Use of disabled HSV-1 vectors to investigate the function of Reg-2

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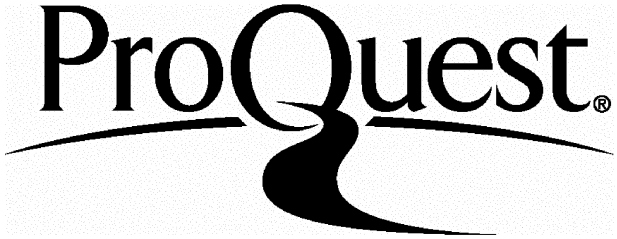

Abstract

Herpes Simplex Virus (HSV) has a number of properties which suggest it may be suitable as a vector for gene delivery to motor neurons. It is naturally neurotrophic and is capable of undergoing retrograde axonal transport in order to reach the neuronal cell body where it may establish latency for the lifetime of the host. Indeed, infection of the motor neuron through a peripheral route would offer an alternative to the more invasive direct approach through the ventral spinal cord. The work presented in this thesis aimed at testing different existing HSV-1 vectors for their capability to efficiently mediate transgene delivery to motor neurons following peripheral inoculation. Attenuated HSV-1 vectors with different combinations in essential and/or non-essential genes were thus identified and were found to vary greatly according to the species and age of animal used. The optimal vectors identified were then used to further elucidate the role of the Schwann cell mitogen, Reg-2. Given that Reg-2 has been strongly implicated in motor neuron regeneration, optimal HSV-1 vectors were used to overexpress Reg-2 in the neonate axotomy model of neuronal death. In this model, Reg-2 appeared to exert a survival effect on the injured motor neurons but not on the sensory neurons of the dorsal root ganglia (DRG). Interestingly, the latter was in accordance with results obtained in studies aimed at analysing the pattern of Reg-2 expression in the DRG. In this case, Reg-2 was found to be strongly upregulated following injury and to follow a dynamic pattern of expression similar to the one observed in the adult animal following peripheral insult. The work described in this thesis provides a basis for the development of vectors for gene delivery to motor neurons and illustrates the use of HSV-1 vectors as tools for the elucidation of gene function.

Acknowledgements

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Finally, I would like to thank my wonderful parents and brother for their unconditional love, encouragement and support. My family means more to me than any thesis I could ever write.

Declaration

I hereby declare that my thesis is not substantially the same as any that I have submitted for a degree or diploma or other qualification at any other University. I further state no part of my thesis has already been or is being concurrently submitted for any such degree, diploma or other qualification.

The work presented in this thesis is entirely my own. Borris Haupt contributed towards the stereotaxic injections described in chapter 4.

Maria Cristina Parsons Perez
London
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Abbreviations

A	adenosine
AAV	adeno-associated virus
Ad	adenovirus
ADA	adenosine deaminase
ALS	amyotrophic lateral sclerosis
APS	ammonium persulphate
BAC	bacterial artificial chromosome
BDNF	brain derived neurotrophic factor
BHK	baby hamster kidney
BHV	bovine herpes virus
bis	n,n'-methylene-bis-acrylamide
bp	base pair
BSA	bovine serum albumin
C	cytosine
CCS	copper chaperone for SOD
cDNA	complementary DNA
CFTR	cystic fibrosis transmembrane conductance regulator
CIAP	calf intestinal alkaline phosphatase
CLC	cardiotrophin like cytokine
CMC	carboxymethylcellulose
CMV	cytomegalovirus
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CPE	cytopathic effect
CT	cardiotrophin
CTL	cytotoxic T-lymphocyte
d	deoxy
dd	dideoxy
ddH_20	double distilled water

DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
ds	double stranded
E	embryonic or early (class of genes)
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetic acid
EHV	equine herpes virus
ELISA	enzyme-linked immunosorbant assay
EMCV	encephalomycocarditis virus
ER	endoplasmic reticulum
EtOH	ethanol
FCS	fetal calf serum
FGF	fibroblast growth factor
FGM	full growth medium
g	gram
G	guanosine
gB	glycoprotein B (similarly for other glycoproteins)
GDNF	glial derived neurotrophic factor
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony stimulating factor
GTP	guanosine triphosphate
HBSS	Hank's balanced salt solution
HCF	host cell factor
HEL	human embryomic lung
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
HMBA	hexamethylene bisacetamide
hr	hour
HRP	horseradish peroxidase
HSV	herpes simplex virus

IAA	isoamyl alcohol
IC	intermediate chain
ICP	infected cell polypeptide
IE	immediate early (class of genes)
Ig	immunoglobulin
IL	interleukin
IRES	internal ribosome entry site
IRL	internal repeat long
IRS	internal repeat short
ITR	inverted terminal repeat
kb	kilobase
kDa	kilo Dalton
l	litre
L	lumbar region of the spinal cord or late (class of genes)
LAP	latency active promoter
LAT	latency associated transcript
LB	Luria Bertani medium
LD	lethal dose
LIF	leukemia inhibitory factor
LMP	low melting point
LTR	long terminal repeat
M	molar
mA	milliamps
mg	milligram
MHC	major histocompatability complex
min	minute
ml	millilitre
mM	millimolar
MMLV	Moloney murine leukaemia virus
MND	motor neuron disease

MOI	multiplicity of infection
mRNA	messenger RNA
MS	master stock
MW	molecular weight
NF	neurofilament
ng	nanogram
NGF	nerve growth factor
NK	natural killer
nm	nanometre
NP40	nonidet P40
NT	neurotrophin
nt	nucleotide
Oct	Octomer
OD	optical density
ORF	open reading frame
P	postnatal
p	plasmid
pA	polyadenylation signal
PAGE	polyacrylamide gel electrophoresis
PB	phosphate buffer
PBS	phosphate buffered saline
PBST	PBS with 0.1% tween-20
PCR	polymerase chain reaction
PFA	paraformaldehyde
pfu	plaque forming units
pmn	progressive motor neuronopathy
PNS	peripheral nervous system
RNA	ribonucleic acid
R	receptor
rpm	revolutions per minute
RSV	Rous sarcoma virus

RT	room temperature
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulphate
SFM	serum free medium
SMA	spinal muscular atrophy
SMN	survival motor neuron
SOD	superoxide dismutase
ss	single stranded
SSC	standard sodium citrate
SV40	simian virus 40
T	thymidine
TAE	Tris-acetate EDTA buffer
TAP	transporter associated with antigen processing
TBE	Tris-borate EDTA buffer
TCA	trichloroacetic acid
TEMED	N,N,N'N'-tetramethylethylenediamine
TK	thymidine kinase
Tris	tris(hydroxyl)aminomethane
Trk	tyrosine kinase receptor
TRL	terminal repeat long
TRS	terminal repeat short
Tween-20	polyoxyethylene-sorbitan monolaurate
UL	unique long
US	unique short
UV	ultraviolet
V	volt
VEGF	vascular endothelial growth factor
Vero	african green monkey cell line
vhs	virion host shut off
VP	virion protein
VSV	vesicular stomatitis virus

v/v	volume for volume
WT	wild type
w/v	weight for volume
X-Gal	4-chloro, 5-bromo, 3-indolyl-β-galactosidase
µg	microgram
µl	microlitre

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Chapter 1

Introduction

Chapter 1 – Introduction

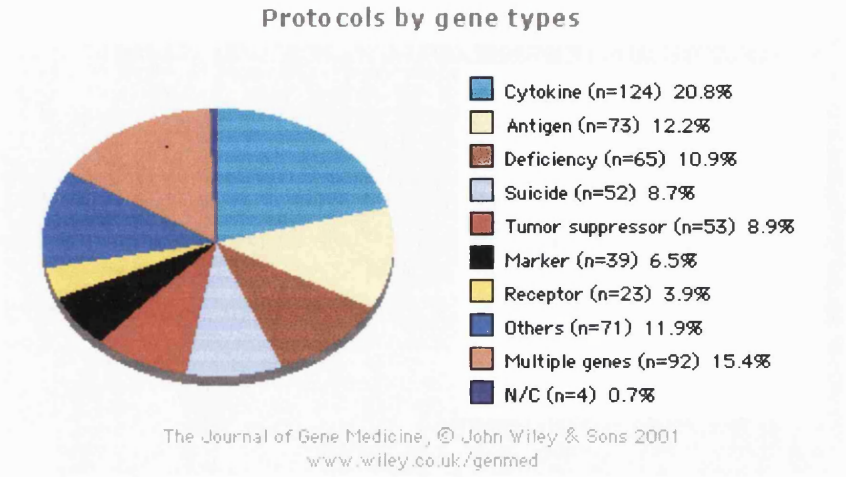
1.1. Gene therapy

Rapid progress brought about by advances in molecular biology techniques and the sequencing of the human genome has resulted in an increased knowledge of the genetic basis of disease and in the establishment of gene therapy as a potentially viable therapeutic option. Indeed, the possibility of expressing therapeutic genetic material in cells by gene therapy techniques has brought about new opportunities in the biological therapy of disease (see reviews by (Somia and Verma 2000; Drew and Martin 1999)). Initially, gene therapy focused on monogenic diseases (genetic disorders in which a single gene is missing or defective) in which the delivery of a correct copy of the gene corrects the genetic defect and thus arrests the disease process. Nowadays, a wide range of genetic disorders and even acquired and infectious diseases are considered as potentially legitimate targets for gene therapy, with genes delivered to augment the functions of existing genes or to provide new functions to the cell (e.g. drug sensitisation) (see chart 1A and chart 1B).

The introduction of genes into gamete cells holds the potential to eradicate hereditary diseases. However, human germline gene therapy is currently considered unacceptable due to ethical implications surrounding the permanent alteration of the human genome. In view of this, only somatic gene therapy is considered with the delivery of genes to the diploid cells of a patient where the genetic material is not passed on to his/her progeny. There are three different routes for gene delivery (Drew and Martin 1999): (1) *ex vivo* transduction or transfection of cells removed from the patient, followed by re-implantation; (2) direct administration to the target tissue - *in vivo* delivery; (3) administration of genetic material systemically – systemic *in vivo* delivery.

This introduction will focus on gene therapy and on the vector exploited in our laboratory, herpes simplex virus type 1. Particular emphasis will be given to Motor Neuron Disease as the work described in this thesis is ultimately aimed at use in this disease.

A.



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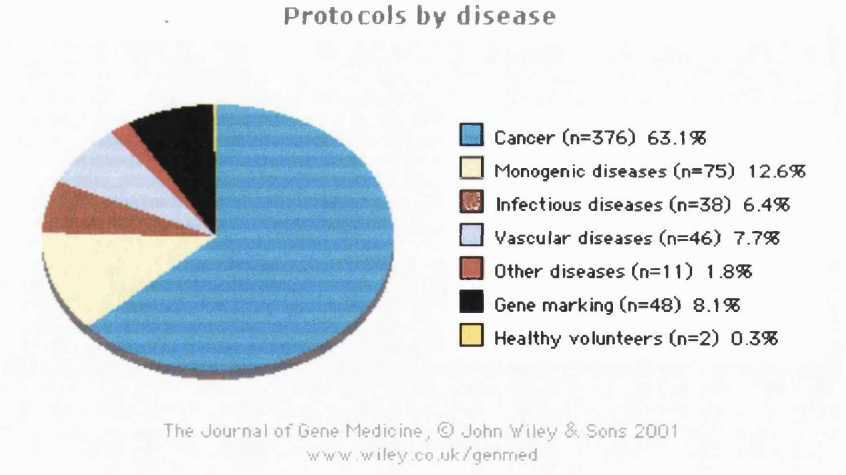


Chart 1- Indication of the gene types currently being delivered in gene therapy trials (A) and the diseases being treated (B). Reproduced with permission from the Wiley Journal of Gene Medicine website http://www.wiley.co.uk/genmed.

1.2. Gene therapy clinical trials

In order for a disease to be treated by gene therapy, several parameters must be fulfilled (Martin and Drew 2001). The disease must be of sufficient seriousness so that potential risks are outweighed by the potential benefits. The symptoms of the disease must be potentially reversible or progress of the disease halted by the treatment, and it must be possible to monitor the physiological effects of the inserted gene. The gene to be delivered must be well characterised and the route of delivery feasible. Since the first clinical trial of gene therapy in 1990 for the treatment of severe combined immunodeficiency (Culver *et al.* 1991), many clinical protocols have been approved world-wide. A full list of these protocols exists on the Internet at http//www.wiley.genetherapy/clinical.

1.2.1. Monogenic diseases

Cystic fibrosis is an autosomal recessive disorder with patients not surviving beyond their mid-thirties. The principal clinical problems are lung damage and respiratory failure, resulting of bacterial colonisation of the thick mucus that accumulates in the lungs of patients. The cause of the disease is the mutated cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, identified in 1989 following an international initiative (Kerem *et al.* 1989). Cystic fibrosis is one of the principal genetic disorders for which gene therapy has been in development. Cystic fibrosis fulfils the parameters mentioned in section 1.2. (Vassaux 1999). As it is a monogenic recessive disorder, it should in principle just require a copy of wild type *CFTR* to be delivered to the affected cells, whereas dominant disorders would also require the removal of the mutated gene. Cystic fibrosis is a lethal disease and the gene involved has been identified and characterised. The system mainly affected in the disease, the respiratory system, is relatively accessible and conveniently isolated target site for *in situ* gene therapy. Finally, electrophysiological measurements in epithelial cells can be used to monitor the efficacy of *CFTR* gene transfer. Cystic fibrosis correction has been attempted delivering *CFTR* in an adenoviral (Crystal *et al.* 1994) or in a cationic liposome vector (Caplen *et al.* 1995). The choice of adenoviral vectors for gene therapy in this case seems an obvious one because of the natural tropism of these viruses for the lung epithelium (reviewed in (Bramson *et al.* 1995)). The first trials using an adenoviral

vector delivered *CFTR* to 3 patients (Crystal *et al.* 1994). Evidence of CFTR function was presented for all 3 patients receiving different vector doses (Zabner *et al.* 1993). Since then, reports of other adenovirus trials described some function of CFTR function in a proportion of the patients involved but evidence of toxicity and immunological responses associated with the treatment were also described, particularly after repeat administration of the vector (Knowles *et al.* 1995; Zabner *et al.* 1996).

Adenosine deaminase (ADA) deficiency is a rare autosomal recessive disorder that results in the syndrome of severe combined immunodeficiency (SCID). Absence of ADA results in the block of T-lymphocyte differentiation in the thymus. ADA-SCID was the first disease to be treated in a phase I gene therapy clinical trial and was the first successful instance of gene therapy (Culver *et al.* 1991). Peripheral T lymphocytes were removed from the patients, transduced *ex vivo* with a retrovirus expressing ADA and re-infused. This procedure was repeated every month for two years and both children involved in the trial demonstrated ADA+ circulating lymphocytes and a normalised immune state. A similar trial conducted in Japan reported similar results, with a 3-year-old boy's immune functions improved (Onodera *et al.* 1998). More recently, a group in Paris successfully treated three babies suffering from a different fatal form of SCID, (SCID-XI) (Cavazzana-Calvo *et al.* 2000). This disease is an X-linked disorder characterised by an early block in T- and natural killer (NK) cell differentiation, due to mutations in the gene encoding the γC cytokine receptor subunit (reviewed in (Somia and Verma 2000)). This subunit is common to several interleukin receptors and mutations in the γC subunit lead to the disruption of signals required for growth, survival and differentiation of lymphoid progenitor cells. Haematopoietic stem cells from the three babies were transduced *ex vivo* with a retrovirus expressing the γC receptor gene and re-infused (Cavazzana-Calvo *et al.* 2000). Ten months later, the patients T-, B-, and NK-cell counts and function were identical to those of their healthy counterparts. After this, the first gene therapy clinic in the UK was opened in the Institute of Child Health, London, with the same treatment being administered to SCID-XI babies by Dr. Adrian Thrasher and colleagues.

1.2.2. Acquired disorders

Most of the approved protocols for gene therapy trials have been for the treatment of cancer (see chart 1B). This might seem surprising as cancer does not seem a suitable target for the classical approach of replacement gene therapy. Indeed, cancer is a multifactorial disease resulting of the interactions between a variety of somatic gene alterations and environmental stimuli. Taking this into account, gene therapy for cancer includes strategies involving augmentation of existing immunotherapeutic and chemotherapeutic approaches (reviewed in (Vile *et al.* 2000; Roth and Cristiano 1997)). These strategies include the use of expression vector constructs that convert inactive prodrugs into active drugs, the protection of bone marrow during chemotherapy by delivering drug-resistant genes to the bone marrow and the induction of immune-mediated destruction via the delivery of immunogenic antigens or cytokines (see chart 1A). Given the advances concerning the genetic basis of cancer, inactivation of oncogene expression and gene replacement for tumour suppresser genes are also included in cancer gene therapy following the more classical approach. One other strategy of particular interest involves the use of replication-competent virus vectors (see also section 1.3.3.5.2) (Vile *et al.* 2000). Probably the most publicised of such vectors has been ONYX-015, an adenovirus that preferentially replicates in cells lacking functional p53 (a tumour suppresser) due to a deletion of the E1B gene (Heise *et al.* 1997). In a clinical trial for head and neck cancer, this virus was found to replicate and lyse tumour cells whilst sparing surrounding healthy cells (Khuri *et al.* 2000). Tumours were treated with ONYX-015 and chemotherapy and it was found that this combination was more effective than each individual method.

1.3. Gene delivery vectors

The identification of the appropriate therapeutic genes, the target tissue and route of delivery is essential for successful gene therapy. However, the delivery system required to transport the genetic material into the cell is still considered the "Achilles heel" of gene therapy (Somia and Verma 2000). There are two categories of vectors used for gene delivery – viral and non-viral vectors (see chart 1 C). Each vector has advantages and disadvantages that make it suited for particular applications.

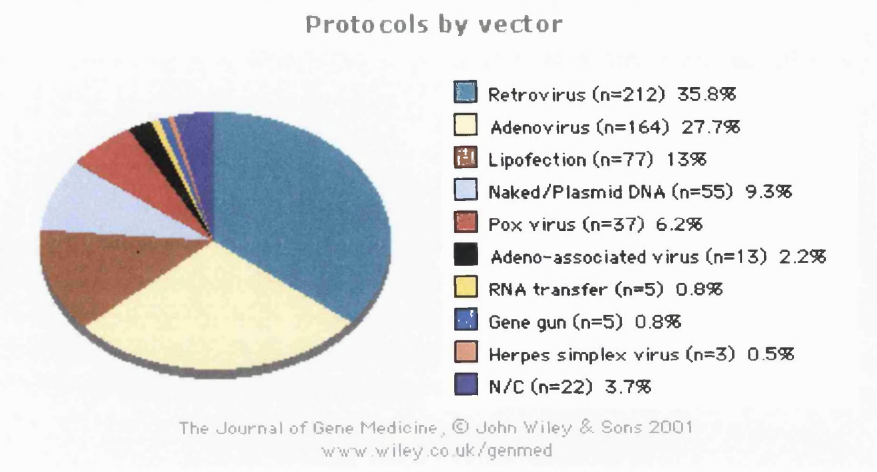


Chart 1C- Vectors currently used for gene delivery protocols. Reproduced with permission from the Wiley Journal of Gene Medicine website http//www.wiley.co.uk/genmed.

1.3.1. Non-viral vectors

Non-viral vectors include naked DNA, cationic lipid- and polymer-based systems. The simplest approach involves direct gene transfer with naked plasmid DNA but the efficiency of transduction observed is not high. To enhance this efficiency several approaches have been developed, such as gene gun technology (Yang *et al.* 1990) and electroporation (Rols *et al.* 1998). These allow the foreign DNA to enter the cell directly thus avoiding endosome-mediated entry. In the case of gene gun technology, DNA can be delivered directly into the cell nucleus. It has been reported that gene transfer efficiency following intramuscular injection of plasmid DNA and electroporation results in high level gene expression (Rizzuto *et al.* 1999). Direct delivery of DNA can thus give transgene expression following *in situ* delivery. For other routes of administration such as intratracheal and intravenous injection, a delivery vector or vehicle is required.

Polycationic based entities, cationic lipid- and polymer-based, have been the most extensively studied non-viral delivery systems (Li and Huang 2000). They cause

compaction of negatively charged nucleic acids accompanied by the formation of nanometric complexes (Miller 1999). These complexes protect the DNA from degradation and are able to enter cells by endocytosis triggered by interactions between the positively charged complex and the negatively charged cell-surface proteoglycans (Miller 1999). In order to increase cellular uptake, a targeting ligand can be added via a covalent or non-covalent linkage to the cationic lipid/DNA complex (lipoplex) (Li and Huang 2000). Before entering the cell, polycationic complexes show instability towards aggregation and have also been shown to be unstable in the presence of certain body fluid components such as serum. Once inside the cell, the majority of complexes become trapped in the endosome and are destroyed. A small percentage of complexes, however, are able to release bound DNA into the cytosol, which must then enter the nucleus. Improved transfection is achieved by increasing the release of plasmid DNA from the endosome to the cytoplasm, by the inclusion of a fusogenic helper lipid such as dioleoyl L-α-phosphoatidylethanolamine (DOPE) (Felgner *et al.* 1987). Lipoplexes have been used in several clinical trials for the treatment of cystic fibrosis. Levels of CFTR activity following cationic lipid- mediated delivery were similar to those observed using adenoviral vectors. In addition, no adverse effects of treatment were detected (Gill *et al.* 1997).

The use of synthetic, non-viral delivery systems is an alternative to viral vectors, as they circumvent the pre-existing immunity problems associated with viral vectors, are easy to use and easy to produce on a large scale (reviewed in (Li and Huang 2000; Miller 1999)).

1.3.2. Viral vectors

Over time, viruses have evolved efficient mechanisms which enable them to evade the host immune response, infect cells and deliver genetic material to the nucleus. These characteristics coupled with our extensive knowledge of viral molecular genetics have allowed attenuated viral vectors to be developed as gene therapy agents (reviewed in (Kay *et al.* 2001; Hermens and Verhaagen 1998; Somia and Verma 2000; Robbins and Ghivizzani 1998; Murphy 1999)). Recombinant viral vectors generally have deletions in genes encoding for pathogenic functions to ensure that cell infection does not lead to cell death and to prevent the spread of viruses to surrounding cells. The genes deleted are often essential for virus growth and consequently must be supplied *in trans* in a packaging cell line which enables the virus to be propagated *in vitro*. There are several ideal properties required of a viral vector for gene therapy purposes: (1) the vector components should be non-immunogenic; (2) the vector should not be toxic in terms of viral components and in terms of viral gene expression; (3) the vector should be replication defective and should be free of wild-type virus or helper virus; (4) the virus vector should be able to carry large sized inserts and should be capable of allowing long-term gene expression or of regulating expression in a precise way; (5) the vector should be easy to produce in large quantities. The most commonly used types of vectors for gene therapy are retroviruses, adenoviruses, adeno-associated viruses and herpes viruses.

1.3.2.1. Retroviruses

Retroviruses are enveloped single-stranded RNA viruses. Most of the retroviral vectors used for gene therapy purposes have been based on the Moloney murine leukaemia virus (MMLV) (reviewed in (Murphy 1999)). The virus genome comprises three core genes *gag*, *pol* and *env* flanked by long terminal repeat (LTR) sequences and a packaging signal (see figure 1.1A) (reviewed in (Kay *et al.* 2001; Murphy 1999)). The *gag* gene encodes viral structural proteins, the *pol* gene encodes the reverse transcriptase and the integrase and the *env* gene encodes the viral envelope glycoprotein. The LTR regions contain the *cis*-acting sequences required for regulation of genome replication, transcription and integration.

A.

5′LTR ψ *gag* *pol* *env* 3′LTR

B.

5′LTR ψ Therapeutic gene 3′LTR

C.

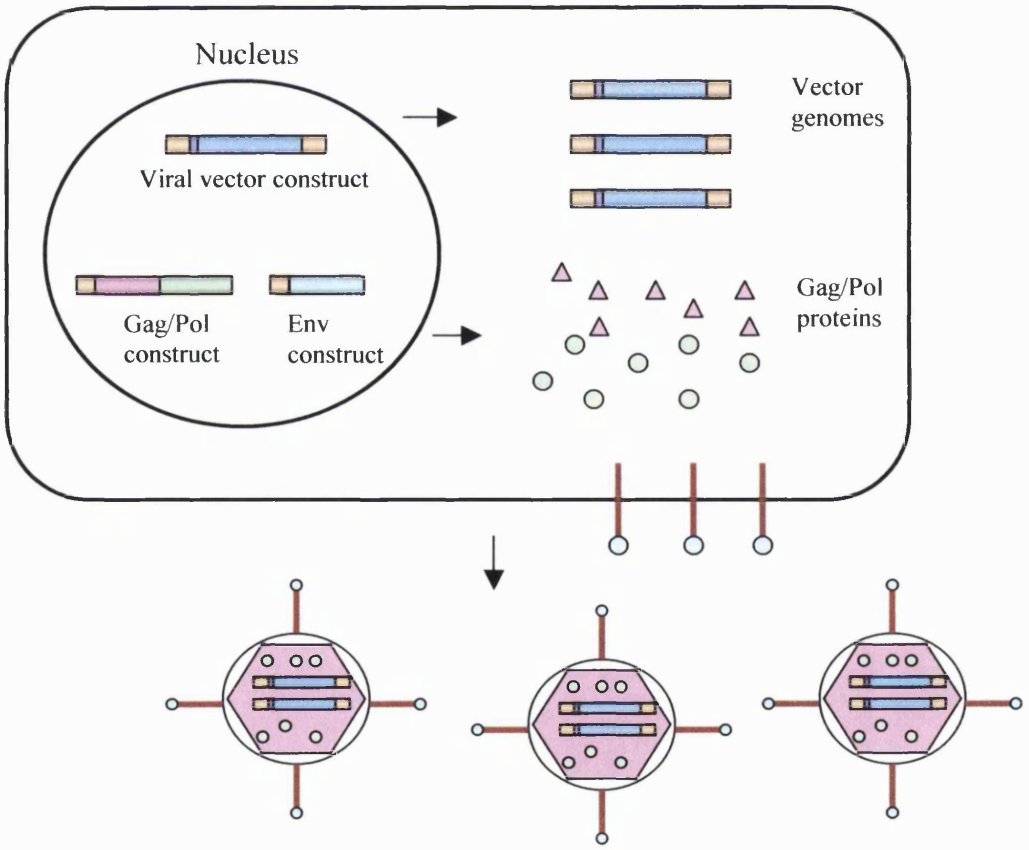


Figure 1.1 – **Retrovirus based vectors** - **A-** The retrovirus genome. The ψ sequence is the packaging sequence which serves to distinguish between viral and cellular RNA, and is recognised by the viral proteins for packaging. **B-** Vector genome. Viral genes *gag*, *pol* and *env* have been replaced by a therapeutic gene. **C-** The packaging cell. Due to the ψ sequence, vector genomes are encapsulated along with Gag and Pol proteins and are assembled under the cell membrane. The retroviral vector is released from the cell by budding through the membrane. Adapted from (Somia and Verma 2000).

