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Ras-MAPK Signaling in Differentiating SH-SY5Y Human Neuroblastoma Cells

BY

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ABSTRACT

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Neuroblastoma is a malignant childhood cancer, originating from sympathetic neuroblasts of the peripheral nervous system. Neuroblastoma is a heterogenous group of tumours, while some are highly malignant others can spontaneosly mature into a more benign form or regress. Less than half of the patients survive and this statistics has improved only modestly over the past 20 years.

SH-SY5Y is a human neuroblastoma cell line established from a highly malignant tumour. The cells have retained a capacity to differentiate *in vitro* in response to low concentrations of the phorbolester 12-O-tetradecanoylphorbol-13-acetate (TPA) in the presence of serum or defined growth factors. Differentiated cells are characterised by neurite formation and upregulation of neuronal marker genes. SH-SY5Y are unresponsive to nerve growth factor (NGF), but when transfected to express the NGF-receptor TrkA, they differentiate in response to NGF. Protein kinase C (PKC) is pivotal for the differentiation response to take place.

We have investigated the role of signaling through the Ras-MAPK pathway in differentiating SH-SY5Y, with respect to neurite formation, expression of neuronal marker genes and growth control. Our results show that differentiation-promoting treatment induced a sustained activation and nuclear accumulation of the MAPK ERK in SH-SY5Y. The nuclear accumulation of ERK was PKC-dependent. However, nuclear accumulation of ERK was not sufficient for a differentiation response to take place in these cells, but ERK activity was needed for the characteristic upregulation of *NPY* and *GAP-43* induced by TPA. ERK activity did not induce neurite formation, neither was it necessary for TPA-induced neurite formation. Instead, stimulation of a pathway distinct from MEK/ERK, but downstream of Ras, was needed for morphological differentiation. We could also show that differentiated cells still entered S-phase and that there was no correlation between expression of the CKI $p21^{cip1}$ (an ERK target), BrdU-incorporation or neurite formation.

To myself

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I **Olsson, A-K.**, Vadhammar, K. and Nånberg, E. 2000. Activation and protein kinase C-dependent nuclear accumulation of ERK in differentiating human neuroblastoma cells. *Exp. Cell Res. 256, 454-467.*

II **Olsson, A-K.** and Nånberg, E. A functional role of ERK in gene induction, but not in neurite outgrowth in differentiating neuroblastoma cells. *Submitted.*

III Söderholm, H., **Olsson, A-K.**, Lavenius, E., Rönnstrand, L. and Nånberg, E. Activation of Ras, Raf-1 and protein kinase C in differentiating human neuroblastoma cells after treatment with phorbolester and NGF. *Submitted.*

IV **Olsson, A-K.**, Vadhammar, K., Arvidsson, L. and Nånberg, E. Growth control in differentiating human neuroblastoma cells. *Manuscript.*

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ABBREVIATIONS

bFGF	basic fibroblast growth factor
cAMP	cyclic adenosine monophosphate
CDK	cyclin-dependent protein kinase
CKI	cyclin-dependent protein kinase inhibitor
CNS	central nervous system
DAG	diacylglycerol
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
ERK	extracellular signal-regulated kinase
GAP-43	growth-associated protein-43
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
IGF-1	insulin-like growth factor-1
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MBP	myelin basic protein
MEK	MAPK/ERK kinase
NB	neuroblastoma
NES	nuclear export signal
NGF	nerve growth factor
NPY	neuropeptide tyrosine
NSE	neuron specific enolase
PDGF	platelet-derived growth factor
PKC	protein kinase C
PLC	phospholipase C
PNS	peripheral nervous system
PTB	phosphotyrosine binding
SNS	sympathetic nervous system
SH2/3	Src-homology 2/3
TH	tyrosine hydroxylase
TPA	12-*O*-tetradecanoylphorbol-13-acetate

INTRODUCTION

This thesis deals with Ras-MAPK signaling during *in vitro* neuronal differentiation of human neuroblastoma cells. These proteins are present in all eucaryotes and are highly conserved. During the last two decades they have been the focus of intense research. The number of hits on Medline when searching for "Ras" or "MAPK" are in August this year 18 316 and 9 468, respectively. Signaling through this pathway has been implicated in very diverse processes, spanning from formation of long term-memory (Brambilla et al., 1997) to a role in the infectious disease anthrax (Duesbery et al., 1998). I have not made an attempt to cover all the described signaling pathways converging to or activated by Ras, but limited the focus to its role in neuronal differentiation. Even so, there are many reports in this area that I have not mentioned due to space. Still, I hope to give the reader a glimpse of the importance of this signaling pathway during neuronal differentiation, mainly based on *in vitro* cell culturing experiments but also some *in vivo* data.

BACKGROUND

Malignant transformation of human cells

Cancer is a genetic disease and evolves through a multistep process. During the last decades it has become evident that transformation of a normal mammalian cell into a cancer cell requires several genetic alterations (for review see Hanahan and Weinberg, 2000). There is also a fundamental difference between cells of different mammalian species; rodent cells are more easily transformed than human. While the introduction of two co-operating oncogenes efficiently transforms primary rodent cells *in vitro* (Land et al., 1983), this is not sufficient for transformation of human cells in culture (Stevenson and Volsky, 1986; Hahn et al., 1999). Possibly even one genetic alteration is sufficient to transform rodent cells *in vivo*, demonstrated by the fact that retroviral introduction of the growth factor PDGF-BB in the brain of mice induces glioma (Uhrbom et al., 1998).

What are the genetic changes needed for a normal human cell to become a cancer cell? Tumour tissue that consist of tumour cells and supporting stromal cells, is characterised by excessive increase in cell number, angiogenesis and the capability of the tumour cells to invade other tissue. For a cell to multiply it needs mitogenic signals, usually supplied by neighbouring cells. Most tumour cells have an unlimited access to growth factors, either by own production (autocrine stimulation) or by inducing neighbouring cells to supply them with growth stimulating factors. Many oncogenes mimic the effect of mitogens. However, a positive proliferation signal is normally not sufficient to create a human tumour cell as mentioned above. The reason for this is mainly apoptosis-programmed cell death. Many tumours suffer massive apoptotic cell death, which efficiently reduces the potential increase in tumour volume. An imbalance in the normal signals in a cell, for instance by an overexpressed oncogene, can trigger apoptosis (Evan et al., 1992; Joneson and Bar-Sagi, 1999; Bordeaux et al., 2000). Therefore, to build up a tumour it is important for the individual tumour cells to escape apoptosis. Accordingly, the most frequently mutated, and thus inactivated, gene in all human cancers is TP53, the tumour suppressor gene encoding the pro-apoptotic protein p53.

Another obstacle for a developing tumour is the built in limited number of divisions and life span of cells. This is due to shortening of the telomeres, structures at the end of the chromosomes, with each round of replication. Telomerase is a reverse transcriptase that maintains the length of the telomeres. In humans, telomerase is expressed in germline but not in somatic cells. In tumours and cell lines it is however frequently upregulated (Harley and Sherwood, 1997). Mice on the other hand have telomerase activity also in their differentiated somatic cells, a possible explanation to why these are more easily

transformed than human cells. In support of this theory is the report by Weinberg an co-workers, where they show that introduction of hTERT (the catalytic subunit of telomerase) into human epithelial cells and fibroblasts, allows transformation of these human cells by two oncogenes (Hahn et al., 1999). However, this issue is still controversial since there is a conflicting report (Morales et al., 1999).

To grow beyond a certain size the tumour needs to induce formation of new blood vessels (angiogenesis). Cells can only survive within a certain distance (approximately 100 μM) from a capillary. Without vasculature in a tumour, the cells in the center would therefore rapidly be short of oxygen and nutrients. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor-2 (FGF-2) are known inducers of angiogenesis (Hanahan and Folkman, 1996) and their expression is upregulated in many tumours.

Finally, the tumour cells also need to change their expression pattern of proteins involved in cell-cell or cell-extracellular matrix contact to spread to other sites in the body. For instance, the pattern of integrin expression can be switched (Keely et al., 1998).

Neuroblastoma

Neuroblastoma (NB) is a pediatric tumour with broad clinical characteristics and patient outcome. Based on phenotype and localisation, the tumour cells are believed to originate from sympathetic neuroblasts of the peripheral nervous system. The disease was first described in 1864 by the german pathologist R.L.K. Virchow (Virchow, 1865). It usually appears in the thoracic and/or gut region, but can arise anywhere in the sympathetic nervous system (SNS) (Figure 1). It is the second most common solid tumour among children and accounts for 7-10 % of the cancers of childhood. In Sweden, approximately 10-15 children under the age of 15 are diagnosed with NB every year. The outcome for a patient affected by neuroblastoma is unfavourable. The two-year survival is around 30% (Breslow and McCann, 1971) and has improved modestly over the past 20 years. This is in sharp contrast to childhood leukemia (the most common group of pediatric tumours), where treatment has improved significantly and approximately 75% of the patients are cured.

Classification

Neuroblastomas are classified according to a clinical staging system; The International Neuroblastoma Staging System (INSS) (Brodeur et al., 1993). This system divides the tumours from stage 1 to 4 depending on degree of differentiation and infiltration of other

organs, where a higher stage indicates a more advanced disease. Stage 1 tumours display a localised growth pattern, while stage 4 tumours show widespread metastatic growth. Stage 4S is a variant that despite its colonisation of several organs has a favourable outcome and can undergo spontaneous regression, as the lower stage tumours. The 4S stage is confined to infants under 1 year of age. The NB tumours can be divided into two main groups; stage 1, 2 and 4S which have a favourable outcome with little or no treatment, and stage 3 and 4 tumours that have a poor prognosis despite treatment. The INSS have proved valuable in prediction of prognosis and avoiding unnecessary radiation and chemotherapy for the individual patient.

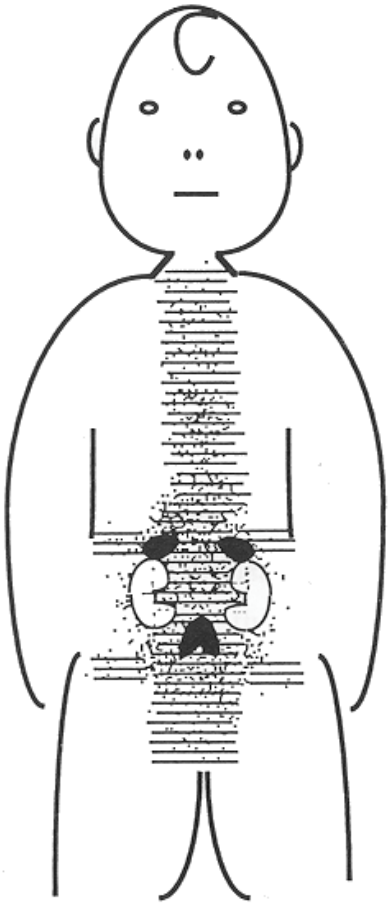

Figure 1. Schematic illustration of possible anatomic localisation of neuroblastoma tumours (dashed areas).

Prognostic factors

Patient age at diagnosis, location of the tumour and of course the clinical stage are the most reliable prognostic factors, but several others of importance are described below. Children diagnosed under the age of two years generally have a better prognosis than older children. Also, patients with extra-adrenal tumours tend to do better than those with an adrenal location of the primary tumour (Coldman et al., 1980).

Genetic alterations

Several genetic changes have been described in NB. The most common and also most versatile in predicting prognosis are DNA content, *N-myc* amplification, allelic loss at chromosome 1p or trisomy for chromosome 17q. Tumour cells with a near-triploid chromosome number have a better prognosis than near-diploid tumours. However, this prognostic factor seems only to be useful for children under 1 year of age (Look et al., 1991). Amplification of the *N-myc* oncogene was originally identified in a series of neuroblastoma cell lines (Schwab et al., 1983) and shortly thereafter in a subset of neuroblastoma tumours, correlating to advanced disease and poor prognosis (Brodeur et al., 1984). Even though not all patients with a poor prognosis have *N-myc* amplification, the majority of those who do have this genetic alteration will die from their disease. There is a strong correlation between *N-myc* amplification and deletion of chromosome 1p (Fong et al., 1989; Fong et al., 1992). Loss of heterozygosity (LOH) at 1p is also associated with a poor outcome (Caron et al., 1996). Abnormalities at chromosome 17q occur with a high incidence in neuroblastoma. Gain of whole chromosome 17 or only 17q are observed. Trisomy for 17q is associated with a more aggressive subset of neuroblastomas (Bown et al., 1999).

