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FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

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M. Sc. (Cellular and Molecular Medicine)

GRADE - DEGREE

Department of Cellular and Molecular Medicine

FACULTÉ, ÉCOLE, DÉPARTEMENT - FACULTY, SCHOOL, DEPARTMENT

TITRE DE LA THÈSE - TITLE OF THE THESIS

The growth-arrest-specific protein gas7 potentiates neuronal differentiation

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The growth-arrest-specific protein gas7 potentiates neuronal differentiation

By

Karine Lortie

This thesis is submitted as a partial fulfillement of the M.Sc. program in

Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine

Faculty of Medicine

University of Ottawa

August 24, 2004

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Abstract

The growth-arrest-specific gas7 protein is involved in neuronal development. Its role in neuronal differentiation and its potential neuroprotective activity were investigated in PC12 and NT2 cells. gas7 overexpression in PC12 cells promoted neurite outgrowth and potentiated nerve growth factor-induced expression of the neuronal markers βIII-tubulin, synaptotagmin, α7 subunit of the acethylcholine receptor, and dihydropyrimidinase related protein-3. This effect was exerted independently of cellular proliferation, as gas7 did not affect cell cycle progression. Endogenous gas7 expression was induced during neuronal differentiation of NT2 cells with retinoic acid, suggesting a role for gas7 in neuronal development. Finally, gas7 overexpression in PC12 cells did not protect against toxicity triggered by oxygen-glucose deprivation, the calcium ionophore A23187 or sodium nitroprusside. The ability of gas7 to potentiate neuritogenesis and neuronal differentiation makes it a potential therapeutic target to promote re-establishment of neuronal connections in the injured or diseased brain, such as following stroke.

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List of Abbreviations

3'-UTR	3' Untranslated region
AChR-α7	Alpha-7 subunit of the nicotinic acetylcholine receptor
AD	Alzheimer's disease
Ad5	Adenovirus 5
ADB	Antibody diluting buffer
ADF	Actin depolymerizing factor
aFGF	Acidic fibroblast growth factor
Amp^R	Gene for ampicilline resistance
ANOVA	Analysis of variance
APS	Ammonium persulfate
ATP	Adenosine triphosphate
βIII-tubulin	Beta-III tubulin
bFGF	Basic fibroblast growth factor
βME	beta-mercaptoethanol
BSA	Bovine serum albumin
BrdU	Bromodeoxyuridine
cAMP	cyclic adenosine monophosphate
cDNA	Complimentary DNA
CMV	Cytomegalovirus promoter
CREB	cAMP response element binding protein
C_T	Threshold cycle
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DRP-3	Dihydropyrimidinase related protein-3
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein

ERK	Extracellular signal related kinase
EtBr	Ethidium bromide
F-actin	Filamentous actin
FBS	Fetal bovine serum
FCH domain	Fes/CIP4 homology domain
FGF-2	Fibroblast growth factor-2
FRET	Fluorescence resonance energy transfer
G0 phase	Resting (or quiescent) phase of the cell cycle
G1 phase	First gap of the cell cycle
G2 phase	Second gap of the cell cycle
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gas	Growth-arrest-specific
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HG-DMEM	High glucose Dubelcco's modified Eagle's medium
HS	Horse serum
IgG	Immunoglobulin G
IP	Immunoprecipitation
ITR	Inverted terminal repeats
kDa	KiloDalton
M phase	Mitosis phase of the cell cycle
MAPII	Microtubule associated protein II
MAPK	Microtubule associated protein kinase
MLL	Mixed lineage leukemia
MOI	Multiplicity of infection
Mo-MuLV	Moloney murine leukemia virus
mRNA	Messenger RNA
MSB	Modified Salter's buffer
NCAM	Neural cell adhesion molecule
NCBI	National Center for Biotechnology Information
NGF	Nerve growth factor

NIH3T3 cells	Murine fibroblast cells
nt	nucleotide
NT2 cells	Human embryonic teratocarcinoma Ntera2/D1 cells
OGD	Oxygen-glucose deprivation
Ori	Origin of replication
ORF	Open reading frame
PA	Polyadenylation site
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC12 cells	Neuronal rat pheochromocytoma cells
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFαR	Platelet-derived growth factor alpha receptor
pfu	Plaque-forming-unit
PI3-kinase	Phosphatidylinositol-3 kinase
PMP-22	Peripheral myelin protein-22
PVDF	Polyvinylidene difluoride
Q-PCR	Real-time quantitative polymerase chain reaction
RA	Retinoic acid
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcriptase-polymerase chain reaction
S phase	DNA synthesis phase of the cell cycle
SDS	Sodium dodecylsulfate
SEM	Standard error of the mean
SNP	Sodium nitroprusside
STG	Synaptotagmin
TBS-T	Tris buffered saline Tween-20
TEMED	Tetramethylethylenediamine
TUJ-1	Neuron-specific beta-III tubulin

Acknowledgments

Special thanks to my supervisor, Dr. Paul Morley, who has been an exceptional mentor. Your guidance and continuous support were, and still are, very valuable to me. Thanks for your advice and for providing me with a rewarding graduate training experience.

I would also like to thank everyone in the Receptors and Ion Channels group at NRC, IBS for making my journey in this lab most enjoyable. Eric, Geoff, Joe, John, Robert, and Tanya - it has been a privilege to work with each one of you. Special thanks to Tania Gendron, who showed me the ropes during my start as a graduate student and with whom I shared a few night shifts in the lab. Adele Nguyen has been of great help in troubleshooting Western blotting and toxicity assays. Michel Ménard, from the Molecular Signaling group at NRC, IBS, has also been a valuable support and work companion. To everyone else at NRC, IBS with whom I have collaborated – it was a pleasure to work with all of you. I would also like to acknowledge my good friends and fellow graduate students Ally and Carmen, for mutual moral support over the last two years.

Thanks to Dr. Sue Lin-Chao and to my advisory committee members, Dr. Balu Chakravarthy, Dr. Danica Stanimirovic, and Dr. William Staines, for helpful discussions, suggestions, and advice.

PC12 cells were kindly given by Dr. Balu Chakravarthy, the gas7 and GFP adenoviruses were provided by Dr. Sheng T. Hou and Dr. Deqi Huang, mass spectrometry analysis was performed by Dr. John Kelly, the gas7 antibodies were provided by Dr. Sue Lin-Chao,

and cDNA and protein samples from differentiating NT2 cells were given by Dr. Dao Ly and Kirsty Malone, respectively.

I would like to acknowledge the University of Ottawa, NSERC, and the NRC for providing me with financial support.

And last but certainly not least, special thanks to the people who are central in my life, my family and my partner, Luc. You have always been supportive of my decisions and have always been there when I needed your encouragement. Thanks for your unconditional patience, understanding, and love.

CHAPTER 1

INTRODUCTION

1.1 Some genes are preferentially expressed during growth arrest

1.1.1 The cell cycle

The cell cycle concept was introduced in 1953 by Howard and Pelc (Howard & Pelc, 1953). They showed, using ^{32}P-labelled sodium phosphate as a precursor for DNA, that replication occurs only during a specific phase of the cell proliferation cycle (Howard & Pelc, 1953). Since DNA synthesis is completed hours before cells start to divide, there is a time gap after replication of the genetic material. Another gap occurs after cell division and before the next round of DNA synthesis. Therefore, the cell cycle is divided into 4 different phases, called G1 (first gap), S (DNA synthesis), G2 (second gap), and M (mitosis) (Lodish *et al.*, 1996). During the G1 phase, cells increase in size, produce RNA, and synthesize proteins. Initiation of DNA replication characterizes the transition to the S phase, at the end of which the cellular genetic material has doubled. Following DNA synthesis, cells continue to grow in the G2 phase and produce new proteins. Cell division takes place during mitosis, which is characterized by chromosome condensation, segregation of the sister chromatids to opposite poles, and division into two sister cells (Lodish *et al.*, 1996). Under appropriate conditions, cells can exit the cell cycle and stop growing. They then enter the G0 state, also called resting or quiescent state. This transition occurs during the G1 phase (Lodish *et al.*, 1996).

Balanced mechanisms regulate whether cells will advance to the G1/S transition, or will leave the cell cycle and become quiescent. Control of cell proliferation thus mainly occurs in G1, and growth arrest of mammalian cells can be accomplished in this phase by depletion of growth factors or serum (Schneider *et al.*, 1988). The majority of the cells in

an organism spend a considerable part of their life span in the resting state (Philipson, 1998). Unless they have reached an end stage of development into a post-mitotic cell type, such as neuronal differentiation, cells can re-enter the cell cycle and proliferate, following appropriate stimulation (Ciccarelli *et al.*, 1990). Regulation of the cell cycle, and in particular of cell cycle exit, is critical for embryonic development, during which growth and differentiation must be tightly regulated (Mullor & Altaba, 2002).

1.1.2 Studying the exit of the cell cycle

A balance between negative and positive stimuli governs the cell cycle (Ciccarelli *et al.*, 1990; Schneider *et al.*, 1988). However, in previous years, major emphasis has first been to study the mechanisms involved in induction of growth from an arrested state, assuming that growth control is positively regulated through transcriptional activation (Ciccarelli *et al.*, 1990). Molecular analysis in mammalian cells then led to the characterization of growth factors involved in the induction of cell division and DNA synthesis (Ciccarelli *et al.*, 1990). Even though a detailed cross-hybridization study carried out in 1975 anticipated the existence of mRNAs specifically expressed during growth arrest, it is only about 15 years ago that studies have begun dealing with the complex pathways involved in cell cycle exit (Williams & Penman, 1975). In 1988, Schneider and coworkers designed a subtraction library enriched in sequences preferentially expressed during growth arrest of murine NIH3T3 fibroblasts. They cloned a set of 6 non-homologous genes whose expression is repressed upon growth stimulation, and called them the growth-arrest-specific genes, or gas genes (Schneider *et al.*, 1988).

1.1.3 The growth-arrest-specific genes

Following Schneider and colleagues' differential screening for growth-arrest-specific genes, other members have been added in the gas family. Eleven gas genes (gas1-11) have now been identified, gas11 being the human version of the murine gas8 gene (Ju *et al.*, 1998; Lih *et al.*, 1996; Schneider *et al.*, 1988; Whitmore *et al.*, 1998; Yeh *et al.*, 2002). Messenger RNAs of the gas genes accumulate when cells reach density inhibition, are induced to exit the cell cycle, or are exposed to a variety of stresses including serum removal and inadequate nutrition (Ciccarelli *et al.*, 1990; Fleming *et al.*, 1998; Fontanier-Razzaq *et al.*, 2002; Schneider *et al.*, 1988).

Other than gas4 and gas10, for which neither the gene nor the product have been characterized, a variety of diverse functions have been attributed to the different gas genes. They include regulation of growth, differentiation, cytoskeletal organization, apoptosis, and cell adhesion. The gas genes are heterogeneous in terms of steady-state levels of expression and there is no sequence or structural similarity among them (Colombo *et al.*, 1992). Their genomic locations, widely dispersed on different chromosomes, are in agreement with the picture of a diverse group of genes (Colombo *et al.*, 1992). The various roles assigned to the characterized gas genes are discussed below, and illustrated in Figure 1.

1.1.3.1 The growth-arrest-specific gene 1 (gas1)

The first identified growth-arrest-specific gene codes for a membrane protein expressed in most organ systems and its expression is upregulated in a variety of growth-arrested

cells (Lee *et al.*, 2001b; Ruaro *et al.*, 2000; Schneider *et al.*, 1988; Stebel *et al.*, 2000). gas1 expression was also observed to be induced in hippocampal neurons subjected to mild excitotoxic insults, in which it is thought to participate to neuronal death (Mellstrom *et al.*, 2002). gas1 reduces cell proliferation and inhibits apoptosis in different cell types (Del Sal *et al.*, 1992; Mellstrom *et al.*, 2002; Ruaro *et al.*, 2000; Schneider *et al.*, 1988; Zamorano *et al.*, 2003). Due to its ability to negatively regulate growth, gas1 has been suggested to be a tumor suppressor gene (Del Sal *et al.*, 1992; Evdokiou & Cowled, 1998; Mullor & Altaba, 2002). However, a knockout mouse model for the gas1 gene raised some controversy regarding its anti-proliferative role. In the retina of mutant animals, cellular overproliferation supported the role for gas1 in inhibiting proliferation, but gas1 was also shown to be required for normal cell proliferation in the cerebellum and the limbs (Liu *et al.*, 2001; Liu *et al.*, 2002). Overall, evidence suggests that gas1 functions might be tissue-specific (Lee *et al.*, 2001a; Mullor & Altaba, 2002).

1.1.3.2 The growth-arrest-specific gene 2 (gas2)

The gas2 gene codes for a component of the microfilament architecture (Brancolini *et al.*, 1992). Upon serum stimulation of quiescent cells, gas2 becomes hyperphosphorylated and this event correlates with the G0 to G1 transition in the cell cycle, as well as with the formation of membrane ruffles (Brancolini *et al.*, 1992; Brancolini & Schneider, 1994). In addition, gas2 expression induces susceptibility to apoptosis and its cleavage by some members of the caspase family of enzymes is thought to regulate morphological changes during programmed cell death (Benetti *et al.*, 2001; Brancolini *et al.*, 1995; Lee *et al.*, 1999; Sgorbissa *et al.*, 1999). gas2 was also suggested to be involved in regulating

cartilage and skeletal muscle differentiation (Cowled *et al.*, 1994; Lee *et al.*, 1999). In opposition to what was observed in murine fibroblasts, gas2 expression is upregulated during exponential cell growth in certain cell types, suggesting a cell-specific regulation of its expression, as previously mentioned for gas1 (Manzow *et al.*, 1996).

1.1.3.3 The growth-arrest-specific gene 3 (gas3)

The product of the gas3 gene is a murine transmembrane glycoprotein preferentially expressed in Schwann cells of myelinated fibers in the peripheral nervous system (Kuhn *et al.*, 1993; Manfioletti *et al.*, 1990; Snipes *et al.*, 1992). Its human and rat homologues are known as peripheral myelin protein-22 (PMP-22) (Snipes *et al.*, 1992). Defects in the gas3/PMP-22 gene are responsible for a set of inherited peripheral neuropathies characterized by demyelination of the peripheral nerves (Fabbretti *et al.*, 1995; Hanemann *et al.*, 1994). The role of gas3/PMP-22 in such diseases was linked to Schwann cell differentiation (Magyar *et al.*, 1996; Spreyer *et al.*, 1991). Overexpression of gas3/PMP-22 reduces proliferation, inhibits DNA synthesis, and induces apoptosis in various cells (Fabbretti *et al.*, 1995; Karlsson *et al.*, 1999; Zoidl *et al.*, 1995; Zoidl *et al.*, 1997). In addition, gas3/PMP-22 alters cell shape and cell spreading, and proper exposure at the cell surface would be essential for its biological functions (Brancolini *et al.*, 2000; Brancolini *et al.*, 1999). Finally, gas3/PMP-22 expression could alter intracellular protein trafficking, by triggering trapping of membrane proteins into vacuoles (Chies *et al.*, 2003).

1.1.3.4 The growth-arrest-specific gene 5 (gas5)

The gas5 gene codes for different spliced mRNAs, with very short open reading frames (ORFs) and no homology to known sequences, or without any ORF (Raho *et al.*, 2000; Vacha *et al.*, 1997). Hence, it was recognized that gas5 does not code for a protein (Raho *et al.*, 2000; Smith & Steitz, 1998; Vacha *et al.*, 1997). However, gas5 spliced mRNAs are thought to act as small nucleolar RNAs involved in ribosomal RNA processing (Raho *et al.*, 2000; Smith & Steitz, 1998). Upregulation of the gas5 transcripts was seen in a variety of growth-arrested cells, as well as in cultured mouse cortical neurons subjected to hypoxia (Coccia *et al.*, 1992; Jin *et al.*, 2002; Rozental *et al.*, 2000; Smith & Steitz, 1998). Functions suggested for the gas5 gene include roles in the inhibition of cell proliferation and regulation of cytoskeleton genes (Feldker *et al.*, 2003; Rozental *et al.*, 2000). In addition, differential analysis of gene expression in two murine strains known to differ in their genetic suceptibility to hyperthermia-induced neural tube defets showed a specific expression of gas5 mRNA in the highly sensitive strain, suggesting a role for gas5 in the susceptibility to hyperthermia (Vacha *et al.*, 1997).

1.1.3.5 The growth-arrest-specific gene 6 (gas6)

The product of the gas6 gene is a vitamin K-dependent ligand for the tyrosine kinases Sky, Axl and Mer, and shows significant homology to the human protein S, a negative regulator of blood coagulation (Manfioletti *et al.*, 1993; Nagata *et al.*, 1996; Ohashi *et al.*, 1995; Stitt *et al.*, 1995). Even though gas6 was first shown to be preferentially expressed in quiescent cells, its overexpression induces cell proliferation in a wide variety of cell types (Goruppi *et al.*, 1996; Li *et al.*, 1996; Schneider *et al.*, 1988; Yanagita *et al.*, 2002).

gas6 has been suggested to be involved in many other cellular phenomena such as cell-cell interactions, anti-inflammatory processes, phagocytosis of apoptotic cells, development/progression of mammary tumors, platelet aggregation and secretion, bone loss, and spermatogenesis (Allen *et al.*, 1999; Angelillo-Scherrer *et al.*, 2001; Avanzi *et al.*, 1998; Bellosta *et al.*, 1997; Goruppi *et al.*, 1996; Ishimoto *et al.*, 2000; Jiang *et al.*, 2001; Katagiri *et al.*, 2001; Lu *et al.*, 1999; McCloskey *et al.*, 1997; Nakano et al., 1996).

1.1.3.6 The growth-arrest-specific gene 7 (gas7)

Since the product of the gas7 gene is the main focus of this thesis, its characteristics are described in detail in a subsequent section (Section 1.2). The main function attributed to the gas7 protein is to be essential for neurite formation in neuronal cells (Chao *et al.*, 2003; Ju *et al.*, 1998; Lazakovitch *et al.*, 1999).

1.1.3.7 The growth-arrest-specific genes 8 and 11 (gas8/gas11)

The gas8 gene was identified in growth-arrested murine NIH3T3 fibroblasts, and gas11 was later shown to be the human counterpart of gas8 (Brenner *et al.*, 1989; Whitmore *et al.*, 1998; Yeh *et al.*, 2002). gas8 is predominantly expressed in the testes of adult mice, and the gas11 protein was detected in human sperm flagella (Yeh *et al.*, 2002). gas8/gas11 was suggested to be involved in force-generating tasks, and specifically in sperm motility (Whitmore *et al.*, 1998; Yeh *et al.*, 2002).

1.1.3.8 The growth-arrest-specific gene 9 (gas9)

gas9 codes for a previously known and characterized protein, the platelet-derived growth factor (PDGF) alpha receptor (PDGFαR) (Lih *et al.*, 1996). PDGFαR can bind all three isoforms of PDGF, and signaling through this receptor induces migration and proliferation of different cell types (Claesson-Welsh, 1996; Laurent *et al.*, 2003). Treatment of growth-arrested cells with PDGF stimulates their re-entry into the cell cycle. Preferential expression of PDGFαR in quiescent cells would thus enable them to re-enter the cell cycle upon appropriate stimulation (Lih *et al.*, 1996).

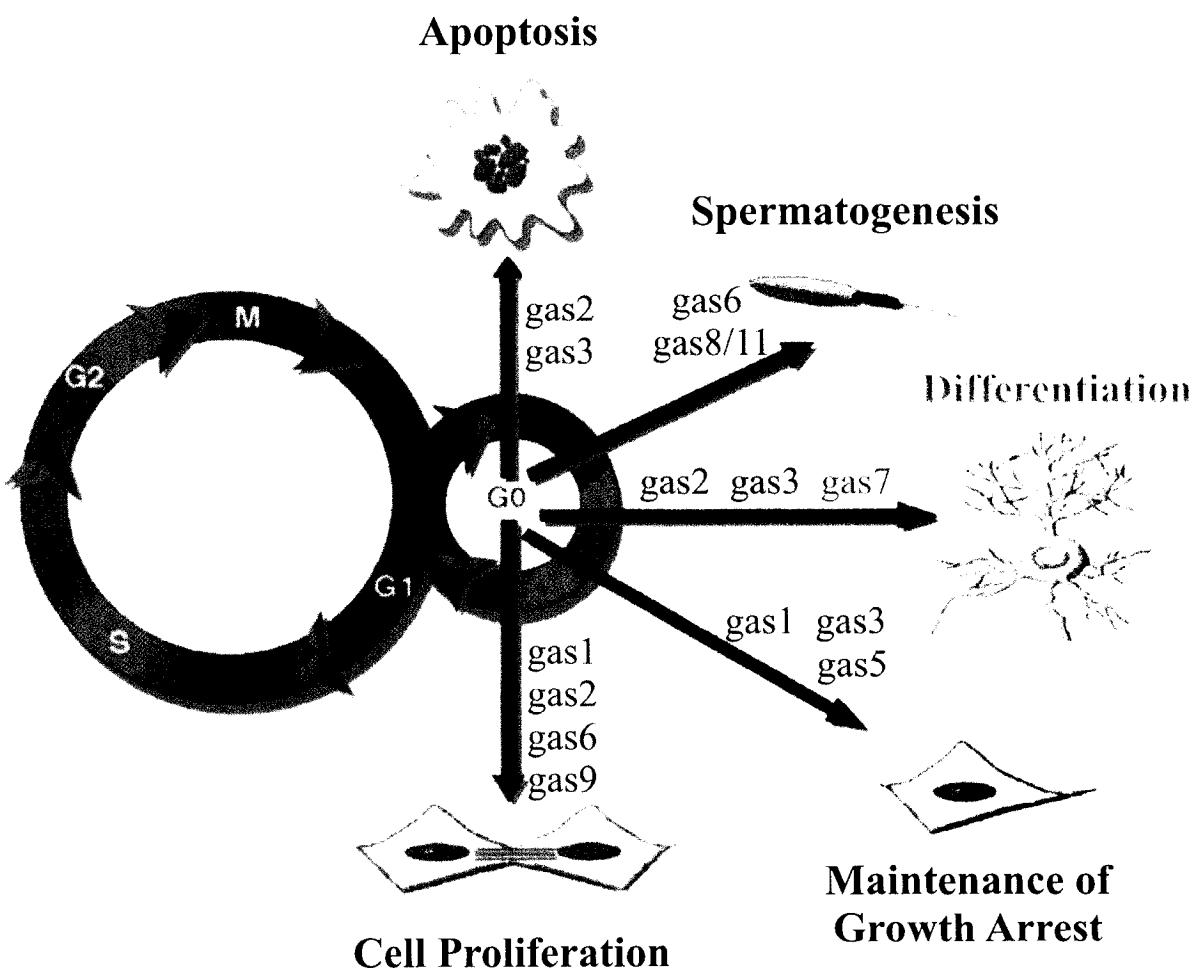
1.2 The growth-arrest-specific gas7 protein

1.2.1 Identification of gas7

The gas7 gene was first cloned in 1989, following a retrovirus-based gene search, which also led to the identification of gas8 (Brenner *et al.*, 1989). Basically, a promoter-less reporter gene, LacZ, was introduced into a Moloney murine leukemia virus (Mo-MuLV) construct, which has the ability to fuse with the host genome, at diverse locations but at a single site in each cell (Brenner *et al.*, 1989). Expression of the reporter gene, also called portable exon, in an infected cell shows that the construct has been inserted into an expressed chromosomal gene. This strategy allows the study of gene expression and regulation under different conditions. For the present case, murine NIH3T3 fibroblasts were infected with the LacZ Mo-MuLV construct and two clones showed increased expression of the reporter gene when cells were subjected to serum deprivation, or grown to confluence (Brenner *et al.*, 1989). This indicated that the genes into which the portable

Figure 1. The growth-arrest-specific genes are involved in diverse functions

As suggested by their family name, many gas genes are involved in regulating the cell cycle. gas2, gas6, and gas9 support cell proliferation, whereas gas3 and gas5 promote growth arrest. Some functions of the gas genes can also be tissue-specific. For example, gas1 was shown to have either a positive or negative effect on cell cycle progression, depending on the cell type in which it is expressed. gas2 and gas3 were suggested to be pro-apoptotic molecules and to be involved in regulation of cell differentiation (cartilage and skeletal muscle cells for gas2, and Schwann cells for gas3). gas8 and gas11, which are respectively murine and human homologues, together with gas6, are thought to be involved in cell motility and spermatogenesis. The main role suggested for gas7 is in neuronal differentiation.


Apoptosis
Spermatogenesis
gas2
gas3
gas6
gas8/11
Differentiation
M
G2
G0
gas2 gas3 gas7
G1
S
gas1 gas3
gas5
gas1
gas2
gas6
gas9
Maintenance of
Growth Arrest
Cell Proliferation

— Figure 1 —

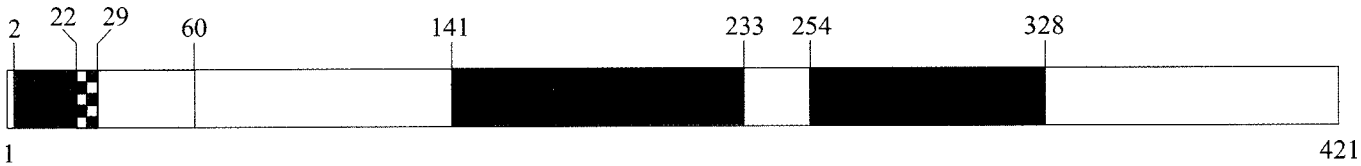
exon had been inserted were upregulated during growth arrest. Therefore, the two loci identified were designated as new members of the growth-arrest-specific family. The genomic DNA portions flanking the retrovirus inserts were sequenced and named gas7 and gas8 (Ju *et al.*, 1998; Yeh *et al.*, 2002). In humans, the gas7 gene maps to the short arm of chromosome 17 (Ju *et al.*, 1998). Its genomic sequence contains 14 exons and codes for a 412-amino acid protein (accession number O60861; Swiss-Prot), which shares 97% homology with the gas7 proteins from the mouse and rat species.

1.2.2 Homologies with other proteins or conserved domains

As previously mentioned, no structural similarity is observed among the different gas genes. However, some regions of the gas7 protein present homologies with known sequences or domains (Figure 2). The amino-terminal region of gas7 contains a WW (Trp-Trp) motif, known to modulate protein-protein interactions through proline-rich regions (Ilsley *et al.*, 2002; Ju *et al.*, 1998; Lazakovitch *et al.*, 1999). The N-terminal portion of gas7 also shows similarities to a region of the synapsins Ia and Ib, proteins involved in the attachment of synaptic vesicles to the cytoskeleton (Ju *et al.*, 1998). In its C-terminal region, gas7 contains a potential coiled-coil domain, which is similar to the region of the cytoskeleton-associated protein PSTPIP responsible for its localization in microfilament-rich structures (Chao *et al.*, 2003; Lazakovitch *et al.*, 1999). Moreover, gas7 has a Fes/CIP4 homology (FCH) domain, often part of proteins involved in regulation of cytoskeleton rearrangements (Chao *et al.*, 2003; Fujita *et al.*, 2002). These features support a potential involvement for gas7 in cytoskeleton-dependent mechanisms. Finally, gas7 has also been observed to be part of a fusion protein with the mixed lineage

Figure 2. Homologies of the mouse gas7 protein with other proteins or domains

Although analysis of gas7's sequence does not allow the attribution of specific functions, some regions of the protein present homologies with known sequences or domains. Numbers refer to the amino acid sequence, from the amino terminal to the carboxy terminal end.



Homology with synapsins 1a and 1b

WW motif

FCH domain

Potential coiled-coil domain

— Figure 2 —

leukemia (MLL) protein, following translocation during treatment-related acute myeloid leukemia (Megonigal *et al.*, 2000). The MLL-gas7 fusion protein impairs differentiation and enhances growth of murine hematopoietic cells (So *et al.*, 2003).

1.2.3 gas7 expression is enriched in the brain

The expression of gas7 in primary tissues was mainly characterized in mice (Ju *et al.*, 1998). Northern analysis, using the murine gas7 full-length cDNA as a probe, showed a preferential expression of gas7-related RNA in the brain (Ju *et al.*, 1998). Furthermore, *in situ* hybridization and immunohistochemistry of brain sections showed that gas7 is expressed in the cerebellum, cerebral cortex, and hippocampus (Ju *et al.*, 1998). Some gas7 RNA sequences were also detected, to a much lower extent, in the heart and testes, but not in spleen, lung, liver, muscle, or kidney tissues (Ju *et al.*, 1998). In mouse cerebella, gas7 was shown to be specifically expressed in neurons, but not in astrocytes, as gas7 expression was observed to colocalize with the neuron-specific microtubule associated protein II (MAPII), but not with the glia marker glial fibrillary acidic protein (GFAP) (Ju *et al.*, 1998). The expression of gas7 was selectively detected in the cytoplasm of both cell bodies and neurites of MAPII-expressing cells (Ju *et al.*, 1998).

1.2.3.1 Differential expression of two gas7 isoforms within the mouse brain

gas7 is expressed in several isoforms (Chao *et al.*, 2003; Ju *et al.*, 1998; Lazakovitch *et al.*, 1999). In the mouse brain, the gas7 gene was shown to produce two different mRNAs, specifying two protein isoforms: gas7 (also called full-length gas7 or gas7-fb) and gas7-cb (Ju *et al.*, 1998; Lazakovitch *et al.*, 1999). The former codes for a 48-kDa

protein mainly found in the forebrain, whereas the latter codes for a 38-kDa protein preferentially expressed in the cerebellum (Lazakovitch *et al.*, 1999). Both proteins share the same C-terminal domain of 321 amino acids (Lazakovitch *et al.*, 1999). The tissue-specific expression of the two isoforms suggested that different promoters might regulate the expression of the two transcripts, but the possibility of alternative splicing could not be ruled out (Lazakovitch *et al.*, 1999). The exon/intron organization of the human gas7 genomic sequence is similar to that of the mouse, and human homologues have been identified for both the gas7 and gas7-cb transcripts (Lazakovitch *et al.*, 1999).

1.2.4 The expression of gas7 is associated with neuronal development

The developmental pattern of expression of gas7 has not been characterized. However, gas7 expression was shown to be induced during neuronal differentiation of cultured pre-neuronal rat pheochromocytoma cells (PC12 cells) (Chao *et al.*, 2003). Treatment of PC12 cells with nerve growth factor (NGF) led to a rapid and transient increase in the endogenous production of a 60-kDa gas7 isoform (Chao *et al.*, 2003). The induction of this gas7 protein, which reached its maximum levels within 3 hours, occured prior to any morphological signs of differentiation, suggesting a role for gas7 in the early stages of NGF-induced PC12 neuronal differentiation (Chao *et al.*, 2003).

1.2.4.1 gas7 is essential for and promotes neurite outgrowth

Downregulation and overexpression studies have shown that gas7 is involved in neurite outgrowth in different neuronal cell systems (Chao *et al.*, 2003; Ju *et al.*, 1998). Antisense-mediated knockdown of gas7 was shown to prevent the formation of neurites

in maturing Purkinje cells and in PC12 cells treated with NGF. gas7 was thus suggested to be essential for neurite outgrowth in developing neurons (Chao *et al.*, 2003; Ju *et al.*, 1998). Accordingly, overproduction of the gas7 protein resulted in an expanded cellular morphology and in the extension of neurite-like processes in NIH3T3 murine fibroblasts and in proliferating Neuro 2A and PC12 cells, two pre-neuronal cell lines (Chao *et al.*, 2003; Ju *et al.*, 1998). Moreover, in gas7-overexpressing PC12 cells, these membrane projections were enhanced in both number and length upon addition of NGF (Chao *et al.*, 2003).

However, the gas7-mediated morphological changes in Neuro 2A cells were not accompanied by biochemical evidence of neuronal differentiation, such as MAPII expression (Ju *et al.*, 1998). It has been suggested that gas7 expression may lead to morphological, but not biochemical, neuronal differentiation (Ju *et al.*, 1998).

1.2.5 gas7 regulates cell morphology partly through binding of F-actin

gas7-induced changes in cellular morphology were hypothesized to depend on actin microfilament reorganization (She *et al.*, 2002). Destruction of microfilaments by the actin depolymerizing agent cytochalasin D was shown to prevent the induction of morphological changes following gas7 overexpression in NIH3T3 fibroblasts (She *et al.*, 2002). This suggested that actin polymerization is required for the formation of gas7-induced membrane extensions (She *et al.*, 2002). Moreover, cell-free assays showed that gas7 directly binds to F-actin and promotes its polymerization (She *et al.*, 2002). Colocalization of gas7 with microfilaments in membrane ruffles of NIH3T3 fibroblasts

further supports the association of gas7 with the actin cytoskeleton (She *et al.*, 2002). Therefore, gas7 could induce neurite outgrowth through reorganization of the cytoskeleton.