The viral genes can be replaced with up to 8 kb of foreign DNA; there fore the transgene of interest may be inserted with its own promoter elements or placed under the transcriptional control of the LTRs (see figure 1.1B) (Murphy 1999). In order to allow viral growth *in vitro*, cell lines that provide *gag*, *pol* and *env* are used. To avoid recombination events resulting in wild-type virus formation, the cell lines provide *gag* and *pol* separated from *env* (see figure 1.1C) (reviewed in (Somia and Verma 2000)). Given the simplicity of manipulation of the vector, it is not surprising that retroviruses have been the most widely used vector system in gene therapy trials (Rosenberg *et al.* 2000) (see chart 1C). The tropism of the virus is dictated by its envelope glycoprotein. This tropism can be changed by pseudotyping, a process that involves replacing one viral Env with one belonging to another virus. Indeed, vectors pseudotyped with the G glycoprotein of the vesicular stomatitis virus (VSV-G) were shown to infect a wide range of cell types and could be grown to high titers (Burns *et al.* 1993). Following cell entry, the retroviral genome is reverse transcribed into double-stranded DNA and randomly integrated into the host chromosomes (Boris-Lawrie and Temin 1994). As a result, the virus can be propagated and the genetic information is maintained even in dividing cells. However, random integration into the host chromosome presents a problem connected with the possibility of oncogene activation or tumour-suppresser gene inactivation. Nuclear membrane disruption is required for entrance into the nucleus and thus productive transduction by most retroviral vectors (see lentiviral vectors, section 1.3.2.2), requires cell division shortly after entry. Consequently, the vectors are unable to transduce non-dividing cells, such as neurons and hepatocytes. Due to the requirement of cell division shortly after entry, gene therapy applications are limited to selected targets *ex vivo* such as lymphocytes. Despite this limitation, the use of retroviral vectors in certain cases has had considerable success, as shown in the SCID-IX clinical trials (Cavazzana-Calvo *et al.* 2000) (see section 1.2.1).

1.3.2.2. Lentiviruses

Lentiviruses, such as the human immunodeficiency virus (HIV), belong to the retrovirus family. In addition to the *gag*, *pol* and *env* genes of simple retroviruses, the HIV genome also carries genes encoding for six accessory proteins: *tat*, *rev*, *vpr*, *vpu*, *nef* and

vif (Naldini 1998). As opposed to retroviruses, lentiviruses possess karyophilic properties that mediate the active transport of the preintegration complex through the target cell nucleopore (Lewis *et al.* 1992). This attribute enables the virus to infect both dividing and non-dividing cells, an obvious advantage for a gene therapy vector. The first major step towards the use of lentiviruses as vectors for gene therapy was achieved by VSV-G pseudotyping, eliminating the viral tropism for cells expressing the CD4 receptor allowing targeting of a wide range of tissues (Naldini *et al.* 1996).

Given the pathogenicity of lentiviruses, there are major safety issues surrounding the use of these vectors in gene therapy protocols. One approach to improve vector safety involves the use of self-inactivating transfer vectors (Miyoshi *et al.* 1998). These vectors have deletions in the 3′LTR that transfer to the 5′LTR during viral replication causing the deletion of the LTR promoter and inhibiting the expression of full length viral RNA (Miyoshi *et al.* 1998). Another approach is based on the observation that the genetic information required for lentiviral vector derived transduction is only a fraction of the parental genome (Naldini 1998). Indeed, Zuffrey *et al* constructed a vector deleted for *env*, *vif*, *vpr*, *vpu* and *nef* (Zufferey *et al.* 1997). The vector was found to still be able to transduce non-dividing cells *in vitro* and found to deliver *β*-gal 2 weeks following vector inoculation into the rat striatum (Zufferey *et al.* 1997).

1.3.2.3. Adenoviruses

Adenoviruses are double-stranded DNA viruses 36 kb in size that cause benign respiratory-tract infections in humans. For gene therapy purposes, adenoviruses of the subgenus C are most commonly used and the members of this subgenus: Ad1, Ad2, Ad5 and Ad6, are endemic in the human population (reviewed in (Murphy 1999)). The viral genome remains episomal following cell infection and is functionally divided into two regions, early and late, as defined by temporal transcription (see figure 1.2). Alternative splicing leads to the RNAs for early, intermediate and late genes being generated from the primary transcripts (Robbins and Ghivizzani 1998). Both of the viral DNA strands are transcribed and they encode more than 50 polypeptides (Kay *et al.* 2001). The early (E) genes encode for proteins involved in host-cell transformation and viral DNA-replication whereas the late (L) genes encode for structural proteins.

One of the main advantages of adenoviruses resides in their broad cell tropism, infecting both dividing and non-dividing cells (Bramson *et al.* 1995).

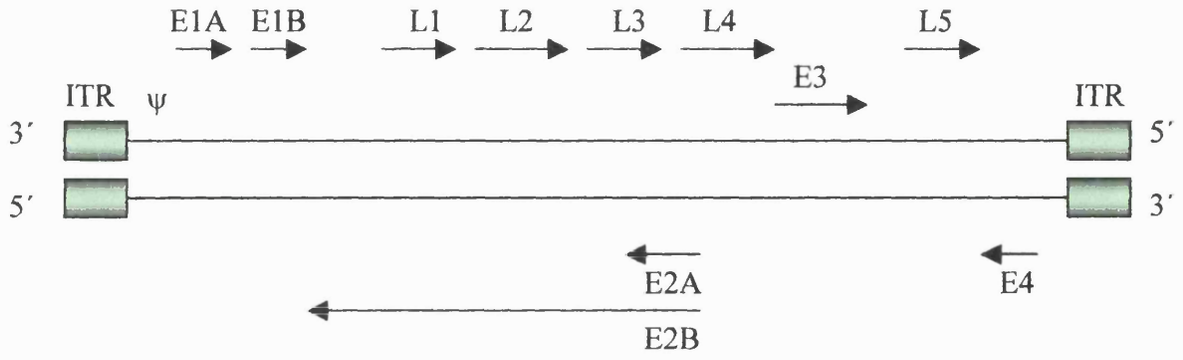


Figure 1.2- Schematic of the adenovirus genome - The double-stranded DNA genome contains overlapping transcriptional units (represented by arrows) on both DNA strands. The inverted terminal repeats (ITRs) function as replication origins and the ψ sequence is required for packaging of the viral genome. Adapted from (Robbins and Ghivizzani 1998).

First-generation adenoviral vectors were made by deleting the early region 1 (E1) to allow for transgene insertion and to render the virus replication defective. In order to increase the vector's cloning capacities to 8 kb, the E3 region was also deleted as it is not required for replication *in vitro* (see more below) (Bett *et al.* 1994). To grow these first-generation vectors, a cell line (293) that provided the E1 polypeptides *in trans*, was used (Graham *et al.* 1977). It soon became apparent that these vectors, although deleted for E1, maintained low-level transcription of the remaining viral genome due to host E1-like transcription factors (Yang *et al.* 1994). The viral proteins produced caused a host immune response, which resulted in cell-mediated destruction of the transduced cells and consequent reduction in gene expression (Yang *et al.* 1995). The cytotoxic capacities of these viruses were harnessed for cancer treatment (see section 1.2.2). For gene delivery purposes however less immunogenic vectors are required and more defective viruses have since been constructed.

The second and third generation adenoviral vectors were deleted for E2 (O'Neal *et al.* 1998) and E4 (Gorziglia *et al.* 1999) in addition to E1. The leaky expression of viral genes and the resulting immunogenic proteins were found to be minimised and decreased toxicity was observed following *in vivo* delivery (O'Neal *et al.* 1998). Both

second and third generation vectors are not fully deleted for E3 as seen in first generation vectors. An E3 19kDa protein encoding sequence is maintained as it is thought to inhibit cytotoxic T lymphocyte (CTL) induced lysis of adenovirus infected cells (Beier *et al.* 1994).

"Gutless" vectors, lacking all viral coding regions except for the ITRs and the packaging recognition signal, have been constructed (Kochanek *et al.* 1996). These vectors are not complemented *in vitro* by a cell line because firstly, adenoviral proteins are cytotoxic and secondly, because they could compromise the viral titres obtained after viral growth in culture (one of the advantages offered by adenoviruses as vectors). Consequently, a helper virus is used, which contains all genes required for replication but has a defect in its packaging domain. Thus the vector genome is the one packaged into the virion (reviewed in (Murphy 1999)). As well as reducing vector immunogenicity, the cloning capacity of the vector is increased to 36 kb. Indeed, Kochanek *et al* delivered a 28.2 kb cassette comprising full length dystrophin under a muscle-specific promoter and a lacZ reporter construct, into primary myoblasts (Kochanek *et al.* 1996). Reduced immunogenicity was reflected in the detection of recombinant dystrophin expression for 4 weeks following intra muscular injection in 6-day-old mice (Clemens *et al.* 1996). One limitation associated with this system resides in the difficulty in separating helper virus from vector (Robbins and Ghivizzani 1998).

The major obstacle to the use of adenoviruses as vectors for gene delivery is related to their significant immunogenicity (Murphy 1999). Humoral immune responses against the virus particles protect the host upon reencounter with the virus (Yei *et al.* 1994). As a result, repeated administration of a vector for sustained gene delivery is not possible due to the presence of neutralising antibodies. Removing the immunogenicity of the vectors can contribute to the decrease of cellular immune responses, but as the capsid structures are identical to wild-type virus the host humoral responses cannot be eliminated (Murphy 1999). Host immunosupression is one alternative. However, it is far from ideal in a scenario in which patients are already in a debilitated state.

1.3.2.4. Adeno-associated virus

Adeno-associated viruses (AAV) are small, single-stranded DNA viruses belonging to the parvovirus family (Robbins and Ghivizzani 1998). They are non-pathogenic and require a helper-virus (adenovirus or herpes simplex virus) for replication. Indeed, AAV was initially discovered as a contaminant in a preparation of adenovirus (reviewed in (Kay *et al.* 2001)). The AAV genome consists of two coding regions, *rep* and *cap*, flanked by the regulatory inverted terminal repeats (ITR) and the packaging signal (ψ) (see figure 1.3) (Murphy 1999). The *rep* genes encode proteins involved in replication whereas the *cap* genes encode the structural proteins (Somia and Verma 2000).

Figure 1.3- Schematic of the AAV genome- The single-stranded DNA genome contains two major coding units, *rep* and *cap*.

AAV-vectors are produced by replacing both *rep* and *cap* with a therapeutic gene, retaining the terminal repeats and the packaging signal. The vectors require *trans*-complementation of Rep and Cap in addition to the helper-virus for replication (Somia and Verma 2000). Due to the cytoxicity of Rep and some of the adenoviral helper proteins, there are no stably transfected cell lines capable of supporting AAV growth (reviewed in (Somia and Verma 2000)). As result, vector production involves co-transfection of vector plasmid with *rep* and *cap* containing-plasmid, followed by super-infection with helper virus. One problem associated with the production of AAV-vectors is the contamination with helper virus and with wild-type AAV (Kremer and Perricaudet 1995). One strategy for eliminating adenovirus infection involves the use of adenoviral DNA transfection, resulting in a triple transfection protocol for AAV-vector production (Xiao *et al.* 1998).

AAV-vectors integrate into a specific site of chromosome 19 following infection, through the action of the Rep protein (Samulski 1993). This insertion site appears to be

a "safe" region of the genome and so this property is of interest in gene therapy applications (reviewed in (Anderson 1998)). The one caveat is that AAV-vectors lack the *rep* gene and are found to integrate in a random fashion (Kotin *et al.* 1990) or remain episomal following cell infection (Duan *et al.* 1998).

AAV has a broad cell tropism and was found to transduce the majority of tissues following systemic administration (reviewed in (Murphy 1999)). It has also been shown to transduce non-dividing cells with long term expression in the muscle being reported (Xiao *et al.* 1996). These results added to the lack of cytotoxicity and immunogenicity hold great promise for AAV as vectors for gene therapy. The main disadvantages concerning these vectors are related to the small cloning capacity (about 4.8 kb) and the difficulty in scale-up of vector production (Robbins and Ghivizzani 1998).

1.3.3. Herpes Simplex Virus

1.3.3.1. Potential of HSV-1 as a vector for gene therapy

Herpes simplex viruses (HSV) are members of the alpha herpes virus family (reviewed by (Roizman *et al.* 1981)) and are among the most intensively investigated of all viruses. The two serotypes HSV-1 and HSV-2 are natural pathogens in man, and are the causative agents of recurrent oro-labial cold sores and genital herpes respectively (Roizman *et al.* 1981). HSV-1 is endemic within the human population, with approximately 80% of individuals testing positive for antibodies to HSV-1, although only a substantially lower percentage show symptoms of the disease. Infection due to spread to the central nervous system (CNS) resulting in an often fatal encephalopathy occurs rarely. HSV-1 has received attention as a candidate vector for gene therapy (reviewed in (Fink *et al.* 1996; Wolfe *et al.* 1999; Coffin and Latchman 1996)) due to a number of unique properties:

- **HSV-1 exhibits a wide host cellular range but during natural infection is neurotropic.** Due to the blood-brain barrier, the nervous system is particularly difficult to access by conventional drugs. In addition, it is physically inaccessible, so *ex-vivo* approaches which might be an appropriate choice in other areas of the

body, like the bone marrow or liver, are not feasible in the nervous system. Neurons, as they are post-mitotic and terminally differentiated cells, cannot be infected by retroviral vectors as these require cell division in order to integrate the genome. HSV-1, on the other hand, has evolved to infect and establish latency in neurons of the peripheral nervous system (PNS). Furthermore, replication-defective viruses are able to establish latency in several areas of the central nervous system (CNS) following stereotaxic injection (Kesari *et al.* 1996). Therefore HSV-1 is potentially an ideal vehicle for gene delivery to the nervous system, both peripheral and central. In addition, HSV-1 can infect other cell types as well as neurons. In dendritic cells and CD34+ve haemapoietic cells, normally refractory to transduction, efficient gene delivery was achieved following HSV-1 infection (Coffin *et al.* 1998).

- **HSV-1 establishes life-long asymptomatic latent infections.** Following an initial peripheral infection, HSV-1 enters latency in sensory neurons (see section 1.3.3.4). Latently infected neurons function normally and are not rejected by the host immune response. This demonstrates the potential for use as a vector which does not damage the neuron, and potentially allows long-term gene expression (reviewed in (Preston 2000)).

- **During latency the viral genome remains in an episomal structure.** Unlike some other viral vectors such as AAV or retroviral vectors, HSV-1 does not integrate into the host genome. As result, there is no possible inactivation/activation of tumour suppresser genes or oncogenes which might occur with integrating viruses.

- **HSV-1 is a large virus** which offers the possibility of accommodating large or multiple transgene cassettes. It is possible to disrupt a series of viral genes and replace them with up to 30 Kb of foreign genetic material.

- **HSV-1 is relatively easy to propagate** and the genetic manipulation of its genome is relatively straightforward.

1.3.3.2. The molecular biology and lifecycle of HSV-1

HSV-1 is a large, enveloped DNA virus. It has a ≈ 152 kb linear double-stranded DNA genome, which encodes at least 80 genes (Roizman and Sears 1996). The genome consists of two unique regions, long and short (U_L and U_S), each flanked by inverted terminal repeats (see figure 1.4). As such, genes present in the repeated regions are present in two copies per genome and those in the unique regions in only one. Approximately half of the viral genes are nonessential for the production of infectious viral particles in highly permissive cell culturing conditions, usually in African Green Monkey kidney (Vero) or Baby Hamster Kidney (BHK) cells. However, these nonessential or accessory genes are necessary to carry out the life cycle of the virus in the host efficiently (Roizman and Sears 1996). The genes that are required for virus replication in culture are said to be essential genes.

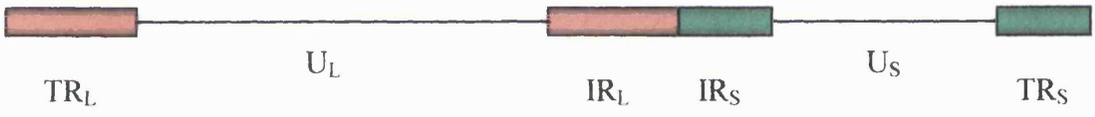


Figure 1.4 – A schematic of the HSV-1 genome. The genome has two regions of unique sequences (U_L and U_S) each flanked by inverted repeats (TR_L, TR_S, IR_L and IR_S)

Upon contact, HSV-1 infects and undergoes a cycle of lytic replication in epithelial cells of skin or mucous membranes (reviewed in (Roizman and Sears 1996)). An epithelial lesion results in the production of large numbers of viral particles that invade the epithelium and spread from cell to cell. Released viral particles are taken up by sensory nerve terminals and undergo retrograde axonal transport to reach the neuronal cell body. At this point, the virus can undergo a further round of lytic replication or can establish a latent infection (see section 1.3.3.4). If the virus enters latency, the viral genome can persist in an episomal state for the lifetime of the host. However, under the influence of several stresses, the virus may be reactivated from latency, reenter the lytic cycle and undergo anterograde axonal transport to skin or mucosal surfaces, leading to a recurrent productive virus infection at or near the original site of primary infection.

The HSV-1 particle consists of four elements arranged in a concentric fashion (see figure 1.5): an electron dense core containing the viral genome, an icosahedral capsid, a protein rich tegument and an outer lipid envelope containing several glycoproteins (gB through gM) (Roizman and Sears 1996). The process by which initial attachment of the viral particle to the cell surface occurs is thought to be mediated by at least three of the envelope glycoproteins (gB, gC and gD) and heparan sulphate moieties on the plasma membrane (Roizman and Sears 1996). Heparin acts as a potent inhibitor of HSV-1 attachment and removal of heparan sulphate from cells either by enzymatic treatment or by use of mutant cell lines lacking heparan sulphate results in a 85% decrease in virus attachment and cell infection (WuDunn and Spear 1989; Shieh and Spear 1994). Interestingly, competition assays with heparin never completely block attachment or infection, leading to the likelihood that heparan sulphate is one of multiple receptors or that it enhances binding of other glycoproteins to cell surface proteins. Indeed, both hypotheses might be true as different cell receptors and glycoproteins are involved in the attachment process and during its normal life cycle, the virus must infect and replicate in multiple cell types. One other receptor that has been implicated in the attachment process has been the basic fibroblast growth factor receptor (FGFR) (Baird *et al.* 1990).

HSV-1 enters cells by a fusion event (Morgan *et al.* 1968) that occurs at the plasma membrane following viral attachment and is mediated by the glycoproteins gB, gD and gH (reviewed in (Fink *et al.* 1996)). The virus can also spread from cell to cell by inducing the fusion of cellular membranes. Glycoproteins gB, gD, gH and gL are capable of inducing cell fusion when expressed from plasmid vectors in the absence of any other virus components, but only when all four glycoproteins are expressed on the same membrane (Browne *et al.* 2001).

Upon cell entry, the capsid-tegument structure is transported retrogradely to the nuclear pores where the viral DNA is released into the cell nucleus (Batterson *et al.* 1983). This retrograde pathway from the membrane to the nucleus is of particular importance in the case of the virus entering axons of dorsal root ganglia (DRG). The transport process involves cytoplasmic dynein, one of the major motor proteins involved in intracellular transport. From a series of glutathione S-transferase pull-down assays, it was found that IC-1a, the neuronal isoform of the IC (intermediate chain) of the dynein complex,

interacts with U_L34, a virion or tegument protein (Ye *et al.* 2000). So, after entry into cells, it seems likely that the U_L34 protein becomes exposed and interacts with the dynein motor and uses the microtubular network for retrograde transport of the capsid-tegument structure to the nuclear pore. At the nuclear pore, by a mechanism as yet unknown, the viral DNA is released into the nucleus.

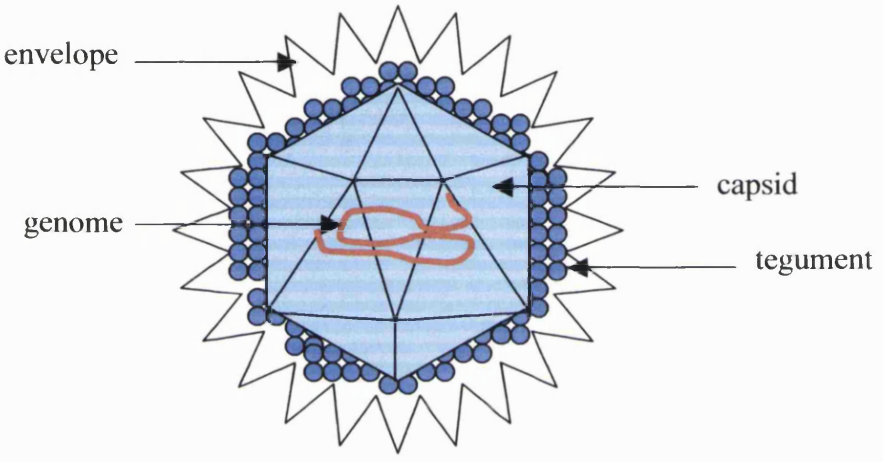


Figure 1.5- Schematic of the HSV-1 virion, showing the envelope surrounding the protein-rich tegument and the icosahedral-shaped capsid containing the electron-dense core with the DNA genome.

1.3.3.3. The lytic lifecycle

Following virus entry into the cell, several tegument proteins act to convert cellular resources from host cell protein synthesis to viral protein synthesis. The virion host shut-off protein (vhs) is released into the cytoplasm following viral cell entry. Early shut-off occurs in the absence of viral gene expression and is due to the presence of approximately 200 copies of vhs within the tegument of the virus (Kwong and Frenkel 1987). Therefore, even though the gene encoding vhs, UL41, is expressed as a late gene, shutoff can occur immediately after virus infection. This protein shuts off host protein synthesis by accelerating mRNA degradation (Kwong and Frenkel 1987). Indeed, a vhs null mutant, UL41NHB was shown to fail to induce the degradation of cellular mRNA

(Strelow and Leib 1995). Vhs is a nuclease which acts as a mRNA degradation factor that is targeted to mRNAs, and to regions of translation initiation through an interaction with the human translation initiation factor, eIF4H (Feng *et al.* 2001). Following the onset of viral transcription, vhs also accelerates the turnover of viral mRNAs (Kwong and Frenkel 1987; Oroskar and Read 1987). By accelerating the half-lives of all mRNAs, it helps redirect the cell from host to viral gene expression facilitating the sequential expression of different classes of viral genes (Feng *et al.* 2001).

A tegument protein, virion protein 16 (VP16, also referred to as α-transinducing factor, α-TIF, or vmw65, as determined by molecular weight) enters the infected cell nucleus together with the viral genome (reviewed by (Preston 2000)). The host cellular protein (HCF) is thought to have an important role in the transport of VP16 to the nucleus (LaBoissiere and O'Hare 2000). VP16 was first identified in 1984 and is a strong transactivator of immediate early (IE) gene expression (Campbell *et al.* 1984). VP16 acts through the target sequence TAATGARAT (R is a purine nucleotide), which is present in at least one copy in all HSV-1 IE promoters (Gaffney *et al.* 1985). The simplest mode of action of this transactivation would entail the direct binding of VP16 to the IE response elements; however, purified VP16 has no affinity for double stranded DNA (Marsden *et al.* 1987). Oct-1, a ubiquitous cellular transcription factor, belongs to a family of proteins which contain a DNA-binding domain, the bipartite POU domain (Herr and Cleary 1995). The POU domain is responsible for the binding to a number of octamer sequences, such as those found in the IE promoters (Herr and Cleary 1995). VP16 in association with the cellular protein HCF binds to the Oct-1/TAATGARAT complex, bringing the C-terminal acidic activation domain of VP16 (Triezenberg *et al.* 1988) into proximity with the preinitiation complex on the IE promoter. Interestingly, the VP16, HCF and Oct-1 complex can strongly bring about activation from a promoter proximal position but cannot induce transcription from a distal downstream enhancer position (Hagmann *et al.* 1997). These authors suggest that this acts as a limitation to the activity of VP16 to the IE genes, avoiding undesired long range effects on other viral promoters in the tightly packed HSV-1 genome. The dependence of VP16 on host proteins Oct-1 and HCF for transactivation of IE viral gene expression implies that the availability of these factors might be of importance in the initiation of the HSV-1 lytic lifecycle (see section 1.3.3.4).

During productive or lytic infection, viral genes are expressed in a well-ordered temporal cascade (see figure 1.6.) of immediate early (IE or α), followed by early (E or β), and subsequently late (L or γ) gene products (Honess and Roizman 1974). There are five IE genes, the infected cell products ICP0, ICP4, ICP22, ICP27 and ICP47. They are expressed in the absence of *de novo* viral protein synthesis and all but ICP47 are involved in the transcriptional and post-transcriptional regulation of the E and L genes.

ICP47 is involved in the evasion of the host immune system by blocking presentation of viral antigens to MHC class I-restricted cells (York *et al.* 1994). This IE gene product was shown to bind to TAP (transporter associated with antigen processing) thus preventing peptide translocation into the endoplasmic reticulum (Hill *et al.* 1995). As result of expressing ICP47 in HeLa cells under an inducible promoter Fruh *et al* showed that nascent class I molecules failed to acquire antigenic peptides (Fruh *et al.* 1995). In addition, this inhibition could be overcome by the transfection of murine TAP (Fruh *et al.* 1995).

ICP0 is a nuclear phosphoprotein required for efficient viral gene expression during lytic infection as it stimulates the expression of all classes of viral genes IE, E and L (Cai and Schaffer 1992; Chen and Silverstein 1992). ICP0 is considered a promiscuous activator of gene expression in transient assays as it can activate expression of viral and cellular genes whose promoter exhibits basal activity and can even turn on previously silent genes (Samaniego *et al.* 1998; Mosca *et al.* 1987; Nabel *et al.* 1988). It has been shown to activate gene expression at the level of mRNA synthesis (Jordan and Schaffer 1997) and shown to interact with a number of cellular factors from a variety of cellular pathways (translational, protein degradation and cell cycle control) capable of contributing to its properties (Maul and Everett 1994; Hobbs and DeLuca 1999; Kawaguchi *et al.* 1997). Although it is not essential for virus replication, ICP0 null mutants show significantly reduced levels of gene expression and virus yields, especially at low multiplicities of infection (MOI) (Cai and Schaffer 1989; Sacks and Schaffer 1987; Everett 1989; Cai and Schaffer 1989). At high MOIs, replication and gene expression are similar in mutant and wild type virus infected cells (Everett 1989). The factor or factors that compensate for the absence of ICP0 at high multiplicities of infection has not yet been identified. Studies by Hobbs and DeLuca have proposed one

other function to ICP0: following cell infection with a replication incompetent virus (*d*106) in which all IE gene expression was restricted to ICP0, cell cycle arrest was observed (Hobbs and DeLuca 1999). This arrest was not observed when cells were infected with a virus (*d*109) which did not express any IE genes (Hobbs and DeLuca 1999). The fact that ICP0 mRNA is antisense and partially complementary to LAT mRNAs is also suggestive of a role in latency (see section 1.3.3.4).

ICP4 is an essential IE protein and the major transcriptional regulator of viral gene expression. It activates the expression of early and late genes, whilst down-regulating its own expression and that of ICP0 (Preston 1979; Roberts *et al.* 1988; DeLuca and Schaffer 1985; Lium *et al.* 1996). Deleting ICP4 causes a dramatic reduction in viral gene expression, with only IE genes being expressed (due to lack of ICP4 repression) (DeLuca *et al.* 1985). ICP4 has been shown to bind DNA directly (Kristie and Roizman 1986) to consensus and non-consensus sites (Michael and Roizman 1989). It has also been shown to interact with the TATA binding protein and TFIIB (Smith *et al.* 1993).

ICP27 is an IE essential protein. It promotes the transition from early to late gene expression by repressing early and activating late gene expression (Sacks *et al.* 1985; Rice *et al.* 1994; Rice and Knipe 1990; McCarthy *et al.* 1989). It is also required for optimal viral DNA replication (Sacks *et al.* 1985; Rice *et al.* 1989). It was shown to act at the post-transcriptional level to inhibit pre-mRNA splicing, providing a selective advantage to the virus whose mRNAs are mainly unspliced when compared to cellular transcripts (Hardwicke and Sandri-Goldin 1994; Hardy and Sandri-Goldin 1994). Indeed, this is the virus' secondary stage of host shut-off (the primary stage being the one induced by vhs), which serves to reduce the remaining levels of host protein synthesis.

ICP27 was shown to inhibit the nuclear localisation of ICP0 and of ICP4, two important transcriptional regulators. Panagiotidis *et al* showed that ICP27 could bind to ICP4 and could modulate its DNA binding activity by affecting its post-translational modification status (Panagiotidis *et al.* 1997). They suggest ICP27 could therefore modulate transcription through its ability to modulate ICP4's DNA binding activity. Evidence of the importance of ICP27 is given by the variety of phenotypes of ICP27 null mutants: overexpression of early gene products, a decrease in DNA synthesis, an

underexpression of late gene products and an impairment in host shut-off (Sacks *et al.* 1985; Rice *et al.* 1989; McCarthy *et al.* 1989).