Expression of TrkA and Ras

TrkA is a tyrosine kinase and the high-affinity receptor for nerve growth factor (NGF). An inverse relationship between TrkA expression and *N-myc* amplification is often found in NB and a combination of high TrkA expression and no *N-myc* amplification gives a good survival prognosis (Nakagawara et al., 1993).

The small GTPase Ras is activated downstream of TrkA (this will be described in more detail below). Mutated active Ras is found in high frequency in many types of cancers, but has so far not been found in primary neuroblastoma material (Ballas et al., 1988; Moley et al., 1991). On the contrary, high expression of normal Ras is associated with lower stage and a better prognosis (Tanaka et al., 1998; Tanaka et al., 1991; Nakada et al., 1993).

Telomerase activity

There is a correlation between telomerase activity and outcome in neuroblastoma patients. In one study the telomerase activity in 79 neuroblastomas were examined (Hiyama et al., 1995). 3 patients had no telomerase activity, 60 had low activity and 16 patients high activity. Interestingly, 12 of the 16 with high activity died whereas only 2 patients out of the 60 with low telomerase activity died. The three patients with no telomerase activity were all stage 4S, and survived. As mentioned earlier, stage 4S tumours can undergo spontaneous regression despite a metastatic growth pattern. Of the

79 tumours, 11 had *N-myc* amplification and all of these displayed high telomerase activity. Recently, hTERT has been shown to be a direct transcriptional target of c-Myc (Wu et al., 1999; Greenberg et al., 1999). But a recent report has suggested a more complex situation explaining the frequently found coincidence of high hTERT and Myc (Wang et al., 2000). The authors report that ectopic expression of hTERT induced overexpression of Myc with increasing passage number. This apparent selection for high expression of c-Myc was retained also after inactivation of hTERT. Thus a mutual effect of hTERT and c-Myc may exist. In a recently published paper Hiyama and co-workers (Hiyama et al., 1999) describes a method for rapid detection of *N-myc* amplification and hTERT expression in NB tumour specimens. The outcome of the study show the usefulness of the analysis for prognostic purposes since *N-myc* amplification and hTERT indicate a poor prognosis.

TP53 and TP73

Even though mutations in the gene encoding the tumour suppressor p53 (TP53) occur in almost 50% of all human cancers, this is rarely found in neuroblastomas (Vogan et al., 1993). However, p53 has been shown to be sequestered in the cytoplasm of undifferentiated tumours (Moll et al., 1995). Another more recent report suggests a correlation between incidence of neuroblastoma and polyomavirus BK infection. The authors also show that p53 in the cytoplasm colocalises and immunoprecipitates with large T antigen of the virus (Flaegstad et al., 1999). p53 contains an nuclear export signal (NES) in the tetramerisation domain and the NES is shown to be necessary and sufficient for nuclear export of the protein (Stommel et al., 1999). The NES is masked in terameric p53, but exposed in monomeric forms of the protein. A tempting speculation is therefore that binding of p53 to large T antigen prevents its tetramerisation and hence nuclear accumulation in neuroblastoma.

TP73 encodes for the protein p73, a homologue to p53. TP73 has been mapped to the 1p 36-3 locus (Kaghad et al., 1997), and since this region is frequently deleted in neuroblastoma it was initially considered a candidate tumour suppressor gene. However, later studies have failed to reveal any mutations in the remaining allele of TP73, which rules it out as a candidate gene in neuroblastoma (Kovalev et al., 1998; Ichimiya et al., 1999).

Treatment

The three major strategies for treatment of neuroblastoma today are surgery, radiation and chemotherapy. Since children have a greater number of dividing cells, the risk of late side effects are considerable. Therfore it would be valuable to find other, milder treatment strategies, based on for instance induction of differentiation or apoptosis in the tumour.

Clinical trials using retinoid therapy (inducer of differentiation of NB *in vitro*) and immunotherapy has given some positive results but there is still many problems left to be solved.

Neuronal differentiation *in vivo* and *in vitro*

Development of the sympathetic nervous system

The nervous system of vertebrates can be divided into two major parts based on location; the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS comprises the brain and spinal cord and the PNS "the rest" of the nervous system. Another division can be made between the somatic and autonomic nervous system, where the autonomic nervous system regulates functions largely without conscious control. The autonomic nervous system lies primarily within the PNS and is divided into the sympathetic, parasympathetic and enteric nervous system. The enteric nervous system innervates the gastrointestinal tract, the gall bladder and the pancreas. The sympathetic and parasympathetic nervous system also innervates these same organs as well as other vital organs such as heart, blood vessels and liver but mediates opposite effects, because of different neurotransmitter release. The sympathetic nervous system (SNS) mediates reactions to stress and danger; the so called "fight-or-flight" reaction.

The SNS originates from neural crest cells budding off from the closing neural tube, which takes place at the fourth week of human development. Neural crest cells are initially pluripotent but as they migrate from the neural tube they become restricted to different sublineages depending on the environment they encounter (LaBonne and Bronner-Fraser, 1998). The sub-lineage that gives rise to the cells within the SNS is called the sympatho-adrenal lineage (Anderson, 1993) (Figure 2). The major cell types within the SNS are neurons and chromaffin cells. The neuronal cells are enclosed by supporting myelinating Schwann cells derived from ectomesenchymal cells in the neural crest. The neurons are primarily located to sympathetic ganglia and the chromaffin cells to the adrenal gland. A third cell type, SIF (small intensely fluorescent) cells, can also be found within the adult SNS and is a type of extra-adrenal chromaffin cell, situated among the neurons in the ganglia. The embryonic and postnatal SNS is also constituted by paraganglia tissue, composed of extra-adrenal chromaffin cells, and neuroblasts, cell types that later regress.

The development of neural crest cells into the sympatho-adrenal lineage and further to the different cell-types of the SNS is tightly regulated by gene expression and sequential exposure to various growth factors. This is an area of intense research and I will only

give a few examples. Transcription factors of the basic helix-loop-helix (bHLH) family and their negative regulators have been shown to play crucial roles in the development of the nervous system (Kageyama and Nakanishi, 1997). The basic domain of these transcription factors binds DNA and the helix-loop-helix region binds other proteins (Murre et al., 1989). *MASH-1* is one of the first bHLH genes expressed in neuronal precursor cells. Studies of *MASH-1* knock-out mice show that they lack sympathetic neurons, as well as some parasympathetic and enteric neurons, while the chromaffin cells of the adrenal gland seem unaffected (Guillemot et al., 1993). There is a human homologue of *MASH-1*; *HASH-1*. Both *MASH-1* and *HASH-1* are expressed transiently at an early stage of development. The finding that some neuroblastoma tumours express *HASH-1* suggests that they are blocked at an early stage of embryonic development (Gestblom et al., 1999; Söderholm et al., 1999). The activity of bHLH transcription factors can be negatively regulated by helix-loop-helix (HLH) proteins, that dimerises with bHLH protein and prevents them from binding DNA. Examples of such proteins are HES-1 (Sasai et al., 1992), an antagonist of MASH-1, and the Id (inhibitor of differentiation) family of proteins (Benezra et al., 1990). The Id proteins are involved in neurogenesis in the developing embryo (Jen et al., 1997). Targeted disruption of Id1 and Id3 results in a premature differentiation in neuroblasts (Lyden et al., 1999).

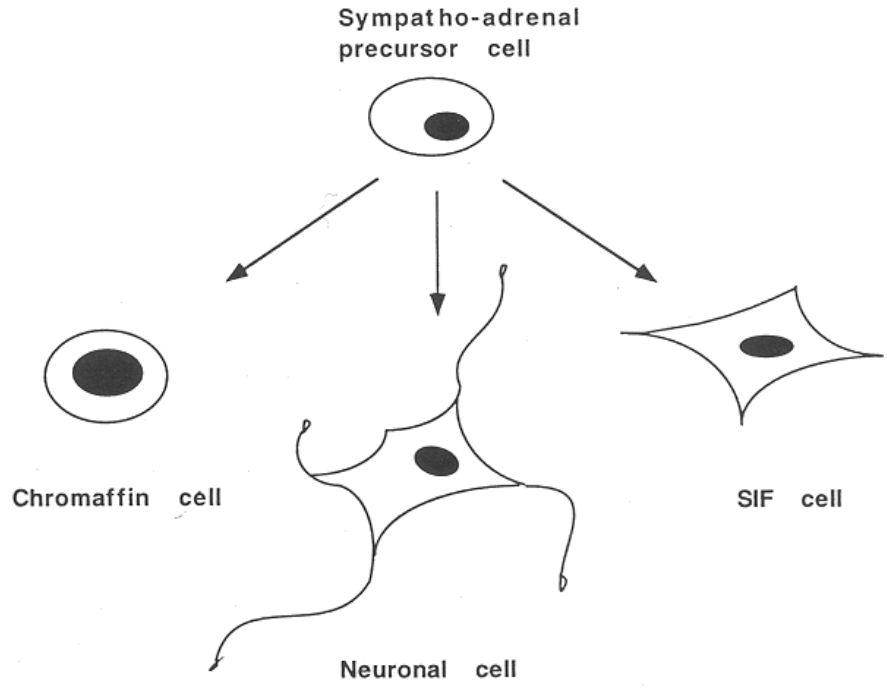

Figure 2. Cell types within the adult sympathetic nervous system derived from the sympatho-adrenal lineage.

The extracellular environment plays a crucial role in regulating the fate of neurons in the developing SNS. Neurotrophins (NT) are growth factors specifically acting on neuronal tissue, among which NGF was the first to be characterised. NGF is also the most well studied NT (for review see Levi-Montalcini, 1987). The NGF-family of NT consists of NGF, brain-derived neurotrophic factor (BDNF) (Barde et al., 1982), neurotrophin-3 (NT-3) (Hohn et al., 1990), NT-4/5 (Hallböök et al., 1991; Berkemeier et al., 1991), NT-6 (Gotz et al., 1994) and NT-7 (Nilsson et al., 1998; Lai et al., 1998). The neurotrophins bind to tyrosine kinase receptors of the Trk-family with high affinity. As mentioned earlier, NGF binds to TrkA (Kaplan et al., 1991; Klein et al., 1991a), while both BDNF and NT-4/5 binds to TrkB (Klein et al., 1991b, Klein et al., 1992). The primary receptor for NT-3 is TrkC (Lamballe et al., 1991), but this neurotrophin can also bind TrkA and B. The receptor for NT-6 and NT-7 has not been found yet. Knock-out studies have revealed an important role for TrkA and TrkC in the development of sympathetic neurons (Crowley et al., 1994; Smeyne et al., 1994; Ernfors et al., 1994; Klein et al., 1994). TrkB on the other hand does not seem to be important for this lineage restriction (Klein et al., 1993; Ernfors et al., 1994; Jones et al., 1994). There is also a low affinity receptor, p75, that can bind all the above mentioned neurotrophins. The function of this receptor has long been an enigma. It has been proposed that p75 cooperatively binds neurotrophins together with the Trk receptors, and thereby creates high affinity binding (Chao, 1994). It has also been shown that the p75 receptor can induce apoptosis in the abscence of TrkA (Frade et al., 1996). Hypothetically, this could be a way of eliminating developing neurons with defect TrkA expression.

It has been suggested that a sequential exposure to certain growth factors and neurotrophins, induces the developing sympathetic neuronal precursor to upregulate neurothropin receptors in a specific pattern, finally leading to the mature neuron. Fibroblast growth factor (FGF) can stimulate proliferation and some differentiation of early SNS precursors (Stemple et al., 1988). Two recent reports also suggest that FGF may be one of the first positive signals directing neural fate (Streit et al., 2000; Wilson et al., 2000). During this early period in the development (approximately the migrating stage) the cells are TrkC positive and dependent on NT-3 for their survival. After a period of NT-3 dependence, TrkC is downregulated, the cells stop proliferating and TrkA is upregulated. It is debated wheather it is the NT-3 treatment or the withdrawal from the cell cycle that induces TrkA upregulation. In this postmitotic state the neurons become dependent on NGF for their survival (Birren et al., 1993; Verdi and Anderson, 1994).

