

INVESTIGATION OF FACTORS CONTROLLING
THE EXPRESSION OF MOLECULES INVOLVED
IN NEURITE OUTGROWTH

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ABSTRACT.

Nerve growth factor (NGF) induced morphological differentiation of the pheochromocytoma PC12 cell line is accompanied by cell surface modulation of the expression of specific cell surface recognition molecules. Changes in the relative expression of the Thy-1 glycoprotein, the neural cell adhesion molecule (N-CAM) and the L1 antigen correlate with the onset of neuritic outgrowth indicating a role for these molecules in the neuritogenic process. NGF induced modulation of cell surface glycoprotein expression is, initially brought about by transcription dependent mechanisms, suggesting that the PC12 cell may be a suitable model for the investigation of primary signal transduction events. Studies utilising activators of adenylate cyclase and protein kinase C demonstrate that NGF induced differentiation events are mediated by a variety of independently acting transduction routes.

Morphological and biochemical responses to NGF are sensitive to secondary cellular signals. Activators of adenylate cyclase acted synergistically with NGF to promote an accelerated phase of neuritic outgrowth while concomitantly causing differential modulation of NGF induced glycoprotein expression. Analysis of the morphological and biochemical status of PC12 cells grown on monolayer cultures of non-neuronal cells provided further evidence that responses to NGF are not immutable. While growth on myotubes and glioma cells provided permissible substrates for differentiation, PC12 cells grown on monolayers of skin fibroblasts failed to respond morphologically to NGF and in addition, NGF induced increases in the expression of neurofilament protein and the Thy-1 antigen were suppressed. The ability of primed PC12 cells to differentiate on fibroblast monolayers indicates that the inhibitory effects of fibroblast contact are directed at a component of primary, transcription dependent NGF-induced gene activation.

Finally, the basic fibroblast growth factor (bFGF) was able to reproduce morphological and biochemical responses associated with NGF induced differentiation. A kinase inhibitor, K252a, was used to discriminate between responses induced by the two growth factors since its effects are specific for NGF.

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INDEX.

	PAGE No.
CHAPTER 1 : GENERAL INTRODUCTION.	
1.1 MOLECULAR ANALYSIS OF THE DEVELOPMENT OF THE NERVOUS SYSTEM.	1.
1.1.1. NON-DIFFUSABLE EPIGENETIC FACTORS.	2-6.
1.1.2. DIFFUSABLE EPIGENETIC FACTORS.	6-8.
1.2 MOLECULAR ASPECTS OF NEURONAL CELL DEVELOPMENT.	8-9.
1.2.1. THE ROLE OF CELL SURFACE RECOGNITION MOLECULES.	9-10.
1.2.2. THE INTEGRINS.	10-12.
1.2.3. NEURAL CELL ADHESION MOLECULES.	12-13.
1.2.4. CALCIUM-INDEPENDENT CELL ADHESION MOLECULES	13-17.
1.2.5. CALCIUM-DEPENDENT CELL ADHESION MOLECULES.	18-21.
1.2.6. GANGLIOSIDES.	21-22.
1.2.7. THE THY-1 ANTIGEN.	23.
1.2.8. THE ROLE OF NEUROTROPHIC MOLECULES.	24.
1.2.9. THE DISCOVERY OF NERVE GROWTH FACTOR (NGF) AND ITS BASIC FUNCTIONS.	24-25.
1.2.10. NGF AS A RETROGRADE NEUROTROPHIC FACTOR.	25-27.
1.2.11. NGF AS A NEUROTROPIC FACTOR AND INDUCER OF NEURONAL DIFFERENTIATION.	27-29.
1.2.12. THE MECHANISM OF ACTION OF NGF.	29-33.
1.2.13. THE PHEOCHROMOCYTOMA (PC12) CELL LINE AS A MODEL FOR NEURONAL DIFFERENTIATION.	33-36.
1.2.14. TRANSCRIPTION DEPENDENT RESPONSES OF PC12 CELLS.	36-39.
1.2.15. THE PRIMING RESPONSE.	39-40.

	PAGE No.
1.2.16. PHYSIOLOGICAL FUNCTION OF THE DIFFERENTIATED PC12 CELL.	40-41.
1.2.17. DISADVANTAGES AND LIMITATIONS OF THE PC12 CELL MODEL.	41-42.
1.3. AIMS OF WORK.	43-44.
 CHAPTER 2 : MATERIALS AND METHODS.	
2.1. APPARATUS.	45.
2.2. MATERIALS.	46.
2.3. CHEMICALS.	46-51.
2.4. BIOCHEMICALS.	51-52.
2.5. SUPPLIERS NAMES AND ADDRESSES.	52-54.
2.6. METHODS.	54.
2.6.1. MAMMALIAN CELL CULTURE.	54-57.
2.6.2. ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA) QUANTITATION OF PC12 CELL SURFACE GLYCOPROTEINS AND NEUROFILAMENT PROTEIN.	57-59.
2.6.3. POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).	59-60.
2.6.4. IMMUNOBLOTTING.	60-61.
2.6.5. IMMUNOFLUORESCENCE MICROSCOPY.	61-62.
2.6.6. METABOLIC LABELLING AND IMMUNOPRECIPITATION OF N-CAM POLYPEPTIDES.	63-64.
2.6.7. MTT COLORIMETRIC ASSAY.	64-65.
2.6.8. PHOSPHATASE TREATMENT OF CELL CULTURES.	65.
2.6.9. PROTEIN QUANTIFICATION.	65.

	PAGE No.
2.6.10. RIBONUCLEIC ACID (RNA) EXTRACTION FROM CELL CULTURES.	65-67.
2.6.11. NORTHERN BLOTTING AND cDNA-mRNA HYBRIDISATION.	67-69.
CHAPTER 3 : REGULATION OF THY-1 EXPRESSION DURING PC12 CELL DIFFERENTIATION.	70.
3.1. INTRODUCTION.	70-73.
3.2. RESULTS.	74.
3.2.1. NGF-INDUCED INCREASES IN THE CELL SURFACE EXPRESSION OF THY-1 : A 4 DAY TIMECOURSE.	74.
3.2.2. 48 HOUR INDUCTION OF THY-1 EXPRESSION : AN NGF DOSE RESPONSE.	74.
3.2.3. EFFECTS OF CORDYCEPIN ON NGF INDUCED THY-1 EXPRESSION.	77.
3.2.4. EFFECTS OF PHORBOL ESTERS ON THE MORPHOLOGICAL STATUS OF PC12 CELLS.	77.
3.2.5. EFFECTS OF PHORBOL ESTERS ON THY-1 EXPRESSION.	80.
3.2.6. EFFECTS OF PHORBOL ESTERS ON NGF INDUCED INCREASES IN THY-1 EXPRESSION.	82.
3.2.7. PHORBOL ESTER MEDIATED INHIBITION OF NGF INDUCED THY-1 EXPRESSION IS NOT ASSOCIATED WITH CYTOTOXIC EFFECTS.	82-85.
3.2.8. DIOCTANOYLGLYCEROL CAN MIMIC THE EFFECTS OF PHORBOL ESTERS ON THY-1 EXPRESSION.	85.
3.2.9. INDUCTION OF INCREASED THY-1 EXPRESSION BY A23187.	85.
3.2.10. INDUCTION OF THY-1 BY NGF AND A23187 IS ADDITIVE.	87.
3.2.11. PHORBOL ESTER AND A23187 MIMIC NGF INDUCTION OF THY-1 AT THE LEVEL OF GENE TRANSCRIPTION.	87-90.
3.2.12. INDUCTION OF THY-1 BY PHORBOL ESTER AND A23187 IS DEPENDENT ON ONGOING GENE TRANSCRIPTION.	90-92.

	PAGE No.
3.2.13. NGF INDUCTION OF THY-1 IS INHIBITED BY CHOLERA TOXIN.	92-94.
3.2.14. CHOLERA TOXIN INHIBITS NGF INDUCED INCREASES IN THE EXPRESSION OF THY-1 mRNA.	94-96.
3.3. DISCUSSION.	96-109.
CHAPTER 4 : REGULATION OF THE EXPRESSION OF N-CAM AND L1 DURING PC12 CELL DIFFERENTIATION.	110.
4.1. INTRODUCTION.	110-113.
4.2. RESULTS.	
4.2.1. EFFECT OF NGF ON THE CELL SURFACE EXPRESSION OF N-CAM : 48 HOUR DOSE RESPONSE.	114.
4.2.2. DEPENDENCE OF THE N-CAM RESPONSE ON ONGOING TRANSCRIPTION.	116.
4.2.3. EFFECTS OF PHORBOL ESTERS AND A23187 ON N-CAM EXPRESSION.	116-119.
4.2.4. EFFECTS OF PHORBOL ESTERS AND A23187 ON NGF INDUCED N-CAM EXPRESSION.	119.
4.2.5. EFFECT OF CHOLERA TOXIN ON N-CAM EXPRESSION IN NAIVE AND NGF TREATED PC12 CELLS.	121.
4.2.6. POTENTIATION OF NGF INDUCED INCREASES IN N-CAM EXPRESSION BY CHOLERA TOXIN IS A TRANSIENT EFFECT.	121-123.
4.2.7. CHOLERA TOXIN POTENTIATES THE DE NOVO SYNTHESIS OF BOTH THE 140 kD AND 180 kD N-CAM POLYPEPTIDES.	123-126.
4.2.8. EFFECT OF NGF ON THE CELL SURFACE EXPRESSION OF L1.	126.
4.2.9. EFFECTS OF PHORBOL ESTER AND A23187 ON L1 EXPRESSION.	127.
4.2.10. EFFECTS OF CHOLERA TOXIN ON THE EXPRESSION OF L1 IN NAIVE AND NGF TREATED PC12 CELLS.	127-130.

	PAGE No.
4.2.11. THE EFFECTS OF CHOLERA TOXIN ARE MIMICKED BY FORSKOLIN.	130-133.
4.3. DISCUSSION.	133-143.
CHAPTER 5 : FIBROBLAST GROWTH FACTOR (FGF) INDUCED DIFFERENTIATION OF PC12 CELLS.	144.
5.1. INTRODUCTION.	144-146.
5.2. RESULTS.	
5.2.1. INDUCTION OF INCREASED THY-1 EXPRESSION BY bFGF IN PRIMED AND NAIVE PC12 CELLS.	146.
5.2.2. INDUCTION OF INCREASES IN N-CAM EXPRESSION BY bFGF.	148.
5.2.3. bFGF INDUCTION OF THY-1 AND N-CAM CAN BE MEDIATED BY BOTH TRANSCRIPTION -DEPENDENT AND -INDEPENDENT MECHANISMS.	150.
5.2.4. NGF AND bFGF INDUCED INCREASES IN THY-1 AND N-CAM ARE NOT MIMICKED BY EPIDERMAL GROWTH FACTOR (EGF).	152-153.
5.2.5. CHOLERA TOXIN DIFFERENTIALLY MODULATES bFGF INDUCED INCREASES IN THE EXPRESSION OF THY-1 AND N-CAM.	153.
5.2.6. K252a IS A SPECIFIC INHIBITOR OF NGF INDUCED RESPONSES.	155-158.
5.2.7. EFFECTS OF PERTUSSIS TOXIN ON NGF AND bFGF INDUCED RESPONSES.	160-162.
5.3. DISCUSSION.	162-168.

	PAGE No.
CHAPTER 6 : MODULATION OF PC12 CELL DIFFERENTIATION BY CELL-CELL INTERACTIONS.	169.
6.1. INTRODUCTION.	169-172.
6.2. RESULTS.	
6.2.1. NGF INDUCED MORPHOLOGICAL DIFFERENTIATION OF PC12 CELLS IN COCULTURE.	172-174.
6.2.2. IMMUNOCHEMICAL QUANTITATION OF NEURITE OUTGROWTH.	174-176.
6.2.3. NGF INDUCED ACCUMULATION OF THE RT97 ANTIGEN.	176-179.
6.2.4. IMMUNOCHEMICAL CHARACTERISATION OF THE RT97 ANTIGEN.	181-183.
6.2.5. RECOGNITION OF THE RT97 EPITOPE IS MODIFIED BY ITS PHOSPHORYLATION STATUS.	183-185.
6.2.6. NGF INDUCED NEUROFILAMENT PROTEIN ACCUMULATION IN COCULTURES GROWN IN SERUM-CONTAINING CULTURE MEDIA.	185.
6.2.7. NGF INDUCED NEUROFILAMENT PROTEIN ACCUMULATION IN COCULTURES GROWN IN SATO MEDIA.	187.
6.2.8. EFFECT OF PRIMING ON NEUROFILAMENT PROTEIN RESPONSE IN COCULTURE.	187-190.
6.2.9. EFFECT OF COCULTURE ON NGF INDUCED EXPRESSION OF THY-1.	190-192.
6.2.10. EFFECTS OF CONDITIONED MEDIA FROM NON- NEURONAL CELLS ON NGF INDUCED INCREASES IN THY-1 AND NEUROFILAMENT PROTEIN EXPRESSION.	192-194.
6.3. DISCUSSION.	195-200.

	PAGE No.
CHAPTER 7 : GENERAL DISCUSSION AND FUTURE PERSPECTIVES.	201.
7.1. SIGNAL TRANSDUCTION.	201-206.
7.2. NEURAL CELL SURFACE RECOGNITION MOLECULES : FUNCTIONS IN THE DIFFERENTIATING PC12 CELL.	206-212.
7.3. MOLECULAR STUDIES OF NGF INDUCED DIFFERENTIATION IN NEURAL CREST CELLS.	212.
REFERENCES.	213-236.

FIGURES.

	PAGE NO
1.1. STRUCTURAL HOMOLOGY BETWEEN NEURAL CELL SURFACE RECOGNITION MOLECULES.	4.
1.2. THE MAJOR STRUCTURAL ISOFORMS OF N-CAM FOUND IN NERVOUS TISSUES.	14.
1.3. WORKING MODEL FOR RECEPTOR MEDIATED GENE ACTIVATION.	32.
3.1. TIMECOURSE :- THY-1 ACCUMULATION.	75.
3.2. NGF DOSE RESPONSE : THY-1 EXPRESSION.	76.
3.3. CORDYCEPIN DOSE RESPONSE :- EFFECT ON NGF INDUCTION OF THY-1.	78.
3.4. COMPARISON OF THE EFFECTS OF NGF AND PMA ON THEMORPHOLOGY OF PC12 CELLS.	79.
3.5. DOSE RESPONSE :- NGF AND PHORBOL ESTER INDUCED THY-1 EXPRESSION.	81.
3.6. EFFECTS OF PHORBOL ESTERS ON NGF INDUCED THY-1 EXPRESSION.	83.
3.7. MTT DETERMINATION ON PMA TREATED CULTURES.	84.
3.8. EFFECTS OF A23187 ON THY-1 EXPRESSION.	86.

	PAGE No.
3.9. EFFECTS OF A23187 ON NGF INDUCED THY-1 EXPRESSION.	88.
3.10. INDUCTION OF INCREASED THY-1 mRNA EXPRESSION BY NGF AND ACTIVATORS OF PROTEIN KINASE C.	89.
3.11. EFFECTS OF CORDYCEPIN ON NGF, PMA AND A23187 INDUCED THY-1 EXPRESSION.	91.
3.12. INHIBITION OF THE THY-1 RESPONSE BY CHOLERA TOXIN.	93.
3.13. MODULATION OF BASAL AND NGF INDUCED mRNA EXPRESSION.	95.
3.14. COMPARISON OF THE STRUCTURAL FEATURES OF PMA WITH A SYNTHETIC DIACYLGLYCEROL.	100.
3.15. PROPOSED MECHANISM FOR NGF ACTIVATION OF INCREASED THY-1 GENE TRANSCRIPTION IN PC12 CELLS.	103.
4.1. NGF DOSE RESPONSE :- N-CAM EXPRESSION.	115.
4.2. CORDYCEPIN DOSE RESPONSE :- EFFECT ON NGF INDUCTION OF N-CAM.	117.
4.3. EFFECTS OF NGF, PMA AND A23187 ON N-CAM EXPRESSION.	118.
4.4. THY-1 AND N-CAM EXPRESSION IN THE PRESENCE OF HIGH DOSES OF PMA.	120.
4.5. DOSE RESPONSE :- POTENTIATION OF THE N-CAM RESPONSE BY CHOLERA TOXIN.	122.
4.6. TIMECOURSE :- MODULATION OF N-CAM EXPRESSION BY CHOLERA TOXIN.	124.
4.7. N-CAM BIOSYNTHESIS IN PC12 CELLS.	125.
4.8. EFFECT OF NGF ON ANTI-L1 Ig BINDING.	128.
4.9. L1 EXPRESSION :- MODULATION BY NGF AND CHOLERA TOXIN.	129.
4.10. EFFECTS OF FORSKOLIN ON THE MORPHOLOGY OF NAIVE AND NGF TREATED PC12 CELL CULTURES.	131.
4.11. MODULATION OF NGF INDUCED GLYCOPROTEIN EXPRESSION BY FORSKOLIN.	132.

	PAGE No.
5.1. bFGF DOSE RESPONSE :- THY-1 EXPRESSION.	147.
5.2. bFGF DOSE RESPONSE :- N-CAM EXPRESSION.	149.
5.3. EFFECT OF CORDYCEPIN ON bFGF INDUCED RESPONSES.	151.
5.4. MODULATION OF bFGF INDUCED RESPONSES BY CHOLERA TOXIN.	154.
5.5. EFFECTS OF K-252a ON NGF AND bFGF INDUCED RESPONSES.	156.
5.6. THE EFFECT OF K-252a ON THE MTT RESPONSE.	159.
6.1. MORPHOLOGY OF NAIVE PC12 CELLS IN COCULTURE.	172.
6.2. MORPHOLOGY OF PRIMED PC12 CELLS IN COCULTURE.	174.
6.3. NGF INDUCTION OF THE RT97 RESPONSE.	176.
6.4. TIMECOURSE :- NGF INDUCED CHANGES IN THE MORPHOLOGY OF NAIVE AND PRIMED PC12 CELLS.	177.
6.5. CORDYCEPIN DOSE RESPONSE :- EFFECT ON NGF INDUCTION OF RT97 ANTIGEN.	179.
6.6. IMMUNOCHEMICAL CHARACTERISATION OF THE RT97 BINDING COMPONENT OF PC12 CELLS.	181.
6.7. RT97 RESPONSE :- EFFECT OF ALKALINE PHOSPHATASE TREATMENT.	183.
6.8. EFFECTS OF COCULTURE ON THE RT97 RESPONSE IN SERUM CONTAINING MEDIUM.	185.
6.9. EFFECTS OF COCULTURE ON THE RT97 RESPONSE IN SATO MEDIUM.	187.
6.10. EFFECTS OF COCULTURE ON THE RT97 RESPONSE IN PRIMED CELLS.	188.
6.11. NGF INDUCTION OF THY-1 IN FIBROBLAST - PC12 COCULTURES.	190.
6.12. EFFECTS OF MUSCLE CELL - PC12 CELL INTERACTION ON THY-1 EXPRESSION IN NAIVE AND NGF INDUCED CULTURES.	192.
7.1. SIGNAL TRANSDUCTION EVENTS IN THE DIFFERENTIATING PC12 CELL.	206.

CHAPTER 1 : GENERAL INTRODUCTION.

1.1 MOLECULAR ANALYSIS OF THE DEVELOPING NERVOUS SYSTEM.

The development of the vertebrate nervous system is a complex yet highly ordered process which is orchestrated by regulatory factors of genetic and epigenetic origin. It is only recently that the molecular mechanisms controlling fundamental events such as cellular proliferation, neural induction, neuroblast migration and differentiation, axonal outgrowth and guidance, and synaptogenesis, have begun to be investigated and understood. Our present understanding of these processes owes much to the development of powerful tissue and cell culture techniques allowing the investigation of the biochemistry and morphology of neural cells in vitro (for review, see Bottenstein and Sato, 1985 ; Parnavelas, Stern and Stirling, 1988), to the production of specific antibodies directed against functional components of the neural cell plasma membrane, and more recently to the application of the techniques of molecular biology to the problems of developmental neurobiology.

It is widely believed that the inherent genetic characteristics of the developing neuron are subject to profound influence by a variety of diverse epigenetic factors encountered in its changing microenvironment. Such influence is also believed to operate at the level of specific changes in gene expression. Environmental factors are thought to dictate the formation of precise and orderly neural connections in both the peripheral (PNS) and central (CNS) nervous systems. To date two major classes of epigenetic factor have been described and investigated at the molecular level.

1.1.1 NON-DIFFUSIBLE EPIGENETIC FACTORS.

Developing neural cells and their pioneering axons encounter a vast range of non-diffusible glycoprotein and glycolipid components of the extracellular matrix and cell surfaces of other neural and non-neural cells. Many of these molecules have been suggested to act as cues for specific developmental events or alternatively to provide essential positional and/or adhesive information (Edelman, 1986 ; Jessell, 1988 ; Bronner-Fraser, 1988).

Macromolecules in the extracellular matrix and the basal lamina that have been functionally implicated in neural development include fibronectin (FN) (Rogers, 1983 ; Salvatore, 1984 ; Bronner-Fraser, 1988), laminin (LN) (Lander et al, 1982 ; Rogers , 1983 ; Salvatore, 1984 ; Bronner-Fraser, 1988), the collagen family (Cohen and Hay, 1971 ; Salvatore, 1984), and a variety of complex proteoglycans including heparan sulphate (Pintar, 1978 ; Denby, 1978 ; Lander, 1982 ; Salvatore, 1984). These molecules primarily mediate interactions between the neural cell and its immediate substratum (Hay, 1982) and have been functionally associated with events such as proliferation of the neuroepithelium (Hay, 1982), neural crest cell migration (Bronner-Fraser, 1988), and axonal outgrowth and guidance (Jessell, 1988 ; Rogers et al, 1983 ; Manthorpe et al, 1983).

The discovery and characterisation of the peptide domains in extracellular matrix proteins that are responsible for mediating their interaction with cell surfaces (Humphries et al, 1987 ; Ruoslahti and Pierschbacher, 1986), and the identification of the specific cell surface receptors (Integrins) that recognise these

protein domains (Ruoslahti and Pierschbacher, 1986 ; Hynes, 1987), has resulted in a far greater understanding of the role of cell-substrate interactions in neural development

Cell-cell interactions involving associations between the neural cell with other neural cells or with non-neural cells are mediated by cell surface components known as cell adhesion molecules (Edelman, 1986 ; Jessell, 1988 ; Lander, 1989). These glycoprotein components include, the neural cell adhesion molecule (N-CAM), the myelin-associated glycoprotein (MAG), N-cadherin, Thy-1, L1, and Po all of which have been functionally implicated with developmental events occurring during the process of neuronal maturation. It is becoming increasingly apparent that these structurally and functionally associated molecules play contributory roles in a variety of physiological processes, with relative cell surface expression of each independent molecule determining the extent of its contribution to any one specific event. The ability of these molecules to apparently share common functions, for example each has been demonstrated to act as a putative receptor for neurite outgrowth and/or cell migration (Jessell, 1988 ; Lander, 1989), is reflected either in their close structural homology or their common mechanisms of recognition. N-CAM, MAG, L1, Thy-1 and Po (Lai et al, 1987) are all structurally related (fig 1.1), each molecule bearing one or more extracellular immunoglobulin related protein domains (Lander, 1989). N-CAM and N-cadherin promote adhesive interactions between apposing cell surfaces by similar mechanisms, both molecules associating by a homophilic binding mechanism (Takeichi, 1988 ; Rutishauser et al, 1982), in which molecules of identical molecular structure bind with one another. N-CAM and N-cadherin do however differ mechanistically in that the former promotes cellular

adhesion by a calcium-independent mechanism while the latter requires calcium for its adhesive function. In contrast to N-CAM and N-cadherin, MAG mediates cell-cell interactions by a heterophilic mechanism (Lander, 1989), while Ll, depending on the cells involved, can promote homophilic or heterophilic interactions (Grumet and Edelman, 1988). These subtle variations in structure and binding mechanisms provide the chemical diversity which formed the basis for Edelman's hypothesis for cell patterning (Edelman, 1983, 1984), in this hypothesis it is assumed that a limited number of surface molecules regulate cell-cell interactions and may generate complex morphogenetic patterns, including axonal guidance and target recognition, by undergoing cell surface modulation (Edelman, 1976). These modifications include tissue- and developmental stage- specific modulation of the number, distribution or chemical structure of each particular CAM during embryonic development, as well as changes in the binding behaviour of CAM's (Edelman et al, 1985).

In addition to glycoproteins there are a large family of complex sialoglycolipids known as gangliosides (Svennerholm et al, 1980) that are a major constituent of the neural cell surface (Urban et al, 1980), and that have been implicated as functional components of many physiological processes including axonal outgrowth (Noisen et al, 1981 ; Gorio et al, 1983 ; Toffano et al, 1983 ; Rybak et al, 1983 ; Facci et al, 1984).

The underlying mechanisms involved in the regulation of neuronal development and differentiation by non-diffusible molecules are still to be determined. Specific functions for individual molecules are still to be described, and the ways in which these independent functions can contribute to

a complex physiological process like neurite outgrowth is a fundamental problem in developmental neurobiology.

DIFFUSIBLE EPIGENETIC FACTORS.

Two major types of soluble epigenetic factor are thought to influence the developing neuron, these being the neurotrophic and neurotropic molecules. Neurotrophic substances, of which nerve growth factor (NGF) (for reviews, see Levi-Montalcini, 1987 ; Thoenen and Barde, 1980 ; Lindsay, 1988) and brain derived neurotrophic factor (BDNF) (Barde et al, 1982 ; Lindsay, 1988) are the best characterised, are believed to act locally at their respective sites of production, these being target sites for innervation in the central or peripheral systems. The major physiological function of neurotrophic factors is to provide support and maintenance for functional neurons, mediating increased cell survival and stimulating differentiation (Varon and Skaper, 1983). The archetypal neurotrophic factor NGF, has provided an ideal subject with which to study the mechanisms by which these epigenetic factors can influence neural cell development and function (see section 1.4). Production and availability of neurotrophic factor at target tissues is proposed to be a limiting factor in the process of innervation (Hollyday and Hamburger, 1976 ; Hamburger et al, 1981 ; Cowen et al, 1984). Experimental evidence (Thoenen and Barde, 1980 ; Hamburger et al, 1981 ; Varon and Skaper, 1983 ; Lumsden and Davies, 1983 ; Hofer and Barde, 1988) suggests that competition between an excess of innervating neurons for a limiting supply of an essential neurotrophic factor at the target tissue may be the basis for naturally occurring cell death during neuronal development, a process which accounts for the loss of between 50 and 80% percent of all postmitotic neurons of a particular neuronal subset (Hamburger and Oppenheim, 1982 ;

Varon and Skaper, 1983). In this way it is thought that innervation can be regulated such that the excess of innervating neurons ensures target cell contact but the cell death resulting from limiting neurotrophic substance ensures only the absolute required degree of innervation is achieved. In a similar manner the neurotrophic theory (Purves, 1988) provides a mechanism for preventing incorrect innervation since it would be assumed that inappropriately directed axons, with their specific trophic requirements, would be unable to recognise and assimilate the target cell's secreted factor and would consequently be unlikely to survive. Evidence for such a mechanism is provided by the inability of specific neuronal populations to respond to certain factors (Lindsay, 1988 ; Varon and Skaper, 1983 ; Purves, 1977).

While a trophic factor is responsible for providing cellular maintenance, a tropic factor acts as an attractant and in the case of neurons mediates axonal guidance by drawing the growing axon along an increasing gradient of substance toward its source or site of secretion. In the past, trophic responses have been mistaken for tropic responses simply because many of the earlier studies in this field were conducted on regenerating neurons that have an absolute requirement for trophic factor in order to survive. In coculture models that are essentially regenerating systems it is difficult to differentiate between a chemotropic attraction of an axon to its source of trophic substance, and the growth of the regenerating axon to its closest supply of trophic factor. Thus a truly tropic response must be independent from any survival response. It is only recently, with the advent of culture techniques and purification strategies for the isolation and growth of primary neurons (Bottenstein and Sato, 1985 ; and Parnavelas

et al, 1988), that authentic neurotropic substances have been identified. Chemoattractants in the nervous system that are biochemically distinct from either NGF, or the extracellular matrix macromolecules have been demonstrated for neurons of the mouse trigeminal ganglia when growing to their target tissue (the developing whisker field) in coculture (Lumsden and Davies, 1983 and 1986), and for the orientation and pattern formation of commissural axon growth in the neural floor plate (Tessier-Lavigne et al, 1988).

The molecular and cellular events that underlie the physiological effects of neurotrophic and neurotropic factors on developing and regenerating neurons are at present poorly understood.

1.2 MOLECULAR CONTROL OF NEURONAL CELL DIFFERENTIATION.

A major challenge in developmental neurobiology is to elucidate the mechanisms that contribute to the generation of the neuronal phenotype. To achieve this it will be necessary to characterise the events that dictate the differentiation process, including those processes that trigger and regulate axonal outgrowth. All neuronal cells are derived from the neuroectoderm (Warner, 1988), being generated by a process known as "neural induction" in which ectodermal cells lying in close proximity to the mesoderm are influenced by the inductive mesodermal tissues to change fate. The end point of the inductive process is the formation of the neural tube (Warner, 1988), a structure that gives rise to the CNS and the entire PNS. Specific components of the neural tube develop to form distinct neural (and non-neuronal) populations (Bronner-Fraser, 1988 ; Keynes and Stern, 1988). Perhaps the most widely studied component is the neural crest which generates sensory, sympathetic and parasympathetic neurons, as well as a

variety of non-neuronal cells including adrenal medullary cells, Schwann cells, glia, and cells of bone and cartilage (Le Douarin, 1982).

The neural crest is essentially a transient population of cells that migrate from the dorsal region of the neural tube shortly after tube closure (Bronner-Fraser, 1988 ; Le Douarin, 1982). After leaving the tube, crest cells follow an extensive migratory pathway with individual cells following a migratory route that is characteristic of their axial level of origin (Le Douarin, 1982). At the beginning of migration the neural crest cells resemble one another morphologically, yet during and after their migration undergo extensive and diverse programs of differentiation to give rise to the various neural and non-neuronal populations described above. The mechanisms that control this differentiation process are believed to include factors of genetic and epigenetic origin (Bronner-Fraser, 1988 ; Le Douarin, 1983 ; Patterson, 1978). In addition it is widely thought that epigenetic factors within the PNS dictate the cessation of neuroblast migration and trigger the events leading to axonal outgrowth.

1.2.1 THE ROLE OF CELL SURFACE RECOGNITION MOLECULES.

Developmentally regulated changes in the adhesive nature of the neuroblast cell surface have been implicated in the regulation of key events including the migration and differentiation of pre-neural cells (Edelman, 1986 ; Jessell, 1988 ; Bronner-Fraser, 1988), and at later stages are believed to influence the growth and guidance of neural axons to their cellular targets (Jessell, 1988 ; Dodd and Jessell, 1988). It has recently been suggested that selection of synaptic contacts may be greatly influenced by cell adhesion molecules present at the surfaces of the

neuron and its target cell (Jessell, 1988 ; Jacobson, 1988). Finally, it is becoming more apparent that many cell surface recognition molecules can function as signal transduction components and thereby provide the cell with mechanisms for detecting and responding to stimuli in its local microenvironment (Bangha et al, 1986 ; Bixby et al, 1988 ; Lander, 1988 ; Saleh et al, 1988 ; Kroczek et al, 1988 ; Schuch et al, 1989).

1.2.2 THE INTEGRINS.

Integrins are a large superfamily of integral cell surface receptor molecules that are responsible for recognition and binding of the cell adhesion domains of a variety of extracellular matrix macromolecules (Ruoslahti and Pierschbacher, 1986 ; Hynes, 1987 ; Buck and Horwitz, 1987). Integrins that interact with FN (Horwitz et al, 1985 ; Takada et al, 1987) have been isolated and characterised by virtue of their affinity with a peptide sequence RGD (Humphries et al, 1988) which has been localised to the cell binding domains of many extracellular matrix molecules (Jessell, 1988 ; Ruoslahti and Pierschbacher, 1986 ; Humphries et al, 1988). Two specific monoclonal antibodies (McAb) called CSAT and JG22 (Neff et al, 1982 ; Greve and Gottlieb, 1982 ; Horwitz et al, 1985 ; Knudsen et al, 1985) have the ability to block the interaction of a variety of cells with FN and LN. CSAT and JG22 have been used for the isolation of the chicken integrin complex known as the "CSAT" complex which binds with both FN and LN (Horwitz et al, 1985 ; Takada et al, 1987 ; Hall et al, 1987). More recently, detailed molecular analyses of the function of the Integrin complex at the surface of developing neurons has been made possible by the utilisation of the CSAT and JG22 McAb's. In vivo experiments in which CSAT and JG22 McAb's were injected into developing chick embryo's showed

that the CSAT antigen plays an important role in neural induction since antibodies directed into the mesenchymal tissue caused severe perturbation to neural tube formation and cranial neural crest migration (Bronner-Fraser, 1986 and 1988). In vitro, both the RGD peptide and the two McAb's block the attachment of neural crest cells onto planar FN substrates (Horwitz et al, 1985 ; Bilozur and Hay, 1988 ; Bronner- Fraser, 1985). Both FN and LN have been localised to presumptive routes for neuroblast migration (Bronner-Fraser, 1988), and furthermore the CSAT antigen has been observed at the surface of migrating neuroblasts (Krotoski et al, 1986) and appears to be found with increasing expression on these cells as a function of their developmental age (Bronner-Fraser, 1988). In contrast, the cell-cell recognition molecules N-CAM and N-cadherin are down regulated and disappear from the neuroblast cell surface during migration (Jessell, 1988) indicating that cell-cell interactions may give way to cell-substrate interactions during the migratory process. In addition to providing a possible guiding mechanism for migratory neuroblasts the integrins have the potential to act as cell surface bound signal transduction molecules (Bangha et al, 1986 ; Bixby et al , 1988) and therefore may contribute to the biochemical events that underlie cellular determination and differentiation.

Extracellular matrix molecules in general, and LN in particular have been suggested to act as cues for the growth and guidance of neurites (Baron-Van Evercooren et al, 1982 ; Carbonetto et al, 1983 ; Tomasseli et al, 1986 ; Martin and Timpl, 1987 ; Cohen et al, 1987 ; Hall et al, 1987 ; Sandrock and Matthew, 1987). LN and other extracellular matrix glycans have been observed along the prospective pathways through which CNS and PNS nerve axons elongate to

reach their targets (Bronner-Fraser, 1986 ; Riggott and Moody, 1987 ; Letourneau et al, 1988). In addition these macromolecules can act as neurite outgrowth promoting substrates in vitro (Carbonetto et al, 1983 ; Tomasselli et al, 1986 ; Cohen et al, 1987 ; Hall et al, 1987 ; Hanaz-Ambroise et al, 1987 ; and Manthorpe et al, 1983), supporting the growth of axons and processes from a variety of neurons. It has recently been suggested that the neurite outgrowth response of chick retinal ganglion cells (RGCs) to laminin is developmentally regulated (Cohen et al,1986) and that a developmental decline of laminin-stimulated neurite outgrowth may be controlled by target tissue dependent modulation of the expression of RGC laminin receptors (Cohen et al,1989). Cell conditioned media from a number of different cell types have been shown to include a LN-heparan sulphate proteoglycan complex (Collins et al, 1978 ; Lander et al, 1982 and 1985 ; Edgar et al, 1984 ; Hanaz-Ambroise et al, 1986) that supports neurite outgrowth from many types of neuron when coated onto poly-L-lysine treated surfaces. Further evidence for the neurite-promoting properties of the LN-heparan sulphate proteoglycan complex was provided by the ability of the McAb INO (Matthew and Patterson, 1983 ; Chiu.A.Y. et al, 1986), raised against the complex, to inhibit neurite outgrowth in vitro (Sandrock and Matthew, 1987a) and in vivo (Sandrock and Matthew, 1987b). Finally, compelling evidence for a role for cell-substrate interactions as components of the neurite outgrowth process has been provided by a series of elegant antibody perturbation studies with cocultures of neural cells grown on substrates provided by either other neural cell or non-neuronal cell surfaces (Tomaselli et al, 1986 and 1988 ; Bixby et al, 1987 ; Chang et al, 1987 ; Neugebauer et al, 1988). CSAT antibodies were found to be important components of a variety of neurite-inhibiting antibody cocktails in

many of the cocultures tested, while in contrast other antibodies (for example, anti-N-CAM, anti-N-cadherin, and anti-L1) appeared to have more specialised effects in specific cocultures.

1.2.3 NEURAL CELL ADHESION MOLECULES.

The cell adhesion molecules (CAM's) promoting cell-cell interactions in the nervous system can be divided into molecules that mediate adhesive interactions by either a calcium-dependent or -independent mechanism (Edelman, 1986 ; Jessell, 1988). The cell surface glycoproteins that comprise these two systems are expressed in the nervous system in both stage and tissue specific forms indicating an important role during neuronal development. Molecules of both adhesion systems promote associations between cells of either the same (e.g. neuron-neuron) or differing (e.g. neuron-muscle) phenotype, with evidence for the existence of both homophilic and heterophilic binding mechanisms (Edelman, 1986 ; Takeichi, 1988 ; Nybroe et al , 1988 ; Rutishauser, 1988 ; Lander, 1989).

1.2.4 CALCIUM-INDEPENDENT NEURAL CELL ADHESION MOLECULES.

The most predominantly expressed and best characterised of the known calcium-independent cell adhesion molecules is N-CAM (for reviews, see Edelman, 1986 ; Cunningham, 1987 ; Rutishauser, 1988), a sialic acid rich glycoprotein that is found in nervous tissues , skeletal and cardiac muscle, and the kidney. N-CAM cDNA sequence data from human (Barton et al, 1988), mouse (Barthels et al, 1987), rat (Small et al, 1987) and chicken (Cunningham et al, 1987), shows the extracellular part of the molecule consists of five domains which exhibit close structural homology with the immunoglobulin domains found in antibody molecules, thus

MAJOR STRUCTURAL ISOFORMS OF N-CAM

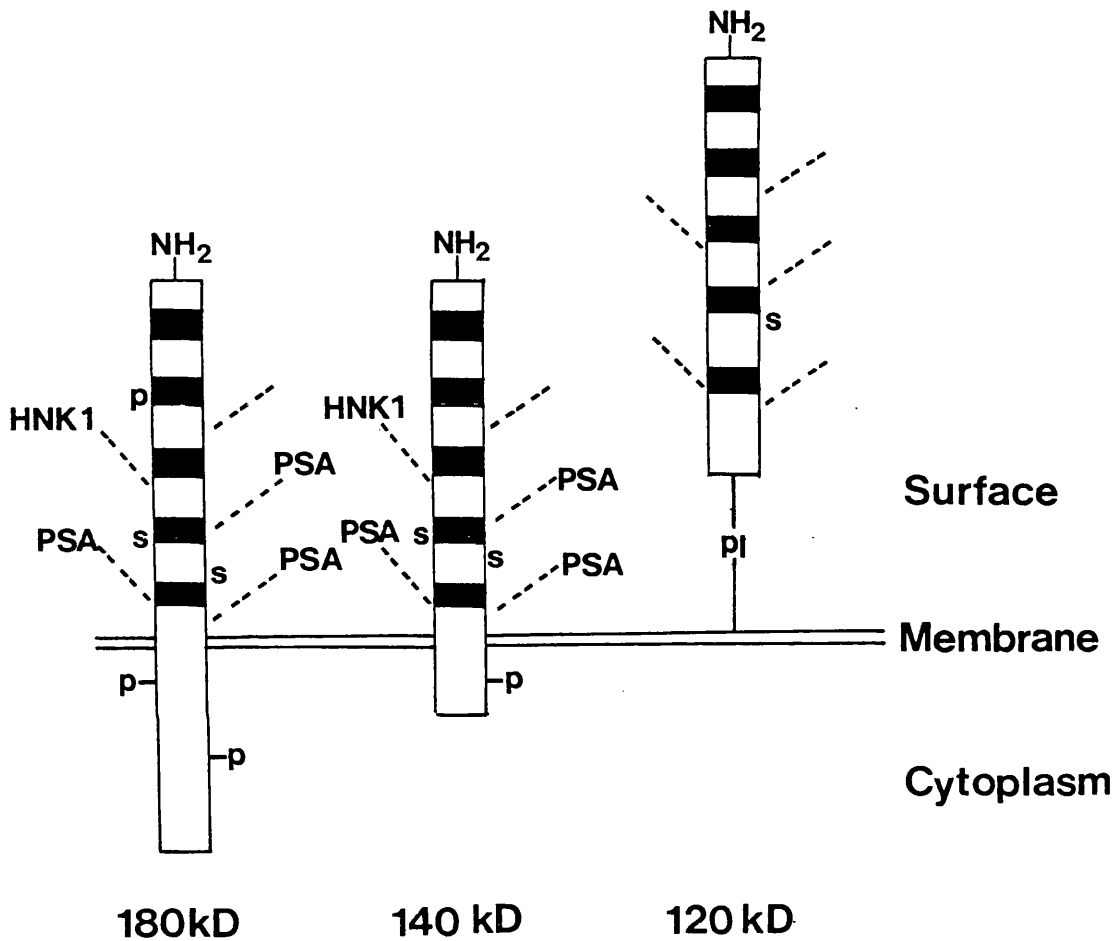


Figure 1.2 Structural features indicated for the N-CAM glycoprotein include C2 type immunoglobulin domains (shaded blocks) ; polysialic acid groups (PSA) ; the HNK1 carbohydrate epitope ; phosphoinositide linkage (PI) ; and sulphated (s) and phosphorylated (p) residues.

making the N-CAM molecule a member of the immunoglobulin gene superfamily (Hunkapiller and Hood, 1986 ; Williams, 1987). The polypeptide chains that form the core of the N-CAM molecule are found in three major size classes in the nervous system, these being called the 180kD, 140kD, and 120kD N-CAM isoforms (Nybroe et al, 1988 ; Goridis and Willie, 1988 ; Cunningham et al, 1987). The major structural variations between the three polypeptides are found at their cytoplasmic domains (fig 1.2). While the 140 and 180 N-CAM isoforms exist as integral membrane molecules with polypeptide domains anchored in the cell cytoplasm (Cunningham et al, 1987), the 120 polypeptide exists purely as a cell-surface molecule (Barthels et al, 1987), being attached to the outer face of the plasma membrane by a phospholipase C sensitive phospholipid anchor which can be spontaneously cleaved (He et al, 1987), or can by alternative exon usage be left out to produce a secreted form of N-CAM (Gower et al, 1988). Recent studies have identified further structural diversity in the N-CAM polypeptides at their extracellular domains which were previously thought to be identical in all N-CAM molecules. Structural heterogeneity has now been demonstrated in human muscle N-CAM (Dickson et al, 1987), chick heart muscle N-CAM (Prediger et al, 1988) and the mouse brain N-CAM's (Santoni et al, 1989). In the latter system up to 16 different N-CAM transcripts have been proposed, with formal proof for the existence of at least eight N-CAM mRNA species. The regulatory mechanisms that control N-CAM expression and isoform usage in the nervous system are not yet fully understood though there is considerable evidence for the existence of tissue and developmental stage specific splicing events (Nybroe et al, 1988 ; Gennarini et al, 1986 ; Murray et al, 1986a and b ; Walsh, 1988 ; Prentice et al, 1987 ; Santoni et al, 1989). In addition little is known

about the effects of structural diversity on N-CAM function in the nervous system and precise functions are still to be described for the three major isoform classes.

N-CAM first appears on pre-neural cells shortly after the formation of the neural tube (Jessell, 1988 ; Jacobson, 1988). Lack of N-CAM expression prior to the inductive process (Jacobson and Rutishauser, 1986) indicates that the molecule is not likely to play a role during this developmental event and indeed is more likely to be expressed as a consequence of the process. Migration of neural crest cells is accompanied by a down-regulation of N-CAM expression (Thiery et al, 1982) leading to a loss of the glycoprotein from the surface of pre-neural cells. At the completion of migration and the onset of gangliogenesis N-CAM is re-expressed at neuroblast cell surfaces (Thiery et al, 1982). These shifts in N-CAM expression suggest that the molecule functions during pre- and post- migratory stages of neuroblast development. It is possible that as a signal for cell-cell associations, the molecule functions during stationary phases of development with its loss of expression during migration allowing such associations to be weakened and broken so as to facilitate cell movement. The regulatory mechanisms that control N-CAM expression in the developing and differentiating neuroblast are at present poorly understood.

Developmental regulation of N-CAM in the nervous system includes modulation at the biochemical level in addition to the changes found at the level of gene regulation. Differentiation of neurons in the chick and *Xenopus* nervous systems is accompanied by an increase in the degree of sialylation of N-CAM polypeptides (Jacobson, 1988 ; Rothbard et al, 1982). Increased sialylation of N-CAM may be

associated with subtle modulation of the molecule's function since the result is to effectively reduce the adhesive force generated between apposing N-CAM molecules (Sadoul et al, 1983 ; Rutishauser et al, 1985). Interestingly it has been demonstrated that N-CAM reverts to its low sialylated form (embryonic form) in adult CNS neurons of the *Xenopus* (Jacobson, 1988), it is yet to be determined if this has any significance to the observed ability of these neurons to regenerate following injury.

N-CAM is expressed at its highest level on neurons during the process of axonal outgrowth (Jacobson, 1988). During axiogenesis N-CAM is found along the entire length of the elongating axon, at the growth cone, and at the neural cell body (Jacobson, 1988). Immunological studies, in which anti-N-CAM antibodies have been used in combination with a variety of other antibodies for cell and substrate adhesion molecules to block neurite outgrowth in coculture, have indicated a prominent role in mediating growth across myotube surfaces from peripheral neurons (Bixby et al, 1987). N-CAM has also been shown to be important in directing the outgrowth of neurites from retinal neurons across monolayer cultures of astrocytes (Neugebauer et al, 1988). In contrast similar studies have demonstrated that N-CAM does not play a prominent role in mediating outgrowth of peripheral neurites across astrocytes, Schwann cells or the surface of other neuronal cells (Tomaselli et al, 1988 ; Bixby et al, 1988 ; Chang et al, 1987). Therefore it would seem that N-CAM can act as a highly specific and versatile neurite-outgrowth-promoting molecule, acting as a cue for outgrowth from specific neurons across specific substrates. As a constituent of an antibody mixture with neurite outgrowth blocking properties, it was clear that N-CAM plays a contributory role in the neuritogenic process, with the

expression of individual components on both the neural and substrate surfaces dictating the level of contribution of specific cell adhesion molecules to the overall process. In vivo expression studies in the developing *Xenopus* peripheral nervous system indicate that N-CAM may play a functional role in axonal growth (Jacobson, 1988). N-CAM is expressed along the shafts and at the growth cones of elongating axons and is found at the surface of recognised peripheral targets. However, N-CAM appears to be absent in the presumptive peripheral axonal pathways. These observations have been suggested to indicate N-CAM function in target recognition, axon fasciculation, and in the guidance of growing axons across other axons, but to argue against a role in the guidance of pioneering axons through specific axonal pathways.

1.2.5 CALCIUM-DEPENDENT CELL ADHESION MOLECULES.

Of the neural cell adhesion molecules that promote cell-cell interactions by a calcium-dependent mechanism, two molecules, namely N-cadherin (Takeichi, 1988) and L1 (Jessell, 1988 ; Rathjen and Schachner, 1984) have received particular attention. N-cadherin, like N-CAM, is a general cell adhesion molecule found in a variety of tissues (Takeichi, 1988) yet is expressed in the developing nervous system in a tissue- and stage- specific manner (Takeichi, 1988 ; Duband et al 1987 ; Hatta et al 1987 ; Hatta et al, 1986). The 130 Kd glycoprotein is found at the cell surface of neural and glial cells in both the PNS and CNS (Hatta et al 1987 ; Hatta et al 1986 ; Crittenden et al, 1988), as well as being found on cells of skeletal and cardiac muscle (Hatta et al, 1987 ; Volk and Geiger, 1984).

The developmental pattern of expression of N-cadherin in pre-neural and neural cells closely parallels that described

for N-CAM. The molecule appears in the neural plate soon after its invagination (Takeichi, 1988) and is predominantly expressed in the neural tube after neural induction. During migration of neural crest cells N-cadherin is lost from the cell surface, yet reappears when neuroblasts reach their destination and differentiate (Hatta et al, 1987). This pattern of expression lends support to the view that during the process of neuroblast migration cells lose their cell surface expression of molecules involved in general cell-cell adhesive interactions so as to favour interaction with extracellular matrix macromolecules fibronectin and laminin which predominate through their presumptive migratory pathways (Bronner-fraser, 1986 ; Riggott and Moody, 1987 ; Letourneau et al, 1988). As with N-CAM, the regulatory mechanisms that control stage-specific expression of N-cadherin are at present not understood.