1.2.5.1 Binding of gas7 to actin is mediated through its C-terminal region

The carboxy-terminal domain of gas7 was shown to mediate its interactions with F-actin (She *et al.*, 2002). She *et al.* (2002) showed that the C-terminal region of gas7 was sufficient to induce actin assembly and crosslinking of microfilaments into bundles. In contrast, the N-terminal region of gas7 did not bind to actin (She *et al.*, 2002). The carboxy-terminal requirement for gas7-induced actin reorganization is consistent with the presence of FCH and coiled-coil domains, respectively known to mediate cytoskeleton rearrangements and localization in microfilament-rich structures (Fujita *et al.*, 2002; Ilsley *et al.*, 2002). However, even though the C-terminal domain was sufficient for gas7 localization at the membrane and for gas7-induced actin reorganization, the full-length protein was shown to be necessary to stimulate morphological changes (She *et al.*, 2002). Therefore, the association of gas7 with microfilament is not sufficient for gas7's role in neurite outgrowth.

1.2.6 Summary of gas7's function

In brief, gas7 was shown to dramatically alter cellular morphology, inducing the formation of membrane extensions similar to those observed during neuronal differentiation (Chao *et al.*, 2003; Ju *et al.*, 1998; She *et al.*, 2002). Downregulation experiments in different neuronal cell types showed that gas7 expression is required for

the acquisition of a neuronal phenotype, during neuronal development (Chao *et al.*, 2003; Ju *et al.*, 1998). Finally, the demonstrated interactions between gas7 and F-actin suggest a potential involvement for gas7 in cytokeleton remodelling and associated neurite plasticity (She *et al.*, 2002).

1.3 Neuronal differentiation

The generation of differentiated neurons is a complex process involving interactions between intrinsic cellular molecules and extrinsic cues, such as growth factors. Understanding the mechanisms by which precursor cells become neurons is of major interest in the treatment of neuronal degeneration. The complete intracellular pathways controlling neuronal differentiation have not been elucidated, but many components have been identified. Some of the principal events occuring during this cellular process, and in which gas7 could potentially be involved, are discussed below.

1.3.1 Neuronal cell fate and the cell cycle

During the vertebrate neuronal development program, various differentiative signals dictate the formation of structures containing neuronal layers made of different types of neurons (Cremisi *et al.*, 2003; Ohnuma & Harris, 2003). Cellular proliferation and differentiation are both tightly regulated at different times during development, and this requires appropriate control of the cell cycle (Cremisi *et al.*, 2003; Ohnuma & Harris, 2003). Work from McConnell and Kaznowski (1991) indicated that the cell cycle status has some influence on cellular differentiation. The number of cycles that a proliferating

cell has been through, as well as the length of the final cycle, are also important factors (Caviness, Jr. *et al.*, 1999; Ohnuma *et al.*, 2001). Some fates, such as whether to be a neuronal or a glial precursor, may be determined when a cell is actively proliferating, whereas other decisions, such as which type of neuron to form, may be made around the time of the final cycle (Ohnuma *et al.*, 2001). In order for neurons to differentiate, precursor cells need to leave the cell cycle in G1 and enter G0 (Ohnuma & Harris, 2003). For example, proteins of the retinoblastoma gene family were shown to be essential for neuronal differentiation, by promoting cell cycle exit (Slack *et al.*, 1998). The time at which a neuronal precursor exits the cell cycle is the neuron's "birth date". Hence, control of cell cycle exit is highly involved in regulating neuronal differentiation. Cell cycle progression is controlled by many positive and negative cues, and different gas genes have been identified as modulators of the cell cycle (Brancolini *et al.*, 1992; Brancolini & Schneider, 1994; Claesson-Welsh, 1996; Goruppi *et al.*, 1996; Laurent *et al.*, 2003; Li *et al.*, 1996; Liu *et al.*, 2001; Liu *et al.*, 2002; Vacha *et al.*, 1997; Zoidl *et al.*, 1995). The possibility that gas7 plays such a role in the nervous system has not been investigated.

1.3.1.1 Coordinated regulation of cell cycle exit and neuronal differentiation

Bidirectional mechanisms are involved in coordinating cell cycle exit with the process of neuronal differentiation (Caviness, Jr. *et al.*, 1999; Ohnuma *et al.*, 2001). Many regulators of the cell cycle are known to be involved in neuronal cell fate, and several differentiation factors were shown to modulate cell cycle progression (Caviness, Jr. *et al.*, 1999; Cremisi *et al.*, 2003; Ohnuma *et al.*, 2001; Ohnuma & Harris, 2003). For example,

downregulation of the cell cycle progression effectors, CDK2 and CDC2, was sufficient to induce neuronal differentiation of PC12 cells (Dobashi *et al.*, 2000). Accordingly, overexpression of some cell cycle inhibitors, such as p73, was also shown to induce differentiation (De Laurenzi *et al.*, 2000). These results suggest that cell cycle arrest can lead to differentiation of neuronal progenitors. However, overexpression of the PC3 protein, a negative regulator of the cell cycle, was not effective in triggering neuronal differentiation of PC12 cells (Corrente *et al.*, 2002). Nevertheless, PC3 potentiated NGF-induced differentiation of these cells, supporting the correlation between cell cycle exit and neuronal differentiation (Corrente *et al.*, 2002). In vivo, PC3 is transiently expressed in the developing neural tube, when the neuroblast undergoes the last proliferative cycle that precedes its differentiation into a mature neuron (Iacopetti *et al*, 1994). PC3's role as an inducer of growth arrest would be required for neuronal differentiation and PC3 was suggested to be a marker for neuronal birth (Tirone, 2001). It might also be involved in differentiaiton of non-neuronal cells, such as muscle and germ cells (Tirone, 2001). The nature of gas7, as a growth-arrest-specific gene, suggests a possible function in regulating cell cycle progression. This could be part of the mechanism by which gas7 acts on neuronal differentiation and/or neurite outgrowth.

1.3.2 Neurons develop a particular morphology

The mature nervous system coordinates the different functions of an organism through a complex arrangement of neurons, which transport information to the various organ systems (Irwin & Madsen, 1997; Nikolic, 2002). For the nervous system to function correctly, each neuron must make appropriate connections with hundreds to thousands of

other cells, and the growth of about one million miles of processes, or neurites, is required for normal wiring of the human brain (Da Silva & Dotti, 2002; Irwin & Madsen, 1997). This underlines the importance for neurons to develop a proper morphology. Round neuronal precursors lose their symmetry upon stimulation of morphological differentiation. The sprouting of neurites, which will later become axons and dendrites, is an important event in early neuronal differentiation (Da Silva & Dotti, 2002). Elongation of neuronal processes, at the right time and in the right direction, forms the basis for neuronal connectivity and, consequently, brain function (Da Silva & Dotti, 2002). Since the gas7 protein was shown to promote neurite outgrowth, better understanding of its role may lead to an improved comprehension of neurite formation.

1.3.2.1 Initiation of neurite outgrowth

More than 100 years ago, Ramon y Cajal (1890) revealed that growing neurites have at their endings actively motile structures, involved in protrusion of membrane extensions. He called them growth cones (Ramon y Cajal, 1890). Their periphery is enriched in actin microfilaments, whereas their central cytoplasmic domain is rich in microtubules and connects with the cell body. These specialized structures are guided by extracellular cues to elongate neurites, change their direction, branch, or retract. They probe their environment for guidance cues, which can be surface-bound molecules or diffusible factors (Landmesser, 1994; Suter & Forscher, 2000). Signals, which are often regulated through members of the small Rho family of GTPases, are then sent to the actin cytoskeleton and result in membrane extension or retraction at the leading edge of neurites (Landmesser, 1994; Van Veen & Van Pelt, 1994).

1.3.3 Actin dynamics during neurite outgrowth

Neurites' shape and motility are regulated by proteins of the cytoskeleton (Wills *et al.*, 1999). Specifically, microtubules are essential for structural integrity and organelle transport to growth cones, and actin filaments are the basis for neurite motility and extension (Mikoshiba *et al.*, 1999; Suter & Forscher, 2000; Van Veen & Van Pelt, 1994). Actin assembly in growth cones causes the formation of lamellipodia, which are thin protrusive sheets filled with actin meshworks, and filopodia, which are finger-like projections made of actin bundles (Da Silva & Dotti, 2002; Meyer & Feldman, 2002). These highly motile structures are found at the leading edge of neurites, and act as sensors for extracellular cues. The elogation of filopodia into growing neurites is mediated through reorganization of the actin cytoskeleton (Letourneau, 1996). Neurite formation and extension is thus directly linked to actin dynamics in growth cones, such as polymerization, crosslinking, and bundling (Letourneau, 1996). These events are locally regulated by a vast array of actin binding proteins, and the gas7 protein could be one of them (Jay, 2000; She *et al.*, 2002).

1.3.3.1 Important roles for actin binding proteins

The actin cytoskeleton is central to every step of morphogenic changes in neurons. More than 100 actin binding proteins control filament dynamics, and many of them have been implicated in the regulation of neurite extension (Hu & Reichardt, 1999; Letourneau, 1996; Pollard & Borisy, 2003). In general, proteins that induce actin assembly, either through polymerization, nucleation of new actin branches, or crosslinking of actin filaments, are believed to increase neurite outgrowth (Goldmann *et al.*, 1999; Hu &

Reichardt, 1999; Meyer & Feldman, 2002; Varnum-Finney & Reichardt, 1994). However, some proteins that promote disassembly of actin filaments, such as the actin depolymerising factor (ADF) and cofilin, have also been shown to facilitate neurite extension (Furnish *et al.*, 2001; Kuhn *et al.*, 2000; Meberg & Bamburg, 2000; Meyer & Feldman, 2002). Consequently, it is thought that increased actin turnover, rather than polymerization *per se*, drives neurite outgrowth mediated by actin binding proteins (Cooper & Schafer, 2000; Kuhn *et al.*, 2000; Meyer & Feldman, 2002). As previously mentioned, gas7 was shown to bind to F-actin and to induce its assembly in *in vitro* cell-free assays (She *et al.*, 2002). Microfilament reorganization was suggested to play an important role in gas7-promoted neurite outgrowth (She *et al.*, 2002).

1.3.4 Neuronal differentiation and neuroprotection

Many neurotrophic factors, such as NGF, cyclic adenosine monophosphate (cAMP), and retinoic acid (RA), have been shown to be pro-survival molecules, in addition to their role in neuronal differentiation (Boniece & Wagner, 1995; Kimura & Schubert, 1992). Several other molecules, such as the cell cycle inhibitor PC3, the Ras-like Rit GTPase, and the neural cell adhesion molecule (NCAM), were also shown to promote differentiation and/or neurite outgrowth, as well as neuroprotection (Corrente *et al.*, 2002; El Ghissassi *et al.*, 2002; Pedersen *et al.*, 2004; Spencer *et al.*, 2002). Therefore, there is a correlation, although not absolute, between the processes of neuronal differentiation and neuroprotection.

Few studies have suggested that NGF-induced differentiation and survival would occur through distinct mechanisms, respectively involving the extracellular signal related kinase (ERK) and the phosphatidylinositol-3 kinase (PI3-kinase) pathways (Barnabe-Heider & Miller, 2003; Klesse *et al.*, 1999). However, others have shown that convergent pathways might lead to differentiation and protection (Boniece & Wagner, 1995; Spencer *et al.*, 2002). For example, blocking the ERK pathway inhibited both Rit-induced survival and differentiation of neuronal pheochromocytoma cells (Spencer *et al.*, 2002). In addition, the ERK and PI3-kinase pathways have common upstream regulators, such as several tyrosine kinases, linking the neuronal differentiation and neuroprotection processes (Barnabe-Heider & Miller, 2003). Hence, the role of the gas7 protein in neuronal development could uncover a potential neuroprotective role. The stimulation of neuronal differentiation and rescue of damaged neurons is an attractive strategy for the treatment of neurodegenerative diseases, such as stroke.

1.4 Promoting neuronal plasticity and neuronal differentiation to repair the brain

Before the 1950's, the scientific community believed that the adult human brain was controlled by static connections and had no regenerative capability. Damage to the brain was therefore thought to result in permanent loss of function (Ramon y Cajal, 1913). However, many clinical observations showing progressive recovery of function following brain injury were in disagreement with this hardwired view of the brain. The concept of brain plasticity, which involves dynamic changes in the brain circuitry, emerged in 1949

when Hebb proposed that cortical connections could be remodeled by experience (Hebb, 1949). Growth of neurites was proposed to continue in the life of a neuron and to be important for memory (Hebb, 1949; Jenkins & Merzenich, 1987). It was also shown that the functional role of a damaged brain region could be "taken over by still intact portions" of the cortex, suggesting that cortical representations could reorganize (Cole & Glees, 1954). Complimentary to the discovery of brain plasticity, Joseph Altman and his colleagues showed, in the late 1960's, that the adult rat brain could also produce new neurons (Altman, 1969; Altman & Das, 1965). Many studies have since dealt with neuronal differentiation in adult brains of different species, including monkeys and humans (Eriksson *et al.*, 1998; Kirschenbaum *et al.*, 1994). These newly-generated neurons were recently shown to form functional connections, suggesting their integration into the existing circuitry (Van Praag *et al.*, 2002). About 10 years ago, neuronal precursor cells were isolated from the adult mammalian central nervous system, providing a cellular mechanism for the production of new neurons in the adult mammalian brain (Gage *et al.*, 1995; Reynolds & Weiss, 1992; Richards *et al.*, 1992).

It is now accepted that cortical map remodeling and neuronal differentiation are continuous, ongoing, normal processes (Eriksson *et al.*, 1998; Jenkins & Merzenich, 1987; Van Praag *et al.*, 2002). Understanding and enhancing the adaptative and regenerative abilities of the brain may lead to improved rehabilitative therapies for neurodegenerative conditions, such as stroke. Recent data raise the possibility that amplification of neuronal plasticity and neuronal differentiation might be of therapeutic value (Adkins-Muir & Jones, 2003; Biernaskie & Corbett, 2001; Jin *et al.*, 2004;

Nakatomi *et al.*, 2002; Plautz *et al.*, 2003; Steiner *et al.*, 1997; Stroemer *et al.*, 1995; Zhang *et al.*, 2001). According to its role in neuronal development, gas7 could be a target for such brain repair approaches.

1.4.1 Brain plasticity: a repair strategy

As previously mentioned, brain injury can result in cortical reorganization. Surrounding areas gain new receptive fields to control the tissues formerly represented by the infarcted zone and this re-emergence of the lost representation is believed to partially account for functional recovery following brain damage (Jenkins & Merzenich, 1987; Merzenich *et al.*, 1983; Merzenich *et al.*, 1984; Stroemer *et al.*, 1998; Wall *et al.*, 1986). Adult human brains also use plasticity to cope with aging. Regions of the brain presenting age-related neuronal loss were shown to undergo extensive dendritic proliferation as a compensatory response to death of neighbouring neurons (Coleman & Flood, 1986; Van Ooyen & Van Pelt, 1994). *In vitro* and *in vivo* experiments have suggested that stimulation of neurite outgrowth can accelerate functional recovery (Adkins-Muir & Jones, 2003; Biernaskie & Corbett, 2001; Keyvani & Schallert, 2002; Plautz *et al.*, 2003; Steiner *et al.*, 1997; Stroemer *et al.*, 1995). Chemical induction of neuritogenesis was shown to promote morphological and functional recovery from sciatic nerve lesion in rats, and to induce dopamine fiber reinnervation to the striatum in models of Parkinson's disease in rats and mice (Steiner *et al.*, 1997). Studies conducted with monkeys have also shown that cortical electrical stimulation and enriched environments lead to increased cortical dendritic surface area, associated with greater functional recovery from cortical ischemia (Adkins-Muir & Jones, 2003; Biernaskie & Corbett, 2001; Keyvani & Schallert, 2002; Plautz *et*

al., 2003). In all cases, increased dendritic branching would provide more surface area for axodendritic connections (Stroemer *et al.*, 1995).

1.4.2 Neuronal differentiation and brain recovery

As for many tissues, such as skin and muscle, it was suggested that the brain could have repair mechanisms allowing the replacement of dying cells (Kokaia & Lindvall, 2003). Various brain insults, including ischemia and epileptic seizures, were shown to upregulate the formation of new neurons in adult mammalian brains. Even though neurogenesis following epileptic insults might play a pathophysiological role, differentiation of neuronal precursors following ischemia was strongly suggested to be involved in functional recovery (Liu *et al.*, 1998; Nakatomi *et al.*, 2002; Parent *et al.*, 1997; Takagi *et al.*, 1999). Administration of growth factors, such as fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF), and of nitric oxide was shown to promote the differentiation of new neurons, which was accompanied by functional improvement (Nakatomi *et al.*, 2002; Zhang *et al.*, 2001). The induction of neuronal differentiation is thus an interesting strategy against neurodegeneration.

1.5 The PC12 cell system as a model to study the role of the gas7 protein

Clonal cell lines expressing neuronal properties are useful models to study the nervous system at the molecular level. The PC12 cell line was established in 1976 and is derived from a pheochromocytoma (tumor of the adrenal gland) passaged subcutaneously in New England Deaconess Hospital strain white rats (Greene & Tischler, 1976). These cells can

be differentiated into neurons by treatment with nerve growth factor (NGF). PC12 cells were chosen as the main cell model for this study for multiple reasons. First, cell lines are easy to transfect and to infect, allowing overexpression and downregulation experiments for functional studies. In addition, neuronal differentiation of this cell model has been extensively characterized, and many neuroprotective strategies have been explored in PC12 cells, two cellular processes in which gas7 could be involved. Finally, as previously mentioned, endogenous expression of gas7 has recently been observed in PC12 cells and was shown to be important for their neuronal differentiation (Chao *et al.*, 2003).

1.5.1 Characteristics of undifferentiated PC12 cells

PC12 cells can be grown in standard serum-containing media, in which they undergo mitosis with an apparent doubling time of 48 to 92 hours (Fujita *et al.*, 1989; Greene & Tischler, 1976). They have a round or polygonal shape, tend to grow in small clumps, attach loosely to plastic culture surfaces, and are not viable in serum-free media (Fujita *et al.*, 1989; Greene, 1978; Greene & Tischler, 1976). PC12 cells present characteristics of chromaffin cells, as they contain intracellular dense core granules and have the ability to synthesize, store, and release the catecholamines dopamine and norepinephrine (Greene, 1978; Greene & Tischler, 1976).

1.5.2 NGF-induced neuronal differentiation of PC12 cells

NGF is a polypeptide involved in the development and maintenance of sympathetic and certain sensory neurons (Gunning *et al.*, 1981). Upon treatment with NGF (usually around 50 ng/mL), PC12 cells cease to divide and extend long, fine, and branching

neuronal-like processes within a week (Greene, 1978; Greene & Tischler, 1976; Gunning *et al.*, 1981). These fibers contain small vesicles, which accumulate in process varicosities and endings (Greene & Tischler, 1976). The PC12 neuronal-like phenotype resembles that of cultured primary sympathetic neurons (Greene & Tischler, 1976). Moreover, NGF treatment of PC12 cells induces electrical excitability. In the absence of NGF, cells express a variety of ion channels (sodium, calcium, potassium), but show low excitability. In the presence of NGF, there is an increase in the density of sodium channels and cells acquire neuronal excitability (Fujita *et al.*, 1989; Greene, 1978; Gunning *et al.*, 1981). Other factors, such as cyclic adenosine monophosphate (cAMP) and basic and acidic fibroblast growth factors (bFGF and aFGF), have also been shown to have neuronal differentiative effects on PC12 cells (Greene & Tischler, 1976; Rydel & Greene, 1987). In contrast, EGF was shown to promote persistent proliferation of PC12 cells (Boonstra *et al.*, 1985).

Briefly, PC12 cells can display characteritics of either chromaffin cells or sympathetic neurons, two neuronal cell types of the adrenal medulla, under appropriate conditions. They therefore possess a certain pluripotency and may be analogus to precursor cells derived from the neural crest (Greene, 1978; Greene & Tischler, 1976).

1.5.2.1 Expression of neuronal markers in differentiating PC12 cells

The neuronal differentiation process is accompanied by the expression of neuron-specific proteins, involved in various roles. For example, the ability of neurons to contact each other and to receive or send information is in part controlled by the expression of

cytoskeleton proteins and neurotransmitter receptors and vesicles. Also, neurons maintain specific membrane properties partly through the expression of tightly regulated ion channels. NGF-mediated neuronal differentiation of PC12 cells induces the expression of several neuronal proteins, or neuronal markers. Cytoskeleton elements, like neurofilament proteins, βIII-tubulin (TUJ-1), and the microtubule associated protein II (MAPII), axonal proteins, like the dihydropyrimidinase-related protein 3 (DRP-3), vesicular proteins, like synaptotagmin (STG), synapsins, and the 25 kDa synaptosomal associated protein (SNAP-25), and receptors, like the alpha-7 subunit of the acetylcholine receptor (AChR-α7) are upregulated by NGF (Byk *et al.*, 1998; Dobashi *et al.*, 2000a; Henderson *et al.*, 1994; Hepp *et al.*, 2001; Minturn *et al.*, 1995; Ohuchi *et al.*, 2002; Romano *et al.*, 1987; Takahashi *et al.*, 1999; Tao-Cheng *et al.*, 1995; Xiao *et al.*, 2002). The physiological roles of the four neuronal markers used in this study, TUJ-1, STG, AChR-α7, and DRP-3 are described below.

TUJ-1 is the only neuron-specific beta tubulin isoform and its expression was shown to increase following stimuli for neurite growth. It is therefore thought to be involved in microtubule dynamics during neurite formation (Moskowitz *et al.*, 1993). STG is a membrane protein of synaptic vesicles and is believed to function as a calcium sensor that triggers calcium-dependent exocytosis (Meldolesi & Chieregatti, 2004). Following depolarization, increases in intracellular levels of calcium would induce conformational changes of STG and allow it to interact with pre-synaptic membrane proteins, resulting in the fusion of vesicles with the plasma membrane (Meldolesi & Chieregatti, 2004). AChR-α7 is a subunit found in neuronal nicotinic acetylcholine receptors. These

receptors are ligand-gated cation channels activated by the neurotransmitter acetylcholine, or by nicotine or other nicotinic agonists (Avila *et al.*, 2003). They are expressed throughout the central nervous system and play a key role in synaptic transmission and information transfer (Avila *et al.*, 2003; Henderson *et al.*, 1994). Finally, DRP-3 (also called TUC-4, CRMP-4, ULIP-1, or TOAD-64) is an axonal protein almost exclusively expressed in the brain and its expression is differentially regulated during development (Byk *et al.*, 1998). DRP-3 was suggested to be involved in neurite outgrowth and axonal guidance (Quinn *et al.*, 1999).

1.5.3 PC12 cells as a model to assess neuroprotection

Even though *in vitro* models are not an exact representation of physiology, they are useful to study the effects of potential clinical drugs on cell biology and biochemistry. Cell cultures can be easily used for screening, characterization, and validation of new protective compounds. PC12 cells have been a popular model in neuroscience research on diseases such as Parkinson's, Alzheimer's, and stroke (Tabakman *et al.*, 2002). Moreover, many signaling pathways have been well characterized in this neuronal cell line. It is therefore possible to dissect mechanisms of cell death involved in different pathological conditions, while the heterogeneity of primary neuronal cultures often complicates the analysis of neuronal death or survival pathways. To reproduce neuropathophysiological situations, different toxicity assays have been developed with both undifferentiated and NGF-differentiated PC12 cells. This study focused on three different insults: oxygen-glucose deprivation (OGD), calcium overload induced by the

calcium ionophore A23187, and oxidative stress mediated by the nitric oxide donor sodium nitroprusside (SNP).

1.5.3.1 Physiological relevance of cytotoxicity induced by OGD, A23187, and SNP in PC12 cells

OGD has been extensively used to mimic ischemic insults. This *in vitro* model of ischemia combines hypoxia/anoxia with glucose deprivation and eventually results in cell death. PC12 cells have been widely used to test the neuroprotective capacities of various compounds (growth factors, antioxidants, and others) against such an insult (Abu-Raya *et al.*, 1999; Abu-Raya *et al.*, 2002; Boniece & Wagner, 1993; Boniece & Wagner, 1995; Tabakman *et al.*, 2002; Tabakman *et al.*, 2004). Calcium is an important signaling agent in mammalian cells. However, sustained high intracellular calcium concentrations initiate cascades of signals leading to activation of phospholipases, endonucleases, and calcium-dependent proteases, which ultimately threaten cell viability (Ray *et al.*, 2000). Calcium overload is known to be part of the pathology of neurodegenerative diseases such as Alzheimer's, Parkinson's, and stroke (Tabakman *et al.*, 2002). The calcium ionophore A23187 is a transporter for divalent cations that gets inserted in the cell membrane and induces calcium influx in the cells (Ray *et al.*, 2000). Finally, exogenous application of SNP is a source of nitric oxide (NO). As for Ca^{2+}, NO is involved in many signaling pathways as a second messenger. Elevated levels of NO combine with cellular superoxide anions (O_2^-) to form a transient metabolite, peroxynitriate ($OONO^-$) (Sarker *et al.*, 2003). As well, NO can induce iron release from ferritin, disrupting iron homeostasis and leading to increased levels of reactive oxygen species (Desole *et al.*, 1998). NO-

induced oxidative stress is known to participate in cell death involved in ischemia and Parkinson's (Sarker *et al.*, 2003).

Studies in PC12 cells using these three toxicity paradigms are not consistent in defining the type of cell death induced. In either undifferentiated or differentiated PC12 cells, OGD was mostly shown to induce apoptosis (Tabakman *et al.*, 2004). However, while some experiments involving calcium or nitric oxide toxicity have shown the presence of apoptotic features, such as caspase-3 activity, DNA fragmentation, and chromatin condensation, others have suggested that the apoptotic pathway is not involved (Desole *et al.*, 1998; Michel *et al.*, 1994; Ray *et al.*, 2000; Sarker *et al.*, 2003; Umegaki *et al.*, 1996; Wada *et al.*, 1996). Therefore, it is not clear if A23187 and SNP induce apoptotic or necrotic cell death in PC12 cells.

1.6 Goals of this thesis

1.6.1 Statement of the problem

The regenerative capacities of the brain are far from being understood. New strategies for brain repair include the exploitation of the brain's intrinsic plasticity and neurogenesis abilities. Identification and characterization of factors and conditions regulating the mechanisms involved in neuritogenesis, neuronal differentiation, and survival are of great importance. Such information could lead to the application of new approaches to repair injured or diseased neuronal tissues. Better understanding of the functions of the gas7 protein might thus come to be of therapeutic value in the treatment of neurodegenerative

conditions. The gas7 gene itself may be a direct therapeutic target or alternatively studying the actions of this gene may lead to a better comprehension of neurodegeneration.

1.6.2 Hypotheses and Objectives

Hypothesis 1: Overexpression of gas7 will enhance neuronal differentiation

The expression of gas7 was shown to be essential for neuronal differentiation of cultured PC12 cells (Chao *et al.*, 2003). In addition, overexpression of gas7 in different neuronal cell lines induces morphological changes similar to those observed during neuronal differentiation (Chao *et al.*, 2003; Ju *et al.*, 1998). Many molecules involved in the process of neuronal differentiation are known to have the ability to potentiate this cellular phenomenon, when overexpressed (Corrente *et al.*, 2002; Liu *et al.*, 2003; Zhou *et al.*, 2003). Therefore, it was proposed that overexpression of gas7 would potentiate neuronal differentiation.

In order to assess this hypothesis, the first objective of this thesis was to determine the effects of gas7 overexpression on the neuronal differentiation of PC12 cells. Overexpression of the gas7 protein aimed at reproducing the published data, suggesting that gas7 induces neurite outgrowth (Chao *et al.*, 2003; Ju *et al.*, 1998). The effects of gas7 on neuronal differentiation, at both the morphological and biochemical levels, were also evaluated in the absence or presence of NGF.

Hypothesis 2: gas7 is differentially regulated during neuronal differentiation

Proteins' functions are often accompanied by changes in their expression. Depending on its function, a protein can be downregulated to reduce inhibitory signals, or upregulated to increase stimulatory signals. During neuronal differentiation, molecules involved in different aspects of the phenomenon are differentially expressed at different stages of differentiation.

The second objective of this thesis was therefore to determine the pattern of endogenous gas7 expression during neuronal differentiation. In addition to PC12 cell extracts, cDNA and protein samples from human NT2 cells, at different time points during the course of neuronal differentiation, were also analyzed for the expression of gas7. This cell system is a relevant human model of neuronal differentiation

Hypothesis 3: gas7 can act as a neuroprotective molecule

As previously discussed, various effectors of neuronal differentiation have been shown to be pro-survival molecules (Boniece & Wagner, 1995; Corrente *et al.*, 2002; El Ghissassi *et al.*, 2002; Kimura & Schubert, 1992; Pedersen *et al.*, 2004; Spencer *et al.*, 2002). It was thus hypothesized that gas7 could protect cells from lethal insults, possibly through mechanisms common with the neuronal differentiation process.

To verify this last hypothesis, the third objective of this thesis was to assess the potential protective role of gas7 in the PC12 cell model. The first step of this objective was to design toxicity paradigms. In order to test neuroprotective strategies, the various toxicity

assays had to generate a mild insult (about 50% cell death), which can be attenuated by the presence of pro-survival molecules. Then, the gas7 protein was overexpressed prior to the insults, to evaluate its ability to protect the cells.

CHAPTER 2

METHODS

2.1 Cell culture

2.1.1 PC12 cell culture

Rat pheochromocytoma cells (PC12; ATCC, CRL-1721) were provided by Dr. Balu Chakravarthy [National Research Council, Institute for Biological Sciences (NRC, IBS), Ottawa, ON, Canada] and used at passages 5-30. Cells were plated in plastic dishes or multiwell plates (VWR International, Chicago, IL, USA) coated with poly-L-lysine (Sigma, St. Louis, MO, USA). Surfaces were treated with 25 μg/mL of poly-L-lysine in phosphate buffered saline (PBS: 140 mM NaCl, 4 mM KCl, 0.5 mM Na_2HPO_4, 0.15 mM KH_2PO_4, pH 7.2; all reagents from Sigma) for 3 hours and washed twice in water. Growth media consisted of high glucose Dulbecco's modified Eagle's medium (HG-DMEM; Invitrogen, Bethesda, MD, USA) supplemented with 10% heat-inactivated horse serum (HS; HyClone, Logan, UT, USA) and 5% heat-inactivated fetal bovine serum (FBS; Wisent Inc., St. Bruno, QC, Canada), and cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cells at low passage were frozen in growth media supplemented with 5% dimethylsulfoxide (DMSO; Sigma) at -80°C overnight, and transferred to liquid nitrogen for long-term storage. When starting a culture, cells were rapidly thawed in a water bath a room temperature and resuspended in growth media. Media was replenished 2-3 times a week and subcultures were performed using trypsin-EDTA (Invitrogen). Cells were washed twice in PBS, to remove all serum proteins which could inhibit the trypsin, and incubated with 0.05% trypsin at 37°C for 5 minutes. Growth media was added to the resulting cell suspension (1:1), to inhibit the trypsin, and repetitive pipetting was used to further dissociate the cells. Cells were then collected by centrifugation (200g, 1 minute), resuspended in growth media, and counted with the use

of a Levy Hemacytometer (VWR International). Specific densities of plating depended on the experiments performed and most treatments were done 16 to 24 hours after plating (see the different experimental sections).

2.1.2 PC12 neuronal differentiation

For neuronal differentiation, PC12 cells were plated at a density of 5 x 10^4 cells/cm^2 in poly-L-lysine-coated plates or dishes. Differentiation media was made up of HG-DMEM supplemented with 2% HS, 1% FBS, and 50 ng/mL of nerve growth factor (NGF, 2.5S; Alomone Labs, Israel) and was added to cultures 16 hours after plating. This media was replenished every 48 hours and observations and treatments were performed at different time points, until up to 5 days.

2.1.3 NT2 culture conditions

cDNA preparations and protein samples from human embryonic teratocarcinoma Ntera2/D1 cells (NT2; Stratagene, La Jolla, CA, USA), extracted at different time points during neuronal differentiation triggered by all-*trans* retinoic acid (RA; Sigma), were provided by Dr. Dao Ly and Kirsty Malone (NRC, IBS), respectively. Briefly, the NT2 precursor cells were plated in 75-cm^2 flasks (VWR International) at 2 x 10^4 cells/cm^2 and the differentiation process involved a 4-week treatment in HG-DMEM supplemented with 10% FBS and 10 μM RA. This differentiation media was replenished 3 times a week and at the end of the fourth week, both neuronal and astrocytic cells and cell precursors were present in the cultures (Sandhu *et al.*, 2002; Sandhu *et al.*, 2003). At this stage, cells were harvested by trypsinization, as described for PC12 subcultures, and

plated at a 1:5 dilution in HG-DMEM and 10% FBS without RA. After 2 days, neuronal cells and cell precursors were separated from the underlying astroglia by gentle trypsinization (0.01% trypsin) and seeded into dishes coated with matrigel (Collaborative Research/Becton Dickinson, Bedford, MA, USA). Cells were placed in HG-DMEM and 10% FBS supplemented with the DNA synthesis inhibitors 1-β-D-arabinofuranosylcytosine (1μM), 5-fluoro-2'-deoxyuridine (10 μM), and uridine (10 μM) (all from Sigma).