ICP22, a protein not essential for viral replication, can alter the phosphorylation state of host RNA polymerase II, which in certain cells is correlated to efficient viral gene expression during lytic infection (Sears *et al.* 1985; Rice *et al.* 1995).

Once IE proteins have been produced to sufficient levels, the infected cell is usually committed to transcription of the remaining viral genome, DNA replication and synthesis of progeny virions. Due to the cascade nature of HSV-1 gene expression, without the IE proteins, productive infection cannot occur.

The early or β genes follow the immediate early genes in the cascade. Early gene products are primarily responsible for viral DNA synthesis, including the enzymes required to produce the appropriate nucleotide pools in nondividing cells (thymidine kinase (TK), ribonucleotide reductase) and the enzymes essential for DNA replication and DNA-interactive proteins (DNA polymerase and origin-binding products). Viral DNA synthesis occurs by a rolling circle, which forms head-to-tail concatemers of U_L and U_S separated by the inverted repeat regions. During this process, due to homologous recombination between inverted repeats (acting as flanking elements), U_L and U_S may independently invert their orientation, resulting in the production of genomes in four of the possible DNA structural isoforms (Jacob *et al.* 1979). Expression of the L or γ genes, which encode many of the structural proteins of the capsid, tegument and envelope, is activated by the IE genes and only after viral DNA synthesis has occurred. Viral DNA concatemers are cleaved into genome length units and packaged into the capsids through the recognition of the packaging sequence "a" located in the internal repeat of the short segment. The capsids assemble in the nucleus of the infected cell and acquire an envelope by budding through the inner nuclear membrane. This is not likely to be the final envelope of the virus: the phospholipid composition of secreted virions is not the same as that of the nuclear membrane (van Genderen *et al.* 1994); non-enveloped virions are observed in axons during egress (Holland *et al.* 1999) and a major tegument protein, VP22, is observed exclusively in the cytoplasm of virus infected cells (Elliott and O'Hare 1999). Skepper *et al* using immunogold electron microscopy showed that the capsids did indeed acquire an

envelope by budding through the inner nuclear membrane, but showed as well that mature progeny virions are deenveloped at the outer nuclear membrane followed by reenvelopment in a post-ER cytoplasmic compartment (Skepper *et al.* 2001).

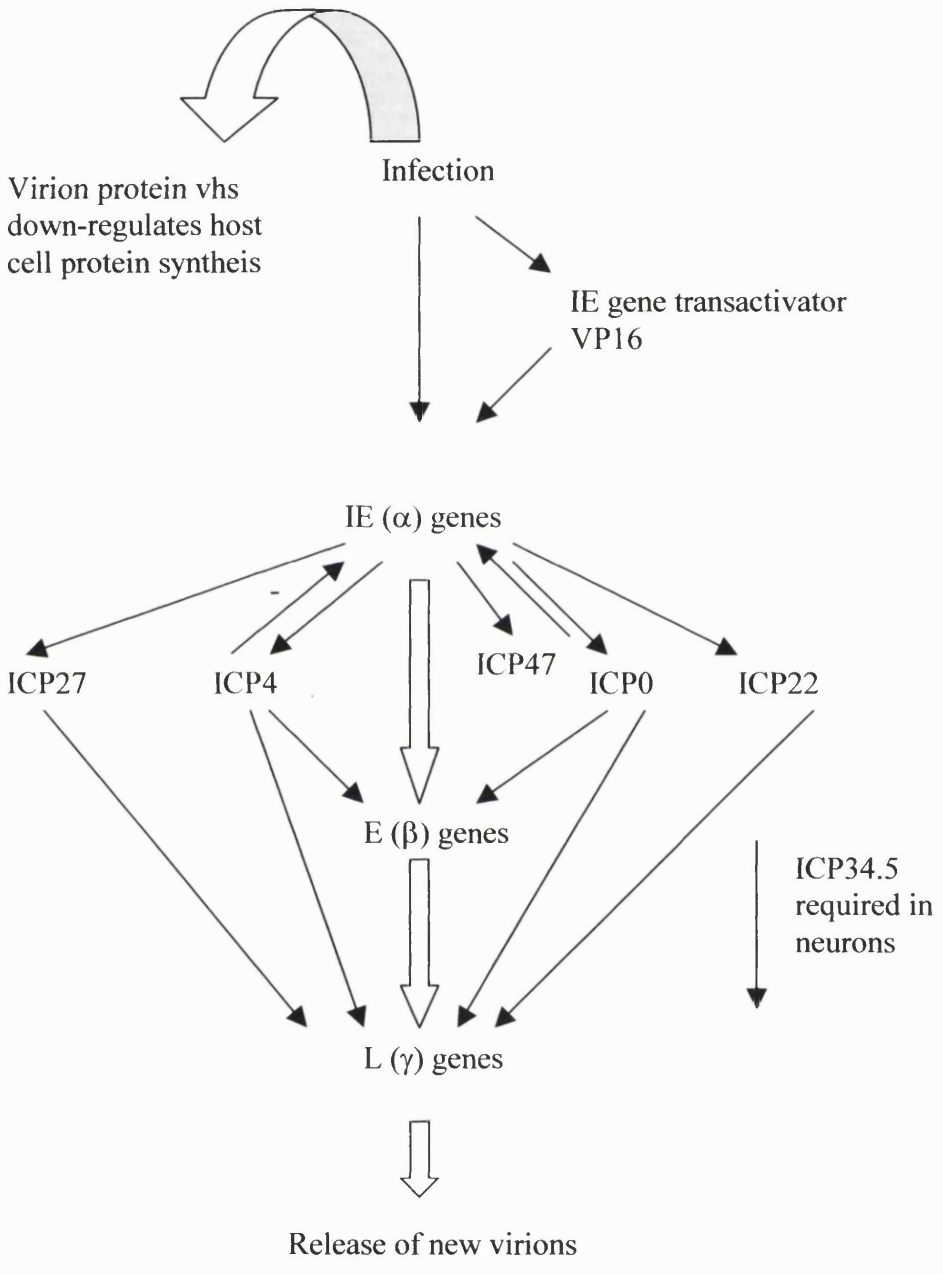


Figure 1.6 – The HSV-1 lytic gene regulatory cascade- Upon virus entry into the cell, vhs shuts off host protein synthesis and VP16 transactivates the IE promoters. Expression of essential IE genes is required for subsequent expression of E and L class genes. Based on a schematic by (Fink *et al.* 1996).

1.3.3.4. The latent lifecycle

Following an initial peripheral infection, HSV-1 infects sensory neurons where in an alternate pathway to infection it can establish a life-long latent state. Periodic reactivation may occur resulting in lytic infection at epithelial cells close to the initial infection site. The precise mechanisms by which HSV-1 establishes, maintains and reactivates from latency are still not entirely understood.

During latency, the viral genome persists as a circular episome, condensed into a chromatin-like structure (Efstathiou *et al.* 1986). No genes of the lytic cycle are expressed and the genome remains quiescent except for a transcriptionally active region mapped within the inverted long repeat sequences, producing the latency associated transcripts (LATs) (Rock *et al.* 1987; Stevens *et al.* 1987). The most abundant LAT species is a 2 kb non-polyadenylated intranuclear RNA, although minor species of 1.45 kb and 1.5 kb can also be detected (Spivack and Fraser 1988). Both major and minor species seem to be stable introns derived by splicing from a large, highly unstable polyadenylated primary LAT transcript (see figure 1.7). This 8.3 kb transcript seems to be found in low abundance in latently infected neurons (Zwaagstra *et al.* 1990) and is antisense to the sequence of IE gene, ICP0. The 2 kb and 1.5 kb species are overlapping and complementary to the 3′ end of the ICP0 gene.

There are two promoter regions controlling LAT expression, LAP1 and LAP2 (see figure 1.7). LAP1, a TATA box containing promoter, is located 28 bp upstream of the LAT primary transcript and 700 -1300 bp upstream of the 2 kb LAT. It contains an enhancer element which gives it increased promoter activity in some cells of neuronal origin (Zwaagstra *et al.* 1990). A recombinant virus in which β-globin was inserted 17 bp downstream of the TATA box was shown to produce β-globin specific mRNA for 3 weeks but at decreased levels to those shown just following infection (Dobson *et al.* 1989). LAP2, located between LAP1 and the 5′end of the 2 kb LAT, lacks a TATA box but contains sequences frequently observed in eukaryotic housekeeping gene promoters (Goins *et al.* 1994). Recombinant viruses containing a LAP2-*lacZ* reporter gene cassette in the glycoprotein C (gC) locus were reported to produce β-galactosidase following latency in trigeminal ganglia (Goins *et al.* 1994). Deletion of both promoters,

LAP1 and LAP2 results in undetectable levels of the 2-kb LAT in culture (Chen *et al.* 1995). These authors showed that both promoters were critical for the 2-kb LAT expression but under different conditions. Using viruses deleted for either LAP1, LAP2 or both, they concluded that LAP1 is essential for LAT expression during latency, whereas LAP2 is primarily responsible for LAT expression under lytic conditions (Chen *et al.* 1995). Latency promoters are discussed more extensively in section 1.3.3.6.

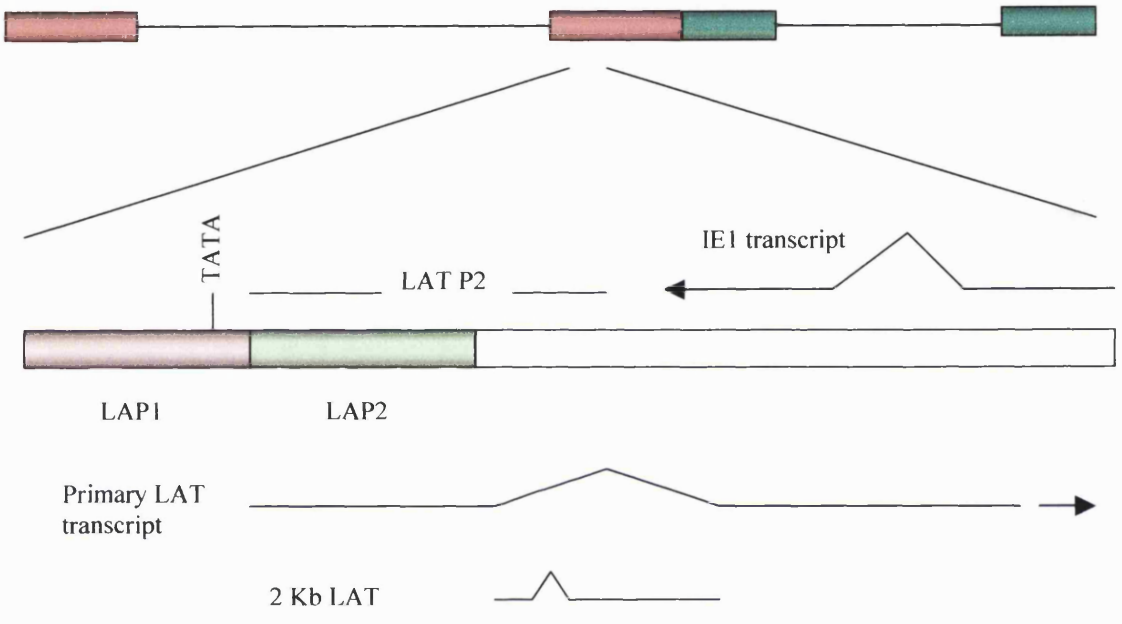


Figure 1.7 - Schematic representation of the latently active region of HSV-1, showing promoter regions LAP1, LAP2 and LAT P2, and relevant RNA transcripts. Adapted from (Palmer *et al.* 2000).

1.3.3.4.1. A block to IE gene expression - function of LATs

Considering no gene products are transcribed other than the LATs during latency, one might assume these have some role in latency, be it establishment, maintenance or reactivation (see review by (Preston 2000)). Some of the first studies using LAT null mutants stated that these were not required for the establishment of latency (Ho and Mocarski 1989; Sedarati *et al.* 1989; Leib *et al.* 1989). However, subsequent studies using LAT promoter mutants, which are incapable of producing any of the LAT mRNAs, showed the mutants to be impaired in the establishment of latency (Perng *et al.*

2000; Thompson and Sawtell 1997). Taking into account that the sequence of the LATs is in part complementary to ICP0, one possible explanation for this phenotype would be that LAT functions as a regulator of ICP0 expression via an antisense mechanism. As such, LATs would act by reducing the amounts of active ICP0, consequently reducing immediate-early gene expression and forcing the establishment of latency. Two lines of argument support the concept of a block to virus replication at the level of IE gene expression. One is that the IE gene products are cytotoxic (Johnson *et al.* 1994); during latency neurons remain undamaged by the virus and no IE gene mRNA can be detected in latently infected neurons. The other is based on the *in* 1814 virus, a virus containing a mutation in VP16 that prevents the interaction of VP16 with Oct-1 and HCF, resulting in low levels of IE gene expression (Ace *et al.* 1989). When inoculated into mice, *in* 1814 establishes latency as effectively as wild-type HSV-1 (Valyi-Nagy *et al.* 1991; Steiner *et al.* 1990), leading to the conclusion that transactivation of IE genes by VP16 is not an absolute requirement for establishment of latency, and that the block to replication during latency occurs before the level of IE gene transcription.

One other function mapped to the LAT region was the ability to reactivate from latency. Several studies have shown that LAT promoter and/or LAT transcription deletion mutants are impaired for reactivation from latency (Perng *et al.* 1994; Hill *et al.* 1990; Leib *et al.* 1989). One possible explanation for this phenotype involves a putative viral protein encoded by the LAT RNAs (Thomas *et al.* 1999). Indeed, there are several open reading frames (ORFs) in the LAT region leading to the idea that LAT might encode for a protein which could be involved in the establishment, maintenance or reactivation from latency. Indeed, the LAT region of a related α-herpesvirus, the bovine herpes virus (BHV), has been found to encode a protein which may have a role in preventing the death of latently infected neurons (Hossain *et al.* 1995; Schang *et al.* 1996). Thomas *et al* found an ORF in the major 2 kb LAT, conserved between HSV-1 strains (Thomas *et al.* 1999). The deregulated expression of this ORF was shown to significantly enhance growth of both wild type viruses and viruses deficient in IE gene synthesis, such as ICP0 and VP16 mutants (Thomas *et al.* 1999). These authors suggest a model by which LAT acts as an antisense transcript to ICP0 during the establishment and maintenance of latency (as mentioned above) but during reactivation, it expresses a protein which compensates for the lack of ICP0 and other IE gene expression, initiating the lytic lifecycle. Although this is an elegant model some caveats must be registered:

no phenotype is observed when the HSV-1 LAT ORFs are mutated (Fareed and Spivack 1994), no protein has ever reliably been detected *in vivo* and the LAT transcripts are intranuclear species. In addition, it was shown that the first 1.5 kb of the primary LAT transcript is entirely sufficient for reactivation (Drolet *et al.* 1999; Perng *et al.* 1996). The complexities involved in latency studies have meant that the exact role of the LATs is still unclear.

1.3.3.4.2. A block to IE gene expression- Oct-1 and HCF

Considering many members of the Oct family have the ability to bind to the TAATGARAT motif, it was hypothesised that other Oct proteins could act as competitors to prevent formation of the VP16/Oct-1/HCF complex and as result repress IE transcription. Using immortalised neuronal cell lines, Lillycrop *et al* showed the binding of neuron-specific forms of Oct-2 (Oct-2.4 and Oct-2.5) to the TAATGARAT element, which competed with the binding of Oct-1 (Lillycrop *et al.* 1991). The Oct-2 isoforms could not bind VP16, which suggested this as the reason for the non-permissiveness of neuronal cells for the viral lytic cycle, thereby allowing the establishment of latency (Lillycrop *et al.* 1994).

One hypothesis which might account for the repressed IE transcription is related to the cell-specific differences in the location of HCF. In most cell types HCF has a general or nuclear distribution (Kristie *et al.* 1995; LaBoissiere and O'Hare 2000). In neurons, however, it was found to be exclusively cytoplasmic (Kristie *et al.* 1999). The inefficient transcription of the IE genes could be due to a lack of HCF to form the VP16/Oct-1/HCF complex or to the consequence of the failure of HCF mediated transport of VP16 to the cell nucleus (LaBoissiere and O'Hare 2000).

1.3.3.4.3. ORFs O and P

Two proteins transcribed from the ORFs O and P have been implicated in the block of IE gene expression (reviewed by (Preston 2000)). The RNA encoding these proteins overlaps in the sense orientation, the LAT primary transcript (Lagunoff and Roizman 1994). Interestingly, ORF P was found to be expressed only during infection with

HSV-1 ICP4 mutants (Lagunoff and Roizman 1994). Furthermore, it was found that ORF P could bind to splicing factors, inhibiting the accumulation of the spliced mRNAs encoding ICP0 and ICP22 early in infection (Bruni and Roizman 1996). Taken together these results can predict a latency model: in the absence of ICP4 expression, ORFs O and P would be produced resulting in the prevention of synthesis or function of IE proteins, maintaining the genome in a nontranscribed state. During lytic infection, ICP4 would repress the production of the RNAs from ORF O and ORF P. One caveat to this model exists in that it has been demonstrated that the two proteins are not necessary for establishment of a latent infection in mice (Randall *et al.* 2000). However, the authors argue that the ORFs O and P are effective in the maintenance rather than the establishment of latency. In a review, Preston argues that the ORFs O an P act as "safety valves" which suppress IE gene expression when the initial block to IE gene transcription has been evaded (Preston 2000).

1.3.3.5. HSV-1 manipulation for gene delivery purposes

In a context of gene transfer, two different approaches have been developed in order to create HSV-1 vectors: the use of disabled viruses and the use of defective viruses. Both will be discussed below, and their respective strengths and weaknesses will be evaluated.

1.3.3.5.1. Defective HSV-1 vectors (amplicons)

The defective HSV-1 vector is based on observations of virus growth: following serial virus propagation in culture at high multiplicity, defective herpes virions accumulate. The genome of these virions consists of reiterations of a small sequence containing a replication origin and a cleavage-packaging signal. Importantly, these virions are incapable of replicating in the absence of a helper virus, which opens the possibility of transgene insertion and delivery to a target cell where they are unable to replicate. Spaete and Frenkel cloned the reiterated sequence (3.9 kb) of one of these defective virions into a plasmid vector (referred to as an amplicon) (Spaete and Frenkel 1982). Following co-transfection in cultured cells of the plasmid and a wild-type helper virus, concatemers of the amplicon up to a viral genome in length were packaged into the

HSV-1 capsid (Spaete and Frenkel 1982). Although this method allows high-level transgene expression (due to the presence of many copies of the transgene) and easy manipulation, the requirement of a helper virus for growth implicates the presence of the latter in the final mixture. Since the defective and helper virus cannot be distinguished physico-chemically, the helper virus cannot be removed from the defective stock (Hermens and Verhaagen 1998). Upon serial passage of stocks from the initial amplicon transfection, the ratio of defective to helper virus increases due to a selective growth advantage of the former as it contains several origins of replication (Spaete and Frenkel 1982). However, complete elimination of the helper virus is impossible.

In order to maximise gene delivery, cytotoxicity limited to the helper virus must be minimised and as a result, disabled helper viruses have been developed (see figure 1.8). One such disabled virus was tsk, a virus containing a temperature sensitive mutation in ICP4 allowing growth at 31°C and inhibiting viral growth at 37°C (Davison *et al.* 1984). Using this virus as a helper, a defective virus containing β-galactosidase driven by the ICP22/47 promoter was produced, giving high levels of gene delivery in cultured rat PNS neurons (Geller and Breakefield 1988) and CNS neurons (Geller and Freese 1990) and in several cultured human cells (Boothman *et al.* 1989). Using the same helper virus, a similar defective virus was produced containing β-galactosidase driven by the CMV promoter (Kaplitt *et al.* 1991). This virus was shown to give transgene expression *in vivo* for at least two weeks, following brain injection in the rat (Kaplitt *et al.* 1991). The relatively high reversion of the tsk helper to wild-type renders this virus less than ideal for *in vivo* use. As such, a helper virus (D30EBA) completely deleted for ICP4 was produced, which could be complemented in culture and which gave lower reversion frequency (10^{-5}) than tsk (Geller and Freese 1990). Using this helper virus, a defective vector was created with human tyrosine hydroxylase cDNA under the control of an HSV immediate early promoter. This vector was injected into the striatum of 6-hydroxydopamine lesioned rats (a model for Parkinson's disease) and found to produce dopamine measurable by microdyalisis and to induce behavioural recovery for up to a year (During *et al.* 1994). This study reflects the advantages and disadvantages of the technology used. On one hand, there is the possibility of long term gene expression at phenotypically active levels; on the other hand, due to the reversion of the helper virus to the wild type phenotype, 10% of the animals used in the experiment died within two

weeks, presumably of HSV-1 encephalitis (During *et al.* 1994). Indeed, this is a problem concerning amplicon technology: although disabled viruses are used to decrease cytotoxicity, in order to increase the ratio of defective virus to helper virus, serial passaging is required, which implies the inevitable recombination and reversion of helper virus to wild type phenotype.

The choice of promoter driving the transgene is not as important as when using disabled vectors (see section 1.3.3.6). This is due to the fact that amplicons contain very little HSV-1 DNA and thus are not expected to shut down gene expression by assuming a stable DNA structure which is transcriptionally inactive, as seen during latency. One would then expect gene expression to continue until the DNA is degraded within the cell. Long-term and cell specific gene expression can thus be obtained, something which has proved to be somewhat more difficult in what disabled vectors are concerned. Two studies exemplify this: an amplicon with a pre-proencephalin promoter driving β-galactosidase was shown to give cell type-specific transgene expression in the rat brain for two months (Kaplitt *et al.* 1994); similarly, an amplicon vector containing the rat tyrosine hydroxylase promoter was found to give tyrosine hydroxylase expression up to 6 weeks and this expression was cell type specific (Song *et al.* 1997). In both these cases, the transgene expression was found to follow the endogenous pattern specified by the promoter.

Two systems have been developed in order to avoid the necessity of helper virus in the propagation of defective vector. The first "helper-free" system is based on the use of a set of cosmids that overlap and represent the whole HSV-1 genome except for the cleavage/packaging signals (Fraefel *et al.* 1996). No contamination of the defective stock exists, because even if the cosmids reconstitute a functional genome by homologous recombination, they cannot be packaged. This system is however difficult to establish due to the instability of cosmid clones. The second "helper-free" system which exhibits increased stability is based on the expression of the entire HSV-1 genome (except for the cleavage/packaging signals) in a bacterial artificial chromosome (BAC) (Saeki *et al.* 1998).

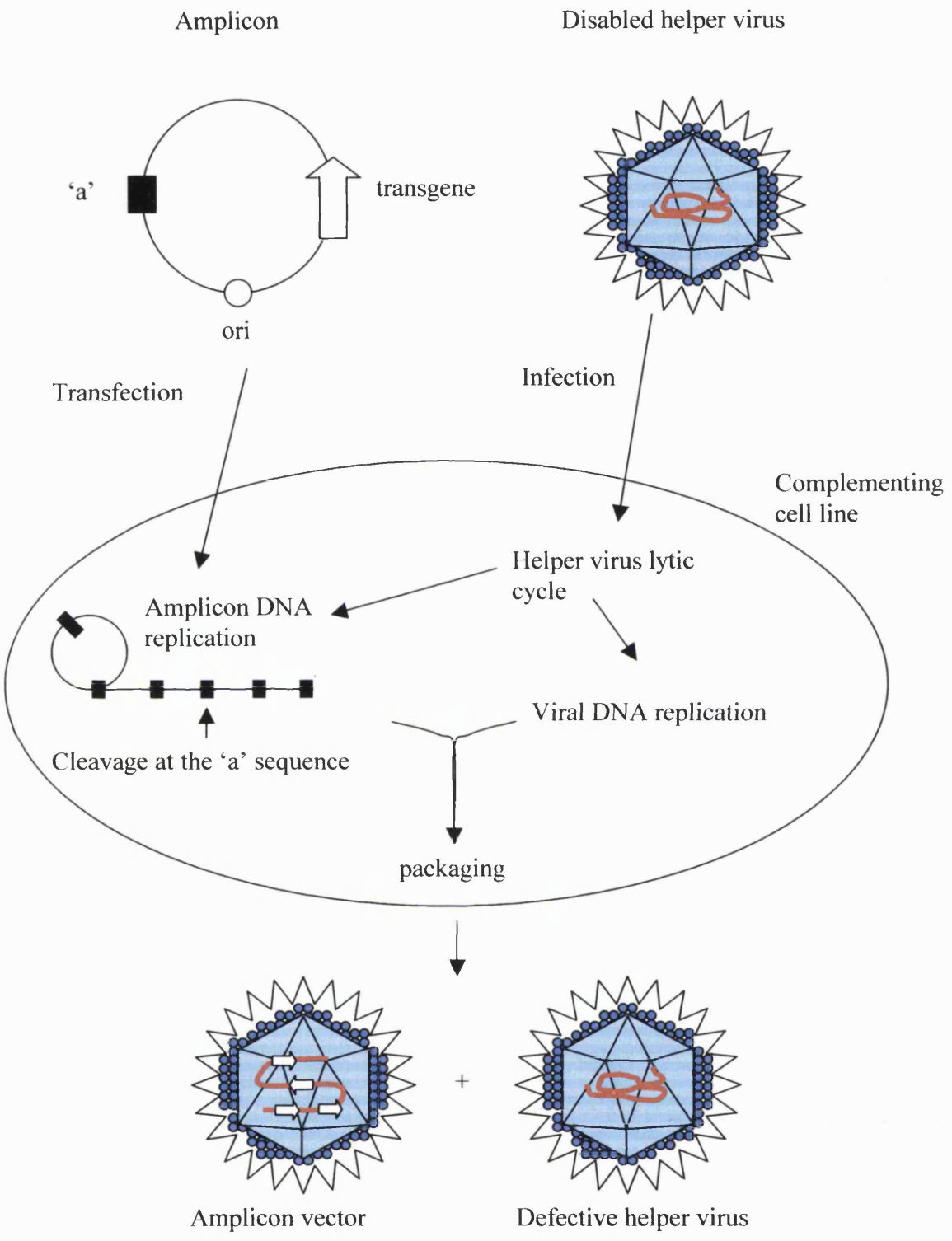


Figure 1.8- The amplicon system- HSV amplicons contain an HSV origin of replication (ori), a packaging signal 'a' and a transgene expression cassette. Following transfection the cells are superinfected with a helper-virus and given that the amplicon has the two essential sequences for replication and packaging, defective HSV vector particles are formed. Since there is always helper virus present in the final mixture, to achieve minimal cytotoxicity disabled helper viruses are used. Depending on the degree of disablement of the helper virus, a complementing cell line may be used. Based on (Fink and Glorioso 1997).

Defective vectors exploit the concept that the delivery of a foreign gene to the nucleus of a cell ideally requires only the HSV-1 capsid and envelope, offering an alternative to the use of modified whole-virus vectors. Amplicons are easy to manipulate genetically due to their reduced size and consequent unique restriction sites; and defective vectors offer the possibility of long term and cell specific gene expression in addition to high levels of transgene expression as the transgene is present in multiple copies. However, these vectors also have limitations. There are differences between individual vectors in the number of transgene concatemers, complicating reproducibility. They present the problem of helper virus contamination, helper virus which might have reverted to wild-type phenotype as result of serial passaging. These vectors and even those obtained in a "helper free" system do not take advantage of the ability of HSV to establish a life long latent infection and only low titres are achieved. It is true that transgene delivery up to one year was obtained (During *et al.* 1994) but as the amplicon DNA is degraded one might expect the levels of transgene to decrease with time (Coffin and Latchman 1996).