Recent immunological studies have suggested that N-cadherin may play a role in guiding the elongation of neurites across monolayer cultures of astrocytes, Schwann cells and myotubes (Tomaselli et al, 1988 ; Bixby et al, 1987 ; Bixby et al 1988 ; Neugebauer et al, 1988). More direct evidence for a guidance function for N-cadherin was provided by a molecular-genetic study in which the ability of the molecule to guide the growth of optic nerve axons was tested by transfection of chicken N-cadherin cDNA into two cell lines, Neuro 2A and L-cells, which were to serve as substrates for axonal growth (Matsunaga et al, 1988). In comparison to their untransfected parental cells which lacked chicken N-cadherin expression, transfected N2A and L-cells promoted greatly improved elongation of neurites, this enhanced outgrowth was apparently blocked by anti-N-cadherin antibodies.

In contrast to the general cell-adhesion molecules such as N-CAM, N-cadherin and the ECM molecules, the calcium-dependent cell adhesion molecule L1 appears late in neural development and is restricted to the nervous system (Lemmon and Mc.Loan, 1986 ; Fushiki and Schachner, 1985). In culture L1 is synthesised as a single glycoprotein in the 200-300 kD range (Salton et al, 1983a ; Salton et al, 1983b ; Stallcup et al, 1983 ; Stallcup et al, 1985). The structure and chemistry of L1 is related to N-CAM and other members of the immunoglobulin superfamily (Moos et al, 1988). Recent biochemical and immunochemical analyses have suggested that L1 is either identical to or otherwise remarkably similar to a number of other cell-surface glycoproteins that are associated with the developing axon , namely NILE (Salton et al, 1983a ; Bock et al, 1985 ; Sajovic et al, 1986), 69A1 (Piggott and Kelly, 1986), ASCS4 (Sweedner, 1983), Ng-CAM (Grumet and Edelman, 1984), G4 (Rathjen et al, 1987a) and 8D9 (Lemmon and Mc.Loan, 1986). L1 and its related proteins are expressed on the surface of post-migratory, post-mitotic neurons of both the PNS and CNS (Rathjen and Schachner, 1984 ; Bock et al, 1985 ; Rathjen et al, 1987b ; Fushiki and Schachner, 1985 ; Salton et al, 1983) and in addition are found at the surface of undifferentiated and non-myelinating Schwann cells (Faissner et al, 1984 ; Nieke and Schachner, 1985). The molecule is predominantly localised to neuronal cell processes with little expression, if any, being associated with the cell body. Restrictions in the expression of L1, both in terms of its late stage specific expression and its association with axons, correlates with its restricted set of roles (Bock et al, 1985). Anti-L1 and anti-Ng-CAM antibodies have been shown to inhibit axonal outgrowth by mouse sensory neurons across mouse Schwann cells (Sajovic et al, 1986), suggesting that the molecule may help provide a permissive

substrate for neurite outgrowth. In a similar study, L1 was identified as a component of the adhesive machinery promoting the outgrowth of processes from peripheral motor neurons across Schwann cell surfaces (Bixby et al, 1988).

Thus L1 appears to act as a highly specialised neurite outgrowth promoting molecule, regulating axonal growth across Schwann cells, this contrasts with N-CAM, N-cadherin, and the integrins which promote axonal elongation over a variety of cell / substrate surfaces. There is also evidence to suggest that L1 plays an important functional role in the process of neurite fasciculation (Stallcup and Beasley, 1985 ; Rathjen et al, 1987b ; Fischer et al, 1985). Unlike N-CAM and N-cadherin (Jessell, 1988), L1 is not involved in the formation of the neuromuscular junction (Mehrke et al, 1984), or in the process of synaptogenesis (Lindner et al, 1985), these deficiencies probably reflecting the highly restricted expression of L1. In the PC12 cell (Greene and Tischler, 1976), a model for neuronal differentiation, the NGF-inducible large external glycoprotein (NILE) which is immunologically indistinguishable from L1 (Bock et al, 1985) has been shown to be induced by NGF in a transcription dependent manner (McGuire et al, 1978 ; Salton et al, 1983). At present little else is known about the mechanisms that may contribute to the restricted tissue specific expression of the molecule.

1.2.6 THE GANGLIOSIDES.

Whilst molecules such as N-CAM ,N-cadherin, L1 and the integrins have been associated with specific developmental events and are implicated with particular functions in the developing neuronal cell, there are many more cell surface molecules whose mode of expression and structural characteristics suggest a possible role in the neuron, yet

await more substantial evidence for a specific function. A unique family of cell-surface bound complex glycolipids known as gangliosides have been implicated in neuronal development and function due to their high abundance in the nervous system (Weigandt, 1971 ; Ledeen, 1978). In addition there is growing evidence for the concept that endogenous gangliosides may function in the process of neurite outgrowth both in developing and regenerating systems. For example, it has been observed that a mixture of brain derived gangliosides can stimulate motor neuron sprouting in vivo (Gorio et al, 1983), and indeed a relatively simple member of the ganglioside family, GM1, has been demonstrated to stimulate neuronal sprouting in vitro (Roisen et al, 1981 ; Toffano et al, 1983 ; Rybak et al, 1983 ; Facci et al, 1984) and to stimulate the survival and / or regenerative capacity of NGF dependent sensory neurons in vitro (Doherty et al, 1985 and 1986). These studies together with the complex carbohydrate structure of the gangliosides suggests that they may function as specific recognition molecules, or, as has been alternatively suggested, act as modulators of ligand- receptor activities (Roisen et al, 1981 ; Facci et al, 1984 ; Doherty et al, 1985 and 1986). One possibility is that gangliosides may facilitate neuronal differentiation by acting as receptors or modulators of receptors for epigenetic factors in the environment such as NGF (Levi-Montalcini, 1987 ; Schwartz and Spiran, 1982). Direct evidence for receptor function of gangliosides is provided by studies demonstrating the ability of GM1 to act as a receptor for cholera toxin (Van Heyningen et al, 1971). As cholera toxin exerts its cellular effects by activating adenylate cyclase (Rybak et al, 1983 ; Limbird, 1981; Cassel and Selinger, 1977), the enzyme that catalyses the synthesis of cyclic AMP (Limbird, 1981) which acts as a mediator of a variety of hormonal

responses (Sutherland and Robinson, 1966), it would appear that GM1 has the potential to function as a receptor for epigenetic factors and to be associated with intracellular signal transduction. It remains to be determined what effects result from triggering of the cholera toxin/ GM1 receptor-ligand event and what are the consequences of such effects on the differentiating neural cell. High order gangliosides such as GQ1b and GT1b which have a more complex carbohydrate structure than GM1 and indeed were found to exhibit more pronounced effects on the survival and regenerative capacity of NGF-dependent sensory neurons (Doherty et al, 1986), may also play functional roles in neuritogenesis (Gorio et al, 1983). Thus, the gangliosides may function as a family of cell surface recognition and / or signal transduction molecules that respond to the binding of specific environmental factors.

1.2.7 THE THY-1 ANTIGEN.

All neuronal cells so far studied have been shown to express the Thy-1 glycoprotein at their cell surface (Morris, 1985). Thy-1 is the simplest member of the immunoglobulin superfamily (Williams and Gagnon, 1982) and is therefore structurally and probably functionally related to N-CAM, L1 and the immunoglobulins. The expression of Thy-1 at the surface of neurons is both stage and tissue specific, (Morris ,1985 ; Reif and Allen, 1964 ; Reif and Allen, 1966 ; Beale and Osborne, 1982 ; Barnstable et al, 1983) indicating a role for the glycoprotein during and after neuronal development and differentiation. Although it has been proposed that Thy-1 may function in the neuritogenic process there is little convincing evidence for such a role (Morris, 1985 ; Leiffer et al, 1984 ; Messer et al, 1984). More recent studies have provided evidence of Thy-1 behaving as a potential signal transduction molecule

(Kroczek et al, 1986 ; Saleh et al, 1988), though direct evidence of such a function in neuronal cells is lacking at the present time. The demonstration that Thy-1 expression can be regulated in PC12 cells by NGF (Richter-Landsberg, 1985 ; Dickson et al, 1986 ; Doherty and Walsh, 1987) suggests that the glycoprotein may function in the differentiated neuron, and indicates that the PC12 cell will provide a useful system for determining the role of the molecule in processes such as neurite outgrowth.

1.2.8. THE ROLE OF NEUROTROPHIC FACTORS.

Trophic interactions are defined as long term interdependent relationships between neurons and the cells they innervate and are thought to involve a specific molecular message that passes between the two cells (Purves, 1988). Most of what is currently understood about the function of neurotrophic factors in neuronal development has been provided by studies with NGF which acts as an intracellular messenger between sympathetic and sensory neurons and their peripheral targets.

1.2.9. THE DISCOVERY OF NGF AND ITS BASIC FUNCTION.

NGF was discovered by Levi-Montalcini and Hamburger (Levi-Montalcini et al, 1954 ; Cohen et al, 1954) as a soluble protein with the ability to cause rapid and extensive growth of nerve fibres from cultured sympathetic and sensory ganglia explants. First isolated from the mouse sarcomas 37 and 180 (Cohen et al, 1954), NGF was later found as a constituent of the mouse salivary gland (Cohen, 1960), and Moccasin snake venom (Cohen and Levi-Montalcini, 1956 ; Cohen, 1959). Initial studies showed that besides its obvious ability to promote axonal outgrowth, the protein was also apparently essential for the survival of developing

sympathetic and sensory neurons (Levi-Montalcini and Angeletti, 1963). Subsequent *in vivo* studies provided dramatic evidence of the absolute requirement of these particular neuronal populations for NGF. For example, administration of anti-NGF antisera to neonatal rodents on a daily basis resulted in the specific and near total disappearance of the entire sympathetic nervous system in what were otherwise normally developed animals (Levi-Montalcini and Angeletti, 1966), while experiments in which pregnant rodents were rendered autoimmune against endogenous NGF (Gorin and Johnson, 1980 ; Johnson et al, 1980) showed that the sensory nervous system of neonates was abnormally developed in the absence of NGF. These pioneering studies were the inspiration for a plethora of subsequent investigations that identified NGF as a trophic and tropic messenger essential to the normal development of the sympathetic and sensory nervous systems.

1.2.10. NGF AS A RETROGRADE NEUROTROPHIC FACTOR.

Surgical and chemical axotomies showed that cell death of innervating neurons could be caused by blocking axonal transport (Angeletti and Levi-Montalcini, 1970 ; Calissano et al, 1976 ; Hendry, 1975), and that this experimental cell death could be successfully alleviated by the administration of a supply of exogenous NGF (Levi-Montalcini et al, 1975 ; Hendry et al, 1975 ; Aloe et al, 1975). Further experiments demonstrated the ability of exogenously supplied NGF to prevent naturally occurring cell death amongst sympathetic neurons innervating the rat superior cervical ganglion (SCG) (Hendry and Campbell et al, 1976), and indeed to actually promote an increase in the number of neurons in the ganglion. Immunosympathectomy studies have demonstrated that the dependency of neurons on NGF for survival declines with the age of the treated animal (Goedert et al, 1978).

In vitro, chick dorsal root ganglia (DRG) neurons have been shown to be dependent on NGF for survival over the E6 to E15 age range, but thereafter to not require NGF for their survival (Levi-Montalcini and Angeletti, 1963 ; Cohen et al, 1964 ; Barde et al, 1980). Similarly, Greene (1977) has reported that NGF is required for the survival of DRG neurons over the age range E8 to E11 and during the E13 to E18 age span cells became gradually less dependent on the factor. Age related dependence for NGF has also been demonstrated for purified neurons from chick (Edgar et al, 1980) and mouse (Coughlin et al, 1978) SG, and rat SCG (Chun and Patterson, 1977).

The observations from the axotomy experiments described above suggested that NGF may be transported along the nerve axon from its point of innervation to the neuronal cell body. This suggestion was confirmed by experiments showing that labelled NGF is taken up from the peripheral tissues at the tip of the nerve axon and is then transported retrogradely along the length of the axon to the cell perikaryon (Stockel et al, 1974 ; Hamburger et al, 1981). Further studies demonstrated that uptake and subsequent internalisation of NGF is dependent on the binding of NGF to NGF-specific receptor molecules (Herrup and Shooter, 1973 ; Sutter et al, 1979 ; Schechter and Bothwell, 1982), present at the surface of the axonal plasma membrane, and to require a process of receptor mediated endocytosis (Burnham and Varon, 1973 ; Norr and Varon, 1975 ; Yankner and Shooter, 1979 and 1982 ; Levi et al, 1980). Taken together these and the earlier pioneering studies of Levi-Montalcini and Hamburger provide convincing evidence for NGF acting as a neurotrophic factor of the PNS, being apparently essential for the survival of several cell types of neural crest origin. With reference to the neurotrophic theory (Purves,

1988), NGF fulfills many of the criteria of a factor that could act as a peripheral signal whose limited supply would serve to select for a limited number of innervating neurons from the excess numbers of axons reaching the target tissue.

Whilst NGF is mainly recognised as a trophic messenger of the sympathetic and sensory ganglia, recent evidence suggests that it may also play a role in the development of the CNS (Levi-Montalcini, 1987). For example, CNS derived neurons from a variety of locations in the brain have been shown to express cell surface receptors for NGF (Szutowicz et al, 1976), to transport NGF retrogradely to the cell body (Seiler and Schwab, 1984), and to be protected by NGF to selective noxious treatments and surgical transections that would otherwise lead to certain cell death (Williams et al, 1986 ; Kromer, 1987). Further evidence for a neurotrophic function for NGF in the brain was provided by studies showing the induction of severe postnatal neuroendocrine syndrome in rat fetuses receiving regular injections of anti-NGF antibodies (Aloe et al, 1981). Furthermore, it has recently been reported that NGF and NGF coding mRNA are synthesized in large quantities by hippocampal nerve cells, thus showing that NGF-producing cells are present in the CNS (Korsching et al, 1985 ; Shelton and Reichardt, 1986).

1.2.11. NGF AS A NEUROTROPIC FACTOR AND INDUCER OF NEURONAL DIFFERENTIATION.

NGF was originally described as a nerve-growth promoting factor (Cohen et al, 1954), and its ability to induce the dramatic outgrowth of fibres from sympathetic and sensory neurons (Levi-Montalcini and Hamburger, 1951 and 1953), as well as from adrenal chromaffin cells (Naujoks et al, 1982) and the pheochromocytoma cell line PC12 (Greene et al, 1977), has become its characteristic feature and indeed is

generally utilised as an assay of NGF activity (Greene et al, 1977). More recently NGF has been recognised as a tropic agent, with in vivo studies demonstrating the ability of microinjected pools of exogenous NGF to stimulate the abnormal growth of sympathetic nerve fibres into the spinal cord and along the dorsal columns to the site of injection (Mensini-Chen et al, 1978). More rigorous evidence of a tropic activity was provided by a series of classical in vitro studies in which neurites were grown along an NGF concentration gradient, and by movement of an NGF-releasing pipette demonstrated to reorientate their growth path in accordance with the repositioned source of growth factor (Letourneau, 1978 ; Gunderson and Barrett, 1980). In reality it is highly unlikely that a concentration gradient of a single tropic factor such as NGF could account for the guidance of axons from multiple populations of sympathetic and sensory neurons through a vast range of specific routes to their widely dispersed target fields. Indeed recent studies with developing sensory nerve fibers indicate that a role for NGF in axonal guidance may be limited, since both the aquirement of axonal NGF receptors and the onset of NGF synthesis by peripheral target tissues appears to be dependent on innervation of peripheral targets by the first pioneering axons to reach the tissue (Davies et al, 1987). However, it is still conceivable that NGF plays a contributory chemotactic role in axonal homing mechanisms and may be of particular importance for the guidance of regenerating nerve fibers following tissue injury.

In addition to its ability to stimulate and guide neurite outgrowth, NGF has been shown to be capable of inducing a number of physical and biochemical changes in sympathetic and sensory neurons (Varon and Skaper, 1983 ; Yankner and Shooter, 1982 ; Guroff, 1985). Of these changes,

the neurotransmitter promoting effects of NGF have been particularly well documented (Chun and Patterson, 1976 ; Patterson, 1978 ; Rohrer et al, 1978 ; Thoenen et al, 1979). These observations indicate that NGF may act as a trigger for specific cellular differentiation responses associated with the generation of the neuronal phenotype. Unfortunately detailed studies on the differentiation promoting effects of NGF have been lacking since most primary neurons that are responsive to the growth factor have been isolated at a stage when they have already been exposed to endogenous NGF. Consequently any effects observed in culture are effectively regenerative responses such as neurite outgrowth. However the primary effects of NGF have been studied in the adrenal chromaffin cell system and in their neoplastic counterparts, the clonal PC12 cell line (for review, see Guroff, 1985). The ability of these cells to respond to NGF treatment with a plethora of biochemical, ultrastructural and morphological changes that result in the generation of a cell with neuronal, rather than glandular phenotypic characteristics, has lead to the PC12 cell line in particular being utilised by many investigators as a model system for probing the mechanism of action of NGF.

1.2.12. THE MECHANISM OF ACTION OF NGF.

NGF is an extremely versatile molecule, acting on a wide variety of neuronal as well as non-neuronal cell types (e.g. Mast cells of the immune system are responsive to NGF). The polypeptide growth factor elicits a diverse range of effects including, increased cell survival (Hamburger et al, 1981), chemotropic attraction (Letourneau, 1978 ; Gunderson and Barrett, 1980), enhancement of neurite outgrowth (Levi-Montalcini and Hamburger, 1951 and 1953), enhanced cellular differentiation (Varon and Skaper, 1983 ; Yankner and Shooter, 1982 ; and Guroff, 1986) and cellular growth (Varon

and Skaper, 1983). In addition NGF promotes the secretion of biologically active molecules such as neurotransmitters and histamine (Chun and Patterson, 1976 ; Patterson, 1978 ; Rohrer, 1978 ; Thoenen et al, 1979 ; Bruni et al, 1982 ; and Mazurek et al, 1986), and has the ability to induce a dramatic phenotypic transformation of the PC12 cell line (Guroff, 1986). At present our understanding of the cellular events that underlie NGF induced activities is poor.

Responsiveness to NGF is primarily dependent on the expression of the cell surface receptor specific for NGF (Yankner and Shooter, 1982 ; Johnson et al, 1986). The NGF receptor is represented on responsive cells by two kinetic classes which differ from one another by approximately 100-fold in their rate of dissociation of NGF (Sutter et al, 1979 ; Schechter and Bothwell, 1982). It is currently believed that the two receptor species are interconvertible forms of the same protein, this is supported by evidence of immunochemical similarity between the low and high affinity molecules and by the identity of a single gene which encodes both forms (Chao et al, 1986 ; Radeke et al, 1987 ; Hempstead et al, 1989). Recent studies show that the NGF-receptor molecule is differentially regulated in different areas of the nervous system during its development and maturation (Buck et al, 1987 ; Yan and Johnson, 1988). NGF-receptor mRNA has been detected in both the PNS and CNS (Buck et al, 1987) and there is the suggestion that the appearance of NGF-receptors at the surface of axons of the sensory nerve ganglia may be closely associated with the onset of synthesis of NGF by target tissues (Davies et al, 1987). Thus, it would appear that regulation of the NGF-receptor at the surface of neuronal cells may act as the key mechanism underlying responsiveness and therefore for many neuronal cells this mechanism will determine their

survival and mode of differentiation. The biochemical basis for this acquisition of NGF-receptor expression is not yet understood.

The active dimeric B-subunit of the 7s NGF complex has been shown to be sufficient for binding to the NGF-receptor and for eliciting the full range of NGF associated responses (Chun and Patterson, 1977 ; Varon et al, 1968). The B-subunit is composed of two 118 amino acid polypeptide chains held together by non-covalent bonds (Scott et al, 1983). In the 7s form the B-subunit coexists in a complex with two other proteins (Varon et al, 1968 ; Stach and Shooter, 1974), one of which has an esteropeptidase activity. Binding of the B-subunit to the NGF-receptor results in the formation of a stable growth factor-receptor complex which is subsequently internalised and transported to the cell body of the neuron where the effects of the growth factor are elicited (Stockel et al, 1974 ; Hamburger et al, 1981). Since retrograde transport of the growth factor-receptor complex has been demonstrated in cells established as totally unresponsive to NGF (Max et al, 1978 ; Ebbott and Hendry, 1978) and as responsiveness to NGF cannot be bestowed upon non-responsive cells by the introduction of the NGF-receptor gene through transfection (Chao et al, 1986 ; Johnson et al, 1986 ; Radeke et al, 1987), it is unlikely that the formation of the growth factor-receptor complex or its subsequent transport are sufficient to elicit the activities of NGF.

The current popular view of the way in which NGF triggers cellular responses is based on evidence for NGF induced activation of specific signal transduction elements in responsive cells (in particular the adrenal chromaffin

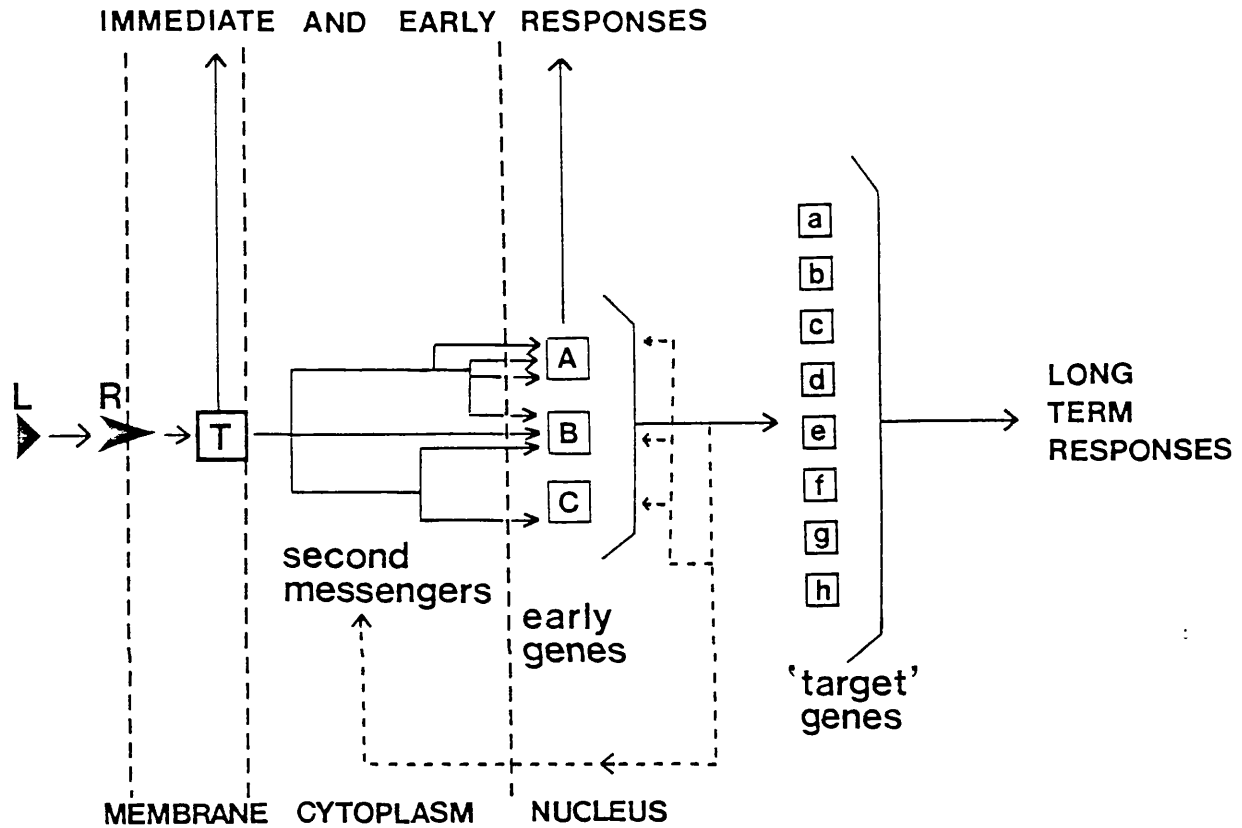


figure 1.3 Working model for receptor mediated gene activation. Key to symbols : L = ligand ; R = Cell surface receptor ; T = Membrane bound signal transduction machinery ; A,B, and C = Early response genes, e.g. c-fos, c-myc, c-jun etc ; a,b,c etc = Target genes, e.g. enzymes, cytoskeletal proteins, cell surface glycoproteins etc.

and PC12 cell types) and envisages the growth factor triggering a series of independently acting but interrelated cascade like pathways that involve multiple reaction steps and produce a vast and diverse range of biologically active molecules that then go on to elicit the actions of NGF (fig 1.3).

Many of the actions of NGF are known to be dependent on the synthesis of new RNA species suggesting that NGF may induce survival and differentiation responses at the level of gene activation (Greene and Tischler, 1982). For example, chick sensory and sympathetic ganglia have been demonstrated to be dependent on both RNA and protein synthesis for NGF mediated neurite outgrowth (Partlow and Larrebee, 1971 ; Burnham and Varon, 1974 ; Mizel and Bamberg, 1976). However studies on neuronal gene regulation have been hampered by the fact that most primary cultures are generated from populations of neurons that have already been exposed to endogenous NGF and are therefore usually dependent on the growth factor for their survival in culture. Consequently it is not possible to study primary inductive events in such cultures, and where differentiation responses may be induced by temporary NGF deprivation and re-exposure, any results should always be interpreted in the light that these responses may alternatively be associated with cell survival as opposed to neuronal differentiation. For these reasons the PC12 cell model has become a very extensively used system for studying the mechanistic details of NGF induced differentiation.

1.2.13. PC12 CELLS AS A MODEL FOR NEURONAL DIFFERENTIATION.

The pheochromocytoma cell line, PC12, has become a popular model for the study of neuronal differentiation and the mechanism of action of NGF. The cell line was originally isolated as a clone of a culture from a norepinephrine producing pheochromocytoma found in New England Deaconess rats (Greene and Tischler, 1976). Its ability to proliferate in the absence of NGF, yet on addition of the growth factor to respond with a dramatic phenotypic transformation from a glandular-type cell to a sensory neuron-type cell has provided the opportunity to study primary NGF induced differentiation responses (for reviews, Greene and Tischler, 1982 ; Guroff, 1985). An additional advantage of the PC12 cell model is its ability in the absence of NGF to generate a large and homogeneous population of cells with which to carry out biochemical studies, it is only very recently that homogeneous populations of primary neurons have been available in numbers that allow biochemical investigation and this requires labour intensive purification and culture methodologies (Bottenstein and Sato, 1985 ; Parnavelas et al, 1988).

In the absence of exogenously supplied NGF the PC12 cell line displays only a few of the characteristics of neuronal cells and more closely resembles the adrenal chromaffin cell, a non- neuronal derivative of the neural crest (Greene and Tischler, 1982 ; Guroff, 1986). Morphologically the PC12 cell appears as a rounded or polygonal body that has a tendency to clump and grow in large aggregates, this being partly due to their poor adhesive properties. Closer morphological analysis reveals the presence of dense-core granules in the cytoplasm. These structures which are believed to be the storage sites for neurotransmitter

substances are also observed in adrenal chromaffin cells and sympathetic neurons (Guroff, 1986). The basic feature of the PC12 cell line which separates it from the adrenal chromaffin cell is its immortality, being able to proliferate indefinitely in the absence of NGF (Greene and Tischler, 1976).

Biochemically the PC12 cell displays many of the characteristic features of neural crest-derived cells (i.e. adrenal chromaffin and sympathetic / sensory neurons), being able to uptake, synthesise, store and secrete the neurotransmitter substances dopamine, norepinephrine, and acetylcholine (Greene and Rein, 1977a,b and c ; Schubert and Kleir, 1977). In addition, PC12 cells have been demonstrated to have the capacity to metabolise the catecholamines and acetylcholine (Greene and Rein, 1978 ; Schubert et al, 1977), and to bear cell surface receptors for acetylcholine and nicotinic cholinergic agonists (Greene and Rein, 1977c), as well as expressing receptors for the muscarinic cholinergic receptors found on adrenal chromaffin cells (Jumblatt and Tischler, 1976).

Addition of NGF to PC12 cell cultures in nanogram amounts results in the induction of a dramatic and complex differentiation response. The differentiation response is time dependent and takes place in specific stages that effectively constitute a differentiation programme. Responses to NGF treatment include morphological and biochemical changes and occur in some cases very rapidly and in others only after a long (days) and continuous period of treatment (Greene and Tischler, 1982 ; Greene, 1984). The most rapid events induced by NGF occur within seconds of its addition to culture medium and are exclusively associated with the plasma membrane suggesting they may occur in the

absence of intracellular signalling or transcription. On addition of NGF the plasma membrane takes on a more simplified surface morphology followed by the appearance of distinct membrane ruffles (Connolly et al, 1979). After only 7 minutes of NGF treatment the membrane ruffles disappear and the cell displays a substantial increase in affinity for its immediate substratum (Schubert and Whitlock, 1977), gradually flattening and spreading out onto its underlying surface. Interestingly, many of these membrane-associated changes have also been observed with cultures of sympathetic neurons starved temporarily of NGF and subsequently reexposed to the growth factor (Connolly et al, 1981), for example, within a few seconds of re-addition of NGF, membrane ruffling is observed and, as with PC12 cells, this effect is lost and superseded by a smoother appearance. Despite an extensive literature on the biochemical changes that accompany these rapid membrane-associated events, including reports of increased uptake of amino acids (McGuire and Greene, 1979), modulation of ion transport mechanisms (Boonstra et al, 1981) and changes in the activity of the $\text{Na}^+\text{-K}^+\text{-ATP}$ dependent pump (Varon and Skaper, 1983), there is little known about the mechanisms responsible for generating the observed morphological events. A candidate mechanism would be NGF induced protein phosphorylations since these events can occur rapidly, with great specificity, and can result in profound alteration of the properties of both catalytic and structural proteins (for reviews see, Cohen, 1985 ; Krebs, 1986). However, although a number of protein phosphorylation events have been observed following NGF binding to its receptor (Nakanishi and Guroff, 1985 ; McTigue et al, 1985 ; Halegoua and Patrick, 1980 ; Hama et al, 1986 ; Hashimoto et al, 1985 ; Landreth and Reiser, 1985) and despite the identification of specific NGF stimulated protein kinases (including

protein kinase C and protein kinase A) (Nakanishi and Guroff, 1985 ; Togari and Guroff, 1985 ; Matsuda and Guroff, 1987 ; Rowland et al, 1987 ; Cremins et al, 1986 ; Halegoua and Patrick, 1980 ; McTigue et al, 1985 ; Hama et al, 1986), there is presently no evidence of phosphorylation playing a mechanistic role in triggering or facilitating the immediate NGF induced differentiation responses.

Long term or so called delayed responses to NGF include, a gradual loss of proliferative activity, cellular hypertrophy and more dramatically the onset and maintenance of neurite outgrowth (Greene and Tischler, 1982 ; Guroff, 1986). These responses are known to be dependent on NGF induced transcriptional changes (Burstein and Greene, 1978 ; Greene, 1984) requiring an apparent lag-phase between addition of NGF and manifestation of the response.

1.2.14. TRANSCRIPTION DEPENDENT RESPONSES OF PC12 CELLS.

The NGF induced responses that occur in PC12 cells with a requirement for new RNA synthesis can be roughly divided into two classes, the early transcription-dependent and late (or delayed) transcription dependent responses (Greene, 1984). The former class of response is on the whole both rapid and transient, and is not a specific effect of NGF. For example, in the case of the c-fos and B-actin genes an increase in transcriptional activity can be detected just 5 and 15 minutes, respectively, after addition of NGF to culture media (Greenberg et al, 1985), these effects peak after 30 minutes of treatment and thereafter decay until transcriptional activity at the gene returns to its basal level. Similar responses have been described for a variety of other genes including ornithine decarboxylase (Greenberg et al, 1985), the proto-oncogenes c-myc and c-jun (Greenberg et al, 1985 ; Bartel et al, 1989), and the transcription

regulation factors NGF1-A and NGF1-B (Milbrandt, 1987 and 1988). In every case these early responses are transient, can be triggered repeatedly by removal and re-addition of NGF to culture medium and occur in the absence of any de-novo protein synthesis. These observations are indicative of the early transcription-dependent responses being direct or primary targets of the NGF mediating intercellular signal. However since EGF, a polypeptide growth factor with mitogenic, but without differentiation effects, on PC12 cells (Huff and Guroff, 1979), has been demonstrated to mimic NGF induction of many of these responses including c-fos, c-myc, c-jun, B-actin and ornithine decarboxylase (Huff and Guroff, 1979 ; Bartel et al, 1989 ; Cho et al, 1989) it is doubtful that their products play any direct role in establishment of the neuronal phenotype. Indeed molecules such as fos, myc, jun, and the NGF1-A and 1-B species have been implicated as regulators of transcription at other genes (Greenberg et al, 1985 ; Changelian et al, 1989 ; Milbrandt, 1987 ; Milbrandt, 1988 ; Rauscher et al, 1988 ; Bartel et al, 1989). Recent evidence suggests that the c-fos and c-jun gene products function together in close association to regulate transcriptional activities at a variety of genes (Rauscher et al, 1988), interacting specifically with these genes at upstream DNA sequences. NGF1-A contains several zinc-finger DNA binding domains which is a characteristic feature of a number of transcription regulation factors found in yeast, Drosophila, Xenopus, rodents and humans (Changelian et al, 1989). Thus, it appears likely that these products of short-term latency responses to NGF are functionally involved in regulating transcriptional events at genes that may be involved in longer-term responses, these being recognised as the target genes for NGF.

Long term or delayed transcription-dependent responses in contrast to the short-latency responses occur over a time course spanning hours to days (Greene, 1984). In addition to the cellular and morphological effects such as loss of cell division and onset of neurite outgrowth there is a vast spectrum of specific biochemical changes that require de-novo RNA and protein synthesis (Burstein and Greene, 1978 ; Garrels and Schubert, 1979 ; Tiery and Shooter, 1986 ; Greene, 1984). These include increases in expression of the TAU and MAP proteins implicated as regulators of neurite outgrowth (Drubin et al, 1985 ; Greene et al, 1983), the neurofilament and vimentin proteins (Varon and Skaper, 1983 ; Dickson et al, 1986 and 1987 ; Lindenbaum et al, 1988), neurotransmitter substances (Guroff, 1985), and cell surface recognition proteins such as Thy-1 (Richter-Landsberg et al, 1985 ; Dickson et al, 1986 ; Doherty and Walsh, 1987), L1 (Mc Guire et al, 1978 ; Fushiki and Schachner, 1985) and N-CAM (Jorgensen and Richter-Landsberg, 1983 ; Prentice et al, 1987). Since these responses occur over a timecourse that is parallel with the timecourse observed for morphological differentiation (Greene et al, 1982 ; Greene, 1984) and as many of the molecules targeted in long-term responses are also associated with neuronal cells, it is likely that they are constituents of the neuronal phenotype and play a functional role in the differentiated cell. At present the intracellular events that mediate NGF induction of long term delayed responses are not clearly understood and the precise roles played by products of these responses in the differentiated cell are still to be defined.

1.2.15. THE PRIMING RESPONSE.

The characteristic morphological response of PC12 cells to NGF, the onset of neurite outgrowth and subsequent

elongation and fasciculation of neuritic extensions to form a dense and complex neuritic network (Greene and Tischler, 1982), can be stimulated by NGF through transcription dependent or transcription independent mechanisms depending on the phenotypic status of the cell (Burstein and Greene, 1978 ; Greene et al, 1982 ; Greene, 1984). In naive cells (previously unexposed to NGF) neurite outgrowth induced by NGF is strictly transcription dependent, as determined by the ability of transcriptional inhibitors to block the response in NGF treated cultures (Burstein and Greene, 1978 ; Greene et al, 1982). PC12 cell cultures that have been grown for several days in the presence of NGF, divested of their neuritic network either by trypsinisation or mechanical disruption , and replated in media containing fresh NGF, display a rapid regenerative response (Greene et al, 1982). In contrast to naive cells, the regenerating (or primed) PC12 cell culture extends neurites within a few hours of replating, generates a neuritic network within 3 to 4 days of further culture, and can mount a full regenerative response to NGF in the presence of transcriptional inhibitors (Greene et al, 1986). It has been proposed that this so called "priming response" reflects the ability of the NGF treated PC12 cell to generate and store "new mRNA species" encoding the proteins required for differentiation (Greene, 1984). Thus, in the replating experiment re-exposure of the primed PC12 cell to NGF results in the tapping of these mRNA stores for de novo protein synthesis and consequently a faster and transcription independent differentiation response. This indicates that NGF has at least two sites of action, one being at the level of activation, the other at the level of protein synthesis. The priming response is not exclusive to the process of neurite outgrowth and in fact appears to operate for all the long-term delayed responses so far studied (Greene, 1984 ;

Doherty and Walsh, 1987 ; Doherty et al, 1987). Consequently it has been suggested that the constituent responses of the priming phenomenon also constitute the essential features of the differentiated phenotype and therefore may resemble the phenotypic features of neural crest derived neurons.

1.2.16. PHYSIOLOGICAL FUNCTION OF DIFFERENTIATED PC12 CELLS.

An important criterion for determining the suitability of the PC12 cell as a model for neuronal differentiation is the ability of the differentiated cell to function in a manner that resembles the normal physiological function of sympathetic and sensory neurons. As already discussed, PC12 cells have the ability to synthesise, secrete, and metabolise neurotransmitter substances and continue to do so following NGF treatment (Guroff, 1985). PC12 cells treated with NGF are electrophysiologically active, with evidence for conductance through voltage-sensitive K^+ , voltage-dependent Ca^{2+} , and Ca^{2+} -dependent potassium mechanisms (O'Lague and Huttner, 1980 ; Arner and Stallcup, 1981), as well as displaying a sodium dependent action potential mechanism not observed in naive cells (Boonstra et al, 1981).

More compelling evidence for the physiological competence of the differentiated PC12 cell is provided by coculture studies in which PC12 cell neurites have been shown to be capable of forming functional synapses with muscle cells (Schubert et al, 1977). Intracellular recordings of micro end-plate potentials from the muscle cells were effectively abolished by bungarotoxin or tubocurarine treatment thus demonstrating that the synapses were physiologically functional, and cholinergic in nature. Therefore, the PC12 cell is of potential use in studies investigating the cell-cell interactions that occur between the differentiated neuron and its peripheral target.

1.2.17. DISADVANTAGES AND LIMITATIONS OF THE PC12 CELL MODEL.

Despite being the most widely used and convenient system for studying NGF induced neuronal differentiation, the PC12 cell line has a number of distinct disadvantages and limitations which dispute its suitability as a neural model. First and foremost PC12 cells are a tumour cell and not a normal derivative of the neural crest, consequently there are a number of conceptual difficulties involved in dealing with the cell line as a model system for an event that is associated with normal neuronal development. Secondly, the PC12 cell is responsive to a variety of biologically active substances that are not currently recognised as effectors to which either adrenal chromaffin cells or sensory and sympathetic neurons respond. For example, EGF (Huff and Guroff, 1979), adenosine (Guroff, 1985), and a number of other less well characterised substances (Edgar et al, 1979 ; Lucas et al, 1980 ; Rieke et al, 1981). A third major disadvantage of the cell is its characteristic plasticity (Guroff, 1985 ; Greene and Reine, 1977a). The cell is extremely susceptible to conditions of culture and will rapidly become heterogeneous in nature under certain conditions. Consequently since independent investigators culture the cells according to the methodology and conditions they favour, the cell line differs in various laboratories. For example, sub-clones of the PC12 cell line have been selected that adhere, grow, and extend neurites across untreated tissue culture plastics (PC12 cells are generally cultured on a substratum consisting of one or more of the extracellular matrix macromolecules). There are also sub-clones that respond to NGF as if it were a mitogen rather than an inhibitor of proliferation (Burstein and Greene, 1983). It is highly likely that between the

original PC12 clone and these extremes are a range of subtle variants, as a consequence, interpretation and comparison of results between independent investigators is very difficult. Therefore, although the PC12 cell provides a powerful tool for probing the mechanistic details of the actions of NGF and for investigating the events that may occur during neuronal differentiation, they must remain a conceptual model from which ideas are eventually tested in a suitable primary neuronal culture.

AIMS OF WORK.

The PC12 cell has been established as a system with which to study the actions of the neurotrophic molecule NGF. Recent studies have determined that NGF induced morphological differentiation of the PC12 cell is associated with changes in the temporal expression of specific cell-surface molecules, of which many have been functionally implicated in events that underlie neuronal development (Prentice et al, 1987 ; Richter-Landsberg et al, 1985 ; Doherty and Walsh, 1987 ; Lemmon and McLoon, 1986 ; Mc Guire et al, 1978). At present the mechanisms by which NGF induces changes in the relative expression of molecules such as N-CAM, Thy-1, L1 and a number of other functional components of the PC12 cell membrane remain poorly defined. In addition the precise role played by these molecules in the differentiating cell is still unclear. The aims of the present study were as follows :-

(a) To investigate the intracellular mechanisms that mediate NGF induced changes in the expression of the specific cell surface recognition molecules Thy-1, N-CAM and L1.

(b) To investigate the effects of pharmacological agents with the ability to modulate intracellular transduction mechanisms, on the expression of cell-surface glycoproteins in naive and NGF treated PC12 cells.

(c) To establish associations, if any, between the morphological status of the PC12 cell and its temporal expression of specific cell surface glycoproteins in an attempt to determine the importance of changes in the relative expression of these functional molecules to the process of neurite outgrowth.

(d) To compare the effects of NGF and fibroblast growth factor (FGF) on the biochemistry of the PC12 cell surface so as to investigate the specificity of the NGF response.

(e) To determine the effects of the putative neuronal microenvironment on NGF induced differentiation of the PC12 cell so as to investigate the potential for epigenetic factors of cellular origin to modulate the neurotrophic activities of NGF.

2.1. APPARATUS.

1. CENTRIFUGES.

- (a) Beckmann J2-21 ultracentrifuge.
- (b) Eppendorf microfuge 5415.
- (c) MSE-CENTAUR 1 bench centrifuge.

2 . ELISA QUANTITATION.

- (a) Dynatech MR700 microtitre plate reader.

3. GEL APPARATUS.

- (a) Biorad vertical gel tank, clamps, glass plates and dividers for SDS-PAGE.
- (b) Biorad protein gel-transfer apparatus.
- (c) BRL horizontal gel tank.
- (d) Hoefffer science instruments slab gel drier.
- (e) Uniscience horizontal mini-gel system.
- (f) U.V. light box.

4. MICROSCOPY.

- (a) Leitz Dialux immunofluorescence microscope.

5. SPECTROPHOTOMETRY.

- (a) Beckmann DU-6 multiscan spectrophotometer.

2.2. MATERIALS.

1. FILTERS

- (a) Acrodisc 0.2 um sterile micropore filters.
- (b) Genescreen nitrocellulose membranes.
- (c) Schleicher and Schull 0.45 um pore nitrocellulose membrane filters.
- (d) Whatman 3mm filter paper.
- (e) Millipore Millicell-HA 0.45 um culture plate inserts.

2. PLASTICS

- (a) Nunc tissue culture plastics. (supplied by GIBCO-BRL)
- (b) Falcon sterile plastic pipettes and tubes.
- (c) Eppendorf centrifuge tubes (supplied by BDH)

2.3. CHEMICALS.

N.B. All chemicals listed were used at AnaLAR grade unless otherwise stated. For convenience chemicals are listed in groups according to their usage in specific methods.

1. ELISA.

- (a) Bovine serum albumin (BSA), TYPE V.....(SIGMA)
- (b) Citric acid.....(BDH)
- (c) Gelatin.....(BDH)
- (d) Hydrogen peroxide (30% V/V).....(BDH)

- (e) Paraformaldehyde.....(BDH)
- (f) O-phenylenediamine.....(SIGMA)
- (g) PBS tablets.....(OXOID)
- (h) Sodium citrate.....(BDH)
- (i) Sulphuric acid.....(BDH)

2. IMMUNOPRECIPITATION AND FLUOROGRAPHY.

- (a) Aprotinin.....(SIGMA)
- (b) Casein Hammerstein.....(BDH)
- (c) Dimethyl sulphoxide (DMSO) fluorography grade..
.....(BDH)
- (d) 2,5-Diphenyloxazole (PPO).....(BDH)
- (e) NP40 detergent.....(BDH)
- (f) Phenyl methyl sulphonyl fluoride (PMSF).(SIGMA)
- (g) Protein A (lysed membranes from culture of
S.aureus).....(SIGMA)
- (h) ³⁵S-methionine.....(Amersham International)

3. POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)
AND IMMUNOBLOTTING.

- (a) Acrylamide monomer (ELECTRAN).....(SIGMA)
- (b) Ammonium persulphate.....(SIGMA)
- (c) 4-chloro-1-naphthol.....(BIORAD)
- (d) Coomassie brilliant blue.....(SIGMA)
- (e) Dithiothreitol (DTT).....(BDH)
- (f) Glacial acetic acid.....(BDH)
- (g) Glycerol.....(BDH)
- (h) Methanol.....(BDH)
- (i) Hydrochloric acid.....(BDH)
- (j) N',N'-methylenebisacrylamide (ELECTRAN).(SIGMA)

- (k) N,N,N',N'-tetramethylethylenediamine (TEMED). (SIGMA)
- (l) Sodium dodecyl sulphate (SDS) (ELECTRAN) (SIGMA)
- (m) TRIZMA base..... (BDH)

N.B. ELECTRAN denotes reagents obtained at high purity.

4. RNA EXTRACTION.

- (a) Antifoam..... (SIGMA)
- (b) Chloroform..... (BDH)
- (c) Diethyl pyrocarbonate (DEPC)..... (BDH)
- (d) DTT..... (SIGMA)
- (e) EDTA..... (BDH)
- (f) Ethanol..... (BDH)
- (g) Heparin..... (SIGMA)
- (h) Hydrochloric acid..... (BDH)
- (i) Isoamyl alcohol..... (BDH)
- (j) Lithium chloride..... (BDH)
- (k) B-mercaptoethanol..... (BDH)
- (l) Phenol..... (BDH)
- (m) SDS..... (BDH)
- (n) TRISMA base..... (BDH)
- (o) Urea..... (BDH)

N.B. All above reagents obtained molecular biology grade and treated as RNAase free.

5. RNA SEPARATION (LARGE AND MINI-SCALE
AGAROSE GEL ELECTROPHORESIS).

- (a) Acridine orange.....(BDH)
- (b) Agarose, Type 11.....(BDH)
- (c) Bromophenol blue.....(BDH)
- (d) DMSO.....(BDH)
- (e) Ethidium bromide.....(BDH)
- (f) Glycerol.....(BDH)
- (g) Glyoxal.....(BDH)
- (h) Sodium dihydrogen phosphate.....(BDH)

6. NORTHERN BLOT AND cDNA PROBE HYBRIDISATIONS.

- (a) BSA.....(BDH)
- (b) Dextran sulphate.....(BDH)
- (c) Ficoll.....(BDH)
- (d) Formamide.....(FISONS)
- (e) Lithium dodecyl sulphate (LDS).....(BDH)
- (f) Magnesium chloride.....(BDH)
- (g) Poly-adenosine (poly-A).....(BRL)
- (h) Poly-guanosine (poly-G).....(BRL)
- (i) Polyvinyl pyrrolidone.....(BDH)
- (j) ^{32}P -deoxycytidine triphosphate (^{32}P -CTP).....
.....(Amersham International)
- (j) Salmon sperm DNA.....(BRL)
- (k) Sodium chloride.....(BDH)
- (l) Sodium citrate.....(BDH)
- (m) Sodium pyrophosphate.....(BDH)

N.B. All above reagents obtained molecular biology
grade.

7. MAMMALIAN CELL CULTURE - BASIC MATERIALS.

- (a) Dulbecco's modified eagle medium (DMEM).....
.....(GIBCO-BRL)
- (b) Foetal calf serum (FCS).....(GIBCO-BRL)
- (c) Glutamine.....(SIGMA)
- (d) Horse serum (HS).....(GIBCO-BRL)
- (e) Insulin, bovine pancreatic.....(SIGMA)
- (f) Methionine-free DMEM.....(GIBCO-BRL)
- (g) Path-O-cyte 4.....(ICN)
- (h) Progesterone.....(SIGMA)
- (i) Putrescine.....(SIGMA)
- (j) Selenium.....(SIGMA)
- (k) Thyroxine.....(SIGMA)
- (l) Transferrin, human.....(SIGMA)
- (m) Triiodothyronine.....(SIGMA)
- (n) Versene solution.....(GIBCO-BRL)
- (o) Vitrogen collagen solution.(Imperial chemicals)

8. MAMMALIAN CELL CULTURE - GROWTH FACTORS, EFFECTORS
AND INHIBITORS.

- (a) A23187 calcium ionophore.....(SIGMA)
- (b) B-cholera toxin.....(SIGMA)
- (c) Cordycepin.....(SIGMA)
- (d) Cholera toxin.....(SIGMA)
- (e) Dibutyryl cAMP.....(SIGMA)
- (f) 1,2-dioctanoyl-sn-glycerol.....(SIGMA)
- (g) EGF.....(SIGMA)
- (h) FGF (recombinant basic bovine).....
.....(Amersham International)
- (i) Forskolin.....(SIGMA)

- (j) NGF (7S B-subunit).....
 .(generous gift of Fidia Research Laboratories)
- (k) Phorbol 12,13-dibutyrate (PdBu).....(SIGMA)
- (l) Phorbol 13-monoacetate.....(SIGMA)
- (m) Phorbol 12-myristate 13-acetate (PMA)...(SIGMA)

2.4. BIOCHEMICALS.

1. PRIMARY ANTIBODIES.

- (a) McAb OX-7 (Anti-Rat Thy-1) (gift of Dr
 N.Barclay)
- (b) McAb RT97 (Anti- Rat neurofilament protein)
 (gift of Prof B.Anderson)
- (c) Rabbit anti-Mouse N-CAM antisera (A4)
- (d) Rabbit anti-Rat Ll antisera (gift of Dr
 F.Rathjen)

2. CONJUGATED ANTIBODIES.

- (a) Sheep Anti-mouse Ig (F(ab')₂ fragment)-HRP.
- (b) Sheep Anti-mouse Ig (F(ab')₂ fragment)-FITC.
- (c) Goat Anti-rabbit Ig (F(ab')₂ fragment)-HRP.
- (d) Goat Anti-rabbit Ig (F(ab')₂ fragment)-FITC.
- (e) Sheep Anti-mouse Ig-Biotin.