2.2 gas7 overexpression in PC12 cells

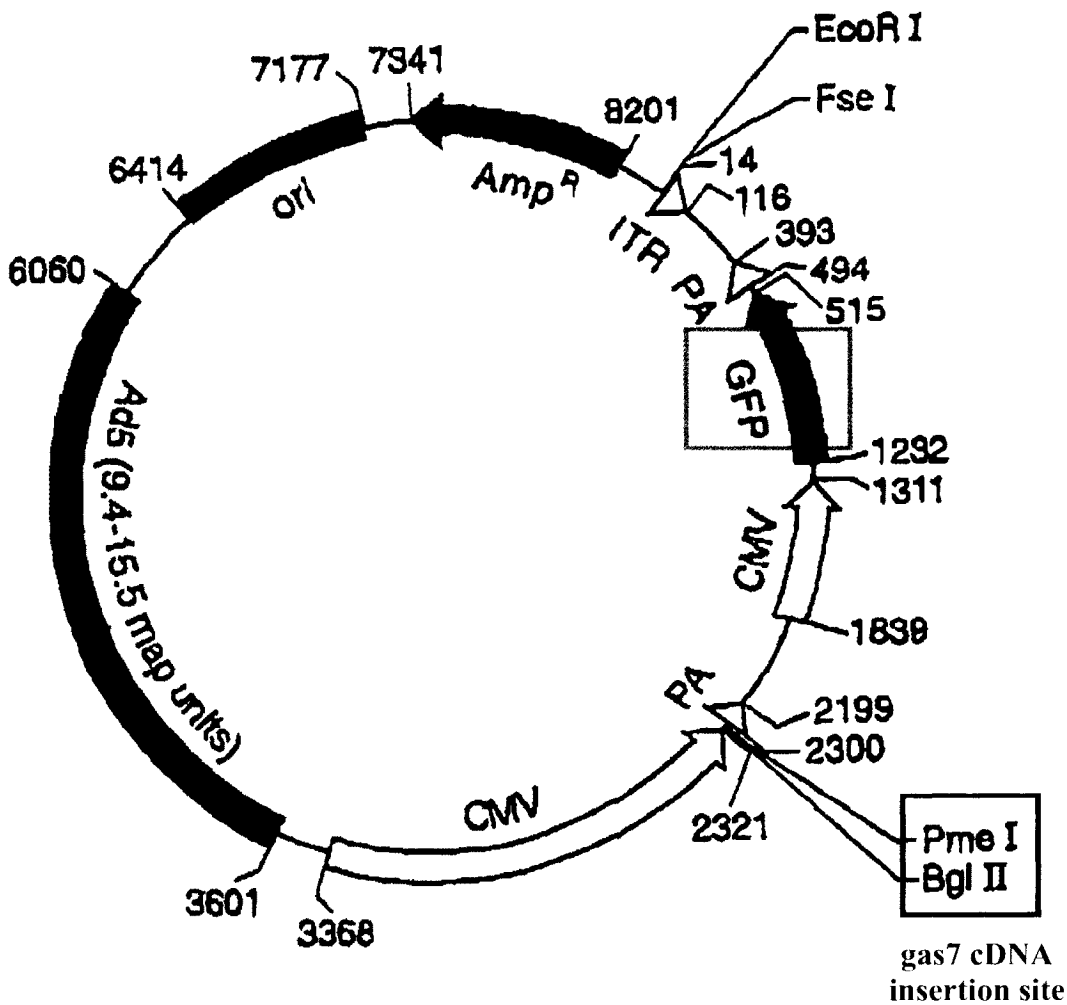
To study the effects of the gas7 protein on neuronal differentiation and neuroprotection, the gas7 protein was overexpressed in PC12 cells using adenovirus infections.

2.2.1 The gas7 adenovirus

A replication-defective adenovirus expressing the green fluorescent protein (GFP) and containing the coding sequence for the murine gas7 full-length protein (mRNA sequence accession number U19860, NCBI) was the kind gift from Dr. Sheng T. Hou and Dr. Deqi Huang (NRC, IBS). Briefly, gas7 cDNA was ligated into a transfer vector (PQBI-AdCMV5-GFP; Qbiogene, Inc., Montreal, QC, Canada) (Figure 3), and the resulting contruct was linearized and introduced into HEK293A cells (Qbiogene), together with linear viral DNA (Qbiogene). The subsequent homologous recombination yielded recombinant viral particles expressing the gas7 gene, which was verified using PCR and

Figure 3. The transfer vector used to create the gas7 adenovirus

A replication-defective adenovirus containing the murine full-length gas7 cDNA was produced for overexpression studies. The gas7 sequence (mRNA sequence accession number U19860, NCBI) was introduced into the construct PQBI-AdCMV5-GFP (Qbiogene, Inc., Montreal, QC, Canada), after digestion with Pme I and Bgl II. The construct also coded for the green fluorescent protein (GFP) and both gas7 and GFP expression were under independent cytomegalovirus promoters (CMV). Both coding sequences were also followed by a polyadenylation signal (PA). A portion of the adenovirus 5 DNA (Ad5) allowed homologus recombination with additional viral DNA, for the production of viral particles. Finally, the transfer vector also contained an origin of replication (ori), a gene for ampicillin resistance (Amp^R), and inverted terminal repeats (ITR). The PQBI-AdCMV5-GFP construct, without the insertion of the gas7 cDNA sequence, was also used to produce the control GFP adenovirus.


EcoR I
Fse I
8201
7177
7341
14
116
393
494
515
6414
ori
Amp R
ITR
PA
6060
GFP
1232
1311
Ad5 (9.4-15.5 map units)
CMV
1839
PA
2199
2300
CMV
2321
Pme I
Bgl II
3601
3368
gas7 cDNA
insertion site

— Figure 3 —

Western blotting. The recombinant adenovirus was propagated in HEK293A cells, purified using a cesium chloride gradient, and titrated using a standard plaque assay. To evaluate non-specific effects resulting from the infection process itself, a similar adenovirus construct without the gas7 cDNA sequence (GFP adenovirus; provided by Dr. Hou and Dr. Huang) was also used.

2.2.2 Adenovirus infection

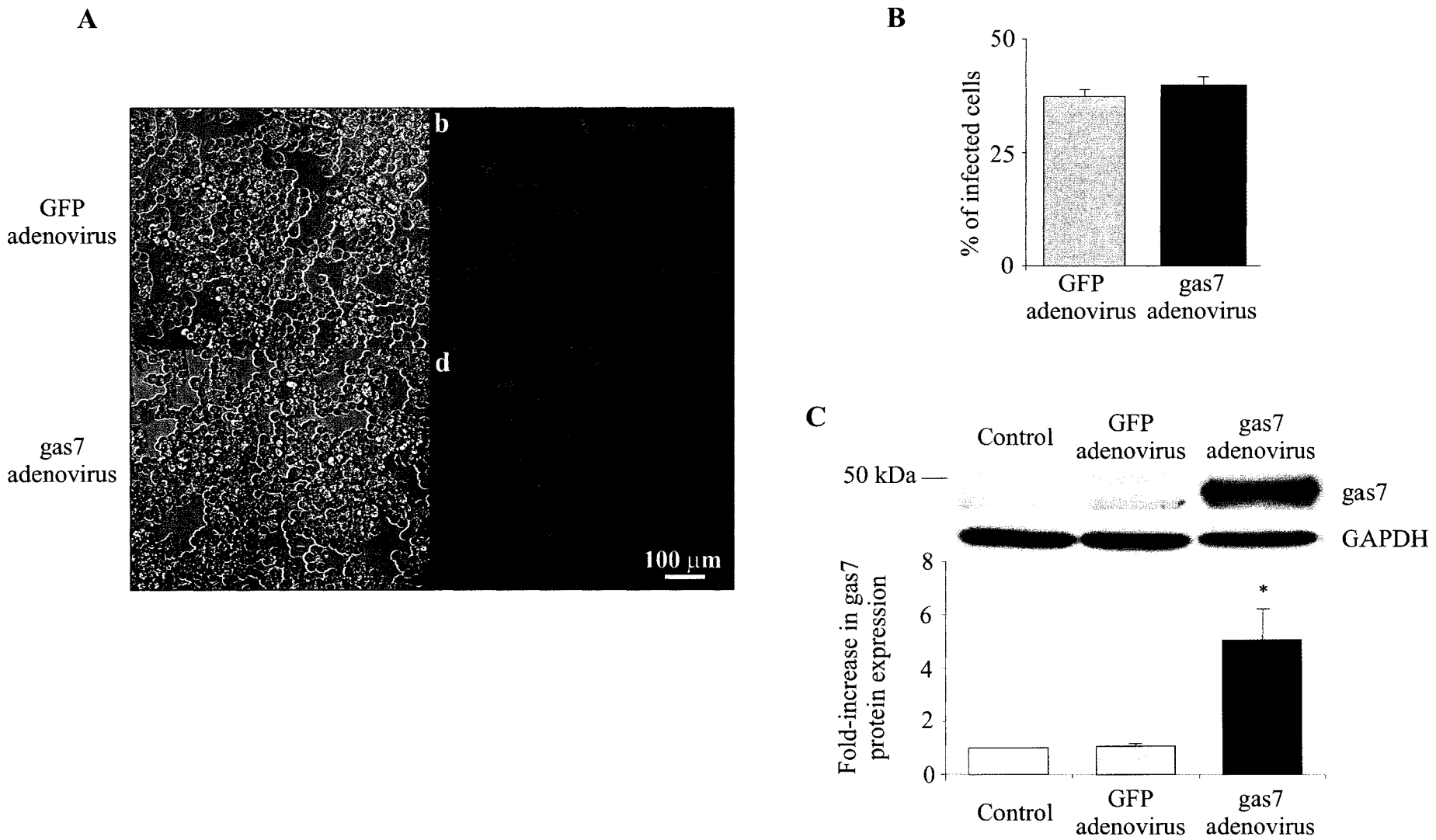
PC12 cells were plated at 10^5 cells/cm^2 and allowed to adhere to culture dishes for 24 hours before infection. For toxicity experiments with differentiated cells, cells were first incubated for 5 days in differentiation media and replated 24 hours before infection, at the same density as the undifferentiated cells. For all of the following steps, differentiated cells were kept in differentiation media, whereas growth media was used for undifferentiated cells. Cells were incubated for 16 hours with the virus diluted in the appropriate media (see Section 2.2.3 for virus concentrations), after which time the media was replenished to remove extra viral particles. Morphological observations and protein extractions were made at different time points and toxicity assays were performed 48 hours post-infection (Section 2.7).

2.2.3 Multiplicities of infection (MOIs)

MOIs of 1, 3, 5, and 10 plaque-forming-units (pfu) per cell were tested to determine the best conditions of infection, for both the GFP and the gas7 adenoviruses. The infection efficiency was evaluated by counting the number of infected cells, or GFP-expressing cells, and dividing by the total number of cells. As shown in Figure 4A-B, 3 MOIs of the

Figure 4. Efficiency of the infection process

PC12 cells were incubated with viral suspensions of the control GFP adenovirus (3 MOIs) or the gas7 adenovirus (1 MOI) and observations were made 48 hours post-infection. **A.** Micrographs of cells infected with the GFP adenovirus (a, b) or the gas7 adenovirus (c, d). a and c (transmitted light) show the same fields as b and d (GFP fluorescence), respectively. **B.** Percentages of infectivity. The infection conditions led to 37% of cells infected with the GFP adenovirus and 40% with the gas7 adenovirus. Data are shown as means ± SEM. Results represent 3 independent experiments performed in duplicate and a total of at least 600 cells were counted per condition. Means were compared by *t* test and were not significantly different ($p > 0.05$). **C.** Analysis of gas7 protein expression following infection. Cells infected with the gas7 adenovirus showed a marked increase in gas7 protein as shown by Western blotting with the gas7 full-length antibody ($p < 0.05$). Similar results were obtained with the gas7 C-terminal antibody (data not shown). gas7 protein levels were normalized to that of GAPDH and results represent 3 independent experiments. Data was analyzed by ANOVA and when significant differences were observed, Tukey's test was used for multiple comparisons. The asterisk indicates a mean significantly different from the other conditions.


A
GFP adenovirus
gas7 adenovirus
b
d
100 μm
B
% of infected cells
50
25
0
GFP adenovirus
gas7 adenovirus
C
Control
GFP adenovirus
gas7 adenovirus
50 kDa
gas7
GAPDH
Fold-increase in gas7 protein expression
8
6
4
2
0
*
Control
GFP adenovirus
gas7 adenovirus

— Figure 4 —

GFP adenovirus and 1 MOI of the gas7 adenovirus resulted in an infectivity of about 40%, 48 hours post-infection. The gas7 adenovirus was also very efficient in overexpressing the gas7 protein (Figure 4C). The observed gas7 band corresponded to the predicted molecular weight for the murine full-length gas7 protein, of 48 kDa (Ju *et al.*, 1998). These conditions were chosen for subsequent infections, as higher MOIs (3 and 5 for the gas7 and GFP adenoviruses, respectively) resulted in some cellular toxicity 3 days after infection (data not shown).

2.3 Evaluation of protein expression

2.3.1 Protein extraction

Cells were rinsed once with PBS, collected in the same buffer by scraping, and pelleted by centrifugation (200g, for 1 minute at 4°C). Cells were then resuspended in hypotonic lysis buffer (1 mM $NaHCO_3$, 5 mM $MgCl_2$, pH 7.5; all reagents from Sigma), supplemented with protease inhibitors (protease inhibitor cocktail, 1:500; Sigma) vortexed for 1 minute, and sonicated for 5 seconds. Cell lysates were centrifuged at 1000g for 10 minutes at 4°C, and supernatants, containing membrane and cytosolic proteins, were transferred to fresh tubes. One-third the volume of 4X modified Salter's buffer (4X MSB; 200 mM Tris-HCl, 4% NP-40, 2% sodium deoxycholate, 0.4% SDS, 600 mM NaCl, 4 mM EDTA, 1:125 protease inhibitor cocktail, pH 7.4; all reagents from Sigma) was added and membrane proteins were allowed to solubilize for 30 minutes at 4°C. Protein concentrations were determined by the Bradford method (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA, USA).

2.3.2 Protein separation and Western blotting

Twenty-five μg of proteins in loading buffer [5 mM Tris-HCl, 5% glycerol (Bio-Rad), 5% β-mercaptoethanol (βME; Invitrogen), 3% sodium dodecylsulfate (SDS; Sigma), 0.5% bromophenol blue (Anachemia, Rouses Point, NY, USA), pH 6.8] were separated by denaturating polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels [separating gel: 375 mM Tris-HCl, 10% polyacrylamide (Sigma), 0.1% SDS, 0.125% ammonium persulfate (APS; Sigma), 0.125% tetramethylethylenediamine (TEMED; Sigma), pH 8.8; stacking gel: 125 mM Tris-HCl, 6% polyacrylamide, 0.1% SDS, 0.1% APS, 0.2% TEMED, pH 6.8], for about 1 hour at 150 volts. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad) by electroblotting for 16 hours at 100 milliamperes.

Membranes were blocked in 1% bovine serum albumin (BSA; Sigma) and 1% dry milk powder in tris-buffered saline Tween-20 [TBS-T: 10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20 (Sigma), pH 8.0] for 1 hour at room temperature and incubated overnight at 4°C with the appropriate primary antibody, diluted in blocking solution (see Table 1 for dilutions). After 3 washes of 10 minutes in TBS-T, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies diluted in TBS-T buffer for 1 hour at room temperature. Goat anti-rabbit IgG and sheep anti-mouse IgG (both from Sigma) were used at 1:60,000 and bovine anti-goat IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), at 1:10,000. After 3 washes in TBS-T, the proteins of interest were visualized with chemiluminescence reagents (ECL-Plus; Amersham Biosciences, Piscataway, NJ, USA). To re-probe a membrane with a different

Table 1. Working dilutions for the different primary antibodies

Target protein	Molecular weight	Antibody Dilution	Source
AChR-α7	40-60 kDa	1:1,000	Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA
DRP-3	64 kDa	1:20,000	BD Biosciences Mississauga, ON, Canada
GAPDH	34 kDa	1:40,000	Chemicon International, Inc. Temecula, CA, USA
gas7 (full-length)	60 kDa in PC12 cells	1:4,500	Dr. Sue Lin-Chao Institute of Molecular Biology Academica Sinica, Nankang Taipei, Taiwan
gas7 (C-terminal)	47-48 kDa in primary tissues	1:10,000	
STG	65 kDa	1:5,000	Novus Biologicals, Inc. Littleton, CO, USA
TUJ-1	53 kDa	1:40	Dr. William Staines University of Ottawa Ottawa, ON, Canada

All primary antibodies were diluted in TBS-T supplemented with 1% BSA and 1% dry milk powder.

antibody, the following stripping procedure was followed. Membranes were washed once in TBS-T, incubated in stripping buffer (62.5mM Tris-HCl, 100 mM βME, 2% SDS, pH 6.7) for 1 hour at 55°C, and washed 3 times in TBS-T. The usual blotting protocol was then performed, starting at the blocking step.

2.3.3 The gas7 antibodies

Two different antibodies for the study of gas7 expression were kindly provided by Dr. Sue Lin-Chao (Institute of Molecular Biology, Academica Sinica, Nankang, Taipei, Taiwan). One of them was directed against the full-length murine gas7 protein. Briefly, a plasmid containing a histidine-tagged sequence of the mouse full-length gas7 cDNA (mRNA sequence accession number U19860, NCBI) was expressed in *E. coli*, purified, and introduced into rabbits to produce the rabbit polyclonal anti-gas7 full-length antibody (Ju *et al.*, 1998). The second antibody was directed against the C-terminal portion of the murine gas7 protein. The peptide NKTEEDIKKARRKSTQAGD, from the carboxy domain of gas7, was introduced into rabbits to produce the rabbit polyclonal anti-gas7 C-terminal antibody (Dr. Sue Lin-Chao, not published).

2.4 Evaluation of mRNA expression

2.4.1 RNA extraction

RNA was isolated by means of the TriReagent method, according to the manufacturer's protocol (Molecular Research Center, Inc. Cincinnati, OH, USA). Cells were homogenized in the extraction reagent and addition of chloroform (1:5, 5 minutes at room

temperature) allowed the separatation of RNA from DNA and protein, after centrifugation (12,000g, 15 minutes, 4°C). RNA was then precipitated from the aqueous phase with isopropanol (1:1, 10 minutes at room temperature) and isolated by centrifugation (12,000g, 10 minutes, 4°C). RNA pellets were washed in 75% ethanol (7,500g, 5 minutes, 4°C) and finally dissolved in water. RNA concentrations were determined by reading the absorbance at 260 nm (1 unit of absorbance = 40 μg/mL).

2.4.2 RT-PCR

To produce single-stranded cDNA, 2 μg of RNA was added to a 10-μL reaction containing 1X reaction buffer (Invitrogen), 200 units of Superscript II reverse transcriptase (Invitrogen), 5 μM of random hexamers (Applied Biosystems, Foster City, CA, USA), 1 mM of deoxynucleotide triphosphate (dNTPs; MBI Fermentas, Hanover, MD, USA), and 10 mM of dithiothreitol (DTT; Applied Biosystems). Tubes were incubated for 10 minutes at room temperature, to allow binding of the random primers to the RNA template, followed by 60 minutes at 42°C, for cDNA synthesis, and 5 minutes at 80°C, to stop the reaction. These cDNA preparations were finally diluted 10-fold in water. Each 25-μL PCR reaction contained 1 μL of diluted cDNA (about 1 μg of cDNA), 1X reaction buffer (Applied Biosystems), 0.4 μM each of forward and reverse primers (see Table 2 for sequences), 200 μM of dNTPs (MBI Fermentas), 1.5 mM of $MgCl_2$ (Applied Biosystems), and 1 unit of *Taq* polymerase (Applied Biosystems). All PCR amplifications were carried out for 42 cycles. Each cycle consisted of 1 minute at 94°C (denaturation), 1 minute at 58°C (annealing), and 1 minute at 72°C (elongation). PCR

Table 2. Primer sequences used for PCR reactions

Target Gene	Sequence	Product size (bp)
Cyclophilin[a]	Fwd: 5'-TGGACCAAACACAAACGGTT-3' Rev: 5'-TGATCTTCTTGCTGGTCTT-3'	161
gas7[b]	Fwd: 5'-AAGTCCACTCAGGCAGGAGA-3' Rev: 5'-CTCGACTGTGCTTTGGTTGA-3'	204
gas7[c]	Fwd: 5'-TAGTGCAGAGTCTCATGGCTGTAGG-3' Rev: 5'-GCTGCCCAAAGGTCCAAGATTAGTA-3'	287
gas7[d]	Fwd: 5'-GCTGGAGATCAAGCTGAGCAA-3' Rev: 5'-AGGTCGTCTCCAGCCTGTGT-3'	81

a: Used for RT-PCR with samples from PC12 cells.

b: Used for RT-PCR with samples from PC12 cells; target: coding region of gas7 mRNA.

c: Used for RT-PCR with samples from PC12 cells; target: 3'-untranslated region of gas7 mRNA. Sequence from Chao *et al.*, 2003.

d: Used for Q-PCR with samples from NT2 cells.

products were separated by electrophoresis on agarose gels (1.5%) and stained with ethidium bromide (EtBr, 0.2 μg/mL; Sigma).

2.4.3 Real-time quantitative PCR (Q-PCR)

Q-PCR reactions were performed with 1.5 ng of cDNA template from NT2 samples added to a reaction of 20 μL containing 1X reaction mix (ABI, Foster City, CA, USA) and 0.15 μM each of forward and reverse primers (see Table 2 for sequences). Thermal cycling was initiated with a 2-minute incubation at 50°C followed by a first denaturation step of 10 minutes at 95°C. Forty cycles of PCR amplification, each consisting of 15 seconds at 95°C (denaturation) and 1 minute at 60°C (annealing and extension), were followed by dissociation of the product (60-90°C) to verify the specificity of amplification. Data were collected continuously during the Q-PCR reactions and were analyzed with the Sequence Detection System (ABI).

For quantification, a threshold amount of amplified product was arbitrarily selected within the linear phase of amplification and the threshold cycle (C_T: number of cycles after which the amount of amplified product reached the threshold) was evaluated for the different conditions. C_T values for the different samples were subtracted from the C_T value for control (undifferentiated) cells (ΔC_T) and changes in expression were calculated using the term $2^{-\Delta CT}$.

2.5 Quantification of protein and mRNA expression

Protein and DNA bands, respectively obtained by Western blotting and RT-PCR, were scanned with a digital camera and band intensities were analyzed with the Northern Eclipse software (Empix, North Tonawanda, NY, USA). The gray scale was inversed, thereby attributing high values to dark pixels, and total gray values were measured for each band. Background values were also measured for each band and subtracted from the band intensities. Results were normalized to that of an internal control (expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for Western blots or of cyclophilin for RT-PCRs) and values were expressed as fold-increase compared to a control condition arbitrarily set to a value of 1.

2.6 Isolation and sequencing the 60-kDa gas7 isoform expressed in PC12 cells

2.6.1 Immunoprecipitation

Four hundred μg of proteins from PC12 cells incubated in differentiation media for 3 hours were incubated with 1 μL of the gas7 C-terminal antibody in modified Salter's buffer (MSB; 50 mM Tris-HCl pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1:500 Sigma protease inhibitor cocktail) for 3 hours at 4°C, in a final volume of 550 μL. This allowed the formation of antibody-antigen interactions. Fifty μL of protein A sepharose bead slurry (Protein A Sepharose CL-4B; Amhersham Biociences) prepared in MSB buffer were then added and the incubation was

continued for another hour, to allow interactions between beads and antibody molecules. Bead-antibody-protein complexes were collected by centrifugation (14,000g, 1 minute at 4°C) and washed 3 times in MSB buffer. The pulled-down proteins were finally solubilized in 40 μL of loading buffer and separated by SDS-PAGE, 10%. After separation on polyacrylamide gels, the immunoprecipitated proteins were either transferred to PVDF membranes and probed with the gas7 C-terminal antibody or stained with silver nitrate and sent for mass spectrometry.

2.6.2 Silver staining

Gels were fixed in 50% ethanol and 5% acetic acid for 30 minutes, washed with 50% ethanol for 10 minutes, and washed 2 times 10 minutes with water. Gels were then placed in a sensitizing solution (0.02% sodium thiosulfate; BDH, Poole, UK) for 2 minutes and washed again twice with water, before a 30-minute incubation at 4°C in the staining solution (0.1% silver nitrate; BDH). Stained gels were briefly washed with water and developed in 0.04% formaldehyde and 2% sodium carbonate (both from BDH). To stop the developing reaction, gels were quickly put in 5% acetic acid and stained gels were stored in 1% acetic acid, at 4°C.

2.6.3 Mass spectrometry analysis

Digestion of the bands of interest and mass spectroscopy analysis of the products (Q-Tof nano-LC-MS/MS) were done by Dr. John Kelly (NRC, IBS). The bands were first excised from the gels and destained with chemical reducers to remove precipitated silver particles. Specifically, gel pieces were incubated in 15 mM potassium ferricyanide and

50 mM sodium thiosulfate (both from BDH) for 3-5 minutes, until the colour disappeared. After 3 washes in water, samples were submitted to in-gel protein digestion. Gels were dried in acetonitrile (5 minutes) and rehydrated in a trypsin solution (Promega, Fitchburg, WI, USA) at 20 ng/mL in 50 mM ammonium bicarbonate (BDH). Digestion was performed overnight at 37°C and digested peptides were transferred to 96-well plates. Peptide fragments were analyzed by nano-LC-MS/MS using a Q-tof Ultima mass spectrometer (Waters, Milford, MA, USA). Samples were separated on a capillary column at a flow rate of about 250 nL/min before being introduced in the Q-tof by elution with an acetonitrile gradient (5 to 50%). The MS/MS spectrum generated was then submitted to database searching using the Mascot Daemon application for protein identification (Matrix Science Inc., Ohio, USA).

2.7 Toxicity assays

Toxicity assays were performed 48 hours post-infection with undifferentiated and differentiated PC12 cells plated in 12-well plates.

2.7.1 Oxygen-glucose deprivation (OGD)

Cells were washed twice in a glucose-free buffer (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2 \cdot 2H_2O$, 0.03 mM glycine, 0.8 mM $MgCl_2$, pH 7.4; all reagents from Sigma) and 500 µL this buffer was added to each well. Lid-free plates were introduced in small hermetic metal chambers, which were placed in a 37°C water bath. Nitrogen (95% N_2, 5% CO_2) was allowed to circulate through the chambers for 30 minutes to purge the

oxygen and sealed chambers were then incubated at 37°C for the indicated periods of time (3-6 hours). Following the OGD insult, plates were removed from the chambers, the glucose-free buffer was replaced with reduced-serum media (HG-DMEM, 3% HS, 2% FBS, with or without 50 ng/mL NGF, for undifferentiated and differentiated cells, respectively), and cells were returned to normoxic conditions at 37°C. Cell viability was assessed 16 hours after reoxygenation. Sister cultures not subjected to the insult were incubated in normoxic conditions with glucose-free buffer supplemented with 3 mM of glucose for the duration of the OGD insult. This was followed by a 16-hour incubation in reduced-serum media.

2.7.2 A23187 and sodium nitroprusside (SNP) toxicity

A23187 and SNP (both from Sigma) were diluted to their final concentrations (3-15 μM for A23187; 0.1-0.75 mM for SNP) in growth or differentiation media, for undifferentiated and differentiated cells, respectively, and added to the cells. Viability was assessed 16 hours later.

2.8 Assessment of cell proliferation and viability

2.8.1 Alamar Blue assay for cell proliferation and viability

The Alamar Blue assay (Medicorp, Montreal, QC, Canada) allowed the quantification of cell growth rates. Basically, reduction of growth media, resulting from cell activity, induced fluorescence and color changes of an oxidation-reduction indicator. To assess the effects of gas7 overexpression on cell proliferation, culture media was supplemented with

10% Alamar Blue 72 hours post-infection and cells were incubated at 37°C for 1 hour. Aliquots of media were then transferred to 96-well plates and the fluorescence intensity (excitation: 530 nm, emition: 590 nm) was measured with a Cytofluor 2350 (Millipore, Beford, MA, USA). Values for non-infected cells were arbitrarily considered as 100% proliferation and were used to normalize the results for the infected cells.

The Alamar Blue assay was also used to quantify cell viability following various insults. To use this proliferation assay for viability assessment, data from the different conditions were normalized to that of sister cultures not subjected to the insults, for which the viability was arbitrarily set to 100%.

2.8.2 KI-67 Immunocytochemistry

KI-67 is a nuclear protein found in proliferating cells. KI-67 is expressed during the G1, S, G2, and M phases of the cell cycle, but not in G0 (Bruno & Darzynkiewicz, 1992). Therefore, positive staining is an indication of cellular proliferation. KI-67 immunocytochemistry was performed 72 hours post-infection to assess the effects of gas7 overexpression on cell cycle progression.

Cells were washed twice for 5 minutes in PBS and fixed in a 10% formalin solution (Sigma) for 10 minutes at room temperature. Cells were then washed in PBS, permeabilized in 0.1% Triton X-100 (Sigma) in PBS for 5 minutes, washed again, and incubated in a serum-free protein block solution (DakoCytomation, Mississauga, ON, Canada) for 60 minutes. The rabbit monoclonal anti-KI-67 antibody (Medicorp) was

added at 1:500 in an Antibody Diluting Buffer (ADB; DakoCytomation) and incubated overnight (16-20 hours) at 4°C. Following incubation with the primary antibody, cells were washed twice and the donkey anti-rabbit Alexa Fluor 568 secondary antibody (Molecular Probes, Eugene, OR, USA) was added at 1:200 in ADB for 1 hour at room temperature. Cells were finally washed and kept at 4°C in 0.01% sodium azide (Sigma) in PBS, to prevent microbial growth until observation.

2.9 Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM) and statistical analysis was performed with the GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). Comparisons between 2 means, in a population of only 2 groups, were made with a two-tailed *t* test. To compare data from a population of more than 2 groups, one-way analysis of variance (ANOVA) was performed and when significant differences were observed, Tukey's or Dunnett's tests were used for multiple comparisons. Statistical significance was inferred at $p < 0.05$.

CHAPTER 3

RESULTS

3.1 Morphological and biochemical characteristics of PC12 neuronal differentiation

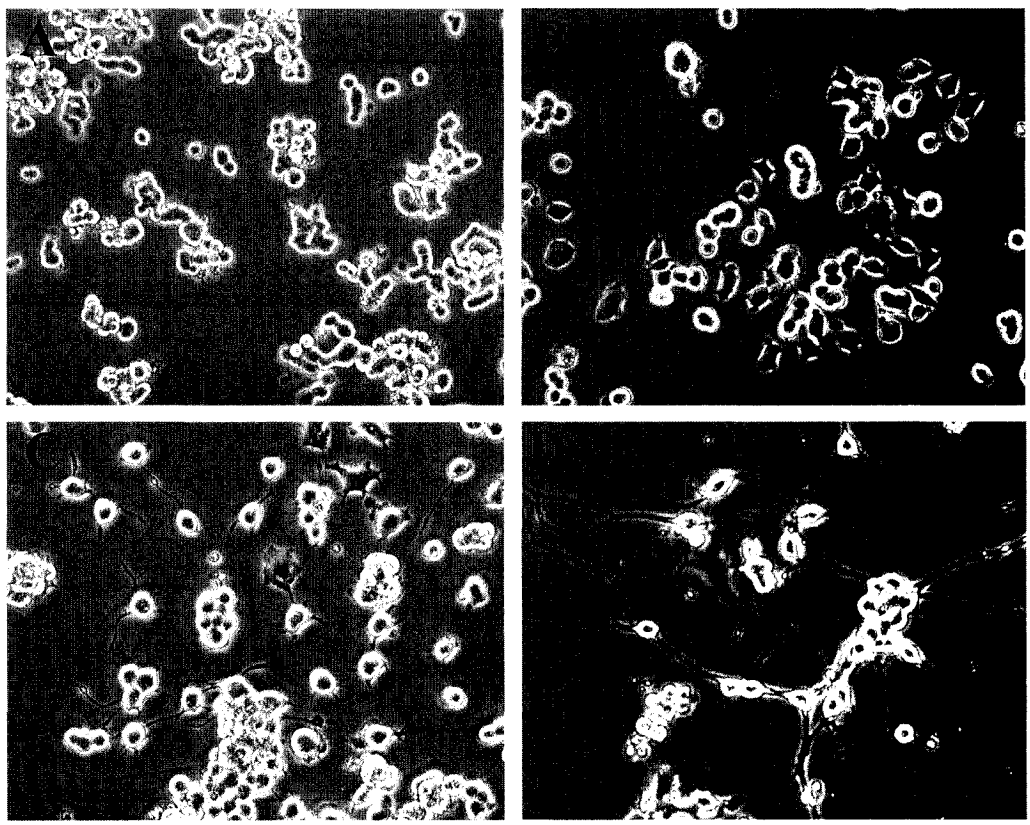
To study the effects of the gas7 protein on neuronal differentiation of PC12 cells, this model first had to be characterized. PC12 cells, derived from the rat adrenal medulla, display specific morphological and biochemical features characteristic of sympathetic neurons when exposed to NGF (Greene & Tischler, 1976). To use specific phenotypic and molecular changes as indicators of the progression of neuronal differentiation, it was necessary to establish the conditions leading to these typical traits.

