from human neurofibromata, would also allow the delineation of second messenger systems. Potentially, acoustic neuromas are a simpler model of up-regulated Schwann cell proliferation. These tumours are almost entirely of Schwann cell origin, whereas the nerve sheath tumours in NF-1 are often a complex mixture of Schwann cells and fibroblasts. In a pilot study, human Schwann cells were successfully purified from normal adult human auditory nerve (obtained from surgical specimens kindly donated by Mr R Brookes; Royal National Ear, Nose and Throat Hospital). Schwann cells derived from acoustic neuromas (from patients with NF-2 or arising as sporadic acoustic neuromas), with Schwann cells from normal auditory nerve, removed for intractable Menière's disease, can thus be compared. Equally, however, panning could be used to yield pure cultures of fibroblasts, Schwann cells, and even macrophages, from neurofibromata. Thus, not only could the mitogen requirements of these cell types be assessed, but also their production of cytokines and growth factors determined from supernatants. We are now in a position to define the mitogenic signals in these tumours, and to develop



treatment strategies accordingly. For instance, immunomodulatory treatments are already available which could influence IL-1 action; corticosteroids and indomethacin block PGE<sub>2</sub> synthesis, IL-1 receptor antagonists are used *in vitro*, and  $\gamma$ -IFN is used therapeutically. Agents that elevate cAMPi inhibit macrophage and T cell activity.  $\beta$ -agonists at high dose, or inhibitors of 24.11, might increase desensitisation, and thereby eventually down-regulate the activation of the cAMPi pathway in Schwann cells. The inhibitory activity of cAMPi elevating ligands is less influenced by desensitisation; in fibroblasts, macrophages, and T cells, inhibition may still occur, even if desensitisation is eventually induced. Inhibitors of 24.11 are currently in use in clinical trials, to assess the impact of enhanced atrial natriuretic peptide (ANP) activity in heart failure, and for use as diuretics. Inhibitors of protein kinases are likely to be too toxic for easy use. Malignant transformation could occur in Schwann cell tumours through mutation in a survival pathway or even in the activity of the cAMPi pathway. Such mutations could be at any level between the receptor and CRE or AP-1 site, or in a cell-cycle control step downstream of cAMPi-stimulated transcription in the nucleus. It seems likely that part of the action of mitogenic combinations or of IL-1 in cultured Schwann cells is due to reduction in cell-death in culture. Therefore, further experiments are essential to define which signals are induced by these agents to promote survival. These survival-promoting factors could be important in the generation of a malignant phenotype.

## ADDITIONAL REFERENCES

Aebischer, P., Salessiotis, A. N., and Winn, S. R. (1989). Basic fibroblast growth factor released from synthetic guidance channels facilitates peripheral nerve regeneration across long nerve gaps. *J. Neurosci. Res.* 23, 282-289.

Aiyar, N., Rand, K., Elshourbagy, N.A., Zeng, Z., Adamou, J.E., Bergsma, D.J., and Li, Y. (1996). A cDNA encoding the calcitonin gene-related peptide type 1 receptor. *J. Biol. Chem.* 10; 271(19), 11325-9

Averill, S., McMahon, S.B., Clary, D.O., Reichardt, L.F., and Priestley, J.V. (1995). Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. *Eur. J. Neurosci.* 7, 1484-94

Bushfield, M., Savage, A., Morris, N.J. and Houslay, M.D. (1993). A mnemonical or negative-co-operativity model for the activation of adenylate cyclase by a common G-protein-coupled calcitonin-gene-related neuropeptide (CGRP)/amylin receptor. *Biochem. J.* 293 (Pt 1), 229-36

Csillik, B., Tajti, L., Kovacs, T., Kukla, E., Rakic, P., and Knyihar-Csillik, E. (1993). Distribution of calcitonin gene-related peptide in vertebrate neuromuscular junctions: relationship to the acetylcholine receptor. *J. Histochem. Cytochem.* 41(10), 1547-55

Grothe, C. (1993). Biphasic increase of calcitonin gene-related peptide-like immunoreactivity in rat hypoglossal motoneurons after nerve transection. *Acta. Histochem.* 94, 20-4

Forsgren, S., Bergh, A., Carlsson, E., and Thornell, L.E. (1993). Calcitonin gene-related peptide expression at endplates of different fibre types in muscles in rat hind limbs. *Cell Tissue Res.* 274(3), 439-46

Lundborg, G. (1989). Experimental research in hand surgery. *Journal of Hand Surgery - British Volume.* 14(1), 3-5.

McWilliam, P.N., Maqbool, A., Battern, T.F., and Kaye, J.C. (1995). Influence of peripheral targets on the expression of calcitonin gene-regulated peptide immunoreactivity in rat cranial motoneurons. *J. Neurobiol.* 28(4): 506-14

Piehl, F., Arvidsson, U., Hokfelt, T., and Cullheim, S. (1993). Calcitonin gene-related peptide-like immunoreactivity in motoneuron pools innervating different hind limb muscles in the rat. *Exp. Brain Res.* 96(2): 291-303

Piehl, F., Ji, R.R., Cullheim, S., Hokfelt, T., Lindholm, D., and Hughes, R.A. (1995). Fibroblast growth factors regulate calcitonin gene-related peptide mRNA expression in rat motoneurons after lesion and in culture. *Eur. J. Neurosci.* 7(8): 1739-50

Stangl, D., Muff, R., Schmolck, C., and Fischer, J.A. (1993). Photoaffinity labeling of rat calcitonin gene-related peptide receptors and adenylate cyclase activation: identification of receptor subtypes. *Endocrinology.* 132(2): 744-50

## REFERENCES

- Aarons, R.D. and Molinoff, P.B. (1982). Changes in the density of beta-adrenergic receptors in rat lymphocytes, heart and lung after chronic treatment with propranolol. *J. Pharmacol. Exp. Ther.* 221, 439-443.
- Abdul-Badi Abou-Samra., Juepper, H., Potts, Jr., J.T., and Segre, G.V. (1989) Inactivation of pertussis toxin-sensitive guanyl nucleotide-binding proteins increase parathyroid hormone receptors and reverse agonist-induced receptor down-regulation in Ros 17/2.8 cells. *Endocrinology* 125, 2594-2599.
- Abercrombie, M., and Johnson, J.L. (1946). Quantitative histology of Wallerian degeneration. Nuclear population in rabbit sciatic nerve. *J. Anat.* 80, 37-50
- Acheson, A., Barker, P.A., Alderson, R.F., Miller, F.D., and Murphy, R.A. (1991). Detection of brain-derived neurotrophic factor-like activity in fibroblasts and Schwann cells: inhibition by antibodies to NGF. *Neuron* 7, 265-275.
- Acheson, A., Conover, J.C., Fandl, J.P., DeChiara, T.M., Russell, M. Thadani, A., Squinto, S.P., Yancopoulos, G.D., and Lindsay, R.M. (1995). A BDNF autocrine loop in adult sensory neurons prevents cell death. *Nature* 374, 450-453.
- Almendral, J.M., Sommer, D., MacDonald-Bravo, H., Burdkhardt, J., Perera, J., and Bravo, R.(1988) Complexity of the early genetic response to growth factors in mouse fibroblasts. *Mol.Cell Biol.* 8, 2140-2148.
- Amara, S.G., Jonas, V., and Rosenfeld, M.G. (1982). Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature,Lond.* 298, 240-244.
- Amara, S.G., Arriza, J.L., Leff, S.E., Swanson, L.W., Evans, R.M., and Rosenfeld, M.G. (1985). Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. *Science* 229, 1094-1097.
- Anderson, N., Maller, J., Tonks, N., and Sturgill, T. (1990). Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature,Lond.* 343, 651-653.
- Andersson, M., Ostman, A., Backstrom, G., Hellman, U., George Nascimento, C., Westermark, B., and Heldin, C.H. (1992). Assignment of interchain disulfide bonds

in platelet-derived growth factor (PDGF) and evidence for agonist activity of monomeric PDGF. *J. Biol. Chem.* 267, 11260-11266.

Aoyagi, K., Takeshige, K., Sumimoto, H., Nunoi, H., and Minakami, S. (1992). Role of  $Mg^{2+}$  in activation of NADPH oxidase of human neutrophils: evidence that  $Mg^{2+}$  acts through G-protein. *Biochem Biophys. Res. Commun.* 186, 391-397.

Appenzeller, O. and Palmer, G. (1972). The cyclic AMP content of sciatic nerve: changes after nerve crush. *Brain Res.* 42, 521-524

Armati, P.J., Pollard, J.D., and Gatenby, P. (1990). Rat and human Schwann cells in vitro can synthesize and express MHC molecules. *Muscle Nerve* 13, 106-116.

Arvidsson, U., Johnson, H., Piehl, F., Cullheim, S., Hokfelt, T., Risling, M., Terenius, L., and Ulfhake, B. (1990). Peripheral nerve section induces increased levels of calcitonin gene-related peptide (CGRP)-like immunoreactivity in axotomized motoneurons. *Exp. Brain Res.* 79, 212-216.

Arvidsson, U., Johnson, H., Piehl, F., Cullheim, S., Hokfelt, T., Risling, M., Terenius, L., and Ulfhake, B., (1990) Peripheral nerve section induces increased levels of calcitonin gene-related peptide (CGRP)-like immunoreactivity in axotomized motoneurons. *Exp-Brain-Res.* 79(1), 212-6.

Assoian, R.K., Fleurdelys, B.E., Stevenson, H.C., Miller, P.J., Madtes, D.K., Raines, E.W., Ross, R., and Sporn, M.B. (1987). Expression and secretion of type beta transforming growth factor by activated human macrophages. *Proc. Natl. Acad. Sci. U S A.* 84, 6020-6024.

Auron, P.E., Webb, A.C., Rosenwasser, L.J., Mucci, S.F., Rich, A., Wolff, S.M., and Dinarello, C.A. (1984). Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. *Proc. Natl. Acad. Sci. USA* 81, 7907-7911.

Avissar, S., Murphy, D.L., and Schreiber, G. (1991). Magnesium reversal of lithium inhibition of beta-adrenergic and muscarinic receptor coupling to G proteins. *Biochem Pharmacol.* 41, 171-175.

Baichwal, R. R., Bigbee, J. W. and DeVries, G. H. (1988) Macrophage mediated myelin related mitogenic factor for cultured Schwann cells. *Proc.Natl.Acad.Sci.USA* 85, 1701-1705.

Ballester, R., Marchuk, D., Boguski, M., Saulino, A., Letcher, R., Wigler, M., and Collins, F. (1990). The NF1 locus encodes a protein functionally related to mammalian GAP and yeast ira proteins. *Cell* 63, 851-859.

Bandtlow, C. E., Heumann, R., Schwab, M. E., Thoenen, H. (1987). Cellular localisation of nerve growth factor synthesis by in situ hybridization. *EMBO J.* 6, 891-899.

Barde, Y. -A., Edgar, D., and Thoenen, H. (1982). Purification of a new neurotrophic factor from mammalian brain. *EMBO J.* 1, 549-553.

Barker, E., Wright, K., Nguyen, K., and et al., (1987). Gene for von Recklinghausen neurofibromatosis in the pericentromeric region of chromosome 17. *Science* 236, 1100.

Barnes, K., Matsas, R., Hooper, N. M., Turner, A.J. and Kenny, A. J. (1988). Endopeptidase 24.11 is striosomally ordered in pig brain and, in contrast to aminopeptidase N and peptidyl dipeptidase A (angiotensin converting enzyme) is a marker for a set of striatal efferent fibres. *Neurosci.* 3, 799-817

Baron-Van Evercooren, A., Kleinmann, H. K., Seppa, H.E., Rentier, B., and Dubois-Dalq, M. (1982) Fibronectin promotes rat Schwann cell growth and motility. *J. Cell Biol.* 93, 211-216

Barres, B.A., Silverstein, B.E., Corey, D.P., and Chun, L.L.Y. (1988). Immunological, morphological, and electrophysiological variation among retinal ganglion cells purified by panning. *Neuron* 1, 791-803.

Barsony, J. and Marx, S. J. (1990). Immunocytochemistry on microwave-fixed cells reveals rapid and agonist-specific changes in subcellular accumulation patterns for cAMP and cGMP. *Proc. Natl. Acad. Sci. USA.* 87, 1188-1192.

Benovic, J.L., Pike, L.J., Cerione, R.A., and et al, (1985). Phosphorylation of the mammalian  $\beta$ -adrenergic receptor by cyclic AMP-dependant protein kinase. *J. Biol. Chem.* 260, 7094-7101.

Benovic, J.L., Strasser, R.H., Caron, M.G., and Lefkowitz, R.J. (1986).  $\beta$ -adrenergic receptor kinase: Identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. *Proc. Natl. Acad. Sci. USA* 83, 2797-2801.

Benovic, J.L., Kuhn, M., Weyand, I., Codina, J., Caron, M., and Lefkowitz, R.J. (1987a). Functional desensitization of the isolated  $\beta$ -adrenergic receptor by the  $\beta$ -adrenergic receptor kinase: Potential role of an analog of the retinal protein arrestin (48-kDa protein). *Proc. Natl. Acad. Sci. USA* *84*, 8879-8882.

Benovic, J.L., Mayor, F.J., Staniszewski, C., Lefkowitz, R.J., and Caron, M.G. (1987b). Purification and characterization of the beta-adrenergic receptor kinase. *J. Biol. Chem.* *262*, 9026-9032.

Berkemeier, L.R., Winslow, J.W., Kaplan, D.R., Nicoliks, K., Goeddel, D.V., and Rosenthal, A. (1991). Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. *Neuron* *7*, 857-866.

Berridge, M. J., Heslop, J.P., Irvine, R.F., and Brown, K. D. (1984). Inositol triphosphate formation and calcium mobilisation in Swiss 3T3 cells in response to platelet-derived growth factor. *Biochem. J.* *222*, 195-201.

Berridge, M.J., and Irvine, R.F.(1989). Inositol phosphates and cell signalling. *Nature* *341*, 197-205.

Bertelsen, H. and Johansen, T. (1991). Dual effect of magnesium on compound 48/80-induced histamine secretion from rat peritoneal mast cells. *Pharmacol. Toxicol.* *69*, 28-33.

Berthold, C.-H. (1973). Local demyelination in developing feline nerves. *Neurobiology* *3*, 339-352.

Beuche, W. and Friede, R.L. (1984). The role of non-resident cells in Wallerian degeneration. *J. Neurocytol.* *13*, 767-796.

Bian, X.P., Seidler, F.J., and Slotkin, T.A. (1991). Promotional role for glucocorticoids in the development of intracellular signalling: enhanced cardiac and renal adenylate cyclase reactivity to beta-adrenergic and non-adrenergic stimuli after low-dose fetal dexamethasone exposure. *J. Dev. Physiol.* *16*, 331-339.

Bijlsma, E.K., Brouwer Mladin, R., Bosch, D.A., Westerveld, A., and Hulsebos, T.J. (1992). Molecular characterization of chromosome 22 deletions in schwannomas. *Genes Chromosom. Cancer* *5*, 201-205.

Birnbaumer, L., Codina, J., Mattera, R., Cerione, R.A., Hildebrandt, D., Sunyer, T., Rojas, F.J., Caron, M., and Lefkowitz, R.J. (1985). Structural basis of adenylate cyclase stimulation and inhibition by distinct guanine nucleotide regulatory proteins. In *Molecular mechanisms of transmembrane signalling*. P. Cohen and N.D. Housley, eds. (pp. 131-182).

Birnbaumer, L., Codina, J., Mattera, R., and et al, (1987). Signal transduction by G-proteins. *kidney Int.* 32, 514-537.

Birnbaumer, L., Abramowitz, J., and Brown, A.M. (1990a). Receptor effector coupling by G proteins. *Biochim. Biophys. Acta* 1031, 163-224.

Blackshear, P., Haupt, D., App, H., and Rapp, U. (1990). Insulin activates the Raf-1 protein kinase. *J. Biol. Chem.* 265, 12131-12134.

Blank, J.L., Ross, A.H., and Exton, J.H. (1991). Purification and characterization of two G-proteins that activate the beta 1 isozyme of phosphoinositide-specific phospholipase C. Identification as members of the Gq class. *J. Biol. Chem.* 266, 18206-18216.

Blank, J.L., Brattain, K.A., and Exton, J.H. (1992). Activation of cytosolic phosphoinositide phospholipase C by G-protein beta gamma subunits. *J. Biol. Chem.* 267, 23069-23075.

Blennerhassett, M.G., Tomioka, M., and Bienenstock, J. (1991). Formation of contacts between mast cells and sympathetic neurons in vitro. *Cell Tissue Res.* 265, 121-128.

Blow, J. and Nurse, P. (1990). A cdc2 like protein is involved in the initiation of DNA replication in xenopus egg extracts. *Cell* 62, 855-862.

Bobik, A. and Little, P.J. (1984). Role of cAMP in cardiac beta-adrenoceptor desensitization: Studies using prenalterol and inhibitors of phosphodiesterase. *J. Cardiovasc. Pharmacol.* 6, 795-801.

Bohm, M., Beuckelmann, D., Brown, L., Feiler, G., Lorenz, B., Nabauer, M., Kemkes, B., and Erdmann, E. (1988). Reduction of  $\beta$ -adrenoceptor density and evaluation of positive inotropic responses in isolated diseased human myocardium. *Eur. Heart J.* 9, 844.

