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Growth, Differentiation and Cell-Cell Coupling in the Human Neuroblastoma Cell Line SH-SY5Y

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ABSTRACT

Neuroblastoma is one of the most common paediatric solid tumours, frequently occurring in infancy with the primary lesion in the adrenal gland and sympathetic nervous system. It originates from primitive neural crest cells. In rare cases, this tumour regresses spontaneously to a more benign ganglioneuroma probably by neuronal differentiation or apoptosis. We investigated the role of induction of neuronal differentiation and apoptosis *in vitro* in SH-SY5Y neuroblastoma cells. A variety of agents that are known to induce neuronal differentiation including retinoic acid, nerve growth factor (NGF), protein kinase C (PKC) inhibitors and a cAMP-dependent protein kinase A (PKA) activator were tested solely or in combination for their capacity to induce terminal differentiation. The cells were characterised for markers of differentiation as well as their ability to withdraw from the cell cycle. We found that the combination of 8-Br-cAMP (PKA activator) with NGF in the presence of the cell cycle inhibitor aphidicolin was the best treatment to induce terminal differentiation in SH-SY5Y cells. Treated cells showed long neurites resembling those of neurones. They expressed markers characteristic of the cytoskeleton (NF200, NF68) and of neuronal function (tyrosine hydroxylase, choline acetyltransferase and the neurone-specific enolase) and showed an increase in the expression of TrkA, the receptor for NGF. Furthermore, the expression of the N-myc oncogene that is normally overexpressed in these cells decreased. Since neurones are dependent on NGF for survival, we tested the ability of terminally differentiated SH-SY5Y cells to survive in the absence of NGF. We found that the cells became NGF-survival dependent and that deprivation from this neurotrophic factor induced programmed cell death. We also tested the effect of PKC

specific and non-specific inhibitors on cell proliferation, differentiation and apoptosis in SH-SY5Y cells. We found that only the non-specific PKC inhibitors staurosporine and H7 induced morphological and molecular differentiation as well as decreased N-myc amplification followed by apoptosis. The effect of differentiation induction on p53 sub-localisation was also investigated in undifferentiated and differentiated SH-SY5Y cells. p53 immunostaining showed that p53 was sequestered in the cytoplasm in undifferentiated SH-SY5Y cells and that the induction of differentiation resulted in the partial transfer of p53 to the nucleus where it probably became able to regulate the cell cycle and apoptosis since p53 is not mutated in neuroblastoma. Gap junction intercellular communication (GJIC) is known to be involved in the regulation of cell growth and homeostasis and has been shown to contribute to many diseases including cancer. We investigated the role of GJIC in neuroblastoma. We found that SH-SY5Y cells have altered GJIC due to an aberrant localisation in the perinuclear region of connexin 43 (Cx43), one of the proteins of GJIC normally present at the plasma membrane. GJ channel formation and gating is regulated by PKC, PKA and /or MAPK. We found that induction of differentiation using the PKA activator 8-Br-cAMP relocalised Cx43 to the plasma membrane region and restored GJIC. Furthermore, inhibition of p38 MAPK induced a block of cell proliferation associated with GJIC proficiency while inhibition of the subfamily of MAPK, Erk1/Erk2, likely promoted the degradation of Cx43.

DEDICATION

À mon père M. Ahmed Belbaraka, à ma mère
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TABLE OF CONTENTS

LIST OF FIGURES AND SCHEMATICS

Chapter one

Chapter two

LIST OF TABLES

LIST OF ABBREVIATIONS

8-Br-cAMP	**8-bromo-cyclic adenosine monophosphate**
AP	**Aphidicolin**
AP-1	**Activator protein 1**
aPKC	**Atypical PKC**
ATP	**Adenosine triphosphate**
BCIP	**5-bromo-4-chloro-3-indolylphosphate**
BDNF	**Brain-derived neurotrophic factor**
BIS	**Bis-indolylmaleimide**
BSA	**Bovine serum albumin**
Cα	**Catalytic subunit**
CA3	**Hyppocampal neurones**
cAMP	**Cyclic adenosine monophosphate**
cdk2	**Cyclin-dependent kinase 2**
Chat	**Choline acetyl transferase**
CMTX	**Charcot Marie Tooth X-linked**
CNPase	**2', 3'-cyclic nucleotide 3'-phosphodiesterase**
CNS	**Central nervous system**
cPKC	**Classical PKC**
CRE	**cAMP-responsive element**
CREB	**cAMP-responsive element binding protein**
Cx	**Connexin**

DAG	**Diacylglycerol**
db-cAMP	**Dibutyryl-cyclic adenosine monophosphate**
DMSO	**Dimethyl sulfoxide**
DNA	**Deoxyribonucleic acid**
EDTA	**Ethylenediaminetetraacetic acid**
EGF	**Epidermal growth factor**
Erk1	**Extracellular response kinase 1**
Erk2	**Extracellular response kinase 2**
FBS	**Fetal bovine serum**
FGF	**Fibroblast growth factor 1**
FITC	**Fluorescein isothiocyanate**
GAP 43	**43 kDa growth associated protein**
GF109209	**PKC inhibitor**
GJ (s)	**Gap Junction (s)**
GJIC	**Gap Junction Intercellular Communication**
Go6983	**PKC inhibitor**
gp140	**Glycoprotein 140 kDa**
gp75	**Glycoprotein 75 kDa**
H33258	**Hoescht fluorochrome 33258**
H7	**1,5-isoquinolinylsulfonyl-2-methylpiperazine**
H-89	**PKA inhibitor**
HL60	**Human leukemia cells**
IMR-32	**Human neuroblastoma cell line**
IP3	**Phosphatidylinositol 3,4,5 trisphosphate**

JNK	**c-Jun N-terminal kinase**
Kd	**Dissociation constant**
kDa	**Kilodalton**
LAN-2	**Human neuroblastoma cells**
LAN-5	**Human neuroblastoma cell line**
MAPK	**Mitogen-activated protein kinase**
MAPKK	**Mitogen-activated protein kinase kinase**
MDCK	**Mardin-Dardy canine kidney cell line**
Mdm2	**Mouse Double Minute 2**
mRNA	**Messenger ribonucleic acid**
NB	**Neuroblastoma**
NBT	**Nitroblue tetrazolium**
Neuro 2a	**Murine neuroblastoma cell line**
NF200	**Neurofilament 200 kDa**
NF68	**Neurofilament 68 kDa**
NG108-15	**Neuroblastoma X glioma hybrid cell line**
NGF	**Nerve growth factor**
NGFR	**Nerve growth factor receptor**
NP40	**Nonidet P40**
nPKC	**Novel PKC**
NSE	**Neurone specific enolase**
NT-3	**Neurotrophin 3**
NT4/5	**Neurotrophin 4/5**
p34cdc2	**Cyclin-dependent p34**

PAGE	**Polyacrylamide gel electrophoresis**
PBS	**phosphate-buffered saline**
PC12	**Rat pheochromocytoma cells**
PCNA	**Proliferating cell nuclear antigen**
PDGF	**Platelet-derived growth factor**
PI3K	**Phosphatidylinisitol-3-kinase**
PKA	**Protein kinase A**
PKC	**Protein kinase C**
PLCγ	**Phospholipase-C γ**
PMSF	**Phenylmethylsulfonyl fluoride**
PNS	**Peripheral nervous system**
RA	**Retinoic acid**
RAR	**Retinoic acid A receptor**
RI	**Regulatory subunit I**
RII	**Regulatory subunit II**
RNA	**Ribonucleic acid**
RPM	**Revolution per minute**
RPMI	**RPMI medium 1640**
RXR	**Retinoic acid X receptor**
SAPK	**Stress-activated protein kinase**
SDS	**Sodium dodecyl sulfate**
Shc	**Adaptor protein**
SH-SY5Y	**Human neuroblastoma cells**

SKNB-E	**Human neuroblastoma cells**
SK-NMC	**Human neuroblastoma cells**
SRE	**Serum responsive element**
SY5Y	**Human neuroblastoma cells**
T cells	**Murine hybridoma cells**
T51B	**Rat epithelial cells**
TBST	**Tris buffered saline + Tween 20**
TGFα	**Transforming growth factor α**
TH	**Tyrosine hydroxylase**
TPA	**12-0-Tetradecanoyl phorbol-13-acetate**
tRA	**All-trans retinoic acid**
TrkA	**Tropomyosin receptor kinase A**
TrkB	**Tropomyosin receptor kinase B**
TrkC	**Tropomyosin receptor kinase C**
Tunel	**TdT-mediated dUTP nick end labelling**

Chapter One

Induction of differentiation and apoptosis in the SH-SY5Y neuroblastoma cell line

1.1. Literature Review

Neuroblastoma (NB) is an embryonic tumour of neural crest origin. This neoplasm is the most prevalent solid tumour in children, excluding brain tumours (Grosfeld *et al.*, 1999). It accounts for 8 to 10% of all childhood cancers and causes 15% of the deaths from childhood malignancy (Castleberry *et al.*, 1997). Each year approximately 525 new cases are reported in the United States (Casteleberry *et al.*, 1997). Canada has an incidence of approximately 65 children per year with a prevalence of about one case per 8000 live births (Brodeur, 2000). Almost 90% of neuroblastomas are diagnosed in children younger than five years of age (Casteleberry *et al.*, 1997). Despite important improvements in care, one-third of these patients die from either disease progression or complications of aggressive therapy (Black & Haase, 1999). A number of environmental factors have been associated with an increased risk of neuroblastoma, however, a definitive link between environmental exposure and neuroblastoma has not been established (Grosfeld, 1999). Neuroblastoma can be detected during intrauterine life by prenatal Ultrasonography and they occasionally contain a cystic component (Black *et al.*, 1999).

Neuroblastoma can arise anywhere in the sympathetic nervous system, including the neck (3%), posterior mediastinum (20%), pelvis (3%), paraspinal ganglia (24%) and adrenal medulla (50%) (Grosfeld, 1998; Black & Haase, 1999). Forty-five percent of children with NB are at high-risk, and often the disease has spread throughout the body when first diagnosed and even with recent improvements in therapy, only 40% of children with high-risk NB survive for five years without recurrence (Brodeur *et al.*, 1997).

The natural history of NB has generated great interest because numerous neuroblasts are seen in the adrenal gland during intrauterine life and may persist over the postpartum period. These neuroblasts usually regress, but may persist and give rise to NB (Grosfeld, 1998; Grosfeld *et al.*, 1993; Black & Haase, 1999). This tumour behaves in an unusual manner and follows one of three clinical courses: 1) spontaneous regression, 2) maturation from malignant tumour to a benign lesion (i.e., ganglioneuroma in 2% to 5% of patients), and 3) a progressive malignant disease (Matthay *et al.*, 1995; Grosfeld *et al.*, 1999).

Neuroblastomas originate in the adrenal medulla or paraspinal sites where sympathetic nervous tissue is present. The most common presentation is an abdominal mass, seen in 50% of patients. Neuroblastoma can also be manifested around the spinal cord or in the chest, neck or pelvis. NB symptoms are due to tumour mass or to bone pain from metastases (Catelberry, 1997).

Neuroblastomas have been classified into low, intermediate, and high-risk tumour categories based on clinical stages (I, II, III, IV-S and IV) according to the International Neuroblastoma Staging System (Brodeur *et* al, 1993). The prognosis for patients with NB is related to their age at diagnosis, clinical stage of the disease, and to

their regional lymph node involvement (Brodeur *et al.*, 1993). Cases associated with good outcomes include patients with stages I, II, and IV-S and less than one year of age. In contrast, poor prognosis may be expected in stage III or IV tumours (Caron *et al.*, 1996; Seeger *et al.*, 1985; Matthay, 1995; Nakagawara *et al.*, 1993; Castle *et al.*, 1993). Other conventional prognostic variables include the site of the primary tumour and tumour histology.

The overall survival rate for neuroblastoma patients is 40%. Survival is affected by age and tumour stage as prognostic factors. Survival is nearly 100% at stage I, 90% for stage II, 37% for stage III, 12% for stage IV and more than 80% for IV-S. Survival is significantly better for children less than one year of age (Black & Haase, 1999; Catelberry, 1997). Patients with primary tumours occurring in the pelvis and neck have an improved prognosis. Patients with the advanced disease (older than one year, stage III (70%) and stage IV (12%) often have amplification of the N-myc oncogene, diploid tumours, 1p36 deletion, low TrkA expression and an unfavourable histology (Grosfeld, 1998).

Some neuroblastomas are associated with neuronal cell differentiation. These include instances of neuroblastomas occurring in the new-born, cystic tumours and cases in infants less than one year with stage IV-S disease (Grosfeld *et al.*, 1993). Many of these patients have elevated levels of the proto-oncogene TrkA, the high-affinity receptor for nerve growth factor that may facilitate differentiation or regression of neuroblastoma (Nakagawara *et al.*, 1993). Regression of neuroblastoma may also be related to tumour apoptosis (Brodeur, 2000; Brown *et al.*, 1998). *In vitro*, neuroblastoma derived cells undergo morphological and biochemical differentiation as well as apoptosis following treatment with a variety of agents such as retinoic acid (Kurie *et al.*,

1996; Lovat *et al.*, 1997), certain protein kinase inhibitors and agents that raise cAMP (Boix *et al.*, 1997; Jalava *et al.*, 1993; Ronca *et al.*, 1997; Sasaki *et al.*, 1997; Slack *et al.*, 1992).

1.1.1 The characteristics of neuroblastomas

1.1.1.1 The molecular abnormalities in neuroblastomas

By virtue of their ability to express many properties of differentiating and mature neurones, neuroblastoma cells represent powerful model systems for studying the sequence of events underlying and accompanying the outgrowth and stabilisation of neurites.

Neuroblastomas are composed of a variety of cell types reflecting the multipotent potential of neural crest tissues. They typically contain heterogeneous cellular sub-populations, including neuroblastic (N), substrate-adherent (S), and intermediate (I) cell types that can be distinguished on the basis of their morphological and biochemical characterisation (Ciccarone *et al.*, 1989; Rettig *et al.*, 1987; Ross *et al.*, 2001).

These tumours show several genetic abnormalities *in vivo* and *in vitro* (Ciccarone *et al.*, 1989; Foley *et al.*, 1991). The N-myc gene has been found amplified in about 20-25% of primary NB tumours associated with poor prognosis irrespective of age and clinical stage. There is evidence that N-myc gene amplification contributes to tumour aggressiveness and progression. (Brodeur *et al.*, 1992, 1993; Brodeur, 1995; Nakagawara *et al.*, 1992). Genomic N-myc amplification manifested by extrachromosomal double minutes or homogeneously staining regions in chromosome 2p is one of the strongest factors that give tumour cells an aggressive malignant

character (Bader et *al.*, 1991; Brodeur et *al.*, 1992). Chromosomal abnormalities represented by chromosome 1p deletions are also common in advanced tumours.

1.1.1.1.1 Chromosome abnormalities

A loss of heterozygocity on chromosome 1 is noted in a large proportion of patients (70%) that have neuroblastoma with a near-diploid karyotype (Brodeur & Fong, 1989). This usually indicates a deletion of the short arm of chromosome 1(1p). The actual site of the deletion may vary. The 1p36 locus is most commonly deleted in neuroblastoma, but not in familial cases (Brodeur *et al.*, 1997a). This may be a site of a suppressor gene that, when affected by deletion or translocation, results in malignant disease or amplification of the N-myc oncogene (Guo *et al.*, 1999). Abnormalities in other chromosomes are also common in neuroblastomas including deletions on chromosomes 11, 14q, and 17q (O'Neill *et al.*, 2001; Brodeur *et al.*, 1997a; Fong *et al.*, 1992). Hyperdiploid tumours are often seen in patients with lower stage disease, with a good prognosis and an excellent response to a lower dosage of chemotherapy; the diploid karyotype carries a worse prognosis and is often resistant to chemotherapy (Grosfeld, 1999).

1.1.1.1.2 N-myc amplification

Amplification of cellular oncogenes is one of the major genetic alterations that appear to contribute to tumourigenesis (Brodeur *et al.*, 1984). The myc gene is expressed in nearly all human and fetal tissues. Mechanisms of deregulation in tumours include chromosomal translocation (Khanna *et al.*, 1995), proviral insertion (DePinho *et al.*, 1991) and gene amplification (Brodeur *et al.*, 1984; Schwab et *al.*, 1998). The N-myc

gene belongs to the myc gene family whose most prominent members include c-myc and L-myc. All three genes encode transcription factors represented as nuclear phosphoproteins with a short half-life of 20-30 min. In contrast to c-myc, N-myc gene expression is limited to tissues of neuroectodermal origin. N-myc expression is high during early embryonic development and declines at later stages (Zimmerman *et al.*, 1986). Approximately 30% of patients present tumours with N-myc amplification, which is correlated with aggressive tumour growth (Brodeur *et al.*, 1986). The N-myc gene product, N-Myc, is an intranuclear specific protein encoded by the human oncogene. The N-myc proto-oncogene is localised to two types of chromosomal abnormalities, double minutes, which represent extrachromosomal elements (Hogarty & Brodeur, 1999; Caron *et al.*, 1995) and homogeneously stained regions integrated within derivative chromosomes (Caron *et al.*, 1996), which are both indicative of gene amplification. More than 90% of patients with N-myc amplification exhibit a rapid progressive disease that is resistant to therapy (Zehnbauer *et al.*, 1988; Brodeur *et al.*, 1984). In contrast, less than 10% of early stage NBs have N-myc amplification. N-myc expression is regulated both at the transcriptional and post-transcriptional levels (Chagnovich & Cohn, 1996; Wada *et al.*, 1988, 1993). Several proteins that interact with myc and are presumably involved in regulating the transcription of myc target genes have been identified. These include Max, the Mad family and Nmi. In NB, Negroni *et al.*, (1991) observed that antisense inhibition of N-myc gene decreases the proliferation rate and the induction of neuronal differentiation. Recently, Galderisi *et al.* (1999) showed that N-myc gene inhibition in neuroblastoma cells can result in one of the three events: 1) suppressed cell proliferation; 2) induced differentiation, and/or 3) apoptosis,

suggesting that N-myc operates through different pathways that involves activation of different genes.

1.1.1.1.3 NGF receptor alteration

Neurotrophic factors and their tyrosine kinase receptors, Trks, play an important role in the pathogenesis, biology and clinical behaviour of neuroblastomas (Nakagawara *et al.*, 1993, 1994; Brodeur *et al.*, 1992). Three Trk genes were identified as genes encoding a receptor tyrosine kinase for the nerve growth factor (NGF) family. TrkA preferentially binds NGF but also weakly binds NT-3 and NT-4/5 (Klein *et al.*, 1991). TrkB can interact with brain-derived growth factor (BDNF) and NT-4/5, and also with NT-3 to a lesser extent. TrkC preferentially binds NT-3 (Kaplan *et al.*, 1991; Klein *et al.*, 1991). Because each of these genes is expressed in a specific sequence during normal neuronal development, it is considered that the Trk family plays a crucial role in neurogenesis (Barde, 1989; Hefti *et al*, 1992). Neuroblastoma arises from embryonic neuronal cells when a strict control of the regulation of these genes is required. Among these three genes, a high level of TrkA gene expression has been accepted as a factor causing favourable outcome in human NB (Brodeur *et al.*, 1997b; Azar *et al.*, 1994). Fresh tumour cells derived from tumours that have favourable outcomes and express a large amount of TrkA mRNA transcripts, were able to respond to NGF and exhibit significant neuronal differentiation *in vitro* (Eggert *et al.*, 2000b). Conversely, NB cell lines with poorly represented TrkA mRNA showed neither expression of c-fos oncogene mRNA nor remarkable neurite outgrowth following NGF exposure (Nakagawara *et al.*, 1993). Furthermore, NB cells are shown to present multiple defects of the nerve growth

factor receptors. Many aggressive neuroblastomas and cell lines expressed both full length TrkB and BDNF instead of TrkA (Nakagawara *et al.*, 1994). Recent studies have supported the hypothesis that TrkA is not a functional receptor in aggressive neuroblastoma even if TrkA is expressed (Azar *et al.*, 1990). *In vitro* studies showed deficiencies in the TrkA signal transduction pathway in aggressive neuroblastoma cells at the level of both protein expression and receptor function and suggest that N-myc overexpression may inhibit TrkA expression (Nakagawara & Brodeur, 1997; Eggert *et al.*, 2000a). Signal transduction pathways used by TrkA have been studied in PC12 cells. Following NGF binding, TrkA receptors rapidly become phosphorylated on tyrosine residues, and their tyrosine domain activated (Klein *et al.*, 1991). Phosphorylated tyrosine residues in the TrkA cytoplasmic domain serve as anchors for binding downstream signalling molecules such as phospholipase-Cγ1, phosphatidylinositol-3-kinase and the adapter protein Shc (Bibel *et al.*, 1999). These proteins couple TrkA to several intracellular signalling pathways such as RAS/MAPK, which activate transcription factors and induce immediate-early genes (Kaplan & Miller, 1997). Activation of Trk receptors by neurotrophins is required for neurite outgrowth and neuronal survival in the peripheral and central nervous systems (Benedetti *et al.*, 1993; Bibel *et al.*, 1999). The expression of the Trk family plays an important role in the regulation of growth and progression into differentiation in NB since these tumours are derived from the peripheral nervous system. In SH-SY5Y NB cells, introduction of an exogenous TrkA gene can restore the ability of this cell line to morphologically differentiate in response to NGF and induce activation of the mitogen activated protein

kinase and immediate-early genes, suggesting that one major defect in NB may be that for the constitutive TrkA, functional expression is too low (Eggert *et al.*, 2000 b, c).

1.1.2 Neuroblastoma treatments

The NB tumour shows a wide range of clinical behavior from spontaneous regression to aggressive malignant disease and a wide morphological diversity and heterogeneity (Evans *et al.*, 1971). One of the unique characteristics of NB is that age at diagnosis and the heterogeneity of the differentiating tumour cells is related to prognosis. Patients younger than 1 year of age have a better prognosis than those older than 1 year of age, even if they have advanced disease. Ganglioneuromas are believed to be the end stage of maturing NBs. A tumour containing immature NB cells is usually highly malignant. All therapeutic modalities used in NB should therefore be adapted to the stages and biological risk factors. There are three main therapies for NB; chemotherapy, radiotherapy and surgery (Brodeur, 2000; Grosfeld, 1999).

Indeed, current protocols for neuroblastoma therapy are based on low, intermediate, and high-risk tumour categories. Cases associated with a good outcome include patients with stages I, II, and IV-S, age less than 1 year, absence of chromosome 1 abnormalities, less N-myc copies, hyperdiploidy, high TrkA expression, normal neurone-specific enolase and low serum ferritin levels. In contrast, poor prognosis may be expected in children with more than 10 copies of N-myc, low TrkA expression, diploid DNA and a loss of heterozygosity at the 1p36 chromosomal locus (Eggert *et al.*, 2000d; Nickerson *et al.*, 2000; DuBois *et al.*, 1999). These patients have very poor outcomes, with less than 5% surviving (Black & Haase, 1999).

Primary surgery is the treatment of choice in patients with localised neuroblastoma. The decision to remove a tumour is based on its location, on how it is impacting major blood vessels, on the child's prognosis, and other factors (Kaneko *et al.*, 1997). Complications can arise as a result of surgery, such as Horner syndrome, but this is often the result of aggressive attempts to remove an abdominal tumour when the tumour is first diagnosed. Low-risk patients can be treated with an operation alone with an expected cure rate greater than 90% (Grosfeld *et al.*, 1993).

Neuroblastoma is a radiosensitive tumour and therefore, local radiotherapy is used for treatment of stages III and IV. Taking into consideration such factors as tumour size and location, as well as the age of the child, radiation is used along with other treatment modalities, such as chemotherapy and/or surgery (Deacon *et al.*, 1985). In addition, radiolabelled antibodies directed against neuroblastoma cells is another therapy for stage IV neuroblastoma (Cheung *et al.*, 1994).

Aggressive chemotherapy is used for high-risk patients, with an intensive dose used early during the therapy phase using multiple chemotherapy agents that include doxorubicin, cyclophosphamide, carboplatin, ifosfamide, carboplatin, iproplatin, epirubicin, vincristine and etoposide. Treatment usually includes a combination of several chemotherapeutic agents (Pritchard *et al.*, 1986).. Retinoic acid was found to decrease the expression of N-myc oncogene amplified in NB. Sidell (1982) first reported that retinoic acid induced growth arrest and differentiation in human NB cell line. Cell infusion and retinoic acid treatment to induce differentiation and stimulate apoptosis are also used in high-risk neuroblastoma patients (Lovat *et al.*, 1997).

Recently, bone marrow and peripheral blood stem cell transplantation have become the treatment of choice for high-risk neuroblastoma. Many children preparing

for transplant will receive total body irradiation. More aggressive therapy such as megadose chemotherapy with rescue by bone morrow transplant can be used clinically. The disease free survival of patients with advanced neuroblastoma has not improved much despite modern therapy (Grosfeld, 1999).

1.1.3 Spontaneous regression in neuroblastomas

Although neuroblastoma are histologically characterised by small, undifferentiated, round cells resembling primitive neuroblasts, some tumours display more differentiated features and contain cells resembling Schwann cells of the glial lineage, ganglion cells of the neuronal lineage, or secretory cells expressing markers of neuroendocrine differentiation (Biedler *et al.*, 1988). The presence of these differentiated features indicates that NB cells retain the molecular mechanisms that regulate the differentiation of neural crest cells along these different physiological lineage (Cooper *et al.*, 1991). This interpretation is compatible with occasional clinical observation of both spontaneous and therapy induced maturation of NB to more benign ganglioneuroma (Matthay, 1995). The role of neuronal differentiation may then be relevant to the clinical course of neuroblastoma progression (Girgert *et al.*, 2000). Advantage may be taken of the fact that neuroblastomas have the highest rate of spontaneous regression among human tumours, probably because of differentiation of tumour cells into neurone-like cells. Neuroblastoma spontaneous regression can also be due to activation of programmed cell death or apoptosis (Black & Haase, 1999).

1.1.4 Cellular differentiation of neuroblastoma cells

Cellular differentiation depends on specific signals present in the extracellular environment that alter intracellular gene expression affecting cell metabolism and growth. Many NB cell lines retain some histopathological features of their normal precursors and the expression of a panel of neuroendocrine markers that resemble those found in normal adrenal medulla histogenesis (Haycock, 1993; Gomez *et al.*, 1998; Girgert *et al.*, 2000). A large body of literature describes the effects of compounds, such as retinoic acid (RA) or cAMP elevating agents, which are able to cause differentiation of NB cells *in vitro* and *in vivo* and reverse their tumourigenicity (Gaetano *et al.*, 1995). (Reynolds, 2000; Malik *et al.*, 2000; Hewson *et al.*, 2000; Hovland *et al.*, 2001). Moreover, a variety of chemical agents and biological response modifiers are known to promote *in vitro* co-ordinated morphological, biochemical and ultrastructural changes in NB cells leading to well differentiated phenotypes. However, the described protocols in the literature for NB differentiation report only morphological differentiation measured by the expression of some neuronal factors without taking in to account that neuronal differentiation should be terminal (Malik *et al.*, 2000). Differentiated NB should not only present neurite extension and express neuronal markers but also evolve to the most important stage, which is the final exit from the cell cycle. Confusion is thus generated in the literature by the application of the term differentiated NB cells, which present morphological differentiation only measured by the expression of neuronal markers. There should be a clear distinction between mature differentiated and terminally differentiating NB cells.

The NGF receptors mainly evoke the MAPK pathway for both proliferation and differentiation. This pathway is also activated by the RAS oncogene product often expressed in NB. Both signals lead to the phosphorylation of the retinoic acid receptor (RAR), which is also, controlled by the antagonistic action of protein kinase C and protein kinase A (PKA) enzymes. These protein kinases play crucial roles in signal transduction, cellular proliferation and differentiation but the pleiotropic effects of these enzymes in cellular regulation and their involvement in tumour promotion underlines the importance of understanding their mechanisms of regulation and specificity. Therefore, the discovery and development of more specific protein kinase inhibitors will enable the functional role of each protein kinase in cells to be more clearly defined.

1.1.4.1 The retinoids and differentiation

Retinoic acid and many of its natural and synthetic derivatives, collectively known as retinoids, have been investigated extensively as potential therapeutic agents because of their effects in inhibiting cell proliferation, inducing cell differentiation and promoting apoptosis in many types of cancers, including neuroblastomas (Reynolds, 2000). The action of retinoic acid is mediated *via* retinoic acid receptors localised in the nucleus (Niles, 2000).

The activity of retinoids is mediated by a network of RA receptors (RARs) and retinoid X receptors (RXRs). Retinoic acid has different isomers: all trans (tRA), 9-cis and 13-cis RA. tRA regulates gene expression by activating nuclear RARs, which function as dimers with RXRs. In contrast to RARs, RXRs also form homodimers in addition to functioning as heterodimer partners for other nuclear receptors (Chambon,

1996). RARs and RXRs differ in their ability to bind different RA isomers: RARs bind tRA and 9-cis RA whereas, RXRs bind only 9-cis RA. 9-cis RA can bind to RXRs and RARs to activate RXR homodimers, or the RXR partners of heterodimers (Levin *et al.*, 1992; 2001). This may generate a range of biological effects not shown by tRA, which activates only RARs. At a pharmacological level, 9-cis RA exhibits different biological properties and has markedly greater effects on neuroblastoma cells than tRA *in vitro* (Redfern *et al.*, 1994; Lovat *et al.*, 1994) and *in vivo* (Miller *et al.*, 1995; 2001; Kurie *et al.*, 1996). However, the toxicity of 9-cis RA may limit its clinical use for neuroblastoma treatment (Lovat *et al.*, 2001).

Retinoic acid exerts important effects during vertebrate development (Morriss-Kay & Ward, 1999; Deluca, 1991). It induces differentiation of neuroblastoma cells under experimental conditions *in vitro* and *in vivo* as well as growth arrest and apoptosis (Sidell, 1982; Han *et al.*, 1995; Lovat *et al.*, 1997). A systematic evaluation of oral 13-cis retinoic acid administration as a single agent in refractory neuroblastoma indicated that there was no significant therapeutic effect (Niles, 2000). *In vitro* studies have shown that other RA isomers have a greater biological activity than 13-cis RA, particularly 9-cis RA and tRA, which have distinct biological properties, and that activation of three distinct RXR/RAR heterodimers induce growth arrest and differentiation in NB cells (Giannini *et al.*, 1997). Furthermore, RARβ, a RAR isoform, is induced by tRA in SH-SY5Y neuroblastoma cells. In these cells, tRA down regulates N-myc expression and up regulates receptors for NGF (Hewson *et al.*, 2000).

1.1.4.2 Protein kinase A activation and neuroblastomas

Protein kinase A is a serine/threonine protein kinase that is cAMP-dependent. It consists of a regulatory (R) subunit dimer and of two catalytic (C) subunits. The two isozymes of PKA, type I and type II, are distinguished by the association of subunit, type I R (RI) or type II R, (RII) with one of the common C isoforms (Cα, Cβ, and Cγ) (Maldonado & Hanks, 1988; Beebe *et al.*, 1990). The regulatory subunits each have 2 isoforms (RIα, RIβ, RIIα, RIIβ) (Sandberg *et al.*, 2001; Oyen *et al.*, 1989). A high level of RI or type I PKA correlates with active cell growth and transformation, and a high level of RII or type II PKA is related to growth arrest and differentiation (Herman *et al.*, 1994; Mena *et al.*, 1995; Johnson *et al.*, 2000; Hovland *et al.*, 2001). RIIβ and type II PKA were shown to increase specifically with RA-induced growth inhibition and neuronal differentiation in SH-SY5Y cells (Kim *et al.*, 2000). 8-Br-cAMP is a cAMP analogue that has a potent proliferation inhibiting effect in a variety of human cancer cells (Kato *et al.*, 1994; Naderi *et al.*, 2000). Neuropeptides or chemicals that act through cAMP-elicited pathways were shown to support the survival of specific types of neurones (Kim *et al.*, 1997). The cellular effect of cAMP is most easily recognised by the induction of neurite outgrowth in cultured neuronal cells and expression of some neuronal markers (Kim *et al.*, 1994). In particular, established cell lines are good model systems for studying biochemical events triggered by cAMP because they are often induced to express neuronal phenotypes in response to agents that raise intracellular cAMP levels. For example Nagasaki *et al.* (1999) identified the vasoactive intestinal polypeptide, a neuronal gene product transiently induced by cAMP stimulation that increases progressively during differentiation. They also examined the protein

expression of tyrosine hydroxylase, the rate-limiting enzyme critical to catecholamine biosynthesis. Furthermore, an increase in intracellular cAMP causes the up-regulation of acetylcholine synthesis in a neuronal cell line derived from the mouse septum (Blusztajn *et al.*, 1992; Misawa *et al.*, 1993).

1.1.4.3 Protein kinase C family and neuroblastomas

1.4.3.1 PKC isoenzyme expression

Protein kinase C is a family composed of 11 isoenzymes, also called isoforms, encoded by at least 10 genes and can be subgrouped into classical (cPKCs: PKCα, βI, βII and γ), novel (nPKC; PKCδ, ε, η, and θ), and atypical (aPKC: PKCι and ζ) isoforms in addition to PKCμ. The classification into subgroups is based on the regulatory and structural properties of the isoforms. Briefly, both classical and novel isoforms are activated by phorbol esters and diacylglycerol but only the activity of the classical isoforms is calcium dependent. Atypical isoforms are insensitive to phorbol esters (for review see Newton & Johnson, 1998). PKC isozymes constitute a family of structurally related serine-threonine lipid-dependent kinases that are encoded by separate genes and ubiquitously expressed. Different isoforms may exert opposite effects on growth within the same cell system. The expression of different isoforms of PKC is altered in astrocytomas, brain tumours, melanomas and other tumours and it has been suggested that this alteration may play a role in malignant transformation (Dempsey *et al.*, 2000). For a given tumour cell type, targeting one specific PKC isoform may possibly influence the growth and malignant behaviour of the cells (Borner *et al.*, 1995). We considered it of great interest to identify the expression patterns of the PKC isoforms during

proliferation and progression into differentiation of human SH-SY5Y NB cells since PKC isoforms were shown to have different functions in proliferation, survival and differentiation of neuroblastoma cells SH-SY5Y, IMR32, LAN-5 and LAN-2 (Zeidman *et al.*, 1999).

1.1.4.3.2 PKC and the control of cell proliferation and differentiation

The involvement of PKC in the control of cell growth was initially suggested by the finding that PKC constitutes the ligand for the mitogenic phorbol esters, which are potent tumour promoters (Pahlman, 1981). Also, calphostin C and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7), two inhibitors of PKC activity, were shown to prevent proliferation of the neuroblastoma cells Neuro-2a in culture (Minana *et al.*, 1994). Furthermore, an antisense oligonucleotide directed against PKCα was shown to inhibit the growth and induce differentiation of several human tumour cell lines in an animal model system including neuroblastoma (Dean *et al.*, 1996). All these facts support the hypothesis that PKC plays a key role in the control of cell proliferation.

PKC is believed to play a key role in neuronal cell differentiation. A number of studies have indicated a negative correlation between PKC activity and NB cell differentiation (Koda *et al.*, 1990; Ponzoni *et al.*, 1993). For example, down-regulation of PKC by prolonged exposure of cells to phorbol esters such as 12-O-tetradecanoyl phorbol 13-acetate (TPA) was shown to induce differentiation of SH-SY5Y human NB cells into neurone-like cells (Jalava et al., 1992; Zeidman *et al.*, 1999). A loss of PKC activity has been observed in the retinoic acid–induced differentiation of the human NB cell line SK-N-SH (Slack *et al.*, 1992) and LAN-5 cells, and in dibutyryl cyclic AMP-

induced differentiation of the neuroblastoma X glioma hybrid cell line NG108-15. A decrease in the mRNA level for cPKC and nPKC was correlated with 8-Br-cAMP induced differentiation of Neuro-2a murine neuroblastoma cells. Furthermore, differential expression of PKC isoforms was observed during the progress into differentiation in many neuroblastoma cell lines, including SH-SY5Y (Leli *et al.*, 1993). Although these results suggested a role for PKC in neuronal differentiation, more direct evidence that a loss of PKC activity is a part of the mechanism by which neuronal cell differentiation is initiated came from studies in which PKC inhibitors such as H7 and staurosporine induced the differentiation of neuroblastoma cells directly (Shea & Beermann, 1991; Felipo *et al.*, 1990; Minana *et al.*, 1989) and from the demonstration that intracellular delivery of PKC-specific antibodies stimulated the differentiation of cultured neuroblastoma cells (Srinivas *et al.*, 1999). In general, there are opposing influences of PKC and PKA toward inducing NB differentiation. Induction of differentiation *in vitro* in NB cell line is associated with an activation of PKA or an inhibition of PKC (Kvanta & Fredholm, 1993; Shea *et al.*, 1992).

1.1.4.4 Nerve growth factors and neuroblastoma differentiation

1.1.4.4.1 Neurotrophic factors involved in neuronal differentiation

Neurones require several trophic factors for growth, differentiation and survival. Nerve growth factor is a member of a family that includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). Despite their sequence homology, the pattern of expression of each of these genes during nervous system development is unique, and their physiological functions differ (Henderson,

1996). Activation of the Trk receptor tyrosine kinase serves to initiate differentiation and survival of selected populations of neurones, but is capable of promoting proliferation in different cellular circumstances (Chao, 1992). NGF binds to its receptor TrkA. TrkB encodes a receptor for BDNF and also for NT-4/5; TrkC encodes a receptor for neurotrophin-3 (Klein *et al.*, 1991). TrkB and BDNF are often expressed in dopaminergic neurones of normal lineage but also in neuroblastoma cell lines like SH-SY5Y (Klein *et al.*, 1990). In neuroblastoma, the role of neurotrophin receptors remains unclear, but the expression of the Trk family of genes is a prognostic indicator (Brodeur, 1993).

1.1.4.4.2 Nerve growth factor receptors

The action of NGF is mediated by two classes of cell surface receptors: TrkA (high affinity NGF receptor), a member of the TrkA family of tyrosine kinase receptors, and gp75 NTR, a low affinity receptor that binds other neurotrophins such as BDNF or NT-3 (Henderson, 1996). The effects of NGF are thus mediated through binding to the high affinity ($Kd=10^{-11}$ M) form and/or the low affinity receptor ($Kd=10^{-9}$ M). The TrkA proto-oncogene product gp140 is required for high affinity NGF binding.

The low affinity NGFR consists of a single transmembrane protein referred to as gp75. The role of gp75 in the functional response to NGF is still controversial. Some reports indicate that gp75 collaborates with TrkA in the formation of the high affinity binding sites and in NGF signal transduction (Verdi & Anderson, 1994) while others suggest that TrkA alone may be sufficient to support the activity of NGF on cell differentiation as shown in the neuroblastoma cell SH-SY5Y (Eggert *et al.*, 2000c). gp75 also possesses unique, Trk-independent, signalling properties that involve ceramide

production and activation of the transcription factor NFkB. Moreover, a recent evidence indicates that NGF uses a two receptor system, TrkA or gp140 and gp75, to activate signalling pathways leading either to cell survival or suicide (Barbacid, 1993); this topic has been a continuous source of controversy. Recently, Urdials *et al.* (1998) demonstrated that TrkA was expressed preferentially at the cell surface in the early G1 and S phases of the cell cycle, whereas gp75 was only detectable during the late G1, S and G2 phases. Signal transduction pathways used by TrkA have been studied mainly in the PC12 rat pheochromocytoma cell line. Following NGF binding, TrkA receptors rapidly become phosphorylated on tyrosine residues, and their tyrosine kinase domain is activated. Phosphorylated tyrosine residues in the cytoplasmic domain serve as anchors for binding signalling molecules (Klein *et al.*, 1991). Proteins known to become phosphorylated and activated in response to NGF include phospholipase-C γ1 (PLCγ), phosphatidylinositol-3-kinase (PI3K), the adapter protein Shc and the associated neurotrophic factor-induced tyrosine phosphorylated target (Maldonado & Hanks, 1988). These proteins couple TrkA to several intracellular signalling pathways like RAS/MAPK pathway (Kaplan & Miller, 1997). Activation of MAPK is followed by activation of transcription factors and the induction of immediate-early genes (Eggert *et al.*, 2000c).

NGF-treated PC12 cells cease proliferating, exhibit somatic hypertrophy, acquire neurites, morphologically differentiate and show a dependence on NGF for survival in serum-free medium. The TrkA signalling pathway may differ in cell lines derived from different tissues. NGF stimulation of TrkA leads to differentiation of neurones, whereas it induces the proliferation of fibroblasts and signals for apoptosis in human

medulloblastoma cells suggesting that the response to TrkA activation by NGF is cell type specific (Nakagawara, 1998a). It is worth noting that blocks of the cell cycle in neuroblastoma triggers the expression of TrkA (Verdi & Anderson, 1994). Some neuroblastomas are associated with neuronal cell differentiation. These include instances of neuroblastoma occurring in the newborn, cystic tumours and cases of bilateral adrenal tumours in infants, and also infants less than one year of age with stage IV-S disease. Many of these patients have elevated levels of TrkA. High levels of TrkA expression may facilitate differentiation or regression of neuroblastoma and is therefore associated with favourable prognosis outcome (Nakagawara *et al.*, 1994; Nakagawara & Brodeur, 1997). Patients who have amplified N-myc oncogene express little or no TrkA activity. Patients whose tumours lack TrkA respond poorly to therapy as mentioned above (Brodeur *et al.*, 1993, 1997b). The human neuroblastoma cell line SH-SY5Y expresses very low levels of TrkA and is not responsive to NGF (Miller *et al.*, 1995; Eggert *et al.*, 2001). Aggressive neuroblastomas with N-myc amplification often display dopaminergic characteristics (Brodeur *et al.*, 1997b). These only express full length TrkB whereas more differentiated tumours (ganglioneuroma and ganglioneuroblastomas) express truncated TrkB, which is lacking tyrosine kinase activity. TrkB is frequently expressed in tumours with N-myc amplification (Nakagawara *et al.*, 1994), that may promote cell survival and increase an invasive activity in an autocrine or paracrine manner. The function of TrkC and NT-3 in neuroblastomas remains unclear. TrkC was shown to be expressed in neuroblastoma with good prognosis but the ligand NT-3 is not expressed in neuroblastomas (Nakagawara, 1998b); its exact role in neuronal differentiation remains unclear. The regression of neuroblastoma, however, may also be related to tumour cell apoptosis and

accumulating evidence suggests that it may be due to the neuronal cell death induced by the deprivation of neurotrophic factors, especially of NGF in the *in vivo* status (Nakagawara, 1998a).

1.1.5 p53 inactivation in neuroblastomas

The p53 phosphoprotein is the product of a tumour suppressor gene, whose inactivation may play a role in the development and progression of many types of cancers (for review see Downing *et al.*, 2001). Its known targets include genes involved in growth arrest (p21 and GADD45), differentiation and apoptosis (Bax, CD95); (Deman & Van, 2001). Mutations of the p53 gene are among the most common genetic disorders in human cancers, including those of the breast, colon, lung and liver (Van *et al.*, 2001). Although mutations disrupt the function of p53, it has been shown in cultured cells that the cytoplasmic sequestration of wild-type p53 protein could also impair its function (Moll *et al.*, 1996). Certain p53 mutations however result in the cytoplasmic sequestration of mutant proteins (McKenzie *et al.*, 1999). NB tumours do not have mutations in the p53 gene but overexpress the wild type p53 protein. The overexpressed p53 accumulates in the cytoplasm in undifferentiated NB cells and it was initially thought that p53 was excluded from the nucleus (Moll *et al.*, 1996). Recently, it has been shown that p53 is not anchored in the cytoplasm, but continually exported from the nucleus by an Mdm-2 dependent pathway (Zaika *et al.*, 1999). The aberrant constitutive p53 accumulation in NB cells may result from resistance to Mdm-2-mediated degradation. This resistance is observed despite normal intracellular levels of Mdm-2 protein, p53-Mdm2 complexes, and p53 ubiquitination (Kusafuka *et al.*, 1997).

1.1.6 Programmed cell death or apoptosis

Apoptosis is an important mechanism for regulating cell numbers within a population to maintain the balance between birth and death during development and cell population homeostasis. It is a mechanism by which the organism eliminates unwanted cells through a safe process (Evan *et al.*, 1995; Gould & McEwen, 1993). Defective regulation of apoptosis contributes to the emergence of various diseases including cancer (for review see Thompson, 1995).

1.1.6.1 Apoptosis during neurogenesis, neurodegeneration and tumourigenesis

1.1.6.1.1 Apoptosis during neurogenesis

The development of the nervous system begins with the birth of about twice as many neurons as are finally present in the adult (for review see Sharma *et al.*, 2000). These neurons die by apoptosis throughout development in a well-defined interval, coincident with the establishment of synaptic connections (Henderson, 1996; Oppenheim, 1991). Recent knockout mouse studies have provided dramatic proof of the occurrence of cell death during early neural development; inactivation of several regulatory or executor molecules in the apoptotic pathway caused embryonic or prenatal lethality. For example, some of the effects of growth factors, originally interpreted as stimulation of proliferation, might be the result of inhibition of cell death (Zupanc, 1999). Results are confusing because proliferation, differentiation and apoptosis coexist

in the early stages of neural development. Complete identification of the signals that control these events has yet to be elucidated.

This natural cell death in the developing nervous system provides a mechanism whereby a neuronal target may determine the extent of its innervation. The process of programmed cell death then should play a critical role in the developing nervous system, ensuring that appropriate innervation of the target neurones and tissues takes place in a specific and timely fashion (Oppenheim, 1991).

Burek and Oppenheim (1996), proposed that embryonic apoptosis or natural cell death might result from the failure of neurones to access locally produced neurotrophic factors that are released in limited amounts. Neurones, which acquire sufficient neurotrophic factors, survive, while those deprived of adequate trophic support die. These neurotrophic factors include NGF, BDNF, NT-3, and NT-4/5. Progression of the cells through neurogenesis appears to require a co-ordinated, sequential action of various survival signals acting in an integrated network.

In the sympathetic nervous system, the immature neuronal precursors are not dependent on NGF for survival (Birren *et al.*, 1993; DiCicco-Bloom *et al.*, 1993). Sympathetic neurones subsequently become acutely dependent on NGF for survival and neurones that do not receive sufficient neurotrophic factors undergo programmed cell death. In the rat, the period of NGF dependence begins at embryonic day 19.5 (Birren *et al.*, 1993), followed by a period where the dependence to NGF decreases progressively, so that adult sympathetic neurones become less dependent on NGF and will die only upon prolonged periods of trophic factor deprivation (Gorin & Johnson, 1980). This developmental process allows the selection of the appropriate number of neurones and rids the nervous system of inappropriate connections (Oppenheim, 1991).

The concept of neurotrophism has broadened and now includes the trophic dependence of different types of neurones, as well as of glial cells, at various developmental stages (Levi-Montalcini *et al.*, 1996). Little attention was paid to cell death affecting neural precursor cells and young neuroblasts at early stages of neural development although the incidence of this regulated form of cell death and its role in building the complex cellular organisation and function of the nervous system seems important. Nerve growth factor regulates the differentiation, growth and survival of responsive neurones, chiefly the sympathetic neurones and many of the sensory neurones of the peripheral nervous system and the cholinergic forebrain neurones of the central nervous system. NGF-responsive neurones are those which bear receptors for NGF, and which respond in some way to the presence of NGF. NGF promotes the survival and differentiation of sympathetic neurones both *in vivo* and *in vitro* (Levi-Montalcini, 1987; Plet *et al.*, 1982). It has been shown *in vitro* that both mitotic and postmitotic differentiated neurones depend on NGF for their survival (Batistatou *et al.*, 1993). The mechanism by which NGF promotes survival and prevents death of responsive neurones is not fully understood. Rat pheochromocytoma (PC12) cells have been used extensively to study neuronal differentiation. These cells can be induced to differentiate and to acquire a sympathetic neuronal like phenotype (Greene & Tischler, 1976). When serum is removed from the culture medium of naïve (not NGF treated) PC12 cells, they die after exhibiting apoptotic-associated DNA fragmentation. NGF rescues PC12 cells from serum-free-induced cell death and prevents DNA fragmentation and the withdrawal of serum and NGF from neuronal (NGF treated) cells results in apoptotic cell death (Martin, 2001).

1.1.6.1.2 Apoptosis and neurodegeneration

Neuronal cell death occurs not only during development, but also as a consequence of acute traumatic events or chronic degenerative disorders that disturb cellular homeostasis, such as trauma, ischemia, Alzheimer's disease and ageing (Levi-Montalcini *et al.*, 1996). In these cases, excessive cell death occurs due to the activation of cell death programs leading to neurodegenerative diseases. A variety of extracellular agents and metabolites produced in the brain such as reactive oxygen species, calcium, ionising radiation, chemotherapeutic agents, and developmental signals, generate stress-related mechanisms in neuronal cells resulting in their apoptosis and/or necrosis. The mechanism(s) by which apoptosis is suppressed in neural tumours such as NBs or activated during neurodegeneration are not well characterised. Previous studies have demonstrated that a wide range of anticancer agents, including chemotherapeutic agents, hormones, and various biological agents, induce apoptosis in malignant cells (Gould & McEwen, 1993). Cerebral ischemia and global ischemia (MacManus & Buchan, 2000), hypoxia, or kainate acid toxicity were reported to induce apoptosis (Martin, 2001). Neuronal apoptosis has also been identified in spinal injury and traumatic models (for review see Zipp *et al.*, 2000).

1.1.6.1.3 Apoptosis and tumourigenesis

Failure to initiate a cell death program is an important mechanism of tumourigenesis in the central and peripheral nervous systems. In contrast, increased apoptosis may contribute to the regression of some neuroblastoma tumours (Shastry *et al.*, 2001). Abnormal regulation of apoptosis has been implicated in tumour development and resistance to cancer therapy. Induction of cell death by activating its

regulating pathways in neuroblastoma might be an attractive new treatment option for this disease. Neuroblastoma cell lines, in particular SH-SY5Y cells, display high levels of Bcl-2 products when they differentiate with phorbol esters, retinoic acid, or FGF1 (Itano *et al., 1996;* Mejia *et al.*, 1998).

1.1.6.2 The pathways contributing to neurotrophic factor-induced apoptosis

Both cell survival and death are likely to be mediated by apoptotic pathways, which have been characterised in other systems. However, there has been little investigation of the defined pathways that act in early neural cell death in vertebrates. Data presented to date suggest an important role for early cell death in neural development. Regulatory genes that have been reported to contribute to apoptosis are some oncogenes and tumour suppressor genes such as p53, bcl-2, c-myc, c-fos and ras (Basu & Haldar, 1998).

1.1.6.2.1 Bcl-2 pathway and apoptosis

Bcl-2, a mitochondrial and perinuclear membrane protein (Reed, 1995) and Bcl-x (Boise *et al.*, 1993) modulate the sensitivity of cells to death. Overexpression of Bcl-2 in neurones or neurone-like cell lines protects from apoptosis in response to death-inducing stimuli (Garcia *et al.*, 1992) . In the central nervous system (CNS), the developmental pattern of Bcl-2 expression has been analysed and it was shown that Bcl-2 expression is at its highest levels in the prenatal brain, but postnatal and adult animals, including humans, express lower levels of Bcl-2 (Ferrer *et al.*, 1994). In the peripheral nervous system (PNS), Bcl-2 is expressed in the prenatal mouse dorsal root ganglion and

is retained into adulthood and postnatal superior cervical ganglion neurones also express Bcl-2 (Merry *et al.*, 1994). Overexpression of Bcl-2 in sympathetic ganglion neurones delays apoptosis induced by trophic factor deprivation (Greenlund *et al.*, 1995). In contrast, ciliary ganglia are not protected by Bcl-2 overexpression from apoptosis induced by ciliary neurotrophic factor-deprivation, indicating that more than one cell death pathway exists within PNS (Allsopp *et al.*, 1993).

1.1.6.2.2 MAPK pathway and apoptosis

The survival-promoting pathway used by neurotrophins consists of the Ras-MEK-MAPK pathway. This pathway has many roles in neurones, including synaptic plasticity, long-term potentiation, and survival. The evidence for the contribution of this pathway to neuronal survival is conflicting. While NGF induces a strong and sustained activation of MAPK in sympathetic neurones and PC12 cells, most studies have found that inhibition of MEK had minimal effects on NGF-dependent neuronal survival. Thus, although the selective activation of MEK/MAPK can promote neuronal survival, MEK seems to be sufficient, but not necessary, for most neurotrophin-mediated survival. MEK may also play a more prominent role in TrkB-induced survival. It was shown that TrkB, unlike TrkA, uses both MEK and protein inositol-3-kinase (PI-3K) to promote the survival of sympathetic neurones (Farinas, 1999). NGF potently increased Bcl-2 levels in sympathetic neurones, which in turn protected the cells from apoptotic cell death (Datta et al., 1999; Hakak *et al.*, 2000). Bcl-2 was shown to be a transcriptional target of the MEK/MAPK pathway (Sheid *et al.*, 1999). cAMP-responsive element binding protein (CREB) activity is also required for Bcl-2 expression and survival induced by

NGF in sympathetic neurones, suggesting an MEK/MAPK-CREB-Bcl-2 survival pathway (for review see Sofroniew *et al.*, 2001). CREB, like Bcl-2, is clearly a key mediator of neuronal survival, as dominant-inhibitory forms of CREB induced apoptosis of virtually all sympathetic neurones grown in the presence of NGF and 25% of cerebellar neurones grown with BDNF. The activation of CREB by survival factors is likely to be due to phosphorylation at Ser133 by multiple kinases, including MAPK activated-Rsk, p38MAPK, and Akt (Zhang *et al.*, 1999; Xing *et al.*, 1998). p38 MAPK is an unlikely candidate for this role since it induces apoptosis or neurite outgrowth in PC12 cells, but not survival. MEK/MAPK is also an unlikely CREB activator in sympathetic neurones, as MEK activity is not required for NGF-mediated survival (Riccio *et al.*, 1999).

1.1.6.2.3 PKC pathway and apoptosis

The role PKC plays in apoptosis is controversial. Phorbol esters that can mimic diacylglycerol and activate both the classical and the novel PKC isozymes have been shown to induce apoptosis in some cells and prevent cell death in others (Hofmann, 2001). They have been implicated in a variety of intracellular processes including exocytosis, differentiation, growth, and survival as well as apoptosis (Rybczynska *et al.*, 2000). The fact that multiple PKC isozymes within the same cell were found to each have unique temporal and spatial patterns of expression suggests that distinct PKC isozymes mediate distinct intracellular processes especially apoptosis.

Non-specific inhibitors of PKC are known inducers of apoptosis. Among them staurosporine and H7 are routinely used. Staurosporine is a natural product, an alkaloid produced by *Streptomyces staurospores*. Its chemical structure contains an indole

carbazole chromophore, which characterises a family of protein kinase inhibitors. The protein kinases include PKC, PKA, a calmodulin-dependent protein kinase, and receptor tyrosine kinase. Each compound of this family displays a different affinity for the ATP binding site of protein kinases and as a consequence, a different ability in the blocking action (Toker, 1998).

1.1.6.3 Apoptosis versus necrosis

Apoptosis is characterised by a precise set of morphological events. There occurs a specific form of chromatin condensation with a preservation of mitochondria and lysosomes until very late in the process. Shrinkage and fragmentation of cells and their nuclei, loss of microvilli, and extensive degradation of chromosomal DNA accompany this process (Blagosklonny, 2000). Time lapse studies have shown that these events occur rapidly and are associated with surface convolutions and a rupture of the cell into a series of membrane bound, condensed apoptotic bodies. In contrast to cells dying by necrosis, mitochondria remain intact and chromatin condenses into dense granular caps under the nuclear membrane. Apoptotic cells do not induce any inflammatory response, but are targets for immediate phagocytosis. In necrotic cells, the primary lesions appear to be in the mitochondria, which become unable to supply sufficient energy for cell metabolism. Cells lose their ability to regulate their osmotic balance; thus they swell and burst. An inflammatory response is elicited by the release of intracellular contents. DNA degradation occurs as a late event. The failure of cells to undergo apoptosis may contribute to the origin and progression of human cancers (Thompson, 1995).

Apoptosis is induced by a variety of agents, which include death factors such as Fas ligand, TNF (tumour necrosis factor), and TRAIL (TNF-related apoptosis-inducing ligand), growth factor starvation, genotoxic agents such as anti-cancer drugs and γ-irradiation and oxidative stress (for review see Abe *et al.*, 2000). Regardless of the origin of the stimulus, the commitment to apoptosis occurs through activation of caspases, a family of cysteine proteases present in growing cells as inactive precursors (Toker, 1998). Cleavage of a selected group of substrates by downstream caspases is responsible for the dismantling of essential cell components, which results in the morphological and biochemical changes characteristic of apoptotic cell death: cytoskeletal rearrangement, cell membrane disruption and blebbing, nuclear condensation, and DNA fragmentation (for review see Martin, 2001). The degradation of nuclear DNA into nucleosomal units is one of the best-characterised biochemical features of apoptotic cell death.

The elucidation of the mechanism (s) involved in induction of the switch from proliferation to apoptosis may provide a new basis for the design of therapeutical targets to increase apoptosis in tumours such as NB and to decrease apoptosis in neurodegenerative processes.

1.1.6.4 Neuroblastoma cell models

Most NB cell lines are characterised by the presence of morphologically distinct cell types (Ciccarone *et al.*, 1989). These include the so-called N-cells, small, rounded cells with short neurite-like processes, that grow as poorly attached aggregates and contain the neurotransmitter biosynthetic enzymes and uptake mechanisms of embryonic

neuronal precursors (Rettig *et al.*, 1987). Such cells exhibit a neuronal phenotype with relatively small nuclei, scant cytoplasm and neurite-like cell processes. Other cell types lack neuronal features. They are known as flat cells (F-cells) or substrate-adherent cells (S-cells) with marked enlargement of nuclei and cytoplasm. They have cytoplasmic and cell surface proteins suggestive of a Schwann/glial/melanocyte or ectomesenchymal precursor cell. A fourth cell type (I-cells) displays intermediate morphology and biochemical activity related to both N and S cells. They most likely represent a transitional state between N and S cells.

Differentiation, occurring spontaneously or following induction by exogenous agents, can proceed towards either a neuronal or non-neuronal neural crest lineage, as previously reported (Ross *et al.*, 2001). NB cells adapted to *in vitro* culture exhibit heterogeneous morphology. They are highly proliferative and capable of causing tumours in nude mice. Despite a number of genetic alterations, treatments by biological response modifiers revert the malignant phenotype of NB cells and induce them to express genes characteristic of different neural crest cell lineage, such as neuronal cells, adrenal medulla cells and melanocytes (Ross *et al.*, 1983).