3.1.1 NGF-treated PC12 cells develop a neuronal morphology

NGF has been widely used to differentiate PC12 cells into neurons and lowering the serum concentration in the media helps the process, mainly by reducing proliferation stimuli (Rudkin *et al.*, 1989). The differentiation media used in this study contained five times less serum than the growth media, and NGF was added at a final concentration of 50 ng/mL. As seen in Figure 5A, undifferentiated PC12 cells grew in small clusters. Within three days of differentiation, cells showed a broad and flattened morphology (sign of growth arrest) and short spike-like membrane protrusions started to extend from the cell bodies (Figure 5B). After five days, these small spikes had elongated into neurite-like outgrowths and most cells displayed such processes (Figure 5C). PC12 cells differentiated for seven days showed very long and thin neurites, with the presence of small blebbings (Figure 5D). These boutons, found within the processes and mainly at their endings most probably corresponded to previously described synaptic-like vesicles

Figure 5. NGF-induced morphological neuronal differentiation of PC12 cells

Transmitted light micrographs of PC12 cells at different time points during differentiation: 0 days (A), 3 days (B), 5 days (C), and 7 days (D). The differentiation media consisted of HG-DMEM supplemented with 2% HS, 1% FBS, and 50 ng/mL NGF and the PC12 neuronal phenotype was characterized by long, thin neurite-like extensions.



— Figure 5 —

in NGF-treated PC12 cells (Greene & Tischler, 1976). This progression of PC12 morphological differentiation was similar to previous reports (Abu-Raya *et al.*, 1999; Abu-Raya *et al.*, 2002; Greene & Tischler, 1976).

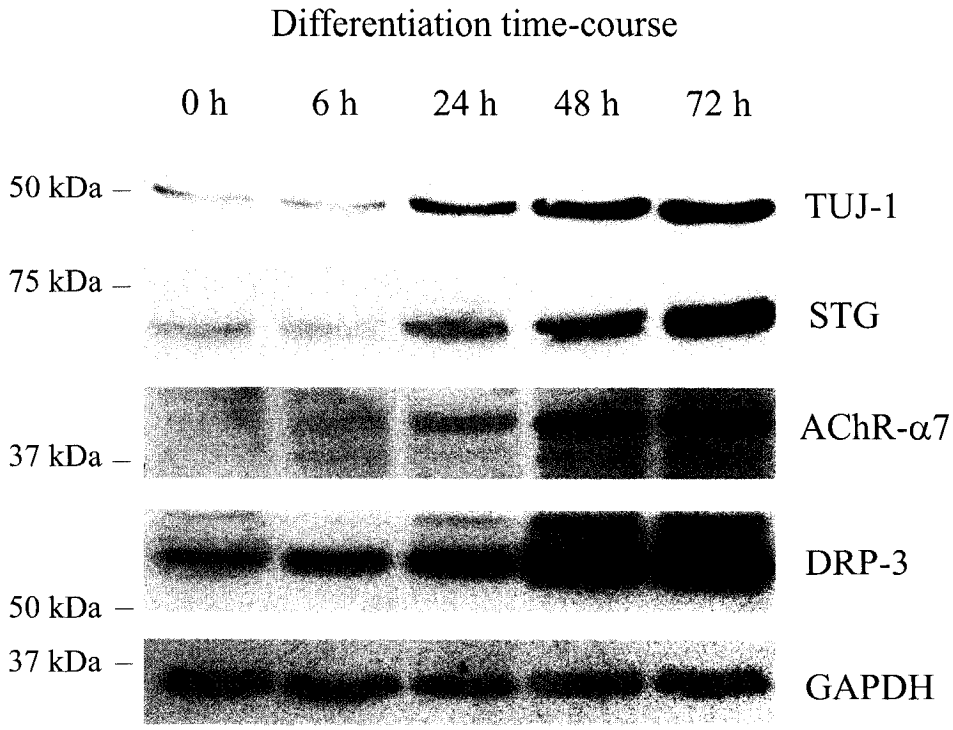
3.1.2 Differentiation of PC12 cells is accompanied by the expression of neuronal markers

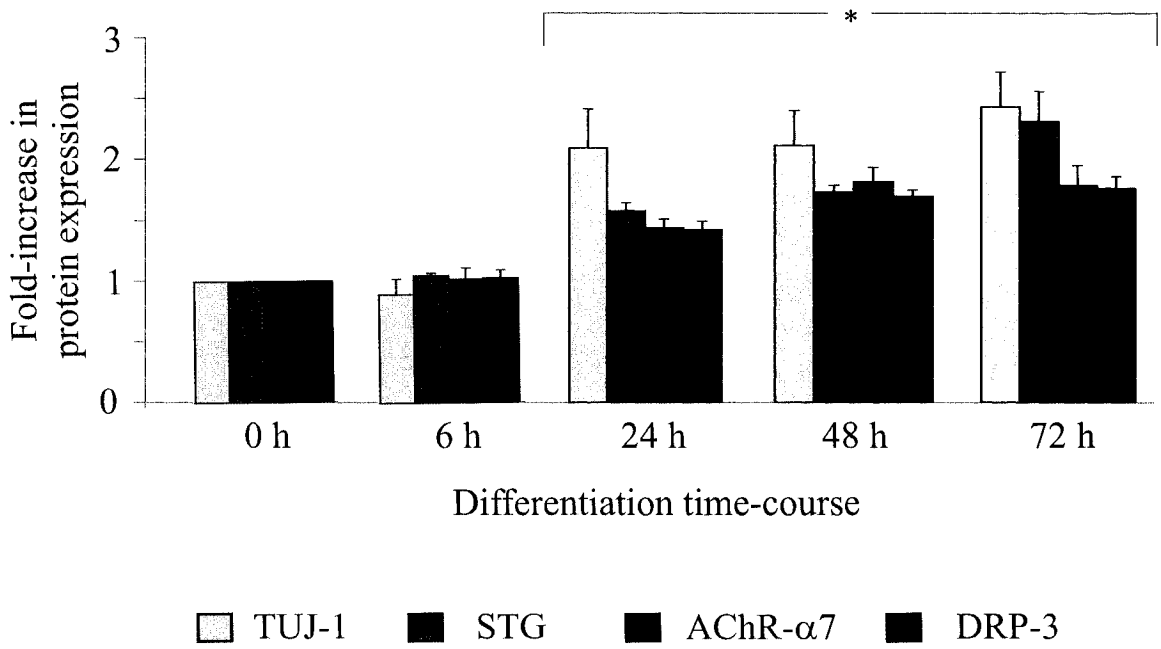
As previously mentioned, neuronal differentiation of PC12 cells can be characterized by the induced expression of several neuronal proteins. Monitoring changes in the expression of these markers, following different treatments, is a useful strategy to determine the effects on the process of neuronal differentiation. The markers of biochemical neuronal differentiation used for the present work were the neuron-specific class III beta-tubulin (βIII-tubulin, or TUJ-1), the pre-synaptic protein synaptotagmin (STG), the alpha-7 subunit of the acetylcholine receptor (AChR-α7), and the dihydropyrimidinase-related protein 3 (DRP-3). The expression of these four proteins is known to be upregulated in PC12 cells during neuronal differentiation with NGF (Byk *et al.*, 1998; Henderson *et al.*, 1994; Ohuchi *et al.*, 2002; Takahashi *et al.*, 1999).

Proteins were extracted at different times during PC12 neuronal differentiation, and the expression of the neuronal markers was analyzed by Western blotting. Within 24 hours, the differentiation conditions (low serum and 50 ng/mL NGF) triggered a significant induction in the expression of TUJ-1, STG, AChR-α7, and DRP-3 ($p < 0.05$; Figure 6). By 48 hours, the expression of all 4 neuronal proteins had increased by close to 2-fold

Figure 6. NGF-mediated differentiation of PC12 cells is accompanied by the expression of neuronal markers

PC12 proteins were extracted at different time points during differentiation in low serum (2% HS and 1% FBS) and 50 ng/mL NGF, and the expression of TUJ-1, STG, AChR-α7, and DRP-3 was analyzed by Western blotting. By 24 hours in differentiating conditions, the expression of the studied neuronal proteins was significantly induced ($p < 0.05$). Data are shown as means ± SEM. Protein levels were normalized to that of GAPDH, and results represent 3 independent experiments. Data were analyzed by ANOVA and when significant differences were observed, Dunnett's test was used for multiple comparisons. The asterisk indicates means significantly different from control values (before differentiaiton, time = 0h).


Differentiation time-course
0 h
6 h
24 h
48 h
72 h
50 kDa
TUJ-1
75 kDa
STG
37 kDa
AChR-α7
DRP-3
50 kDa
37 kDa
GAPDH


*
Fold-increase in protein expression
3
2
1
0
0 h
6 h
24 h
48 h
72 h
Differentiation time-course
TUJ-1
STG
AChR-α7
DRP-3

— Figure 6 —

compared to control (undifferentiated) cells. However, as previously mentioned, cells did not show any morphological indication of differentiaiton before 72 hours (Figure 5). The neuronal proteins studied here could therefore be involved in the induction of the neuronal phenotype. Alternatively, their expression might be concomittant with the activation of signaling pathways leading to morphological changes.

3.2 gas7 potentiates neuronal differentiation of PC12 cells

To evaluate the role of gas7 in neuronal differentiation, the murine full-length gas7 protein was overexpressed in PC12 cells using an adenoviral construct. A control virus, referred to as the GFP adenovirus, was used to assess non-specific effects due to the infection process. Following gas7 overexpression, changes in morphology and in the expression of biochemical markers of neuronal differentiation were assessed in both growing and differentiating conditions. Infected cells were identified by the expression of the green fluorescent protein (GFP).

3.2.1 gas7 promotes neurite-like outgrowth

Studies on the function of the gas7 protein have reported that overexpression of gas7 leads to dramatic morphological changes in different cell types (Chao *et al.*, 2003; Ju *et al.*, 1998; She *et al.*, 2002). Specifically, gas7 expression in neuroblastoma cells was shown to promote neurite outgrowth (Chao *et al.*, 2003; Ju *et al.*, 1998). In this study, overexpression of gas7 in PC12 cells resulted in the formation of short, spike-like membrane extensions, which could be observed 72 hours post-infection (Figure 7E-F).

These changes were specifically due to the overproduction of gas7, since infection with the GFP adenovirus did not have any effect on cell morphology (Figure 7A-B). Moreover, gas7-overexpressing cells were of larger diameter than control cells, which correlates with the documented expanded cell shape characteristic of PC12 cell differentiation (Greene & Tischler, 1976). These observations suggest that gas7 might induce neuronal differentiation.

3.2.1.1 In the presence of NGF, gas7 promotes neurite branching and extension

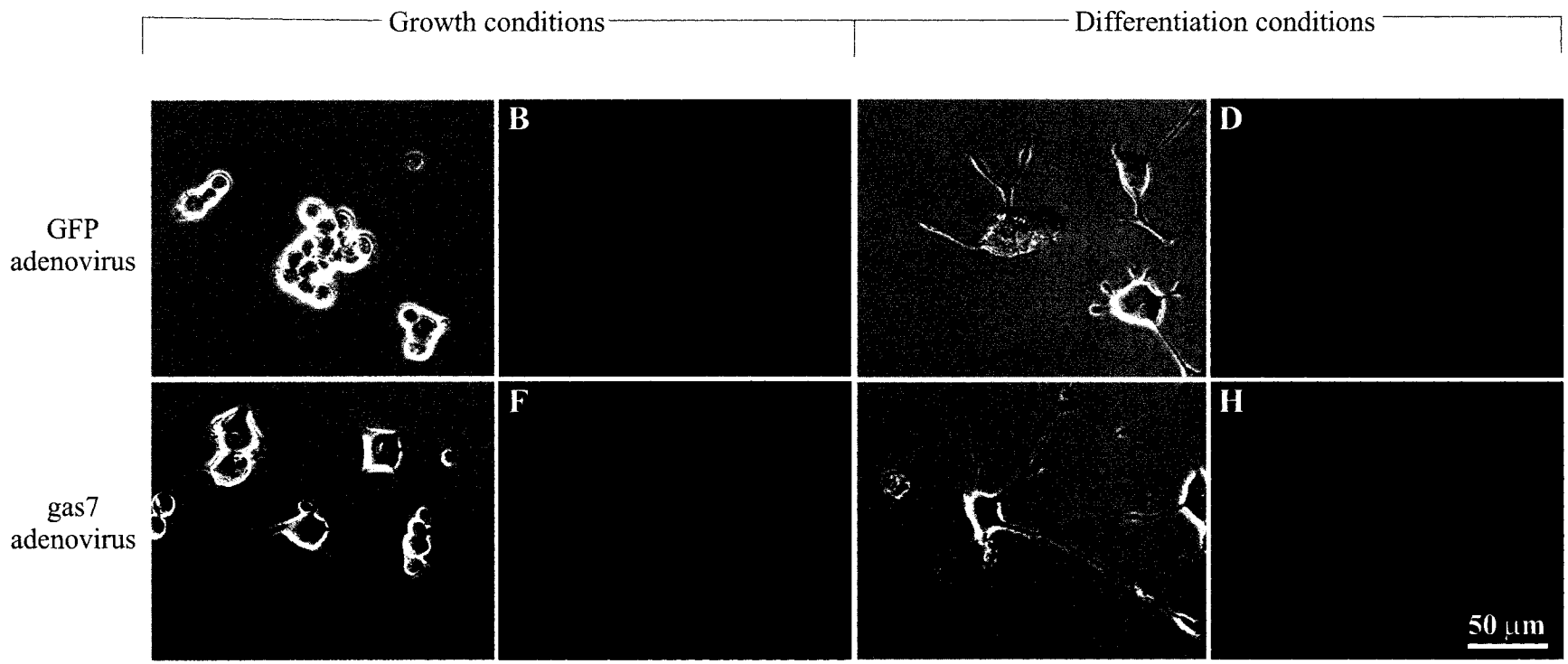
To further evaluate the ability of gas7 to induce neurite growth, the effects of gas7 on cell morphology were studied in differentiating PC12 cells. Sixteen hours after infection, differentiation media was added. As shown in Figure 7C-D, cells infected with the control virus extended simple and short neurites after 4 days. However, overexpression of gas7 triggered a different morphology. Cells were bearing more neurites, which were longer, highly branched, and displayed many vesicle-like boutons (Figure 7G-H). The expression of gas7 thus sped up the neuronal differentiation process at the morphological level.

3.2.2 gas7 potentiates NGF-induced biochemical neuronal differentiation

The next step in studying the effects of gas7 on neuronal differentiation was to determine if gas7 overproduction affects the expression of neuronal markers, another hallmark of differentiation, in addition to induce morphological changes. PC12 cells were infected with the gas7 adenovirus and incubated in either growing or differentiating conditions. Proteins were extracted after 72 hours and analyzed by Western blotting for the

Figure 7. Overexpression of gas7 induces neurite-like outgrowth

Undifferentiated PC12 cells were infected with the GFP (A, B, C, D) or gas7 (E, F, G, H) adenovirus for 16 hours and incubated in either growing or differentiating media (low serum and 50 ng/mL NGF). In growth conditions, overproduction of gas7 resulted in the formation of short membrane extensions 72 hours post-infection (E, F), whereas infection with the GFP adenovirus did not affect cell morphology (A, B). After 4 days in differentiation conditions, gas7-overexpressing cells developed a very complex branching pattern (G, H), whereas cells infected with the control virus showed simple neurite outgrowth (C, D). A, C, E, and G (transmitted light) respectively show the same fields as B, D, F, and H (GFP fluorescence, showing infected cells).


Growth conditions
Differentiation conditions
GFP adenovirus
gas7 adenovirus
B
D
F
H
50 μm

— Figure 7 —

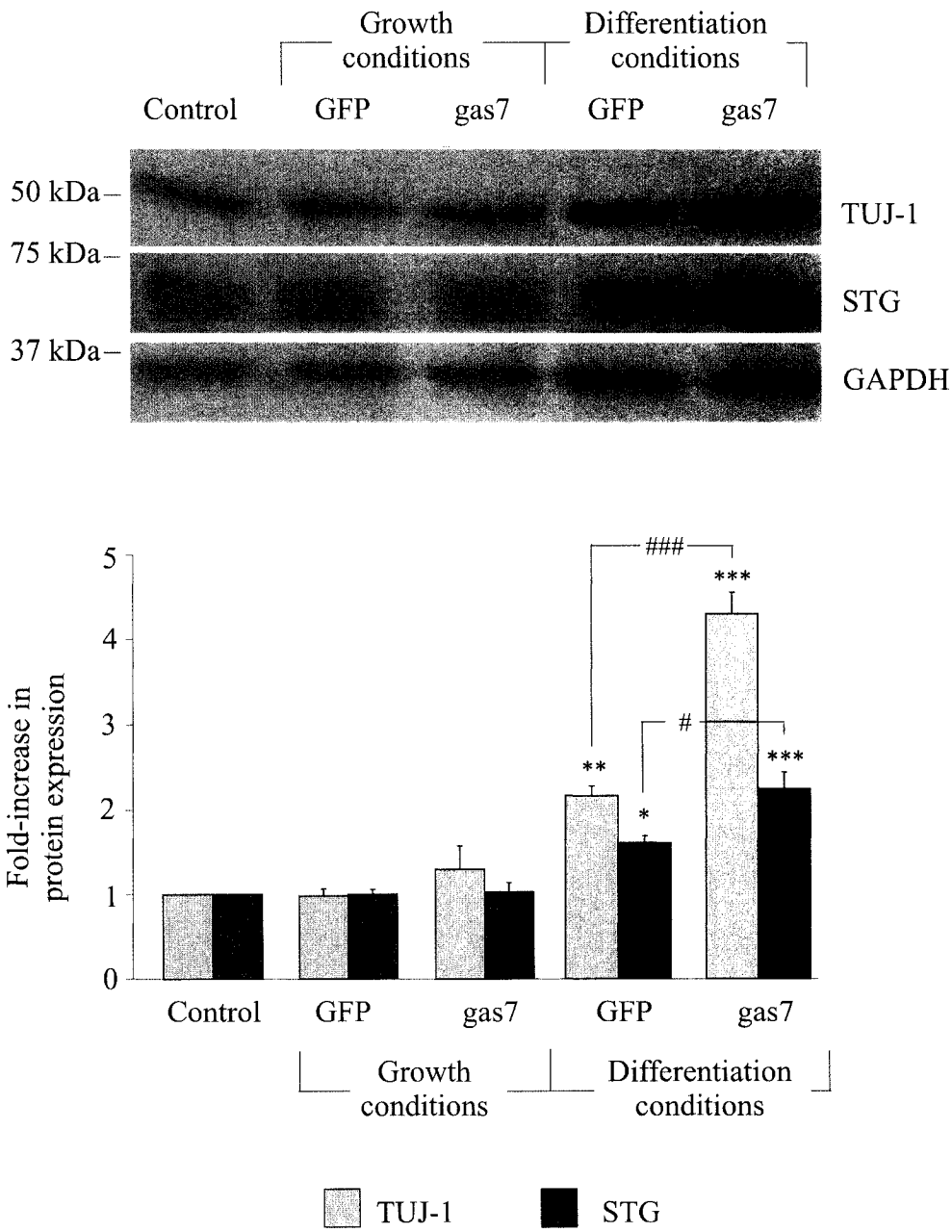
expression of the neuronal markers TUJ-1 and STG. This time point was selected because it is when gas7 induced morphological changes in growing conditions (Figure 7E-F). Although the expression of TUJ-1 and STG was not affected by gas7 overexpression alone ($p > 0.05$) in differentiating conditions (low serum and 50 ng/mL NGF), gas7 overexpression potentiated the induction of the expression of neuronal markers (Figure 8). After 72 hours, the expression of TUJ-1 increased by 2.2-fold in cells infected with the control adenovirus and this induction was significantly augmented to 4.3-fold in gas7-overexpressing cells ($p < 0.001$) (Figure 8). Similarly, the expression of STG went from a 1.6-fold increase in GFP-infected cells, to a 2.3-fold increase in gas7-infected cells ($p < 0.05$) (Figure 8). Therefore, even though gas7 did not induce biochemical neuronal differentiation on its own, it potentiated the induction of neuronal markers upon differentiation with NGF.

3.2.3 gas7 synergizes with sub-optimal concentrations of NGF to induce the expression of neuronal markers

To further characterize the ability of gas7 to potentiate neuronal differentiation, its overexpression in PC12 cells was followed by incubation in sub-optimal differentiation conditions. gas7 overexpression was thus combined with conditions that are not sufficient on their own to trigger differentiation. Sixteen hours after infection of PC12 cells with the GFP or gas7 adenovirus, the media was replaced by HG-DMEM supplemented with 2% HS, 1% FBS, and 10 ng/mL NGF. This sub-optimal differentiation media contained five times less NGF than the usual differentiation conditions (50 ng/mL). As previously shown, the expression of neuronal markers was significantly induced within 24 hours

Figure 8. Overexpression of gas7 during NGF-induced neuronal differentiation potentiates the expression of neuronal markers

Undifferentiated PC12 cells were infected with the GFP or gas7 adenovirus for 16 hours and then incubated in either growing or differentiating conditions (low serum and 50 ng/mL NGF) for another 72 hours. Proteins were then extracted and the expression of the neuronal markers TUJ-1 and STG was analyzed by Western blotting. In growing conditions, gas7 overproduction did not affect the expression of the markers ($p > 0.05$). However, in differentiating conditions, gas7 overexpression potentiated the induction of TUJ-1 ($p < 0.001$) and STG ($p < 0.05$), compared to levels in GFP-infected cells. Data are shown as means ± SEM. Protein levels were normalized to that of GAPDH and results represent 3 independent experiments. Data were analyzed by ANOVA and when significant differences were observed, Tukey's test was used for multiple comparions. Asterisks indicate means significantly different from control values, and # symbols indicate significant differences between selected means. Control: untreated non-infected cells. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$.


Growth conditions
Differentiation conditions
Control
GFP
gas7
GFP
gas7
50 kDa
75 kDa
37 kDa
TUJ-1
STG
GAPDH
Fold-increase in protein expression
0
1
2
3
4
5
###
***
#
**
*
***
Control
GFP
gas7
GFP
gas7
Growth conditions
Differentiation conditions
TUJ-1
STG

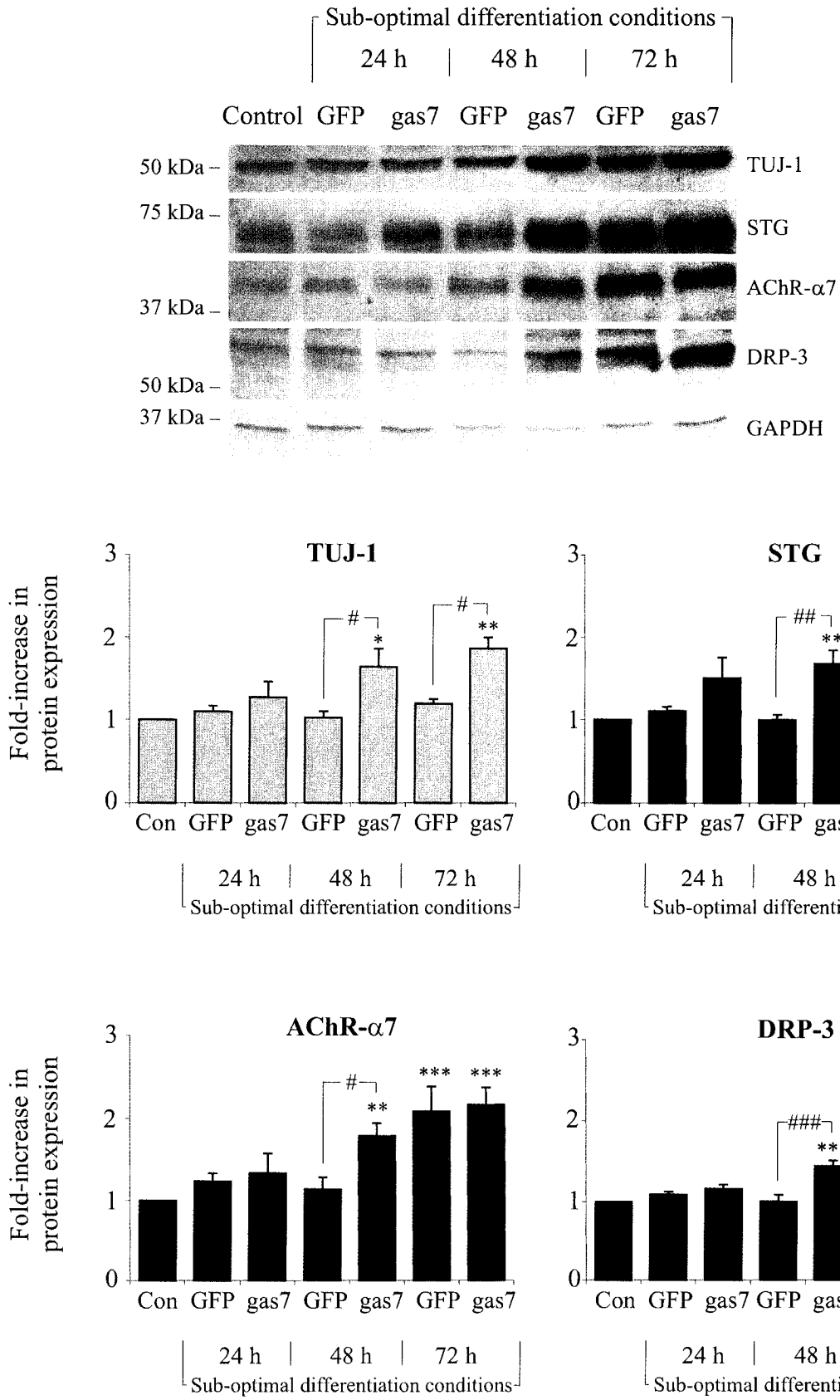
— Figure 8 —

with 50 ng/mL NGF (Figure 6). With the sub-optimal conditions described above, it took 3 days to significantly promote the expression of AChR-α7 ($p < 0.001$) and DRP-3 ($p < 0.01$) in cells infected with the control virus, whereas the expression of TUJ-1 and STG was still at control levels ($p > 0.05$) (Figure 9). Overexpressing the gas7 protein did not have any effect after 24 hours in sub-optimal differentiating conditions ($p > 0.05$), but markedly induced the expression of the four neuronal proteins after 48 hours (Figure 9). At this time, the expression of TUJ-1, STG, AChR-α7, and DRP-3 was respectively induced by 1.6-fold ($p < 0.05$), 1.7-fold ($p < 0.01$), 1.8-fold ($p < 0.01$), and 1.4-fold ($p < 0.001$) compared to control non-infected cells. The levels of expression of the neuronal markers in gas7-infected cells were thus significantly increased compared to those of GFP-infected cells ($p < 0.05$, $p < 0.01$, $p < 0.05$, and $p < 0.001$ for TUJ-1, STG, AChR-α7, and DRP-3, respectively). This confirmed the specificity of induction following gas7 overexpression. After 72 hours, gas7 overexpression still promoted the expression of TUJ-1 and STG (respectively $p < 0.05$ and $p < 0.01$, compared to GFP-infected cells), but AChR-α7 and DRP-3 were expressed to levels similar as in cells infected with the control virus (Figure 9).

In brief, the gas7 protein acted in synergy with low concentrations of NGF to induce the expression of neuronal markers, supporting the ability of gas7 to potentiate neuronal differentiation. Despite the marked induction in the expression of the neuronal proteins, gas7 overexpression combined with sub-optimal differentiating conditions did not affect cell morphology (data not shown). This might be explained by the fact that the incubation period was not long enough to induce morphological changes.

Figure 9. Overexpression of gas7 combined with sub-optimal concentrations of NGF induces the expression of neuronal markers

Undifferentiated PC12 cells were infected with the GFP or gas7 adenovirus for 16 hours and the media was then replaced with HG-DMEM supplemented with 2% HS, 1% FBS, and 10 ng/mL NGF. The expression of the differentiation markers TUJ-1, STG, AChR-α7, and DRP-3 was analyzed by Western blotting at different time points following media change. By 48 hours, gas7-overexpressing cells showed a significant induction in the expression of the 4 markers ($p < 0.05$, $p < 0.01$, $p < 0.01$, and $p < 0.001$ for TUJ-1, STG, AChR-α7, and DRP-3, respectively), whereas their expression in cells infected with the control virus was not different from that of control cells ($p > 0.05$). Accordingly, gas7-infected cells showed significantly higher levels of TUJ-1, STG, AChR-α7, and DRP-3 than GFP-infected cells ($p < 0.05$, $p < 0.01$, $p < 0.05$, and $p < 0.001$, respectively). At 72 hours, the expression of TUJ-1 and STG in gas7-overexpressing cells was still higher than in cells infected with the control virus ($p < 0.05$ and $p < 0.01$, respectively), but the expression of AChR-α7 and DRP-3 was similar in gas7- and GFP-infected cells ($p > 0.05$). Data are shown as means ± SEM. Protein levels were normalized to that of GAPDH and results represent 4 independent experiments. Data were analyzed by ANOVA and when significant differences were observed, Tukey's test was used for multiple comparions. Asterisks indicate means significantly different from control values and # symbols indicate significant differences between selected means. Con: untreated non-infected (control) cells. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$.


Sub-optimal differentiation conditions
24 h
48 h
72 h
Control GFP gas7 GFP gas7 GFP gas7
50 kDa
75 kDa
37 kDa
50 kDa
37 kDa
TUJ-1
STG
AChR-α7
DRP-3
GAPDH
TUJ-1
STG
AChR-α7
DRP-3
Fold-increase in protein expression
Con GFP gas7 GFP gas7 GFP gas7
Sub-optimal differentiation conditions

— Figure 9 —

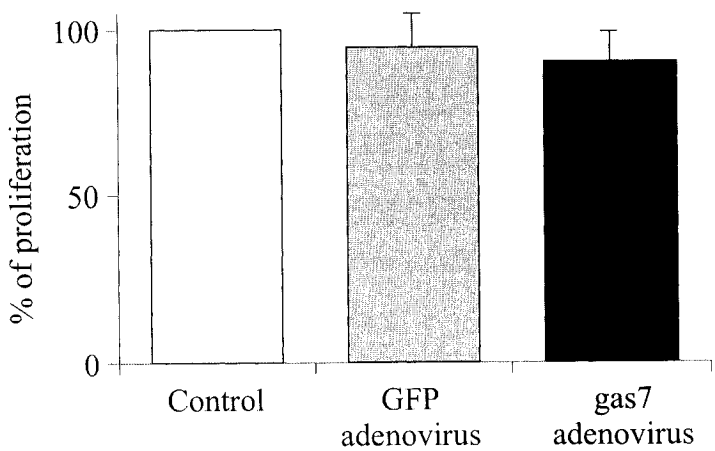
3.2.4 gas7's role in potentiating neuronal differentiation does not involve modulation of cell cycle progression

As previously mentioned, control of the cell cycle is crucial for the regulation of neuronal differentiation (Ohnuma & Harris, 2003). Therefore, it was possible that the potentiating effects exerted by gas7 were mediated through regulation of cell cycle progression, most probably by inducing arrest of cell division. This hypothesis was supported by the intrinsic nature of the gas7 protein being a member of the growth-arrest-specific family, among which many genes are involved in cell cycle control (Brancolini & Schneider, 1994; Brenner *et al.*, 1989; Claesson-Welsh, 1996; Goruppi *et al.*, 1996; Karlsson *et al.*, 1999; Li *et al.*, 1996; Rozental *et al.*, 2000; Ruaro *et al.*, 2000; Zoidl *et al.*, 1995; Zoidl *et al.*, 1997).

To test the possibility that gas7 overexpression could cause arrest of the cell cycle, the proliferation of gas7-overexpressing PC12 cells was compared to that of control cells (non-infected or infected with the control virus), 72 hours post-infection. The Alamar Blue assay incorporates a fluorometric oxidation-reduction indicator in the culture media and the degree of change of the media color is reflective of the extent of cellular proliferation. Values obtained for non-infected cells were arbitrarily correlated to a 100% proliferation rate and were used to normalize results for the infected cells. As shown in Figure 10, neither the control virus nor the gas7 virus affected proliferation of PC12 cells, when compared to non-infected cells ($p > 0.05$). These results suggested that the gas7 protein does not modulate cell cycle progression. However, about 40% of the cells were infected and the Alamar Blue assay quantifies proliferation of the entire cell population.

Figure 10. Overexpression of gas7 does not affect cellular proliferation at the level of the total cell population

Undifferentiated PC12 cells were infected with the GFP or gas7 adenovirus and proliferation was assessed 72 hours post-infection using the Alamar Blue assay. No change in proliferation rates could be detected between the different conditions ($p > 0.05$). Data are shown as means ± SEM. Results represent 4 independent experiments and differences between means were analyzed by ANOVA.