In vitro models for neuronal differentiation

Several different *in vitro* systems are used to study signals regulating neuronal differentiation. Primary cultures of nervous system precursor cells from mouse, rat and chicken are frequently used. To study differentiation of PNS neurons, rat embryonic superior cervical ganglion (SCG) (Carnahan and Patterson, 1991) or dorsal root ganglia (DRG) from embryonic chicken (Ernsberger and Rohrer, 1988) or rat (van Dorp et al., 1990) are used. NGF treatment of SCG induces a sympathetic phenotype, while DRG differentiate towards a sensory phenotype. Oligodendroglia cells from rat can be used for studies of neuronal CNS differentiation (Collarini et al., 1991). A problem with biochemical studies on primary primary cultures is the limited access to cells. Therefore, immortalised cell lines offer an alternative. One such example is the MAH (Myc-infected, adrenal derived, HNK1+) cell line, established by immortalising sympatho-adrenal progenitor cells with *v-myc* (Birren and Anderson, 1990).

Cell lines derived from tumours of the nervous system can also serve as model system for studies of neuronal differentiation, as well as contributing with information about possible defects in pathways controlling differentiation in these tumours. The most frequently used tumour-derived cell line for studying neuronal differentiation is the rat pheochromocytoma cell line PC12 (Greene and Tischler, 1976). Pheochromocytoma is a SNS tumour of the adrenal medulla, and unlike neuroblastoma of chromaffin origin. The vast majority of pheochromocytomas are identified during adulthood. The chromaffin cells of the SNS are endocrine rather than neuronal, since they secrete their neurotransmitter directly into the bloodstream, and lack axons and dendrites. However, cultured PC12 cells treated with NGF respond with neurite outgrowth and expression of genes indicative for a sympathetic phenotype (Greene, 1978). A switch from chromaffin to neuronal phenotype can also occur *in vivo* in rat after injection with NGF (Aloe and Levi-Montalcini, 1979). Several cell lines have been established from high-stage (3 and 4) neuroblastoma tumours. Attempts to establish clones from low-stage tumours have failed. The majority of the NB cell lines have an *N-myc* amplification, although this genetic alteration is only present in approximately 25% of all NB-tumours (Brodeur, 1995). Frequently used NB cell lines are IMR-32, LA-N-2, LA-N-5 and SH-SY5Y, which all except SH-SY5Y contains amplified *N-myc*. The phorbolester 12-O-tetradecanoylphorbol-13-acetate (TPA), retinoic acid (RA) and certain growth factors are all factors used to induce differentiation of these cells (Påhlman et al., 1981; Spinelli et al., 1982; Sidell, 1982; Thiele et al., 1985; Påhlman et al., 1995). Below I will describe *in vitro* differentiation of SH-SY5Y, the system on which the work presented in this thesis is based.

SH-SY5Y

SH-SY5Y is a subclone of the SK-N-SH cell line that was established from a bone-marrow aspirate of a 4 year old girl with a highly malignant tumour (Biedler et al., 1973). In addition to SH-SY5Y with a neuronal phenotype, subcloning of SK-N-SH resulted in establishment of an epithelial-like clone, SH-EP, and cells with an intermediate fibroblast-like phenotype. SH-SY5Y is a well characterised system for studies of neuronal differentiation. When treated with nanomolar concentrations of TPA in the presence of serum the cells differentiate morphologically by sending out neurites with growth cones and varicosities (Påhlman et al., 1981). A number of characteristics strongly suggests that the cells differentiate towards a sympathetic neuronal phenotype. After TPA treatment, the expression of neuronal markers such as *growth-associated protein-43 (GAP-43)* and *neuron specific enolase (NSE)* is increased. Also TPA induces expression of the neurotransmitter *neuropeptide Y (NPY)* and *tyrosine hydroxylase (TH)*, an enzyme involved in catecholamine synthesis. Acetylcholine causes depolarisation of differentiated SH-SY5Y and release of noradrenaline (for review see Påhlman et al., 1995). Another characteristic of cells induced to differentiate with TPA is an elevated biphasic *c-fos* transcription (Hammerling et al., 1987).

TPA in the absence of serum does not induce morphological differentiation of SH-SY5Y (Påhlman et al., 1991 and paper I) and only a smaller increase in *GAP-43* and *NPY* trancripts is seen under those conditions (Påhlman et al., 1991; Lavenius et al., 1994). The requirement for serum can be substituted with certain growth factors like platelet-derived growth factor (PDGF) (Påhlman et al., 1992), insulin-like growth factor-1 (IGF-1) (Påhlman et al., 1991) or basic fibroblast growth factor (bFGF) (Lavenius et al., 1994). Also a combination of the growth factors IGF-1 and bFGF induce morphological differentiation and upregulation of primarily *NPY* (Lavenius et al., 1994). These growth factors are on their own mitogens for SH-SY5Y. Many high-stage NB cell lines are unresponsive to NGF (Azar et al., 1990), like SH-SY5Y, or lack TrkA expression (Suzuki et al., 1993). When SH-SY5Y are stably or transiently transfected with cDNA encoding human TrkA (SH-SY5Y/TrkA) they differentiate in response to NGF both morphologically and biochemically (Lavenius et al., 1995; Poluha et al., 1995; Eggert et al., 2000). SH-SY5Y express low amounts of a 140 kDa protein recognised by TrkA specific antibodies. Despite efforts, no mutation was identified in the endogenous TrkA gene (Påhlman and Söderholm, unpublished). Alternatively, the unresponsiveness of SH-SY5Y to NGF may be due to too low expression levels of endogenous TrkA. To avoid confusion, untransfected SH-SY5Y will be referred to as SH-SY5Y/wt. SH-SY5Y will be used to designate SH-SY5Y/wt and SH-SY5Y/TrkA collectively.

SH-SY5Y undergoing TPA-induced differentiation are reported to have a decreased proliferation (Påhlman et al., 1981; Spinelli et al., 1982), although the latter report claims it to be transient. There are conflicting reports about the situation in SH-SY5Y undergoing growth factor induced differentiation. Påhlman and co-workers report no decrease in proliferation in SH-SY5Y/wt treated with bFGF/IGF-1 or SH-SY5Y/TrkA undergoing NGF-induced differentiation (Lavenius et al., 1994; Lavenius et al., 1995). However, Poluha et. al. report that SH-SY5Y transfected to express TrkA cease proliferating in response to NGF (Poluha et al., 1995). A decreased proliferation is also seen in TrkA transfected HTLA230 neuroblastoma cells treated with NGF (Matsushima and Bogenmann, 1993). One recent paper reports a decreased proliferation in the absence of NGF in SH-SY5Y transfected to express TrkA. NGF stimulation of these cells increased the proliferation rate to the same level as untransfected SH-SY5Y (Eggert et al., 2000). A qualitative difference between SH-SY5Y treated with TPA or growth factors is that the phorbol ester induces down regulation of *c-myc* (Hammerling et al., 1987; Påhlman et al., 1991; Lavenius et al., 1994). This seems to be a dominant effect, insensitive to the presence or absence of serum. Cell cycle regulation of differentiating SH-SY5Y cells will be discussed in paper IV.

Protein kinase C (PKC) is a family of serine/threonine kinases that plays a central role in the differentiation response of SH-SY5Y/wt and SH-SY5Y/TrkA. The members of the PKC family can be divided into three subgroups; classical, novel and atypical (Newton, 1997). SH-SY5Y express at least α and βII of the classical isoforms, δ, ε and μ of the novel type and ζ of the atypical PKC isoforms (Parrow et al., 1992; Leli et al., 1993; Fagerström et al., 1996; Zeidman et al., 1999). TPA activates classical and novel type PKC's by mimicking the effect of diacylglycerol (DAG), the *in vivo* activator. High concentrations of TPA (in the μM range) downregulates PKC and does not induce differentiation of SH-SY5Y, while the two established differentiation protocols 16 nM TPA in the presence of serum or the combination of bFGF/IGF-1 both induce sustained activation of PKC (Parrow et al., 1992; Lavenius et al., 1994 and paper III). There is an enrichment of PKC in the growth cone of the developing neurite (Parrow et al., 1995) and all three described differentiation protocols are dependent on PKC activity for the induction of neurite outgrowth. Also, maintenance of the growth cone structure is PKC-dependent (Fagerström et al., 1996). Inhibition of PKC activity with the compound GF109203X is reported to prevent bFGF/IGF-1 induced *NPY* and *GAP-43* expression, but only about 30% of the NGF-induced expression of these genes (Fagerström et al., 1996). Another recent report however, claims that bFGF/IGF-1 induced expression of *NPY* and *GAP-43* is not PKC-dependent (Zeidman et al., 1999).

RA induces a strong morphological differentiation response in SH-SY5Y, but the phenotype is unclear. There is a slight increase in NSE activity and *GAP-43* expression, suggesting a neuronal differentiation, but no upregulation of *NPY* (Påhlman et al., 1984). Moreover, TH is downregulated (Lavenius, 1996), which speaks against a sympathetic neuronal differentiation. RA treatment of SH-SY5Y leads to upregulation of TrkB (Kaplan et al., 1993). This neurotrophin receptor seems not to be involved in neuronal development, but rather chromaffin differentiation (Barbacid, 1995; Hoehner et al., 1995).

Tyrosine kinase receptor signaling

The intracellular responses elicited by growth factors binding to their cognate receptors has been extensively studied. Growth factors bind to transmembrane protein-tyrosine kinase receptors (TKR) or, as in the case of the TGF-β superfamily, to serine/threonine kinase receptors. The receptors transduce signals from the extracellular environment to the cytoplasm and the nucleus of the cell and are involved in such diverse cellular processes as proliferation, differentiation, cell motility and apoptosis. TKR and members of their downstream signaling pathways are also frequently deregulated in tumours. Growth factors like NGF, PDGF, FGF and epidermal growth factor (EGF) all bind to tyrosine kinase receptors and, to a large extent, activate common intracellular signaling pathways. Much effort has therefore been made to understand why distinct growth factors have different effects in the same cell, or why the same growth factor elicits unique responses in two different cell types, i.e. to find out where the specificity lies. Below I will describe the sequence of events that takes place from growth factor binding of a TKR to transcriptional activation in the nucleus, with the NGF-receptor TrkA as model.

TrkA - from the cell surface to the nucleus

Like most polypeptide growth factors, NGF is a dimer, consisting of two identical subunits (McDonald et al., 1991). This in turn offers more than one binding site to the receptor and facilitates TrkA dimerisation, which is required for receptor activation. A unique featue of TKR is that they contain a single or split tyrosine kinase domain in their intracellular part. The proximity of the two receptor polypeptides promotes autophosphorylation in trans, i.e. one member of the pair phosphorylates the other, on specific tyrosine residues. Autophosphorylation activates the receptors intrinsic kinase activity towards other substrates. The process of TKR dimerisation and autophosphorylation is thoroughly reviwed in (Heldin, 1995; Heldin et al., 1998). The autophosphorylated tyrosine residues serve as specific docking sites for proteins

containing Src-homology 2 (SH2) (Koch et al., 1991) or phosphotyrosine binding (PTB) (Kavanaugh and Williams, 1994) domains. Tyrosine (Y) 490 and Y785 are autophosphorylation sites in the TrkA receptor, which couple to well characterised signaling pathways. Y490 binds the adapter protein Shc (Obermeier et al., 1993), leading to activation of Ras and its downstream targets. Y785 associates with phospholipase C-γ (Obermeier et al., 1993) resulting in PKC activation. SNT/FRS2 (Rabin et al., 1993; Klint et al., 1995; Kouhara et al., 1997) has been shown to associate directly with TrkA via a specific sequence motif (Peng et al., 1995), or by competing with Shc for the Y490 binding site (Meakin et al., 1999). More recently, SH2-B has been characterised as a substrate for TrkA (Qian et al., 1998), as well as the Csk homologous kinase (CHK), which has been shown to also associate to the receptor via Y785 (Yamashita et al., 1999). Apparently, new substrates for TrkA are still found as evidenced by three very recent reports showing an association between TrkA and the tyrosine kinase c-Abl (Yano et al., 2000; Koch et al., 2000; Brown et al., 2000). The signaling pathways activated downstream of Shc and PLCγ will now be described in more detail.