1.3.3.5.2. Disabled vectors

The other strategy for the development of herpes vectors involves the insertion of the foreign gene directly into the viral genome and the deletion of genes involved in the production of cytopathic effects. Due to the large size of the HSV-1 genome, it is not possible to clone a restriction fragment of foreign DNA directly into it. As result, a different strategy is used, which involves the introduction into cells of the viral DNA together with the foreign DNA flanked on each side by at least 1 kb of unaltered viral sequence (reviewed in (Coffin and Latchman 1996)) (see figure 1.9). Since the viral DNA is still infectious, a lytic cycle ensues and by homologous recombination, the foreign DNA is introduced into the virus at a site dictated by the flanking viral sequences. Of the viral plaques produced, a small percentage will be of recombinant virus which can then be selected due to a phenotypic difference (marker gene activity or in the case of an essential gene deletion, growth in a complementing cell line).

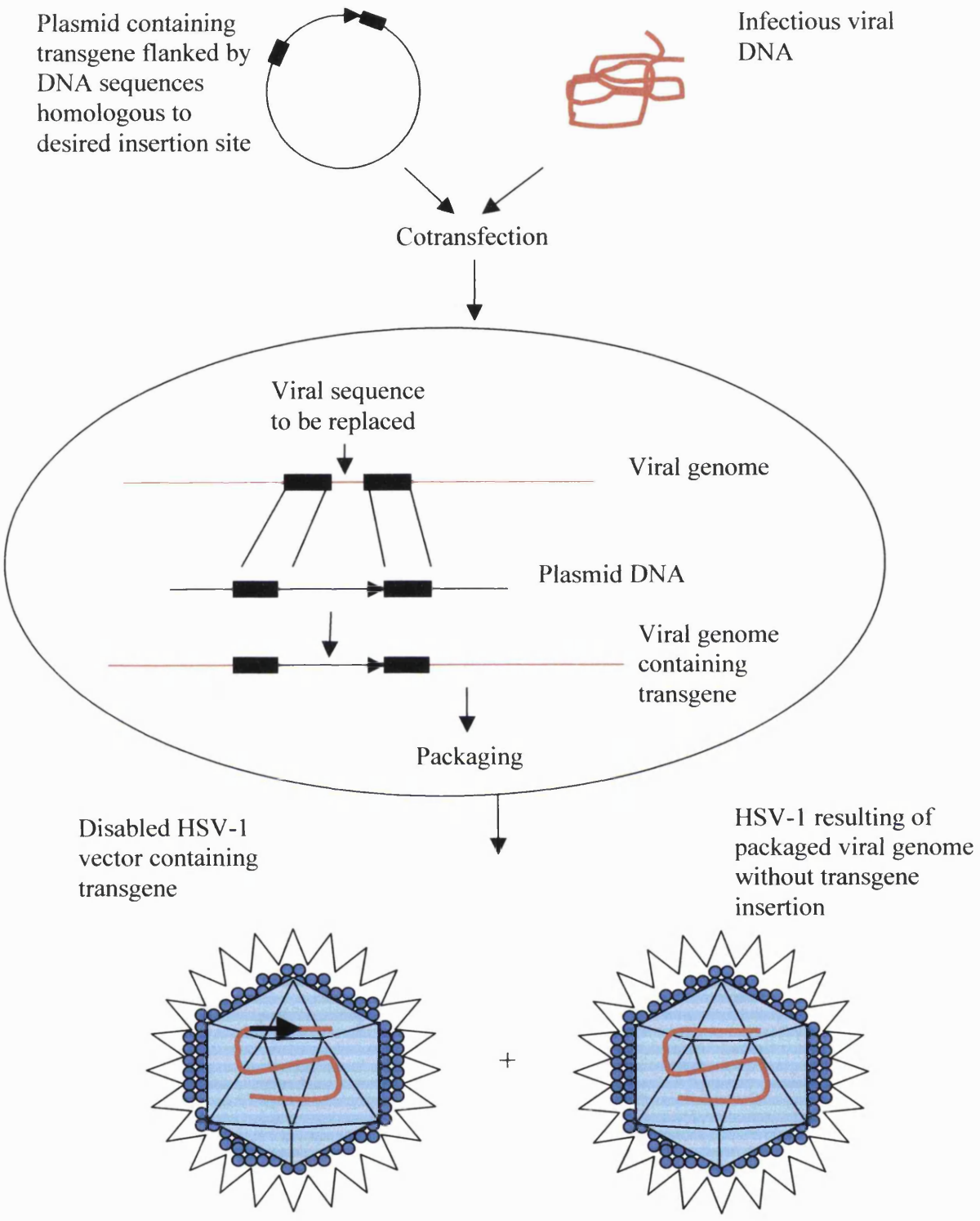


Figure 1.9- Production of disabled viruses- Cells are cotransfected with plasmid containing the transgene of interest and with viral DNA. Due to the flanking of the transgene with the viral DNA of interest, the transgene is inserted into the viral genome by homologous recombination. Transgene insertion results in the deletion of the viral sequence in between the flanking regions. The viral mixture resulting of the cotransfection can be distinguished phenotypically and the vector is selected for accordingly.

Following direct brain injection of wild-type HSV in test animals, lytic replication and fatal encephalitis occurs. In order to consider HSV-1 as a vector for gene delivery, the virus must be disabled in order not to enter lytic replication and to be forced into a latent state (reviewed in (Fink *et al.* 1996; Marconi *et al.* 1996; Hermens and Verhaagen 1998; Coffin and Latchman 1996)). When disabling the virus, two types of genes may be deleted, essential and non-essential. The essential genes are absolutely required for growth and pathogenicity and their deletion requires complementation for viral growth in culture. In the case of non-essential genes, these are required for growth *in vivo* but not *in vitro.* The first disabled vectors produced were deleted for non-essential genes, which rendered the vectors non pathogenic *in vivo* and did not require complementation in culture. Initial vectors were deleted for the thymidine kinase (tk) gene, with the insertion of either β-galactosidase (Ho and Mocarski 1988) or hypoxanthine phosphoribosyltranferase (Palella *et al.* 1988a). Both vectors gave expression of the transgene for 5 days in mouse DRG (Palella *et al.* 1988a) or brain (Ho and Mocarski 1988). The viruses showed decreased virulence in neurons although they were still lethal after inoculation at high titers (Palella *et al.* 1989b). Of all non-essential genes, the deletion of ICP34.5 was found to give the biggest decrease in neurovirulence, with an LD_{50} (lethal dose) of $>10^6$ p.f.u. (plaque forming units) following intracerebral inoculation in mice as compared to an LD_{50} of $<10^2$ p.f.u. for wild-type virus (Chou *et al.* 1990). Indeed, the disabled virus was found to be replication defective in the CNS and sensory ganglia of mice (Chou *et al.* 1990; MacLean *et al.* 1991). Interestingly, at some primary sites of infection, (e.g. footpad) the mutant was still found to be capable of replication (Robertson *et al.* 1992). It has become a general consensus that ICP34.5's effect on viral replication is cell type and tissue specific and that ICP34.5 null mutants permit viral replication in actively dividing cells but not in terminally differentiated cells (Brown *et al.* 1994). Based on these observations, an ICP34.5-deleted mutant (1716) containing a *lac*Z gene under the control of the LAT promoter (see below) was constructed and injected into the footpad and into the mouse brain (Coffin *et al.* 1996). It was found to give transitory transgene expression in the sensory ganglia and brain (Coffin *et al.* 1996). This virus, however, was found to still express viral antigens, eliciting a host immune response and causing inflammation in the brain (McMenamin *et al.* 1998; McMenamin *et al.* 1998). For gene delivery purposes therefore, ICP34.5-deleted viruses require further disablement. However, one application for these vectors, based on the ability of the mutants to allow productive HSV-1 replication in dividing

cells but not in non-dividing cells, has been in the treatment of brain tumours. These mutants have been found to replicate within tumour cells, causing their death, whilst sparing the surrounding brain tissue (Kesari *et al.* 1998). Indeed, the 1716 virus and a virus deleted for ICP34.5 and ICP6 are currently being used in clinical trials in patients with recurrent high grade gliomas (Markert *et al.* 2000; Rampling *et al.* 2000).

Considering the cascade nature of HSV-1 gene expression, gene therapy approaches have been devised based on the removal of IE genes, preventing the lytic cycle and promoting the establishment of latency. Deletion of essential genes ICP4 and ICP27 effectively blocks the lytic lifecycle preventing virus growth in any cell type. Some of the first vectors developed were deleted for ICP4, preventing viral replication unless grown on a complementing cell line providing ICP4 *in trans* (Dobson *et al.* 1990; DeLuca *et al.* 1985; Chiocca *et al.* 1990). Infection of non-permissive cells with an ICP4 deleted virus was associated with expression of the remaining IE genes (ICP0, ICP22, ICP27 and ICP47) and the large subunit of ribonucleotide reductase (ICP6) but not with early and late genes (DeLuca *et al.* 1985). In addition, these mutants were still able to enter a latent state as observed by β-galactosidase expression in mouse sensory neurons albeit in a very small number of cells (Dobson *et al.* 1990). Interestingly, despite the limited gene expression observed in the ICP4 mutant background, these first vectors proved to be cytotoxic (Johnson *et al.* 1992; Chiocca *et al.* 1990). Injection of these viruses in the brain resulted in transient expression of β-galactosidase and despite reduced pathogenicity when compared to wild-type virus, necrosis at the site of injection was observed (Chiocca *et al.* 1990). *In vitro*, one to three days after infection with an ICP4 mutant, cytoplasmic blebbing, fragmentation of cellular DNA and appearance of chromosomal damage was observed in the virally infected cells (Johnson *et al.* 1992). These cytopathic effects were found to be reduced by two methods which reduced viral gene expression: UV irradiation and pretreatment of cells with interferon (Johnson *et al.* 1992). This led to the conclusion that the cytopathic effects observed were not due to the toxicity of the virion itself but due to viral gene expression (which implied the involvement of one or more of the remaining IE genes expressed). Following infection with viruses deleted in one or more of the five individual IE genes, cytotoxicity was still observed (Johnson *et al.* 1992). In addition, the remaining IE genes reduced the transformation of cells when cotransfected individually with a selectable marker, implying that the IE genes were cytotoxic with no single gene

product being entirely responsible (Johnson *et al.* 1994). In this assay system, ICP6 and ICP47 were found not to inhibit transformation. The vhs protein, which was initially thought to contribute to the cytotoxicity of the ICP4 null mutants, was found not to do so as Johnson *et al* showed that no decrease in cytotoxicity was observed when comparing the ICP4 deleted virus with a vhs/ICP4 deleted virus (Johnson *et al.* 1994).

Based on these observations, two different groups constructed a vector deficient in IE gene expression, deleted for ICP4, ICP27 and ICP22, grown on a cell line providing ICP4 and ICP27 *in trans*. Krisky *et al* studied the effect of infection of primary rat cortical and dorsal root neurons in culture. They found that the virus deleted for ICP4, ICP27 and ICP22 showed reduced cytotoxicity when compared with viruses deleted for ICP4 alone or in combination with either ICP22, ICP27 or ICP47 (Krisky *et al.* 1998). Furthermore, cultured neurons infected at a low multiplicity with the triple mutant survived over 21 days in culture and allowed transgene expression for over 14 days (Krisky *et al.* 1998). Wu *et al* showed that following infection of Vero cells with this mutant at an MOI of 10, the cells retained a relatively normal morphology and expressed genes from the viral and cellular genomes up to 3 days postinfection (Wu *et al.* 1996). Viruses deleted for ICP4 alone or in combination with ICP27 or with ICP22 were too toxic to allow gene expression 1 day postinfection (Wu *et al.* 1996). However, in cells infected with the triple mutant, cellular DNA replication and cell division were found to be inhibited (Wu *et al.* 1996). Upon electron microscopy analysis of the nucleus of these infected cells, large ICP0-containing nuclear inclusions were found (Wu *et al.* 1996). Pooling the results obtained by both groups, one concludes that the virus deleted for ICP4, ICP27 and ICP22 offers decreased cytotoxicity and the possibility of high level transgene expression. It is hypothesised that such transgene expression is due to the accumulation of ICP0, acting as an activator of gene expression, but the same ICP0 causes cell cycle arrest and alterations in infected cell nuclear morphology. In accordance with these observations are the results obtained using a virus deleted for ICP4, ICP27 and ICP0 (Samaniego *et al.* 1997). The authors found that the virus was significantly less toxic than the virus deleted for ICP4, ICP27 and ICP22 with no inhibition of cell division observed. In addition, β-galactosidase inserted under the ICP0 promoter was detected up to 14 days postinfection, with the levels dropping dramatically after 1 day. In a cell line complementing ICP0 in addition to ICP4 and ICP27 (see below), the transgene levels were increased but the cells were

destroyed 3 days postinfection (Samaniego *et al.* 1997). The triple mutant was also found to be less cytotoxic than a virus deleted for ICP4 and ICP27. However, at a high MOI, cell survival was reduced to 40%, thought to be due to the increased accumulation of ICP22. This suggested that further deletion of ICP22 would render the vector less cytotoxic.

The next step in disabling HSV-1 would be the deletion of all IE genes, for an ultimately non-cytotoxic and safe vector (Lilley *et al.* 2001). Samaniego *et al* created a virus, *d*109, deleted for all IE genes (Samaniego *et al.* 1998). It was found to be nontoxic to Vero and human embryonic lung (HEL) cells, even at a MOI of 30. The viral genome thus remained in a quiescent but fully functional state for at least 28 days, as shown by transgene expression (*lacZ* under the control of HCMVIEp in the ICP27 locus) upon superinfection with a virus expressing ICP0 (Samaniego *et al.* 1998). This study highlights two aspects concerning a vector fully deleted for all IE genes. The first concerns transgene expression. A vector which is fully disabled for all IE gene expression but which cannot provide expression of a foreign gene (probably due to promoter shutdown) is as a result of limited use in gene therapy. Ideally, one would achieve long-term gene expression by exploiting the promoters involved in latency (see section 1.3.3.4). The second aspect concerns the growth of these viruses. Deletion of ICP4 and ICP27 requires complementation for virus production (DeLuca *et al.* 1985; Sacks *et al.* 1985). The deletion of ICP22 and ICP0, whilst not essential genes, impairs viral replication, especially at a low MOI (Cai and Schaffer 1992; Chen and Silverstein 1992). A virus deleted for all IE genes would ideally be complemented by a cell line providing these 4 gene products *in trans*. The major problem is that given the toxicity of the IE genes (Johnson *et al.* 1992), such a cell line is hard to generate (Krisky *et al.* 1998). One alternative has been the generation of cell lines with one or more IE genes under the control of their own promoter, since these are practically inactive in the absence of viral infection (Samaniego *et al.* 1997; DeLuca and Schaffer 1987). Using this strategy, the cell line made by Samaniego and colleagues to allow growth of the virus deleted for all IE genes, expressed ICP4, ICP27 and ICP0 (Samaniego *et al.* 1997). As mentioned above, in the study of transgene expression by the virus deleted for ICP4, ICP27 and ICP0, when the virus was grown on the cell line containing all these IE gene products, the cells were dead within 3 days probably due to the presence of ICP0 expressed by the cells themselves following viral infection (Samaniego *et al.*

1997). In addition, the authors report that the cell line could not offer full and consistent complementation of ICP0 (Samaniego *et al.* 1997). As shown, the construction of a cell line offering complementation for all IE genes is a complex endeavour, with none having been reported so far.

A different approach for the creation of a virus deficient in IE gene expression involves the inactivation of the gene encoding VP16, the transactivator of IE promoters following virus infection (see section 1.3.3.3) (Lilley *et al.* 2001; Palmer *et al.* 2000; Thomas *et al.* 1999; Marshall *et al.* 2000; Johnson *et al.* 1994; Preston and Nicholl 1997). This strategy allows for the potential reduction of IE gene expression without the need to delete individually all the IE genes and arrange for a complementing cell line. As VP16 is an essential structural component of the virion, it cannot be deleted. An approach like the one used for IE genes in which the cell line provides the gene product is of no use in this case, because the newly formed virions would carry in their tegument the fully functional VP16 provided by the cell line. Upon infection of non-complementing cells, these virions would be able to transactivate the IE promoters and thus the effect desired from the VP16 mutation would have been abolished. As result, instead of a deletion of the gene, an insertional mutation is used. A 12 bp insertion within the coding sequence for VP16 disrupts a domain of the protein responsible for the interaction with Oct-1 and HCF (see above) whilst allowing it to retain its structural role (Ace *et al.* 1989). This VP16 insertion mutation was inserted into HSV-1 strain 17^{+}, and the resulting virus (*in* 1814) was unable to transinduce IE gene expression, expressed IE genes at a reduced efficiency and was avirulent in mice (Ace *et al.* 1989). This virus was shown to be able to establish latency *in vivo* (Steiner *et al.* 1990), and from *in vitro* experiments it was found that *in* 1814 was much less toxic than wild-type virus and preferred latent to lytic growth (Harris and Preston 1991). The VP16 mutation does not require complementation *in vitro* as the inclusion in the media of hexamethylbisacetamide (HMBA) can transactivate the IE genes (McFarlane *et al.* 1992). At a high MOI *in* 1814 can still initiate infection (Ace *et al.* 1989) and as such, mutant viruses have been constructed with further deletions. Johnson *et al* combined the VP16 mutation with a deletion for ICP4 and found it to be considerably less cytotoxic than a virus deleted for ICP4 alone (Johnson *et al.* 1994). This is thought to be due to the reduction in expression of the IE genes ICP0, ICP27 and ICP22 (Johnson *et al.* 1994). A combination of the VP16 mutation with a temperature sensitive

mutation of ICP4 and a promoter exchange to reduce expression of ICP0 was also reported (Preston and Nicholl 1997). No cytotoxicity was observed in Vero cells following infection with this virus at an MOI of 5 and incubation at the non-permissive temperature of 38.5° (Preston and Nicholl 1997). A similar virus with a deletion of the RING domain of ICP0 as opposed to a promoter exchange was reported to be non-pathogenic in mouse DRG following footpad inoculation (Preston *et al.* 1998). Exploiting the LAT promoters, long-term transgene expression was achieved (Marshall *et al.* 2000). Taking one step forward, a virus which is completely replication-incompetent was obtained by combining the VP16 mutation with deletions in ICP4, ICP27 and ICP34.5 (Lilley *et al.* 2001). In non-complementing cells, minimal amounts of IE genes ICP0, ICP22 and ICP47 were detected. ICP6 was detected to significant levels, but this IE protein has been shown to be non-toxic (Johnson *et al.* 1994). Similarly to a mutant with all IE genes deleted individually (Samaniego *et al.* 1998), it was found to persist in Vero cells in a quiescent state. As opposed to the results reported by Samaniego *et al*, GFP was detected up to 23 days post-infection (reflecting the difference in promoters chosen to drive transgene expression, see below) (Samaniego *et al.* 1998). Indeed, the replication-incompetent virus containing a reporter cassette either in the vhs or LAT locus was found to be non-toxic following infection of primary DRG cultures (Lilley *et al.* 2001). In addition, it was found to give transgene expression for at least one month following direct brain and spinal injection (Lilley *et al.* 2001). The cell line used to complement this virus contained ICP4, ICP27 and the equine herpesvirus homologue of VP16 (Thomas *et al.* 1999). The latter was used because it was found that HMBA couldn't provide satisfactory transactivation for sufficient viral growth, and the equine homologue was able to grow the replication incompetent virus in culture. In addition, the equine homologue presents minimal sequence homology to HSV-1 VP16 and as such is not packaged into the HSV-1 virions (Thomas *et al.* 1999).

1.3.3.6. Achieving transgene expression

Hand in hand with the creation of non-cytotoxic vectors has been the effort to achieve stable long-term gene delivery (reviewed in (Lachmann and Efstathiou 1999; Fink *et al.* 1996; Coffin and Latchman 1996)). Early studies revealed that insertion of foreign genes into areas of the genome not usually active during latency or under the control of

promoters not usually active during latency, be it HSV-1 or heterologous non-HSV-1 promoters, did not allow long-term gene expression (see table 1).

Transgene inserted	**Promoter**	**Position of insertion**	**Reference**
HPRT	ICP4 ICP8	Tk	(Ho and Mocarski 1988)
LacZ	tk	Tk	(Palella *et al.* 1988a)
	gC IE CMV	US3	(Fink *et al.* 1992)
	ICP0	ICP0	(Chiocca *et al.* 1990)
	ICP6	ICP4	
	Metallothienin	gC	(Lokensgard *et al.* 1994)
	Phosphoglycerate kinase		
	MMLV LTR		

Table 1.1- Studies driving transgene expression from a lytic gene locus, using heterologous non HSV-1 promoters or HSV-1 promoters of genes expressed during the lytic lifecycle. All experiments resulted in short term marker gene expression. CMV – cytomegalovirus; MMLV LTR – Moloney murine leukaemia virus long terminal repeat long terminal repeat.

In all studies mentioned in table 1.1, gene expression was obtained at high levels in the mouse CNS or PNS (Palella *et al.* 1988a) but only for 4-5 days post infection. As the virus enters latency, all transcription is shut down including that of the marker gene, limiting foreign gene expression to that observed (during lytic infection). This shutdown might be related to unusual conformations taken up by the HSV-1 genome, which is >80% G+C rich, in order to minimise gene expression. One interesting study, in which the Moloney murine leukaemia virus long terminal repeat (MMLV LTR) driving *lacZ* was inserted into the ICP4 locus, allowed long term expression for at least 24 weeks in the PNS and 5 weeks in the PNS (Dobson *et al.* 1990). These results seemed surprising at the time, as when inserted into the gC locus only short term

expression was obtained (Lokensgard *et al.* 1994). With the knowledge currently available, one explanation of these results is linked with the fact that MMLV LTR/*lac*Z was inserted in a position antisense to ICP4, placing its 3' end in the same orientation as the LAT promoters (the MMLV promoter will be discussed in greater depth below).

As previously mentioned, one advantage in using HSV as a vector for gene delivery resides in its ability to establish life long latency in the host. As a result, exploring long term gene delivery using an HSV-1 vector, has long involved an exploitation of this natural ability. The expression of LATs is controlled by two promoters LAP1 and LAP2 (see figure 1.7). The insertion of β-globin just downstream of the LAP1's TATA box resulted in the detection of β-globin specific mRNA for 3 weeks after footpad injection in the mouse, although the RNA levels were reduced significantly with the establishment of latency (Dobson *et al.* 1989). Similarly, the insertion of *lac*Z or the nerve growth factor (NGF) gene just downstream of the TATA box showed abundant transgene expression in the mouse DRG 4 days following footpad inoculation, but none following 21 days (Margolis *et al.* 1993). The inability of LAP1 to drive gene expression through latency was also shown when the promoter was inserted at an ectopic site in the genome: a LAP1/*lac*Z cassette inserted into the gC locus only gave short -term expression with the β-galactosidase staining decreasing over a few weeks (Dobson *et al.* 1995; Margolis *et al.* 1992). In conclusion, LAP1 seems to drive high-level short-term transgene expression, within the LAT region or ectopically from another region in the genome. *In vivo* experiments using a virus in which the *lac*Z gene was inserted into the LAT region 136 bp upstream of the 5' end of the 2-kb LAT showed that the mutant was unable to produce β-galactosidase during productive infection, but in the trigeminal ganglia of infected mice, *lac*Z expression could be detected up to 8 weeks (Ho and Mocarski 1989). The staining observed was of a punctate appearance within the latently infected neurons (Ho and Mocarski 1989). Following this, plasmid transfection experiments by Goins *et al* identified LAP2, located between LAP1 and the 5'end of the 2-kb LAT (where Ho and Mocarski had inserted *lac*Z) (Goins *et al.* 1994). A LAP2/*lac*Z reporter cassette inserted into an ectopic site, the gC locus, was reported to drive low level transgene expression in the mouse DRG for at least 300 days (results shown up to 150 days) (Goins *et al.* 1994). No transgene expression was obtained from productive infection *in vitro* and the staining observed *in vivo* was of a punctate nature as seen by Ho and Mocarski with the

insertion of *lacZ* after LAP2 in the LAT region. In conclusion, LAP2 drives low level extended gene expression, either from within the LAT region or at an ectopic site.

Ideally, one would combine the high-level expression capabilities of LAP1 with the long-term activity associated with LAP2. Indeed, insertion of a *lacZ* gene linked to the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) 750 bp downstream of LAP2 did not disrupt the structural integrity of the LAT region, allowing the transgene to be expressed (Lachmann and Efstathiou 1997). Furthermore, β-galactosidase could be detected in mice DRG up to 190 days, and in specific brainstem areas up to 307 days (Lachmann and Efstathiou 1997). The same reporter cassette was inserted into a virus impaired for IE gene expression with a mutation in VP16, a temperature conditional mutation of ICP4 and deletion of the RING domain of ICP0 (Marshall *et al.* 2000). This mutant established latency in the DRG and β-galactosidase was expressed in increasing numbers of neurons over the first 25 days of infection (onset of latency) and then remaining constant over 5 months (Marshall *et al.* 2000). The same virus when injected into the rat striatum gave transgene expression for 4 weeks, after which transgene expression dramatically decreased (Scarpini *et al.* 2001). Viral genomes were detected by PCR up to 180 days following delivery, indicating that in the CNS there is a slow down-regulation of the viral promoter resulting in loss of transgene expression (Scarpini *et al.* 2001). Lokensgard *et al* reported the ability of LAP2 region to confer long term activity onto LAP1 at ectopic site, gC (Lokensgard *et al.* 1997). β-galactosidase was detected in the DRG of mice at 28 days post footpad inoculation. Long term expression was also obtained when the LAP2 region was placed upstream and in reverse direction of LAP1 (Lokensgard *et al.* 1997). However, the best results obtained were those when the LAP2 region was in its natural orientation and position, 3'of LAP1 (Lokensgard *et al.* 1997). The LAP2 region was explored by the construction of viruses containing either an NSE promoter, a CMV promoter or an MMLV promoter inserted at 1.5 kb downstream from the start of the primary LAT transcript (Palmer *et al.* 2000). Strong and long term expression in the mouse PNS was obtained. Work by Palmer *et al* showed that long-term elements were contained within the LAT P2 fragment (LAP2 plus 700 bp at the 3′end) and exploited this to use the fragment to confer long-term ability onto adjacent promoters facing away in a back-to-back orientation (the pR20.9 and pR20.5 cassettes described in section 3.3.1) (Palmer *et al.* 2000). The ability of LAT P2 to drive strong expression from 2 heterologous

promoters driving *lacZ* and GFP was demonstrated for the LAT region and for at least 2 months at ectopic sites within the genome (Palmer *et al.* 2000).

As previously mentioned, the MMLV promoter alone when inserted antisense into ICP4 was capable of driving expression through latency, thought to be due to the proximity to the endogenous LAT region (Dobson *et al.* 1990). Indeed, when inserted into the gC locus (distant to the LAT region), only short term transgene expression was observed (Lokensgard *et al.* 1994). Other promoters when inserted into ICP4 do not exhibit the same long term capabilities of MMLV (Chiocca *et al.* 1990), which suggests something unique about this promoter. Based on these observations, Lokensgard *et al* linked downstream of a TATA-less LAP1, the MMLV LTR promoter driving *lacZ* and inserted this fusion into the ectopic site, gC (Lokensgard *et al.* 1994). High level transgene expression was observed and expression lasted for 42 days post injection in the PNS. The only other promoter known to confer long term expression onto LAP1 is LAP2 (mentioned above), and as such it seems the MMLV LTR promoter has unique properties which allow it to functionally replace the LAP2 region in conferring long term properties to LAP1. The function of LAP2 and MMLV promoters might be a structural one, conferring a secondary structure which allows transcription by allowing continued access of transcription factors to LAP1 (Palmer *et al.* 2000).