100
50
0
% of proliferation
Control
GFP
adenovirus
gas7
adenovirus

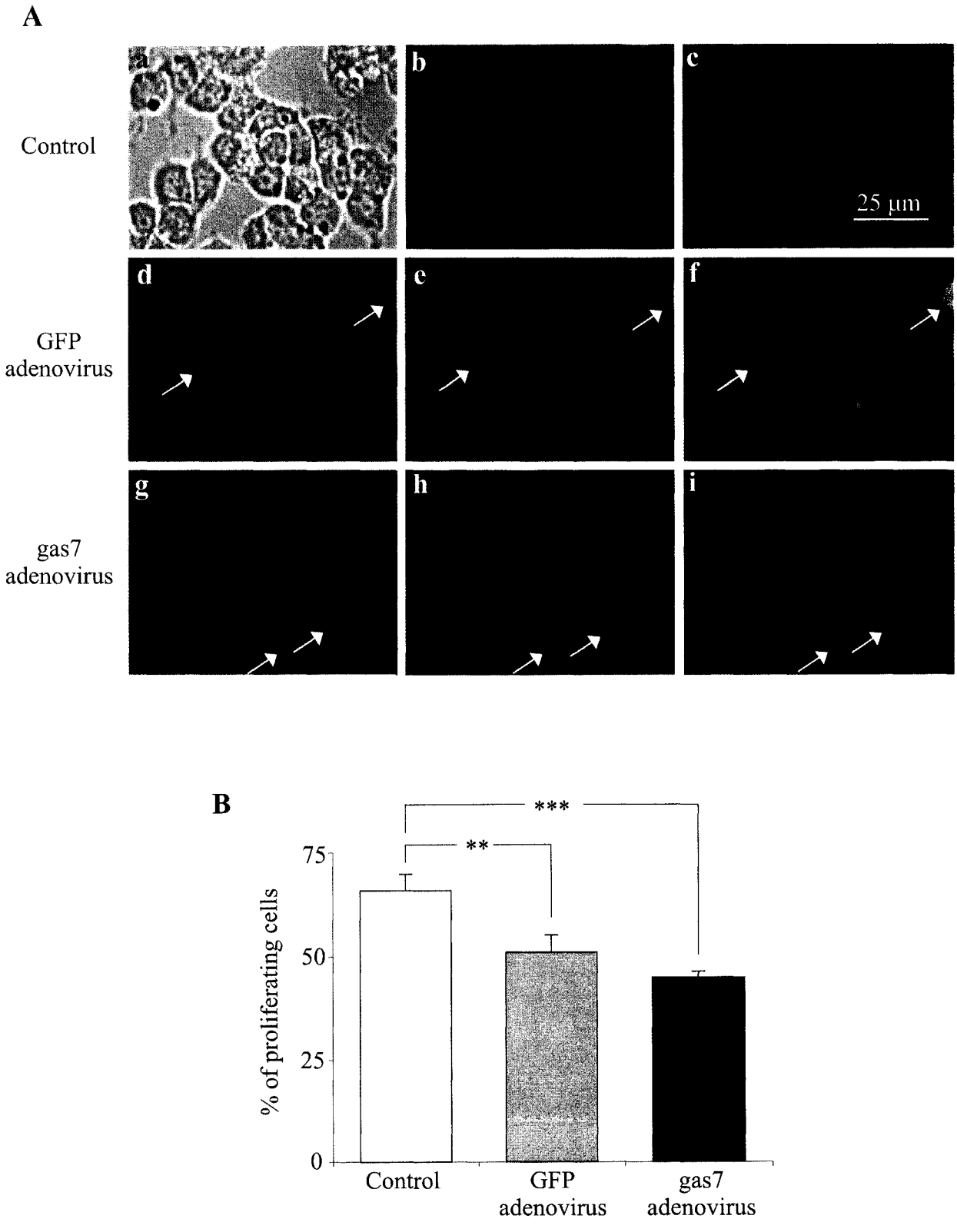
— Figure 10 —

There were thus chances that significant changes in cell proliferation among the infected population could have been missed.

To assess proliferation of only the infected cells, staining for the proliferation marker KI-67 was performed. The KI-67 protein is expressed during all phases of the cell cycle, but not in G0 (Bruno & Darzynkiewicz, 1992). Therefore, positive immunocytochemistry staining for KI-67 indicates active cell proliferation. Cells were stained 72 hours post-infection and proliferation of infected cells was visualized by KI-67 and GFP colocalization (Figure 11A). A small, but significant decrease in cell proliferation was detected when cells were infected with the GFP or gas7 adenovirus, compared to non-infected cells. Among the latter, 66% expressed the KI-67 protein, or were cycling, and this proliferation rate decreased to 51% upon infection with the control adenovirus ($p < 0.01$), and to 45% with the gas7 adenovirus ($p < 0.001$) (Figure 11B). This observation implies that the infection process itself resulted in decreased cellular proliferation. However, the difference between the proliferation rates of cells infected with either of the two viruses was not statistically different ($p > 0.05$) (Figure 11B). Therefore, overexpression of the gas7 protein did not significantly affect cell cycle progression.

Figure 11. Overexpression of gas7 does not affect proliferation

Undifferentiated PC12 cells were infected with the GFP or gas7 adenovirus and stained for KI-67 72 hours post-infection. **A.** Visualization of cycling cells with the proliferation marker KI-67. GFP expression (d, g) shows infected cells, KI-67 staining (b, e, h) shows cycling cells, and colocalization of GFP and KI-67 (overlay: f, i) shows infected cells actively proliferating. a (transmitted light) and b: non-infected cells of the same field. c: negative control for KI-67 staining (secondary antibody only). d-f: GFP-infected cells. g-i: gas7-infected cells. Arrows point to examples of infected cells expressing KI-67. **B.** Quantification of proliferation. The number of KI-67 positive cells was divided by the total number of cells for control (non-infected) cells, whereas the number of double-positive cells for GFP and KI-67 was divided by the number of GFP-expressing cells, for the infected conditions. The infection process caused a decrease in cellular proliferation ($p < 0.01$ and $p < 0.001$ for GFP- and gas7-infected cells, respectively), but overexpression of gas7 did not affect cell cycle progression, when compared to cells infected with the control virus ($p > 0.05$). Data are shown as means ± SEM. Results represent 3 experiments performed in duplicate and a total of about 600 cells were counted per condition. Data were analyzed by ANOVA and when significant differences were observed, Tukey's test was used for multiple compatisons. Asterisks indicate significant differences between selected means. ** $p < 0.01$; *** $p < 0.001$.


A
Control
GFP adenovirus
gas7 adenovirus
a
b
c
d
e
f
g
h
i
25 µm
B
***
**
75
50
25
0
% of proliferating cells
Control
GFP adenovirus
gas7 adenovirus

— Figure 11 —

3.3 Patterns of endogenous gas7 expression during neuronal differentiation

To further assess the role of gas7, its endogenous expression was quantified at various times during neuronal differentiation of two neuronal cell lines: PC12 and NT2 cells. PC12 cells were induced to differentiate into neurons by reducing the media serum content and adding NGF at a final concentration of 50 ng/mL. Samples from human NT2 cells had been collected at different stages of retinoic acid (RA)-mediated neuronal differentiation. Differential expression of a protein, in different conditions, often gives insights into the possible roles played by the molecule. Therefore, the possibility that gas7's role in inducing neurite outgrowth and supporting neuronal differentiation could be reflected by changes in expression was investigated.

3.3.1 The gas7 protein is not endogenously expressed in PC12 cells

As previously mentioned, Chao *et al.* (2003) showed that the expression of a 60-kDa gas7 isoform was induced in PC12 cells during differentiation. Moreover, they suggested that the expression of this protein is essential for NGF-mediated neuronal differentiation of PC12 cells (Chao *et al.*, 2003). The following experiments aimed at reproducing these results in order to eventually use the PC12 model to study the mechanisms of action of gas7 and the pathways in which it is involved.

3.3.1.1 The 60-kDa gas7 isoform is not differentially regulated during NGF treatment of PC12 cells

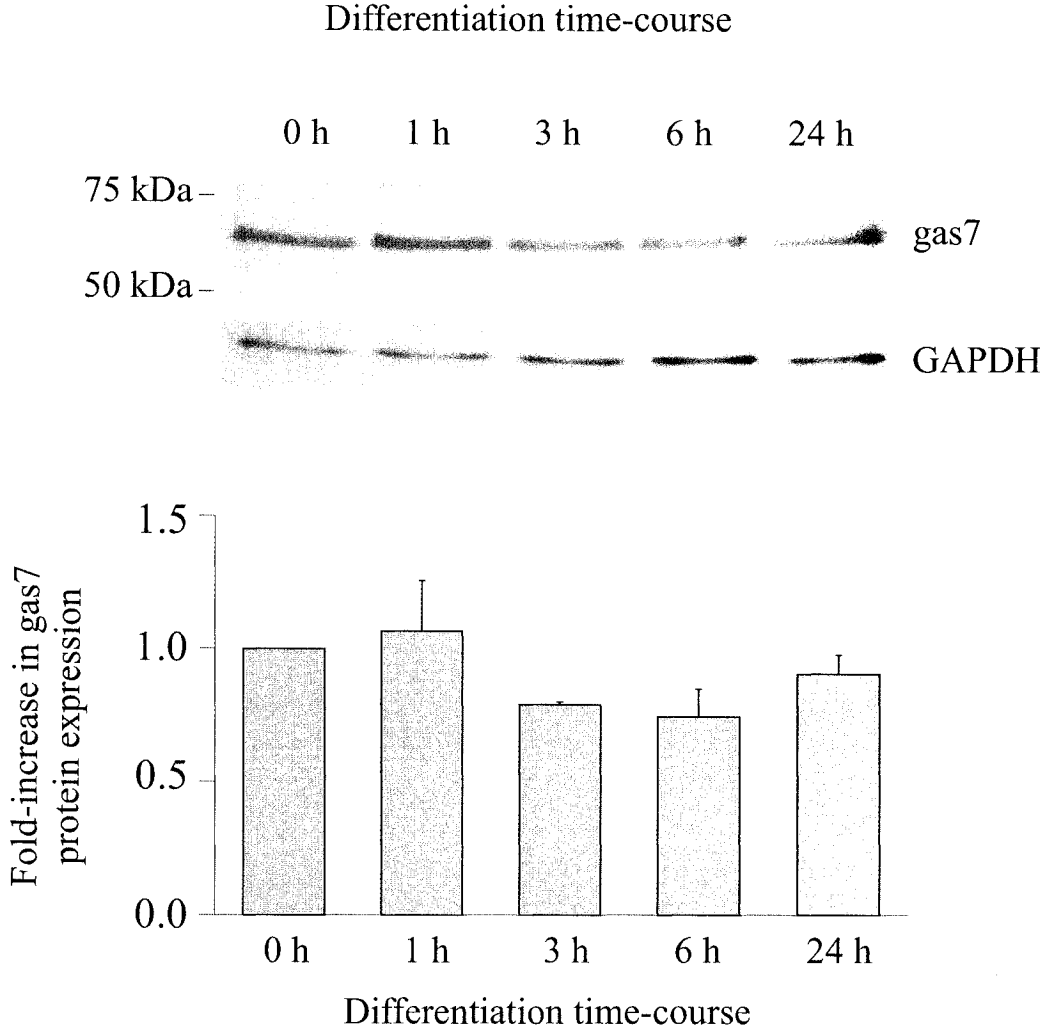
Protein extracts from PC12 cells were collected at different stages during NGF-mediated neuronal differentiation and analyzed by Western blotting for the expression of gas7. Even though the full-length gas7 antibody showed multiple non-specific bands (data not shown), a protein of about 60 kDa was detected with the antibody against the C-terminal portion of gas7 (Figure 12). This correlated with the recent observation from Chao *et al.* (2003) showing the expression of a 60-kDa gas7 isoform in PC12 cells. The gas7 C-terminal antibody was thus used for subsequent analysis of endogenous gas7 expression in PC12 cells. However, the reported increase in the expression of this 60-kDa gas7 isoform, at 3 hours following NGF treatment (Chao *et al.*, 2003), could not be reproduced. The expression of the 60-kDa protein remained constant following addition of NGF ($p > 0.05$) (Figure 12). Proteins were also extracted after differentiation periods additional to those represented in Figure 11 (from 30 minutes to five days), but the expression of the 60-kDa band never changed (data not shown).

3.3.1.2 PC12 cells do not express gas7 mRNA

Following the inability to see an induction in the expression of the 60-kDa gas7 protein during neuronal differentiation of PC12 cells, the expression of gas7 was evaluated at the message level. RNA was extracted at different time points during differentiation (from 10 minutes to 24 hours) and the expression of gas7 mRNA was studied by RT-PCR. Two different sets of primers, targeting either the coding region or the 3' untranslated region (3'-UTR) of the rat gas7 mRNA, were used. However, no mRNA for gas7 could be

Figure 12. The 60-kDa band recognized by the gas7 C-terminal antibody is not differentially regulated during NGF treatment of PC12 cells

PC12 proteins were extracted after different periods of time in differentiating conditions (low serum and 50 ng/mL NGF) and analyzed by Western blotting with the gas7 C-terminal antibody. A protein of ~60 kDa was detected in all samples, but its expression remained constant following NGF treatment ($p > 0.05$). Data are shown as means ± SEM. Levels of gas7 were normalized to that of GAPDH and results represent 6 independent experiments. Differences between means were analyzed by ANOVA.


Differentiation time-course
0 h
1 h
3 h
6 h
24 h
75 kDa
50 kDa
gas7
GAPDH
Fold-increase in gas7 protein expression
1.5
1.0
0.5
0.0
0 h
1 h
3 h
6 h
24 h
Differentiation time-course

— Figure 12—

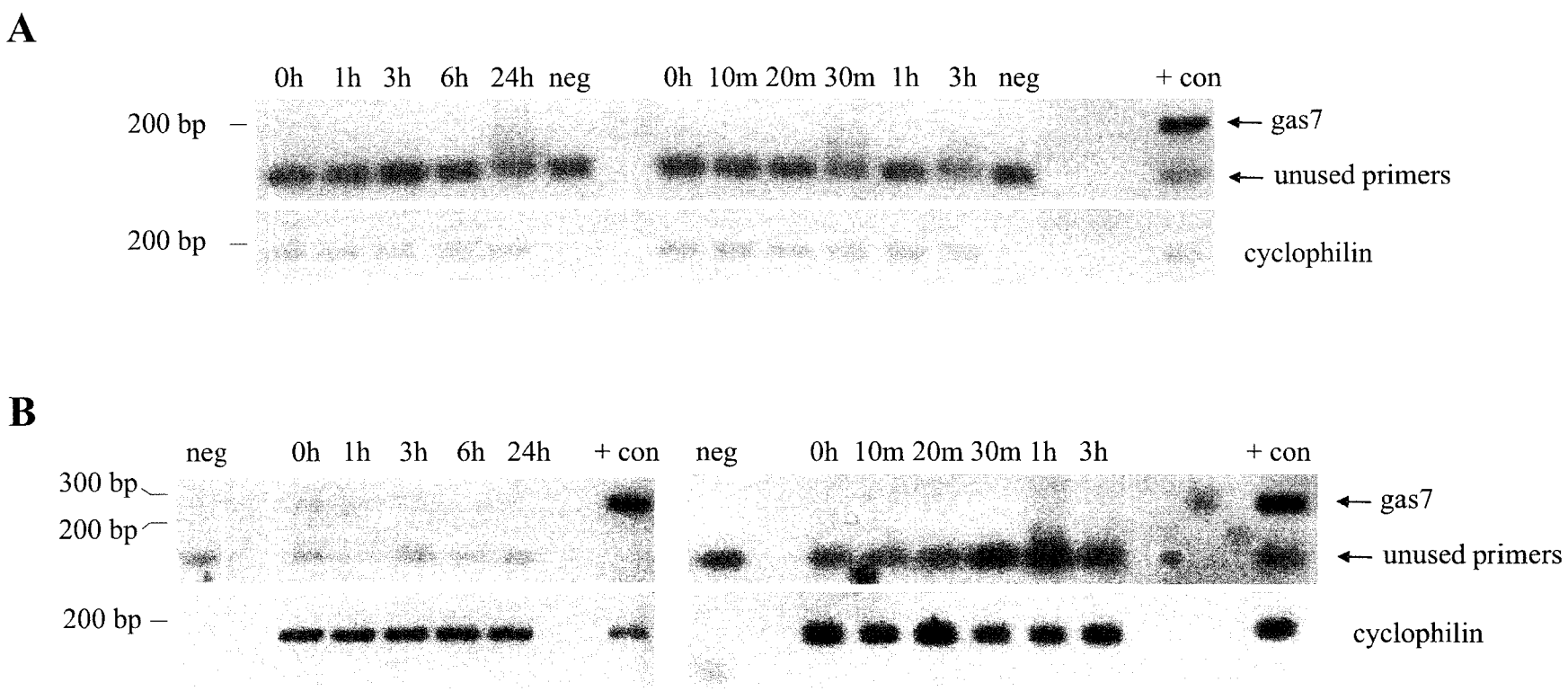
detected in any of the samples (Figure 13). mRNA for the house-keeping gene cyclophilin was ubiquitously expressed, showing the integrity of the samples. These results raised concerns about the identity of the 60-kDa band recognized by the gas7 C-terminal antibody.

3.3.1.3 The 60-kDa protein observed in PC12 cells is not gas7

Since the expression of gas7 could not be confirmed by RT-PCR, the 60-kDa protein observed by Western blotting with the gas7 C-terminal antibody was analyzed by mass spectrometry, to verify its identity. The band of interest was first purified by immunoprecipitation (IP) from PC12 cell lysates after 3 hours of differentiation. This time point was chosen because Chao *et al.* (2003) observed a peak in the expression of the 60-kDa gas7 isoform in similar conditions (Chao *et al.*, 2003). The IP procedure allows the formation of antigen-antibody-bead complexes, which can be isolated by centrifugation. Immunoprecipitated samples were separated by SDS-PAGE and transferred onto PVDF membranes for Western blotting analysis. The 60-kDa band was present in the immune sample and the specificity of the IP reaction was shown by the absence of the 60-kDa band in the control samples (Figure 14A). When the supernatants resulting from the IP reaction were analyzed for the expression of gas7, the 60-kDa band was markedly decreased, showing the efficiency of the IP reaction to pull down the band of interest (Figure 14B). The next step was to determine the sequence of this 60-kDa band.

Figure 13. PC12 cells do not express gas7 mRNA

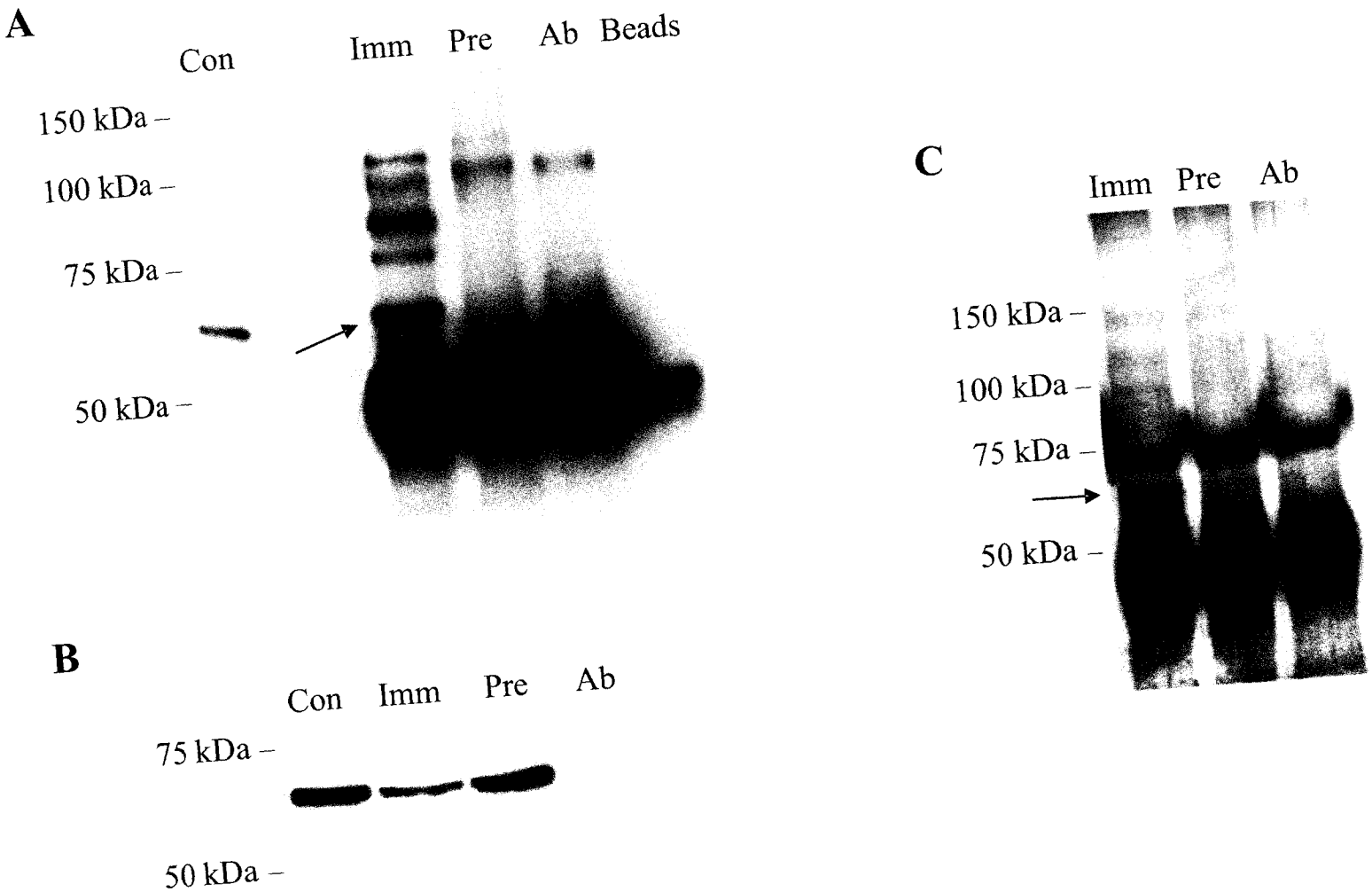
PC12 RNA extracts were collected after different periods of time in differentiating conditions (low serum and 50 ng/mL NGF) and analyzed by RT-PCR for the expression of gas7 mRNA. **A.** Amplification of a region in the coding sequence of the gas7 mRNA. **B.** Amplification of a 3'-unstranslated portion of the gas7 mRNA. No expression of gas7 mRNA was detected in any condition. + con: positive control with RNA extracted from primary rat cortical cultures; neg: negative control without RNA template.


A
0h 1h 3h 6h 24h neg 0h 10m 20m 30m 1h 3h neg + con
200 bp
gas7
unused primers
200 bp
cyclophilin
B
neg 0h 1h 3h 6h 24h + con neg 0h 10m 20m 30m 1h 3h + con
300 bp
200 bp
gas7
unused primers
200 bp
cyclophilin

— Figure 13 —

Figure 14. Isolation of the 60-kDa band recognized by the gas7 C-terminal antibody

Protein extracts from PC12 cells treated with differentiating conditions for 3 hours (low serum and 50 ng/mL NGF) were subjected to immunoprecipitation (IP) with the gas7 C-terminal antibody. **A.** Immunoprecipitated samples were analyzed by Western blotting with the gas7 C-terminal antibody. The band of interest was successfully pulled-down during the IP, as shown by its presence in the immune fraction. **B.** Supernatants were collected after the IP (30 μL) and analyzed by Western blotting with the gas7 C-terminal antibody. The expression of the 60-kDa band was markedly decreased in the immune supernatants, showing the efficiency of the IP reaction in pulling down the band of interest. **C.** Silver staining of immunoprecipitated samples separated by SDS-PAGE. The 60-kDa band was successfully purified. Con: whole cell lysate; Imm: immune fraction with the gas7 C-terminal antibody; Pre: samples treated with pre-immune serum; Ab: gas7 C-terminal antibody incubated with beads; Beads: beads alone. Arrows point to the band of interest.


A
Con
Imm
Pre
Ab
Beads
150 kDa –
100 kDa –
75 kDa –
50 kDa –
B
Con
Imm
Pre
Ab
75 kDa –
50 kDa –
C
Imm
Pre
Ab
150 kDa –
100 kDa –
75 kDa –
50 kDa –

— Figure 14 —

Immunoprecipitated samples were separated by SDS-PAGE, and gels were stained with silver nitrate. The conditions used for silver staining allowed the detection of proteins at the nanogram scale. Staining thus ensured that proteins were present in appreciable amounts to be analyzed. As shown in Figure 14C, the IP reaction was efficient in purifying the 60-kDa band. The silver-stained band of interest, as well as corresponding areas of similar migration in control lanes, were cut off the gel and samples from two different gels were sent for mass spectrometry analysis. The proteins present in the samples were identified from the peptidic sequences characterized after digestion. Among the different samples, the only protein that was specific to the immune reaction was the protein coronin.

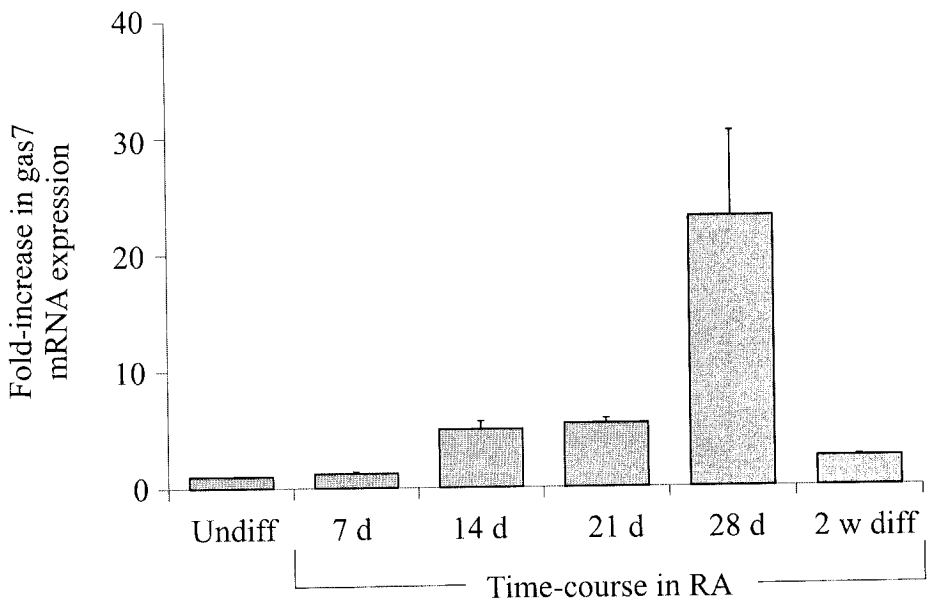
Coronin is an actin-binding protein of 53-57 kDa involved in cell motility and phagocytosis and is expressed in many tissues such as spleen, lung, liver, brain, and kidney (De Hostos, 1999). Sequence analysis with databases from the National Center for Biotechnology Information (NCBI) revealed that coronin does not show any similarity to gas7, at either the nucleic acid or protein levels. No possible link with the gas7 protein could be found from the peptidic sequences identified, suggesting that gas7 was not present in the 60-kDa band recognized by the gas7 C-terminal antibody. Together with the RT-PCR results, showing no expression of gas7 mRNA, this strongly suggests that gas7 is not expressed in PC12 cells.

3.3.2 The expression of gas7 is induced during neuronal differentiation of NT2 cells

cDNA preparations from human NT2 cells, extracted at different stages of neuronal differentiation with retinoic acid (RA), were a kind gift from Dr. Dao Ly (NRC, IBS). Cells were treated with 10 μM of all-trans RA for four weeks and neuronal cells were isolated from the resulting mixed culture and allowed to mature for 2 weeks in RA-free media. Samples were analyzed by Q-PCR to quantify the expression of gas7 mRNA. A prononced increase in gas7 mRNA expression was observed after four weeks of RA treatment (Figure 15). At this time point, the levels of message for gas7 were augmented by 23-fold, compared to that of undifferentiated cells. This induction of gas7 mRNA was transient, as levels in 2-week differentiated NT2 neurons had returned to those in undifferentiated cells. To confirm these results, the expression of gas7 in RA-treated NT2 cells was also analyzed at the protein level. NT2 protein samples, from cells subjected to the same conditions as described above, were provided by Kirsty Malone (NRC, IBS). Proteins from undifferentiated NT2 cells showed a major band below 50 kDa when analyzed by Western blotting with the gas7 full-length antibody (Figure 16A). This protein, which correlated to the predicted molecular weight of the human gas7 protein (47 kDa), was also detected by the gas7 C-terminal antibody, in addition to a major band of about 60 kDa (Figure 16A). The latter probably corresponded to the protein coronin, as identified in PC12 cells. Since the gas7 full-length antibody gave cleaner results, it was used for subsequent analysis of endogenous gas7 expression in NT2 cells. The expression of gas7 protein was increased by 3.6-fold after 4 weeks in RA ($p < 0.001$) and returned to control levels in 2-week mature NT2 neurons ($p > 0.05$) (Figure 15B). The intense induction in gas7 expression during RA treatment of NT2 cells, at both the message and

Figure 15. The expression of gas7 mRNA is induced during retinoic acid treatment of human NT2 cells

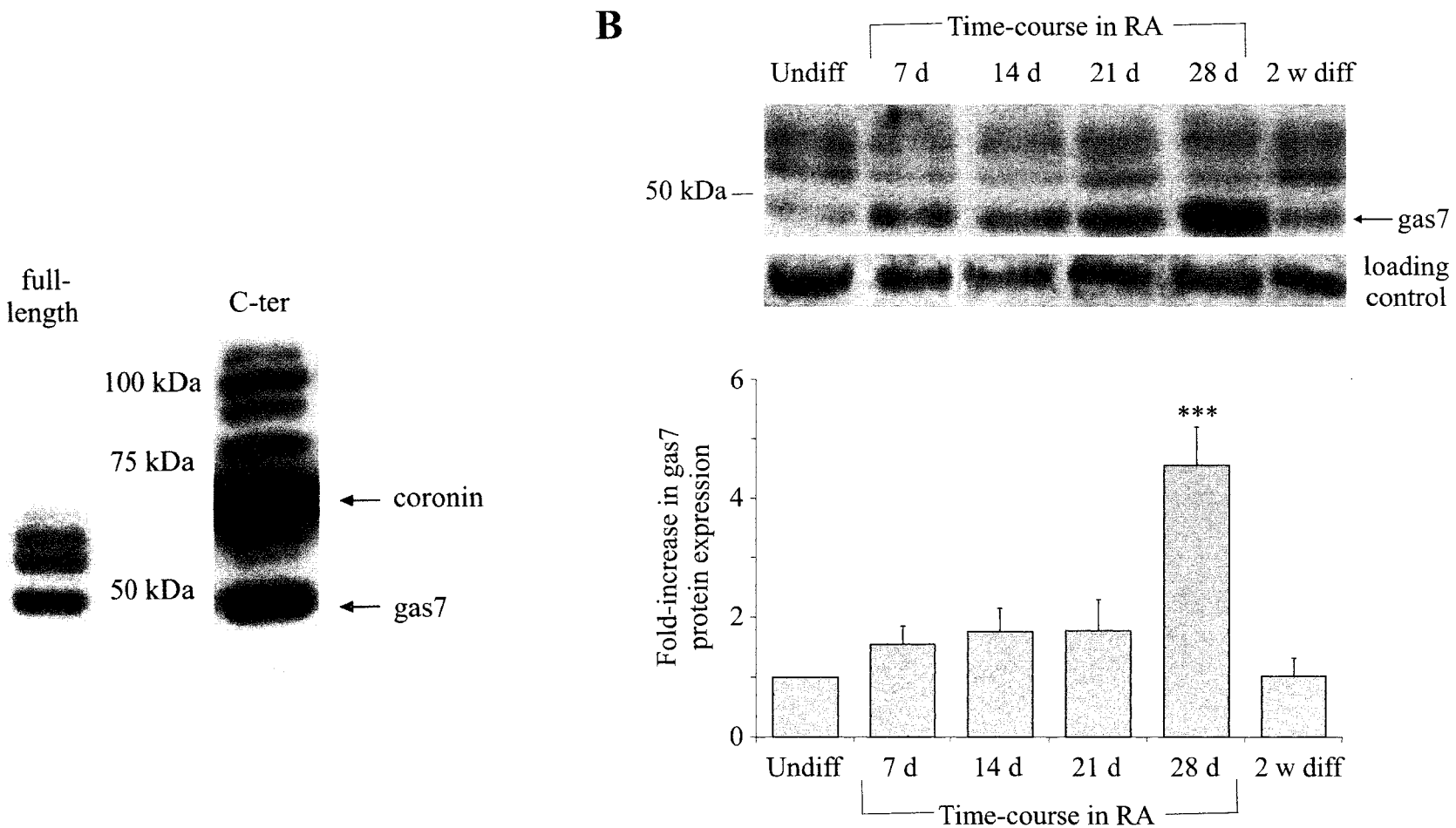
NT2 cDNA samples were obtained at different time points during neuronal differentiation with RA and analyzed by Q-PCR for the expression of gas7 mRNA. A major increase in gas7 mRNA was observed after 4 weeks of RA treatment, while 2-week mature NT2 neuronal cultures (2 w diff) presented similar amounts of gas7 mRNA as undifferentiated cells (no statistical analysis possible due to the unavailability of samples). Data are shown as means ± SEM. Results represent one experiment done in triplicate. Undiff: control undifferentiated NT2 cells.