Ras

As mentioned above, Shc couples TrkA to activation of Ras, a protein that plays a central role in the regulation of diverse cellular processes. Two major effects of Ras are activation of mitogen-activated protein kinase (MAPK) and phosphoinositide 3OH-kinase (PI3K) (see below). There are four highly conserved Ras proteins in the Ras subfamily of small GTPases; H-Ras, N-Ras, K-Ras4A and K-Ras4B. Other members of the Ras subfamily include R-Ras, Ral, Rap, Rheb, Rin and Rit (Campbell et al., 1998). Ras proteins are localised to the plasma membrane by two post-translational modificatons in the C-terminal. Ras proteins bind guanine nucleotides (GDP or GTP) with high affiniy and is active in the GTP-bound state. Ras is activated by an increased nucleotide exchange and the function of GEF's (guanine nucleotide exchange factors) is to release GDP from Ras. Since there is more GTP than GDP in the cytoplasm, the net result of an increased nucleotide exchange is more Ras-GTP. Oncogenically activated forms of Ras exist with point mutations in positions 12, 13 and 61. The activity of Ras is negatively regulated by GTPase-activating proteins (GAP's) like p120GAP and NF1 (neurofibromin-1) (Bourne et al., 1991). Recently, SynGAP has been identified as a novel RasGAP. SynGAP is negatively regulated by calcium/calmodulin-dependent kinase II (CaM kinase II) (Chen et al., 1998; Kim et al., 1998). GAP's stimulate the intrinsic GTPase activity of Ras thus promoting a GDP-bound inactive state of Ras. Shc binds to Y490 of TrkA either by its PTB domain (Kavanaugh and Williams, 1994) or its phosphotyrosine interaction (PI) domain (Dikic et al., 1995) and becomes tyrosine phosphorylated by the receptor. This phosphorylated tyrosine recruits the Grb2-Sos complex, by binding to the SH2 domain of the adapter Grb2 (Basu et al., 1994). Sos, a

RasGEF, is constitutively active, but comes in contact with Ras upon recruitment to the membrane by the receptor interaction via Shc, or alternatively FRS2 (described later). Although Sos is the most well characterised RasGEF, at least two more exist; RasGRF, activated downstream of G-protein coupled receptors and calcium/calmodulin (CaM) (Farnsworth et al., 1995; Mattingly and Macara, 1996) and RasGRP that can be activated by PLC induced production of DAG and calcium (Ebinu et al., 1998; Tognon et al., 1998). The binding of GTP to Ras induces a conformational change and exposure of the effector domain of Ras (Barbacid, 1987). Several proteins have been shown to interact with the Ras effector domain including p120GAP (Yatani et al., 1990), Raf (Warne et al., 1993; Zhang et al., 1993), PI3K (Rodriguez-Viciana et al., 1994; Kodaki et al., 1994), RalGDS (Feig et al., 1996) and Rin1 (Han and Colicelli, 1995). RalGDS, Rin1 and p120GAP will not be further discussed.

Raf

Mammalian Raf proteins contains three family members; Raf-1, A-Raf and B-Raf (Campbell et al., 1998), with B-Raf existing in multiple spliced forms (Barnier et al., 1995). Of these, Raf-1 is the most frequently studied isoform. Raf is a serine/threonine kinase coupling Ras to the mitogen-activated protein kinase (MAPK) pathway, constituting the now classical "Ras-MAPK" pathway. Although the players in this pathway has been extensively studied, the mechanisms of Raf activation has not been conclusively elucidated. It is clear that membrane translocation of Raf by interaction with active Ras is a critical step (Leevers et al., 1994; Marais et al., 1995). At the membrane, Raf is thought to meet its activating kinase. However, this model might be too simplified. It has been suggested that Ras is involved in direct activation of Raf-1 and not only in its recruitment to the plasma membrane (Kuroda et al., 1996; Akasaka et al., 1996). Apart from the first Ras binding site (RBS) in Raf-1 (residues 55-131) reported by Rodrigues-Viciana et al., a second RBS has been identified in the cystein-rich domain (CRD) (For scematic figure of Raf-1 see paper III, figure 1). A model has been proposed where the first RBS is needed for membrane translocation and the second RBS required for full activation of Raf-1 by Ras (Clark et al., 1996; Mineo et al., 1997; Roy et al., 1997). Phosphorylation is an important regulatory mechanism of Raf-1 activity. Several *in vivo* phosphorylation sites have been described, affecting Raf-1 activity both positively and negatively (Morrison and Cutler, 1997). PKC has been implied in both activating (Howe et al., 1992) and inhibitory (Morrison et al., 1993) phosphorylation of Raf-1. Other kinases involved in phosphorylation positively regulating Raf-1 are Src (Marais et al., 1995) and Pak1 and 3 (King et al., 1998; Sun et al., 2000; Chaudhary et al., 2000), while PKB/Akt has been suggested to negatively regulate Raf-1 activity (Zimmermann and Moelling, 1999). Recently it has been suggested that dimerisation of Ras is required for Raf-1 activation (Inouye et al., 2000). Other factors that may influence the activation

of Raf-1 are p50, Hsp90 and kinase suppressor of Ras (Ksr) (Morrison and Cutler, 1997). 14-3-3 proteins have also been implicated in binding of Raf-1 and regulation of its activity. The exact function of these interactions are not known, but functions regulating both the inactive and active state of Raf-1 have been proposed (Fantl et al., 1994; Clark et al., 1997). Also, dimerisation of Raf-1 itself might be a regulatory mechanism (Farrar et al., 1996; Luo et al., 1996).

MAPK

Catalytically active Raf-1 complexes with, phosphorylates and hence activates the dual specificity kinase MEK (MAPK/ERK kinase). Two isoforms of MEK have been identified; MEK1 and MEK2. Active MEK phosphorylates and activates the serine/threonine kinase ERK1 and ERK2 (extracellular signal-regulated kinase) on tyrosine and threonine. The Raf/MEK/ERK pathway constitutes a highly conserved signaling module where ERK is a MAPK (mitogen-activated protein kinase), MEK is a MAPKK and Raf a MAPKKK. MAPK cascades are evolutionary conserved among eucaryotes and they have a central role in transducing signals from the extracellular environment to transcription and cytoplasmic events. In mammals, five distinct MAPK families have been identified (Schaeffer and Weber, 1999). The ERK1/2 proteins have been extensively studied and are major transducers of growth factor stimulation. There is a large body of literature demonstrating their involvement in proliferation, differentiation and development. The JNK (c-jun N-terminal kinase) and p38MAPK are primarily activated by different types of cellular stress (UV, osmosis) and inflammatory cytokines. Activators and responders of the last two groups of MAPK's; ERK3 and ERK5, are still largely unknown. The interaction of MEK with Raf is dependent on a proline-rich sequence unique to MEK's and not found in other MAPKK's (Catling et al., 1995). Raf-1 activates MEK1 and MEK2 to the same degree, while A-Raf preferentially activates MEK1 (Wu et al., 1996). In the case of B-Raf there may be two splice variants that have opposing preferences on MEK1 and MEK2 activation (Schaeffer and Weber, 1999). One way of achieving specificity within a given MAPK module is the use of scaffolding proteins. Examples of these are JIP1 and MP1. JIP1 functions in the JNK signaling module, where it physically couples four members of the pathway (Whitmarsh et al., 1998). MP1 selectively couples MEK1 and ERK1 in the Ras-MAPK pathway (Schaeffer et al., 1998). Activated MAPK's can phosphorylate substrates in the cytoplasm or translocate into the nucleus where they affect gene expression by phosphorylation of transcription factors (Schaeffer and Weber, 1999). Cytoplasmic substrates for ERK include phospholipase A_2 (Lin et al., 1993), p90Rsk (Dalby et al., 1998) and tyrosine hydroxylase (Haycock et al., 1992). Nuclear localisation of ERK has been suggested to be a critical event in the decision between proliferation and differentiation in neuronal cells. Therefore this topic will be discussed in a later section.

PI3K

Another target of active Ras is PI3K. PI3K is a lipid kinase that phosphorylates phosphoinositides (PI) at the 3-position of the inositol ring, generating $PI(3,4)P_2$ and $PI(3,4,5)P_3$ (Haycock et al., 1992). PI3K consists of two subunits; the p85 regulatory subunit and the p110 catalytic domain. Ras-GTP activates PI3K by binding to p110 and recruiting it to the plasma membrane, where it meets its substrates; the PI's. PI3K can also be activated by direct interaction of the SH2 domain of p85 and a phosphorylated TKR. In the case of TrkA however, PI3K does not bind directly to the receptor. In addition to Ras-GTP, the adapter protein Gab-1 might be another way of activating PI3K. Gab-1 can bind Grb2 and also PI3K (Holgado-Madruga et al., 1997; Korhonen et al., 1999). The phosphorylated lipids mediates signals from active PI3K. Well-established targets for PI3K are protein kinase B (PKB), also called Akt, and the RhoGTPases. Several targets of PKB/Akt that promote survival have been identified (Datta et al., 1999). Activation of PKB/Akt is dependent on PIP_2 and PIP_3. The family of RhoGTPases are modulators of cytoskeletal events. The RhoGTPases (Rho, Rac and Cdc42) are like Ras activated by nucleotide exchange induced by GEF's. All GEF's specific for RhoGTPases contain pleckstrin homology (PH) domains that binds inositol lipids and regulate the GEF activity and specificity (Leevers et al., 1999).

PLCγ

Phosphorylated Y785 in TrkA can interact with the SH2 domain of PLCγ, which is then phosphorylated and activated by the receptor. As for PI3K, PIP_2 is a substrate for PLCγ. PIP_2 is hydrolysed by PLCγ to generate DAG and inositol-trisphosphate ($InsP_3$). As previously mentioned, DAG activates PKC (Nishizuka, 1992). $InsP_3$ mediates release of calcium from intracellular compartments. PKC can feed into the Ras-MAPK pathway by phosphorylation of Raf as previously mentioned. Also, atypical PKC's has been shown to activate MEK independently of Raf (Schönwasser et al., 1998).

The Ras-MAPK pathway in neuronal differentiation

Several proteins in the above delineated signaling pathways have been implicated in neuronal differentiation, both *in vitro* and *in vivo*, which will be discussed in this section.

Ras

The properties of Ras as a growth promoting and transforming protein are well established. Ras is a potent oncogene and mutated forms are found in approximately 30%

of all human cancers (Bos, 1989). In contrast to its growth promoting effect in cultured murine fibroblasts, Ras can induce growth arrest in human fibroblast and differentiation of cells with neuronal origin. Microinjection of active Ras in PC12 cells cause morphological differentiation and neurite outgrowth (Bar-Sagi and Feramisco, 1985). Also, NGF- and bFGF-induced neurite formation in PC12 cells is blocked by microinjection of a neutralising Ras-antibody (Altin et al., 1991) or by the expression of dominant negative (N17Ras) (Szeberenyi et al., 1990). Sustained activation of Ras is seen in PC12 cells stimulated to differentiate with NGF or bFGF, but not when the cells are treated with epidermal growth factor (EGF), which acts a mitogen for these cells (Qiu and Green, 1992).

In primary cultures of sympathetic and sensory neurons Ras has been shown to regulate survival. Peripheral neurons can survive in culture without neurotrophins if they have an elevated Ras activity (Vogel et al., 1995; Nobes et al., 1996), whereas the survival is decreased if the Ras activity is blocked (Borasio et al., 1993; Nobes and Tolkovsky, 1995). In Caenorhabditis elegans (C. elegans) a role of the Ras-MAPK pathway in the maturation of olfactory neurons has recently been demonstrated (Hirotsu et al., 2000). As mentioned above, high expression of normal Ras in neuroblastomas is generally associated with a better prognosis. The effects of elevated Ras activity in SH-SY5Y neuroblastoma differentiation are discussed in papers II and IV.

MAPK

The same pattern as for sustained Ras activation seen after NGF and bFGF treatment of PC12 cells, but not after EGF stimulation, was also demonstrated for ERK (Qiu and Green, 1992; Traverse et al., 1992). In addition, differentiating cells, but not proliferating, showed an accumulation of ERK in the nucleus (Traverse et al., 1992). The theory emanated that sustained activation of ERK was required to induce nuclear accumulation of the kinase. In the nucleus ERK could activate neuronal specific genes (Marshall, 1995). Several studies support this hypothesis; PC12 cells normally respond to insulin and EGF stimulation with proliferation, but when transfected to overexpress the receptors for insulin and EGF, they respond to these stimuli with sustained activation and nuclear accumulation of ERK and differentiation (Dikic et al., 1994; Traverse et al., 1994). Constitutively active forms of Raf (Wood et al., 1993), MEK (Cowley et al., 1994; Fukuda et al., 1995) and ERK (Fukuda et al., 1995; Robinson et al., 1998) is sufficient to induce PC12 differentiation. Expression of inactive forms of MEK have demonstrated the requirement of MAPK activation in NGF-induced differentiation of these cells (Cowley et al., 1994) and selective inhibition of MEK activation by the compound PD98059 (Alessi et al., 1995; Pang et al., 1995) blocks NGF-induced neurite outgrowth. However, there are conflicting data on the differentiation promoting capacity

of MAPK in PC12 cells (Vaillancourt et al., 1995; Sano and Kitajima, 1998). In the H19-7 cell line, immortalised from rat hippocampal neurons, constitutive activation of MEK or ERK is not sufficient for differentiation (Kuo et al., 1996). In DRG sensory and sympathetic neurons derived from chicken PNS, activation of ERK is not necessary for NGF-induced neurite outgrowth (Klinz et al., 1996). This demonstrates a variation between different cell types, but also an inconsistency exists within reported results from the PC12 cell line.