SH-SY5Y is a human neuroblastoma cell line derived from a neural crest-derived tumour of the sympathetic nervous system. It belongs to the N-type (neuroblastic) subtype of NB cell lines following subcloning from the parental SK-N-SH cell line. These cells express low levels of both the low-affinity nerve growth factor receptor and the TrkA NGF receptor (Miller *et al.*, 1995). Recently, it was reported that bcl-2 was richly expressed in SH-SY5Y cells (Itano *et al.*, 1996).

1.1.6.4.1 SH-SY5Y as a cell model for neuronal differentiation

Human NB cell lines differ widely in morphological response to commonly used differentiation inducers (Yu *et al.*, 1988) and SH-SY5Y has been shown to be one of the most responsive NB cells lines. This characteristic may be related to the lack of N-myc gene amplification (Brodeur & Fong, 1989; Cohn *et al.*, 1990). These cells express genes associated with neuronal differentiation and are considered to represent immature neuroblasts at different stages of differentiation (Wada *et al.*, 1988, 1993). The human adrenergic SH-SY5Y neuroblastoma cell line is widely used as a model for neuronal differentiation since it is believed to recapitulate *in vitro*, the *in vivo* differentiation pattern of PNS neuroblasts. Thus, SH-SY5Y cells are used in a large number of studies concerning human neuronal function.

Upon treatment with all-trans-retinoic acid, SH-SY5Y cells differentiate towards the neuronal phenotype as seen by the significant neurite outgrowth and the differentiated SH-SY5Y cells are ultrastructurally, biochemically and electro-physiologically similar to sympathetic neurones (Cooper *et al.*, 1991; Encinas *et al.*, 2000; Guarneri *et al.*, 2000; Malik *et al.*, 2000). They also differentiate into a neuronal phenotype on treatment with the tumour promoter 12-O-tetradecanoyl phorbol 13-acetate, and Insulin growth factor I (Pahlman, 1981). However they have never been enzymatically characterised in molecular terms during the progress to the differentiated state and at the terminal stage after withdrawal from the cell cycle. The protocols described in the literature for NB differentiation generally do not lead the cells to terminal differentiation but rather just to differentiated cells. Terminally differentiated NB should not only demonstrate neurite extension and express neuronal markers of advanced differentiation but also evolve to the most important stage, which is the final

exit from the cell cycle. A lack of clarity is present in the literature through the use of the term differentiated to refer to NB cells which present extensive morphological differentiation and growth arrest (that we call mature stage) but have not terminally exited from the cell division cycle (that we call terminal differentiation). In the following pages we make a clear distinction between differentiating, mature and terminally differentiated NB cells. Inducing NB cells to exit from the cell cycle may provide a new tool to NB therapy providing that it could be applicable *in vivo*. The goal of the following study is to determine whether NB cells are amenable to terminal differentiation, to determine the conditions that would lead these cells to exit from the cell cycle and to possibly identify some of the key contributors to this process. It is anticipated that, if NB cells can be forced into terminal differentiation, they may be more prone to apoptosis.

1.2. Objectives

1.2.1. To optimise the differentiation conditions in SH-SY5Y cells using various differentiation inducers in order to promote terminal differentiation.

1.2.2 To characterise the progression into differentiation of SH-SY5Y cells at the morphological and the molecular levels.

1.2.3 To examine whether terminally differentiated SH-SY5Y cells become NGF- survival dependent.

1.2.4 To examine the role of PKC in cell proliferation, differentiation and cell death in SH-SY5Y cells.

1.3 Materials and Methods

1.3.1 Materials

All the chemicals were purchased from Sigma and the cell culture components from Gibco unless mentioned otherwise.

1.3.2 Methods

1.3.2.1 Cell culture

The origin of human adrenergic neuroblastoma cells (SH-SY5Y, ATCC) is well-documented (Biedler et *al.*, 1978). Cells were maintained in a complete medium containing RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS) containing penicillin (100 U/ml) and streptomycin (100 U/ml). The medium was replaced every third day. They were grown in 100 mm^2 culture dishes or 25 cm^2 flasks from Corning (NY, USA). Cells were maintained at 37°C in a saturating humidity atmosphere containing 95% air and 5% CO_2.

1.3.2.2 Cell treatment

tRA was dissolved in absolute ethanol to a concentration of 10 mM and further diluted to a final concentration of 1μM in complete medium. Control cells were treated with an equivalent concentration of ethanol. Cells were plated in Falcon dishes and were treated with 100 ng of NGF (2.5S) per ml and/or 0.3 μM aphidicolin and/or 0.5 mM 8-Br-cAMP and/or 1 μM tRA. Fresh medium containing aphidicolin and NGF was added

every 2 days. For long term differentiation, SH-SY5Y cells were seeded at $1x10^5$ cells per 100 mm^2 and were treated for 2 weeks with 0.3 μM aphidicolin, 0.5 mM 8-Br-cAMP and 100 ng of NGF (2.5S) per ml as described above, followed by fresh medium containing NGF alone for up to 6 weeks. Alterations in morphology and cytotoxicity were assessed microscopically. Increased number and length of neurite-like processes and pseudoganglia formation were used as markers for neuroblastic or neuronal differentiation, and cell floating as evidence of cytotoxicity. The cells were microphotographed and the percent of differentiated cells as well as the length of neurites quantified using Image Pro software.

1.3.2.3 Estimation of cell population

SH-SY5Y were seeded in six well plates at a density of $1x10^4$ cells per well (1 cm^2); 24 h later cells were treated with the various differentiation induces as reported in the figure legends. At given times following exposure to the differentiation inducers, cells were rinsed with PBS, trypsinized, harvested and counted by using the trypan blue dye exclusion assay with a hematocytometer. The average count of three wells was used to obtain the growth curves.

1.3.2.4 Immunofluorescence microscopy

The following antibodies were used: anti-N-Myc monoclonal antibody (Santa Cruz), human anti-neurone-specific enolase (NSE) rabbit polyclonal antibody, anti-neurofilament 200 (NF200) and anti-neurofilament 68 (NF68) rabbit polyclonal

antibodies (Chemicon), human anti-TrkA, anti-TrkB and anti-TrkC goat polyclonal antibodies (Santa Cruz), and human anti-p53 goat polyclonal antibody (Santa Cruz).

For immunofluorescence microscopy, cells were rinsed twice with phosphate buffered saline (PBS), fixed at room temperature for 20 min with 4 % paraformaldehyde in PBS and then post-fixed with methanol (-20°C) for 10 min. The cells were then blocked at room temperature for 30 min with 0.1 % bovine serum albumin (BSA) in PBS. The fixed cells were incubated at room temperature for 1 h with the primary antibody at the following dilutions: anti-N-myc: 1/100, anti-TrkA: 1/200, anti-p53: 1/100, anti-NSE: 1/40, anti-NF200 and anti-NF68: 1/100. After washing twice with PBS, the cells were incubated with the corresponding secondary antibodies conjugated to rhodamine or fluorescein fluorochromes for 1 hour at a 1/100 dilution in PBS. Cells were washed with PBS to eliminate the excess of secondary IgG, mounted with Vectorshield mounting medium (Vector laboratories) and observed with a NIKON microscope set with epifluorescence and the corresponding fluorochrome filters. Microphotographs were taken using a 35mm Kodak camera.

1.3.2.5 Flow cytometry

Cells were harvested by treatment with trypsin (0.05%) and ethylenediaminetetracetic acid (EDTA) (0.02%), washed, resuspended as single cell suspensions, fixed as described above, treated with PBS containing 2% BSA and then exposed from 1 hour to overnight at 4°C to the corresponding primary antibody (anti-N-myc, anti-TrkA, anti-NSE, anti-NF200, anti-NF68, anti-H-ras). After three washes in PBS, the cells were exposed to the corresponding secondary antibodies (Jackson

laboratories) at a 1/100 dilution in PBS containing 2% BSA and 0.02% sodium azide. The analysis was performed using on a Coulter Elite flow cytometer. Statistical error on a 10,000 cell analysis amounts to 0.8%. All the experiments were repeated at least three times in duplicate.

1.3.2.6 Immunoblotting and immunoprecipitation

Subconfluent cultures in 100 mm^2 tissue culture plastic dishes were washed once with PBS containing 0.5 mM sodium orthovanadate, lysed by addition of 0.6 ml of RIPA buffer (PBS, 1 % NP40, 0.5% sodium deoxycholate, 0.1% SDS) with freshly added protease inhibitors (0.57 mM phenylmethylsulfonyl fluoride (PMSF), 1 U/ml aprotonin, 1 U/ml leupeptin, 1 mM sodium orthovanadate) on ice, and passed several times through a 21-gauge needle. The cell lysate was incubated for 30 min on ice. After adding 0.57 mM of fresh PMSF, insoluble material was removed by centrifugation at 4°C for 20 min at 10,000 g. Protein concentration was determined by the Bradford method using a protein assay kit (Bio-Rad Laboratories, Richmond, CA). One hundred micrograms of protein were mixed with an equal volume of electrophoresis sample buffer (250 mM Tris-HCL, pH 6.7; 20% glycerol; 10% β-mercaptoethanol; 6% SDS; 0.04% bromophenol blue, boiled for 90 s, and separated by SDS-PAGE using 10% acrylamide gels. After electrophoresis, proteins were transferred to nitocellulose membranes (Bio-Rad Laboratories, Richmond, CA). Blots were incubated with monoclonal antibodies, which recognised the relevant antigen. Bound antibody was detected with the use of an alkaline phosphatase conjugated secondary antibody and colorimetric detection.

For immunoprecipitation, subconfluent cells were washed once with PBS containing 0.5 mM sodium orthovanadate, incubated in 1 ml of lysis buffer (50 mM Hepes, pH 7.5; 1% Triton X-100; 50 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA; 0.5 mM sodium orthovanadate and 0.57 mM PMSF) on ice for 10 min and passed several times through a 21 gauge needle. Cellular debris was removed by centrifugation at 4°C for 10 min at 10,000 g. Protein concentration was determined as described above, and 1 ml of lysate was incubated with 1µg of anti-phosphotyrosine antibody (Sigma) for 1 hr at 4°C. The resulting immunocomplex was collected by precipitation with protein A-agarose, washed four times with RIPA buffer and mixed with an equal volume of electrophoresis sample buffer. After boiling for 3 min, protein samples were normalised to the same quantity (100µg protein) and were separated by SDS-PAGE 10% gels, transferred to nitrocellulose membranes, and probed with relevant antibodies.

1.3.2.7 Determination of the concentration-dependent death profile

SH-SY5Y cells were plated in 96-well dishes, after a 24 hr period, they were treated with PKC inhibitors (staurosporine, H7, Go6976 and Bis) at the following concentrations 100 nM, 1 µM, 160 nM and 2 µM respectively. The treatment was performed in order to have eight wells per experimental condition. Following 24 h, 48 h, 72 h and 7 days incubation periods, cells were counted using Trypan Blue dye exclusion.

1.3.2.8 Chromatin staining with Hoechst 33258

Control or treated cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, exposed to 1 μg/ml Hoechst 33258 in PBS for 30 min at room temperature, washed three times with PBS and, finally, mounted. The cell preparations were examined under UV illumination with an Olympus microscope equipped with epifluorescence and photographed using a 35mm camera.

1.3.2.9 Chromatin staining with TUNEL reagent

SH-SY5Y cells were seeded on polylysine coated coverslips ($1x10^4$ cells /well) in RPMI with 10 % FBS and grown for 24 h. Culture medium was then aspirated and replaced with fresh medium containing differentiating agents (0.5 mM 8-Br-cAMP, 100 ng/ml NGF) that was supplemented with 0.3 μM aphidicolin. Once terminal differentiation was reached (approximately 21 days with treatment every 3 days), the cells were rinsed with PBS and fresh medium was added supplemented with an anti-NGF antibody. Every 15 min, a slide was rinsed with PBS and fixed for 30 min at room temperature with a 4 % paraformaldehyde solution. The cells were then rinsed twice with PBS and measurement of DNA fragmentation by the terminal-deoxynucleotide (TnT)-mediated dUTP-biotin nick end labelling assay (TUNEL) was carried out using the *in situ* cell death detection kit POD (Boehring Mannheim, Germany) according to the supplier instructions. Briefly, residues of dioxigenin-nucleotides are added to the 3'-OH end of DNA fragments by terminal deoxynucleotidyltransferase, and the anti-

digoxigenin antibody fluoroscein conjugate is used to stain the apoptotic DNA fragments.

1.3.2.10 Statistical Analysis

Cell growth and flow cytometry data were analysed using one-way ANOVA followed by Tukey's post-hoc tests using MyAnova software (Carleton University). $P < 0.05$ was considered statistically significant.

1.4. Results

1.4.1 Induction of differentiation of SH-SY5Y cells: optimisation of cell culture and treatment conditions

In order to optimise cell culture conditions and treatments to obtain terminally differentiated SH-SY5Y cells, known differentiation inducers including: all trans retinoic acid (tRA), 8-Br-cAMP, NGF and aphidicolin (AP; a specific and reversible inhibitor of DNA polymerases α and δ), were tested for their ability to induce morphological differentiation and to limit growth. These treatments were also combined in some instances to verify whether their effects were additive or synergistic in inducing differentiation. tRA, 8-Br-cAMP, AP and NGF were first tested separately and in combination as follow: 8-Br-cAMP + tRA, NGF + AP and NGF + 8-Br-cAMP + AP for their ability to induce neurite extension in SH-SY5Y (Figs. 1.1, 1.2 and 1.2a). At the morphological level, the cAMP analogue-treated SH-SY5Y cells (Fig.1.1.B) showed longer neurite extension when compared to NGF alone, tRA alone or NGF + AP, during the first two weeks of progress into differentiation (Fig. 1.1). SH-SY5Y cells did not respond to NGF treatment alone at the morphological level (Fig. 1.1.D) while NGF + AP treated cells showed the beginning of neurite emission (Fig. 1.1.E). tRA treated cells showed small neurite extension (Fig. 1.1.C) but when tRA was combined with 8-Br-cAMP, cells emitted longer neurites for up to 2 weeks of treatment (Fig. 1.1.F). After 2 weeks, cells started to float and die (data not shown). However when SH-SY5Y cells were exposed to the combination treatment of 8-Br-cAMP + NGF + AP, the treatment

Figure 1.1 Effects of differentiation inducers on SH-SY5Y cell neurite emission

Phase-contrast photographs of SH-SY5Y cells exposed for 2 weeks to the following treatments; Control (A), 8-Br-cAMP (B), tRA (C), NGF (D), Aphidicolin + NGF (E) and 8-Br-cAMP + tRA (F). SH-SY5Y cells were plated at $1x10^5$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Cells were treated every 2 days, starting 24 h after plating, with 100 ng of NGF (2.5S) per ml and/or 0.3 μM aphidicolin and/or 0.5mM 8-Br-cAMP and/or 1 μM tRA for 2 weeks. Cells were viewed under phase contrast with a Nikon microscope and photographed with Kodak ektachrome 400 film.

resulted in very long neurite extensions and cells were able to maintain their differentiation and have longer neuritic extensions when compared to other treatments (Fig. 1.2.a) such as 8-Br-cAMP + tRA (Fig. 1.1.F). Furthermore, 8-Br-cAMP + NGF + AP differentiated cells were stable for up to 6 weeks if supplemented with NGF alone and did not show any signs of cell death (Fig. 1.2.C). SH-SY5Y cells were exposed 8-Br-cAMP + NGF + AP for 3 weeks (Fig. 1.2.B) and then were supplemented with NGF alone for an additional 3 weeks. At this point of differentiation, NGF seems to maintain SH-SY5Y at their fully differentiated state without inducing cell growth in contrast to the increased cell numbers seen in undifferentiated cells (Fig. 1.1.D) and (Fig. 1.3). In addition, fully differentiated SH-SY5Y cells change in appearance to cells with small cell bodies and longer neurites that are bipolar or multipolar, which is a characteristic of neuronal phenotypes. They also revealed axonal connections indicating synaptic formation (Fig. 1.2.B, C). It will be described later how prolonged 8-Br-cAMP + NGF + AP exposure renders cells survival–dependent on NGF. Under these conditions, the cultures remain stable for up to 6 weeks if supplemented with NGF alone (Fig. 1.2.C) and will die of apoptosis upon NGF withdrawal (Chapter1, section 4.3).

1.4.2 Characterisation of differentiating SH-SY5Y cells

1.4.2.1 Cell proliferation

The kinetics of SH-SY5Y cell growth was measured after induction of differentiation. Cells were enumerated every 24 h for each treatment for up to 2 weeks. Relative to control cultures, all the treatments tested induced a decrease in SH-SY5Y

Figure 1.2 Terminal differentiation of SH-SY5Y cells

Phase-contrast photographs of terminally differentiated SH-SY5Y cells.
Cells were maintained in aphidicolin + NGF + 8-Br-cAMP for 2 weeks (A) or 3 weeks in (B). In (C) SH-SY5Y cells were exposed to aphidicolin + NGF + 8-Br-cAMP for 3 weeks, then cells were maintained in a medium containing NGF alone for another 3 weeks. SH-SY5Y cells were plated at $1x10^5$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Cells were treated every 2 days, starting 24 h after plating, with a combination of 100 ng per ml NGF, 0.3 μM aphidicolin and 0.5 mM 8-Br-cAMP for 3 weeks, followed by a sequential treatment every 2 days with 100 ng/ml NGF for another 3 weeks. Cells were viewed under phase contrast with a Nikon microscope and photographed with Kodak ektachrome 400 film.

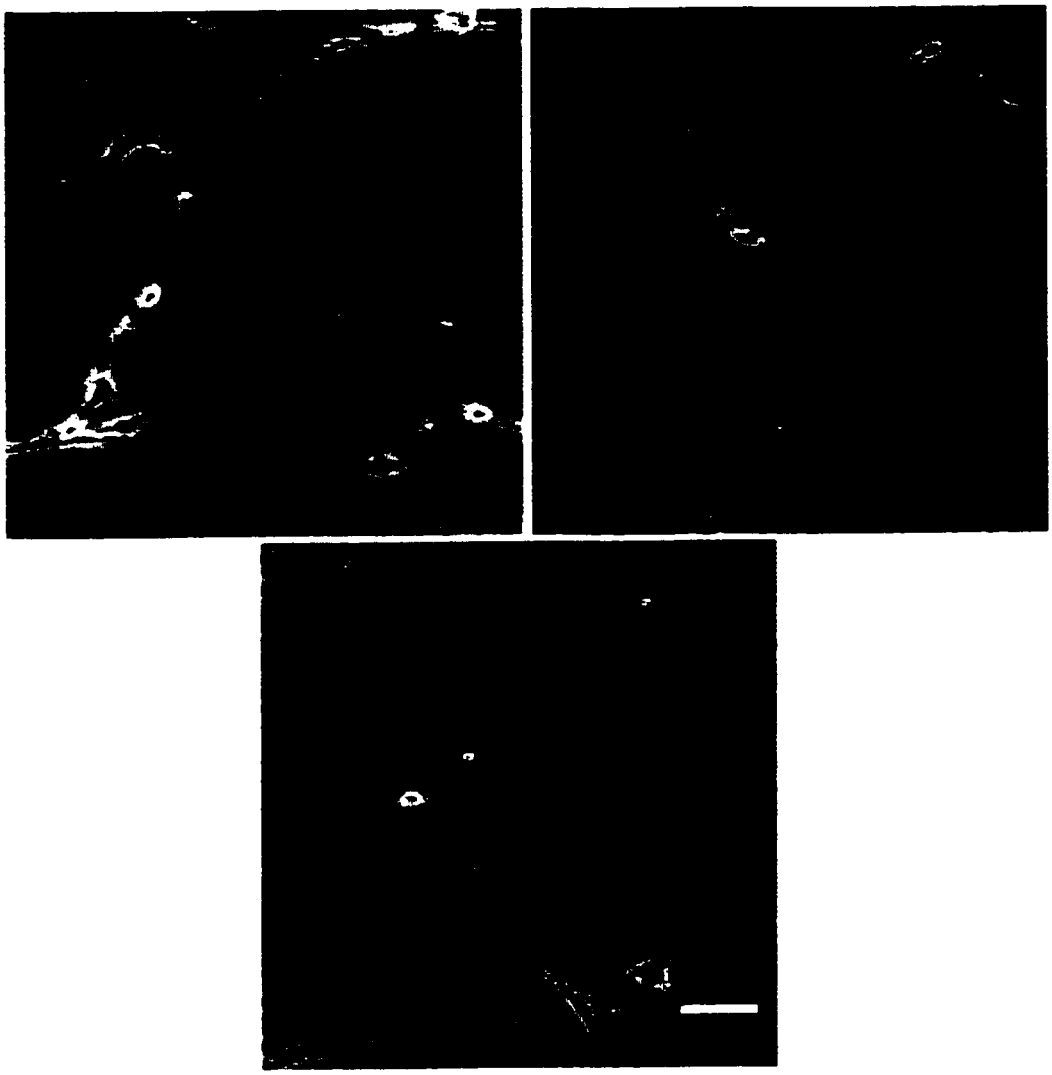
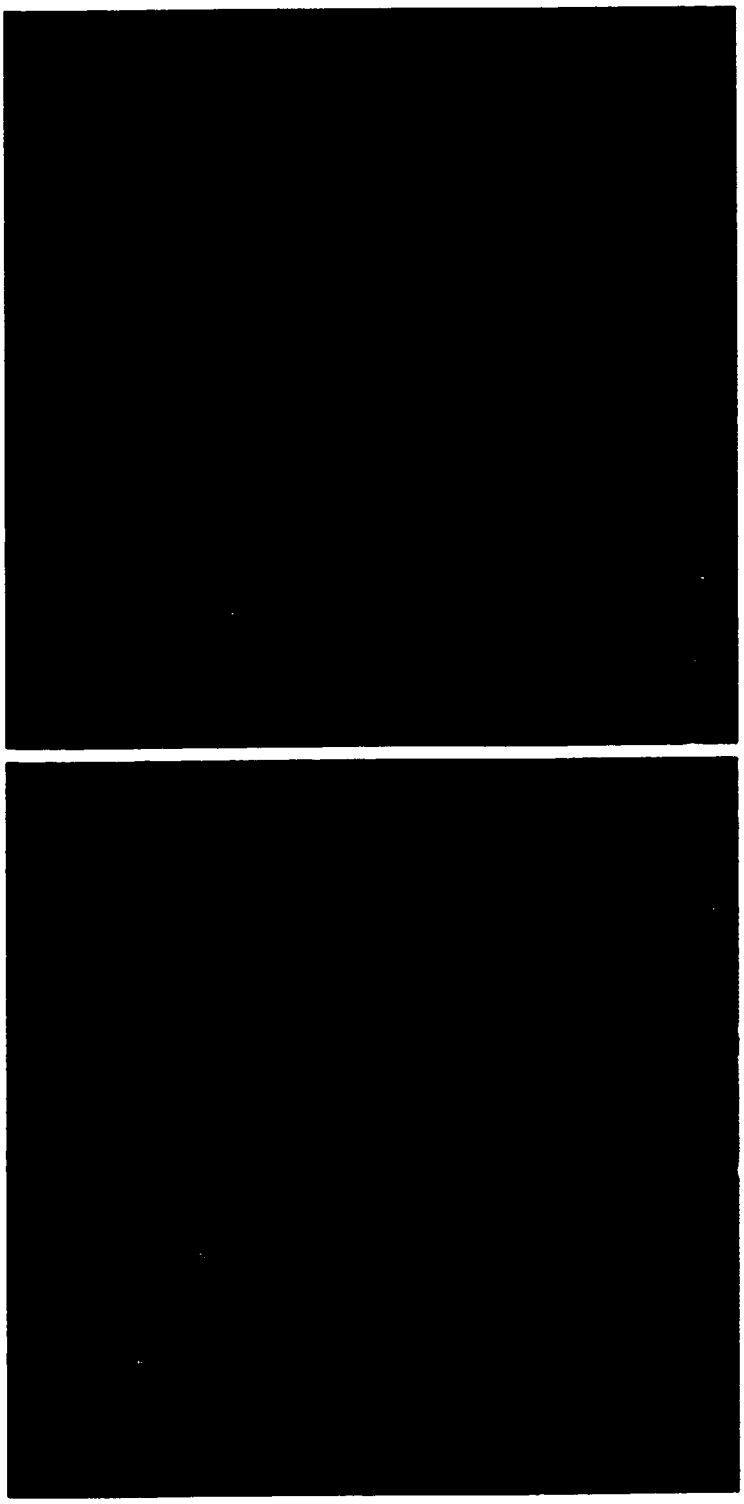


Figure 1.2a Terminal differentiation of SH-SY5Y cells

Phase-contrast photographs of terminally differentiated SH-SY5Y cells stained with giemza stain. Cells were maintained in aphidicolin + NGF + 8-Br-cAMP for 3 weeks. Then cells were maintained in a medium containing NGF alone for another 6 weeks (A and B). SH-SY5Y cells were plated at $1x10^5$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Cells were treated every 2 days, starting 24 h after plating, with a combination of 100 ng per ml NGF, 0.3μM aphidicolin and 0.5 mM 8-Br-cAMP for 3 weeks, followed by a sequential treatment every 2 days with 100 ng/ml NGF for another 3 weeks. Cells were fixed and stained with giemza stain as described in methods. Cells were viewed under phase contrast with a Nikon microscope and photographed with Kodak ektachrome 400 film.



cell proliferation except for cells treated with NGF individually (Fig. 1.3). When cells were supplemented with aphidicolin every second day, cell proliferation stopped. By itself, treatment with aphidicolin did not induce neurite extension and the cells resumed proliferation following removal of the compound (data not shown). tRA inhibited cell proliferation by approximately 60% but induced only slight neurite extension. When 8-Br-cAMP was combined with RA, we noticed an almost 70 % decrease in cell growth; these cells could be maintained for up to 2 weeks, after which the differentiated cells died (data not shown). Treatment with 8-Br-cAMP and NGF in the presence of AP was the most efficient differentiation-inducing treatment in decreasing cell growth. We noticed the lowest level of cell proliferation after 2 weeks of treatment and cells then continued to emit neurites if maintained in NGF alone.

SH-SY5Y cell proliferation was also assessed by flow cytometry using the proliferating cell nuclear antigen (PCNA), as a proliferation marker (Fig. 1.4). The level of PCNA expression was assayed using either the differentiation agents alone or in combination as described above. Treatment with 8-Br-cAMP alone, RA alone, RA + 8-Br-cAMP for 2 weeks and NGF + 8-Br-cAMP + AP for 2 weeks, all caused a decrease in cell proliferation as demonstrated by a decrease in the expression of PCNA. SH-SY5Y cells exposed to NGF alone had a similar growth rate to the untreated cells (Fig. 1.4). Since no dead cells were noticed in the treated cultures this suggests a decrease in cell cycling rather than cell loss.

Figure 1.3 Effects of differentiation inducers on SH-SY5Y cell proliferation

The growth curve of SH-SY5Y cells exposed for 2 weeks to the following treatments: aphidicolin, 8-Br-cAMP (cAMP), tRA (RA), NGF, 8-Br-cAMP + tRA (cAMP + RA) and aphidicolin + NGF + 8- Br-cAMP (NGF + cAMP). Controls were untreated. SH-SY5Y cells were plated at $1x10^4$ cells per well in six well plates in RPMI medium supplemented with 10% FBS. Cells were treated every 2 days, starting 24 h after plating, with 100 ng of NGF (2.5S) per ml and/or 0.3 μM Aphidicolin and/or 0.5 mM 8-Br-cAMP and/or 1 μM tRA. Cells were counted every 24 h for 2 weeks using Trypan blue dye exclusion and a hemotocytometer. The average of three wells was used for each set of experiments. Each experiment was repeated in triplicate. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).

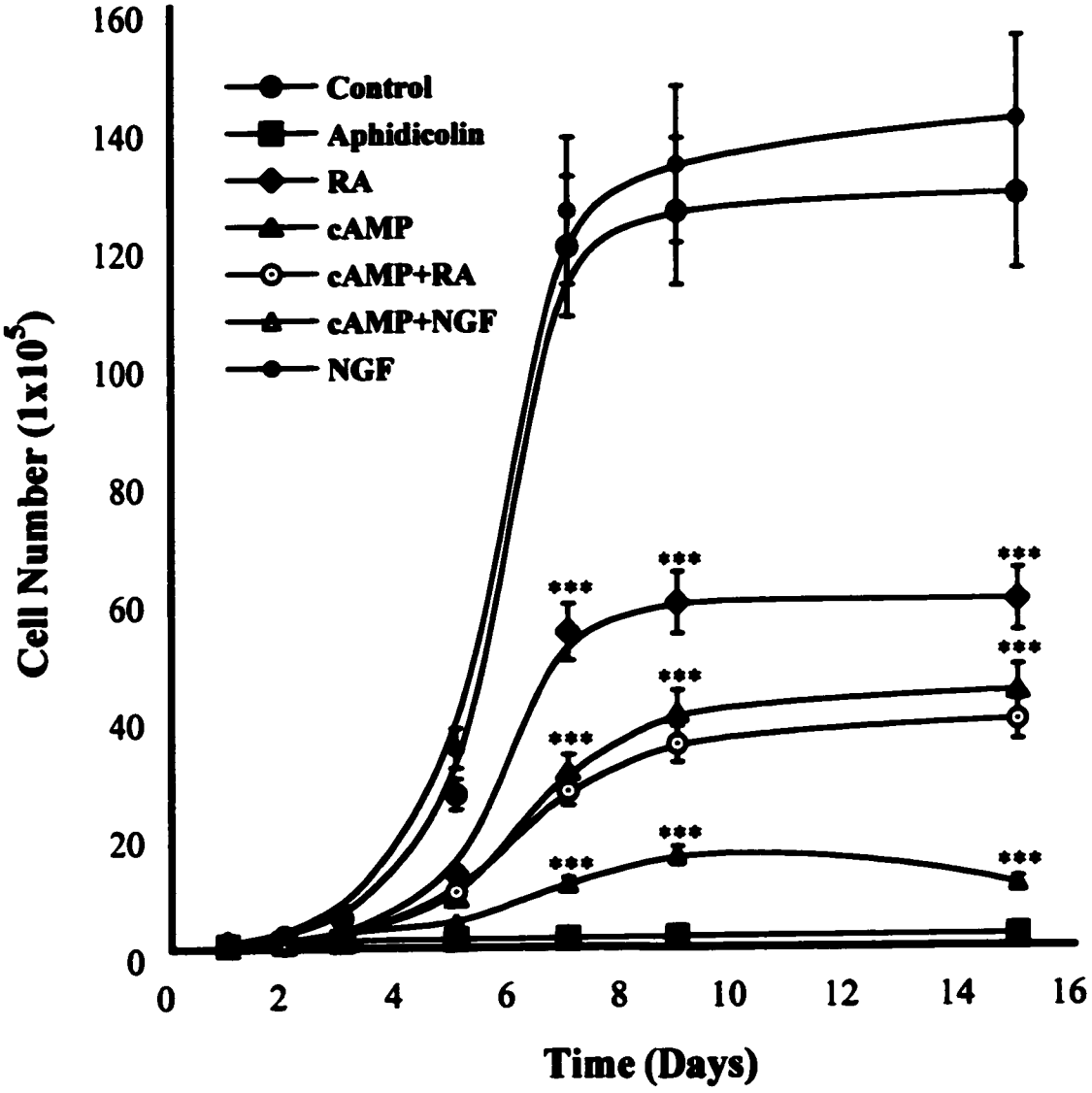
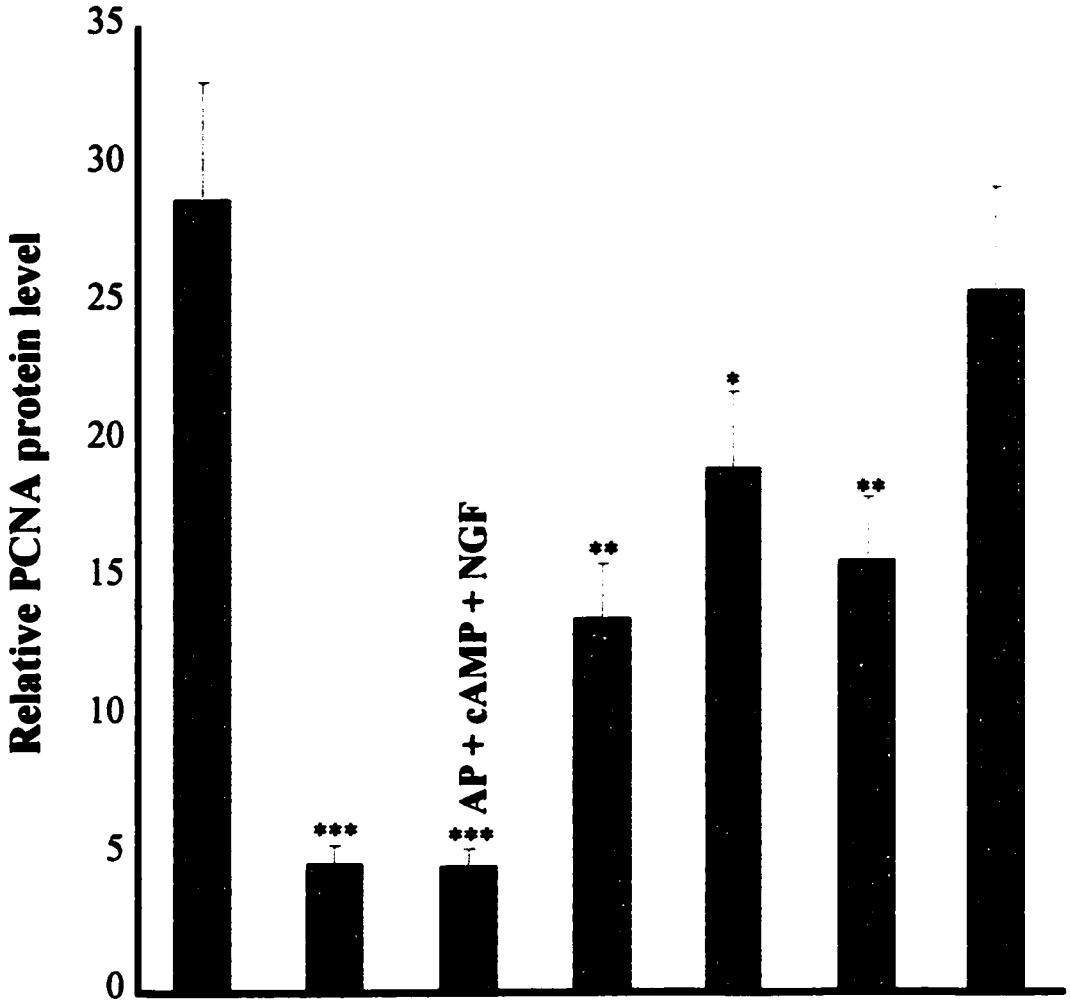

Control
Aphidicolin
RA
cAMP
cAMP+RA
cAMP+NGF
NGF
Cell Number (1x10^5)
Time (Days)
0
20
40
60
80
100
120
140
160
0
2
4
6
8
10
12
14
16
***

Figure 1.4 Comparison of PCNA expression in response to differentiation inducers in SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24h later cells were treated for 2 weeks with 100 ng of NGF (2.5S) per ml and/or 0.3 μM aphidicolin and/or 0.5 mM 8-Br-cAMP and/or 1 μM tRA every second day. Cells were harvested by treatment with 0.05% trypsin and 0.02% EDTA, washed 3 times with PBS and fixed as described in Methods. Fixed cells were incubated in PBS containing 2% BSA and immunolabelled for 1 hour to overnight with PCNA monoclonal antibody at a dilution of 1:100 in PBS containing 0.2% BSA at 4^0C then washed 3 times with PBS and incubated with an FITC conjugated secondary antibody at a dilution of 1:100 for one hour in PBS. Immunostained cells were washed and analysed using a Coulter Elite flow cytometer. The measured fluorescence intensity provides a relative estimation of the protein level per cell. Statistical error on 10,000 cell analysed amounts to 0.8%. All the experiments were repeated at least three times in duplicate. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).


Relative PCNA protein level
35
30
25
20
15
10
5
0
***
***
AP + cAMP + NGF
**
*
**

1.4.2.2 Expression of differentiation markers

Although a number of neuroblastoma cell lines appear to differentiate into neurones morphologically, they do not always express gene products consistent with neuronal differentiation. To determine the level of differentiation of SH-SY5Y cells, we used immunohistochemistry and flow cytometry to assay for markers known to be associated with the differentiation of neural crest cells. In order to investigate whether the effect of the inducers on morphological differentiation was accompanied by an effect on molecular differentiation, we measured the expression of differentiation markers known to be expressed in neurones. We chose the neurofilament proteins neurone-specific enolase (NSE), tyrosine hydroxylase (TH), and choline acetyl-transferase (Chat). Neurofilament proteins are neurone-specific proteins belonging to the class IV gene family of intermediate filament proteins and are the major constituents of the neuronal cytoskeleton (Pant & Veeranna, 1995). They are assembled as 10 nm neurofilaments concentrated principally in the axons. Together with the intermediate filaments, NFs form a cross-linked, three dimensional filamentous network that supports the nerve cell, helps to define its shape and size, and organises and anchors the constituents within the axonal cytoplasm. Mature mammalian neurones contain three NF protein subunits varying in molecular mass: low, NF-L (NF68, 68 kDa); medium, NF-M (NF150, 150 kDa); and high, NF-H (NF200, 200 kDa). The head and tail domains of NF-L, NF-M, and NF-H contain the principal phosphorylation sites, with each domain playing a different role in regulating the synthesis, assembly, and transport of NFs from perikaryon to the axon in the mature neurone. The N-terminal head domain of each NF subunit can be phosphorylated by second messenger-dependent protein kinases such as

PKA, PKC, and Ca^{2+}/CaM kinases. NSE specifies neuro-secretory cells. NSE antibody reacts selectively with the gamma subunits of NSE of neuronal origin and is also specific to peripheral endocrine tumours. After 2 weeks of treatment with differentiation agents, FACS analysis showed a two-fold increase in the expression of NF200 (Fig. 1.5), and a substantial elevation from essentially undetectable levels in the expression of NF68 (Fig. 1.6) and NSE (Fig. 1.7). Differentiated cells stained positively for all these neuronal proteins. In contrast, undifferentiated cells were negatively or weakly stained for NF200, NF68 and NSE. NSE expression increased with progress into differentiation (Fig. 1.7), it was also shown to increase with cell confluency in cultures of proliferating cells (results not shown). These results indicate that the expression of neuronal proteins paralleled the development of neurites. Taken together, our results show that neuronal cytoskeletal intermediate filaments were present in all differentiation stages of SH-SY5Y cells and evolved with progression into differentiation. The combination treatment of 8-Br-cAMP, NGF and aphidicolin induced the highest level of neuronal marker expression.

The expression of NF68, NF200 and TH as well as their localisation were analysed by immunohistochemistry in SH-SYSY cells treated for 3 weeks with 8-Br-cAMP + NGF + AP to confirm the results obtained by FACS analysis. We noticed a significant increase in fluorescence intensity for NF68 immunostaining when compared to the untreated cells (Fig. 1.8). Undifferentiated SH-SY5Y cells seem to express more NF200 (Fig. 1.9.A) than NF68 (Fig. 1.8.A) proteins. When cells were treated for 3 weeks with the combination treatment 8-Br-cAMP + NGF + AP, they still express both neurofilament proteins, but the NF68 was expressed at a higher level than control cells (Fig. 1.8) if compared to NF200 (Fig. 1.9).

Figure 1.5 Comparison of the neuronal protein NF200 expression in response to differentiation inducers in SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated for 2 weeks with 100 ng of NGF (2.5S) per ml and/or 0.3 μM aphidicolin and/or 0.5 mM 8-Br-cAMP and/or 1 μM tRA every 2 days. Cells were harvested by treatment with 0.05% trypsin and 0.02% EDTA, washed 3 times with PBS and fixed as described in Methods Fixed cells were incubated in PBS containing 2% BSA and immunolabelled for 1 hour to overnight with a rabbit anti-NF200 polyclonal antibody at a dilution of 1:200 in PBS containing 0.2% BSA at 4^0C then washed 3 times with PBS and incubated with a goat anti rabbit secondary antibody conjugated to FITC at a dilution of 1:100 for one hour in PBS. Immunostained cells were washed and analysed using a Coulter Elite flow cytometer. The measured fluorescence intensity provides a relative estimation of protein level per cell. All the experiments were repeated at least three times in duplicate. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).

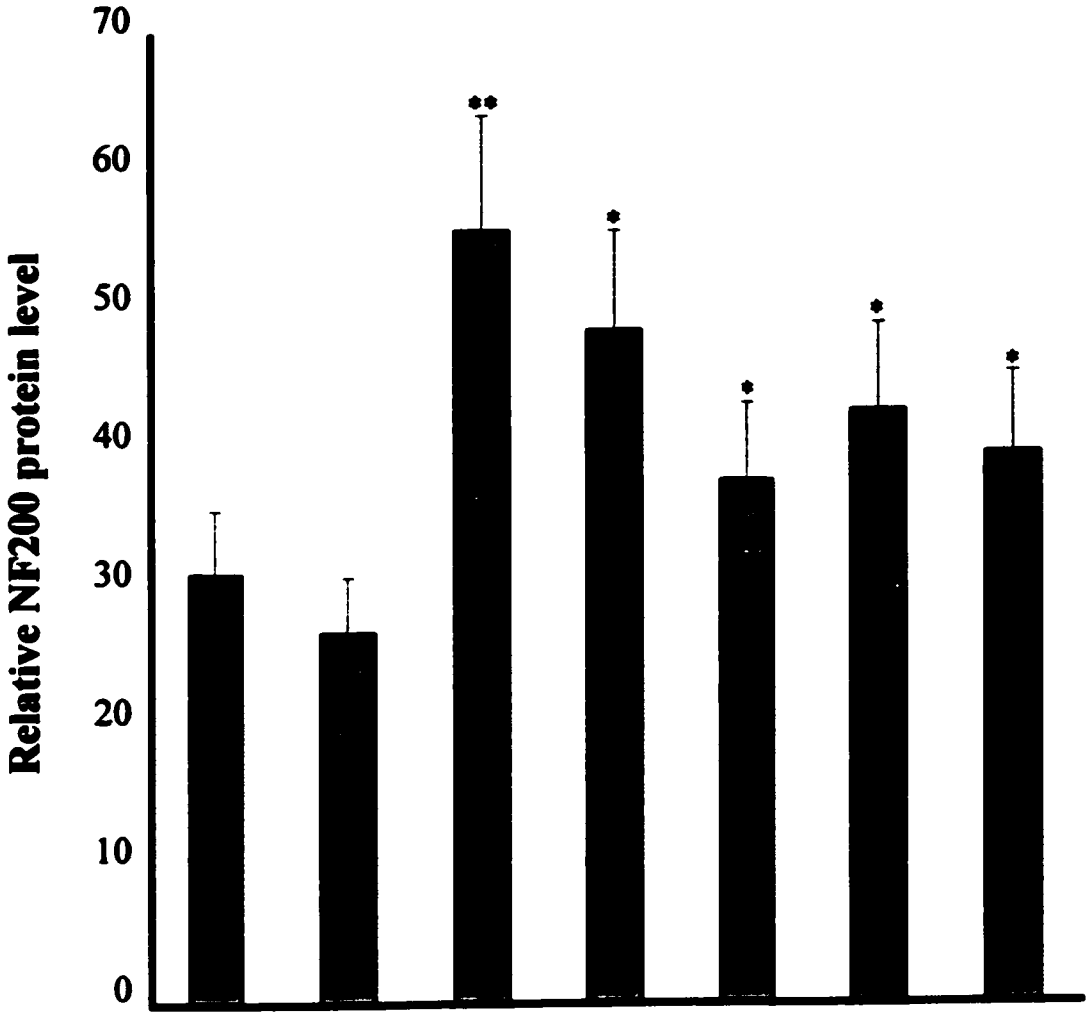

Relative NF200 protein level
70
60
50
40
30
20
10
0
**
*
*
*
*

Figure 1.6 Comparison of NF68 expression in response to differentiation inducers in SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated for 2 weeks with 100 ng of NGF (2.5S) per ml and/or 0.3 μM aphidicolin and/or 0.5 mM 8-Br-cAMP and/or 1 μM tRA every 2 days. Cells were harvested by treatment with 0.05% trypsin and 0.02% EDTA, washed 3 times with PBS and fixed as described in Methods. Fixed cells were incubated in PBS containing 2% BSA and immunolabelled for 1 hour to overnight with a mouse anti-NF68 antibody at a dilution of 1:250 in PBS containing 0.2% BSA at 4^0C then washed 3 times with PBS and incubated with a rabbit anti-mouse antibody conjugated to rhodamine at a dilution of 1:100 for one hour in PBS. Immunostained cells were washed and analysed using a Coulter Elite flow cytometer. The measured fluorescence intensity provides a relative estimation of protein level per cell. Statistical error on 10,000 cell analysed amounts to 0.8%. All the experiments were repeated at least three times in duplicate. ($* = p < 0.05$, $** = p < 0.001$, $*** = p < 0.0001$).

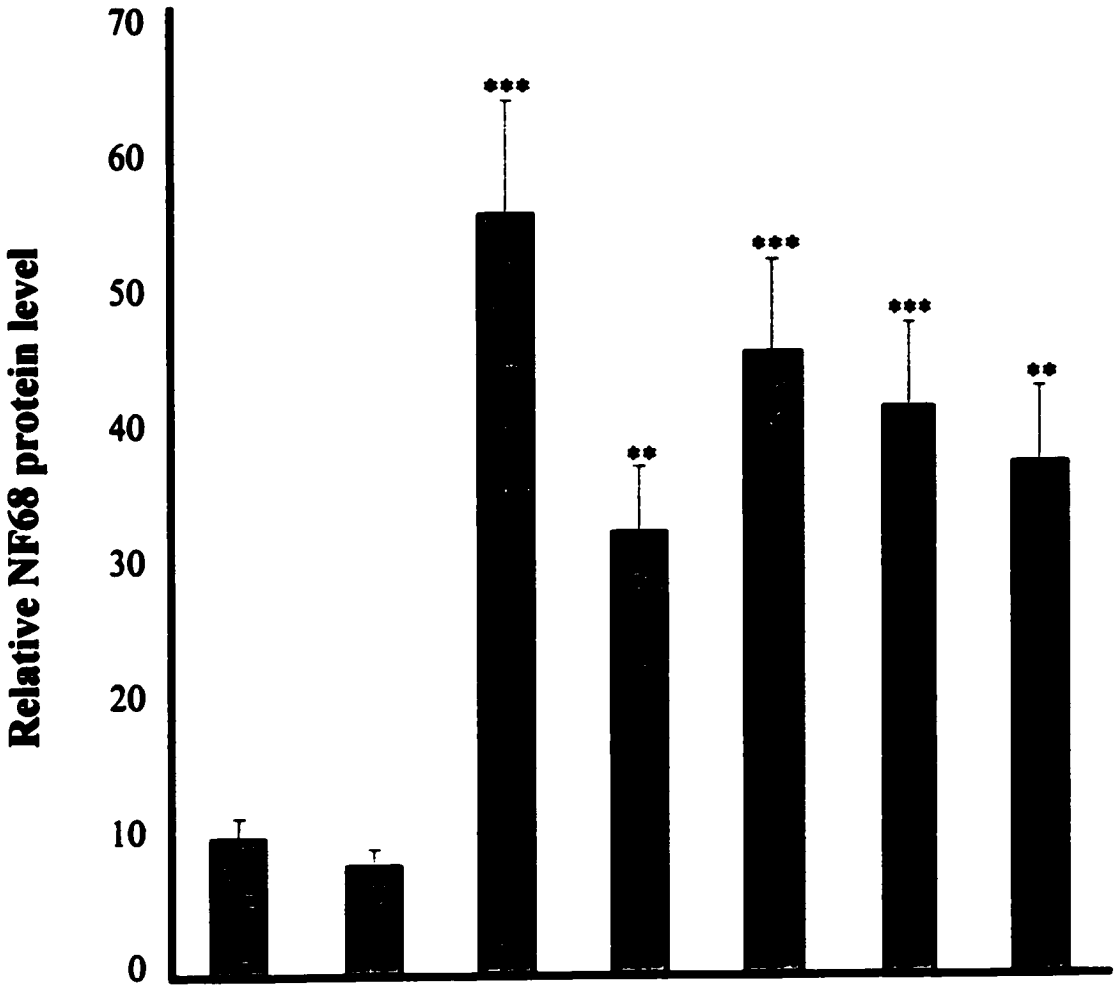

Relative NF68 protein level
70
60
50
40
30
20
10
0
***
**
***
***
**

Figure 1.7 Comparison of NSE expression in response to differentiation inducers in SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated for 2 weeks with 100 ng of NGF (2.5S) per ml and/or 0.3 μM aphidicolin and/or 0.5 mM 8-Br-cAMP and/or 1 μM tRA every 2 days. Cells were harvested by treatment with 0.05% trypsin and 0.02% EDTA, washed 3 times with PBS and fixed as described in Methods. Fixed cells were incubated in PBS containing 2% BSA and immunolabelled for 1 hour to overnight with a mouse anti-NSE antibody at a dilution of 1:40 in PBS containing 0.2% BSA at 4^0C then washed 3 times with PBS and incubated with a goat anti-mouse antibody conjugated to rhodamine at a dilution of 1:100 for one hour in PBS. Immunostained cells were washed and analysed using a Coulter Elite flow cytometer. The measured fluorescence intensity provides a relative estimation of protein level per cell. Statistical error on 10,000 cell analysed amounts to 0.8%. All the experiments were repeated at least three times in duplicate. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).

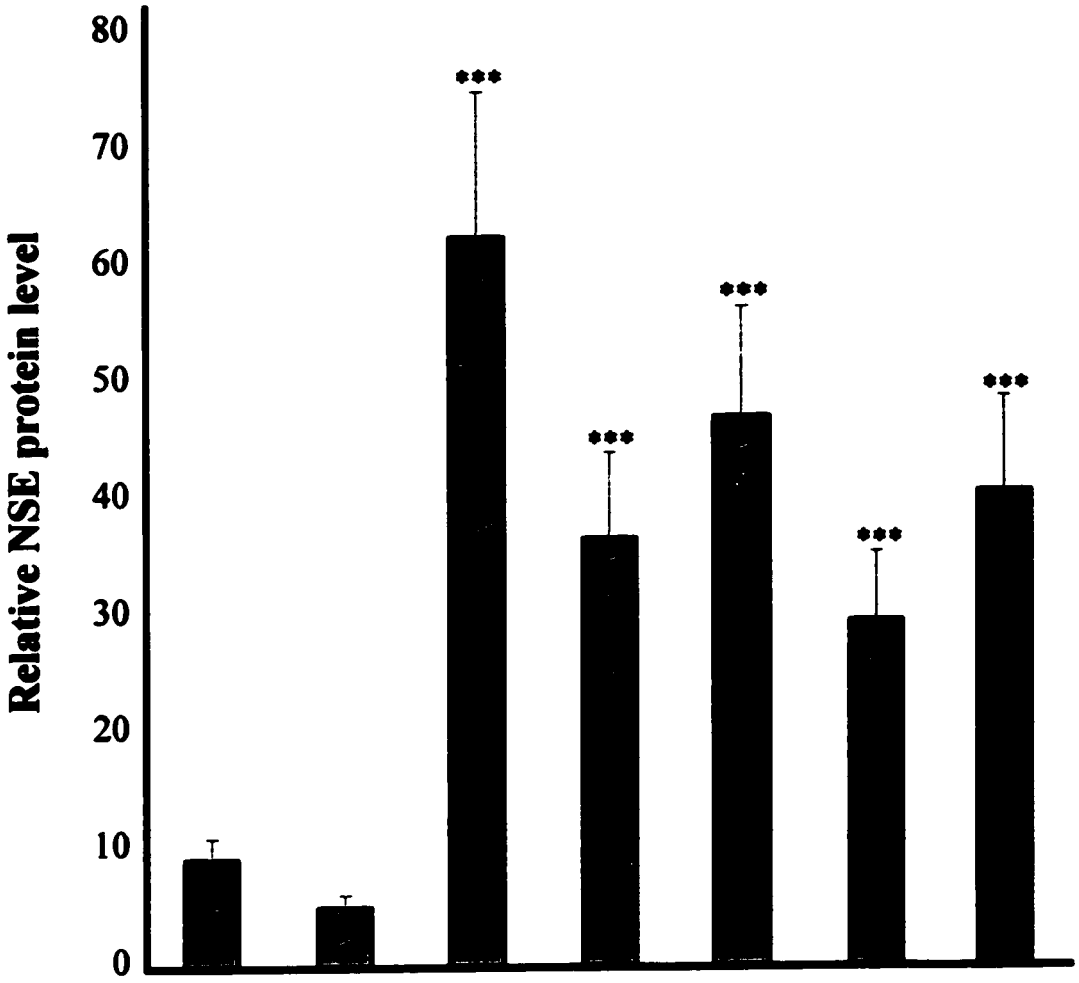

Relative NSE protein level
80
70
60
50
40
30
20
10
0
***
***
***
***
***

Figure 1.8 Expression of neuronal protein NF68 in undifferentiated and differentiated SH-SY5Y cells

Immunocytochemical analysis of NF68 in undifferentiated (A) and differentiated SH-SY5Y cells (B). SH-SY5Y cells were plated at $1x10^5$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Cells were treated every 2 days, starting 24 h after plating, with a combination of 100 ng per ml NGF, 0.3 μM aphidicolin and 0.5 mM 8-Br-cAMP for 3 weeks, followed by a sequential treatment every 2 days with 100 ng/ml NGF for another 3 weeks. Cells were washed, fixed and incubated with NF68-specific antibody and antigen-antibody was detected with rhodamine-conjugated secondary antibody as described in Methods. Cells were viewed under epifluorescence with a Nikon microscope equipped with the appropriate filter and photographed with Kodak ektachrome 400 film.

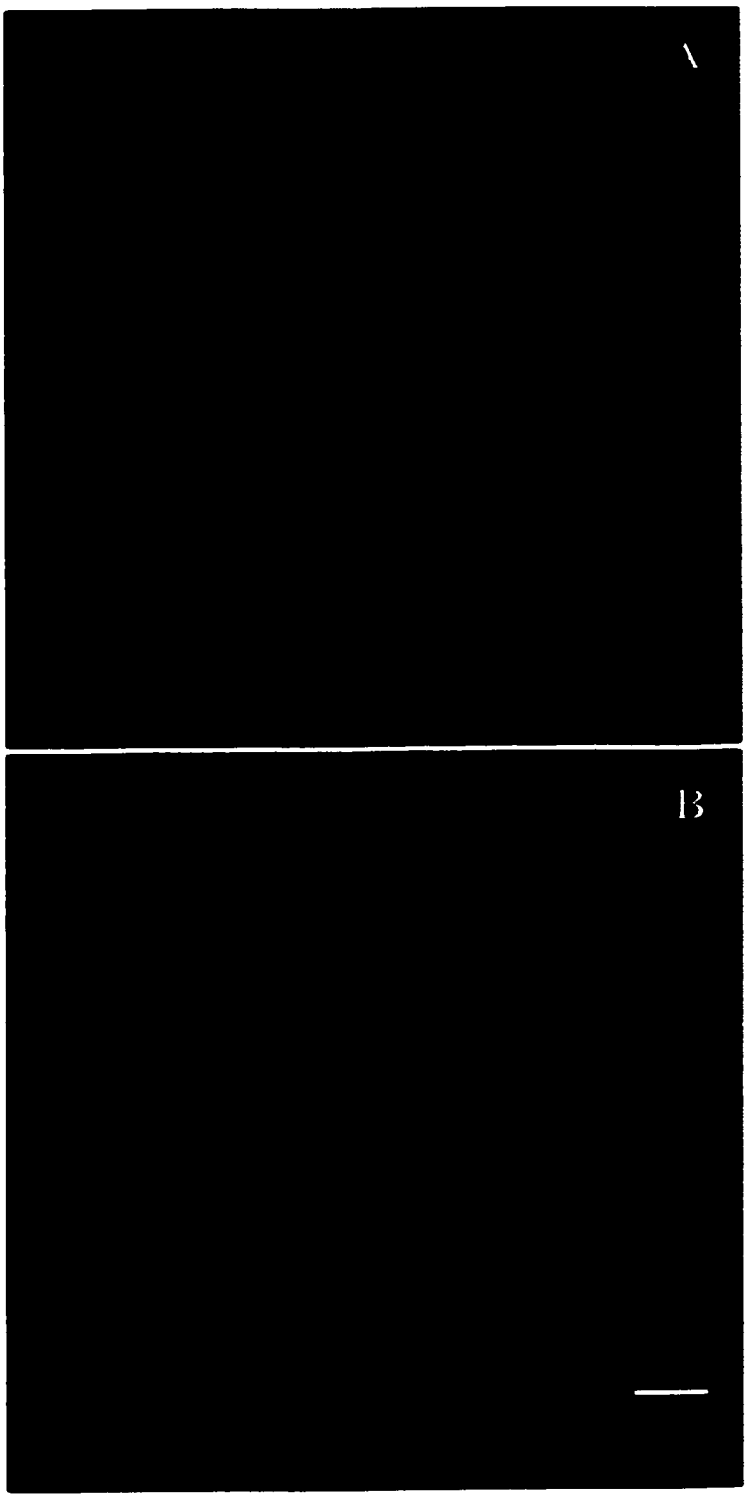
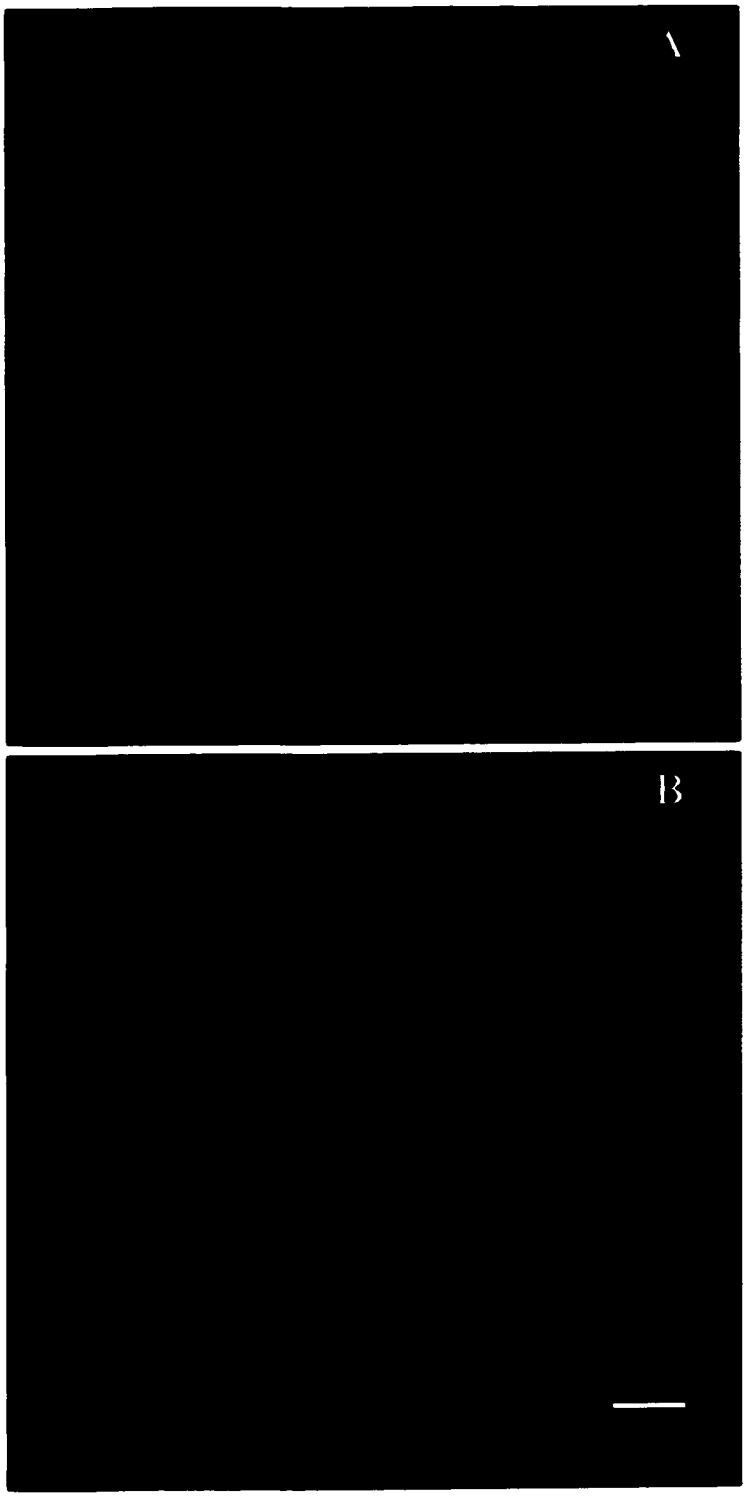

A
B

Figure 1.9 Expression of neuronal protein NF200 in undifferentiated and differentiated SH-SY5Y cells

Immunocytochemical analysis of NF200 in undifferentiated (A) and differentiated SH-SY5Y cells (B). SH-SY5Y cells were plated at $1x10^5$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Cells were treated every 2 days, starting 24 h after plating, with a combination of 100 ng per ml NGF, 0.3μM aphidicolin and 0.5 mM 8-Br-cAMP for 3 weeks, followed by a sequential treatment every 2 days with 100 ng/ml NGF for another 3 weeks. Cells were washed, fixed and incubated with NF200-specific antibody and antigen-antibody detected with rhodamine-conjugated secondary antibody as described in Methods. Cells were viewed under a fluorescence microscopy with a Nikon microscope equipped with the appropriate filter and photographed with Kodak ektachrome 400 film.