N.B. All conjugated antibody reagents were supplied
 by SIGMA.

3. cDNA PROBES.

- (a) B-actin : a full length chick B-actin cDNA fragment.
- (b) NGF-receptor (gift of Dr M.Chao)
- (c) NF68 : a 600-bp Blg/Xho1 fragment from the rat NF68 cDNA p567c.
- (d) Thy-1 : a 355-bp Alu fragment corresponding to bases 34-389 of the rat Thy-1 cDNA pT64 (obtained from Dr N.Barclay).

4. ENZYMES.

- (a) Alkaline phosphatase.....(SIGMA)
- (b) Collagenase.....(SIGMA)
- (c) Trypsin.....(GIBCO-BRL)
- (d) DNAase, RNAase free.....(GIBCO-BRL)

2.5. SUPPLIERS ADDRESSES.

Amersham International PLC, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA.

Beckman RIIC LTD, Progress road, Sands Industrial Estate, High Wycombe, Buckinghamshire, HP12 4JL.

BIORAD Laboratories LTD, Caxton Way, Watford Business Park, Watford, Hertfordshire, WD1 8RP.

British Drug House (BDH) Chemicals LTD, Freshwater Road, Dagenham, Essex, RM8 1RZ

FALCON Labware, Decton Dickinson U.K. LTD, Between Towns Road, Cowley, Oxford, OX4 3LY.

FIDIA Research Laboratories, 35031 Abano Terme (PD), Italy.

FISONS, FSA Laboratory Supplies, Bishop Meadow Road, Loughborough, LE11 0RG.

Gelman Sciences LTD, 10 Harrowden Road, Brackmills, Northampton, NN4 0E2.

GIBCO-BRL, Cowley Mill Trading Estate, Longbridge Way, Uxbridge, UB8 2ZC.

ICN Biomedicals LTD, Limold Road, Cressex Industrial Estate, High Wycombe, Buckinghamshire, HP12 3XJ.

Imperial Laboratories (Europe) LTD, West Portway, Andover, Hants, SP10 3LF.

Millipore (U.K.) LTD, 11-15 Peterborough Road, Harrow, Middlesex, HA1 2YH.

New England Nuclear (NEN) Research Products, Dupont (U.K.) LTD, Biotechnology Systems Division, Wedgewood Way, Stevenage, Hertfordshire, SG1 4QN.

Pharmacia LTD, Pharmacia LKB Biotechnology Div, Midsummer Boulevard, Central Milton Keynes, Bucks, MK9 3HP.

SIGMA Chemical Company LTD, Fancy Road, Poole, Dorset, BH17 7NH.

Whatman Laboratory Sales LTD, Unit 1, Coldred Road,
Parkwood, Maidstone, Kent, ME15 9XN.

2.6. METHODS.

2.6.1. MAMMALIAN CELL CULTURE.

(A) PC12 CELLS : PC12 cells (obtained from L. Greene) were, unless otherwise stated, grown and maintained in a chemically defined medium SATO (Bottenstein, 1985). The medium was comprised of DMEM supplemented with (in mg/L), progesterone, 0.0624 ; putrescine, 16.1 ; thyroxine, 0.4 ; selenium, 0.039 ; transferrin (human), 100 ; Insulin (bovine pancreas), 10 ; and triiodothyronine, 0.377. The medium was further supplemented with path-O-cyte 4 bovine serum albumin solution to a final concentration of 1% (V/V).

Cells were grown at 37°C in an atmosphere of approximately 8% CO₂ on collagen (vitrogen) coated tissue culture plates. Collagen coating being achieved by incubating tissue culture plastics for 2 hours at 37°C with DMEM containing vitrogen solution at a concentration of 7 % (V/V). On every third day of culture, a 50% media exchange was carried out with fresh SATO medium to ensure cell growth.

After reaching subconfluence or after 7 days of continuous culture, cells were subcultured onto freshly collagen coated dishes. For subculture, cells were washed twice in warm sterile PBS, then incubated for 5 to 7 min at 37°C with versene solution containing trypsin at a final concentration of 0.05% (W/V). Detached cells were transferred by 10 ml pipette to 20 ml sterile centrifuge tubes and collected by centrifugation at 1000 rpm for 10 min

in an MSE-CENTAUR bench centrifuge. The cell pellet was resuspended into SATO medium by trituration with a 10 ml pipette followed by further trituration with a syringe and 19-gauge needle. Single cells were then counted using either a haemocytometer or a coulter counter, and seeded onto collagen coated dishes at appropriate densities (For a 100mm dish, approximately 4×10^6 cells). For storage, cells were resuspended into SATO medium supplemented with DMSO at a concentration of 10% (V/V) and maintained in liquid nitrogen.

For ELISA determinations cells were seeded into collagen coated 96 well microtitre plates at a density of between 10 and 20×10^3 cells per individual well.

For NGF-priming, cells were seeded at a density of 4×10^6 cells on collagen coated 100 mm dishes in SATO medium containing 50 ng/ml NGF. Fresh NGF was added to cultures every third day, with cells being utilised after 5-7 days of continuous treatment.

(B). HUMAN SKIN FIBROBLASTS : Primary cultures of skin fibroblasts were prepared from human skin biopsies (obtained from the Royal Marsden Hospital, South Kensington, London) by incubation and agitation at 37°C in a solution of versene containing BSA (0.1%, W/V), collagenase (0.1%, W/V) and trypsin (0.15% W/V). After 15 min single cells were collected by filtration (through 50 and 20 gauge muslin filters) and centrifugation, and were then resuspended into DMEM medium supplemented with FCS at 10% (V/V) and plated onto a 150 mm plastic culture dish. Cells were grown at 37°C in an atmosphere containing 8% CO_2 until reaching confluence. Harvesting and subculturing of primary cultures

was achieved by trypsinisation as described above for PC12 cells.

For coculture studies, fibroblasts were grown to confluence in the individual wells of 96 well microtitre plates, or on 35 mm culture dishes. Prior to addition of PC12 cells, fibroblast monolayers were washed twice with sterile PBS followed by a further two washes with SATO medium. PC12 cells were then seeded onto monolayers in SATO medium at a density of 20,000 cells (microtitre wells) or 50,000 cells (35 mm dishes).

(C). MUSCLE AND GLIOMA CELL LINES : The muscle cell lines G8-1 (Christian et al,1977) and C2 (Yaffe and Saxel,1978), were grown in DMEM supplemented with foetal calf serum (20% V/V) in an atmosphere of 8% CO₂ at 37°C until confluent. Cells were subcultured as described above for PC12 cells. Myotubes were established by exchanging culture medium for DMEM supplemented with horse serum (5% V/V) and foetal calf serum (2.5% V/V) when cultures had just reached confluency.

The C6 glioma cell line was grown under identical conditions as those described for the muscle cell lines.

For coculture studies, muscle and glioma cell lines were treated in the way described for human skin fibroblasts.

(D) FREEZING AND THAWING CELL CULTURES : For long term storage of cell lines and primary cultures, cells were suspended (at 2.5×10^6 cells per ml) in culture media (DMEM / 10% (V/V) FCS or SATO) supplemented with 10% (V/V) sterile DMSO and frozen and stored in liquid nitrogen. When required for culture, frozen stocks were removed from liquid

nitrogen, allowed to reach room temperature, cells were then recovered by centrifugation (1000 rpm, 10 min at 20°C) and washed twice with fresh DMEM or SATO media to remove all traces of DMSO before being plated onto culture dishes.

(E) PREPARATION AND CONCENTRATION OF CONDITIONED MEDIA : SATO medium was conditioned over confluent cultures of skin fibroblasts and C2 myotubes for 1 or 4 days. The conditioned medium was collected, filtered through a 0.2 um filter to remove debris and then added directly to PC12 cells. In addition, DMEM was conditioned over G8-1 myotubes and C6 glioma monolayers for 24 hr, concentrated approximately 10-fold by ultrafiltration using a vacuum pump and dialysis membrane system, then added to PC12 cell cultures.

2.6.2. ELISA QUANTITATION OF PC12 CELL SURFACE GLYCOPROTEINS AND NEUROFILAMENT (NF) PROTEIN.

PC12 cell cultures growing in the individual wells of 96 well microtitre plates were fixed by a 50% medium exchange with a 4% paraformaldehyde solution made up in fresh DMEM. After a period of 60 min fixation at 20°C, a 100% medium exchange was carried out with the same fixative solution. After a further 60 min fixation cells were washed three times with PBS or, in the case of cultures for neurofilament determinations, were permeabilised by incubation with methanol (100%) at -20°C. Fixed cultures were then blocked for non-specific protein binding by incubation with a 0.5% gelatin/PBS solution for 60 min at 20°C. Cultures were then incubated with primary antibody solutions in PBS containing carrier BSA (0.5%) for either 60 min at 20°C or alternatively overnight at 4°C (see materials section 2.31 for N-CAM, Thy-1, L1 and neurofilament antibodies used in ELISA determinations). The anti-N-CAM antisera was used at

a 1 in 2000 fold dilution, anti-L1 antisera at 1 in 1000, McAb OX-7 at 1 in 50 dilution of tissue culture supernatant, and McAb RT97 at a 1 in 1500 dilution of ascites. After the incubation period primary antibodies were removed from cultures by three 5 minute washes with a BSA/PBS solution. Cultures were then incubated with horseradish peroxidase (HRP) conjugated secondary antibodies for a further 60 min at 20 c, for rabbit antisera a 1:1000 dilution of HRP-conjugated goat anti-rabbit IgG (F (ab')₂ fragment) was used while McAb were detected using a 1:1000 dilution of HRP-conjugated Sheep anti-mouse IgG (F (ab')₂ fragment). Second antibodies were removed by four 5 minute washes with PBS followed by two further washes with distilled water to remove phosphate which inhibits the OPD reaction. Finally cultures were incubated with 50 ul of a 0.2% (W/V) solution of OPD in citrate buffer (pH 5.0) containing 0.02% hydrogen peroxide. After 10 to 30 min incubation in the dark at 20^o C conversion of OPD to its oxidised product was stopped by the addition of 50 ul of a 12.5% solution of sulphuric acid. The optical density of the final reaction product was determined at 490 nm (using a reference filter at 630 nm) using a Dynatech MR700 microtitre plate reader. Background binding of secondary antibodies was normalised in all readings by subtraction of the O.D. value for cultures incubated with secondary antibody only.

2.6.3. POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

(A) PREPARATION OF SAMPLES : Cell cultures (approximately 4 x 10⁶ cells) were solubilised by scraping into a small volume (300 ul) of SDS-protein sample buffer (0.125 mM Tris-HCl pH 6.8 containing 2% (W/V) SDS and 10% (V/V) glycerol) supplemented with 50 ug/ml PMSF to inhibit proteolysis. The total protein content of the soluble fraction was then

determined by the Bradford protein assay (Bradford,1976) and approximately 50ug was denatured by boiling for 2 min in the presence of 0.1 M DTT and 0.2% (W/V) bromophenol blue. The soluble fraction was recovered by centrifugation (5 min, 14,000 rpm in an Eppendorf microcentrifuge) and stored at -20°C until use.

(B) PREPARATION OF SDS-POLYACRYLAMIDE SLAB GELS :

Solubilised, denatured proteins were separated according to molecular size by SDS-PAGE using the tris-glycine discontinuous buffer system of Laemmli (1970). A 1.5 mm thick, 14 cm long, 7.5% polyacrylamide gel was prepared by the addition of 11.25 ml 30% acrylamide mixture (30% to 0.8% mixture of acrylamide monomer and bisacrylamide) to 16.8 ml 1M Tris-HCl (pH 8.8), 16.8 ml distilled water, 225 ul 20% (W/V) SDS solution, 150 ul 10% (W/V) ammonium persulphate solution (freshly prepared) and 30 ul TEMED (always added last and after filtering and degassing of the acrylamide mix). A 2.0 cm long, 3.0% stacking gel was then poured and set on top of the 7.5% running gel by the addition of 2.0 ml acrylamide mixture to 2.5 ml 1M Tris-HCl (pH 6.8), 15.4 ml distilled water, 100 ul 20% SDS solution, 100 ul ammonium persulphate solution and 20 ul TEMED.

(C) ELECTROPHORESIS : Samples were electrophoresed through the 3% stacking gel at a constant current of 25 mA and then through the 7.5% running gel at a constant current of 40 mA, using a running buffer consisting of 3.03 g/L Tris base, 14.42 g/L glycine and 1.0 g/L SDS (final pH adjusted to 8.3).

(D) COOMASSIE STAINING : For visualisation of all soluble PC12 proteins, gels were incubated at 20°C overnight in a 0.0125% (W/V) Coomassie brilliant blue stain (solubilised

in a 7.5 : 40.5 : 52 mixture of methanol : glacial acetic acid : water). Estimation of molecular sizes for component proteins was made by reference to molecular weight size markers (obtained from SIGMA) electrophoresed adjacent with the PC12 protein sample.

For analysis of specific PC12 cell proteins by antibody staining, gels were treated as described below.

2.6.4. IMMUNOBLOTTING.

Size separated PC12 cell proteins were transferred from 7.5% SDS-PAGE slab gels to 0.45 um pore nitrocellulose membrane filters (supplied by Schleicher and Schull) electrophoretically in Laemmli buffer (in the absence of SDS) at a constant 30 V for 18 hr at 4°C. To ensure transfer had taken place, the presence of pre-stained molecular weight size markers (SIGMA) on the nitrocellulose filter were determined, and post-transfer gels were stained for protein with Coomassie blue.

Transfers were prepared for immunostaining by first, washing in an excess of PBS and then incubating for 60 min in PBS containing 2% (W/V) casein Hammerstein to block non-specific protein binding. Transfers were then sealed in plastic bags with a volume of PBS containing 1% (W/V) casein and primary antibody at its appropriate dilution (1:1000 of McAb RT97 ascites), and were incubated for 60 min at 20°C. Primary antibody solutions were decanted and the transfers washed for three 10 minute (minimum) periods in a 0.5% casein/PBS solution before being sealed in a fresh plastic bag containing the latter solution supplemented with HRP-conjugated second antibody at a dilution of 1:1000. After a further 60 minute incubation at 20°C transfers were washed

twice in PBS followed by four 10 minute washes in TRIS-buffered saline (pH 7.4). Finally, the transfers were incubated in a reaction mixture composed of 50 ml TBS containing 30 ul 30% hydrogen peroxide solution and 30 mg of 4-chloro-1-naphthol dissolved in 10 ml methanol. On the visualisation of reactive protein bands the reaction was stopped by washing transfers in an excess of distilled water.

2.6.5. IMMUNOFLUORESCENCE MICROSCOPY.

Plate cultures (35mm) of PC12 cells (50,000 cells) growing on a substratum of either collagen or a non-neuronal cell monolayer were stained for immunoreactive Thy-1 and neurofilament protein by one of three alternative methods :

(A) INDIRECT IMMUNOFLUORESCENCE STAINING OF FIXED CELL CULTURES USING FITC CONJUGATED IgG.

Cultures were treated essentially in the same manner as for an ELISA estimation. Cells were fixed by a 50% media change with DMEM containing paraformaldehyde (4%) for a minimum of 60 min after which they were fixed for a further 60 min following a 100% media change with the same fixative. Cultures were then blocked for non-specific binding with gelatin and incubated with primary and secondary antibodies as previously described (see ELISA protocol), with the exception that an FITC-conjugated antibody (1:1000 dilution) was used instead of an HRP-conjugated antibody. After extensive washing cultures were mounted with coverslips in 80% (V/V) glycerol/PBS and viewed on x20 and x40 objectives with a Leitz Dialux microscope. For control staining, omission of primary antibodies gave an indication of background immunofluorescence.

(B) INDIRECT IMMUNOFLUORESCENCE STAINING OF FIXED CELL CULTURES USING A BIOTIN CONJUGATED IgG.

Cultures were treated in an identical manner to that described above with the exception that the FITC-conjugated antibody was replaced with a biotin-conjugated sheep anti-mouse IgG (3 ug/ml specific antibody) and staining was then visualised by a final incubation (60 min at 20°C) with fluorescein labelled streptavidin (1 ug/ml) (SIGMA).

(C) INDIRECT IMMUNOFLUORESCENCE STAINING OF LIVE CULTURES.

Cultures were washed twice with a Hepes/DMEM staining media (5 ml of 10 times concentrated DMEM added to 45 ml distilled water containing 120 mg Hepes and 18.5 mg sodium hydrogen carbonate) supplemented with 10% foetal calf serum. Cultures were then incubated with primary and secondary antibodies in the same media with careful washing between incubations. Stained cells were mounted and viewed as described above.

2.6.6. METABOLIC LABELLING AND IMMUNOPRECIPITATION OF N-CAM POLYPEPTIDES.

PC12 cell cultures (approximately 4 million cells on a collagen coated 100mm plate) were grown for 44 hr and then for a further 4 hr in SATO medium made up in methionine-free DMEM supplemented with 10 uCi/ml ³⁵-S labelled methionine. Cultures were then washed four times (for a minimum of 10 min each time) with warm PBS and extracted into a minimum volume (usually 300 ul) of a 10 mM TRIS-HCl buffer (pH 7.4) containing 2.5 mM EDTA, 1% (W/V) NP40, 1 mM PMSF and 2000 kallikrein inactivation units of trasylol (aprotinin). Extracts were centrifuged at 14,000 rpm for 15 min at 4°C in an Eppendorf microcentrifuge, pellets discarded, and supernatants incubated for 30 min on ice with 50 ul of protein A that had been preabsorbed with an equal volume of

rabbit pre-immune sera. Supernatants were cleared by centrifugation (2 min at 14,000 rpm, 4°C) transferred to fresh eppendorf tubes and made up to 0.2 M with respect to casein and 0.5 M with respect to sodium chloride. Supernatants were then incubated at 4°C for 18 hr after the addition of rabbit anti-mouse N-CAM antisera at a final dilution of 1:50. After 18 hr, 50 ul pre-washed (with 10 mM TRIS-HCl/2.5 mM EDTA buffer, pH 7.4) protein A was added to supernatants which were then incubated for a further 60 min at 4°C with constant mixing. Bound protein A / Ig complexes were collected by centrifugation (maximum rpm for 2 min at 4°C) and washed four times (10 min minimum each washing) in extraction buffer supplemented with 0.5 M sodium chloride followed by two further washes in the same buffer further supplemented with 0.1% SDS. Washed protein A / Ig complexes were pelleted, resuspended in protein sample buffer containing 0.1 mM DTT and 0.2 % (W/V) bromophenol blue, boiled for 10 min, centrifuged (maximum rpm for 2 min at 20°C) and separated on 7.5% polyacrylamide SDS-PAGE gels as previously described.

For visualisation of separated ³⁵S-methionine labelled immunoprecipitates, polyacrylamide gels were fixed for 18 hr in standard fixative (Methanol : Glacial acetic acid : Water, 40 : 7 : 53), and treated for fluorography as follows. The fixed gel was shaken gently for 60 min at 20°C in neat DMSO, this wash was repeated with fresh DMSO followed by a further 60 minute wash in a PPO (20% W/V) / DMSO mixture. The gel was then shaken gently in distilled water for 60 min (during which fresh water was added every 15 min), dried under vacuum onto 3 mm Whatman filter paper, and exposed to Fuji-RX X-ray film at -70°C in the presence of an intensifying screen.

2.6.7. MTT COLORIMETRIC ASSAY.

A colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay based on the method described by Mosmann (1983) and including the modifications of Manthorpe et al (1986) was adapted for use with microtitre plate cultures of PC12 cells. The assay is based upon the cleavage of the tetrazolium ring of MTT, a reaction that will only take place in active mitochondria and will thereby determine the activity of a variety of dehydrogenase enzymes, as well as providing an index of the viability of a cell culture. After a given period of growth, cultures were supplemented with MTT to a final concentration of 0.5 mg/ml by the addition of a concentrated solution of MTT (dissolved in DMEM) to their culture medium. After an incubation of 2 hr at 37°C, conversion of MTT to its formazin product was stopped by the addition of a volume of a 0.08 N HCl solution in isopropanol equal to the total reaction volume of the microwell. Cells were lysed and the MTT reaction product dissolved by vigorous pipetting and shaking to yield a dark blue color with an O.D. which was measured at 570 nm.

2.5.8. PHOSPHATASE TREATMENT OF CELL CULTURES.

Cultures were thoroughly washed with TBS (pH 8.0) and incubated with the same buffer containing alkaline phosphatase (up to 100 ug/ml), for up to 24 hr at 37°C. Control cultures were treated with alkaline phosphatase under similar conditions but in PBS instead of TBS.

2.6.9. PROTEIN QUANTIFICATION.

Total cellular protein contents of cultures were determined by the Bradford protein assay (Bradford, 1976) using the Bradford reagent supplied by Biorad. PC12 cell cultures (35 mm dishes) were washed in PBS and lysed in a 0.2% solution of SDS (made up in PBS). Lysates were serially diluted, made up to a final volume of 800 ul with PBS and mixed with 200 ul of the Bradford reagent. The O.D. of the reaction product was measured at 580 nm and protein concentrations estimated from a freshly prepared protein (BSA) standard curve in the 0 to 25 ug/ml range.

2.6.10. RIBONUCLEIC ACID (RNA) EXTRACTION FROM CELL CULTURES.

(A) PREPERATION OF EQUIPMENT AND MATERIALS : All glassware, pipette tips, eppendorf centrifuge tubes and distilled water were treated prior to use for removal of RNAase enzymes. DEPC was added to distilled water at a 1:1000 (V/V) dilution, left to stand for 18 hr at 20°C and then autoclaved. All glasswear was baked at 80°C for 2 hr, all plastics were immersed in distilled water containig fresh DEPC and after 18 hr treatment autoclaved.

(B) CELL LYSIS : 150 mm dish cultures of PC12 cells (seeded at a density of 12×10^6 cells) were washed in warm PBS and then scraped into 10 ml lysis buffer consisting of 6M urea, 3M lithium chloride, 10 mM sodium acetate (pH 5.2), 200 ug/ml heparin, 0.1% (W/V) SDS, and antifoam reagent (at a final dilution of 1:340). Cell lysis was aided by trituration through a 19-gauge needle and syringe followed by sonication (two 30 sec, 7 um exposures). Lysates were left to stand at 4°C for 18 hr, then triturated and sonicated as before. Precipitates were collected by

centrifugation (20,000g for 30minutes at 4°C), resuspended in 5 ml of a freshly prepared 8M urea / 4M lithium chloride solution, then incubated for 60 min at 4°C.

N.B. All following steps carried out using RNAase free solutions and equipment (see, preparation of equipment and materials).

(C) PHENOL:CHLOROFORM EXTRACTION : Precipitates were collected by centrifugation (20,000g for 30 min at 4°C) and resuspended in 2.5 ml of 10 mM TRIS-HCl buffer (pH 7.5) containing 1 mM EDTA, 0.2M sodium acetate, and 0.1% (W/V) SDS. This aqueous phase was extracted with an equal volume of a 50:50 (V/V) mixture of phenol (freshly equilibrated in 0.1M TRIS-HCl, pH 7.5, and containing 0.1% (W/V) hydroquinone and 0.2% (W/V) B-mercaptoethanol) / chloroform (containing isoamylalcohol, 1 part to 24 parts chloroform) by vortexing for a few seconds. Aqueous and organic phases were resolved by centrifugation (3000g for 10 min at 4°C). The aqueous phase was carefully transferred to a fresh tube and re-extracted with a fresh volume of phenol / chloroform mixture. Organic phases were back-extracted with a second aqueous phase to ensure maximal RNA recovery, aqueous phases were pooled and RNA precipitated for 18 hr at -20°C by the addition of 2.5 volumes of absolute alcohol and 0.1 volumes 3M sodium acetate.

(D) QUALITATIVE AND QUANTITATIVE ANALYSIS OF RNA EXTRACTS: Precipitated RNA samples were recovered by centrifugation (20,000g for 30 min at 4°C), salts removed by one wash in 70% ethanol and pellets dried under vacuum. RNA pellets were resuspended in 50 ul DEP-treated distilled water and the total RNA content of 1 ul determined by an O.D. measurement at 260 nM (1.0 O.D = 40 ug RNA). Qualitative

analysis of RNA extracts was determined by examining an ethidium bromide stained aliquot (1 ug) on an agarose gel (1% W/V). Agarose minigels were prepared by boiling 0.5 grams type 111 agarose into 50 mls of 90mM Tris-borate buffer containing 0.2 mM EDTA (pH 8.0), cooling to 50°C, adding 5 ng/ml ethidium bromide and pouring while still warm into a horizontal minigel tank. Samples were loaded into wells and electrophoresed for 30 min at a constant 30 volts. Ethidium stained RNA bands and any contaminating DNA were visualised by viewing gels on a U.V light box. Samples with a high contamination of DNA were treated for 30 min at 37°C with 200 units of RNAase free DNAase, phenol extracted, ethanol precipitated and their RNA content reassessed.

2.6.11. NORTHERN BLOTTING AND DETECTION OF mRNA SPECIES BY SPECIFIC cDNA PROBE HYBRIDISATION.

(A) NORTHERN BLOTTING : Samples of total cellular RNA were denatured with glyoxal as described by Thomas (1983). RNA samples (up to 10 ug) in 3.7 ul DEP-treated water were incubated in the presence of 2.7 ul 6M glyoxal, 8.0 ul DMSO, and 1.6 ul 0.1 M sodium dihydrogen phosphate buffer (pH 6.5) at 50°C for 60 min. Samples were cooled to room temperature and added to 4 ul sterile loading buffer (0.01 M sodium dihydrogen phosphate buffer (pH 6.5), 50% glycerol (V/V), and 0.4% (W/V) bromophenol blue). Electrophoretic separation of denatured RNA samples on 1.2% agarose gels was performed in 10 mM sodium dihydrogen phosphate buffer (pH 6.5) at a constant 35 volts for approximately 18 hr with a continuous circulation of running buffer and with cooling. For RNA size estimation a sample of total mouse brain RNA was run parallel with samples for northern transfer. The size marker track was removed and stained in a 33 ug/ml solution of acridine orange for 30 min and 28S and 18S ribosomal RNA bands were visualised under UV light. Separated RNA species

were transferred to Genescreen nitrocellulose membranes by capillary blotting in a 25 mM sodium dihydrogen phosphate buffer (pH 6.5) for 18 hr. Filters were removed and baked at 80°C for 2 hr.

(B) ³²P dCTP LABELLING OF cDNA PROBES : Complementary DNA probes were radioactively labelled using the Amersham Nick Translation kit. Approximately 100 ng DNA insert or plasmid in 12 ul DEP-treated water was boiled for 2 min, cooled on ice and then incubated at 15 c for 90 min with 1 ul E.coli DNA polymerase I, 2 ul nucleotide buffer solution (containing dATP, dGTP and dTTP at 25mM concentrations) and 5 ul ³²P dCTP (50 uCi). Samples were heat inactivated for 10 min at 70°C and labelled DNA seperated from unincorporated nucleotides on a Pharmacia Nick column, single stranded DNA inserts being eluted with 10 mM Tris-HCl (pH 7.5)/ 1 mM EDTA buffer in a volume of 400 ul after an initial 400 ul pre-elution volume. Efficiency of incorporation of radiolabelled dCTP into DNA probes was determined by liquid scintillation counting using a Beckman LS-700 B-particle counter.

(C) HYBRIDISATION OF ³²P-dCTP LABELLED cDNA PROBES TO RNA

TRANSFERS : Baked Genescreen filters were soaked in 0.025 sodium dihydrogen phosphate buffer (pH 6.5) for 5 min, transferred to a dry dish and soaked with boiling 20 mM Tris-HCl buffer (pH 8.0) and left to cool for 60 min. Filters were then pre-hybridised for 4 to 16 hr at 42°C in a sealed plastic bag containing prehybridisation buffer (50% V/V) freshly deionised Formamide, 0.2% (W/V) Polyvinyl-Pyrrolidone, 0.2% BSA (W/V), 0.2% (W/V) Ficoll, 0.05 M Tris-HCl (pH 7.5), 1.0 M Sodium Chloride, 0.1% (W/V) Sodium Pyrophosphate, 1% (W/V) SDS, 10% (W/V) Dextran Sulphate and 100 ug/ml denatured salmon sperm DNA. Radioactively

labelled cDNA probes in 400 ul Tris-HCl / EDTA buffer were boiled for 10 min, mixed with 5 ml prehybridisation buffer and then added directly to the bag containing the filter. Hybridisations were carried out for 18 hr at 42°C after which the filter was removed from the bag and washed in 2 X Saline Sodium Citrate (pH 7.0) (1 X SSC = 0.15 M Sodium Chloride, 15mM Sodium Citrate) at 20°C for 1 hour, 2 X SSC with 1% (W/V) SDS at 65°C for 1 hour and finally with 0.1 X SSC at 20°C for 1 hour. For visualisation of specific cDNA / RNA hybridisations, filters were exposed to Fuji X-ray film for between 1 and 14 days at -70°C in the presence of an intensifying filter.

(D) STRIPPING FILTERS : For re-use of RNA filters, radiolabelled DNA probes were stripped by incubation for 1 hour at 65°C in a solution of 0.005 M Tris-HCl (pH 8.0), 0.2 uM EDTA, 0.05% (W/V) Sodium pyrophosphate, 0.002% Polyvinylpyrrolidone, 0.02% BSA and 0.002% Ficoll. Filters were then probed with a second cDNA or stored at -70°C for future use.

CHAPTER 3 : REGULATION OF THY-1 EXPRESSION DURING PC12 CELL DIFFERENTIATION.

3.1. INTRODUCTION.

The Thy-1 glycoprotein is one of the most extensively characterised molecules of the neuronal cell surface (Morris, 1985). First described in lymphoid tissue (Reif and Allen, 1964), the glycoprotein is now recognised as a major constituent of the plasma membrane of neuronal cells and their axons, accounting for between 2.5 and 7.5 % of the total exposed protein of nervous tissue (Beech et al, 1983). Consequently Thy-1 is thought to be a potentially important functional component of the neuronal cell surface. Evidence of its tissue specific regulation during neuronal development (Reif and Allen, 1964 and 1966), and data from carbohydrate analyses suggesting a unique "glycoform" for brain Thy-1 relative to thymus Thy-1 (Parekh et al, 1987), indicate that a specific function may exist for Thy-1 in the developing nervous system.

The structure and biochemistry of Thy-1 has been extensively documented (for review, see Morris, 1985). In the mouse, Thy-1 exists in two allelic forms (Thy-1.1 and Thy-1.2) which are immunologically distinct and are generally considered to be independent molecules (Zhou et al, 1985). Mouse brain Thy-1 consists of a 111 amino acid core polypeptide (Campbell et al, 1981) which adopts a secondary structure that closely resembles the B-pleated sheet arrangement found in the variable domains of immunoglobulin molecules (Cohen et al, 1981) and indeed is widely believed to be the simplest and possibly the ancestral member of the immunoglobulin superfamily (Williams and Gagnon, 1982). The allelic difference between Thy1.1 and Thy1.2 has been shown to be due to variation at amino acid

89 (Williams and Gagnon, 1982). Post-translational modifications include the addition of three large N-linked carbohydrate chains at the asparagine residues 23, 75 and 99, with the effect that the apparent molecular weight of the molecule is increased from 17.5 kd to approximately 25 kd (Kuchel et al, 1978) (as determined by SDS-PAGE). It has been suggested that a substantial proportion of the protein's external face is probably obscured by carbohydrate (Burton, 1985). Thus the evidence that brain Thy-1 may possess a unique carbohydrate structure (Parekh et al, 1987) and that Thy-1 carbohydrate content can be modulated during cellular differentiation (Morrison M.H. et al, 1986), strongly suggests that a specific form of the Thy-1 glycoprotein may exist in nervous tissues and that this molecule may play an important role in the developing nervous system. One of the most remarkable features of the Thy-1 molecule is a glycosyl-phosphatidylinositol membrane anchor which is covalently attached to the carboxyl terminus of the core protein (Low and Kincade, 1985 ; Tse et al, 1985) and serves to attach the entire molecule to the plasma membrane (Low, 1987). Structures of this type have been associated with important physiological mechanisms, such as the generation of second messengers and modulation of cell surface glycoprotein expression (Low, 1987).

Despite the extensive literature describing the biochemistry of Thy-1 its function remains obscure. The molecule's close structural resemblance to the immunoglobulins (Williams and Gagnon, 1982), its extensive glycosylation (Williams and Gagnon, 1982), and novel membrane anchoring (Low and Kincade, 1985 ; Tse et al, 1985), are indicative of Thy-1 functioning as a recognition molecule, possibly being involved as a component of the neural cell's adhesive and / or homing machinery. There is

indirect evidence from monoclonal antibody studies (Leiffer et al, 1984 ; Messer et al, 1984) to suggest that Thy-1 may play a role in the neuritogenic process possibly acting as a receptor for growing neurites, however there is currently no direct evidence to support this theory. More recently it has been demonstrated that Thy-1 can mediate the modulation of intracellular levels of calcium in cells of both nervous (Saleh et al, 1988) and thymus (Kroczek et al, 1986) origins and in B-lymphocytes transfected with Thy-1 cDNA (Kroczek et al, 1986). These observations strongly implicate Thy-1 as a signal transduction element though mechanisms of action and physiological consequences of its activities remain unknown.

The Thy-1 core protein is encoded by mRNA species derived from a single gene composed of four exons (Giguere et al, 1985). An understanding of the regulatory mechanisms that control the synthesis and expression of Thy-1 during neuronal differentiation may provide important clues as to its function in the differentiating cell. At present, despite the isolation and characterisation of the regulatory sequences present at the 5' upstream end of the Thy-1 gene (Giguere et al, 1985), little is known about the cellular events that lead to the changes in expression of Thy-1 in developing nervous tissues (Morris, 1985). The PC12 cell system is a potential system for studying regulation of Thy-1 expression during trophic factor induced differentiation, since NGF induced morphological differentiation of the cell line is accompanied by an elevation of cell surface immunoreactive Thy-1 (Doherty and Walsh, 1987 ; Richter-Landsberg et al, 1985), and by an increase in the rate of synthesis and accumulation of the mRNA species encoding the protein (Dickson et al, 1986) . Furthermore, since Thy-1 is associated with the cytoskeleton of the PC12 cell (Richter-Landsberg et al, 1985 ; Bourgignon

et al, 1987), a structure which must undergo extensive alterations during PC12 cell differentiation as indicated by the observed morphological changes and accompanying alterations in cytoskeletal protein abundance (Greene et al, 1983 ; Lee and Page, 1984 ; Drubin et al, 1985), the PC12 cell system may also provide a model with which to determine the contribution made by Thy-1 to the changes in cellular shape and morphology in the differentiating cell.

In the current study the relationship between cell surface Thy-1 expression and morphological status of the PC12 cell has been investigated. In addition attempts have been made to elucidate the cellular signals that mediate NGF induced changes in Thy-1 gene activity.

An ideal strategy for these studies was to characterise the effects on Thy-1 expression of agents that are able to partially mimic NGF induced differentiation and which have been identified as agonists of specific second messenger molecules. Phorbol esters (for review, see Blumberg, 1980), calcium ionophore A23187 (Contreras and Guroff, 1987), and cholera toxin (Hynie and Sharp, 1972 ; Levinson and Blume, 1977 ; Cassel and Salinger, 1977), stimulate the protein kinase C, phosphoinositide, and protein kinase A / cAMP signal transduction systems respectively, and were found to mimic NGF induced morphological differentiation to varying degrees. The effects of these agents on Thy-1 expression in naive and NGF treated cells are described below.

3.2. RESULTS

3.2.1. NGF-INDUCED INCREASES IN THE CELL SURFACE EXPRESSION OF THY-1 : A 4 DAY TIMECOURSE.

PC12 cells were cultured for a total of 4 days in the presence and absence of NGF (50 ng/ml). At a 16 hr, 40 hr, 72 hr, and 4 days, cells of sister cultures were fixed and assayed for their relative cell surface level of Thy-1 glycoprotein as described in Materials and Methods. Figure 3.1 shows that whereas cultures growing in the absence of NGF undergo only minor changes in Thy-1 expression over the entire 4 day period, NGF treated cultures undergo significant increases in expression of the glycoprotein displaying up to 2 and 3 fold enhanced expression relative to untreated cultures at 40 hr and 4 days respectively.

3.2.2. 48 HOUR INDUCTION OF THY-1 EXPRESSION : AN NGF DOSE RESPONSE.

Naive and primed PC12 cells were grown for a period of 48 hr in the presence of NGF over a concentration range between 0 and 50 ng/ml, and then fixed and assayed for their relative binding of Thy-1 reactive McAb OX7. Figures 3.2 a and b show the relative increase in Thy-1 antibody binding for NGF treated cultures compared with control cells grown in the absence of growth factor. Both naive and primed PC12 cell cultures underwent dose dependent increase in their cell surface Thy-1 antigen content. For naive cells , half maximal and maximal responses were estimated at 0.8 and 3 to 12.5 ng/ml respectively. For primed cultures half maximal responses were observed at 0.2 and 3.0 ng/ml respectively. Thus as expected from previous studies (Doherty and Walsh, 1987) the primed PC12 response is induced at lower concentrations of NGF.

TIMECOURSE :- THY-1 ACCUMULATION

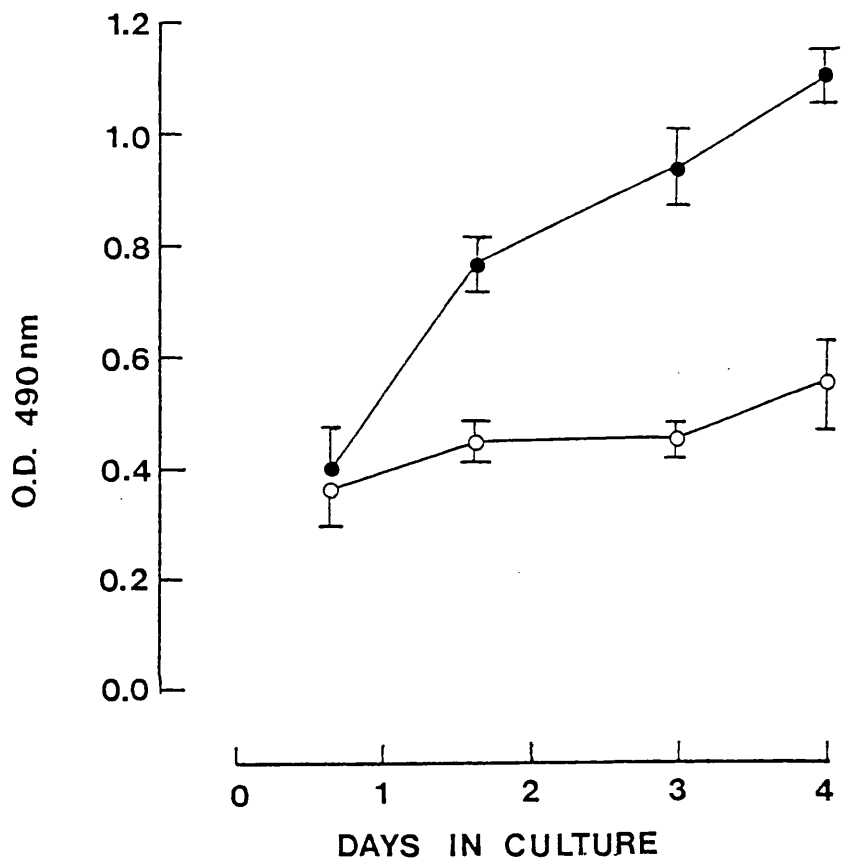


Figure 3.1. Cell surface Thy-1 antigen levels were determined for PC12 cells cultured for between 16 hours and 4 days in the absence (O) or presence (●) of 50 ng/ml NGF. The results show the absolute increase in the binding of the McAb OX7 to cell cultures with each value representing the mean +/- SE of six independent determinations.

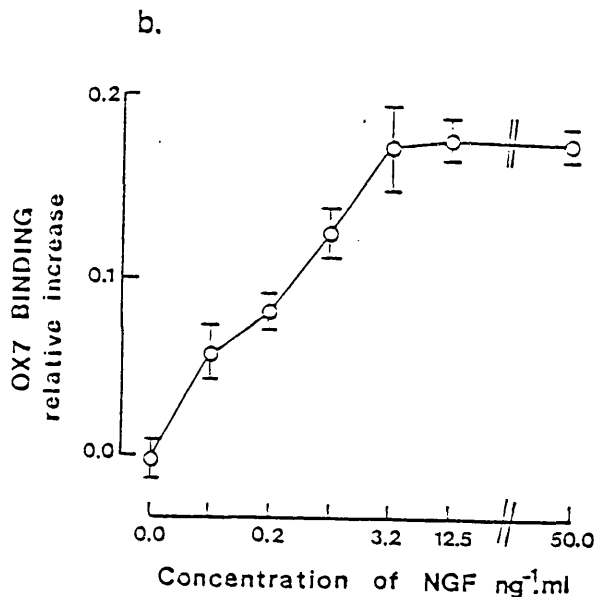
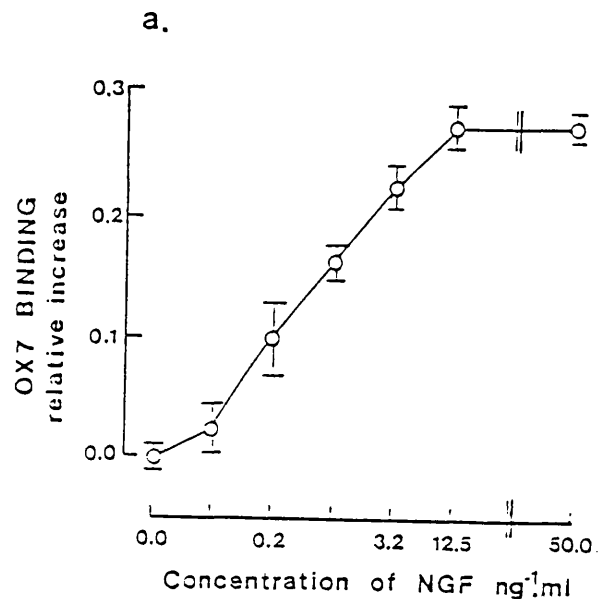


Figure 3.2. Naive (a) and primed (b) PC12 cells were grown for 48 hours over a wide range of NGF concentrations. Cultures were analysed for Thy-1 cell surface expression by determining their ability to bind the OX7 McAb. The results show the increase in binding of OX7 to cultures relative to sister cultures growing in the absence of NGF. Each value is the mean \pm S.E. of six independent determinations. Control values (0 ng/ml NGF) were 0.331 ± 0.017 and 0.105 ± 0.007 O.D. units for naive and primed cells respectively.

3.2.3. EFFECTS OF CORDYCEPIN ON NGF INDUCED THY-1 EXPRESSION

Naive and primed cells were maintained for a 48 hour period in NGF (50 ng/ml) supplemented media containing the transcriptional inhibitor cordycepin over a concentration range of 0 to 1.0 ng/ml. Measurement of Thy-1 antigen expression in sister cultures of primed cells (fig. 3.3) show that cordycepin has no effect on NGF induced increases, whilst in contrast similar measurements in naive cultures show that cordycepin inhibits the same NGF induced response in a dose dependent manner. These data support earlier studies in which induction of the Thy-1 response by NGF in naive PC12 cells was associated with increased transcriptional activity at the Thy-1 gene (Dickson et al, 1986), but in primed cells appeared to operate independently of the lag-phase associated with RNA synthesis dependent induction (Doherty and Walsh, 1987).

3.2.4. EFFECTS OF PHORBOL ESTERS ON THE MORPHOLOGICAL STATUS OF PC12 CELLS.

Naive PC12 cells were cultured for 24 hr in SATO media either alone, or in the presence of NGF (10 ng/ml), PMA (phorbol 12-myristate,13-acetate) (20nM) or PdBu (phorbol 12,13,-dibutryate) (20nM). While NGF treatment is associated with a clear morphological response including the early signs of generation of neurites (fig. 3.4 b), in contrast neither phorbol ester was able to elicit a similar effect (fig. 3.4 c) with cultures more closely resembling cells grown in SATO media alone (fig. 3.4 a).

CORDYCEPIN DOSE RESPONSE:- EFFECT
ON NGF INDUCTION OF THY-1

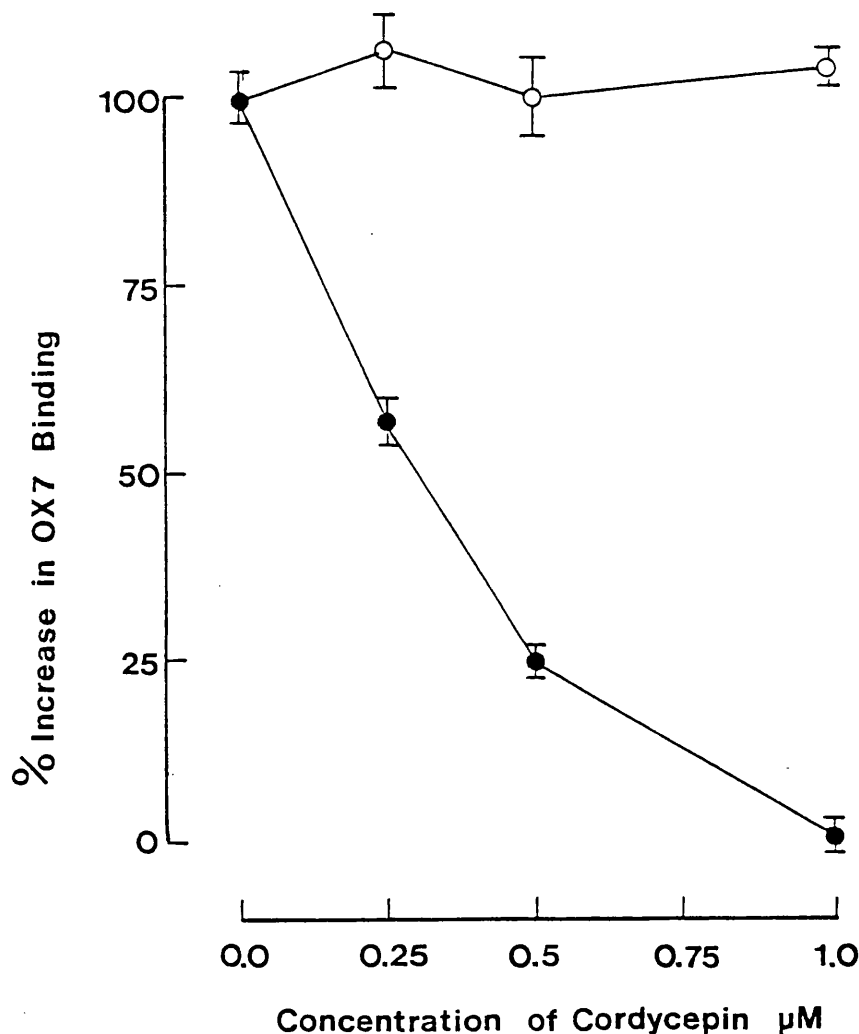


Figure 3.3. Naive (●) and primed (○) PC12 cells were grown for 48 hours in media containing the transcriptional inhibitor cordycepin (0 to 1.0 μM) and 50 ng/ml NGF. Cultures were assessed for Thy-1 antibody binding, with the results showing the percentage increase in OX7 McAb binding over cultures growing in control media (SATO only). Each value is the mean \pm S.E. of six independent determinations. 100 % control value was 0.508 \pm 0.018 O.D. (mean \pm SE, n = 6).