In addition to these studies of differentiation and developing phenotypes, there are several reports providing evidence for a role of ERK in signaling cascades that are required for synaptic plasticity, learning and memory (Thomas et al., 1994; English and Sweatt, 1997; Coogan et al., 1999; Selcher et al., 1999). ERK has also been demonstrated to play a direct role in neuronal survival by phosphorylation and downregulation of the pro-apoptotic molecule Hid, in Drosophila photoreceptor cells (Bergmann et al., 1998; Kurada and White, 1998).

What are the mechanisms behind a sustained activation of MAPK? How is the nuclear accumulation of the kinase regulated? What are the qualitative differences between NGF and EGF stimulation of PC12, i.e. which genes are induced? The answers to these questions are still largely unknown, but there are accumulating data clarifying these issues.

Duration of ERK activity

The ERK activating signal can be counteracted by ERK specific phosphatases, like MKP-3 (MAPK phosphatase 3) (Muda et al., 1996a). MKP-1 and MKP-2 are not selectively ERK specific and can dephosphorylate other MAPK's also (Chu et al., 1996). MKP's are dual-specificity phosphatases, dephosphorylating both the threonine and tyrosine of active MAPK. There is a difference in subcellular distribution of individual MKP's, indicating a spatial regulation of the signal duration. For instance, MKP-3 is cytoplasmic (Muda et al., 1996b), while MKP-1 is localised to the nucleus (Brondello et al., 1995).

Feedback phosphorylation can also be a way to regulate the duration of a signal. One example is the phosphorylation of Sos by ERK, which leads to a dissociation of the Grb2-Sos complex and termination of the activating signal (Cherniack et al., 1994; Dong et al., 1996).

Recently a role of the small GTPase Rap1 and B-Raf in sustained activation of ERK was proposed by Stork and co-workers (York et al., 1998). The authors reported that Ras is responsible for the initial activation of ERK in response to NGF, whereas Rap1, via B-

Raf, mediates the later sustained activation (Figure 3). The same group has also demonstrated that NGF-induced sustained activation and nuclear accumulation of ERK is dependent on cAMP-dependent protein kinase (PKA), an effect mediated by Rap1 (Yao et al., 1998). However, expression of a mutant Rap1 that blocks the sustained ERK activation, does not prevent NGF-induced neurite formation, but it inhibits features of neuronal differentiation such as gene expression and sodium channel function (York et al., 1998). Rap1 is activated by stimulated nucleotide exchange and one GEF with specificity for Rap1 is C3G. This exchange factor is found in complex with the adapter protein Crk, analogous to the Sos-Grb2 complex (Figure 3). Another mechanism for activation of Rap1 is via cAMP that can mediate PKA-dependent activation of Rap1 (Vossler et al., 1997). PKA-independent activation of Rap1 has also been described, via direct interaction of cAMP with the RapGEF. Two recently descibed GEF's for Rap1 regulated in this way are Epac (de Rooij et al., 1998) and cAMP-GEFII (Kawasaki et al., 1998). Recent studies in Drosophila suggest that C3G can activate ERK via Rap1 (Ishimaru et al., 1999).

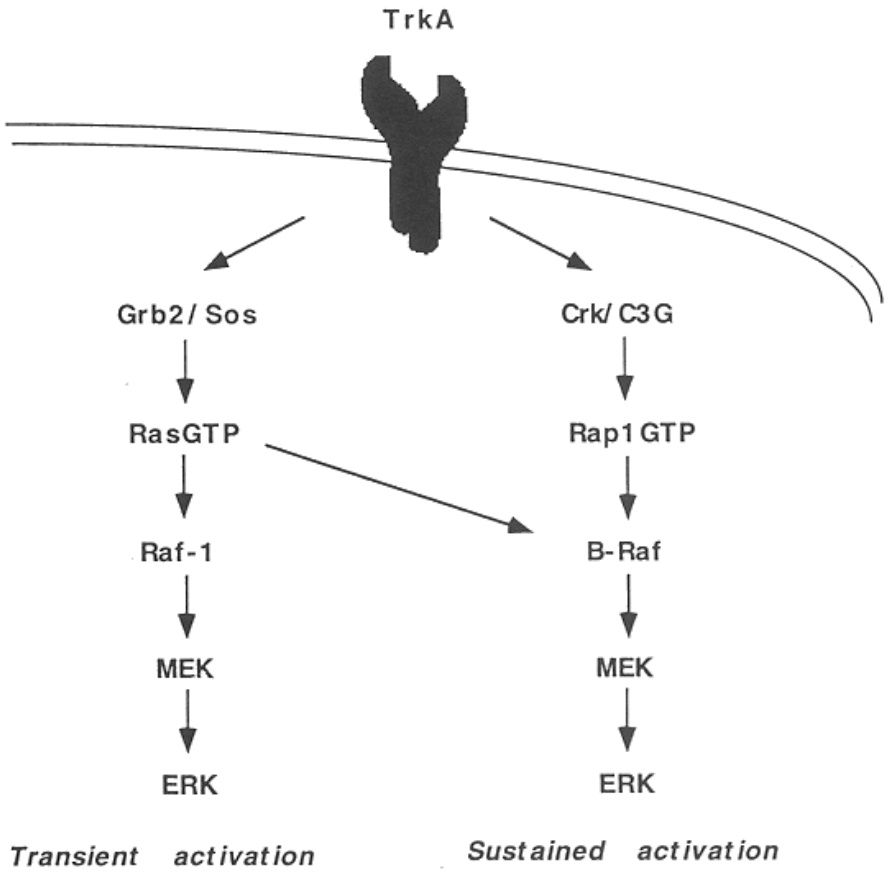

Figure 3. Proposed model for NGF-induced sustained activation of ERK, mediated via Rap1 and B-Raf (York et al., 1998).

PC12 cells stimulated with NGF or bFGF, but not EGF, display tyrosine phosphorylation of a protein originally named SNT. This protein was shown to bind to a juxtamembrane sequence in TrkA essential for NGF-induced neuritogenesis (Peng et al., 1995). Later Schlessinger and co-workers cloned FRS2, which proved identical to SNT (Kouhara et al., 1997). SNT/FRS2 is a lipid-anchored protein and contains a PTB domain that mediates direct binding to the FGF and NGF receptors. Binding to the FGF-receptor is constitutive and independent of ligand stimulation and tyrosine phosphorylation. Binding to TrkA on the other hand, is strongly dependent on receptor activation (Ong et al., 2000). SNT/FRS2 contains four Grb2 binding sites and two binding sites recognising the protein tyrosine phosphatase Shp2. Moreover, the adapter protein Crk and the SH3 domain of Src are other binding partners of SNT/FRS2 (Meakin et al., 1999; Ong et al., 2000). Recruitment of Shp2 is essential for FGF-induced sustained activation of MAPK (Hadari et al., 1998) and the binding to Crk indicates a role for FRS2 in the activation of Rap1 and sustained ERK activity. SNT/FRS2 binds to phosphorylated Y490 in TrkA, and competes with Shc for binding to this site (Meakin et al., 1999). The authors suggest a model where the competitive binding between SNT/FRS2 and Shc regulates the decision between differentiation and proliferation.

Subcellular localisation of ERK

A number of mechanisms regulating nuclear versus cytoplasmic localisation of ERK have been identified. ERK is retained in the cytoplasm by association with MEK, which contains a nuclear export sequence (NES) in its N-terminal (Fukuda et al., 1996; Fukuda et al., 1997). Tyrosine phosphorylation of ERK by MEK is required for dissociation of the two molecules (Adachi et al., 1999). Substitution of residues 312-319 in ERK to alanine prevents its association with MEK as well as its cytosolic retention, without affecting its activity, as demonstrated by a GFP (green fluorescent protein) fusion construct. If instead residues 321-327 in ERK are substituted to alanine, its nuclear translocation upon peroxyvanadate stimulation is impaired (Rubinfeld et al., 1999). It has been demonstated that ERK dimerises in a phosphorylation-dependent manner and that dimerisation is essential for ligand-induced localisation of ERK to the nucleus. One phosphorylated ERK molecule in the pair is sufficent for dimerisation to occur (Khokhlatchev et al., 1998). There are evidence that the dimerisation takes place in an isoform-specific manner, that is, ERK2 binds to ERK2 and ERK1 to ERK1 (Hochholdinger et al., 1999). Nishida and co-workers report that nuclear translocation of dimeric ERK requires an active transport mechanism, but that monomeric ERK also can enter the nucleus, then by passive diffusion (Adachi et al., 1999). The same group has also shown that export of ERK from the nucleus is dependent on the NES in MEK and require active transport (Adachi et al., 2000). The retention of ERK in the nucleus has

been suggested to require synthesis of nuclear anchoring proteins (Lenormand et al., 1998).

As discussed above, NGF-induced sustained activation and nuclear accumulation of ERK has been reported to be dependent on PKA (York et al., 1998). There is also another study showing that calcium-induced activation of CREB- (cAMP response element binding protein-) dependent transcription requires ERK, and that calcium-induced nuclear translocation of both ERK and its target Rsk2 (a member of the p90Rsk family) is dependent on PKA in PC12 cells (Impey et al., 1998). Recently, two articles were published that offers an explanation for the mechanism. ERK associates with the brain-specific tyrosine phosphatase PTP-SL via a kinase interaction motif (KIM) located in the juxtamembrane region of PTP-SL. The binding of ERK to the KIM of PTP-SL inactivates ERK by dephosphorylation and retains ERK in the cytoplasm (Zuniga et al., 1999). The KIM contains a PKA consensus phosphorylation site and phosphorylation of serine 231 by PKA dissociates the ERK/PTP-SL complex and allows ERK to localise to the nucleus (Blanco-Aparicio et al., 1999). In analogy to this, another group reported that that ERK binding to the hematopoietic tyrosine phosphatase (HePTP) via a KIM was abolished when the corresponding serine was phosphorylated by PKA (Saxena et al., 1999). Possibly this type of mechanism could exist in cells of other origin as well.

The involvement of PKC in nuclear accumulation of ERK and ERK-induced transcription in differentiating SH-SY5Y are discussed in paper I.

Rsk and CREB

NGF-induced, Ras-dependent, phosphorylation of the key regulatory site serine 133 in the transcription factor CREB is well documented (Ginty et al., 1994; Bonni et al., 1995). In 1995 Greenberg and co-workers showed that neurotrophins induced a sustained phosphorylation of serine 133 compared to other growth factors that elicited a transient CREB phosphorylation, indicating a role for CREB in neuronal signaling (Bonni et al., 1995). Shortly thereafter a signaling pathway from ERK to CREB, via Rsk2 as the CREB kinase, was delineated (Xing et al., 1996). It has also been shown that NGF can induce serine 133 phosphorylation via the MAPK p38 and its downstream effector MAPK-activated protein kinase 2 (MAPKAP kinase 2). NGF-induced activation of p38 is also Ras-dependent but transient, in contrast to NGF-induced ERK activation (Xing et al., 1998). At later time-points it is therefore likely that the ERK/Rsk2 pathway is solely responsible for CREB phosphorylation. Recently it was shown that CREB-mediated gene expression was sufficient on its own and necessary for NGF-dependent survival of sympathetic neurons. This effect was attributed to CREB-mediated transcriptional upregulation of Bcl-2, which is an anti-apoptotic protein (Riccio et al.,

1999). Both Rsk1 and Rsk2 can also mediate survival of neurons via direct phosphorylation of Bad. This phosphorylation abrogates the pro-apoptotic function of Bad (Bonni et al., 1999), (Shimamura et al., 2000). These data demonstrates that the p90Rsk family members are critical mediators of MAPK induced survival, acting both via transcriptional-dependent and -independent mechanisms (Figure 4).

CREB is also a target of PKB/Akt (Du and Montminy, 1998; Pugazhenthi et al., 2000) (Figure 4). Possibly this pathway is the most important in sympathetic neurons, since there are reports that MEK activation is not crucial for survival in this type of cell (Creedon et al., 1996; Virdee and Tolkovsky, 1996; Klesse et al., 1999).