A
B

Both choline acetyl transferase and tyrosine hydroxylase are indicative of cholinergic and adrenergic neuronal function respectively. Tyrosine hydroxylase is the rate-limiting enzyme critical to catecholamine biosynthesis. In order to measure the neuronal function of differentiated SH-SY5Y cells, the expression of both TH and Chat were measured by FACS analysis for all differentiation inducers as described above. The 8-Br-cAMP alone or in combination with tRA or NGF seems to induce an increase in TH expression (Fig. 1.10). 8-Br-cAMP and NGF or tRA in the presence of AP seem to have a additive effect on TH expression while RA alone and NGF alone did not have any significant effect on TH modulation ($P>0.05$). In contrast, 8-Br-cAMP treatment alone seemed to significantly up-regulate TH protein expression ($P<0.05$) (Fig. 1.10). TH immunostaining using a TH monoclonal antibody showed an increase in TH protein expression in 8-Br-cAMP + NGF + AP differentiated SH-SY5Y cells when compared to control cells (Fig. 1.11). Chat protein expression was also compared in differentiated and undifferentiated SH-SY5Y cells using FACS. Results showed that while tRA did not seem to have any significant effect on Chat, NGF as well as 8-Br-cAMP increased the expression of Chat ($P<0.001$) (Fig. 1.12).

1.4.2.3 Effect of differentiation on markers of neuroblastoma tumours

1.4.2.3.1 Effect of differentiation on N-myc expression

The N-myc oncogene is a marker for poor prognosis in NB. Experiments were designed to determine if the induction of both morphological and biochemical differentiation impacts on neuroblastoma tumourogenicity as assessed by the high expression of the N-myc oncogene. In contrast with some NB cell lines, the N-myc

Figure 1.10 Comparison of tyrosine hydroxylase expression in response to differentiation inducers in SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated for 2 weeks with 100 ng of NGF (2.5S) per ml and/or 0.3 μM aphidicolin and/or 0.5 mM 8-Br-cAMP and/or 1 μM tRA every 2 days. Cells were harvested by treatment with 0.05% trypsin and 0.02% EDTA, washed 3 times with PBS and fixed as described in Methods. Fixed cells were incubated in PBS containing 2% BSA and immunolabelled for 1 hour to overnight with a rabbit anti-TH antibody at a dilution of 1:400 in PBS containing 0.2% BSA at 4^0C then washed 3 times with PBS and incubated with a goat anti-rabbit antibody conjugated to fluorescein at a dilution of 1:100 for one hour in PBS. Immunostained cells were washed and analysed using on a Coulter Elite flow cytometer. The measured fluorescence intensity provides a relative estimation of protein level per cell. Statistical error on 10,000 cell analysed amounts to 0.8%. All the experiments were repeated at least three times in duplicate. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).

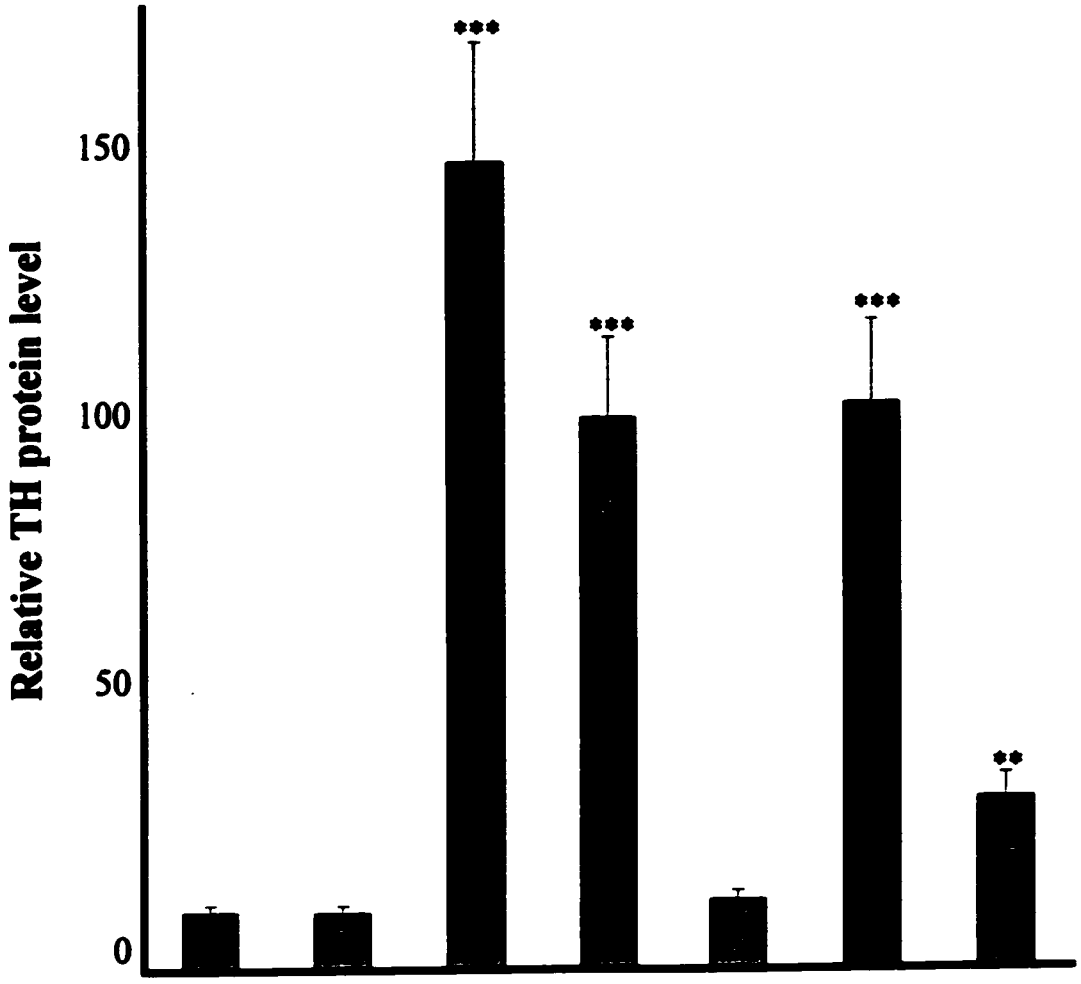

Relative TH protein level
150
100
50
0
***
***
***
**

Figure 1.11 Expression of neuronal protein tyrosine hydroxylase (TH) in undifferentiated and differentiated SH-SY5Y cells

Immunocytochemical analysis of TH expression in undifferentiated (A) and differentiated SH-SY5Y cells (B). SH-SY5Y cells were plated at $1x10^5$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Cells were treated every 2 days, starting 24h after plating, with a combination of 100 ng per ml NGF, 0.3 μM aphidicolin and 0.5 mM 8-Br-cAMP for 2 weeks. Cells were washed, fixed and incubated with TH-specific antibody and antigen-antibody detected with fluorescein-conjugated secondary antibody as described in Methods. Cells were viewed under fluorescence with a Nikon microscope equipped with the appropriate filter and photographed with Kodak ektachrome 400 film. Magnification is x200.

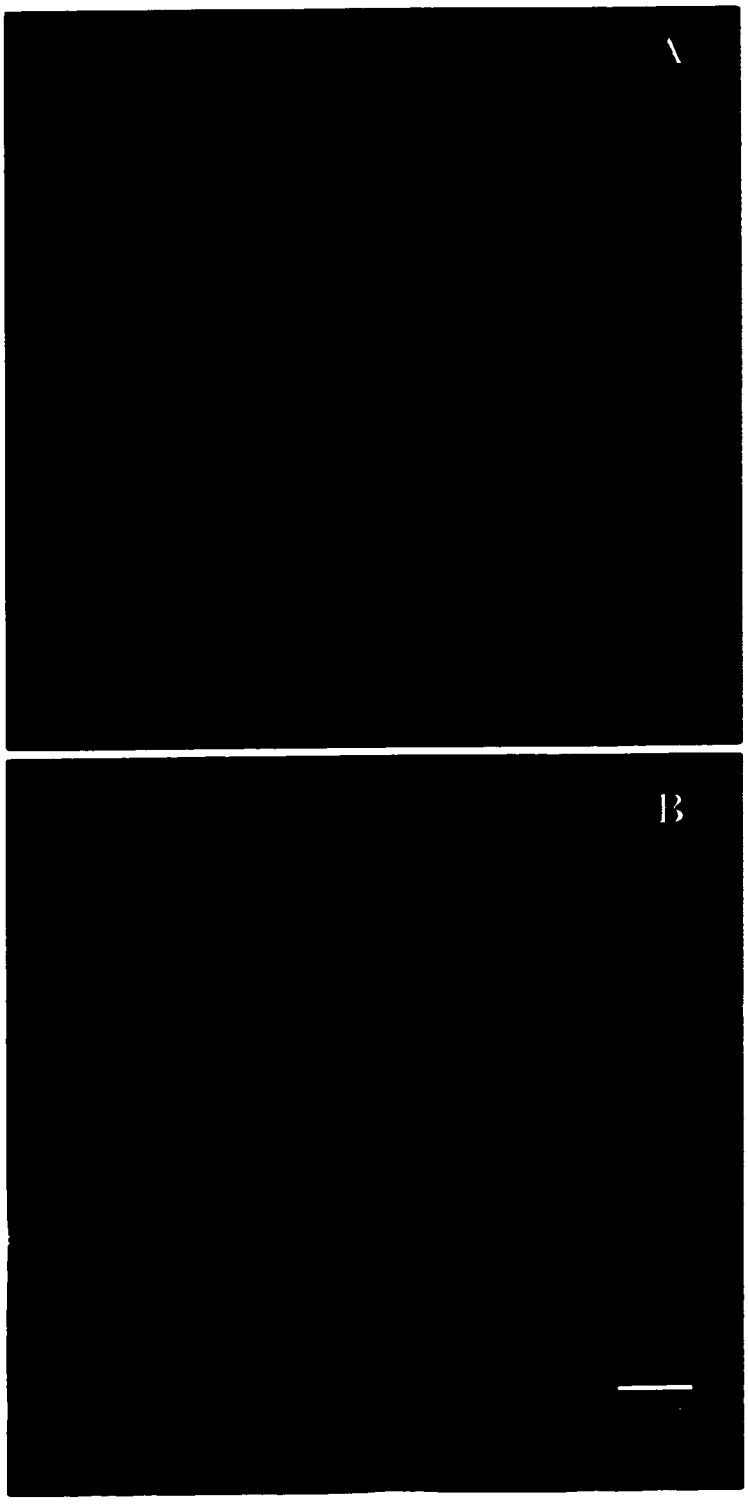
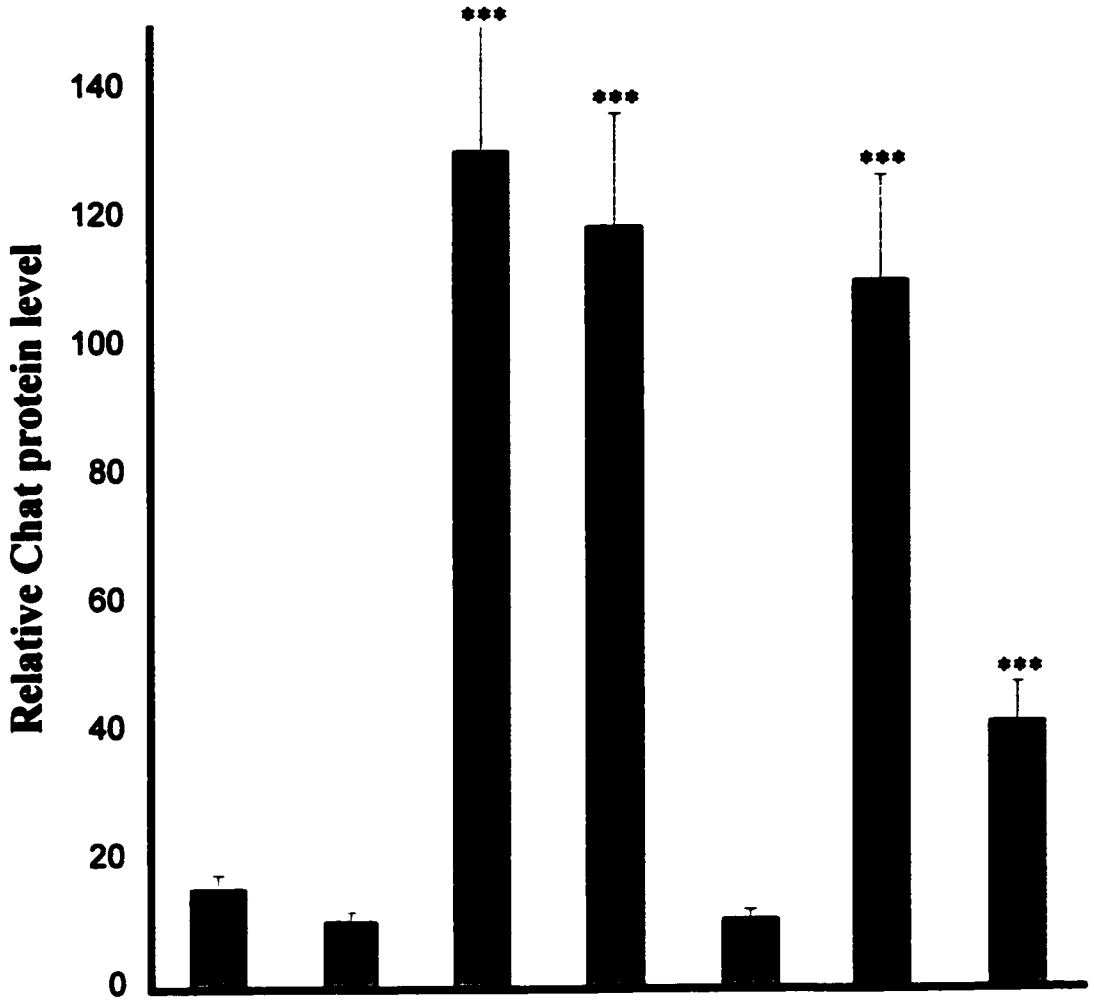

A
B

Figure 1.12 Comparison of choline acetyl transferase expression in response to differentiation inducers in SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Starting 24 h later cells were treated for 2 weeks with 100 ng of NGF (2.5S) per ml and/or 0.3 μM aphidicolin and/or 0.5 mM 8-Br-cAMP and/or 1 μM tRA every 2 days. Cells were harvested by treatment with 0.05% trypsin and 0.02% EDTA, washed 3 times with PBS and fixed as described in Methods. Fixed cells were incubated in PBS containing 2% BSA and immunolabelled for 1 hour to overnight with a mouse anti-Chat monoclonal antibody at a dilution at of 1: 500 in PBS containing 0.2% BSA at 4^0C then washed 3 times with PBS and incubated with a rabbit anti-mouse antibody conjugated to fluorescein at a dilution of 1:100 for one hour in PBS. Immunostained cells were washed and analysed using a Coulter Elite flow cytometer. The measured fluorescence intensity provides a relative estimation of protein level per cell. Statistical error on 10,000 cell analysed amounts to 0.8%. All the experiments were repeated at least three times in duplicate. ($* = p < 0.05$, $** = p < 0.001$, $*** = p < 0.0001$).


Relative Chat protein level
140
120
100
80
60
40
20
0
***
***
***
***

oncogene is not amplified in the SH-SY5Y NB cell line, but undifferentiated SH-SY5Y cells express a high amount of N-myc protein as measured by FACS (Fig. 1.13). Fig. 1.13 shows that when SH-SY5Y cells were induced to differentiate using the cAMP analogue 8-Br-cAMP alone or in the presence of RA or NGF, N-myc oncogene expression decreased. Moreover, the combination treatment (8-Br-cAMP + NGF + AP) resulted in the lowest expression of N-myc protein when compared to the other treatments. This expression reached undetectable levels as the cells progressed towards differentiation and in terminally differentiated SH-SY5Y cells after 6 weeks of treatment with 8-Br-cAMP + NGF + AP (data not shown).

1.4.2.3.2. Effect of differentiation on H-ras p21 expression

The H-ras gene product is a member of the intracellular signal transducing G proteins with a molecular weight of 21 kDa. A large amount of H-ras p21 is found in normal rat brain (Sweetser *et al.*, 1997). The expression of H-ras p21 in rat neural cells progresses upward from the paravertebral ganglions to the brain, depending on the gestational age. The possibility of using p21 H-ras as a new factor for assessing NB prognosis has been proposed since the level of this protein is higher in patients with a good prognosis. The level of expression of H-ras protein was measured in differentiated and undifferentiated SH-SY5Y cells by FACS analysis. We found that H-ras protein is expressed at a lower level in undifferentiated SH-SY5Y cells than in differentiated cells. Upon treatment with differentiation inducers, we noticed an increase, albeit not always significant, in H-ras protein expression that was maintained for up to 2 weeks (Fig. 1.14). The combination treatment consisting of 8-Br-cAMP + NGF in the presence of

Figure 1.13 Comparison of N-myc protein expression in response to differentiation inducers in SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Starting 24 h later cells were treated for 2 weeks with 100 ng of NGF (2.5S) per ml and/or 0.3 μM aphidicolin and/or 0.5 mM 8-Br-cAMP and/or 1 μM tRA every 2 days. Cells were harvested by treatment with 0.05% trypsin and 0.02% EDTA, washed 3 times with PBS and fixed as described in Methods. Fixed cells were incubated in PBS containing 2% BSA and immunolabelled for 1 hour to overnight with a mouse anti-N-myc monoclonal antibody at a dilution at of 1:50 in PBS containing 0.2% BSA at 4^0C then washed 3 times with PBS and incubated with a rabbit anti-mouse antibody conjugated to fluorescein at a dilution of 1:100 for one hour in PBS. Immunostained cells were washed and analysed using on a Coulter Elite flow cytometer. The measured fluorescence intensity provides a relative estimation of protein level per cell. Statistical error on 10,000 cell analyses amounts to 0.8%. All the experiments were repeated at least three times in duplicate. ($* = p < 0.05$, $** = p < 0.001$, $*** = p < 0.0001$).

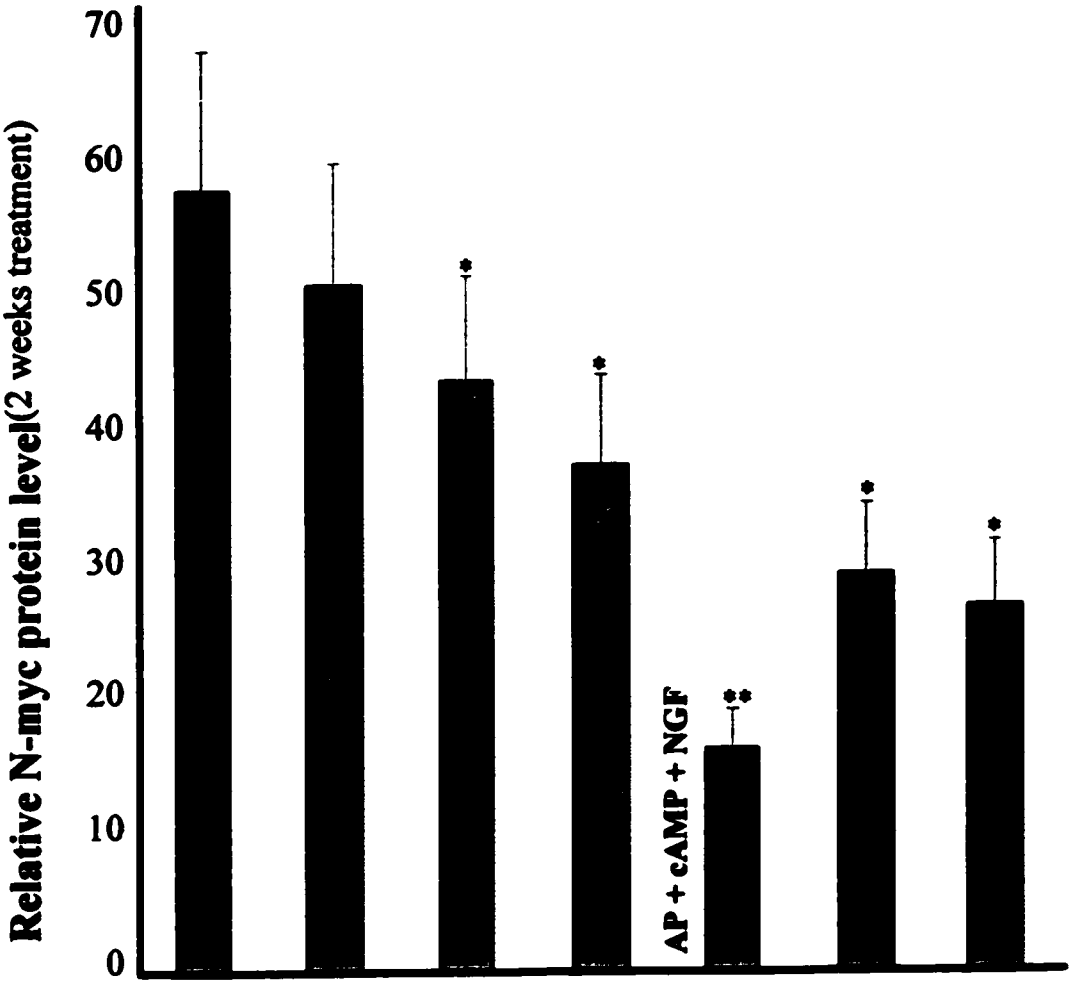
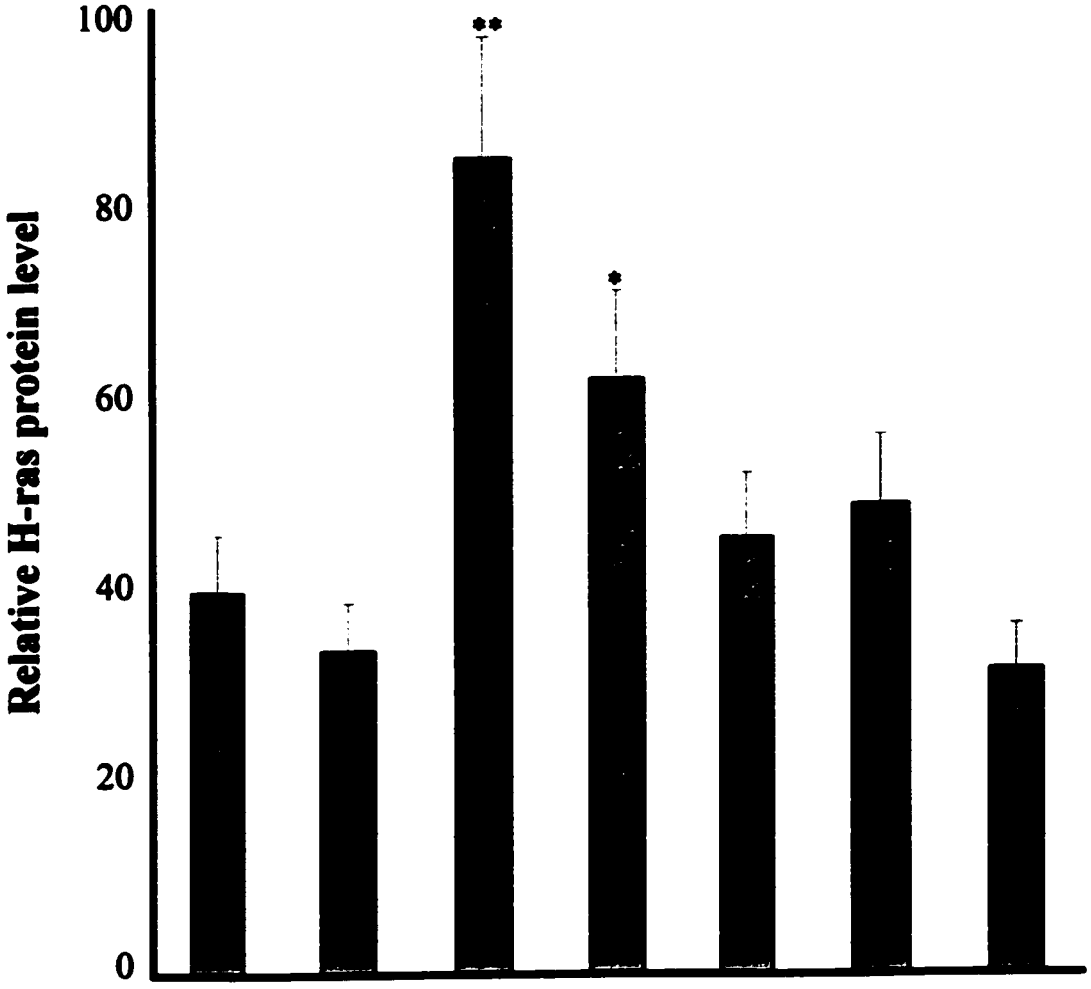

Relative N-myc protein level(2 weeks treatment)
70
60
50
40
30
20
10
0
*
*
*
AP + cAMP + NGF
**
*
*

Figure 1.14 Comparison of H-ras expression in response to differentiation inducers in SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Starting 24 h later cells were treated for 2 weeks with 100 ng of NGF (2.5S) per ml and/or 0.3 μM aphidicolin and/or 0.5 mM 8-Br-cAMP and/or 1 μM tRA every 2 days. Cells were harvested by treatment with 0.05% trypsin and 0.02% EDTA, washed 3 times with PBS and fixed as described in Methods. Fixed cells were incubated in PBS containing 2% BSA and immunolabelled for 1 hour to overnight with a rabbit anti-H-ras monoclonal antibody at a dilution at of 1: 50 in PBS containing 0.2% BSA at 4^0C then washed 3 times with PBS and incubated with a goat anti-mouse antibody conjugated to rhodamine at a dilution of 1:100 for one hour in PBS. Immunostained cells were washed and analysed using a Coulter Elite flow cytometer. The measured fluorescence intensity provides a relative estimation of protein level per cell. Statistical error on 10,000 cell analysed amounts to 0.8%. All the experiments were repeated at least three times in duplicate. ($* = p < 0.05$, $** = p < 0.001$, $*** = p < 0.0001$).


Relative H-ras protein level
100
80
60
40
20
0
**
*

aphidicolin induced the highest level of H-ras protein expression, which was significantly different from that of the controls (Fig. 1.14).

1.4.2.3.3 Effect of differentiation on TrkA, TrkC and gp75 expression and TrkA function

One of the markers presently used for NB prognosis is TrkA, the high affinity receptor of NGF (Brodeur *et al.*, 1997b). It is a tyrosine kinase, which is autophosphorylated upon binding its ligand NGF. TrkC is also a receptor tyrosine kinase that responds to NT3/4 growth factor (for review see Poo, 2001). gp75, in contrast to gp140 (TrkA), is a low affinity receptor for NGF. It is not a tyrosine kinase and it is reported to contain a death domain in its sequence, which may implicate it in apoptosis (for review see Sofroniew *et al.*, 2001). Both TrkA and TrkC protein expression have been reported to be markers for good prognosis in neuroblastomas (Nakagawara et al., 1998b; Poluha *et al.*, 1995). We first compared the expression of TrkA using differentiation inducers alone or in combination using FACS analysis as described (Fig. 1.15). We observed that only the combination treatment of 8-Br-cAMP + NGF + AP seems to induce a significant increase in the expression of TrkA ($P>0.05$). Furthermore, none of the others differentiation inducers including, NGF, 8-Br-cAMP, tRA alone or in combination with 8-Br-cAMP seems to have a significant effect on TrkA expression (Fig. 1.15). However, TrkA, TrkC and gp75 protein expression were also analysed by Western blot in terminally differentiated cells using monoclonal antibodies that detected only the non-phosphorylated forms (Fig. 1.16). The levels of both TrkA and TrkC increased following treatment with AP + NGF + 8-Br-cAMP for 3 weeks. Further differentiation of these cells (6 weeks) did not show any further enhancement of TrkA

expression while TrkC continued to increase (Fig. 1.16). Furthermore, gp75 protein expression showed a significant increase after 3 weeks followed by a decrease at the 6 weeks treatment. Since TrkA is a tyrosine kinase and it responds to its ligand NGF by autophosphorylation, we analysed differentiated SH-SY5Y cells by immunoblot analysis using an antibody to TrkA that detects both its phosphorylated and unphosphorylated forms. The effects of combination treatments AP + NGF and AP + NGF + 8-Br-cAMP (Fig. 1.17) were assessed. Undifferentiated SH-SY5Y cells express both TrkA (gp140) and gp75 proteins. AP + NGF treatment for 2 weeks seems to increase the expression of the phosphorylated form of TrkA (Fig. 1.17). Furthermore, 8-Br-cAMP when added with AP and NGF increased both the induction of TrkA expression and its phosphorylation after 2 and 3 weeks treatment. These combination treatments seem to enhance the level of gp75 when compared with the undifferentiated SH-SY5Y cells to its highest level after 2 weeks of treatment (Fig. 1.17).

1.4.3 Effects of NGF withdrawal on terminally differentiated SH-SY5Y cells

1.4.3.1 Cell morphology

SH-SY5Y cells were induced to terminal differentiation as described earlier using 8-Br-cAMP + NGF + AP for 3 weeks followed by treatment with NGF alone for up to 6 weeks. In order to confirm that SH-SY5Y cells were terminally differentiated, their dependency to NGF survival was tested. Terminally differentiated neurones become NGF-dependent for survival (for review see Sofroniew *et al.*, 2001). In one set

Figure 1.15 Comparison of the expression of the high affinity NGF receptor TrkA, in response to differentiation inducers in SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Starting 24 h later cells were treated for 2 weeks with 100 ng of NGF (2.5S) per ml and/or 0.3 μM aphidicolin and/or 0.5 mM 8-Br-cAMP and/or 1 μM tRA every 2 days. Cells were harvested by treatment with 0.05% trypsin and 0.02% EDTA, washed 3 times with PBS and fixed as described in Methods. Fixed cells were incubated in PBS containing 2% BSA and immunolabelled for 1 hour to overnight with a 1 μg/ml goat anti-TrkA polyclonal antibody in PBS containing 0.2% BSA at 4^0C, then washed 3 times with PBS and incubated with a rabbit anti-mouse antibody conjugated to fluorescein at a dilution of 1:100 for one hour in PBS. Immunostained cells were washed and analysed using a Coulter Elite flow cytometer. The measured fluorescence intensity provides a relative estimation of protein level per cell. Statistical error on 10,000 cell analysed amounts to 0.8%. All the experiments were repeated at least three times in duplicate. ($* = p < 0.05$, $** = p < 0.001$, $*** = p < 0.0001$).

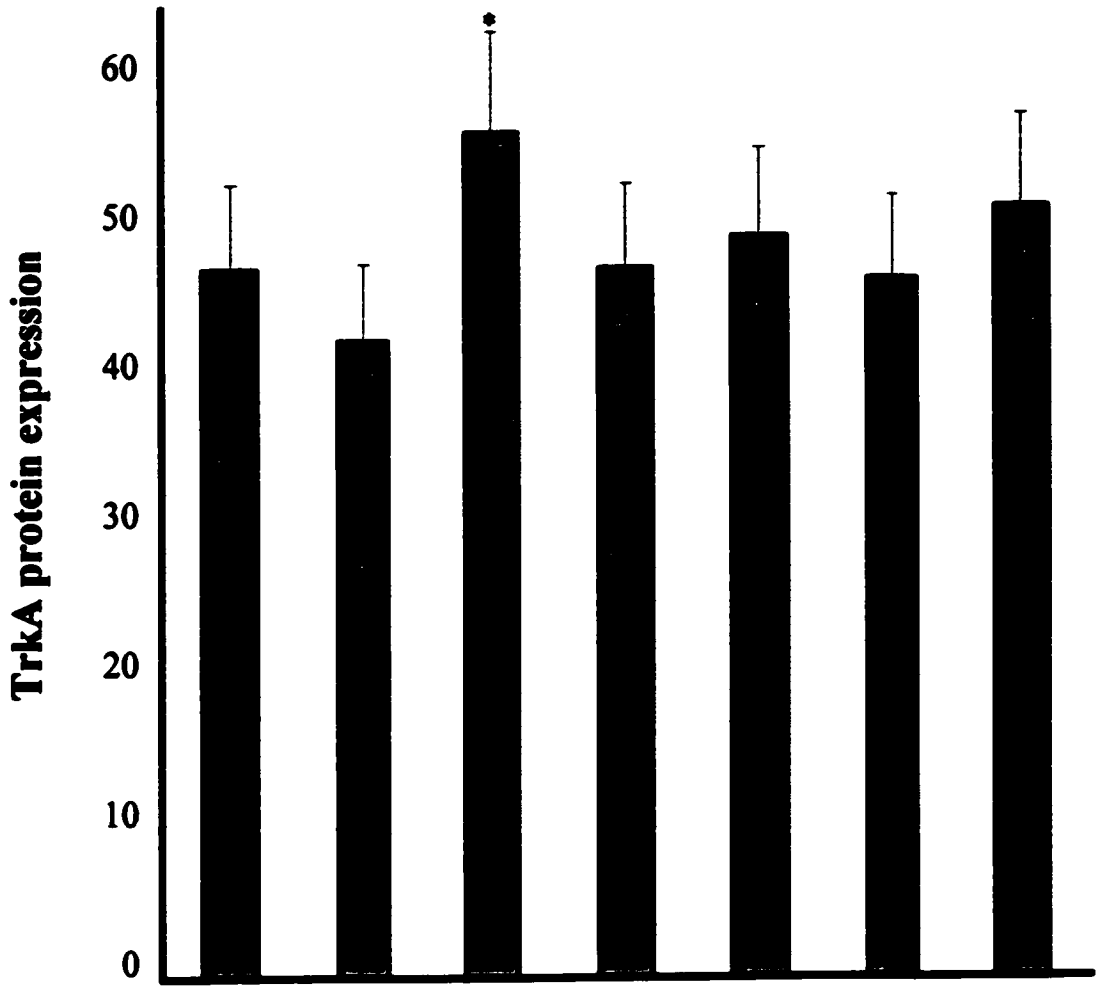
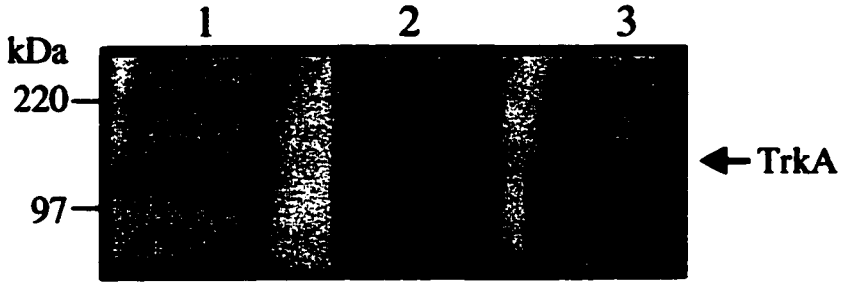

TrkA protein expression
60
50
40
30
20
10
0
*

Figure 1.16 Expression of TrkA, TrkC and gp75 in terminally differentiated SH-SY5Y cells

(A) Immunoblot analysis of TrkA in undifferentiated (1) and terminally differentiated SH-SY5Y cells for 3 weeks (2) and 6 weeks (3). (B) Immunoblot analysis of TrkC and gp75 in undifferentiated (1) and terminally differentiated SH-SY5Y cells for 3 weeks (2) and 6 weeks (3). SH-SY5Y cells were plated at $1x10^5$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Cells were treated every 2 days, starting 24 h after plating, with a combination of 100 ng per ml NGF, 0.3 μM aphidicolin and 0.5 mM 8-Br-cAMP for 3 weeks, followed by a sequential treatment every 2 days with 100 ng/ml NGF for another 3 weeks. Treated and untreated cells were homogenised, 100 μg protein per lane was separated by electrophoresis on 10% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with TrkA (A) and TrkC and gp75 (B) specific antibodies, then detected using alkaline phosphatase-conjugated secondary antibodies as described in Methods. The molecular weight is expressed as kilodalton (kDa).

A



B

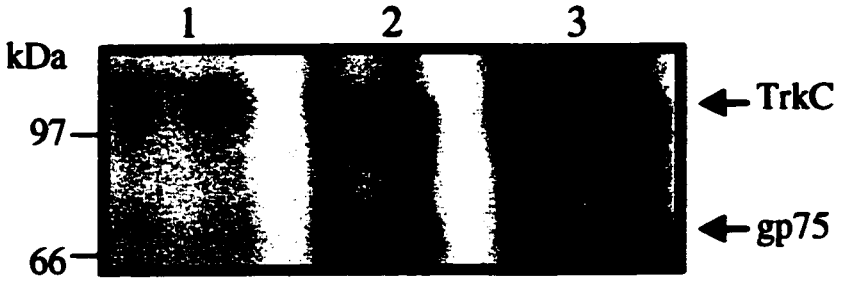


Figure 1.17 Expression of TrkA and gp75 in differentiating SH-SY5Y cells

Immunoblot analysis of TrkA in undifferentiated (1), AP + NGF (2) and AP + NGF + 8-Br-cAMP (3) treated cells for 2 weeks and 3 weeks (4). SH-SY5Y cells were plated at $1x10^5$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Cells were treated every 2 days, starting 24 h after plating, with the combination treatments using the following concentration: 100 ng per ml NGF, 0.3 μM aphidicolin; and 0.5 mM 8-Br-cAMP for 2 weeks. Treated and untreated cells were homogenised, 100 μg proteins per lane was separated by electrophoresis on 10% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with TrkA and gp75 specific antibodies, then detected using alkaline phosphatase-conjugated secondary antibodies as described in Methods. The molecular weight is expressed as kilodalton (kDa).

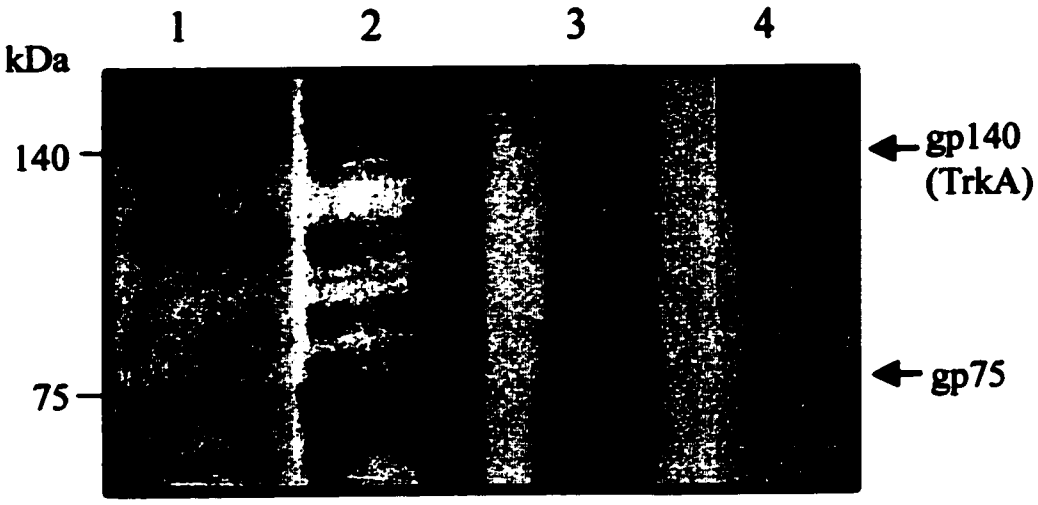
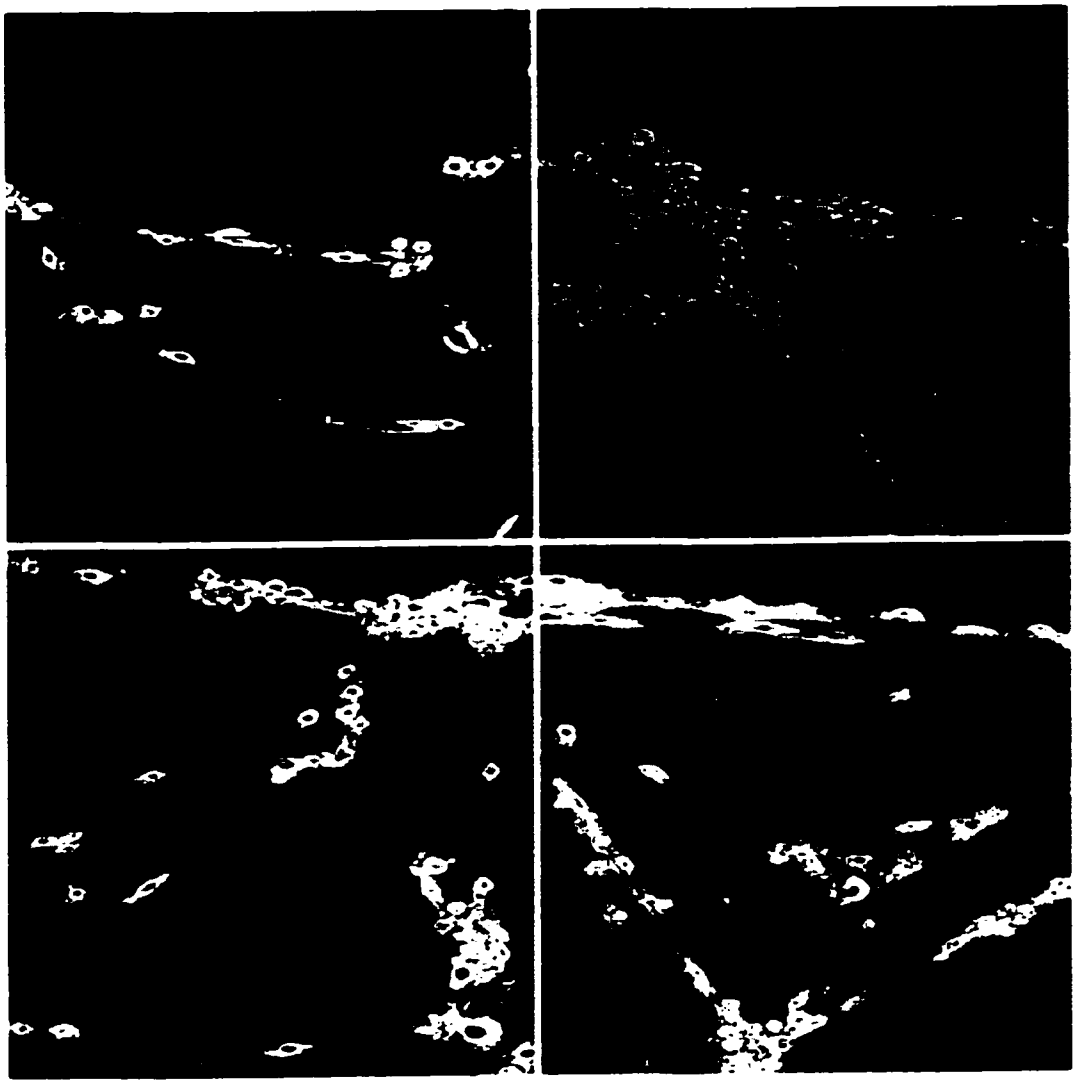

1
2
3
4
kDa
140
75
gp140
(TrkA)
gp75

Figure 1.18 Effect of NGF withdrawal on the cell morphology of terminally differentiated SH-SY5Y cells

Phase-contrast photographs of NGF-deprived terminally differentiated SH-SY5Y cells. **(A)** terminally differentiated cells were incubated in fresh medium without NGF for 3 h and for 6 h **(B)**. **(C)** terminally differentiated cells were incubated in fresh medium containing anti-NGF serum for 3 h and for 6 h **(D)**. SH-SY5Y cells were plated at $1x10^5$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Cells were treated every 2 days, starting 24h after plating, with a combination of 100 ng per ml NGF, 0.3 μM aphidicolin and 0.5 mM 8-Br-cAMP for 3 weeks, followed by a sequential treatment every 2 days with 100 ng/ml NGF for another 3 weeks. The cell culture medium was replaced with fresh medium lacking NGF or with fresh medium containing 50 μg/ml anti-NGF serum. Cells were viewed under phase contrast with a Nikon microscope and photographed with a Kodak ektachrome 400 film after 3 h and 6 h incubation periods.



of experiments, terminally differentiated SH-SY5Y cells were incubated with anti-NGF antibody by replacing the differentiation medium with a fresh medium containing 50 μg/ml anti-NGF. In another set of experiments we replaced the medium with fresh medium without NGF. In both experiments, the terminally differentiated cells revealed morphological signs of cell death that could be either to necrosis or apoptosis. We observed also signs of apoptotic cell death such as cell blebbing and axonal breakage after only 3 h of NGF withdrawal (Fig. 1.18). Cell viability was assayed for the two experiments. After 12 h of NGF deprivation, almost 50 % of the terminally differentiated cells remained viable while most of them died after 48h (Fig. 1.19) in both experiments. However, we noticed that terminally differentiated SH-SY5Y cells incubated with anti-NGF serum are less viable than those where the NGF was simply removed from the medium (Fig.1.19). We concluded that terminally differentiated SH-SY5Y became NGF survival-dependent.

1.4.3.2 Cell death characterisation

NGF withdrawal-induced apoptosis was demonstrated in terminally differentiated SH-SY5Y cells by staining nuclei with the Hoescht reagent and checking for condensed chromatin. Terminally differentiated cells became apoptotic after only 3 hours of NGF withdrawal as demonstrated by the appearance of chromatin condensation and an increase in the intensity of fluorescence due to the compaction of chromatin (Fig. 1.20). Nuclear fragmentation was also confirmed using the TUNEL assay (Gavrieli *et al.*, 1992) (Fig. 1.21). Differentiated cells containing NGF in the medium did not show any chromatin compaction (Fig. 1.20A) or DNA fragmentation (Fig. 1.21A).

Figure 1.19 Effect of NGF withdrawal on terminally differentiated SH-SY5Y cell viability

Cell death follows NGF deprivation in terminally differentiated SH-SY5Y cells. SH-SY5Y cells were plated at $1x10^5$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Cells were treated every 2 days, starting 24 h after plating, with a combination of 100 ng per ml NGF, 0.3 μM aphidicolin and 0.5 mM 8-Br-cAMP for 3 weeks, followed by a sequential treatment every 2 days with 100 ng/ml NGF for another 3 weeks. The cell culture medium was replaced with a fresh medium lacking NGF (NGF withdrawal) or with a fresh medium containing 50 μg/ml anti-NGF serum (anti-NGF). Viable cells were enumerated with the Tryptan blue dye exclusion method using an hemocytometer, after 0, 3, 6, 12, 24 and 48 of NGF deprivation or addition of antibody. Viability is expressed as a percent of SH-SY5Y terminally differentiated cells supplemented with NGF. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).

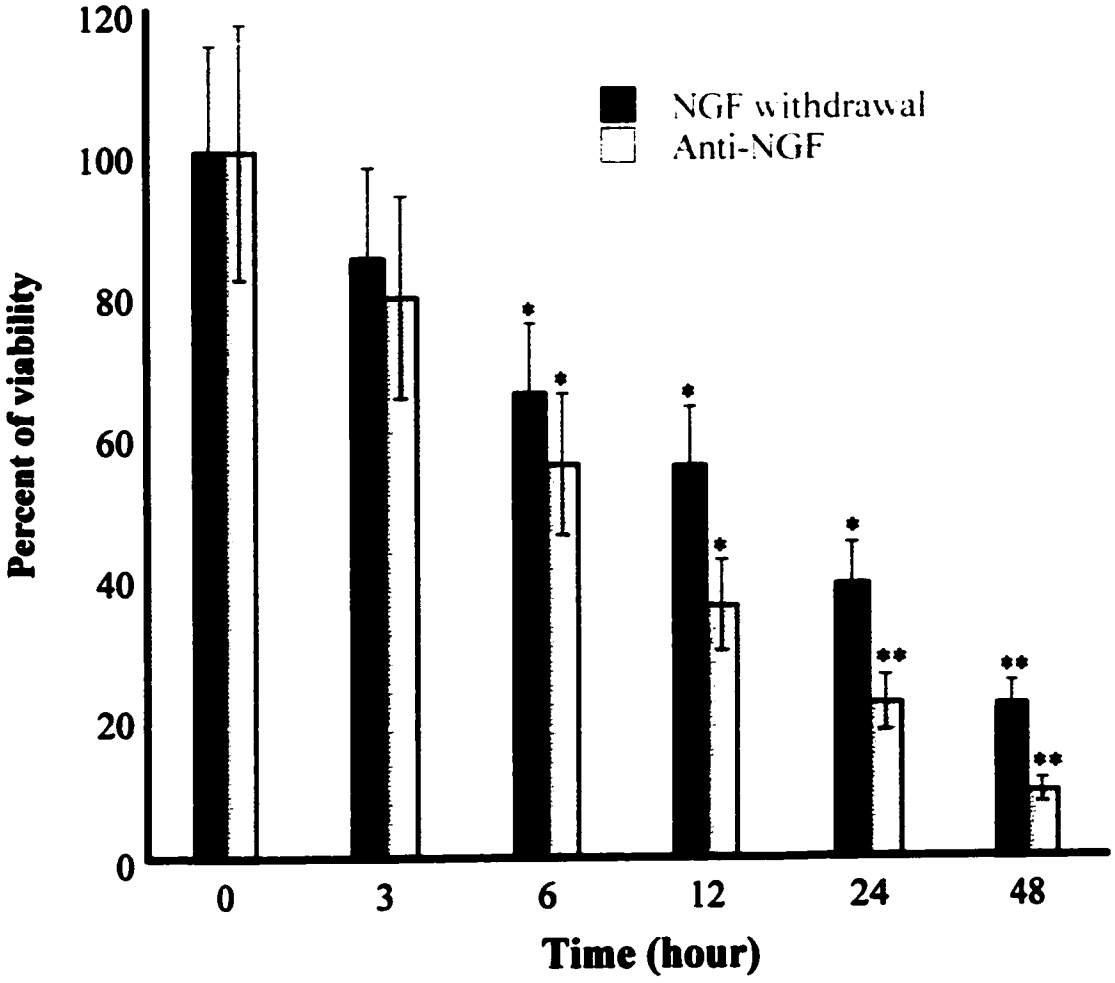

120
100
80
60
40
20
0
Percent of viability
NGF withdrawal
Anti-NGF
*
*
*
*
**
**
**
0
3
6
12
24
48
Time (hour)

Figure 1.20 Chromatin condensation in NGF-deprived terminally differentiated SH-SY5Y cells

Fluorescence photographs of Hoeschst-stained terminally differentiated cells in the presence of NGF (A), and in the absence of NGF (B, C). SH-SY5Y cells were plated at $1x10^5$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Cells were treated every 2 days, starting 24 h after plating, with a combination of 100 ng per ml NGF, 0.3 μM aphidicolin and 0.5 mM 8-Br-cAMP for 3 weeks, followed by a sequential treatment every 2 days with 100 ng/ml NGF for another 3 weeks. The cell cultures were incubated in fresh medium lacking NGF for 3 h (B) or with fresh medium containing 50 μg/ml anti-NGF serum for 3 h (C). Cells were washed, fixed, permeabilised and stained with Hoeschst as described in Methods. Cells were viewed under fluorescence with a Nikon microscope equipped with an UV filter and photographed with a Kodak ektachrome 400 film.

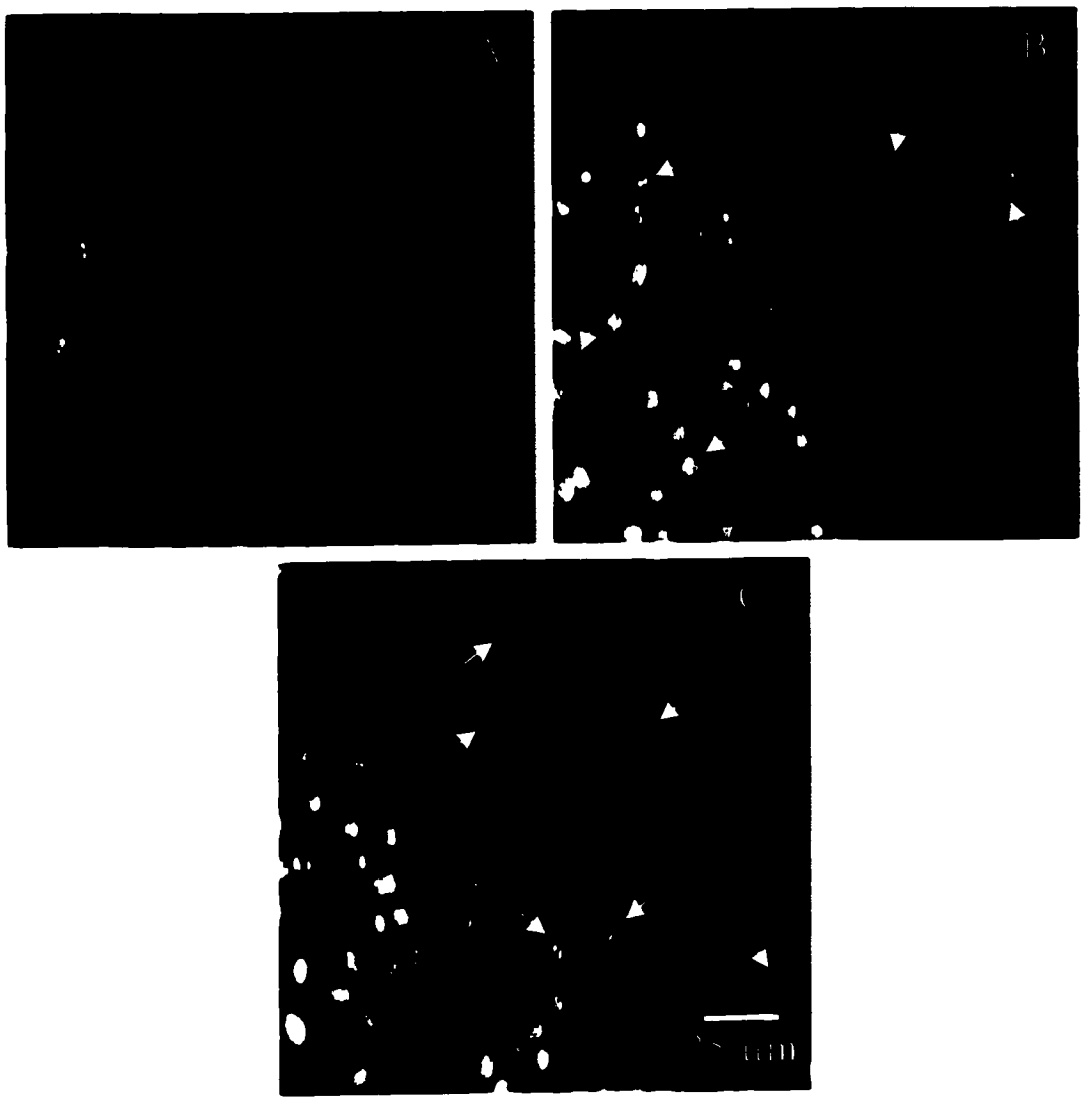
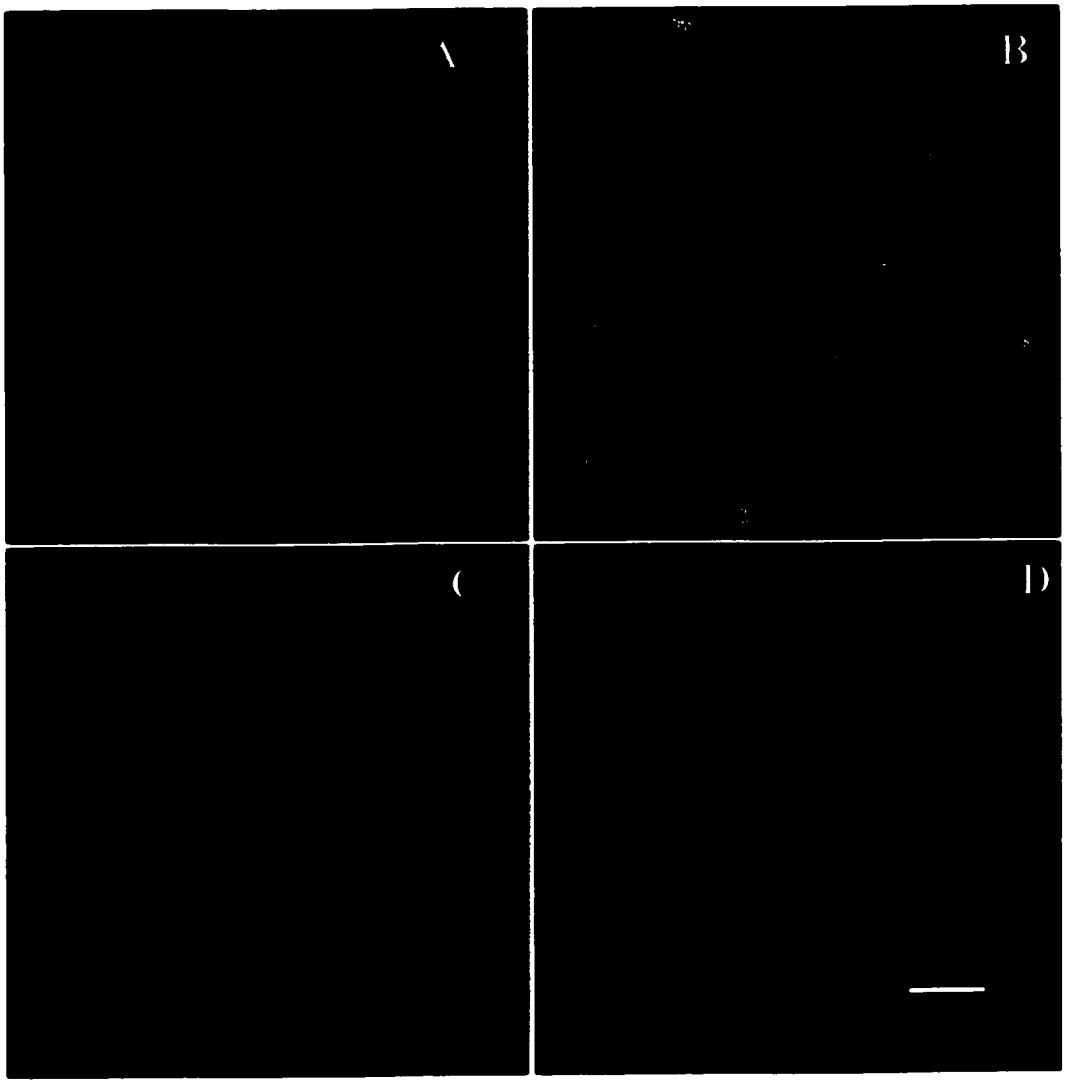


Figure 1.21 Chromatin degradation in NGF-deprived terminally differentiated SH-SY5Y cells

Fluorescence photographs of terminally differentiated SH-SY5Y cells stained by the TUNEL assay in the presence of NGF (**A**), and in the absence of NGF (**C, D**). (**B**) is a positive control (100 nM staurosporine treated SH-SY5Y cells for 3 h). SH-SY5Y cells were plated at $1x10^5$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Cells were treated every 2 days, starting 24 h after plating, with a combination of 100 ng per ml NGF, 0.3 μM aphidicolin and 0.5 mM 8-Br-cAMP for 3 weeks, followed by a sequential treatment every 2 days with 100 ng/ml NGF for another 3 weeks. The cell cultures were incubated in fresh medium lacking NGF for 3 h (**C**) or with fresh medium containing 50 μg/ml anti-NGF serum for 3 h (**D**). Cells were washed, permeabilised and incubated with TUNEL reaction mixture as described in Methods. Cells were viewed under fluorescence with a Nikon microscope equipped with the appropriate filter and photographed with a Kodak ektachrome 400 film.