Bosche, E. P., Assouline, J. G., Miller, J. F. and Lim, R. (1984). Glial maturation factor promotes proliferation and morphologic expression of rat Schwann cells. *Brain Res.* 304, 311-319

Boulton, T., Yancopoulos, G., Gregory, J., Slaughter, C., Moomaw, C., Hsu, J., and Cobb, M. (1990). An insulin stimulated protein kinase similar to yeast kinases involved in cell cycle control. *Science* 249, 64-67.

Bourne, H.R., Sanders, D.A., and McCormick, F. (1991). The GTPase superfamily: conserved structure and molecular mechanism. *Nature* 349, 117-127.

Bouvier, M., Leeb-Lundberg, L.M.F., Benovic, J.L., Caron, M.G., and Lefkowitz, R.J. (1987). Regulation of adrenergic receptor function by phosphorylation. *J. Biol. Chem.* 262, 3106-3113.

Bouvier, M., Hausdorff, W.P., De Blasi, A., O'Dowd, B.F., Kobilka, B.K., Caron, M.G., and Lefkowitz, R.J. (1988). Removal of phosphorylation sites from the beta 2-adrenergic receptor delays onset of agonist-promoted desensitization. *Nature* 333, 370-373.

Bouvier, M., Hnatowich, M., Collins, S., Kobilka, B.K., DeBlasi, A., Lefkowitz, R.J., and Caron, M.G. (1988). Expression of a human cDNA encoding the beta 2-adrenergic receptor in Chinese hamster fibroblasts (CHW): functionality and regulation of the expressed receptors. *Mol. Pharmacol.* 33, 133-139.

Boudard, F. and Bastide, M. (1991). Inhibition of mouse T-cell proliferation by CGRP and VIP: effects of these neuropeptides on IL-2 production and cAMP synthesis. *J. Neurosci. Res.* 29, 29-41.

Branden, K. R. and Poduslo, J. F. (1987). Lysosomal delivery of the major myelin glycoprotein in the absence of myelin assembly: Posttranslational regulation of expression by Schwann cells. *J. Cell Biol.* 104,, 661- 669.

Brandt, D.R. and Ross, E.M. (1985). GTPase activity of the stimulatory GTP-binding regulatory protein of adenylate cyclase. *J. Biol. Chem.* 260, 266-272.

Brandt, D.R. and Ross, E.M. (1986). Catecholamine stimulated GTPase cycle: multiple sites of regulation by  $\beta$ -adrenergic receptor and  $Mg^{++}$  studied in reconstituted receptor-Gs vesicles. *J. Biol. Chem.* 261, 1656-1664.



Bravo, R., Neuberg, M., Burckhardt, J., Almendral, J., Wallich, R., and Muller, R. (1987). Involvement of common and cell type-specific pathways in c-fos gene control: stable induction of cAMP in macrophages. *Cell* 48, 251-260.

Bray, G.M., Rasminsky, M., and Aguayo, A.J. (1981). Interactions between axons and their sheath cells. *Ann. Rev. Neurosci.* 4, 127-162.

Bray, P., Carter, A., Simons, C., Guo, V., Puckett, C., Kamholz, J., Spiegel, A., and Nirenberg, M. (1986). Human cDNA clones for four species of Gs signal transduction protein. *Proc. Natl. Acad. Sci. USA* 83, 8893-8897.

Bressler, J. P. and Tinsely, P. (1990) Regulation of cAMP levels by protein kinase C in C6 rat glioma cells. *J. Neurosci. Res.* 25, 81-86.

Bristow, M.R., Ginsberg, R., Minobe, W., Cubicciotti, R.S., Sageman, W.S., Lurie, M.S.K., Billingham, M.E., Harrison, D.L., and Stinson, E.B. (1982). Decreased catecholamine sensitivity and  $\beta$ -adrenoceptor density in failing human heart. *N. Engl. J. Med.* 307, 2052-11.

Bristow, M.R., Kantrowitz, N.E., Ginsburg, R., and Fowler, M.B. (1985).  $\beta$ -adrenergic function in heart muscle disease and heart failure. *J. Mol. Cell. Cardiol.* 17(2), 41.

Bristow, M.R., Ginsberg, R., Umans, V., Fowler, M., Minobe, W., Rasmussen, R., Zera, P., Menlove, R., Shah, P., Jamieson, S., and Stinson, E.B. (1986).  $\beta$ 1- and  $\beta$ 2-adrenergic receptor subpopulations in nonfailing and failing human ventricular myocardium: Coupling of both receptor subtypes to muscle contraction and selective  $\beta$ 1-receptor down-regulation in heart failure. *Circ. Res.* 59, 297-309.

Bristow, M.R., Herschberger, R.E., and Port, J.D. (1989).  $\beta$ 1- and  $\beta$ 2-adrenergic receptor mediated adenylate cyclase stimulation in nonfailing and failing human ventricular myocardium. *Mol. Pharmacol.* 35, 295-303.

Brockes, J. P., Fields, K. L. and Raff, M. C. (1979) Studies on cultured rat Schwann cells. 1. establishment of purified populations from cultures of peripheral nerve. *Brain Res.* 165, 105-118

Brockes, J.P., Lemke, G.E., and Balzar, D.R. (1980). Purification and preliminary characterization of glial growth factor from bovine pituitary. *J. Biol. Chem.* 255, 8374-8377.

- Brockes, J.P., Breakefield, X.O., and Martuza, R.L. (1986). Glial growth factor-like activity in Schwann cell tumors. *Ann. Neurol.* 20, 317-322.
- Brodde, O.E., Kretsch, R., Ikezona, K., Zerkowski, H.R., and Reidemeister, J.C. (1986). Human beta-adrenoceptors: Relation of myocardial and lymphocyte beta-adrenoceptor density. *Science* 231, 1584-1586.
- Brown, A.M. and Birnbaumer, L.B. (1988). Direct G protein gating of ion channels. *Am. J. Physiol.* 23, H401-H410.
- Brown, E., Enyedi, P., LeBoff, M., Rotberg, J., Preston, J., and Chen, C. (1987). High extracellular  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  stimulate accumulation of inositol phosphates in bovine parathyroid cells. *FEBS Lett.* 218, 113-118.
- Brown, L., Lorenz, B., and Erdmann, E. (1986). Reduced positive inotropic effects in diseased human ventricular myocardium. *Cardiovasc. Res.* 20, 516.
- Brown, M.C., Perry, V.H., Lunn, E.R., Gordon, S., and Heumann, R. (1991). Macrophage dependence of peripheral sensory nerve regeneration: possible involvement of nerve growth factor. *Neuron* 6, 359-370.
- Brown, M.C., Lunn, E.R., and Perry, V.H. (1992). Consequences of slow Wallerian degeneration for regenerating motor and sensory axons. *J. Neurobiol.* 23, 521-536.
- Brown, M.J. and Asbury, A.K. (1981). Schwann cell proliferation in the postnatal mouse: timing and topography. *Exp. Neurol.* 74, 170-186.
- Bruck, W. and Friede, R.L. (1990). Anti-macrophage CR3 antibody blocks myelin phagocytosis by macrophages in vitro. *Neuropath.* 80, 415-418.
- Bryckaert, M.C., Eldor, A., Fontenay, M., Gazit, A., Osherov, N., Gilon, C., Levitzki, A., and Tobelem, G. (1992). Inhibition of platelet-derived growth factor-induced mitogenesis and tyrosine kinase activity in cultured bone marrow fibroblasts by tyrphostins. *Exp. Cell Res.* 199, 255-261.
- Buchberg, A.M., Cleveland, L.S., Jenkins, N.A., and Copeland, N.G. (1990). Sequence homology shared by neurofibromatosis type-1 gene and IRA-1 and IRA-2 negative regulators of the RAS cyclic AMP pathway. *Nature, Lond.* 347, 291-294.

Bunge, R.P., Wood, P.M., Tynan, L.B., Bates, M.L., and Sanes, J.R. (1989). Perineurium originates from fibroblasts: demonstration in vitro with a retroviral marker. *Science* 243, 229-231.

Butcher, R.W., Barber, R., and Goka, T.J. (1986). cAMP metabolism in an S49 mouse lymphoma variant with diminished phosphodiesterase activity. *J. Cyclic Nucleotide Protein Phosphor. Res.* 11, 275-290.

Carlsson, E., Ablad, B., Brandstrom, A., and Carlsson, B. (1972). Differential blockade of the chronotropic effects of various adrenergic stimuli in the cat heart. *Life Sci.* 11, 953-958.

Carlson, L.L., Weaver, D.R., and Reppert, S.M. (1989). Melatonin signal transduction in hamster brain: Inhibition of adenylyl cyclase by a pertussis toxin-sensitive G protein. *Endocrinology* 125 (5), 2670-2676.

Carman Krzan, M., Vige, X., and Wise, B.C. (1991). Regulation by interleukin-1 of nerve growth factor secretion and nerve growth factor mRNA expression in rat primary astroglial cultures. *J. Neurochem.* 56, 636-643.

Chang, D.M., Baptiste, P., and Schur, P.H. (1990). The effect of antirheumatic drugs on interleukin 1 (IL-1) activity and IL-1 and IL-1 inhibitor production by human monocytes. *J. Rheumatol.* 17, 1148-1157.

Chatterjee, T.K., Moy, J.A., and Fisher, R.A. (1991). Characterization and regulation of high affinity calcitonin gene-related peptide receptors in cultured neonatal rat cardiac myocytes. *Endocrinology* 128,, 2731-2738.

Chedid, M., Shirakawa, F., Naylor, P., and Mizel, S.B. (1989). Involvement of a pertussis toxin-sensitive GTP-binding protein in the activation of adenylate cyclase. *J. Immunol.* 142, 4301-4306.

Chedid, M., Yoza, B.K., Brooks, J.W., and Mizel, S.B. (1991). Activation of AP-1 by IL-1 and phorbol esters in T cells. Role of protein kinase A and protein phosphatases. *J. Immunol.* 147, 867-873.

Chellappan, S., Hiebert, S., Mudryj, M., Horowitz, J., and Nevins, J., (1991) The E2F transcription factor is a cellular target for the Rb protein. *Cell.* 65, 1053-1061.

Chen, J.K., Yao, L.L., and Jenq, C.B. (1991). Mitogenic response of rat Schwann cells to fibroblast growth factors is potentiated by increased intracellular cyclic AMP levels. *J. Neurosci. Res.* 30, 321-327.

Chen, P., Scully, P., Shew, J., Wang, J., and Lee, W. (1989). Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell* 58,, 1193-1198.

Cisar, L.A., Schimmel, R.J., and Mochan, E. (1991). Interleukin-1 stimulation of arachidonic acid release from human synovial fibroblasts; blockade by inhibitors of protein kinases and protein synthesis. *Cell Signal.* 3, 189-199.

Claesson-Welsh, L., Eriksson, A., Morén, A., Severinson, L., Ek, B., Östman, A., Betsholtz, C., and Heldin, C.-H. (1988). cDNA cloning and expression of a human platelet-derived growth factor (PDGF) receptor specific for  $\beta$ -chain containing PDGF molecules. *Mol. Cell. Biol.* 8 ,3476-3486.

Clark Lewis, I., Sanghera, J.S., and Pelech, S.L. (1991). Definition of a consensus sequence for peptide substrate recognition by p44mpk, the meiosis-activated myelin basic protein kinase. *J. Biol. Chem.* 266, 15180-15184.

Clark, R.B. (1986). Desensitization of hormonal stimuli coupled to regulation of cyclic AMP levels. *Adv. Cycl. Nucl. Phosph. Res.* 20, 155-209.

Clark, R.B., Friedman, J., Johnson, J.A., and Kunkel, M.W. (1987). Beta-adrenergic receptor desensitization of wild-type but not cyc lymphoma cells unmasked by submillimolar  $Mg^{2+}$ . *FASEB J.* 1, 289-297.

Clark, R.B., Kunkel, M.W., Friedman, J., Goka, T.J., and Johnson, J.A. (1988). Activation of cAMP-dependant protein kinase is required for heterologous desensitization of adenyly cyclase in S49 wild-type lymphoma cells. *Proc. Natl. Acad. Sci. USA* 85, 1442-1446.

Clemence, A., Mirsky, R., and Jessen, K.R. (1989). Non-myelin-forming Schwann cells proliferate rapidly during Wallerian degeneration in the rat sciatic nerve. *J. Neurocytol.* 18, 185-192.

Clements, J.M., Bawden, L.J., Bloxidge, R.E., Catlin, G., Cook, A.L., Craig, S., Drummond, A.H., Edwards, R.M., Fallon, A., Green, D.R., and et al, (1991). Two

PDGF-B chain residues, arginine 27 and isoleucine 30, mediate receptor binding and activation. *EMBO J.* 10, 4113-4120.

Climenti, E., Malgaretti, N., Meldolesi, J., and Toramelli, R. (1990). A new constitutively activating mutation of Gs protein  $\alpha$  subunit-gsp oncogene is found in human pituitary tumours. *Oncogene* 5, 1059-1061.

Cohen, J.A., Yachnis, A.T., Arai, M., Davis, J.G., and Scherer, S.S. (1992). Expression of the *neu* proto-oncogene by Schwann cells during peripheral nerve development and Wallerian degeneration. *J. Neurosci. Res.* 31, 622-634.

Cocchia, D. (1981) Immunocytochemical localisation of S100 protein in the brain of adult rat. *Cell and Tissue res.* 214, 529-40.

Cocchia, D. and Michetti, F. (1981). S-100 antigen in satellite cells of the adrenal medulla and the superior cervical ganglion of the rat. An immunochemical and immunocytochemical study. *Cell and tissue res.* 215, 103-12.

Cohn, J.N., Levine, T.B., Olivari, M.T., Garberg, V., Lura, D., Francis, G.S., Simon, A.B., and Rector, T. (1984). Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. *N. Engl. J. Med.* 311, 819.

Colucci, W.S., Alexander, R.W., Williams, G.H., Rude, R.E., Holman, B.L., Konstam, M.A., Wynne, J., Mudge, G.H., and Braunwald, E. (1981). Decreased lymphocyte beta-adrenergic receptor density in patients with heart failure and tolerance to the beta adrenergic agonist pirbuterol. *N. Engl. J. Med.* 305, 185-190.

Colucci, W.S., Ribeiro, J.P., Rocco, M.B., Quigg, R.J., Creager, M.A., Marsh, J.D., Gauthier, D.F., and Hartley, L.H. (1989). Impaired chronotropic response to exercise in patients with congestive cardiac failure. Role of post-synaptic beta-adrenergic desensitization. *Circulation* 80, 314-323.

Colucci, W.S. (1990). In vivo studies of myocardial  $\beta$ -adrenergic receptor pharmacology in patients with congestive heart failure. *Circulation* 82(suppl.1), I-44-I-51.

Comelli, M.C., Seren, M.S., Guidolin, D., Manev, R.M., Favaron, M., Rimland, J.M., Canella, R., Negro, A., and Manev, H. (1992). Photochemical stroke and brain-derived neurotrophic factor (BDNF) mRNA expression. *Neuroreport* 3, 473-476.

Cooper, J. A., Bowen-Pope, D.F., Raines, E., Ross, R., and Hunter, T. (1982). Similar effects of platelet-derived growth factor and epidermal growth factor on the phosphorylation tyrosine in cellular proteins. *Cell* 31, 263-273.

Corbin, J.D., Sugden, P.G., West, L., and et al, (1978). Studies on the properties and mode of action of the purified regulatory subunit of bovine heart adenosine 3',5'-monophosphate-dependant protein kinase. *J. Biol. Chem.* 253, 3997-4003.

Cordon-Cardo, C., Tapley, P., Jing, S., Nanduri, V., O'Rourke, E., Lamballe, F., Kovary, K., Klein, R., Jones, K.R., Reichardt, L., and Barbacid, M. (1991). The *trk* tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. *Cell* 66, 173-183.

Cornblath, D.R., Griffin, D.E., Welch, D., Griffin, J.W., and McArthur, J.C. (1990). Quantitative analysis of endoneurial T-cells in human sural nerve biopsies. *J. Neuroimmunol.* 26, 113-118.

Coyne, D.W. and Morrison, A.R. (1990). Effect of the tyrosine kinase inhibitor, genistein, on interleukin-1 stimulated PGE<sub>2</sub> production in mesangial cells. *Biochem. Biophys. Res. Commun.* 173, 718-724.

Crowe, F.W., Schull, W.J., and Neel, J.V. (1956). A clinical, pathological and genetic study of multiple neurofibromatosis (Illinois: Springfield).

Dahlstrom, A.B. and Booj, S. (1988). Rapid axonal transport as a chromatographic process: the use of immunocytochemistry of ligated nerves to investigate the biochemistry of anterogradely versus retrogradely transported organelles. *Cell Motil. Cytoskeleton* 10, 309-320.

Daston, M.M., Scrabble, H., Nordlund, M., Sturbaum, A.K., Nissen, L.M., and Ratner, N. (1992). The protein product of the neurofibromatosis type 1 gene is expressed at highest abundance in neurons, Schwann cells and oligodendrocytes. *Neuron* 8, 415-428.