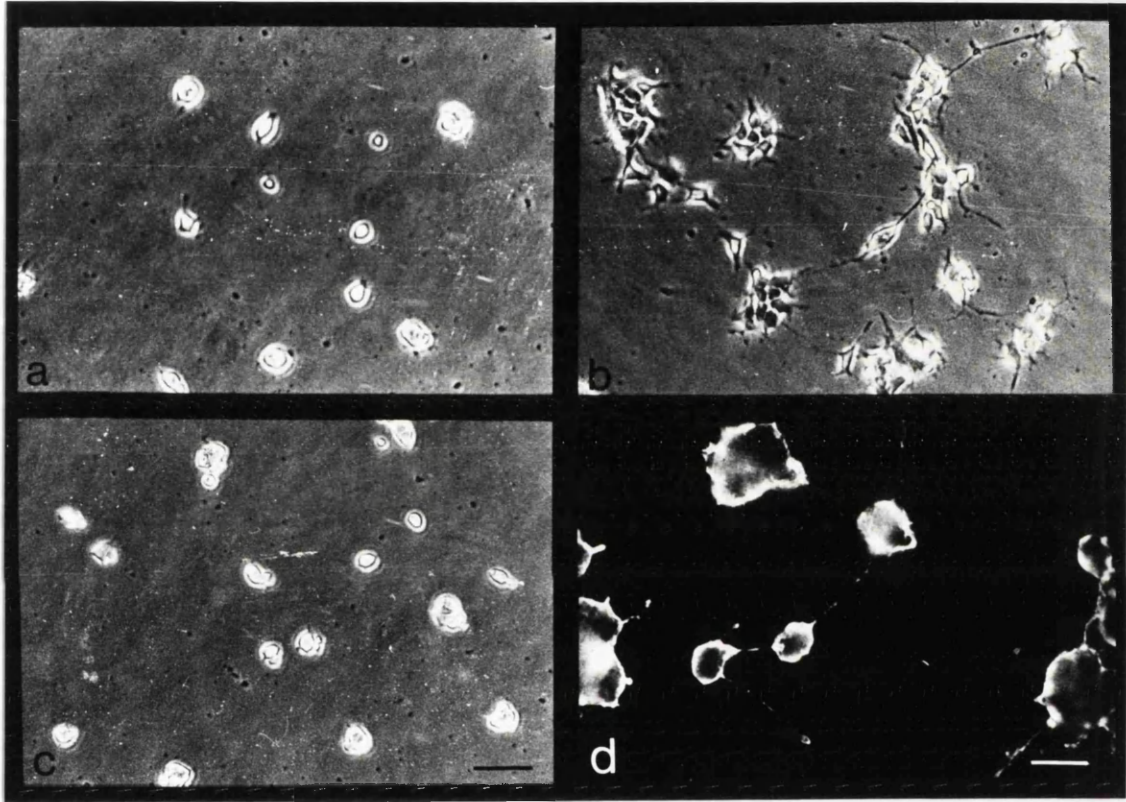


Figure 3.4. PC12 cells were grown for 24 hr in control media (a), media supplemented with 10 ng/ml NGF (b), or 20 nM PMA (c). Frames a to c show representative fields following assay for OX7 binding. Frame d shows an NGF treated culture stained with McAb OX7. Immunoreactivity is present over the entire surface and is additionally found in association with growth cones. Bars : (a to c) 50 um ; (d) 200 um.

3.2.5. EFFECTS OF PHORBOL ESTERS ON THY-1 EXPRESSION.

Cultures of naive PC12 cells were grown for 24 hr in SATO media containing either the phorbol esters PMA or PdBu, and were then fixed and assayed for Thy-1 expression relative to control cultures grown in the absence of either agent. Figure 3.5 b shows that both PMA and PdBu can induce dose dependent increases in Thy-1 expression, with PMA being the slightly more active of the two compounds at sub-optimal doses. At their optimal dose of 20 nM both PMA and PdBu induced a maximal Thy-1 response of 74.4% relative to the Thy-1 response measured in cultures of sister cells treated with NGF (5 ng/ml), (fig. 3.5a). PMA in contrast to NGF was unable to promote continued increases in Thy-1 expression in cultures treated for periods of in excess of 26 hr.

At much higher concentrations (greater than 200 nM) both PMA and PdBu showed a reduced ability to induce the Thy-1 response (results not shown). Similar biphasic responses with these phorbol esters have been reported in other systems (Blumberg et al, 1985) and are believed to be the result of phorbol ester induced desensitisation of protein kinase C (Rodriguez-Pena and Rozengurt, 1984). In contrast with PMA and PdBu , a third phorbol ester 13-monoacetate which does not activate protein kinase C had no effect on Thy-1 expression (data not shown).

DOSE RESPONSE:- NGF AND PHORBOL ESTER INDUCED THY-1 EXPRESSION

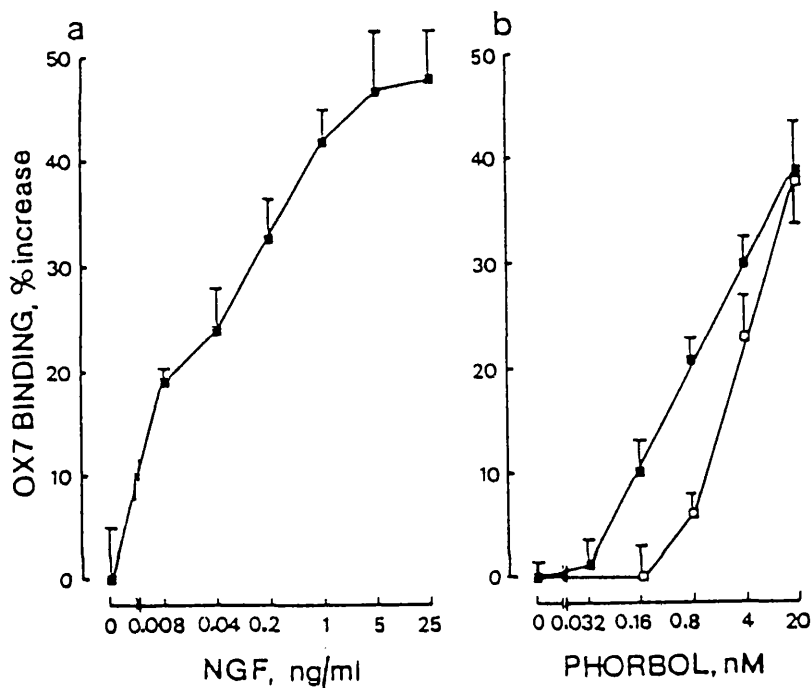


Figure 3.5. Cell surface-associated Thy-1 antigen levels were determined for PC12 cultures grown over 24 hours in the presence of 0-25 ng/ml NGF (a) or 0-20 nM PMA (■) and PdBu (□) (b). The results show the percentage increase in McAb OX7 binding over cultures grown in control media, with each value representing the mean \pm S.E. of six independent determinations. 100 % control binding was measured as 0.383 \pm 0.008 O.D. units (mean \pm S.E., n=12)

3.2.6. EFFECTS OF PHORBOL ESTERS ON NGF INDUCED INCREASES IN THY-1 EXPRESSION.

In the presence of NGF (0 to 25 ng/ml concentration range) and a submaximally active concentration of PMA (0.33 nM), 24 hour cultures of PC12 cells were shown to have undergone increases in Thy-1 expression that were additive (not synergistic) to the enhancement promoted by NGF on its own (fig. 3.6a). The effects of varying phorbol ester dosage on NGF induction were determined by measuring Thy-1 expression in PC12 cell cultures grown for 24 hr in NGF containing media (5 ng/ml) over a wide range of concentrations of PdBu (fig. 3.6b). At low concentrations (less than 1.0 nM) PdBu had additive effects with NGF, however at higher concentrations (greater than 1.0 nM) the phorbol ester was found to inhibit NGF induced increases in Thy-1 in a progressive manner until the response was eventually abolished. When PdBu was replaced with PMA similar results were obtained (data not shown).

3.2.7. PMA MEDIATED INHIBITION OF NGF INDUCED THY-1 EXPRESSION IS NOT ASSOCIATED WITH CYTOTOXIC EFFECTS.

The ability of cultures to convert MTT to its formazan product (Mosmann, 1983) appears to be dependent on the general metabolic state of the cell population. For example NGF can induce an elevated MTT activation state relative to that seen in untreated control cultures, and this effect can be inhibited in a dose dependent manner by cordycepin (fig. 3.11). Cultures treated continuously for a period of 40 hr with either NGF (50 ng/ml), PMA (500 ng/ml), or NGF and PMA were tested for their ability to activate MTT relative to cultures grown for the same period in SATO media alone (fig. 3.7). In both the presence and absence of NGF, PMA had no detectable effects on cellular MTT activity, neither

EFFECTS OF PHORBOL ESTER ON NGF INDUCED THY-1 EXPRESSION

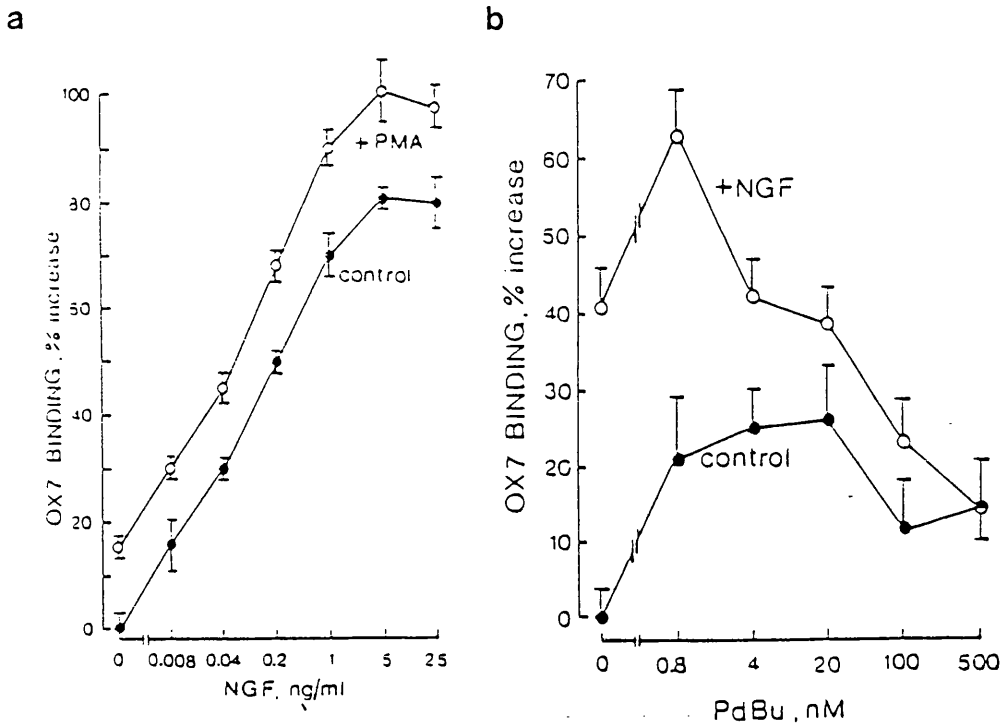


Figure 3.6. (a) Immunoreactive Thy-1 levels were determined for PC12 cells grown for 24 hours over a wide range of NGF concentrations in the presence (O) and absence (●) of 0.33 nM PMA. Results show the percentage increase in McAb OX7 binding relative to cultures grown in control media and each value is the mean \pm S.E. of six independent determinations. 100 % control value was 0.432 \pm 0.011 O.D. units (mean \pm S.E., n = 12). (b) PC12 cells were grown over a wide range of PdBu concentrations (0-500 nM) in control media (●) or alternatively, media supplemented with 5 ng/ml NGF (O). After 24 hours the cultures were analysed for Thy-1 binding and the results show the percentage increase in McAb OX7 binding over cultures maintained only in SATO media. Each value is the mean \pm S.E. of six independent determinations. 100 % control was 0.355 \pm 0.012 O.D. units (mean \pm S.E. n = 6).

MTT DETERMINATION ON PMA TREATED CULTURES

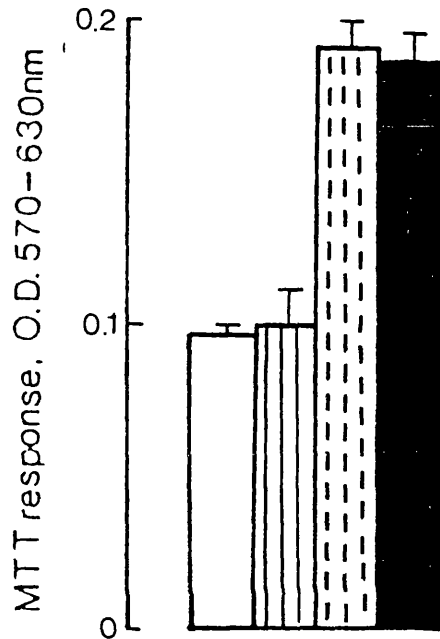


Figure 3.7. PC12 cells were grown for 40 hours in control media (□), or media supplemented with 500 nM PMA (▨), or 50 ng/ml NGF (▤) or 500 nM PMA and 50 ng/ml NGF (■). Results show the absolute O.D. units for conversion of MTT to its formazan product, with each value representing the mean \pm S.E. of five independent determinations.

reducing basal activity in naive cultures nor inhibiting induced activity in NGF treated cultures. These results eliminate the possibility that inhibitory effects of high concentrations of phorbol esters on NGF induction of Thy-1 could be due to a general cytotoxic effect.

3.2.8. DIOCTANOYLGLYCEROL CAN MIMIC THE EFFECTS OF PHORBOL ESTERS ON THY-1 EXPRESSION.

The synthetic diacylglycerol, dioctanoylglycerol (DOG) has been shown to be a highly specific and potent activator of protein kinase C (Lepetina et al, 1985). In three independent experiments (n = 7 per experiment), treatment with DOG for 24 hr resulted in a significant (< 0.1%) increase in Thy-1 expression, giving mean increases of 30.7% +/- S.E. 3.2%.

3.2.9. INDUCTION OF INCREASED THY-1 EXPRESSION BY A23187.

An NGF-stimulated influx of extracellular calcium has been postulated to mediate stimulation of the metabolism of membrane bound phosphoinositides in PC12 cells (Contreras and Guroff, 1987), a process which gives rise to the generation of diacylglycerol the physiological activator of protein kinase C (Nishizuka, 1984). The calcium ionophore A23187 can cause an increased influx of extracellular calcium into a variety of cells (Contreras and Guroff). PC12 cells were cultured for 24 hr in SATO media supplemented with A23187 (60 to 250 ng/ml). At a concentration of 250 ng/ml, the ionophore promoted a Thy-1 response comparable to that observed in sister cultures treated with NGF (50 ng/ml) (fig.3.8). As observed for phorbol ester treated cultures, and in contrast to NGF treated cells, A23187 had no effect on the general morphological status of PC12 cell cultures (data not shown).

EFFECTS OF A23187 ON THY-1 EXPRESSION

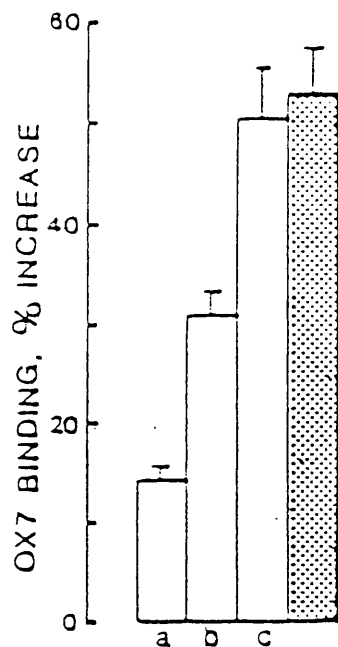


Figure 3.8. Cell surface associated Thy-1 levels were determined in sister cultures of PC12 cells grown over 24 hours in the presence of (\square) A23187 (a to c : 62, 125 and 250 ng/ml respectively), or alternatively (\boxtimes) 50 ng/ml NGF. Results show the percentage increase in McAb OX7 binding relative to control cultures (SATO only) and each value is the mean \pm S.E. of seven independent determinations. 100% value was 0.423 \pm 0.027 O.D. units (n = 7).

3.2.10. INDUCTION OF THY-1 BY NGF AND A23187 IS ADDITIVE.

Naive PC12 cells treated with NGF (0 to 17 ng/ml) in SATO media supplemented with and without A23187 (125 ng/ml) were assayed for Thy-1 expression after 24 hr. A23187 was found to have an additive effect on the NGF-induced response across the entire dose range (fig. 3.9).

3.2.11. PHORBOL ESTER AND A23187 MIMIC NGF INDUCTION OF THY-1 AT THE LEVEL OF GENE TRANSCRIPTION.

NGF induced increases in the cell surface accumulation of Thy-1 are associated with increases in the rate of transcription of the Thy-1 gene and in the accumulation of mRNA species encoding the Thy-1 protein (Dickson et al, 1986). To determine if induction of Thy-1 by phorbol ester and A23187 is also associated with elevation of Thy-1 mRNA expression, Northern blot analysis was carried out on lysates prepared from cultures grown for 24 hr in SATO media alone or supplemented with either NGF (10 ng/ml), PMA (20 nm), or A23187 (125 ng/ml). A single RNA band of approximately 1.8 Kb was detected following hybridisation with a cDNA probe specific for Thy-1 (Koriuchi et al, 1983), this is in agreement with previous studies investigating Thy-1 mRNA expression in PC12 cells (Dickson et al, 1986). Basal expression of Thy-1 mRNA (fig. 3.10) is clearly elevated in cultures treated with NGF, PMA and A23187. As a control the same transfer was stripped (see Methods and Materials), and reprobbed with a cDNA specific to the 68 kD neurofilament mRNA species (Julien et al, 1985 ; Dickson et al, 1986). The resulting hybridisation showed essentially no increase in basal mRNA expression for either the 2.4 or 3.4 kb species previously identified in PC12 cells (Dickson et al, 1986) with any one of the three agents. As neurofilament mRNA levels are similar in all tracks it is assumed that

EFFECTS OF A23187 ON NGF INDUCED THY-1 EXPRESSION

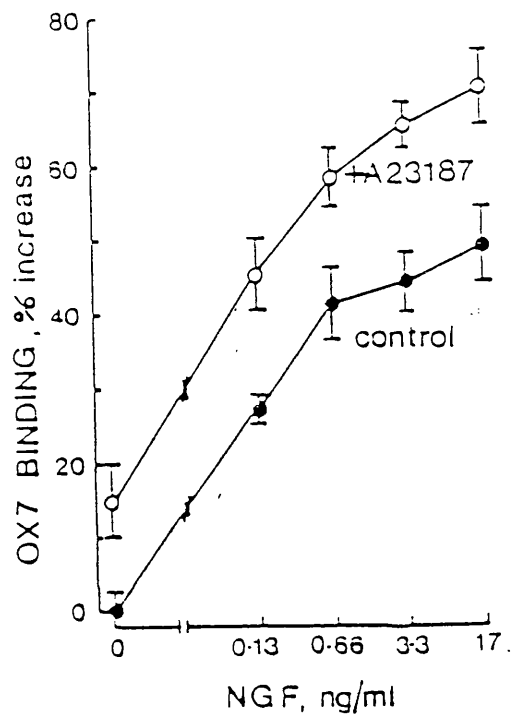


Figure 3.9. Immunoreactive Thy-1 levels were determined for PC12 cells grown for 24 hours over a wide concentration range of NGF (0-17 ng/ml) in the presence (○) and absence (●) of A23187. The results show the percentage increase in the binding of McAb OX7 over cultures grown in control media and each value is the mean \pm S.E. of four independent determinations 100% control value was 0.610 \pm 0.017 O.D. units (mean \pm S.E., n = 4).

INDUCTION OF INCREASED THY-1 mRNA EXPRESSION
BY NGF AND ACTIVATORS OF PROTEIN KINASE C

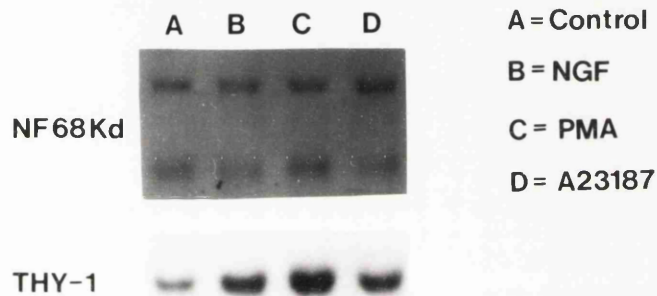


Figure 3.10. Levels of mRNA encoding Thy-1 and the 68 kd neurofilament protein subunit in control (a) NGF- (b), PMA- (c), and A23187 (d) treated PC12 cells. Cultures were grown over 24 hr and total RNA samples collected. RNA samples (10 ug) were processed for Northern blot analysis, hybridised to cDNA probes, and the filter was autoradiographed at -70 c in the presence of an intensifying screen.

roughly equivalent amounts of total RNA were added to sample wells, thus, confirming that the observed increases in Thy-1 mRNA were genuine. Lack of response for neurofilament contradicts a previous report in which an increase in the mRNA species encoding the 68kD subunit was found after 21 hr of NGF treatment (Dickson et al, 1986). The most plausible explanation of this discrepancy is that the authors used poly-A enriched RNA while in this present study analysis was performed on total cellular RNA. It has been suggested that induction of some weakly induced genes by NGF can be masked during Northern analysis due to the ability of NGF to induce increased expression of ribosomal RNA's (Gunning et al, 1981) thereby effectively lowering the ratio of poly-A to total RNA in lysates relative to their uninduced counterparts.

3.2.12. INDUCTION OF THY-1 BY PHORBOL ESTER AND A23187 IS DEPENDENT ON ONGOING GENE TRANSCRIPTION.

The Thy-1 response induced by NGF is dependent upon ongoing gene transcription (section 3.3), and can be suppressed by inhibitors of the transcription process such as cordycepin (fig. 3.2). To confirm that Thy-1 induction by phorbol esters and A23187 involves a transcription dependent mechanism, the effects of cordycepin (0.1 μ M) on PMA (20 nM) and A23187 (125 ng/ml) induced increases in the cell surface level of Thy-1 were determined. As previously observed, 0.1 μ M cordycepin significantly reduced the increase in Thy-1 expression induced by NGF (10 ng/ml) in naive cells (fig 3.11a). Induction of Thy-1 by PMA and A23187 was also inhibited by cordycepin, effectively reducing the increased expression in both cultures by 50% relative to cultures

EFFECTS OF CORDYCEPIN ON NGF, PMA
AND A23187 INDUCED THY-1 EXPRESSION

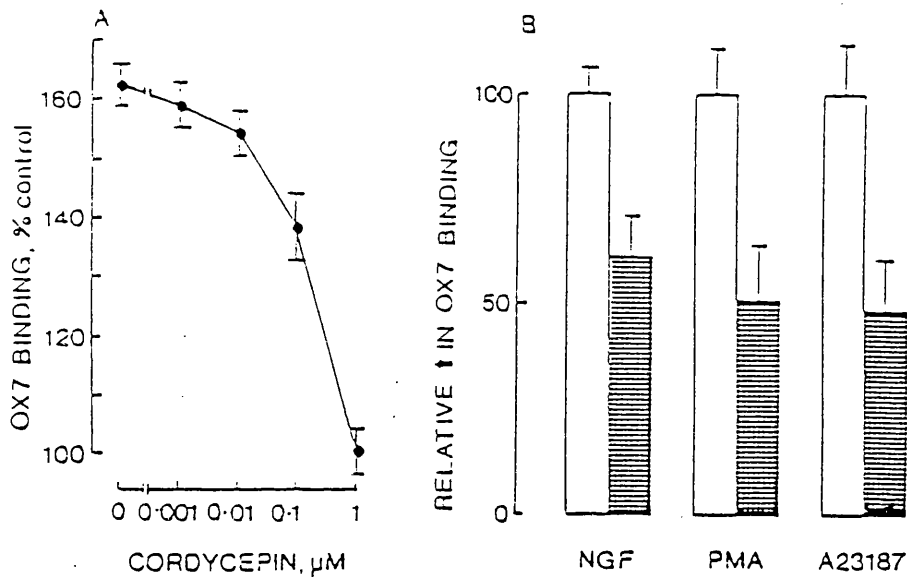


Figure 3.11. (A) Cell surface Thy-1 antigen levels were determined for PC12 cells grown in the presence of cordycepin (0 to 1 μ M) in media supplemented with 10 ng/ml NGF. McAb OX7 binding was assayed after 24 hours of culture, with each value representing the mean \pm S.E. of six independent determinations. Results are expressed as percentage of McAb OX7 binding to cultures grown only in SATO media, with this value measured as 0.316 \pm 0.004 OD units (mean \pm S.E., n = 12). (B) PC12 cells were grown in media supplemented with 10 ng/ml NGF, 125 ng/ml A23187, or 20 nM PMA. Cells were grown in both the presence (▨) and absence (□) of 0.1 μ M cordycepin. In each instance the increase in OX7 binding induced by test agent in the absence of cordycepin has been normalised to 100 arbitrary units. Each value is the mean \pm S.E. of six independent determinations.

treated with inducers in the absence of inhibitor (fig. 3.11 b). These results suggest that PMA and A23187 induce Thy-1 by mechanisms that are similar to the transcription dependent process described for the NGF induced response.

3.2.13. NGF INDUCTION OF THY-1 IS INHIBITED BY CHOLERA TOXIN.

Despite its ability to act synergistically with NGF in promoting a rapid neuritogenic response, cholera toxin has been shown to cause an inhibition of NGF induced accumulation of neurofilament protein, NGF-receptor and Thy-1 (Doherty and Walsh,1987 ; Doherty et al, 1987 ; Doherty et al,1988). In naive PC12 cells induction of Thy-1 by NGF over a three period of culture can be inhibited by cholera toxin in a dose dependent manner, with maximal inhibition achieved at between 0.1 and 1.0 ng/ml toxin (Doherty and Walsh,1987), with induction of neurite outgrowth being apparent in cultures over the same concentration range.

Having demonstrated that changes in temporal expression of Thy-1 can be detected and quantitated at timepoints earlier than day 3 of NGF treatment (fig 3.1), it was of interest to determine if inhibitory effects of cholera toxin can be seen at these early timepoints so as to assess whether they are imposed immediately or are the result of long term treatment. Figure 3.12 shows that cholera toxin (0.1 ng/ml) on its own has limited ability to directly effect Thy-1 expression, promoting a weak induction of the glycoprotein relative to the increases seen in sister cultures treated with NGF. However, NGF induced increases at both 24 and 48 hr were reduced in the presence of cholera toxin. Since the Thy-1 response is transcription dependent,

INHIBITION OF THE THY-1 RESPONSE BY CHOLERA TOXIN

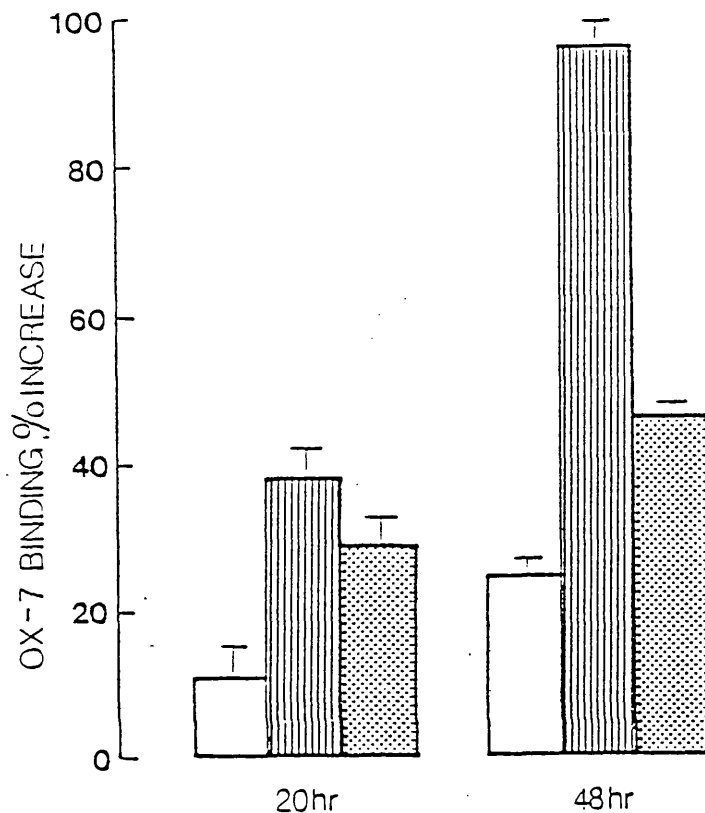


Figure 3.12. Cell surface Thy-1 levels were determined for PC12 cell cultures grown for 20 and 40 hours in media supplemented with 0.1 ng/ml cholera toxin (□), 50 ng/ml NGF (▨), or 0.1 ng/ml cholera toxin and 50 ng/ml NGF (▩). Results show the percentage increase in McAb OX7 binding relative to cultures grown in SATO media only. Each value represents the mean \pm S.E. of seven independent determinations. Binding of OX7 to control cultures was measured as 0.26 ± 0.015 and 0.23 ± 0.015 O.D. units for 20 and 40 hour cultures respectively (mean \pm S.E., $n = 7$).

and as seen with other delayed responses requires a lag-phase of several hr of NGF treatment (Greene,1984), it appears likely that the effects elicited by cholera toxin are directed at the level of primary induction.

3.2.14. CHOLERA TOXIN INHIBITS NGF INDUCED INCREASES IN THE EXPRESSION OF THY-1 mRNA.

Since the inhibitory effects of cholera toxin are exerted on the Thy-1 response within a few hr of induction, it is possible that the effects arise from modulation of the NGF signal transduction process rather than changes occurring at later stages of Thy-1 synthesis and turnover. To test this possibility, Northern blot analysis was carried out on total RNA extracts prepared from cultures grown for 48 hr in the presence of either NGF (25ng/ml), cholera toxin (0.1ng/ml), NGF and cholera toxin, or SATO media alone. The transfer was hybridised with the same cDNA probe used in an earlier study (fig 3.10), and subsequently revealed the same 1.8kb mRNA band identified as encoding the Thy-1 molecule (fig 3.13a). While NGF induced a clear increase in the abundance of Thy-1 message relative to control cultures, cholera toxin had a much weaker inductive effect. Relative to NGF treated cultures, cells grown in the presence of both NGF and cholera toxin showed a dramatically reduced increase in Thy-1 mRNA.

The same RNA transfer was stripped and probed sequentially with three more cDNA probes, these having specificity to NGF-receptor (fig 3.13b), B-actin (fig 3.13c) and the 68kd neurofilament subunit (fig 3.13d). The NGF-receptor, as expected from ELISA data (Doherty et al,1988),

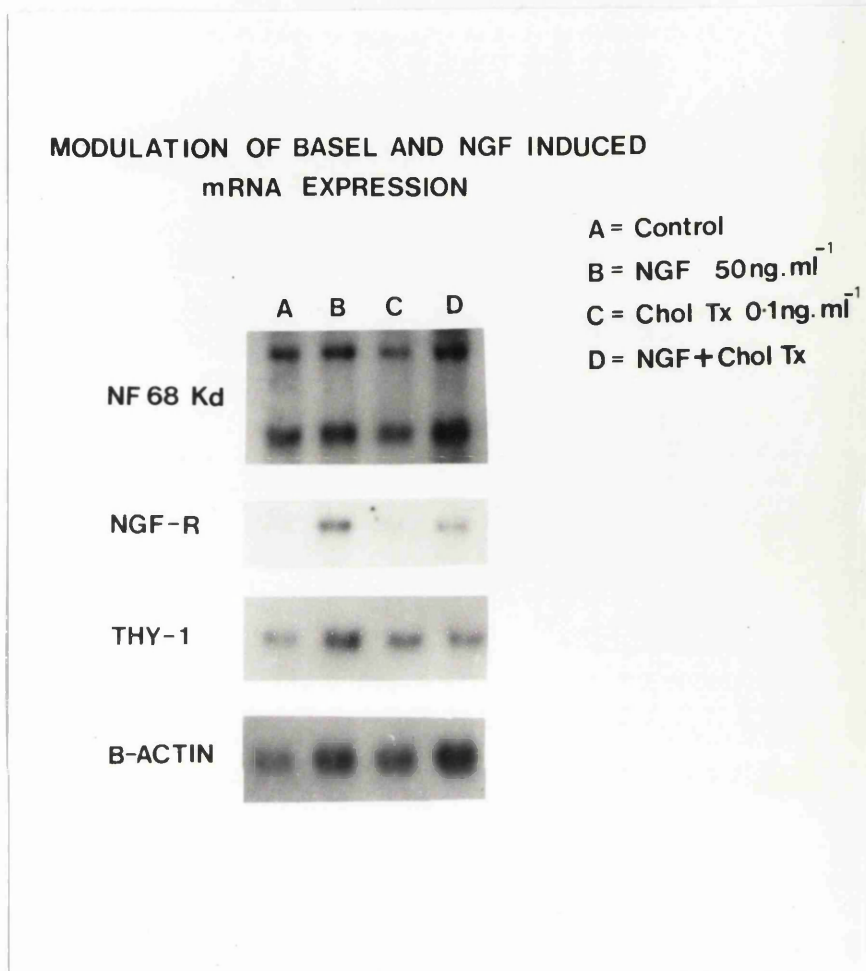


Figure 3.13. Levels of mRNA encoding Thy-1, NGF-receptor, B-actin and NF68 were determined by Northern blot and cDNA hybridisation analysis of total cellular RNA (10 ug) from PC12 cells grown for 48 hr in media supplemented with either (B) 25 ng/ml NGF, or (C) 0.1 ng/ml cholera toxin, or (D) 25 ng/ml NGF and 0.1 ng/ml cholera toxin, and in cultures grown in control media. After each cDNA hybridisation the filter was exposed to X-ray film at -70 c in the presence of an intensifying screen.

was induced at the mRNA level by NGF but not by cholera toxin, and as with Thy-1 these NGF induced increases were greatly reduced in cultures co-treated with NGF and cholera toxin. Levels of mRNA encoding the 68kd neurofilament subunit were apparently the same in all four sample tracks providing a convenient control for the experiment. In contrast to Thy-1 and NGF-receptor, induction of B-actin mRNA accumulation by NGF appeared to be stimulated by cholera toxin. Since basal levels of B-actin mRNA were not directly affected by the toxin on its own it would seem that this potentiation is a synergistic effect.

3.3. DISCUSSION.

NGF can induce in a dose dependent manner, increases in the cell surface expression of the Thy-1 glycoprotein (Doherty and Walsh, 1987). These increases occur over a timecourse that is similar to that observed for NGF induced neurite outgrowth and neurite regeneration in naive and primed PC12 cells respectively, suggesting that the biochemical response (Thy-1) and the morphological response (neurite outgrowth) may be closely linked. Indeed it has been suggested that Thy-1 may function as a component of the neuritogenic response (Leiffer et al,1984 ; Messer et al,1984 ; Doherty and Walsh,1987), though a precise role has not yet been identified.

The current study has investigated the regulation of Thy-1 expression in PC12 cells growing in conditions that impose different constraints on their morphological status than those observed with NGF treatment. When challenged with activators of protein kinase C such as the phorbol esters PMA and PdBu (Rodriguez-Pena and Rozengurt,1984 ; Rozengurt et al,1985), or when cultured in the presence of the calcium

ionophore A23187, PC12 cells fail to respond morphologically in any way that resembles their response to NGF and totally lack the ability to extend neuritic processes. In contrast cells treated with cholera toxin, an activator of adenylate cyclase (Hynie and Sharp,1972 ; Levinson and Blume,1977 ; Cassel and Salinger,1977), undergo morphological changes that for a limited period of time (3 or 4 days) are almost indistinguishable from the NGF induced response. Moreover, cholera toxin can act synergistically with NGF to produce, over the same 3 day timecourse, a more rapid neuritogenic response than that induced by either agent alone (Doherty et al,1987).

NGF induction of increases in the cell surface expression of Thy-1 can be conveniently quantitated by an ELISA methodology described elsewhere (Doherty and Walsh,1987). In the present study this binding assay has been used to quantitate changes in Thy-1 expression at early timepoints of NGF treatment. In the absence of significant changes in cell numbers (Doherty and Walsh, 1987) and against a background increase in total cellular protein of just 10% at 24 hr and 30% at 48 hr of NGF treatment respective increases of up to 30% and 70% of basal Thy-1 expression have been measured. These highly specific increases have been shown here and elsewhere to be mediated by a transcription dependent mechanism, being reduced in the presence of the transcriptional inhibitor cordycepin, and being associated with an elevation of mRNA's encoding the glycoprotein (Dickson et al, 1986). Therefore, the Thy-1 response provides an ideal system with which to study early transcription dependent changes and to probe the signal transduction mechanisms mediating NGF induced gene activation.

There is accumulating evidence to suggest that the calcium and phospholipid-dependent protein kinase, protein kinase C (Lakshmanan, 1978 ; Traynor et al, 1982 ; Hama et al, 1986 ; Contreras and Guroff, 1987) is involved in transduction of the NGF signal and may possibly mediate certain NGF induced gene activation events. In its active form (Takai et al, 1979 a and b ; Kishimoto et al, 1980) as a constituent of a membrane associated quaternary complex (Ganong et al, 1986) including the kinase, Ca^{2+} , phospholipid and diacylglycerol (DAG), protein kinase C has a broad substrate specificity, phosphorylating in a rapid and highly selective manner a wide variety of cellular proteins in many tissues (Nishizuka, 1984). Protein phosphorylation is widely recognised as a major route for signal transduction in both normal and transformed cells (Mochly-Rosen and Koshland, 1987). Activated protein kinase C has been demonstrated in PC12 cells directly and has been associated with a number of NGF induced phosphorylation events (Hama et al, 1986), though the absolute requirement for activated protein kinase C during NGF induction of these responses is not known.

Activation of protein kinase C is coupled to receptor mediated breakdown of membrane bound polyphosphoinositides (Takai et al, 1979 b ; Kishimoto et al, 1980 ; Mochly-Rosen and Koshland, 1987) and more specifically to the appearance of DAG in the cell plasma membrane (Nishizuka, 1984). Small quantities of DAG appear to increase the kinase affinity for calcium and phospholipid (Takai et al, 1979 b). Treatment of PC12 cells and sympathetic neurons with NGF has been shown to stimulate phospholipase C mediated hydrolysis of inositol phosphates (Lakshmanan, 1978 ; Traynor et al, 1982 ; Contreras and Guroff, 1987), and to subsequently give rise

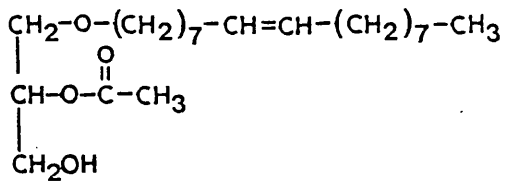
to an elevation of intracellular levels of DAG.

To investigate the possible involvement of the phosphoinositide / protein kinase C pathway in mediating NGF induction of Thy-1, the ability of direct activators of protein kinase C, such as phorbol esters and the synthetic diacylglycerol DOG, to mimic the NGF induced Thy-1 response was determined.

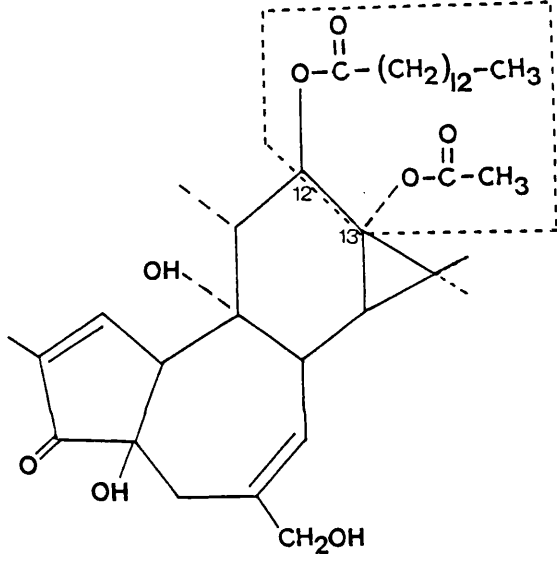
Active tumour promoting phorbol esters such as PMA and PdBu bind to protein kinase C and can at extremely low concentrations substitute for DAG and activate the enzyme both in vitro and in vivo (Castagna et al, 1982 ; Kraft and Anderson, 1983 ; Yamanishi et al, 1983). Binding of phorbol esters to the kinase is, as with DAG, dependent upon the presence of Ca^{2+} and an excess of phospholipid (Ganong et al, 1986). Under these conditions PMA and PdBu, which resemble DAG structurally (Nishizuka, 1984 ; see fig 3.14), combine with the kinase, calcium and phospholipid to form a quaternary complex in which the kinase is biologically active (Ganong et al, 1986).

PC12 cells treated for 24 hr with either PMA or PdBu were found to undergo dose dependent increases in their cell surface expression of Thy-1. At an optimal concentration of 20nM both phorbol esters promoted a quantitatively similar Thy-1 response to that observed in the NGF treated cells. In contrast phorbol-13-monoacetate which is unable to activate protein kinase C was without effect.

NGF induced increases in Thy-1 expression were elevated in cultures co-treated with sub-optimal doses of PMA or



SYNTHETIC DIACYLGLYCEROL



PHORBOL ESTER (PMA)

Figure 3.14. Comparison of the structural features of PMA with a synthetic diacylglycerol.

PdBu. This effect was essentially additive rather than synergistic indicating that induction by phorbol esters and NGF operates via a common mechanism of action. The observation that higher doses of phorbol esters actually inhibited, leading eventually to a complete suppression of NGF induced increases in Thy-1 expression, was not addressed at the mechanistic level. However, other investigators describing a similar effect in other systems (Blumberg et al, 1985 ; Rodriguez-Pena and Rozengurt, 1984), have demonstrated a desensitisation of protein kinase C leading to a suppression of the enzyme's activity. Since Thy-1 induction by NGF can be suppressed by high doses of PMA or PdBu it would appear that activation of protein kinase C may be an absolute requirement for the Thy-1 response in PC12 cells. However, there are indications that phorbol esters may have effects on cells other than the activation of protein kinase C (Kreutter et al, 1985), and may therefore induce changes in Thy-1 expression by an alternative mechanism in which protein kinase C is not involved.

The direct activator of protein kinase C, DOG, and the calcium ionophore A23187, were both able to mimic NGF induction of Thy-1, thus confirming the involvement of protein kinase C in the Thy-1 response. A23187 can fully mimic NGF induced increases in the accumulation of IP3 and IP2 (Contreras and Guroff, 1987) by a mechanism requiring the the presence of extracellular calcium. Increased influx of calcium into the PC12 cell following its exposure to either NGF or A23187 was shown to result in a stimulation of the metabolism of phosphoinositides (Contreras and Guroff,1987), and therefore indirectly to the activation of protein kinase C. This common mechanism of action was indicated in the present study by the ability of NGF and A23187 to act additively in promoting increases in the

expression of Thy-1. These data also indicate that the inhibitory effects of PMA and PdBu must be due to an inactivation of protein kinase C rather than an inhibitory effect of protein kinase C itself on an alternative pathway involved in the induction of Thy-1, otherwise A23187 would have been expected to have displayed the same inhibitory capabilities.

Signal transduction pathways that are involved in mediating the actions of growth factors and/or hormones are generally conceived as being responsible for generating the cellular changes associated with the agonists activities, these changes including transcriptional, translational, and post-translational effects. The demonstration that induction of cell surface changes in the expression of Thy-1 by both phorbol esters and A23187 were associated with the accumulation of Thy-1 mRNA indicates that protein kinase C operates in a signal transduction route that conducts stimuli from the cell surface to the Thy-1 gene. The identity of the signal transduction elements that precede protein kinase C in this pathway are at present unknown. On the basis of the studies with activators of protein kinase C and A23187 it is possible to propose a working model for NGF activation of the Thy-1 gene in PC12 cells. Figure 3.15 illustrates the proposed model for the transcription dependent response in naive cells. Binding of NGF to its cell surface receptor (Yankner and Shooter,1982) leads to an increased influx of extracellular calcium (Contreras and Guroff,1987), which leads to the stimulation of the hydrolysis of phosphoinositides. Subsequent accumulation of IP3 and DAG promotes the formation of the active protein

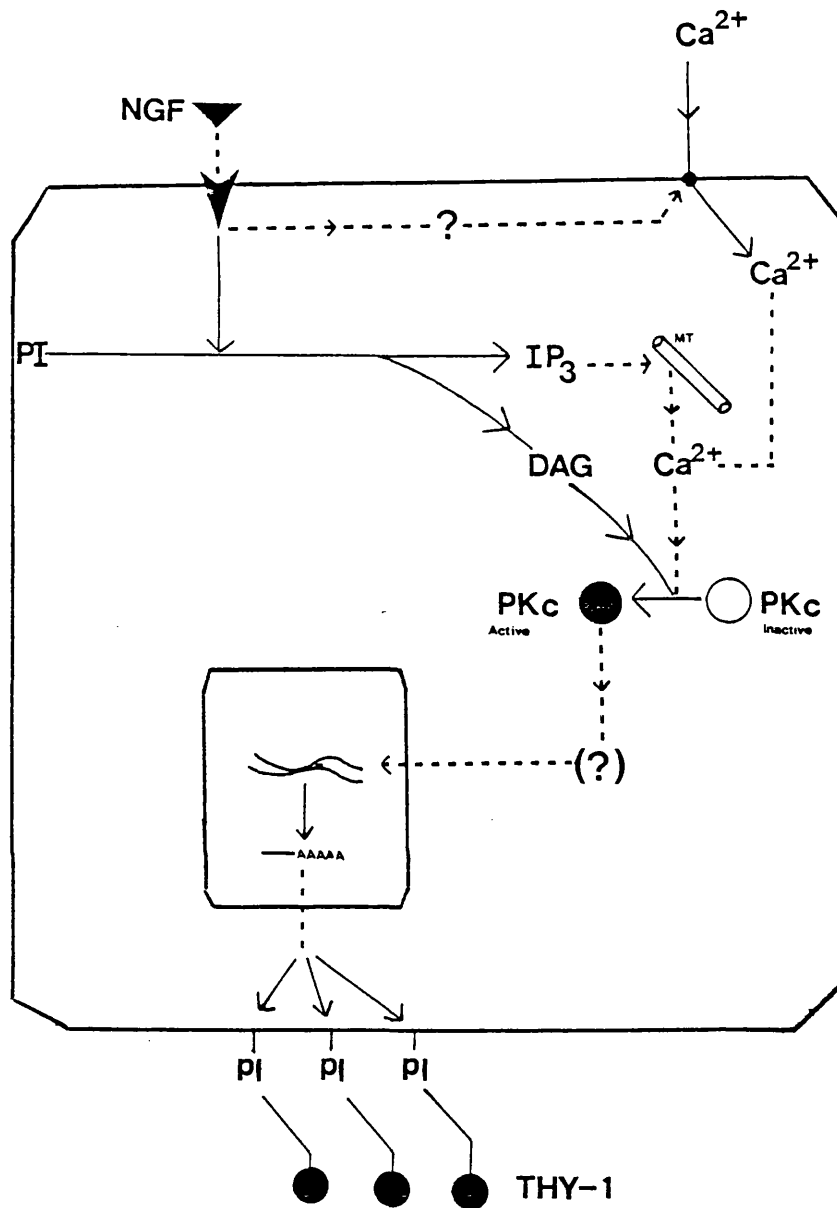


Figure 3.15. Proposed mechanism for NGF activation of increased Thy-1 gene transcription in PC12 cells.

kinase C complex. Signal transduction events occurring immediately after binding of NGF to the NGF-receptor, and after the activation of protein kinase C remain obscure.

Precisely how NGF mediates an increased calcium influx is not known and there are conflicting reports regarding the role of extracellular calcium in the mechanism of action of NGF (Schubert et al,1978 ; Landreth et al,1980). Clearly, further studies are required to resolve the question of the involvement of extracellular calcium in early signalling, with one potential area of study being the identification and characterisation of calcium channels at the PC12 cell membrane and to determine their association with regulatory species such as G-proteins (Need and Clapham,1988 ; Graziano and Gilman, 1987), which have been shown in other systems to potentiate growth factor and hormone induced changes in ion channel activity. At the opposite end of the NGF induced Thy-1 transduction pathway, i.e. transcriptional activation, future studies may be directed towards identifying NGF induced trans-acting factors that interact with regulatory DNA sequences at the 5' end of the Thy-1 gene (Giguere et al, 1985). Once identified, the association of such transcriptional factors with the protein kinase C transduction system would be investigated.

As already discussed, the biochemical effects of phorbol esters and A23187 were elicited in the absence of any 'NGF like' morphological differentiation. Similar results can be demonstrated in PC12 cell cultures grown on substrates that are non-permissive for neurite outgrowth (Data not shown). NGF treatment of cells cultured on laminin or fibronectin substrata results in a full induction of Thy-1 expression in the complete absence of any morphological differentiation. These observations suggest that the Thy-1 response is indeed

a primary cellular response to NGF and, that the ability of phorbol esters to independently activate the Thy-1 response in the absence of morphological differentiation reflects the independent nature of the components responsible for regulation of Thy-1 expression and neurite outgrowth in the PC12 cell, rather than any peculiarity associated with the biochemistry of these protein kinase C activators. In support it has been recently reported that the protein kinase C signal transduction pathway is not responsible for mediating NGF induced neurite outgrowth in PC12 cells (Reinhold and Neet, 1989). The implication to be drawn from these data is that Thy-1 is certainly not directly responsible for triggering the neuritogenic response or indeed any of the major changes in cell shape associated with the NGF induced differentiation response. However, these studies do not preclude the possibility that Thy-1 may act in concert with other as yet unidentified molecules to help orchestrate events underlying the neuritogenic response.