A
B
C
D

However, terminally differentiated SH-SY5Y cells deprived from NGF showed both chromatin condensation (Fig. 1.20.B, C) and DNA fragmentation (Fig. 1.21.C, D).

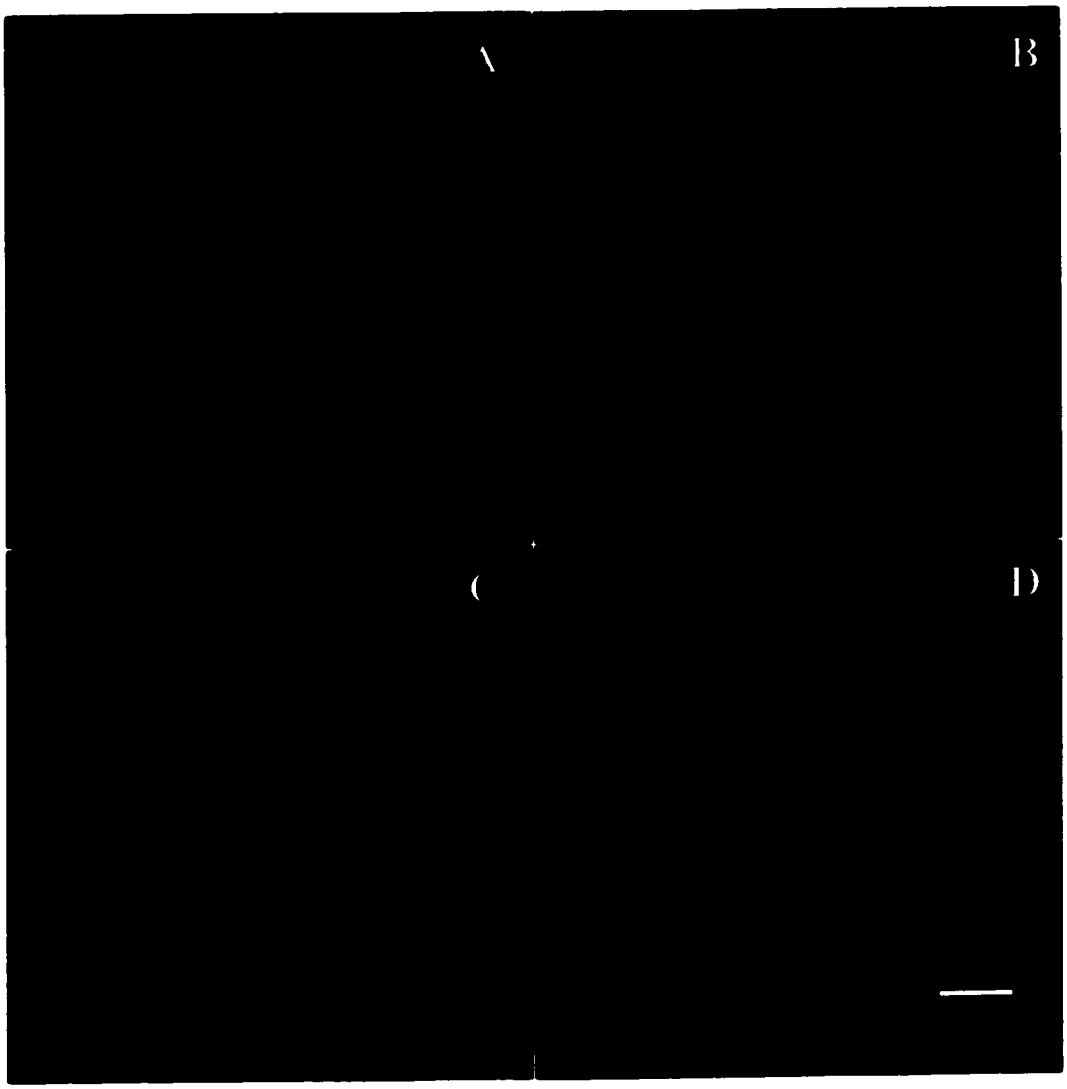
1.4.3.3 Effect of differentiation on p53 sub-cellular localisation

We investigated the possibility that a relationship may exist between non-mutational inactivation of p53 in the human NB cell line SH-SY5Y and cell differentiation since growth arrest favours differentiation as well as apoptosis induction. Fixed cells were analysed by immunohistochemistry using a p53 polyclonal antibody. We observed exclusively cytoplasmic immunostaining in the undifferentiated cells (Fig. 1.22.A). The secondary antibody conjugated to p53 was conjugated to the rhodamine fluorochrome. This permitted the demonstration that p53 is restricted to the cytoplasm and is absent from the nucleus (Fig. 1.22.A). We investigated whether the arrest of cell proliferation observed as SH-SY5Y cells undergo differentiation would impact on p53 sub-localisation. We found that when SH-SY5Y cells were treated with 8-Br-cAMP for 2 days, p53 immunostaining was re-located partially to the nucleus rather than being expressed solely in the cytoplasm (Fig. 1.22.B). This was also demonstrated using a green fluorochrome that stained only the nucleus (data not shown). We noticed that as SH-SY5Y cells progress towards differentiation, there was a progressive shift of p53 from the cytoplasm to the nucleus although p53 did not completely disappear from the cytoplasm, even when cells reached the state of terminal differentiation (Fig.1.22.C, D).

Wild type-p53 protein is known to accumulate in NB, this is due to the dramatic increase in p53 half-life, thereby stabilising the protein. It was recently shown that the aberrant constitutive p53 accumulation in IMR-32, LAN-5 and SK-NSH, the SH-SY5Y

Figure 1.22 p53 sub-localisation in undifferentiated and differentiated SH-SY5Y cells

Immunocytochemical analysis of p53 in undifferentiated (A) and differentiated SH-SY5Y cells for 2 days (B), 3 weeks (C) and 6 weeks (D). SH-SY5Y cells were plated at $1x10^5$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Cells were treated every 2 days, starting 24 h after plating, with a combination of 100 ng per ml NGF, 0.3 μM aphidicolin and 0.5 mM 8-Br-cAMP for 3 weeks, followed by a sequential treatment every 2 days with 100 ng/ml NGF for another 3 weeks. Cells were washed, fixed and incubated with p53-specific antibody and antigen-antibody detected with rhodamine-conjugated secondary antibody as described in Methods. Cells were viewed under fluorescence with a Nikon microscope equipped with the appropriate filter and photographed with a Kodak ektachrome 400 film.


A
B
C
D

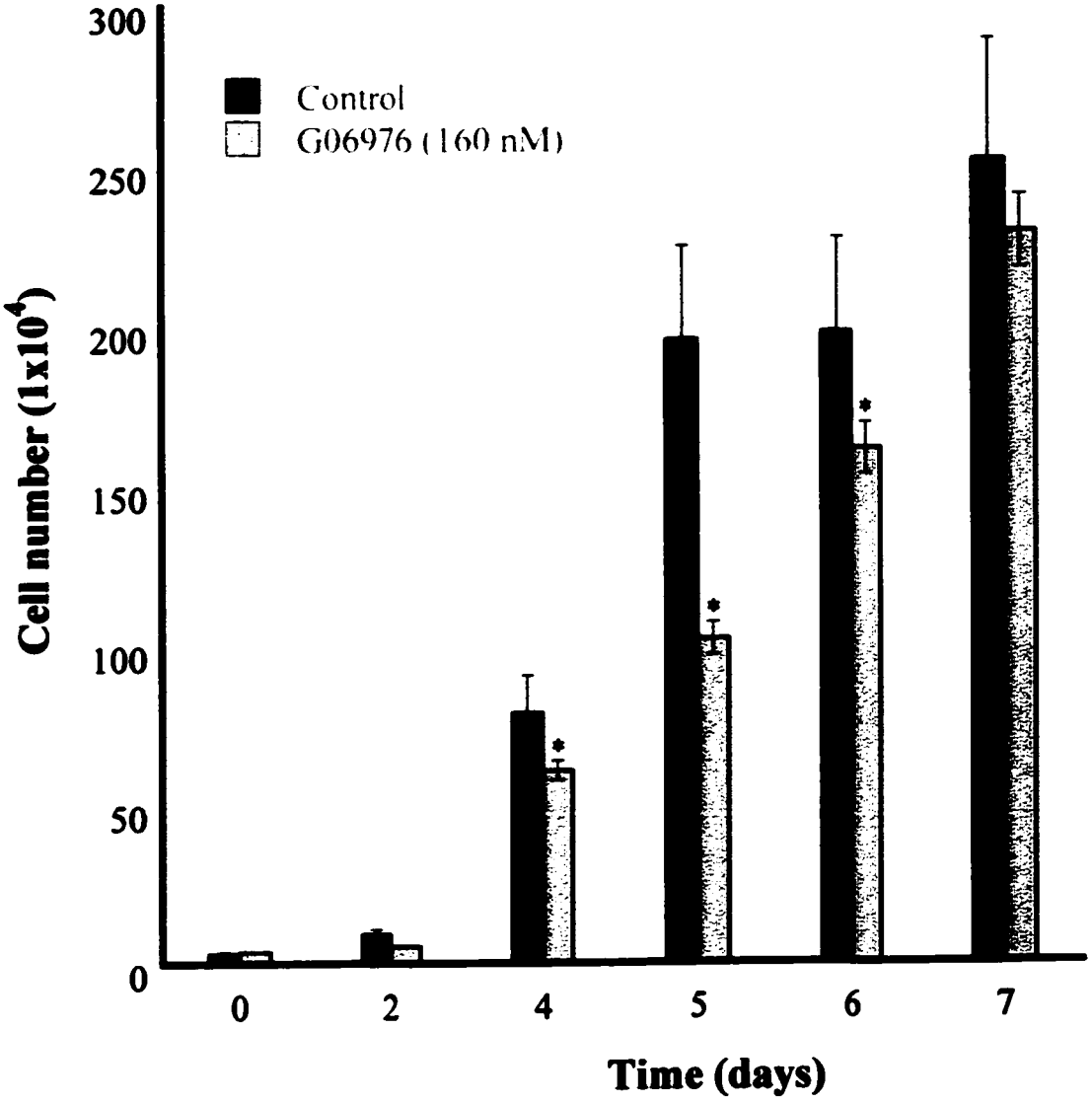
parental cell line, and other NB cell lines is due to the resistance to MDM-2 mediated degradation (Tweddle *et al.*, 2001; Isaacs *et al.*, 2001). In conclusion, during the progression into differentiation, the level of p53 decreased significantly in SH-SY5Y cells when compared to the undifferentiated cells as p53 was partially re-located to its normal nuclear localisation. This suggests that these conditions are favourable to the induction of cell cycle arrest and apoptosis since p53 is involved in the regulations of both cell growth and apoptosis (Gottlieb *et al.*, 1996; Ko *et al.*, 1996).

1.4.4 Effect of differentiation on PKC

Previous studies have demonstrated that the SH-SY5Y NB cell line can be induced to differentiate in the presence of serum following activation of PKC with phorbol esters or its inactivation by PKC inhibitors (Shea *et al.*, 1991; 1992). This differentiation process is accompanied by a decrease in growth rate, increased neurite outgrowth and enhanced expression of differentiation markers such as the neuropeptide tyrosine hydroxylase (Pahlman, 1981). We used PKC inhibitors to investigate whether PKCs contributed to induced differentiation of SH-SY5Y cells under the conditions previously defined. Firstly, PKC specific and non-specific inhibitors were assayed for their effect on SH-SY5Y cell proliferation and differentiation. Secondly, the pattern of PKC isoform expression was determined in both undifferentiated and differentiated SH-SY5Y cells.

Figure 1.23 Effect of the cPKC inhibitor Go6976 on SH-SY5Y cell proliferation

Cell growth of untreated and treated SH-SY5Y cells with 160 nM Go6976 for 0, 2, 4, 5, 6 and 7 days. SH-SY5Y cells were plated at $1x10^4$ cells per 20 mm^2 six in well plates in RPMI medium supplemented with 10% FBS. 24 h later cells were treated with 160 nM Go6976 for the selected periods of time. Cells were washed, trypsinized and counted using Trypan blue dye exclusion and a hematocytometer as described in Methods. The average of three wells was used for each set of experiments. Each experiment was repeated 3 times. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).


300
250
200
150
100
50
0
Cell number (1x10⁴)
Control
G06976 (160 nM)
*
*
*
0
2
4
5
6
7
Time (days)

1.4.4.1 Effect of PKC inhibitors on SH-SY5Y cell growth and differentiation

In order to assess the putative involvement of PKC in NB growth and differentiation, undifferentiated SH-SY5Y cells were cultured in the presence of the non-specific PKC inhibitors H7 and staurosporine, the more specific PKC inhibitors Bis and Calphostin C or an inhibitor of the cPKCs, Go6976. The effect on cell proliferation was assayed for all these PKC inhibitors. The PKC inhibitors Bis and Calphostin C had no effect on cell proliferation at concentrations of 2 mM and 1 mM respectively (data not shown). However, Go6976 inhibited cell proliferation by 50% at a concentration of 160 nM following 5 days of treatment but this difference was no longer significant by 7 days of treatment (Fig. 1.23). In contrast, when undifferentiated SH-SY5Y cells were exposed to the non-specific PKC inhibitors staurosporine or H7 at 100 nM and 100 μM respectively, cells stopped growing (Fig. 1.24) and started dying by apoptosis as will be discussed later. At the morphological level, none of the specific PKC inhibitors (Bis and Calphostin C) used induced morphological differentiation in SH-SY5Y cells (Fig. 1.25). However, the non-specific PKC inhibitors, staurosporine and H7 both induced morphological differentiation in SH-SY5Y cells (Fig. 1.26) and (Fig. 1.27). Undifferentiated SH-SY5Y cells were treated with 100 nM staurosporine for 3, 6, 12, 24 and 48 hours. Staurosporine-treated cells started emitting short filopodia-like neurites within 15 min of treatment (data not shown). These cells rapidly elaborated an extensive network of neurite-like processes if exposed for longer period of time to staurosporine

Figure 1.24 Effect of the PKC non-specific inhibitors staurosporine and H7 on SH-SY5Y cell proliferation

Cell growth of untreated (CT) and treated SH-SY5Y cells with 100 nM staurosporine (ST) or with 100 μM H7 (H7) after 12, 24 and 48 hours. SH-SY5Y cells were plated at $5x10^4$ cells per well in six well plates in RPMI medium supplemented with 10% FBS. Starting 24 h later cells were treated with 100 nM staurosporine or 100 μM H7 for the appropriate periods of time. Cells were washed, trypsinized and counted using the Trypan blue dye exclusion and a hematocytometer as described in Methods. The average of three wells was used for each set of experiments. Each experiment was repeated 3 times. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).

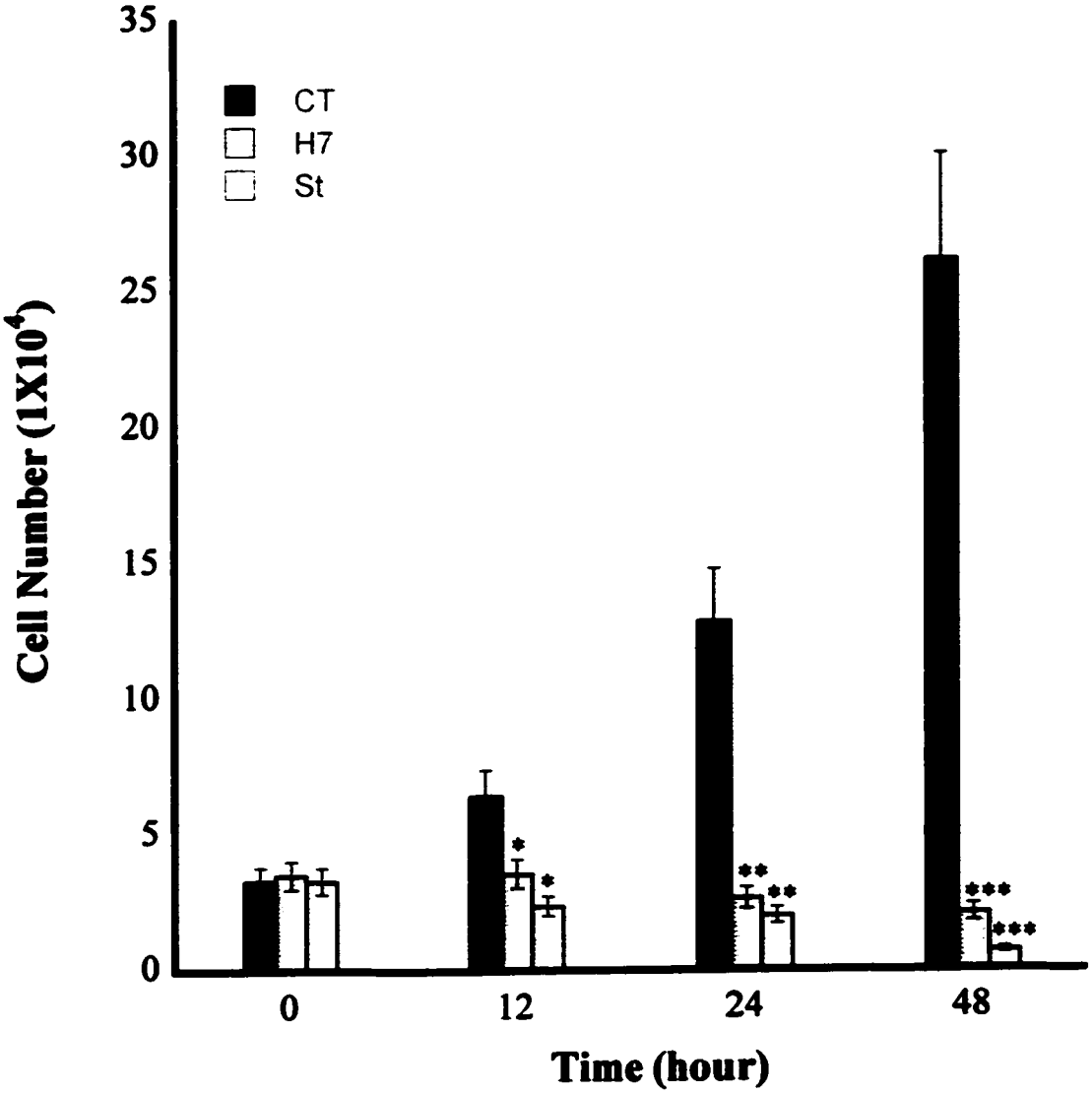

35
30
25
20
15
10
5
0
CT
H7
St
Cell Number (1X10⁴)
*
*
**
**
***
***
0
12
24
48
Time (hour)

Figure 1.25 Effect of the PKC inhibitors Bis, Calphostin C and Go6976 on SH-SY5Y cell morphology

Phase-contrast photographs of untreated controls (A) or SH-SY5Y cells exposed for 24 h to 2 mM Bis (B), 160 nM Go6976 (C) and 1 mM calphostin C (D). SH-SY5Y cells were seeded at $1x10^4$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated with 2 mM Bis (B), 160 nM Go6976 (C) or 1 mM Calphostin C (D) (final concentrations) for a 24 h time period. Cells were viewed under phase contrast with a Nikon microscope and photographed with a Kodak ektachrome 400 film.

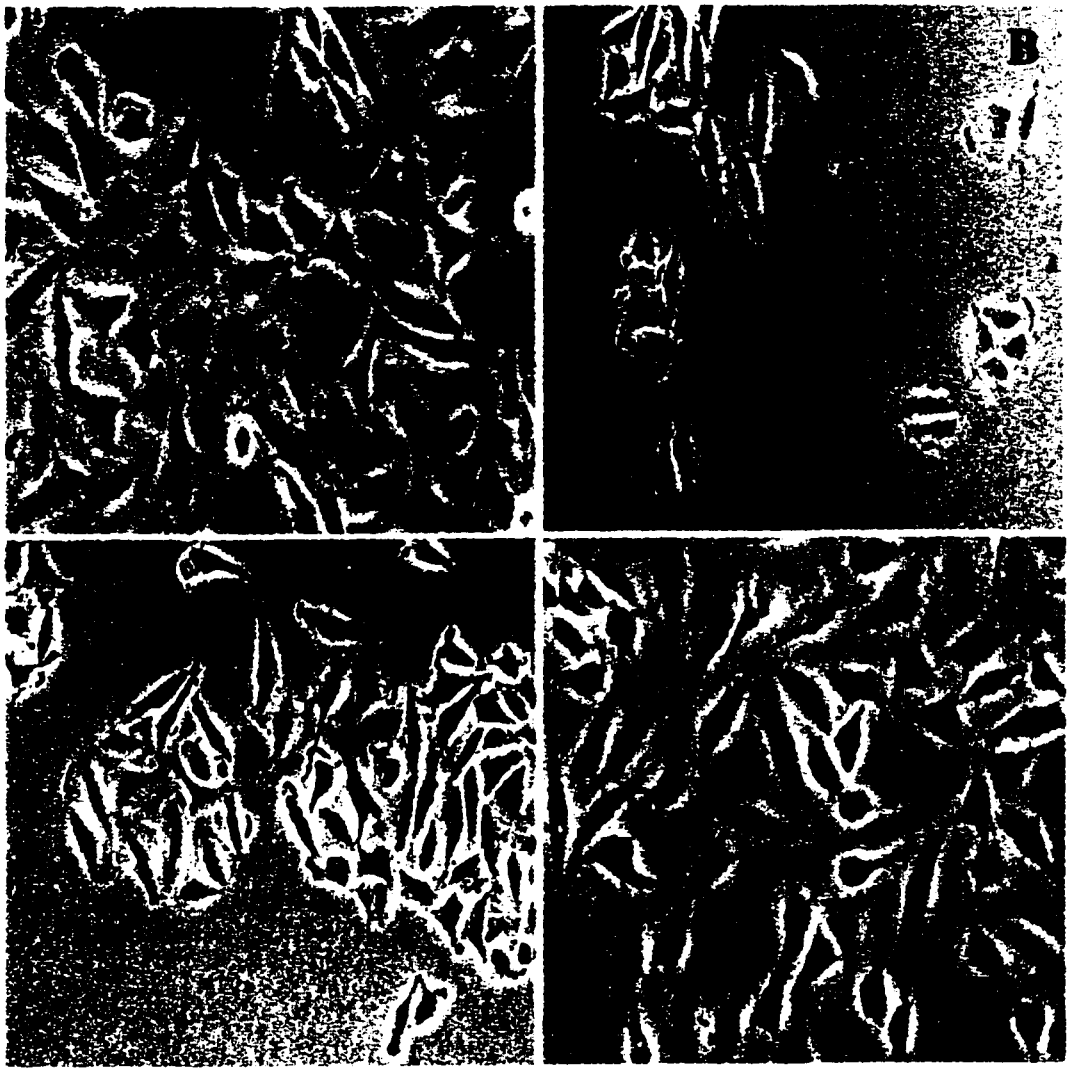

B
P

Figure 1.26 Effect of the PKC non-specific inhibitor staurosporine on SH-SY5Y cell morphology

Phase-contrast photographs of untreated controls (**A**) or staurosporine treated SH-SY5Y cells for 0, 3, 6, 12, 24 and 48 h time periods. SH-SY5Y cells were seeded at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated with staurosporine at 100 nM final concentration for 0 h (**A**), 3 h (**B**), 6 h (**C**), 12 h (**D**), 24 h (**E**) and 48 h (**F**) time periods. Cells were viewed under a phase contrast with a Nikon microscope and photographed with a Kodak ektachrome 400 film.

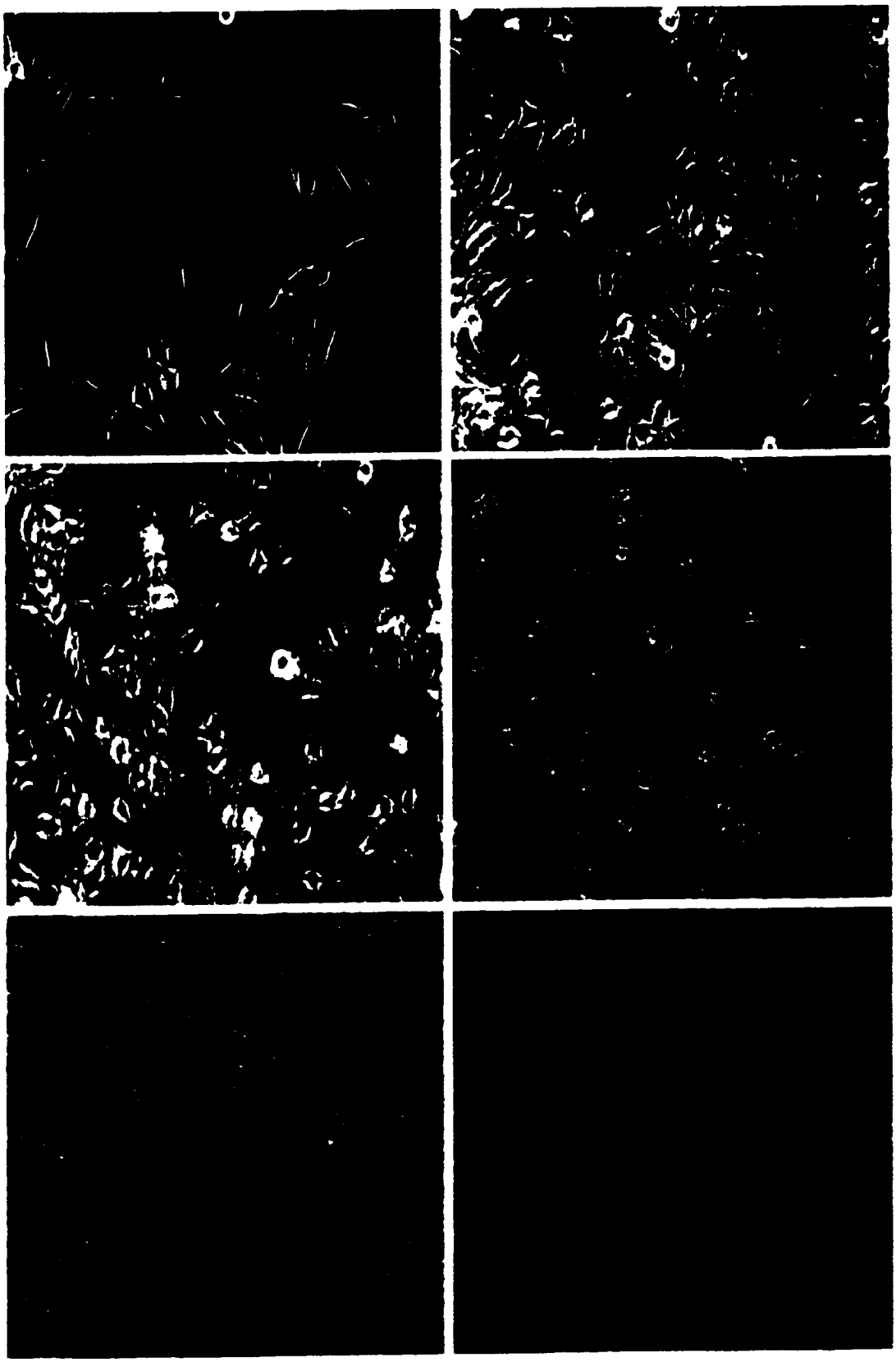


Figure 1.27 Effect of the PKC non-specific inhibitor H7 on SH-SY5Y cell morphology

Phase-contrast photographs of untreated controls (**A**) or H7 treated SH-SY5Y cells for 0, 3, 6, 12, 24 and 48 h time periods. SH-SY5Y cells were seeded at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated with H7 at a final concentration of 100 μM for 0 h (**A**), 3 h (**B**), 6 h (**C**), 12 h (**D**), 24 h (**E**) and 48 h (**F**) time periods. Cells were viewed under phase contrast with a Nikon microscope and photographed with a Kodak ektachrome 400 film.

(Fig. 1.26B, C, D). Despite this extensive outgrowth, these neurites retracted within 24 h following removal of the inhibitor (data not shown). Cells treated with 100 nM staurosporine for 24 and 48 hours started showing extensive axonal breakage and cell death (Fig. 1.26E, F). The other non-specific inhibitor of PKC, H7 also induced rapid neurite outgrowth that was maintained for up to 48 hours treatment, after which time H7 treated cells started showing the characteristics of cell death by apoptosis (Fig. 1.27). Although both inhibitors induced neurite outgrowth, the morphology of the neurites differed: staurosporine treated cells elaborated a branching network of neurites and long unbranched neurites, while H7-treated cells elaborated mostly long unbranched neurites, and lacked the network of shorter neurites.

SH-SY5Y exposure to the cPKC isoform inhibitor, Go6976 had no effect on cell morphology (data not shown). In contrast, when combined with staurosporine and H7, Go6976 rescued cells from the cell death induced by staurosporine and H7 and maintained the cell in a differentiated morphological state for up to 3 weeks of treatment if they were supplemented with fresh medium containing these inhibitors (Fig. 1.28).

The two PKC inhibitors that induced morphological differentiation of SH-SY5Y cells, H7 and staurosporine, were analysed for their induction of neuronal markers and their effects on N-myc, H-ras and TrkA expression by FACS analysis as described above. Treatment with 100 nM staurosporine induced an almost 3 to 4 times increase in the expression of NF200 and NF68 after 3 hours (Fig. 1.29). Later during differentiation, we noticed a decrease in NF200 and NF68 that may be due to the beginning of programmed cell death induced by staurosporine (Fig. 1.29). Staurosporine treated SH-SY5Y cells were also assayed for the expression of TrkA and H-Ras protein as indicators for good NB prognosis. We found that staurosporine has no effect on the

Figure 1.28 Effect of combining staurosporine, H7 and Go6976 PKC inhibitors on SH-SY5Y cell morphology

Phase-contrast photographs of undifferentiated SH-SY5Y cells (**A**) and differentiated cells following 1 week (**B**) and 3 weeks (**C**) exposure to a mixture of staurospaurine, H7 and Go6976. SH-SY5Y cells were seeded at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24h later cells were treated with 100 nM staurospaurine, 100 μM H7 and 160 nM Go6976 (final concentrations). Cells were viewed under phase contrast with a Nikon microscope and photographed with a Kodak ektachrome 400 film.

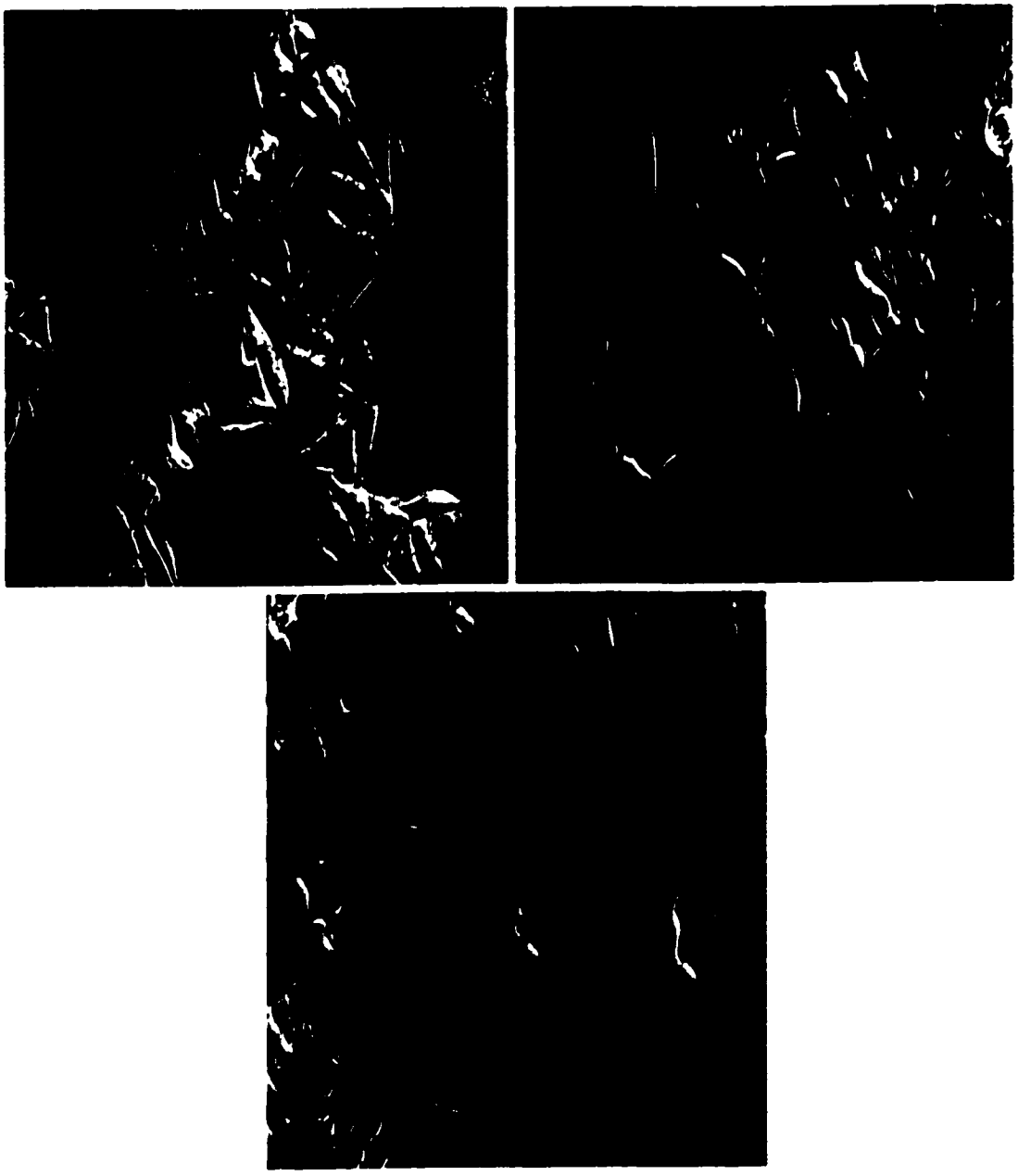


Figure 1.29 Expression of the neuronal proteins NF200 and NF68 in staurosporine treated SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24h later cells were treated with 100 nM staurosporine for 0, 1, 2, 3, 6 and 12 hours. Cells were harvested by treatment with 0.05% trypsin and 0.02% EDTA, washed 3 times with PBS and fixed as described in Methods. Fixed cells were incubated in PBS containing 2% BSA and immunolabelled for 1 hour to overnight with NF200 and NF68 specific antibodies at a dilution of 1:200 and 1:250 respectively in PBS containing 0.2% BSA at 4°C then washed 3 times with PBS and incubated with rhodamine conjugated secondary antibodies at a dilution of 1:100 for one hour in PBS. Immunostained cells were washed and analysed using a Coulter Elite flow cytometer. The measured fluorescence intensity provides a relative estimation of protein level per cell. Statistical error on 10,000 cell analyses amounts to 0.8%. All the experiments were repeated at least three times in duplicate. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).

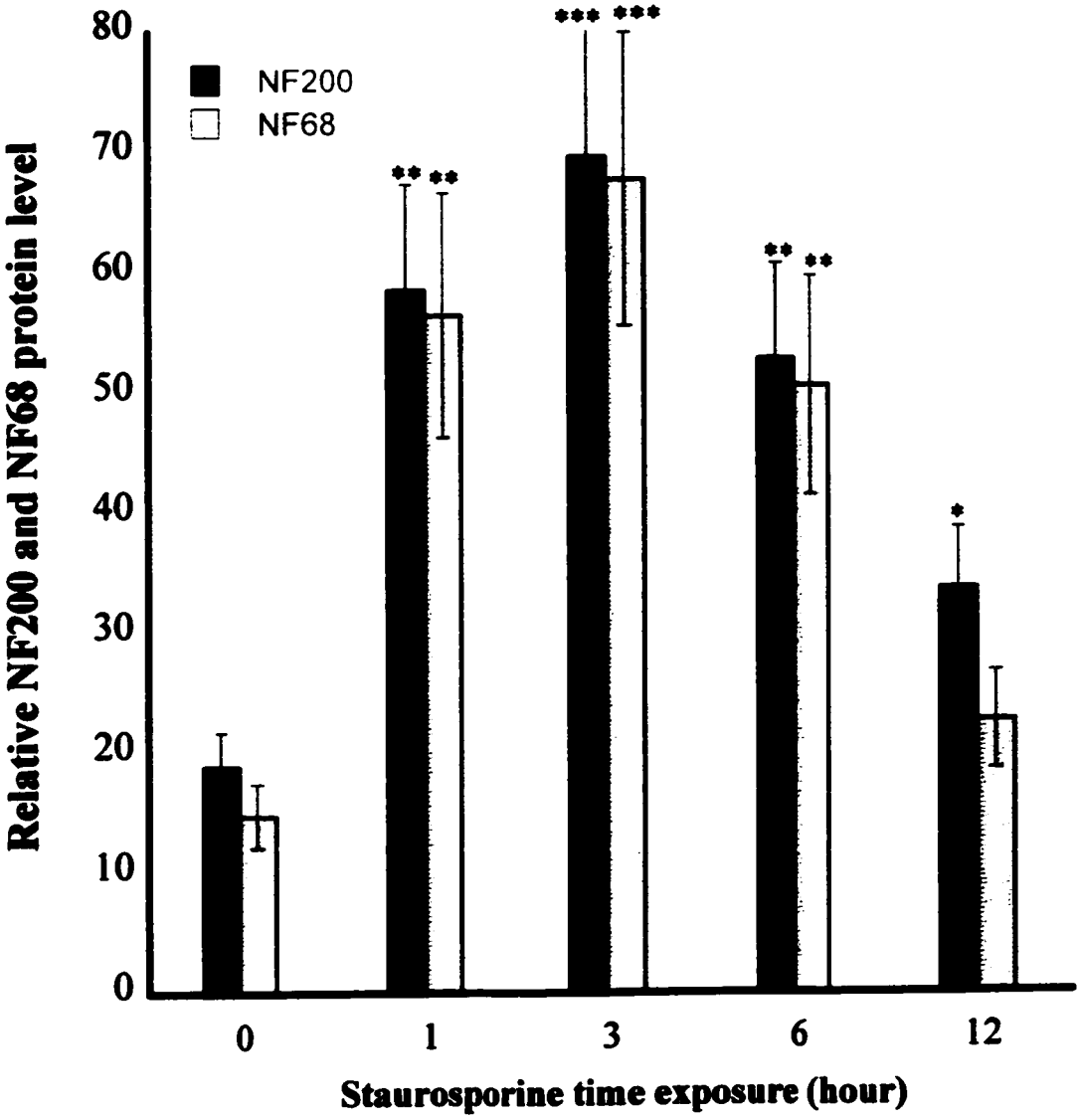

80
70
60
50
40
30
20
10
0
NF200
NF68
***
***
**
**
**
**
*
Relative NF200 and NF68 protein level
0
1
3
6
12
Staurosporine time exposure (hour)

Figure 1.30 Expression of H-ras protein in staurosporine treated SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24h later cells were treated with 100 nM staurosporine for 0.5, 1, 3, 6, 8, 12 and 24 hours. Cells were harvested by treatment with 0.05% trypsin and 0.02% EDTA, washed 3 times with PBS and fixed as described in Methods. Fixed cells were incubated in PBS containing 2% BSA and immunolabelled for 1 hour to overnight with H-ras specific antibody at a dilution of 1:50 in PBS containing 0.2% BSA at 4°C then washed 3 times with PBS and incubated with fluorescein conjugated secondary antibodies at a dilution of 1:100 for one hour in PBS. Immunostained cells were washed and analysed using a Coulter Elite flow cytometer. The measured fluorescence intensity provides a relative estimation of protein level per cell. All the experiments were repeated at least three times in duplicate. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).

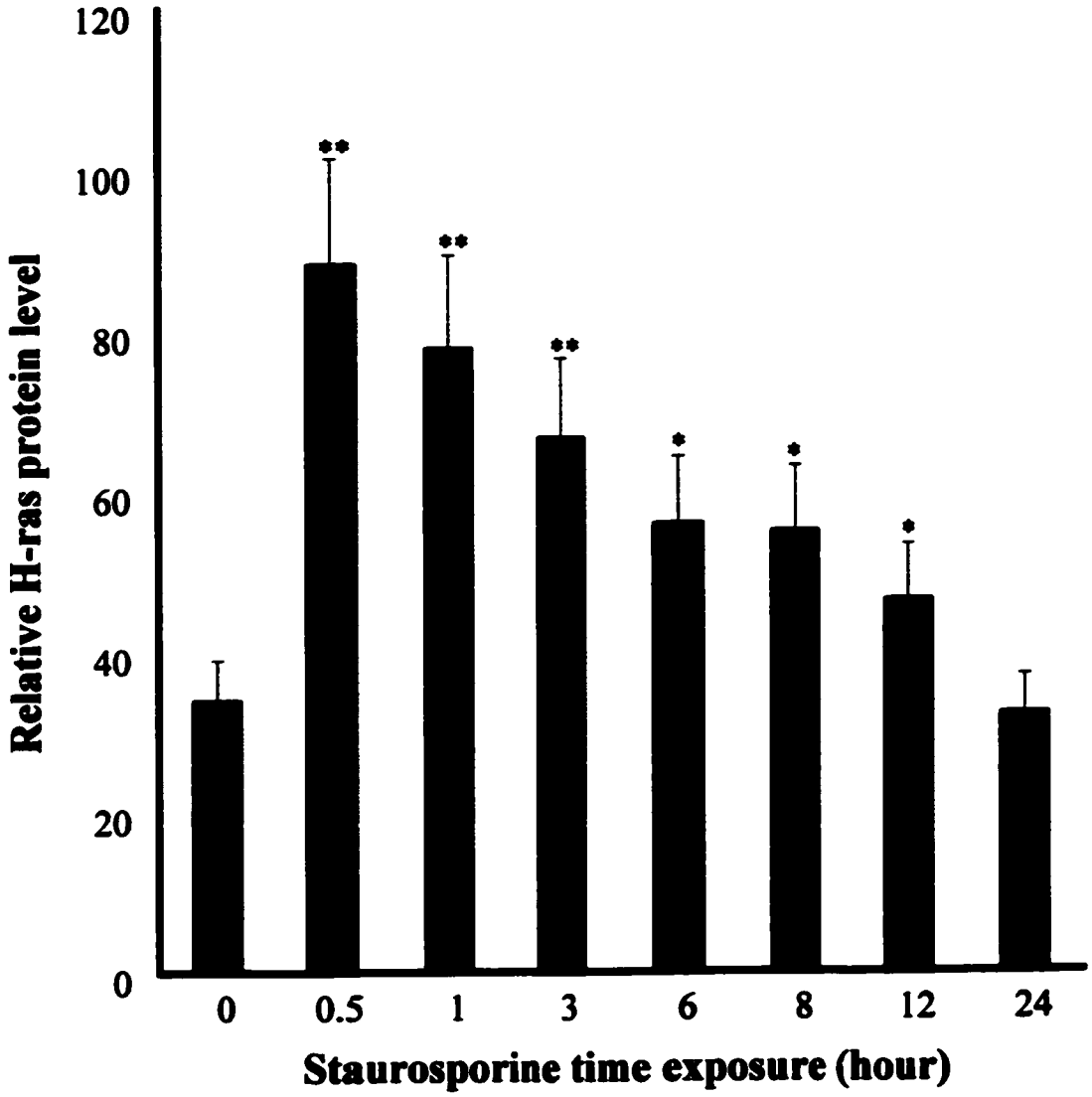
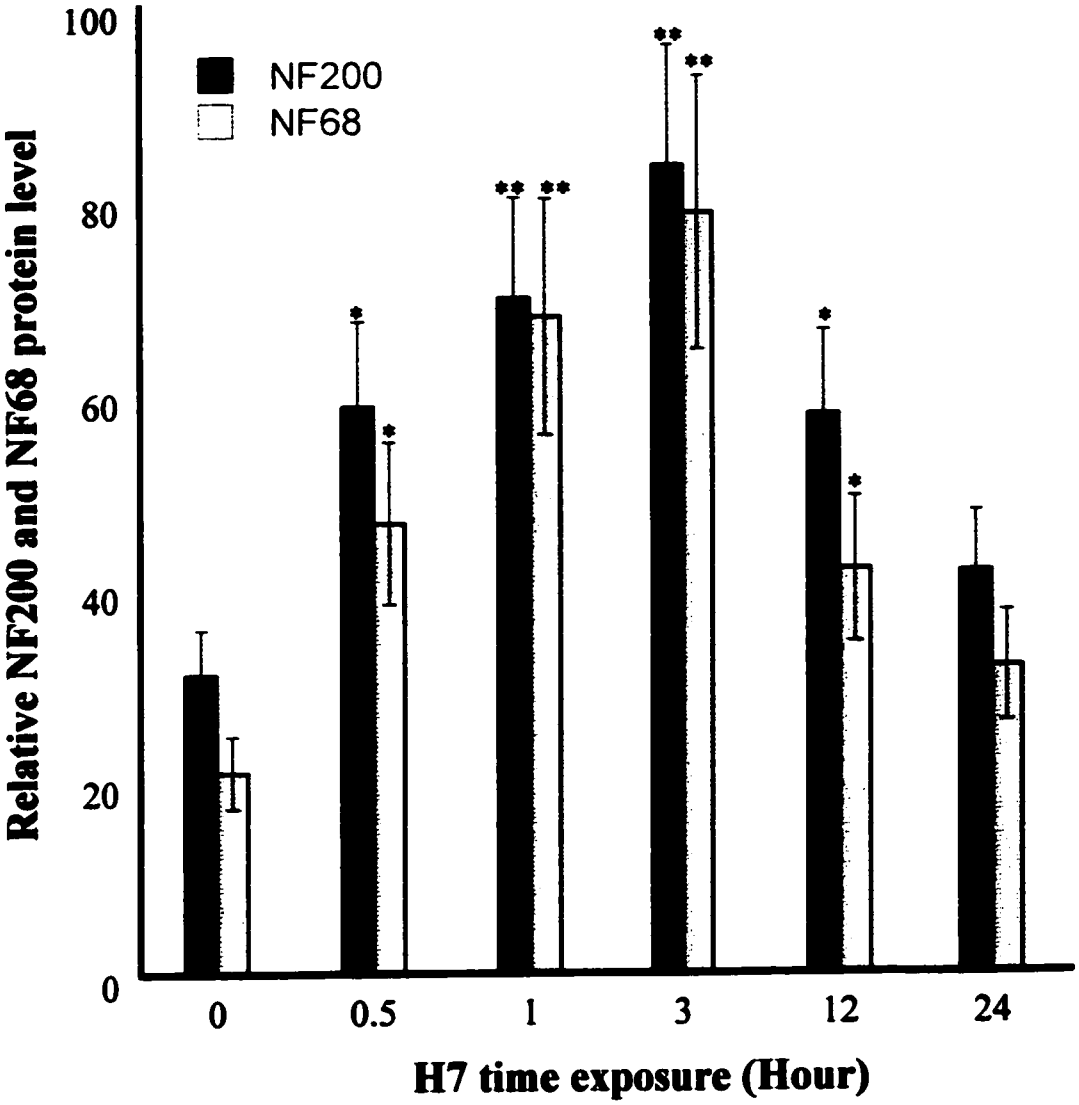

120
100
80
60
40
20
0
Relative H-ras protein level
**
**
**
*
*
*
0
0.5
1
3
6
8
12
24
Staurosporine time exposure (hour)

Figure 1.31 Expression of the neuronal proteins NF200 and NF68 in H7 treated SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated with 100 μM H7 for 0, 0.5, 3, 12 and 24 hours. Cells were harvested by treatment with 0.05% trypsin and 0.02% EDTA, washed 3 times with PBS and fixed as described in Methods. Fixed cells were incubated in PBS containing 2% BSA and immunolabelled for 1 hour to overnight with NF200 and NF68 specific antibodies at a dilution at of 1:200 and 1:250 respectively in PBS containing 0.2% BSA at 4°C then washed 3 times with PBS and incubated with rhodamine conjugated secondary antibodies at a dilution of 1:100 for one hour in PBS. Immunostained cells were washed and analysed using a Coulter Elite flow cytometer. The measured fluorescence intensity provides a relative estimation of protein level per cell. All the experiments were repeated three times in duplicate. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).


100
80
60
40
20
0
NF200
NF68
Relative NF200 and NF68 protein level
0
0.5
1
3
12
24
H7 time exposure (Hour)

expression of the high affinity NGF receptor TrkA (data not shown). In contrast, the protein level of H-Ras doubled within only 30 min of treatment with staurosporine. H-Ras protein level decreased slowly and returned to its original level after 24 hours of treatment with 100 nM staurosporine (Fig. 1.30). This time period coincided with staurosporine induced cell death. H7 was also assayed for its induction of neuronal marker expression. We found that H7 also induced an increase in the expression of NF200, NF68 (Fig. 1.31) similar to that of staurosporine but have no significant effect on the expression of H-Ras (data not shown).

Both non-specific PKC inhibitors, staurosporine and H7, down-regulated N-myc expression as shown by FACS. Staurosporine induced a decrease by 50% in N-myc expression after only 30 min of treatment. N-myc level increased again after 3 hours of treatment and reached its lowest level by 8 hours treatment (even when cells were dying, the level of N-myc remained low, Fig. 1.32). This may be due to an irreversible effect of staurosporine on N-myc expression or a general effect on protein synthesis down-regulation. In contrast, when SH-SY5Y cells were exposed to 100 μM H7, they showed a decrease in N-myc protein expression, which occurred after only 30 min of treatment (Fig. 1.33) and reached its minimum level following 3 hours H7 treatment. After that time, the levels of N-myc gradually increased and reached control levels by 24 hours after treatment (Fig. 1.33).

1.4.4.2 PKC isozyme expression pattern during SH-SY5Y cell differentiation

Although neuronal cells are a major target of phorbol ester action, the activity of the various PKC isozymes has not been extensively studied in neuroblastoma.

Figure 1.32 Expression of the N-myc gene product in staurosporine treated SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24h later cells were treated with 100 nM staurosporine for 0, 0.5, 1, 3, 6, 8, 12 and 48 hours. Cells were harvested by treatment with 0.05% tyrosine and 0.02% EDTA, washed 3 times with PBS and fixed as described in Methods. Fixed cells were incubated in PBS containing 2% BSA and immunolabelled for 1 hour to overnight with N-myc specific antibody at a dilution of 1:100 in PBS containing 0.2% BSA at 4°C then washed 3 times with PBS and incubated with fluorescein conjugated secondary antibodies at a dilution of 1:100 for one hour in PBS. Immunostained cells were washed and analysed using a Coulter Elite flow cytometer. The measured fluorescent intensity provides a relative estimation of protein level per cell. All the experiments were repeated three times in duplicate. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).

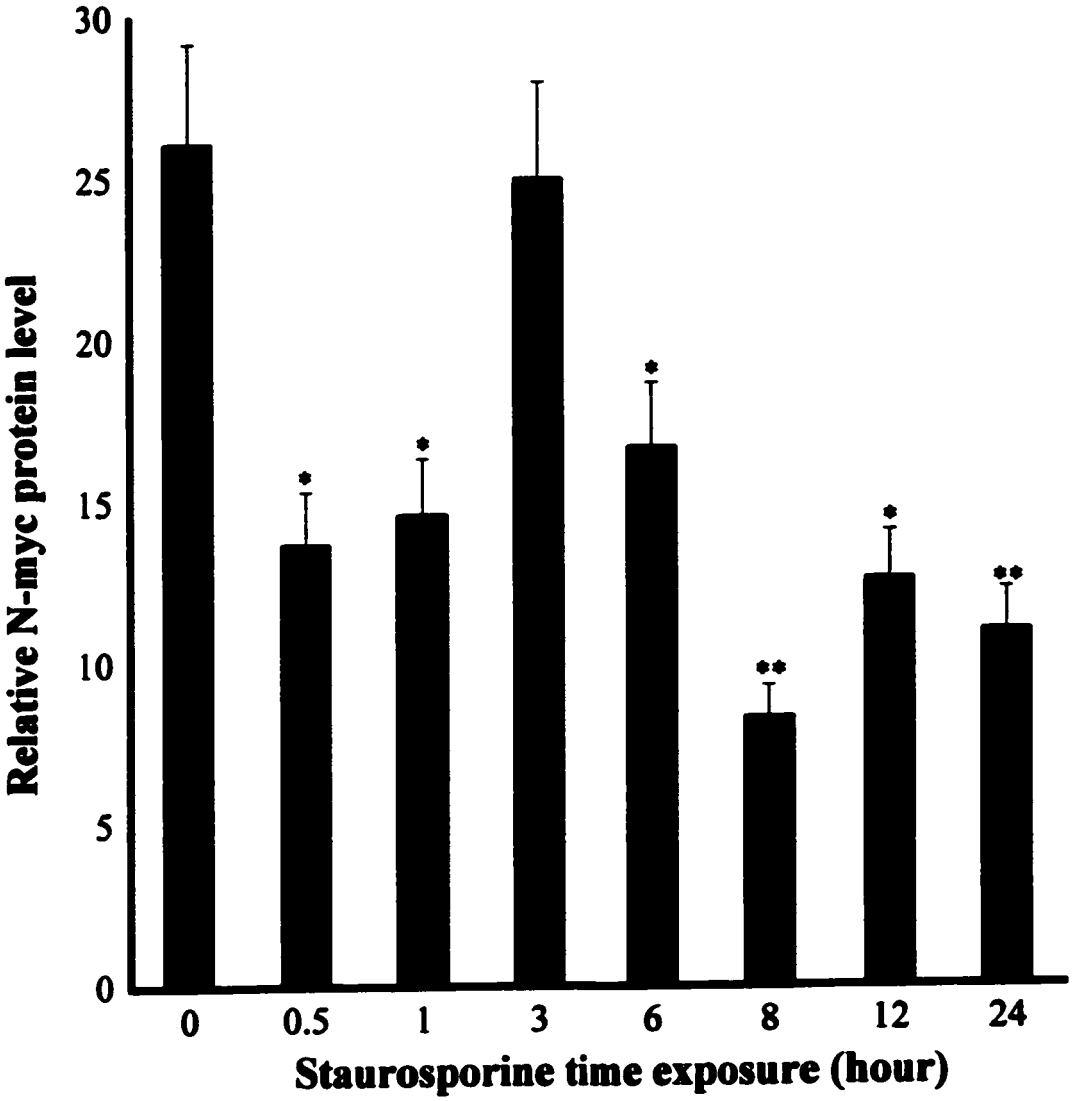

30
25
20
15
10
5
0
Relative N-myc protein level
*
*
*
*
**
*
**
0
0.5
1
3
6
8
12
24
Staurosporine time exposure (hour)

Figure 1.33 Expression of the N-myc gene product in H7 treated SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated with 100 μM H7 for 0, 0.5, 1, 3, 12 and 24 hours. Cells were harvested by treatment with 0.05% trypsin and 0.02% EDTA, washed 3 times with PBS and fixed as described in Methods. Fixed cells were incubated in PBS containing 2% BSA and immunolabelled for 1 hour to overnight with N-myc specific antibody at a dilution at of 1:100 in PBS containing 0.2% BSA at 4°C then washed 3 times with PBS and incubated with fluorescein conjugated secondary antibodies at a dilution of 1:100 for one hour in PBS. Immunostained cells were washed and analysed using a Coulter Elite flow cytometer. The measured fluorescence intensity provides a relative estimation of the protein level per cell. All the experiments were repeated at least three times in duplicate. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).