1.4. Motor Neuron Disease

Motor neuron diseases can be considered to be a family of conditions centred on the destruction in various ways, at various ages, and with various effects on motor neurons (reviewed by (Eisen and Krieger 1998)). The particular disease usually referred to as "motor neuron disease" (MND) in Britain, is often referred to as "amyotrophic lateral sclerosis" (ALS) in North America (Swash and Schwartz 1992). It was first described by Charcot in 1865 and is the most common adult-onset motor neuron disease (Charcot 1865). Other motor neuron diseases include spinal muscular atrophy (SMA), the most frequent fatal autosomal-recessive disorder in infants, which is almost invariably caused by mutations in the survival motor neuron gene (*SMN*) (Lefebvre *et al.* 1995) and spinobulbar muscular atrophy, also known as Kennedy's disease.

MND is a progressive neurodegenerative disorder resulting from the selective dysfunction and loss of motor neurons in the anterior horn cells of the spinal cord and the motor nuclei of the brain stem, and in upper motor neurons forming the corticospinal tracts (Mulder *et al.* 1986). There are two forms of the disease: sporadic and familial (Mulder *et al.* 1986). The former comprises 90-95% of all cases and is distinguished by the absence of any family history of the condition. It is often further sub-divided for clinical purposes into (reviewed by (Swash and Schwartz 1992)):

- Classical MND, the form described by Charcot, involving upper and lower motor neurons, which accounts for 2/3 of all sporadic cases.
- Progressive bulbar palsy, a form of the disease initially affecting the brainstem and clinically affecting speech and swallowing in the early stages. Approximately 25% of the sporadic cases begin in this form.
- Progressive muscular atrophy, accounting for 10% of sporadic cases, involves mainly lower motor neurons in the spinal cord but might progress to involve the upper motor neurons.
- Primary lateral sclerosis, a rare form of the disease in which only upper motor neurons are involved.

It is now generally agreed that these subgroups represent part of a clinicopathological spectrum based on a common neurodegenerative pathway (Shaw 2001). The other form of the disease, familial MND, occurs in 5-10% of all cases. It is usually transmitted by an autosomal dominant trait and is clinically and pathologically indistinguishable from sporadic MND (Mulder *et al.* 1986). One very rare form of inherited MND presents itself as an autosomal recessive disorder affecting children. Recently, two groups have just reported mutations in the *ALS2* gene in affected individuals (Yang *et al.* 2001; Hadano *et al.* 2001). The mRNA for *ALS2* was found to be widely expressed in several areas of the brain and spinal cord (Hadano *et al.* 2001). The protein encoded by the gene has been named alsin (Yang *et al.* 2001) and both groups suggest loss of function as the mechanism by which it may cause disease (Yang *et al.* 2001; Hadano *et al.* 2001). The functions of alsin have been predicted from its sequence and as result it has been grouped with the GTPase regulatory proteins (Yang *et al.* 2001; Hadano *et al.* 2001). These proteins play important roles in cytoskeleton organisation, intracellular trafficking and signalling pathways, possibly indicative of the role of alsin itself (Shaw 2001).

1.4.1. Clinical features and neuropathology

MND has a worldwide prevalence of ~5 per 100,000 individuals. It usually afflicts adults in mid-to-late life, is more common in men than in women, and the median survival for all cases is 3-4 years (Jablecki *et al.* 1989). Generally, the earlier the age of onset the longer the survival (Eisen *et al.* 1993). It is characterised by progressive muscle weakness accompanied by hyperreflexia and spasticity associated with fibrillations and fasciculations (Brooks 1994). Muscular atrophy and weakness reflects selective degeneration of large motor neurons of the brain stem and spinal cord. Spasticity, hyperreflexia and extensor plantar signs are attributable to lesions in the upper motor neurons.

Regardless of the form, MND is characterised by variable losses of bulbar and spinal motor neurons with reductions in descending tract axons (Eisen and Krieger 1998). Both α and γ motor neurons disappear in MND (Tsukagoshi *et al.* 1979). Neuronal atrophy is the most common cellular change seen in MND, with atrophic cells appearing shrunken and dark with fragmented and irregular dendrites (reviewed in (Eisen and Krieger 1998)). Several neuronal inclusions are seen in MND (reviewed by (Eisen and Krieger 1998)). Indeed, one hallmark of the pathology of MND is the accumulation of often abnormally phosphorylated neurofilaments (Munoz *et al.* 1988) in the cell bodies and proximal axons of motor neurons (Carpenter 1968) (see section 1.4.2.3). These neurofilament inclusions can be diffused or focal, which can result in axonal enlargements referred to as spheroids (Carpenter 1968). Other frequently found inclusions in MND are ubiquitin-immunoreactive deposits (Lowe 1994) and the Bunina body (a 1-10 μm cytoplasmic inclusion) (Hirano 1996). Rarely observed is a Lewy-body like intracytoplasmic inclusion (Hirano 1996).

1.4.2. MND is a multifactorial disease

Epidemiological and biochemical data support the view that MND is a multifactorial disease with interactive pathogenic mechanisms (reviewed by (Cleveland and Rothstein 2001; Brown, Jr. 1995; Shaw and Eggett 2000)). Among the mechanisms suggested are: oxidation/nitration mediated damage, aggregation of critical components,

excitotoxicity, dysfunction of critical components such as mitochondria and neurofilaments.

1.4.2.1. SOD1

Approximately 15-20% of patients with autosomal-dominant familial MND have missense point mutations in the gene encoding for Cu/Zn superoxide dismutase (SOD1) (Rosen 1993). Reflecting the amount of research in MND in the past eight years, at least 60 different point mutations throughout the five exons of the gene have now been reported (Shaw 2001). This metalloenzyme acts as a free-radical scavenger catalysing the dismutation of superoxide anion into hydrogen peroxide and water. In 1994, Gurney and colleagues, reported the production of transgenic mice expressing a human Cu/Zn SOD1 mutation, which developed weakness and neuronal degeneration similar to that seen in MND patients (Gurney *et al.* 1994). Since then, other transgenic mice have been produced expressing different SOD1 mutations and have developed motor neuron degeneration similar to that seen in MND (Ripps *et al.* 1995; Bruijn *et al.* 1997; Wong *et al.* 1995). In view of the function of SOD1 and studies on free-radical toxicity, it was initially proposed that the pathogenesis of SOD1-linked familial MND was a result from diminished free-radical scavenging due to a reduction in the activity of SOD1 (Deng *et al.* 1993). However, several lines of evidence point in the direction that the familial MND-linked mutations in SOD1 may act through cytotoxic, newly acquired functions (reviewed in (Cleveland and Rothstein 2001; Brown, Jr. 1995; Shaw and Eggett 2000)). Transgenic mice expressing familial MND-linked mutant SOD1 develop a progressive motor neuron disease, despite elevated SOD1 activity levels (Gurney *et al.* 1994; Wong *et al.* 1995) or unchanged SOD1 activity (Ripps *et al.* 1995; Bruijn *et al.* 1997). In addition, SOD1 null mice do not develop motor neuron disease (Reaume *et al.* 1996) and no clear null mutations in SOD1 have yet been reported in familial MND (Brown, Jr. 1995). So, the inevitable question arises: what are the cytotoxic mechanisms of the mutant SOD1 molecule? Different answers have been forwarded.

Mutant SOD1 may allow greater access of abnormal substrates to the copper active site (Deng *et al.* 1993). The peroxidase activity hypothesis suggests mutant SOD1 has an enhanced ability to use hydrogen peroxide (H_2O_2) as a substrate to generate highly reactive toxic hydroxyl radicals, which could damage cellular targets (Wiedau-Pazos *et*

al. 1996). The nitration hypothesis suggests that the mutant SOD1 molecule might have an enhanced affinity for peroxynitrite ($^{-}$ONOO) leading to the production of nitronium ions which catalyse the nitration of tyrosine residues on proteins such as neurofilaments and tyrosine kinase receptors (Beckman *et al.* 1993).

It was reported that in yeast, the acquisition of copper by SOD1 was mediated by CCS (copper chaperone for SOD1) (Culotta *et al.* 1997) via a direct physical interaction (Casareno *et al.* 1998). In CCS knockout mice, reduction of SOD1 activity due to impaired copper incorporation suggests CCS is essential to activate SOD1 *in vivo* (Wong *et al.* 2000). Wong and colleagues also propose CCS is essential to protect copper for delivery to SOD1 under low levels of intracellular free copper (Wong *et al.* 2000). It is possible that copper could be exposed during the transfer from the CCS to the mutated SOD1, with neurotoxic consequences (Cleveland and Rothstein 2001; Wong *et al.* 1998). The idea of copper-mediated oxidative damage in MND can now ideally be tested by crossbreeding the CCS knockout with SOD1 mutated mice (Wong *et al.* 2000).

One other theory for gain of function involves the co-aggregation of SOD1 mutants in astrocytes and motor neurons with an unidentified essential component or components (Bruijn *et al.* 1998). SOD1-containing aggregates were found to coincide with disease onset, increased in abundance as disease progressed and were common to the disease caused by different SOD1 mutants (Bruijn *et al.* 1998; Bruijn *et al.* 1997). However, this mechanism is only suggested to account for a portion of mutant-mediated toxicity as other mutant-mediated defects are seen at least 5 months before the aggregates (Bruijn *et al.* 1998).

1.4.2.2. Excitotoxicity

Excitotoxicity has been suggested as one of the mechanisms by which motor neurons are affected in MND (Brown, Jr. 1995). Glutamate is the major CNS excitatory amino acid and its receptors are present throughout the neocortex (reviewed in (Eisen 1995)). Excessive release of glutamate or impaired re-uptake of this neurotransmitter from the synaptic cleft induces excessive influx of calcium into post-synaptic neurons, leading to their death (Plaitakis 1990). About 60-75% of sporadic MND patients show reduction

in the astroglial glutamate transporter EAAT2, also referred to as GLT-1, in the motor cortex and spinal cord (Rothstein *et al.* 1995). This reduction could lead to excitotoxic degeneration of motor neurons given GLT-1 is the main mechanism by which the synaptic action of glutamate is inactivated (Rothstein *et al.* 1996). In accordance, one study has shown that a subset of sporadic MND patients have GLT-1 specific abnormal mRNA editing which could lead to loss of expression (Lin *et al.* 1998). Of particular interest are the results obtained by Trotti and colleagues using *Xenopus laevis* oocytes (Trotti *et al.* 1999). These correlate the activity of familial MND-linked SOD1 mutants with the loss of function of GLT-1, upon intracellular administration of H_2O_2. Indeed, the familial MND-linked SOD1 mutants, but not wild type SOD1, specifically inactivated GLT-1 as monitored electrophysiologically and biochemically (Trotti *et al.* 1999). Furthermore, chelation of the copper ion prevented inactivation (Trotti *et al.* 1999). Thus, these studies bring together the copper-mediated peroxidase hypothesis mentioned above with excitotoxic neuronal degeneration, reinforcing the concept of interactive pathogenic mechanisms in MND. These results also point out astrocytes as possible culprits in MND as these are in charge of producing GLT-1 and have been shown to contain SOD1 aggregates in mutant SOD1 mice (Bruijn *et al.* 1997; Trotti *et al.* 1999). However, in defence of astrocytes are the results obtained using mice expressing mutant SOD1 restricted to astrocytes (Gong *et al.* 2000). These mice did indeed have hypertrophied astrocytes but they developed normally and did not experience progressive motor neuron degeneration. In fact, histological analysis revealed normal motor neuron and microglial morphology (Gong *et al.* 2000). These results suggest that familial MND is not a primary disease of astrocytes, however, these might contribute to disease progression after a different cell type has initiated the disease process (Gong *et al.* 2000).

1.4.2.3. Neurofilaments

Neurofilament proteins are a major component of the neuronal cytoskeletal structure and play an essential role in axonal transport as well as maintaining cell shape and axon calibre (reviewed in (Shaw and Eggett 2000). Given the uniqueness of the motor neuron, with its large soma (50-60 μm) and exceptionally large axonal process (up to one meter in length in humans), these cells have a very large content of neurofilaments when compared to others (Shaw and Eggett 2000; Bruijn and Cleveland 1996).

Neurofilaments consist of three protein subunits of different molecular weights, light (NF-L), medium (NF-M) and heavy (NF-H). NF-L is required for filament assembly and composes the core of the neurofilament, while NF-M and NF-H are arranged around this core and project outwards their long tail domains (Nixon and Shea 1992). Neurofilament accumulation in motor neurons is considered an early pathological hallmark in MND (Carpenter 1968). Mutations of the NF-H gene have been found in a small number of sporadic MND patients (Al Chalabi *et al.* 1999). A plethora of information on the role of neurofilaments in MND has been given by the study of transgenic mice (reviewed in (Newbery and Abbott 2001)). Transgenic mice which overexpress mouse NF-L (Xu *et al.* 1993) or human NF-H (Cote *et al.* 1993) subunits develop motor neuron pathology, as well as mice expressing mutations in the NF-L subunit (Lee *et al.* 1994; Canete-Soler *et al.* 1999). Taking together the results obtained from the study of sporadic MND patients and those obtained from the study of transgenic mice, one proposes that disruption of neurofilament assembly is involved in MND pathogenesis. The mechanism of pathogenesis of the neurofilament accumulations could be axonal strangulation and deficits caused in axonal transport (Cleveland and Rothstein 2001). Surprising results were obtained from the crossing of mutant SOD1 mice with NF-H overexpressing mice (Couillard-Despres *et al.* 1998). Most of the neurofilaments were trapped in the cell body, decreasing the axonal neurofilament content, and a 6 month delayed onset of disease was observed (Couillard-Despres *et al.* 1998). This delay might be due to the neurofilament accumulation forming a "phosphorylation sink", being phosphorylated preferentially to other neuronal proteins which might be cytotoxic in the phosphorylated form (Newbery and Abbott 2001).

Of particular interest are the results recently published reporting a deletion of the hypoxia-response element in the vascular endothelial growth factor (VEGF) promoter (Oosthuyse *et al.* 2001). This deletion resulted in a reduction of baseline and hypoxic expression of VEGF and caused adult-onset progressive motor neuron degeneration similar to that seen in mutant SOD1 mice. From 5-7 months of age spheroids (abnormal accumulation of neurofilaments in motor neuron axons) were observed and axonal transport was found to be impaired. The authors found that physiological concentrations of VEGF protected a mouse motor neuron cell line against apoptosis induced by hypoxia, serum deprivation, oxidative stress and tumour necrosis factor α.

The select degeneration of motor neurons seen *in vivo* was found to be due to chronic vascular insufficiency and/or to insufficient protection levels of VEGF. It is now of interest to look for VEGF differences between MND patients and healthy counterparts.

1.4.2.4. Mitochondrial dysfunction

Due to the large size of motor neurons, these cells have high energy demands and as result have high levels of mitochondrial activity (reviewed in (Shaw and Eggett 2000)). Enlarged mitochondria in hepatocytes and mitochondrial dysfunction in lymphocytes of MND patients have been reported (Curti *et al.* 1996). Mutant SOD1-expressing mice contain vacuoles derived from dilated mitochondria (Dal Canto and Gurney 1995; Wong *et al.* 1995) and dilated endoplasmic reticulum (Dal Canto and Gurney 1995). These vacuoles appear at an early stage in the cascade of motor neuron injury previous to disease onset (Kong and Xu 1998). Metabolic function was found to decrease previous to the observation of mitochondrial pathological changes and this metabolic impairment was found to increase with the age of mutant SOD1 mice (Browne *et al.* 2001). Both wild type and mutant SOD1 were shown to localise within mitochondria in the spinal cord in studies using confocal immunofluorescence microscopy and immuno-electron microscopy (Higgins *et al.* 2001). In conclusion, high metabolic activity in motor neurons together with the acquired oxidative properties of mutant SOD1 may cause motor neuron degeneration by directly damaging mitochondria, triggering the onset of MND.

1.4.3. MND therapy

The multifactorial nature of MND, including the interaction of different pathogenic mechanisms linked to the lack of a clear indication when the disease begins or its exact cause, render the development of therapeutic strategies a complex task. Those that have been developed so far have been based on known or suspected mechanisms. One might speculate that the greatest therapeutic success would derive from a combination of substances targeted at different pathogenic aspects.

As discussed above, the synaptic action of glutamate appears to be potentiated in MND. Hence, attenuation of glutamatergic transmission is relevant as a therapeutic

intervention in the disease. To reduce the levels of glutamate in the synaptic cleft, one can modulate the release of glutamate from the nerve terminals, enhance glutamate transport and modify glutamate receptor activity (for a review see (Eisen and Krieger 1998)). In 1996, Riluzole was approved by the Food and Drug Administration for use in patients with MND, after 2 placebo-controlled multicenter trials demonstrated a statistically significant benefit on survival times (Lacomblez *et al.* 1996; Miller *et al.* 1996). Unfortunately, Riluzole is not associated with improved muscle strength nor improved quality of life (Lacomblez *et al.* 1996; Miller *et al.* 1996). Riluzole acts by inhibiting synaptic release and blocking voltage-dependent sodium channels (Hebert *et al.* 1994).

An impressive result was seen with the addition of creatine to the drinking water of mutant SOD1 mice, increasing survival time by 26 days to a total of 157 days (Klivenyi *et al.* 1999). Creatine supplementation was also found to affect oxidative injury seen in the mutant SOD1 mice (Klivenyi *et al.* 1999). The mode of action of creatine is not clear but it may help to buffer intracellular energy stores and it is thought to stabilise mitochondrial creatine kinase in an octomeric form, which inhibits the opening of the mitochondrial transition pore by calcium (O'Gorman *et al.* 1997). Athletes wanting to boost the energy reserves in muscle have used creatine for a long time. Given its availability, there is no doubt that many MND patients are using creatine as well. A placebo-controlled trial of creatine is currently recruiting patients (see NIH link, http://www.clinicaltrials.gov).

1.4.4. Neurotrophic factors

During the early development of higher vertebrates, motor neurons are produced in excess and about 50% of the neurons which have made contact with skeletal muscle die between embryonic day 14 and postnatal day 3, by a process called physiological motor neuron death (see review by (Sendtner *et al.* 2000)). The removal of limbs from developing chick embryos massively enhances the neuronal death observed and limb transplantation reduces the number of dying motor neurons (Oppenheim 1991; Hamburger 1992). The neurotrophic factor hypothesis suggests that target tissues produce a limited supply of neurotrophic factors, which select and sustain the fittest neurons while the surplus neurons undergo cell death (the physiological motor neuron

death observed) (Oppenheim 1989). Indeed, the dependence of developing motor neurons on target tissue derived neurotrophic factors is reflected by the extensive neuronal death observed by interruption of target tissue contact by nerve axotomy (Snider *et al.* 1992). In adult life, neurotrophic factors also play an important role: adult motor neurons are able to regenerate following nerve axotomy but successful regeneration is dependent on neurotrophic factors (reviewed by (Terenghi 1999)). In addition, complete interruption with the periphery by ventral root avulsion leads to motor neuron loss (Koliatsos *et al.* 1994).

The discovery of neurotrophic factors has prompted the experimenting for protective effects in several models. The most commonly used models have been axotomy-induced cell death in neonate animals (Sendtner *et al.* 1992; Sendtner *et al.* 1990; Pennica *et al.* 1996; Cheema *et al.* 1994; Oppenheim *et al.* 1995; Schmalbruch and Rosenthal 1995), adult ventral root avulsion (Novikov *et al.* 1997) and murine models for inherited progressive motor-neuron degeneration, such as the *wobbler* and the *pmn* (progressive motor neuronopathy) mice (Sendtner *et al.* 1992; Mitsumoto *et al.* 1994; Mitsumoto *et al.* 1994; Bordet *et al.* 1999). From these studies, no single factor was found that could support survival of > 20-30% of the total number of motor neurons that undergo programmed cell death (Oppenheim *et al.* 2001). One other interesting observation was that neurotrophic factors can potentiate each other in supporting motor neuron survival (Mitsumoto *et al.* 1994; Haase *et al.* 1997). One interpretation of these results is that individual motor neurons may require more than one factor for survival and subpopulations of motor neurons may vary their trophic requirements depending on their maturation stage, peripheral target, afferent inputs or functional properties (reviewed in (Oppenheim 1996)).

Nerve-growth factor (NGF), which is specific for subpopulations of sensory sympathetic neurons, was the first neurotrophic factor to be identified (reviewed in (Levi-Montalcini 1987)). It is considered the prototype neurotrophic factor as it is synthesised in the target tissue and undergoes retrograde axonal transport to reach the neuronal cell body where it promotes a trophic and survival effect (Levi-Montalcini 1987). The discovery of NGF sparked a "gold rush" for the discovery of other neurotrophic factors. Among several described, of particular interest are some members

of the neurotrophin family; the glial-derived neurotrophic factor (GDNF); members of the CNTF/LIF family and the member of the Reg family, Reg-2.

1.4.4.1. Neurotrophins

The neurotrophins are a related family of neurotrophic peptides which include NGF, brain -derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin 4/5 (NT-4/5) (Terenghi 1999). They share a low-affinity receptor, p75, and use a high-affinity tyrosine receptor kinase (Trk). Each neurotrophin preferentially binds to and activates one Trk receptor, NGF to TrkA, BDNF and NT-4/5 to TrkB and NT-3 to TrkC (Segal and Greenberg 1996). Neurotrophins exert their trophic actions via receptor binding, by activating the intrinsic tyrosine kinase activity of the Trk receptor via ligand-induced dimerisation, leading to a series of events culminating in modification of gene expression and protein synthesis (Segal and Greenberg 1996). BDNF, NT-3 and NT-4/5 are thought to exert trophic actions on motor neurons. All three are expressed by skeletal muscle (Griesbeck *et al.* 1995) and are reported to be expressed by motor neurons (Buck *et al.* 2000) suggesting a role in neuronal synaptic modulation (reviewed by (Poo 2001)). BDNF is also found in Schwann cells and BDNF expression is upregulated following nerve lesion (Meyer *et al.* 1992). Each individual factor was found to promote survival of motor neurons *in vitro* (Henderson *et al.* 1993).

NT-3 was found to enhance nerve regeneration and reinervation following denervation (Sterne *et al.* 1997) and to stimulate neurite outgrowth and formation of motor endplates in cocultures of neonatal motor neurons and muscle (Braun *et al.* 1996). The effects of exogenous NT-3 administration on axotomised nerves of neonate animals are not clear according to the literature, spanning from potent (Vejsada *et al.* 1995) to absent (Koliatsos *et al.* 1993), possibly reflecting different experimental conditions. Further results elucidating the role of NT-3 were obtained by gene delivery experiments (see section 1.4.5).

NT-4/5 is a very strong survival factor for embryonic spinal motor neurons in culture (Henderson *et al.* 1993). *In vivo*, it was found to promote motor neuron survival following neonate axotomy, but only transiently (Li *et al.* 1994; Schmalbruch and

Rosenthal 1995). This indicates that other factors are possibly required for long term motor neuron rescue.

In the chick, BDNF promotes motor neuron survival but only for the last half of the normal period of programmed cell death, reflecting how motor neurons can change their trophic requirements depending on their developmental stage (Becker *et al.* 1998). BDNF was found to promote axonal regeneration and long term survival of adult rat spinal motor neurons following ventral root avulsion (Novikov *et al.* 1997). In the neonate rat, following axotomy of the facial nerve, repeated application of BDNF resulted in temporary motor neuron survival (Vejsada *et al.* 1995), but this survival was found to decrease with increasing concentrations of BDNF (Vejsada *et al.* 1994). One possible explanation for these results lies in the fact that BDNF concentration might downregulate the expression of high-affinity BDNF binding sites (Carter *et al.* 1995). Subcutaneous administration of BDNF to *wobbler* mice was found to slow disease progression (Ikeda *et al.* 1995) and co-administration with CNTF was found to arrest the disease altogether (Mitsumoto *et al.* 1994) (see section 1.4.4.). In light of these results, recombinant BDNF has been tested in clinical trials for MND (see section 1.4.5).

1.4.4.2. GDNF

GDNF belongs to the transforming growth factor-β superfamily (TGF-β) (Lin *et al.* 1993) and acts through a complex involving the transmembrane Ret receptor tyrosine kinase and the specific receptor GFRα1 (Jing *et al.* 1996). GDNF is expressed in skeletal muscle and Schwann cells of developing and adult mammals (Henderson *et al.* 1994). It is a potent neurotrophic factor for cultured embryonic motor neurons (Henderson *et al.* 1994), transiently rescues axotomised neonatal motor neurons (Yan *et al.* 1995) and prevents motor neuron death after adult ventral root avulsion (Li *et al.* 1995). In addition, it has been shown to act synergistically with cardiotrophin-1 (CT-1) to promote cultured motor neuron survival (Arce *et al.* 1998) (see section 1.4.4.3.2). *Pmn* mice treated with encapsulated GDNF-secreting cells showed significantly reduced motor neuron loss but no effect on axonal degeneration neither on the lifespan (Sagot *et al.* 1996). Thus, GDNF seems to rescue motor neuron cell bodies but is unable to

promote axonal regeneration (Sagot *et al.* 1996). In studies by Oppenheim and colleagues, motor neuron programmed cell death was examined in GDNF-deficient mice, in mice exposed to GDNF *in utero* at E14 and in transgenic mice overexpressing GDNF under the control of the muscle-specific promoter myogenin (Oppenheim *et al.* 2000). GDNF-deficient embryos showed a reduction in the number of motor neurons surviving the period of programmed cell death whereas overexpression of GDNF in both the transgenic myogenin-GDNF mice and in the mice exposed to GDNF *in utero* showed increased motor neuron survival (Oppenheim *et al.* 2000). This survival was seen in virtually all motor neuron populations (spinal and cranial) but in only a subpopulation (20-40 %) of each region or nucleus. The authors suggest that this reflects distinct motor neuron phenotypes related to the pattern of GDNF receptor expression.

1.4.4.3. The CNTF/LIF family

Members of the CNTF/LIF family of neurotrophic cytokines promote motor neuron survival through receptor complexes involving gp130 and LIFRβ (Davis *et al.* 1993). Neurotrophic factors belonging to this family are ciliary neurotrophic factor (CNTF), leukaemia-inhibitory factor (LIF), cardiotrophin-1 (CT-1) and the cardiotrophin-like cytokine (CLC) (Sendtner *et al.* 2000).

1.4.4.3.1. LIF

LIF is a secreted cytokine that promotes the survival of embryonic rat motor neurons in culture (Martinou *et al.* 1992) and stimulates muscle cell proliferation (Austin *et al.* 1992). It was shown to promote muscle regeneration *in vivo* (Kurek *et al.* 1997) and to prevent the death of neonate axotomised motor neurons (Cheema *et al.* 1994; Ikeda *et al.* 1996). Following peripheral nerve injury, LIF mRNA is upregulated in Schwann cells and denervated muscle and is retrogradely transported by motor neurons (Curtis *et al.* 1994). These results taken together suggest LIF acts as a lesion factor (reviewed in (Kurek *et al.* 1998)). The administration of LIF by subcutaneous injection to *wobbler* mice slows the deterioration of the walking pattern and grip strength (Ikeda *et al.* 1995). LIF knockout mice, however, did not have any apparent changes in number and

structure of motor neurons and showed no function impairment (Sendtner *et al.* 1996). A transgenic mouse lacking both CNTF and LIF shows an effect of LIF on the phenotype observed (see section 1.4.4.3.3).

1.4.4.3.2. CT-1

CT-1 is a potent neurotrophic factor produced by differentiated muscle cells and expressed at high levels in embryonic limb bud (Pennica *et al.* 1996). Although it lacks a signal peptide, muscle cells have been found to secrete CT-1(Pennica *et al.* 1996). It exerts strong survival effects on rat embryonic motor neurons *in vitro* (Pennica *et al.* 1996), on embryonic chick motor neurons following *in ovo* administration (Oppenheim *et al.* 2001) and on embryonic mouse motor neurons after *in utero* administration (Oppenheim *et al.* 2001). It also reduces motor neuron death after neonatal axotomy (Pennica *et al.* 1996) but this effect is transient as shown for other neurotrophic factors (Vejsada *et al.* 1995). The actions of CT-1 on cultured motor neurons were enhanced when combined with GDNF, possibly reflecting the dependence of motor neurons on more than one neurotrophic factor (Arce *et al.* 1998). It is of interest to note in this particular case, one factor was Schwann cell-derived whereas the other was muscle-derived. Some very interesting results were obtained with the CT-1 knockout mouse, with increased motor neuron death in spinal cord and brain stem being observed between embryonic day 14 and the first postnatal week (Oppenheim *et al.* 2001). No further loss of motor neurons was observed in the subsequent postnatal period. Thus, CT-1 is the only member of the CNTF/LIF family to be required for the survival of developing motor neurons (see transgenic animal studies in the following section). In conclusion, Oppenheim and colleagues propose CT-1 to be a specific muscle-derived neurotrophic factor for a subpopulation of embryonic motor neurons (Oppenheim *et al.* 2001).