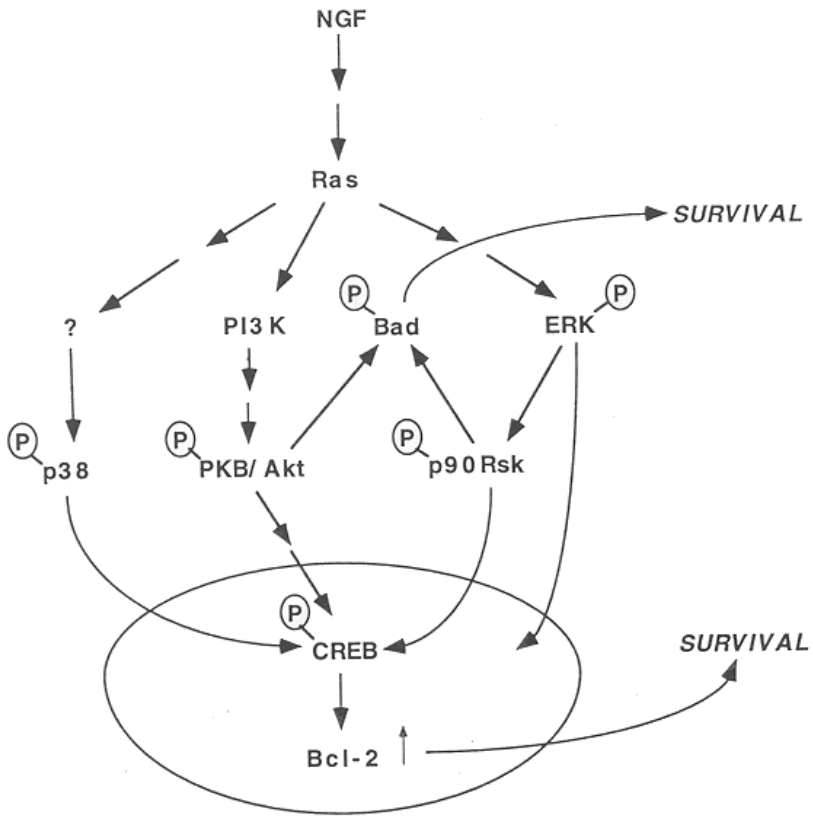

Figure 4. Involvement of p90Rsk and CREB in NGF-mediated cell survival.

PKC

As previously discussed, there are strong evidences of the involvment of PKC in neuronal differentiation of the neuroblastoma cell line SH-SY5Y. TPA as a sole inducer of differentiation in PC12 cells has not been reported. However, an enhancement of NGF-induced neuritogenesis by TPA has been shown (O'Driscoll et al., 1995). PKCδ has been implicated in growth factor induced differentiation in PC12 and H19-7 cells. NGF treatment of PC12 leads to membrane-translocation of PKCδ, which is not seen after EGF stimulation (O'Driscoll et al., 1995). It has also been shown that in both PC12 and H19-7 cells, ERK activation by NGF and bFGF, but not EGF, was dependent on PKCδ. This conclusion was drawn using antisense PKCδ oligonucleotides and rottlerin, an inhibitor of PKCδ. Neurite outgrowth induced by NGF, bFGF or activated Raf was also prevented by PKCδ inhibition. EGF-induced activation of ERK was shown to be PI3K-dependent (Corbit et al., 1999). In a very recent report the same group show that EGF-induced ERK activation is dependent on PKCζ, an isoform activated downstream of PI3K and PDK1 (phosphoinositide-dependent kinase 1). The authors also demonstrate that in cultures of embryonic rat hippocampal cells, EGF and bFGF induced ERK activity is suppressed by inhibitors of PKCζ and PKCδ, respectively (Corbit et al., 2000).

A result possibly in contradiction to those reported above is that deletion of Y785 in TrkA, the binding site for PLCγ, does not prevent NGF-induced neurite formation in PC12 cells (Stephens et al., 1994). PLCγ catalyzes the formation of DAG, involved in the activation of novel and classical isoforms of PKC. However, additional mechanisms for NGF-induced activation of PKCδ can not be ruled out. Neither in SH-SY5Y/TrkA did deletion of Y785 prevent NGF-induced neuritogenesis (Eggert et al., 2000).

PKC can also interact with signals regulating differentiation associated transcription. One report demonstrates that inhibition of the bHLH transcriptional repressor HES-1 induces neurite outgrowth in the absence of NGF and expression of wild-type HES-1 attenuates the NGF-response. NGF induces a post-translational inhibitory modification of HES-1 and it is shown that phosphorylation of HES-1 by PKC prevents its DNA-binding (Strom et al., 1997). Also, an involvement of PKC has been implied in NGF-induced expression of *NPY* (Balbi and Allen, 1994) and stabilisation of *GAP-43* mRNA (Perrone-Bizzozero et al., 1993) in PC12.

SH2-B

SH2-B is a newly identified TrkA substrate in sympathetic neurons. It can bind Grb2 and activate the Ras-MAPK pathway (Qian et al., 1998). SH2-B is expressed in SH-SY5Y and phosphorylated after NGF stimulation, which is not the case in PC12 cells (Eggert et al., 2000). This might be an explanation to the finding that NGF-induced ERK activation

and neurite formation are not prevented in SH-SY5Y/TrkA, lacking both Y785 and Y490 (Eggert et al., 2000). In PC12 cells however, the Y785/Y490 double mutant is defective in both NGF stimulated ERK activation and neurite formation (Stephens et al., 1994).

CHK

CHK is a tyrosine kinase expressed primarily in the nervous system and in hematopoietic cells (Brinkley et al., 1995). As mentioned above, CHK is reported to bind to phosphorylated Y785 in TrkA, via its SH2 domain. Overexpression of CHK is reported to enhance NGF-induced MAPK activation in PC12 cells and microinjection of an anti-CHK antibody prevents NGF-induced neurite outgrowth (Yamashita et al., 1999).

PI3K

The role of PI3K as an inducer of survival in neuronal and other cell types via its downstream target PKB/Akt, is well documented (Datta et al., 1999). The RhoGTPases are another important target of PI3K, among which the most well known and characterised members of this family are RhoA, Rac1 and Cdc42. Rho regulates the formation of actin stress fibers and assembly of focal contacts, while Rac and Cdc42 controls formation of lamellipodia and filopodia, respectively (Hall, 1998). Neurite outgrowth can be considered as a particular form of cell motility, involving actin dynamics during growth cone navigation and neurite elongation. Several reports demonstrate an involvement of RhoGTPases in the formation of neurites and growth cones and there seem to be an inverse relationship between Rho and Rac/Cdc42 in this aspect. Lim and co-workers have studied neurite outgrowth and growth cone morphology in N1E-115 neuroblastoma cells. They show that neurite outgrowth stimulated by serum withdrawal, or growth cone development stimulated by acetylcholine, both required Cdc42 and Rac1 activity. Clostridium botulinum C3 exoenzyme, which inhibits RhoA activity, also induced neurite formation in a Rac1 and Cdc42 dependent manner (Kozma et al., 1997). The same group has recently shown that Ras, via the sequential activation of PI3K, Cdc42 and Rac1 mediates integrin-dependent neurite outgrowth in N1E-115 neuroblastoma cells and that a mutated active Ras that preferentially binds PI3K could promote neurite formation (Sarner et al., 2000). In a study using chicken DRG, conflicting data were reported. Constitutively active Rac1 increased the proportion of collapsed growth cones. Injection of C3 stimulated axonal outgrowth, but the growth cones of these processes were devoid of filopodia and lamellae in contrast to the C3-induced growth cones in the N1E cells (Jin and Strittmatter, 1997). However, there are reports from PC12 cells showing that RhoA is involved in neurite retraction (Katoh et al., 1998) and Rac, via JNK, stimulates outgrowth (Kita et al., 1998).

Cell cycle regulation

The ultimate decision for a cell to multiply or cease proliferating is regulated by the proteins comprising the cell cycle. The mammalian cell cycle is divided into four phases; G_1, S, G_2 and M. During S-phase the chromosomes are replicated and later separated in the M-phase by mitosis. To proceed into S-phase, a restriction point late in G_1 has to be passed. Until a cell has reached that point, it needs continous mitogenic stimulation. Progression through the cell cycle is mediated by sequential actvation of cyclin-dependent kinases (CDK's). Association with a cyclin is required for the CDK to be active. Distinct complexes of cyclins/CDK's regulate different phases of the cell cycle; cyclin D in complex with CDK 4/6 regulate the early events in G_1, while cyclin E in complex with CDK2 is active in the transition from G_1 to S. Cyclin A/CDK2 controls the activities during S-phase, while cyclin B in complex with CDK1 is active during G_2. Cyclin-dependent kinase inhibitors (CKI's) can negatively regulate the CDK's. Two classes of CKI's are found; INK4 proteins ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$) that bind only to CDK4 and CDK6, and the Cip/Kip family ($p21^{cip1}$, $p27^{kip1}$ and $p57^{kip2}$), that interacts with cyclin D-, E- and A-dependent kinases. The primary target of the G_1 CDK's is phosphorylation of the retinoblastoma protein, pRb. In its unphosphorylated form, pRb binds the transcription factor E2F and prevents it from activating genes required for S-phase entry. Upon pRb phosphorylation E2F is released (Sherr, 1996; Sherr and Roberts, 1999).

Traditionally it has been considered to exist an inverse relationship between cell differentiation and proliferation. Terminal differentiation has also been associated with irreversible growth arrest. However, later experiments have demonstrated that differentiated neurons are more plastic than previously believed (Raina et al., 1999). Even though signals inducing differentiation and growth arrest most often occur simultaneously *in vivo*, these processes might be regulated via separate pathways.

Cell cycle targets of the Ras-MAPK pathway

Signaling through the Ras-MAPK pathway can have either positive or negative effects on progression through the cell cycle. Several papers have established the connection between Ras signaling and pRb phosphorylation, via MAPK-induced upregulation of cyclin D expression, resulting in an accelerated G_1-phase (Filmus et al., 1994; Liu et al., 1995; Winston et al., 1996; Lavoie et al., 1996; Peeper et al., 1997; Mittnacht et al., 1997).

A requirement for Ras activity late in G_1 for G_1/S progression has also been demonstrated (Taylor and Shalloway, 1996). This is probably due to the ability of active

Ras to induce decreased stability and hence reduced levels of the CKI $p27^{kip1}$ (Kawada et al., 1997; Leone et al., 1997; Takuwa and Takuwa, 1997). ERK can phosphorylate $p27^{kip1}$ *in vitro*, which prevents its binding to cdk2. However, Ras activity late in G1 does not coincide with ERK activation as shown in NIH3T3 and HeLa cells (Taylor and Shalloway, 1996). Instead a role for PI3K has been implicated in the downregulation of $p27^{kip1}$ (Takuwa and Takuwa, 1997).

Ras signaling via Raf-MAPK can also cause growth arrest, an effect attributed to the induced expression of the CKI $p21^{cip1}$ (Sewing et al., 1997; Woods et al., 1997). In these papers it was shown that a strong sustained Raf signal could upregulate $p21^{cip1}$ expression, both via p53-dependent and -independent mechanisms. The Raf-induced p53-independent effect was probably mediated via ERK, since a direct transcriptional activation of the $p21^{cip1}$ gene by ERK has been demonstrated (Liu et al., 1996). In some situations Ras needs the cooperation of Rho to transform cells (Qiu et al., 1995). An explanation for this observation has been offered by the work of Marshall and co-workers. They show that Rho inactivates $p21^{cip1}$ and thus allows cells with activated Ras to enter S-phase (Olson et al., 1998).

Ras can also arrest cells by inducing senescence in cultured human cells, a process that involves the upregulation of $p16^{INK4a}$ (Serrano et al., 1997). The molecular mechanism is not clear but seems to involve the Raf-MAPK pathway (Lin et al., 1998).

$p21^{cip1}$ in differentiation

The CKI $p21^{cip1}$ has been implicated in terminal differentiation of several cell types including myocytes (Naya and Olson, 1999) and hematopoietic cells (Parker et al., 1995). In myogenic differentiation, a highly ordered sequence of events takes place. Upon growth factor withdrawal, proliferating myocytes start to express differentiation markers as myogenin, thereafter $p21^{cip1}$ is upregulated and the cells become growth arrested. When this post-mitotic state is achieved, phenotypic differentiation takes place (Andres and Walsh, 1996; Walsh and Perlman, 1997).