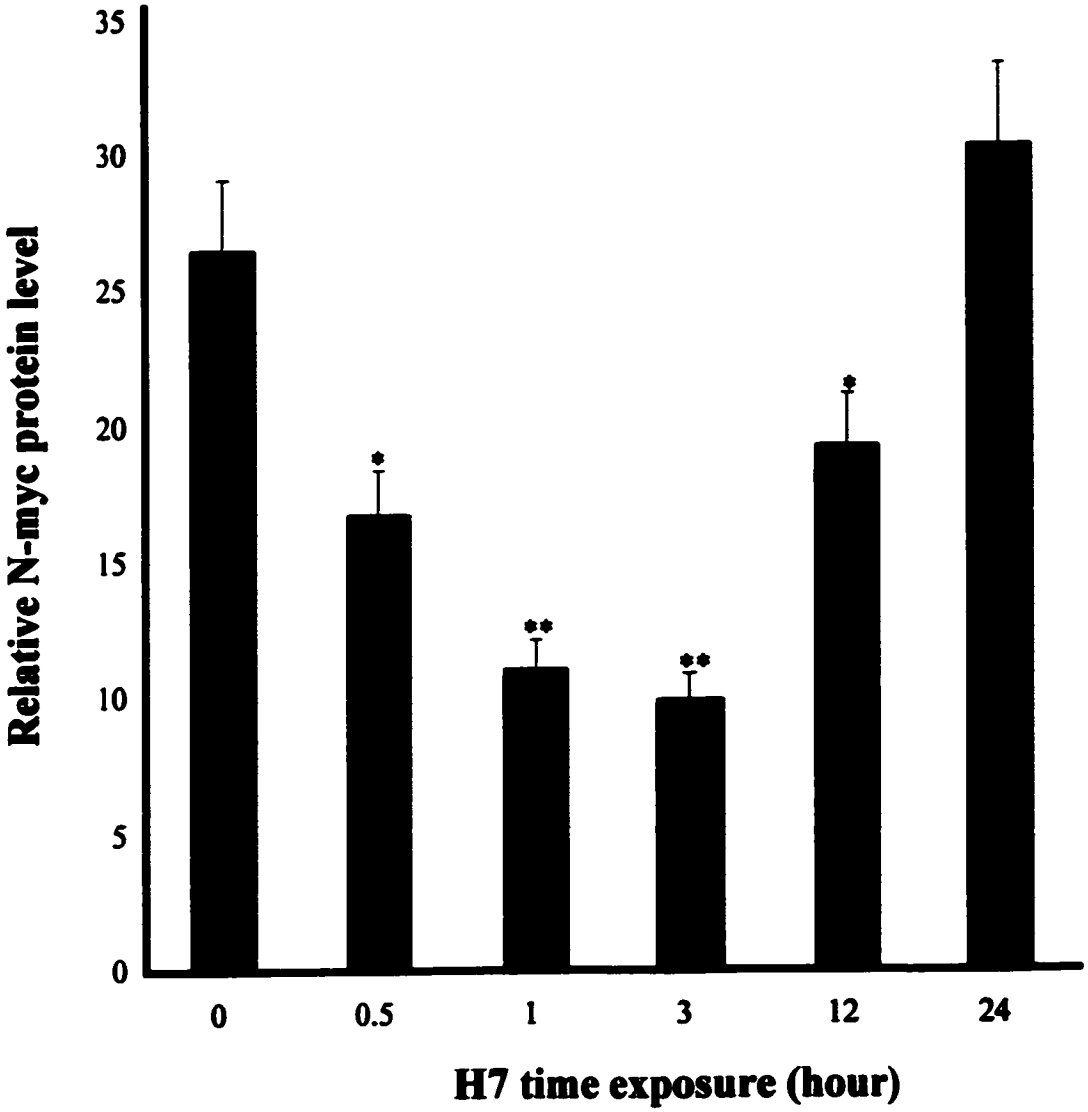
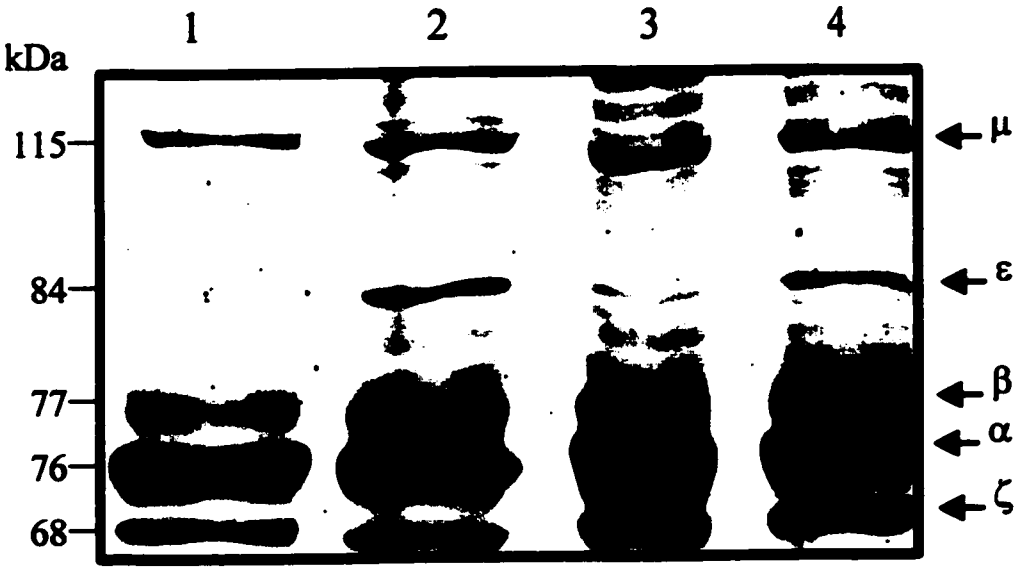

Relative N-myc protein level
35
30
25
20
15
10
5
0
*
*
**
**
0
0.5
1
3
12
24
H7 time exposure (hour)

Figure 1.34 Comparison of the profile of PKC isoforms in undifferentiated and differentiated SH-SY5Y cells

Immunoblot analysis of PKC isoforms in undifferentiated (1) and differentiated cells for 2 weeks with 8-Br-cAMP (2), AP + NGF (3) and AP + NGF + 8-Br-cAMP (4). SH-SY5Y cells were plated at $1x10^5$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Cells were treated every 2 days, starting 24 h after plating, with a combination of 100 ng per ml NGF, 0.3 μM aphidicolin and/or 0.5 mM 8-Br-cAMP for 2 weeks. Treated and untreated cells were homogenised and 100 μg protein per lane was separated by electrophoresis on 10% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with PKCα, PKCβ, PKCε, PKCζ, PKCδ, PKCη, PKCθ PKCμ and PKCγ specific antibodies, and detected using alkaline phosphatase-conjugated secondary antibodies as described in Methods. The molecular weight is expressed as kilodalton (kDa).


1
2
3
4
kDa
115
84
77
76
68
μ
ε
β
α
ζ

Differentiation of SH-SY5Y cells with the various inducers resulted in an increase in whole cell PKC activity (data not shown). Since PKC is a multigene family, we investigated which isoforms were expressed in control and differentiated cells. The expression pattern of novel and classical PKC isoforms in the SH-SY5Y cell line was analysed at the protein level to investigate which PKC isoform(s) may be involved in growth control and differentiation. Western blot analyses of extracts from undifferentiated SH-SY5Y cells revealed the presence of PKCα, β, μ and ζ isoforms. PKCε, δ, η, θ and PKCγ appear not to be expressed in the SH-SY5Y cell line (Fig. 1.34). Treatment with 8-Br-cAMP induces an increase in PKCα, β, μ with the appareance of PKCε. NGF treated-SH-SY5Y cells show a further increase in the expression of PKCα, β, μ and ζ with a lower expression of PKCε when compared to treatment with 8-Br-cAMP. The combination treatment of 8-Br-cAMP, NGF and aphidicolin induces an increase in the expression of all the PKC isoforms α, β, ε, μ and ζ. Induction of differentiation using 8-Br-cAMP alone or in combination with NGF seems to induce the expression of PKCε, β, μ and ζ (Fig. 1.34). The expression of these isoforms is probably coupled to neuronal differentiation in SH-SY5Y cells, and may play a role in neuronal differentiation.

1.4.5 PKC inhibition induced apoptosis in SH-SY5Y cells

Phenotypic changes in SH-SY5Y cells were evaluated by examining the overall morphological changes that take place up to 48h following treatments with PKC inhibitors as previously described. With time, both staurosporine (100 nM) and H7 (50 μM) induced cell death as shown in (Fig. 1.26 and Fig. 1.27). The death induced by

Figure 1.35 Determination of the cell survival profile for staurosporine treated SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated with 50, 100, 200, 400, 600, 800 and 1000 nM staurosporine for 24 h. Cells were washed, trypsinized and counted using Trypan blue dye exclusion and a hematocytometer as described in Methods. The average of three wells was used for each set of experiments. Each experiment was repeated 3 times. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).

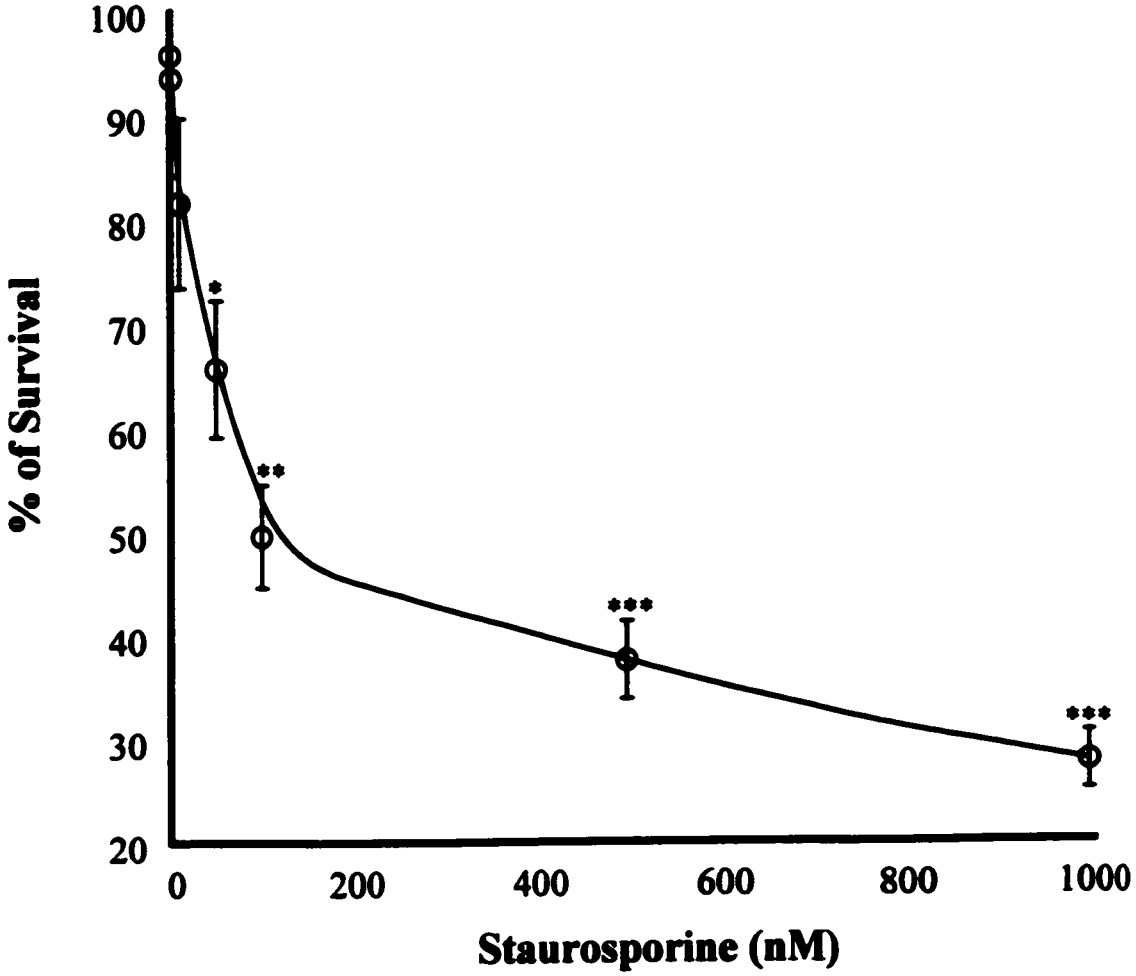

100
90
80
70
60
50
40
30
20
% of Survival
*
**
***
***
0
200
400
600
800
1000
Staurosporine (nM)

Figure 1.36 Chromatin condensation in staurosporine or H7 treated SH-SY5Y cells

Fluorescence photographs of Hoeschst- stained cells treated with staurosporine or H7. Untreated SH-SY5Y cells (**A**) were treated with 100 nM staurosporine for 1h (**B**) or 100 μM H7 for 3 h (**C**). SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated with 100 nM staurosporine or 100 μM H7 for the appropriate time periods. Cells were washed, fixed, permeabilised and stained with Hoeschst as described in Methods. Cells were viewed under fluorescence with a Nikon microscope equipped with an UV filter and photographed with a Kodak ektachrome 400 film.

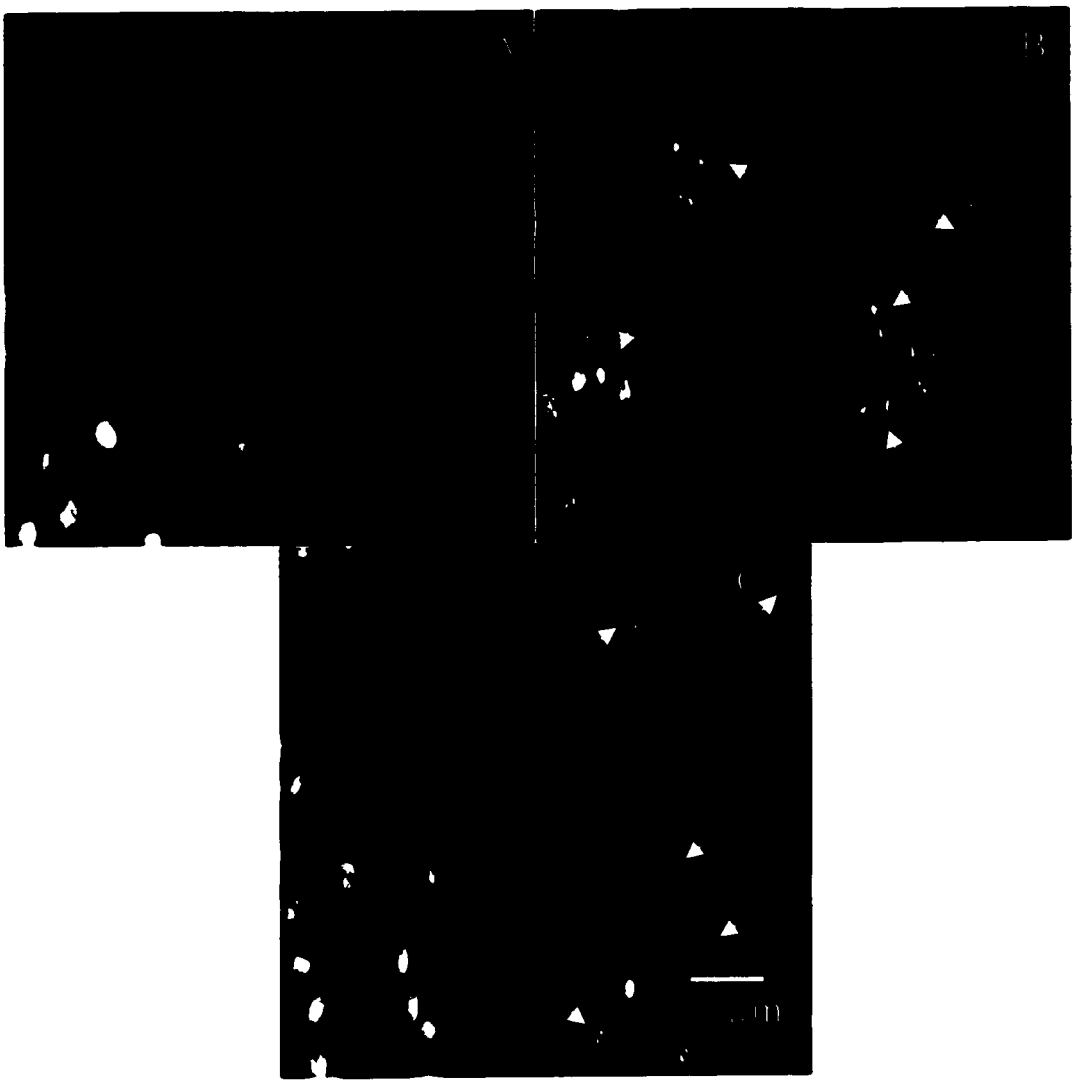
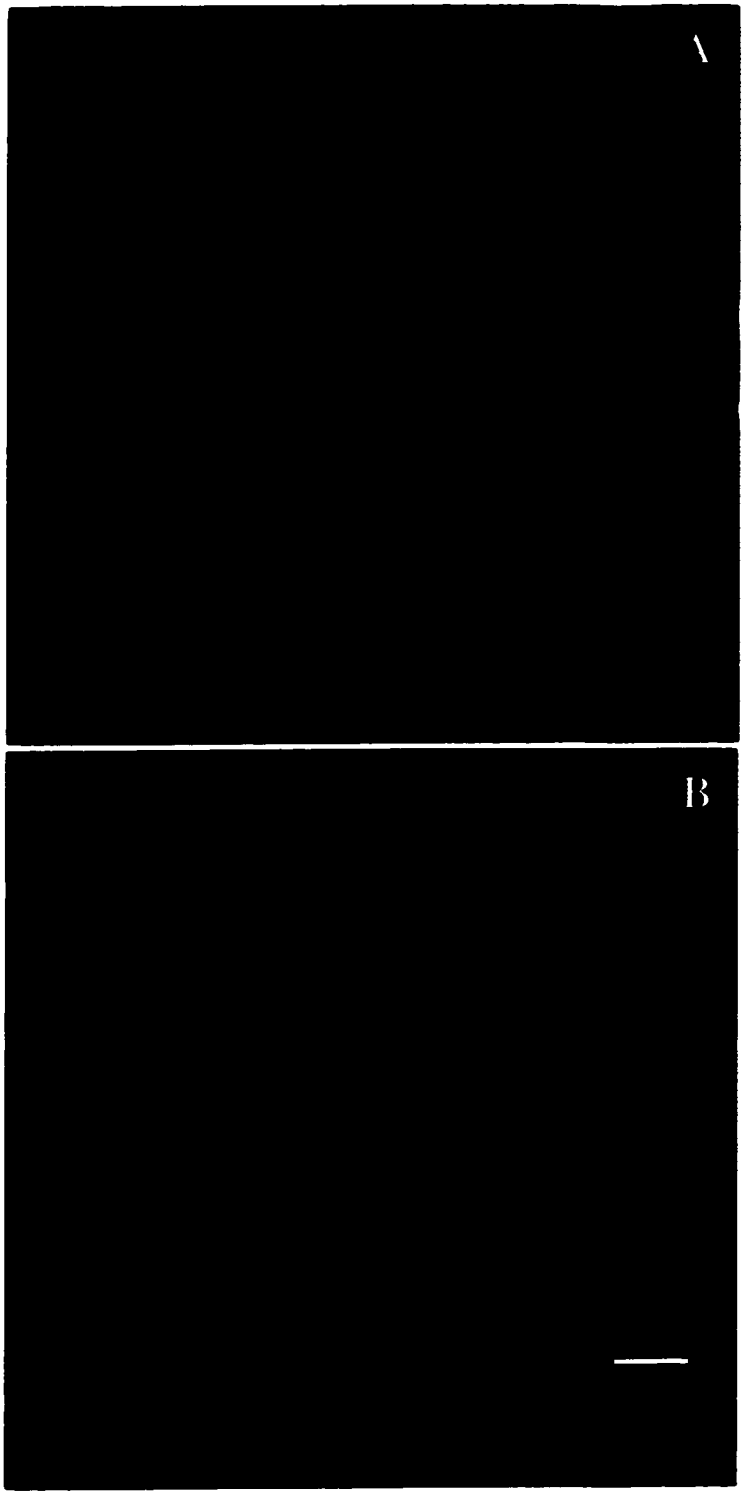


Figure 1.37 Hoechst staining of SH-SY5Y cells treated with Bis and calphostin C

Fluorescence photographs of Hoescht-stained cells treated with Bis or calphostin C. SH-SY5Y cells were treated with 2 mM Bis for 24 h (**A**) and 1 mM calphostin C for 24 h (**B**). SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated with 2 mM Bis or 1 mM calphostin for 24 hours. Cells were washed, fixed, permeabilised and stained with Hoeschst as described in Methods. Cells were viewed under fluorescence with a Nikon microscope equipped with an UV filter and photographed with a Kodak ektachrome 400 film.


A
B

staurosporine in the SH-SY5Y cell line was initially characterized by determining a concentration–dependency profile according to the procedure described in materials and methods. The cell death phenomenon appeared to be concentration-dependent (Fig. 1.35).

Hoescht staining of cells treated with staurosporine and H7 showed chromatin condensation only after 3h of treatment (Fig. 1.36 and Fig. 1.37 respectively) while Bis or calphostin C treatment did not induce any nuclear condensation (Fig. 1.38).

Bcl-2 expression was measured in both staurosporine and H7 treated cells (Fig. 1.39 and Fig. 1.40). Staurosporine treatment induces a significant decrease in Bcl-2 expression only after 12h of treatment coincident with cells dying of apoptosis (Fig. 1.39). Although H7 treatment induced a slight increase (1h) followed by a dcrease (12h) in Bcl-2, there was obvious pattern to the changes. We thus conclude that H7 has minimal effects on Bcl-2 expression (Fig. 1.40). The low number of viable cells in the presence of PKC inhibitors could be caused by an increase in apoptosis, which was investigated by staining the nuclei with Hoescht and checking for condensed and fragmented nuclei. One of the features of apoptosis is a specific pattern of chromatin condensation. When Hoechst 33258 reagent was applied, a characteristic apoptotic pattern was observed under UV illumination by means of fluorescence microscopy. Normal growing cells display a nucleus with homogenous fluorescent chromatin. In apoptotic cells, chromatin is found mostly in clustered, different sized and intensely bright round bodies of a globular appearance. Such analysis revealed a significant increase in the number of apoptotic nuclei after both treatments with staurosporine and H7 (Fig. 1.36B, C). In contrast, SH-SY5Y cells exposed to Go6976, Bis or calphostin C

Figure 1.38 Expression of the Bcl-2 gene product in staurosporine treated SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated with 100 nM staurosporine for 0, 0.5, 1, 3, 6, 8, 12 and 24 hours. Cells were harvested by treatment with 0.05% trypsin and 0.02% EDTA, washed 3 times with PBS and fixed as described in Methods. Fixed cells were incubated in PBS containing 2% BSA and immunolabelled for 1 hour to overnight with Bcl-2 specific antibody at 1 µg/ml dilution in PBS containing 0.2% BSA at 4°C then washed 3 times with PBS and incubated with fluorescein conjugated secondary antibodies at a dilution of 1:100 for one hour in PBS. Immunostained cells were washed and analysed using a Coulter Elite flow cytometer. The measured fluorescence intensity provides a relative estimation of the protein level per cell. All the experiments were repeated at least three times in duplicate. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).

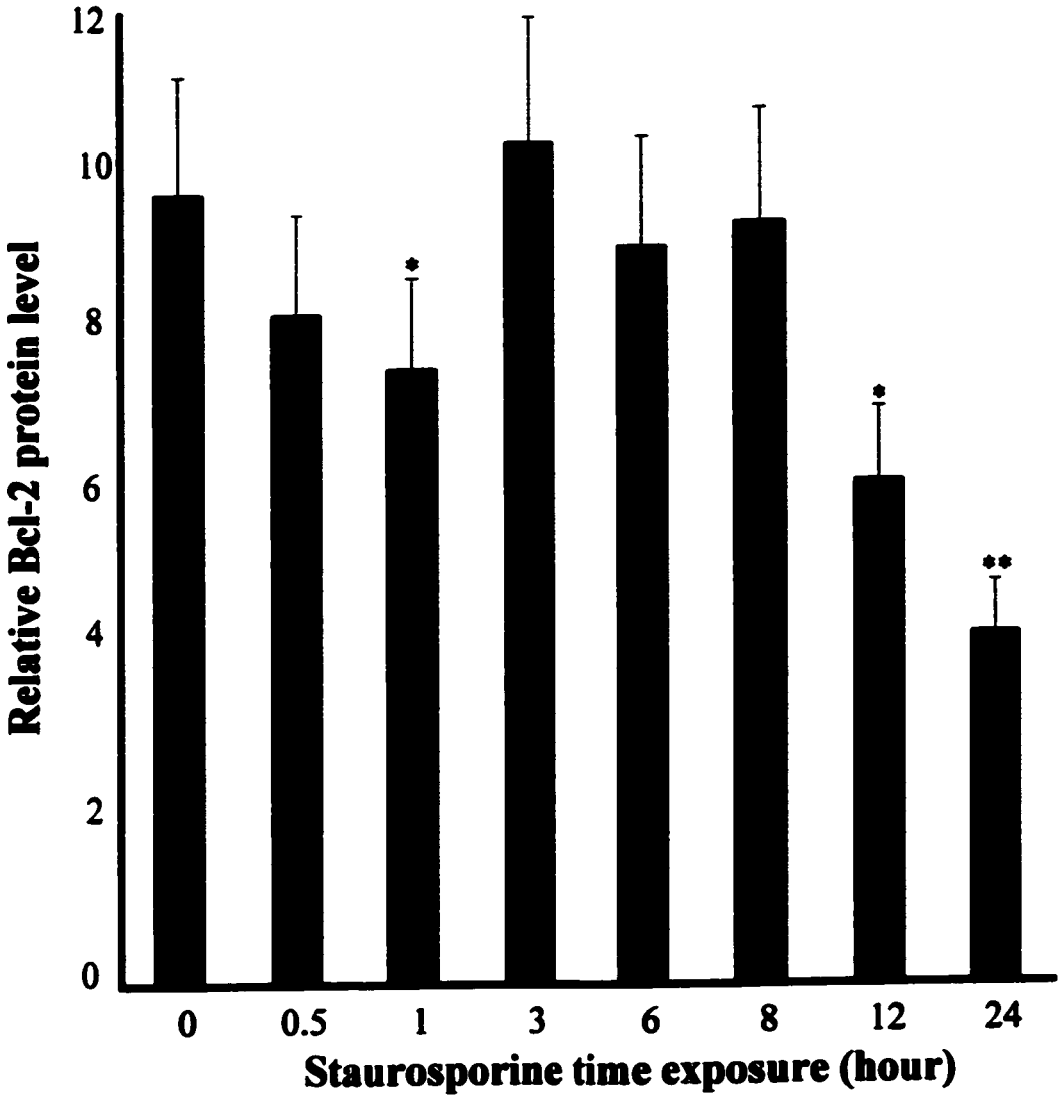
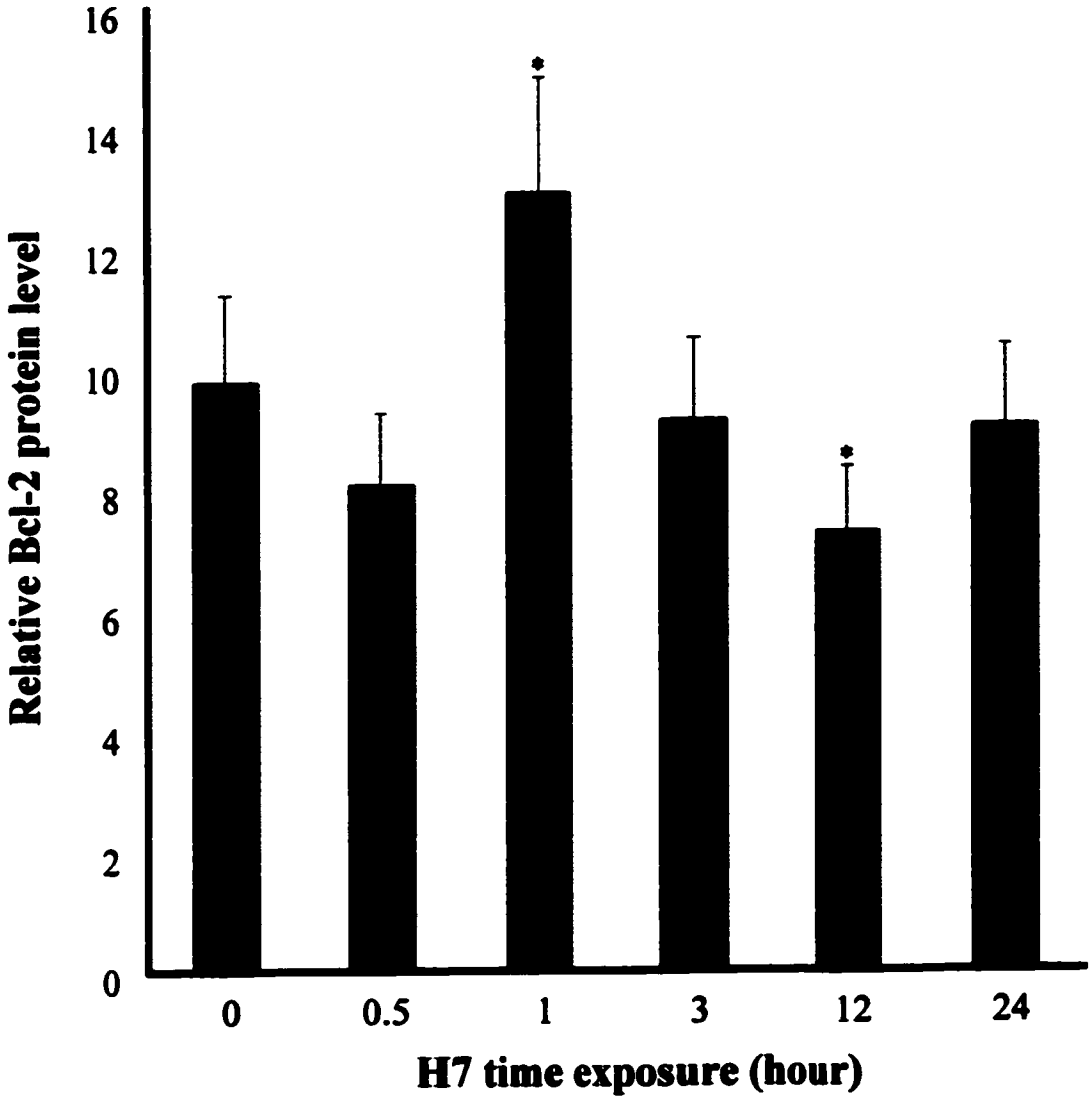

12
10
8
6
4
2
0
Relative Bcl-2 protein level
*
*
**
0
0.5
1
3
6
8
12
24
Staurosporine time exposure (hour)

Figure 1.39 Expression of the Bcl-2 gene product in H7 treated SH-SY5Y cells

SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated with 100 μM H7 for 0, 0.5, 1, 3, 12 and 24 hours. Cells were harvested by treatment with 0.05% trypsin and 0.02% EDTA, washed 3 times with PBS and fixed as described in Methods. Fixed cells were incubated in PBS containing 2% BSA and immunolabelled for 1 hour to overnight with Bcl-2 specific antibody at 1 μg/ml dilution in PBS containing 0.2% BSA at 4°C then washed 3 times with PBS and incubated with fluorescein conjugated secondary antibodies at a dilution of 1:100 for one hour in PBS. Immunostained cells were washed and analysed using a Coulter Elite flow cytometer. The measured fluorescence intensity provides a relative estimation of the protein level per cell. All the experiments were repeated at least three times in duplicate. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).


16
14
12
10
8
6
4
2
0
Relative Bcl-2 protein level
*
*
0
0.5
1
3
12
24
H7 time exposure (hour)

did not show any chromatin condensation after staining with Hoechst. Further analyses of the cell samples documented that the cells were undergoing apoptotic death. Most of the morphological hallmarks associated with apoptosis were detectable, including cell shrinkage, intranucleosomal fragmentation and chromatin condensation.

1.5. Discussion

1.5.1 Neural crest cells, NB and differentiation

NB is a tumour derived from an immature neural crest cell that can be induced to differentiate *in vivo* (Black & Haase, 1999; Catelberry, 1997; Cooper *et al.*, 1991). In general, the literature describes NB differentiation *in vitro* by measuring the extent of neurite extension and the expression of some neuronal markers with little regard to other important differentiation criteria (Brodeur & Goldstein, 1976). These criteria include the expression of neuronal markers of the cystoskeleton, neurone function and final withdrawal from the division cell cycle in addition to the emission of neurites (Brodeur & Goldstein, 1976; Brodeur et al., 1992; Haycock, 1993; Malik *et al.*, 2000). An important criterion is that if the differentiation inducers are withdrawn from terminally differentiated neurones, the cells should now be dependent on neurotrophins for the maintenance of the differentiated state and for survival. During embryonic development, it is generally held that morphological differentiation of neural progenitors of the CNS, in contrast with those of the PNS, occurs after cells have ceased proliferating. Neoplastic cell lines of neural crest origin, such as various neuroblastomas, have been used as models for investigating neuronal differentiation and proliferation (Pahlman *et al.*, 1990; Pahlman 1981). *In vitro*, several agents can induce NB to differentiate, presumably by activating distinct biochemical signal transduction pathways that have not yet been fully deciphered. Signalling involving activation of PKA and PKC plays a leading role in inducing neuritogenesis and the arrest of cell proliferation (Carter, 2000; Kanashiro *et al.*, 1998; dos Santos *et al.*, 2000; Kim *et al.*, 1997; Lee *et al.*, 1999).

The peripheral nervous system consists of a large number of neuronal cell types that vary in their morphology and repertoire of neurotransmitters synthesised. To date, there are a limited number of cell lines that have been characterised as models for differentiation of specific neuronal cell types of the peripheral nervous system. For example, PC12 cells provide a model for adrenergic sympathetic neurones. P19 embryonic carcinoma cells provide a model for primitive cells that can give rise to both neurones and glia. Since the neurones of the peripheral nervous system are complex, it would be beneficial to identify other cell lines that may represent subsets of neurones. The neural crest-derived childhood tumour, neuroblastoma, is a potential source of a large variety of committed neural crest cells that have been arrested in their differentiation. These tumours have been observed to spontaneously differentiate *in vivo* into derivatives of the neural crest following exposure to differentiation agents or transfection with TrkA (Ciccarone *et al.*, 1989; Brodeur *et al.*, 2000; Eggert *et al.*, 2000b).

Anderson (1997), have hypothesised that the differentiation of neural crest cells is a multistep process where crest cells are progressively restricted in their developmental fates as they migrate and are subjected to different environments containing a variety of growth factors. Our results suggest that SH-SY5Y cells represent one of these populations of cells that have been committed to a neuronal fate but have not yet fully differentiated.

In this study, terminal differentiation of SH-SY5Y cells was induced. The differentiation conditions were optimised in order to obtain terminally differentiated cells by testing several differentiation inducers. These included 8-Br-cAMP, retinoic

acid and the neurotrophic factor NGF, supplemented individually or in combination. The most potent treatment was a combination of 8-Br-cAMP, aphidicolin and NGF.

1.5.2 Effect of neuronal differentiation on cell growth

1.5.2.1 Effect of differentiation inducers on SH-SY5Y cell growth

RA is a naturally occurring compound that has been shown to have a wide variety of effects on vertebrate morphogenesis (for review see Morriss-Kay & Ward, 1999). In particular, the teratogenic properties of RA appear to affect many cell types including derivatives of the neural crest (Reynolds, 2000). Furthermore, RA has clinically been used to treat acute promyelocytic leukaemia and neuroblastoma by inducing terminal differentiation in these malignant cells. RA affects the neurogenic component of neural crest cells by either promoting their survival or inducing their differentiation (Giannini *et al.*, 1997).

In this study, we showed that SH-SY5Y cells treated with 10^{-6} M tRA for 2 weeks, responded by extending short neurites after only 2 days of treatment when compared to the other differentiation inducers (Fig. 1.1). The cellular differentiation observed in SH-SY5Y cells was associated with a considerable reduction in proliferation rate (Fig. 1.3) that was estimated by measuring the expression of PCNA (Fig. 1.4). It was previously reported that both tRA and its isomer 9-cis RA induce SH-SY5Y cells to extend neurite-like processes in a time- and dose-dependent manner (Lovat *et al.*, 1994). Moreover, 9-cis RA was shown to have a greater effect on the reduction of cell proliferation than its isomer tRA in LAN-5 and SH-SY5Y cells (Redfern *et al.*, 1994; 1995). We found that the tRA isomer 9-cis RA induced longer neurites but was toxic to

the cells.13-cis RA isomer had no effect on either cell, differentiation or cell growth (data not shown). 9-cis RA toxicity may limit its clinical use for neuroblastoma (Hewson *et al.* 2000).

tRA exerts its effects by binding to its nuclear receptors RAR and RXR that are part of the steroid/thyroid/vitamin D superfamily. These receptors function as dimers and directly modulate transcription activity by interacting with DNA sequences that contain retinoic acid response elements, RAREs (Niles, 2000). RAR specifically binds tRA and 9-cis RA, whereas RXR have affinity for only 9-cis RA (Levin *et al.*, 1992). Both tRA and 9-cis retinoic acid induce the expression of RARβ in a number of NB cell lines (Redfern *et al.*, 1995). This suggests that the pathway by which tRA regulates gene expression is intact in most NBs. 9-cis RA and tRA were shown to enhance the potency in the induction of neuroblastoma differentiation and apoptosis (Han *et al.*, 1995), (Lovat *et al.*, 1997).

In this study tRA induced a decrease in cell proliferation. SH-SY5Y cells continued to proliferate albeit less than control cells. Proliferation of cells treated for one week with tRA was inhibited by 50% when compared to control cells (Fig. 1.3) and the level of PCNA was decreased by 30% (Fig. 1.4). In most sensitive cells, retinoic acid blocks cell-cycle progression in the G1 phase of the division cycle by modulating the expression of the cell-cycle regulatory proteins. In human neuroblastoma cells, G1 arrest was reported to occur within 2 days of retinoid treatment and there was an increase in cyclin-dependent kinase (cdk) inhibitor $p27^{kip1}$ but not in $p21^{Waf1/cip1}$ (Matsuo & Thiele, 1998). This increase was coincident with a decrease in cdk activity and an increase in G1 cyclin/kinase-bound p27 (kip1). The reason for induction of $p27^{kip1}$ instead of

p21Waf1/cip1 is not known but may be due to a direct regulation of p27 gene expression by RAR (Niles, 2000). In immortalised human bronchial epithelial cells, RA induces cell growth arrest through a marked decline in cyclin D1 protein but not in cyclin D1 mRNA. This was due to enhanced ubiquitin-dependent proteosomal degradation of cyclin D1 (Lagenfeld *et al.*, 1997). The mechanism by which retinoic acid enhanced this proteolysis remains unknown. In a human breast cancer cell line, tRA was reported to reduce the mRNA level of cyclin D1 and the cyclin-dependent kinase 2 (cdk-2), followed by a decrease in their protein levels. Extracts from retinoid treated cells also contained a cdk-2 inhibitory activity (Teoxeora *et al.*, 1997). None of these studies addressed the mechanism by which RA changed the level of cyclin, cdk, or the cdk inhibitor.

We also showed that tRA induced a significant decrease in the expression of the N-myc oncogene product (Fig. 1.13), consistent with its effect on cell proliferation and its use for neuroblastoma therapy. However, the expression of the NGF high affinity receptor TrkA, one of the prognostic factors used for NB, remained unchanged (Fig. 1.15) while the expression of H-ras increased in tRA treated cells (Fig. 1.14). N-myc was found to be expressed at a high level in SH-SY5Y cells. N-myc amplification correlates with a poor prognosis in NB (Brodeur, 1984). RA was found to decrease the expression of N-myc and this was synergistic with the addition of the interferon-γ to NB cells (Wada *et al.*, 1997). Transfection of SH-EP NB cells with N-myc oncogene enhanced its expression and induced an advanced malignant phenotype in these cells (Schweigerer *et al.*, 1990). Furthermore, antisense inhibition of N-myc expression decreases the proliferation rate and the induction of neuronal differentiation in SH-

SY5Y cells (Negroni *et al.*, 1991). The mechanism by which tRA modulates N-myc expression is still unclear. tRA was shown to block cell cycle progression in G1 through many mechanisms. Down-regulation of N-myc expression in NB cells is probably one of these mechanisms.

It is accepted that RA affects differentiation through transcriptional regulation of genes directly involved in the differentiation process (Niles, 2000). SH-SY5Y cells treated with tRA for two weeks showed a substantial enhancement of the neuronal marker, neurofilament NF68 and the neurone-specific enolase (Fig. 1.6; Fig. 1.7) and a slight increase in neurofilament NF200 (Fig. 1.5) protein expression. However, tRA treatment did not enhance the expression of TH or Chat, the markers that are characteristic for neuronal function (Fig. 1.10; Fig. 1.12). tRA seems to modulate more differentiation associated with cytoskeleton rearrangement than neuronal markers associated with neuronal function such as TH and Chat (Iwase *et al.*, 1994; Brodeur & Goldstein, 1976) except for NSE (Ishiwata *et al.*, 1989). NSE was shown to be expressed in some neuroblastoma biopsies (Abdennebi *et al.*, 2000; Berthold *et al.*, 1991). While RA down-regulation of N-myc expression may be mediated via an indirect effect on cell cycle elongation or on the N-myc promoter, RARα, β or δ are likely to play some role in the down-regulation of N-myc by tRA as shown in breast cancer (Liu *et al.*, 1996). Treatment with RA induced the resistance of NB cells to cytotoxic agents (Niles, 2000). This effect was associated with the suppression of the apoptotic response to anti-neoplastic agents in differentiated cells that expressed very high levels of Bcl-2 (Fotsis *et al.*, 1999). This is what prompted us to investigate whether we could lead cells

to terminal differentiation *in vitro*, using a combination of known differentiation inducers.

8-Br-cAMP-treated SH-SY5Y cells showed a decrease in their proliferation when compared to controls. The cell growth was inhibited by almost 50%. Furthermore, the tRA and 8-Br-cAMP combined treatment induced a further down-regulation of the cell cycle progression. In contrast, NGF alone did not have any effect on cell proliferation. Aphidicolin, the cell cycle inhibitor, stopped cell growth as expected. The most efficient treatment on the reduction of cell proliferation was 8-Br-cAMP combined with aphidicolin and NGF (8-Br-cAMP + NGF + AP) (Fig. 1.3). 8-Br-cAMP is an analogue of cAMP, a protein kinase A activator. It is a cell permeable molecule that activates the cAMP-mediated signal transduction pathway. Differential expression of PKA isoforms have been linked to the control of cell growth (Cho-Chung, 2001) An enhanced expression of RI PKA correlates with active cell growth and transformation, whereas an increase of RII PKA is related to growth inhibition and differentiation (Kim *et al.*, 2000). In NB cells, 8-Br-cAMP has been shown to arrest cell proliferation and induce differentiation (Prasad *et al.*, 1994). It is not known whether cell cycle arrest resulted from PKA activation triggering differentiation or, conversely, whether it is the progress into differentiation that controls proliferation. There are reports that RA signals change the activity and intracellular distribution of PKA in F9 teratocarcinoma cells (Plet *et al.*, 1982; 1987). In addition, F9 cells respond to db-cAMP, another PKA activator, only when treated with RA, suggesting that the tRA signal may change the responsiveness to cAMP. Recently, it was found that RA-induced growth arrest and

neuronal differentiation was mediated by an increase of the RII β activity in SH-SY5Y cells (Kim *et al.*, 2000).

1.5.2.2 Effects of differentiation inducers on neurite extension and expression of neuronal markers

In this study, we have shown that activation of PKA through treatment with the cAMP analogue, 8-Br-cAMP, induced morphological and biochemical differentiation and down-regulated cell proliferation of the SH-SY5Y cells. Long-term treatment of several neural crest cells and NB cell lines with forskolin or dibutyryl cAMP arrests cell proliferation and induces morphological changes with neurite spreading (Mena *et al.*, 1995; Galderisi *et al.*, 1999; Girgert *et al.*, 2000).

At the morphological level, both cAMP and tRA induced neurite extension after only 2 days treatment that were maintained for 2 weeks (Fig. 1.1). Other cell types, such as ras-transformed mouse fibroblasts and human leukaemia cells showed differentiated characteristics upon treatment with 8-Br-cAMP. In neural cells, cAMP exerted a control on the mechanism for neurite initiation (Mena *et al.*, 1995). In PC12 cells, cAMP induced initiation of neurite outgrowth, involving a rapid but unstable re-organisation of the cytoskeleton that is independent of RNA synthesis. In contrast, NGF alone achieved a more stable cytoskeletal reorganisation inducing an RNA-dependent mechanism in the same cells (Nakagawara *et al.*, 1998). In this study, SH-SY5Y cells did not seem to respond to NGF treatment, since no neurite extensions were observed. This is in agreement with data previously reported (Lavenus *et al.*, 1994; Pohula *et al.*, 1995) and suggests that the failure of SH-SY5Y cells to respond to NGF treatment is a consequence of low TrkA receptor expression, although the TrkA signalling pathway

itself seems to be intact in these cells (Eggert *et al.*, 2000c). When cells were treated with a combination of aphidicolin and NGF, we noticed a NGF response through an induction of neuronal differentiation. This is probably due to an enhancement of TrkA expression by aphidicolin. Indeed, antimitotic agents such aphidicolin and mitomycin C were shown to induce TrkA even more efficiently than high doses of NT-3 and CNTF in sympathetic neuroblasts, suggesting that this neurotrophin receptor is primarily a consequence of mitotic arrest (Verdi & Anderson, 1994). Furthermore, SY5Y, the SH-SY5Y parental cell was shown to be induced to differentiate by a combination of aphidicolin and NGF.

Treated cells expressed neuronal markers such as synaptophysin and tau and showed neurite outgrowth. Furthermore, we noticed that the NGF + 8-Br-cAMP treatment also had a synergistic effect on SH-SY5Y cells morphological differentiation and growth arrest. When cells were treated for 3 weeks with the combination treatment NGF + 8-Br-cAMP + AP, they showed the longest neurite extension and the lowest level of cell growth (Fig. 1.2, Fig. 1.3, Fig. 1.4). In addition, cells could be maintained for another 3 weeks if supplemented with NGF, and they started forming a neuronal network (Fig. 1.2; Fig. 1.2a). It has been shown in PC12 cells that NGF and cAMP have a synergistic effect on neurite outgrowth. Aphidicolin combined with NGF + 8-Br-cAMP, induced the highest level of morphological differentiation in SH-SY5Y cells. This was accompanied by an increase in molecular differentiation markers. The synergistic effect of aphidicolin with NGF + 8-Br-cAMP is probably due to its effect on proliferation since it is reported in the literature that growth arrest favours the expression of TrkA (Verdi & Anderson, 1994; Bulseco *et al.*, 2001; Kokunai *et al.*, 1999). Furthermore, cessation of proliferation due to aphidicolin was reported to increase

intracellular choline and acetylcholine levels in the absence of any change in choline acetyltransferase activity in neuroblastoma cells.

Morphological differentiation was characterised at the molecular level by measuring the expression of neuronal markers. We have chosen three differentiation markers of the cytoskeleton of neurones and two markers for neurone function. These markers are NF200, NF68, NSE, Chat, and TH. 8-Br-cAMP and NGF treatments increased the level of NF200, NF68 and NSE. Furthermore, a combined treatment of 8-Br-cAMP + NGF + AP gave the highest level for neurofilaments and NSE. The presence of the neuronal cytoskeletal protein NF200 suggests that outgrowth from differentiated NB cells belong to the axonal neuritic class of dendrites (Kong *et al.*, 1998). The expression of NF200 proteins is known to characterise neurites as axons (Wu *et al.*, 1998). NSE is the neurone-specific enolase, an enzyme expressed in neurones. It is also expressed in NB cells but at a relatively low level (Abdennebi *et al.*, 2000).

Acetylcholinesterase is a key component in the transmission of the nerve impulse and is also expressed non-synaptically during embryonic development (Henderson, 1996). Abnormalities in Chat expression are seen in neuronal tumours and degenerative disorders (Brodeur & Goldstein, 1976). This unusual expression is believed to be associated with a novel function of the enzyme related to differentiation and cell-adhesion (De *et al.*, 2000). The cAMP dependent pathway contributes to the regulation of human acetylcholinesterase expression (Wan *et al.*, 2000).

Tyrosine hydroxylase is the rate-limiting enzyme critical to catecholamine biosynthesis. It has been established that cAMP causes up-regulation of this protein (Kim *et al.*, 1994). Treatment with 8-Br-cAMP alone and with 8-Br-cAMP + NGF + AP caused a 50 and 70-fold increase in TH expression respectively (Fig. 1.10). Forskolin, an

intracellular cAMP inducer, was also reported to induce a 30-fold increase in the expression of this enzyme. In adult mammals, TH expression is confined to a small number of neuronal groups in the central nervous system and to the neurones of the sympathetic nervous system and neuroendocrine chromaffin cells of the adrenal medulla in the PNS (Ginns *et al.*, 1988). Since neuroblastomas are derived from the PNS, the expression of this enzyme in differentiated SH-SY5Y cells at high levels compared to that of undifferentiated cells indicates that these cells have ongoing catecholamine biosynthesis. The levels and activity of TH are finely and differentially regulated in these cells by a large variety of pharmacological and physiological stimuli (Iwase *et al.*, 1994; Kim *et al.*, 1994; Rabinovsky *et al.*, 1995). Our results demonstrated that differentiated SH-SY5Y cells possess a cast of molecular players induced in the display of neuronal functions and features.

1.5.2.3 Characterisation of morphologically differentiated SH-SY5Y cells

N-myc amplification in human neuroblastoma has been correlated with advances of disease and rapid tumour progression, suggesting that N-myc plays an important role in determining the biological behaviour of NB (Brodeur *et al.*, 1984), and that it is involved in determining tumour progression (Schweigerer *et al.*, 1990; Fotsis *et al.*, 1999). SH-SY5Y cells have a single copy of N-myc (Wada *et al.*, 1988). The N-myc gene, a member of the myc gene family of transcription factors, encodes a nuclear phosphoprotein frequently amplified in neuroblastomas. The N-myc protein is a normally short-lived transcription factor with a half life ranging between 30 and 50 min as determined in several N-myc amplified NB cell lines (reviewed in Henriksson &

Luscher, 1996). N-myc is expressed transiently during development and differentiation of neurones (Mugrauer *et al.*, 1988).

In the present study, we measured the expression of the N-myc protein in both undifferentiated NB and in cells that had been induced to differentiate using various differentiation protocols. We showed that both tRA and 8-Br-cAMP treatment decreased the level of N-myc expression consistently with cells undergoing neuronal differentiation (Mugrauer *et al.*, 1988). Furthermore, a combination treatment of 8-Br-cAMP+tRA induced the extension of longer neurites (Fig. 1.1D) and the expression of neuronal markers including NF200, NF68, TH and Chat (Figs. 1.5, 1.6, 1.7, 1.11, 1.12).

The 8-Br-cAMP, NGF and AP combination demonstrated an additive effect on the decrease in N-myc protein expression (Fig. 1.13). Negroni *et al.*, (1991) observed that antisense inhibition of N-myc expression decreases the proliferation rate and the induction of neurone differentiation. In this treatment, aphidicolin seemed to enhance TrkA expression and SH-SY5Y cells responsiveness to NGF. In addition, it was shown that PKA activation also increased cell expression of TrkA (data not shown). Recently, it was shown that transfection of SH-SY5Y cells with the TrkA receptor and subsequent NGF treatment restores the NGF signal from the membrane receptor to the nucleus independent of the N-myc status. NGF-mediated neuronal differentiation, growth inhibition and survival is suppressed in N-myc amplified NB. N-myc could therefore alter the repertoire of cell surface receptors involved in mediating growth and differentiation (Eggert *et al.*, 2000b).

It has been suggested that N-myc may participate in the control of early differentiation events in the developing embryo (Khanna *et al.*, 1995). Expression of myc oncogenes might be required to retain the cells in an undifferentiated state

(Henriksson & Luscher, 1996). The various members of the myc family, including N-myc and c-myc proteins, have been implicated in the control of transcription and differentiation, but the physiological target for myc-mediated transcriptional activation has not been identified (Bannasch *et al.*, 1999). We demonstrated that differentiation induced a decrease in N-myc expression. This is probably due to an increase of N-myc degradation since N-myc was shown to be a substrate for the 26S proteosome in neuroblastoma cells (Bonvini *et al.*, 1998). There are reports that support our data. Chen *et al.*, (1990) found that three of six NB cell lines which have a single copy of N-myc as does SH-SY5Y cells could differentiate in response to NGF, whereas nine N-myc-amplified cell lines did not respond to NGF. The human NB cell line SH-SY5Y was reported to have a normal N-myc gene copy, express very low levels of TrkA, and not to be responsive to NGF (Azar *et al.*, 1994). It was thus intriguing that SH-SY5Y cells responded only to NGF following 8-Br-cAMP and AP treatment or in the presence of aphidicolin. It was shown that NGF + AP and 8-Br-cAMP + NGF + AP induced phosphorylation of the TrkA in SH-SY5Y (Fig. 1.17). The response of SH-SY5Y to NGF may be related to the expression of TrkA to a higher level. We, indeed, have evidenced that 8-Br-cAMP treatment potentiates the expression of TrkA and that the NGF response was associated with phosphorylation of TrkA (data not shown). In contrast, cells that were treated with NGF alone did not show any neurite outgrowth and immunoblot analysis did not show any phosphorylation (data not shown).

TrkA is present in NB with favourable prognosis and is highly correlated with patient survival (Nakagawara, 1993). TrkA mediates the NGF response. TrkA expression in NB cells was shown to promote growth inhibition and neuronal differentiation when expressed in single copy N-myc cell lines (Eggert *et al.*, 2000c).

Transfection of SH-SY5Y with TrkA gene restored TrkA autophosphorylation, phosphorylation of MAPK/Erk, and immediate-early gene induction in response to NGF (Eggert *et al.*, 2001). When the combined treatment was continued for another three weeks SH-SY5Y cells continued emitting neurites and when 8-Br-cAMP treatment was stopped, cells did not show any characteristics of cell death. This observation supported the idea that the presence of NGF is sufficient to support the survival of differentiated SH-SY5Y cells. Neuroblastoma regression observed in patients with favourable prognosis is associated with the expression of TrkA (Nakagawara *et al.*, 1993). In addition, the NGF/TrkA signalling system in the aggressive NBs seems to be impaired (Nakagawara *et al*, 1994; Nakagawara & Brodeur, 1997). Thus, expression of TrkA with normal signalling function may be indispensable to lead the neuroblastoma to regress by undergoing differentiation and /or apoptosis.

NBs respond to other neurotrophic factors which include glial cell line-derived neurotrophic factor (Hishiki *et al.*, 1998), neurturin (Hishiki *et al.*, 1998), pleitrophin (Nakagawara *et al.*, 2001), midkine (Nakagawara *et al.*, 2001), basic fibroblast growth factor (Wanaka *et al.*, 1990), and insulin growth factor (El-badry, 1991). These factors function to promote survival and differentiation in NB in an autocrine/paracrine manner (Hishiki *et al.*, 1998). According to the literature, NGF is the strongest factor to induce differentiation of the favourable NB cells expressing TrkA (Hishiki *et al.*, 1998; Nakagawara & Brodeur, 1997). Furthermore, introduction of TrkA into NB cells with amplified N-myc leads to a marked growth inhibition in the absence of NGF, whereas NGF treatment results in increased proliferation. However, introduction of TrkA in SH-SY5Y, which have only one copy of N-myc resulted in growth inhibition and neuronal

differentiation in the presence of NGF (Eggert *et al.*, 2001). This indicates that N-myc expression might affect neurotrophin signal transduction pathways.

During sympathetic neuronal development *in vitro*, TrkA expression occurs before growth arrest, suggesting that differentiation and growth arrest are two distinctly regulated phenomena in neuroblasts (Anderson, 1997). This might explain the increase in cell proliferation observed when SH-SY5Y cells were treated with NGF alone (Fig. 1.5) and their response to NGF when cell growth was blocked by aphidicolin (Fig. 1.17). Therefore, *in vivo*, the signalling through the NGF/TrkA system may be the main regulator of differentiation and survival in NB with favourable prognosis. However, retinoic acid pre-treatment was shown to trigger BDNF signalling and induce neuronal differentiation in these cells, (Encinas *et al.*, 1999 and 2000).

The RAS/MAPK signalling transduction pathway was shown to mediate NGF-induced differentiation, whereas inhibition of the PI3K survival pathway has no effect on differentiation signalling (Eggert *et al.*, 2000c; Kaplan & Miler, 1997). H-ras protein is used as a marker for favourable NB prognosis (Brodeur et al., 2000; Eggert *et al.*, 2000d). We investigated the expression of H-ras in differentiated and undifferentiated SH-SY5Y cells. We found that differentiation induced either by PKA activation and NGF treatment or by PKC inhibition induced an increase in H-ras expression. The mechanism by which H-ras is involved in neuronal differentiation in NB has not been studied yet. However, it may be related to the TrkA signalling pathway. In order to confirm this we needed not only to measure the expression of H-ras, but also its activation in terminally differentiated SH-SY5Y cells. In addition, $p21^{ras}$ is the product of the cellular oncogene ras, it is known to play an important role in the regulation of signal transduction that has been implicated in transformation *in vitro*, and

tumourigenesis *in vivo* (Marshall, 1991). Furthermore, microinjection of the oncogene protein in PC12 cells induces morphological differentiation (Bar-Sagi & Feramisco, 1985). This is consistent with the enhanced H-ras protein expression observed following SH-SY5Y neuronal differentiation and cell growth arrest.