Davies, D., Medeiros, M.S., Keen, J., Turner, A.J., and Haynes, L.W. (1992). Eosinophil chemotactic peptide sequences in rat CGRP. *Ann. N. Y. Acad. Sci.* 657, 405-411.

Davies, S.W., Fussell, A., Poole-Wilson, P.A., and Lipkin, D.P. (1989). Diastolic function and exercise capacity in chronic heart failure. *Circulation* 80(suppl.II), II-75.

Davis, J.B. and Stroobant, P. (1990a). Platelet-derived growth factors and fibroblast growth factors are mitogens for rat Schwann cells. *J. Cell Biol.* 110, 1353-1360.

Davis, J.B. and Stroobant, P. (1990b). Platelet-derived growth factors and fibroblast growth factors are mitogens for rat Schwann cells. *J. Cell Biol.* 110, 1353-1360.

Davis, S., Aldrich, T.H., Valenzuela, D.M., Wong, V.V., Furth, M.E., Squinto, S.P., and Yancopoulos, G.D. (1991). The receptor for ciliary neurotrophic factor. *Science* 253, 59-63.

de Vos, A.M., Tong, L., Milburn, M.V., Matias, P.M., Jancarik, J., Noguchi, S., Nishimura, S., Miura, K., Ohtsuka, E., and Kim, S.-H. (1988). Three-dimensional structure of an oncogene protein: catalytic domain of human c-H-ras p21. *Science* 239, 888-893.

Devos, C., Robberecht, P., Waelbroeck, M., Clignet, M., Beaufort, P., Schoenfeld, P., and Christophe, J. (1985). Uncoupling between  $\beta$ -adrenoceptors and adenylate cyclase in dog ischaemic myocardium. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 331, 71-75.

de Vries Smits, A.M., Burgering, B.M., Leervers, S.J., Marshall, C.J., and Bos, J.L. (1992). Involvement of p21ras in activation of extracellular signal-regulated kinase 2. *Nature, Lond.* 357, 602-604.

Di Scala Guenot, D. and Strosser, M.T. (1992). Oxytocin receptors on cultured astroglial cells. Regulation by a guanine-nucleotide-binding protein and effect of  $Mg^{2+}$ . *Biochem J.* 284, 499-505.

DiStefano, P.S. and Johnson, E.M.J. (1988). Nerve growth factor receptors on cultured rat Schwann cells. *J. Neurosci.* 8, 231-241.

Dobrea, G.M., Unnerstall, J.R., and Rao, M.S. (1992). The expression of CNTF message and immunoreactivity in the central and peripheral nervous system of the rat. *Brain Res. Dev. Brain Res.* 66, 209-219.

Dohlman, H.G., Caron, M.G., and Lefkowitz, R.J. (1987). A family of receptors coupled to guanine nucleotide regulatory proteins. *Biochemistry* 26, 2656-2664.

Drozdoiff, V. and Pledger, W.J. (1991). Cellular response to platelet-derived growth factor (PDGF)-AB after down-regulation of PDGF  $\alpha$ -receptors: evidence that functional binding does not require  $\alpha$ -receptors. *J. Biol. Chem.* 266, 17165-17172.

Duchatelle Gourdon, I., Lagrutta, A.A., and Hartzell, H.C. (1991). Effects of  $Mg^{2+}$  on basal and beta-adrenergic-stimulated delayed rectifier potassium current in frog atrial myocytes. *J. Physiol. Lond.* 435, 333-347.

Dulic, V., Lees, E., and Reed, S.I. (1992). Association of human cyclin E with a periodic G1-S phase protein kinase. *Science* 257, 1958-1961.

Dumont, J.E., Jauniaux, J.C., and Roger, P.P. (1989). The cyclic AMP mediated stimulation of cell proliferation. *Trends Biochem. Sci.* 14, 67-71.

Eccleston, P.A., Jessen, K.R., and Mirsky, R. (1989). Transforming growth factor-beta and gamma-interferon have dual effects on growth of peripheral glia. *J. Neurosci. Res.* 24, 524-530.

Eccleston, P.A., Collarini, E.J., Jessen, K.R., Mirsky, R., and Richardson, W.D. (1990). Schwann cells secrete a PDGF-like factor: evidence for an autocrine growth mechanism involving PDGF. *Eur. J. Neurosci.* 2, 985-992.

Eckenstein, F.P., Shipley, G.D., and Nishi, R. (1991). Acidic and basic fibroblast growth factors in the nervous system: distribution and differential alteration of levels after injury of central versus peripheral nerve. *J. Neurosci.* 11, 412-419.

El-Deiry, W.S., Tokino, T., Velculesco, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumour suppression. *Cell.* 75, 817-825.

Elias, J.A., Gustilo, K., Baeder, W., and Freundlich, B. (1987). Synergistic stimulation of fibroblast prostaglandin production by recombinant interleukin 1 and tumor necrosis factor. *J. Immunol.* 138, 3812-3816.

Eneroth, A., Andersson, T., Olsson, T., Orvell, C., Norrby, E., and Kristensson, K. (1992). Interferon- $\tau$ -like immunoreactivity in sensory neurons may influence the replication of sendai and mumps viruses. *J. Neurosci. Res.* 31, 487-493.



Englemeier, R.S., O'Connell, J.B., Walsh, R., Rad, N., Scanlon, P.J., and Gunnar, R.M. (1985). Improvement in symptoms and exercise tolerance by metoprolol in patients with dilated cardiomyopathy: A double blind, randomized, placebo controlled trial. *Circulation* 72, 536-546.

Eriksson, A., Nister, M., Leveen, P., Westermark, B., Heldin, C.H., and Claesson Welsh, L. (1991). Induction of platelet-derived growth factor alpha- and beta-receptor mRNA and protein by platelet-derived growth factor BB. *J. Biol. Chem.* 266, 21138-21144.

Eriksson, A., Rorsman, C., Ernlund, A., Claesson Welsh, L., and Heldin, C.H. (1992a). Ligand-induced homo- and hetero-dimerization of platelet-derived growth factor alpha- and beta-receptors in intact cells. *Growth Factors*. 6, 1-14.

Eriksson, A., Siegbahn, A., Westermark, B., Heldin, C.-H., and Claesson-Welsh, L. (1992b). PDGF  $\alpha$ - and  $\beta$ -receptors activate unique and common signal transduction pathways. *EMBO J.* 11, 543-550.

Escobedo, J.A., Barr, P.J., and Williams, L.T. (1988). Role of tyrosine kinase and membrane spanning domains in signal transduction by the platelet-derived growth factor receptor. *Mol. Cell Biol.* 8, 5126-5131.

Escobedo, J.A. and Williams, L.T. (1988). A PDGF receptor domain essential for mitogenesis but not for many other responses to PDGF. *Nature, Lond.* 335, 85-87.

Falls, D.L., Rosen, K.M., Corfas, G., Lane, W.S., and Fischbach, G.D. (1993). *Cell*. 72, 801-815.

Fantl, W.J., Escobedo, J.A., Martin, G.A., Turck, C.W., Del Rosario, M., McCormick, F., and Williams, L.T. (1992). Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. *Cell* 69, 413-423.

Feldman, A.M., Cates, A.E., Veazey, W.B., Herschberger, R.E., Bristow, M.R., Baughman, K.L., Baumgartner, W.A., and Van Dop, C. (1988). Increase of the 40,000-mol wt. pertussis toxin substrate(G protein) in the failing human heart. *J. Clin. Invest.* 82, 189.

Feldman, M.D., Copelas, L., Gwanthmey, J.K., Phillips, P., Warren, S.E., Schoen, F.J., Grossman, W., and Morgan, J.P. (1987). Deficient production of cyclic AMP: pharmacologic evidence of an important cause of contractile dysfunction in patients with endstage heart failure. *Circulation* 75, 331-339.

Fenton, M.J., Clark, B.D., Collins, K.L., Webb, A.C., Rich, A., and Auron, P.E. (1987). Transcriptional regulation of the human prointerleukin 1 beta gene. *J. Immunol.* 138, 3972-3979.

Fields, A.P., Tyler, G., Kraft, A.S., and May, W.S. (1990). Role of nuclear protein kinase C in the mitogenic response to platelet-derived growth factor. *J. Cell Sci.* 96, 107-114.

Findlay, J.B.C. and Pappin, D.J.C. (1986). The opsin family of proteins. *Biochem. J.* 238, 625-642.

Fowler, M.B., Laser, J.A., Hopkins, G.L., Minobe, W., and Bristow, M.R. (1986). Assessment of the  $\beta$ -adrenergic receptor pathway in the intact failing human heart: progressive receptor downregulation and subsensitivity to agonist response. *Circulation* 74, 1290.

Fontaine, B., Klarsfeld, A., Hokfelt, T., and Changeux, J-P. (1986). Calcitonin gene-related peptide present in spinal cord motoneurons, increases the number of acetylcholine receptors in primary cultures of chick embryo myotubes. *Neurosci. Lett.* 71, 59-65.

Fontenay, M., Bryckaert, M., and Tobelem, G. (1992). Transforming growth factor-beta 1 inhibitory effect of platelet-derived growth factor-induced signal transduction on human bone marrow fibroblasts: possible involvement of protein phosphatases. *J. Cell Physiol.* 152, 507-519.

Franke, R., Konig, B., Sakmar, T.P., Khorana, H.G., and Hofmann, K.P. (1990). Rhodopsin mutants that bind but fail to activate transducin. *Science* 250, 123-125.

Freid, G., Franck, J., Brodin, E., Born, W., Fischer, J.A., Hiort, W., and Hokfelt, T. (1989). Evidence for differential storage of calcitonin gene-related peptide, substance P and serotonin in synaptosomal vesicles of rat spinal cord. *Brain Res.* 499, 315-324.

Frick, K.K., Womer, R.B., and Scher, C.D. (1988). Platelet-derived growth factor-induced c-myc RNA expression. Analysis of an inducible pathway independent of protein kinase C. *J. Biol. Chem.* 263, 2948-2952.

Fried, K., Brodin, E. and Theodorsson, E. (1989). Substance P-, CGRP- and NPY-immunoreactive nerve fibers in rat sciatic nerve-end neuromas. *Regul-Pept.* 25(1), 11-24.

Friedman, W.J., Larkfors, L., Ayer LeLievre, C., Ebendal, T., Olson, L., and Persson, H. (1990). Regulation of beta-nerve growth factor expression by inflammatory mediators in hippocampal cultures. *J. Neurosci. Res.* 27, 374-382.

Freissmuth, M., Casey, P.J., and Gilman, A.G. (1989). G Proteins control diverse pathways of transmembrane signaling. *FASEB J.* 3, 2125-2131.

Furukawa, Y., Piwnica-Worms, H., Ernst, T., Kanakura, Y., and Griffin, J.D. (1990). cdc2 gene expression at the G1 to S transition in human T lymphocytes. *Science* 250, 805-808.

Gadient, R.A., Cron, K.C., and Otten, U. (1990). Interleukin-1 beta and tumor necrosis factor-alpha synergistically stimulate nerve growth factor (NGF) release from cultured rat astrocytes. *Neurosci. Lett.* 117, 335-340.

Gamble, H. J. and Goldby, S. (1961). Mast cells in peripheral nerve trunks. *Nature* 189, 766.

Gibson, S.J., Polak, J.M., Giaid, A., Hamid, Q.A., Kar, S., Jones, P.M., Denny, P., Legon, S., Amara, S.G., Craig, R.K., Bloom, S.R., Penketh, R.J.A., Rodek, C., Ibrahim, N.B.N., and Dawson, A. (1988). Calcitonin gene-related peptide messenger RNA is expressed in sensory neurons of the dorsal root ganglia and also in spinal motoneurons in man and rat. *Neurosci. Lett.* 91, 283-288.

Gilman, A.G. (1987). G-Proteins: Transducers of receptor generated signals. *Ann. Rev. Biochem.* 55, 615-649.

Ginsburg, R., Bristow, M.R., Billingham, M.E., Stinson, E.B., Schroeder, J.S., and Harrison, D.C. (1983). Study of the normal and failing isolated human heart: decreased response of the failing heart to isoproterenol. *Am. Heart J.* 106, 535.

Glutzer, M., Murray, A.W., and Kirschner, M.W. (1991). Cyclin is degraded by the ubiquitin pathway. *Nature*. 349, 132-138.

Gomez, N., and Cohen, P. (1991). *Nature* 351, 69-72.

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

Gould, K. and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. *Nature, Lond.* 342, 39-45.

Greenberg, B., Rhoden, K., and Barnes, P. (1987). CGRP is a potent non-endothelium-dependant inhibitor of coronary vasomotor tone. *Br. J. Pharmacol.* 92, 789-794.

Greenberg, M.E., and Ziff, E.B. (1984). Stimulation of 3T3 cells induces transcription of the c-fos proto oncogene. *Nature* 311, 433-438.

Griffin, J.W., Stocks, E.A., Fahnestock, K., Van Praagh, A., and Trapp, B.D. (1990a). Schwann cell proliferation following lysolecithin-induced demyelination. *J. Neurocytol.* 19, 367-384.

Griffin, J.W., Stoll, G., Li, C.Y., Tyor, W., and Cornblath, D.R. (1990b). Macrophage responses in inflammatory demyelinating neuropathies. *Ann. Neurol.* 27 Suppl, S64-S68.

Gronwald, R.G., Grant, F.J., Haldeman, B.A., Hart, C.E., O'Hara, P.J., Hagen, F.S., Ross, R., Bowen Pope, D.F., and Murray, M.J. (1988). Cloning and expression of a cDNA coding for the human platelet-derived growth factor receptor: evidence for more than one receptor class. *Proc. Natl. Acad. Sci. USA.* 85, 3435-3439.

Guenard, V., Dinarello, C.A., Weston, P.J., and Aebischer, P. (1991). Peripheral nerve regeneration is impeded by interleukin-1 receptor antagonist released from a polymeric guidance channel. *J. Neurosci. Res.* 29, 396-400.

Gulbenkian, S., Merighi, A., Wharton, J., Varndell, I.M., and Polak, J.M. (1986). Ultrastructural evidence for the coexistence of calcitonin gene-related peptide and substance P in secretory vesicles of peripheral nerves in the guinea pig. *J. Neurocytol.* 15, 535-542.

- Guttman, E., Guttman, L., Medawar, P.B., and Young, J.Z. (1942). The rate of regeneration of nerve. *J. Exp. Biol.* 19, 14-44.
- Haas, C.A., Streit, W.J., and Kreutzberg, G.W. (1990). Rat facial motoneurons express increased levels of calcitonin gene-related peptide mRNA in response to axotomy. *J. Neurosci. Res.* 27, 270-275.
- Habenicht, A. J. R., Glomset, J. A., King, W. C., Nist, C., Mitchell, C. D., and Ross, R. (1981) *J. Cell Biol.* 256, 12329-12335
- Hadcock, J. R., Ros, M., and Malbon, C.C. (1989). Agonist regulation of  $\beta$ -adrenergic receptor mRNA: analysis in S49 mouse lymphoma mutants. *J. Biol. Chem.* 264, 19928-19933.
- Hadcock, J.R. and Malbon, C.C. (1988). Down-regulation of  $\beta$ -adrenergic receptors: agonist-induced reduction in receptor mRNA levels. *Proc. Natl. Acad. Sci. USA* 85, 5021-5025.
- Haegerstrand, A., Dalsgaard, C.J., Jonzon, B., Larsson, O., and Nilsson, J. (1990). Calcitonin gene-related peptide stimulates proliferation of human endothelial cells. *Proc. Natl. Acad. Sci. USA* 87, 3299-3303.
- Haftck, J. and Thomas, P.K. (1968). Electron-microscopic observations on the effects of localised crush injuries on the connective tissues of peripheral nerve. *J. Anat.* 103, 233-243.
- Hall, A. and Self, A.J. (1986). The effect of  $Mg^{2+}$  on the guanine nucleotide exchange rate of p21N-ras. *J. Biol. Chem.* 261, 10963-10965.
- Hall, F., Braun, R., Carbonaro-Hall, D., and Vulliet, P. (1990). Evidence that proline directed protein kinase is a cytosolic growth factor sensitive form of p34cdc2. *J. Cell Biol.* 111, 341a.
- Hall, S.M. (1986b). The effect of inhibiting Schwann cell mitosis on the re-innervation of acellular autografts in the peripheral nervous system of the mouse. *Neuropathol. Appl. Neurobiol.* 12, 401-414.
- Han, S.-p., Naes, L., and Westfall, T.C. (1990). Inhibition of periarterial nerve stimulation induced vasodilation of the mesenteric arterial bed by CGRP (8-37) and CGRP receptor desensitization. *Biochem. Biophys. Res. Commun.* 168, 786-791.

Hannon, G.J., and Beach, D. (1994). p15<sup>INK4B</sup> is a potential effector of TGF $\beta$ -induced cell cycle arrest. *Nature* 371, 257-261.

Harden, T.K. (1983). Agonist-induced desensitization of the beta-adrenergic receptor-linked adenylate cyclase. *Pharmacol. Rev.* 35, 5-32.

Hardy, M., Reddy, U.R., and Pleasure, D. (1992). Platelet-Derived Growth factor and regulation of Schwann cell proliferation in vivo. *J. Neurosci. Res.* 31, 254-262.