TPA has been reported to induce $p21^{cip1}$ expression in human keratinocytes (Todd and Reynolds, 1998) and endothelial cells (Zezula et al., 1997). As previously mentioned, TPA can down-regulate *c-myc* in SH-SY5Y (Hammerling et al., 1987; Påhlman et al., 1991; Lavenius et al., 1994), and in other cell types (Mitchell and El-Deiry, 1999). A functional connection between these two TPA-induced effects may be explained by the recently reported Myc-dependent repression of the $p21^{cip1}$ gene (Coller et al., 2000). Forced cell cycle arrest by the DNA polymerase inhibitor aphidicholine has been reported to sensitise SH-SY5Y to differentiation promoting agents (LoPresti et al., 1992; Poluha

et al., 1995). Also, a requirement for $p21^{cip1}$ for survival of differentiated SH-SY5Y has been demonstrated (Poluha et al., 1996). The role of $p21^{cip1}$ in NGF- and TPA-induced differentiation of SH-SY5Y will be discussed in paper IV.

THE PRESENT INVESTIGATION

Aims

The general aim of this work has been to investigate the role of the Ras-MAPK pathway in neuronal differentiation of cultured SH-SY5Y human neuroblastoma cells. The reason for this is of course to gain more information about the signals that control differentiation of these tumour cells and hopefully make a contribution to the development of better treatment strategies of neuroblastoma.

Specific aims were:

- To study if and how differentiation promoting treatment activate the Ras-MAPK pathway in SH-SY5Y cells.

- To investigate the importance of the Ras-MAPK pathway for morphological changes, gene induction and growth control coupled to differentiation in SH-SY5Y.

The role of ERK in SH-SY5Y differentiation (paper I and II)

Results (I)

In paper I we have addressed the existence of a differentiation-specific mode of activation and subcellular distribution of ERK1 and 2 in SH-SY5Y. We have also investigated the involvement of PKC in these processes.

ERK activity was measured in SH-SY5Y/TrkA cells after differentiation promoting treatment with NGF and TPA in the presence of serum (TPA/FCS), or mitogenic stimulation with PDGF-BB. The cells were also treated with TPA in the absence of serum, which does not induce differentiation. The ERK activity was estimated using either an *in vitro* kinase assay against myelin basic protein (MBP) or immunoblotting for phosphorylated active ERK. The two methods gave equal results. All four treatments induced activation of ERK. However, the effect of NGF, TPA/FCS or TPA alone was sustained, lasting for several hours, while PDGF-BB only gave a transient activation of ERK.

To evaluate whether there was a differentiation-specific pattern of subcellular distribution of ERK, we did immunocytochemical staining for total ERK in cultures stimulated for four hours with the same additions as for the ERK activity measurements. The treatments that gave a prolonged activation of ERK also induced nuclear accumulation of the kinase. The strongest inducer of nuclear ERK was TPA, either in the presence or absence of serum. We also performed immunocytochemical stainings against phosphorylated active ERK after treatment with NGF and TPA in the absence of serum. The result of these studies were the same as when staining for total ERK; TPA was a stronger inducer of nuclear accumulation of ERK than NGF, also in the absence of serum.

In addition to the immunocytochemical stainings, we analysed nuclear ERK activity. ERK-induced transcription was measured using a luciferase reporter, p3TD-lux, driven by the SRE-element from the human *c-fos* promoter. Both JNK and p38 MAPK's have been shown to induce SRE-dependent transcription. However, we could demonstrate that transcriptional activation of p3TD-lux in TPA- and NGF-stimulated SH-SY5Y was totally ERK-dependent. This makes this assay a useful reporter for nuclear ERK activity. In agreement with the ERK stainings, NGF and TPA both in the absence and presence of serum, but not PDGF-BB induced transactivation of p3TD-lux. Also in this respect TPA was the most potent. Interestingly, TPA in the absence of serum induced higher luciferase activity compared to when serum was present.

We next investigated the contribution of PKC in both TPA- and NGF-stimulated activation and subcellular distribution of ERK. As expected, a PKC-inhibitor (GF109203X) prevented both ERK activation and nuclear accumulation induced by TPA. NGF-induced activation of ERK was however not dependent on PKC. On the contrary, ERK activity in unstimulated and NGF stimulated cells was slightly elevated after PKC-inhibition. Therefore it was somewhat of a surprise that PKC-inhibition prevented NGF-induced nuclear accumulation of ERK, judged by immunocytochemical staining. This result was further supported by the fact that GF109203X and another PKC-inhibitor, Ro-32-0432, completely prevented NGF-induced SRE transactivation. To further investigate this finding, we studied the effect of PKC-inhibition on NGF induction of a ERK-responsive gene; *c-fos*. Nuclear translocation of activated ERK is essential for transcriptional activation of mitogen-induced genes like *c-fos* (Hochholdinger et al., 1999; Brunet et al., 1999). NGF-induced upregulation of *c-fos* was reduced to 40% in the presence of GF109203X. This result is in agreement with a role for PKC in NGF-induced nuclear accumulation of ERK.

Results (II)

We next addressed the functional role of ERK in differentiating SH-SY5Y. We also wanted to find out whether the requirement for PKC in nuclear accumulation of ERK could be one reason for the previously demonstrated PKC-dependency in the differentiation process.

To turn the ERK activity on or off, we used a constitutively active form of MEK (MEKS222D/S218D), the kinase immediately upstream of ERK and the specific MEK inhibitor PD98059. We looked at TPA-induced neurite formation and expression of the neuronal marker genes *NPY* and *GAP-43* in the presence of PD98059. The results showed that ERK was needed for a full upregulation of *NPY* and *GAP-43*, but was not necessary for neurite formation under those conditions. Expression of constitutively active MEK did not induce neurite outgrowth either in SH-SY5Y/wt or SH-SY5Y/TrkA, while constitutively active Ras (V12Ras) had a pronounced effect on neurite formation in both cell types.

We could also show that there was no correlation between nuclear ERK activity, measured as SRE transactivation, and neurite formation. Interestingly, there was a difference between SH-SY5Y/wt and SH-SY5Y/TrkA in the level of SRE-mediated transcription induced by the active constructs. MEKS222D/S218D gave a strong induction of SRE-dependent transcription in SH-SY5Y/TrkA, while there was almost no effect in SH-SY5Y/wt. Expression of the HA-tagged MEKS222D/S218D at protein level was easily detected in both cell types. Also SRE transactivation induced by active Ras

varied between the two cell types; a strong response in SH-SY5Y/TrkA and a very limited effect in SH-SY5Y/wt. However, Ras was a strong inducer of neurite formation in both cell types. TPA was equally potent inducing SRE-dependent transcription in both SH-SY5Y/wt and SH-SY5Y/TrkA.

Discussion (I and II)

The differentiation promoting treatments NGF and TPA/FCS both induced sustained activation and nuclear accumulation of ERK in SH-SY5Y. TPA in the absence of serum, which does not induce differentiation, was equally potent as TPA/FCS in promoting activation and nuclear accumulation of ERK. This result demonstrates that sustained activation and nuclear accumulation of ERK is not sufficient for a differentiation response to take place in these cells. In contrast to PC12 cells where nuclear active ERK is sufficient for differentiation to take place (Cowley et al., 1994; Fukuda et al., 1995; Robinson et al., 1998), additional signals are needed in SH-SY5Y. In agreement with the PC12 data is however our finding that mitogenic treatment (PDGF-BB) of SH-SY5Y only induced a transient ERK activation and no nuclear accumulation of the kinase.

With respect to ERK-induced transcription, TPA in the absence of serum was even more potent than TPA/FCS, which was surprising. Transactivation of the SRE-element requires cooperation of an Ets-transcription factor like Elk-1 and the serum response factor (SRF). Besides ERK-induced phosphorylation of Elk-1, TPA must activate a pathway leading to SRF activation. In serum, a possible pathway to SRF is via lysophosphatidic acid (LPA) induced activation of Rho, leading to SRF phosphorylation. However, in SH-SY5Y/TrkA serum had an inhibitory effect on TPA-induced SRE transactivation, a result which we don't have a good explanation for. One possibility could be that a MAPK specific phosphatase is activated in serum, but not under serum-free conditions.

The observed PKC-dependent nuclear accumulation of ERK is interesting, especially considering the central role that PKC plays during SH-SY5Y differentiation. PKC is known to have regulatory effects on the cytoskeleton. A simple explanation would therefore be that PKC inhibition abrogates some intracellular transport mechanism preventing nuclear import. However, both previous data (Fagerström et al., 1996) and paper I in this thesis shows that PKC-inhibition does not completely block NGF-induced transcription, indicating that some of the NGF-induced signalling pathways under those conditions are still intact. Another explanation would be that PKC phosphorylation, in analogy to PKA, targets a protein residue involved in regulating the subcellular localisation of ERK. We therefore investigated whether ERK dimerisation maybe was dependent on PKC activity, but could not find any evidence for this hypothesis

(unpublished observation). The PKC-dependent mechanism regulating nuclear accumulation of ERK remains to be solved.

The experiments in paper II revealed that ERK was not sufficient on its own or necessary for TPA-induced neurite formation. However, a tendency to somewhat less developed growth cones was seen in cultures treated with TPA in the presence of the MEK-inhibitor. In another paper where one studied the role of ERK for NGF-induced neurites in chicken DRG, the authors found no inhibitory effect on neurite outgrowth by PD98059, but a slight inhibiton on the branching of the neurite network (Klinz et al., 1996). These results indicate that even if neurite outgrowth is not completely prevented by MEK-inhibition as in PC12 cells, more subtle changes can be observed. The characteristic upregulation of the neuronal marker genes *NPY* and especially *GAP-43*, was on the other hand dependent on ERK. The large inhibitory effect of PD98059 on TPA-induced *GAP-43* expression can be one explanation for the observed effect on the growth cones. GAP-43 is a PKC substrate located in the growth cone and has been implicated in growth cone functionality. Overexpression of GAP-43 in transgenic mice induces nerve sprouting (Aigner et al., 1995). In another study using knock-out mice, GAP-43 was shown to play an important role in the pathfinding of the growth cone, but not for axonal outgrowth or growth cone formation per se (Strittmatter et al., 1995).

From the present and previously reported data it is also possible to draw the conclusion that sustained activation and nuclear accumulation is not sufficient for a full upregulation of *NPY* and *GAP-43*. As demonstrated in paper I, TPA in the absence of serum is as potent as TPA/FCS in promoting nuclear accumulation of ERK and ERK-induced transcription. However, under these conditions there is only a partial induction of *NPY* and *GAP-43* transcription (Påhlman et al., 1991; Lavenius et al., 1994) and no morphological differentiation response (Påhlman et al., 1991; Lavenius et al., 1994 and paper I).

Active MEK did not induce neurite outgrowth in SH-SY5Y, in contrast to PC12 cells. Active Ras on the other hand promoted a distinct differentiated morphology with extending neurites with growth cones and varicosities. This is in agreement with a recent report using N1E-115 neuroblastoma cells, showing that Ras and the downstream targets PI3K, Cdc42 and Rac1 were each sufficient to promote neurite outgrowth. Moreover, the authors reported that expression of dominant negative ERK did not inhibit integrin-dependent neurite formation in these cells (Sarner et al., 2000).

As described above Stork and co-workers have shown that sustained activation of ERK in response to NGF was mediated by Rap1 in PC12 cells (York et al., 1998). Inhibition

of Rap1 activity prevented NGF-mediated gene expression, but not neurite formation. This indicates that neurite formation and differentiation-coupled gene expression are regulated by separate pathways, a model that could apply to SH-SY5Y as well.

An interesting observation was the difference between SH-SY5Y/wt and SH-SY5Y/TrkA in respect to SRE-driven transcription induced by active MEK and active Ras. The very limited SRE-transactivation seen in SH-SY5Y/wt was not due to a defect protein expression in these cell. On the contrary, HA-tagged MEKS222D/S218D was easily detected in both cell types. One possible explanation is that SH-SY5Y/TrkA cells has a higher basal activity of a signalling pathway required for ERK to activate transcription. In unstimulated cells, the basal SRE-mediated luciferse activity is approximately 5-10 times higher in SH-SY5Y/TrkA compared to SH-SY5Y/wt. An interesting experiment would be to compare the subcellular distribution of ERK in SH-SY5Y/wt and SH-SY5Y/TrkA transfected with HA-tagged MEKS222D/S218D.

In summary, paper I and II shows that sustained activation and PKC-dependent nuclear accumulation of ERK may be an important event during neuronal differentiation of SH-SY5Y, since ERK had a role in regulating expression of the neuronal marker genes *NPY* and *GAP-43*. Sustained activation and nuclear accumulation of ERK is however not sufficient for a differentiation response to take place in SH-SY5Y. This requires additional signals.