In conclusion, it was clearly demonstrated that the inhibition of the cell cycle might stimulate the expression of TrkA in response to NGF and that 8-Br-cAMP and NGF together gave the most pronounced effect in both the expression of TrkA and its phosphorylation in order to mediate neuronal differentiation in these cells. This differentiation was estimated at the level of neurite extension, growth inhibition, expression of the neuronal markers and the dependency on NGF for survival. These cells expressed neuronal markers of mature neurones and cytologically resemble sympathetic neurones (Fig. 1.2 and Fig. 1.2a), especially when exposed to a long-term treatment (6 weeks). In addition, treatment of SH-SH5Y cells with aphidicolin and NGF was shown to up-regulate the mRNAs for the low affinity NGF receptor and TrkA. A similar mechanism is proposed for developing neurones, in which aphidicolin enhances differentiation of sympathetic neuroblasts by inducing expression of TrkA (Verdi & Anderson, 1994). In addition, PC12, a rat adrenal medullary pheochromocytoma cell line, responds to NGF in a cell cycle-specific manner. PC12 cells treated with NGF showed a down-regulation of proliferation caused by a G1 block after 2 weeks of treatment (Klein *et al.*, 1991). Induction of p21 by NGF has also been shown to occur under certain conditions in SH-SY5Y cells and in fibroblasts ectopically expressing TrkA (Nakagawara, 1998a). Superior cervical ganglia sympathetic neurones require NGF for survival and in these neurones a number of cell cycle protein mRNAs such as p53 and pRb persist after differentiation (Henderson, 1996). Evidence was also brought

that the mature SH-SY5Y cells were terminally differentiated by the combined treatment and mimic the steps of embryonic neuronal development. Finally, this model of cell differentiation might be useful to study transduction pathways during neurogenesis.

1.5.2.4 Terminally differentiated SH-SY5Y and NGF-survival dependency

Trk molecules and their ligands are utilised in the developing nervous system in regulating the survival of developing neurones (Barbacid, 1993). During the development of the nervous system, many more neurones are made than are required for proper neuronal patterning and these extra neurones undergo programmed cell death if proper signals are not received at their target sites (Henderson, 1996). Following NGF withdrawal, terminally differentiated SH-SY5Y cells also undergo cell death subsequent to differentiation (Fig. 1.19, Fig. 1.20, and Fig. 1.21). One simple hypothesis to explain this cell death is: if the neurones that differentiate fail to receive the signals that would be required for their survival *in vivo*, they undergo programmed cell death (Anderson, 1997). Three likely candidates exist that would be capable of promoting the survival of SH-SY5Y derived neurones, NGF, BDNF and NT-5. These ligands bind to the TrkA and TrkB receptors, which are induced in terminally differentiated SH-SY5Y cells (Eggert *et al.*, 2000c; Nakagawara *et al.*, 1994). It has recently been suggested that the differentiation and survival of SH-SY5Y cells is controlled by BDNF (Marcus & Tarbell, 1997) and that withdrawal of BDNF from retinoic acid differentiated SH-SY5Y induced apoptosis (Encinas *et al.*, 1999). If this is the case, nerve growth factor may play a role in the survival of the cells. This is consistent with the expression of TrkA being

induced late in the differentiation process (Fig. 1.17). The observed cell death in terminally differentiated cells after deprivation from NGF, (confirmed by the elevated numbers of cell death, apoptotic bodies, i.e., cells with fragmented nuclei and/or condensed chromatin (Fig. 1.19, 1.20 and 1.21)), demonstrated that SH-SY5Y cells became NGF-survival–dependent like neurones.

The mechanisms of cell death following withdrawal of trophic factors may be quite distinct from those that occur in response to p75 signalling. The p75 neurotrophin receptor was shown to mediate neuronal apoptosis and to be essential for naturally occurring sympathetic neurone death (Bamji *et al.*, 1998). Cell death following NGF deprivation can be inhibited by Bcl-2, whereas p75-induced death can not be inhibited by bcl-2 (Ahn *et al.*, 2000). Bcl-2 was shown to be overexpressed in many NB cell lines including SH-SY5Y (Raguenez *et al.*, 1999). In neurones which express TrkA as well as p75, it is widely held that NGF withdrawal leads to cell death due to a lack of anti-apoptotic signalling through TrkA (for review see Sofroniew, 2001). One of the ways Trk signalling opposes apoptosis may be by inducing phosphorylation of Bad, which inhibits the formation of heterodimers of Bad and Bcl-xl (Chao, 1992). Withdrawal of Trk signalling may effectively lead to sequestration of Bcl-xl, allowing apoptotic signals to proceed unopposed (Huang *et al.*, 2001). This still begs the question of what gives rise to the pro-apoptotic signal (Miller *et al.*, 2001). Evidences were obtained in sensory neurones (Bamji *et al.*, 1998) and in PC12 cells, that p75 is required for cell death to occur after NGF withdrawal. Reduced levels of p75 protected both sensory neurones and PC12 cells against cell death after NGF withdrawal, whereas overexpression of p75 accelerated PC12 cell death after NGF withdrawal (Barde, 1989). Therefore, a consideration of the mechanisms of NGF-withdrawal induced apoptosis may be relevant

to the mechanisms used by p75. NGF withdrawal in PC12 cells leads to changes in the expression of many genes (Henderson, 1996).

It was shown that changes in Bcl-xl, Bcl-xs, bax and caspase-3 mRNA levels were potently regulated by NGF and NGF withdrawal in PC12 cells (Batistatou *et al.*, 1993; Reed, 1995). NGF withdrawal led to a precipitous decrease in bcl-xl mRNA, a relative increase in bcl-xs mRNA, and an absolute increase in bax mRNA. Forced expression of bcl-xl was sufficient to prevent NGF-induced cell death. Bax has also been shown to be important (Deckwerth *et al.*, 1996), as have the mitogen-activated protein kinases p38, JNK and Erk (Kyriakis & Avruch, 2001). It remains to be seen whether any of these NGF-dependent factors are regulated through p75 as well as TrkA.

Apoptosis can also be activated through the p53 pathway (Bamji *et al.*, 1998). As will be discussed later, p53 is sequestered in the cytoplasm in neuroblastoma and we showed that undifferentiated SH-SY5Y cells have nuclear p53 immunostaining (data not shown). Under conditions of terminal differentiation, we showed that p53 became localised to the nucleus (Fig. 1.22). NGF-withdrawal induced cell death in these cells is probably due to the activation of the p53 pathway.

CREB is a transcription factor that mediates NGF survival signals. CREB regulates many aspects of neuronal function such as nerve cell excitation, CNS development, long term memory formation and circadian rhythms (for review see Martin, 2001). CREB plays a key role in regulating neuronal survival and differentiation in response to the neurotrophic factors NGF, BDNF, FGF and IGF (Riccio *et al.*, 1999). CREB is activated through phosphorylation by the MAPK and the PI3K pathways. Recently, CREB was reported to be a target for caspases during apoptosis in neuroblastoma extracts (François *et al.*, 2000). Furthermore, inhibition of CREB activity

induced apoptosis in sympathetic neurones. The induced apoptosis in SH-SY5Y cells following NGF withdrawal is likely due to CREB destruction by caspase since CREB was reported to be a substrate for caspase-3 or 7. SH-SY5Y cells treatment by 8-Br-cAMP activates CREB which mediates NGF survival signals in differentiated cells. Fully differentiated NB cells become NGF survival-dependent. NGF withdrawal induces apoptosis probably *via* the destruction of CREB transcription factor by caspases.

1.5.3 Effect of differentiation on p53 sub-cellular localisation

Although NB is one of a small group of early onset tumours that do not have a mutation of the p53 gene, overexpression of p53 is the most universal change observed in the disease (Moll *et al.*, 1995). Overexpressed p53 accumulates in the cytoplasm of NB cells and it was initially thought that p53 is excluded from the nucleus. Immunolocalisation studies of p53 in these SH-SY5Y cells showed that undifferentiated SH-SY5Y cells exhibited a marked cytoplasmic p53 accumulation with concomitant nuclear exclusion. Cytoplasmic accumulation of p53 in undifferentiated SH-SY5Y cells may reflect an aberrant nuclear translocation pathway due to a block in maturation (Isaacs *et al.*, 2001. We confirmed that SH-SY5Y cells carry the wild type p53 gene. Wild type p53 protein was shown to be expressed in many of the cell lines derived from NB including SH-SY5Y cells. When cells were induced to differentiate using 8-Br-cAMP, we noticed an increase in the accumulation of p53 in the nucleus and an overall decrease in p53 expression. Further differentiation for up to 3 weeks induced a relocalisation of p53 in the nucleus with sparse staining in the cytoplasm (Fig. 1.22).

Similar effects were observed when H7 was used to induce neuronal differentiation (Jeoung *et al.*, 1995). It has been reported that the PKC inhibitor H7 possesses novel activities such as inducing nuclear accumulation of the p53 protein and triggering p53-dependent apoptosis in several cell lines that carry the wild type p53 gene (Chernov *et al.*, 1998; 2001). H7, similarly to DNA damaging agents, increases the steady state level of p53 in NB cells, which in part accounts for the dramatic nuclear accumulation of p53 in these cells (Nakamura *et al.*, 2000). In addition, NGF treatment of PC12 cells caused an increase in p53 protein in the nucleus and subsequently in the cytoplasm (Pohula *et al.*, 1997; Hughes *et al.*, 2000). The increase of p53 protein in PC12 cells, following NGF treatment, indicates a role for this protein in NGF signal transduction, either by acting on cessation of proliferation or by having a more direct effect on differentiation (Hughes *et al.*, 2000). Although we did not directly reveal the contribution of p53 to terminal exit from the cell cycle and in the apoptosis triggered by NGF withdrawal, our results certainly indicated that p53 translocation to the nucleus was associated with the progression of induced SH-SY5Y cells to the mature stage.

1.5.4 Effects of differentiation on PKC activity and isozyme expression

The regulation of neurite outgrowth during neuronal differentiation is complex and likely to involve multiple signal transduction components. One group of enzymes that has been suggested to be involved in this process is the protein kinase C family (for review see Pahlman *et al.*, 1995).

In order to determine which PKC isoform may contribute to NB differentiation, we compared the expression pattern of the isoforms in differentiated and

undifferentiated SH-SY5Y cells following various protocols to induce NB differentiation. We found that both differentiated and undifferentiated SH-SY5Y cells express PKCα, βII, μ and ζ. In addition, when induced to differentiate by 8-Br-cAMP or by 8-Br-cAMP + NGF, cells started to express PKCε isoform. This suggests a role for PKCε in the regulation of neurite outgrowth. We also observed an increase in the expression of PKCα, βII, μ and ζ (Fig. 1.34). Transfection of different PKC isoforms into SH-SY5Y cells showed that among the classical and novel PKC isoforms that are consistently expressed in human neuroblastoma cells (PKCα, β, δ, and ε) (Zeidman *et al.*, 1999), only overexpression of PKCε induced neurite-like processes. PKCε has been suggested to be important for neurite outgrowth in PC12 cells where overexpression of PKCε potentiated NGF-induced neurite outgrowth (Borgatti *et al.*, 1996). This involvement of PKCε in NB differentiation was shown to be independent of its kinase activity (Zeidman *et al.*, 1999). In neuroblastoma cells several PKC isoforms are enriched in growth cones (for review see Akinori *et al.*, 1998). This effect was attributed to a region encompassing the pseudosubstrate, the two C1 domains, and parts of the V3 domain. There are other reports where parts of the entire PKC regulatory domain exert the same effects as the complete enzyme (Cabedo *et al.*, 1996).

Overexpression of PKCε in SH-SY5Y cells induced processes in these cells. PKCα, β, δ and ε isoforms were also found to be expressed in other NB cell lines such as IMR32, LAN-2, LAN-5 and SKNB-E (Brodie *et al.*, 1999). Also, we have shown that PKC inhibition impacts SH-SY5Y cell proliferation and differentiation. Neurite growth is associated with a down regulation of PKCα in Neuro-2A and PC12 (Cabedo *et al.*, 1996). It was demonstrated that PKC enhances noradrenaline release in SH-

SY5Y cells and that PKCα is the isozyme responsible for this effect (Jalava *et al.*, 1993).

The decreased number of NB cells resulting from addition of PKC inhibitors in the growth medium could be due to either inhibition of proliferation and/or increased apoptosis. The fact that the expression of PKC isoforms was modulated during SH-SY5Y cell differentiation and growth arrest suggests the possibility that specific modulation of PKC isoforms expression may be a fruitful approach to improve NB therapy.

1.5.5 PKC inhibition induces differentiation in SH-SY5Y cells

PKC is regarded as a marker enzyme for tumourigenesis (for review see Newton & Johnson, 1998; Dempsey *et al.*, 2000; Borgatti *et al.*, 1996; Borner *et al.*, 1995). PKC activity has been linked to metastasis through studies using PKC inhibitors that inhibited cell growth and induced cell differentiation (Jalava *et al.*, 1993; Shea & Beermann, 1991).

We tested different PKC blockers for their ability to induce both neuronal differentiation and cell cycle growth in the SH-SY5Y cell. These PKC inhibitors include those that are more specific to PKC such as Bis-indolylmaleimide, calphostin C and others that are not specific such as staurosporine and H7. Among the PKC inhibitors tested, only two inhibitors, staurosporine (100 nM) and H7 (100 μM), induced the outgrowth of morphologically distinct neurites (Fig. 1.26, 1.27). Staurosporine-treated cells elaborated branched neurites, while H7-treated cells elaborated mostly long,

unbranched neurites, and lacked a network of shorter neurites. These morphological differences may reflect the inhibition of different protein kinase subtypes.

Shea *et al.*, (1991) found that the neurite outgrowth observed after staurosporine treatment was unaffected by cycloheximide, indicating that outgrowth is independent of protein synthesis. In contrast neurite outgrowth was blocked by colchicine, indicating the requirement for microtuble assembly as a driving force. We observed that staurosporine and H7-treated cells were blocked in their proliferation (data not shown). Cells could survive for 24 h when treated with 100 nM staurosporine and 48 h when treated with 100 μM H7. Bis and calphostin C, the specific PKC inhibitors, did not produce any effect on SH-SY5Y cell morphology nor on cell proliferation. Staurosporine is a potent inhibitor of multiple protein kinases. These include PKC, cAMP-dependent protein kinase, a calmodulin-dependent protein kinase and a receptor tyrosine kinase (Shea & Beermann, 1991).

Neuronal proliferation has been shown to be associated with changes in PKC activity and in the expression of specific isozymes (Jalava *et al.*, 1993; Ponzoni *et al.*, 1993; Slack *et al.*, 1992; Parodi *et al.*, 1990). In our study, SH-SY5Y cells were shown to emit neurites when treated with staurosporine or H7. This is probably due to the inhibition of protein kinases other than PKC since the more PKC specific inhibitors have no effect on SH-SY5Y morphology. Another possible mechanism would be the type of inhibition exerted by these various PKC inhibitors on (catalytic site versus regulatory domain) the appropriate PKC isoforms that are necessary for SH-SY5Y cell differentiation. Identifying the PKC isoforms responsible for neuronal differentiation and cell growth arrest may provide a powerful tool for NB therapy.

The morphological differentiation observed following treatment with staurosporine and H7 allowed for the expression of neuronal markers characteristic of neurones (NF200, NF68) that reach maximum levels after 3h staurosporine and H7 treatment. Longer treatment with these inhibitors down-regulated the expression of the neuronal markers, coincident with the time where SH-SY5Y cell underwent apoptosis (Fig. 1.32 and Fig. 1.33). N-myc protein level fluctuated during treatment with staurosporine and H7. It decreased significantly after half an hour treatment for both the PKC inhibitors and it increased after 3h and decreased again after 24h in staurosporine treated cells.

In H7 treated cells, the expression of N-myc increased again to return to the control level after 24h. The differential expression of N-myc during the progression into differentiation was probably due to the presence of a heterogeneous population in SH-SY5Y cells that respond to the PKC inhibitors H7 and staurosporine in different manners. In addition, we noticed an increase in the expression of H-ras after only half an hour treatment with staurosporine. $p21^{ras}$ protein expression decreased during the progress into staurosporine induced-differentiation. This suggests that staurosporine-induced differentiation probably activated the H-ras pathway. Staurosporine and H7 treatment had no effect on the expression of TrkA (data not shown), suggesting that staurosporine and H7 did not induce differentiation through activation of the TrkA expression even if they induced a cell cycle block similar to aphidicolin. Furthermore, this effect is probably due to cells subsequently undergoing programmed cell death and the expression of another set of genes involved in apoptosis. Staurosporine was shown to increase the steady-state level of VGF mRNA, a neuronal specific gene transiently induced by cAMP stimulation of PKA (Jalava *et al.*, 1992; Nagasaki *et al.*, 1999). The

VGF gene was shown to encode a secretory protein localised in large dense core vesicles, which also contain neuropeptides and monoamines (Sasaki *et al.*, 1996). Staurosporine was also shown to induce the expression of TH and to have a synergistic effect with cAMP (Nagasaki *et al.*, 1999). Furthermore, it was reported that staurosporine has a promoting effect on cAMP-induced transcription of the vasoactive intestinal polypeptide and dopamine β-hydroxylase genes (Sasaki *et al.*, 1997). Taken together, these results may suggest that the staurosporine effect on inducing neuronal differentiation is mediated by the cAMP responsive element CRE. Staurosporine is reported to have paradoxical effects on PC12 cells: at concentrations as low as 5 nM, it inhibits NGF-induced differentiation (Raffioni & Bradshaw, 1995) but stimulates epidermal growth factor-induced differentiation, whereas at high concentrations of 10 to 100 nM, staurosporine can induce differentiation, independently of growth factors. Staurosporine, which belongs to the K-252a family of glycosylated indole carbazole alkaloids, inhibits a number of protein kinases and prevents the autophosphorylation of protein tyrosine kinases, including the NGF Trk receptor (Shea & Beermann, 1991).

Staurosporine was shown to induce cell growth arrest in SH-SY5Y cells after only 15 min treatment (Fig. 1.35). It was reported to exert its effects on cell proliferation of primary and cultured cells in either the G1 or G2/M phases of the cell cycle (Osda, 1993). Staurosporine inhibits the activity of purified cdc2 kinase with an IC50 value as low as 4-5 nM and, hence, could block the cell cycle through this effect on a cell cycle kinase. In PC12 cells, staurosporine treatment causes a cessation of replication and thus has an anti-proliferative effect analogous to that of NGF (Raffioni & Bradshaw, 1995).

In SH-SY5Y cells, staurosporine does not affect the protein level or subcellular localisation of p53 (Tieu *et al.*, 1999). H7, a PKC non-specific inhibitor, was reported to induce differentiation in SH-SY5Y cells (Felipo *et al.*, 1990). The exact mechanism by which H7 exerts its effect on cell proliferation and induction of differentiation is still unknown. One of the possible mechanisms of H7 induced cell growth arrest could be through p53. H7 was shown to modulate the localisation of p53 from the cytoplasm to the nucleus, where it would exert its role on the regulation of cell cycle progression and induction of apoptosis through a p53-dependent pathway. In contrast, staurosporine probably acts on cell proliferation through a p53 independent pathway since staurosporine treatment has no effect on p53 subcellular localisation in SH-SY5Y cells.

1.5.5.1 Induction of morphological differentiation and apoptosis by the non-specific PKC inhibitors H7 and staurosporine

PKC is regarded as a marker enzyme for tumourigenesis (for review see Carter, 2000). PKC activity has been linked to metastasis through studies of PKC blockers that inhibited cell adhesion, growth of tumour cells, and growth factor phosphorylation (Dempsey *et al.*, 2000). These findings suggest that PKC may be a potent target for therapeutic intervention. Potent kinase inhibitors (staurosporine, H7, Bis, Go6976 and calphostin C) have been used in the present study to examine their effects on SH-SY5Y cells with regards to PKC influence on cell proliferation, differentiation and apoptosis.

In the present study, we have used staurosporine, a drug that has proven to be very effective in many cell types to induce apoptosis (Boix *et al.*, 1997). We have determined the concentrations required to reproducibly cause cell death in SH-SY5Y cells (Fig. 1.35). We have also observed that staurosporine is able to induce a prominent

neuritogenesis in this cell line. Because of this neurotrophic-like effect, staurosporine has been proposed as a potential prototype that could stimulate the process of regeneration and neurite outgrowth in damaged neurones or induce morphological differentiation in NB. Little is known about the mechanisms underlying staurosporine induction of cell death and neuritogenesis. Staurosporine is known to be a potent and non-specific PKC inhibitor. There are published results about neuritogenesis as a consequence of PKC inhibition, but staurosporine induction of neurite outgrowth seems to be independent of PKC (Klein *et al.*, 1991). This was supported in our work by using a PKC specific inhibitor, Bis, which did not induce neuritogenesis. The involvement of PKC in cell survival and of its inhibitors in triggering apoptosis has been reported (Dempsey *et al.*, 2000). Other protein kinases are likely involved in the mechanisms of staurosporine action. There is evidence of staurosporine disrupting the cycling of several cell lines (Jalava *et al.*, 1992). If CDK1 activity is absent, the ratios of staurosporine-induced apoptosis are reduced. Staurosporine displays an IC50 value of 5 nM for PKC. The evidences of strong interrelationships between cell cycle and apoptosis regulation are rapidly accumulating.

In PC12 cells, staurosporine has a dual action: at 10 nM concentration, it inhibits the NGF induction of neurite formation whereas at 100 nM, it triggers neurite outgrowth (Brodeur *et al.*, 1997b). The 10 nM effect of staurosporine in PC12 cells is accounted for by the inhibition of the tyrosine kinase activity, which characterises gp140 trk, the NGF high affinity receptor, upon activation. However, the 100 nM neurotrophin-like effect is not so readily explained. It has been reported to be independent of staurosporine-induced PKC inhibition (Brodeur *et al.*, 1997b). On the other hand, staurosporine induces apoptotic cell death in immature rat thymocytes (Goswami *et al.*, 1998) and many other

cell lines. Staurosporine concentrations ranging from 100 nM to 1 μM induce apoptosis in Jurkat T cells, promyelocytic leukemia HL-60 cells, fibroblasts and rat oligodendrocyte precursors (Boix *et al.*, 1997). Furthermore, in a murine T cell hybridoma, 500 nM staurosporine surpassed the apoptotic ratios found by the most common apoptotic stimuli. Staurosporine was found to induce apoptosis in NB cells. This is not surprising, as others have suggested that it is a universal inducer of apoptosis in all mammalian cell lines tested, regardless of the state of differentiation and cell cycle phase. The broad apoptotic activity of demonstrated by staurosporine appears to be unique among other protein kinase inhibitors, raising the question of the involvement of a kinase mechanism in the effect.

1.6. Conclusions

The overall conclusion of this chapter is that the combination treatment of 8-Br-cAMP and NGF in the presence of aphidicolin induces terminal differentiation in SH-SY5Y cells. The differentiation was characterised by the enhanced expression of neuronal markers of cytotoskeleton and neuronal function. Final withdrawal from the cell cycle and NGF-survival dependency in SH-SY5Y cells was demonstrated. The non-specific PKC inhibitors staurosporine and H7 induced a block in cell proliferation as well as morphological cell differentiation followed by apoptosis.

The specific conclusions are:

1.6.1 SH-SY5Y cells differentiate terminally following treatment with the combination of 8-Br-cAMP, NGF and aphidicolin expressed elevated levels of the neuronal markers NF68, NF200, NSE, TH and Chat. The level of the N-myc oncogene that was over-expressed in untreated SH-SY5Y cells decreased significantly following this treatment. Furthermore, the levels of H-ras and TrkA, the two markers for good NB prognosis increased in terminally differentiated cells.

1.6.2 Terminally differentiated SH-SY5Y cells became NGF-survival-dependent and deprivation of NGF induced programmed cell death.

1.6.3 The PKC non-specific inhibitors staurosporine and H7 induced morphological and partial molecular differentiation followed by apoptosis.

1.6.4 p53 is sequesterted in the cytoplasm of undifferentiated SH-SY5Y cells. The induction of differentiation following exposure to a soluble PKA activator, cAMP

homologue, promoted the translocation of p53 from the cytoplasmic region to the nucleus.

CHAPTER TWO

Gap Junction Intercellular Communication in the Human Neuroblastoma Cell Line SH-SY5Y

2.Literature review

Cancer is viewed as a multistage and sequential process. While mutations play a principal role in the multistage nature of carcinogenesis, nongenotoxic mechanistic types of events also play key roles in carcinogenesis. Nongenotoxic mechanisms include changes in gene expression, cell proliferation, cell organisation, cell interrelationships including intercellular communication mediated by gap junctions (GJIC), cell death, and cell renewal (Trosko & Ruch, 1998). It is through gap junction channels that neighbouring cells communicate with each other directly. Furthermore, cancer has been described as a disease of differentiation and as a disease of cell regulation control. Since GJIC has been linked in healthy cells to the regulation of development, differentiation and growth, it was suggested that there is a relationship between cancer and GJIC (Yamasaki *et al.*, 1999a). Among the universal features associated with all cancers are the loss of contact inhibition and failure to terminally differentiate (Yamasaki *et al.*, 1999c). NB originate from the neural crest and emerge from a failure of peripheral nervous system neural crest cells to terminally differentiate during embryonic development, due to a blockage in the embryonic differentiation program (Grosfeld, 1998). In the first part of this project, the focus was on exploring the mechanisms that may pertain to NB therapeutic approaches: the induction of terminal differentiation and

apoptosis. In the last part of this project, the possibility was explored that intercellular communication may contribute to uncontrolled growth in NB. In order to examine a possible role for GJIC in NB, we focused on the identification of potential cell signalling pathways that regulate GJIC: gene expression, channel formation, gating and degradation. Understanding the mechanisms of GJIC regulation in NB may allow us to suggest a new therapeutic approach for treatment of this disease.

The gap junction (GJ) was first clearly observed by electron microscopy as a septalaminar structure with a 2-4 nm space or "gap" between two opposing cells (Robertson, 1963). In a facial view, a polygonal lattice of 7.5 Å subunits could be visualised by using electron-opaque tracers. These structural features of GJs are conserved over a wide range of organisms (Kato, 1999). Strategies were developed for the isolation of enriched subcellular fractions containing GJ and their characterisation at the molecular level was carried out (Kumar & Gilula, 1986). This effort resulted in the identification of the gene family of connexins (Cx), which are the protein structural components of gap junctions. Among various forms of cell-cell interactions, GJIC is the only one that provides for direct two-way cytoplasmic exchange between neighbouring cells. Thus GJIC is considered to play a crucial role in maintaining homeostasis by averaging growth control signals among GJIC-connected cells (Trosko & Ruch, 1998).

Gap junctions are found in most normal cell types with the known exceptions of skeletal muscle fibers (for review see Trosko et al., 1998), certain neurones (Bennett *et al.*, 1990), and circulating blood cells (Beyer & Steinberg, 1991).

2.1 Connexin classification

Gap junctions have been detected in primitive invertebrates such as jellyfish and hydra and similar structures known as plasmodesmata are found in plants (Meiners et al, 1987, 1991; Robards & Lucas, 1990). In evolutionary terms, connexins can be grouped into three superfamilies, alpha, beta and gamma. However, the most common nomenclature is based on the molecular weight of each connexin, e.g. Cx26, Cx43, where the figure corresponds to the molecular weight (Table 2.1) (Kumar & Gilula, 1992).

Of the 17 connexin genes so far identified in mammals, nine belong to the alpha class and 6 belong to the beta class. A connexin cDNA, termed Cx35, has been identified from a primitive vertebrate, the skate (O'Brien *et al.*, 1996). The homologue of skate Cx35 has been identified in mouse neurones (Cx36). Amino acid alignment indicates that Cx36 falls near the divergence between the alpha and beta classes. The gene structure of this connexin is also different from the other 15, since it has an intron within the coding region, whereas the other 15 Cx genes contain a single intron in the 5` non-translated region. The unusual gene structure of Cx36, as well as its divergence from the alpha and beta classes, suggest that this connexin may belong to a third class of connexins (Srinivas *et al.*, 1999). Recently, two mouse connexins Cx47 and Cx29 were cloned. They are expressed in the mouse brain and sciatic nerve respectively (Teubner *et al.*, 2001; Sohl *et al.*, 2001). So far, 17 connexins have been identified in rodents, and each presumably has a human counterpart. In mammals, Group I (beta) connexins include Cx26, Cx30, Cx30.3, Cx31, Cx31.1 and Cx32; Group II (alpha) connexins include Cx33, Cx37, Cx40, Cx43, Cx45, Cx46, Cx50 and Cx57 (Manthey *et al.*, 1999).

Table 2.1 Chromosomal locations of mammalian connexin genes

Connexin-type	Human chromosome	Mouse chromosome
Group I (β connexins)		
Cx26	13q11-12	14
Cx30	-	-
Cx30.3	-	4
Cx31	1p35.1	4
Cx31.1	-	4
Cx32	Xq13.1	X
Group II (α connexins)		
Cx33	-	-
Cx37	1p35.1	4
Cx40	1q21.1	3
Cx43	6q21-23.2	10
Cx45	-	11
Cx46	13q11-12	14
Cx50	1q21.1	3
Cx57	-	4
Group III (γ connexins)		
Cx36	15q14	-

(Rozental *et al.*, 2000)

In addition, Cx36, the newly described connexin type, has been identified in the mammalian brain and assigned to a new group, Group III or the gamma class of connexins (Sohl *et al.*, 1998). This connexin was shown to be functional and to code for a neurone-specific gap junction protein (Teubner *et al.*, 2000).

2.2 Connexin diversity and distribution

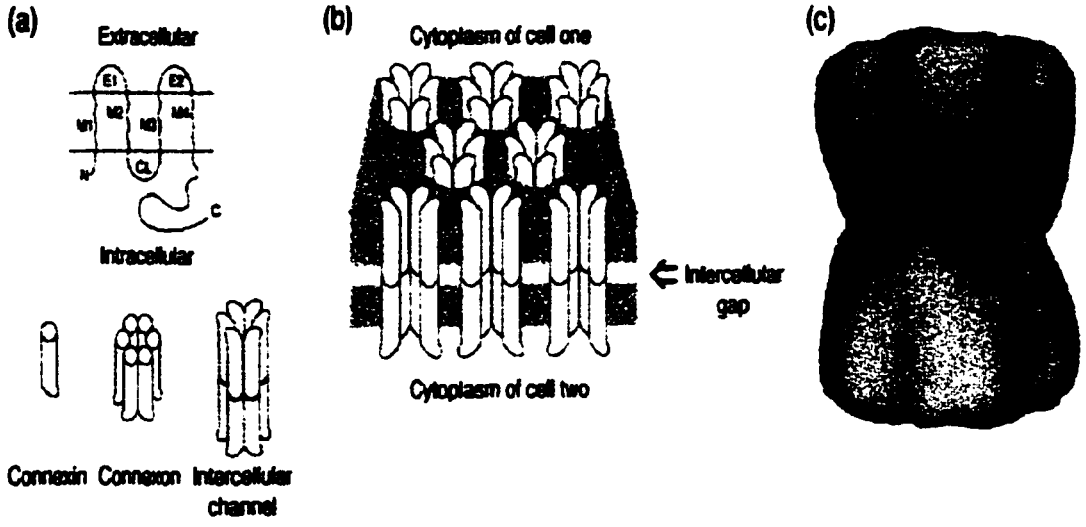
Connexins present a high degree of diversity. The different members of the connexin multigene family selectively create diversity by producing channels that have different permeability (Brissette *et al.*, 1994; Veenstra, 2001). Connexins are expressed in an overlapping pattern of tissue distribution (Simon & Goodenough, 1998); more than one Cx type is generally expressed per individual cell. Connexins are expressed in cell-, tissue- (Plum *et al.*, 2000) and in a development-specific manner in many organisms (Simon, 1999; Lo, 2000). Connexin 43 is widely distributed among different tissues and is developmentally regulated. It is the most expressed of the connexins during embryonic development (Makarenkova & Patel, 1999; Lo *et al.*, 1997).

In the nervous system, at least eight connexin genes are expressed, these include Cx26, Cx32, Cx43, Cx36, Cx37, Cx45 (Bruzzone & Ressot, 1997). The pattern of connexin expression is related to cellular differentiation. For example, the types of connexins expressed by the nervous system is related to stage of embryonic development and to differentiation state (Nadarajah *et al.*, 1997). This is true also for keratinocyte differentiation (Choudhry *et al.*, 1997; Gibson *et al.*, 1997). Cx43 is characteristic of embryogenic stages of development (Delorme *et al.*, 1997). The differential expression is not due to alternative splicing of RNAs but to the fact that there is one gene for each connexin. Many connexin genes have been mapped and are located

on several chromosomes suggesting that their distribution is random throughout the genome (Bennett *et al.*, 1994). The reason for the diversity of connexins is not clear. It may reflect differences in their functions and/or regulation of their expression, formation, and channel permeability. It may also reflect their importance in multicellular organisms. The factors that govern these connexin patterns of distribution, which are temporally and spatially regulated during development are not completely elucidated. Retinoids, some hormones and signalling molecules were reported to be involved in this regulation (Atkinson *et al.*, 1995; Bex *et al.*, 1995).

2.2.1 Gap junction channel

Each gap junction channel is comprised of two hemichannels or connexons and each connexon is formed by the aggregation of six protein subunits known as connexins (Schematic 2.1) (Sosinsky, 1996). In those cells where multiple connexins are expressed, gap junction hemichannels may be heteromeric or homomeric. Heteromeric connexons may display different permeability and regulatory properties than homomeric ones and may provide numerous additional options for regulating the type of signals that pass through the gap junction channel (Diez *et al.*, 1999). In addition heterotypic channels consisting of two different connexons have also been described (Rubin *et al.*, 1992). Only certain heterotypic channels are functional. When the connexon is made by one type of connexins, it is called a homologous channel; if it is formed by more than one connexin it is called a heterologous channel. When two homologous connexons form the channel, it is called homotypic and when it is formed by two heterologous connexons, it



(Simon et al. 1998)

Scheme 2.1. Gap junction channel structure

(a) The transmembrane topology of a generic connexin polypeptide creates four membrane domains (M1-M4), one cytoplasmic (CL) and two extracellular (E1 and E2) loops, and the N- and C-termini face the cytoplasm. Fourier microscopy shows that M1-M4 are packed helices, creating a single connexin subunit. These subunits oligomerize into a connexon within the membrane of a single cell, which docks with a counterpart in an adjacent cell to form the intercellular channel. (b) Gap junctions are variable numbers of intercellular channels clustered at close appositions of the plasma membranes of two cells, leaving a 3-nm `intercellular gap' and forming axial channels connecting the cytoplasms of the cells. The shaded planes represent the lipid bilayers. (c) Model of the docking interactions between interacting connexons.

is called a heretotypic channel. All these types of combinations exist and may regulate channel permeability (Bruzzone *et al.*, 1996). Each connexon is a hexamer of connexins (Schematic 2.1) that may be homologous or not (Yeager *et al.*, 1998). All the connexins have similar structure topology: they are folded in the plasma membrane in the approximate shape of an "M". They have four membrane spanning domains separated by two extracellular and one intracellular loop and two intracytoplasmic tails (Schematic 2.1) (for review see Simon *et al.*, 1998). The amino and carboxyl termini project into the cytoplasm. The membrane-spanning regions lie in parallel. The third one contains a high proportion of hydrophilic amino acids and is thought to line the interior of the channel. The four membrane spanning domains and the extracellular loops are highly conserved between the many different connexins that have been cloned. More variety is found in the cytoplasmic regions (Falk, 2000). As will be discussed later, these different regions may be involved in the cellular regulation of gap junction formation and channel gating. Connexin folding as well as connexin-connexin and connexon-connexon interactions are mediated through disulphide bonds, hydrophobic protein interactions, and other less understood mechanisms (for review see Kumar *et al.*, 1999; Falk *et al.*, 2001).

2.2.2 Gap junction channel function

Gap junction channels have a diameter of approximately 1.5-2 nm depending upon the type of connexin forming the junctions. This allows cytoplasmic water-soluble molecules such as ions, water, sugars, nucleotides, amino acids, fatty acids, small peptides, drugs, carcinogens and second messengers such as cAMP, calcium and inositol 1,4,5-trisphosphate, to diffuse directly into the cytoplasm of neighbouring cells.

However, proteins, complex lipids, polysaccharides, RNA, and other large molecules cannot diffuse through the GJ channel (for review see Goodenough *et al.*, 1996). Molecular transfer does not require ATP and appears to result from passive diffusion. One of the most significant physiological implication of GJIC is that GJ "coupled cells within a tissue are not individual, but highly integrated within their neighbourhood". This aspect facilitates homeostasis and also permits the rapid, direct transfer of second messengers between cells to co-ordinate cellular responses within the tissue (Trosko & Ruch, 1998). Thus GJIC can be viewed as an important mechanism of cellular homeostasis which permits the integration of the different cellular functions within tissues and organs and the regulation of cell growth (Yamasaki *et al.*, 1996).

2.2.3 Connexin structure

Connexin family members are quite homologous, with about 50% sequence identity at the amino acid level. All members of the connexin protein family share a very similar tertiary structure (Schematic 2.1). The most divergent sequence in structure between connexin proteins is the C-terminal tails, followed by the amino acid sequences of the cytoplasmic loop (Goodenough *et al.*, 1996). Cx26 is the smallest connexin and has almost no C-terminal tail (Zhang *et al.*, 1994). The size of the C-terminal end reflects on connexin molecular size. The C-terminal tail of Cx43 has been extensively analysed, leading to the conclusion that this domain contains phosphorylation sites for protein kinases that may be involved in the control of channel formation, gating and degradation. These kinases include c-Src, MAP kinases, PKC, Cdc2 and PKA (Cooper *et al.*, 2000; Hertzberg *et al.*, 2000).

2.3 Connexin gene expression

The structure of most connexin genes is similar. It consists of two exons separated by a long intron (Bruzzone *et al.*, 1996). The first exon is about 100 base pairs and does not contain any protein coding information. The connexin gene promoters are located upstream of exon 1. Two additional promoters have been identified in cx32 gene and were shown to be active in neuronal tissue only and not in liver tissue (Neuhaus *et al.*, 1995; Sohl *et al.*, 1996). This may provide another cell specific control mechanism for connexin expression and function in a tissue-specific manner. The promoters of the human, mouse and rat cx43 genes have been cloned (Sullivan *et al.*, 1993; De *et al.*, 2001). They contain a TATA box located near the transcription start site and several activator proteins (AP-1) site(s) further upstream (Seul *et al.*, 1997; 2000) The rodent and human Cx43 promoters also contain several half-palindromic estrogen-responsive elements that enhance Cx43 transcription in response to estrogens and progesterone (Petrocelli *et al.*, 1993; Yu *et al.*, 1994; Risek *et al.*, 1995). Several physiological, pharmacological, and dietary conditions alter the expression of connexins, often in a cell-specific manner. For example, cyclic AMP increases Cx43 transcription, presumably through a putative cAMP-response-element located in the Cx32 and Cx43 promoters (Atkinson *et al.*, 1995). Retinoic acid also increased gene expression of Cx43 (Bex *et al.*, 1995). Stabilisation of connexin messenger RNA and protein stability is another mechanism that appears to control Cx expression (Theodorakis *et al.*, 1999; Lee *et al.*, 1998; Kren *et al.*, 1993). Finally, connexin degradation, either through the proteosome or lysosome pathways was reported to be important in the regulation of

GJIC (Musil *et al.*, 2000). Sequences located in the 3'-untranslated region of the Cx43 mRNA appear to stabilise its message. Besides increasing connexin gene transcription (Banoub *et al.*, 1996), cAMP was shown to increase the permeability of pre-existing gap junction channels (Burghardt *et al.*, 1995), and to decrease connexin degradation (Carystinos *et al.*, 2001). These effects may involve connexin phosphorylation by cAMP-dependent protein kinase.

In summary, several transcriptional and post-transcriptional mechanisms are involved in the regulation of connexin expression. A better understanding of these mechanisms will lead to the development of therapies designed to alter connexin expression for treatment of diseases such as neuroblastoma.

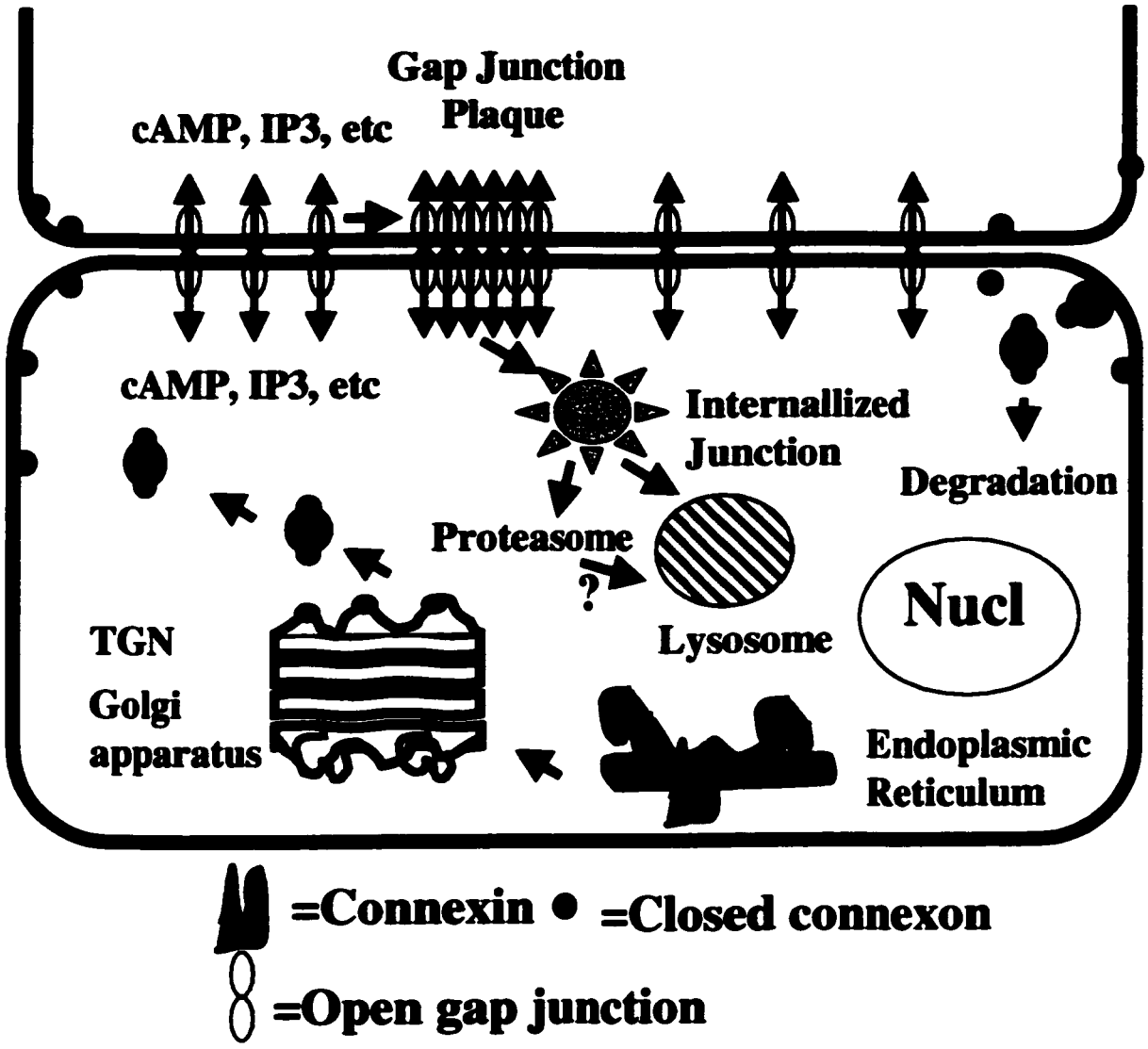
2.3.1 Factors that influence GJ channel formation

Rates of connexin synthesis and degradation and the disassembly and removal of gap junctions from the cell surface are important in the regulation of GJIC (Diez *et al.*, 1999; Evans *et al.*, 1999). In response to certain physiological agents and prior to aggregation into plaques, gap junction particles are dispersed in the plasma membrane. Connexin degradation was shown to be induced through both the ubiquitin-proteosome pathway and the lysosomal pathway for proteolysis of the connexin 43 polypetide (Musil *et al.*, 2000). The half-life for Cx43 is between 1 and 5 hours (Laing *et al.*, 1995; Laird *et al.*, 1991). Gap junctions containing Cx43 are internalised and degraded in the lysosomes or are ubiquitinated and degraded in proteosomes in response to various physiological or toxicological agents. These pathways may be controlled by phosphorylation with protein kinases. The mechanisms that trigger connexin degradation

are still unknown. Cx43 has also been reported to be ubiquitinated via the proteosomal pathway and to be further degraded by lysosomes (VanSlyke *et al.*, 2000; Laing *et al.*, 1997). The relative contribution of each pathway may be cell specific. Degradation of Cx32-containing gap junctions can be mediated by the calcium activated proteases, μ-calpain and m-caspain. Thus, there appear to be several pathways for connexin degradation and the contribution of each are cell-and connexin-specific (Schematic 2.2) (Musil *et al.*, 2000).

2.3.2 Connexin trafficking

Subcellular fractionation and the detection of connexins in different subcellular fractions as well as immunohistochemistry provide information regarding connexin trafficking. Cx43 was found to be distributed in plasma membranes, in microsomal, Golgi and lysosomal fractions. This reflects Cx43 trafficking and an underlying rapid synthesis and breakdown of gap junctions. Cx43 and Cx32 from the heart and liver of the pig were found to be present in Golgi and plasma membrane in their oligomeric form and as monomers and oligomeric intermediates in endoplasmic reticulum derived vesicles, as well as in a fraction containing vesicles derived from a location between the endoplasmic reticulum and Golgi (Revel & Karnovsky, 1967; Musil & Goodenough, 1993). These results demonstrate that connexins oligomerize upon entry into the Golgi apparatus. Connexins are proteins that lack a conventional signal sequence and Cx43 and Cx32 are inserted co-translationally into endoplasmic reticulum-derived canine pancreatic microsomal membranes (Falk *et al.*, 1994; Wang & Rose, 1995b). Cx26 is also inserted into membranes post-translationally (Zhang *et al.*, 1996; Falk *et al.*, 1997). The Golgi apparatus was shown to play a central role in the trafficking that is followed



(adapted from VanSlyke et al. 2000)

Scheme 2.2. Schematic representation of the synthesis, assembly, and degradation of gap junction membrane channels.

by connexins and where post-translational modifications such as phosphorylation are important (Laird *et al.*, 1995). Cx26 is also able to traffic to the plasma membrane and to form GJ channels that are functional with minimal involvement of the Golgi (Evans *et al.*, 1999). Cx26 transmenbrane domain was shown to be important for Cx assembly (Martin *et al.*, 1999).

2.3.3 Gap junction formation, control of channel permeability, and mechanisms of GJIC disruption

The mechanisms of gap junction formation and regulation of channel permeability are poorly understood. The most complete knowledge regarding gap junction formation has been acquired for Cx43 and is based largely upon the work of Musil and Goodenough (Musil *et al.*, 1990a; Musil & Goodenough, 1990b; Cotrina *et al.*, 1998). Their data suggest that six connexin subunits oligomerize into connexons in the Golgi apparatus and are then transported to the plasma membrane (Musil & Goodenough, 1993). At this point, the connexons are closed to prevent leakage of cellular contents and entry of extracellular material. At the plasma membrane, the connexons are attracted to those on adjacent cells by poorly understood forces and two connexons join in an end-to-end manner to form a complete channel. Subsequently, the channels aggregate into large gap junction plaques and the channels open to connect the two cells, although the order of these last two steps is still controversial. Coincident with the formation of open gap junctional channels and aggregation of particles into junctional plaques, Cx43 is phosphorylated at many consensus sequences for protein kinases. The kinases that perform these phosphorylations are the MAPK, PKC, PKA, p34cdc2 or pp60v-src (Warm-Cramer, 1996; Takeda *et al.*, 1987; Godwin *et al.*, 1993; Kanemitsu *et al.*, 1998;

Loo *et al.*, 1995). These phosphorylations may increase particle aggregation, channel permeability, Cx43 stability and/or degradation (Kwak *et al.*, 1995; Saez *et al.*, 1998).

Gap junction channel formation also requires appropriate cell-cell adhesion as a prerequisite. The cadherins appear to be especially important in this regard since gap junction formation can be induced in connexin-expressing, cadherin deficient, non-communicating cells following transfection with cadherin expression vectors (Woodward et al., 1998). Furthermore, cadherin antibodies blocked gap junction formation. Proteins such as calmodulin (Torok *et al.*, 1997) and adhesion protein cadherins (Frenzel & Johnson, 1996) were shown to ensure the fidelity and rapidity of assembly turnover since their blockage was shown to inhibit GJIC (Jansen *et al.*, 1996). Cadherins were shown to be involved in the determination of the connexin pattern of expression during melanoma development (Hsu *et al.*, 2000). In addition, membrane protein glycosylation can also impair gap junction formation. Treatment of non-communicating cells with an inhibitor of glycosylation induced gap junction formation (Wang *et al.*, 1995a)

2.3.4 Gene knockout mice

During the past several years, mouse genetics have demonstrated that cell-cell communication via gap junction channels has an important role during early development. Gene knockout involves the disruption of a target gene by the insertion of a non-coding sequence through homologous recombination. Loss of the Cx43 gene results in the death of mice after birth due to abnormal heart function (Reaume et al., 1995; Chen *et al.*, 1995; Ewart *et al.*, 1997; Ya *et al.*, 1998). This mutation resulted in

offspring that had abnormally enlarged hearts. Furthermore, Cx deficiency causes delayed ossifivation, craniofacial abnormality and osteoblast dysfunction (Lecanda *et al.*, 2000). No neoplasm was evident in the embryos, possibly because of the young age at death or because of compensatory communication by the expression of other connexins. However cell lines developed from the embryos exhibited abnormal patterns of growth (Martyn *et al.*, 1997; Naus *et al.*, 1997). Furthermore, $Cx43^{-/-}$ clones grew at faster rates and to higher saturation densities, had a more spindly morphology, were more refractive and adhered less well to the substratum than did the $Cx43^{+/+}$ clones (Naus *et al.*, 1997). Re-expression of Cx43 in these $Cx43^{-/-}$ clones suggested that partial restoration of Cx43–mediated GJIC was sufficient to restore a degree of growth control but was insufficient to restore normal morphology or cell substratum adhesion. Taken together, the $Cx43^{-/-}$ experiments suggest that the absence of Cx43 function in early development may contribute to the acquisition of biological properties indicative of advancement towards neoplastic transformation. This is in support of the hypothesis that the loss of Cx43 function may play a role in the multistep process of carcinogenesis (Naus *et al.*, 1997). Connexin 32 knockout mice have also been developed (Anzini *et al.*, 1997). These mice exhibited a high degree of hepatocyte proliferation and were vulnerable to carcinogen–induced hepatic tumour formation without showing any alteration in the process of myelination in Schwann cells. A mutation in Cx32 was shown to be responsible for the Marie Charcot disease, indicating that probably another connexin such as Cx26 was responsible for Schawnn cells myelination in Cx32 knockout mice (Fairweather *et al.*, 1994; Hahn *et al.*, 1999). Furthermore, female mice lacking the Cx37 gene were found to be sterile due to meiotic maturational arrest of the oocytes (Simon *et al.*,

1997). In mice lacking the Cx40 connexin gene, there was a reduction in the electrical coupling in the conduction system of the heart (Kirchhoff *et al.*, 1998; Simon *et al.*, 1998). Homozygous Cx32 deficient mice developed several defects, including both late onset neuropathy (Anzini *et al.*, 1997) and liver defects (Nelles *et al.*, 1996). Loss of the Cx26 gene results in autosomal deafness, presumably due to the loss of function of this connexin in the placenta of these mice and to the observed dysfunction in glucose uptake (Gabriel *et al.*, 1998). Mice with a disrupted Cx46 gene developed nuclear lens cataracts (Gong *et al.*, 1997). Cx45-deficient mice present a defective vascular development (Kruger *et al.*, 2000) and Cx43 and Cx40 double mutant mice showed cardiac malformation associated with Cx channel conductance (Kirchhoff *et al.*, 2000). Table 2 summarizes the phenotypes of connexin knockout mice. The mechanisms by which these different phenotypes arise in the different connexin gene knockout mice are poorly understood. It emphasizes the important role of the connexin gene family in development and regulation of cell growth and homeostasis.

2.4 Gap junctions in cell growth and differentiation

Gap junctions have been hypothesised to have an important role in modulating cell growth and differentiation. It is proposed that cell signalling molecules involved in regulating growth and differentiation can pass from cell to cell through gap junctions, and the loss of gap junctional communication will lead to uncontrolled growth and aberrant differentiation (Pitts *et al.*, 1988). Recent studies of Cx32 knockout mice

Table 2.2 Phenotypes of mice in which specific connexin genes are homozygously deleted

Gene	Phenotype in -/- mice	Reference
Cx43	Heart malfunction	Reaume *et al.*, 1995
Cx26	Malfunction of placenta	Martin *et al.*, 1999
Cx32	Increased tumours and DNA synthesis in the liver	Moennikes *et al.*, 1999
Cx37	Malfunction of ovary	Simon *et al.*, 1997
Cx40	Cardiac conduction abnormalities	Bevilacqua *et al.*, 2000
Cx46	Cataract	Gong *et al.*, 1999
Cx50[a]	Cataract	Gong *et al.*, 1998

[a] Strain of mice carrying a mutation in the Cx50 gene.

(Yamasaki *et al.* 1999)

suggest that GJIC may play a role in carcinogenesis (Temme *et al.*, 1997). The appearance of chemically induced hepatic tumours was accelerated in Cx32 null mice (Willicke *et al.*, 2000). They have also shown that establishment of coupling between cells can suppress the growth of human prostate cancer cells (Habermann *et al.*, 2001).

2.5 Role of GJIC in development

Furshpan and Lennox (Potter *et al.*, 1966) showed that the cells of the squid embryo are linked through electrical coupling via gap junctions and consequently proposed the idea that direct cell-cell communication permits the transmission of regulatory signals between developing cells through gap junctions. Presently, there are numerous data confirming that GJIC is a universal feature among early embryos. Warner (Warner, 1999) supplied the most direct evidence by demonstrating that blockage of cell-cell communication by introducing GJ protein antibodies into the amphibian embryo generates developmental defects especially during the early stages of development. These results suggested the primary role of GJIC in ensuring normal pattern formation. The link between embryonic patterning and direct cell-cell communication is reinforced by the realisation that gap junctions between cells in different regions of the embryo are not identical and are formed by different types of connexins. The differential expression of connexins during various stages of development as well as in different cell types controls the passage of signalling molecules such as growth factors and ions necessary for the development of the embryo. Also, the presence of channels with incompatible connexins that are not able to communicate permits the isolation of compartments that permits cell differentiation.

2.6 GJIC in the nervous system

2.6.1 Connexin distribution in the nervous system

Of the 17 rodent connexins so far identified, at least nine (Cx26, Cx29, Cx32, Cx33, Cx36, Cx37, Cx40, Cx43, Cx45, Cx46, Cx47) are expressed to various degrees in the nervous system (Bruzzone & Ressot, 1997; Srinivas *et al.*, 1999). In the brain, gap junctions represent an important means of intercellular communication especially in the astrocytic compartment. Electrotonic coupling, mediated by gap junctions, has been proposed to be responsible for synchronisation of signals in the inferior olive (Llinas *et al.*, 1974), among hippocampal CA3 neurones (MacVicar & Dudek, 1981), in the retina (Becker & Mobbs, 1999; Dermietzel *et al.*, 2000) and during neuronal development, (Kandler & Katz, 1998; Becker & Rozental *et al.*, 1998). In the central nervous system, Cx43 has been identified in astrocytes and neurones, Cx32 has been described in both oligodendrocytes and neurones, and recent studies identified Cx30 in astrocytic gap junctions (Dermietzel *et al.*, 1989; Mehta *et al.*, 1992; Nadarajah & Parnavelas, 1999).

During neuronal development and in the mature brain, different cellular elements of the nervous system are characterised by discrete patterns of connexin expression. Junctional communication has been observed between different cell types and between similar cell types (Nadarajah *et al.*, 1997; Nadarajah & Parnavelas, 1999). Adult neurones are relatively poorly coupled, the predominant connexin type in neuroblasts appears to be Cx43 which is also present in certain neuronal populations of the brain stem, in cerebral cortical layers, in the basal ganglia, and in the substantia nigra. (Aberg *et al.*, 1999; Bittman *et al.*, 1999). Cx36 was detected in adult neurones and shown to be

tissue-specific to adult neurones (Srinivas *et al.*, 1999). In fact Cx26, Cx33, Cx37, Cx40 and Cx43 have been shown to be present in specific neuronal populations *in vivo* or in culture at specific developmental stages (Nadarajah & Parnavelas, 1999). Recently, Cx47 and Cx29 were detected in mouse brain and sciatic nerve respectively (Teubner *et al.*, 2001; Sohl *et al.*, 2001).

2.6.2 Gap junctions in the peripheral nervous system

In the adult PNS, Cx32 is expressed in myelinating Schwann cells, where it is located in the lamellar layers at the nodes of Ranvier and at Schmidt-Lanterman incisures; it is absent from compact myelin (Bergoffen *et al.*, 1993). Mutations in the gene encoding Cx32 are responsible for the inherited human X-linked form of Charcot-Marie-Tooth disease, (CMTX) implicating this gap junction protein in normal adult neural function (Bergoffen *et al.*, 1993; Chance & Lupski, 1994; Fairweather *et al.*, 1994). The resulting peripheral neuropathy is characterised by progressive nerve demyelination and impeded nerve conduction (Hahn *et al.*, 1999). Surprisingly, in Cx32 null-knockout mice, there is no evidence of behavioural or neurological disorders in mice studied up to 3 months of age (Nelles *et al.*, 1996); however, they are characterised by a late-onset and progressive peripheral neuropathy (Anzini *et al.*, 1997). It remains to be determined if other connexins identified in rodent Schwann cells, such as Cx46, Cx43 or Cx26, play a compensatory role in Cx32-deficient mice (Zhao *et al.*, 1999). Interestingly, although Cx32 is expressed by a variety of other mammalian cells including oligodendroglia (Dermietzel *et al.*, 1989; Scherer *et al.*, 1995; Li *et al.*, 1997), hepatocytes (Paul, 1986), renal cells (Wilgenbus *et al.*, 1992), and mammary tissue (Lee

et al., 1992), there are no reported abnormalities in brain, liver, kidney, or breast of patients with CMTX, suggesting that Cx32 plays a unique role in the peripheral nerve.