Harper, J.W., Adami, N., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993). The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75, 805-816.

Hashimoto, K., Watanabe, Y., Uchida, S. and Yoshida, H. (1989). Increase in the amount of adenylate cyclase in rat gastrocnemius muscle after denervation. *Life-Sci.* 44(24), 1887-95.

Hausdorff, W.P., Bouvier, M., O'Dowd, B.F., Irons, G.P., Caron, M.G., and Lefkowitz, R.J. (1989). *J. Biol. Chem.* 264, 12657-12665.

Hausdorff, W.P., Caron, M.G., and Lefkowitz, R.J. (1990a). Turning off the signal: desensitization of  $\beta$ -adrenergic receptor function. *FASEB J.* 4, 2881-2889.

Hausdorff, W.P., Hnatowich, M., O'Dowd, B.F., Caron, M.G., and Lefkowitz, R.J. (1990b). A mutation of the  $\beta$ -adrenergic receptor impairs agonist activation of adenylate cyclase without affecting high affinity agonist binding. *J. Biol. Chem.* 265, 1388-1393.

Hayakawa, Y., Obata, K.I., Itoh, N., Yanaihara, N. and Okamoto, H. (1984). cAMP regulation of pro-VIP/PMH-27 synthesis in human neuroblastoma cells. *J. Biol. Chem.* 259, 9207-9211.

Hart, C.E., Forstrom, J.W., Kelly, J.D., Seifert, R.A., Smith, R.A., Ross, R., Murray, M.J., and Bowen-Pope, D.F. (1988). Two classes of PDGF receptor recognize different isoforms of PDGF. *Science* 240, 1529-1531.

He, X., Treacy, M.N., Simmons, D.M., Ingraham, H.A., Swanson, L.W., and Rosenfeld, M.G. (1989). Expression of a large family of POU-domain regulatory genes in mammalian brain development. *Nature, Lond.* 340, 35-42.

He, X., Gerrero, R., Simmons, D.M., Park, R.E., Lin, C.R., Swanson, L.W., and Rosenfeld, M.G. (1991). Tst-1, a member of the POU domain gene family, binds the promoter of the gene encoding the cell surface adhesion molecule Po. *Molec. cell. Biol.* 11, 1739-1744.

Heidaran, M.A., Yu, J.C., Jensen, R.A., Pierce, J.H., and Aaronson, S.A. (1992). A deletion in the extracellular domain of the alpha platelet-derived growth factor (PDGF) receptor differentially impairs PDGF-AA and PDGF-BB binding affinities. *J. Biol. Chem.* 267, 2884-2887.

Heilbrunn, S.M., Shah, P., and Bristow, M.R. (1989). Increased  $\beta$ -receptor density and improved haemodynamic response to catecholamine stimulation during long term metoprolol therapy in heart failure from dilated cardiomyopathy. *Circulation* 79, 483-490.

Heithier, H., Frohlich, M., Dees, C., Baumann, M., Haring, M., Gierschik, P., Schiltz, E., Vaz, W.L., Hekman, M., and Helmreich, E.J. (1992). Subunit interactions of GTP-binding proteins. *Eur. J. Biochem* 204, 1169-1181.

Heldin, N-E., Paulsson, Y., Forsberg, K., Heldin, C-H., and Westermark, B. (1989). *J. Cell Physiol.* 138:17-23.

Heldin, C.-H., Bäckström, G., Östman, A., Hammacher, A., Rönnstrand, L., Rubin, K., Nistér, M., and Westermark, B. (1988). Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. *EMBO J.* 7, 1387-1393.

Hempstead, B.L., Martin-Zanca, D., Kaplan, D.R., Parada, L.F., and Chao, M.V. (1991). High affinity NGF binding requires co-expression of the *trk* protooncogene and the low affinity NGF receptor. *Nature* 350, 678-683.

Hen, R., Axel, R., and Obibi, S. (1989). Activation of the  $\beta_2$  adrenergic receptor promotes growth and differentiation in thyroid cells. *Proc. Natl. Acad. Sci. USA* 86, 4785-4788.

Henderson, C.E., Camu, W., Mettling, C., et al., (1993). Neurotrophins promote motor neuron survival and are present in embryonic limb bud. *Nature* 363, 266-270.

Hengst, L., Dulic, V., Slingerland, J.M., Lees, E., and Reed, S.I. (1994). A cell cycle-regulated inhibitor of cyclin-dependent kinases. *Proc.Natl.Acad.Sci.USA*. *91*, 5291-5295.

Hess, K., Eames, R.A., Danveniza, P. and Gilliatt, R.W. (1979). Acute ischaemic neuropathy in the rabbit. *J.Neurol.Sci*. *44*, 19-43.

Heumann, R. (1987a). Regulation of the synthesis of nerve growth factor. *J. Exp. Biol.* *132*, 133-150.

Heumann, R., Lindholm, D., Bandtlow, C., Meyer, M., Radeke, M. J., Misko, T. P., Shooter, E., and Thoenen, H. (1987b). Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development, degeneration, and regeneration: role of macrophages. *Proc.Natl.Acad.Sci.USA* *84*, 8735-8739.

Heumann, R., Korsching, S., Bandtlow, C., and Thoenen, H. (1987). Changes of nerve growth factor synthesis in non neuronal cells in response to sciatic nerve transection. *J. Cell Biol.* *104*, 1623-1631.

Higashijima, T., Ferguson, K.M., Sternweis, P.C., Smigel, M.D., and Gilman, A.G. (1987a). Effects of  $Mg^{2+}$  and the beta gamma-subunit complex on the interactions of guanine nucleotides with G proteins. *J. Biol. Chem.* *262*, 762-766.

Higashijima, T., Ferguson, K.M., Smigel, M.D., and Gilman, A.G. (1987b). The effect of GTP and  $Mg^{2+}$  on the GTPase activity and the fluorescent properties of Go. *J. Biol. Chem.* *262*, 757-761.

Higashijima, T., Graziano, M.P., Suga, H., Kainosho, M., and Gilman, A.G. (1991).  $^{19}F$  and  $^{31}P$  NMR spectroscopy of G protein alpha subunits. Mechanism of activation by  $Al^{3+}$  and F-. *J. Biol. Chem.* *266*, 3396-3401.

Hill, T.D., Dean, N.M., Mordan, L.J., Lau, A.F., Kanemitsu, M.Y., and Boynton, A.L. (1990). PDGF-induced activation of phospholipase C is not required for induction of DNA synthesis. *Science* *248*, 1660-1663.

Hinds, P.W., Mitnacht, S., Dulic, V., Arnold, A., Reed, S.I., and Weinberg, R.A. (1992). Regulation of Retinoblastoma protein functions by ectopic expression of human cyclins. *Cell*. *70*, 993-1006.



Hiroshima, O., Sano, Y., Yuzuriha, T., Yamato, C., Saito, A., Okamura, N., Uchiyama, Y., Kimura, S., and Goto, K. (1988). Solubilization and characterization of calcitonin gene-related peptide binding site from porcine spinal cord. *J. Neurochem.* *50*, 480-485.

Hokfelt, T., Wiesenfeld-Hallin, Z., Villar, M., and Melander, T. (1987). Increase in galanin-like immunoreactivity in rat dorsal root ganglion cells after peripheral axotomy. *Neurosci. Lett.* *83*, 217-220.

Holbrook, S.R. and Kim, S.-H. (1989). Molecular model of the G protein  $\alpha$  subunit based on the crystal structure of the HRAS protein. *Proc. Natl. Acad. Sci. USA* *86*, 1751-1755.

Holmes, W.E., et al., (1992). *Science*. *256*, 1205-1210.

Horn, E.M. and Bilezikian, J.P. (1990). Mechanisms of abnormal transmembrane signaling of the  $\beta$ -adrenergic receptor in congestive heart failure. *Circulation* *82(suppl.1)*, I-26-I-34.

Hughes, D. A., Ashworth, A., and Marshall, C. J. (1993) Complementation of *byr1* in fission yeast by mammalian MAP kinase kinase requires coexpression of Raf kinase. *Nature* *364*, 349-352.

Hunter, T. (1995). Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signalling. *Cell* *80*, 225-236.

Ip, N.Y., Li, Y.P., van de Stadt, I., Panayotatos, N., Alderson, R.F., and Lindsay, R.M. (1991). Ciliary neurotrophic factor enhances neuronal survival in embryonic rat hippocampal cultures. *J. Neurosci.* *11*, 3124-3134.

Ip, N.Y., Ibanez, C.F., Nye, S.H., McClain, J., Jones, P.F., Gies, D.R., Belluscio, L., LeBeau, M.M., Espinosa, R., Squinto, S.P., Persson, H., and Yancopoulos, G.D. (1992). Mammalian neurotrophin-4: structure, chromosomal localisation, tissue distribution, and receptor specificity. *Proc. Natl. Acad. Sci. USA* *89*, 3060-3064.

Ishikawa, T., Okamura, N., Saito, A., and Goto, K. (1987). Effects of calcitonin gene-related peptide (CGRP) and isoproterenol on the contractility and adenylate cyclase activity in the rat heart. *J. Mol. Cell Cardiol.* *19*, 723- 727.

Jamal, S. and Ziff, E. (1990). Transactivation of c-fos and  $\beta$ -Actin genes by Raf as a step in early response to transmembrane signals. *Nature, Lond.* 344, 463-466.

Johnson, D.G., Schwartz, J.K., Cress, W.D., and Nevins, J.R. (1993). Expression of E2F1 induces quiescent cells to enter S phase. *Nature* 365, 349-352.

Johnson, G.L., Wolfe, B.B., Harden, T.K., Molinoff, P.B. and Perkins, J.P. (1978). Role of  $\beta$ -adrenergic receptors in catecholamine induced desensitisation of adenylate cyclase in human astrocytoma cells. *J.Biol.Chem.* 253:1472-1480.

Jones, S.M., Hunt, N.A., Del Monte, F., and Harding, S.E. (1990). Contraction of cardiac myocytes from noradrenaline treated rats in response to isoprenaline, forskolin, and dibutyryl cAMP. *Eur. J. Pharmacol.* 129, 140.

Kafiluddi, R., Kennedy, R.H., and Seifen, E. (1989). Effects of buffer magnesium on positive inotropic agents in guinea pig cardiac muscle. *Eur. J. Pharmacol.* 165, 181-189.

Kaplan, D.R., Morrison, D.K., Wong, G., McCormick, F., and Williams, L.T. (1990). PDGF beta-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signalling complex. *Cell* 61, 125-133.

Karliner, J.S., Simpson, P.C., Honbo, N., and Woloszyn, W. (1986). Mechanisms and timecourse of beta1 adrenoceptor desensitisation in mammalian cardiac myocytes. *Cardiovasc. Res.* 20, 221-228.

Karliner, J.S. and Scheinman, M. (1988). Adenylate cyclase activity coupled to the stimulatory guanine nucleotide binding protein in patients having electrophysiological studies and either structurally normal hearts or idiopathic myocardial disease. *Am. J. Cardiol.* 62, 1129-1130.

Kashihara, Y., Sakaguchi, M., Kuno, M.S.O.J., and 9(11): 3796, P.Y. (1989). Axonal transport and distribution of endogenous calcitonin gene-related peptide in rat peripheral nerve. *J. Neurosci.* 9(11), 3796-3802.

Kashishian, A., Kazlauskas, A., and Cooper, J.A. (1992). Phosphorylation sites in the PDGF receptor with different specificities for binding GAP and PI3 kinase in vivo. *EMBO J.* 11, 1373-1382.

Kassis, S. and Fischman, P.H. (1984). Functional alteration of the  $\beta$ -adrenergic receptor during desensitization of mammalian adenylyl cyclase by  $\beta$ -agonist. *Proc. Natl. Acad. Sci. USA* 6686-6690.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R.W. (1991). Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.* 51, 6304-6311.

Kastan, M.B., et al., (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD 45 is defective in ataxia-telangiectasia. *Cell* 71, 587-597.

Kato, J., Matsushime, H., Hiebert, S.W., Ewen, M.E., and Sherr, C.J. (1993). Direct binding of cyclin D to the retinoblastoma gene product(pRb) and pRb phosphorylation by the cyclin D dependent kinase CDK4. *Genes Dev.* 7, 331-342.

Katz, A.M. (1979). Role of the contractile proteins and sarcoplasmic reticulum in the response of the heart to catecholamines: An historical perspective. *Adv. Cyclic Nucleotide Res.* 11, 303-343.

Kaumann, A.J., Lemoine, H., Morris, T.H., and Schwederski, U. (1982). An initial characterisation of human heart  $\beta$ -adrenoceptors and their mediation of the positive inotropic effects of catecholamines. *Naunyn-Schmiedebergs Arch. Pharmacol.* 319, 216-221.

Kaumann, A.J., Morris, T.H., and Bojar, H. (1983). Heart  $\beta$ -receptors. On the functional role of heterogeneous binding sites. *J. Receptor Res.* 3, 61-70.

Kaumann, A.J., Murray, K.J., Hall, J.A., and et al, (1988). Stimulation of cAMP-dependant protein kinase through both  $\beta$ 1 and  $\beta$ 2 adrenoceptors in human atrial myocardium. *Br. J. Pharmacol.* 95, 565p.

Kaumann, A.J., Lemoine, H., Schwederski-Menke, U., and Ehle, B. (1989). Relations between  $\beta$ -adrenoceptor occupancy and increases in contractile force and adenylate cyclase activity induced by catecholamines in human ventricular myocardium. Acute desensitization and comparison with feline ventricle. *Naunyn-Schmiedebergs Arch. Pharmacol.* 339, 99-112.

Kaumann, A.J. and Birnbaumer, L. (1974). Characteristics of the adrenergic receptor coupled to myocardial adenylate cyclase. Stereospecificity for ligands and determination of affinity constants. *J. Biol. Chem.* 249, 7874-7885.

Kaumann, A.J. and Birnbaumer, L. (1976). Desensitization of kitten atria to chronotropic, inotropic and adenylate cyclase stimulating effect of (-)-isoprenaline. *Naunyn- Schmiedebergs Arch. Pharmacol.* 293, 199-202.

Kaumann, A.J. and Lemoine, H. (1987).  $\beta$ - adrenoceptor mediated positive inotropic effect of adrenaline in human ventricular myocardium: Quantitive discrepancies with binding and adenylate cyclase stimulation. *Naunyn- Schmiedebergs Arch. Pharmacol.* 335, 403-411.

Kaumann, A.J. and Lobnig, B.M. (1986). Mode of action of (-)-pindolol on feline and human myocardium. *Br. J. Pharmacol.* 89, 207-218.

Kawasaki, H., Takasaki, A., Saito, A., and Goto, K. (1988). Calcitonin gene-related peptide acts as a novel vasodilator neurotransmitter in mesenteric resistance vessels of the rat. *Nature* 335, 164-167.

Kazlauskas, A., Bowen Pope, D., Seifert, R., Hart, C.E., and Cooper, J.A. (1988). Different effects of homo- and heterodimers of platelet-derived growth factor A and B chains on human and mouse fibroblasts. *EMBO J.* 7, 3727-3735.

Kazlauskas, A., Ellis, C., Pawson, T., and Cooper, J.A. (1990). Binding of GAP to activated PDGF receptors. *Science* 247, 1578-1581.

Kazlauskas, A., Kashishian, A., Cooper, J.A., and Valius, M. (1992). GTPase-activating protein and phosphatidylinositol 3-kinase bind to distinct regions of the platelet-derived growth factor receptor beta subunit. *Mol. Cell Biol.* 12, 2534-2544.

Kenny, A. J. and Bourne, A. (1991). Cellular reorganization of membrane peptidases in Wallerian degeneration of pig peripheral nerve. *J.Neurocytol.* 20:875-885

Keyomarsi, K., and Pardee, A.B. (1993). Redundant cyclin overexpression and gene amplification in breast cancer cells. *Proc. Natl. Acad. Sci. USA.* 90, 1112-1116.

Kiefer, R. and Kreutzberg, G.W. (1990). Gamma interferon-like immunoreactivity in the rat nervous system. *Neuroscience* 37, 725-734.

Kim, H.K., Kim, J.W., Zilberstein, A., Margolis, B., Kim, J.G., Schlessinger, J., and Rhee, S.G. (1991). PDGF stimulation of inositol phospholipid hydrolysis requires PLC- $\tau$ 1 phosphorylation on tyrosine residues 783 and 1254. *Cell* 65, 435-441.

Kimura, H., Fischer, W.H., and Schubert, D. (1990). Structure, expression and function of a schwannoma-derived growth factor. *Nature,Lond.* 348, 257-260.

Kioussi,C., and Matsas,R.(1991) *J.Neurochem.* 57:431-440. Endopeptidase 24.11,a cell surface peptidase of central nervous sytem neurons, is expressed by Schwann cells in the pig nervous sytem.

Kioussi, C., Crine, P., and Matsas, R. (1992). Endopeptidase-24.11 is suppressed in myelin-forming but not in non-myelin-forming Schwann cells during development of the rat sciatic nerve. *Neuroscience* 50, 69-83.

Kioussi, C. and Matsas, R. (1991). Endopeptidase-24.11, a cell-surface peptidase of central nervous system neurons, is expressed by Schwann cells in the pig peripheral nervous system. *J. Neurochem.* 57, 431-440.