The second messenger cAMP has been suggested, like protein kinase C, to be a potential cellular mediator of NGF induced differentiation responses in PC12 cells. This suggestion was based on the ability of agents such as db-cAMP and the activator of adenylate cyclase, cholera toxin, to mimic and potentiate NGF-induced morphological differentiation of PC12 cell cultures (Gunning et al, 1981 a and b ; Drubin et al, 1985 ; Richter-Landsberg and Jasteroff, 1986). However, more recent studies have demonstrated that the effects of these and similar agents (e.g. forskolin) are limited at the biochemical level of the differentiation response and are only short lived at the morphological level (Heidermann et al, 1985 ; Greene et al, 1986). For example, db-cAMP , cholera toxin and forskolin

are unable promote the formation of a stable neuritic network which is a classic feature of NGF induced differentiation (Greene et al, 1986), and cannot induce associated increases in the accumulation of neurofilament proteins that is also a feature of the NGF induced response (Doherty et al, 1987). Moreover whilst such agents can act synergistically with NGF to promote a more rapid neuritogenic response (Gunning et al, 1981 a and b), this effect is associated with an inhibition of NGF induced accumulation of neurofilament protein (Doherty et al, 1987), and is eventually superseded in longer term cultures (longer than 6 days) by an inhibitory effect on NGF induced elongation of neurites (Richter-Landsberg and Jasteroff, 1986). Thus, it would appear to be highly unlikely that cAMP acts as the key cellular mediator of NGF-induced differentiation events. It is however still possible that cAMP may act as a component of the signal transduction machinery that underlies NGF induced activation of specific responses that contribute to the neuritogenic process.

In the present study the effects of cholera toxin on NGF induction of Thy-1 were determined at timepoints that are closer to the primary inductive event than can be studied with neurite outgrowth. Earlier studies have demonstrated that cholera toxin cannot directly affect Thy-1 expression in PC12 cells and can cause an inhibition of NGF induced accumulation of Thy-1 in cultures of generating and regenerating PC12 cells (Doherty and Walsh, 1987). These studies were carried out after relatively long term periods of culture (day 3), and did not investigate the possibility that the observed effects may be time dependent and / or may be preceded by stimulatory effects in the manner described for the morphological response. Therefore the direct and indirect (NGF-dependent) effects of cholera toxin were

investigated at 24 and 48 hour timepoints of culture.

Cholera toxin was shown to have no direct effect on the basal level of expression of Thy-1 at either the 24 or 48 hour timepoints suggesting that cAMP is unlikely to be the dominant mediator of the NGF induced response. In the presence of cholera toxin, NGF induced increases in Thy-1 glycoprotein expression were significantly reduced at both timepoints indicating that cAMP is probably not involved in mediating the NGF-induced Thy-1 response. These data show that the inhibitory effects of cAMP on NGF induced increases in Thy-1 expression are an early and persistent phenomenon. Since the Thy-1 response is transcription dependent it was of interest to determine the effects of cholera toxin on NGF induced expression of cellular mRNA encoding the glycoprotein. By demonstrating that cholera toxin had no effect on the basal levels of Thy-1 mRNA yet had the ability to inhibit NGF promoted elevation of the expression of the Thy-1 message, the study shows that the inhibitory effects of cAMP are likely to be directed at a component of the signal transduction pathway mediating primary induction of the Thy-1 gene. Since the toxin concomitantly inhibited a second PC12 cell response to NGF, increased expression of NGF-receptor mRNA's (Doherty et al ,1988), whilst also potentiating NGF induced accumulation of mRNA species encoding the B-actin protein, it is clear that the effects of cAMP on NGF induced transcriptional responses are highly specific. Differential modulation of NGF stimulated responses by cAMP has been reported elsewhere (Greene et al, 1986) and may indicate a regulatory role for the molecule in PC12 cell differentiation rather than the mediatory role suggested in earlier discussions.

The precise mechanism underlying cAMP's modulation of the Thy-1 response was not investigated. However, other investigators have shown that the cAMP and protein kinase C signal transduction systems can interact in a modulatory manner (Nishizuka, 1986 ; Berridge, 1984 ; Rasmussen and Barrett, 1984 ; Yoshimasa et al, 1987). This process of "cross talk" is possible because of the ability of protein kinase C and protein kinase A (cAMP dependent protein kinase) to catalyse the phosphorylation of components of both transduction systems and consequently alter the biological activity of these transduction elements (Yoshimasa et al, 1987 ; Katada et al, 1985 ; Suppattapone et al, 1988). For example, under the conditions of elevated intracellular cAMP, protein kinase A can mediate the inhibition of the hydrolysis of inositol polyphosphates (Nishizuka, 1984 ; Billah et al, 1979). More recently it has been reported that protein kinase A can catalyse a highly specific phosphorylation of the membrane bound receptor for IP3, causing a substantial reduction in the capacity of the phospholipid to stimulate release of calcium from the endoplasmic reticulum (Supattapone et al, 1988). As the phosphoinositide / protein kinase C pathway has been reported to be responsible for mediating NGF induced activation of the Thy-1 gene, regulation of the activity of IP3 by protein kinase A may be the mechanism that underlies the inhibitory effect of cAMP on the Thy-1 response.

Since cholera toxin was able to stimulate an "NGF like" morphological response in the absence of any increased expression of Thy-1 and as the synergistic effects of cholera toxin and NGF on neurite outgrowth were demonstrated to be associated with an inhibition of NGF induced increases in Thy-1 , it would seem to be unlikely for Thy-1 to function as an essential component of the neuritogenic

machinery. Taken together with the observations with phorbol esters and A23187 it appears that increased expression of Thy-1 is independent from mechanisms regulating NGF induced changes in cell shape and moreover does not itself trigger these mechanisms or contribute any essential feature to the resulting morphological response. Finally, although it may be argued that the absence of a full Thy-1 induction may be a causative factor toward the poor long-term effects of cAMP on neurite outgrowth, many other responses are similarly affected and indeed cAMP inhibition of NGF-receptor turnover is likely to have the more significant long-term effects on NGF induced differentiation.

CHAPTER 4 : REGULATION OF THE EXPRESSION OF N-CAM AND L1 DURING PC12 CELL DIFFERENTIATION.

4.1. INTRODUCTION.

The observations showing the N-CAM and L1 glycoproteins to be expressed in the developing nervous system in both a tissue specific and stage specific manner suggest that they play important functional roles at the neuronal cell surface and that complex regulatory mechanisms operate to modulate their expression (Jessell,1988 ; Dodd and Jessell,1988 ; Lander, 1989). While N-CAM and L1 are related both structurally and functionally it is apparent from expression studies that the two molecules are likely to play independent roles in neuronal development. This suggestion is supported by antibody perturbation studies which, while showing that both N-CAM and L1 have potent neurite outgrowth promoting properties, provide evidence that the two molecules function in a tissue specific manner (Bixby et al,1987 and 1988). For example, N-CAM appears to be an important component of the machinery driving neuritic outgrowth across muscle cell surfaces with L1 apparently playing no significant role (Bixby et al, 1987), whilst in contrast, neuritic outgrowth from peripheral motor neurons across Schwann cell surfaces can be regulated by L1 but apparently not by N-CAM (Bixby et al,1988). An understanding of the regulatory mechanisms that operate in the control of the cell surface expression of N-CAM and L1 during neuronal development is important in determining their precise role in the differentiating neuron.

As already discussed (chapter 1) neuronal development is associated with both gross changes in N-CAM expression and subtle changes in N-CAM sialylation. In addition, neural development appears to be accompanied by a novel tissue

specific splicing process that allows the three N-CAM polypeptides (N-CAM's 180, 140 and 120) to be expressed in a stage specific manner (Nybroe et al, 1988 ; Gennarini et al, 1986 ; Murray et al, 1986 a and b ; Walsh, 1988 ; Prentice et al, 1987). N-CAM 140 is expressed on pre-neural cells at the time of neural tube formation, whereas in contrast the 180 kD isoform only appears on differentiating post-mitotic and post-migratory neurons (Murray et al, 1986 b). In the chick nervous system a 6.7 kb transcript encoding the 140 kD polypeptide has been shown to be the first N-CAM transcript to appear (Murray et al, 1986 b). This transcript is derived from a 7.2 kb transcript which encodes the larger 180 kD N-CAM polypeptide (Murray et al, 1986 b). Thus a splicing process must operate in the developing neuroblast to allow expression of the 140 kD isoform and prevent premature expression of the 180 kD polypeptide. The cellular mechanisms that control N-CAM expression in the differentiating neural cell are not understood. The demonstration that NGF treatment of PC12 cells can induce both an increase in the expression of N-CAM 140 and trigger the regulatory events that lead to the appearance of N-CAM 180 (Prentice et al, 1987) indicates that the PC12 cell line is an excellent system in which to study the events that underlie NGF induced N-CAM gene activation.

There is increasing evidence to suggest that N-CAM plays a major functional role in the process of axonal growth and guidance (Jessell, 1988 ; Jacobson, 1988 ; Bixby et al, 1987 ; Neugebauer et al, 1988). In addition to the indirect evidence provided by antibody perturbation studies, more direct proof has been provided by a molecular - genetic approach (Doherty et al, 1989, 1990). Fibroblasts transfected with the cDNA coding for a variety of N-CAM (human) isoforms have been shown to be more effective in

supporting axonal growth from dissociated cultures of human and rat DRG neurons compared to the outgrowth seen on their untransfected parental cells (Doherty et al, 1989, 1990). Both transmembrane and GPI linked N-CAM polypeptides improved the neurite promoting ability of the fibroblast cell surface suggesting they are likely to be important components of the machinery controlling axonal growth in vivo. The demonstration that the N-CAM 180kd isoform is associated with the differentiated state of the NGF treated PC12 cell (Prentice et al, 1987) suggests a role for the molecule in the process of morphological differentiation and indicates that the PC12 cell is a potential system for investigating the function of N-CAM in neuritogenesis.

The L1 antigen (Rathjen and Schachner, 1984) appears on neuroblasts much later than N-CAM (Fushiki and Schachner, 1985), appearing initially in the ventral region of the neural tube at the 31 somite stage and being primarily associated with neuritic processes (Thiery et al, 1985). L1 which is now thought to be identical to the chick antigens G4 and 8D9 (Rathjen et al, 1987 ; Lemmon and McCloon, 1986), to the neural-glial cell adhesion molecule (Ng-CAM) (Grumet and Edelman, 1984) and to the PC12 cell surface antigen NILE (Bock et al, 1985), has been shown to be developmentally regulated in neuronal and glial cells (Martini and Schachner, 1986), though the molecular details of this regulation are at present unknown.

There is a wealth of evidence suggesting that L1 is an important component of the neurite outgrowth mechanism, contributing to the neurite promoting properties of Schwann cells (Bixby et al, 1988 ; Seilheimer and Schachner, 1988), as well as promoting neurite extension from a variety of neurons when used in culture as a purified substrate

(Lagenaur and Lemmon, 1987). The mechanism underlying this neurite outgrowth promoting property is poorly characterised.

The L1 molecule found at the surface of NGF induced PC12 cells (McGuire et al, 1978 ; Lee et al, 1981 ; Salton et al 1983 a and b), is synthesised as a single high molecular weight (200 to 230 Kd) glycoprotein. While the antigen was also found on both CNS and PNS neurons (Salton et al, 1983 a and b), its size appeared to vary, with the CNS form being smaller (200 to 210 kd) than the PNS and PC12 cell forms. NGF treatment of PC12 cells is associated with a transcription dependent increase in the incorporation of radioactive fucose or glucosamine into the L1 molecule (McGuire et al, 1978), and more recently has been demonstrated to be accompanied by an approximately three fold increase in the incorporation of radioactive amino acids into the protein (Guroff, 1985). These observations suggest that NGF induced PC12 cell differentiation is concomitant with the increased expression of the L1 antigen and indicate that the PC12 cell may provide a model with which to probe the regulation and function of the molecule during neuronal differentiation.

4.2. RESULTS.

4.2.1. EFFECT OF NGF ON THE CELL SURFACE EXPRESSION OF N-CAM : 48 HOUR DOSE RESPONSE.

Naive and primed PC12 cells were cultured for 48 hr in SATO media supplemented with NGF (0 to 100 ng/ml). Cultures were then fixed and assayed for their relative cell surface levels of immunoreactive N-CAM (as described in materials and methods). At concentrations exceeding 1 ng/ml, NGF induced increases in N-CAM expression at the cell surface of naive cells (fig 4.1a). Half maximal and maximal responses were recorded at NGF concentrations of 6 and 50 ng/ml respectively, with the latter response yielding a 2.5 fold increase in N-CAM expression relative to basal levels recorded on untreated cultures (0 ng/ml NGF). In comparison to the Thy-1 induction (chapter 3, fig 3.2) , the N-CAM response has a higher dose dependancy. While increases in N-CAM levels are not detectable at concentrations of NGF below a 1 ng/ml threshold, a dose of 0.8 ng/ml promotes a half-maximal Thy-1 response.

Primed PC12 cells responded to NGF at much lower doses of NGF than observed with naive cells, with increases in cell surface N-CAM expression being induced at the lowest concentration tested (0.05 ng/ml), however an optimal dose of 50 ng/ml was similar to that estimated for naive cells (fig 4.1b).

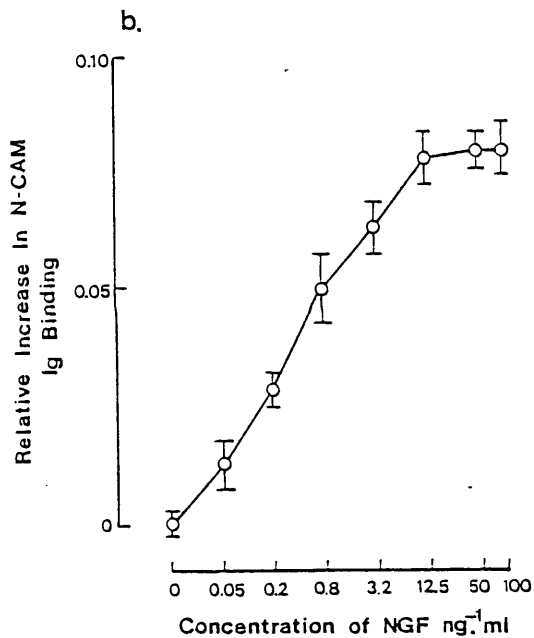
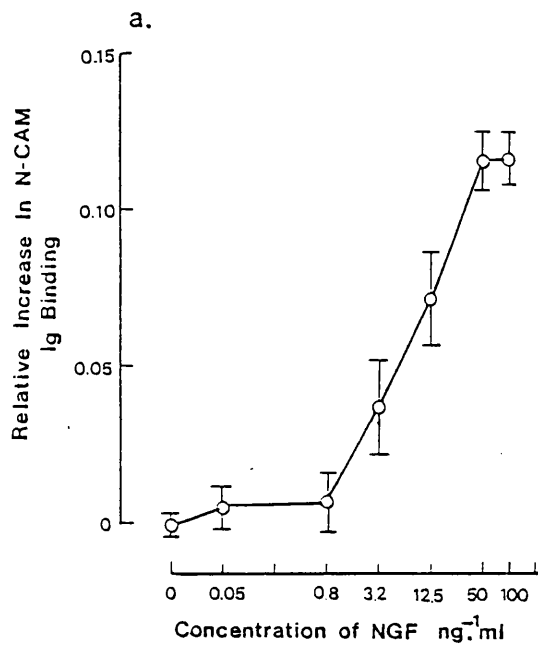


Figure 4.1. Naive (a) and primed (b) PC12 cells were grown for 48 hours in media supplemented with NGF over a wide range of concentrations (0-100 ng/ml). Cell surface immunoreactive N-CAM levels were assayed by determination of the binding of an anti-N-CAM Ig to cell cultures. Results are expressed in terms of relative increases in anti-N-CAM Ig binding over cells grown in control media and represent the mean \pm S.E. of six independent determinations. Values for control cultures (0 ng/ml NGF) were 0.087 \pm 0.006 and 0.016 \pm 0.002 for naive and primed cells respectively (mean \pm S.E., n = 6).

4.2.2. DEPENDENCE OF THE N-CAM RESPONSE ON ONGOING TRANSCRIPTION.

Naive and primed PC12 cells were cultured for a period of 48 hr in the presence of NGF (50 ng/ml), in media supplemented with cordycepin (0 to 1.0 ng/ml). Increases in N-CAM expression were determined relative to control cells grown in the absence of NGF. While NGF induced increases were unaffected by cordycepin in primed cells (fig 4.2), in contrast, increases in N-CAM expression in naive cells were inhibited in a dose dependent manner. These observations are in agreement with those seen for the Thy-1 response (fig 3.3). At the highest concentration tested, 1.0 ng/ml, cordycepin inhibited the NGF induced increase in N-CAM expression by more than 60%. These data lend support to the proposal that NGF induction of N-CAM in naive PC12 cells is a transcription dependent response (Prentice et al,1987).

4.2.3. EFFECTS OF PHORBOL ESTERS AND CALCIUM IONOPHORE A23187 ON N-CAM EXPRESSION.

Naive cells were cultured for 24 hr in media supplemented with NGF (0 to 50 ng/ml), or PMA (up to 200 nM), then assayed for their cell surface N-CAM expression relative to cultures grown in the absence of an inducer. As previously demonstrated over a 48 hour period of culture (fig 4.1a), NGF induces increases in the expression of immunoreactive N-CAM in a dose dependent manner (fig 4.3a). However, while a 50 ng/ml dose of NGF can promote a greater than two fold increase in cell surface N-CAM levels, PMA at either 20 or 200nM concentrations was completely without effect (fig 4.3b).

CORDYCEPIN DOSE RESPONSE :- EFFECT
ON NGF INDUCTION OF N-CAM

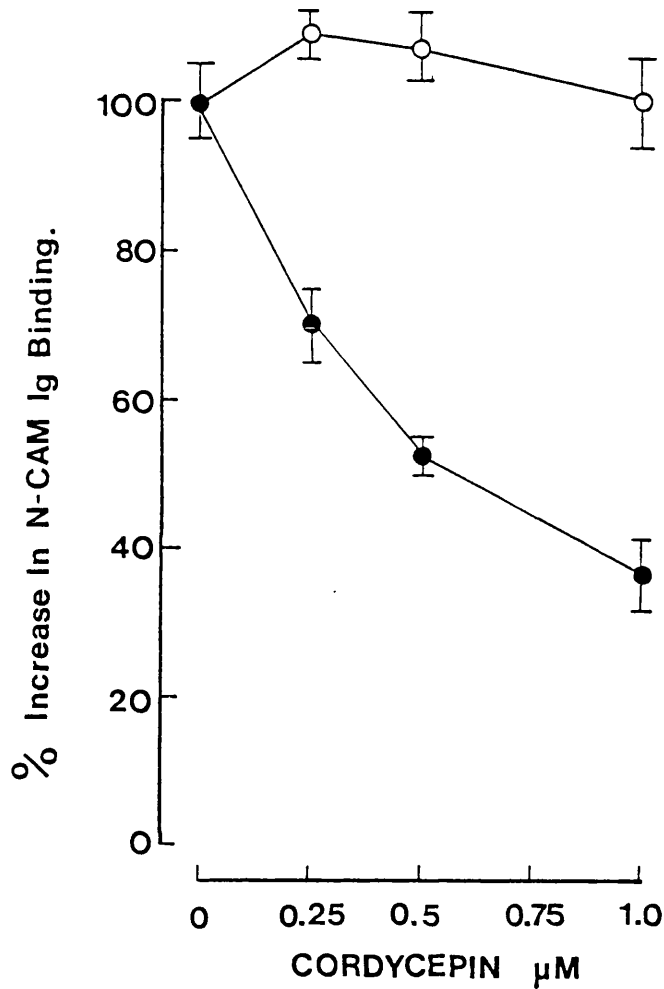


Figure 4.2. Immunoreactive levels of N-CAM were determined in cultures of naive (●) and primed (○) PC12 cells grown for 48 hours in NGF (50 ng/ml) supplemented media containing cordycepin over a range of doses (0 to 1.0 μM). Results show the percentage increase of binding of anti-N-CAM Ig to treated cultures, over cells grown in control media and are the mean \pm S.E. values of six independent determinations. 100% values were 0.086 \pm 0.004 and 0.023 \pm 0.002 O.D. units (mean \pm S.E. n = 6) for naive and primed cells respectively.

EFFECTS OF NGF, PMA AND A23187 ON N-CAM EXPRESSION

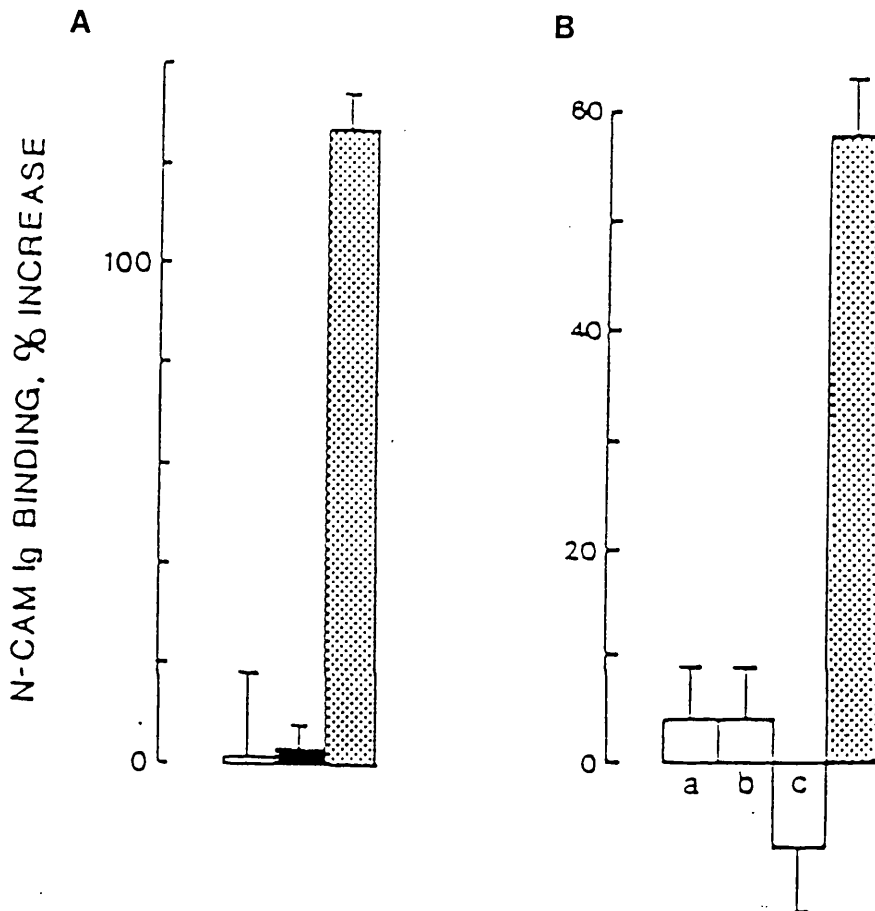


Figure 4.3. N-CAM levels were determined for PC12 cells grown over 24 hours in the presence of (A) 20 nM PMA (□), 200 nM PMA (■) or 50 ng/ml NGF (▣); and (B) 62, 125, or 250 ng/ml A23187 (a to c), or 50 ng/ml NGF (▣). The results show the percentage increase in anti-N-CAM Ig binding over cultures grown in control media, and each value is the mean \pm S.E. of seven independent determinations. 100 % control values for (A) and (B) were 0.184 ± 0.006 and 0.126 ± 0.008 O.D. units respectively (mean \pm S.E., $n = 12$).

Cells cultured over the same 24 hour period at optimal concentrations of the calcium ionophore A23187 were also tested for increased N-CAM expression (fig 4.4). While sister cultures treated with NGF (50 ng/ml) demonstrated an 80% increase in their cell surface level of N-CAM relative to control cells, cultures treated with A23187 at 125 ng/ml showed no change in their basal levels of N-CAM expression.

4.2.4. EFFECTS OF PHORBOL ESTERS AND A23187 ON NGF INDUCED N-CAM EXPRESSION.

PC12 cells were maintained over a 40 hour culture period in media containing NGF (50 ng/ml) in the presence and absence of PMA (500nM). Sister cultures were fixed and assayed for their relative cell surface levels of N-CAM and Thy-1. Figure 4.4 shows that while NGF induced increases in Thy-1 expression are inhibited in the presence of high concentrations of PMA, the same dose of PMA has little or no effect on NGF induced increases of N-CAM.

Cells cultured for 24 hr in the presence of NGF (50 ng/ml) in media supplemented with and without A23187 (125 ng/ml) were also assessed for their levels of cell surface N-CAM and Thy-1. While A23187 significantly elevated NGF induced expression of Thy-1, it had neither additive or inhibitory effects on NGF induced N-CAM expression (data not shown).

These data confirm that PMA and A23187 are without effect on N-CAM regulation in PC12 cells and indicate that while the phosphoinositide/ protein kinase C signal transduction system may mediate NGF induction of Thy-1 it is unlikely to mediate NGF activation of the N-CAM gene or play a direct role in promoting translation of N-CAM mRNA's.

THY-1 AND N-CAM EXPRESSION IN THE PRESENCE OF HIGH DOSES OF PMA

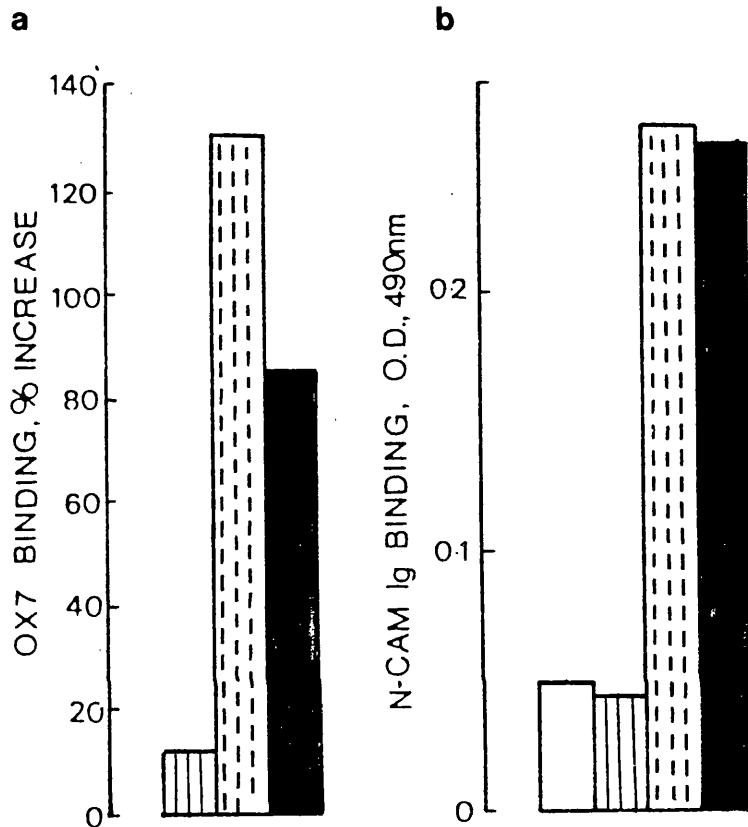


Figure 4.4. PC12 cells were grown over 40 hours in control media (no shading), media supplemented with 500 nM PMA (unbroken lines), 50 ng/ml NGF (broken lines), or 500 nM PMA and 50 ng/ml NGF (shaded). **a** shows the percentage increase in McAb OX7 binding over control cultures with 100 % control measured as 0.373 ± 0.028 (mean \pm S.E., $n = 7$). **b** shows the absolute binding of anti-N-CAM Ig to the four sets of cultures, with each value being the mean \pm S.E. of seven independent determinations.

4.2.5. THE EFFECT OF CHOLERA TOXIN ON N-CAM EXPRESSION IN NAIVE AND NGF TREATED PC12 CELLS.

PC12 cells were grown in the presence of and absence of NGF in media containing cholera toxin at concentrations ranging between 0 and 1.0 ng / ml. After a culture period of 48 hr cells were fixed and assayed for N-CAM immunoreactivity. In the absence of NGF the level of expression of N-CAM was essentially unchanged over the full range of cholera toxin concentrations (fig. 4.5), thus confirming as previously reported (Prentice et al, 1987) that cholera toxin has no direct effect on N-CAM expression. However, in the presence of NGF, cholera toxin promoted further increases in the induced level of expression of N-CAM. This effect was dose dependent, being quantifiable at the lowest cholera toxin concentration tested (10^{-6} ng / ml) with half maximal (100% increases over cultures treated with NGF alone) and maximal (greater than 2 - fold increase) effects being observed at 0.1 pg/ml and 0.1 ng/ml respectively. This result suggests that cholera toxin can act synergistically with NGF to promote greater accumulation of N-CAM over the 48 hour period, this resembles the effect of NGF and cholera toxin cotreatment on morphological differentiation over the same period of culture (Doherty and Walsh, 1987 ; Doherty et al, 1987).

4.2.6. POTENTIATION OF NGF INDUCED INCREASES IN N-CAM EXPRESSION BY CHOLERA TOXIN IS A TRANSIENT EFFECT.

The synergistic effects of NGF and cholera toxin on the morphological differentiation of PC12 cells is a transient phenomenon (Doherty et al, 1987 ; Richter-Landsberg and Jastorff, 1986), being superseded in long term cultures (approximately 6 days) by an inhibitory effect in which further elongation of neurites appears to be reduced relative to cultures treated with NGF alone. To determine

DOSE RESPONSE:- POTENTIATION OF THE N-CAM
RESPONSE BY CHOLERA TOXIN

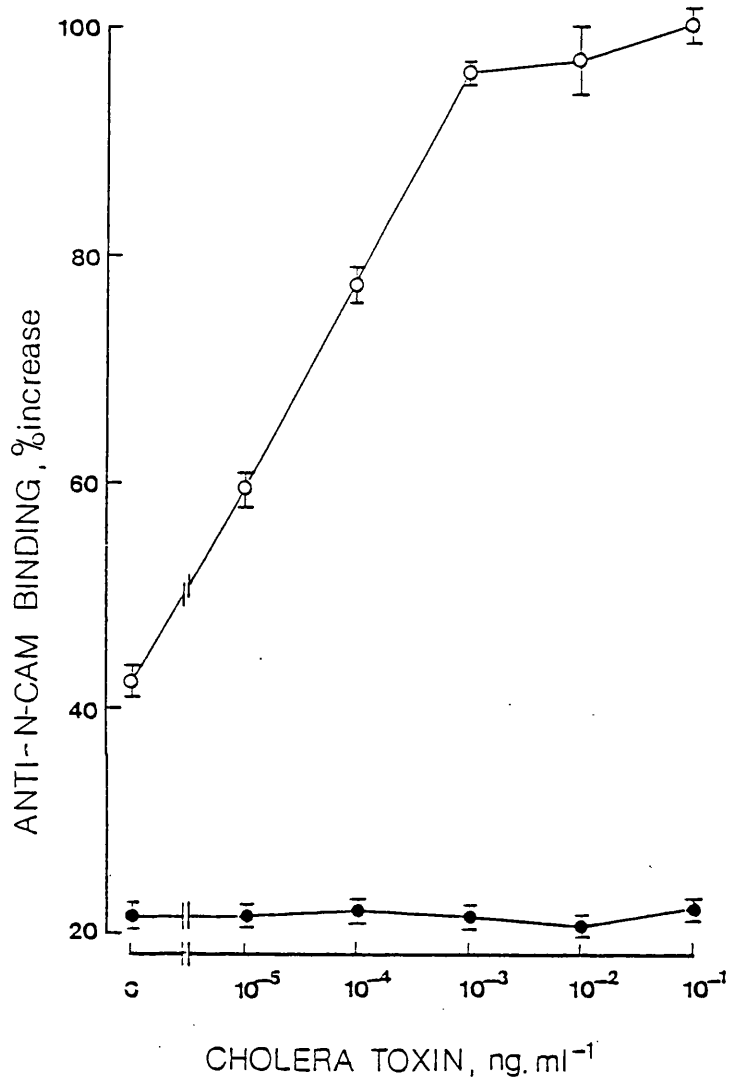


Figure 4.5. Immunoreactive N-CAM levels were determined for PC12 cells grown for 48 hours over a wide range of doses of cholera toxin in the presence () and absence () of 50 ng/ml NGF. The results show the percentage increase in anti-N-CAM Ig binding over cultures grown in control media, each value representing the mean +/- S.E. of seven independent determinations. 100 % control value was 0.476 +/- 0.029 O.D. units (mean +/- S.E., n = 7).

if similar time dependent changes are associated with the induction of N-CAM, cells were cultured for between 1 and 5 days in media supplemented with NGF (50 ng / ml), cholera toxin (0.1 ng / ml), or NGF and cholera toxin, fixed at daily intervals and assayed for increases in N-CAM immunoreactivity relative to control cultures (SATO only). Figure 4.6 shows that on its own cholera toxin has no effect on N-CAM expression over the entire 5 day period. In the presence of NGF, cholera toxin acted synergistically to yield over the initial 72 hr of culture up to 100% higher expression of N-CAM than in cultures treated with NGF alone. However the potentiation was transient and was followed by a clear inhibitory effect on NGF induced increases between day 4 and 5 of the timecourse.

4.2.7. CHOLERA TOXIN POTENTIATES THE DE NOVO SYNTHESIS OF BOTH THE 140 Kd AND 180 Kd N-CAM POLYPEPTIDES.

NGF induction of PC12 cells is associated with qualitative as well as quantitative changes in N-CAM expression, involving increased synthesis of the 140 kd polypeptide and the appearance of the 180 kd polypeptide (Prentice et al, 1987). It was of interest to determine if both NGF induced events are subject to modulation by cholera toxin. Figure 4.7 shows a detailed analysis of the molecular forms of N-CAM synthesised over a 4 hour period of radiolabelling following 44 hr of pretreatment with NGF (50 ng / ml), cholera toxin (0.1 ng / ml), cholera toxin and NGF, or SATO media alone. The fluorograph (see Materials and Methods) shows that control cultures synthesise the 140 kd isoform alone. Cholera toxin on its own had no effect on this pattern of expression while in contrast NGF increased the amount of ³⁵S-methionine incorporated into both the 140 kd and 180 kd polypeptides. A further increase in the rate of

TIMCOURSE :- MODULATION OF THE N-CAM
RESPONSE BY CHOLERA TOXIN

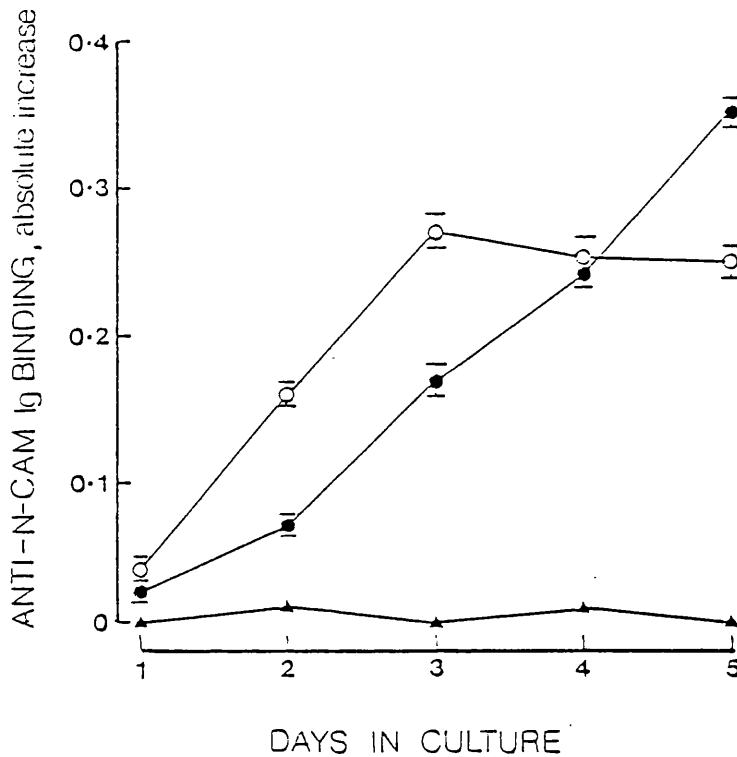
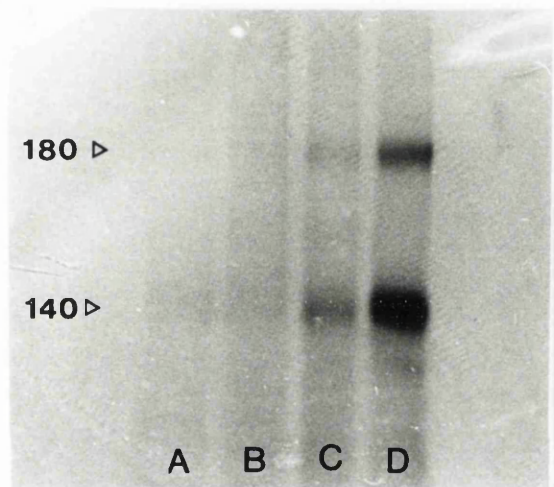


Figure 4.6. Cell surface immunoreactive N-CAM levels were determined for PC12 cells grown for up to five days in media supplemented with 50 ng/ml NGF (●), 0.1 ng/ml cholera toxin (▲), or 50 ng/ml NGF and 0.1 ng/ml cholera toxin (○). The results show the increases in anti-N-CAM Ig binding in treated cultures relative to control cultures and each value represents the mean \pm S.E. of seven independent determinations. Binding of anti-N-CAM Ig to control cultures did not change over the five day period and was measured as 0.045 \pm 0.003 O.D. units (mean \pm S.E., n = 7).

N-CAM BIOSYNTHESIS IN PC12 CELLS



- A. Control
- B. Cholera Tx
- C. NGF
- D. NGF + Cholera Tx

Figure 4.7. Accumulation of ^{35}S -labelled N-CAM over 4 hr in cultures treated for 48 hr in control media (a), media supplemented with 0.1 ng/ml cholera toxin (b), 50 ng/ml NGF (c), or 0.1 ng/ml cholera toxin and 50 ng/ml NGF (d). Immunoprecipitated N-CAM polypeptides of 140 and 180 Kd molecular weights were resolved by SDS-PAGE (7%) and visualised by fluorography at -70°C in the presence of an intensifying screen.

synthesis of the 140 kd and 180 kd isoforms is apparent in cultures treated with both NGF and cholera toxin.

Sister cultures were treated in an identical manner to those used in the protein synthesis experiment, with the exception being that radiolabelling was not carried out. After 48 hr culture these cells were lysed and assayed for total cellular protein content (see Materials and Methods). Increases of 37%, 34%, and 12% in total protein were determined for cultures treated with NGF, cholera toxin, and NGF plus cholera toxin respectively, relative to control cultures. Therefore potentiation of NGF induced N-CAM synthesis cannot be explained by a general stimulation of protein synthesis.

4.2.8. EFFECT OF NGF ON THE CELL SURFACE EXPRESSION OF L1.

Naive PC12 cells were cultured in the presence and absence of NGF (50 ng/ ml) for 48 hr. Cells were then fixed and assayed for their ability to bind anti-L1 Ig utilising an ELISA based binding assay in which cells were incubated with an anti-L1 antisera over a wide range of titrations (200 to 80,000 fold dilution of an ammonium sulphate cut of rabbit antisera). Fig 4.8 shows an increase in the anti-L1 Ig binding profile in NGF treated cultures compared with sister cultures growing in the absence of growth factor. An increase of approximately two-fold was detectable at an anti-L1 Ig titre of 1:200 and 1:800, this difference being substantially reduced at lower Ig concentrations. Scarcity of the antisera did not allow the investigation of relative binding profiles at lower titres and therefore it is not possible to determine whether the observed two-fold increase at the 1:200 dilution is maximal.

4.2.9. EFFECTS OF PHORBOL ESTER AND A23187 ON L1 EXPRESSION.

PC12 cells were cultured for 24 hr in the presence of either NGF (50 ng/ml), PMA (20 nM), A23187 (125 ng/ml) or SATO alone. Cells were fixed and assayed for their relative cell surface expression of L1 antigen by ELISA using a 1:800 dilution of anti-L1 antisera. While NGF induced a two-fold increase in L1 expression relative to control cultures, PMA induced only a 36% increase while A23187 was without effect (data not shown). These data indicate that the calcium-dependent protein kinase C signal transduction pathway is unlikely to play a major role in mediating NGF induced increases in the expression of L1.

4.2.10. EFFECTS OF CHOLERA TOXIN ON THE EXPRESSION OF L1 IN NAIVE AND NGF TREATED PC12 CELLS.

Sister cultures of naive PC12 cells were grown for 48 hr in SATO media alone, or supplemented with NGF (50 ng/ml), cholera toxin (0.1 ng/ml), or NGF and cholera toxin. Cultures were fixed and assayed for their relative cell surface abundance of L1 antigen as described above (fig 4.9). In contrast to N-CAM (Fig 4.6), Thy-1 (fig 3.12) and the NGF-receptor (Doherty et al,1988), L1 expression was significantly increased in cholera toxin treated cultures, with an 80% increase in total cell surface antigen relative to control cultures (SATO alone). NGF treated cultures demonstrated higher levels of cell surface L1 than their sister cultures treated with either SATO alone or with cholera toxin, increases in expression of approximately two to three fold over control cultures. In cultures co-treated with NGF and cholera toxin there was a marked inhibition of the NGF induced levels of L1 found in sister cultures treated with NGF alone, increases in L1 expression were reduced to a level similar to that seen in cholera toxin treated cultures (at 0 ng/ml NGF).

EFFECT OF NGF ON ANTI-L1 Ig BINDING

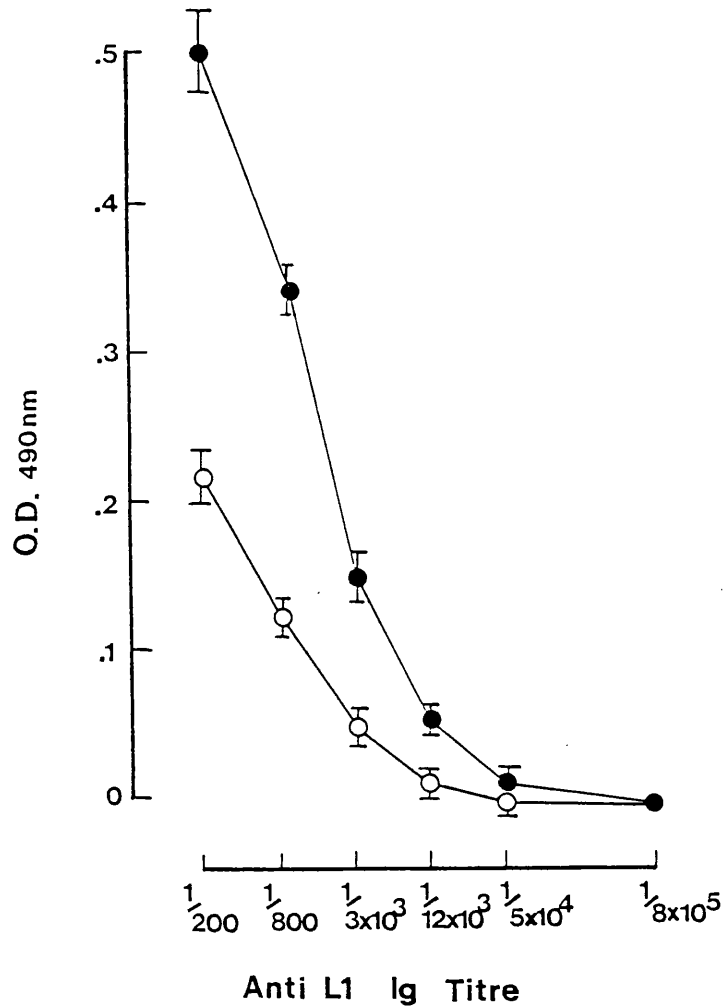


Figure 4.8. PC12 cells were cultured over 48 hours in the presence (●) and absence (○) of 50 ng/ml NGF. Cultures were then assayed for their ability to bind anti-L1 Ig. The results show the effects of NGF treatment on the binding of anti-L1 Ig over a wide range of antibody titrations. Each data point is the mean +/- S.E. of six independent cell binding determinations.

L1 EXPRESSION :- MODULATION BY NGF AND CHOLERA TOXIN

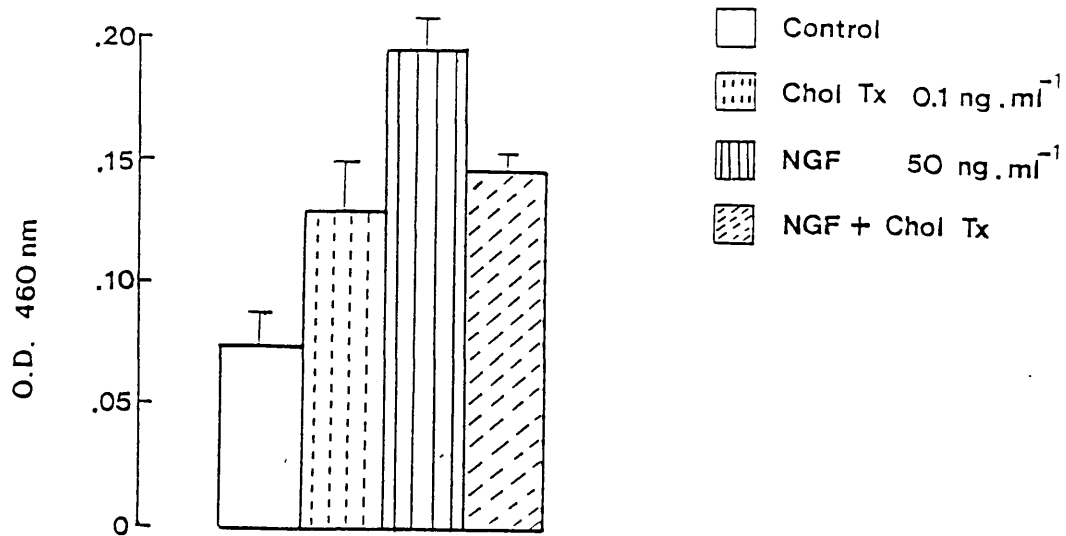


Figure 4.9. Cell surface immunoreactive L1 antigen levels were determined for PC12 cell cultures grown for 48 hours in SATO media alone (control), or with cholera toxin (0.1 ng/ml), NGF (50 ng/ml), or cholera toxin and NGF as indicated. The results show the binding of anti-L1 Ig to cultures expressed in OD units and represent the mean +/- S.E. of seven independent determinations.

These data suggest that cholera toxin can induce L1 expression independantly of NGF and can in addition cause an inhibition of NGF induced increases in the same molecule. Thus it would appear that L1 expression can be regulated in PC12 cells by at least two independent mechanisms, one being transduced by a cholera toxin inducable pathway, the other by a cholera toxin sensitive NGF inducable route.

4.2.11. THE EFFECTS OF CHOLERA TOXIN ARE MIMICKED BY FORSKOLIN.

Cholera toxin and forskolin share a common property, the ability to activate adenylate cyclase and thereby elevate intracellular levels of cAMP (Seamon.K.B. et al, 1981). In the presence of forskolin, NGF treated and untreated PC12 cells respond in a similar manner to that observed with cholera toxin treatment (Doherty et al, 1987). As shown in figure 4.10, forskolin (10uM) mimics NGF induced morphological differentiation (50 ng/ml) over a 48 hour period of culture. In addition forskolin appears to act synergistically with NGF to promote more rapid neurite outgrowth over the 48 hour period. Cells growing for the same 48 hour period and under the same conditions as those described for morphological studies were fixed and assayed for cell surface N-CAM, L1 and Thy-1 immunoreactivities (fig 4.11). The effects elicited on the three cell surface glycoproteins were essentially the same as described with cholera toxin. Of the three molecules, L1 was induced by forskolin to the greatest extent (156% increase relative to control cultures), whearas relatively minor changes (50% or less) were detected for N-CAM and Thy-1. NGF induced increases in Thy-1 and L1 expression were inhibited by forskolin, whilst in contrast NGF induction of N-CAM was potentiated to yield a two-fold higher level of expression

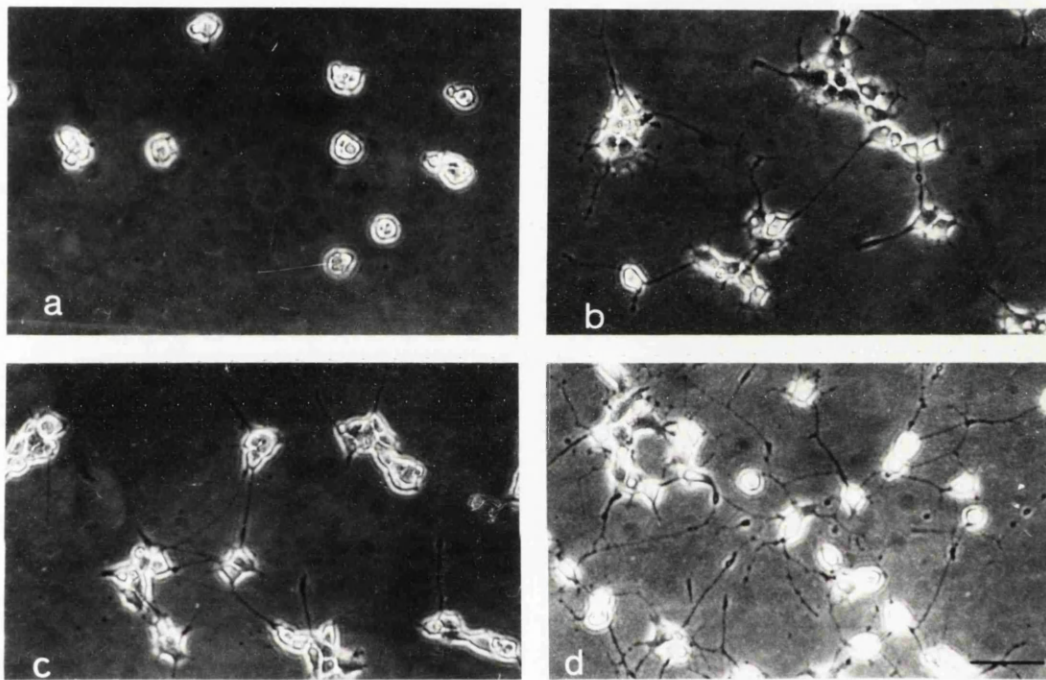


Figure 4.10. PC12 cells were grown over 48 hours in control media (a), or, media supplemented with 50 ng/ml NGF (b), 10 uM forskolin (c), or 50 ng/ml NGF and 10 uM forskolin (d). Photomicrographs show representative fields from cultures of naive PC12 cells seeded at a density of 40,000 cells / chamber. Scale bar represents 50 uM.