Cx43 is expressed in several cellular elements of adult peripheral nerves, including fibroblasts (Beyer *et al.*, 1989), macrophages (Polacek *et al.*, 1993) and blood vessel cell walls (Moore *et al.*, 1991). In the sciatic nerve, Cx43 forms a lattice-like pattern among perineuronal fibroblasts, which contributes to the formation of the blood nerve barrier. However, low level of Cx43 is present in adult mice Schwann cell cytoplasm and nodes of Ranvier, and is absent from myelin (Zhao *et al.*, 1999). Distinct changes in connexin expression and intercellular coupling strength are observed after crush-injury of the rat sciatic nerve within 3 days. This event was coincident with the downregulation of Cx32 and increased expression of Cx43 in Schwann cells (Zhao *et al.*, 1999).

2.6.3 GJIC in the developing nervous system

A number of electrophysiological studies in the developing nervous system have shown the extensive cell-cell coupling between neurones in the cortex. The authors proposed that GJs play a role in the formation of neuronal domains, which eventually leads to the formation of cortical columns (LoTurco *et al.*, 1991; Peinado *et al.*, 1993; Yuste *et al.*, 1995) and interneuronal communication in the adult cortex (Gutnick & Prince, 1981). The critical role of gap junctions during embryogenesis may be to provide intercellular pathways for the diffusion of morphogens and other developmentally relevant factors, including Ca^{2+} and other second messenger molecules. Early in corticogenesis, neuroepithelial cells of the ventricular zone are coupled into clusters by Lucifer yellow-permeable gap junctions; these clusters may be analogous to the

"proliferative units" described in the primate neocortex (Rakic *et al.*, 1988; LoTurco *et al.*, 1991). Although the precise role of cell clustering during corticogenesis is unclear, it is thought to spatially restrict the interactions between ventricular zone cells. The pattern of expression of connexins and the distribution of GJ in the proliferative regions of the developing cerebral cortex have been characterised (Nadarajah & Parnavelas, 1999). Experiments using Cx43 knockout mice showed a defect in cell migration during early development (Lo, 1999). However, a follow up study was not possible since the mice died at birth due to a heart malformation that was shown to be due to a failure in the heart conduction system in these mice (Lo, 2000).

Time courses for the expression of connexins in the brain during early postnatal development have so far only been published for Cx26, Cx32, Cx43 and Cx36. The expression of some of these connexins has been localised to specific cell populations, for example Cx43 in astrocytes and some neurones, Cx32 in oligodendrocytes and some neurones and Cx36 in some neurone subpopulations (for review see Nagy & Rash, 2000; Dermietzel, 1998).

Numerous studies have demonstrated that the incidence of interneuronal coupling decreases dramatically during the process of neuronal maturation. For instance, in rats, Cx26 is highly expressed in foetal as compared to adult brain while Cx32 shows a developmentally regulated increase in abundance that only reaches maximal levels after birth (Nadarajah *et al.*, 1997). Because these changes in coupling strength and in pattern of connexin expression coincide with the progression into differentiation and commitment of cells and of cell groups to the neuronal lineage, it has been hypothesised that the presence of GJ provides for the diffusion of signals among the requisite cell types (Nadarajah & Parnavelas, 1999; Trosko *et al.*, 2000). Even after neuronal circuits

are formed, coupling among neurones persists to various degrees in different brain regions (Batter *et al.*, 1992; Peinado *et al.*, 1993; LoTurco *et al.*, 1991; Spray, 1998). For example in the neocortex, GJ coupling becomes gradually more and more restricted during early neonatal life, as the necessity for synchrony in the establishment of projections onto adjacent cells declines (Peinado *et al.*, 1993; Yuste *et al.*, 1995). Recently, it was found that the formation of circuits in the cortex and in the hippocampus coincides with the peak of expression of the newly discovered neuronal-specific gap junction protein Cx36 and formation of its channel (Prime *et al.*, 2000).

2.6.4 Cx43 expression during neuronal differentiation

Imunohistochemical analysis using Cx specific antibodies revealed that early rat migratory neural crest cells express the gap junction constituents Cx43 and Cx46 and that they are functional (Lo *et al.*, 1996; 1997; Bannerman, 2000). Furthermore, the authors provided evidence that neural crest cell survival requires gap junctions and that GJ modulates neural crest migration in mouse (Huang *et al.*, 1998). According to Rozental *et al.*, (1998), during early CNS development, neuronal precursor cells are extensively coupled by gap junction channels composed of Cx43 and that uncoupling is necessary to permit cells to exit from the cell cycle and to differentiate. Thereafter, there is progressive expression of gap junction channels composed of Cx40, Cx33, Cx45 and Cx36, which influence further neuronal formation and circuit formation.

2.7 Gap junction intercellular communication and carcinogenesis

The role of gap junctional communication in growth control and neoplasia was first proposed by Loewenstein and Kanno in the 1960s (Loewenstein, 1965; Loewenstein & Kanno, 1966). The authors observed that neoplastically transformed cells in culture were deficient in junctional communication. They provided a hypothesis that normal cells form communicating compartments, the size of which is monitored via GJ transferred signals. In this model the loss of communication by neoplastic cells would result in decreased growth regulation. This hypothesis was reinforced by the discovery that tumour promoting agents such as phorbol esters strongly inhibited GJIC and that anticancer agents such as retinoids have the ability to upregulate GJIC and to down regulate neoplastic proliferation (Li *et al.*, 1998). Furthermore, all trans retinoic acid treatment was shown to prevent the disruption of GJIC function in MDCK (Mardin-Darby canine kidney) cells exposed to TPA (Watanabe *et al.*, 1999). TPA is a well-known tumour promotor for skin and liver. It disrupts GJIC by inducing Cx43 phosphorylation (Berthoud *et al.*, 1992).

In MDCK cells a non-transformed renal tubular epithelial cell line, tRA increased Cx43 expression levels at the plasma membrane, and some of the Cx43 protein remained localised in the perinuclear region. Furthermore, anticancer compounds such as flavoids and Lycopene were shown to counteract TPA-induced GJIC blockage and to increase their channel permeability (Chaumontet *et al.*, 1997; Stahl *et al.*, 2000). Also, other lines of evidence have indicated that connexin genes form a family of tumour-suppressor

genes. For example Cx26 was shown to have tumour suppressor activity as does Cx32 (Mesnil *et al.*, 1997; Temme *et al.*, 1997).

Abnormalities of gap junction channels or of connexin proteins, that are commonly seen in tumour tissues, include the loss or reduced expression of connexins and aberrant localisation of connexin molecules or of connexons (for review see Yamasaki *et al.*, 1999b). Moreover, transfection of various connexin genes into tumourigenic cells has revealed that connexins suppress cell growth and tumourigenicity in a cell-type-specific manner (Yamasaki *et al.*, 1999c).

Cancer may be viewed as the result of the disruption of the regulatory mechanisms that affect the cell's ability to respond appropriately to extracellular signals of the body. These extracellular signals trigger intracellular signal transducing mechanisms, which modulate GJIC between the cells within the tissue. Normal homeostatic control of these different types of cell communication determines whether the cell remains quiescent, proliferates, is induced to differentiate or will be committed to programmed cell death (Spray, 1998).

Gap junction size and number, connexin expression, and cell-cell coupling have been studied in many neoplastic cells. The vast majority of neoplastic cells have fewer and smaller gap junctions, express less connexins, and have reduced GJIC compared to their normal counterparts. This can be due to an alteration of connexin expression at the level of gene expression, connexin assembly or channel gating permeability (Trosko & Ruch, 1998).

2.7.1 Effects of oncogenes, growth factors and viruses on GJIC

Many oncogene products such as *Ras*, *Neu*, and *Src* were reported to block GJIC in specific cell lines (Trosko & Ruch, 1998; Khoo *et al.*, 1998). pp60 v-src was shown to block GJIC by phosphorylation (Loo *et al.*, 1995; Zhou *et al.*, 1999). Some growth factors such as transforming growth factor beta enhance GJIC in some types of cells but decrease it in others (Robe *et al.*, 2000).

Several oncogenes code for growth factors, growth factor receptors, or mitogenic signal transducing elements and several tumour promoters act as growth factors since they induce cell proliferation. These oncogenes can also cooperate in their ability to reduce GJIC and transform cells (Yamasaki *et al.*, 1999c).

Many growth factors such as epidermal growth factor, platelet derived growth factor, basic fibroblast growth factor, hepatocyte growth factor, and transforming growth factor alpha (TGFα) inhibit GJIC when applied to cultured cells (Kanemitsu & Lau, 1993; Yamakage *et al.*, 1998; Robe *et al.*, 2000). This effect may occur rapidly in minutes to hours (Hossain *et al.*, 1998).

The mechanism(s) of inhibition of GJIC by EGF was shown to be related to the stimulation of connexin phosphorylation by MAPK and closure of gap junctional channels (Yamakage *et al.*, 1998). PDGF induced a rapid inhibition of GJIC coincident with the phosphorylation of connexin 43 through the MAPK and PKC pathways (Hossain *et al.*, 1998). Basic FGF, however, inhibited GJIC after long exposure coincident with decreased Cx43 expression and was shown to be important during neuronal development (Warner, 1999).

Table 2.4 Examples of cell-cell interaction genes involved in carcinogenesis

Gene	Tumours or cells	Changes observed
Integrin	Skin, liver, lung, osteosarcoma	Reduced expression
E-cadherin	Stomach, colon, breast, prostate, esophagus, kidney, bladder, etc.	Mutations: reduced expression
α–catenin	Stomach, colon, breast, prostate, esophagus, kidney, bladder, etc.	Reduced expression[a]
	Human cancer cells	Mutations
β–catenin	Melanoma, colon	Mutations : reduced expression
γ–catenin	Breasts, colon	Loss of expression , translocation into nuclei
	Human cancer cells	Loss of expression (suppression of tumourigenicity by enforced expression)
Connexins	Liver, skin, etc.	Reduced expression Aberrant localisation

[a] With very few exceptions, expression altered along with E-cadherin.

(Yamasaki *et al.* 1999c)

Hormones such as estrogens were shown to modulate Cx43 (Yu *et al.*, 1994) and Cx32 gene expression in female rats (Shinohara *et al.*, 2000) and trafficking (Hendrix *et al.*, 1995). Estrogens alter Cx43 expression in the myotrium during pregnancy. Other hormones such as the parathyroid hormone were shown to up-regulate Cx43 expression by an unknown mechanism (Mitchell *et al.*, 2001).

As noted above, oncogenes and tumour promoters have also been associated with dysfunctional GJIC. Thus, it is evident that growth factors, oncogenes, tumour promoters and viruses share the common properties of increasing cell proliferation and inhibiting GJIC.

2.7.2 Growth inhibitors stimulate GJIC

In contrast to the effect of growth factors, oncogenes and tumour promoters on GJIC, many growth inhibitors and anticancer agents increase GJIC and connexin expression in target cells. Retinoids, carotenoids, green tea extract, certain flavinoids, dexamethasone and cAMP analogues and agonists inhibit neoplastic transformation and/or tumour cell growth and can block neoplastic transformation in some tissues (Trosko & Ruch, 1998). These agents also increase connexin expression and gap junction formation in target tissues or block the inhibitory effects of tumour promoters on GJIC. Certain tumour suppressor gene products also increase GJIC in neoplastic cells.

Introduction of chromosome 11 that carries one or more tumour suppressor gene into neoplastic cells restored normal growth control, reduced tumourigenicity, and

enhanced GJIC mediated by connexins despite the fact that the connexin 43 gene is located on human chromosome 6. This result also suggests that tumour suppressor gene products inhibit neoplastic transformation by enhancing GJIC in addition to their known action on cell cycle genes, signal transduction pathways, and gene expression (Zhou *et al.*, 1999).

2.7.3 Cell cycle-related changes in GJIC

GJIC may also have a role in the progression of dividing cells through the cell cycle. In several model systems, cell cycle-related changes in GJIC have been noted. Cell culture studies have demonstrated changes in GJIC at specific stages in the cell cycle. Blockage of cell cycle by chemicals and growth factor deprivation showed a reduction in GJIC in late G1 and in mitosis (Evan *et al.*, 1995).

PKC was reported to be involved in this cell cycle–arrest induced GJIC reduction (Kato, 1999). Furthermore, the Cx43 sequence was shown to have a consensus phosphorylation site for pp34cdc2 that may be involved in the regulation of GJ function during cell cycle phases (Warn-Cramer *et al.*, 2001). Thus both *in vivo* and *in vitro* studies have documented cell cycle-related changes in GJIC. Connexin expression and phosphorylation may be involved in this reduced GJIC, but the exact mechanism by which GJIC regulates the cell cycle is still not fully understood (Trosko & Ruch, 1998).

2.7.4 Modulation of GJIC for cancer therapy: the bystander effect

As suggested by Trosko (Trosko & Ruch, 1998), one possible mechanism to increase drug penetration and dispersal in tumours would be to increase GJIC. This can

be achieved through increasing tumour cell connexin expression, by introducing connexin genes or by modulating key regulatory kinases involved in the control of channel assembly, transfer to the membrane or the gating of GJ channels. As shown in many cancer cells, enhancement of GJIC might have the additional benefit of reducing tumour cell growth (for review see Omari *et al.*, 2001).

Mesnil *et al.*, (1997) suggested the possibility of using connexins as both tumour suppressor genes and as diffusers of ganciclovir toxicity as a therapeutic approach. The presence of functional GJs opens the way to a novel type of cancer therapy that involves introducing a lethal gene such as the herpes simplex virus thymidine kinase gene into tumour cells. Expression of this gene renders the tumour cells susceptible to the thymidine analogue ganciclovir, which is readily phosphorylated by herpes simplex virus thymidine kinase, but not by the endogenous human tyrosine kinase. The phosphorylated metabolite, which is incorporated into the DNA of proliferating cells such as cancer cells, is cytotoxic (for review see Link *et al.*, 2000). Cells that are herpes simplex virus thymidine kinase positive are sensitive to ganciclovir, but non-expressing and non-proliferating cells are very resistant. The percentage of dead cells following ganciclovir treatment is higher than expected from the small number of transfected cells. The dead cells are apposed to the transfected ones. This effect known as the "bystander effect" has been attributed to GJIC function (Mesnil *et al.*, 2000). It was demonstrated that connexin transfection of neoplastic cells largely increases the bystander effect and acquires a *quasi* normal morphology (Yamasaki *et al.*, 1999b). Furthermore, agents that increase GJIC function such as cAMP and retinoic acids were shown to enhance the bystander effect in neuroblastoma cells (Carystinos *et al.*, 1999).

2.8 Signal transduction mechanisms involved in GJIC regulation

It was proposed that some molecules traversing gap junctions act as regulatory signals (Loewenstein *et al.*, 1978; Schiller *et al.*, 1992) and that the cellular concentration of these signals was primarily adjusted by the opening or the closure of the junctional channels. These hypotheses demand that gap junctions are subjected to strict regulatory controls.

Studies on the regulation of gap junctions composed of Cx43 revealed a complex mechanism (Holm *et al.*, 1999). The establishment of functional gap junctions is regulated by transcriptional as well as post-translational controls (Warn-Cramer *et al.*, 1998). Disruption of GJIC can also be achieved by multiple mechanisms. Permanent or chronic blockade of GJIC can be caused by tyrosine phosphorylation of Cx43, while acute and/or transient blockade of GJIC by growth factors and tumour promoters is correlated with the phosphorylation of Cx43 at serine/threonine residues (for review see Dermietzel & Spray, 1993). Cx43 contains putative phosphorylation sites for several known protein kinases, including PKC and MAPK which were suggested to be involved in Cx43 phosphorylation leading to the closure of GJs (Hertzberg *et al.*, 2000; Warn-Cramer *et al.*, 2001). Additionally, blockade of Cx43 gap junctions can also be achieved by phosphorylation-independent mechanisms, presumably via protein-protein interactions (Cooper *et al.*, 2000). Recently, it was demonstrated that a PDGF induced rapid and transient interruption of GJIC and phosphorylation of Cx43 in T51B rat epithelial cells expressing the PDGF receptor β (Hossain *et al.*, 1998). Further examination of the PDGF-signalling pathway revealed that activation of PKC and

MAPK were critical steps in PDGF-induced Cx43 phosphorylation and GJIC blockade in these cells (Hossain *et al.*, 1999b). MAPK is also involved in EGF mediated GJIC blockade (Oh *et al.*, 1993).

2.8.1 Mitogen Activated Protein Kinases

Numerous studies of cells coupled via gap junctions composed of Cx43 indicate that channel gating is regulated by connexin phosphorylation (Warn-Cramer *et al.*, 2001). Induction of Cx43 dephosphorylation by various methods has been correlated with either a reduction in GJIC or an increase in unitary channel conductance (Trosko & Ruch, 1998). Cx43 is phosphorylated on serine and threonine residues, although tyrosine phosphorylation is also found in $pp60^{v\text{-}src}$ transformed cells (Goodenough *et al.*, 1996; Bruce-Staskal & Bouton, 2001; Cai *et al.*, 1998; Lau *et al.*, 1996). Phosphorylation of Cx43 is thought to be required for proper synthesis and assembly of connexins into GJ (Laird *et al.*, 1995). Although tyrosine phosphorylation of Cx43 has been linked to decreased GJIC, phosphorylation of Cx43 has been associated with both increased and decreased GJIC (Goodenough *et al.*, 1996).

Mitogen-activated protein kinases are a family of serine/threonine kinases involved in the regulation of a wide range of cellular responses, including cell proliferation, differentiation, and survival (for review see Sebolt *et al.*, 2000). To date, several distinct MAP kinases have been identified that act in independent signalling pathways to effect the pleiotropic functions of this kinase family. These include p42/p44 extracellular signal-related kinases (Erk1 and 2), c-jun N-terminal protein kinase (JNK)/stress-activated protein kinase (SAPK), and p38 MAP kinase. These kinases represent the

terminal stages of signalling cascades, initiated by ligation of growth/survival factors or death receptors (Beyer & Steinberg, 1991).

A number of p38 inhibitors have now been identified, all acting at the ATP binding site. The p38 inhibitor, SB202190, has been observed to induce apoptosis through activation of caspase-like activity (Nemato *et al.*, 1998; Manthey *et al.*, 1998). Thus, the roles of p38 in the induction of apoptosis driven diseases have yet to be fully elucidated. Activation of MAPK has been associated with decreased GJIC (Warn-Cramer *et al.*, 1996). MAPKs are activated downstream via the Ras-Raf pathway by many mitogens (Mulder *et al.*, 2000). MAPK was shown to be responsible for increasing phosphorylation of Cx43 on serine residues leading to the inhibition of communication in epithelial cell lines (Hossain *et al.*, 1999a).

2.8.2 Protein kinase C

PKC is a family of several protein kinases, involved in the regulation of growth, differentiation, cell death, and neurotransmission (for review see Newton & Johnson, 1998). PKC was reported to regulate GJIC by phosphorylation on connexin phosphorylation sites (Budunova *et al.*, 1994; Godwin *et al.*, 1993). Different isoforms were reported to be involved in different functions; this was suggested by their differential pattern of localisation and function. TPA-induced GJIC impaired function was to modulate PKC isoform localisation, indicating that different PKC isoforms have different functions on the regulation of GJIC (Berthoud *et al.*, 2000). PDGF-induced disruption of GJIC was shown to be due to phosphorylation by both MAPK and PKC. Furthermore, the FGF-2 mediated decrease in GJIC in cardiomyoytes was shown to be

mediated by PKC phosphorylation of Cx43 on serine residues; PKCε was shown to be involved directly in Cx43 phosphorylation (Doble *et al.*, 2000). PKC isoforms α and ε were shown to modulate directly connexin expression in human heart (Bowling *et al.*, 2001). PKC isoform differential expression in relation with their role to GJIC regulation needs to be further clarified in order to understand whether GJIC regulation is a consequence of PKC isoforms distribution or the inverse.

2.8.3 Protein kinase A

As suggested by Loewenstein in 1969 (Loewenstein, 1969), cAMP might be a regulatory molecule that regulates GJIC. Furthermore, cAMP-dependent kinase PKA, which is activated by cAMP, was reported to phosphorylate Cx43 in the heart myocardium (Warn-Cramer *et al.*, 2001). Treatment with cAMP increased junctional permeability and connexin distribution in mammary cells (Burghardt *et al.*, 1995). Furthermore, cAMP-induced differentiation during stem cells development was shown to modulate GJ channel expression and function (Trosko *et al.*, 2000). PKA activation regulates also the clustering of the channel into GJ plaques in rat tumour cells (Wang & Rose, 1995b). PKA activation may then modulate GJIC at the level of gene expression or mRNA stability by increasing connexin expression (Carystinos *et al.*, 1999), it can also increase channel permeability in other cells. This activation is associated with induced differentiation and an arrest in cell proliferation (Trosko *et al.*, 2000). The mechanism by which PKA regulates GJIC is cell and tissue specific and its molecular basis is still unclear.

2.9 Neuroblastoma as a model to study GJIC regulation

Neuroblastoma is the most common extracranial tumour found in children. It is an interesting tumour since it is blocked or frozen at certain stages during its embryonic development. As shown in chapter one, the induction of *in vitro* differentiation and apoptosis would be one way to treat NB. It was also demonstrated that NB could be used as a model to mimic embryonic differentiation. Since GJIC plays a pivotal role during embryonic development, experiments were designed to use NB as an *in vitro* model to investigate the regulation of GJIC during proliferation and differentiation and to possibly identify the key regulatory transduction pathways and the components that may be involved in the regulation of GJ channel permeability and connexin expression. We hypothesised that neuroblastoma is a cancer with minimal or non-existent intercellular communication and that the modulation of the regulatory mechanisms that control GJ channel formation and function may lead to the re-establishment of intercellular communication and thus the control of cell proliferation.

2.2 Objectives

2.2.1 To examine for the presence and function of GJIC in neuroblastomas

2.2.2 To identify the signal transduction mechanisms that modulate GJIC gene expression and function in neuroblastomas

2.3. Materials and Methods

2.3.1 Materials

All the chemicals used in this study were purchased from Sigma unless mentioned otherwise. Cell culture plasticware and glassware was purchased from Gibco and protein kinase inhibitors (Go6976, PD98059, staurospaurine, H7, SB202190, Bis, calphostin C) were obtained from Calbiochem.

2.3.2 Methods

2.3.2.1 Cell culture

SH-SY5Y human neuroblastoma cells were kindly provided by Dr. Pierre Proulx (University of Ottawa, Ottawa, Ont., Canada). Cells were maintained in complete RPMI 1640 medium containing 10% foetal bovine serum (GIBCO, Burlington, and Ont.), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.24 μg/ml gentamycin.

2.3.2.2 Cell differentiation

SH-SY5Y cells were seeded on glass coverslips at a cell density of 1×10^5 per well in 6 well plates. The growth medium was exchanged 24 h later for a differentiation medium containing 0.5 mM 8-Br-cAMP or/and aphidicolin or/and 100 ng/ml NGF in RPMI 1640 with 10% FBS. Differentiation medium was changed every 2 days.

2.3.2.3 Cell treatment

H7 and 8-Br-cAMP were dissolved in water; staurosporine, SB202190, PD98059 and TPA were dissolved in dimethylsulfoxide and then diluted in PBS. When reagents containing DMSO were used, an equal volume of DMSO was added to the control cells. SH-SY5Y cells in culture medium were treated individually for the appropriate period of time with various protein kinase inhibitors at the following concentrations (H7, 200 μM; staurosporine, 250 nM; SB202190, 50 μM; PD98059, 10 or 50 μM and TPA, 100 nM).

SH-SY5Y cells were cultured as previously described in chapter one. Cells were seeded in 6 well dishes on glass coverslips at a cell density 1×10^5 cells in 20 mm^2 for dye coupling experiments, $1x10^4$ cells per 6 well plates for dose/effect experiments and $1x10^6$ cells in 100 mm^2 for immunoblotting experiments. 24 h after plating, cells were treated with 10 μM or 50 μM PD98059. In other experiments, they were treated with either 160 nM Go6976, 2 μM Bis or 50 μM SB202190.

2.3.2.4 Proliferation assay

SH-SY5Y cells were treated 24 hours after plating with either SB202190 at concentrations ranging from 3 to 100 μM or PD98059 at concentrations ranging from 1 to 50 μM in triplicate for the appropriate period of time depending on the experiment (see figures legends). The cells were harvested and counted using the Trypan blue dye exclusion method.

2.3.2.5 Connexin protein extraction

Treated and untreated SH-SY5Y cells ($1x\ 10^7$) were washed twice with PBS and harvested using a rubber policeman. Connexins were extracted using a previously

described technique (Kumar & Gilula, 1992). Briefly, crude gap junction preparations were obtained by NaOH fractionation. Cells were washed in PBS, scraped in 0.02% EDTA, centrifuged (5 min at 1000 g, Eppendorf) to pellet the cells and suspended in 0.375 ml of 0.1 M $NaHCO_3$ pH 7 containing 1 mM phenylmethylsulphonyl fluoride, 1mM sodium orthovanadate, 10 mM sodium molybdate, 10 mM sodium fluoride and 10 mM sodium pyrophosphate. An equal volume of 40 mM NaOH (freshly prepared) was added; the sample was sheared by passage trough a 21 gauge needle, incubated on ice for 30 min, and centrifuged for 30 min at 15,000 g speed (Beckman centrifuge). The NaOH insoluble pellets were washed with the $NaHCO_3$ buffer described above, recentrifuged for 30 min and suspended in 30 µl of Laemmli sample buffer. Proteins (25 µg) were separated by SDS gel electrophoresis (12.5% acrylamide at 100 V). The separated proteins were transferred to a nitrocellulose membrane and immunoblotted with anti Cx43 antibodies.

2.3.2.6 Antibodies

The following antibodies were used: anti-Cx43 and anti-Cx32, a rabbit polyclonal IgG at a dilution of 1:1000 (Zymed Laboratories, San Francisco, CA), and the secondary antibody Alexa 568 goat anti-rabbit IgG (1/200) (Molecular Probes, Eugene, OR), anti-phosphotyrosine, anti-phosphoserine, anti-phosphothreonine (1:100) dilutions and anti-p44, p42 MAP kinase at 1/100 dilution.

2.3.2.7 Immunofluorescence

Cells were grown on glass coverslips until they reached 70 to 90 % confluence. Cells were then gently rinsed three times with PBS, and fixed with a freshly prepared solution of 4% paraformaldehyde in PBS for about 20 min. Permeabilization of fixed samples was accomplished using 0.1% Triton X-100 in PBS for 5 min. Cells were rinsed 3 times with PBS for 5 min each and incubated with the primary antibody in 0.1 % Triton X-100 in PBS in a humidified chamber for 1 h. The secondary antibody, Alexa 568, a goat anti-rabbit IgG, was used to detect the anti-Cx43 primary antibody. The cells were rinsed three times with PBS for 5 min each and incubated with the secondary antibodies for 45 min. The cells were again rinsed three times with PBS for 5 min each time, then once with distilled water to prevent crystal formation. All samples were mounted in Vectashield mounting medium (Vector Laboratories). Samples were viewed with a Zeiss Photomicroscope III with a plan-Neofluor X63, N.A 1.25 oil immersion objective under epifluorescence, using a mercury lamp, and photographed using ILford XP2 - 400 film.

2.3.2.8 Measurement of gap junctional intercellular communication: Scrape loading

We examined gap junction-mediated intercellular communication using a scrape loading dye transfer technique (Trosko & Ruch, 1998). A scrape causes slight temporary damage to cells, thereby allowing the dye to enter. Only the cells along the scrape take up the dye. SH-SY5Y cells were seeded on coverslips until a cell confluency of between 70 and 90 % was obtained. Each dish was rinsed three times with PBS, incubated with 2 ml of 1 mg/ml (final concentration) Lucifer Yellow (Molecular Probes, Eugene, OR),

scrape loaded and left undisturbed for 10 min. After removing the dye, cells were rinsed three more times and subsequently fixed with 4% paraformaldehyde in PBS for 30 min. Fixed cells were again rinsed with PBS three times and mounted in Vectashield mounting medium (Vector Laboratories). Samples were viewed with a Zeiss Photomicroscope III with a 10x objective.

2.4. Results

2.4.1 Characterisation of GJIC in the human neuroblastoma cell line SH-SY5Y

2.4.1.1 Detection of connexin during SH-SY5Y cell proliferation

The presence of Cx43 and Cx32 was studied using immunofluorescence and immunoblotting techniques with Cx43 and Cx32 specific antibodies. We found that the human NB cells, SH-SY5Y, express Cx43 (Fig. 2.1 and Fig. 2.1a) but not Cx32 (data not shown). Immunofluorescence experiments showed that Cx43 is aberrantly localised in the cytoplasm of undifferentiated cells but is not found at the cell boundaries where it is expected to be present (Fig. 2.2). Cx43 immunostaining showed that Cx43 is localised in the perinuclear region and that the staining at the membrane in differentiated cells was punctate suggestive of the formation of GJ plaques at the boundaries of cell-cell contact (Fig. 2.1.a). Immunoblotting showed that Cx43 was phosphorylated. In fact, 3 bands are observed: the non phosphorylated (NP), phosphorylated 1 (P1) and phosphorylated 2 (P2) (Fig. 2.1).

Cell coupling was estimated using the scrape loading technique which consists of introducing a small molecular weight dye, Lucifer yellow, via a scrape and examining the transfer of the dye to neighbouring cells. In proliferating SH-SY5Y cells, the dye remained in the cells along the scrape line and was not passed on to neighbouring cells (Fig. 2.3). This indicates that these cells do not communicate via GJs. Thus, this experiment showed that GJ channels are non-functional in the NB cell line SH-SY5Y, which confirmed the results obtained in the Cx43 immunolocalisation studies (Fig. 2.2.A) that demonstrated the absence of Cx43 at areas of cell-cell contact and its

Figure 2.1 Cx43 expression in differentiated and undifferentiated SH-SY5Y cells

Immunoblot analysis of Cx43 in undifferentiated (1) and cells differentiated with 8-Br-cAMP for 24 h (2), 48 h (3) and one week (4). SH-SY5Y cells were plated at $1X10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated with 0.5 mM 8-Br-cAMP for 24 h (2), 48h (3) and one week (4). Treated and untreated cells were homogenised, extracted and 25 μg protein per lane was separated by electrophoresis on 12.5% SDS-PAGE, transferred to nitrocellulose membrane, immunoblotted with Cx43 specific antibody, and detected using alkaline phosphatase-conjugated secondary antibodies as described in Methods. The molecular weight is expressed as kilodalton (kDa). NP, P1 and P2 refer to non-phosphorylated, phosphorylated form1 and 2, respectively.

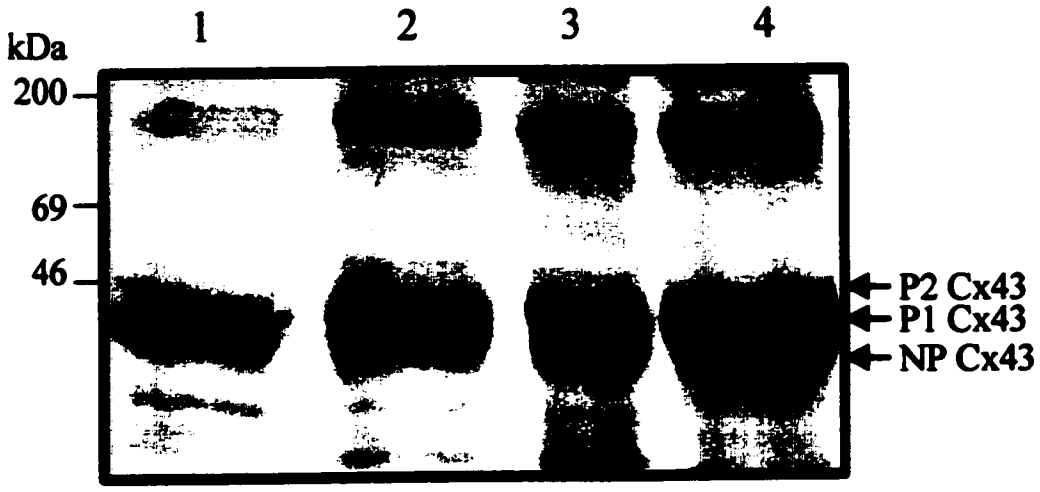

kDa
1
2
3
4
200
69
46
P2 Cx43
P1 Cx43
NP Cx43

Figure 2.1a Cx43 expression in differentiated and terminally differentiated SH-SY5Y cells

Immunoblot analysis of Cx43 in undifferentiated (1) and cells differentiated with 8-Br-cAMP + NGF+ AP for 3 weeks (2). SH-SY5Y cells were plated at $1X10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. Starting 24 h later cells were treated with 0.5 mM 8-Br-cAMP and 100 ng/ml NGF and 0.3 μM aphidicolin every 2 days for 3 weeks. Treated and untreated cells were homogenised, extracted and 25 μg protein per lane was separated by electrophoresis on 12.5% SDS-PAGE, transferred to nitrocellulose membranes, immunoblotted with Cx43 specific antibody, and detected using alkaline phosphatase-conjugated secondary antibodies as described in Methods. The molecular weight is expressed as kilodalton (kDa).

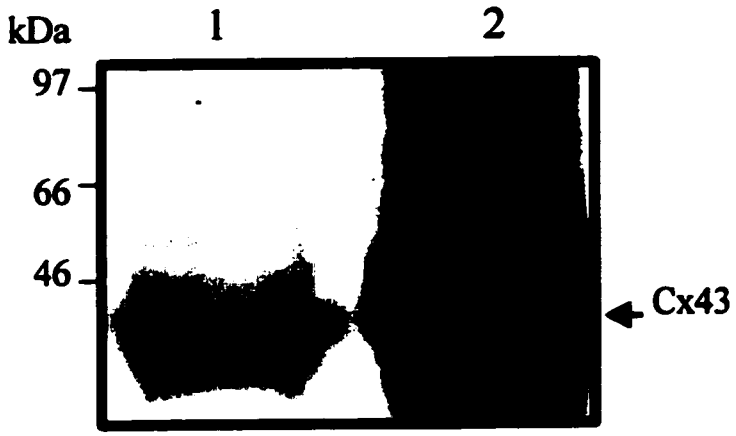

kDa
1
2
97
66
46
Cx43

Figure 2.2 Cx43 subcellular localisation in differentiated and undifferentiated SH-SY5Y cells

Immunocytochemical analysis of Cx43 in undifferentiated SH-SY5Y cells (A) and differentiated cells for 24 h (B), 48 h (C), one week (D) and 2 week time periods (E, F). SH-SY5Y cells seeded on coverslips at $1X10^5$ cells per 20 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated with 0.5 mM 8-Br-cAMP for 24 h (B), 48 h (C), one week (D), and 2 weeks (E and F). Cells were washed, fixed and incubated with Cx43-specific antibody and antigen-antibody detected with rhodamine-conjugated secondary antibody and mounted as described in Methods. Cells were viewed under fluorescence with a Nikon microscope equipped with the appropriate filter and photographed with a Kodak ektachrome 400 film.

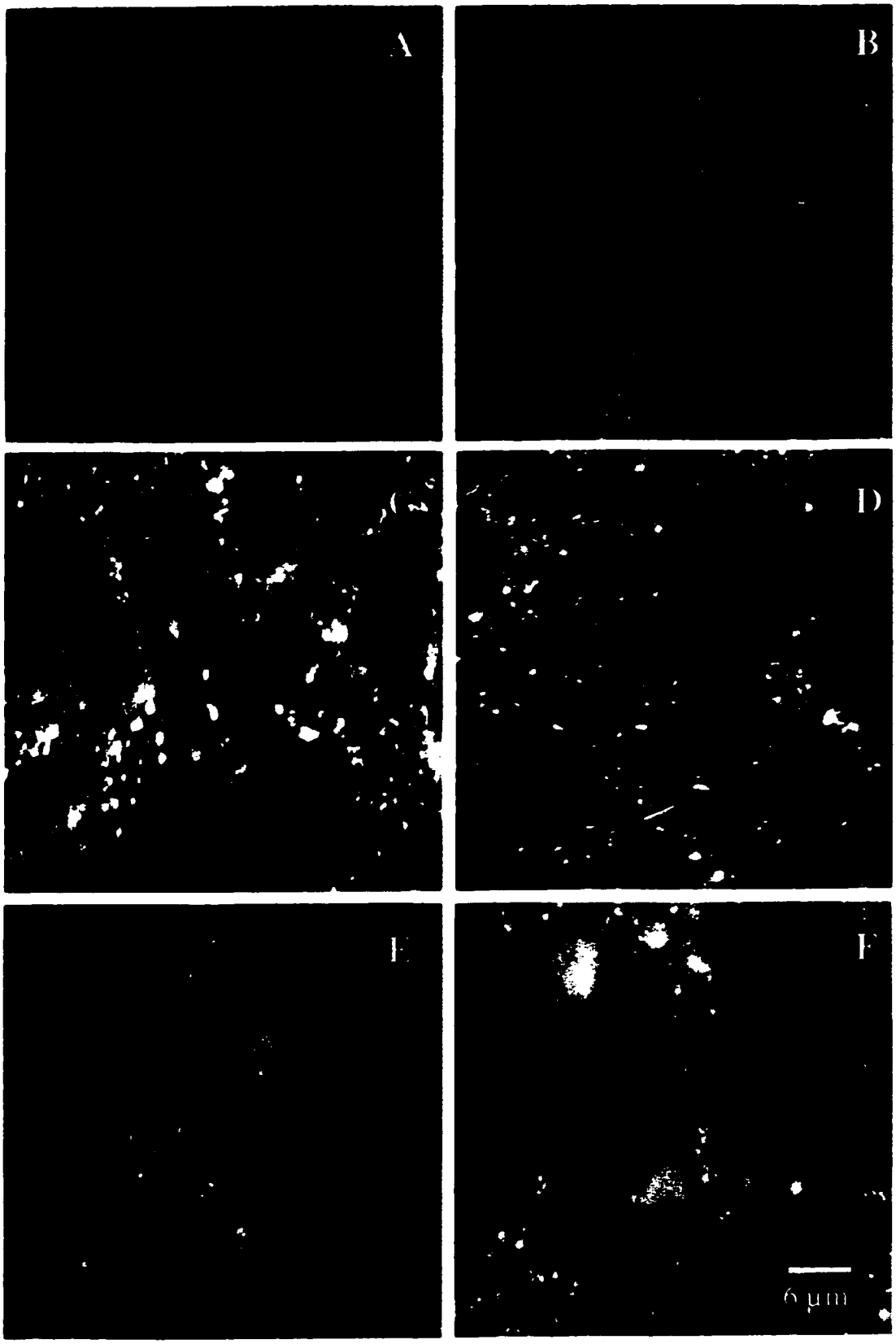
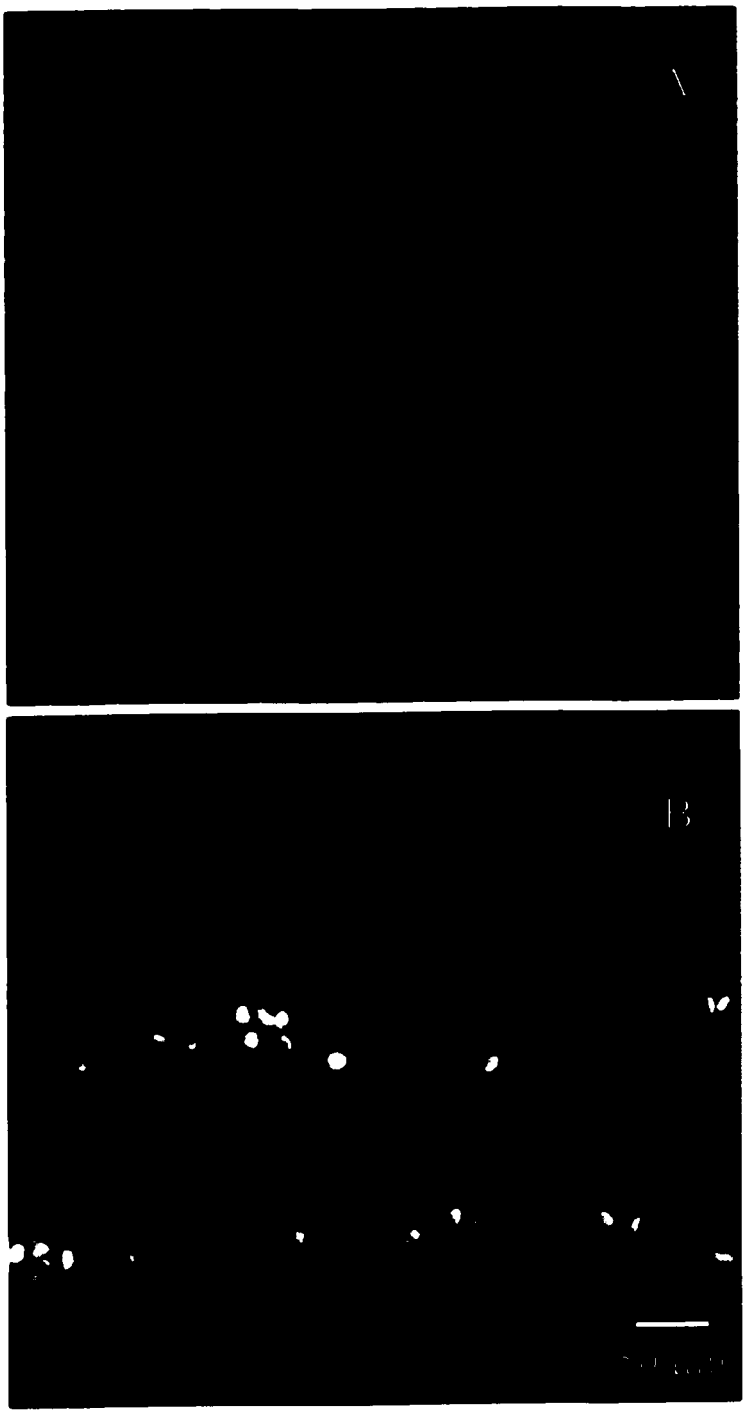

A
B
C
D
E
F
6 µm

Figure 2.3 GJ channel permeability in neuroblastoma SH-SY5Y cells

Fluorescence photographs of scrape loaded untreated SH-SY5Y. GJIC in SH-SY5Y cells was assayed by the scrape loading dye transfer technique. SH-SY5Y cells were seeded on coverslips until 70-90 % confluence was obtained. Cultured cells were washed, incubated in 1 mg/ml Lucifer Yellow for 10 min, scrape loaded, fixed as described in Methods. Mounted cells were viewed under fluorescence with a Nikon microscope equipped with the appropriate filter and photographed with a Kodak ektachrome 400 film.


A
B

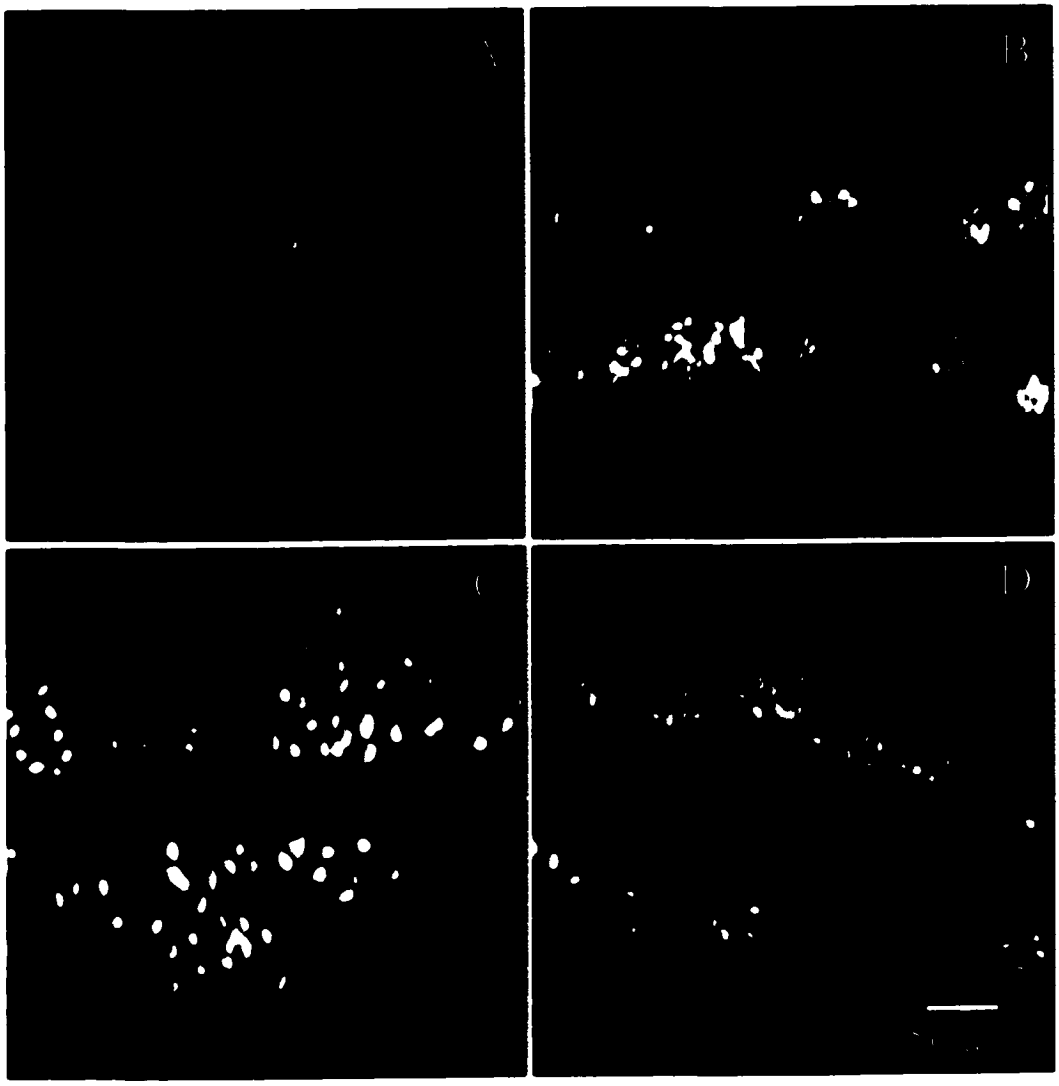
inability to form a GJ channel. We concluded that GJs in SH-SY5Y cells are composed of Cx43 and not of Cx32, although the expression of another GJ protein, such as Cx26, is not excluded. The cells have non-functional GJs due to an aberrant Cx43 localisation in the perinuclear region and its absence at areas of cell-cell contacts. We hypothesised that Cx43 phosphorylation may be responsible for GJIC dysfunction in SH-SY5Y cells. In many other cell types, phosphorylation has been associated with altered GJIC (Kumar & Gilula, 1992; Hossain *et al.*, 1998; Holm *et al.*, 1999; Bruce-Staskal & Bouton, 2001).

2.4.1.2 GJIC in differentiated SH-SY5Y cells

GJIC has been hypothesised to play an important role in cell growth and differentiation (Lo, 2000; Yamasaki *et al.*, 1999a). SH-SY5Y cells were induced to differentiate using 8-Br-cAMP. From our earlier differentiation studies, we knew that this PKA activator caused both morphological and molecular differentiation as well as cell cycle arrest. We monitored both the expression and sub-localisation of Cx43 as well as the function of GJIC in differentiated and undifferentiated SH-SY5Y cells. We found that treatment with 8-Br-cAMP for 24 h caused an increase of Cx43 protein expression, as shown by Western blot analysis. This overexpression of Cx43 was seen only after 24 h of treatment (Fig. 2.1) and increased with the progression into differentiation and reached a maximal level after 1 week of treatment. The long-term differentiation using the combination treatment of 8-Br-cAMP + NGF + AP for 3 weeks showed also an overexpression of Cx43 when compared to the control (Fig. 2.1a).

Figure 2.4 GJIC function in differentiated SH-SY5Y cells

Fluorescence photographs of scrape loaded undifferentiated SH-SY5Y cells (A) and differentiated SH-SY5Y cells for 24 h (B), 48 h (C) and one week (D). GJIC in SH-SY5Y cells was assayed by scrape loading dye transfer technique. SH-SY5Y cells were seeded on coverslips at $1x10^5$ cells per 20 mm^2. 24 h later cells were treated with 0.5mM 8-Br-cAMP for 24 h (B), 48h (C) and one week (D). Cultured cells were washed, incubated in 1 mg/ml Lucifer Yellow, scrape loaded, fixed as described in Methods. Mounted cells were viewed under fluorescence with a Nikon microscope equipped with the appropriate filter and photographed with a Kodak ektachrome 400 film.


A
B
C
D

Induction of differentiation also caused a shift of Cx43 from the perinuclear region to the plasma membrane region and an accumulation of this protein in GJ plaques (Fig. 2.2). The progression into differentiation was coincident with an increase in the level of NP and P1 forms of Cx43 after 24 and 48 hours, while one week and long term differentiation seems to increase the level of P2 form of Cx43 (Fig. 2.1; 2.1a). Untreated SH-SY5Y cells showed a different pattern of phosphorylation with approximately equal amounts of the 3 forms NP, P1 and P2 (Fig. 2.1). This is probably due to the asynchronous population of SH-SY5Y cells since it was shown that the phosphorylation patterns depend on cell cycle phases (Saez *et al.*, 1998).

We also measured the level of dye coupling between SH-SY5Y coupled cells and found that 8-Br-cAMP restored GJ function in SH-SY5Y cells (Fig. 2.4). The number of dye coupled cells increased with the progress into differentiation (24 h, 48 h and 7 days). In Fig. 2.4, we measured GJ channel permeability using the dye coupling technique. As expected undifferentiated SH-SY5Y cells present a non-functional GJ channel since the scraped cells could not transfer the Lucifer yellow dye to neighbouring cells (Fig. 2.4.A). Thus, differentiation of SH-SY5Y cells caused an increase in GJ channel permeability as well as an increase in Cx43 expression and Cx43 transport from the cytoplasm to the plasma membrane. Channel gating and the level of Cx43 expression both continued to increase as differentiation progresses (Fig. 2.4.B, C, D; Fig. 2.1 and Fig. 2.1a).

2.4.1.3 Cx43 phosphorylation in SH-SY5Y cells

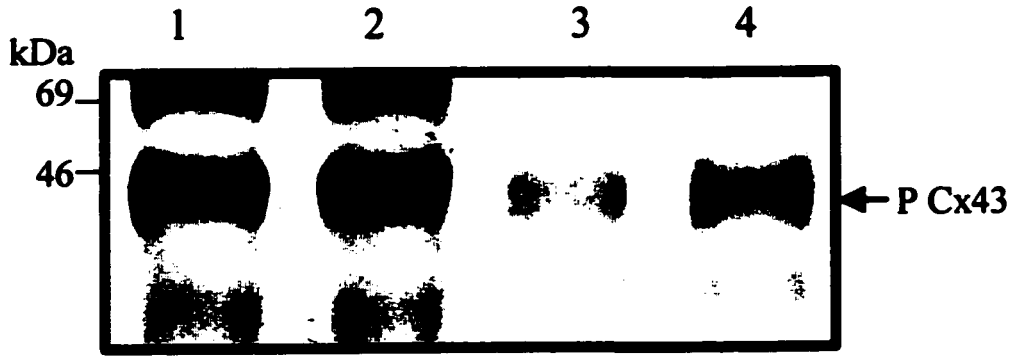
Phosphorylation of Cx43 has been postulated to be a critical regulatory element for GJIC (Lau *et al.*, 1992; Warn-Cramer *et al.*, 1998; 2001). Identification of the responsible kinases constitutes a key step in elucidating signalling pathways influencing junctional permeability. The central issue we attempted to address in the present part of this study was the evaluation of kinases responsible for GJ blockade in SH-SY5Y cells.

According to our results, SH-SY5Y cells contain at least 2 forms of phosphorylated Cx43: P1 and P2 (Fig. 2.1). The protein kinases that are known to phosphorylate Cx43 are PKC, PKA, $pp60^{v\text{-}src}$, cdc34 and MAP kinase. Examination of PDGF signalling pathways in liver cells showed that activation of PKC and MAPK were critical steps in PDGF induced Cx phosphorylation and GJIC blockage (Hossain *et al.*, 1999b). Phosphorylation may be involved in the control of connexon assembly in the trans-Golgi, connexon transfer to the plasma membrane, GJ channel formation or GJ channel gating since in other cell lines phosphorylation was shown to take part in each of these steps (Musil *et al.*, 2000). In order to identify the protein kinase(s) that phosphorylate(s) Cx43 in SH-SY5Y cells, we took two different approaches:

1. Analysis of immunoprecipitated Cx43 with phosphoserine, phosphotyrosine and phosphothreonine antibodies. This would allow us to discriminate between tyrosine kinases ($pp60^{v\text{-}src}$) and ser/threonine kinases (PKA, MAPK and PKC) that are known to regulate GJ in other cell lines.
2. Specific modulation of protein kinase activity through use of protein kinase inhibitors or activators: PD98059 (Erk1/Erk2 inhibitor), SB202190 (p38 inhibitor),

Figure 2.5 Cx43 phosphorylation in undifferentiated SH-SY5Y cells

Immunoprecipitation analysis of Cx43 with phosphotyrosine (1), (2), with phosphoserine (3) and with phosphothreonine (4) antibodies. SH-SY5Y cells were plated at $1x10^6$ cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 3 days later cells were homogenised, 100 µg of protein was immunoprecipitated with Cx43 antibody and separated by electrophoresis on 12.5% SDS-PAGE, transferred to a nitrocellulose membrane, immunoblotted with phosphotyrosine, phosphoserine and phosphothreonine specific antibodies and antigen-antibody reactions were detected using alkaline phosphatase-conjugated secondary antibodies as described in Methods. The molecular weight is expressed as kilodalton (kDa).


1
2
3
4
kDa
69
46
P Cx43

Go6976, Bis, calphostin C, staurosporine and H7 (PKC inhibitors) and finally through activation of PKA using an analogue of cAMP: 8-Br-cAMP.

2.4.1.3.1 Analysis of Connexin 43 phosphorylation pattern

In order to determine which kinase phosphorylates Cx43, we analysed immunoprecipitated Cx43 with phosphotyrosine, phosphothreonine and phosphoserine antibodies. Fig. 2.5 shows that Cx43 is phosphorylated at a higher level on tyrosine residues compared to serine or threonine residues. One candidate kinase for inducing phosphotyrosine is the src oncogene (Lau *et al.*, 1996; Zhou *et al.*, 1999). The observed phosphorylation on serine and threonine could be due to phosphorylation by PKC, MAPK or PKA since Cx 43 has consensus sites for all these kinases (Warn-Cramer *et al.*, 2001). In order to discriminate between these three kinases and to point out their respective roles in GJIC mediated tumourigenicity in SH-SY5Y cells, we used a pharmacological approach by using specific kinase inhibitors and monitoring their effects on Cx43 expression, phosphorylation and on GJIC function

2.4.1.3.2 Determination of the kinases that regulate GJ channel permeability in the SH-SY5Y cell line

MAP kinase is a serine/threonine kinase that is reported to phosphorylate Cx43 (Warn-Cramer *et al.*, 1996). MAP kinase activation is associated with disruption of GJIC in PDGF and EGF treated cells (Vikhamar *et al.*, 1998; Lau *et al.*, 1992).We investigated if MAP kinase activation was also involved in the altered expression of Cx43 in SH-SY5Y cells. We used two specific inhibitors of MAP kinase, one specific to the p38 subfamily (SB202190), and the other specific to the Erk1/Erk2 subfamily

(PD98059). We also monitored the effect of these inhibitors on SH-SY5Y cell proliferation.

2.4.1.3.2.1 MAP kinase regulation of GJ channel permeability and cell proliferation

2.4.1.3.2.1.1 Effect of the Erk1/Erk2 MAP kinase inhibitor PD98058 on SH-SY5Y cell proliferation and GJ function

PD98058 is a specific Erk1/Erk2 MAP kinase inhibitor (Beyer & Steinberg, 1991). To determine the range of PD98058 concentrations that affect SH-SY5Y cell proliferation, cells were treated with PD98058 at different concentrations ranging from 1 μM to 50 μM for 1 h and 24 h. After each time period, treated cells were counted. As shown in Fig. 2.6, PD98059 decreased cell proliferation by almost 50% at concentrations ranging between 2.5 and 10 μM. Surprisingly however, 50 μM PD98059 had no effect on cell proliferation. Since, PD98059 is an inhibitor for both Erk1 and Erk2; we considered the possibility that PD98059 could inhibit both of these kinases differentially at different concentrations. In order to monitor the effect of PD98059 on Erk1/Erk2 MAP kinases activity, SH-SY5Y cells were treated with either 10 μM or 50 μM PD98058 and the activity of each of the isozymes was determined by measuring the expression of the active forms of theses kinases; phosphoErk1 and phosphoErk2. Immunoblot analyses in (Fig. 2.7) showed that at 10 μM of PD98059 only Erk2 was active because the presence of its phosphorylated form of p42 MAPK while 50 μM PD98059 treatment inactivated both Erk1 and Erk2 kinases. We concluded that double inhibition of Erk1 and Erk2 kinases is associated with the maintenance of cell proliferation while the inhibition of Erk2 only is associated with cell growth arrest. The

growth inhibition resulting from inactivation of p44Erk1 at the 10μM dose of PD98059 suggests that p44/Erk2 elicits growth arrest in our cell model.

GJIC is known to regulate cell homeostasis and we have demonstrated that GJs are non-functional in SH-SY5Y cells and showed that Cx43 is phosphorylated at a low level by a serine/threonine kinase which may be MAP kinase, and that Erk1/Erk2 MAP kinase can be involved in the regulation of cell proliferation. To determine whether these kinases regulate GJIC in SH-SY5Y cells, we measured GJ function in PD98059 treated cells. We found that 10 μM PD98059 seemed to have no effect on GJ function since there was no difference in dye coupling observed between control and PD98059 treated cells at 10 μM or at 50 μM (Fig. 2.8).

Figure 2.6 Effect of Erk1/Erk2 MAPK inhibition on SH-SY5Y cell proliferation

Cell growth of untreated (CT) and SH-SY5Y cells treated with a concentration range of PD98059 between 1 μM and 50 μM for 1 h (1h PD98059) and for 24 h (24 h PD98059). SH-SY5Y cells were plated at $1x10^5$ cells per well in six well plates in RPMI medium supplemented with 10% FBS. 24 h later cells were treated with PD98059 (1-50 μM). After appropriate incubation periods, cells were washed, trypsinised and counted using Trypan blue dye exclusion and a hematocytometer as described in Methods. The average of three wells was used for each set of experiments. Each experiment was repeated 3 times. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).