Klein, R., Parada, L.F., Coulier, F., and Barbacid, M. (1989). *trkB*, a novel tyrosine protein kinase receptor expressed during mouse neural development. *EMBO J.* 8, 3701-3709.

Klippel, A., Escobedo, J.A., Fantl, W.J., and Williams, L.T. (1992). The C-terminal SH2 domain of p85 accounts for the high affinity and specificity of the binding of phosphatidylinositol 3-kinase to phosphorylated platelet-derived growth factor beta receptor. *Mol. Cell Biol.* 12, 1451-1459.

Knudsen, P.J., Dinarello, C.A., and Strom, T.B. (1986). Prostaglandins posttranscriptionally inhibit monocyte expression of interleukin 1 activity by increasing intracellular cyclic adenosine monophosphate. *J. Immunol.* 137, 3189-3194.

Kobilka, B.K., Kobilka, T.S., Daniel, K., Regan, J.W., Caron, M.G., and Lefkowitz, R.J. (1988). Chimeric  $\alpha 2$ - $\beta$ - adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. *Science* 240, 1310-1316.

Koff, A., Cross, F., Fisher, A., Schumacher, J., Phillipe, M., and Roberts, J.M. (1993). Negative regulation of G1 in mammalian cells: inhibition of cyclin E dependant kinase by TGF-beta. *Science.* 260, 536-539.

Kohase, M., May, L.T., Tamm, I., Vilcek, J., and Sehgal, P.B. (1987). . *Mol. Cell Biol.* 7, 273-280.

- Kolch, W., Heidecker, G., Lloyd, P., and Rapp, U.R. (1991). *Nature* 349, 426-428.
- Korsching, S. and Thoenen, H. (1983). Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: correlation with density of sympathetic innervation. *Proc. Natl. Acad. Sci. USA* 80, 3513-3516.
- Kovacina, K., Yonezawa, K., Brautigan, D., Tonks, N., Rapp, U., and Roth, R. (1990). Insulin activates the kinase activity of the Raf-1 proto-oncogene by increasing its serine phosphorylation. *J. Biol. Chem.* 265, 12115-12118.
- Kozasa, T., Itoh, H., Tsukamoto, T., and Kaziro, Y. (1988). Isolation and characterisation of the human Gs $\alpha$  gene. *Proc. Natl. Acad. Sci. USA* 85, 2081-2085.
- Kubo, T., Bujo, H., Akiba, I., Nakai, J., Mishina, M., and Numa, S. (1988). Location of a region of the muscarinic acetylcholine receptor involved in selective effector coupling. *FEBS Lett.* 241, 119-125.
- Kuerbitz, S.J., Plunkett, B.S., Walsh, W.V., and Kastan, M.B. (1992). Wild type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natl. Acad. Sci. USA* 89, 7491-7495.
- Kuhn, R., Monuki, E.S., and Lemke, G. (1991). The gene encoding the transcription factor SCIP has features of an expressed retroposon. *Mol. Cell. Biol.* 11, 4642-4650.
- Kunkel, M.W., Friedman, J., Shenolikar, S., and Clark, R.B. (1989). . *FASEB J.* 3, 2067-2074.
- Kyle, R.A. (1992). Monoclonal proteins in neuropathy. *Neurol. Clin.* 10, 713-734.
- Laiho, M., DeCapiro, J., Ludlow, J., Livingston, D., and Massague, J. (1990). Growth inhibition by TGF- $\beta$  linked to suppression of retinoblastoma protein phosphorylation. *Cell* 62, 175-185.
- Lambertz, H., Meyer, J., and Erbel, R. (1984). Long-term effects of prenalterol in patients with severe congestive heart failure. *Circulation* 69, 298-305.
- Lamph, W.W., Dwarki, V.J., Ofir, R., Montminy, M., and Verma, I.M. (1990). Negative and positive regulation by transcription factor cAMP response element-binding protein is modulated by phosphorylation. *Proc. Natl. Acad. Sci. USA* 87, 4320-4324.

Landis, C.A., Masters, S.B., Spada, A., Pace, A.M., Bourne, H.R., and Vallar, L. (1989). GTPase inhibiting mutations activate the  $\alpha$  chain of Gs and stimulate adenylyl cyclase in human pituitary tumours. *Nature* **340**, 692-696.

Lappin, D. and Whaley, K. (1989). Regulation of C1-inhibitor synthesis by interferons and other agents. *Behring. Inst. Mitt.* 180-192.

Laufer, R. and Changeux, J-P. (1987). Calcitonin gene-related peptide elevates cyclic AMP levels in chick skeletal muscle: possible neurotrophic role for a coexisting neuronal messenger. *EMBO J.* **6**, 901-906.

Le Beau, J.M., LaCorbiere, M., Powell, H.C., Ellisman, M.H., and Schubert, D. (1988). Extracellular fluid conditioned during peripheral nerve regeneration stimulates Schwann cell adhesion, migration and proliferation. *Brain Res.* **459**, 93-104.

LeBlanc, A.C., Poduslo, J.F. and Mezei, C. (1987). Gene expression in the presence or absence of myelin assembly. *Mol. Brain Res.* **2**:57-67.

Lee, E., Taussig, R., and Gilman, A.G. (1992). The G226A mutant of Gs alpha highlights the requirement for dissociation of G protein subunits. *J. Biol. Chem.* **267**, 1212-1218.

Lehtola, L., Nister, M., Holttä, E., Westermarck, B., and Alitalo, K. (1991). Down-regulation of cellular platelet-derived growth factor receptors induced by an activated neu receptor tyrosine kinase. *Cell Regul.* **2**, 651-661.

Lefkowitz, R.J., Caron, M.G., and Stiles, G.L. (1984). Mechanism of membrane-receptor regulation. Biochemical, physiological, and clinical insights derived from studies of the adrenergic receptors. *N. Engl. J. Med.* **310**, 1570- 1579.

Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengeler, B., Masiakowski, P., Thoenen, H., and Barde, Y. -A. (1989). Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* **341**, 149-152.

Lemke, G. and Chao, M. (1988). Axons regulate Schwann cell expression of the major myelin and NGF receptor genes. *Development* **102**, 499-504.

Lemoine, H., Schonell, H., and Kaumann, A.J. (1988). Contribution of  $\beta 1$  and  $\beta 2$ -adrenoceptors of human atrium and ventricle to the effects of noradrenaline and adrenaline as assessed with (-)-atenolol. *Br. J. Pharmacol.* 95, 55-66.

Libby, P., Ordovas, J.M., Auger, K.R., Robbins, A.H., Birinyi, L.K., and Dinarello, C.A. (1986a). Endotoxin and tumor necrosis factor induce interleukin-1 gene expression in adult human vascular endothelial cells. *Am. J. Pathol.* 124, 179-185.

Libby, P., Ordovas, J.M., Birinyi, L.K., Auger, K.R., and Dinarello, C.A. (1986b). Inducible interleukin-1 gene expression in human vascular smooth muscle cells. *J. Clin. Invest.* 78, 1432-1438.

Liggett, S. B., Bouvier, M., Hausdorff, W.P., O'Dowd, B., Caron, M.G. and Lefkowitz, R.J.(1989). Altered patterns of agonist-stimulated cAMP accumulation in cells expressing mutant beta 2-adrenergic receptors lacking phosphorylation sites. *Mol.Pharmacol.* 36(4): 641-6.

Liggett, S.B., Bouvier, M., O'Dowd, B.F., Caron, M.G., Lefkowitz, R.J., and DeBlasi, A. (1989). Substitution of an extracellular cysteine in the  $\beta 2$ -adrenergic receptor enhances agonist-promoted phosphorylation and receptor desensitization. *Biochem. Biophys. Res. Commun.* 1, 257-263.

Limas, C.J., Goldenberg, I.F., and Limas, C. (1990). Influence of anti-beta-receptor antibodies on cardiac adenylate cyclase in patients with idiopathic dilated cardiomyopathy. *Am. Heart J.* 119, 1322-1328.

Limas, C.J., Goldenberg, I.F., and Limas, C. (1991). Effect of antireceptor antibodies in dilated cardiomyopathy on the cycling of cardiac beta receptors. *Am. Heart J.* 122), 108-114.

Lin, L.F., Mismer, D., Lile, J.D., Armes, L.G., Butler, E.T., Vannice, J.L., and Collins, F. (1989). Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). *Science* 246, 1023-1025.

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



Lindholm, D., Heumann, R., and Thoenen, H. (1988). Interleukin 1 increases stability and transcription of mRNA encoding nerve growth factor in cultured rat fibroblasts. *J. Biol. Chem.* 263, 16348-16351.

Lisak, R.P., Sobue, G., Kuchmy, D., Burns, J.B., and Pleasure, D.E. (1985). Products of activated lymphocytes stimulate Schwann cell mitosis in vitro. *Neurosci. Lett.* 57, 105-111.

Lisak, R.P. and Bealmear, B. (1991). Antibodies to interleukin-1 inhibit cytokine-induced proliferation of neonatal rat Schwann cells in vitro. *J. Neuroimmunol.* 31, 123-132.

Litosch, I. (1991). G protein regulation of phospholipase C activity in a membrane-solubilized system occurs through a Mg<sup>2+</sup>(+)- and time-dependent mechanism. *J. Biol. Chem.* 266, 4764-4771.

Lohse, M.J., Lefkowitz, R.J., Caron, M.G., and Benovic, J.L. (1989). Inhibition of  $\beta$ -adrenergic receptor kinase prevents rapid homologous desensitization of  $\beta$ -adrenergic receptors. *Proc. Natl. Acad. Sci. USA* 86, 3011-3015.

Lohse, M.J., Benovic, J.L., Caron, M.G., and Lefkowitz, R.J. (1990). Multiple pathways of rapid beta 2-adrenergic receptor desensitization. Delineation with specific inhibitors. *J. Biol. Chem.* 265, 3202-3211.

Lohse, M.J., Andexinger, S., Pitcher, J., Trukawinski, S., Codina, J., Faure, J.P., Caron, M.G., and Lefkowitz, R.J. (1992). Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of beta-arrestin and arrestin in the beta 2-adrenergic receptor and rhodopsin systems. *J. Biol. Chem.* 267, 8558-8564.

Ludlow, J., Shon, J., Pipas, J., Livingston, D., and DeCapiro, J. (1990). The retinoblastoma susceptibility gene product undergoes cell-cycle dependant dephosphorylation and binding to and release from SV40 large T. *Cell* 60, 387-396.

Lunn, E.R., Perry, V.H., Brown, M.C., Rosen, H., and Gordon, S. (1989). Absence of Wallerian degeneration does not hinder regeneration in peripheral nerve. *Eur. J. Neurosci.* 1, 27-33.

Lunn, E.R., Brown, M.C., and Perry, V.H. (1990). The pattern of axonal degeneration in the peripheral nervous system varies with different types of lesion. *Neurosci.* 35, 157-165.

Maehlen, J., Olsson, T., Zachau, A., Klareskog, L., and Kristensson, K. (1989). Local enhancement of major histocompatibility complex(MHC) class I and II expression and cell infiltration in experimental allergic encephalomyelitis around axotomized motor neurons. *J. Neuroimmunol.* 23, 125-132.

Maguire, M.E. (1988). Magnesium and cell proliferation. *Ann. N. Y. Acad. Sci.* 551, 201-215.

Maisel, A.S., Wright, C.M., Carter, S.M., Ziegler, M., and Motulsky, H.J. (1989b). Tachyphylaxis with amrinone therapy: Association with sequestration and down-regulation of lymphocyte beta-adrenergic receptors. *Ann. Intern. Med.* 110, 195-201.

Maisonpierre, P.C., Belluscio, L., Squinto, S., Ip, N.Y., Furth, M.E., Lindsay, R.M., and Yancopoulos, G.D. (1990). Neurotrophin-3: a new neurotrophic factor related NGF and BDNF. *Science* 247, 1446-1451.

Mancini, D., Frey, M.J., Fishberg, D., Molinoff, P., and Wilson, J.R. (1989). Characterization of lymphocyte beta-adrenergic receptors at rest and during exercise in ambulatory patients with chronic congestive cardiac failure. *Am. J. Cardiol.* 63, 307-312.

Marchionni, M.A., Goodearl, A.D.J., Chen, M.S., et al. (1993). Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature.* 362, 312-318

Malkin, D., et al., (1990). Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas and other neoplasms. *Science* 250, 1233-1238.

Martin, G.A., Viskochil, D., Bollag, G., McCabe, P.C., Crosier, W.J., Haubruck, H., Conroy, L., Clark, R., O'Connell, P., Cawthorne, R.M., Innis, M.A., and McCormick, F. (1990). The GAP-related domain of the NF-1 gene product interacts with ras p21. *Cell* 63, 843-849.

Martinet, Y., Bitterman, P.B., Mornex, J.-F., Grotendorst, G.R., Martin, G.R., and Crystal, R.G. (1986). Activated human monocytes express the c-sis proto-oncogene and release a mediator showing PDGF-like activity. *Nature,Lond.* 319, 158-160.

Martin-Zanca, D., Barbacid, M., and Parada, L.F. (1990). Expression of the *trk* protooncogene is restricted to sensory cranial and spinal ganglia of neural crest origin in mouse development. *Genes Dev.* 4, 683-694.

Mathew, J., Date, S., and Eichberg, J. (1992). Activity and distribution of phosphoinositidase C in rat sciatic nerve. *J. Neurosci. Res.* 33, 122-128.

Matsas, R., Kenny, A.J. and Turner, A. (1984). The metabolism of neuropeptides. The hydrolysis of peptides, including enkephalins, tachykinins and their analogues, by endopeptidase 24.11. *Biochem.J.* 223:433-440.

Matsas, R., Kenny, A.J., and Turner, A.J. (1986). An immunohistochemical study of endopeptidase-24.11 ("enkephalinase") in the pig nervous system. *Neurosci.* 4:991-1012

Matsuda, H., Kannan, Y., Ushio, H., Kiso, Y., Kanemoto, T., Suzuki, H., and Kitamura, Y. (1991). Nerve growth factor induces development of connective tissue-type mast cells in vitro from murine bone marrow cells. *J. Exp. Med.* 174, 7-14.

Matsui, T., Heidaran, M., Miki, T., Popescu, N., La Rochelle, W., Kraus, M., Pierce, J., and Aaronson, S. (1989). Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. *Science* 243, 800-804.

Matsuoka, I., Meyer, M., and Thoenen, H. (1991a). Cell-type-specific regulation of nerve growth factor (NGF) synthesis in non-neuronal cells: comparison of Schwann cells with other cell types. *J. Neurosci.* 11, 3165-3177.

Matsuoka, I., Meyer, M., and Thoenen, H. (1991b). Cell-type specific regulation of NGF synthesis in non-neuronal cells: comparison of Schwann cells with other cell types. *J. Neurosci.* 11(10), 3165-3177.

Matsushime, H., Roussel, M.F., Ashmun, R.A., and Sherr, C.J. (1991). CSF 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell.* 65, 701-713.

Matsushime, H., Ewen, M.E., Strom, D.K., Kato, J.Y., Hanks, S.K., Roussel, M.F. and Sherr, C.J. (1992). Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins. *Cell.* 71, 323-334.

Mattera, R., Graziano, M.P., Yatani, A., Graf, R., Codina, J., Gilman, A.G., Birnbaumer, L., and Brown, A.M. (1989). Bacterially synthesised splice variants of the  $\alpha$  subunit of the G protein Gs activate both adenylyl cyclase and dihydropyridine sensitive calcium channels. *Science* 243, 804-807.

Mattera, R., Graziano, M.P., Yatani, A., Graf, R., Codina, J., Gilman, A.G., Birnbaumer, L., and Brown, A.M. (1989). Bacterially synthesized splice variants of the  $\alpha$  subunit of the G protein Gs activate both adenylate cyclase and the dihydropyridine sensitive calcium channels. *Science* 243, 804-807.

Mauro, A., Bulfone, A., Turco, E., and Schiffer, D. (1991). Coexpression of platelet-derived growth factor (PDGF) B chain and PDGF B-type receptor in human gliomas. *Childs. Nerv. Syst.* 7, 432-436.

McGarvey, M.L., Baron VanEvercooren, A., Kleinmann, H.K. and Dubois-Dalq, M. (1984). Synthesis and effects of basement membrane components in cultured rat schwann cells. *Dev.biol.* 105:18-28

McGlade, C.J., Ellis, C., Reedijk, M., Anderson, D., Mbamalu, G., Reith, A.D., Panayotou, G., End, P., Bernstein, A., Kazlauskas, A., and et al, (1992). SH2 domains of the p85 alpha subunit of phosphatidylinositol 3-kinase regulate binding to growth factor receptors. *Mol. Cell Biol.* 12, 991-997.

McMahon, S.B., Armanini, M.P., Ling, L.H., and Phillips, H.S. (1994). *Neuron.* 12, 1161-1171.

Meakin, S.O., and Shooter, E.M. (1992). *Trends Neurosci.* 15, 323-331.

Mihara, K., Cao, X., Yen, A., Chandler, S., Driscoll, B., Murphree, A., T'Ang, A., and Fung, Y. (1989). Cell cycle dependant regulation of phosphorylation of the human retinoblastoma gene product. *Science* 246, 1300-1303.