MODULATION OF NGF INDUCED GLYCOPROTEIN EXPRESSION BY FORSKOLIN

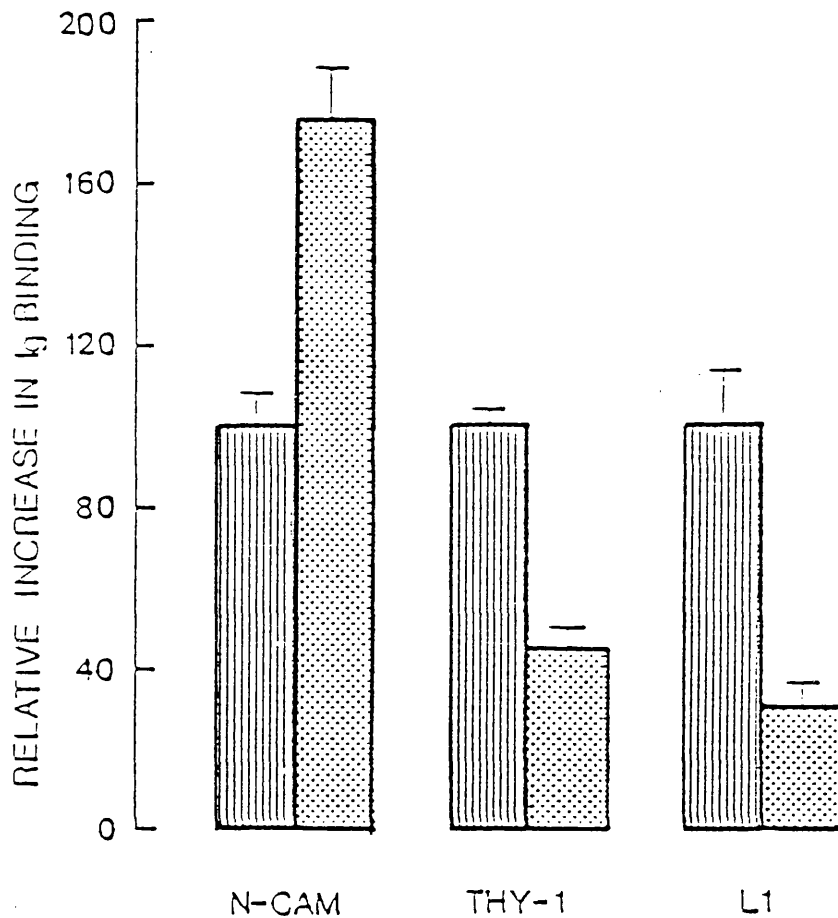


Figure 4.11. Immunoreactive N-CAM, Thy-1, and L1 levels were determined for PC12 cells grown for 48 hours in the presence of 50 ng/ml NGF (▨) or 50 ng/ml NGF and 10 uM forskolin (▩). Data show the mean \pm S.E. (bars) from seven independent determinations and are expressed as relative increases in the binding of anti-N-CAM Ig, OX7 McAb, or anti-L1 Ig over cultures grown in SATO media alone. A relative increase in binding of 100 units for anti-N-CAM, OX7, and anti-L1 represented O.D. values of 0.201 ± 0.010 , 0.764 ± 0.010 , and 0.489 ± 0.027 (mean \pm S.E.) respectively

than that seen in cultures treated with NGF alone. These data suggest that the differential effects of cholera toxin (and forskolin) on NGF induction of Thy-1 and N-CAM are the result of stimulation of adenylate cyclase activity and a subsequent rise in the intracellular level of cAMP.

4.3. DISCUSSION

There is increasing evidence to suggest that the neural cell recognition molecules N-CAM and L1 may function as environmental cues for the support and guidance of axons during development of the nervous system (Edelman, 1986 ; Jessell, 1988 ; Jacobson, 1988 ; Dodd and Jessell, 1988 ; Bixby et al, 1987 and 1988). N-CAM is an integral glycoprotein component of all axonal surfaces (Dodd and Jessell, 1988 ; Silver and Rutishauser ; Edelman et al, 1983), and is also expressed in abundance at the surface of the neuroepithelial and mesenchymal cells that act as substrates for pioneering axons (Dodd and Jessell, 1988 ; Thiery et al, 1982 ; Silver and Rutishauser, 1984 ; Crossin et al, 1985 ; Balak et al, 1987). L1 can be detected at the axonal surface of a variety of CNS and PNS neurons and can also be found at the surface of the Schwann cells and glial cells which extending axons may encounter and interact with in nervous tissues (Rathjen and Schachner, 1984 ; Grumet et al, 1984 ; Faissner et al, 1984 ; Nieke and Schachner, 1985 ; Noble et al, 1985).

Neurite outgrowth is essentially an adhesion based mechanism (Bray, 1987), being driven by the ability of the advancing growth cone to adhere to substrate, and by a process described as "push pull" can extend the length of the growing axon. N-CAM can promote adhesive interactions

between apposing cell surfaces providing that it is expressed at both cell membranes (Duband et al, 1987). Thus N-CAM mediated homophilic binding could contribute to the mechanism of neurite extension. Support for this contention is provided by studies in which anti-N-CAM antibodies have been demonstrated to significantly reduce the outgrowth of central and peripheral axons on permissive substrates in vitro (Tomaselli et al, 1986 ; Bixby et al, 1987 ; Chang et al, 1987 ; Neugebauer et al, 1988). Similar effects have been described for neuritic outgrowth across Schwann cell surfaces when anti-L1 antibodies are introduced (Bixby et al, 1988), suggesting that L1 mediated binding (can be homophilic or heterophilic, see chapter 1) may also promote neurite extension. Modulation of the spatio-temporal expression of cell surface recognition molecules may allow for subtle changes in the affinity of apposing cell surfaces (Edelman, 1986). The ability of the growing axon to reach its appropriate destination may depend on a mechanism of differential adhesion in which the axon chooses a path of preferential adhesive interactions. The relative expression of N-CAM and L1 on neuronal cells is regulated by tissue and developmental programmes. For example, in the developing nervous system the appearance of the different N-CAM isoforms is strictly controlled such that the ratio of the three N-CAM polypeptides is altered during the development of nervous tissue (Murray et al, 1986 b ; Pollerberg et al, 1985) and, while N-CAM is present at the surface of undifferentiated pre-neural cells (Edelman et al, 1983) L1 is exclusively associated with the axonal surfaces of differentiating neurons (Thiery et al, 1985). Elucidation of the cellular signals that mediate control of neuronal cell expression of these recognition molecules may provide key insights into the mechanisms underlying development of the nervous system.

NGF induced differentiation of PC12 cells is accompanied by increases in mRNA and polypeptide species encoding the N-CAM isoforms found in nervous tissue (Prentice et al, 1985). In the present study, changes in PC12 cell surface levels of N-CAM were quantitated after only 24 hr of NGF treatment and were found to accumulate over a similar timecourse to that for neurite outgrowth (Doherty et al, 1987). The N-CAM response was shown to be transcription dependent and to require significantly higher concentrations of NGF for induction than those necessary for activation of the Thy-1 response. For example, while concentrations of less than 1 ng/ml NGF had no effect on N-CAM expression, a concentration of 0.8 ng/ml NGF is sufficient to induce a half-maximal Thy-1 response (Ch. 3). These differences in absolute requirement of each response for NGF suggest that N-CAM transcription may be activated by NGF through an independent signal transduction pathway from that mediating the Thy-1 response. This suggestion was confirmed by the inability of phorbol ester PMA, and calcium ionophore A23187 to mimic or modulate NGF induced increases in N-CAM expression. Thus N-CAM induction by NGF must be mediated by intracellular signals that act independently from the phosphoinositide and calcium dependent protein kinase C transduction machinery that mediates Thy-1 induction.

Previous studies have provided evidence for the ability of NGF to regulate the cell surface expression of the L1 molecule in PC12 cells (Lee et al, 1981 ; McGuire et al, 1978 ; Salton et al, 1983 a and b). In the present study NGF was shown to induce increases of between two- and three-fold in the level of immunoreactive L1 antigen at the surface of differentiating PC12 cells after 24, 48 and 72 hr of treatment. Limited supply of antibody restricted these studies thus disallowing extensive dose and timecourse

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analyses, however in an attempt to determine the possibility that the protein kinase C signal transduction route may play a role in mediating NGF induction of the L1 antigen, the ability of PMA and A23187 to mimic the NGF response was investigated. Neither PMA or A23187 were able to induce an NGF-like L1 response, the former reagent promoting a relatively minor (30% of NGF response) induction while the latter was completely without effect. The small effect induced by PMA is unlikely to be mediated through activation of protein kinase C since the effect was not mimicked by A23187, however it cannot be ruled out that the induction is mediated by an alternative signal transduction route that is sensitive to PMA (phorbol esters are known to have biochemical effects other than their activation of protein kinase C, see Nishizuka, 1986) and which may contribute to NGF activation of the L1 gene. In conclusion, NGF induction of increased L1 expression in PC12 cells is unlikely to be mediated by the phosphoinositide and calcium dependent protein kinase C signal transduction pathway and is therefore, like the N-CAM response, regulated by an independent transduction route from that controlling Thy-1 expression.

It was of interest to determine the potential for the cAMP signal transduction system to be functionally involved in regulation of N-CAM and L1 expression, since it has been suggested that the protein kinase C and protein kinase A pathways may act in concert to orchestrate the NGF differentiation response as a whole. In a previous study it was reported that cholera toxin was unable to mimic NGF induced increases in N-CAM expression (Prentice et al, 1987). This observation was confirmed in the current study, indicating that cAMP has no direct effects on N-CAM expression and therefore is unlikely to be the dominant

mediator of this NGF induced response. In contrast, L1 expression in naive cells was directly affected by cholera toxin which induced an 80% (relative to control cells) increase in cell surface expression of the antigen. The involvement of the second-messenger cAMP as the intracellular mediator of this response was given further support by the demonstration of a similar direct induction by forskolin, a direct activator of adenylate cyclase (Seamon et al,1981). Furthermore, the inability of cholera toxin to directly induce changes in the expression of Thy-1 (Doherty and Walsh, 1987), N-CAM (Prentice et al, 1987) and the NGF-receptor, (Doherty et al, 1988) demonstrates that the L1 response to activators of adenylate cyclase is specific and not a consequence of a general stimulation of glycoprotein synthesis. Since cAMP is unable to induce a full NGF-like morphological response and cannot promote the formation of the dense and highly branched neuritic network associated with NGF induced differentiation (Greene et al, 1986), it would seem that induction of increases in L1 expression alone are not sufficient for supporting morphological differentiation of PC12 cells.

Although cAMP does not apparently play a dominant mediatory role in NGF induced differentiation of PC12 cells, it does have the capacity to act as a powerful modulatory signal upon NGF induced events. For example, cholera toxin and dibutryl cAMP can act in synergism with NGF to promote rapid neuritogenic responses (Gunning et al, 1981 a and b ; Drubin et al, 1985 ; Doherty et al, 1987). As discussed in chapter 3, the modulatory effects of cAMP are differential and highly specific for a given response, while NGF induced increases in the synthesis and expression of B-actin and the MAP and TAU proteins are potentiated by cAMP (Drubin et al, 1985), other responses functionally implicated in the

neuritogenic process such as accumulation of neurofilament protein (Doherty et al, 1987), increased expression of Thy-1 (Doherty and Walsh, 1987) and increased synthesis of the NGF receptor (Doherty et al, 1988), are actually inhibited by cAMP. Since N-CAM and L1 have been proposed to be potential neurite promoting molecules it was of interest to determine the status of their cell surface expression during the phase of accelerated neurite outgrowth generated by the synergistic effects of cAMP and NGF.

In the presence of either cholera toxin or forskolin, NGF induced increases in the cell surface expression of L1 antigen were reduced to levels similar to those seen in sister cultures exposed to adenylate cyclase activators in the absence of growth factor. Thus whilst directly mediating an induction of the L1 antigen, cholera toxin and forskolin are able to concomitantly inhibit an alternative and as yet unidentified transduction component that mediates NGF activation of L1 gene expression. Since induction by NGF promotes higher levels of L1 antigen than those induced by activators of adenylate cyclase, it is unlikely that cAMP acts as the dominant mediator of NGF induced changes in L1 expression. Moreover, as co-treatment of PC12 cells with NGF and, either cholera toxin or forskolin, is associated with a synergistic effect on morphological differentiation, it is unlikely that L1 plays a dominant role in the process of neurite outgrowth since this phase of enhanced neurite extension is accompanied by a significantly reduced level of cell surface L1 expression relative to the levels observed in sister cultures differentiating less rapidly in the presence of NGF alone.

In direct contrast to the effects observed with Thy-1 (Doherty and Walsh, 1987 ; chapter 3) L1, neurofilament

protein (Doherty et al, 1987), and the NGF-receptor (Doherty et al, 1988), cholera toxin acted synergistically with NGF to promote further increases in the cell surface expression of N-CAM. At an optimal dose of 0.1 ng / ml, the toxin alone had no effect on N-CAM expression, yet in the presence of NGF promoted up to 2-fold higher levels of N-CAM than recorded in cells treated with NGF alone. Metabolic labeling revealed the increased de novo synthesis of both the 140 and 180 kd isoforms stimulated by NGF in PC12 cells (Prentice et al, 1987). Therefore the effects of cholera toxin are directed at both of the N-CAM responses induced by NGF, one being the increased expression of the 140 kd polypeptide which is present in naive cells at a low basal level of expression and the other being the activation of a new splicing pattern allowing the 180 kd isoform to emerge in the differentiated cell (Prentice et al, 1987).

The ability of cholera toxin to differentially modulate NGF induction of N-CAM, L1 and Thy-1 provides further evidence that NGF induced differentiation of PC12 cells is mediated by more than one signal transduction cascade. L1 resembles Thy-1 in that its induction by NGF is inhibited by cholera toxin, however it differs in that its induction by NGF cannot be mimicked by activators of protein kinase C, similarly N-CAM induction by NGF differs from both L1 and Thy-1 in that it is potentiated by cholera toxin. Thus, L1, Thy-1 and N-CAM gene activation by NGF appears to be mediated via at least three independantly acting signal transduction pathways.

The synergistic effect of cholera toxin and NGF on N-CAM synthesis was found to be transient. Over the initial 72 hr of culture, cells co-treated with cholera toxin and NGF expressed higher levels of N-CAM than sister cultures

treated with NGF alone, however at by days 4 and 5 of culture the potentiating effects of cholera toxin were lost and replaced by a small, but significant inhibitory effect on the NGF induced response. This pattern of potentiation followed by inhibition resembles the effects observed morphologically in which the synergistic effects of cAMP and NGF on neurite outgrowth are eventually superseded by inhibitory effects (Richter-Landsberg and Jastorff, 1986). Thus it would appear that of the cell adhesion molecules so far studied, changes in the cell surface expression of N-CAM correlate best with the morphological status of the PC12 cell. The mechanism responsible for causing the potentiation / inhibition effects of cholera toxin on the N-CAM response was not investigated. The demonstration that the potentiation of NGF induced increases in the expression of N-CAM could be reproduced by forskolin strongly supports the view that the modulatory effects of cholera toxin on the NGF induced response may be mediated by cAMP. In chapter 3, cholera toxin was shown to concomitantly potentiate B-actin gene transcription and inhibit Thy-1 and NGF receptor transcription. Thus it is possible that cAMP may also enhance transcription of the N-CAM gene, although this remains to be demonstrated. In the case of the Thy-1 response it appears likely that the inhibitory effect of cAMP is due to protein kinase A mediated inhibition of the hydrolysis of inositol polyphosphates (Nishizuka, 1984 ; Supattapone et al, 1988 ; Billah et al, 1979 ; Isakov and Altman, 1985 ; Omann et al, 1987). The pleiotropic effects of cAMP are believed to reflect the ability of protein kinase A to act on a variety of substrates, producing in some cases a more active protein, and in others a less active protein (Krebs, 1986). Therefore a plausible explanation of the ability of cholera toxin and forskolin to potentiate NGF induced N-CAM synthesis would be that

activation of protein kinase A by cAMP leads to a protein phosphorylation event that increases the activity of an already active component of the signal transduction pathway responsible for mediating NGF increases in N-CAM synthesis. Future studies would be directed towards the identification of intermediates of this signal transduction route and determining the effects of phosphorylation on their biological activities in an effort to determine the mechanism of action of cAMP as a potentiator of the NGF induced response. Additional studies may be aimed at determining the ability of the phosphorylated intermediate(s) to stimulate N-CAM synthesis at translational and / or transcriptional levels.

The events that underlie cAMP's inhibitory effects on neurite outgrowth and on N-CAM synthesis in long term cultures were not investigated. However, one possibility would be that the inhibitory effects of cAMP on NGF induced increases in NGF receptor expression (Doherty et al, 1988) lead to a gradual loss of expression at the cell surface with the result that all NGF induced responses are eventually inhibited.

An association between cell surface N-CAM expression and morphological status of the cell was observed and may be indicative of the glycoprotein playing a major functional role in the process of neurite outgrowth. However a precise function for N-CAM and its different isoforms in the neuritogenic process was not determined. Coculture studies using fibroblasts transfected with cDNA's encoding a variety of N-CAM isoforms as substrates for neurite outgrowth (Doherty et al, 1989 a and b) provide evidence for N-CAM promoting neurite outgrowth via a homophilic binding mechanism. It is unlikely that a similar mechanism operates

in the current study as all PC12 cell cultures were grown and differentiated on collagen coated substrates. Recent investigations with a variety of neurite outgrowth promoting CAM's and integrins have demonstrated that these integral membrane molecules may stimulate process outgrowth by mechanisms other than cell adhesion (Bixby et al, 1988). It has been suggested that integral membrane cell adhesion molecules such as N-CAM 180 and 140, and L1, can act as signal transduction elements and may promote neurite outgrowth by transducing a cue for outgrowth in the microenvironment, to the cytoskeleton via the interaction of their cytoplasmic domains with cytoskeletal -associated proteins (Horwitz et al, 1985 ; Togari et al, 1983 ; Pollerberg et al, 1986 ; Hirano et al, 1987 ; Volk et al, 1987 ; Bixby et al, 1988). For example, the CSAT antigen is thought to promote cytoskeletal stabilisation through its association with fibronectin or / and laminin at the outer face of the cell, and at the cytoplasmic domain with cytoskeletal elements via its association with talin (Horwitz et al, 1986) a cytoskeletal -associated protein. A similar stabilising effect has been proposed for the 180 kd N-CAM isoform (Pollerberg et al, 1986) in the membrane of the differentiating PC12 cell. It has been proposed that the large cytoplasmic domain of N-CAM 180 may provide an anchorage for cytoskeletal components of the cytoplasm with the plasma membrane and in doing so effectively reduce lateral mobility within the plasma membrane. Such an interaction would therefore stabilise any cytoskeletal interactions. Since the 180 kd isoform only appears in the NGF induced cell (Prentice et al, 1987) it may play a specific role in providing stabilisation for cytoskeletal elements involved in formation and maintenance of neurites. In support it is noteworthy that cAMP treatment of NGF induced cells results in the stimulation of NGF induced

increases in a variety of cytoskeletal associated proteins including the MAP and TAU proteins (Drubin et al, 1985) which are believed to be key regulators of the neuritogenic response.

CHAPTER 5 : FIBROBLAST GROWTH FACTOR (FGF) INDUCED DIFFERENTIATION OF PC12 CELLS.

5.1. INTRODUCTION.

Recent studies have indicated the potential for the acidic and basic fibroblast growth factors (aFGF and bFGF) to act as neurotrophic and neurotropic substances in the CNS (Walicke et al, 1986 ; Morrison et al, 1986 ; Morrison, 1987 ; Anderson et al, 1988 ; Lipton et al, 1988). For example, bFGF has been reported to promote the survival of dissociated hippocampal neurons and enhance neuritic extension from these cells in culture (Walicke et al, 1986), to support the survival of cerebral cortical neurons in culture (Morrison et al, 1986) and to prevent the death of lesioned cholinergic neurons in vivo (Anderson et al, 1988), while aFGF has been demonstrated to stimulate the regeneration of processes by postnatal rat retinal ganglion cells in culture (Lipton et al, 1988). Coupled to the finding that nervous tissues provide a rich source of both aFGF (Lobb and Fett, 1984; Thomas et al, 1984 ; Gospodarowicz et al, 1986) and bFGF (Gospodarowicz et al, 1984), there appears to be increasing evidence for a role for the FGF's in the development and maintenance of the nervous system.

The FGF's are polypeptide hormones (Gospodarowicz et al, 1986 ; Thomas and Gimenez-Gallego, 1986) that have potent angiogenic and mitogenic activities for a wide variety of cells including those of mesoderm and neuroectoderm origins. The FGF's have a high degree of structural homology with one another (Esch et al, 1985 ; Gimenez-Gallego et al, 1985) and act as mitogens on the same cell types, this latter property reflecting their ability to interact with a common cell surface receptor (Neufeld and Gospodarowicz, 1986). The

bioactivity of the FGF's is known to be modulated by heparin for which both polypeptides have a high affinity (Gimenez-Gallego et al, 1985 ; Neufeld et al, 1987 ; Lipton et al, 1988) and indeed it has been suggested that the FGF's may exist in an active form as components of a complex including a heparin like molecule such as the heparan-sulphate proteoglycan (Neufeld et al, 1987 ; Lipton et al, 1988).

In contrast to their mitogenic activities the FGF's cause a cessation of the proliferation of PC12 cells and promote the induction of the vast majority of biochemical responses attributed to NGF (Togari et al, 1983 and 1985 ; Rydel and Greene, 1987 ; Neufeld et al, 1987). Included amongst these effects is the induction of neurite outgrowth, which has also been demonstrated to be stimulated by FGF's in some CNS derived neuronal cultures (Lipton et al, 1988). The FGF's have been shown to reproduce both the rapid, transcription independent, and the delayed, transcription dependent, responses characteristic of NGF induced differentiation (Rydel and Greene, 1987), suggesting that the FGF's and NGF may share a similar mechanism of action and may utilise similar or perhaps common signal transduction components.

Amongst the biochemical responses of the PC12 cell to NGF that can be mimicked by the FGF's is the incorporation of radiolabelled fucose into the Thy-1 and L1 (or NILE) cell surface glycoproteins (Rydel and Greene, 1987). The aims of the present study were to determine the effects of FGF on cell surface glycoprotein expression and investigate the possibility that induction of specific biochemical changes by NGF and FGF may involve the utilisation of similar signal transduction pathways.

The source of FGF used in these studies was a highly purified recombinant bovine bFGF (obtained from Amersham International).

5.2. RESULTS.

5.2.1. INDUCTION OF INCREASED THY-1 EXPRESSION BY bFGF IN PRIMED AND NAIVE PC12 CELLS.

Naive and primed PC12 cells were cultured for 48 hr in the presence of bFGF over a wide range of concentrations (0 to 100 ng/ml). Fixed cultures were assayed for changes in cell surface expression of Thy-1, as determined by the relative binding of the McAb OX7. Treatment with bFGF was found to induce increases in antibody binding capacity of cultures (fig 5.1), with a dose-dependent effect on both naive and NGF primed cells. In naive cells (a), increases Thy-1 expression were detected at the lowest concentration of bFGF tested (0.0025 ng/ml), with a maximal response elicited at a concentration of 0.2 ng/ml. Higher concentrations of bFGF appeared to have slightly reduced effects on Thy-1 expression relative to the response observed at optimal concentrations. In primed cells (b), a maximal Thy-1 response was induced at a bFGF dose of 0.04 ng/ml indicating that the dose response curve is shifted toward lower concentrations than recorded with naive cells.

At their optimal doses, NGF (10 ng/ml) and bFGF (0.2 ng/ml) elicit comparable increases in Thy-1 expression, with bFGF being the slightly weaker agent of the two, promoting increases of approximately 86% and 72% relative to the NGF induced responses (100%) in naive and primed cells respectively.

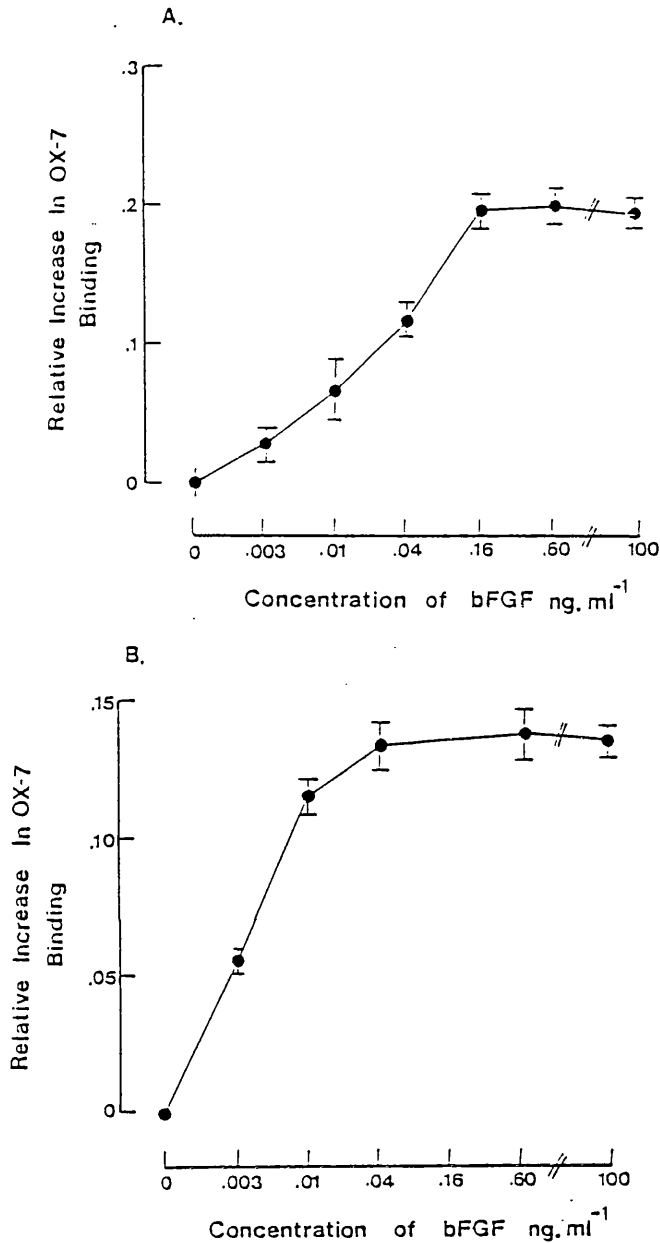


Figure 5.1. Immunoreactive cell surface Thy-1 levels were determined in cultures of naive (A) and primed (B) PC12 cells grown over 48 hours in media supplemented with bFGF over a wide range of concentrations (0 to 100 ng). The results show the relative increase in McAb OX7 binding to cultures over cells maintained in control media and each value is the mean \pm S.E. of seven independent determinations. Values for control cultures were 0.318 ± 0.006 and 0.105 ± 0.005 OD units (mean \pm S.E., $n=7$) for naive and primed cells respectively.

5.2.2. INDUCTION OF INCREASES IN N-CAM EXPRESSION BY bFGF.

Binding of anti-N-CAM antibody to PC12 cell cultures (NGF primed and naive) was determined in cultures treated for 48 hr with a range of bFGF concentrations (0 to 100 ng/ml). Both primed and naive cultures responded to bFGF by undergoing dose-dependent increases in their cell surface abundance of immunoreactive N-CAM (fig 5.2). In naive cultures an induction was detectable at concentrations of less than 10 pg/ml, with a maximal N-CAM response recorded at 0.6 ng/ml. At bFGF concentrations higher than 0.6 ng/ml the induction was markedly reduced, with increasing concentrations promoting increasingly reduced responses. This latter effect appeared to be more significant than the slight fluctuations observed with the Thy-1 response at high bFGF doses. Primed PC12 cells (b) responded to bFGF in a similar manner to naive cultures though the dose response curve was shifted toward lower concentrations, with the maximal response recorded at a dose of approximately 0.2 ng/ml.

N-CAM responses induced by optimal doses of bFGF were roughly comparable to those induced by NGF (50 ng/ml), with the former being the slightly weaker inducer of the two factors.

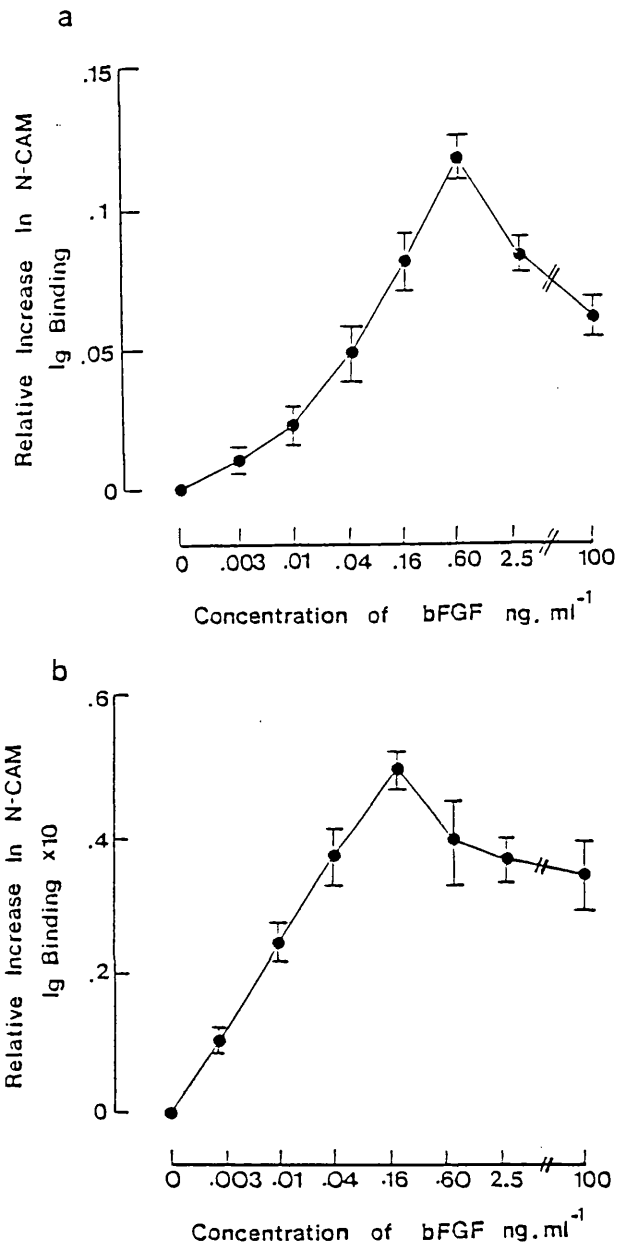


Figure 5.2. Cell surface immunoreactive levels of N-CAM were determined for naive (a) and primed (b) PC12 cells grown over 48 hours in the presence of bFGF (0 to 100 ng/ml). Results show the increase in anti-N-CAM Ig binding to cultures relative to binding measured in cell cultures maintained in control media, with each value representing the mean \pm S.E. of seven independent determinations. Values for control cells were 0.074 ± 0.003 and 0.034 ± 0.002 (mean \pm S.E., $n = 7$) respectively for naive and primed cells.

5.2.3. bFGF INDUCTION OF THY-1 AND N-CAM CAN BE MEDIATED BY BOTH TRANSCRIPTION -DEPENDENT AND -INDEPENDENT MECHANISMS.

To determine the dependency of bFGF induction of Thy-1 and N-CAM on transcription, naive and NGF primed PC12 cells were cultured in the presence of bFGF (1.0 ng/ml) in media supplemented with and without the transcriptional inhibitor cordycepin (1 μ M). After a period of 48 hr cells were fixed and assayed for their increased cell surface expression of both glycoproteins relative to basal levels on control cells (SATO only). In primed cell cultures increases in Thy-1 and N-CAM abundance were comparable in cordycepin treated and untreated populations (fig 5.3). However, in contrast naive cells treated with cordycepin showed essentially no change from their basal level of expression of either Thy-1 or N-CAM, while both glycoproteins were found at elevated levels of expression in sister cultures grown in the absence of the inhibitor. These results suggest that bFGF-induced increases in Thy-1 and N-CAM expression can, like their NGF induced responses, be mediated by either transcription -dependent or -independent mechanisms, with both responses in naive cultures being dependent on ongoing gene transcription. In addition, since NGF primed cells can respond to bFGF in a transcription -independent manner, the data indicates that the cellular signals mediating translational control of cell surface glycoprotein expression must be similar or the same for NGF and bFGF.

EFFECT OF CORDYCEPIN ON bFGF INDUCED RESPONSES

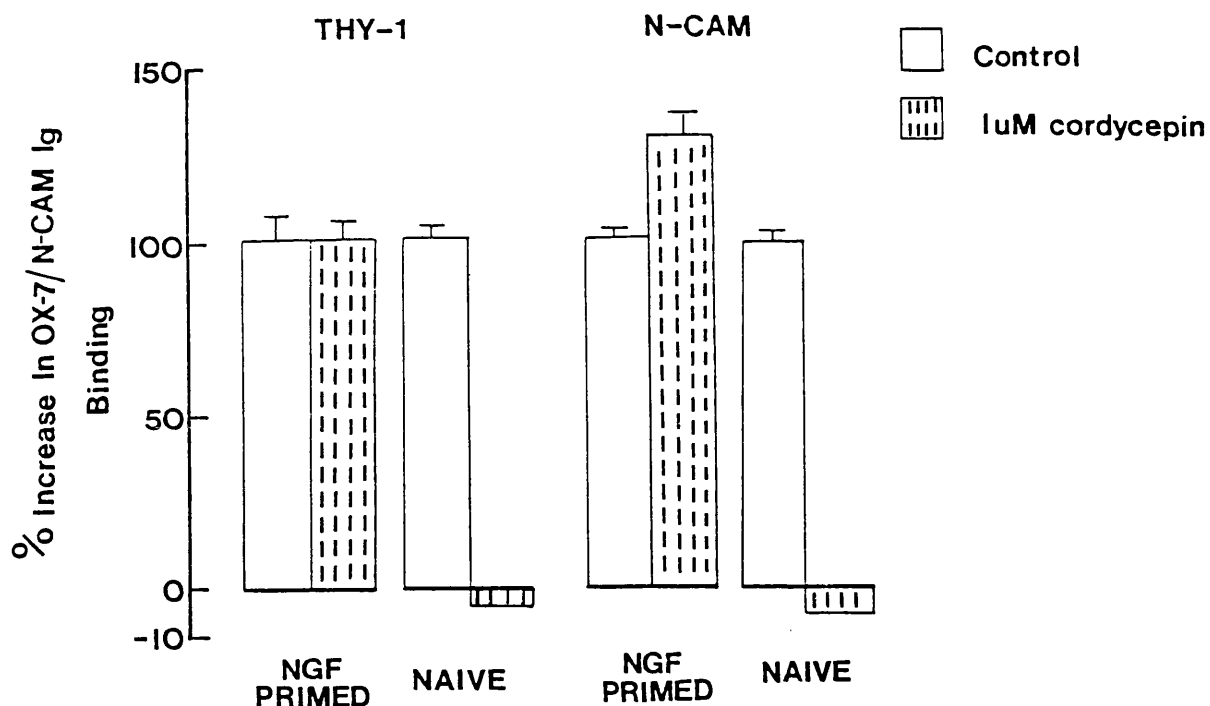


Figure 5.3. Naive and primed PC12 cells were cultured for 48 hours in media supplemented with bFGF in the presence and absence of 1 μ M cordycepin. Cultures were assayed for cell surface expression of immunoreactive Thy-1 and N-CAM. Results are expressed as the percentage increase in binding of either the McAb OX7 or anti-N-CAM Ig over cells maintained in control media (SATO only). Each value is the mean \pm S.E. of seven independent determinations. 100 % control values for Thy-1 were 0.150 ± 0.008 and 0.130 ± 0.010 OD units (mean \pm S.E. $n = 7$) for naive and primed cells respectively. 100 % control values for N-CAM were 0.009 ± 0.003 and 0.080 ± 0.002 OD units (mean \pm S.E. $n = 7$) for naive and primed cells respectively.

5.2.4. NGF AND bFGF INDUCED INCREASES IN THY-1 AND N-CAM ARE NOT MIMICKED BY THE EPIDERMAL GROWTH FACTOR (EGF).

While both NGF and bFGF promote dramatic changes in the morphological status of PC12 cells, a third polypeptide growth factor, EGF (Carpenter and Cohen, 1979), is without any comparable effect (Boonstra et al, 1983). However, EGF is able to mimic a limited number of NGF responses at the biochemical level, such as the induction of the proto-oncogenes c-fos and c-myc (Greenberg et al, 1985) and a variety of other early transcriptional responses (Bartel et al, 1989), increased ornithine decarboxylase (ODC) activity (Huff et al, 1981 ; Koizumi et al, 1988), and stimulation of the hydrolysis of inositol phosphates (Koizumi et al, 1988). In addition, long-term exposure of PC12 cells to EGF is associated with a down-regulation of its own cell surface receptor (Koizumi et al, 1988), an effect which is also promoted by NGF (Huff et al, 1981).

To determine the ability of EGF to mimic NGF/bFGF induced increases in the expression of Thy-1 and N-CAM, naive cells were cultured for 48 hr in media supplemented with a wide range of concentrations of the growth factor (0 to 200 ng/ml), fixed and assayed for changes in cell surface glycoprotein expression. While cells treated with NGF (50 ng/ml) showed increased expression of both Thy-1 and N-CAM, cultures treated with EGF showed no change from their basal expression of N-CAM and, relative to NGF underwent only a 30% increase in their cell surface level of Thy-1 (data not shown). This latter response was apparently not dose-dependent, being induced to the same degree at all doses of EGF tested. In the presence of NGF, EGF (50 ng/ml) had no detectable effects on NGF-induced increases of either Thy-1 or N-CAM (data not shown).

NGF and EGF treated cultures induced 21% and 18% increases in MTT conversion relative to control cultures (SATO only), indicating that both growth factors affect cell survival and/or general cell metabolism, and providing evidence that the source of EGF used in all of the above experiments was biologically active.

5.2.5. CHOLERA TOXIN DIFFERENTIALLY MODULATES bFGF INDUCED INCREASES IN THE EXPRESSION OF THY-1 AND N-CAM.

PC12 cells were grown over a period of 48 hr in control media (SATO only) or media supplemented with 1 ng/ml bFGF in the presence and absence of 0.1 ng/ml cholera toxin. Cultures were assayed for their relative cell surface expression of Thy-1 and N-CAM. Figure 5.4 shows that cholera toxin promotes an inhibition of the bFGF induced increases in Thy-1 expression whilst concomitantly potentiating bFGF induced increases in the cell surface abundance of N-CAM.

These data suggest that cholera toxin, and therefore cAMP, is able to modulate differentiation responses induced by either NGF or bFGF, and since effects on the responses are similar in each case it is possible that the mechanisms underlying modulation of these growth factor responses are common.

MODULATION OF bFGF INDUCED RESPONSES
BY CHOLERA TOXIN

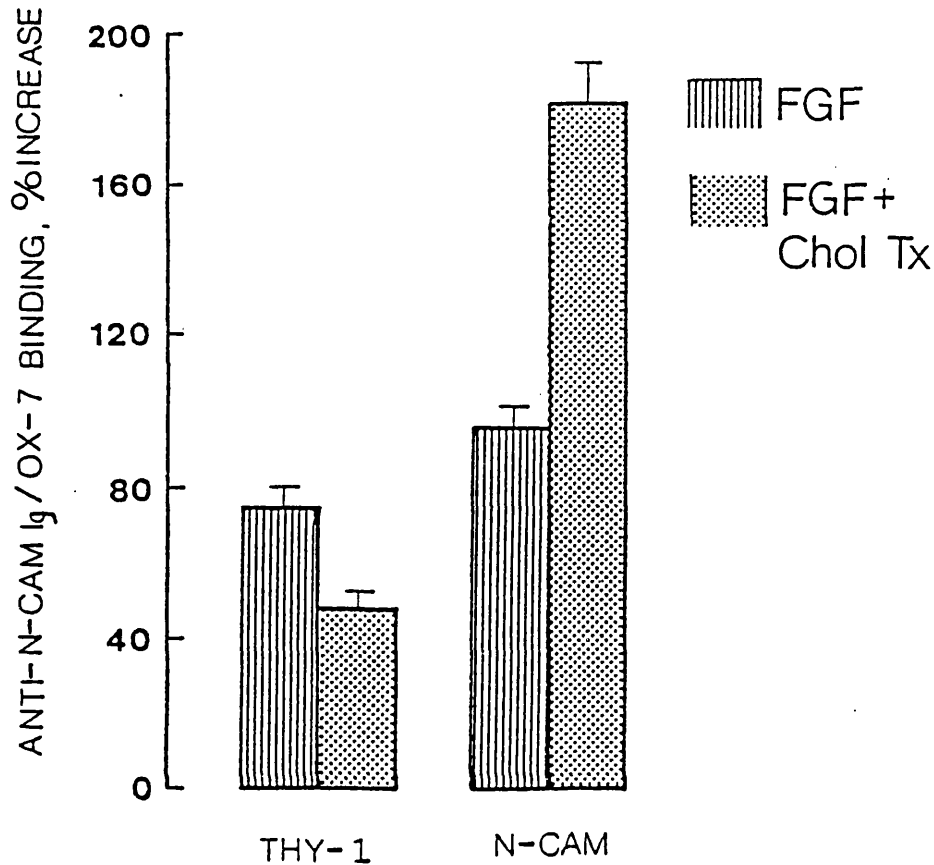


Figure 5.4. Immunoreactive Thy-1 and N-CAM levels were determined for PC12 cells grown over 48 hours in media supplemented with 1 ng/ml bFGF in the presence or absence of 0.1 ng/ml cholera toxin as indicated. Results show the percentage increase in McAb OX7 or anti-N-CAM Ig binding over cultures grown in control media. Each value is the mean \pm S.E. of seven independent determinations. OX7 and anti-N-CAM Ig binding to control cultures was 0.200 ± 0.006 and 0.100 ± 0.007 OD units respectively (mean \pm S.E. $n = 7$).

5.2.6. K252a IS A SPECIFIC INHIBITOR OF NGF INDUCED RESPONSES.

K252a, a microbial alkaloid isolated from the culture broth of *Nocardioopsis* sp, is a potent inhibitor of the activities of both protein kinase A and C (Yamada et al, 1987 ; Kase et al, 1987). K252a inhibits these protein kinases competitively with respect to ATP (Koizumi et al, 1988). These effects are elicited at μM concentrations of the alkaloid, however at lower concentrations (200 nM) K252a appears to act as a highly specific inhibitor of NGF induced responses (Yamada et al, 1987 ; Hashimoto et al, 1988 ; Doherty and Walsh, 1989).

To determine the effects of K252a treatment on NGF and bFGF induced increases in Thy-1 and N-CAM expression, PC12 cells were grown for 48 hr in media supplemented with either NGF (10 ng/ml) or bFGF (5 ng/ml) in the presence of K252a over a range of concentrations (0 to 100 nM). Figure 5.5 a and b show dose response curves for the effects of K252a on NGF/bFGF induced increases in Thy-1 and N-CAM expression respectively. NGF induced increases of both glycoproteins were inhibited by K252a in a dose dependent manner, with full inhibition of both responses achieved at a concentration of 100 nM. In contrast the alkaloid had relatively little effect on bFGF induction of N-CAM, and intriguingly had a slight potentiating effect on bFGF induced increases in Thy-1 expression.

At a concentration of 100 nM, K252a completely inhibited NGF induced morphological differentiation of PC12 cells (fig 5.6a), whilst having essentially no effects on the morphological status of differentiating bFGF induced

EFFECTS OF K-252a ON NGF AND bFGF INDUCED RESPONSES

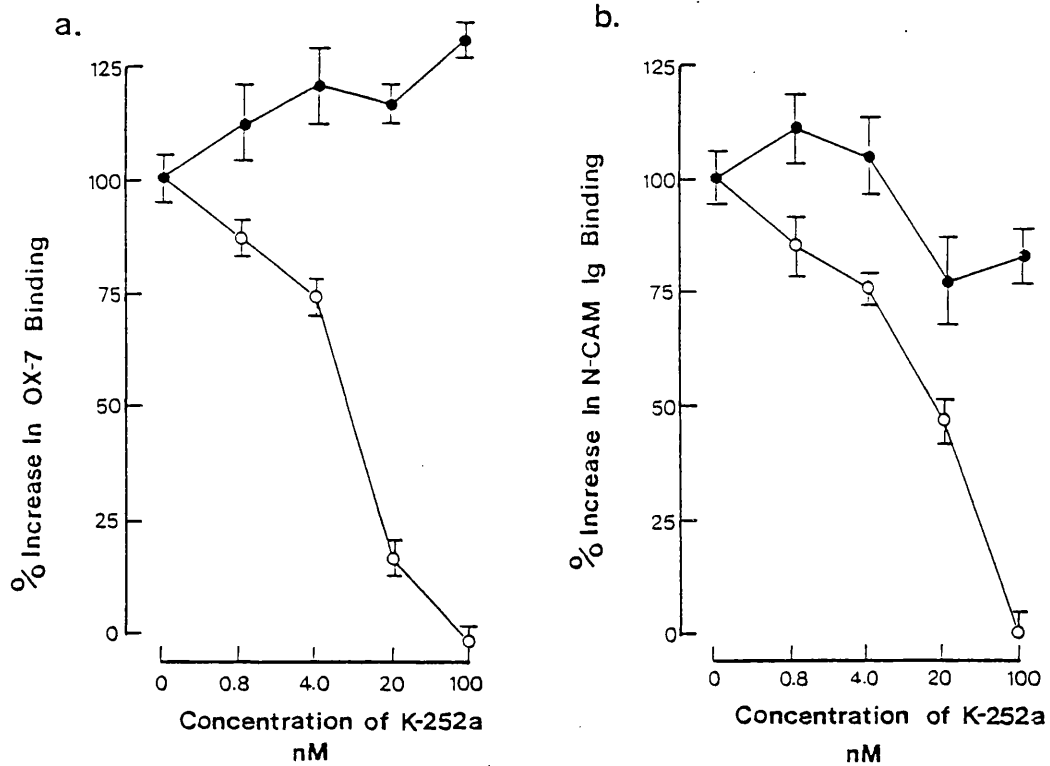


Figure 5.5. PC12 cells were grown for 48 hours in the presence of either 5 ng/ml bFGF (●) or 10 ng/ml NGF (○) in media supplemented with K-252a over a wide range of concentrations (0 to 100 nM). Cultures were assayed for cell surface Thy-1 (a) and N-CAM (b). Results show the percentage increases in McAb OX7 or anti-N-CAM Ig binding to treated cultures over control cells grown in SATO media alone. Each value represents the mean \pm S.E. of seven independent determinations. For bFGF treated cultures, 100 % control values were 0.130 \pm 0.100 and 0.166 \pm 0.160 (mean \pm S.E. n = 7) for Thy-1 and N-CAM respectively. For NGF treated cells 100 % control values were 0.288 \pm 0.130 and 0.271 \pm 0.008 (mean \pm S.E. n = 7) for Thy-1 and N-CAM respectively.