Fold-increase in gas7 mRNA expression
40
30
20
10
0
Undiff
7 d
14 d
21 d
28 d
2 w diff
Time-course in RA

— Figure 15 —

Figure 16. The expression of gas7 protein is induced during retinoic acid treatment of human NT2 cells

A. Analysis of gas7 expression in undifferentiated NT2 cells by Western blotting with with the gas7 full-length and C-terminal antibodies. The 60-kDa band, identified as the protein coronin, was recognized by the gas7 C-terminal antibody. A major band below 50 kDa, corresponding to the gas7 protein, was recognized by both gas7 antibodies. **B.** Analysis of gas7 protein expression during neuronal differentiation of NT2 cells with RA. Protein samples were analyzed by Western blotting with the gas7 full-length antibody. A major increase in gas7 protein was observed after 4 weeks of RA treatment ($p < 0.001$), while 2-week mature NT2 neuronal cultures (2 w diff) presented similar amounts of gas7 protein as undifferentiated cells ($p > 0.05$). Data are shown as means ± SEM. Levels of gas7 protein were normalized to the intensity of Ponceau S staining, as a loading control, and results represent 3 independent experiments. Data were analyzed by ANOVA and when significant differences were observed, Tukey's test was used for multiple comparisons. Asterisks indicate a mean significantly different from other conditions.


A
full-length
C-ter
100 kDa
75 kDa
50 kDa
coronin
gas7
B
Time-course in RA
Undiff
7 d
14 d
21 d
28 d
2 w diff
50 kDa
gas7
loading control
Fold-increase in gas7 protein expression
6
4
2
0
***
Undiff
7 d
14 d
21 d
28 d
2 w diff
Time-course in RA

— Figure 16—

protein levels, suggests that gas7 could be involved in the differentiaiton process of these cells. These results therefore support a role for gas7 in neuronal differentiation.

3.4 Overexpression of gas7 is not neuroprotective

As previously mentioned, many factors involved in neuronal differentiation were shown to also be involved in pro-survival pathways (Boniece & Wagner, 1995; Corrente *et al.*, 2002; El Ghissassi *et al.*, 2002; Kimura & Schubert, 1992; Pedersen *et al.*, 2004; Spencer *et al.*, 2002). Therefore, in addition to playing an important role in neuronal differentiation, gas7 could have neuroprotective properties. Three different toxicity assays were established, on the basis of their relevance to neuronal damage. Oxygen-glucose deprivation (OGD) is a paradigm that mimics the lack of energy experienced when global or local cerebral circulation is disrupted, such as during stroke. The calcium ionophore A23187 induces calcium influx, and therefore elevated intracellular calcium concentrations, while sodium nitroprusside is a nitric oxide donor and results in oxidative stress. These last two insults are known to contribute to cell damage in pathophysiological conditions including stroke, Parkinson's, and Alzheimer's (Honda *et al.*, 2004; Tabakman *et al.*, 2002; Ter Host & Postigo, 1997; Verkhratsky & Toescu, 2003).

Undifferentiated and differentiated (6 days in differentiation media) cells were subjected to the insults at various severities, to determine their potential to cause cell death. Viability was assessed with the Alamar Blue assay. To be able to assess the potential

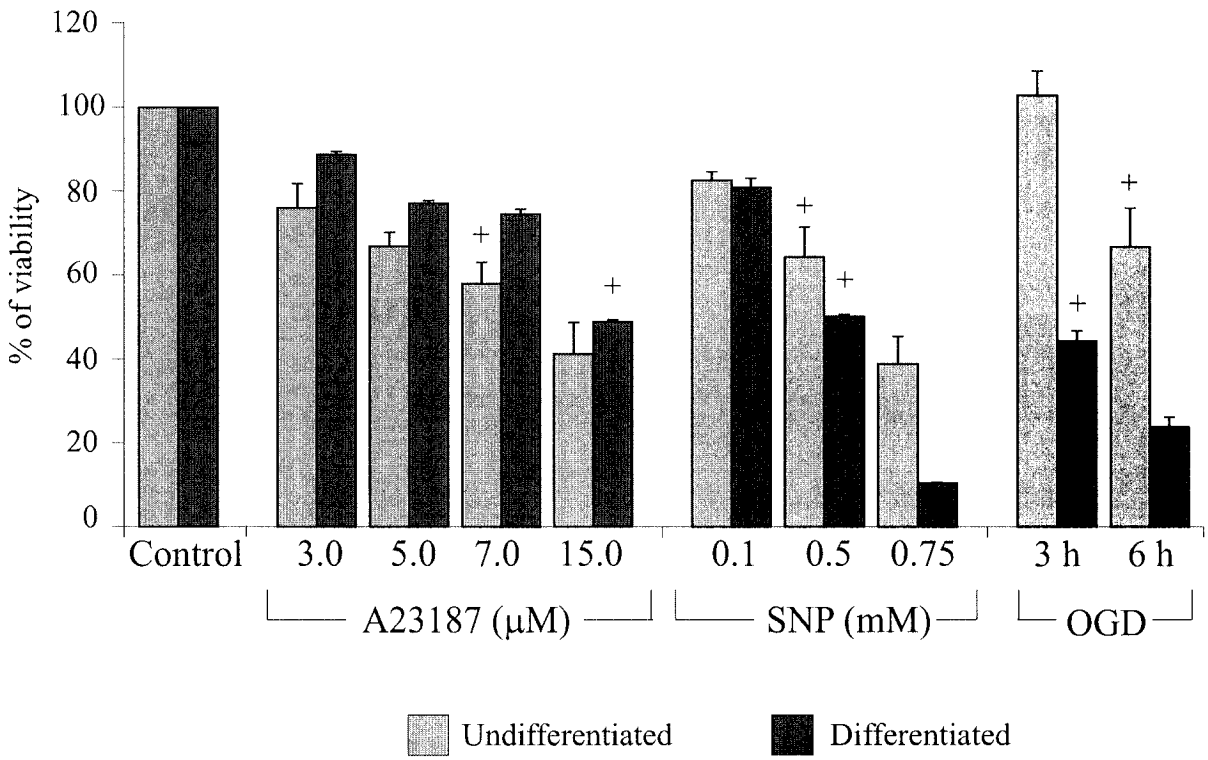
neuroprotective role of gas7, conditions leading to 40-60% cell death were established (Figure 17). The conditions selected for undifferentiated cells were 7.0 μM A23187, 0.5 mM SNP (both for 16 hours), and 6 hours of OGD. For the differentiated cells, the chosen insults were 15.0 μM A23187, 0.5 mM SNP (both for 16 hours), and 3 hours of OGD.

3.4.1 Gas7 overexpression does not protect PC12 cells against lethal insults

Once the toxicity paradigms were defined, the objective was to determine if gas7 could diminish the damage. Undifferentiated and 4-days differentiated PC12 cells were infected with the GFP or gas7 adenoviruses and toxicity assays were performed 48 hours later. The infection process itself did not change the cells' response to the insults, since cells infected with the control virus showed similar viability percentages as non-infected cells ($p > 0.05$) (Figure 18). Overexpressing the gas7 protein did not have any effect either ($p > 0.05$), suggesting that gas7 is not neuroprotective in the models used (Figure 18).

Figure 17. PC12 cell viability is challenged by A23187, sodium nitroprusside (SNP), and oxygen-glucose deprivation (OGD)

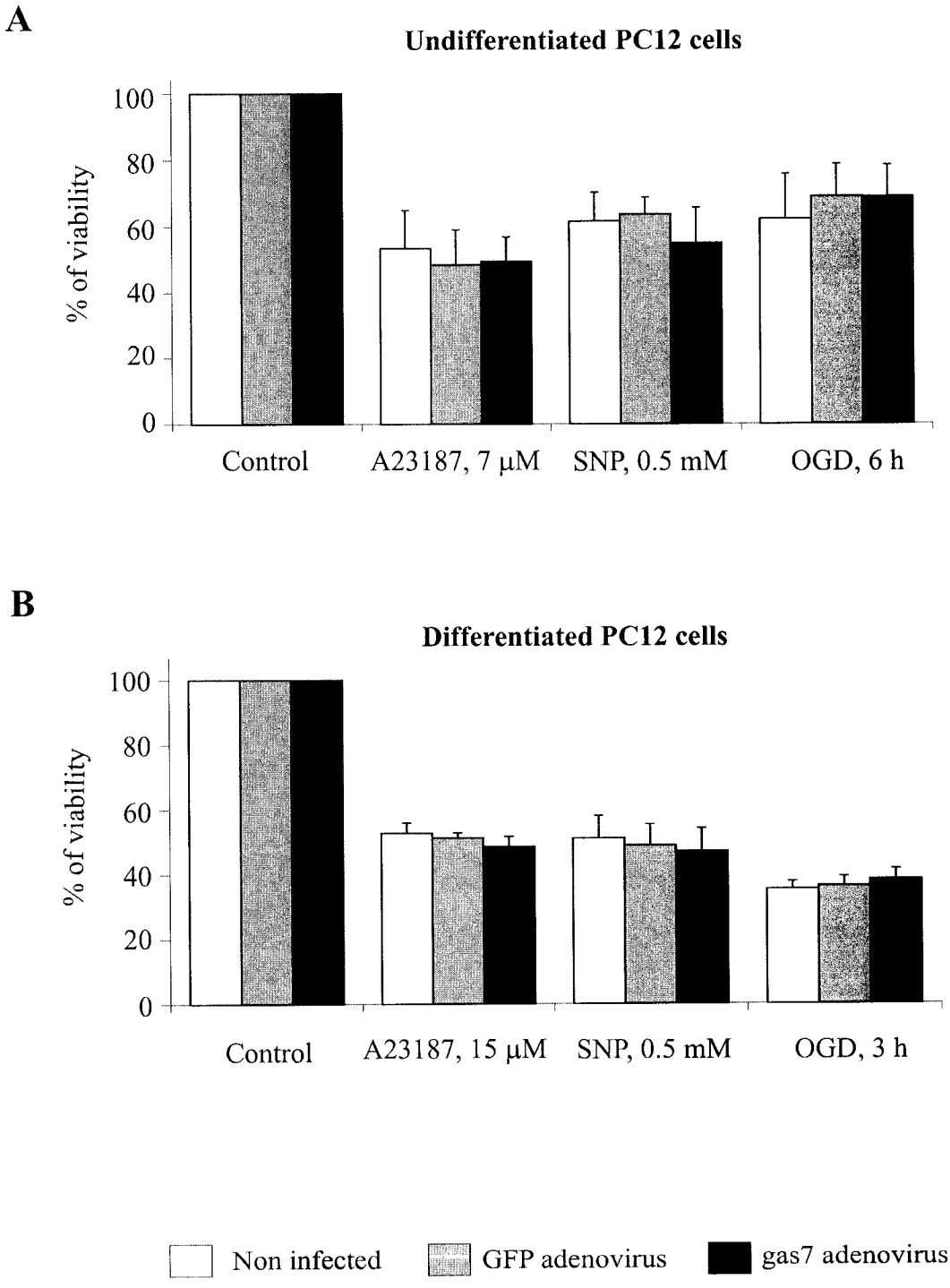
Undifferentiated and differentiated (6 days in differentiation media) PC12 cells were treated with different concentrations of A23187 and SNP for 16 hours, or subjected to OGD for different periods of time followed by 16 hours of reoxygenation. Cell viability was then assessed with the Alamar Blue assay. Other than for 3h of OGD with the undifferentiated cells, all conditions resulted in a significant decrease of viability ($p < 0.05$) and increased intensities of the insults further reduced cell viability. Data are shown as means ± SEM. Results represent 3 independent experiments. Data were analyzed by ANOVA and when significant differences were observed, Dunnett's test was used for multiple comparisons. Control: untreated cells; + : conditions chosen for subsequent experiments.


120
100
80
60
40
20
0
% of viability
Control
3.0
5.0
7.0
15.0
0.1
0.5
0.75
3 h
6 h
A23187 (μM)
SNP (mM)
OGD
Undifferentiated
Differentiated

— Figure 17 —

Figure 18. Overexpression of gas7 does not protect against lethal insults

Undifferentiated and differentiated (4 days in differentiation media) PC12 cells were infected with the GFP or gas7 adenoviruses and toxicity assays were performed 48 hours post-infection. Cells were incubated with A23187 or SNP at the indicated concentrations for 16 hours, or subjected to OGD for the indicated periods of time followed by 16 hours of reoxygenation. Viability was assessed using the Alamar Blue assay. Overexpression of gas7 did not affect the cells' response to the different insults ($p > 0.05$). Data are shown as means ± SEM. Results represent 4 independent experiments and were analyzed by ANOVA. Control: untreated cells.


A
Undifferentiated PC12 cells
% of viability
100
80
60
40
20
0
Control
A23187, 7 μM
SNP, 0.5 mM
OGD, 6 h
B
Differentiated PC12 cells
% of viability
100
80
60
40
20
0
Control
A23187, 15 μM
SNP, 0.5 mM
OGD, 3 h
Non infected
GFP adenovirus
gas7 adenovirus

— Figure 18—

CHAPTER 4

DISCUSSION

Neurodegenerative conditions including stroke, Alzheimer's, and Parkinson's are associated with high morbidity, affecting various functions such as cognition and behavior. Since few or no effective options are available for their treatment, there is a great need for new rehabilitative strategies. Re-establishment of neuronal connections and formation of alternate communication pathways, through neurite sprouting from uninjured neurons as well as migration and differentiation of neuronal precursors, are believed to be important for brain functional recovery (Stroemer *et al.*, 1995). Better understanding of the mechanisms involved in neuron formation and neurite outgrowth could therefore lead to improvements in neuronal regeneration strategies. The gas7 protein, which was shown to be involved in neuronal differentiation and neuritogenesis, is an interesting candidate for such approaches to treat neurodegeneration (Chao *et al.*, 2003; Ju *et al.*, 1998). This thesis aimed at characterizing the role of gas7 in neuronal differentiation and to assess its potential neuroprotective role.

4.1 gas7 potentiates neuronal differentiation

4.1.1 gas7 promotes neurite outgrowth

Adenovirus-mediated overexpression of the murine gas7 full-length protein induced the formation of short neurite-like processes in PC12 cells. Even though the expression of gas7 was detected 48 hours after infection, these morphological changes appeared only after 72 hours, suggesting that a certain time is required for gas7 to exert its effects on cell morphology. This observation is similar to that of Chao *et al.* (2003), who saw

expanded cell shape and neurite-like outgrowth 36 hours after transfection of PC12 cells with a gas7-expressing construct (pmGas7-EGFP; contained the murine gas7 full-length cDNA fused to enhanced GFP), while the expression of gas7 could be detected as early as 24 hours following transfection. Overproduction of gas7 with another gas7-expressing plasmid (pcDNA3gas7; contained the murine gas7 full-length cDNA) was also shown to promote the formation of abundant and lengthy neuronal-like extensions in Neuro 2A mouse neuroblastoma cells (Ju *et al.*, 1998). The results obtained in this thesis thus support the suggested role for gas7 in neurite outgrowth, by showing that gas7 promotes the development of a phenotype corresponding to the early stages of neuronal development. Short membrane outgrowths have also been reported to be induced following transfection of NIH3T3 murine fibroblasts with pcDNA3gas7, suggesting that the role of gas7 in the formation of cell processes might not be limited to neuronal cell types (She *et al.*, 2002).

When PC12 cells infected with the gas7 adenovirus were treated with NGF for neuronal differentiation (50 ng/mL, 72 hours), cells displayed long and highly branched processes, compared to the simple neurite outgrowth elicited in cells infected with a control virus. This was also observed by Chao's group, who showed that gas7-induced membrane extensions in pmGas7-EGFP-transfected PC12 cells were longer and more abundant in the presence of NGF (Chao *et al.*, 2003). The formation of complex neurite networks further indicates that gas7 can promote neurite outgrowth and suggests that gas7's effects on morphology are additive to those of NGF.

She and colleagues (2002) suggested that the dramatic effects of gas7 on cell morphology could be mediated through its ability to induce reorganization of the actin cytoskeleton. Changes in actin organization are indeed essential for neurite outgrowth. Rapid extension of neurites and their exploration of the environment require polymerization of actin filaments towards the tips of filopodia, whereas actin meshworks in lamellipodia provide adhesion and tension for growth cone movement (Da Silva & Dotti, 2002; Jay, 2000). She *et al.* (2002) showed that gas7 directly binds to F-actin. Protein samples from contact-inhibited NIH3T3 fibroblasts were run on F-actin affinity chromatography columns and the gas7 protein was only eluted in very stringent conditions, suggesting tight interactions (She *et al.*, 2002). *In vitro* actin assembly assays revealed that gas7 promotes actin polymerization and electron microscopy analysis showed that gas7 can also induce the formation of actin filament bundles, by mediating crosslinking of actin filaments (She *et al.*, 2002). In addition, the observed localization of gas7 at the cell membrane, which was essential for gas7 to promote changes in morphology, supports the model in which gas7 directly interacts with actin to promote the formation of membrane extensions (Chao *et al.*, 2003; She *et al.*, 2002). The requirement for actin polymerization during gas7-induced morphological changes was confirmed by experiments with the actin depolymerizing agent, cytochalasin D. Disruption of microfilament organization prevented the membrane-localized expression of ectopic gas7 and inhibited the formation of membrane protrusions following overexpression (She *et al.*, 2002). Therefore, actin polymerization was suggested to be essential for gas7's effects on cell morphology.

gas7 was shown to directly bind F-actin through its carboxy domain, since in addition to the full-length gas7 protein, a purified C-terminal gas7 peptide also bound the F-actin column, but not a gas7 N-terminal peptide (She *et al.*, 2002). Polymerization of actin and crosslinking of filaments were also observed in the presence of the C-terminal peptide, but not with the N-terminal one (She *et al.*, 2002). In addition, the carboxy domain was shown to be responsible for the membrane localization of gas7, since the gas7-related protein produced after transfection of NIH3T3 cells with the gas7 C-terminal sequence, but not with the N-terminal, was enriched at the membrane (She *et al.*, 2002). The potential coiled-coil domain in the carboxy region of gas7 is thought to be involved in the interactions between gas7 and F-actin (She *et al.*, 2002). Coiled coils are known to promote protein-protein interactions and to participate in various molecular recognition systems, such as oligomerization of transcription factors and formation of core complexes involved in membrane fusion (Burkhard *et al.*, 2001; Lupas, 1996). The C-terminal region of gas7 shows homology with the domain of the cytoskeleton-associated protein PSTPIP responsible for the protein localization in actin-rich regions (She *et al.*, 2002; Wu *et al.*, 1998). This supports the role of the carboxy domain in gas7 membrane localization. The presence of a FCH domain in the C-terminal portion of gas7 could also participate in mediating gas7-induced morphological changes, since many FCH domain-containing proteins regulate cytoskeletal rearrangements (Fujita *et al.*, 2002; Takahashi *et al.*, 2003; Tian *et al.*, 2000). The above observations support the hypothesis that the carboxy region of gas7 would mediate its binding to F-actin, and thereby its effects on cell morphology (She *et al.*, 2002).

However, there are a few uncertainties about the role played by gas7-actin interactions in gas7-induced changes in cell morphology. First, gas7 does not possess any known actin-binding motif, and the binding of gas7 to actin was only shown in cell-free assays (She *et al.*, 2002). Even though the membrane localization of gas7 supports its possible interaction with actin, the direct binding has not been confirmed in a cellular context (Chao *et al.*, 2003; She *et al.*, 2002). Co-immunoprecipitation assays have failed because of the tendency of gas7 to aggregate (She *et al.*, 2002). Other methods, such as enzyme complementation and fluorescence resonance energy transfer (FRET) assays could be useful tools to study the interactions between gas7 and actin in living cells. Moreover, gas7 binding to actin was shown not to be sufficient for gas7-induced morphological changes. Indeed, transfection of either the C-terminal or the N-terminal sequences of gas7 did not affect cell morphology, suggesting that the carboxy-mediated interactions with actin are not sufficient for gas7-induced changes in morphology (She *et al.*, 2002). The effects of gas7 on cell shape could thus only partly be explained by its interactions with actin.

Since only the full-length gas7 protein could induce membrane protrusions, the N-terminal domain is probably also involved in gas7-mediated neurite outgrowth (She *et al.*, 2002). The WW motif in the amino domain of gas7, known to mediate protein-protein interactions through binding of proline-rich regions, might be important for gas7-induced morphological changes (Ilsley *et al.*, 2002).

Another mechanism involved in neuronal morphogenesis is signaling through the Rho GTPases Rho, Rac, and Cdc42. These proteins are known to be key regulators of neurite growth, guidance, and branching (Luo, 2000). Rho activity induces neurite retraction, while Rac and Cdc42 promote elongation. The balance between these positive and negative stimuli results in neurite navigation towards specific targets (Luo, 2000). Regulation of neurite outgrowth by Rho GTPases is mediated by selective stabilization of the actin cytoskeleton, through activation of various kinases and actin binding proteins, such as cofilin, profilin, and others (Luo, 2000). There is thus a possibility that the role of gas7 in the development of neuronal morphology could be linked to the Rho GTPases pathways. It would be interesting to study the effects of dominant negative mutant forms of Rho, Rac, and Cdc42 on gas7-induced morphological changes. Moreover, since gas7 was shown to have numerous potential phosphorylation sites, it would also be of interest to determine if Rho GTPases affect the phosphorylation state of gas7 (Chao *et al.*, 2003).

4.1.2 gas7 potentiates NGF induction of neuronal markers

Following the demonstration of gas7's ability to promote neurite outgrowth, the next step consisted of the study of gas7's effects on the expression of neuronal markers. During the neuronal differentiation process, precursor cells are induced to express various neuronal proteins to become functional neurons. Evaluating the levels of expression of such proteins can be used to determine the effects of potential drugs on the progression of neuronal differentiation. When treated with NGF, PC12 cells are induced to express neuron-specific proteins such as TUJ-1, a protein expressed in growing neurites, STG, a pre-synaptic vesicle protein, AChR-α7, a subunit of neuron-specific nicotinic

acethylcholine receptors, and DRP-3, an axonal protein (Byk *et al.*, 1998; Henderson *et al.*, 1994; Minturn *et al.*, 1995; Ohuchi *et al.*, 2002; Takahashi *et al.*, 1999). The effects of gas7 overexpression on PC12 neuronal differentiation were evaluated by studying changes in the expression of these neuronal markers.

Even though adenovirus-mediated overexpression of gas7 in undifferentiated PC12 cells induced neurite-like outgrowth, these changes were not accompanied by the induction of neuronal marker expression. After 72 hours, cells infected with the gas7 adenovirus expressed TUJ-1 and STG levels that were similar to those observed in cells infected with the control adenovirus and in non-infected cells. These results agree with the findings of Ju *et al.* (1998), who showed that gas7 overexpression in Neuro 2A cells (with pcDNA3gas7) induced neuronal-like morphological changes, but did not promote the expression of the neuronal marker MAPII, which is usually associated with retinoic acid-mediated differentiation of these cells (Fischer *et al.*, 1986). In addition, downregulation of gas7 in cultured mouse cerebellar Purkinje neurons was shown to prevent the development of neuronal morphology, but did not inhibit the expression of calbindin, a marker of neuronal maturation (Ju *et al.*, 1998). gas7 was thus suggested to be involved in morphological, but not biochemical, neuronal differentiation (Ju *et al.*, 1998). In other words, gas7 might only act on the cytoskeleton structure, and not on the regulation of neuronal gene expression.

However, while gas7 did not induce the expression of neuronal markers on its own, results from this thesis showed that it potentiated the induction of TUJ-1 and STG

expression triggered by NGF (50 ng/mL, 72 hours) in PC12 cells. The complex neuritic pattern induced in the same conditions thus correlated with an increased expression of neuronal proteins. These results further suggest that the effects of gas7 and NGF on neuronal development are additive. To further characterize the ability of gas7 to potentiate neuronal differentiation, the effects of gas7 overexpression were studied in sub-optimal differentiation conditions. Undifferentiated PC12 cells were infected with the gas7 adenovirus and treated with low concentrations of NGF (10 ng/mL) for different periods of time. After 48 hours, gas7-overexpressing PC12 cells showed a significant induction in the expression of the neuronal markers TUJ-1, STG, AChR-α7, and DRP-3. These results suggest that gas7 synergized with low concentrations of NGF to trigger biochemical differentiation. In the same conditions, no indication of morphological differentiation was observed, which is consistent with the findings that neuronal markers are usually expressed before the appearance of neuronal morphology. The potential synergy between gas7 and NGF after longer periods of time could not be studied because the infection process was eventually toxic. Despite the fact that gas7 overexpression alone was not sufficient to induce neuronal differentiation, it sensitized the cells to respond more readily to NGF and therefore potentiated differentiation in sub-optimal conditions.

Various molecules, such as the cell cycle inhibitor PC3, ascorbic acid derivatives, and different plant extracts, have been shown to potentiate NGF differentiation of PC12 cells (Corrente *et al.*, 2002; Li *et al.*, 2000; Liu *et al.*, 2003; Shigeta *et al.*, 2002; Zhou *et al.*, 2003). PC3 was suggested to "prime" neuronal precursors for differentiation by its ability

to arrest the cell cycle (Corrente *et al.*, 2002). Ascorbate derivatives and plant alkaloid extracts are thought to enhance NGF-induced differentiation by increasing phosphorylation of the microtubule associated protein kinases (MAPKs) p42 and p44, involved in NGF signaling, while picrosides I and II, natural substances with anti-inflammatory effects, would amplify a downstream step of the same MAPKs (Li *et al.*, 2000; Liu *et al.*, 2003; Zhou *et al.*, 2003).

The expression of gas7 protein and mRNA in NIH3T3 fibroblasts was shown to be induced by contact inhibition or serum starvation, and to return to control levels upon replating or addition of serum (Brenner *et al.*, 1989; Ju *et al.*, 1998; She *et al.*, 2002). This suggests a potential link between gas7 expression and cell cycle regulation. Moreover, various members of the growth-arrest-specific family have been shown to be involved in controlling cell cycle progression (Brancolini & Schneider, 1994; Brenner *et al.*, 1989; Claesson-Welsh, 1996; Goruppi *et al.*, 1996; Karlsson *et al.*, 1999; Li *et al.*, 1996; Rozental *et al.*, 2000; Ruaro *et al.*, 2000; Zoidl *et al.*, 1995; Zoidl *et al.*, 1997). The possibility that gas7 potentiates neuronal differentiation through regulation of the cell cycle was investigated. However, results showed that gas7 does not affect cell cycle progression. Rates of cell growth were evaluated by measuring chemical reduction of the Alamar Blue dye, and the number of proliferating cells was quantified by staining for the proliferation marker KI-67. Since the KI-67 protein is expressed in all phases of the cell cycle except G0, it is a better indicator of cell proliferation than bromodeoxyuridine (BrdU) incorporation. BrdU is only incorporated during the S phase of the cell cycle and therefore neglects the presence of proliferating cells in the G1, G2, and M phases. Alamar

Blue assay and KI-67 staining showed that gas7-overexpressing PC12 cells proliferated at a similar rate as control cells. Therefore, the potentiating effects of gas7 on neuronal differentiation do not involve regulation of the cell cycle.

As previously mentioned, gas7 was suggested to induce morphological changes partly through binding of F-actin, promoting assembly of microfilaments (She *et al.*, 2002). Could this mechanism also be implicated in the potentiation of neuronal marker expression during differentiation of PC12 cells? The cytoskeleton organization and state of actin polymerization were shown to affect expression and/or phosphorylation of different proteins, such as vinculin, Rho, c-Abl tyrosine kinase, and cortactin (Bershadsky *et al.*, 1995; Fan *et al.*, 2004; Laufs *et al.*, 2000; Woodring *et al.*, 2001). The Fyn and Fer kinases are thought to be involved in actin depolymerization-mediated phosphorylation of cortactin, but the complete mechanisms by which the state of actin polymerization regulates gene expression and protein tyrosine phosphorylation are still unclear (Bershadsky *et al.*, 1995; Fan *et al.*, 2004; Laufs *et al.*, 2000; Woodring *et al.*, 2001). There is thus a possibility that gas7-induced remodeling of the actin cytoskeleton could trigger gene expression signals. Although the above examples of actin-mediated regulation of gene expression and protein phosphorylation all involved proteins controlling the cytoskeleton, it is possible that during neuronal differentiation, gas7-actin interactions could regulate neuronal genes and/or proteins such as TUJ-1, STG, AChR-α7, and DRP-3. This possibility could be assessed by evaluating the ability of gas7 to potentiate neuronal marker expression in the presence of actin depolymerizing agents, such as cytochalasin D.

Another potential site of action for gas7 to act on neuronal gene expression could be the MAPK signaling to the extracellular signal related kinases (ERKs). This pathway is considered to be a major mediator of NGF differentiation, as ERK activity was shown to be essential for NGF-mediated differentiation of PC12 cells (Marshall, 1995; Traverse *et al.*, 1992; Xiao & Liu, 2003). Upon NGF stimulation, ERKs are translocated to the nucleus and regulate gene expression by phosphorylating transcription factors either directly, as for Elk-1, or indirectly, as for the cAMP response element binding protein (CREB) (Ahn *et al.*, 1998; Horgan & Stork, 2003; Traverse *et al.*, 1992; Vanhoutte *et al.*, 2001). Gene expression mediated by ERK-activated Elk-1 and CREB was suggested to be highly involved in NGF-induced differentiation of PC12 cells (Ahn *et al.*, 1998; Vanhoutte *et al.*, 2001). In the human neuroblastoma SH-SY5Y cell line, ERK activity was shown to mediate the expression of the neuronal proteins neuropeptide Y and GAP-43, whereas in rat striatal cultured neurons, inhibition of ERK blocked the expression of tyrosine hydroxylase, an enzyme essential for the production of dopamine (Guo *et al.*, 1998; Olsson & Nanberg, 2001). These observations suggest that the ERK pathway could also be involved in the expression of other neuronal markers, such as TUJ-1, STG, AChR-α7, and DRP-3. It is thus possible that the potentiating effects of gas7 on the expression of these proteins be linked to the ERK pathway. To further investigate this hypothesis, it would be interesting to study the effects of MAPK inhibitors on gas7-potentiation of neuronal differentiation and on gas7 phosphorylation state, as well as to determine the effects of gas7 overexpression on MAPK phosphorylation.

Since gas7 triggered the expression of neuronal markers only in the presence of NGF, gas7's signals for neuronal gene expression might require interactions with molecules induced or activated by NGF, such as specific kinases or transcription factors. It is also possible that gas7 and NGF act on common target proteins. To dissect the pathways in which gas7 is involved, its effect on neuronal differentiation triggered by factors other than NGF, for example cAMP and RA, should be determined.

4.2 gas7 is not endogenously expressed in PC12 cells

Since the first identification of gas7 in murine NIH3T3 fibroblasts, its endogenous expression has also been shown in mouse and rat brain tissues and in rat PC12 cells (Brenner *et al.*, 1989; Chao *et al.*, 2003; Ju *et al.*, 1998; She *et al.*, 2002). Because PC12 cells were the only neuronal cell line in which gas7 had been shown to be expressed, and because cell lines are easy to manipulate, PC12 cells were the ideal model to study the role of endogenous gas7 in neuronal differentiation.

Western blot analysis of PC12 protein extracts with the gas7 C-terminal antibody showed the expression of a 60-kDa gas7 isoform as mentioned by Chao *et al.* (2003). Because the antibody used in their study was coming from the same source as the one used here (Dr. Sue Lin-Chao, Institute for Molecular Biology, Academica Sinica, Nankang, Taipei, Taiwan), it was believed that the 60-kDa band represented the protein observed by Chao's group (Chao *et al.*, 2003). However, while Chao and colleagues (2003) showed a rapid and transient increase in the expression of this so-called gas7 band during treatment

of PC12 cells with NGF, no differential pattern of expression was seen in this study. Furthermore, no gas7 mRNA could be detected in PC12 cells. The 60-kDa protein was enriched by immunoprecipitation and mass spectrometry analysis identified it as the protein coronin, which does not present any similarity to gas7. It is possible that the gas7 C-terminal antibody might not have been 100% pure and that it bound non-specifically to coronin. Since the murine full-length gas7 protein overexpressed in PC12 cells with the gas7 adenovirus was specifically recognized by this antibody, the gas7 protein would probably have been detected if it had been expressed in the PC12 cells. Together with the absence of gas7 mRNA, these observations suggest that gas7 was not expressed in the PC12 cell model studied.