1.4.4.3.3. CNTF

CNTF is a cytosolic protein present in postnatal myelinating Schwann cells and a subset of astrocytes (Stockli *et al.* 1989). It has strong neurotrophic properties and is thought to act through a two component receptor complex (reviewed in (Sleeman *et al.* 2000)).

The specificity of action is conferred by the alpha component, CNTFRα, localised to muscle and spinal cord (Davis *et al.* 1991). Binding of CNTF to the alpha component recruits the beta component, gp130 and LIFRβ, leading to tyrosine phosphorylation of gp130 by the JAK family of kinases (Stahl *et al.* 1995). The activated receptor then provides docking sites for STAT3, which once activated, dimerises and translocates to the nucleus where it activates gene expression (Sleeman *et al.* 2000).

CNTF mRNA only becomes apparent in Schwann cells after postnatal day 4 (Stockli *et al.* 1989) but nevertheless exogenous CNTF can exert survival effects on cultured embryonic motor neurons of chick origin (Arakawa *et al.* 1990) and of rat origin (Magal *et al.* 1991). It has also been shown to rescue chick motor neurons following administration *in ovo* (Oppenheim *et al.* 1991) and to prevent degeneration of neonate rat motor neurons following nerve axotomy (Sendtner *et al.* 1990). In the case of the latter, CNTF was shown to rescue most of the motor neurons with transected axons but this effect however, was transient. In *pmn* mice, treatment with CNTF was found to prolong survival and to greatly improve motor function (Sendtner *et al.* 1992). Indeed, a reduction in the degeneration of facial motor neurons was observed in addition to a reduction in loss of motor axons of the phrenic nerve (Sendtner *et al.* 1992). In *wobbler* mice, synergistic effects of BDNF and CNTF were studied by subcutaneous injection of the two factors on alternate days (Mitsumoto *et al.* 1994). BDNF and CNTF cotreatment was found to arrest progression of motor neuron dysfunction whereas each factor individually was only capable of slowing down disease progression (Mitsumoto *et al.* 1994).

CNTF lacks a signal sequence, is located in the cytosol and as result is characterised as a non-secreted protein (Stockli *et al.* 1989). However, an unconventional, regulated release has been suggested as a means by which CNTF is transferred from the Schwann cells to its motor neuron targets. Indeed, given it is a highly potent neurotrophic factor only small amounts would be required for biological action, and so its release from the cytosol could result from the incidental discontinuity of the plasma membrane due to nerve injury (Masu *et al.* 1993; Stockli *et al.* 1989). The lack of signal sequence by CNTF together with its postnatal expression by Schwann cells leads us to believe CNTF is a lesion factor (Sendtner *et al.* 1990). Genetic deletion of CNTF did not result in enhanced death of developing motor neurons, motor neuron degeneration was only

observed in adult mice reflected by a small but statistically significant reduction in muscle strength (Masu *et al.* 1993). The combined deletion of CNTF and LIF, on the other hand, caused enhanced postnatal loss of motor neurons (Sendtner *et al.* 1996). Indeed, the degenerative changes were more extensive and appeared earlier than in the CNTF knockout and enhanced cell death was observed following axotomy, reflecting how LIF and CNTF act together to support survival of lesioned postnatal motor neurons (Sendtner *et al.* 1996). As opposed to animals lacking CNTF and/or LIF, developmental cell death of motor neurons is enhanced in mice lacking gp130 (Nakashima *et al.* 1999), LIFRβ (Li *et al.* 1995) or CNTFRα (DeChiara *et al.* 1995). Comparing the results obtained for the CNTF or LIF knockout with the observation that CNTFRα-deficient mice show similar loss of motor neurons as LIFRβ and even gp130-deficient mice, suggests the presence of other factors that act through CNTFRα, LIFRβ and gp130 on developing motor neurons. Programmed cell death of motor neurons was enhanced in mice lacking CT-1 but this loss was less than that observed in CNTFRα, LIFRβ and gp130-deficient mice (Oppenheim *et al.* 2001). One concludes CT-1 is required for the survival of subgroups of developing motor neurons and that another unknown CNTF/LIF family member is involved in the motor neuron loss in CNTFRα-deficient mice, as CT-1 has been shown not to bind CNTFRα (Pennica *et al.* 1996).

1.4.4.4. Reg-2

The Reg family consists of structurally related proteins belonging to the calcium (C-type) dependent lectin superfamily. Indeed, many residues important in the three-dimensional structure of the C-lectins, such as hydrophobic core residues and disulphide-bonded cysteines are conserved in the structure of the Reg family (Hartupee *et al.* 2001). In 1988, the first member of the Reg family was found by screening a regenerating pancreatic islet derived cDNA library (Terazono *et al.* 1988). The novel gene was named *Reg* (i.e. the *reg*enerating gene) and was found to encode a 16 kDa secreted protein present in regenerating islets but not in normal pancreatic islets (Terazono *et al.* 1988). Since then, several other *Reg* and *Reg*- related genes have been described and based on the primary structures of the proteins encoded, they have been assigned to the *Reg* family and grouped into three subclasses, type I, II and III (see review by (Okamoto 1999)). Type I Reg proteins, which contain the original described

rat Reg and its human homologue are thought to be regenerating or growth factors for pancreatic β-cells (Okamoto 1999). Indeed, Reg (now known as Reg-1) was shown to cause proliferation of insulin-producing pancreatic β-cells (Watanabe *et al.* 1994) and to ameliorate diabetes in surgical or autoimmune mouse models of diabetes (Gross *et al.* 1998; Watanabe *et al.* 1994). Type I Reg expression has also been reported to be involved in pathological conditions such as in human colorectal carcinoma (Macadam *et al.* 2000) and in rat gastric mucosa (Fukui *et al.* 1998), likely due to its mitogenic properties. Reg-1 has also been found to be expressed in the developing and Alzheimer's disease affected human brain (de la Monte *et al.* 1990). Type II Reg proteins refer to mouse RegII (Unno *et al.* 1993) but no biological function has yet been determined. Type III Reg proteins include Reg-2/PAP1, RegIIIβ and HIP/PAP. Reflecting the varied nomenclature preferences in the field, Reg-2/PAP1, RegIIIβ and HIP/PAP actually refer to the same protein expressed in rat, mouse and human respectively (reviewed in (Okamoto 1999)). This protein was first identified as a transcript upregulated during pancreatitis (Iovanna *et al.* 1991; Orelle *et al.* 1992). HIP/PAP has been suggested to be involved in cellular proliferation in intestinal Paneth's granular cells and in hepatocellular carcinomas (Christa *et al.* 1996). In the case of the latter, it was shown to act as an adhesion molecule for rat hepatocytes interacting with extracellular matrix proteins such as laminin-1 and fibronectin (Christa *et al.* 1996). In the pancreatic acinar cell line, AR4-2J, Reg-2/PAP1 was shown to be induced by free radicals and to increase cell resistance to apoptosis {Ortiz, Dusetti, et al. 1998 ID: 1387}. In the nervous system, Reg-2 has been described as a pro-regenerative factor for sensory and motor neurons following peripheral nerve injury in the rat (see more below) (Livesey *et al.* 1997). A recent paper reports the isolation and characterisation of a cDNA encoding a protein belonging to the Reg family (Hartupee *et al.* 2001). The authors suggest that due to specific protein structural differences the protein does not belong to any of the previously described subclasses and as result, they have suggested a forth subclass and have named the protein, Reg IV. The authors also reported that Reg IV mRNA is up regulated following mucosal injury from active Crohn's disease or ulcerative colitis and are currently looking for mitogenic activity (Hartupee *et al.* 2001).

In 1997, Livesey and colleagues reported Reg-2 had pro-regenerative effects in motor and sensory neurons in the rat (Livesey *et al.* 1997). Messenger RNA differential

display was used to compare gene expression in resting dorsal root ganglia (DRG) with that in regenerating DRG, and Reg-2 mRNA was found solely in the latter. Reg-2 expression in the adult rat central or peripheral nervous system was not seen unless in a regeneration scenario. Reg-2 was shown to be a strong Schwann cell mitogen and Reg-2 mRNA was not found in resting nor regenerating peripheral nerve. Within 24 hours of adult rat sciatic nerve crush, virtually all motor neurons and a subpopulation of sensory neurons expressed high levels of Reg-2 mRNA and protein. Intraneural administration of an anti-Reg-2 polyclonal antiserum following nerve crush resulted in a reduction of regeneration of Reg-2-containing axons.

Ventral root avulsion results in motor neuron death (Koliatsos *et al.* 1994) but surgical reimplantation of the roots can improve motor neuron survival (Chai *et al.* 2000). Following ventral root avulsion (L4-L6) in the adult rat, Reg-2 is expressed in motor neurons with and without root reimplantation (Bergerot *et al.* 2002). Expression levels peak 3-7 days after avulsion and interestingly, in only the cases with reimplanted roots, Reg-2 expression is detected up to 3 months. Sustained Reg-2 expression in the avulsed motor neuron following reimplantation might be the factor that allows for motor neuron prolonged survival in this scenario (Bergerot *et al.* 2002).

During development, Reg-2 is expressed in motor and sensory neurons between embryonic day 15 and postnatal day 5 (Livesey *et al.* 1997). Within 24 hours of neonatal axotomy, levels of Reg-2 protein drop, reflecting the requirement of peripheral target contact for Reg-2 expression in the developing motor neuron (Livesey *et al.* 1997). Based on this result and on the presence of two interleukin-6 response elements in the promoter region of the rat Reg-2 gene (Dusetti *et al.* 1995), given that the CNTF/LIF family belongs to the interleukin-6 family, transgenic mice lacking LIFRβ were analysed (Livesey *et al.* 1997). E15 embryos homozygous for the disrupted LIFRβ gene were found not to express Reg-2 in motor neurons and in sensory neurons, whereas Reg-2 is expressed in both cell types in wild type mice and in mice heterozygous for the disrupted LIFRβ gene (Livesey *et al.* 1997). In developing motor and sensory neurons, Reg-2 expression is thus dependent on factors acting through LIFRβ.

These results prompted a series of studies by the group of Henderson at INSERM (Nishimune *et al.* 2000), due to the existence of an established working model of cultured embryonic motor neurons and an interest in the CNTF/LIF family. Rat embryonic day 14 spinal cord motor neurons were found to express low levels of Reg-2 that became undetectable after one day in culture in the presence of BDNF. In these cultures, Reg-2 protein and Reg-2 cDNA were induced by the addition of CNTF; CT-1 or LIF, indicating a dependence on members of the CNTF/LIF family. The addition of purified Reg-2 to the culture media promoted survival in a saturable, dose-dependent manner. The number of motor neurons maintained was less than that observed for BDNF or CNTF, and the cotreatment of motor neurons with Reg-2+BDNF or Reg-2+CNTF didn't increase the number of surviving neurons for just BDNF or CNTF. Thus, Reg-2 acts on a subpopulation of CNTF-responsive motor neurons and considering it is produced by motor neurons *in vivo* (Livesey *et al.* 1997) and motor neurons *in vitro* have been shown to be responsive to exogenous Reg-2, it can act as a paracrine survival factor. The infection of low-density cultured embryonic motor neurons with an adenovirus expressing Reg-2, promoted cell survival suggesting Reg-2 can also act in an autocrine manner. The infection of motor neurons with an adenoviral vector encoding antisense Reg-2 eliminated the survival effect mediated by the presence of CNTF and this was reversed by the addition of exogenous Reg-2 to the media, indicating Reg-2 is required for the neurotrophic activity of CNTF on motor neurons.

Given the requirement of a LIFRβ ligand for Reg-2 expression *in vivo* (Livesey *et al.* 1997), *in situ* hybridisation was performed on spinal cords of mice lacking CNTF, CT-1 and CNTF/LIF. Interestingly, all animals still expressed normal levels of Reg-2 implying the involvement of an unyet identified member of the CNTF/LIF family in the induction of Reg-2 *in vivo.*

Henderson and colleagues also analysed Reg-2 expression during embryonic development (Nishimune *et al.* 2000). Reg-2 is expressed in motor neurons during the period of naturally occurring cell death but it was never found to be expressed in 100% of the motor neurons in any given pool. Furthermore, it was found that Reg-2 expressing cells were often located next to non-expressing cells leading the authors to conclude that Reg-2 during development is tightly regulated on a cell-by-cell basis. The observation that only some motor neurons express Reg-2 *in vivo* led to further

experiments. Reg-2-expressing cells *in vivo* were found to be a subgroup of LIFRβ and CNTFRα-expressing cells and *in vitro*, 85 % of LIFRβ-expressing cells produced Reg-2 in response to CNTF indicating that the restricted expression of Reg-2 within motor pools is not due to the lack of cytokine response machinery (cytokine receptors or signal transduction machinery). Considering a much higher percentage of LIFRβ-containing cells expressed Reg-2 *in vitro* after exposure to CNTF than that observed *in vivo*, the restricted Reg-2 expression observed could be due to the limited access to members of the CNTF/LIF family.

The neurotrophic effect of Reg-2 on motor neurons suggests the existence of a high affinity receptor due to the saturable, dose–response curve seen following addition of exogenous Reg-2 (Nishimune *et al.* 2000). One receptor for another member of the Reg family, Reg-1, has been identified as the transmembrane protein EXTL3 (Kobayashi *et al.* 2000). However, EXTL3 mRNA was not detected in rat embryonic motor neurons cultured in the presence of CNTF (Nishimune *et al.* 2000) and so the receptor for Reg-2 has not yet been identified. The signalling pathway of Reg-2 was found to involve the PI(3)K/Akt kinase and NF-κB (Nishimune *et al.* 2000). The suggested mode of action of Reg-2 is represented in Figure 1.10.

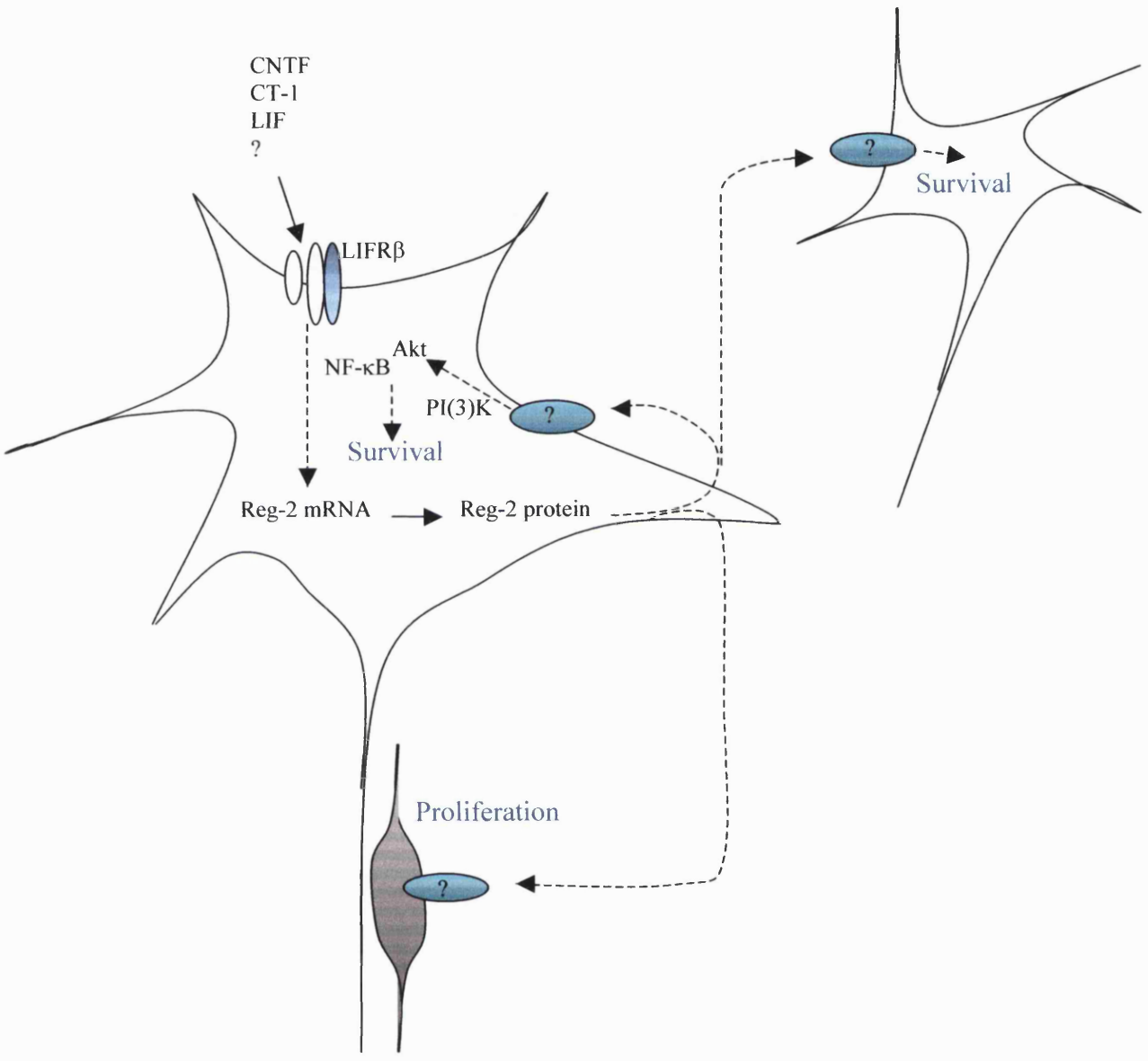


Figure 1.10 – Schematic of the mode of action of Reg-2 – Members of the CNTF/LIF family act through their receptors and the JAK/STAT pathway to induce Reg-2 expression. Secreted Reg-2 can act on the same motor neuron (autocrine effect) or on neighbouring motor neurons (paracrine effect) to promote survival via the PI(3)K/Akt kinase and NF-κB pathway after binding to an unknown receptor (Nishimune *et al.* 2000). Secreted Reg-2 can also promote Schwann cell proliferation to aid peripheral nerve regeneration (Livesey *et al.* 1997). Based on (Nishimune *et al.* 2000).

1.4.5. Gene therapy for motor neuron disease

It has been speculated (and to some extent tested in the clinic) that neurotrophic factors of interest in motor neuron disease may be able to prevent further neuronal degeneration in addition to improving the function of degenerating motor neurons by reinervating denervated muscle fibres. Some promising results of neurotrophic factors in animal studies prompted the first clinical trials based on subcutaneous administration of these factors as recombinant proteins. The results however, were very disappointing. In the case of CNTF, the very short half-life of the protein required large amounts of the protein to be administered and severe side effects were observed as result (Miller *et al.* 1996). The injection of high doses of BDNF also failed to slow progression of the disease (Eisen and Krieger 1998) perhaps due to a downregulation of TrkB receptors (Carter *et al.* 1995). The main problem associated with these trials is the poor bioavailability of the drug, as in order to ensure a minute amount will reach the nervous system substantial amounts must be delivered systemically and inevitable toxicity problems arise. In order to circumvent these problems, increased attention has been given to gene therapy as it ideally allows continuous and targeted gene delivery. The first report of gene therapy to treat the *pmn* model involved *ex vivo* transfection of tumour cells with a plasmid encoding a secretable from of CNTF followed by implantation in the mouse peritoneum (Sendtner *et al.* 1992). Improvement of survival time was observed, axonal degeneration was reduced and loss of facial nerve motor neurons was also reduced (Sendtner *et al.* 1992). Another group who delivered microencapsulated fibroblasts transfected with CNTF by subcutaneous injection, obtained the same results (Sagot *et al.* 1995). Using the same system, encapsulated cells secreting GDNF were delivered and a reduction in loss of facial nerve motor neurons was observed (Sagot *et al.* 1996). In contrast to CNTF however, there was no reduction of axonal degeneration or an increase in the lifespan (Sagot *et al.* 1996). This indicates GDNF should be delivered with another neurotrophic factor that acts on nerve processes.

In 1997, the first attempt of gene therapy using a viral vector to treat *pmn* mice was reported (Haase *et al.* 1997). A first generation adenovirus (deleted for the E1 and E3 regions, see section 1.3.2.3) was used to deliver NT-3 under the control of the RSV

promoter. The virus was injected ($1x10^9$ PFU) into three muscle groups of *pmn* mice aged 3-5 days: the gastrocnemius, the triceps brachii and the long muscles of the thoracic trunk. Adenoviral NT-3 transcripts were detected in the injected muscles up to 55 days later. A 50% increase in lifespan was observed in addition to a reduction in axonal degeneration, improved neuromuscular function and efficient reinervation of muscle fibres. Interestingly, the effect of NT-3 on motor neurons was further elucidated finding it stimulated sprouting of surviving motor neurons and contributed to the efficient reinervation of axon-depleted endplates. The effects observed for NT-3 were due to the production of NT-3 in infected muscle and the resulting presence in the systemic circulation (Haase *et al.* 1998; Haase *et al.* 1997). The results obtained were further potentiated by the coinjection of an adenovirus vector encoding a secretable form of CNTF. Indeed, maximum lifespan and effect on axonal degeneration was greater for both factors coadministered compared to each individually (Haase *et al.* 1997). This is an example of the future therapeutic orientation for motor neuron disease, neurotrophic factors from different families acting synergistically to promote neuronal survival.

A first generation adenovirus was used to deliver CT-1 to *pmn* mice in a manner identical to the one described above for NT-3 (Bordet *et al.* 1999). As for NT-3, CT-1 was distributed systemically following infection of muscle. It was found to prevent motor neuron loss (not observed for NT-3) and to prevent degeneration and loss of motor units, however, it only marginally increased lifespan. The coinjection of CT-1 vectors with GDNF expressing vectors, based on the synergistic activity seen *in vitro* (Arce *et al.* 1998), did not improve lifespan either. The likely reason for this is due to toxic effects stemming from systemic CT-1 as a reduction in weight gain and a dilation of the heart left ventricle was observed in CT-1 treated animals. Thus, the beneficial effects of CT-1 require more targeted gene transfer, perhaps to the motor neurons themselves.

In the model of neonate axotomy, a first generation adenovirus was used to deliver CNTF and/or BDNF to the facial nucleus motor neurons (Gravel *et al.* 1997). 1.2×10^8 PFU of control virus expressing *lacZ* under the control of the CMV promoter was injected into the proximal nerve stump and surrounding tissue. Gene delivery to Schwann cells and motor neurons was observed and in the latter, gene expression lasted

up to 5 weeks. Injection of adenoviruses expressing secretable CNTF or BDNF under the control of the CMV promoter resulted in survival of axotomised motor neurons for 5 weeks. These long-term results differ from the transient effects seen in studies delivering recombinant protein following neonate axotomy (Sendtner *et al.* 1990; Vejsada *et al.* 1995). This could be due to increased availability of the factors to the motor neurons, avoidance of receptor downregulation (as previously observed for BDNF (Carter *et al.* 1995), or simply by helping bridge the gap for endogenous upregulation of neurotrophic factors. Indeed, CNTF expression is only seen after postnatal day 4 and increases to adult levels over 2 weeks (Stockli *et al.* 1989). Although CNTF and BDNF synergise for motor neuron survival, it would be of interest to use a muscle-derived factor (as NT-3) to promote reinervation, as no reinervation was observed in these experiments by the lack of whisker pad movement. In the same year, two other similar studies reported the increased motor neuron survival following facial nerve axotomy in the neonate (Baumgartner and Shine 1997; Gimenez *et al.* 1997). First generation adenoviruses delivering neurotrophic factors (BDNF, GDNF) or CNTF (Baumgartner and Shine 1997) under the control of the RSV promoter were injected into the neonate facial muscles prior to nerve cut.

1.5. Thesis aims

The discovery of neurotrophic factors and the therapeutic potential they hold has been very encouraging for both the medical/scientific community and to the patients with motor neuron disease themselves. A lesson learnt from the first clinical trials using these factors is that effective methods of delivery are essential if the full potential of neurotrophic factors is to be unlocked. Gene therapy for motor neuron disease has mainly included approaches based on the delivery of genes to muscle and the resulting release of the therapeutic factor. Alternatively, the delivery of genes to the motor neurons themselves has been considered. The work presented in this thesis aims to test different disabled HSV-1 vectors for their ability to deliver genes to motor neurons following peripheral administration of the vector *in vivo*. The potential of each vector will be evaluated in both mice and rats of different ages in order to optimise systems by which potential therapeutic gene products can be tested in models of neuronal death. Optimised vectors will then be modified to express Reg-2 in order to further characterise its functions and to test for any neuroprotective properties in damaged motor neurons.

Additionally, in order to gain a better understanding of the role of Reg-2 in the nervous system its pattern of expression will be further characterised.

Chapter 2

Materials and Methods

Chapter 2 – Materials and Methods

2.1. Materials

Unless otherwise stated, all chemicals were obtained from Merck Ltd. (Poole, Dorset, U.K.), Boehringer Mannheim (Lewes, East Sussex, U.K.) or Sigma Chemical Company Ltd. (Poole, Dorset, U.K.) and were of analytical grade.

2.1.1. Standard buffers and solutions

PBS - NaCl (137 mM), KCL (2.7 mM), $Na_2HPO_4.7H_2O$ (4.3 mM), KH_2PO_4 (1.4 mM), pH 7.4

PB - Na_2HPO_4 (72 mM), NaH_2PO_4 (28 mM), pH 7.2

TE - Tris-HCl (10 mM), EDTA (1mM), pH 8.0

TAE - Tris base (400 mM), sodium acetate (200 mM), EDTA (20 mM), pH 8.3

TBE - Tris base (89 mM), boric acid (89 mM), EDTA (2 mM), pH 8.0

SSC - NaCl (150 mM), sodium citrate (15 mM)

Luria Bertani (LB) media - 1% (w/v) Bacto®-tryptone
1% (w/v) NaCl
0.5% Bacto®-yeast extract

LB media was autoclaved at 120°C for 20 minutes at 10lb/square inch (psi)

2.1.2. Bacterial strains

E. coli XL1-Blue of genotype recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proAB $LacI^q$ ZΔM15, TN 10 $(Tet)^r$] was obtained from Stratagene Ltd. (Cambridge, U.K.).

2.1.3. Animals

All animals were obtained from breeding colonies within the Department of Biological Services, UCL or from Harlan Laboratories (Oxon, U.K.).

2.1.4. Materials for molecular biology

General disposable plasticware was supplied by Greiner (Stonehouse, Gloucester, U.K.) or Sterilin (Stone, Staffordshire, U.K.)

Restriction and modifying enzymes and buffers (Promega Corporation, Madison, Wisconsin, USA)

Bacto®-agar, Bacto®-tryptone, yeast extract (Difco Laboratories, Basingstoke, U.K.)

Ammonium persulphate, N,N′-methylene-bis-acrylamide,N,N,N′,N′-tetramethylethylene-diamine (TEMED) (Bio-Rad, Hemel Hempstead, U.K.)

Concert™ "midi-prep" plasmid extraction kit, 1Kb DNA ladder (Gibco-BRL-Life Technologies Ltd., Renfrewshire, Scotland, U.K.)

α-^{32}P-dCTP (3000Ci/mM), α-^{35}S-dATP (400Ci/mM), Rainbow™ coloured protein molecular weight markers, Hybond-C and Hybond-N membranes, Sequenase version 2.0 sequencing kit, Ready-To-Go™DNA Labelling kit (-dCTP) (Amersham International plc., Little Chalfont, U.K.)