TPA- and NGF-induced activation of Ras, Raf-1 and PKC in SH-SY5Y/TrkA (paper III)

Results (III)

In paper III we compared the ability of TPA and NGF to activate Ras and Raf-1 in SH-SY5Y/TrkA, i.e. events upstream of ERK. We also addressed the involvement of PKC.

Ras activity was measured using the Ras Binding Domain of Raf-1 (RafRBD), which specifically binds to active Ras-GTP and not inactive Ras-GDP. NGF induced a strong increase in the amount of Ras-GTP, while TPA was without effect. Similar results were obtained with or without the presence of serum. We also analysed the relative amount of Ras-GTP in [^{32}Pi]-ortophosphate labelled cultures after NGF and TPA stimulation in serum-free medium, with the same result.

Raf-1 activity was measured in an *in vitro* kinase assay, using recominant GST-MEK1 as a substrate. The effect of TPA and NGF on Raf-1 activation was measured in the

presence of serum, because we wanted to use differentiation promoting treatment and still compare the effect of TPA and NGF under similar conditions. Both TPA and NGF caused a rapid activation of Raf-1, an effect that was sustained for at least two hours. The effect of TPA on the amplitude of Raf-1 activation was approximately half of the response seen after NGF stimulation.

When we looked at NGF- and TPA-induced phosphorylation of Raf-1, the situation was the opposite. Phosphorylation of Raf-1 was assayed by its mobility in a polyacrylamide gel, and by two-dimensional tryptic phosphopeptide analysis. In serum, TPA caused a more potent and sustained retarded mobility of Raf-1 than NGF. The two-dimensional tryptic phosphopeptide analysis was performed under serum-free conditions for technical reasons. This assay revealed that TPA induced an increased phosphorylation of five peptides in Raf-1. In NGF stimulated cells on the other hand, increased phosphorylation was only found in one peptide. Analysis of the peptides revealed that they all exclusively contained phosphoserine. The more pronounced effect of TPA on Raf-1 phosphorylation probably reflects that PKC can phosphorylate Raf-1 on several residues, with stimulatory and inhibitory function on the catalytic activity of Raf-1.

We also investigated the involvement of PKC in NGF-induced activation and phosphorylation of Raf-1, since activation of PKC apparently could lead to Raf-1 activation in SH-SY5Y/TrkA and PKC can be activated downstream of TrkA. To prevent PKC-activation we used the compound GF109203X. No inhibitory effect on NGF-induced Raf-1 activity by GF109203X was seen. On the contrary, basal Raf-1 activity was elevated in unstimulated cultures treated with GF109203X. Phosphorylation of Raf-1 in unstimulated and to some extent in NGF stimulated cells was decreased after PKC-inhibition, measured as an increased mobility of Raf-1. These results suggest that GF109203X abolishes an inhibitory phosphorylation present on Raf-1 in unstimulated cells.

Since NGF-induced activation of Raf-1 was not dependent on PKC, we were curious to see to what extent NGF stimulated PKC-activity. We measured PKC activity by phosphorylation of the endogenous PKC substrate MARCKS (myristoylated alanine-rich C-kinase substrate) and by membrane translocation of PKC-α, -δ and ε. NGF had a very limited effect on PKC activation measured as MARCKS phosphorylation and no translocation of any of the three PKC-isoforms was seen after NGF-stimulation. This result was a bit surprising, but in good agreement with the lack of a PKC component in NGF-induced Raf-1 activation.

Discussion (III)

In conclusion, the data in this study showed that Raf-1 is a common target for NGF and TPA in differentiating SH-SY5Y/TrkA, although NGF was a more potent inducer of Raf-1 activity. Upstream of Raf-1, at the level of Ras, these stimuli differ in their activating capacity. While TPA has been reported to increase Ras-GTP levels in COS cells (Marais et al., 1998), this is not the case in SH-SY5Y/TrkA.

There was also a big difference between NGF and TPA in their capacity to induce phosphorylation of Raf-1, TPA being the more potent in this respect. The poor activation of PKC induced by NGF, might be one explanation for the fewer phosphorylated peptides seen after NGF stimulation. Interestingly, all peptides were phosphorylated on serine. This result rules out Src as the Raf-1 activating kinase since it is tyrosine specific. Interesting candidates are instead the serine/threonine kinases Pak1 and Pak3, which have been reported to phosphorylate Raf-1 on serine 338 and increase its catalytic activity (King et al., 1998; Sun et al., 2000; Chaudhary et al., 2000). Further studies are needed to identify the phosphorylated peptides.

NGF and TPA also differ largely in their capacity to activate PKC in SH-SY5Y/TrkA. While TPA is a strong activator of PKC, NGF stimulated only a very small increase in MARCKS phosphorylation and no detectable translocation of PKC-α, -δ or -ε was seen. This was unexpected, especially since we have seen NGF-induced tyrosine phosphorylation of PLCγ in these cells and generation of inositol phosphates (unpublished). However, there seem to be a basal PKC activity in these cells, negatively regulating both ERK and Raf-1 activity in the absence of ligands or TPA.

In conclusion, NGF and TPA both activate Raf-1 in SH-SY5Y/TrkA, although via different mechanisms. At the level of Raf-1, NGF is a more potent activator than TPA and NGF-induced activation of Raf-1 is independent of PKC. Further down in the Ras-MAPK pathway, NGF and TPA are equally potent activators of ERK. In the next step; nuclear accumulation of ERK, TPA is a more potent inducer than NGF, and the effect of NGF is PKC-dependent.

Growth control in differentiating SH-SY5Y (paper IV)

Results (IV)

In this paper we have investigated the cell cycle activity of SH-SY5Y cells undergoing NGF- or TPA-induced differentiation. As mentioned previously there are some conflicting data on this matter in the literature.

Cells entering S-phase were detected by their incorporation of BrdU, a thymidine analogue. Cultures of SH-SY5Y/TrkA that had been treated with TPA/FCS or NGF (with or without serum) for eight days showed a decreased incorporation of BrdU compared to unstimulated cells, after exposure to BrdU for 2 hours. Addition of a mitogen, PDGF-BB, to cultures treated with TPA for eight days did not reverse the observed decrease in BrdU incorporation. When cultures treated with TPA/FCS for eight days were allowed to incorporate BrdU for 2 up to 48 hours, another picture emerged. As previously, the BrdU incorporation was decreased in TPA treated cells challenged with BrdU for 2 hours. However, after longer exposure to BrdU this difference disappeared. After 48 hours in the presence of BrdU, the labelling index for unstimulated and TPA-treated cells was 96% and 93%, respectively. This indicated that almost all cells entered S-phase under both conditions. The same was true for NGF-treated cultures (data not shown). The most likely explanation for these findings is that the differentiated cells still entered S-phase but at a slower rate compared with control cells.

When studying the cell morphology in the BrdU-labelled cultures, we found that both neurite bearing and undifferentiated cells were BrdU poitive. This was seen in both SH-SY5Y/wt and SH-SY5Y/TrkA cells and indicates that morphological differentiation did not require growth arrest.

Since the CKI $p21^{cip1}$ has been demonstrated to regulate cell cycle activity in cells undergoing differentiation, we investigated the expression of $p21^{cip1}$ in SH-SY5Y/TrkA treated with TPA or NGF in the presence of serum. Both TPA and NGF induced an upregulation of $p21^{cip1}$ that was sustained over at least eight days. This upregulation was largely mediated by a MEK-dependent pathway, as demonstrated by the inhibitory effect of PD98059 on $p21^{cip1}$ expression. However, inhibition of $p21^{cip1}$ expression had no effect on the rate of BrdU incorporation after TPA- or NGF-treatment. Immunocytochemical staining for $p21^{cip1}$ after TPA-, NGF- or PDGF-BB-treatment revealed that all three additions induced expression of $p21^{cip1}$. Thus, there was no direct correlation between $p21^{cip1}$ expression and morphological differentiation. Interestingly, $p21^{cip1}$ localised primarily to the cytoplasm.

We also investigated $p21^{cip1}$ expression and cell cycle activity in SH-SY5Y cells transfected to stably express constitutively active Ras (SH-SY5Y/V12Ras). As the cells transiently transfected with V12Ras in paper II, these cells show a highly differentiated morphology, with long neurite extensions. SH-SY5Y/V12Ras cells do not show any sign of decreased proliferation compared with SH-SY5Y not transfected to express active Ras, even though they have an increased expression of $p21^{cip1}$. This result further

supports the conclusion that $p21^{cip1}$ does not induce growth arrest in SH-SY5Y neuroblastoma cells. However, SH-SY5Y/V12Ras responded to TPA-treatment with the same lowering of BrdU incorporation seen after a 2 hours chase as SH-SY5Y/TrkA. Moreover, the morphological differentiation of SH-SY5Y/V12Ras cells was even more pronounced in after TPA treatment, indicating a synergistic effect between Ras and PKC in this respect.

Discussion (IV)

The results from this study show that SH-SY5Y cells induced to undergo differentiation by NGF or TPA can still enter S-phase, although at a slower rate. There was no correlation between $p21^{cip1}$ expression, BrdU-incorporation or neurite formation. We have observed neurite-bearing cells in culture undergoing mitosis, which supports the data showing BrdU-positive cells with extending neurites. These results indicates that SH-SY5Y are not defect in their differentiation-response, but rather in their growth control. Maybe the level of $p21^{cip1}$ expression is not sufficient to induce growth arrest. Another possible explanation is that the predominant cytoplasmic localisation of $p21^{cip1}$ that we found, prevents it from interacting properly with CDK's.

The different results concerning cell cycle activity in differentiating SH-SY5Y that have been reported are difficult to explain. In our hands, unstimulated SH-SY5Y/wt and SH-SY5Y/TrkA does not differ in their proliferation rate. Although our results show that the differentiating cells still entered S-phase, a decreased cell density was seen in those cultures due to their slower cell cycle rate. However, when counting the number of cells a plateau would not be reached. This is in agreement with the findings in a previous report (Påhlman et al., 1981). The ceased proliferation reported in NGF-treated cultures of SH-SY5Y transfected to express TrkA (Poluha et al., 1995), differs from our results. However, in this study they do not mention for how long the cells were treated with BrdU. In two previous reports with members of our own group among the authors, no decrease in proliferation was seen in SH-SY5Y/wt cells treated with the combination bFGF/IGF-1 (Lavenius et al., 1994) or SH-SY5Y/TrkA treated with NGF (Lavenius et al., 1995). In the bFGF/IGF-1 study, proliferation was estimated by measuring the amount of protein. We have later observed that differentiated cells contain more protein/cell than undifferentiated, due to their neurites. Therefore, protein measurement as an estimate of cell number may give misleading results. In the NGF study, a short pulse (1 hour) of [^{3}H] thymidine was added to NGF-differentiated SH-SY5Y/TrkA, but no decrease in S-phase entry was seen. According to our present data, a lowered thymidine incorporation would have been expected under those conditions. However, one reason for this discrepancy might be the density at which the cells are seeded. We have noticed that the decreased proliferation rate induced by NGF is only seen if the cells are seeded

very sparsly. Another explanation for the contradictory results can of course be subcloning of the cells over time and in different laboratories due to different culture conditions.

CONCLUSIONS

- The differentiation promoting treatments NGF and TPA/FCS induced sustained activation and nuclear accumulation of the MAPK ERK in SH-SY5Y, while the mitogen PDGF-BB only activated ERK transiently and induced no nuclear accumulation of the kinase. Sustained activation and nuclear accumulation of ERK was not sufficient for a differentiation response to take place in these cells, as demonstrated by TPA in the absence of serum, but additional signals are required. NGF-induced nuclear accumulation of ERK was PKC-dependent.

- ERK activation was needed for a full upregulation of the neuronal marker genes *NPY* and *GAP-43* by TPA in SH-SY5Y/TrkA. However, ERK activation was not sufficient on its own or necessary for TPA-induced neurite formation. There was no correlation between nuclear ERK activity and neurite outgrowth. Instead, stimulation of a pathway probably distinct from MEK/ERK, downstream of active Ras, was needed for morphological differentiation of these cells.

- Raf-1 was a common target for TPA and NGF in the Ras-MAPK pathway in SH-SY5Y/TrkA, although they activated Raf-1 by different mechanisms.

- SH-SY5Y induced to differentiate with NGF or TPA/FCS continued to enter S-phase, but with a slower kinetics. Growth arrest was not required for morphological differentiation. There was no correlation between $p21^{cip1}$ expression, BrdU-incorporation or neurite formation.

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