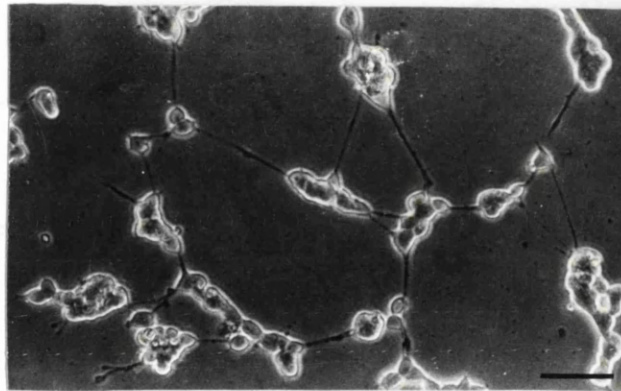
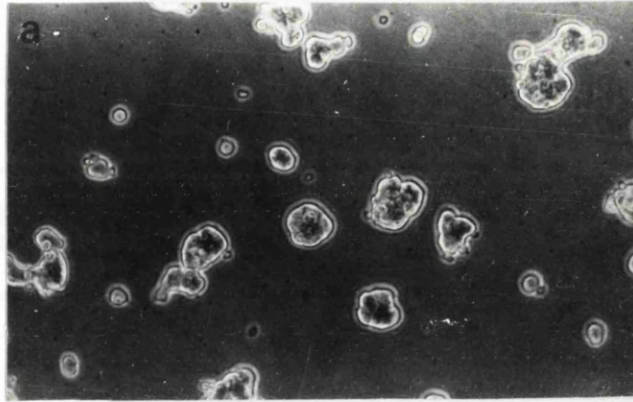


Figure 5.6. PC12 cells were cultured for 48 hours in media containing 100 nM K-252a supplemented with either 10 ng/ml NGF (**a**) or 5 ng/ml bFGF (**b**). Photomicrographs show representative fields of microwell cultures. Bars represent 50 μ M.

cultures (fig 5.6b). Thus the effects on PC12 cell differentiation at the biochemical level are consistent with effects observed at the morphological level. One possible explanation for the effects observed with K252a on NGF induced differentiation is that the alkaloid is acting as a general toxin, with bFGF somehow protecting cultures by an activity not promoted by NGF. Toxicity of K252a was determined by growing PC12 cells in the presence of NGF (10 ng/ml) over a range of K252a concentrations, and after 48 hr continuous culture testing the ability of each population to convert MTT to its formazan product relative to the conversion found in control cultures grown in the absence of growth factor or inhibitor. The data (fig 5.7) show that at a concentration of K252a that is optimal for selective inhibition of NGF-induced responses (100 nM), the treated cultures are still in a higher state of metabolic activity than control cultures and are only slightly (less than 10%) less active than NGF treated cultures grown in the absence of K252a. At higher concentrations of (400 nM), K252a was found to reduce the level of metabolic activity of cultures below the basal activity recorded in control cultures suggesting that the effects of the alkaloid on non-specific cellular kinases at these higher concentrations (Kase et al, 1987 ; Yamada et al, 1987) causes a general toxic response.

THE EFFECT OF K-252a ON THE MTT RESPONSE

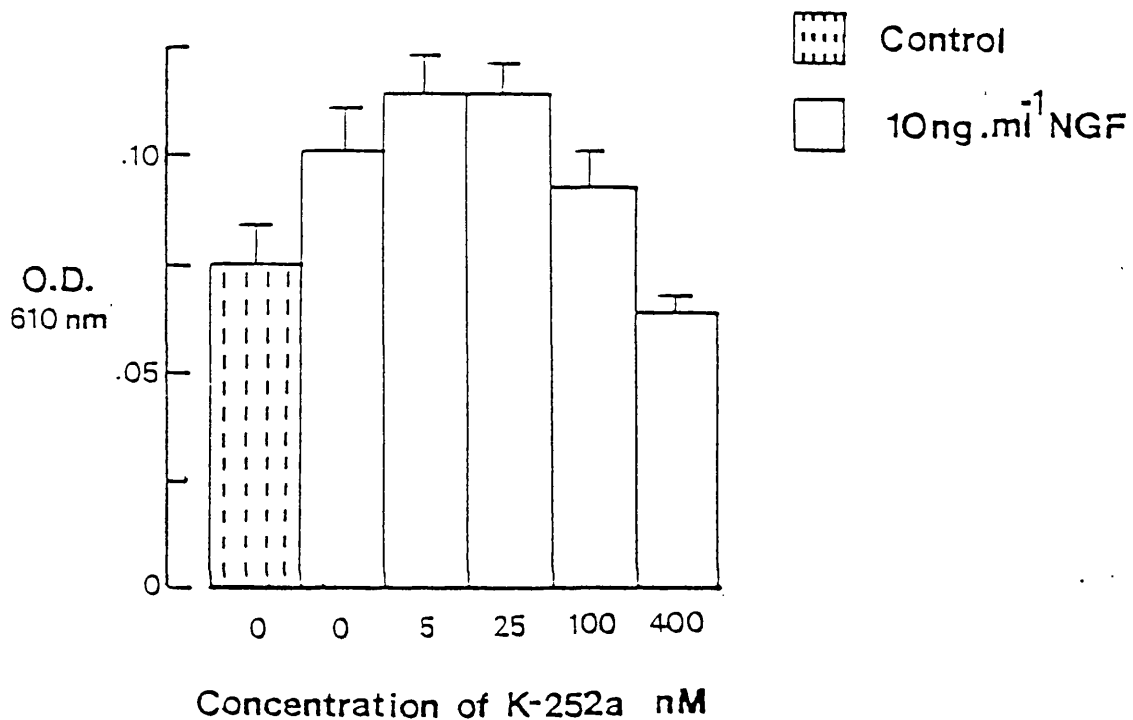


Figure 5.7. PC12 cells were grown over 48 hours in control media or in NGF supplemented media containing K-252a (0 to 400 nM). Results show the ability of cultures to convert MTT to its formazan product which was determined by assaying changes in OD at 610 uM.

5.2.7. EFFECTS OF PERTUSSIS TOXIN ON NGF AND bFGF INDUCED RESPONSES.

The bacterial toxin from *Bordetella pertussis* can covalently modify G-proteins by the addition of an ADP-ribose group to the alpha subunit of the membrane bound transducing protein (Neer and Clapham, 1988). This resembles the mechanism proposed for the action of cholera toxin on the G-protein associated with the adenylate cyclase system (Limbird, 1981). Thus, like cholera toxin, pertussis toxin can mediate a range of effects on a variety of cell types, causing both stimulatory and inhibitory responses by modulating the activity of kinase catalysed transduction signals (Esch et al, 1985 ; Neer and Clapham, 1988). There is evidence to suggest that G-proteins can be categorized according to their susceptibility to ADP-ribosylation : substrates for cholera toxin only, substrates for pertussis toxin only, substrates for both toxins or neither toxin (Neer and Clapham, 1988). It was of interest to determine if a pertussis toxin sensitive G-protein was involved in any of the signal transduction mechanisms mediating either NGF and/or bFGF induced responses.

Pertussis toxin has been shown to have profound effects in a variety of cells and to have the capacity to inhibit growth factor induced responses including cell proliferation and DNA replication (Chambard et al, 1987). The source of toxin used in the present study was shown to be biologically active at ng/ml concentrations (data not shown). PC12 cells were grown for 48 hr in control media (SATO only), NGF supplemented media (10 ng/ml), and media containing bFGF (1 ng/ml), in the presence and absence of 1-100 ug/ml pertussis toxin. Cultures were assayed for their relative expression Thy-1, N-CAM, and L1. In the presence of pertussis toxin neither the NGF or the bFGF induced

glycoprotein responses were modulated and in addition morphological differentiation of cultures was also unaffected, whether induced by NGF or bFGF (data not shown). These results indicate that transduction of the NGF and bFGF signals for transcription dependent "delayed" responses does not involve the direct activity of a G-protein with pertussis toxin sensitivity.

5.3 DISCUSSION.

The FGF's have been shown to be capable of reproducing the entire spectrum of responses of the PC12 cell line that were previously shown to be components of NGF induced differentiation (Rydel and Greene, 1987). These responses include the rapid, transcription independent responses and the delayed, transcription dependent responses. The action of the FGF's on PC12 cells has been demonstrated to be the result of their interaction with independent cell surface receptors and not as a result of their competing with NGF for binding with the NGF-receptor (Togari et al, 1985). The signal transduction mechanisms underlying FGF induction of 'NGF-like' responses has so far not been investigated. The aim of the current study was to determine the similarity between the transduction mechanisms mediating NGF and bFGF induced modulation of PC12 cell surface glycoprotein expression.

NGF induction of Thy-1 and N-CAM are mediated by protein kinase C dependent and independent transduction systems respectively. Temporal changes in the expression of the two glycoproteins at the cell surface have been demonstrated to be mediated by both transcription dependent and independent mechanisms depending on the differentiation status of the PC12 cell (see chapters 3 and 4). In addition their

induction has been shown to be dose dependent with respect to the external concentration of NGF.

bFGF can induce changes in the cell surface expression of both Thy-1 and N-CAM, as well as promoting increased cell surface expression of two other NGF inducible glycoproteins, namely the L1 glycoprotein and the NGF-receptor. Regulation of the Thy-1 molecule in PC12 cell cultures by bFGF has been reported previously (Rydel and Greene, 1987), however these studies did not address the question of cell surface expression of the molecule since the authors measured changes in carbohydrate uptake into Thy-1 rather than changes in the level of cell surface glycoprotein. No previous studies have investigated the effects of bFGF on N-CAM expression. Both the Thy-1 and N-CAM responses were induced by bFGF in a dose-dependent manner and were found, at optimal bFGF concentrations, to be comparable to the responses induced at optimal concentrations of NGF. While bFGF exerted maximal Thy-1 increases at a concentration of 0.2 ng/ml, maximal N-CAM increases were recorded at a dose of 0.6 ng/ml. Concentrations exceeding these optimal doses caused a slight fluctuation in the intensity of the Thy-1 response and produced a dramatic dose-dependent reduction in the N-CAM response. The mechanisms underlying these dual effects were not investigated, though it is possible they are the consequence of an inhibitory substance in the bFGF source, or may be a genuine regulatory effect of the growth factor. If the latter is the case then it would appear that bFGF, like NGF, regulates Thy-1 and N-CAM independently from one another considering the differences in sensitivity of each response to high doses of bFGF.

NGF primed PC12 cells were found to respond maximally to bFGF at lower concentrations than naive cells both in the

case of the Thy-1 and the N-CAM response. This observation indicates that the sensitivity of the PC12 cell to bFGF induced differentiation may be enhanced following prior exposure to NGF.

bFGF induced increases in Thy-1 and N-CAM expression were blocked in naive PC12 cells by the transcriptional inhibitor cordycepin, as found with the NGF induced responses, the inhibitor had no effect on bFGF induction of either Thy-1 or N-CAM in NGF-primed PC12 cells. Thus, like NGF, bFGF can stimulate increased glycoprotein expression by either a transcription dependent (naive PC12 cells) or independent (primed PC12 cells) mechanism of action. Similarly, Rydel and Greene (1987) demonstrated that bFGF can promote neurite outgrowth by a transcription-independent mechanism in regenerating PC12 cell cultures, and furthermore were able to demonstrate the ability of bFGF to induce the 'priming' response. The present study demonstrates that bFGF induced biochemical responses associated with the regenerative response are also subject to priming by NGF. The priming phenomenon is believed to reflect the ability of the PC12 cell to respond to growth factor with long term regulation of a number of mRNA species that are associated with the generation of the neuronal phenotype (Greene, 1984). Subsequent exposures to growth factor result in stimulation of translation of these mRNA's allowing a rapid, transcription-independent regenerative response. Since there appears to be a degree of redundancy in terms of which of two independently acting growth factors, NGF and bFGF, primes or stimulates regeneration, it is likely that certain mechanistic elements may be shared by the NGF and bFGF signals at least at the level of translation.

Studies in previous chapters have shown that the adenylate cyclase activator cholera toxin can be used to distinguish and identify responses that are mediated by independently functioning signal transduction pathways. Protein kinase C dependent NGF induction of Thy-1 can be inhibited by cholera toxin, while in contrast NGF induction of N-CAM by a protein kinase C independent mechanism is potentiated by the toxin in a synergistic manner. The effects of cholera toxin on bFGF induced Thy-1 and N-CAM responses were found to be qualitatively identical to its effects on the NGF responses, Thy-1 induction being inhibited while N-CAM increases were stimulated. In a similar study, cholera toxin has been reported to completely block transcriptional regulation of the expression of the NGF-receptor induced by either NGF or bFGF in naive PC12 cells (Doherty et al, 1988). Thy-1, N-CAM, and the NGF-receptor appear to be regulated by NGF via independently acting signal transduction pathways (NGF regulation of the NGF-receptor protein cannot be mimicked by either phorbol esters or A23187, and are in contrast to N-CAM inhibited by cholera toxin). The effects of cholera toxin on bFGF induction of these responses indicate that similar or possibly common transduction systems are utilised by both polypeptide growth factors. The inability of pertussis toxin, a G-protein effector (Esch et al, 1985 ; Neer and Clapham, 1988), to modulate induction of Thy-1, N-CAM or L1 by either NGF or bFGF adds further support to the evidence for common growth factor transduction systems. Taken together, these data indicate that PC12 cell responses to NGF and bFGF are more likely to be determined by the inherited transduction machinery of the cell as opposed to any inherent activity associated with either factor.

While NGF and bFGF trigger similar or common transduction elements in PC12 cells, previous studies have demonstrated that cellular responses are activated by the interaction of each growth factor with independent cell surface receptors (Togari et al, 1983). Evidently signals arising from these two receptors must eventually converge, at a stage prior to their activation of common transduction elements such as protein kinases A and C, and stimulate the activity of a similar or common transduction intermediate. Recent evidence suggests that the kinase inhibitor K252a can act as a highly specific inhibitor of an NGF-specific kinase activity that is likely to be very closely associated with the NGF-receptor (Koizumi et al, 1988 ; Hashimoto et al, 1988 ; Doherty and Walsh, 1989). In the present study K252a was shown to be capable of completely blocking NGF-induced increases in the expression of both Thy-1 and N-CAM without affecting the general metabolic activity of cultures. In contrast the same responses when induced by bFGF were either unaffected or surprisingly, slightly potentiated by the alkaloid. Similar effects have been reported elsewhere (Koizumi et al, 1988 ; Hashimoto et al, 1988), and at present only NGF induced responses have been suppressed by K252a when at concentrations in the 200nM range. Thus, the PC12 cell must have a mechanism by which specific growth factor signals can be recognised and assimilated independently of one another. In the case of NGF it is possible that a highly specific protein kinase is responsible for linking signals emanating from the NGF-receptor to later signal transduction elements that are commonly utilised by effectors of the neuronal differentiation programme. It remains to be determined if such a kinase exists, if it is a target for K252a, and if as expected a similar specific kinase is closely associated with the bFGF/aFGF receptor.

In contrast to NGF and bFGF, the polypeptide growth factor EGF is unable to induce morphological differentiation of PC12 cells and has a limited range of biochemical effects (Koizumi et al, 1988). EGF was able to induce a small but significant and reproducible induction of Thy-1 (36% increase relative to the induction recorded in sister cultures treated with NGF), whilst having no detectable effect on N-CAM expression. EGF has been demonstrated to be capable of stimulating the hydrolysis of inositol polyphosphates by a mechanism that is apparently stimulated by K252a (Koizumi et al, 1988). This effect may explain the observed induction of Thy-1, however it is unusual that no characteristic dose response was observed for the induction and that a stronger response was not seen since accumulation of radiolabelled inositol phosphate in EGF treated cells is known to be almost as high as the accumulation in NGF treated cells (Koizumi et al, 1988). One possibility is that the source of EGF used was not of great purity and may have contained trace amounts of an inhibitory material that reduced the biological activity of the growth factor. Alternatively, it has been suggested that EGF may be incapable of promoting the long-term changes in intracellular levels of free calcium which are essential for maintaining the activation of the phosphoinositide/ protein kinase C pathway (Eberhard and Holz, 1987). A third explanation may be that the observed increase in Thy-1 expression is an indirect consequence of the increased cell volume and metabolic activity promoted by EGF.

The inability of EGF to mimic NGF or bFGF induced responses that are characteristic of the neuronal phenotype of the PC12 cell provides evidence that the cell has evolved a transduction mechanism that allows independent ligand-receptor events to be recognised and assessed as either

signals for differentiation or signals for other as yet unspecified growth related events. As EGF can mimic many of the short-term transcription dependent events including the induction of a battery of proto-oncogenes (Greenberg et al, 1985 ; Bartel et al, 1989), it is clear that these primary signal transduction events are, on their own, insufficient to trigger PC12 cell differentiation. Elucidation of the transduction events that augment proto-oncogene induction in NGF and EGF treated cells may provide important clues regarding the growth factor specific transduction mechanisms for differentiation and growth respectively.

PC12 cells are unusual in that they express cell surface receptors for a wide variety of effectors and can respond to both NGF and the FGF's by undergoing phenotypic transformation to a neuronal like cell. The biological significance of the effects described for FGF and EGF in the PC12 cell system is difficult to determine in terms of primary neurons. To date only neurons of the CNS have been shown to be responsive to the FGF's (Morrison, 1987 ; Lipton et al, 1988 ; Anderson et al, 1988) and it has not been unequivocally determined that their neurotrophic effects are not produced indirectly through a stimulation of the activity of neurotrophic factor secreting endothelial cells.

CHAPTER 6 MODULATION OF PC12 CELL DIFFERENTIATION BY CELL-CELL INTERACTIONS.

6.1 INTRODUCTION.

During the development of the nervous system, axons must grow out from their cell of origin, across a variety of substrata, and then make contact with their specific synaptic targets. This process, though not yet fully understood, is known to be highly ordered and to be achieved with a remarkable degree of precision and reproducibility (Parnavelas et al, 1988 ; Dodd and Jessell, 1988). The mobile organelle of the axon, the growth cone, is guided along its migratory pathway by, in part its selective contacts with other neuronal and non-neuronal cells, and by the molecules these cells secrete (Jessell, 1988 ; Eisen, 1988 ; Lindsay, 1988 ; Dodd and Jessell, 1988). It is currently believed that cell-surface molecules with the ability to influence the extent and/or orientation of axonal growth do so by recognising and interacting with receptors present at the surface of the axonal plasma membrane (Dodd and Jessell, 1988 ; Jacobson, 1988 ; Eisen, 1988).

Two main classes of non-soluble neurite-promoting molecules have been described, the extracellular matrix macromolecules (Bronner-Fraser, 1988) and the CAMS (Edelman, 1986). As discussed earlier (chapter 1), the extracellular matrix molecules interact with the neurite by a heterophilic binding mechanism, involving a functional cell-binding domain (RGD) recognising and adhering to a specific receptor (an integrin) present at the surface of the neuronal membrane (Ruoslahti and Pierschbacher, 1986 ; Hynes, 1987). In contrast, the CAMS promote neurite outgrowth by an adhesion based mechanism involving, in the main, a homophilic binding mechanism between CAMS present at the two

apposing surfaces (Takeichi, 1988 ; Rutishauser et al, 1982). The availability of highly specific antibody reagents to components of both classes of neurite-promoting molecule, together with the development of coculture systems in which differentiating neurons can be cultured on other neuronal and non-neuronal cellular substrata, has allowed studies to be carried out in which the contribution of individual molecules on neurite outgrowth can be assessed in multi-component models (Bixby et al, 1987 and 1988 ; Tomaselli et al, 1988 ; Chang et al, 1987 ; Neugebauer et al, 1988). These studies have shown that no one molecule in any single nerve /cellular-substratum model acts totally independently. It appears that the extracellular matrix components and the CAMS, despite their very different structures, can serve as complementary functional components of the neurite directing machinery, with subtle modulation in the relative spatio-temporal distribution of individual components dictating the ability of a particular substratum to support the growth of a specific neuronal cell type.

Recent studies with regenerative CNS neurons have shown that certain so-called 'non-permissive' substrata , such as CNS myelin and the myelin-forming oligodendrocytes, contain membrane bound proteins whose properties seem to account for the inability of these substrata to support neuritic outgrowth (Caroni and Schwab, 1988a and b). Size fractionation revealed the presence of two minor myelin proteins of 35- and 250-kD which when removed from myelin proteins or when absorbed with monoclonal antibodies IN-1 and IN-2, raised against the two fractions, generated a permissive substrate. When introduced into liposomes the two proteins yielded highly non-permissive substrates, furthermore these proteins were demonstrated to have the ability to generate a non-permissive substrate for neurite

outgrowth from a normally permissive substrate (Caroni and Schwab, 1988a). Thus there is evidence to suggest, at least in the CNS, that the pioneering growth cone may encounter non-adhesive as well as adhesive signals from cell surface substrates encountered during its passage to an appropriate target cell.

The differentiating PC12 cell has been shown to be capable of forming physiologically functional synapses with cultured muscle cells and in such cocultures to respond biochemically to substances secreted by the muscle culture (Schubert et al, 1977). The molecular basis for this synaptic interaction was not investigated nor was the ability of the PC12 cell to form functional interactions with cells of origins other than muscle.

Studies carried out in the preceding chapters have determined that NGF treatment of PC12 cells results in changes in the biochemical character of the outer surface of the cell's plasma membrane and in the nature of the shape and adhesive properties of the cell as a whole. In addition it has been convincingly demonstrated that external cues or signals other than NGF can have profound effects on both the biochemistry and general morphology of the PC12 cell, both when under the influence of NGF and when growing in the absence of the growth factor. It was therefore of interest to investigate the responsiveness of naive and primed PC12 cells to NGF when cultured on a cellular substratum rather than the relatively simple homogenous collagen substratum used in the earlier studies. To this end, the ability of non-neuronal cellular monolayers to either support and/or modulate NGF induced differentiation was assessed. As well as determining the extent of morphological differentiation of PC12 cells in these coculture systems, the effects of the

cellular substrata on NGF induced increases in the expression of Thy-1 and neurofilament protein were established, with the latter response being utilised as a more accurate means of assaying neurite outgrowth than purely morphological parameters.

6.2. RESULTS.

6.2.1. NGF INDUCED MORPHOLOGICAL DIFFERENTIATION OF PC12 CELLS IN COCULTURE.

The morphological differentiation status of PC12 cells cultured on monolayer substrates of C2 myotube/myoblast mixed cultures and primary human fibroblasts was determined by both phase-contrast and immunofluorescence microscopy of live cultures. Immunofluorescence studies were carried out using the rat specific Thy-1 McAb OX7 (as described in materials and methods). In the absence of NGF there is little, if any, sign of morphological differentiation in naive cultures following three days continuous culture on either muscle (fig 6.1 e and f) or fibroblast (fig 6.1 a and b) monolayers. In the presence of NGF there was a clear indication of neurite outgrowth from the vast majority of PC12 cells cultured on muscle cells (fig. 6.1 g and h). Staining with OX7 McAb shows that the PC12 cells can project long and highly branched neurites bearing highly differentiated growth cones which appear to be in close contact with the muscle surface. In contrast, PC12 cells cultured on monolayers of skin fibroblasts showed no signs of morphological differentiation (fig 6.1 c and d).

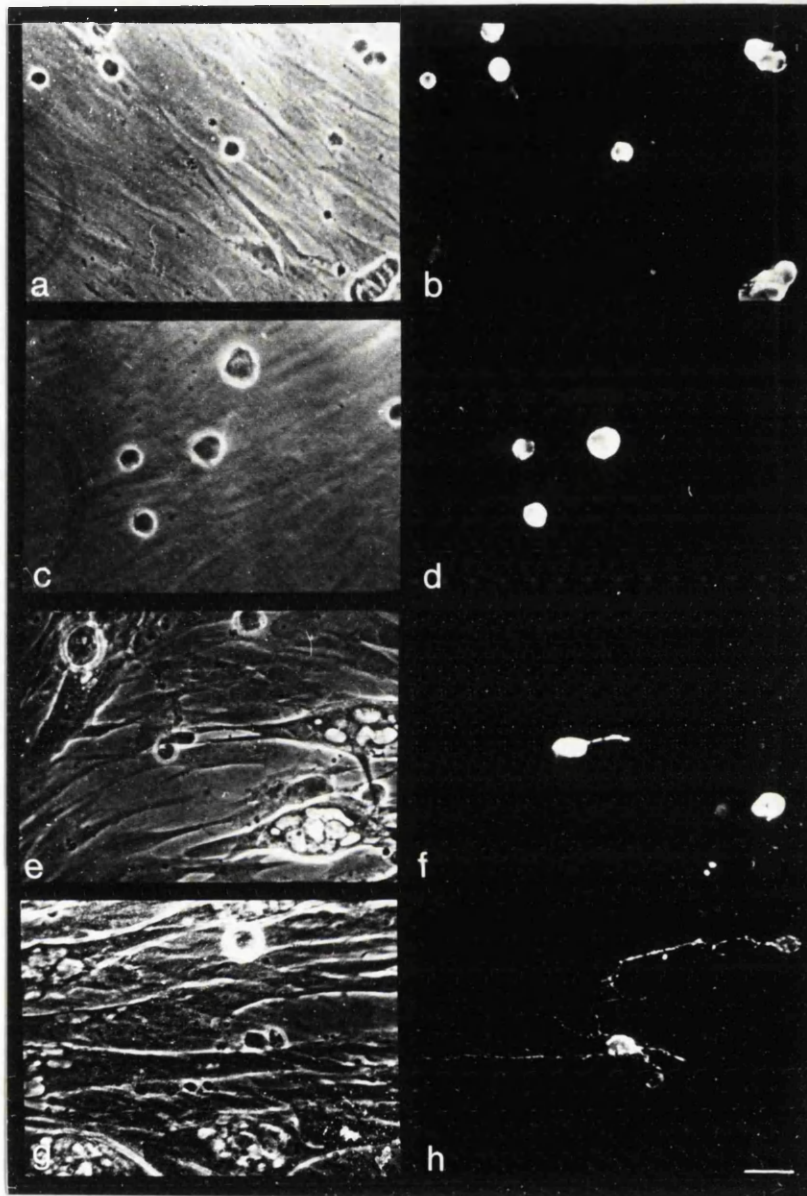


Figure 6.1. Naive PC12 cells were grown for 3 days on monolayer cultures of skin fibroblasts and C2 muscle. PC12 cell morphology for the respective cultures was highlighted by staining live cultures with the OX7 McAb. The cultures were fixed with methanol and viewed under phase-contrast and epifluorescence optics. The micrographs show representative fields for PC12 cells grown in the absence of NGF on fibroblasts (**a,b**) and muscle (**e,f**), and in the presence of NGF on fibroblasts (**c,d**) and muscle (**g,h**). The scale bar represents 40 μ m.

In cocultures containing primed PC12 cells (fig. 6.2) it was confirmed that neither muscle (fig. 6.2 e and f) nor fibroblast (fig. 6.2 a and b) substratum could directly stimulate neurite outgrowth in the absence of NGF. In the presence of NGF, C2 monolayers supported extensive neurite outgrowth showing essentially the same morphological response as observed with naive cells (fig. 6.2 g and h). In direct contrast to naive cells, NGF treated primed PC12 cells were able to project long and multi-branched neurites across the surface of fibroblasts, which were apparent in both stained and phase-contrast micrographs (fig. 6.2 c and d).

These results indicate that primary morphological differentiation of PC12 cells may be modulated by cell-cell interactions.

6.2.2. IMMUNOCHEMICAL QUANTITATION OF NEURITE OUTGROWTH.

Quantitation of neurite outgrowth by purely morphological criteria is a difficult task and often yields highly inaccurate results. In cocultures the determination of parameters such as the length of neurites, outgrowths per cell, and extent of branching becomes an almost impossible task against the complex background of a cellular substratum. A novel bioassay for quantitation of NGF-induced neurite outgrowth has been described (Doherty et al, 1984 a and b) and shown to be an accurate method for determining the extent of neurite outgrowth in a variety of neuronal systems including PC12 cells (Doherty et al, 1987). This bioassay is essentially an ELISA based determination utilising the McAb RT97 (Wood and Anderton, 1981) which recognizes the 210 kD and 150 kD neurofilament protein subunit of human and rat neurofilaments respectively (McHaugh et al, 1986 ; Doherty et al, 1987). In order to

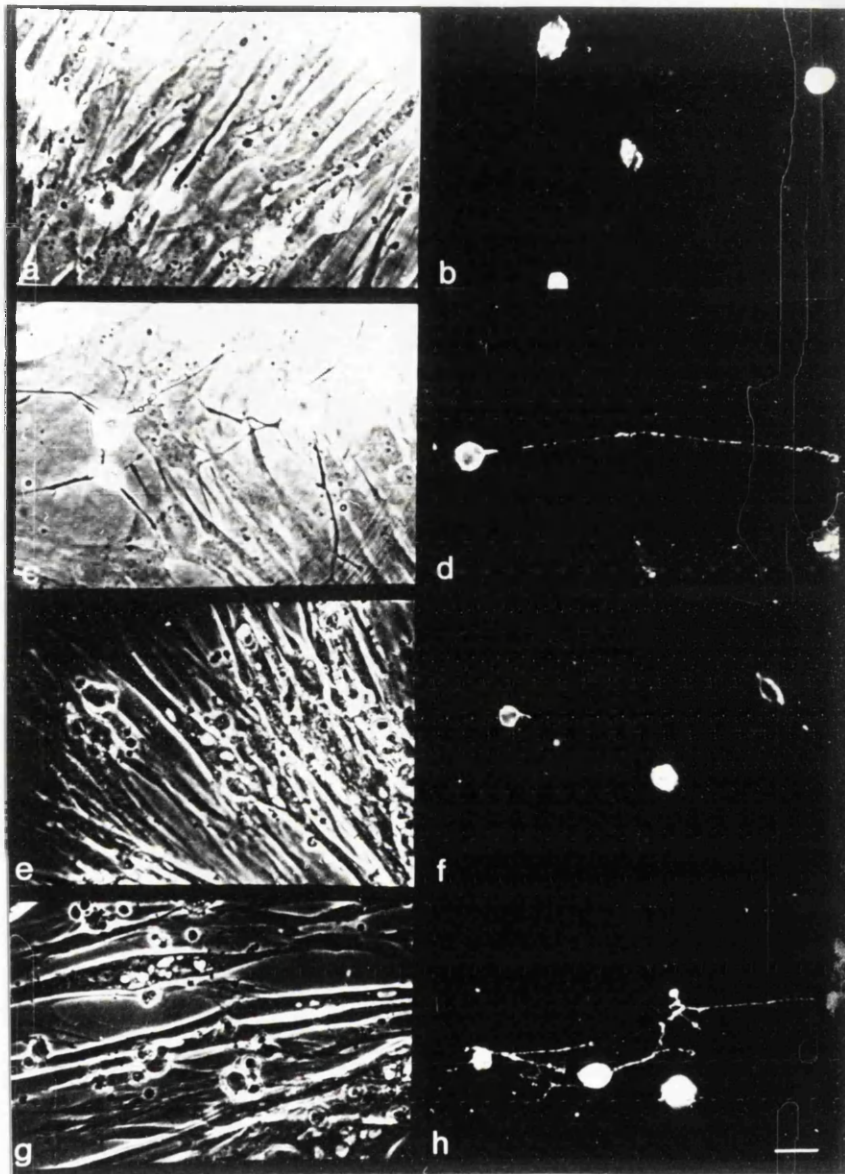


Figure 6.2. Primed PC12 cells were subcultured onto monolayers of fibroblasts or C2 muscle for 48 hours. Phase contrast micrographs show representative fields of live cultures grown in the absence (a) and presence (c) of NGF on fibroblast monolayers. Representative fields of these cultures stained with McAb OX7 are shown in (b) (- NGF) and (d) (+ NGF). Phase contrast and epifluorescence micrographs of OX7 stained PC12 cells grown on C2 monolayers are shown for cells grown in the absence (e, f) and presence (g, h) of NGF.

assess the suitability of this bioassay for measurement of neurofilament and neurite outgrowth in PC12 cocultures, extensive biochemical and immunological characterisation of both the reactivity of the RT97 antibody with differentiating PC12 cells, and the NGF induced RT97 antigen response was carried out.

6.2.3. NGF INDUCED ACCUMULATION OF RT97 ANTIGEN.

Binding of McAb RT97 to naive and primed PC12 cells was determined as described in Materials and Methods following growth in the presence and absence of NGF (50 ng/ml) for between 1 and 13 days. NGF treatment was found to be associated with time dependent accumulation of the RT97 antigen in cultures of both naive and primed PC12 cells (fig 6.3 a). Binding profiles are very similar for naive and primed cultures with the main difference being the onset of their respective responses. For naive cells, no increase in RT97 binding relative to that observed in control cultures was detectable until after 72 hr of continuous NGF treatment, RT97 binding was then increased maximally over the period spanning day 5 to day 8, reaching a maximum level of accumulation of RT97 antigen after 9 days of culture. In contrast, primed cells displayed quantitative increases in RT97 binding after only 20 hr of exposure to NGF, and showed maximal increases between day 1 and day 4 of culture. Concomitant morphological assessments (fig 6.4) showed that these periods of maximal increases in RT97 binding roughly corresponded with the maximal neuritogenic growth phases for both cell types. In a second experiment, naive and primed cells were cultured in the presence of NGF over a range of concentrations (0 to 50 ng/ml) and after 3 days treatment, assayed for relative increases in RT97 binding. Figure 6.3 b shows that both naive and primed cells underwent dose

NGF INDUCTION OF THE RT97 RESPONSE.

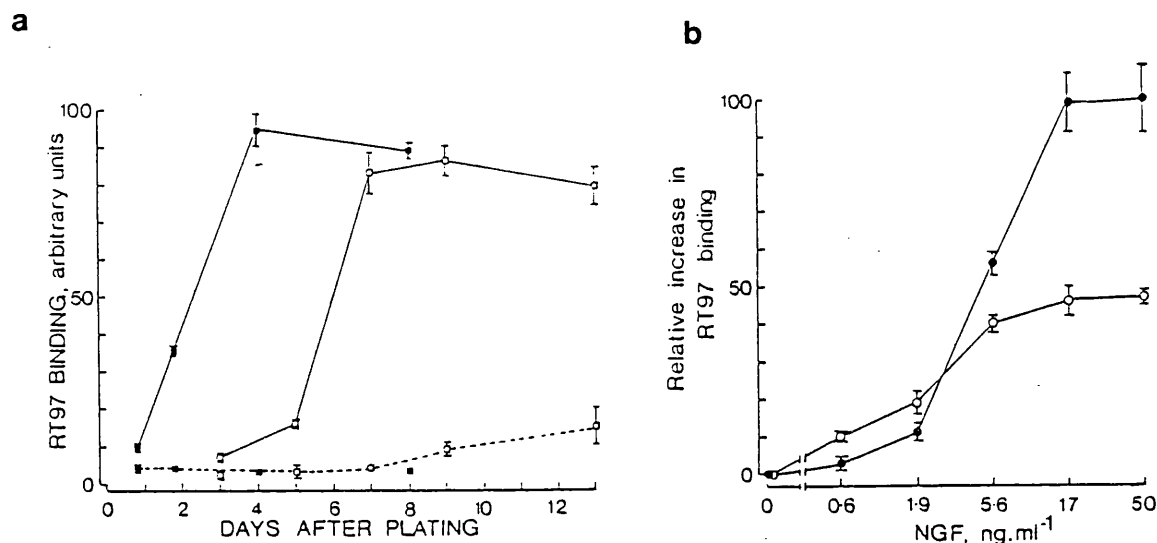


Figure 6.3. (a) Binding of McAb RT97 was determined for naive (□) and primed (■) PC12 cells grown over a 12 day timecourse in the presence (solid lines) and absence (dashed lines) of 50 ng/ml. RT97 binding was assayed as described in Materials and Methods, with each value representing the mean \pm S.E. of four independent determinations. 100 arbitrary units were equivalent to 1.00 OD unit of reaction product at 492 nm. (b) RT97 binding to naive (○) and primed (●) PC12 cells was determined as a function of NGF dosage. Cells were grown for 3 days in media supplemented with NGF (0 to 50 ng/ml) and assayed for capacity to bind RT97. Results show the absolute increase in binding over control cultures (0 ng/ml NGF) and each value is the mean \pm S.E. of four independent determinations. 100 arbitrary units were equivalent to 0.102 OD units.

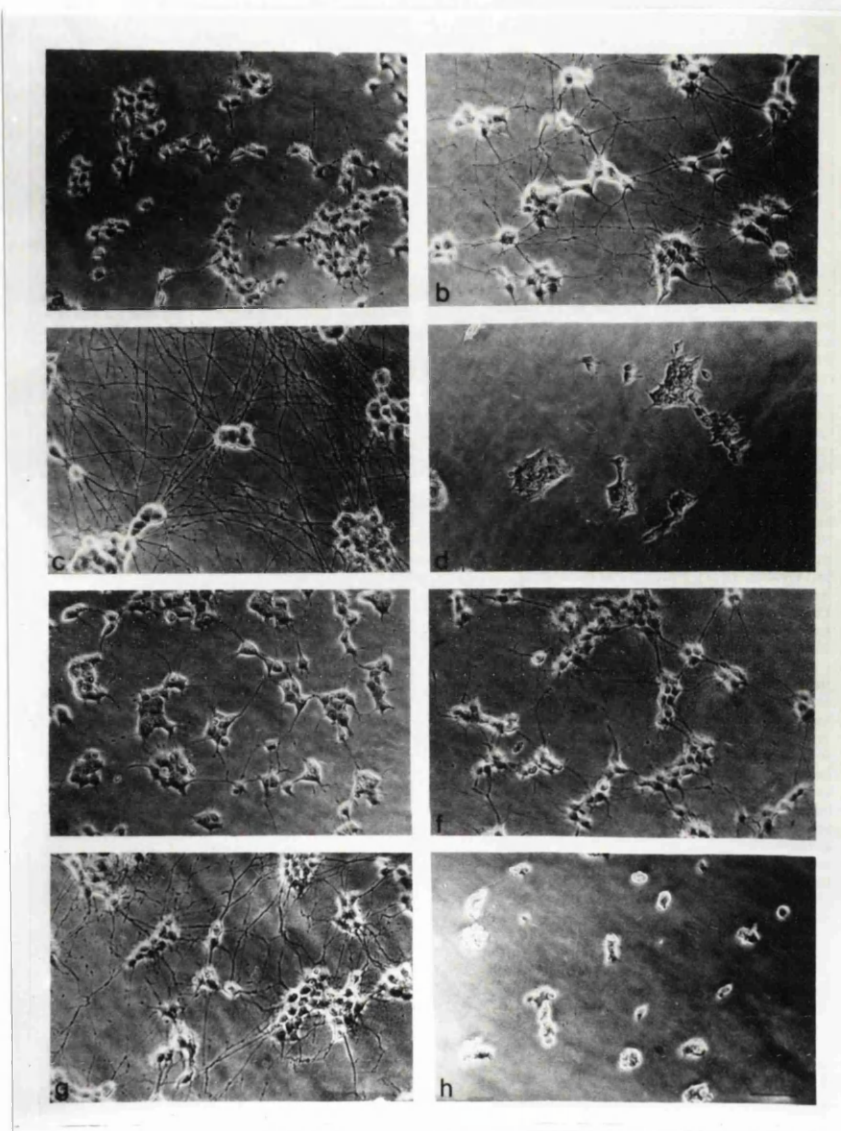


Figure 6.4. Morphological appearance of naive and primed PC12 cells grown in the presence and absence of NGF. Representative photomicrographs show naive cells grown in control media at day 13 of culture (**d**) as compared to sister cultures grown in the presence of 50 ng/ml NGF for 3 (**a**), 5 (**b**) and 13 (**c**) days. Photomicrographs are also shown for primed cells at 20 hours (**e**), 44 hours (**f**) and day 4 (**g**) following replating into NGF supplemented media, and for 4 days (**h**) in control media. Cells were photographed after assaying for RT97 in microwell cultures. Scale bar represents 140 μm .

dependent increases in their expression of RT97.

Half-maximal and maximal responses were estimated at 2 and 6 ng/ml respectively for naive cells and at 6 and 20 ng/ml respectively for primed cells. Therefore it would appear that the ability of primed cells to respond more rapidly to NGF than naive cells in terms of RT97 antigen accumulation is due to the ability of primed cells to generate a quantitatively greater response over a fixed period of time rather than any improved sensitivity to the growth factor.

Dependency of the RT97 response on ongoing transcription was determined by comparing RT97 binding in sister cultures treated with NGF (50 ng/ml) in the presence and absence of cordycepin (0 to 1 μ M). Cordycepin was found to inhibit NGF induced accumulation of RT97 antigen in naive cells in a dose dependent manner, causing up to a 90% reduction of the response at the maximal concentration tested (fig 6.5). As with other NGF induced responses in primed PC12 cells, the RT97 response was completely unaffected by cordycepin over the entire dose range.

The above experiments show that NGF can induce transcription dependent accumulation of the RT97 antigen over a timecourse that coincides with its induction of neurite outgrowth. In regenerative or primed PC12 cells, NGF can induce a rapid, transcription independent RT97 response which again closely correlates with changes in the morphological status of the cell.

**CORDYCEPIN DOSE RESPONSE :- EFFECT
ON NGF INDUCTION OF RT97 Ag**

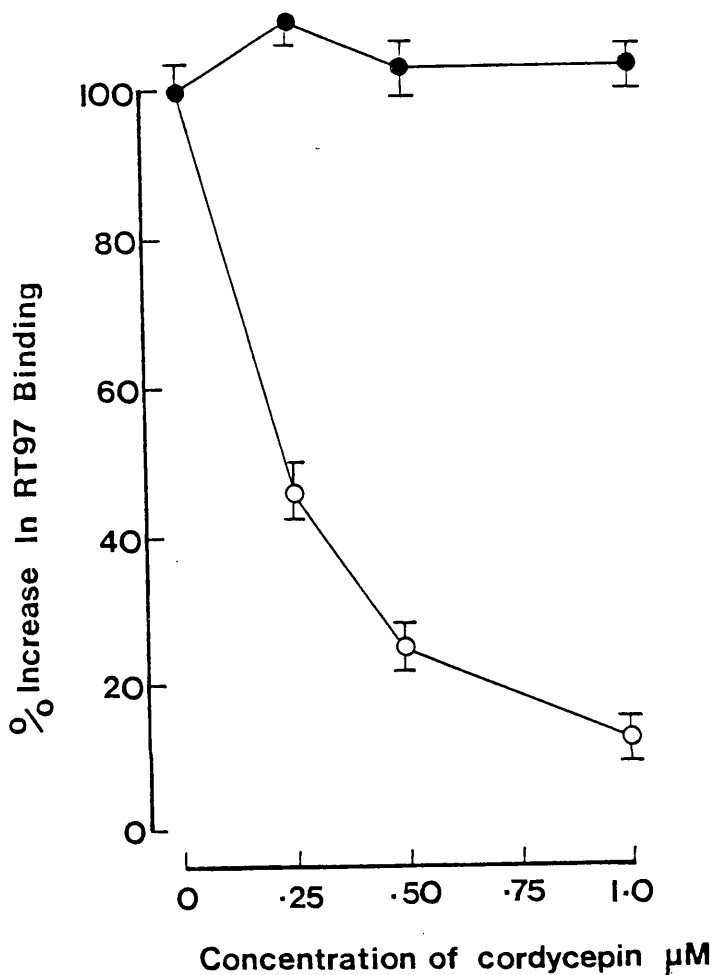


Figure 6.5. RT97 McAb binding was determined for naive (○) and primed (●) PC12 cells grown over 3 days in the presence of 50 ng/ml NGF, in media supplemented with cordycepin over a range of concentrations (0 to 1.00 μM). Results show the percentage increase for RT97 binding over cultures grown in SATO media alone and each value is the mean +/- S.E. of five independent determinations. 100 % control values were 0.192 +/- 0.011 OD and 0.110 +/- 0.009 units (mean +/- S.E. n = 5) for primed and naive cells respectively.

6.2.4. IMMUNOCHEMICAL CHARACTERISATION OF THE RT97 ANTIGEN.

The physical components of the differentiating cell responsible for binding the RT97 McAb were visualised by immunofluorescence microscopy (for detailed protocol, see Materials and Methods). Figure 6.6 shows a culture of primed PC12 cells stained for the RT97 antigen following three days of NGF treatment. RT97 staining appears to be localised to the advancing growth cones and along the entire length of the neurites. In contrast very little staining is evident in the cell bodies (although some staining is visible at higher magnifications). Thus, RT97 is apparently recognising a component of the neurite and its mobile organelle that is not present in such great abundance or alternatively, in a mature antigenic form, in the cell body. Consequently increased expression of RT97 is closely related to the extent of neuritic development and therefore to the morphological differentiation status of the PC12 cell. Immunoblotting of the RT97 antigen was carried out on PC12 cell cultures growing for 3 and 5 days in the presence and absence of NGF (50 ng/ml). No reactivity was found for RT97 binding with protein extracts (Triton X-100 soluble fraction) from control cultures at either of the two timepoints (fig 6.6), however in the case of the NGF treated cultures specific reactivity was demonstrated at a protein band with an approximate molecular weight of 150 kD. In extracts taken from 5 day cultures this reactive component appeared heterogeneous, being composed of at least three reactive species. Previous studies have indicated that the 150 kD neurofilament protein subunit may exist in at least three isoforms, these being the result of subtle differences in post-translational modifications which account for their

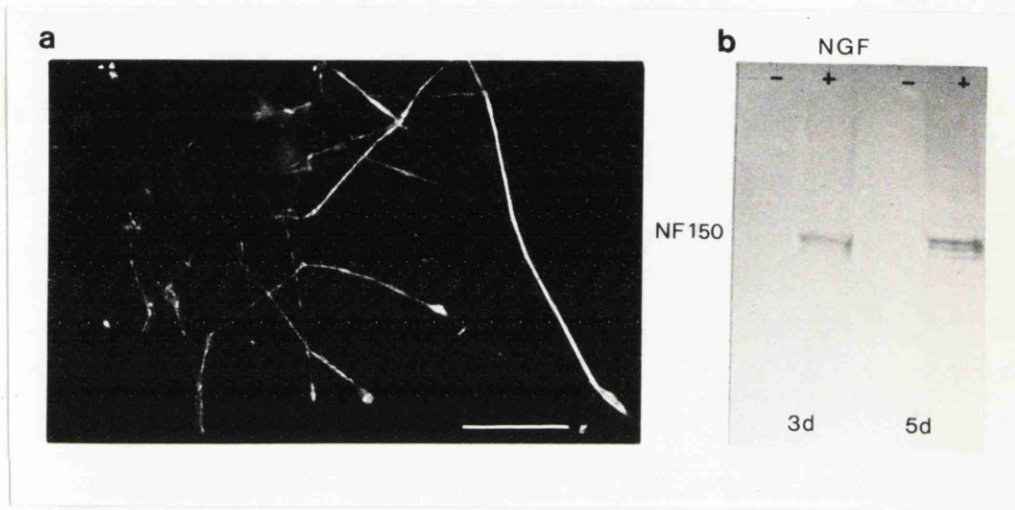


Figure 6.6. (a) Localisation of RT97 binding component. PC12 cells were primed with 50 ng/ml NGF for 5 days, divested of their neuritic network and replated in the presence of fresh NGF (50 ng/ml) for a further 3 days. RT97 McAb staining was carried out as described in Materials and Methods, and can be seen to be localised to axonal processes and growth cones. Scale bar is 40 μ m. (b) Immunoblotting of RT97 antigen. PC12 cells were grown for 3 and 5 days in the presence (+) and absence (-) of 50 ng/ml NGF. Cells were harvested and 50 μ g total cellular protein subjected to electrophoresis and immunoblotting as described in Materials and Methods.

difference in relative SDS-PAGE mobility (Lee, 1986). As the RT97 McAb was originally described as a neurofilament antibody and since it recognises the 150 kD subunit of neurofilament protein in other cell types (Anderton et al, 1982) then it is highly likely that the antibody recognises the equivalent component of the PC12 cell axonal cytoskeleton.

6.2.5. RECOGNITION OF THE RT97 EPITOPE IS MODIFIED BY ITS PHOSPHORYLATION STATUS.

Earlier studies have demonstrated that RT97 specifically recognises and interacts with a phosphorylated epitope of neurofilament polypeptides (McHaugh et al, 1986). Figure 6.7a shows the effects of increasing concentration of the enzyme alkaline phosphatase on RT97 binding to NGF treated PC12 cell cultures (4 days culture at 50 ng/ml). Treatment of cultures with the enzyme (at 37° C for 2.5 hr) prior to incubation with RT97 leads to a dose dependent decrease in the capacity of cultures to bind antibody, with a maximal loss of RT97 binding (approximately 50%) at a concentration of 1 ug/ml. In contrast, cultures treated for the same period of time with 100 ug/ml alkaline phosphatase in a 0.1M sodium phosphate buffer (pH 8.0) showed only a 14% loss of RT97 binding which, since phosphate inhibits alkaline phosphatase, confirms that the reduced RT97 binding activity in phosphatase treated cultures is probably due to the loss of phosphate groups from the RT97 epitope.