The results presented here are not in agreement with the publication from Chao *et al.* (2003), who proposed that gas7 could be essential for neuronal differentiation of PC12 cells. Were they really studying the gas7 protein, or were they looking at coronin? The only indication from Dr. Chao's work supporting the identity of the 60-kDa gas7 isoform comes from downregulation experiments. They showed that treatment of PC12 cells with a gas7 antisense DNA oligonucleotide reduced the expression of the 60-kDa band (Chao *et al.*, 2003). The properties of cell lines can vary from one clone to another, and so it is possible that the PC12 cells used in this study could have been different from those used by Dr. Chao's group. gas7 might have been expressed in their PC12 cells, but not in the ones used for this thesis.

The predicted molecular weight for the rat gas7 protein is 48 kDa (accession number O55148; Swiss-Prot). Chao *et al.* (2003) mentioned that the gas7 isoform observed in their study (60 kDa) was larger than other isoforms observed in brain tissues, but they did not comment on this difference. Various types of post-translational modifications could explain such an increase in molecular weight. However, the gas7 protein observed by Ju *et al.* (1998) in mouse brain tissue and the band recognized by the gas7 antibodies in NT2 cells were of the predicted molecular weight (48 and 47 kDa, respectively). These observations suggest that the gas7 protein is usually not subjected to post-translational modification. Moreover, Dr. Chao's group did not do any sequence analysis to confirm the identity of the observed protein, as opposed to Ju *et al.* (1998), who showed that the N-terminal portion of the 48-kDa gas7 band observed in mouse brain tissue corresponded to the sequence predicted by the gas7 cDNA isolated in growth-arrested NIH3T3 fibroblasts. In addition, Chao and colleagues (2003) did not validate the expression of gas7 at the RNA level. While they also mentioned the presence of the 60-kDa gas7 isoform in mouse kidney tissue, and thus suggested that gas7 could also have a function in non-neuronal cells, Ju *et al.* (1998) showed that gas7-related RNA species were absent from mouse kidney tissue. This discrepancy adds to the concerns about the identity of the protein observed by Dr. Chao's group.

Overall, the results presented in this thesis showed that gas7 was not expressed in the PC12 cell model. Furthermore, since these cells could be differentiated into neuron-like cells with NGF, it can also be concluded that gas7 was not essential for their differentiation.

4.3 gas7 could be involved in neuronal differentiation of NT2 cells

The NT2 cell line is a human model of neuronal precursor cells. The embryonic nature of these cells, which are considered closely related to human embryonic stem cells, makes them a relevant system to study the mechanisms involved in neuron formation (Freemantle *et al.*, 2002). Since cDNA and protein samples were available, it was of interest to investigate the endogenous expression of gas7 during their neuronal differentiation with RA.

A marked augmentation in both gas7 mRNA and protein was shown after 4 weeks of RA treatment, and the expression had returned to control levels in 2-week mature NT2 neurons. This upregulation suggests that gas7 could be involved in RA-mediated neuronal differentiation of NT2 cells. Even though the study of endogenous gas7 expression in PC12 cells raised some concerns about the specificity of the gas7 antibodies, evidence strongly supports that the protein of detected in NT2 samples (just below 50 kDa) was gas7. First, the clear correlation between the patterns of gas7 mRNA and protein expression suggests that they represent the same gene. Moreover, the observed band corresponded to the molecular weight predicted for the human gas7 protein, of 47 kDa (accession nnumber O60861; Swiss-Prot). Finally, this gas7 isoform was recognized by both the gas7 full-length and C-terminal antibodies, further supporting that the detected protein was gas7.

After 4 weeks in RA, NT2 cultures contain commited neuronal and astrocyte precursors, as well as different types of neurons, such as dopaminergic and GABAergic neurons, and

differentiated astrocytes (Sandhu *et al.*, 2002; Sandhu *et al.*, 2003; Zigova *et al.*, 1999). Therefore, one could argue that the increase in gas7 cannot be linked to neuronal differentiation. However, gas7 expression in the mouse brain was shown to be restricted to neurons and was not seen in glial cells (Ju *et al.*, 1998). This suggests that gas7 expression in NT2-derived cells is probably specific to neurons and thus that it might be associated with their neuronal differentiation.

NT2 cells were shown to receive growth arrest signals very quickly following the addition of RA. The expression of the following anti-proliferative genes was shown to be upregulated after 8 hours of RA treatment: chimerin 2, a GTPase activating protein downregulated in highly proliferating gliomas, PC3, an inhibitor of cyclin D1, and hepatocyte growth factor-regulated tyrosine kinase substrate, a negative regulator of cytokine-induced DNA synthesis (Asao *et al.*, 1997; Corrente *et al.*, 2002; Freemantle *et al.*, 2002; Yuan *et al.*, 1995). The late induction of gas7 expression, which occurred only 4 weeks after addition of RA, would thus indicate that gas7 is probably not involved in regulating the NT2 cell cycle. This is in agreement with previous findings in this thesis, showing that gas7 does not affect cell proliferation of PC12 cells. Neuronal fate commitment of RA-treated NT2 cells was suggested to happen within 24 to 48 hours, whereas terminal differentiation into different neuronal types would occur after more than 3 weeks of RA treatment (Cheung *et al.*, 1997; Freemantle *et al.*, 2002). Therefore, gas7 is probably involved in late stages of NT2 neuronal differentiation, such as development and maturation of the neuronal phenotype. Data from the literature and in this thesis show that gas7 can promote neurite outgrowth (Chao *et al.*, 2003; Ju *et al.*,

1998). After 4 weeks of RA treatment, the morphology of NT2 cultures could not be examined since in addition to forming different cell types (neurons and glia), the cells were organized in a dense multi-layered structure (Sandhu *et al.*, 2002). It was therefore not possible to establish a correlation between the expression of gas7 and specific changes in morphology. However, when NT2 cells are replated after 4 weeks in RA, neuron-like cells with short membrane extensions can be observed immediately after replating (Sandhu *et al.*, 2002). It is thus possible that the high expression of gas7 in NT2 cells after 4 weeks in RA is involved in neurite formation. As it was shown for NGF-treated PC12 cells, gas7 expression in NT2 cells might also be involved in promoting the expression of neuronal proteins. Finally, since the expression of gas7 had returned to control levels in 2-week mature NT2 neurons, gas7 probably does not play any role in maintenance and survival of neurons.

NT2 cells were thus shown to endogenously express gas7, at both the message and protein levels, and would be a good model for future research on the role of gas7 in neuronal differentiation. However, before pursuing such work it would be important to confirm the identity of the gas7 band detected in NT2 protein samples. Immunoprecipitation followed by mass spectrometry analysis, as used in this study to identify the so-called 60-kDa gas7 isoform expressed in PC12 cells, would be suitable. In addition, even though gas7 expression in the mouse brain was previously shown to be neuron-specific, this should be confirmed in the NT2 model since as mentioned above, NT2 cells can differentiate into both neurons and astrocytes upon RA treatment (Ju *et al.*, 1998; Sandhu *et al.*, 2002; Sandhu *et al.*, 2003). Finally, it would be interesting to

determine if gas7 expression in NT2 cells is specific to certain neuronal types, for example if it specifically localizes in dopamine- or GABA-expressing neurons (Zigova *et al.*, 1999). To understand the mechanisms leading to the induction of gas7, the effects of growth factors other than RA, for example NGF, EGF, and FGF, on the expression of gas7 in NT2 cells should also be evaluated. Furthermore, the developmental pattern of gas7 expression in the mammalian brain could be studied, to correlate the induction of gas7 during neuronal differentiation of NT2 cells to the mammalian neuronal development program.

4.4 gas7 is not neuroprotective

The ability to promote neuronal differentiation is often accompanied by a neuroprotective role. Various neurotrophic molecules, such as NGF, cAMP, RA, PC3, Rit, and NCAM, were shown to protect cells against different insults including ischemia, oxidative stress, and growth factor withdrawal (Boniece & Wagner, 1995; Corrente *et al.*, 2002; El Ghissassi *et al.*, 2002; Kimura & Schubert, 1992; Pedersen *et al.*, 2004; Spencer *et al.*, 2002; Wada *et al.*, 1996). The possibility for gas7 to protect cells against various insults was thus investigated. Oxygen-glucose deprivation (OGD) and incubation with the calcium ionophore A23187 or the nitric oxide donor sodium nitroprusside (SNP) were selected as the insults, to cover a range or cell death triggers: energy depletion, calcium overload, and oxidative stress, respectively. These toxicity assays have been widely characterized in the PC12 system (Abu-Raya *et al.*, 1993; Boniece & Wagner, 1993; Desole *et al.*, 1998; Ray *et al.*, 2000; Tabakman *et al.*, 2002; Wada *et al.*, 1996).

However, adenoviral-mediated gas7 overexpression in PC12 cells did not have any effect on OGD, A23187, and SNP toxicity, in either undifferentiated or differentiated cells. This suggests that gas7 is not a neuroprotective molecule.

Even though gas7 overexpression did not protect the cells, the possibility that it could potentiate the protection elicited by other factors, such as NGF, was not investigated. NGF is known to promote its survival effect mainly through the activation of PI3-kinase. Various inhibitors of this pathway, such as wortmannin and LY294002, prevent neurotrophin-mediated neuroprotection (Klesse & Parada, 1999; Spear *et al.*, 1997). The mechanisms of protection are not clear, but could involve inhibition of caspase-9, an initiator of apoptosis, and the anti-apoptotic protein Bcl-2 (Klesse & Parada, 1999). Since it was shown that gas7 can potentiate the effects of NGF on neuronal differentiation, it is possible that it could also potentiate NGF-induced neuroprotection. In order to test such a role, it would be of interest to evaluate if gas7 overexpression promotes cell viability of NGF-treated undifferentiated cells subjected to lethal insults. Alternatively, gas7 might be endowed with neuroprotective properties that in PC12 cells, where it is not endogenously expressed, do not find the proper context, such as appropriate target proteins. The potential protective role of gas7 should thus be assessed in a cell model which endogenously express gas7, such as human NT2 cells and primary cortical cultures.

4.5 Conclusion and future research directions

In the present thesis, the role of gas7 in neuronal differentiation and its potential ability to promote neuroprotection were investigated. As hypothesized, gas7 was shown to potentiate NGF-induced neuronal differentiation of PC12 cells, and the induction of endogenous gas7 expression during neuronal differentiation of NT2 cells suggested a possible role for gas7 in this process. However, the hypothesis that gas7 could be neuroprotective was not supported, since gas7 overexpression in PC12 cells did not protect against lethal insults. This study reinforces the role of gas7 in neuronal development. The ability of gas7 to potentiate neuronal differentiation makes it a very interesting molecule for brain repair, promoting neuron formation and neurite outgrowth for the re-establishement of neuronal connections in the injured or diseased brain. Neuronal circuit reorganization can improve recovery, based on the evidence that deafferented brain areas do not lose their ability to integrate functionally with other regions and that accordingly, neurons that have lost efferent targets may integrate into new neuronal circuitry (Stroemer *et al.*, 1995). Better understanding of the mechanisms of action underlying gas7's role might lead to improved therapeutic strategies to treat diseased or injured neuronal tissues.

The gas7 protein is very novel, and there is still a great deal to learn about it. Many experiments will be required to fully understand the function of gas7, and eventually to understand its potential application to neuroregeneration. In addition to the future experiments suggested in this discussion, it would be essential to assess the effects of gas7 on functional neuronal differentiation and electrical excitability of neurons, such as

through the evaluation of ion channel expression and cell currents, following gas7 overexpression. Moreover, downregulation of gas7, in a system where it is endogenously expressed, would lead to insights into the roles of gas7. RNA interference (RNAi) experiments, for example, would allow one to evaluate if gas7 expression is essential for proper neuronal development of primary cultured neurons, as suggested by Ju *et al.* (1998). Finally. knockout animals would also be a great tool to further assess the function of gas7 in all different organ systems, in addition to the brain.

Bibliography

Abu-Raya,S., Blaugrund,E., Trembovler,V., Shilderman-Bloch,E., Shohami,E. & Lazarovici,P. (1999) Rasagiline, a monoamine oxidase-B inhibitor, protects NGF-differentiated PC12 cells against oxygen-glucose deprivation. *J.Neurosci.Res.*, **58,** 456-463.

Abu-Raya,S., Tabakman,R., Blaugrund,E., Trembovler,V. & Lazarovici,P. (2002) Neuroprotective and neurotoxic effects of monoamine oxidase-B inhibitors and derived metabolites under ischemia in PC12 cells. *Eur.J.Pharmacol.*, **434,** 109-116.

Abu-Raya,S., Trembovler,V., Shohami,E. & Lazarovici,P. (1993) A tissue culture ischemic device to study eicosanoid release by pheochromocytoma PC12 cultures. *J.Neurosci.Methods*, **50,** 197-203.

Adkins-Muir,D.L. & Jones,T.A. (2003) Cortical electrical stimulation combined with rehabilitative training: enhanced functional recovery and dendritic plasticity following focal cortical ischemia in rats. *Neurol.Res.*, **25,** 780-788.

Ahn,S., Olive,M., Aggarwal,S., Krylov,D., Ginty,D.D. & Vinson,C. (1998) A dominant-negative inhibitor of CREB reveals that it is a general mediator of stimulus-dependent transcription of c-fos. *Mol.Cell Biol.*, **18,** 967-977.

Allen,M.P., Zeng,C., Schneider,K., Xiong,X., Meintzer,M.K., Bellosta,P., Basilico,C., Varnum,B., Heidenreich,K.A. & Wierman,M.E. (1999) Growth arrest-specific gene 6 (Gas6)/adhesion related kinase (Ark) signaling promotes gonadotropin-releasing hormone neuronal survival via extracellular signal-regulated kinase (ERK) and Akt. *Mol.Endocrinol.*, **13,** 191-201.

Altman,J. (1969) Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. *J.Comp.Neurol.*, **137,** 433-457.

Altman,J. & Das,G.D. (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J.Comp.Neurol.*, **124,** 319-335.

Angelillo-Scherrer,A., de Frutos,P., Aparicio,C., Melis,E., Savi,P., Lupu,F., Arnout,J., Dewerchin,M., Hoylaerts,M., Herbert,J., Collen,D., Dahlback,B. & Carmeliet,P. (2001) Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. *Nat.Med.*, **7,** 215-221.

Asao, H., Sasaki,Y., Arita,T., Tanaka,N., Endo,K., Kasai,H., Takeshita,T., Endo,Y., Fujita,T. & Sugamura,K. (1997) Hrs is associated with STAM, a signal-transducing adaptor molecule. *J.Biol.Chem.*, **272,** 32785-32791.

Avanzi,G.C., Gallicchio,M., Bottarel,F., Gammaitoni,L., Cavalloni,G., Buonfiglio,D., Bragardo,M., Bellomo,G., Albano,E., Fantozzi,R., Garbarino,G., Varnum,B., Aglietta,M., Saglio,G., Dianzani,U. & Dianzani,C. (1998) GAS6 inhibits granulocyte adhesion to endothelial cells. *Blood*, **91,** 2334-2340.

Avila,A.M., Davila-Garcia,M.I., Ascarrunz,V.S., Xiao,Y. & Kellar,K.J. (2003) Differential regulation of nicotinic acetylcholine receptors in PC12 cells by nicotine and nerve growth factor. *Mol.Pharmacol.*, **64,** 974-986.

Barnabe-Heider,F. & Miller,F.D. (2003) Endogenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathways. *J.Neurosci.*, **23,** 5149-5160.

Bellosta,P., Zhang,Q., Goff,S.P. & Basilico,C. (1997) Signaling through the ARK tyrosine kinase receptor protects from apoptosis in the absence of growth stimulation. *Oncogene*, **15,** 2387-2397.

Benetti,R., Del Sal,G., Monte,M., Paroni,G., Brancolini,C. & Schneider,C. (2001) The death substrate Gas2 binds m-calpain and increases susceptibility to p53-dependent apoptosis. *EMBO J.*, **20,** 2702-2714.

Bershadsky,A.D., Gluck,U., Denisenko,O.N., Sklyarova,T.V., Spector,I. & Ben Ze'ev,A. (1995) The state of actin assembly regulates actin and vinculin expression by a feedback loop. *J.Cell Sci.*, **108 (Pt 3),** 1183-1193.

Biernaskie,J. & Corbett,D. (2001) Enriched rehabilitative training promotes improved forelimb motor function and enhanced dendritic growth after focal ischemic injury. *J.Neurosci.*, **21,** 5272-5280.

Boniece,I.R. & Wagner,J.A. (1993) Growth factors protect PC12 cells against ischemia by a mechanism that is independent of PKA, PKC, and protein synthesis. *J.Neurosci.*, **13,** 4220-4228.

Boniece,I.R. & Wagner,J.A. (1995) NGF protects PC12 cells against ischemia by a mechanism that requires the N-kinase. *J.Neurosci.Res.*, **40,** 1-9.

Boonstra,L., Van Der Saag,P.T., Feijen,A., Bisschop,A. & De Laat,S. (1985) Epidermal growth factor, but not nerve growth factor, stimulates tyrosine-specific protein-kinase activity in pheochromocytoma (PC12) plasma membranes. *Biochimie*, **67**, 1177-1183.

Brancolini,C., Benedetti,M. & Schneider,C. (1995) Microfilament reorganization during apoptosis: the role of Gas2, a possible substrate for ICE-like proteases. *EMBO J.*, **14,** 5179-5190.

Brancolini,C., Bottega,S. & Schneider,C. (1992) Gas2, a growth arrest-specific protein, is a component of the microfilament network system. *J.Cell Biol.*, **117,** 1251-1261.

Brancolini,C., Edomi,P., Marzinotto,S. & Schneider,C. (2000) Exposure at the cell surface is required for gas3/PMP22 To regulate both cell death and cell spreading: implication for the Charcot-Marie-Tooth type 1A and Dejerine-Sottas diseases. *Mol.Biol.Cell*, **11,** 2901-2914.

Brancolini,C., Marzinotto,S., Edomi,P., Agostoni,E., Fiorentini,C., Muller,H.W. & Schneider,C. (1999) Rho-dependent regulation of cell spreading by the tetraspan membrane protein Gas3/PMP22. *Mol.Biol.Cell*, **10,** 2441-2459.

Brancolini,C. & Schneider,C. (1994) Phosphorylation of the growth arrest-specific protein Gas2 is coupled to actin rearrangements during G0 to G1 transition in NIH 3T3 cells. *J.Cell Biol.*, **124,** 743-756.

Brenner,D.G., Lin-Chao,S. & Cohen,S.N. (1989) Analysis of mammalian cell genetic regulation in situ by using retrovirus-derived "portable exons" carrying the Escherichia coli lacZ gene. *Proc.Natl.Acad.Sci.U.S.A*, **86,** 5517-5521.

Bruno,S. & Darzynkiewicz,Z. (1992) Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells. *Cell Prolif.*, **25,** 31-40.

Buonomano,D.V. & Merzenich,M.M. (1998) Cortical plasticity: from synapses to maps. *Annu.Rev.Neurosci.*, **21,** 149-186.

Burkhard,P., Stetefeld,J. & Strelkov,S.V. (2001) Coiled coils: a highly versatile protein folding motif. *Trends Cell Biol.*, **11,** 82-88.

Byk,T., Ozon,S. & Sobel,A. (1998) The Ulip family phosphoproteins. Common and specific properties. *Eur.J.Biochem.*, **254,** 14-24.

Caviness,V.S., Jr., Takahashi,T. & Nowakowski,R.S. (1999) The G1 restriction point as critical regulator of neocortical neuronogenesis. *Neurochem.Res.*, **24,** 497-506.

Chao,C.C., Su,L.J., Sun,N.K., Ju,Y.T., Lih,J.C. & Lin-Chao,S. (2003) Involvement of Gas7 in nerve growth factor-independent and dependent cell processes in PC12 cells. *J.Neurosci.Res.*, **74,** 248-254.

Cheung,W.M., Chu,A.H. & Ip,N.Y. (1997) Identification of candidate genes induced by retinoic acid in embryonal carcinoma cells. *J.Neurochem.*, **68,** 1882-1888.

Chies,R., Nobbio,L., Edomi,P., Schenone,A., Schneider,C. & Brancolini,C. (2003) Alterations in the Arf6-regulated plasma membrane endosomal recycling pathway in cells overexpressing the tetraspan protein Gas3/PMP22. *J.Cell Sci.*, **116,** 987-999.

Ciccarelli,C., Philipson,L. & Sorrentino,V. (1990) Regulation of expression of growth arrest-specific genes in mouse fibroblasts. *Mol.Cell Biol.*, **10,** 1525-1529.

Claesson-Welsh,L. (1996) Mechanism of action of platelet-derived growth factor. *Int.J.Biochem.Cell Biol.*, **28,** 373-385.

Coccia,E.M., Cicala,C., Charlesworth,A., Ciccarelli,C., Rossi,G.B., Philipson,L. & Sorrentino,V. (1992) Regulation and expression of a growth arrest-specific gene (gas5) during growth, differentiation, and development. *Mol.Cell Biol.*, **12,** 3514-3521.

Cole,J. & Glees,P. (1954) Effects of small lesions in sensory cortex in trained monkeys. *J.Neurophysiol.*, **17,** 1-13.

Coleman,P.D. & Flood,D.G. (1986) Dendritic proliferation in the aging brain as a compensatory repair mechanism. *Prog.Brain Res.*, **70,** 227-237.

Colombo,M.P., Marfinotti,A., Howard,T.A., Schneider,C., D'Eustachio,P. & Seldin,M.F. (1992) Localization of growth arrest-specific genes on mouse chromosomes 1, 7, 8, 11, 13, and 16. *Mamm.Genome*, **2,** 130-134.

Cooper,J.A. & Schafer,D.A. (2000) Control of actin assembly and disassembly at filament ends. *Curr.Opin.Cell Biol.*, **12,** 97-103.

Corrente,G., Guardavaccaro,D. & Tirone,F. (2002) PC3 potentiates NGF-induced differentiation and protects neurons from apoptosis. *Neuroreport*, **13,** 417-422.

Cowled,P.A., Ciccarelli,C., Coccia,E., Philipson,L. & Sorrentino,V. (1994) Expression of growth arrest-specific (gas) genes in senescent murine cells. *Exp.Cell Res.*, **211,** 197-202.

Cremisi,F., Philpott,A. & Ohnuma,S. (2003) Cell cycle and cell fate interactions in neural development. *Curr.Opin.Neurobiol.*, **13,** 26-33.

Da Silva,J.S. & Dotti,C.G. (2002) Breaking the neuronal sphere: regulation of the actin cytoskeleton in neuritogenesis. *Nat.Rev.Neurosci.*, **3,** 694-704.

De Hostos,E.L. (1999) The coronin family of actin-associated proteins. *Trends Cell Biol.*, **9,** 345-350.

De Laurenzi,V., Raschella,G., Barcaroli,D., Annicchiarico-Petruzzelli,M., Ranalli,M., Catani,M.V., Tanno,B., Costanzo,A., Levrero,M. & Melino,G. (2000) Induction of neuronal differentiation by p73 in a neuroblastoma cell line. *J.Biol.Chem.*, **275,** 15226-15231.

Del Sal,G., Ruaro,M.E., Philipson,L. & Schneider,C. (1992) The growth arrest-specific gene, gas1, is involved in growth suppression. *Cell*, **70,** 595-607.

Desole,M.S., Sciola,L., Sircana,S., Godani,C., Migheli,R., Delogu,M.R., Piras,G., De Natale,G. & Miele,E. (1998) Protective effect of deferoxamine on sodium nitroprusside-induced apoptosis in PC12 cells. *Neurosci.Lett.*, **247,** 1-4.

Dobashi,Y., Katayama,K., Kawai,M., Akiyama,T. & Kameya,T. (2000a) APC protein is required for initiation of neuronal differentiation in rat pheochromocytoma PC12 cells. *Biochem.Biophys.Res.Commun.*, **279,** 685-691.

Dobashi,Y., Shoji,M., Kitagawa,M., Noguchi,T., Kameya,T. (2000b) Simultaneous suppression of cdc2 and cdk2 activities induces neuronal differentiation of PC12 cells. *J.Biol.Chem.*, **275,** 12572-12580.

El Ghissassi,F., Valsesia-Wittmann,S., Falette,N., Duriez,C., Walden,P.D. & Puisieux,A. (2002) BTG2(TIS21/PC3) induces neuronal differentiation and prevents apoptosis of terminally differentiated PC12 cells. *Oncogene*, **21,** 6772-6778.

Eriksson,P.S., Perfilieva,E., Bjork-Eriksson,T., Alborn,A.M., Nordborg,C., Peterson,D.A. & Gage,F.H. (1998) Neurogenesis in the adult human hippocampus. *Nat.Med.*, **4,** 1313-1317.

Evdokiou,A. & Cowled,P.A. (1998) Growth-regulatory activity of the growth arrest-specific gene, GAS1, in NIH3T3 fibroblasts. *Exp.Cell Res.*, **240,** 359-367.

Fabbretti,E., Edomi,P., Brancolini,C. & Schneider,C. (1995) Apoptotic phenotype induced by overexpression of wild-type gas3/PMP22: its relation to the demyelinating peripheral neuropathy CMT1A. *Genes Dev.*, **9,** 1846-1856.

Fan,L., Ciano-Oliveira,C., Weed,S.A., Craig,A.W., Greer,P.A., Rotstein,O.D. & Kapus,A. (2004) Actin depolymerization-induced tyrosine phosphorylation of cortactin: the role of Fer kinase. *Biochem.J.*, **380,** 581-591.

Feldker,D.E., Datson,N.A., Veenema,A.H., Proutski,V., Lathouwers,D., De Kloet,E.R. & Vreugdenhil,E. (2003) GeneChip analysis of hippocampal gene expression profiles of short- and long-attack-latency mice: technical and biological implications. *J.Neurosci.Res.*, **74,** 701-716.

Fischer,I., Shea,T.B., Sapirstein,V.S. & Kosik,K.S. (1986) Expression and distribution of microtubule-associated protein 2 (MAP2) in neuroblastoma and primary neuronal cells. *Brain Res.*, **390,** 99-109.

Fleming,J.V., Hay,S.M., Harries,D.N. & Rees,W.D. (1998) Effects of nutrient deprivation and differentiation on the expression of growth-arrest genes (gas and gadd) in F9 embryonal carcinoma cells. *Biochem.J.*, **330,** 573-579.

Fontanier-Razzaq,N., Harries,D.N., Hay,S.M. & Rees,W.D. (2002) Amino acid deficiency up-regulates specific mRNAs in murine embryonic cells. *J.Nutr.*, **132,** 2137-2142.

Freemantle,S.J., Kerley,J.S., Olsen,S.L., Gross,R.H. & Spinella,M.J. (2002) Developmentally-related candidate retinoic acid target genes regulated early during neuronal differentiation of human embryonal carcinoma. *Oncogene*, **21,** 2880-2889.

Fujita,H., Katoh,H., Ishikawa,Y., Mori,K. & Negishi,M. (2002) Rapostlin is a novel effector of Rnd2 GTPase inducing neurite branching. *J.Biol.Chem.*, **277,** 45428-45434.

Fujita,K., Lazarovici,P. & Guroff,G. (1989) Regulation of the differentiation of PC12 pheochromocytoma cells. *Environ.Health Perspect.*, **80,** 127-142.

Furnish,E.J., Zhou,W., Cunningham,C.C., Kas,J.A. & Schmidt,C.E. (2001) Gelsolin overexpression enhances neurite outgrowth in PC12 cells. *FEBS Lett.*, **508,** 282-286.

Gage,F.H., Coates,P.W., Palmer,T.D., Kuhn,H.G., Fisher,L.J., Suhonen,J.O., Peterson,D.A., Suhr,S.T. & Ray,J. (1995) Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc.Natl.Acad.Sci.U.S.A*, **92,** 11879-11883.

Goldmann,W.H., Hess,D. & Isenberg,G. (1999) The effect of intact talin and talin tail fragment on actin filament dynamics and structure depends on pH and ionic strength. *Eur.J.Biochem.*, **260,** 439-445.

Goruppi,S., Ruaro,E. & Schneider,C. (1996) Gas6, the ligand of Axl tyrosine kinase receptor, has mitogenic and survival activities for serum starved NIH3T3 fibroblasts. *Oncogene*, **12,** 471-480.

Greene,L.A. (1978) Nerve growth factor prevents the death and stimulates the neuronal differentiation of clonal PC12 pheochromocytoma cells in serum-free medium. *J.Cell Biol.*, **78,** 747-755.

Greene,L.A. & Tischler,A.S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc.Natl.Acad.Sci.U.S.A*, **73,** 2424-2428.

Gunning,P.W., Landreth,G.E., Bothwell,M.A. & Shooter,E.M. (1981) Differential and synergistic actions of nerve growth factor and cyclic AMP in PC12 cells. *J.Cell Biol.*, **89,** 240-245.

Guo,Z., Du,X. & Iacovitti,L. (1998) Regulation of tyrosine hydroxylase gene expression during transdifferentiation of striatal neurons: changes in transcription factors binding the AP-1 site. *J.Neurosci.*, **18,** 8163-8174.

Hanemann,C.O., Stoll,G., D'Urso,D., Fricke,W., Martin,J.J., Van Broeckhoven,C., Mancardi,G.L., Bartke,I. & Muller,H.W. (1994) Peripheral myelin protein-22 expression

in Charcot-Marie-Tooth disease type 1a sural nerve biopsies. *J.Neurosci.Res.*, **37,** 654-659.

Hebb,D.O. (1949) *The Organization of Behavior*. John Wiley and Sons, New York.

Henderson,L.P., Gdovin,M.J., Liu,C., Gardner,P.D. & Maue,R.A. (1994) Nerve growth factor increases nicotinic ACh receptor gene expression and current density in wild-type and protein kinase A-deficient PC12 cells. *J.Neurosci.*, **14,** 1153-1163.

Hepp,R., Dupont,J.L., Aunis,D., Langley,K. & Grant,N.J. (2001) NGF enhances depolarization effects on SNAP-25 expression: induction of SNAP-25b isoform. *Neuroreport*, **12,** 673-677.

Honda,K., Casadesus,G., Petersen,R.B., Perry,G. & Smith,M.A. (2004) Oxidative stress and redox-active iron in Alzheimer's disease. *Ann.N.Y.Acad.Sci.*, **1012,** 179-182.

Horgan,A.M. & Stork,P.J. (2003) Examining the mechanism of Erk nuclear translocation using green fluorescent protein. *Exp.Cell Res.*, **285,** 208-220.

Howard,A. & Pelc,S.P. (1953) Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. *Heredity*, **6,** 261-273.

Hu,S. & Reichardt,L.F. (1999) From membrane to cytoskeleton: enabling a connection. *Neuron*, **22,** 419-422.

Iacopetti,P., Barsacchi, G., Tirone, F., Maffei, L. & Cremisi, F. (1994) Developmental expression of PC3 gene is correlated with neuronal cell birthday. *Mech. Dev.*, **47,** 127-137.

Ilsley,J.L., Sudol,M. & Winder,S.J. (2002) The WW domain: linking cell signalling to the membrane cytoskeleton. *Cell Signal.*, **14,** 183-189.

Irwin,N. & Madsen,J.R. (1997) Molecular biology of axonal outgrowth. 1. Growth cones and GAP-43. *Pediatr.Neurosurg.*, **27,** 113-120.

Ishimoto,Y., Ohashi,K., Mizuno,K. & Nakano,T. (2000) Promotion of the uptake of PS liposomes and apoptotic cells by a product of growth arrest-specific gene, gas6. *J.Biochem.(Tokyo)*, **127,** 411-417.

Jay,D.G. (2000) The clutch hypothesis revisited: ascribing the roles of actin-associated proteins in filopodial protrusion in the nerve growth cone. *J.Neurobiol.*, **44,** 114-125.

Jenkins,W.M. & Merzenich,M.M. (1987) Reorganization of neocortical representations after brain injury: a neurophysiological model of the bases of recovery from stroke. *Prog.Brain Res.*, **71,** 249-266.

Jiang,H.Y., Lee,K.H., Schneider,C., WS,O., Tang,P.L. & Chow,P.H. (2001) The growth arrest specific gene (gas6) protein is expressed in abnormal embryos sired by male golden hamsters with accessory sex glands removed. *Anat.Embryol.(Berl)*, **203,** 343-355.

Jin,K., Mao,X.O., Eshoo,M.W., del Rio,G., Rao,R., Chen,D., Simon,R.P. & Greenberg,D.A. (2002) cDNA microarray analysis of changes in gene expression induced by neuronal hypoxia in vitro. *Neurochem.Res.*, **27,** 1105-1112.

Jin,K., Sun,Y., Xie,L., Childs,J., Mao,X.O. & Greenberg,D.A. (2004) Post-ischemic administration of heparin-binding epidermal growth factor-like growth factor (HB-EGF) reduces infarct size and modifies neurogenesis after focal cerebral ischemia in the rat. *J.Cereb.Blood Flow Metab*, **24,** 399-408.

Ju,Y.T., Chang,A.C., She,B.R., Tsaur,M.L., Hwang,H.M., Chao,C.C., Cohen,S.N. & Lin-Chao,S. (1998) gas7: A gene expressed preferentially in growth-arrested fibroblasts and terminally differentiated Purkinje neurons affects neurite formation. *Proc.Natl.Acad.Sci.U.S.A*, **95,** 11423-11428.