GeneClean II Gel Extraction Kit (Bio 101, Vista, CA, USA)

Rat recombinant CNTF (for use in Western blots) (Promega Corporation, Madison, Wisconsin, USA)

All oligonucleotide primers were synthesised by Genosys (Pampisford, U.K.)

2.1.5. Materials for tissue culture

Tissue culture plasticware (Nunc, Roskilde, Denmark)

Roller bottles (Corning Glass Works, Corning, New York, USA)

Tissue culture media and supplements (Gibco-BRL-Life Technologies Ltd., Renfrewshire, Scotland, U.K.)

Zeocin™ - phleomycin derivative - (Cayla, Toulouse, France)

Disposable 0.45 μm and 0.2 μm filters (Gelman Life Sciences, Ann Arbor, Michigan, U.S.A.)

Polydisc™ HD disposable 5 μm filters (Whatman International Ltd., Maidstone, Kent, U.K.)

4-chloro, 5-bromo, 3-indolyl-β-galactosidase (X-Gal), Recombinant human granulocyte macrophage colony stimulating factor (GM-CSF) (Insight Biotechnology Ltd, Wembley, U.K.)

Rat recombinant CNTF (Sigma, Poole, Dorset, U.K.)

2.1.6. Materials for surgery and dissection

Halothane (Rhône Mérieux Ltd., Harlow, Essex, U.K.)

Dissecting microscope, Olympus SZ40

Hamilton microliter syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland)

Ligation material – 4.0 Mersilk® (Ethicon Ltd. Edinburgh, U.K.)

Vetbond™ (3M, St. Paul, Germany)

Surgical instruments (Fine Science Tools, Heidelberg, Germany)

Expiral (Sanofi Animal Health, Watford, U.K.)

Paraformaldehyde (PFA)

Dextran, tetramethylrhodamine, 110,000 MW, lysine fixable (fluororuby) (Molecular Probes, Eugene, Oregon, U.S.A)

2.1.7. Materials for tissue preparation

Cryostat, Leica CM1900 (Leica Instruments, Nusloch, Germany)

Freezing microtome, Leica SM 2000R (Leica Instruments, Nusloch, Germany)

Poly-L-lysine hydrobromide MW 350,000 (Sigma, Poole, Dorset, U.K.)

Microscope slides and coverslips (BDH Laboratory supplies, Poole, Dorset, U.K.)

Histoclear (National Diagnostics, Atlanta, GA, U.S.A.)

DPX (BDH Laboratory supplies, Poole, Dorset, U.K.)

Vectashield® antifade mounting medium (Vector, Burlingame, CA, U.S.A.)

OCT Embedding matrix (CellPath plc, Powys, Wales, U.K.)

2.1.8. Materials for immunohistochemistry

Anti-Reg-2 (generously provided by Prof. S. P. Hunt)

Biotinylated anti-rabbit IgG (Vector, Burlingame, CA, U.S.A.)

Oregon green anti-mouse H+L conjugate (Molecular Probes, Eugene, Oregon, U.S.A.)

FITC- *bandeiraea simplicifolia* isolectin IB4 (Sigma, Poole, Dorset, U.K.)

N52 mouse monoclonal antibody against the 200 k-Da neurofilament subunit (Sigma, Poole, Dorset, U.K.)

Mouse anti-actin antibody (Chemicon, Temecula, CA, U.S.A.)

Goat polyclonal anti-rat CNTF (Santa Cruz Biotechnology, Santa Cruz, California, U.S.A.)

Anti-goat IgG horseradish peroxidase (HRP) conjugated (Dako Ltd, High Wycombe, Bucks, U.K.)

Normal goat serum and Vectastain® *Elite* ABC kit (Vector laboratories, Burlingame, CA, U.S.A.)

2.2. Methods

2.2.1. Molecular biology

2.2.1.1. Bacterial growth conditions

XL1-Blue (XL1-B) cells were grown either in liquid LB media or on plates prepared from LB media containing 2% Bacto®-agar. When appropriate, the media contained ampicillin at a final concentration of 100μg/ml. Liquid cultures were grown overnight at 37°C in a Gallenkamp orbital shaker at 200rpm. Bacterial plates were incubated overnight at 37°C in a standard incubator.

2.2.1.2. Transformation of bacteria

Competent XL1-Blue cells were prepared using a standard calcium chloride technique . 100ml of LB (containing no antibiotic) were inoculated with 100μl of a starter culture of XL1-Blue cells. The culture was then grown to an OD_{580} of 0.4-0.55 units and the bacteria pelleted by centrifugation at 2000rpm for 10min at 4°C. The cells were resuspended in 10ml of ice-cold 100mM $CaCl_2$ and incubated on ice for 1hr. The cells were then pelleted as before, resuspended in 4ml of ice cold $CaCl_2$ and incubated on ice until required.

200µl of competent XL1-Blue cells were transformed by addition of DNA and subsequent incubation on ice for 30min. The cells were then heat shocked for 90 seconds at 42°C and returned to ice for a further 2min. 800µl of LB was then added to the cells and the cells incubated in an orbital shaker for 1hr at 37°C/200rpm. The cells were then pelleted and resuspended in 100µl of LB and plated onto LB agar plates containing 100µg/ml of ampicillin. If detection of β-galactosidase was required, the plates used contained 50µl of a 20mg/ml stock of X-Gal dissolved in dimethyl formamide.

2.2.1.3. Small scale plasmid DNA extraction from transformed bacteria

The "mini-prep" extraction method used is based on an alkaline lysis method previously described (Birnboim and Doly 1979). Single colonies of XL1-Blue cells were used to inoculate 5ml of LB containing 100µg/ml ampicillin and were incubated in an orbital shaker overnight at 37°C/200rpm. The cells from 1.5ml of culture were then pelleted by centrifugation at 13000rpm for 2min and resuspended in 100µl of resuspension buffer (50mM Tris-HCl pH 7.5, 10mM EDTA pH 8, 100µg/ml RNase-A). Cells were lysed by addition of 200µl of lysis buffer (200mM NaOH, 1% (v/v) Triton X-100) and neutralised by the addition of 150µl of neutralisation buffer (3M Sodium acetate pH 5.5). The cell lysate was then centrifuged for 5min at 13000rpm and the pelleted precipitate removed with a hypodermic needle bent at the tip. 500µl of isopropanol was then added to the supernatant and the mixture was centrifuged for 5min at 15000 rpm. The supernatant was removed and the DNA pellet washed with 500µl of 70% ethanol. The DNA pellet was air dried and then resuspended in 50µl of double-distilled water (ddH_20) containing 20µg/ml RNase-A. Plasmid DNA was stored at –20°C.

2.2.1.4. Large scale plasmid DNA extraction

A single bacterial colony or 100µl of a liquid bacterial culture were used to inoculate 400ml of LB containing 100µg/ml ampicillin. After an overnight incubation at 37°C at 200rpm, 100ml of the culture were spun down at 3000rpm for 10min. Plasmid DNA was then extracted using the Concert™ midi-prep plasmid extraction kit following manufacturer's instructions. A typical yield of DNA was 100µg, which was

resuspended in 100µl of ddH_2O. The DNA obtained was used for cloning and for transfections.

2.2.1.5. Restriction enzyme digestion

Restriction digests were performed on plasmid DNA for either clonal analysis or isolation of DNA fragments. Analytical digests were performed in a total volume of 20µl, containing either 5µl of mini-prep DNA (section 2.2.1.3) or 1µl of midi-prep DNA (section 2.2.1.4). 10 units of each enzyme were added and the buffer recommended by the manufacturer was used at 1x concentration. The volume was made up to 20µl using ddH_20. Digests were incubated for 1-2hr at the appropriate temperature. The digests were then run on a 1% agarose gel and the bands visualised on a UV transilluminator.

Restriction digests required for isolation of DNA fragments were carried out in a total volume of 20µl containing ~5µg of midi-prep DNA, 10 units of each enzyme and the buffer recommended by the manufacturer used at 1x concentration. The volume was made up to 20µl using ddH_20. Digests were incubated overnight and then run on a 1% agarose gel. DNA bands were visualised on a UV transilluminator and the required bands were carefully excised using a scalpel. DNA was then extracted from the agarose using the GeneClean II Gel Extraction Kit, as per manufacturer's instructions. The DNA was then eluted in a final volume of 20µl.

2.2.1.6. Agarose gel electrophoresis

1% (w/v) agarose in 1xTAE gels were cast. Ethidium bromide was added to a final concentration of 0.5 µg/ml. Approximately 0.1 volume of 10x loading buffer (1xTAE, 50% v/v glycerol, 0.025% bromophenol blue) was added to DNA samples prior to loading. In order to estimate the size of the DNA fragments detected, a DNA marker was used (1 kilobase ladder). DNA was electrophoresed at approximately 120mA until the fragments were well separated. Bands were visualised on a UV transilluminator and photographed onto Polaroid film.

2.2.1.7. Blunt-end reactions

When there were no compatible restrictions sites for cloning, sticky-ends were blunted by filling in their 3' overhangs using T4 DNA polymerase. After restriction digest, 1µl of a 25mM stock of dNTPs (dATP, dCTP, dGTP, dTTP) and 15 units of T4 DNA polymerase were added directly to the reaction mix. The reaction was incubated for 30 min at 37°C. If subsequent restriction digests were to be performed, the reaction was heat inactivated at 80°C for 30min and then cooled on ice for 20min prior to addition of further restriction enzymes.

2.2.1.8. Phosphatase treatment of plasmid DNA

When religation of vector ends was possible during insert/vector ligation, the vector DNA was dephosphorylated by calf intestinal alkaline phosphatase (CIAP). Restriction enzyme/blunt ending reactions were made up to 400µl and 1 unit of CIAP was added directly to the restriction endonuclease digest. The reaction was incubated as follows:

. For 5′ recessed - 50°C for 5min

. For blunt end - 50°C for 5min

. For 5′overhang - 37°C for 5min

CIAP was then inactivated by organic extraction (see section 2.2.1.9).

2.2.1.9. Phenol extraction of digested DNA

When DNA restriction digestions needed to be purified, a phenol extraction was performed by the standard technique (Sambrook *et al.* 1989). This was necessary if a digestion required two enzymes and a compatible buffer could not be found, when linearised DNA was used in transfections or when CIAP inactivation was required. Digestions were made up to a total volume of 400µl with ddH_2O. An equal volume of phenol/chloroform/IAA (1:1:24) was added and the mixture vortexed thoroughly before centrifugation at 12000 rpm for 2min. The aqueous layer was removed and re-extracted with one volume of chloroform/IAA (1:24). The mixture was vortexed thoroughly and pulse spun. The aqueuos layer was removed and to this, 40µl ($1/10^{th}$ total volume) of 3M Sodium acetate (pH 5.5) and 880µl (2 volumes) of ice cold 100% ethanol, were added. The mixture was vortexed and then left at –20°C for 10min, before

centrifugation for 15min at 13000prm to pellet the DNA. The supernatant was then removed and the DNA washed in 70% ethanol before being left to dry. The extracted DNA was then resuspended in 10µl of ddH_20.

2.2.1.10. DNA ligations

Ligations were performed in thin walled 0.5ml reaction tubes in a total volume of 30µl. Reactions contained 1-3µl of gel-purified vector, 7-9µl of gel-purified insert, 1x ligase buffer and 1-3 units of T4 DNA ligase in ddH_2O. Reactions were placed either at RT for ~2hr or in the case of blunt end ligations in a thermocycler (Eppendorf Thermocycler). The thermocycler reactions were incubated at 16°C for 1min followed by 37°C for 1min, for 30 cycles. The reactions were then submitted to a final incubation at 22°C for 30min. In each case the ligation reaction was transformed into 200µl of competent XL1-B cells as described earlier (section 2.2.1.2).

2.2.1.11. DNA sequencing

1µg of phenol/chloroform extracted plasmid DNA was sequenced using the Sequenase™ kit v2.0 according to the manufacturers instructions. The Sequenase kit is based on the dideoxy chain termination method of sequencing (Sanger *et al.* 1977).

2.2.1.11.1. Polyacrylamide gel electrophoresis

Sequencing reactions were run on an 8% 19:1 acrylamide: bisacrylamide, 50% urea gel in 1xTBE. The gel was pre-run at 1600 volts for at least 20min prior to sample loading. 5µl of each sequencing reaction was loaded and the gel was run at 1800 volts for a period of time appropriate to the distance of the required sequence from the primer. The gel was then immersed in fixing solution (10 % (v/v) methanol, 10 % (v/v) acetic acid) for 20min and placed between 3mm Whatman paper and Saran wrap to dry under vacuum at 80°C in a Biorad gel drier. The gel was then exposed to X-ray film overnight.

2.2.1.12. Southern blot analysis of viral DNA

Southern blots (Southern 1975) were performed on viral DNA in order to confirm correct homologous recombination events had occurred when producing recombinant HSV vectors.

2.2.1.12.1. Small scale viral DNA extraction

A well of a 6-well plate of virally infected cells at complete cytopathic effect (CPE) was harvested and pelleted by centrifugation at 1000rpm for 10min. The cells were resuspended in 200µl of TES buffer (50mM Tris pH 7.8, 1mM EDTA, 30% (v/v) sucrose). 200µl of each solutions A and B (A: 2% (w/v) SDS, B: 100mM β-mercaptoethanol) were added and the resulting solution incubated on ice for 30min. 10µl of proteinase K (stock at 20mg/ml) was then added and the cells were incubated at 55°C overnight. Extractions were performed twice using phenol/chloroform (1:1 v/v) and then once with chloroform/isoamyl alcohol (24:1 v/v). Viral DNA was then precipitated by addition of 75µl of 7.5M ammonium acetate and 2.5 volumes of ice-cold 95% ethanol and subsequent centrifugation at 15000rpm for 10min. The viral DNA pellet was washed with 500µl of 70% ethanol, pelleted and air-dried for 5min. Viral DNA was then resuspended in 50µl of ddH_20.

2.2.1.12.2. Sample preparation

10µl of each viral DNA prep, including a negative control, were digested overnight with the appropriate enzymes and buffers in a total volume of 50µl. 0.1µg of plasmid DNA (positive control) was digested in a total volume of 20µl. The digested reactions were then run on a 1% TAE agarose gel until DNA fragments were well separated. The DNA was visualised on a UV transilluminator and photographed against a fluorescent ruler.

2.2.1.12.3. DNA transfer (Southern blot)

The gel was left on the transilluminator for 2min and for further denaturation was placed in denaturing solution (1.5M NaCl, 0.5M NaOH) for 45min. The gel was then

transferred to neutralising solution (2M NaCl, 1M Tris pH 5.5) for a further 45min. The gel was then placed upside-down on a plastic support which was covered in a layer of 3MM Whatman paper, which was used as a wick placed in a reservoir of 20x SSC. A piece of Hybond N+ nylon membrane cut to the same size as the gel was pre-soaked in the neutralising solution and then carefully placed on the gel ensuring there were no air bubbles present. 10 pieces of 3MM Whatman paper, pre-soaked in 20x SSC, were placed on the nylon membrane and a stack of dry paper towels was placed on top with a suitable weight. The DNA was then transferred by capillary action to the nylon membrane overnight. The membrane was removed and washed in 6x SSC and the DNA cross-linked to the membrane twice (UV Stratalinker 2400). The membrane was then air dried for 30min. The membrane could be stored at this point if placed between a layer of 3MM Whatman paper, wrapped in Saran wrap and placed at 4°C.

2.2.1.12.4. DNA radiolabelling

Southern blot membrane analysis was performed using a radiolabelled probe designed to distinguish recombinant vector from non-recombinant vector. Approximately 1μg of the DNA to be used as a probe was digested and run on a 1% low-melting-point agarose gel. The desired band was cut out from the gel with a minimum of excess agarose and the DNA fragment labelled with α-[^{32}P]-dCTP. "Oligolabelling" was done using the Ready-To-Go™DNA Labelling Kit (-dCTP), which is based on the random prime labelling reaction previously described (Feinberg and Vogelstein 1983). Unincorporated nucleotides were removed by filtration on a Sephadex G-50 DNA grade column. The radiolabelled DNA probe was then denatured by heating at 95-100°C for 2min and snap cooled on ice.

2.2.1.12.5. Hybridisation

The nylon membranes were pre-hybridised for between 2-5 hours at 65°C with 30ml of pre-hybridisation solution (6x SSC, 5 x Denhardt's reagent, 0.5% w/v SDS in ddH_2O containing 100μg/ml of denatured herring sperm DNA [100 x Denhardt's reagent: 2% w/v bovine serum albumin, 2% Ficoll® (type 400), 2% w/v polyvinylpyrrolidone in ddH_2O]). The denatured probe was added to the pre-hybridisation solution and left overnight at 65°C. The membrane was then washed twice for 10min in 2x SSC/0.1%

w/v SDS. The filters were then wrapped in cling film and exposed to X-ray film at –70°C. If necessary the blot was washed further in 0.5x SSC/0.1% SDS and then 0.1x SSC/0.1% SDS.

2.2.1.13. Polymerase chain reaction (PCR)

PCR reactions were performed in a total volume of 50 µl containing the following: 10 ng of plasmid DNA, 50 pmol of each primer, 200 µM dNTP mix, 1 mM MgCl2, 1 x reaction buffer and 1 U of Taq DNA polymerase. Samples were then placed in a thermocycler where they were incubated as follows: 94°C for 3 minutes, (94°C for 1 minute, 55°C for 45 seconds and 72°C for 1 minute for 25 cycles) and 72°C for 10 minutes. PCR products were electrophoresed on a 1.5% agarose gel.

2.2.2. Tissue culture

All cell lines were stored in long term in liquid nitrogen and during culture were maintained at 37°C in a 5% CO_2 incubator with a humidified atmosphere. All manipulations were carried out under sterile conditions using standard aseptic techniques. Viral preparations were carried out under Health and Safety Category 2 conditions.

2.2.2.1. Description and culturing conditions of cell lines

2.2.2.1.1. Baby hamster kidney cells

Baby Hamster Kidney cells (BHKs) Clone 13 (ATCC* CCL-10) have been previously described (Macpherson and Stoker 1962). BHK cells were cultured in full growth media (FGM): Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100units/ml penicillin/streptomycin. Cells were passaged when they were 80-90% confluent by rinsing with Hanks Balanced Salt Solution (HBSS) at room temperature and then incubated with a minimal volume (enough to cover the area of flask) of 10% (v/v) trypsin in versene. The disaggregated cells were then resuspended in an appropriate volume of FGM and fresh flasks seeded at a ratio of 1:10.

2.2.2.1.2. 27/12/M:4 cells (MAM49)

27/12/M:4 cells are a stable cell line derived from BHK cells (Thomas *et al.* 1999). These cells can complement mutations in HSV-1 genes ICP4, ICP27 and VP16, by virtue of providing these gene products *in trans*. In the case of VP16, the cell line expresses a highly homologous gene, the EHV-1 gene 12. Cells were cultured in FGM containing 5% (v/v) tryptose phosphate broth, 800μg/ml of neomycin and 750μg/ml of zeocin. Cells were passaged as for BHK cells (section 2.2.2.1.1). When cells were grown for viral infection all antibiotics were omitted.

2.2.2.1.3. TF-1.CN5a.1 cells

TF-1.CN5a.1 (ATCC* CRL-2512) is a human erythroleukemic cell line expressing the alpha subunit of human ciliary neurotrophic factor receptor. These cells have been previously described (Kitamura *et al.* 1989). They are grown in suspension in RPMI 1640 medium with 25mM Hepes bufffer and with L-Glutamate, supplemented with 2ng/ml of GM-CSF (granulocyte macrophage colony stimulating factor), 0.4mg/ml of neomycin and 20% of FCS. Cells are maintained at a concentration between 1 x 10^5 and 1 x 10^6 cells/ml. Addition of fresh medium or replacement of medium maintains the cultures. Alternatively, cells are pelleted at 1600rpm for 4min and resuspended in fresh media at 1 x 10^5 viable cells/ml.

*ATCC® is a registered trademark of the American Type Culture Collection.

2.2.2.2. Cell line storage

Cells were trypsinised and spun at 1500rpm for 10min to pellet. The cells were then resuspended in freezing media A (DMEM supplemented with 20% FCS) followed by addition of an equal volume of freezing media B (DMEM supplemented with 40% FCS and 16% dimethlysulphoxide [DMSO]). For a 175cm^2 flask, cells were frozen in 18ml total volume. Cells were aliquoted into 1.5ml cryovials and were maintained at –70°c for 24hr prior to storage in liquid nitrogen. Frozen cell stocks were rapidly thawed at 37°C, transferred immediately to 10ml of fresh FGM and pelleted by centrifugation at 1500rpm for 10min. The cells were then resuspended in 5ml of FGM and transferred to a 25cm^2 flask.

2.2.3. Virus construction and propagation

2.2.3.1. Virus strain

All HSV-1 vectors described here were developed from the 17+ HSV-1 strain (Brown *et al.* 1973). Vector 1764 (Coffin *et al.* 1996) refers to 17+ deleted for both copies of ICP34.5 and deficient in the C-terminal transactivation domain of VP16 (Ace *et al.* 1989).

2.2.3.2. Production of recombinant HSV-1 vectors

Hexamethylene *bis*-acetamide (HMBA) was added to the growth media to a final concentration of 3mM in order to induce immediate early gene transcription in HSV-1 mutants with a disrupted VP16 gene (McFarlane *et al.* 1992). BHK cells were used in all cases.

2.2.3.2.1. Homologous recombination transfections

BHKs were co-transfected with 5-10μg of supercoiled plasmid DNA and 10-30μg of purified viral DNA per well of a 6-well plate. The transfections were based on the calcium phosphate-mediated method as previously described (Stow and Wilkie 1976). Cells were grown in 35mm wells until they were 70% confluent. Two tubes were set up, A and B. Tube A contained 31μl 2M $CaCl_2$, 10μg plasmid DNA (prepared by the midi-prep method (section 2.2.1.4)), 30μg of viral DNA (prepared as described in section 2.2.3.5) and 20μg of herring sperm DNA (phenol/chloroform extracted). Tube B contained 400μl of HEBES transfection buffer.

HEBES Transfection Buffer:

140mM NaCl
5mM KCl
0.7mM Na_2HPO_4
5.5mM D-glucose
20mM Hepes, pH 7.05 with NaOH
Filter sterilised with a 0.2 μm filter. Stored at 4°C.

The contents of tube A were mixed by gentle pipetting and added to tube B drop-wise whilst it was being vortexed. The mixture was then left for 20-40min at room temperature to allow the DNA to precipitate. Media was removed from the cell monolayer and the precipitated DNA mixture added. The cells were incubated for 20-40min at 37°C. 1ml of growth media without selection was added to the cells and the plate incubated for 7hr prior to DMSO shocking. To DMSO shock, media was removed from the cells and the cells washed twice with 2ml of FGM. 1ml of 25% (v/v) DMSO in HEBES transfection buffer (previously prepared and left at 4°C) was then added to the cells and left precisely for 1.5min. The DMSO solution was removed and the cells quickly washed twice with 2ml of FGM. A final 2ml of FGM with 3mM HMBA was added to the cells which were then incubated at 37°C/5% CO_2 for 3-5 days until complete CPE was observed. The cells were then harvested and freeze-thawed. The harvested cells were then titred (see section 2.2.3.2.2) and the efficiency of the recombination determined by assaying for loss or gain of a reporter gene.

2.2.3.2.2. Viral titre assay

Cells were grown in 6-well plates until 80% confluent. Serial decimal dilutions of virus stock (either obtained from harvested transfections or from pure stock) were added to 0.5ml of serum free media and plated on the cells. The virus was allowed to adsorb for 40-60 min at 37°C/5%CO_2 after which 2ml of 1:2 of 1.6% (v/v) carboxymethyl cellulose (CMC): FGM supplemented with 3mM HMBA was added. The cells were then incubated for a further 48hr at 37°C/5%CO_2 and the number of plaques in each well counted, in order to determine the titre of the virus in plaque forming units per ml (pfu/ml). If the reporter gene was β-galactosidase, then viral titre was determined following X-Gal staining (section 2.2.3.2.3), if GFP was the reporter gene then plaques were detected following direct observation of GFP (section 2.2.3.2.4).

2.2.3.2.3. Detection of β-galactosidase by X-Gal staining

Media was removed and the cells washed twice with 2ml of 1x PBS. The cells were then fixed with 1ml of 1x PBS containing 0.05% glutaraldehyde for 10min at room temperature. The cells were washed twice with 2ml of 1x PBS before the addition of 2ml of X-Gal solution (5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.6H_2O$, 1mM $MgCl_2$, and

150μg X-Gal - dissolved in DMSO in 1x PBS). The cells were then incubated overnight. The X-Gal stain was removed and the cells were washed twice in 1x PBS followed by addition of 2ml of 70% v/v glycerol.

2.2.3.2.4. Detection of GFP expression

Cells expressing GFP required no pre-treatment and were visualised directly under an inverted fluorescent microscope at a wavelength of 500nm.

2.2.3.2.5. Purification of viral recombinants by plaque selection

Harvested wells from homologous recombination transfections were freeze-thawed in order to disrupt the cells and titred out as described in section 2.2.3.2.2. If the transfection was designed to result in loss or gain of the β-galactosidase gene, then the plaques were stained as in section 2.2.3.2.3. except that the cells were not fixed. If the transfection was designed to result in the loss or gain of GFP, the plaques were visualised directly under an inverted fluorescent microsocope. Once identified, the appropriate plaques were picked from the cell monolayer using a P20 Gilson micropipette (set at 3μl). Plaques containing the required recombinant vector were then transferred to an eppendorf containing 100μl of DMEM without FCS. The plaque suspension was then freeze-thawed to disrupt the cells. 10μl and 90μl of the plaque suspension were then used to infect 2 wells of a 6-well plate containing the appropriate complementing cells at 80% confluency. The virus was allowed to adsorb for 40-60 min at 37°C/5%CO_2 and the cells overlaid with 2ml of 1:2 of 1.6% (v/v) carboxymethyl cellulose: FGM supplemented with 3mM HMBA. The cells were then incubated for 48hr. The plaque purification process was repeated until a pure population of recombinant vector was obtained. When a pure recombinant population was observed a single plaque was used to infect two wells of a 6-well plate and left for 48 hours before harvesting. The virus stock was referred to as the master stock (MS) of virus, stored at –80°C and used as a starting stock for large-scale propagation of the recombinant virus.

2.2.3.3. High titre stock production of replication competent virus

100 μl of MS were used to infect 90% confluent BHK cells in 6-well dishes. The virus was made up to 0.5ml with SFM and the cells left for 1hr at 37°C/5%CO2. Cells were

then overlaid with FGM containing 3mM HMBA. After complete CPE was observed, the cells were harvested and the virus was grown in increasing quantities until 2x 175cm^2 flasks were obtained. The cells were then harvested and the viral titre determined. This stock was termed the sub-master stock (SMS). 10 x 850cm^2 roller bottles containing BHK cells grown to 90% confluency, were infected at an MOI (multiplicity of infection) of 0.01 (approximately 2x10^6 pfu of virus per roller bottle with extended surface area). The virus was added in a total volume of 100ml of FGM supplemented with 3mM HMBA per roller bottle. The cells were then incubated at 32°C/0.5rpm for 3-5 days until complete CPE was observed. The cells and supernatant were harvested by vigorous shaking of the roller bottle and stored at –80°C. After defrosting, the cellular debris was pelleted by centrifugation at 3000rpm for 30min at 4°C. The supernatants were then combined and the virus particles pelleted by centrifugation at 12000rpm for 2hr at 4°C. The supernatant was then removed and 1-3ml of this supernatant was used to resuspend the viral pellet by gentle pipetting. The resuspended viral pellet was then sonicated for 1min in a water bath sonicator. 100µl aliquots of the resuspended virus were stored in liquid nitrogen. The titre of the resuspended virus was determined using the standard viral titre assay.