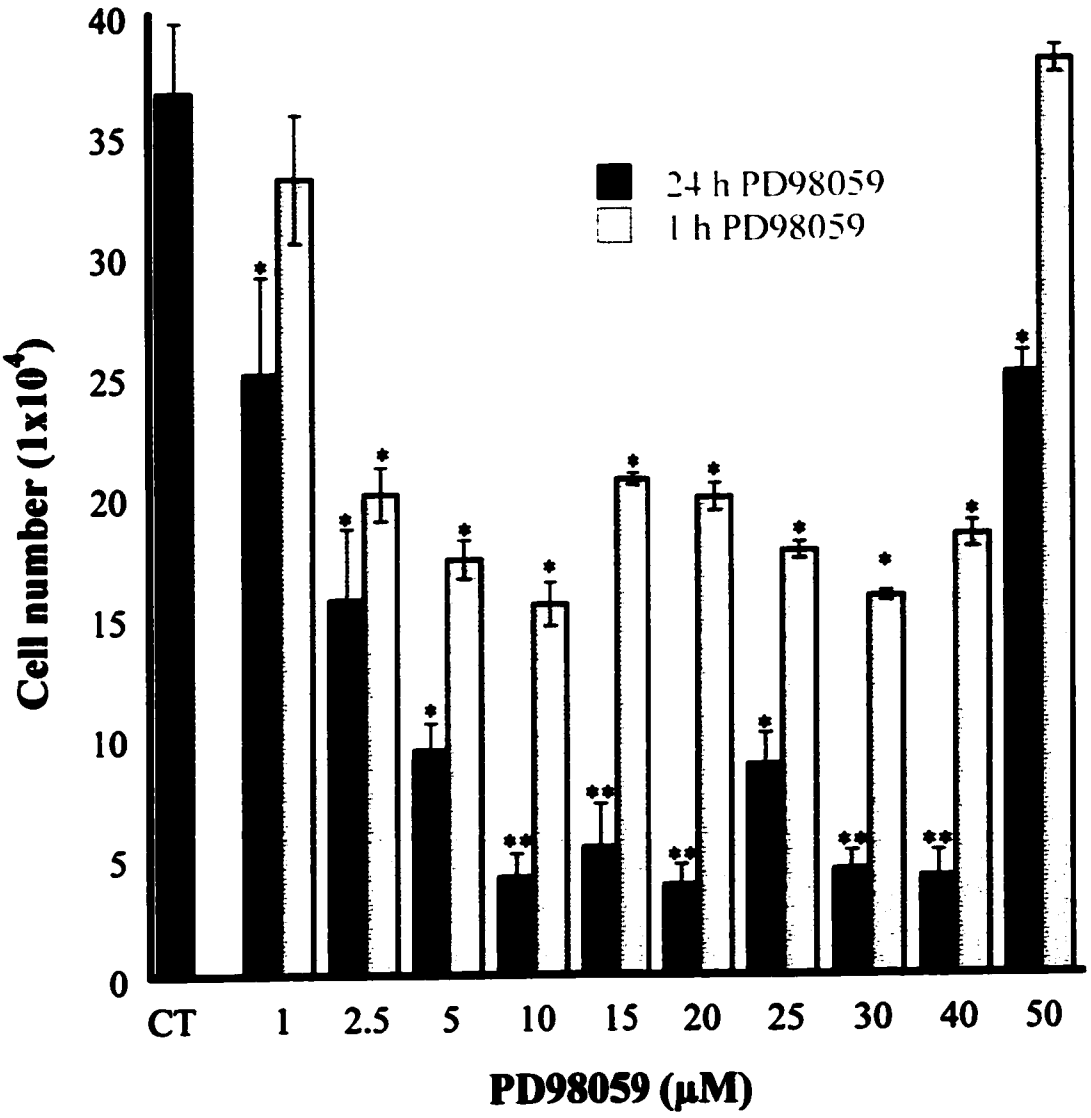

40
35
30
25
20
15
10
5
0
Cell number (1x10⁴)
24 h PD98059
1 h PD98059
CT
1
2.5
5
10
15
20
25
30
40
50
PD98059 (μM)

Figure 2.7 Erk1/Erk2 MAPK activity in PD98059-treated SH-SY5Y cells

Immunoblot analysis of phosphoErk1 (p44) and phosphoErk2 (p42) in undifferentiated (1), 10 μM (2), and 50 μM (3) PD98059-treated SH-SY5Y cells for 24 h. SH-SY5Y cells were plated at $1x10^6$cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later, cells were treated with 10 μM (2) and 50 μM (3) PD98059 for 24 h. Treated and untreated cells were homogenised, extracted and 100μg protein per lane was separated by electrophoresis by SDS-PAGE on 10% gels, transferred a to nitrocellulose membrane, immunoblotted with phospho-p42/p44 specific antibody, and detected using alkaline phosphatase-conjugated secondary antibodies as described in Methods. The molecular weight is expressed as kilodalton (kDa).

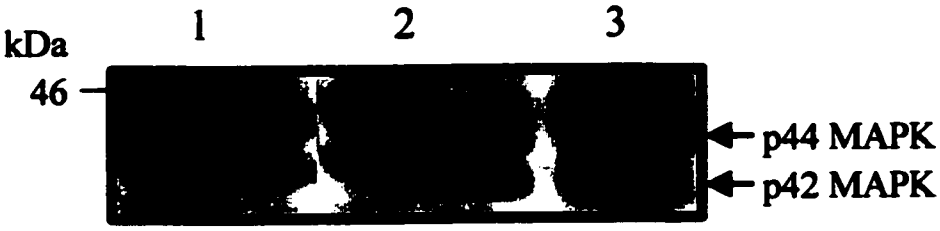

kDa
1
2
3
46
p44 MAPK
p42 MAPK

Figure 2.8 Effect of Erk1/2 inhibition on GJIC function in SH-SY5Y cells

Fluorescence photographs of scrape loaded SH-SY5Y treated for 1 hour with 10 μM PD98059 (A) and with 50 μM PD98059 (B). GJIC in SH-SY5Y cells was assayed by the scrape loading dye transfer technique. SH-SY5Y cells were seeded on coverslips at $1x10^5$ cells per 20 mm^2 until confluent. Cells were treated, washed, incubated in 1 mg/ml Lucifer yellow and scrape loaded, fixed as described in Methods. Mounted cells were viewed under fluorescence with a Nikon microscope equipped with the appropriate filter and photographed with Kodak ektachrome 400.

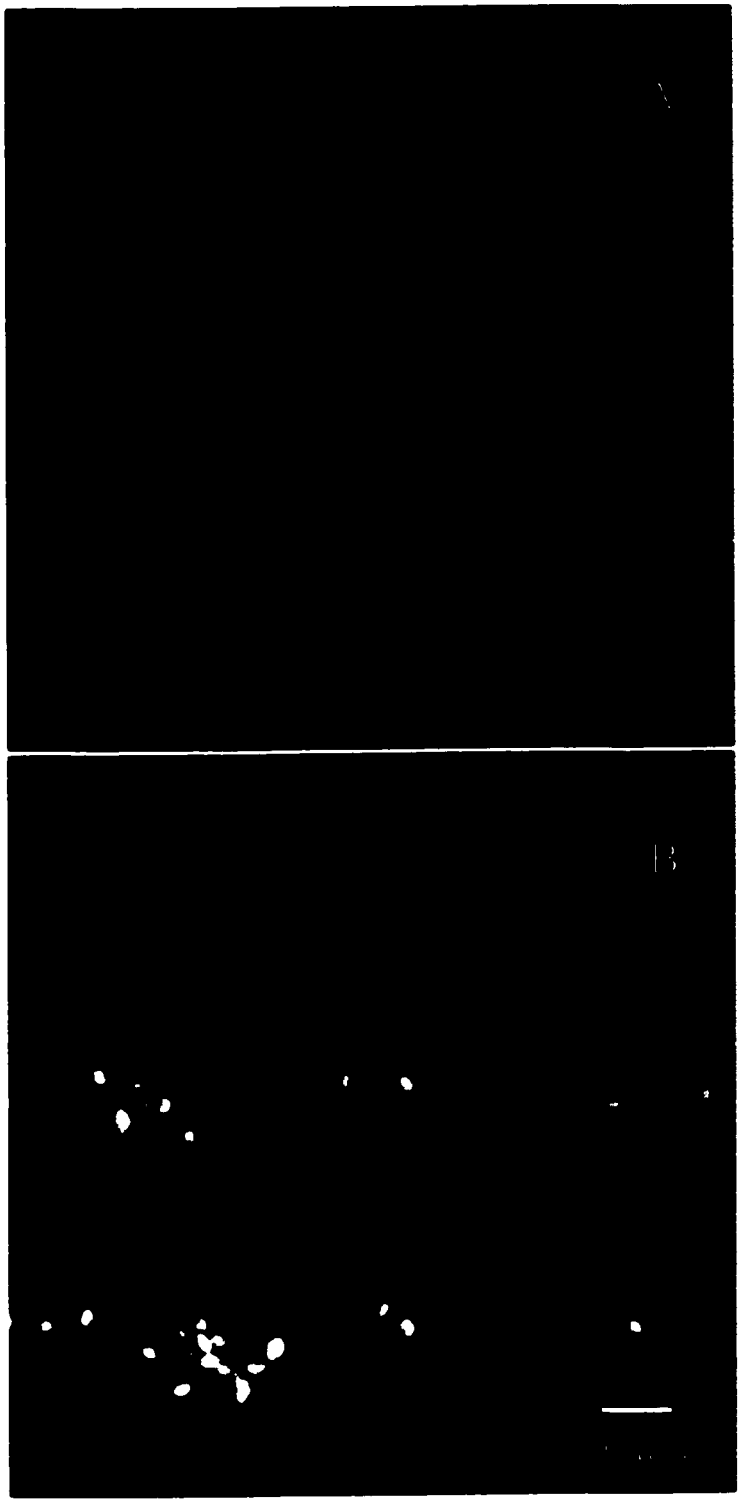
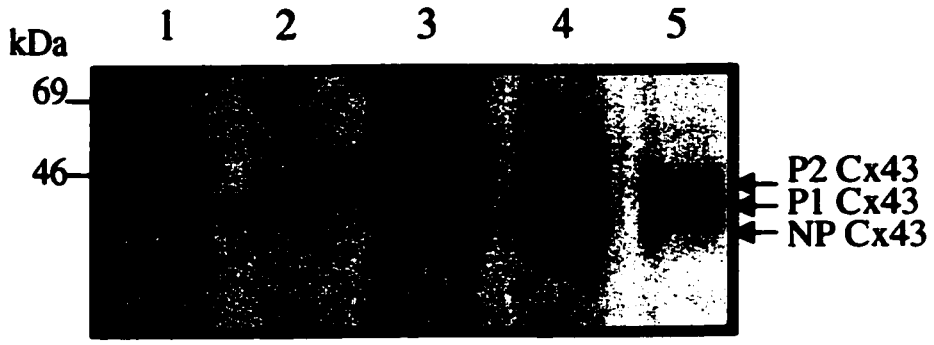

A
B

Figure 2.9 Effects of kinase inhibitors PD98059 and Go6976 on Cx43 expression in SH-SY5Y cells

Immunoblot analysis of Cx43 in untreated (1) and cells treated with 50 μM (2), 10 μM PD98059 (3, 4) or with 160 nM Go6976 (5) for 1 h. SH-SY5Y cells were plated at $1x10^6$cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h after seeding cells were treated; homogenised, extracted and 25 μg protein per lane was separated by electrophoresis on 12.5% SDS-PAGE, transferred to a nitrocellulose membrane, immunoblotted with Cx43 specific antibody, and detected using alkaline phosphatase-conjugated secondary antibodies as described in Methods. The molecular weight is expressed as kilodalton (kDa).


kDa
1
2
3
4
5
69
46
P2 Cx43
P1 Cx43
NP Cx43

This observation lead us to conclude that even if inhibition of Erk1 MAP kinase down regulates cell proliferation, it does not modulate cell coupling. Since Erk1/Erk2 MAP kinases may control other downstream events in GJIC regulation, we measured GJIC gene expression (Cx43) in PD98059 treated SH-SY5Y cells.

Cx43 immunoblot analysis showed that Cx43 protein level is very low in PD98059 treated cells at the dose of 50 μM even after only 1 hour of treatment. However, 10 μM PD98059 treated SH-SY5Y cells showed an increased level of Cx43 intracellular levels when compared to the control level (Fig. 2.9). These data confirmed what we obtained previously and showed that double inhibition of Erk1/Erk2 achieved at the 50 μM PD98059 concentration is associated to loss of both Cx expression and cell growth control. Cx43 protein is likely degraded since the Cx43 band was absent. At 50 μM, SH-SY5Y cells seem to grow normally, which is consistent with the idea that poor or no cell-cell communication favours proliferation (for review Yamasaki *et al.*, 1998).

2.4.1.3.2.1.2 Effect of p38 MAP kinase on SH-SY5Y cell proliferation and GJIC function

p38 MAP kinase is another candidate that may phosphorylate Cx43 since it is a serine/threonine kinase (Allemain *et al.*, 1997; Bennett *et al.*, 1990). p38 was reported to play a pivotal role in the control of cell proliferation and induction of apoptosis. One of the most specific inhibitors for p38 MAP kinase is SB202190.

GJIC was found to be altered in NB SH-SY5Y cells. We also showed that undifferentiated SH-SY5Y cells express the phosphorylated forms of Cx43 (Fig. 2.5). Hyperphosphorylation is reported to be linked to a blockage in GJ channel and to isolation from external stimuli that leads to an increase in cell proliferation. In order to

investigate whether p38 MAPK is responsible for these phenomena in SH-SY5Y cells, we used a p38 MAPK inhibitor. If our hypothesis is correct, it means that p38 MAPK inhibition will induce GJ opening, which will impact upon cell proliferation.

A. Effect of SB202190 on cell growth

Since the increase of channel permeability is proportional to a decrease in cell proliferation, SH-SY5Y cells were treated for 24 h with SB202190 at different concentrations ranging from 0 to 100 μM and cell a proliferation assay was performed (Fig. 2.10). The cell growth curve showed that at 3 μM, SB202190 induced a decrease in cell proliferation by almost 50%, a concentration coincident with the open state of GJ channels (Fig. 2.11). This decrease in cell number could be due either to a decrease in cell proliferation or to an increase in cell death. Cells were then visualised by phase-contrast microscopy and did not show any cell death. Furthermore, the percentage of dead cells was estimated and did not exceed that found in proliferating control counterparts.

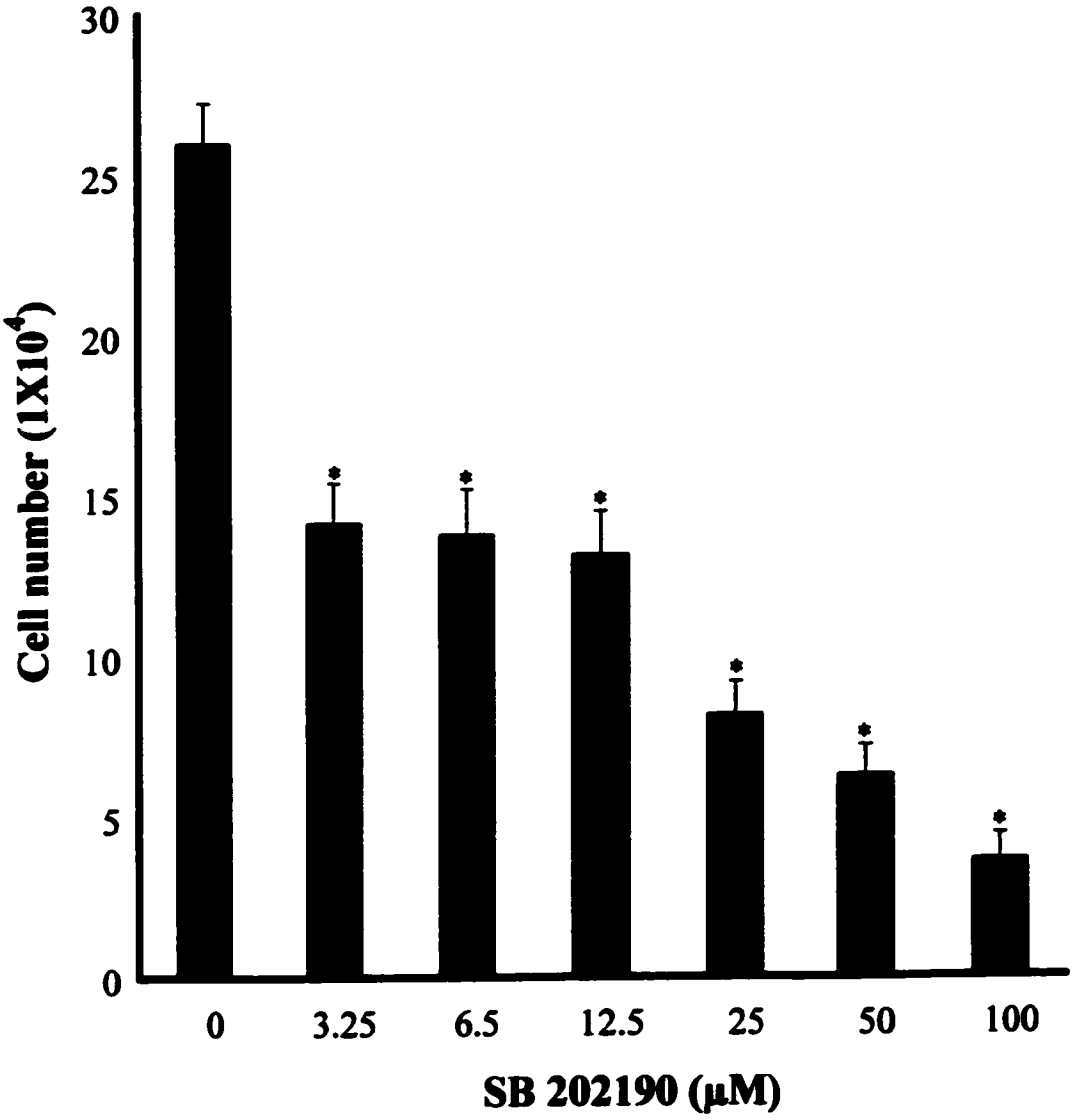
B. Effect of SB202190 on GJIC function

In order to determine the dose response of GJIC function, cells were treated with different SB202190 concentrations and the subsequent GJIC function was measured by scrape loading. We found that at a 2 μM concentration, there was no effect on GJ function and that the concentration of SB202190 that leads to the opening of the GJ channel was 3 μM after only 1 hour treatment (Fig. 2.11).

Image Pro-analysis software combined with imaging was used to quantify the extent of channel permeability. The highest level of channel permeability was coincident with the

Figure 2.10 Effect of p38 MAPK inhibition on SH-SY5Y cell proliferation

Cell growth of untreated and SH-SY5Y cells treated with SB202190 at a concentration range between 0 and 100 μM for 24 h. SH-SY5Y cells were plated at $1x10^5$ cells per well in six well plates in RPMI medium supplemented with 10% FBS. 24 h later cells were treated with SB202190 (0-100 μM). After a 24 h incubation period, cells were washed, trypsinized and counted using trypan blue dye exclusion and a hematocytometer as described in Methods. The average of three wells was used for each set of experiments. Each experiment was repeated 3 times. (* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$).


30
25
20
15
10
5
0
Cell number (1X10⁴)
*
*
*
*
*
*
0
3.25
6.5
12.5
25
50
100
SB 202190 (μM)

highest degree of the cell survival and occurred after a 24 h treatment (data not shown). We conclude that p38 MAP kinase is involved in the regulation of channel permeability.

Since Cx43 has a very short half life, we wanted to explore whether the increase in dye coupling may result from an increase in the expression of Cx43. Western blot analysis showed that the level of protein expression did not change when cells were treated with SB202190 at a concentration of 3 μM (data not shown).

We conclude that p38 MAPK is involved in the regulation of GJs at the level of channel gating given that its inhibition has no effect on gene expression following short periods of exposure to SB2022190. The opening of GJ channels made of Cx43 coincided with the proliferation arrest in SH-SY5Y NB cells.

2.4.1.3.2.2 Effect of PKC inhibition on cell proliferation and GJIC function

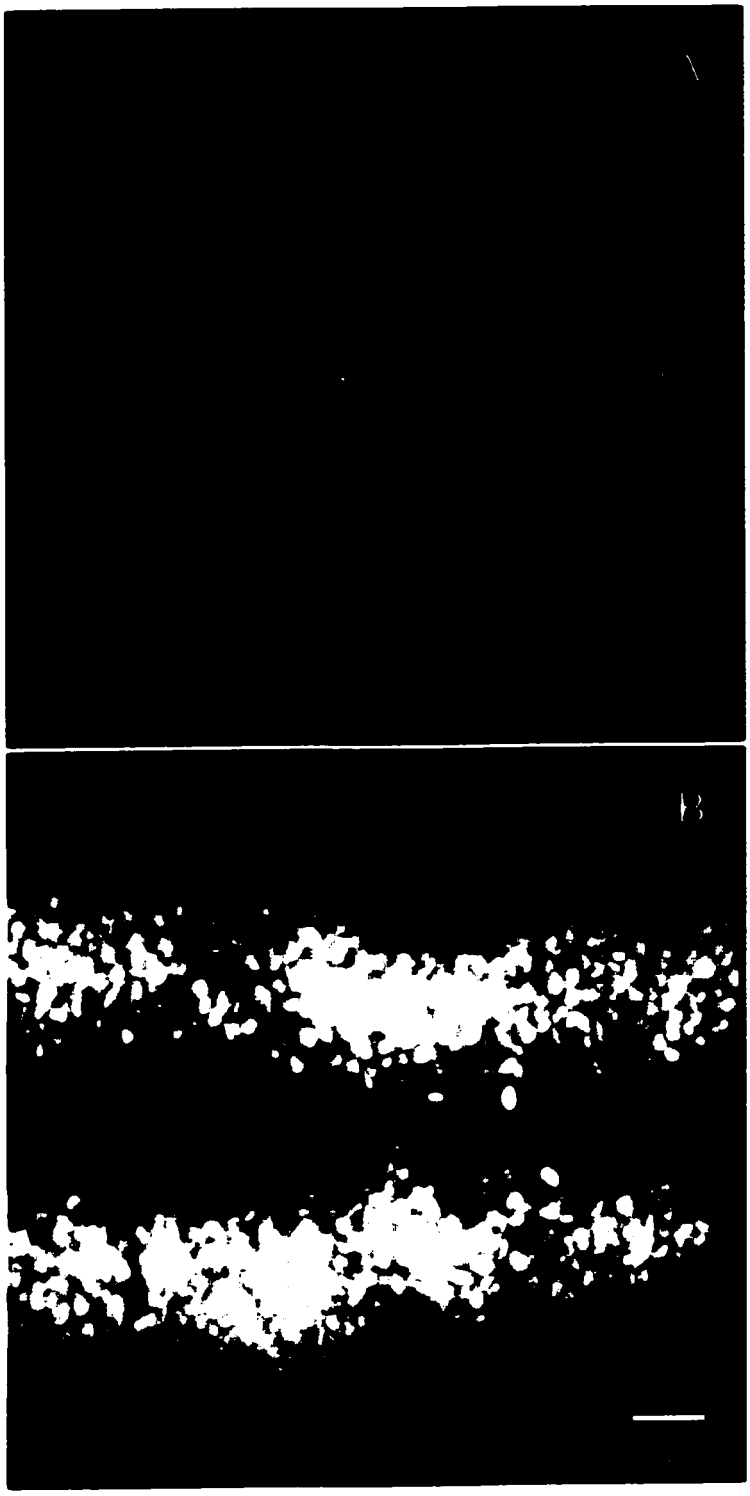
In order to investigate the role of PKC on GJIC function in SH-SY5Y cells, we treated these cells with specific and non-specific inhibitors of PKC. We used Go6976 as specific PKC inhibitor and staurosporine and H7 as non-specific PKC inhibitors. Go6976 inhibits the cPKC class, thus in SH-SY5Y cells it inhibits isoforms α and β.

We found that treatment with staurosporine (100 nM) or H7 (1 μM) restored GJIC function after only 1 hour treatment (Fig. 2.12A,B). Dye coupling seems to be higher in H7 treated cells. This was accompanied by a decrease in Cx43 expression after 1 and 4 h treatment and increase in Cx43 expression after 24 h (Fig. 2.13). We did not observe any change at the level of Cx phosphorylation pattern (Fig. 2.13).

SH-SY5Y cells express PKC isoforms α, βII, ε, μ and ζ (see chapter one), and we wanted to investigate which PKC group of isoforms was responsible for GJIC blockage

Figure 2.11 Effect of p38 MAPK inhibition on GJIC function in SH-SY5Y cells

Fluorescence photographs of scrape loaded untreated SH-SY5Y cells (A), treated with 3 μM SB202190 for 1 h (B). GJIC in SH-SY5Y cells was assayed by scrape loading dye transfer technique. SH-SY5Y cells were seeded on coverslips at $1x10^5$ cells per 20 mm^2 until confluent. 24 h later cells were treated, washed, incubated in 1 mg/ml Lucifer yellow, scrape loaded, and fixed as described in Methods. Mounted cells were viewed under fluorescence with a Nikon microscope equipped with the appropriate filter and photographed with a Kodak ektachrome 400 film.


A
B

in SH-SY5Y cells. We used Go6976 since it is specific for PKC α, β, and γ. We found that 160 nM Go6976 treated-cells showed a decrease in cell proliferation after 24 h treatment (see chapter one Fig. 1.23) without causing any morphological differentiation or any sign of apoptosis (Fig. 1.25 and Fig. 1.26) and induced an increase in cell-cell coupling via GJ as shown by dye coupling experiments (Fig. 2.11.C). Since PKC α, βII are expressed in this cell line, we can conclude that PKC α and/or βII are likely responsible of GJ blockage in NB SH-SY5Y cells.

Go6976 did not seem to affect Cx43 expression (Fig. 2.9, lane 5). Treatment with PKC non-specific inhibitors, staurosporine and H7 induced a modulation of Cx43 expression that was time-dependent (Fig. 2.13). The expression of Cx43 increased after 4 hours H7 treatment and 24 h staurosporine treatment, which is coincident with cells undergoing apoptosis.

Figure 2.12 Effect of PKC non-specific kinase inhibition on GJIC function in SH-SY5Y cells

Fluorescence photographs of scrape loaded SH-SY5Y treated with 100 nM staurosporine (A), 1 μM H7 (B) and 160nM Go6976 (C) for 1 hour. GJIC in SH-SY5Y cells was assayed by the scrape loading dye transfer technique. SH-SY5Y cells were seeded on coverslips at $1x10^5$ cells per 20 mm^2. 24 h later cells were treated for 1 h with staurosporine or H7, washed, incubated in 1 mg/ml Lucifer Yellow and scrape-loaded, and fixed as described in Methods. Mounted cells were viewed under fluorescence microscopy with a Nikon microscope equipped with the appropriate filter and photographed with a Kodak ektachrome 400 film.

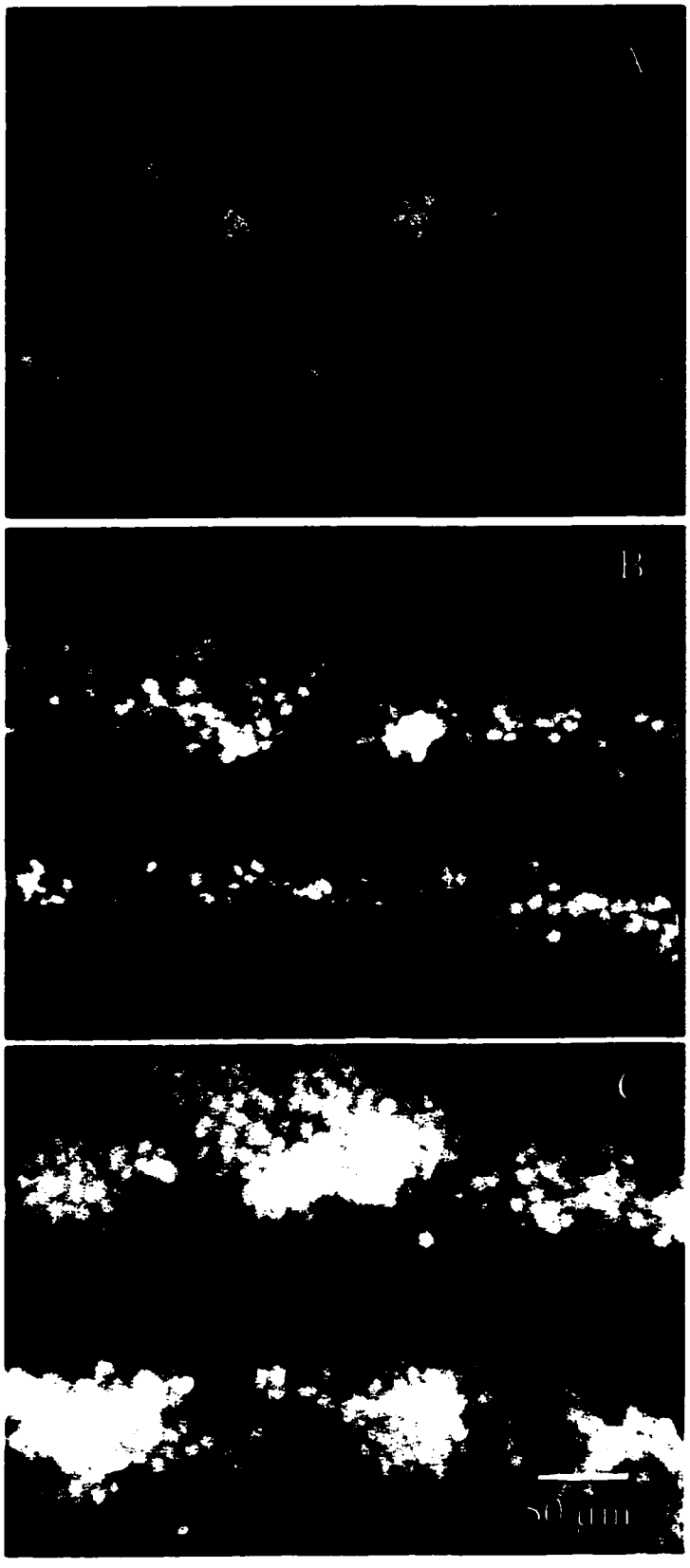

A
B
C
50 μm

Figure 2.13 Effect of PKC non-specific kinase inhibitors on Cx43 expression in SH-SY5Y cells

Immunoblot analysis of Cx43 in untreated (1) and treated cells with 100 nM staurosporine for 1 h (2), 4 h (3) and 24 h (4) or with 100 μM H7 for 1 h (5), 4 h (6), and 24 h (7). SH-SY5Y cells were plated at $1x10^6$cells per 100 mm^2 in RPMI medium supplemented with 10% FBS. 24 h later cells were treated, homogenised, extracted and 25 μg protein per lane was separated by electrophoresis on 12.5% SDS-PAGE gels, transferred to a nitrocellulose membrane, immunoblotted with Cx43 specific antibody, and detected using alkaline phosphatase-conjugated secondary antibodies as described in Methods. The molecular weight is expressed as kilodalton (kDa).


1
2
3
4
5
6
7
kDa
200
69
46
Cx43

2.5. Discussion

GJIC is thought to play a key regulatory role in growth control and differentiation (Lo, 2000; Trosko & Ruch, 1998; Trosko *et al.*, 2000; Yamasaki *et al.*, 1999b). The regulating mechanisms are not fully elucidated. Our objectives were dual: first to identify the connexins potentially expressed and second to determine which signal transduction pathway(s) was involved in the regulation of GJIC in NB SH-SY5Y cells. New findings from the present study include the following,

1) Neuroblastoma SH-SY5Y cells express Cx43 that is abnormally located around the nucleus and are GJIC deficient like most other cancer cells. GJIC is non-functional even through phosphorylated and unphosphorylated forms of Cx43 were expressed. The blockage in GJ channel permeability seemed to be due to the aberrant Cx43 sublocalisation in the perinuclear region.
2) Induction of differentiation via activation of the upstream PKA pathway through an increase in intracellular cAMP resulted in increased Cx43 expression, re-localisation of Cx43 to the membranes and progressive restoration of GJ channel permeability. This process paralleled the morphological and molecular neuronal differentiation.
3) The inhibition of cPKC isoforms α and/or βII restored GJ function.
4) Erk1/Erk2 MAPK inhibition had no effect on GJ channel permeability but may affect cell proliferation.
5) p38 MAPK inhibition induced an increase in channel permeability and a decrease in cell proliferation.

2.5.1 The alteration of connexin 43 localisation and GJIC function in SH-SY5Y neuroblastoma cells

Neuronal precursor cells are extensively connected by GJ channels made of Cx43 during the early development of the nervous system (Lo *et al.*, 1997; LoTurco *et al.*, 1991). Cx43 expression and channels were shown to be closely linked to the migratory behaviour of those cells. This is true for CNS neuroblasts (LoTurco *et al.*, 1991) as well as PNS neuroblasts (Waldo *et al.*, 1999; Lo, 1996).The presence of Cx43 in NB cells is therefore not surprising since NB may be considered as neuroblasts that were blocked during progression into differentiation. Neural crest cells express Cx43 which is developmentally regulated (Bannerman *et al.*, 2000). Furthermore, neural crest cells have functional GJIC (Ewart *et al.*, 1997). By itself, the lack of expression of Cx32, that is controversial in PNS mature neurones (Nagy *et al.*, 1999; Nagy & Rash, 2000), does not rule out the expression of other connexins that we did not to investigate in this study. In opposition, the altered expression, localisation and GJIC in NB cells is not a normal feature of neuronal precursors from which NB are derived (Lo *et al.*, 1997). This alteration starts at the level of gene expression since Cx43 is found to be expressed at low levels in proliferating NB cells.

A second defect seems to arise at the level of gap junction channel formation and a third one at the level of channel gating. Since immunostaining analysis showed that GJ were absent from cell-cell contacts in the SH-SY5Y cell line (Fig. 2.2), the alteration in channel formation and translocation becomes a key factor explaining the observed GJIC deficiency in these cells. Connexins pass through many steps before they are transferred to the plasma membrane to accomplish their function. Firstly, connexin assembly into

connexons occurs in the trans-Golgi network through an unknown mechanism (Musil & Goodenough, 1993). Secondly, connexons are transferred to the plasma membrane where each connexon docks with the connexon of a neighbouring cell to form the GJ channel. It is believed that GJ channels are not functional if they are arranged as single GJ channels. Only GJ plaques have been shown to have functional GJ channels (Musil *et al.*, 2000).

A number of studies have documented a correlation between neoplastic transformation and reduced GJIC (for review see Trosko & Ruch, 1998; Yamasaki *et al.*, 1999a). These neoplastic cells have fewer and smaller gap junctions, express less connexins, and have reduced GJIC compared to their normal counterparts. GJIC was shown to be reduced in human glioma (Soroceanu *et al.*, 2001), in adrenocortical tumours (Murray *et al.*, 2000) and in human prostate cells (Hossain *et al.*, 1999). There are, however, some neoplastic cells with normal or greater GJ expression and cell-cell coupling (Trosko & Ruch, 1998). Abnormal perinuclear localisation of Cx has been observed in a number of cancer cell lines (Yamasaki *et al.*, 1999a), an example is the prostate ovarian cancer cells where connexin43 was present in the cytoplasm (Hossain *et al.*, 1999c). It would be of interest to investigate whether the mechanisms underlying this defect are common to all neoplastic cells.

2.5.2. Relationship between growth control, differentiation and GJIC function in neuroblastoma SH-SY5Y cells

TPA, a specific regulator of PKC activity, affects a wide variety of cellular processes related to deficiency in gap junctional communication (Brissette *et al.*, 1991).

Signalling involving PKA and PKC activation plays a leading role in inducing neuritogenesis and arrest of cell proliferation (Shea *et al.*, 1992; Newton & Johnson., 1998). Long term treatment of several neuroblastoma cell lines with forskolin or dibutyryl cAMP arrests proliferation and induces morphological changes with neurite spreading (Shea & Beermann, 1991).

Outgrowth is associated with a down regulation of PKC kinase activity (Shea & Beermann, 1991; Pahlman, 1981; Zeidman *et al.*, 1999). PKC isoform α was shown to regulate cell proliferation (Wada *et al.*, 1989). The two classes of kinases, PKC and PKA, are indeed also controlling the several steps that lead to GJ formation and function (Saez *et al.*, 1998). The association with cellular differentiation is not fortuitous but it is not known, however, whether this association is a cause or the consequence of cell differentiation. We showed that during proliferation and differentiation events, GJIC is modulated both at the level of gene expression and regulation. Activation of PKA induced both down regulation of cell proliferation and morphological and molecular induction of differentiation that were associated with modulation of Cx43 expression and function. These events were shown to be irreversible during long term treatment. We did not provide evidence of a direct involvement of GJIC in these events, however we demonstrated that they are closely associated with an evolution of Cx43 expression and GJIC function in NB *in vitro*.

The fact that Cx43 knockout mice present morphologically differentiated CNS neurones (Naus *et al.*, 1997) seems to reinforce the generally held opinion that Cx43 relates more to neuronal migration than to differentiation in developing neuroblasts. Let us stress however that the authors did not provide any molecular proof that the neurones

of knockout mice were terminally differentiated. In addition, Cx43 is no longer expressed in mature neurones of CNS and PNS (Nadarajah *et al.*, 1997; Lo *et al.*, 1997). It means that its expression may evolve in parallel with differentiation as it does in NB cells. In the latter stages, however, the Cx43 remains expressed and the channels operational up to the mature stage of differentiation. Cx43 was present at a higher level in terminally differentiated SH-SY5Y cells after 3 weeks of treatment with 8-Br-cAMP and NGF.

According to the literature, most of cancer cells are GJIC aberrant. Cx43 was found to be expressed in its phosphorylated form in SH-SY5Y cells (Fig. 2.1). Dye coupling experiments showed that SH-SY5Y cells have defective GJIC (Fig. 2.3) but that the channels become functional upon differentiation induction by treatment with 8-Br-cAMP. Therefore there is a molecular defect with the Cx43 or the channels themselves. It is the aberrant localisation of Cx43 that seems responsible for the GJIC deficiency in these cells. Induction of terminal differentiation leads to both GJIC proficiency and down-regulation of SH-SY5Y cell proliferation (see chapter 1). We speculate that the down-regulation of proliferation to which GJIC proficiency contributes may relate directly to progress into differentiation and its association with the end of neuroblast migration.

2.5.3 Signalling pathways that may control GJIC function in the neuroblastoma cell line SH-SY5Y

A number of serine phosphorylation sites have been identified within the Cx43 sequence (Warn-Cramer *et al.*, 2001) and induction of Cx dephosphorylation has been correlated with either a reduction (Cotrina *et al.*, 1998; Godwin *et al.*, 1993) or an

increase in unitary channel conductance (Moreno *et al.*, 1994). Phosphorylation is thought to be required for proper synthesis and assembly of connexins into GJs and the regulation of electrotonic conductance as well as permeability to small molecules (Saez *et al.*, 1998).

All GJIC-competent cells expressing Cx43 have at least one phosphorylated Cx43 species in addition to the unphosphorylated form (Bruzzone *et al.*, 1996). In most cells, Western blot analysis indicates three major Cx43 species, but the exact pattern deviates somewhat between various cell types. Thus, kinase activity is necessary to obtain functional GJIC. Cx43 is phosphorylated on serine and threonine residues, although tyrosine phosphorylation is also found in some instances (Warn-Cramer *et al.*, 2001). Tyrosine phosphorylation has been linked to decreased GJ intercellular communication and phosphorylation on serine and threonine has been linked with both increased and decreased GJIC (Lau *et al.*, 1996).

2.5.3.1 Effect of PKA activation

8-Br-cAMP is a PKA activator. PKA is a serine/threonine kinase and PKA phosphorylation sites were identified in the carboxyl terminal end of rat Cx43 (Lau *et al.*, 1996). We have shown that increasing the intracellular level of cAMP by treating SH-SY5Y cells with the cAMP membrane soluble analogue 8-Br-cAMP not only induced cell growth arrest but also promoted differentiation (see chapter 1). These events are also associated with both an increase of Cx43 gene expression and the transfer of Cx43 from the perinuclear region to the plasma membrane (Fig. 2.3). Cx43 was probably located either in the Golgi apparatus where Cx43 is reported to be

assembled into connexons or in the endoplasmic reticulum vesicles where Cx43 is synthesised in undifferentiated cells (Srinivas *et al.*, 1999; Spray, 1998).

Cyclic AMP was shown to regulate cell growth in many cell types including neuroblastoma. In addition, 8-Br-cAMP induced cell differentiation in NB and other neuronal precursor cells as shown by others and us. It has a molecular weight of 329 kDa and thus can pass through gap junction channels. Cyclic AMP was shown to inhibit the growth of many cell lines due to a block in cell cycle progression in the G1 phase. This was shown to be due to at least two mechanisms. First, cAMP decreases the expression of cyclin D1 which is associated with cyclin-dependent kinase (cdk) 4 or 6, depending upon the cell type and is active in G1 (Trosko & Ruch, 1998). The active complex phosphorylates the retinoblastoma protein which releases E2F transcription factors and in turn these activate the expression of genes needed for the S phase. Secondly, cAMP increases the expression of the cyclin-dependent kinase inhibitor, p27/kip-1 (Kato, 1999)and its intracellular accumulation (Shen *et al.*, 2000).

The increase in intracellular cAMP in neoplastic cells was reported to induce cell growth arrest through activation of p27 or decrease of cyclin D3 (Trosko & Ruch, 1998). Furthermore, cAMP was shown to induce an accumulation of cyclin B1 (Kurokawa & Kato, 1998). cAMP activates PKA and probably acts at the level of connexin assembly into connexons or connexon transfer to the plasma membrane. Both these events were shown to be regulated by phosphorylation during connexin trafficking. Recently, cAMP was reported to increase the level of GJ assembly within one hour of treatment in lung epithelial cells (Banoub *et al.*, 1996). Reaggregating cells in the presence of agents that increase cAMP levels (8-Br-cAMP, forskolin) enhanced both dye transfer rate between

cells and the extent of gap junction formation by 2 to 3 fold (Naus *et al.*, 2000; Trosko *et al.*, 2000).

Studies with the PKA inhibitor H-89 indicated that cAMP signalling plays a key role in enhancing assembly of connexon (Saez *et al.*, 1998). Furthermore, agents such as, brefeldin A, monesin and nocodazole that blocks GJ assembly inhibit the intracellular trafficking that is triggered by elevated cAMP. These agents act through different mechanisms (Musil & Goodenough, 1993; De *et al.*, 1993; Laird *et al.*, 1995; George *et al.*, 1999). This is consistent with the pattern of changes occurring upon exposure to 8-Br-cAMP as illustrated by our results. In mouse preimplantation embryos, treatment with db-cAMP increased the phosphorylation of GJ and Cx43 positive spots, whereas, TPA treatment increased Cx phosphorylation but decreased Cx43 positive spots, suggesting that Cx43 phosphorylation induced by different kinases leads to different effects on GJ formation in the mouse preimplantation embryo (Ogawa *et al.*, 2000).

Taken together, the literature and our data support PKA phosphorylation activity as being essential to Cx trafficking and localisation. In many neoplastic cells, the level of cAMP was shown to be reduced (Trosko & Ruch, 1998). This is probably the reason why GJIC was altered in these cells. In some tumour cells, connexins, when expressed, were reported to be aberrantly localised. Thus, in human hepatocellular carcinoma, Cx32 proteins were localised intracytoplasmically and sometimes perinuclearly (Krutovskikh *et al.*, 2000). Aberrant localisation of connexins also occurs in cells exposed to tumour promoting agents. When rat liver fibroblastic cells were exposed to TPA, Cx43 became intracytoplasmically located (Husoy *et al.*, 2001). This was associated with the appearance of a new phosphorylated form of Cx43, suggesting that PKC activation by

TPA may cause phosphorylation of Cx43, which in turn also resulted in inhibition of GJIC. Furthermore, gap junctions were shown to be internalised in keratoris foliculoris (Darer's disease) (Haftek *et al.*, 1999). Although PKA seems to be primarily responsible for the translocation of Cx43 to the plasma membrane, the contribution of PKC cannot be ruled out from our experiments.

2.5.3.2 Effect of PKC inhibition on cell growth and Cx expression

Phorbol esters such as TPA activate PKC, increase Cx43 phosphorylation, and decrease cell-cell communication via gap junction in many cell types (Lampe *et al.*, 2000). Recently, ser368 was found to be the residue on which Cx43 is phosphorylated after TPA treatment. The authors brought evidence that PKC directly phosphorylated Cx43 on ser368 *in vivo*, which resulted in single channel behaviour that in turn contributed to a decrease in intercellular communications (Lampe *et al.*, 2000). Furthermore, serine phosphorylation by PKC was found to be responsible for the hepatocarcinogen nafenopin-mediated loss of Cx32 function in rat hepatocytes (Elcock *et al.*, 2000).

It has long been known that the two classes of classical and novel PKC both respond to phorbol ester exposure. It was shown that in human heart cells, not only PKCα but also PKCε modulates Cx43 phosphorylation (Granot & Dekel, 1994). Both PKC isoforms increased phosphorylation within the Cx43 immuno-complex, but only PKCε directly phosphorylated Cx43. Furthermore, PKCε was found to be responsible for FGF-mediated decreases in GJ permeabilty by stimulating Cx43 phosphorylation in cardiomyocyte cells (Doble *et al.*, 2000). However, in the SH-SY5Y cell model, PKCε

was shown to be up regulated in differentiated SH-SY5Y, which is associated with an increase in GJ permeability. This is probably due to tissue-specific regulation of PKC isoforms in the regulation of GJ.

Oncogenic Ras blocks GJIC in E9 cells apparently through a PKC-dependent mechanism that does not directly involve Cx43 expression or phosphorylation (Trosko & Ruch, 1998). Taken together these results point to the complexity of the interaction of PKC with GJ and seems to indicate that PKC effects are likely to be both isoform and cell type specific.

SH-SY5Y cells express classical isoforms of PKC α and βII, μ, ζ and novel PKCδ and ε at the protein level as demonstrated earlier by Shea and colleagues (Shea & Beermann, 1991; Shea *et al.*, 1992) and confirmed in our study. All PKC inhibitors used suppressed SH-SY5Y cell growth. Go6976 was originally described as a selective inhibitor of classical PKC inhibitors and it has been shown to also inhibit PKCμ (Dempsey *et al.*, 2000; Newton & Johnson, 1998). Since Go6976 does not affect novel PKC isoforms and that inhibitors that do not show potency toward PKCμ (GF109203X and Go6983) also suppressed NB growth, a logical interpretation is that one or several classical PKC isoforms are important for NB cell growth. Classical isoforms, particularly PKCα, have been implicated in growth stimulation and tumour growth in several cell types. An antisense oligonucleotide directed toward PKCα has been shown to inhibit growth of several human tumour cell lines in an animal model (Dean *et al.*, 1996). The fact that PKCα and βII were found in the NB cell line SH-SY5Y and that their inhibition by Go6976 also acted as a growth suppressor indicates that either PKCα

or βII may be contributing directly or indirectly to NB proliferation. These isoforms may constitute interesting targets for controlling the growth of NB.

Zeidman *et al.* (1999) reported that supplementing with Go6976 caused apoptosis in SH-SY5Y cell cultures, suggesting that increased apoptosis may also be involved in the decreased number of viable cells. It is possible that long-term treatment with Go6976 could cause apoptotic death.

Keeping in mind that non-specific inhibitors of PKC such as staurosporine and H7 act upon several protein kinases, we examined their effect on gap junctions in SH-SY5Y cells. Staurosporine is more potent than H7 on PKA at the concentrations used in our experiments. Staurosporine and H7 both induced differentiation and apoptosis in SH-SY5Y cells. In chapter one, we wanted to investigate whether gap junctions may be involved in this effect. The effect of staurosporine and H7 was explored at the level of the Cx43 protein induction and gap junction permeability. As shown by immunoblotting in Fig. 2.14, staurosporine treatment induced an increase in Cx43 levels coincident with the induction of neurites. This was followed by a decrease in Cx43 and cell apoptosis. The same pattern of Cx43 expression was observed when cells were treated with H7. However, there was no change in the pattern of Cx43 phosphorylation with neither staurosporine nor H7 treated cells. The treatment with staurosporine had no effect on GJ channel function while H7 induced an increase in GJIC channel permeability.

As explained earlier, staurosporine and H7 differ in their ability to inhibit different kinases. Furthermore, H7 was reported to induce SH-SY5Y cell differentiation and apoptosis through the p53 pathway, unlike staurosporine (Perron & Bixby, 1999).

Except for the observations reported, no conclusion could be drawn from our experiments with regard to GJs due to the lack of selectivity of the two inhibitors.

2.5.3.3 MAP Kinase

The involvement of MAPK in GJIC regulation was suggested by the following observations. Firstly, various GJIC inhibitory agents, including EGF, PDGF and TPA, are also activators of MAPK (Kanemitsu & Lau, 1993; Hossain *et al.*, 1998). Secondly, Cx43 contains consensus sites for MAPK phosphorylation and MAPK is capable of phosphorylating Cx43 *in vitro*. Finally, mutations of the putative MAPK phosphorylation site in Cx43 abrogated disruption of GJIC by EGF (Warn-Cramer *et al.*, 1996). Since many of the above-mentioned ligands and growth factors activate other protein kinases, it is not certain whether activation of MAPK is itself sufficient for Cx43 phosphorylation or whether factors additional to MAPK are also needed.

MAPK activation was reported to cause GJIC blockage through PDGF or EGF elicited pathways in rat liver epithelial cells. We wanted to know if MAPK activation contributed to the blockage of GJIC in our cell model. MAP kinase is a family of kinases including the Erk family and p38 family of kinases. p38 MAP kinase contributes to GJ blockade in NB SH-SY5Y cells since treatment with SB202190, a specific inhibitor of p38 MAP kinase, leads to a restoration of cell-cell coupling.

SB202190 was reported to induce a decrease in cell proliferation due to induced programmed cell death in SH-SY5Y cells. A subsequent treatment with NGF restored cell proliferation by preventing cell death. We observed that upon longer exposure of the cells to the p38 specific inhibitor, apoptosis was induced. Therefore SB202190 induced

increases in GJIC permeability is likely a consequence (or a cause) of SB202190 induced apoptosis in SH-SY5Y cells. The role of GJIC in apoptosis is not well established.

2.5.3.3.1 Erk1/Erk2 family of kinases in gene expression and GJIC

Very intriguing data were obtained with regard to Erk1/Erk2 effects on GJIC. By using phospho-specific anti Erk1 and Erk2 antibodies, it was clearly shown (Fig. 2.8) that both Erk isoforms are phosphorylated in control proliferating cells. p44 Erk1 phosphorylation is inhibited by the 10 μM dose of the PD98059 inhibitor. It coincides with growth inhibition of the treated cultures. A dose of 50 μM inactivated both Erks but did not alter the population growth. This indicates that another pathway contributes to proliferation of SH-SY5Y cells besides the MAPK pathway. We speculate it could be PKC acting on the serum responsive element SRE independently from MAPK. The decrease in Cx43 subsequent to Erk double inactivation is consistent with the maintenance of proliferation.

The data also suggest that proliferation in SH-SY5Y cells be regulated by p44 Erk1 and a second unidentified pathway. Disruption of GJs can be achieved by multiple mechanisms. Permanent or chronic blockage of GJ can be caused by tyrosine phosphorylation. We showed that Cx43 was highly phosphorylated on its tyrosine site. This may be attributed to pp60 $^{v\text{-}src}$ present in many transformed cells, which caused the tyrosine phosphorylation.

The pp60$^{v\text{-}src}$ tyrosine kinase disrupts GJIC in transformed fibroblasts and induced the phosphorylation of Cx43 on tyrosine (Loo *et al.*, 1995). Recently, another tyrosine kinase, the p130gag-fps kinase was reported to phosphorylate Cx43 and to

induce disruption of GJIC. Both pp60$^{v\text{-}src}$ and p130gag-fps were shown to trigger other signalling pathways through serine kinases (Kurata & Lau, 1994). Further work aimed at deciphering how pp60 $^{v\text{-}src}$ may affect Cx43 phosphorylation in NB would be of interest.

2.6. Conclusions

The overall conclusions of this chapter are that gap junction intercellular communication is altered in SH-SY5Y neuroblastoma cells. SH-SY5Y cells were found to express Cx43, a protein forming GJ however, we could not detect any GJIC function. Immunolocalisation of Cx43 showed that this connexin was sub-localised in the perinuclear region and was absent from the membrane contact regions. Modulation of protein kinases known to regulate GJIC function induced changes in GJIC permeability. The specific conclusions are:

2.6.1 The activation of cAMP-dependent protein kinase directly or indirectly induced an increase in GJ channel permeability after a 48 h treatment due to a re-localisation of Cx43 from the perinuclear region to cell-cell contact areas. This event was associated with progression of the cells into differentiation.

2.6.2 The inhibition of cPKC by Go6976 treatment was associated with an increase in GJ channel permeability and a decrease in cell proliferation.

2.6.3 The inhibition of the p38 MAPK by treatment with SB202190 resulted in an increase in GJ channel permeability and a decrease in cell proliferation.

2.6.4 The inhibition of p44 Erk1 was associated with cell cycle arrest. In contrast, double inhibition of p44 Erk1 and Erk2 did not have any effect on cell proliferation and was associated with a drastic decrease in Cx43 intracellular levels.

GENERAL CONCLUSION

Neuroblastoma is an enigmatic tumour of childhood originating from the neural crest cells. Approximately 60% of the children diagnosed are in the advanced stages. When diagnosed in infants less than one year old, however, spontaneous regression of the tumour is often observed. The molecular mechanism of NB regression is unclear. One of the hypothesis used to explain this phenomenon is that NB cells are blocked during their progression into differentiation or to apoptosis during embryogenesis but still possess an intact machinery to support differentiation. Understanding the molecular mechanisms that lead to the control of proliferation, induction of differentiation and apoptosis will permit investigation into new therapeutic approaches to manage this solid tumour.

In chapter one, the cell culture conditions for the induction of terminal differentiation were optimised *in vitro* using SH-SY5Y neuroblastoma cells as a cell model of neuronal differentiation. Undifferentiated SH-SY5Y cells are highly proliferative, they display an epithelial-like morphology and they do not respond to NGF since they express the receptor TrkA at low levels. Exposure to 8-Br-cAMP, a modulator of PKA activity, and inhibition of the cell cycle using aphidicolin increased the expression level of TrkA and rendered the cells responsive to NGF. The combination treatment aphidicolin, 8-Br-cAMP and NGF triggered extensive neurite outgrowth (further characterised as axons), cell growth arrest and expression of neuronal markers characteristic of neuronal function. This combination treatment was more potent in inducing neuronal differentiation than retinoic acid, a conventional inducer. Furthermore, terminal differentiation of SH-SY5Y cells was achieved as demonstrated

by the widely accepted criteria of NGF-survival dependence. Cells died of apoptosis following NGF withdrawal indicating that the cells had exited the cell cycle. According to the literature, the expression of TrkA may be the final step towards terminal differentiation of sympathetic neurones. Its ligand, NGF, may control terminal differentiation and/or cell death. The model of terminal differentiation of SH-SY5Y cells presented in this work is reproducible and the various stages from proliferation to differentiation were well characterised at the molecular level. The model may then be used to decipher signalling pathways leading to terminal differentiation and NGF deprivation-dependent apoptosis. It may also constitute a suitable model to screen new drugs or design new approaches for neuroblastoma therapy. The model may also apply to investigations relative to diseases involving apoptosis in the nervous system such as Alzheimer's disease.

The possible contribution of the PKC pathway was investigated in the first chapter. It was shown that the non-specific PKC inhibitors staurosporine and H7 induced morphological differentiation and apoptosis in SH-SY5Y cells. We found that staurosporine- and H7-induced differentiation led to the expression of neuronal markers and to a decrease of N-myc expression. None of the non-selective inhibitors, however, increased the expression of the NGF receptor TrkA, indicating that their effect on differentiation is limited to the very first step (neurite outgrowth). In contrast, more specific cPKC and nPKC inhibitors such as Bis and calphostin C did not affect proliferation or differentiation. These results suggest that the PKA pathway may be contributing to growth control in SH-SY5Y cells and that more detailed and specific experiments addressing the PKC isoforms are needed to obtain valid conclusions.

Gap junctional intercellular communications may be another mechanism to control cell growth, differentiation and apoptosis in neuroblastoma cell lines. Furthermore, much evidence has implicated GJIC in carcinogenesis. The expression and function of connexins, the proteins of gap junctions, are developmentally regulated. Since neuroblastoma are likely blocked during their embryonic development, we hypothesised that gap junctions were probably expressing Cx43 similar to PNS neuroblasts. The modulation of protein kinases involved in their regulation may lead to changes in function and/or expression of connexins. Our purpose was not to decipher these pathways but rather to determine which of these pathways may contribute to GJIC control.

In Chapter two, it was shown for the first time that neuroblastoma cells SH-SY5Y express Cx43 characteristic of the embryonic lineage of neurones, and that they do not express Cx32, which is present in some adult neurone sub-populations. Even if Cx43 is present under phosphorylated and non-phosphorylated forms, it was shown that the cell-cell communication in SH-SY5Y cells was altered and that the alteration was due to an aberrant perinuclear localisation of Cx43 instead being localised in the cell-cell contact regions. This was not surprising since many cancers are reported to exhibit GJ alterations at different levels. In order to investigate a mechanism that would restore GJIC function, we modulated signalling pathways involved in GJIC regulation, since PKA, PKC and MAPK are reported to be involved in GJIC regulation and Cx43 phosphorylation. A pharmacological approach based on specific kinase inhibitors and activators was used. It was shown that treatment with 8-Br-cAMP; an activator of PKA induced a re-localisation of Cx43 to the plasma membrane with an increase in both Cx43 expression and GJIC permeability. This was associated with the translocation of

cytoplasmically sequestered p53 to the nucleus, cell growth arrest and induction of morphological and molecular differentiation. It was concluded that PKA may regulate Cx43 gene expression and trafficking in SH-SY5Y cells. Furthermore, it was shown that p38 MAP kinase inhibition induced an increase in GJIC channel permeability within hours of treatment and that Erk1/Erk2 double inhibition probably triggered Cx43 degradation thus being ineffective in blocking proliferation. This last result may also be indicative of the presence of an alternative MAPK-independent pathway regulating SH-SY5Y cell proliferation.

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