Treatment of cultures in an NGF timecourse experiment (0 to 10 days) with alkaline phosphatase (50 ug/ml) for 24 hr prior to ELISA resulted in a dramatic loss of the classical time dependent RT97 binding profile observed here (fig 6.7b) and elsewhere (Doherty et al, 1987) in the

**RT97 RESPONSE :- EFFECT OF ALKALINE
PHOSPHATASE TREATMENT**

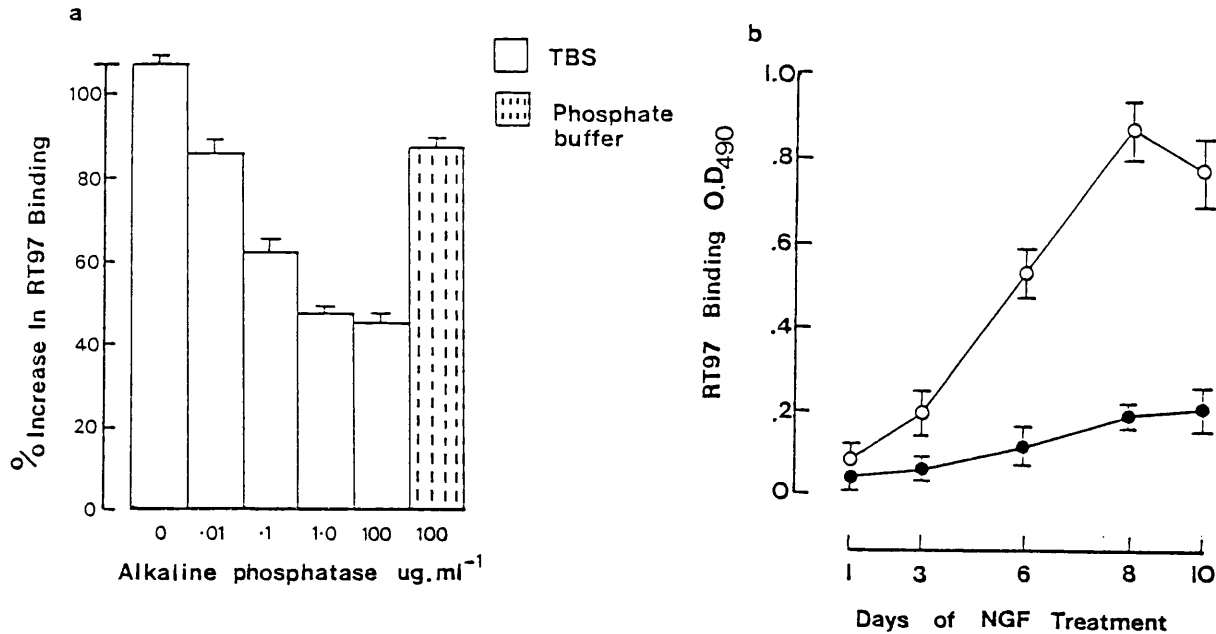


Figure 6.7. (a) PC12 cells grown over 4 days in the presence of 50 ng/ml NGF were treated for 2.5 hours at 37° C with a range of concentrations of alkaline phosphatase in either phosphate buffer or TBS, then assayed for capacity to bind McAb RT97. The results show the percentage increase in RT97 binding over cells grown in control media (0 ng/ml). Each value is the mean +/- S.E. of seven independent determinations. 100% control value was 0.650 +/- 0.010 OD units (mean +/- S.E. n = 7). (b) PC12 cells were grown for between 1 and 10 days in the presence of 50 ng/ml NGF and then incubated for 24 hours at 37° C with TBS in the absence (O) or presence (●) of 50 ug/ml alkaline phosphatase. Cultures were assayed for McAb RT97 binding. Results show increase in RT97 binding as a function of length of exposure to NGF and represent the mean +/- S.E. of seven independent determinations.

absence of phosphatase treatment. This demonstrates that the neurofilament component accumulated in neurites during NGF induced differentiation is a phosphorylated species and furthermore it is a phosphorylated epitope of the induced polypeptide that is recognised by RT97.

6.2.6. NGF INDUCED NEUROFILAMENT PROTEIN ACCUMULATION IN COCULTURES GROWN IN SERUM-CONTAINING CULTURE MEDIUM.

PC12 cells were seeded onto confluent monolayers of either C6 glioma, G8-1 skeletal myotubes, or human skin fibroblasts. Cocultures were maintained in a serum containing medium (10% FCS /DMEM for C6 glioma and fibroblasts, 5% HS for myotubes), in the presence and absence of NGF (50 ng/ml). In the absence of exogenous NGF, significant time-dependent changes in RT97 binding activity were only seen in the C6 glioma/ PC12 cocultures (fig 6.8). This was not unexpected since C6 glioma have previously been demonstrated to synthesise and secrete an NGF-like activity (Westermann and Unsicker,1986). NGF-dependent increases in RT97 binding were apparent by day 5 of culture in both C6 glioma and G8 myotube cocultures, but in contrast were absent in fibroblast cocultures. By the final day of culture (day 7) NGF treated PC12 cells grown on C6 glioma or G8 myotubes expressed a tenfold and fivefold greater level of RT97 antigen respectively than sister cultures treated with NGF on monolayers of skin fibroblasts.

EFFECT OF COCULTURE ON THE RT97 RESPONSE IN SERUM CONTAINING MEDIUM.

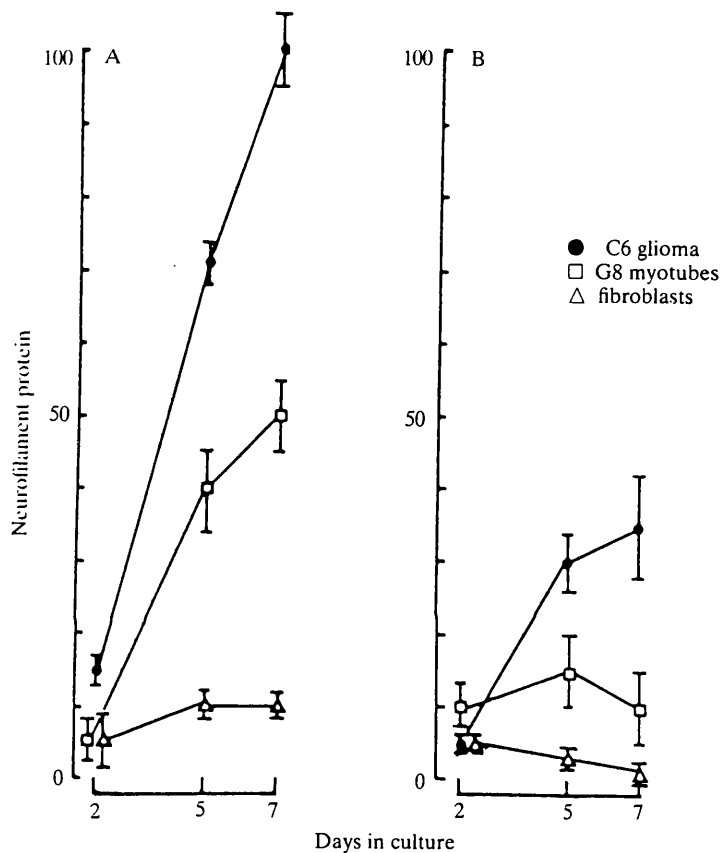


Figure 6.8. Timecourse for RT97 binding to PC12 cells grown on monolayer cultures of C6 glioma, G8-1 myotubes and skin fibroblasts in the presence (A) and absence (B) of 20 ng/ml NGF. Each data point was obtained as the mean \pm S.E. of eight independent RT97 McAb binding determinations, non-specific binding was measured in sister cultures and was subtracted for all measurements. 100 arbitrary units = 0.202 OD units.

6.2.7. NGF INDUCED NEUROFILAMENT PROTEIN ACCUMULATION IN COCULTURES GROWN IN SATO MEDIUM.

PC12 cells were grown for 3 days in SATO medium on collagen, or monolayer cultures of C2 myotubes or human skin fibroblasts, in the presence of NGF over a wide range of concentrations (0 to 50 ng/ml). PC12 cells growing on collagen and C2 myotubes demonstrated similar dose response curves for NGF induced increases in RT97 binding indicating that a monolayer of C2 myotubes can provide a suitable substrate for neurite outgrowth (fig 6.9). However, when grown on monolayer cultures of skin fibroblasts, the RT97 response was clearly suppressed, reaching only a third of the level of expression seen in sister cultures growing on collagen or C2 myotubes. The dose response experiment indicates that the fibroblast/ PC12 cocultures are still responsive to NGF since a neurofilament response is seen, however this response is obviously greatly reduced relative to the response in control cultures and myotube/ PC12 cocultures.

6.2.8. THE EFFECT OF PRIMING ON THE NEUROFILAMENT PROTEIN RESPONSE IN COCULTURES.

Accumulation of neurofilament protein as determined by the time dependent NGF induced increase in RT97 binding to PC12 cells is like many other transcription-dependent delayed responses, more rapidly achieved in primed cultures than in their naive counterparts (Doherty et al, 1987). Figure 6.10 shows the effect of priming on the neurofilament protein response of PC12 cells grown in coculture. In serum containing media (a) the responsiveness of the PC12 cell was essentially the same whether maintained on a monolayer of C6 glioma, G8 myotubes, or skin fibroblasts. In each coculture

EFFECTS OF COCULTURE ON THE RT97 RESPONSE IN SATO MEDIUM.

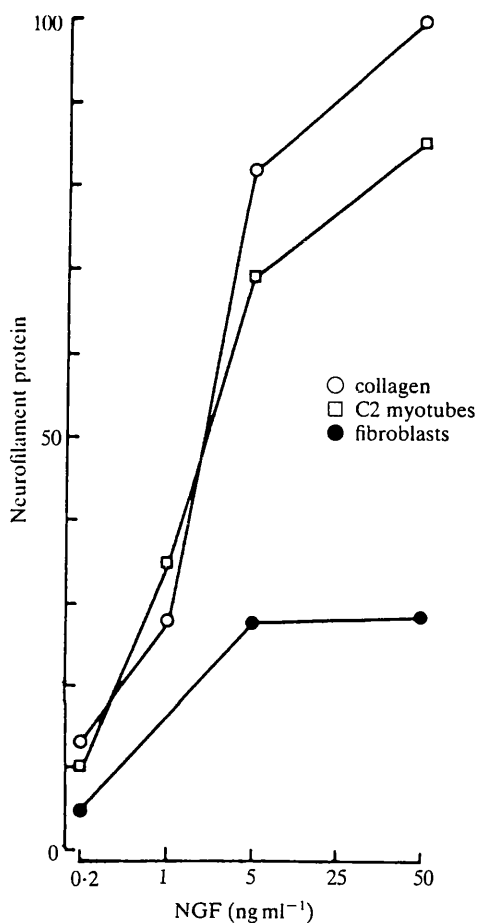


Figure 6.9. PC12 cells were grown for 3 days on collagen, C2 myotube monolayers, or on fibroblast monolayers. Results show the absolute increase in RT97 binding to cocultures grown in the presence of NGF as compared to sister cultures grown in the absence of NGF. Each value represents the mean \pm S.E. of four independent determinations. 100 arbitrary units = 0.078 OD units with standard errors of means within 2 to 20 arbitrary units.

EFFECT OF COCULTURE ON THE RT97 RESPONSE IN PRIMED CELLS

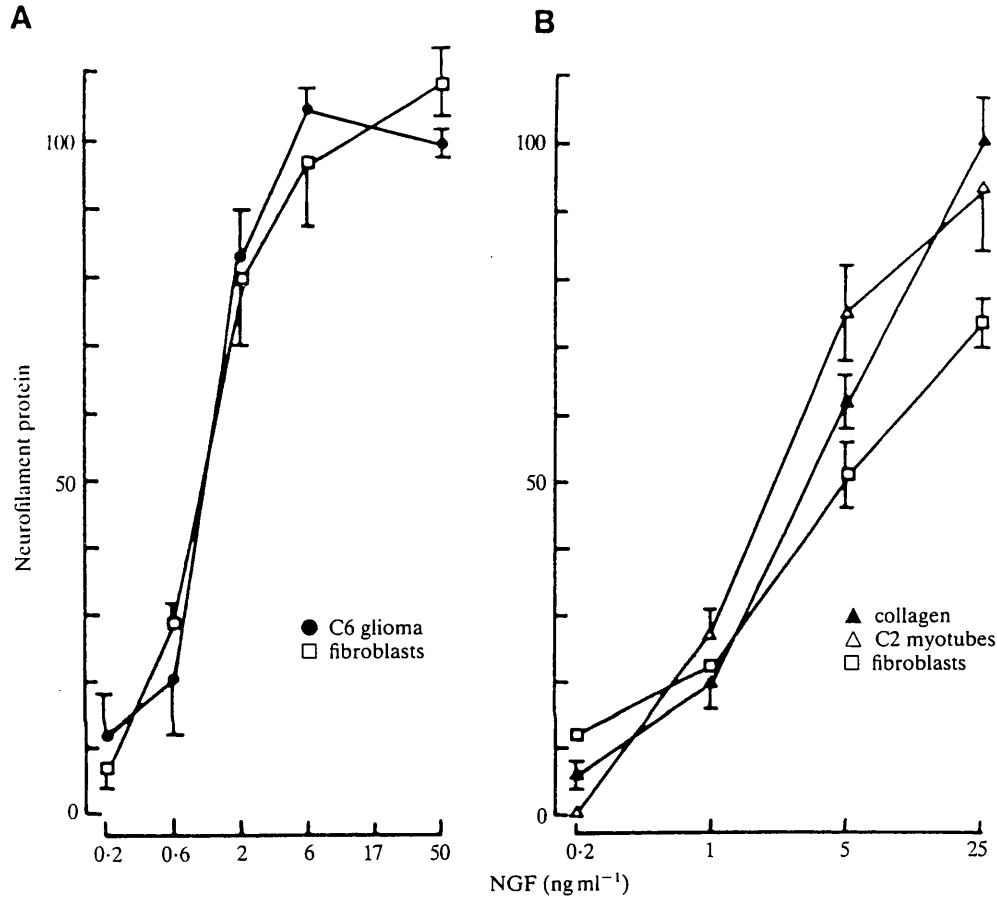


Figure 6.10. PC12 cells were primed for 5 days with 50 ng/ml NGF and then subcultured onto monolayers of C6 glioma and skin fibroblasts in experiment A (with serum containing media), or monolayers of C2 myotubes, skin fibroblasts and a collagen substratum in experiment B (with SATO medium). After 48 hours coculture in the presence of 50 ng/ml NGF, increases in the binding of McAb RT97 binding were determined. Each data point is the mean \pm S.E. of four independent determinations. 100 arbitrary units were 0.263 and 0.138 OD units for experiments A and B respectively.

RT97 binding was increased by NGF treatment in a dose dependent manner, with submaximal and maximal effects being similar for all three models. Similar observations were made in cocultures grown in SATO medium, with responsiveness to NGF being comparable on fibroblast, myotube and collagen substrates. In the absence of NGF, primed cultures showed no ability to differentiate morphologically or to accumulate RT97 on any monolayer in either of the culture media. These data support the observation that morphological differentiation of primed PC12 cells was comparable on all monolayer substrates tested, with skin fibroblasts providing as suitable a substrate for neurite regeneration as glioma, myotubes or collagen.

6.2.9. THE EFFECT OF COCULTURE ON NGF INDUCED EXPRESSION OF THY-1.

Naive PC12 cells were grown for 3 days on monolayers of either C2 myotubes or human skin fibroblasts in SATO medium supplemented with NGF over a wide concentration range (0 to 20 ng/ml). Control cultures were grown under identical conditions except for being seeded onto a pure collagen substratum. After the 3 day period cells were fixed and assayed for their relative level of expression of Thy-1, as determined by their capacity to bind the McAb OX7. Figure 6.11 shows the relative expression of Thy-1 for PC12 cells cultured on fibroblasts or collagen.

As reported here (see chapter 3) and elsewhere (Doherty and Walsh, 1987), NGF induces a 2 to 3 fold increase in the level of expression of Thy-1 when cells are grown on a collagen substratum. However, in contrast to the dose dependent increases observed in the latter cultures, PC12

NGF INDUCTION OF THY-1 IN FIBROBLAST - PC12 COCULTURES

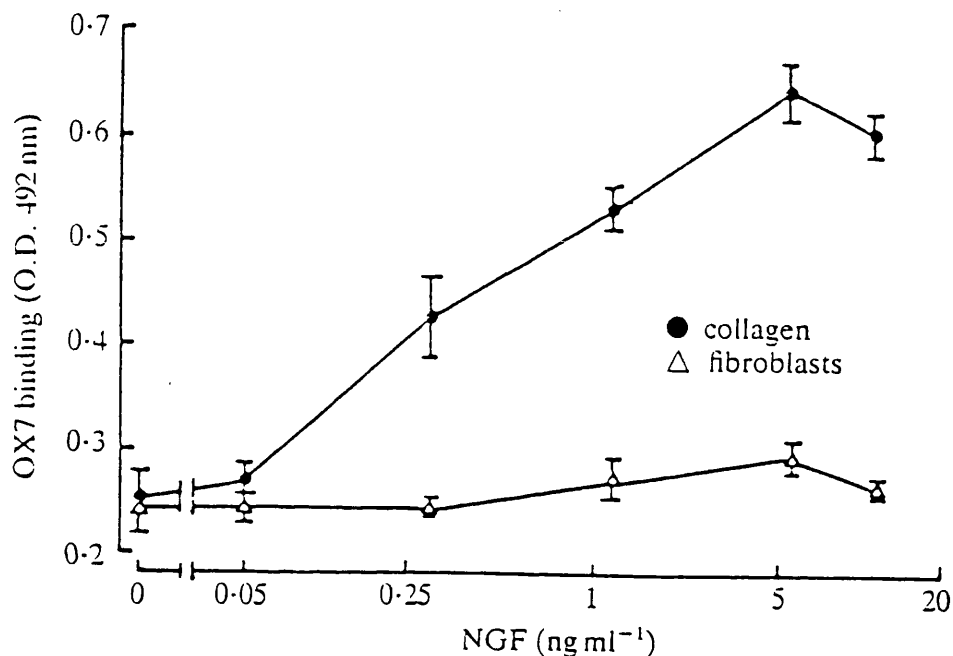


Figure 6.11. Naive PC12 cells were seeded onto a collagen substratum or a fibroblast monolayer and grown for 3 days in media supplemented with up to 20 ng/ml NGF. For each data point, OX7 McAb binding was determined for three independent cocultures with non-specific binding to sister monolayer cultures subtracted. The results show the mean \pm S.E. of three independent determinations. Non-specific binding to fibroblasts was measured as 0.06 ± 0.003 OD units and this value did not vary as a function of NGF concentration.

cells cultured on fibroblast monolayers underwent essentially no change in their cell surface level of expression of Thy-1 over the entire NGF concentration range. Thus, it would appear that the Thy-1 response is totally suppressed in the fibroblast/ PC12 cocultures.

Over the same 3 day timecourse and in the absence of exogenously added NGF, there was a 2 fold increase in the expression of Thy-1 in PC12 cell cultures growing on myotubes compared to the level of expression seen on control cultures growing on collagen (fig 6.12). The addition of NGF to these cultures promoted increases in Thy-1 expression that were only 20% greater than the increase observed in myotube cocultures grown in the absence of growth factor. NGF induced expression did not differ appreciably between PC12 cells cultured on a substratum of collagen or C2 myotubes.

Taken together these data suggest that the nature of the substratum that a PC12 cell is grown upon has profound effects on both its basal and NGF induced level of expression of Thy-1, and indicate that C2 muscle has the ability to directly induce increased expression of Thy-1 in a manner that is apparently not additive to the response induced by NGF.

6.2.10. EFFECTS OF CONDITIONED MEDIA FROM NON-NEURONAL CELLS ON NGF INDUCED INCREASES IN THY-1 AND NEUROFILAMENT PROTEIN EXPRESSION.

SATO medium was conditioned over monolayer cultures of skin fibroblasts and C2 myotubes for 1 or 4 days and added at up to 50% (V/V) to the culture medium of naive PC12 cells growing on collagen. Cells were grown for three days in the

EFFECT OF MUSCLE CELL - PC12 CELL INTERACTION ON THY-1
EXPRESSION IN NAIVE AND NGF INDUCED CULTURES

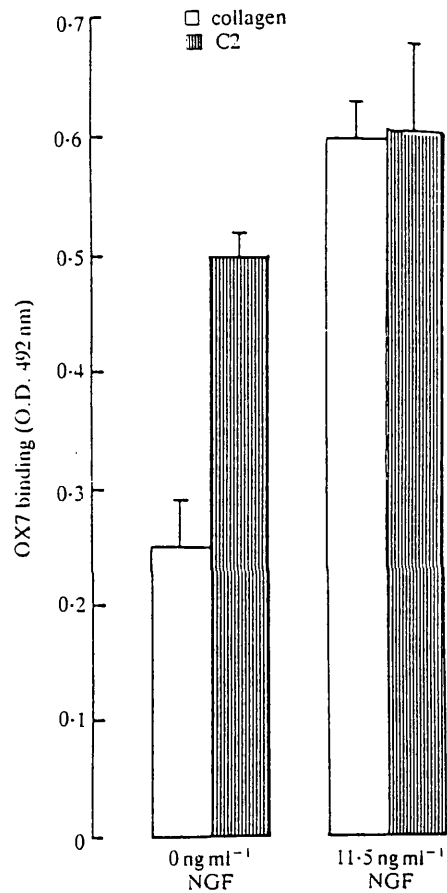


Figure 6.12. Naive PC12 cells were seeded onto a collagen substratum or C2 myotube monolayers and grown in the presence and absence of 11.5 ng/ml NGF for 3 days. The results show determinations for the absolute binding of the OX7 McAb to PC12 cells and each value represents the mean \pm S.E. of 3 independent determinations with binding to the monolayer subtracted. The latter was measured as 0.060 \pm 0.004 OD units.

presence and absence of NGF (50 ng/ml), fixed and used for

ELISA determinations of Thy-1 and neurofilament protein expression. Conditioned medium from either non-neuronal cell culture was without effect on Thy-1 or neurofilament expression at the basal or NGF-induced level. Similarly DMEM conditioned over G8 myoblasts and myotubes or C6 glioma for 24 hours and concentrated 10 - fold was found to be without effect on the binding of RT97 to naive and primed cells grown for 3 days on collagen (data not shown). In contrast , Doherty et al (1986) reported that G8 conditioned media contained an activity that increases the survival of spinal neurons.

To test the effects of fibroblast and myotube secretions on PC12 cells more directly , monolayer cultures of non-neuronal cells were grown with cultures of PC12 cells at the same time only separated by means of a porous cellulose membrane. Briefly, PC12 cells were seeded onto the individual wells of a 24-well collagen coated tissue plate into which were carefully placed cellular membranes (Millicell-H.A, 0.45 μ M diameter) on which monolayers of either fibroblasts or C2 myotubes had been previously established. Under these conditions and over a 5 day period, neither fibroblasts nor myotubes had any effect on either basal or NGF induced expression of neurofilament protein (Data not shown).

6.3 DISCUSSION.

NGF can induce distinct changes in both the morphology and biochemistry of the PC12 cell, including changes in cellular shape that lead ultimately to the onset of neurite outgrowth. In the present study the effects of the cellular microenvironment on three potentially related NGF induced events, namely (1) neurite outgrowth, (2) neurofilament protein accumulation, and (3) induction of increases in Thy-1 glycoprotein expression, have been investigated. To this end a simple coculture system was developed in the microwell culture model utilised for ELISA determinations. Briefly, monolayer cultures of fibroblasts, skeletal muscle myotubes (differentiated cultures of C2 and G8-1 cell lines) and C6 glioma, were established in microwells and utilised as substrates for the growth of sister cultures of PC12 cells by seeding the latter directly onto the surface of the different monolayers.

The most striking observation of the studies presented was the relative failure of NGF to induce morphological differentiation of PC12 cells cultured on a fibroblast monolayer. This total suppression of neurite outgrowth was also associated with a reduced ability of NGF to induce increases in the expression of the mature phosphorylated 150 kD neurofilament protein subunit recognised by McAb RT97. A thorough investigation of the RT97 response showed that increased expression of the phosphorylated neurofilament subunit was closely associated with the morphological differentiation status of the cell, with the precise physical location of the antigen being the neurite and its pioneering growth cone. Hence, by inference, it can be assumed that with increasing neurite length and branching there will be increased requirement for, and expression of, the neurofilament proteins. In support, it has been

demonstrated that mRNA species encoding all three neurofilament protein subunits found in the PC12 cell are increased quantitatively with long term NGF treatment (Lee, 1986 ; Lee and Page, 1984 ; Lindenbaum et al, 1987 and 1988). In fibroblast cocultures the differential neurofilament response was made relative to C6 glioma and two independent muscle cell lines under two differing culture conditions (serum containing media and SATO media). PC12 cells cultured on monolayer cultures of C6 glioma were directly induced in the absence of exogenously added growth factor, generating increases of up to tenfold in their basal level of expression of neurofilament protein. This direct effect is probably the result of induction triggered by an "NGF-like" activity reported to be actively secreted by C6 glioma cells in serum containing but not defined media (Westermann and Unsicker, 1986). In contrast while C2 and G8 myotube cultures were able to support NGF induced neurite outgrowth and increased neurofilament protein expression they were unable to directly induce changes in cell morphology or cellular expression of neurofilament protein.

In contrast to naive cells , primed PC12 cells were able to extend neurites equally well across fibroblast monolayers as PC12 cells grown on a collagen substratum or muscle monolayers. In addition primed PC12 cells were able to generate a neurofilament protein response in fibroblast cocultures comparable to the response observed in C2 myotube cocultures or with cells grown on collagen. Therefore, the relative inability of naive PC12 cells to differentiate in response to NGF when cocultured with fibroblasts cannot be attributed to an inherent inability of the fibroblast cell surface to support neurite outgrowth. The possibility that fibroblasts may actively secrete an inhibitory substance that would modulate NGF induced differentiation is unlikely since

(a) primed cells respond to NGF equally well whether growing on a monolayer of fibroblasts or myotubes, and (b) medium conditioned by fibroblasts was ineffective in modulating NGF induced responses in naive cells.

Recent studies have shown that the ability of axons to grow across neuronal and non-neuronal tissues and cell surfaces in a differential manner reflects their choice of adhesive interactions on a given cell surface (Bixby et al, 1987 and 1988 ; Tomaselli et al, 1988 ; Chang et al, 1987 ; Neugebauer et al, 1988). For example, it has been demonstrated that a cocktail of antibodies to cell adhesion molecules including N-CAM, N-cadherin, and the integrin receptor are required to successfully block neurite outgrowth from chick ciliary ganglion neurons across monolayers of muscle (Bixby et al, 1987). In a similar study, N-cadherin and integrins, but not N-CAM were identified as the major recognition systems involved in the promotion of outgrowth from the same neuronal cell type across astrocyte or Schwann cell monolayers (Bixby et al, 1988 ; Tomaselli et al, 1988). These studies show that neurite outgrowth across a tissue may be influenced by the adhesive input of a variety of cell surface components including Ca^{2+} -dependent and -independent CAM's and integrin receptor molecules, and that the relative expression of these adhesive components at the surface of the neuron and its substratum may influence the ability of the neuron to extend an axon in any given direction. Molecular genetic approaches to the study of neurite outgrowth have shown that transfection of Neuro-2a and 3T3 fibroblasts with N-cadherin and N-CAM respectively, lead to these transfectants acting as better substrates for neurite outgrowth than their untransfected parental cells (Matsunaga et al, 1988 ; Doherty et al, 1989).

Molecules such as N-cadherin, N-CAM and L1 are involved in homophilic interactions, recognising and binding to themselves (Takeichi, 1988 ; Rutishauser et al, 1988 ; Lander, 1989). Therefore, these studies provide evidence that modulation of homophilic binding molecules at the surface of the neuron and / or its apposing cell surface can directly influence the extent of neurite outgrowth. NGF treatment has been shown to profoundly alter the adhesive properties of PC12 cells, increasing both their affinity for one another as well as increasing their ability to adhere and spread onto tissue culture plastic (Schubert and Whitlock, 1977). In turn PC12 cell differentiation can be modulated by their immediate substratum. Thus it might be expected that the ability of primed PC12 cells to extend neurites across fibroblast monolayers, in contrast to their naive counterparts, may be due to differences in the relative adhesiveness of the two cell types to fibroblast cell surfaces. However, Doherty et al (1988) demonstrated that PC12 cell adhesiveness to fibroblasts was unaffected by NGF pretreatment, and indeed PC12-fibroblast adhesion did not appear to be mediated by the common extracellular matrix molecules. Thus, it is likely that more subtle mechanisms than gross differences in cell-cell adhesion account for the relative differences in the ability of primed and naive cells to extend axons across fibroblast substrates. Neurite outgrowth has been demonstrated to be influenced by specialised cell-cell adhesion molecules which have been proposed to play both receptor and transduction like roles in the neurite outgrowth process (Bixby et al, 1988 ; Lander, 1989). As many of these molecules (e.g. N-CAM, L1 and Thy-1) have been demonstrated to be expressed at higher levels at the surface of NGF treated PC12 cells, it is possible that the increased expression of adhesive glycoproteins at the primed PC12 cell surface, relative to

their low basal expression on naive cells, renders the primed cell to be capable of differentiating on fibroblast surfaces. Alternatively, the ability of the primed cell to respond both morphologically and biochemically to NGF in a transcription-independent manner may have a bearing on this capability.

Cell shape and biochemical status of the PC12 cell have been shown in the preceding chapters to be closely associated phenomena. The demonstration that induction of Thy-1 in naive PC12 cells by NGF can be modulated by cell-cell interactions in the absence of detectable diffusible substances suggests that interactions influencing cell adhesion and spreading may also have profound effects on early signal transduction events. Intriguingly, while fibroblast-PC12 cell interactions suppressed NGF induced Thy-1 expression, muscle-PC12 interactions triggered a direct 'NGF-like' Thy-1 response in the absence of NGF and independently of any overt morphological differentiation. Thus, direct membrane-membrane contact may influence both the resting and activated phosphoinositide / protein kinase C transduction system that mediates Thy-1 induction in PC12 cells. Such a mechanism may also be responsible for modulating other NGF induced responses, and in the case of fibroblast-PC12 cell association may cause the complete suppression of morphological differentiation and neurofilament protein expression as observed in coculture. If such a mechanism operated on transcription dependent transduction only, then similar transcription independent transduction signalling in primed PC12 cells would be unaffected. Clearly such a hypothesis should be tested before being given serious consideration, one possibility would be to measure inositol phosphate accumulation in PC12 cell cocultures so as to determine the direct effects of

cell-cell interactions on a known NGF inducible transduction system.

Recent studies by Caroni and Schwab (1988 a and b) provide compelling evidence for the existence of two membrane proteins in CNS white matter with the ability to inhibit neurite outgrowth. Thus at least for CNS neurons, a pioneering axon will encounter non-permissive growth signals. It is conceivable, though not yet proven, that similar molecules may exist in peripheral tissues and may have the ability to modulate primary transduction signals generated by growth factors.

Taken together, the data presented in this chapter demonstrate that PC12 cell responsiveness to NGF is critically dependent on the nature of its immediate microenvironment, and moreover that responsiveness to cues in the microenvironment is itself not immutable and can in turn be modified by pre-exposure of the cell to NGF. Importantly the data illustrate how differentiation is dependent on the concerted actions of both diffusible and non-diffusable factors encountered by the cell. The novel demonstration that cell-cell interactions can modulate NGF induced transcription-dependent responses occurring independently of morphological differentiation may be indicative of modulation of cellular signal transduction mechanisms being an important route for cellular communication in developing tissues.

CHAPTER 7. GENERAL DISCUSSION AND FUTURE PERSPECTIVES.

The PC12 cell line provides a convenient and versatile model with which to study mechanistic and functional aspects of NGF induced neuronal differentiation. The PC12 cell lends itself to studies at both the morphological and molecular level, and allows investigation of events operating during signal transduction and gene regulation. In addition, the control of expression and function of specific neural cell surface molecules may be analysed during events that may parallel similar processes in developing and sympathetic neurons. Finally, the PC12 cell was shown to be a suitable candidate for detailed biochemical studies on differentiation in a complex multi-cellular coculture system in which a variety of environmental factors may come into play. The following discussion summarises the study and provides suggestions for the direction of future studies in the field.

7.1 SIGNAL TRANSDUCTION.

Investigation of the mechanisms that underlie NGF induced increases in the expression of Thy-1, N-CAM and L1 in the differentiating PC12 cell have provided an indication as to the complexity of the signal transduction machinery mediating the cellular actions of NGF. It is clear that multiple biochemical responses to NGF are generated by amplification and divergence of the initial signal yielding an as yet undetermined number of sub-signals. Studies by other investigators (Milbrandt, 1987 ; Cho et al, 1989 ; Changelian et al, 1989) have shown that the initial targets for these signal transduction pathways may be genes encoding transcriptional regulators, and that it is likely to be the products of these transduction events that are ultimately

responsible for regulating the activity of the so-called " target genes " that constitute the neural phenotype of the PC12 cell.

Having shown that NGF induction of the Thy-1 gene requires the activation of the protein kinase C-dependent intracellular transduction pathway, it will be of interest to determine the sequence of events that follow activation of protein kinase C. Upstream regulatory sequences have been fully characterised at the 5' end of the rat Thy-1 gene (Giguere et al, 1985) and do not include either the sequence known as the serum-response element (SRE), which has been suggested to act as a potential NGF-response element (Milbrandt et al, 1987 ; Changelian et al, 1989), or the control element TRE (TPA responsive element) that is associated with other phorbol ester inducible genes (Angel et al, 1987). The transcriptional factor AP1 was recently shown to recognise the phorbol ester inducible element (TRE) and therefore have the potential to act as a signal transduction element at the receiving end of the protein kinase C pathway (Angel, 1987 ; Lee, 1987). Since the TRE is not present in the regulatory region of the Thy-1 gene it is unlikely that AP1 acts directly as a regulatory factor for NGF induced THY-1 transcription. However, AP1 may regulate THY-1 gene activity indirectly, influencing transcription at genes encoding other trans-acting factors which interact with regulatory cis-acting elements of the Thy-1 gene. It remains to be determined if AP1 is a component of protein kinase C induced THY-1 expression and moreover, if AP1 is inducible by NGF.

In addition to AP1 , potential mediators of the Thy-1 response and indeed other NGF induced responses at the level of gene transcription, include the transcriptional

factors c-fos, c-myc, c-jun, NGF1-A and NGF1-B (Greenberg et al, 1985 ; Bartel et al, 1989 ; Milbrandt, 1987 and 1988), which have been shown to be rapidly induced by NGF in PC12 cells and to have the potential to bind to regulatory DNA sequences found at the 5' end of a variety of inducible genes. NGF1-A has recently been characterised at the gene sequence level (Changelian et al, 1989) and has been shown to encode protein domains for the zinc-finger structure which is a common feature of many DNA-binding proteins, in addition sequence analysis at the 5' end of the gene revealed a group of SRE's which inferred NGF responsiveness to the gene. Target genes for regulation by NGF1-A have yet to be identified.

Future studies with N-CAM would include the identification of specific signal transduction elements involved in mediating increased synthesis of the 140 kD form and the appearance of the 180 kD form. The demonstration that neither protein kinase C nor protein kinase A are directly responsible for mediating NGF induction of N-CAM discounts the view that the two transduction systems orchestrate the NGF induced differentiation response in PC12 cells (Cremins et al, 1986) and illustrates the complexity of the cell's transduction machinery. Characterisation of the upstream sequences of the N-CAM gene (Barton et al, 1990) may reveal clues as to the nature of the intracellular signals that regulate transcription and control the splicing event that yields the 140 and 180 kD polypeptides. Of perhaps greater importance will be the identification of the splicing factors that control N-CAM isoform expression in PC12 cells, and elucidation of the mechanism(s) regulating their activity during NGF induced differentiation.

In addition to the existence of multiple transduction pathways, a further level of complexity was revealed by the demonstration that cholera toxin induced increases in the intracellular level of cAMP, resulted in highly specific modulation of NGF induced responses. NGF induction of Thy-1 and L1 was inhibited while N-CAM synthesis was potentiated. Thus the intracellular signals triggered by NGF are clearly not immutable and appear to be sensitive to secondary cellular signals. Since these effects were shown to be closely associated with an acceleration of the neuritogenic response there is evidence that secondary cellular signals can profoundly alter the behaviour of the NGF treated cell. As cholera toxin triggers these events by binding to cell surface ganglioside GM1 (Van Heyningen et al, 1971 ; Wiegandt et al, 1978 ; Ribi et al, 1988), it is clear that the NGF treated PC12 is sensitive to factors in the environment other than classical growth factors and can modulate its activity in response to such factors. The demonstration in coculture studies that Thy-1 expression can be modulated at the surface of PC12 cells when in direct contact with the surface of muscle cells and fibroblasts supports the contention that a variety of environmental factors are likely to regulate differentiation.

Elucidation of the mechanisms that modulate NGF induced transduction events in response to external stimuli, may reveal a unique set of processes that allows the neuronal cell and / or its growing axon to respond to more than one external factor at the same time.

Great emphasis is likely to be placed on the transduction events that take place in close proximity to the NGF-receptor since there is growing evidence that these events are probably specific to NGF whilst later

transduction processes are likely to be common as they are also activated by the FGF's and activators of protein kinase C. The ability of K252a to distinguish between NGF and bFGF activation of the Thy-1 and N-CAM transduction pathways indicates the existence of growth factor specific transduction elements that may be responsible for directing NGF and bFGF signals into common transduction routes that constitute the differentiation programme. In contrast similar EGF specific transduction elements must direct the EGF signal into an alternative cellular transduction system that promotes cell growth as opposed to differentiation. Chemical analogues of K252a (e.g. molecules that are unable to enter the cell) may reveal further clues regarding the events that occur soon after the binding of NGF to its receptor and help identify transduction elements that mediate the early membrane-associated events that occur within a few seconds of the binding event (Guroff, 1985). Identification and characterisation of similar elements specific for FGF and EGF may provide details of the mechanisms that allow the PC12 cell to distinguish between external cues for differentiation and cues for growth and proliferation.

Finally, it is becoming increasingly apparent that proto-oncogenes may play important roles in both neural maturation and signal transduction (for review, see Hanley, 1988). The int-1 and int-2 oncogene products have been implicated as potential neural growth factors, with the latter product bearing a close resemblance to the FGF's (Dickson and Peters, 1987). The product of the ras proto-oncogene p21, has an immunoreactivity which is highly concentrated in neurons (Chesa et al, 1987), has GTP-binding activity, and is closely associated with the inner surface of plasma membranes indicating it may function in early

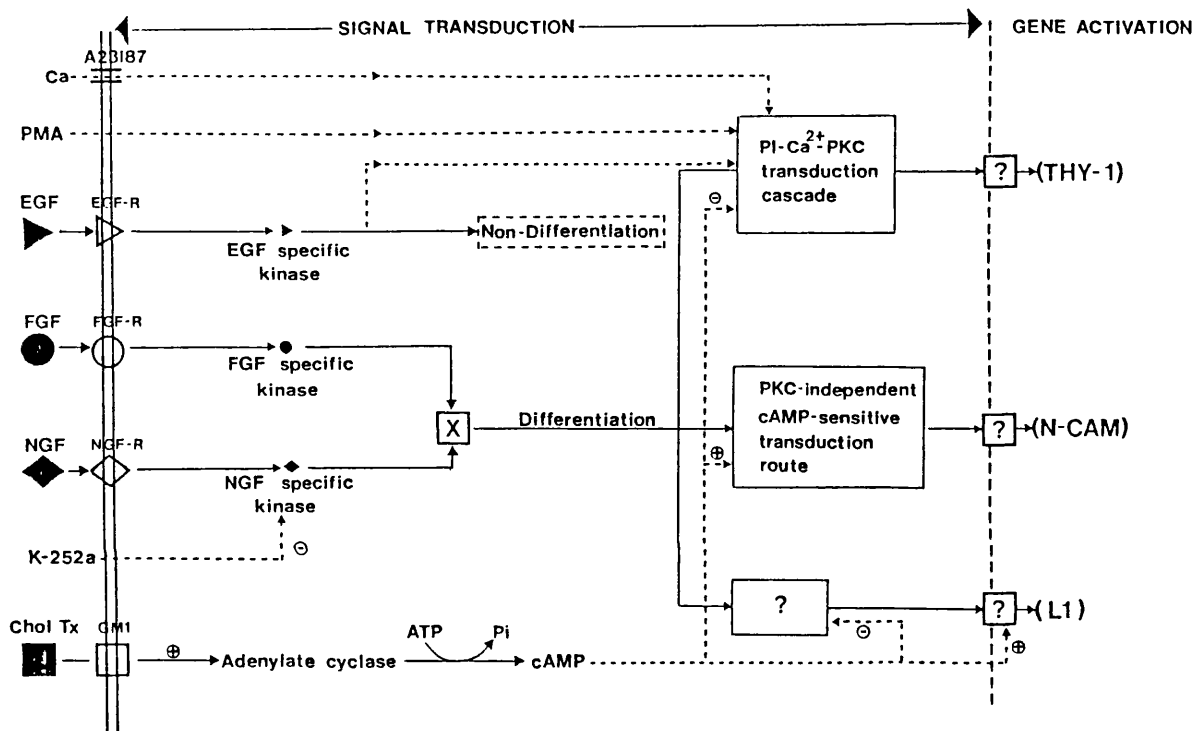
trans-membrane signalling events. Interestingly, the actions of NGF in PC12 cell cultures can be blocked by microinjection of anti-ras antibodies (Hagag et al, 1986) and can be mimicked by mutationally activated ras (Bar-Sagi and Feramisco, 1985). It will be of importance in future studies to determine the potential for the ras protein to act as a coupling element between events occurring at the NFG-receptor, and transduction events occurring in the cell cytoplasm.

Figure 7.1 summarises the details of the NGF induced transduction process resulting in stimulation of Thy-1, L1 and N-CAM synthesis and includes the actions of FGF, cholera toxin, PMA, A23187 and K252a.

7.2 NEURAL CELL SURFACE RECOGNITION MOLECULES : FUNCTIONS IN THE DIFFERENTIATING PC12 CELL.

Neuronal differentiation has been shown to be dependent on the interaction of the neuron and its growing axon with other cellular and extracellular surfaces (Bixby et al, 1987 and 1988 ; Chang et al, 1987 ; Caroni and Schwab, 1988a and b ; Tomaselli et al, 1988 ; Neugebauer et al, 1988 ; Doherty et al, 1989). These studies have provided strong evidence for the role of cell adhesion and therefore CAMS in the neuritogenic process and indicate that growth and guidance of nerve axons may be regulated by mechanisms involving the selection of preferential cell surface interactions. In the current study this view was supported by the demonstration that (a) morphological differentiation of NGF treated PC12 cells is dependent on the type of cellular substratum chosen for supporting growth (muscle monolayers and glial monolayers support neurite outgrowth while in contrast fibroblast monolayers do not) and on the phenotypic status

FIG 7.1. SIGNAL TRANSDUCTION EVENTS IN THE DIFFERENTIATING PC12 CELL.



of the PC12 cell (primed PC12 cells extend neurites across all tested monolayer cultures including fibroblasts), and (b) cell-cell interactions between PC12 cells and non-neuronal cell surfaces can result in the modulation of primary signal transduction events (expression of Thy-1 in naive PC12 cells was elevated in myotube cocultures, while NGF induced Thy-1 increases in fibroblast cocultures were completely suppressed). In both cases the effects were shown to be elicited in the absence of detectable diffusible factors suggesting a role for cell-cell recognition molecules in both signal transduction and the establishment of morphological differentiation.

Thy-1, L1 and N-CAM were investigated for their potential for a role in the differentiating PC12. Their relative contribution to morphological differentiation and neurite outgrowth was assessed by correlation of glycoprotein expression with distinct stages of morphological status of the cell. Since Thy-1 expression could be induced in the absence of any detectable cellular differentiation and as the phase of accelerated neurite outgrowth observed in cultures co-treated with NGF and cholera toxin was accompanied by an inhibition of the NGF-induced Thy-1 response it appeared unlikely that Thy-1 played a major neuritogenic role. Similarly inhibition of NGF induced increases in the expression of the L1 antigen were demonstrated to be concomitant with accelerated neuritic outgrowth. In contrast N-CAM expression was closely correlated to the morphological status of the cell suggesting a role for the molecule in the establishment of neural morphology.

Future studies may be directed toward determining the precise contribution of the differing N-CAM isoforms to the

morphological status of the PC12 cell. As the PC12 cells utilised in the N-CAM expression studies were cultured on a substratum consisting of the type 1 and type 111 collagens only, any function of the N-CAM isoforms in these conditions must be mediated by a process other than homophilic recognition. Involvement of the 180 kd isoform in stabilisation of cytoskeletal structure in the differentiating cell has been suggested here (see chapter 4) and elsewhere (Pollerberg et al, 1986). Similar studies with other CAMS and integrins indicate that they may transduce the signal for neurite outgrowth through their cytoplasmic domains to cytoskeletal proteins (Togari et al, 1983 ; Pollerberg et al, 1986 ; Horwitz et al, 1985 and 1986 ; Hirano et al, 1987 ; Volk et al, 1987 ; Bixby et al, 1988), it remains to be determined whether the long cytoplasmic domain of N-CAM plays a transduction role in the neuritogenic process. Immunological studies utilising isoform-specific antibodies may help to elucidate the specific function of each N-CAM isoform, or alternatively a strategy could be developed using a molecular-genetic approach either utilising cDNA constructs for specific N-CAM polypeptides (Doherty et al, 1989) or by the production of PC12 cell mutants lacking the ability to synthesise a specific N-CAM isoform. In addition, the elucidation of the mechanisms controlling N-CAM mRNA splicing during cellular differentiation will provide important clues regarding isoform function.

A role for the Thy-1 and L1 glycoproteins in the process of neuronal development and neuritic outgrowth, though not shown here, cannot be ruled out since, (a) exhaustive functional studies were not carried out, and (b) expression and differentiation studies were performed on a simple collagen substratum where subtle cell-cell recognition

events are impossible to assess. Indeed investigation of Thy-1 expression in coculture models established that the molecule is sensitive to environmental influence, with both basal and NGF induced expression being susceptible to modulation by direct cell-cell interaction with non-neuronal cell surfaces. It will be of interest to investigate the molecular mechanisms underlying this cell surface modulation so as to establish whether it is a contributory factor in determining the ability of non-neuronal cell surfaces to support NGF induced differentiation of naive PC12 cells. Thy-1 has been implicated as a signal transduction molecule (Kroczek et al, 1986 ; Saleh et al, 1988), the possibility that Thy-1 plays a similar role at the junction formed between the PC12 cell and an apposing non-neuronal cell should be investigated further.

Availability of antibodies specific to other neuronal cell surface recognition molecules such as N-cadherin ,MAG and Po will allow comparable studies to those carried out with Thy-1, L1 and N-CAM. As each recognition molecule is cloned and characterised at the gene level it should be possible to combine antibody studies with cDNA transfection studies and thereby develop initiatives to determine the independent and contributory roles of individual components in the differentiation process. As such it will be important to consider molecules that may provide negative cues for differentiation as well as those that promote neuronal development, since these signals will also contribute to specificity and order in the developing nervous system. The relative inability of fibroblast cell surfaces to support NGF induced differentiation of naive PC12 cells may reflect the presence of negative cues that actively inhibit primary differentiation. Proteins that are able to infer a non-permissive nature on substrates with regard to their ability

to support axonal outgrowth have been identified and isolated from the myelin protein of oligodendrocytes and from white brain matter (Caroni and Schwab, 1988 a and b). The presence or absence of similar molecules at the fibroblast cell surface should be established together with an investigation of the properties these molecules utilise as negative cues.

In addition to glycoproteins, the glycolipid components of the neuronal cell surface have also been shown to be of importance in recognition and cellular function. In the current study the recognition molecule GM1 was studied in its capacity as a receptor for cholera toxin (Wiegandt et al, 1978). GM1 binds with the 12 kD B-subunit of external cholera toxin allowing insertion of the toxins 27 kD A-subunit through the plasma membrane (Ribi et al, 1988), leading to ADP-ribosylation of the stimulatory G component of adenylate cyclase. This mechanism results in the triggering of a spectrum of biochemical and morphological changes in both naive and NGF treated cells. Of greatest interest was the ability of cholera toxin, via its intracellular second messenger cAMP, to modulate biochemical and behavioural responses induced by NGF. The observed acceleration of neurite outgrowth for a limited period in NGF and cholera toxin co-treated cultures supports the growing evidence for a role for GM1 in the neuritogenic process (Gorio et al, 1983 ; Roisen et al, 1981 ; Toffano et al, 1983 ; Rybak et al, 1983 ; Facci et al , 1984 ; Doherty et al, 1985). By searching for physiological ligands for GM1 and the more complex members of the ganglioside family, investigators may identify previously uncharacterised trophic and/or tropic factors, or alternatively may reveal subtle mechanisms that provide the developing neuron and its growing axon with a process that

allows several external signals to be processed and assimilated into a behavioural response.

7.3 MOLECULAR STUDIES OF NGF INDUCED DIFFERENTIATION IN PRIMARY NEURONAL CULTURES.

Investigators now have the ability to purify and culture relatively homogenous populations of neurons that can survive in the absence of externally added trophic factors yet will on addition of such factors respond at the morphological and biochemical level. Populations of NGF responsive rat DRG neurons can be cultured in the absence of NGF (Lindsay, 1988b) and have recently been reported to undergo NGF induced transcriptional regulation of the expression of mRNA's encoding the precursors of two neuropeptides, substance P and the calcitonin gene-related peptide (Lindsay and Harmer, 1989). This neural crest-derived system may be useful in establishing the relevance of the findings made with the PC12 cell model to the mechanisms of action of NGF in normal neuronal populations and should stimulate further studies in both culture systems.

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