Miyamoto, Y., Higaki, T., Sugita, T., Ikuta, Y., and Tsuge, K. (1986). Morphological reaction of cellular elements and the endoneurium following nerve section. *Peripheral Nerve Regeneration Repair* 3, 7-18.

Miles, K., Greengard, P. and Huganir, R.L. (1989). *Neuron.* 2(5):1517-24. Calcitonin gene-related peptide regulates phosphorylation of the nicotinic acetylcholine receptor in rat myotubes.

Miller, J.L., Litman, B.J., and Dratz, E.A. (1987). Binding and activation of rod outer segment phosphodiesterase and guanosine triphosphate binding protein by disc membranes: influence of reassociation method and divalent cations. *Biochim. Biophys. Acta* 898, 81-89.

Minnemann, K.P., Pittman, R.N., and Molinoff, P.B. (1981).  $\beta$ -adrenergic receptor subtypes: properties, distribution and regulation. *Ann. Rev. Neurosci.* 4, 419-461.

Mirsky, R., Dubois, C., Morgan, L., and Jessen, K. R. (1990). O4 and A007- sulfatide antibodies bind to embryonic Schwann cells prior to the appearance of galactocerebroside; regulation of the antigen by axon-Schwann cell signals and cyclic AMP. *Development* 109, 105-116.

Miyauchi, T., Sano, Y., Hiroshima, O., Yuzuriha, T., Sugishita, Y., Ishikawa, T., Saito, S., and Goto, K. (1988). Positive inotropic effects and receptors of CGRP in porcine ventricular muscle. *Biochem. Biophys. Res. Commun.* 155, 289-294.

Molloy, C.J., Fleming, T.P., Bottaro, D.P., Cuadrado, A., and Aaronson, S.A. (1992). Platelet-derived growth factor stimulation of GTPase-activating protein tyrosine phosphorylation in control and c-H-ras-expressing NIH 3T3 cells correlates with p21ras activation. *Mol. Cell Biol.* 12, 3903-3909.

Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G., and Goodman, R.H. (1986). Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc. Natl. Acad. Sci. U. S. A.* 83, 6682-6686.

Monuki, E.S., Weinmaster, G., Kuhn, R., and Lemke, G. (1989). SCIP: a glial POU domain gene regulated by cyclic AMP. *Neuron* 3, 783-793.

Monuki, E.S., Kuhn, R., Weinmaster, G., Trapp, B.D., and Lemke, G. (1990). Expression and activity of the POU transcription factor SCIP. *Science* 249, 1300-1303.

Moore, B.W. (1965). *Biochemical and Biophysical Research Communications* 6, 739-744. A soluble protein characteristic of the nervous system.

Moore, B.W. (1982). Chemistry and biology of the S100 protein. *Scandinavian J. Immunol.* 15 (suppl.9) 53-74.

Morgan, L., Jessen, K.R., and Mirsky, R. (1991). The effects of cAMP on differentiation of cultured Schwann cells: progression from an early phenotype (04+) to a myelin phenotype (Po+, GFAP-, N-CAM-, NGF-receptor-) depends on growth inhibition. *J. Cell Biol.* 112, 457-467.

Mornex, J.F., Martinet, Y., Yamauchi, K., Bitterman, P.B., Grotendorst, G.R., Chytil Weir, A., Martin, G.R., and Crystal, R.G. (1986). Spontaneous expression of the c-sis gene and release of a platelet-derived growth factorlike molecule by human alveolar macrophages. *J. Clin. Invest.* 78, 61-66.

Morrison, D., Kaplan, D., Escobedo, J., Rapp, U., Roberts, T., and Williams, L. (1989). Direct activation of the serine/threonine kinase activity of Raf-1 through tyrosine phosphorylation by the PDGF- $\beta$ -receptor. *Cell* 58, 649-657.

Motokura, T., Bloom, T., Kim, H.G., Juppner, H., Ruderman, J.V., Kronenberg, H.M. and Arnold, A. (1991). A novel cyclin encoded by a bcl-linked candidate oncogene. *Nature*. 350, 512-515.

Motokura, T., and Arnold, A., (1993). Cyclin D and oncogenesis. *Curr. Opin. Genet. Dev.* 3, 5-10.

Muir, D., Gennrich, C., Varon, S., and Manthorpe, M. (1989). Rat sciatic nerve Schwann cell microcultures: responses to mitogens and production of trophic and neurite-promoting factors. *Neurochem. Res.* 14, 1003-1012.

Muir, D., Varon, S., and Manthorpe, M. (1990). Schwann cell proliferation in vitro is under negative autocrine control. *J. Cell Biol.* 111, 2663-2671.

Muir, D., and Manthorpe, M. (1992). Stromelysin generates a fibronectin fragment that inhibits Schwann cell proliferation. *J. Cell Biol.* 116, 177-185.

Murphree, S.S. and Saffitz, J.E. (1989). Distribution of beta-adrenergic receptors in failing human myocardium: Implications for mechanisms of downregulation. *Circulation* 79, 1214-1225.

Murphy, P.R., Myal, Y., Sato, Y., Sato, R., West, M., and Friesen, H.G. (1989). Elevated expression of basic fibroblast growth factor messenger ribonucleic acid in acoustic neuromas. *Mol. Endocrinol.* 3, 225-231.

Murray, A. and Kirschner, M. (1989a). cyclin synthesis drives the early embryonic cell cycle. *Nature, Lond.* 339, 275-280.

Murray, A. and Kirschner, M. (1989b). Dominoes and clocks: the union of two views of the cell cycle. *Science* 246, 614-621.

Negro, A., Tolosano, E., Skaper, S.D., Martini, I., Callegaro, L., Silengo, L., Fiorini, F., and Altruda, F. (1991). Cloning and expression of human ciliary neurotrophic factor. *Eur. J. Biochem.* 201, 289-294.

Nennesmo, I. and Reinholt, F. (1986). Mast cells in nerve end neuromas of mice. *Neurosci. Lett.* 69, 296-301.

Neve, K.A. and Molinoff, P.B. (1986). Turnover of  $\beta 1$  and  $\beta 2$ -adrenergic receptors after down-regulation or irreversible blockade. *Mol. Pharmacol.* 30, 104-111.

New, H. and Mudge, A. (1986). Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. *Nature, Lond.* 323, 809-811.

Ng, L.L., Garrido, M.C., Davies, J.E., Brochwicz Lewinski, M.J., and Tan, L.B. (1992). Intracellular free magnesium in lymphocytes from patients with congestive cardiac failure treated with loop diuretics with and without amiloride. *Br. J. Clin. Pharmacol.* 33, 329-332.

Nielsen, U. and Keen, P. (1989). Reciprocal regulation of tachykinin- and vasoactive intestinal peptide-gene expression in rat sensory neurons following cut and crush injuries. *Brain Res.* 481, 25-30.

Nikitin, A.Y., Ballering, L.A., Lyons, J., and Rajewsky, M.F. (1991). Early mutation of the neu (erbB-2) gene during ethylnitrosourea-induced oncogenesis in the rat Schwann cell lineage. *Proc. Natl. Acad. Sci. USA.* 88, 9939-9943.

Nishizawa, N., Okano, Y., Chatani, Y., Amano, F., Tanaka, E., Nomoto, H., Nozawa, Y., and Kohno, M. (1990). Mitogenic signaling pathways of growth factors can be distinguished by the involvement of pertussis toxin-sensitive guanosine triphosphate-binding protein and of protein kinase C. *Cell Regul.* 1, 747-761.

Nister, M., Hammacher, A., Mellstrom, K., Siegbahn, A., Ronnstrand, L., Westermarck, B., and Heldin, C.H. (1988). A glioma-derived PDGF A chain

homodimer has different functional activities from a PDGF AB heterodimer purified from human platelets. *Cell* 52, 791-799.

Nishizuka, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* 334:661-665.

Noguchi, K., Senba, E., Morita, Y., Sato, M., and Tohyama, M. (1989). Prepro-VIP and preprotachykinin mRNAs in the rat dorsal root ganglion cells following peripheral axotomy. *Brain Res. Mol. Brain Res.* 6, 327-330.

Noguchi, K., Senba, E., Morita, Y., Sato, M., and Tohyama, M. (1990). Alpha-CGRP and beta-CGRP mRNAs are differentially regulated in the rat spinal cord and dorsal root ganglion. *Brain Res. Mol. Brain Res.* 7, 299-304.

Noguchi, M., Taniya, T., Koyasaki, N., Kumaki, T., Miyazaki, I., and Mizukami, Y. (1991). Effects of the prostaglandin synthetase inhibitor indomethacin on tumorigenesis, tumor proliferation, cell kinetics, and receptor contents of 7,12-dimethylbenz(a)anthracene-induced mammary carcinoma in Sprague-Dawley rats fed a high- or low-fat diet. *Cancer Res.* 51, 2683-2689.

Northup, J.K., Sternweiss, P.C., and Gilman, A.G. (1983). The subunits of the stimulatory regulatory component of adenylate cyclase. Resolution, activity and properties of the 35,00-dalton  $\beta$  subunit. *J. Biol. Chem.* 258, 11361-11368.

O'Dowd, B.F., Hnatowich, M., Regan, J.W., Leader, W.M., Caron, M.G., and Lefkowitz, R.J. (1988). Site directed mutagenesis of the cytoplasmic domains of the human  $\beta_2$ -adrenergic receptor. *J. Biol. Chem.* 263, 15985-15992.

Okamoto, K., Okazawa, H., Okuda, A., Sakai, M., Muramatsu, M., and Hamada, H. (1990). A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. *Cell* 60, 461-472.

Okamoto, T., Murayama, Y., Hayashi, Y., Inagaki, M., Ogata, E., and Nishimoto, I. (1991a). Identification of a Gs activator region of the beta 2-adrenergic receptor that is autoregulated via protein kinase A-dependent phosphorylation. *Cell* 67, 723-730.

Okamoto, T., Ohkuni, Y., Ogata, E., and Nishimoto, I. (1991b). Distinct mode of G protein activation due to single residue substitution of active IGF-II receptor peptide Arg2410-Lys2423: evidence for stimulation acceptor region other than C-terminus of Gi alpha. *Biochem Biophys. Res. Commun.* 179, 10-16.



Oldfors, A. (1980). Macrophages in peripheral nerves. An ultrastructural study on rats. *Acta Neuropath. (Berlin)* 49, 43-49.

Oliver, M.H., Harrison, N.K., Bishop, J.E., Cole, P.J., Laurent, G.J. (1989). A rapid and convenient assay for counting cells cultured in microwell plates: assessment of growth factors. *J.Cell.Sci.* 92, 513-518.

Olsson, Y. (1968). Mast cell in the nervous system. *Int.Rev. Cytol.* 24, 27

Olsson, T., Kristensson, K., Ljungdahl, A., Maehlen, J., Holmdahl, R., and Klareskog, L. (1989). Gamma-interferon-like immunoreactivity in axotomized rat motor neurons. *J. Neurosci.* 9, 3870-3875.

Ono, K., Delay, M., Nakajima, T., Isisawa, H., and Giles, W. (1989). Calcitonin gene-related peptide regulates calcium current in heart muscle. *Nature* 340, 721-724.

Osterieder, W., Brown, C., Hescheler, J., and et al, (1982). Injection of subunits of cAMP dependant protein kinase into cardiac myocytes modulates  $Ca^{++}$  current. *Nature* 298, 576-578.

Otto-Erich, B., Hans-Reichard, Z., Doetsch, N., Motomura, S., Khamssi, M., and Michel, M.C. (1989). Myocardial beta-adrenoceptor changes in heart failure: concomitant reduction in beta-1 and beta-2 adrenoceptor function related to the degree of heart failure in patients with mitral valve disease. *J. Am. Coll. Cardiol.* 14, 323-331.

Packer, M. (1990a). Abnormalities of diastolic function as a potential cause of exercise intolerance in chronic heart failure. *Circulation* 81(*suppl.III*), III-78-III-86.

Packer, M. (1990b). Pathophysiological mechanisms underlying the effects of  $\beta$ -adrenergic agonists and antagonists on functional capacity and survival in chronic heart failure. *Circulation* 82(*suppl.I*), I-77-I-88.

Pardee, A. (1989). G1 events and regulation of cell proliferation. *Science* 246, 603-608.

Parker, E.M. and Ross, E.M. (1989). G protein-coupled receptors: structure and function of signal transducing proteins. In *Current topics in membranes and transport*. T. Claudio, ed. (New York: Acad.Press Inc.),

Parker, J.D., Bittl, J.A., and Colucci, W.S. (1989). The effect of betaadrenergic stimulation with dobutamine on isovolaemic relaxation in the normal and failing human ventricle. *Circulation* 80(suppl.II), II-176.

Pellegrino, R.G., Politis, M.J., Ritchie, J.M., and Spencer, P.S. (1986). Events in degenerating cat peripheral nerve: induction of Schwann cell S phase and its relation to nerve fibre degeneration. *J.Neurocytol.* 15, 17-28.

Perkins, J.P., Hausdorff, W.P., and Lefkowitz, R.J. (1990). The  $\beta$ -adrenergic receptor(Perkins,J.P.,ed) Humana press,New York.

Perry, G.J. and Brown, M.J. (1981). Arachidonate-induced experimental nerve infarction. *J.Neurol.Sci.* 50, 123-133.

Perry, V.H., Brown, M.C., and Gordon, S. (1987). The macrophage response to central and peripheral nerve injury: a possible role for macrophages in regeneration. *J. Exp. Med.* 165, 1218-1223.

Perry, V.H., Lunn, E.R., Brown, M.C., Cahusac, S.and Gordon, S. (1990) Eur.J.Neurosci.2:408-413. Evidence that the Rate of Wallerian Degeneration is Controlled by a single Autosomal Dominant Gene. J

Peters, A. and Muir, A.R. (1959). *Quart. J. Exp. Physiol.* 44, 117.

Pierart, M.E., Najdovski, T., Appelboom, T.E., and Deschodt Lanckman, M.M. (1988). Effect of human endopeptidase 24.11 ("enkephalinase") on IL-1-induced thymocyte proliferation activity. *J. Immunol.* 140, 3808-3811.

Pitcher, J., Lohse, M.J., Codina, J., Caron, M.G., and Lefkowitz, R.J. (1992). Desensitization of the isolated beta 2-adrenergic receptor by beta-adrenergic receptor kinase, cAMP-dependent protein kinase, and protein kinase C occurs via distinct molecular mechanisms. *Biochemistry* 31, 3193-3197.

Pollack, I.F., Randall, M.S., Kristofik, M.P., Kelly, R.H., Selker, R.G., and Vertosick, F.T.J. (1990). Response of malignant glioma cell lines to activation and inhibition of protein kinase C-mediated pathways. *J. Neurosurg.* 73, 98-105.

Politis, M.J., Ederle, K., and Spencer, P.S. (1982). Tropism in nerve regeneration in vivo. Attraction of regenerating axons by diffusible factors derived from cells in distal stumps of transected peripheral nerves. *Brain Res.* 253, 1-12.

Polyak, K. et al., (1994). p27, a cyclin-CDK inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev.* 8, 9-22.

Polyak, K. et al., (1994). Cloning of p27, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* 78, 67-74.

Pouleur, H., van Eyll, C., Hanet, C., Cheron, P., Charlier, A.A., and Rousseau, M.F. (1988). Long-term effects of xamoterol on left ventricular diastolic function and late remodelling: A study in patients with anterior myocardial infarction and single vessel disease. *Circulation* 77, 1081-1089.

Powell, H.C., Myers, R.R., Costello, M.L. and Lampert, P.W. (1979). Endoneurial fluid pressure in Wallerian degeneration. *Ann.Neurol.* 5, 550-557.

Pratt, B.L., and Takahashi, J.S. (1988). A pertussis toxin sensitive G-protein mediates the  $\beta$ 2-adrenergic receptor inhibition of melatonin release in photoreceptive chick pineal cell cultures. *Endocrinology* 123, 277

Pruss, R. M., Moskal, J.R., Leiden, L.E. and Beinfeld, M.C. (1985). Specific regulation of VIP biosynthesis by phorbol ester in bovine chromaffin cells. *Endocrinology* 117, 1020-1026.

Raff, M.C., Abney, E., Brockes, J.P., and Hornby-Smith, A. (1978a). Schwann Cell Growth Factors. *Cell* 15, 813-822.

Raff, M.C., Hornby-Smith, A., and Brockes, J.P. (1978b). Cyclic AMP as a mitogenic signal for cultured rat Schwann cells. *Nature,Lond.* 273, 672-673.

Raines, E.W., Lane, T.F., Iruela Arispe, M.L., Ross, R., and Sage, E.H. (1992). The extracellular glycoprotein SPARC interacts with platelet-derived growth factor (PDGF)-AB and -BB and inhibits the binding of PDGF to its receptors. *Proc. Natl. Acad. Sci. U. S. A.* 89, 1281-1285.

Rappolee, D.A., Mark, D., Banda, M.J., and Werb, Z. (1988). Wound macrophages express TGF- $\alpha$  and other growth factors in vivo: analysis by mRNA phenotyping. *Science* 241, 708-712.

Rapoport, B., Kaufman, K.D., and Chazenbalk, G.D. (1992). Cloning of a member of the arrestin family from a human thyroid cDNA library. *Mol. Cell Endocrinol.* **84**, R39-R43.