Karlsson,C., Afrakhte,M., Westermark,B. & Paulsson,Y. (1999) Elevated level of gas3 gene expression is correlated with G0 growth arrest in human fibroblasts. *Cell Biol.Int.*, **23,** 351-358.

Katagiri,M., Hakeda,Y., Chikazu,D., Ogasawara,T., Takato,T., Kumegawa,M., Nakamura,K. & Kawaguchi,H. (2001) Mechanism of stimulation of osteoclastic bone

resorption through Gas6/Tyro 3, a receptor tyrosine kinase signaling, in mouse osteoclasts. *J.Biol.Chem.*, **276,** 7376-7382.

Keyvani,K. & Schallert,T. (2002) Plasticity-associated molecular and structural events in the injured brain. *J.Neuropathol.Exp.Neurol.*, **61,** 831-840.

Kimura,H. & Schubert,D. (1992) Schwannoma-derived growth factor promotes the neuronal differentiation and survival of PC12 cells. *J.Cell Biol.*, **116,** 777-783.

Kirschenbaum,B., Nedergaard,M., Preuss,A., Barami,K., Fraser,R.A. & Goldman,S.A. (1994) In vitro neuronal production and differentiation by precursor cells derived from the adult human forebrain. *Cereb.Cortex*, **4,** 576-589.

Klesse,L.J., Meyers,K.A., Marshall,C.J. & Parada,L.F. (1999) Nerve growth factor induces survival and differentiation through two distinct signaling cascades in PC12 cells. *Oncogene*, **18,** 2055-2068.

Klesse,L.J. & Parada,L.F. (1999) Trks: signal transduction and intracellular pathways. *Microsc.Res.Tech.*, **45,** 210-216.

Kokaia,Z. & Lindvall,O. (2003) Neurogenesis after ischaemic brain insults. *Curr.Opin.Neurobiol.*, **13,** 127-132.

Kuhn,G., Lie,A., Wilms,S. & Muller,H.W. (1993) Coexpression of PMP22 gene with MBP and P0 during de novo myelination and nerve repair. *Glia*, **8,** 256-264.

Kuhn,T.B., Meberg,P.J., Brown,M.D., Bernstein,B.W., Minamide,L.S., Jensen,J.R., Okada,K., Soda,E.A. & Bamburg,J.R. (2000) Regulating actin dynamics in neuronal growth cones by ADF/cofilin and rho family GTPases. *J.Neurobiol.*, **44,** 126-144.

Landmesser,L. (1994) Axonal outgrowth and pathfinding. *Prog.Brain Res.*, **103,** 67-73.

Laufs,U., Endres,M., Custodis,F., Gertz,K., Nickenig,G., Liao,J.K. & Bohm,M. (2000) Suppression of endothelial nitric oxide production after withdrawal of statin treatment is

mediated by negative feedback regulation of rho GTPase gene transcription. *Circulation*, **102,** 3104-3110.

Laurent,M., Martinerie,C., Thibout,H., Hoffman,M.P., Verrecchia,F., Le Bouc,Y., Mauviel,A. & Kleinman,H.K. (2003) NOVH increases MMP3 expression and cell migration in glioblastoma cells via a PDGFR-alpha-dependent mechanism. *FASEB J.*, **17,** 1919-1921.

Lazakovitch,E.M., She,B.R., Lien,C.L., Woo,W.M., Ju,Y.T. & Lin-Chao,S. (1999) The Gas7 gene encodes two protein isoforms differentially expressed within the brain. *Genomics*, **61,** 298-306.

Lee,C.S., May,N.R. & Fan,C.M. (2001a) Transdifferentiation of the ventral retinal pigmented epithelium to neural retina in the growth arrest specific gene 1 mutant. *Dev.Biol.*, **236,** 17-29.

Lee,K.K., Leung,A.K., Tang,M.K., Cai,D.Q., Schneider,C., Brancolini,C. & Chow,P.H. (2001b) Functions of the growth arrest specific 1 gene in the development of the mouse embryo. *Dev.Biol.*, **234,** 188-203.

Lee,K.K., Tang,M.K., Yew,D.T., Chow,P.H., Yee,S.P., Schneider,C. & Brancolini,C. (1999) gas2 is a multifunctional gene involved in the regulation of apoptosis and chondrogenesis in the developing mouse limb. *Dev.Biol.*, **207,** 14-25.

Letourneau,P.C. (1996) The cytoskeleton in nerve growth cone motility and axonal pathfinding. *Perspect.Dev.Neurobiol.*, **4,** 111-123.

Li,P., Matsunaga,K., Yamakuni,T. & Ohizumi,Y. (2000) Potentiation of nerve growth factor-action by picrosides I and II, natural iridoids, in PC12D cells. *Eur.J.Pharmacol.*, **406**, 203-208.

Li,R., Chen,J., Hammonds,G., Phillips,H., Armanini,M., Wood,P., Bunge,R., Godowski,P.J., Sliwkowski,M.X. & Mather,J.P. (1996) Identification of Gas6 as a growth factor for human Schwann cells. *J.Neurosci.*, **16,** 2012-2019.

Lih,C.J., Cohen,S.N., Wang,C. & Lin-Chao,S. (1996) The platelet-derived growth factor alpha-receptor is encoded by a growth-arrest-specific (gas) gene. *Proc.Natl.Acad.Sci.U.S.A*, **93,** 4617-4622.

Liu,J., Solway,K., Messing,R.O. & Sharp,F.R. (1998) Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. *J.Neurosci.*, **18,** 7768-7778.

Liu,J.H., Bao,Y.M., Song,J.J. & An,L.J. (2003) *Codonopsis pilosula* (Franch) Nannf total alkaloids potentiate neurite outgrowth induced by nerve growth factor in PC12 cells. *Acta Pharmacol.Sin.*, **24,** 913-917.

Liu,Y., Liu,C., Yamada,Y. & Fan,C.M. (2002) Growth arrest specific gene 1 acts as a region-specific mediator of the Fgf10/Fgf8 regulatory loop in the limb. *Development*, **129,** 5289-5300.

Liu,Y., May,N.R. & Fan,C.M. (2001) Growth arrest specific gene 1 is a positive growth regulator for the cerebellum. *Dev.Biol.*, **236,** 30-45.

Lodish,H., Baltimore,D., Berk,A., Zipursky,S.L., Matsudaira,P. & Darnell,J. (1996) *Molecular Cell Biology*. Scientific American Books, Inc., New York.

Lu,Q., Gore,M., Zhang,Q., Camenisch,T., Boast,S., Casagranda,F., Lai,C., Skinner,M.K., Klein,R., Matsushima,G.K., Earp,H.S., Goff,S.P. & Lemke,G. (1999) Tyro-3 family receptors are essential regulators of mammalian spermatogenesis. *Nature*, **398,** 723-728.

Luo,L. (2000) Rho GTPases in neuronal morphogenesis. *Nat.Rev.Neurosci.*, **1,** 173-180.

Lupas,A. (1996) Coiled coils: new structures and new functions. *Trends Biochem.Sci.*, **21,** 375-382.

Magyar,J.P., Martini,R., Ruelicke,T., Aguzzi,A., Adlkofer,K., Dembic,Z., Zielasek,J., Toyka,K.V. & Suter,U. (1996) Impaired differentiation of Schwann cells in transgenic mice with increased PMP22 gene dosage. *J.Neurosci.*, **16,** 5351-5360.

Manfioletti,G., Brancolini,C., Avanzi,G. & Schneider,C. (1993) The protein encoded by a growth arrest-specific gene (gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade. *Mol.Cell Biol.*, **13,** 4976-4985.

Manfioletti,G., Ruaro,M.E., Del Sal,G., Philipson,L. & Schneider,C. (1990) A growth arrest-specific (gas) gene codes for a membrane protein. *Mol.Cell Biol.*, **10,** 2924-2930.

Manzow,S., Brancolini,C., Marks,F. & Richter,K.H. (1996) Expression of growth arrest-specific (Gas) genes in murine keratinocytes: Gas2 is specifically regulated. *Exp.Cell Res.*, **224,** 200-203.

Marshall,C.J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*, **80,** 179-185.

McCloskey,P., Fridell,Y.W., Attar,E., Villa,J., Jin,Y., Varnum,B. & Liu,E.T. (1997) GAS6 mediates adhesion of cells expressing the receptor tyrosine kinase Axl. *J.Biol.Chem.*, **272,** 23285-23291.

McConnell,S.K. & Kaznowski,C.E. (1991) Cell cycle dependence of laminar determination in developing neocortex. *Science*, **254,** 282-285.

Meberg,P.J. & Bamburg,J.R. (2000) Increase in neurite outgrowth mediated by overexpression of actin depolymerizing factor. *J.Neurosci.*, **20,** 2459-2469.

Megonigal,M.D., Cheung,N.K., Rappaport,E.F., Nowell,P.C., Wilson,R.B., Jones,D.H., Addya,K., Leonard,D.G., Kushner,B.H., Williams,T.M., Lange,B.J. & Felix,C.A. (2000) Detection of leukemia-associated MLL-GAS7 translocation early during chemotherapy with DNA topoisomerase II inhibitors. *Proc.Natl.Acad.Sci.U.S.A*, **97,** 2814-2819.

Meldolesi,J. & Chieregatti,E. (2004) Fusion has found its calcium sensor. *Nature Cell Biol.*, **6**, 476-478.

Mellstrom,B., Cena,V., Lamas,M., Perales,C., Gonzalez,C. & Naranjo,J.R. (2002) Gas1 is induced during and participates in excitotoxic neuronal death. *Mol.Cell Neurosci.*, **19,** 417-429.

Merzenich,M.M., Kaas,J.H., Wall,J.T., Sur,M., Nelson,R.J. & Felleman,D.J. (1983) Progression of change following median nerve section in the cortical representation of the hand in areas 3b and 1 in adult owl and squirrel monkeys. *Neuroscience*, **10,** 639-665.

Merzenich,M.M., Nelson,R.J., Stryker,M.P., Cynader,M.S., Schoppmann,A. & Zook,J.M. (1984) Somatosensory cortical map changes following digit amputation in adult monkeys. *J.Comp.Neurol.*, **224,** 591-605.

Meyer,G. & Feldman,E.L. (2002) Signaling mechanisms that regulate actin-based motility processes in the nervous system. *J.Neurochem.*, **83,** 490-503.

Michel,P.P., Vyas,S., Anglade,P., Ruberg,M. & Agid,Y. (1994) Morphological and molecular characterization of the response of differentiated PC12 cells to calcium stress. *Eur.J.Neurosci.*, **6,** 577-586.

Mikoshiba,K., Fukuda,M., Ibata,K., Kabayama,H. & Mizutani,A. (1999) Role of synaptotagmin, a Ca2+ and inositol polyphosphate binding protein, in neurotransmitter release and neurite outgrowth. *Chem.Phys.Lipids*, **98,** 59-67.

Minturn,J.E., Fryer,H.J., Geschwind,D.H. & Hockfield,S. (1995) TOAD-64, a gene expressed early in neuronal differentiation in the rat, is related to unc-33, a C. elegans gene involved in axon outgrowth. *J.Neurosci.*, **15,** 6757-6766.

Moskowitz,P.F., Smith,R., Pickett,J., Frankfurter,A. & Oblinger,M.M. (1993) Expression of the class III beta-tubulin gene during axonal regeneration of rat dorsal root ganglion neurons. *J.Neurosci.Res.*, **34,** 129-134.

Mullor,J.L. & Altaba,A. (2002) Growth, hedgehog and the price of GAS. *Bioessays*, **24,** 22-26.

Nagata,K., Ohashi,K., Nakano,T., Arita,H., Zong,C., Hanafusa,H. & Mizuno,K. (1996) Identification of the product of growth arrest-specific gene 6 as a common ligand for Axl, Sky, and Mer receptor tyrosine kinases. *J.Biol.Chem.*, **271,** 30022-30027.

Nakano,T., Kawamoto,K., Higashino,K. & Arita,H. (1996) Prevention of growth arrest-induced cell death of vascular smooth muscle cells by a product of growth arrest-specific gene, gas6. *FEBS Lett.*, **387,** 78-80.

Nakatomi,H., Kuriu,T., Okabe,S., Yamamoto,S., Hatano,O., Kawahara,N., Tamura,A., Kirino,T. & Nakafuku,M. (2002) Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell,* **110,** 429-441.

Nikolic,M. (2002) The role of Rho GTPases and associated kinases in regulating neurite outgrowth. *Int.J.Biochem.Cell Biol.*, **34,** 731-745.

Ohashi,K., Nagata,K., Toshima,J., Nakano,T., Arita,H., Tsuda,H., Suzuki,K. & Mizuno,K. (1995) Stimulation of sky receptor tyrosine kinase by the product of growth arrest-specific gene 6. *J.Biol.Chem.*, **270,** 22681-22684.

Ohnuma,S. & Harris,W.A. (2003) Neurogenesis and the cell cycle. *Neuron,* **40,** 199-208.

Ohnuma,S., Philpott,A. & Harris,W.A. (2001) Cell cycle and cell fate in the nervous system. *Curr.Opin.Neurobiol.*, **11,** 66-73.

Ohuchi,T., Maruoka,S., Sakudo,A. & Arai,T. (2002) Assay-based quantitative analysis of PC12 cell differentiation. *J.Neurosci.Methods*, **118,** 1-8.

Olsson,A.K. & Nanberg,E. (2001) A functional role for ERK in gene induction, but not in neurite outgrowth in differentiating neuroblastoma cells. *Exp.Cell Res.*, **265,** 21-30.

Parent,J.M., Yu,T.W., Leibowitz,R.T., Geschwind,D.H., Sloviter,R.S. & Lowenstein,D.H. (1997) Dentate granule cell neurogenesis is increased by seizures and

contributes to aberrant network reorganization in the adult rat hippocampus. *J.Neurosci.*, **17,** 3727-3738.

Pedersen,M.V., Kohler,L.B., Ditlevsen,D.K., Li,S., Berezin,V. & Bock,E. (2004) Neuritogenic and survival-promoting effects of the P2 peptide derived from a homophilic binding site in the neural cell adhesion molecule. *J.Neurosci.Res.*, **75,** 55-65.

Philipson,L. (1998) Cell cycle exit: growth arrest, apoptosis, and tumor suppression revisited. *Mol.Med.*, **4,** 205-213.

Plautz,E.J., Barbay,S., Frost,S.B., Friel,K.M., Dancause,N., Zoubina,E.V., Stowe,A.M., Quaney,B.M. & Nudo,R.J. (2003) Post-infarct cortical plasticity and behavioral recovery using concurrent cortical stimulation and rehabilitative training: a feasibility study in primates. *Neurol.Res.*, **25,** 801-810.

Pollard,T.D. & Borisy,G.G. (2003) Cellular motility driven by assembly and disassembly of actin filaments. *Cell*, **112,** 453-465.

Quinn,C.C., Gray,G.E. & Hockfield,S. (1999) A family of proteins implicated in axon guidance and outgrowth. *J.Neurobiol.*, **41,** 158-164.

Raho,G., Barone,V., Rossi,D., Philipson,L. & Sorrentino,V. (2000) The gas 5 gene shows four alternative splicing patterns without coding for a protein. *Gene*, **256,** 13-17.

Ramon y Cajal,S. (1890) À quelle époque apparaissent les expansions des cellules nerveuses de la möelle épinière du poulet? *Anat.Anz.*, **21,** 606-611.

Ramon y Cajal,S. (1913) *Estudios Sobre la Degeneracion y Regeneracion del Sistema Nervioso (Degeneration and Regeneration of the Nervous System).* Moya, Madrid.

Ray,S.K., Fidan,M., Nowak,M.W., Wilford,G.G., Hogan,E.L. & Banik,N.L. (2000) Oxidative stress and Ca2+ influx upregulate calpain and induce apoptosis in PC12 cells. *Brain Res.*, **852,** 326-334.

Reynolds,B.A. & Weiss,S. (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science*, **255,** 1707-1710.

Richards,L.J., Kilpatrick,T.J. & Bartlett,P.F. (1992) De novo generation of neuronal cells from the adult mouse brain. *Proc.Natl.Acad.Sci.U.S.A*, **89,** 8591-8595.

Romano,C., Nichols,R.A., Greengard,P. & Greene,L.A. (1987) Synapsin I in PC12 cells. I. Characterization of the phosphoprotein and effect of chronic NGF treatment. *J.Neurosci.*, **7,** 1294-1299.

Rozental,R., Srinivas,M., Gokhan,S., Urban,M., Dermietzel,R., Kessler,J.A., Spray,D.C. & Mehler,M.F. (2000) Temporal expression of neuronal connexins during hippocampal ontogeny. *Brain Res.Brain Res.Rev.*, **32,** 57-71.

Ruaro,M.E., Stebel,M., Vatta,P., Marzinotto,S. & Schneider,C. (2000) Analysis of the domain requirement in Gas1 growth suppressing activity. *FEBS Lett.*, **481,** 159-163.

Rudkin,B.B., Lazarovici,P., Levi,B.Z., Abe,Y., Fujita,K. & Guroff, G. (1989) Cell cycle-specific action of nerve growth factor in PC12 cells: differentiation without proliferation. *EMBO J.*, **8,** 3319-3325.

Rydel,R.E. & Greene,L.A. (1987) Acidic and basic fibroblast growth factors promote stable neurite outgrowth and neuronal differentiation in cultures of PC12 cells. *J.Neurosci.*, **7,** 3639-3653.

Sandhu,J.K., Pandey,S., Ribecco-Lutkiewicz,M., Monette,R., Borowy-Borowski,H., Walker,P.R. & Sikorska,M. (2003) Molecular mechanisms of glutamate neurotoxicity in mixed cultures of NT2-derived neurons and astrocytes: protective effects of coenzyme Q10. *J.Neurosci.Res.*, **72,** 691-703.

Sandhu,J.K., Sikorska,M. & Walker,P.R. (2002) Characterization of astrocytes derived from human NTera-2/D1 embryonal carcinoma cells. *J.Neurosci.Res.*, **68,** 604-614.

Sarker,K.P., Biswas,K.K., Rosales,J.L., Yamaji,K., Hashiguchi,T., Lee,K.Y. & Maruyama,I. (2003) Ebselen inhibits NO-induced apoptosis of differentiated PC12 cells

via inhibition of ASK1-p38 MAPK-p53 and JNK signaling and activation of p44/42 MAPK and Bcl-2. *J.Neurochem.*, **87,** 1345-1353.

Schneider,C., King,R.M. & Philipson,L. (1988) Genes specifically expressed at growth arrest of mammalian cells. *Cell*, **54,** 787-793.

Schubert,D., Heinemann,S. & Kidokoro,Y. (1977) Cholinergic metabolism and synapse formation by a rat nerve cell line. *Proc.Natl.Acad.Sci.U.S.A*, **74,** 2579-2583.

Sgorbissa,A., Benetti,R., Marzinotto,S., Schneider,C. & Brancolini,C. (1999) caspase-3 and caspase-7 but not caspase-6 cleave gas2 in vitro: implications for microfilament reorganization during apoptosis. *J.Cell Sci.*, **112,** 4475-4482.

She,B.R., Liou,G.G. & Lin-Chao,S. (2002) Association of the growth-arrest-specific protein Gas7 with F-actin induces reorganization of microfilaments and promotes membrane outgrowth. *Exp.Cell Res.*, **273,** 34-44.

Shigeta,K., Ootaki, K., Tatemoto,H., Nakanishi,T., Inada,A. & Muto,N. (2002) Potentiation of nerve growth factor-induced neurite outgrowth in PC12 cells by a Coptidis Rhizoma extract and protoberberine alkaloids. *Biosci.Biotechnol.Biochem.*, **66,** 2491-2494.

Slack,R.S., El Bizri,H., Wong,J., Belliveau,D.J. & Miller,F.D. (1998) A critical temporal requirement for the retinoblastoma protein family during neuronal determination. *J.Cell Biol.*, **140,** 1497-1509.

Smith,C.M. & Steitz,J.A. (1998) Classification of gas5 as a multi-small-nucleolar-RNA (snoRNA) host gene and a member of the 5'-terminal oligopyrimidine gene family reveals common features of snoRNA host genes. *Mol.Cell Biol.*, **18,** 6897-6909.

Snipes,G.J., Suter,U., Welcher,A.A. & Shooter,E.M. (1992) Characterization of a novel peripheral nervous system myelin protein (PMP-22/SR13). *J.Cell Biol.*, **117,** 225-238.

So,C.W., Karsunky,H., Passegue,E., Cozzio,A., Weissman,I.L. & Cleary,M.L. (2003) MLL-GAS7 transforms multipotent hematopoietic progenitors and induces mixed lineage leukemias in mice. *Cancer Cell*, **3,** 161-171.

Spear,N., Estevez,A.G., Barbeito,L., Beckman,J.S. & Johnson,G.V. (1997) Nerve growth factor protects PC12 cells against peroxynitrite-induced apoptosis via a mechanism dependent on phosphatidylinositol 3-kinase. *J.Neurochem.*, **69,** 53-59.

Spencer,M.L., Shao,H. & Andres,D.A. (2002) Induction of neurite extension and survival in pheochromocytoma cells by the Rit GTPase. *J.Biol.Chem.*, **277,** 20160-20168.

Spreyer,P., Kuhn,G., Hanemann,C.O., Gillen,C., Schaal,H., Kuhn,R., Lemke,G. & Muller,H.W. (1991) Axon-regulated expression of a Schwann cell transcript that is homologous to a 'growth arrest-specific' gene. *EMBO J.*, **10,** 3661-3668.

Stebel,M., Vatta,P., Ruaro,M.E., Del Sal,G., Parton,R.G. & Schneider,C. (2000) The growth suppressing gas1 product is a GPI-linked protein. *FEBS Lett.*, **481,** 152-158.

Steiner,J.P., Hamilton,G.S., Ross,D.T., Valentine,H.L., Guo,H., Connolly,M.A., Liang,S., Ramsey,C., Li,J.H., Huang,W., Howorth,P., Soni,R., Fuller,M., Sauer,H., Nowotnik,A.C. & Suzdak,P.D. (1997) Neurotrophic immunophilin ligands stimulate structural and functional recovery in neurodegenerative animal models. *Proc.Natl.Acad.Sci.U.S.A*, **94,** 2019-2024.

Stitt,T.N., Conn,G., Gore,M., Lai,C., Bruno,J., Radziejewski,C., Mattsson,K., Fisher,J., Gies,D.R., Jones,P.F. & . (1995) The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. *Cell*, **80,** 661-670.

Stroemer,R.P., Kent,T.A. & Hulsebosch,C.E. (1995) Neocortical neural sprouting, synaptogenesis, and behavioral recovery after neocortical infarction in rats. *Stroke*, **26,** 2135-2144.

Stroemer,R.P., Kent,T.A. & Hulsebosch,C.E. (1998) Enhanced neocortical neural sprouting, synaptogenesis, and behavioral recovery with D-amphetamine therapy after neocortical infarction in rats. *Stroke*, **29,** 2381-2393.

Suter,D.M. & Forscher,P. (2000) Substrate-cytoskeletal coupling as a mechanism for the regulation of growth cone motility and guidance. *J.Neurobiol.*, **44,** 97-113.

Tabakman,R., Jiang,H., Levine,R.A., Kohen,R. & Lazarovici,P. (2004) Apoptotic characteristics of cell death and the neuroprotective effect of homocarnosine on pheochromocytoma PC12 cells exposed to ischemia. *J.Neurosci.Res.*, **75,** 499-507.

Tabakman,R., Lazarovici,P. & Kohen,R. (2002) Neuroprotective effects of carnosine and homocarnosine on pheochromocytoma PC12 cells exposed to ischemia. *J.Neurosci.Res.*, **68,** 463-469.

Takagi,Y., Nozaki,K., Takahashi,J., Yodoi,J., Ishikawa,M. & Hashimoto,N. (1999) Proliferation of neuronal precursor cells in the dentate gyrus is accelerated after transient forebrain ischemia in mice. *Brain Res.*, **831,** 283-287.

Takahashi,S., Inatome,R., Hotta,A., Qin,Q., Hackenmiller,R., Simon,M.C., Yamamura,H. & Yanagi,S. (2003) Role for Fes/Fps tyrosine kinase in microtubule nucleation through is Fes/CIP4 homology domain. *J.Biol.Chem.*, **278,** 49129-49133.

Takahashi,T., Yamashita,H., Nakamura,S., Ishiguro,H., Nagatsu,T. & Kawakami,H. (1999) Effects of nerve growth factor and nicotine on the expression of nicotinic acetylcholine receptor subunits in PC12 cells. *Neurosci.Res.*, **35,** 175-181.

Tao-Cheng,J.H., Dosemeci,A., Bressler,J.P., Brightman,M.W. & Simpson,D.L. (1995) Characterization of synaptic vesicles and related neuronal features in nerve growth factor and ras oncogene differentiated PC12 cells. *J.Neurosci.Res.*, **42,** 323-334.

Ter Host,G.J. & Postigo,A. (1997) Prevalence and mechanisms of cell death, in *Clinical Pharmacology of Cerebral Ischemia.*Ter Host,G.J. & Postigo,A. pp. 1-30. Humana Press, Totowa.

Tian,L., Nelson,D.L. & Stewart,D.M. (2000) Cdc42-interacting protein 4 mediates binding of the Wiskott-Aldrich syndrome protein to microtubules. *J.Biol.Chem.*, **275,** 7854-7861.

Tirone, F. (2001) The gene $PC3^{TIS21/BTG2}$, prototype member of the PC3/BTG/TOB family: Regulator in control of cell growth, differentiation, and DNA repair? *J. Cell Physiol.*, **187**, 155-165.

Tonchev,A.B., Yamashima,T., Zhao,L., Okano,H.J. & Okano,H. (2003) Proliferation of neural and neuronal progenitors after global brain ischemia in young adult macaque monkeys. *Mol.Cell Neurosci.*, **23,** 292-301.

Traverse,S., Gomez,N., Paterson,H., Marshall,C. & Cohen,P. (1992) Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochem.J.*, **288,** 351-355.

Umegaki,H., Yamada,K., Naito,M., Kameyama,T., Iguchi,A. & Nabeshima,T. (1996) Protective effect of interleukin-6 against the death of PC12 cells caused by serum deprivation or by the addition of a calcium ionophore. *Biochem.Pharmacol.*, **52,** 911-916.

Vacha,S.J., Bennett,G.D., Mackler,S.A., Koebbe,M.J. & Finnell,R.H. (1997) Identification of a growth arrest specific (gas 5) gene by differential display as a candidate gene for determining susceptibility to hyperthermia-induced exencephaly in mice. *Dev.Genet.*, **21,** 212-222.

Vanhoutte,P., Nissen,J.L., Brugg,B., Gaspera,B.D., Besson,M.J., Hipskind,R.A. & Caboche,J. (2001) Opposing roles of Elk-1 and its brain-specific isoform, short Elk-1, in nerve growth factor-induced PC12 differentiation. *J.Biol.Chem.*, **276,** 5189-5196.

Van Ooyen,A. & Van Pelt,J. (1994) Activity-dependent neurite outgrowth and neural network development. *Prog.Brain Res.*, **102,** 245-259.

Van Praag,H., Schinder,A.F., Christie,B.R., Toni,N., Palmer,T.D. & Gage,F.H. (2002) Functional neurogenesis in the adult hippocampus. *Nature*, **415,** 1030-1034.

Van Veen,M.P. & Van Pelt,J. (1994) Dynamic mechanisms of neuronal outgrowth. *Prog.Brain Res.*, **102,** 95-108.

Varnum-Finney,B. & Reichardt,L.F. (1994) Vinculin-deficient PC12 cell lines extend unstable lamellipodia and filopodia and have a reduced rate of neurite outgrowth. *J.Cell Biol.*, **127,** 1071-1084.

Verkhratsky,A. & Toescu,E.C. (2003) Endoplasmic reticulum Ca(2+) homeostasis and neuronal death. *J.Cell Mol.Med.*, **7,** 351-361.

Wada,K., Okada,N., Yamamura,T. & Koizumi,S. (1996) Nerve growth factor induces resistance of PC12 cells to nitric oxide cytotoxicity. *Neurochem.Int.*, **29,** 461-467.

Wall,J.T., Kaas,J.H., Sur,M., Nelson,R.J., Felleman,D.J. & Merzenich,M.M. (1986) Functional reorganization in somatosensory cortical areas 3b and 1 of adult monkeys after median nerve repair: possible relationships to sensory recovery in humans. *J.Neurosci.*, **6,** 218-233.

Whitmore,S.A., Settasatian,C., Crawford,J., Lower,K.M., McCallum,B., Seshadri,R., Cornelisse,C.J., Moerland,E.W., Cleton-Jansen,A.M., Tipping,A.J., Mathew,C.G., Savnio,M., Savoia,A., Verlander,P., Auerbach,A.D., Van Berkel,C., Pronk,J.C., Doggett,N.A. & Callen,D.F. (1998) Characterization and screening for mutations of the growth arrest-specific 11 (GAS11) and C16orf3 genes at 16q24.3 in breast cancer. *Genomics*, **52,** 325-331.

Williams,J.G. & Penman,S. (1975) The messenger RNA sequences in growing and resting mouse fibroblasts. *Cell*, **6,** 197-206.

Wills,Z., Marr,L., Zinn,K., Goodman,C.S. & Van Vactor,D. (1999) Profilin and the Abl tyrosine kinase are required for motor axon outgrowth in the Drosophila embryo. *Neuron*, **22,** 291-299.

Woodring,P.J., Hunter,T. & Wang,J.Y. (2001) Inhibition of c-Abl tyrosine kinase activity by filamentous actin. *J.Biol.Chem.*, **276,** 27104-27110.

Wu,Y., Spencer,S.D. & Lasky,L.A. (1998) Tyrosine phosphorylation regulates the SH3-mediated binding of the Wiskott-Aldrich syndrome protein to PSTPIP, a cytoskeletal-associated protein. *J.Biol.Chem.*, **273,** 5765-5770.

Xiao,J. & Liu,Y. (2003) Differential roles of ERK and JNK in early and late stages of neuritogenesis: a study in a novel PC12 model system. *J.Neurochem.*, **86,** 1516-1523.

Xiao,J., Zhou,Q. & Liu,Y. (2002) Variant PC12 cell line that spontaneously differentiates and extends neuritic processes. *J.Neurosci.Res.*, **69,** 104-109.

Yanagita,M., Ishimoto,Y., Arai,H., Nagai,K., Ito,T., Nakano,T., Salant,D.J., Fukatsu,A., Doi,T. & Kita,T. (2002) Essential role of Gas6 for glomerular injury in nephrotoxic nephritis. *J.Clin.Invest*, **110,** 239-246.

Yeh,S.D., Chen,Y.J., Chang,A.C., Ray,R., She,B.R., Lee,W.S., Chiang,H.S., Cohen,S.N. & Lin-Chao,S. (2002) Isolation and properties of Gas8, a growth arrest-specific gene regulated during male gametogenesis to produce a protein associated with the sperm motility apparatus. *J.Biol.Chem.*, **277,** 6311-6317.

Yuan,S., Miller,D.W., Barnett,G.H., Hahn,J.F. & Williams,B.R. (1995) Identification and characterization of human beta 2-chimaerin: association with malignant transformation in astrocytoma. *Cancer Res.*, **55**, 3456-3461.

Zamorano,A., Lamas,M., Vergara,P., Naranjo,J.R. & Segovia,J. (2003) Transcriptionally mediated gene targeting of gas1 to glioma cells elicits growth arrest and apoptosis. *J.Neurosci.Res.*, **71,** 256-263.

Zhang,R., Zhang,L., Zhang,Z., Wang,Y., Lu,M., Lapointe,M. & Chopp,M. (2001) A nitric oxide donor induces neurogenesis and reduces functional deficits after stroke in rats. *Ann.Neurol.*, **50,** 602-611.

Zhou,X., Tai,A. & Yamamoto,I. (2003) Enhancement of neurite outgrowth in PC12 cells stimulated with cyclic AMP and NGF by 6-acylated ascorbic acid 2-O-alpha-glucosides (6-Acyl-AA-2G), novel lipophilic ascorbate derivatives. *Biol.Pharm.Bull.*, **26,** 341-346.

Zigova,T., Willing,A.E., Tedesco,E.M., Borlongan,C.V., Saporta,S., Snable,G.L. & Sanberg,P.R. (1999) Lithium chloride induces the expression of tyrosine hydroxylase in hNT neurons. *Exp.Neurol.*, **157,** 251-258.

Zoidl,G., Blass-Kampmann,S., D'Urso,D., Schmalenbach,C. & Muller,H.W. (1995) Retroviral-mediated gene transfer of the peripheral myelin protein PMP22 in Schwann cells: modulation of cell growth. *EMBO J.*, **14,** 1122-1128.

Zoidl,G., D'Urso,D., Blass-Kampmann,S., Schmalenbach,C., Kuhn,R. & Muller,H.W. (1997) Influence of elevated expression of rat wild-type PMP22 and its mutant PMP22Trembler on cell growth of NIH3T3 fibroblasts. *Cell Tissue Res.*, **287,** 459-470.