Rapp, U., et al., (1988). *Cold Spring Harbour Symp. quant. Biol.* **53**, 173-181.

Ratner, N., Hong, D.M., Lieberman, M.A., Bunge, R.P., and Glaser, L. (1988). The neuronal cell-surface molecule mitogenic for Schwann cells is a heparin-binding protein. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 6992-6996.

Ratner, N., Lieberman, M.A., Riccardi, V.M., and Hong, D.M. (1990a). Mitogen accumulation in von Recklinghausen neurofibromatosis. *Ann. Neurol.* **27**, 298-303.

Ratner, N., Lieberman, M.A., Riccardi, V.M., and Hong, D.M. (1990b). Mitogen accumulation in von Recklinghausen neurofibromatosis. *Ann. Neurol.* **27**, 298-303.

Salhany, K.E., Robinson Benion, C., Candia, A.F., Pledger, W.J., and Holt, J.T. (1992). Differential induction of the c-fos promoter through distinct PDGF receptor-mediated signaling pathways. *J. Cell Physiol.* **150**, 386-395.

Reier, P.J. and Hughes, A. (1972). *Am. J. Anat.* **135**, 147-152.

Reithmann, C., Gierschik, P., Sidiropoulos, D., Werdan, K., and Jakobs, K.H. (1989). Mechanism of noradrenaline-induced heterologous desensitisation of adenylate cyclase stimulation in rat heart muscle cells: increase in the level of inhibitory G-protein alpha sub-units. *Eur. J. Pharmacol.* **172**, 211.

Reithmann, C. and Werdan, K. (1988). Homologous vs heterologous desensitization of the adenylate cyclase system in heart cells. *Eur. J. Pharmacol.* **154**, 99-104.

Reithmann, C. and Werdan, K. (1989). Noradrenaline-induced desensitization in cultured heart cells as a model for the defects of the adenylate cyclase system in severe heart failure. *Naunyn-Schmiedeberg Arch. Pharmacol.* **339**, 138.

Rende, M., Muir, D., Ruoslahti, E., Hagg, T., Varon, S., and Manthorpe, M. (1992). Immunolocalization of ciliary neuronotrophic factor in adult rat sciatic nerve. *glia* **5**, 25-32.

Reuter, H. (1983). Calcium channels modulation by neurotransmitters, enzymes and drugs. *Nature* **301**, 569-574.

Reznitsky, D., Gossen, M., Bujard, H., and Reed, S.I. (1994). Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. *Mol. Cell Biol.* 14, 1669-1679.

Riabowol, K.T., Vosatka, R.J., Ziff, E.B., Lamb, N.J., and Feramisco, J.R. (1988). Microinjection of fos-specific antibodies blocks DNA synthesis in fibroblast cells. *Molec. cell. Biol.* 8, 1670-1676.

Riccardi, V.M. (1981). Von Recklinghausen neurofibromatosis. *N. Engl. J. Med.* 305, 1617.

Rich, K.M., Alexander, T.D., Pryor, J.C., and Hollowell, J.P. (1989). Nerve growth factor enhances regeneration through silicone chambers. *Exp. Neurol.* 105, 162-170.

Ridley, A. and Rainbird, S. (1978). An immunoflourescent study of the passive Arthus reaction in rat sciatic nerve. *Neuropath.Appl.Neurobiol.* 4, 225-234.

Ridley, A.J., Davis, J.B., Stroobant, P., and Land, H. (1989). Transforming growth factors-beta 1 and beta 2 are mitogens for rat Schwann cells. *J. Cell Biol.* 109, 3419-3424.

Roa, M. and Changeux, J-P. (1991). Characterisation and developmental evolution of a high-affinity binding site for calcitonin gene-related peptide on chick skeletal muscle membrane. *Neuroscience* 41, 563-570.

Robbins, D.J. et al., (1992). *Proc. Natl. Acad. Sci. USA.* 89, 6924-6928.

Rodbell, M., Birnbaumer, L., Pohl, S.L., and Krans, H.M. (1971). The glucagon sensitive adenylyl cyclase system in plasma membranes of rat liver.V.An obligatory role of guanyl nucleotides in glucagon action. *J. Biol. Chem.* 246, 1877-1882.

Rodriguez, F.D., Bardaji, E., and Traynor, J.R. (1992). Differential effects of Mg<sup>2+</sup> and other divalent cations on the binding of tritiated opioid ligands. *J. Neurochem.* 59, 467-472.

Rollins, P., Witham, S., Ray, K., Thompson, N., Sadler, H., Smithers, N., Grenfell, S., and Solari, R. (1991). Modification of biological responses to interleukin-1 by agents that perturb signal transduction pathways. *Cytokine.* 3, 42-53.

Romani, A. and Scarpa, A. (1990). Hormonal control of  $Mg^{2+}$  transport in the heart. *Nature, Lond.* 346, 841-844.

Ross, E.M. (1989). Signal sorting and amplification through G protein-coupled receptors. *Neuron* 3, 141-152.

Ross, R., Masuda, J., Raines, E.W., Gown, A.M., Katsuda, S., Sasahara, M., Malden, L.T., Masuko, H., and Sato, H. (1990). Localization of PDGF-B protein in macrophages in all phases of atherogenesis. *Science* 248, 1009-1012.

Rossomando, A., Payne, D., Weber, M., and Sturgill, T. (1989). Evidence that pp42, a major tyrosine kinase target protein is a mitogen-activated serine/threonine protein kinase. *Proc. Natl. Acad. Sci. USA* 86, 6940-6943.

Roth, M., Emmons, L.R., Perruchoud, A., and Block, L.H. (1991). Expressions of the low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase genes are stimulated by recombinant platelet-derived growth factor isomers. *Proc. Natl. Acad. Sci. USA* 88, 1888-1892.

Rothenberg, P.L. and Kahn, C.R. (1988). Insulin inhibits pertussis toxin-catalyzed ADP-ribosylation of G-proteins. Evidence for a novel interaction between insulin receptors and G-proteins. *J. Biol. Chem.* 263, 15546-15552.

Rouleau, G.A., Wertelecki, W., and Haines, J.L. (1987). Genetic linkage of bilateral acoustic neurofibromatosis to a DNA marker on chromosome 22. *Nature, Lond.* 329, 246.

Roytta, M., Salonen, V., and Peltonen, J. (1987). Reversible endoneurial changes after nerve injury. *Acta Neuropathol. Berl.* 73, 323-329.

Sakoda, T., Kaibuchi, K., Kishi, K., Kishida, S., Doi, K., Hoshino, M., Hattori, S., and Takai, Y. (1992). smg/rap1/Krev-1 p21s inhibit the signal pathway to the c-fos promoter/enhancer from c-Ki-ras p21 but not from c-raf-1 kinase in NIH3T3 cells. *Oncogene* 7, 1705-1711.

Salo, P.T. and Tatton, W.G. (1992). Deprenyl reduces the death of motoneurons caused by axotomy. *J. Neurosci. Res.* 31, 394-400.

Salonen, V., (1987). Connective tissue reactions to peripheral nerve injury. *Ann. Univ. Turku.* 28:1.

Salonen, V., Roytta, M., and Peltonen, J. (1987). The effects of nerve transection on the endoneurial collagen fibril sheaths. *Acta Neuropathol. Berl.* 74, 13-21.

Salonen, V., Aho, H., Roytta, M., and Peltonen, J. (1988). Quantitation of Schwann cells and endoneurial fibroblast-like cells after experimental nerve trauma. *Acta Neuropathol. Berl.* 75, 331-336.

Samuel, N.M., Jessen, K.R., Grange, J.M., and Mirsky, R. (1987a). Gamma interferon, but not *Mycobacterium leprae*, induces major histocompatibility class II antigens on cultured rat Schwann cells. *J. Neurocytol.* 16, 281-287.

Samuel, N.M., Mirsky, R., Grange, J.M., and Jessen, K.R. (1987b). Expression of major histocompatibility complex class I and class II antigens in human Schwann cell cultures and effects of infection with *Mycobacterium leprae*. *Clin. Exp. Immunol.* 68, 500-509.

Santala, P. and Heino, J. (1991). Regulation of integrin-type cell adhesion receptors by cytokines. *J. Biol. Chem.* 266, 23505-23509.

Sasahara, M., Fries, J.W.U., Raines, E.W., Gown, A.M., Westrum, L.E., Frosch, M.P., Bonthron, D.T., Ross, R., and Collins, T. (1991). PDGF B-chain in neurons of the central nervous system, posterior pituitary, and in a transgenic model. *Cell* 64, 217-227.

Scarpini, E., Lisak, R. P., Beretta, S., Velicogna, M., Doronzo, R., Moggio, M., Jann, S., and Scarlato, G. (1990). Quantitative assessment of class II molecules in normal and pathological nerves. Immunocytochemical studies in vivo and in tissue culture. *Brain* 113, 659-675.

Schatteman, G.C., Gibbs, L., Lanahan, A.A., Claude, P., and Bothwell, M. (1988). Expression of NGF receptor in developing and adult primate central nervous system. *J. Neurosci.* 8, 860-873.

Schechterton, L.C., and Bothwell, M. (1992). Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons.

Schlessinger, J. (1988). The epidermal growth factor receptor as a multifunctional allosteric protein. *Biochemistry* 27: 3119-3123

Schmidt, B., Stoll, G., Toyka, K.V., and Hartung, H.P. (1990). Rat astrocytes express interferon-gamma immunoreactivity in normal optic nerve and after nerve transection. *Brain Res.* 515, 347-350.

Schomig, A., Dart, A.M., Dietz, R., Mayer, E., and Kubler, W. (1984). Release of endogenous catecholamines in the ischaemic myocardium of the rat. *Circ. Res.* 55, 689-701.

Seehafer, J.G. and Shaw, A.R. (1991). Evidence that the signal-initiating membrane protein CD9 is associated with small GTP-binding proteins. *Biochem. Biophys. Res. Commun.* 179, 401-406.

Seilheimer, B., Persohn, E., and Scachner, M. (1989). Neural cell adhesion molecule expression is regulated by Schwann cell-neuron interactions in culture. *J. Cell Biol.* 108, 1909-1915.

Seizinger, B.R., Martuza, R.L., Rouleau, G., Breakefield, X.O., and Gusella, J.F. (1987). Models for inherited susceptibility to cancer in the nervous system: a molecular-genetic approach to neurofibromatosis. *Dev. Neurosci.* 9, 144-153.

Sendtner, M., Kreutzberg, G.W., and Thoenen, H. (1990). Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. *Nature, Lond.* 345, 440-441.

Serrano, M., Hannon, G.J. and Beach, D. (1994). A new regulatory motif in cell cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 366, 704-707.

Shear, M., Insel, P.A., Melmon, K.L. and Coffino, P. (1976). *J.Biol.Chem.* 251:7572-7576.

Shehab, S.A. and Atkinson, M.E. (1986). Vasoactive intestinal polypeptide increases in areas of the dorsal horn of the spinal cord from which other neuropeptides are depleted following peripheral axotomy. *Exp. Brain Res.* 62, 422-430.

Shimokado, K., Raines, E.W., Madtes, D.K., Barrett, T.B., Benditt, E.P., and Ross, R. (1985a). A significant part of macrophage-derived growth factor consists of at least two forms of PDGF. *Cell* 43, 277-286.

Shirakawa, F., Yamashita, U., Chedid, M., and Mizel, S.B. (1988). CAMP -an intracellular second messenger for IL-1. *Proc. Natl. Acad. Sci. USA* 8, 8281.



Sibley, D. R., Benovic, J.L., Caron, M.G. and Lefkowitz, R.J. (1987). *Cell* 48:913-922

Sibley, D.R. and Lefkowitz, R.J. (1985). Molecular mechanisms of receptor desensitization using the  $\beta$ -adrenergic receptor-coupled adenylate cyclase system as a model. *Nature* 317, 124-129.

Sigrist, S., Franco-Cereceda, A., Muff, R., Henke, H., Lundberg, J.M., and Fischer, J.A. (1986). *Endocrinology* 119, 381-389.

Skene, J.H.P. and Shooter, E.M. (1983). Denervated sheath cells secrete a new protein after nerve injury. *Proc. Natl. Acad. Sci. USA* 80, 4169.

Skofitsch, G. and Jacobowitz, D.M. (1985). Calcitonin gene-related peptide: detailed immunohistochemical distribution in the central nervous system. *Peptides* 6, 721-745.

Smith, M.L., et al., (1994). Interaction of the p53-regulated protein GADD 45 with proliferating cell nuclear antigen. *Science* 266, 1376-1380.

Smits, A., Funa, K., Vassbotn, F.S., Beausang Linder, M., af Ekenstam, F., Heldin, C.H., Westermark, B., and Nister, M. (1992). Expression of platelet-derived growth factor and its receptors in proliferative disorders of fibroblastic origin. *Am. J. Pathol.* 140, 639-648.

Sperelakis, N. (1984). Cyclic AMP and phosphorylation in regulation of  $\text{Ca}^{++}$  influx into myocardial cells and blockade by calcium antagonistic drugs. *Am. Heart J.* 107, 347-357.

Sporn, M.B., Roberts, A.B., Wakefield, L.M., DeCrombrughe, B.(1987). Some recent advances in the chemistry and biology of transforming growth factor-beta. *J.Cell Biol.* 105, 1039-1045.

Staugaitis, S.M., Bernier, L., Smith, P.R., and Colman, D.R. (1990). Expression of the oligodendrocyte marker 2'3'-cyclic nucleotide 3'-phosphodiesterase in non-glial cells. *J-Neurosci-Res.* 25(4): 556-60.

Steenbergh, P.H., Hoppener, J.W.M., Zandberg, J., Lips, C.J.M., and Jansz, H.S. (1985). A second human calcitonin\CGRP gene. *FEBS Lett.* 183(2), 403-407.

Stepien, H., Lyson, K., Stanis., and Pawlikowski, M. (1991). The effect of nerve growth factor on DNA synthesis, cyclic AMP and cyclic GMP accumulation by mouse spleen lymphocytes. *Int.J.Immunopharmacology* 13(1):51-56.

Stern, D.F. and Kamps, M.P. (1988). EGF-stimulated tyrosine phosphorylation of p185neu: a potential model for receptor interactions. *EMBO J.* 7, 995-1001.

Stewart, H.J., Eccleston, P.A., Jessen, K.R., and Mirsky, R. (1991). Interaction between cAMP elevation, identified growth factors, and serum components in regulating Schwann cell growth. *J. Neurosci. Res.* 30, 346-352.

Stiles, G.L., Taylor, S., and Lefkowitz, R.J. (1983). Human cardiac  $\beta$ -adrenergic receptors: Subtype heterogeneity delineated by direct ligand binding. *Life Sci.* 33, 467-473.

Stockli, K.A., Lottspeich, F., Sendtner, M., Masiakowski, P., Carroll, P., Gotz, R., Lindholm, D., and Thoenen, H. (1989). Molecular cloning, expression and regional distribution of rat ciliary neurotrophic factor. *Nature, Lond.* 342, 920-923.

Stoll, G., Griffin, J.W., Li, C.Y., and Trapp, B.D. (1989). Wallerian degeneration in the peripheral nervous system: participation of both Schwann cells and macrophages in myelin degradation. *J. Neurocytol.* 18, 671-683.

Stoll, G., Meuller, H.W., Trapp, B.D. and Griffin, J.W. (1989). Oligodendrocytes but not astrocytes express apolipoprotein E after injury of rat optic nerve. *GLIA*.2(3): 170-6.

Strader, C.D., Sigal, I.S., Candelore, M.R., Rands, E., Hill, W.S., and Dixon, R.A.F. (1988). Conserved aspartic acid residues 79 and 113 of the  $\beta$ -adrenergic receptor have different roles in receptor function. *J. Biol. Chem.* 263, 10267-10271.

Strader, C.D., Candelore, M.R., Hill, W.S., Sigal, I.S., and Dixon, R.A.F. (1989a). Identification of two serine residues involved in agonist activation of the  $\beta$ -adrenergic receptor. *J. Biol. Chem.* 264, 13572-13578.

Strader, C.D., Sigal, I.S., and Dixon, R.A.F. (1989b). Structural basis of  $\beta$ -adrenergic receptor function. *FASEB J.* 3, 1825-1832.

Strasser, R.H., Stiles, G.L., and Lefkowitz, R.J. (1984). Translocation and uncoupling of the beta-adrenergic receptor in rat lung after catecholamine induced desensitization in vivo. *Endocrinology* 115, 1392-1400.

Strasser, R.H., Benovic, J.L., Caron, M.G., and Lefkowitz, R.J. (1986a). Beta agonist and prostaglandin E1- induced translocation of the beta-adrenergic receptor kinase: Evidence that the kinase may act on multiple adenylate cyclase coupled receptors. *Proc. Natl. Acad. Sci. USA* 83, 2797-2801.

Strasser, R.H., Sibley, D.R., and Lefkowitz, R.J. (1986b). A novel catecholamine activated cAMP-independant pathway for beta-adrenergic phosphorylation in wild type and mutant S49 lymphoma cells: Mechanisms of homologous desensitization of adenylate cyclase. *Biochemistry* 25, 1371-1377.

Strasser, R.H. (1989). Phosphorylation of the beta-adrenergic receptor: Mechanisms of desensitization. In *Receptor phosphorylation*. V.K. Moudgil, ed. (Boca Raton, Fla: CRC Press), pp. 199-226.

Strasser, R.H., Marquetant, M.D., and Kubler, W. (1990). Adrenergic receptors and sensitization of adenylyl cyclase in acute myocardial ischemia. *Circulation* 82(supp.2), II-23-II-29.

Streit, W.J., Dumoulin, F.L., Raivich, G., and Kreutzberg, G.W. (1989). Calcitonin gene-related peptide increases in rat facial motoneurons after peripheral nerve transection. *Neurosci. Lett.* 10, 143-148.

Stryer, L. and Bourne, H.R. (1986). G proteins: a family of signal transducers. *Annu. Rev. Cell Biol.* 2, 391-419.

Sturgill, T., Ray, L., Erikson, E., and Maller, J. (1988). Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal S6 kinase II. *Nature, Lond.* 334, 715-718.

Su, Y. -F., Harden, T.K. and Perkins, J.P. (1979) *J.Biol.Chem.* 254:38-41.

Sullivan, K.A., Miller, R.T., Masters, S.B., Beidermann, B., Heidemann, W., and Bourne, H.R. (1987). Identification of receptor contact site involved in receptor-G protein coupling. *Nature* 330, 758-760.

- Sussani, E.E., Manders, W.T., Knight, D.R., Vatner, D.E., Vatner, S.F., and Homcy, C.J. (1989). One hour of myocardial ischaemia decreases the activity of the stimulatory guanine-nucleotide regulatory protein Gs. *Circ. Res.* 65, 1145-1150.
- Swinnen, J.V., Joseph, D.R. and Conti, M. (1989). *Proc. Natl. Acad. Sci. USA.* 86, 8197-8201.
- Tada, M. and Katz, A.M. (1982). Phosphorylation of the sarcoplasmic reticulum and sarcolemma. *Am. Rev. Physiol.* 44, 401-423.
- Tang, W.-J. and Gilman, A.G. (1991). *Science* 254, 1500-1503.
- Tang, W.-J. and Gilman, A.G. (1992). Adenylyl cyclases. *Cell* 70, 869-872.
- Taniuchi, M., Clark, H.B., and Johnson, E.M.J. (1986). Induction of nerve growth factor receptor in Schwann cells after axotomy. *Proc. Natl. Acad. Sci. USA.* 83, 4094-4098.
- Taniuchi, M., Clark, H.B., Schweitzer, J.B., and Johnson, E.M.J. (1988). Expression of nerve growth factor receptors by Schwann cells of axotomized peripheral nerves: ultrastructural location, suppression by axonal contact, and binding properties. *J. Neurosci.* 8, 664-681.
- Taya, Y., Yasuda, H., Kamijo, M., Nakaya, K., Nakamura, Y., Ohba, Y., and Nishimura, S. (1989). In vitro phosphorylation of the tumour suppressor gene Rb protein by mitosis-specific histone H1 kinase. *Biochem. Biophys. Res. Commun.* 164, 580-586.
- Theoharides, T.C. (1990). Mast cells: the immune gate to the brain. *Life Sci.* 46, 607-617.
- Thomas, P.K., (1966). The cellular response to injury. I. The cellular outgrowth from the distal stump of transected nerve. *J. Anat.* 100, 287-303.
- Thomas, J.A. and Marks, B.H. (1978). Plasma norepinephrine in congestive heart failure. *Am. J. Cardiol.* 41, 233-243.
- Tingstrom, A., Reuter Dahl, C., Lindahl, P., Heldin, C.H., and Rubin, K. (1992). Expression of platelet-derived growth factor-beta receptors on human fibroblasts.

Regulation by recombinant platelet-derived growth factor-BB, IL-1, and tumor necrosis factor-alpha. *J. Immunol.* *148*, 546-554.

Tota, M.R. and Strader, C.D. (1990). Characterization of the binding domain of the  $\beta$ -adrenergic receptor with the fluorescent antagonist carazolol. *J. Biol. Chem.* *265*, 16891-16897.

Trahey, M., and McCormick, F. (1987). *Science* *238*, 542-545.

Trapp, B.D., Hauer, P. and Lemke, G. (1988). Axonal regulation of myelin protein mRNA levels in actively myelinating Schwann cells. *J. Neurosci.* *8*, 3515- 3521.

Treacy, M.N., He, X., and Rosenfeld, M.G. (1991). I-POU: a POU-domain protein that inhibits neuron-specific gene activation. *Nature, Lond.* *350*, 577-584.

Tse, J., Powell, J.R., Baste, C.A., Priest, R.E., and Kuo, F. (1979). Isoproterenol-induced cardiac hypertrophy: modifications in characteristics of beta-adrenergic receptors, adenylate cyclase, and ventricular contraction. *Endocrinology* *105*, 246-255.

Tsuda, L. et al., (1993). *Cell* *72*, 407-414.

Uchida, S., Yamamoto, H., Iio, S., Matsumoto, N., Wang, X-B., Yonehara, N., Imai, Y., Inoki, R., and Yoshida, H. (1990). Release of calcitonin gene-related peptide-like immunoreactive substances from neuromuscular junction by nerve excitation and its action on striated muscle. *J. Neurochem.* *54*, 1000-1003.

Ullrich, A., et al., (1984) *Nature.* *309*, 418-425.

Umeda, Y., Takamiya, M., Yoshizaki, H., and Arisawa, M. (1988). *Biochem. Biophys. Res. Commun.* *154*, 227-235.

Umeda, Y. and Arisawa, M. (1989). *neuropeptides* *14*, 237-242.

Unsicker, K., Skaper, S.D., and Varon, S. (1985). Neuronotrophic and neurite-promoting factors: effects on early postnatal chromaffin cells from rat adrenal medulla. *Brain Res.* *349*, 117-129.

Unsicker, K., Flanders, K.C., Cissel, D.S., Lafyatis, R., and Sporn, M.B. (1991). Transforming growth factor beta isoforms in the adult rat central and peripheral nervous system. *Neuroscience* 44, 613-625.

Vairo, G., Argyriou, S., Bordun, A.M., Whitty, G., and Hamilton, J.A. (1990). Inhibition of the signaling pathways for macrophage proliferation by cyclic AMP. Lack of effect on early responses to colony stimulating factor-1. *J. Biol. Chem.* 265, 2692-2701.

Vatner, D.E., Vatner, S.F., Fujii, A.M., and Homcy, C.J. (1985). Loss of high affinity cardiac beta adrenergic receptors in dogs with heart failure. *J. Clin. Invest.* 76, 2259-2264.

Vatner, D.E., Knight, D.R., Shen, Y.T., Thomas, JxJ., Homcy, C.J., and Vatner, S.F. (1988). One hour of myocardial ischaemia in conscious dogs increases beta-adrenergic receptors, but decreases adenylate cyclase activity. *J. Mol. Cell Cardiol.* 20, 75-82.

Vatner, D.E., Vatner, S.F., Nejima, J., Uemura, N., Susanni, E.E., Hintze, T.H., and Homcy, C.J. (1989). Chronic norepinephrine elicits desensitization by uncoupling the  $\beta$ -receptor. *J. Clin. Invest.* 84, 1741-1748.

Verge, V.M., Tetzlaff, W., Richardson, P.M., and Bisby, M.A. (1990). Correlation between GAP43 and nerve growth factor receptors in rat sensory neurons. *J. Neurosci.* 10, 926-934.

Vige, X., Costa, E., and Wise, B.C. (1991). Mechanism of nerve growth factor mRNA regulation by interleukin-1 and basic fibroblast growth factor in primary cultures of rat astrocytes. *Mol. Pharmacol.* 40, 186-192.

Villar, M.J., Cortes, R., Theodorsson, E., Wiesenfeld Hallin, Z., Schalling, M., Fahrenkrug, J., Emson, P.C., and Hokfelt, T. (1989). Neuropeptide expression in rat dorsal root ganglion cells and spinal cord after peripheral nerve injury with special reference to galanin. *Neuroscience* 33, 587-604.

Villar, M.J., Wiesenfeld-Hallin, Z., Xu, X.J., Theodorsson, E., Emson, P.C., and Hokfelt, T. (1991). Further studies on galanin-, substance P-, and CGRP-like immunoreactivities in primary sensory neurons and spinal cord: effects of dorsal rhizotomies and sciatic nerve lesions. *Exp. Neurol.* 112, 29-39.

Virchow, R. (1854). Über das ausgebreitete Vorkommen einer der Nervenmark analogen Substanz in den tierischen Geweben. *Virchow's Arch. Path. Anat.* 6, 562

Viskochil, D. et al., (1990) *Cell* 62, 187-192.

Vulliet, P.R., Hall, F.L., Mitchell, J.P., and Hardie, D.G. (1989). Identification of a novel proline directed serine/threonine protein kinase in rat pheochromocytoma. *J. Biol. Chem.* 264, 16293-16298.

Waga, S., Hannon, G.J., Beach, D., and Stillman, B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* 369, 574-578.

Wahl, S.M., Hunt, D.A., Wakefield, L.M., McCartney Francis, N., Wahl, L.M., Roberts, A.B., and Sporn, M.B. (1987). Transforming growth factor type beta induces monocyte chemotaxis and growth factor production. *Proc. Natl. Acad. Sci. USA.* 84, 5788-5792.

Wallace, M.R., et al., (1990). *Science* 249, 181-186.

Waller, A.V. (1850). *Philos. Trans. R. Soc. Lond. [Biol.]* 140:423

Wang, H., Lipfert, L., Malbon, C.C., and Bahouth, S. (1990). Site-directed anti-peptide antibodies define the topography of the  $\beta$ -adrenergic receptor. *J. Biol. Chem.* 264, 14424-14431.

Warne, P. H., Vician, P. R. and Downward, J. (1993). Direct interaction of Ras and the amino-terminal region of Raf-1 *in vitro*. *Nature* 364, 352-355.

Weinmaster, G. and Lemke, G. (1990). Cell-specific cyclic AMP-mediated induction of the PDGF receptor. *EMBO J.* 9, 915-920.

West, R.E., Moss, J., Vaughan, M., Liu, T., and Liu, T.-Y. (1985). Pertussis toxin catalysed adp-ribosylation of transducin. *J. Biol. Chem.* 260, 14428-14430.

Wewetzer, K., MacDonald, J.R., Collins, F., and Unsicker, K. (1990). CNTF rescues motoneurons from ontogenetic cell death in-vivo, but not in-vitro. *Neuroreport.* 1, 203-206.

Wheeler, M.B. and Veldhuis, J.D. (1989). *Endocrinology* 125:2414- 2420

Whiteway, M., Hougan, L., Dignard, D., Thomas, D.Y., Bell, L., Saari, G.C., Grant, F.J., O'Hara, P., and MacKay, V.L. (1989). The STE4 and STE18 genes of yeast encode potential  $\beta$  and  $\alpha$  subunits of the mating factor receptor-coupled G protein. *Cell* 56, 467-477.

Whitman, M., Kaplan, D., Roberts, T., and Cantley, L. (1987). Evidence for two distinct phosphatidylinositol kinases in fibroblasts. Implications for cellular regulation. *Biochem J.* 247, 165-174.

Whitman, M., Downes, C.P., Keeler, M., Keller, T., and Cantley, L. (1988). Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. *Nature, Lond.* 332, 644-646.

Wiik, P. (1989). Vasoactive intestinal polypeptide inhibits the respiratory burst in human monocytes by a cyclic AMP-mediated mechanism. *Regulatory Peptides* 25, 187-197.

Williams, P.F., Zilkens, R.R., Caterson, I.D., and Cooney, G.J. (1991a). Cation-induced restoration of insulin action in insulin-desensitized HTC cells. *Endocrinology* 129, 249-255.

Williams, P.F., Zilkens, R.R., Caterson, I.D., and Cooney, G.J. (1991b). Cation-induced restoration of insulin action in insulin-desensitized HTC cells. *Endocrinology* 129, 249-255.

Wilson, P.D., Dixon, B.S., Dillingham, M.A., Garcia-Sainz, J.A. and Anderson, R.J. (1986). *J.Biol.Chem.* 261:1503-1506.

Winston, J.T., Olashaw, N.E., and Pledger, W.J. (1991). Regulation of the transmodulated epidermal growth factor receptor by cholera toxin and the protein phosphatase inhibitor okadaic acid. *J. Cell Biochem* 47, 79-89.

Wong, S.K.F., Slaughter, C., Ruoho, A.E., and Ross, E.M. (1988). The catecholamine binding site of the  $\beta$ -adrenergic receptor is formed by juxtaposed membrane spanning domains. *J. Biol. Chem.* 263, 7925-7928.

Wood, K.W., Sarnecki, C., Roberts, T.M., and Blenis, J. (1992). *Cell* 68, 1041-1050.



Xiong, Y., et al., (1993). p21 is a universal inhibitor of cyclin kinases. *Nature* 366, 700-704.

Xu, G., Lin, B., Tanaka, K., Dunn, D., Wood, D., Gesteland, R., White, R., Weiss, R., and Tamanoi, F. (1990a). The catalytic domain of the neurofibromatosis type 1 gene product stimulates ras GTPase and complements ira mutants of *S.cerevisiae*. *Cell* 63, 835-841.

Xu, G., O'Connel, P., Viskochil, D., Cawthon, R., Robertson, M., Culver, M., Dunn, D., Stevens, J., Gesteland, R., White, R., and Weiss, R. (1990b). The neurofibromatosis type 1 gene encodes a protein related to GAP. *Cell* 62, 599-608.

Yamaguchi, A., Chiba, T., Yamatani, T., Inui, T., Morishita, T., Nakamura, A., Kadowaki, S., Fukase, M., and Fujita, T. (1988). Calcitonin gene-related peptide stimulates adenylate cyclase activation via a guanine nucleotide-dependent process in rat liver plasma membranes. *Endocrinology* 123, 2591-2596.

Yamamoto, A.I. and Tohyama, M. (1989). CGRP in the peripheral nervous system. *Prog. Neurobiol.* 33, 353-386.

Yamayoshi, M., Ohue, M., Kawashima, H., Kotani, H., Iida, M., Kawata, S., and Yamada, M. (1990). A human IL-1 alpha derivative which lacks prostaglandin E2 inducing activity and inhibits the activity of IL-1 through receptor competition. *Lymphokine Res.* 9, 405-413.

Yang, S.D., Benovic, J.L., Fong, Y.L., Caron, M.G., and Lefkowitz, R.J. (1991). Cyclic phosphorylation-dephosphorylation of rhodopsin in retina by protein kinase FA (the activator of ATP.Mg-dependent protein phosphatase). *Biochem. Biophys. Res. Commun.* 178, 1306-1311.

Yang, Y.C., Vu, N.D., and Wagner, P.D. (1992). Guanine nucleotide stimulation of norepinephrine secretion from permeabilized PC12 cells: effects of Mg<sup>2+</sup>, other nucleotide triphosphates and N-ethylmaleimide. *Biochim. Biophys. Acta* 1134, 285-291.

Yasuda, T., Sobue, G., Mitsuma, T., and Takahashi, A. (1988a). Peptidergic and adrenergic regulation of the intracellular 3',5'-cyclic adenosine monophosphate content in cultured rat Schwann cells. *J. Neurol. Sci.* 88, 315-325.

- Yasuda, T., Sobue, G., Mitsuma, T., and Takahashi, A. (1988b). Peptidergic and adrenergic regulation of the intracellular 3,5-cyclic adenosine monophosphate content in cultured rat Schwann cells. *J. Neurol. Sci.* 88, 315-325.
- Yatani, A. and Brown, A.M. (1989). Rapid  $\beta$ -adrenergic modulation of cardiac calcium channel currents by a fast G protein pathway. *Science* 245, 71-74.
- Yeh, H.-J., Ruit, K.G., Wang, Y.-X., Parks, W.C., Snider, W.D., and Deuel, T.F. (1991). PDGF A-chain gene is expressed by mammalian neurons during development and in maturity. *Cell* 64, 209-216.
- Yoshida, K. and Gage, F.H. (1991). Fibroblast growth factors stimulate nerve growth factor synthesis and secretion by astrocytes. *Brain Res.* 538, 118-126.
- Yu, J.C., Heidaran, M.A., Pierce, J.H., Gutkind, J.S., Lombardi, D., Ruggiero, M., and Aaronson, S.A. (1991). Tyrosine mutations within the alpha platelet-derived growth factor receptor kinase insert domain abrogate receptor-associated phosphatidylinositol-3 kinase activity without affecting mitogenic or chemotactic signal transduction. *Mol. Cell Biol.* 11, 3780-3785.
- Zhang, X-F., Settleman, J., Kyriakis, J. M., Takiuchi-Suzuki, E., Elledge, S. J., Marshall, M. S., Bruder, J. T., Rapp, U. R., and Avruch, J. (1993). Normal and oncogenic p21<sup>ras</sup> proteins bind to the amino-terminal regulatory domain of c-Raf-1. *Nature* 364, 308-313.
- Zhu, L., Van den Heuvel, S., Helin, K., Fattaey, A., Ewen, M., Livingstone, D., Dyson, N., and Harlow, E. (1993). Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. *Genes Dev.* 7, 1111-1125.