2.2.3.4. High titre stock production of replication incompetent virus

Approximately 300 µl of MS was used to infect 90% confluent 27/12/M:4 cells in 6-well dishes. The virus was made up to 0.5ml with SFM and the cells left for 1hr at 37°C/5%CO_2. Cells were then overlaid with FGM containing 3mM HMBA. After complete CPE was observed, the cells were harvested and virus was then grown in increasing quantities in 80cm^2 flasks and 175cm^2 flasks until enough virus was obtained to infect 10 x 224mm^2 plates of 27/12/M:4 cells grown to 90% confluency. Each plate was infected with 5x10^{6}pfu of virus stock suspension in a total volume of 50ml of FGM supplemented with HMBA. Cells were harvested when complete CPE was observed. Cells were harvested using a sterile cell scraper and freeze thawed before the suspension was spun at 3500rpm for 45min to remove the cell debris. The supernatant was then sequentially filtered through a 5.0µm filter and a 0.45µm filter. To pellet the virus particles, the supernatant was spun at 12000rpm for 2hr at 4°C. The supernatant was removed and the virus pellet was gently resuspended (avoiding air bubble formation) in approximately 1.5 ml of supernatant volume. This was then centrifuged at 12000 rpm

for 1hour at 4°C in a bench top centrifuge. The supernatant was then discarded and the virus pellet gently resuspended in approximately 100 µl of DMEM. The resuspended viral pellet was sonicated for 1min in a water bath sonicator. 15µl aliquots of the resuspended virus were stored in liquid nitrogen. The titre of the resuspended virus was determined using the standard viral titre assay (see section 2.2.3.2.2).

2.2.3.5. Large scale viral DNA extraction

Large-scale viral DNA extractions were carried out for use in homologous recombination transfections. Virally infected cells at complete CPE were harvested from 4-8 x 175cm^2 tissue culture flasks. Replication competent and incompetent virus required 4 or 8 flasks respectively. Cells and virus were pelleted by centrifugation at 12000rpm for 2hr in a Beckman JA14 or equivalent. The supernatant was then discarded and the pellet was resuspended in 10ml of proteinase K buffer (0.01M Tris pH 8.0, 5mM EDTA, 0.5% SDS) and proteinase K was added to a final concentration of 50µg/ml. The resuspended pellet was then incubated in an orbital shaker (200rpm, 37°C) overnight or until the lysate became clear. 10ml of dd$H_2$0 were then added to the lysate. Extractions were performed by addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) followed by gentle mixing, by inversion, for 10min. The mixture was then divided equally into Beckman polyallomer tubes and ultracentrifugation performed at 15000rpm for 30min in an SW41 rotor or equivalent. Repeated extractions steps were performed on the aqueous layer, with ultracentifugation for 10min only, until no white protein interface appeared. The aqueous layer was then transferred into 50ml tubes and a final extraction was performed using an equal volume of chloroform/isoamyl alcohol (24:1). The tubes were mixed by gentle inversion for 5min and spun on a benchtop centrifuge at 2000rpm for 5min. The aqueous layer was removed and the viral DNA was then precipitated with 2 volumes of room temperature 100% ethanol, by running the ethanol gently down the side of the tube, layering on the aqueous solution and then gently mixing. The precipitated viral DNA was then pelleted by centrifugation at 3000rpm for 10min. The viral DNA was washed with 5ml of 70% ethanol and the pellet air-dried overnight. The pellet was then resuspended in an appropriate volume of dd$H_2$0 and the integrity and approximate concentration of the DNA determined by running 5µl on a 1% agarose gel. Replication competent vector

DNA was resuspended in 3ml of water and replication incompetent vector DNA in between 100-500µl.

2.2.4. Protein analysis

2.2.4.1. Western blotting

2.2.4.1.1. Extraction of "fragile" proteins from cultured cells

Cells were washed twice with 1x PBS and harvested in 1ml of PBS (per well of a 6-well plate). Cells were pelleted at 2000rpm for 3min and the pellet resuspended in 100µl of 2x extraction buffer (4.8% (w/v) SDS, 2M urea, 8% (w/v) sucrose, 1mM PMSF, 10µg/ml leupeptin and 10µg/ml pepstatin). The cells were then sheared by passing 10x through a 21-gauge needle. Samples could then be stored at -20°C or further processed by adding 100µl of 2x TM protein sample buffer (4.8% (w/v) SDS, 2M urea, 8% (w/v) sucrose, 350mM β-mercaptoethanol). The samples were then stored at -20°C. Before loading onto an SDS-polyacrylamide gel, samples were incubated at 37°C for 15min. This method was developed by Dr. Caroline Lilley (The Salk Institute). Both resolving and stacking gels contained a final concentration of 2M urea (see section 2.2.4.1.2).

2.2.4.1.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Approximately 10µl of sample and 5µl of coloured molecular weight protein standards were loaded per lane. SDS-polyacrylamide gels were prepared and run in a vertical gel electrophoresis system according to the method of (Laemmli 1970). The composition of stacking and resolving gels can be seen in table 2.1. To both the resolving and stacking gel, urea was added to a final concentration of 2M. The resolving gel used was of 15%. Gels were run at a constant current (30-40mA/gel) with variable voltage in 1x running buffer (25mM Tris, 250mM glycine and 0.1% (w/v) SDS, pH 8.3). Gels were run until the protein of interest was approximately in the middle of the gel, as determined by the migration of the coloured molecular weight markers.

Table 2.1. Composition of stacking and resolving gels used in SDS-PAGE *Acrylamide mix = acrylamide-bis (30:1.5). **TEMED = NNNN-tetraethylethalinediamine

	4 x resolving gel buffer pH 8.8	**4 x stacking gel buffer pH 6.8**
Tris-base	36.3 g	12.1 g
SDS	0.8 g	0.8 g
ddH_2O to	200 ml	200 ml

	Resolving gel (15%)	**Stacking gel (5%)**
Acrylamide mix*	15.75 ml	1.6 ml
4 x buffer	7.9 ml	2.6 ml
10 % APS	150 µl	100 µl
TEMED**	9 µl	5 µl
10M urea	10 ml	3 ml
ddH_2O	32.5 ml	10.55 ml

2.2.4.1.3. Transfer of proteins to nitrocellulose membranes (Western blot)

Proteins were transferred from the SDS-polyacrylamide gel to Hybond-C nitrocellulose membranes by a wet transfer method, based on that previously described. Briefly, the SDS-PAGE gel and the nitrocellulose membrane were pre-soaked in transfer buffer (50mM Tris, 180mM glycine, 0.1% (w/v) SDS and 20% (v/v) methanol) and then sandwiched between sheets of 3MM Whatman paper and a Trans-Blot™ cell (BioRad, Hemel Hempstead, Herts) assembled according to the manufacturer's instructions. Transfer was carried out overnight at 200mA and 4°C.

2.2.4.1.4. Immunodetection of proteins on western blots

Throughout the protocol the blots were shaken constantly at room temperature. Following transfer, the membranes were blocked by incubation in 5% (w/v) skimmed milk powder in 1 x PBS for 1 hour. The membrane was then incubated in primary antibody diluted in 3% (w/v) skimmed milk powder for 1-2 hours. The primary antibody used was goat polyclonal anti-rat CNTF at a 1:500 dilution. A monoclonal antibody against actin was also used at a 1:500 dilution. Unbound antibody was removed by washing the membrane for 3x 10 minutes in 1 x PBS with 0.1% Tween-20 (PBST). The membrane was then incubated with secondary antibody (anti-goat or anti-mouse IgG horseradish peroxidase (HRP) conjugated) diluted 1:2,000 in 3% (w/v) skimmed milk powder in 1 x PBS. Unbound antibody was removed by washing the membrane for 3x 10 minutes in 1 x PBST. The bound HRP was then detected using an enhanced chemiluminescence system (ECL™) according to manufacturer's instructions. The membrane was then exposed to X-ray film, from 1 second to 15 minutes, depending on the signal intensity.

2.2.4.2. Immunohistochemistry

2.2.4.2.1. Tissue preparation

Animals were transcardially perfused with ice-cold 4% paraformaldehyde (PFA), the tissues of interest dissected and post-fixed for approximately 1 hour in ice-cold 4% PFA. The tissues were then stored at 4°C in 30% sucrose containing 0.02% sodium azide. When the tissues had been cryoprotected (confirmed by sinking to the bottom of the tube), they were sectioned. In the case of spinal cord and brain, sections were cut at 30μm on the freezing microtome. In the case of DRG, they were mounted in OCT and cryostat cut at 20 μm. Sections were kept free floating in 5% sucrose containing 0.02% sodium azide, at 4°C.

2.2.4.2.2. Immunodetection

Throughout the immunodetection protocol, antibodies were always prepared in standard block solution (1 x PBS, 3% Goat Serum, 0.02% sodium azide, 0.5% Triton-X). Between steps, sections were washed in PB for 3 x 10min at room temperature on a

shaking platform. The presence of antigen was visualised using the avidin-biotin immunohistochemical procedure, with two different methods used depending on whether a colourimetric or fluorescent endpoint was required. Reg-2 detection required the sections to be incubated in primary antibody (1:10,000 to 1:20,000 depending on aliquot used) for 2 days, at 4°C on a shaking platform. The sections were then incubated in secondary antibody (biotinylated anti-rabbit IgG, 1:200) for 1 hour at room temperature on a shaking platform. For a fluorescent endpoint, sections were incubated with Cy3™- conjugated streptavidin (1:4,000) for 45min. Sections were mounted onto subbed slides (see section 2.2.5.5.2) and allowed to briefly air-dry before mounting in antifade mounting medium. For a colourimetric endpoint, sections were incubated with avidin-horseradish peroxidase using the Vectastain® *Elite* ABC kit, following manufacturers instructions. Bound peroxidase was visualised by reaction with hydrogen peroxide in the presence of diaminobenzidine (DAB). Sections were incubated briefly in 3ml of 0.2mg/ml of DAB made in 0.15M Tris pH 7.4. 100µl of a 0.0075% hydrogen peroxide solution were added and left for 5min. If no colour developed, 100µl of a 0.030% hydrogen peroxide solution were added and left for 5min. If still no colour developed then consecutive 100µl of a 0.075% hydrogen peroxide solution were added and left for 5min. The reaction was stopped by washing in 0.15M PB followed by 0.01M PB. The sections were then dehydrated through a series of ascending alcohols, cleared in histoclear and mounted in DPX.

2.2.4.2.3. Double immunohistochemistry

Double immunohistochemistry for Reg-2 and IB4 or N52 was carried out consecutively, following Reg-2 detection by fluorescence. For IB4, the sections were incubated in fluorescein conjugated *bandeiraea simplicifolia* lectin I, 1:1,000 overnight at 4°C on a shaking platform. For N52, sections were incubated in primary antibody, 1:1,000 overnight at 4°C on a shaking platform, followed by a 1 hour incubation with Oregon green anti mouse H+L conjugate (1:500) at room temperature. After washing with PB, sections were mounted onto subbed slides (see section 2.2.5.2.2) and allowed to briefly air-dry before mounting in antifade mounting medium.

2.2.5. *In vivo* work

All experiments were carried out in accordance with the UK Animals (Scientific Procedures) act 1986.

Except otherwise indicated, Balb/c mice and Lewis rats were used. Adult mice were 3 weeks old and adult rats weighed at least 200g. All adult animals used were female. All adult animals were anaesthetised by halothane inhalation whereas neonate animals (up to 5 days old) were hypothermia anaesthetised. In neonatal animals, P refers to postnatal day.

2.2.5.1. Axotomy

Sciatic transection was carried out on P0 Lewis rat pups (less than 24 h old, 7g) of either sex. The animals were hypothermia anaesthetised and the left sciatic nerve transected at midthigh level. The wounds were closed using Vetbond, the animals warmed and, when revived, allowed to return to their mother. Animals were sacrificed at various time-points after axotomy and processed as described in section 2.2.4.2.1.

2.2.5.2. *In vivo* gene delivery

2.2.5.2.1. Footpad inoculation

Animals were lightly anaesthetised and inoculated in the left rear footpad, as previously described (Coffin *et al.* 1996), with 20µl of a stock of recombinant vector (inoculum titres ranged from 1x 10^8 to 5x 10^8pfu/ml). In the case of neonates, the animals were warmed to 37°C and, when revived returned to their mother. At various times post-inoculation, the animals were deeply anaesthetised and transcardially perfused with ice cold 4% PFA. The spinal cord and ipsilateral lumbar dorsal root ganglia (DRG) L3-L5 were removed and post-fixed in ice cold 4% PFA for a further 1-2 hours. Reporter gene expression was then determined (sections 2.2.5.5 and 2.2.5.6).

2.2.5.2.2. Sciatic nerve inoculation

Animals were anaesthetised by halothane inhalation or by hypothermia for as long as necessary. A skin incision was made over the left gluteal muscles and the sciatic nerve

exposed following blunt dissection of overlying muscle. The sciatic nerve was placed under tension and 2-5µl of a stock of recombinant vector was injected using a glass micropipette (inoculum titres ranged from 1x 10^7 to 5x 10^8pfu/ml). Tension was then released, the wound was closed using surgical clips (for neonate animals Vetbond was used) and animals allowed recovering. In the case of neonates, the animals were warmed to 37°C and, when revived returned to their mother. At various time points post-inoculation animals were sacrificed and processed as in section 2.2.5.2.1.

2.2.5.2.3. Striatal inoculation

Adult rats received stereotaxic injections unilaterally into the striatum. The co-ordinates used were: anterior-posterior +0.18; lateral +0.3 and ventral –0.45. Injections were made through a 25µl Hamilton syringe with a ceramic tip connected to a pump at a rate of 0.5µl/min. 5µl of virus stock of 1x10^8 pfu/ml were injected. Following virus injection, the needle was left in place for an additional 5min to allow the injectate to diffuse from the needle tip. The needle was then removed over a period of 3min. The wound was closed using clips and the animals were allowed to recover. Two days following virus inoculation the animals were sacrificed and processed as in section 2.2.5.2.1.

2.2.5.3. Retrograde labelling

In order to specifically label the sciatic motor neuron pool, a fluorescent retrograde tracer, Fluororuby, was used. P2 Lewis rats were anaesthetised on ice, the sciatic nerve exposed at mid thigh level and transected. The proximal nerve stump was then immersed in a 10% (w/v) Fluororuby solution prepared in saline containing 2% DMSO, as prepared in (Richmond *et al.* 1994). The nerve stump was immersed for 5min in the tracer using parafilm as a platform. The wound was then closed using Vetbond, the animals warmed and returned to their mothers. At various time-points following axotomy, the animals were sacrificed. The animals were transcardially perfused with ice cold 4% PFA. The spinal cord and ipsilateral lumbar dorsal root ganglia (DRG) L3-L5 were removed and post-fixed in ice cold 4% PFA for a further 1-2 hours. The tissues were cryoprotected in 30% sucrose and the spinal cords trimmed down to the lumbar segments L3-L6. The spinal cords were embedded in OCT and transverse

sectioned (30μm) on the cryostat. Serial sections were cover-slipped with antifade mounting medium and profiles of labelled cells were counted on each section. In the case of the DRG, these were either observed as a full mount (directly at the fluorescent microscope) or they were embedded in OCT and serially sectioned at 15 μm on the crysotat.

2.2.5.4. *In vivo* gene delivery with sciatic nerve axotomy and retrograde labelling

P2 Lewis rats were injected in the sciatic nerve as described in section 2.2.5.2.2, except prior to inoculation, a loose knot was tied around the nerve using some suture (this was done in order to mark the injection site in the nerve). Ten minutes following vector injection, the nerve was transected below the knot. The proximal nerve stump was then labelled with retrograde tracer as described in section 2.2.5.3.

P1 Lewis rats were injected in the footpad as described in section 2.2.5.2.1. The following day, the sciatic nerve was axotomised and retrograde labelling performed as described in section 2.2.5.3.

2.2.5.5. Detection of β-galactosidase activity in tissue

2.2.5.5.1. DRG

After post-fixing, the DRG were washed 3 times with 1x PBS for 15min each wash. The DRG were then placed in 100μl of DRG X-Gal solution (5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.6H_2O$, 1mM $MgCl_2$, 0.02% sodium deoxycholate, 0.02% NP-40, and 40mg/ml X-Gal [dissolved in DMSO] in 1x PBS) and incubated at 37°C overnight. The X-Gal solution was then removed and the DRG placed in 70% v/v glycerol and stored at 4°C prior to photography. DRG were photographed by placing in 1 x PBS on a glass slide and placing a coverslip on top. Photographs were taken at either x5 or x10 magnification on a Zeiss Axiophot microscope.

2.2.5.5.2. Spinal cord and brain

After post-fixing, the tissues were stored at 4°C in 30% sucrose containing 0.02% sodium azide. When the tissues had been cryoprotected (confirmed by sinking to the

bottom of the tube), they were sectioned at 30μm on the freezing microtome. Sections were placed in X-Gal solution (5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.6H_2O$, 1mM $MgCl_2$, and 150μg X-Gal - dissolved in DMSO in 1x PBS) and incubated overnight at 37°C. The X-Gal stain was then removed, the sections washed in PB and mounted on subbed slides. The slides were prepared by dissolving 6g of gelatin in 350ml of water at 50°C. When the mixture had cooled, 4g of Chromium Potassium Sulphate previously dissolved in 50ml of water, was added. Microscope slides were dipped in the mix for at least 1min, wrapped in foil and baked overnight at 50°C. The mounted sections were allowed to dry overnight and were counterstained with neutral red. 5ml of 1% (w/v in dH_2O) neutral red was added to 1ml of 1M NaOAc (pH 8.5) and 1ml of 1.2M acetic acid (pH 2.4) and made up to 250ml in dH_2O. Sections were placed in this solution for at least 30min. The sections were then dehydrated through a series of ascending alcohols, cleared in histoclear and mounted in DPX.

2.2.5.6. Detection of green fluorescent protein in the DRG

DRG were removed and placed in 1x PBS. The DRG were then placed on a glass slide using a plastic pipette tip and coverslipped. GFP expression was then visualised under UV light (520nm) under either x5 or x10 magnification and photographed.

Chapter 3

Comparison of disabled HSV-1 vectors for gene delivery to motor neurons following peripheral administration

Chapter 3 – Comparison of disabled HSV-1 vectors for gene delivery to motor neurons following peripheral administration

3.1. Introduction

Several neurotrophic factors have emerged as potential candidates of therapeutic use for motor neuron disease. The repeated subcutaneous administration of these factors has resulted in either no therapeutic effect due to poor bioavailability or in some cases serious side-effects due to toxic systemic levels of the factor (Dittrich *et al.* 1996; Miller *et al.* 1996). Gene therapy in this case could ideally allow specific, efficient targeting of the affected motor neurons.

Motor neurons are unique cells with large soma located in the CNS and with long axonal processes that innervate skeletal muscles. One approach for gene delivery to motor neurons has involved direct administration of the vector to the cell bodies via spinal cord injection. To this effect, adeno-associated virus (Hermens 99), adenovirus (Lisovoski *et al.* 1994), herpes simplex virus (Lilley *et al.* 2001; Yamada *et al.* 2001) and lentivirus (Hottinger *et al.* 2000) have been used. Although this method does indeed result in the transduction of a large number of motor neurons, some trauma to the CNS inevitably occurs due to direct injection. In addition, precise targeting of the vector is required in order to avoid infection of other cell types. An alternative method for gene delivery takes advantage of the physical characteristics of the motor neuron and involves vector administration at the periphery, i.e. at the axon level or at the neuromuscular junction. The vector can then undergo retrograde axonal transport to reach the motor neuron cell body to deliver the therapeutic gene to the nucleus. This is of particular use in the experimental evaluation of potential neuroprotective factors in an axotomy or ventral root avulsion model where the factor is delivered to a restricted pool of motor neurons (see chapter 5).

The use of HSV-1 vectors for gene delivery to motor neurons following peripheral inoculation has not been extensively studied. One study by Neve and colleagues used an amplicon system to deliver a glutamate receptor subunit to motor neurons by sciatic nerve injection (Neve *et al.* 1997). The promoter chosen to drive the transgene was the

HSV-1 ICP22/47 promoter and transgene expression was achieved for one week with the observed decrease in gene expression being attributed to promoter shutdown (Neve *et al.* 1997). In addition, the transduction obtained was of low efficiency. Dobson *et al* reported in 1990 the use of a disabled HSV-1 vector with *lacZ* under the control of the MMLV LTR promoter inserted into the ICP4 region in a position antisense to ICP4 (Dobson *et al.* 1990a). When injected into the tongue of adult mice, expression of β-galactosidase was observed over a limited period in motor neurons of the hypoglossal nucleus in the brainstem (Dobson *et al.* 1990a). In 1995, Keir *et al* reported the use of an HSV-1 vector with an MMLV LTR fused downstream of a TATA-less LAP1, driving *lacZ* (Keir *et al.* 1995). Following intramuscular injection in mice, short-term transgene expression was obtained in motor neurons in the spinal cord. Yamamura and colleagues adopted a different approach in order to achieve motor neuron transduction: here a highly neuroattenuated HSV-1 strain was selected from an HSV-1 collection of clinical isolates and the *lacZ* gene inserted just downstream of LAP2 (Yamamura *et al.* 2000; Shiraki *et al.* 1998). β-galactosidase expressing motor neurons were detected 5 days after intramuscular inoculation (Yamamura *et al.* 2000). 14 days after virus administration, the virus had replicated and spread so that motor neurons throughout spinal levels, lumbar 1 to sacral 1 expressed the transgene (Yamamura *et al.* 2000). Transgene expression was maintained up to at least 182 days after inoculation and no pathological changes were observed, leading to the conclusion that the virus was non-neurovirulent (Yamamura *et al.* 2000). While these results are impressive, the virus used has not yet been fully characterised. Hence it is not possible to extrapolate from this study any information on the HSV genes it is important to delete or retain for the transduction of motor neurons by HSV vectors.

In contrast to the few studies on motor neurons, there are many reports of HSV-1 mediated gene delivery to the DRG after peripheral administration. Indeed, our laboratory has extensively characterised vectors for this purpose. Initial studies showed that the 1764 backbone (see figure 3.2), a vector deleted for both copies of ICP34.5 (MacLean *et al.* 1991) and with an inactivating mutation in VP16 (Ace *et al.* 1989), was capable of gene delivery to the DRG after footpad inoculation (Coffin *et al.* 1996). In further studies testing combinations of mutations in ICP34.5, VP16 and vhs, the 1764 backbone gave the most efficient gene delivery after either sciatic nerve or footpad inoculation (Palmer *et al.* 2000). Work in our laboratory also aimed at identifying

promoters capable of driving long term transgene expression. Based on work done on the LAP2 region indicative of the presence of long-term elements (Lachmann and Efstathiou 1997), a series of promoter constructs were tested within the 1764 backbone (Palmer *et al.* 2000). As result, LAT P2 was identified. LAT P2 is a 1.4 kb region immediately downstream of the LAP1 TATA capable of conferring long term activity onto promoters including the CMV promoter, a minimal neuron-specific enolase (NSE) promoter and the MMLV promoter (Palmer *et al.* 2000). In addition, reporter gene cassettes where LAT P2 was flanked by pairs of promoters in a back-to-back orientation were constructed (pR20.5 and pR20.9, see figure 3.2). These were found to maintain latent gene expression when placed at a non-LAT site within the HSV genome (cassettes were inserted into the UL43 gene)(Palmer *et al.* 2000).

The removal of the non-essential genes ICP34.5 and VP16 reduces viral pathogenicity *in vivo* whilst maintaining some capabilities for replication. This replication-competence is required for viral gene delivery to the DRG following either sciatic nerve or footpad inoculation (Palmer *et al.* 2000). Indeed, a number of replication-incompetent viruses were tested by these routes and were found incapable of gene delivery to the DRG, unless when injected at high titres directly into the nerve (Palmer *et al.* 2000). A replication-incompetent vector based on the 1764 backbone but further deleted for essential gene products ICP4 and ICP27 gave high levels of gene delivery in the DRG that remained up to one month following sciatic nerve inoculation at high titres. The *lacZ* transgene was under the control of the CMV promoter, which in turn was inserted just downstream of LAT P2 (Palmer *et al.* 2000). These series of experiments formed the starting point for this thesis which aimed to explore the ability of the vectors developed to mediate gene delivery to spinal motor neurons following peripheral administration. Given the variety of animal models used for the study of trophic substances on degenerating motor neurons (transgenic mice, neonate axotomy and adult rat ventral root avulsion (see section 1.4.4), the vectors were tested in adult mice, adult rats, and neonate rats.

3.2. Rationale

As discussed in 3.1, promising results previously obtained in DRG following peripheral administration of various HSV vectors prompted their testing for delivery to motor

neurons. Indeed, footpad injection leading to DRG transduction might be anticipated to result in the transduction of motor neurons due to retrograde transport of the virus from neuromuscular junctions. Similarly, motor neurons might be transduced after sciatic nerve injection by delivery of the vector to the axons of motor neurons (see figure 3.1). An analysis of the results obtained by Palmer *et al* with different HSV-1 vectors is presented below, and in table 3.1.

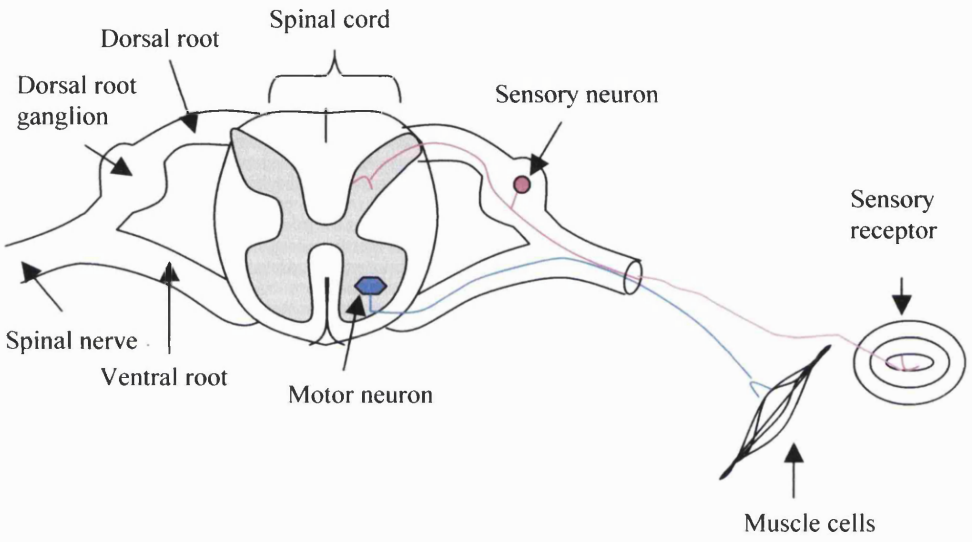


Figure 3.1. Representative schematic indicating the ganglionic sensory neurons and the spinal motor neurons.

Footpad injection of 1764 containing either the pR20.9 or pR20.5 cassette (see figure 3.2) resulted in long term expression of GFP and *lacZ* in DRG in both cases. 1764pR20.9, however, gave more transgene expressing cells than did 1764pR20.5, indicating the possible presence of long term gene expression elements in LAP1 as well as LAT P2 (Palmer *et al.* 2000).

Sciatic nerve injection of 1764pR20.5 resulted in very high efficiency gene delivery to the DRG (much higher than by footpad), but after 2 weeks this had decreased to levels comparable to those seen after footpad inoculation. With 1764pR20.9, very low levels of gene delivery were obtained shortly after injection which increased to a maximum at 2 weeks and remained constant from then on at levels similar to those seen after footpad

inoculation. One explanation for these different results with the two vectors may be connected with the only low-level *lacZ* activity seen with 1764pR20.9 in BHK cells in which the virus can replicate (Palmer *et al.* 2000). Here it was proposed that ICP4 and ICP0 might repress LAT P2, as when replication was inhibited by the addition of acyclovir, *lacZ* activity was greatly increased. In the sciatic nerve, a higher level of ICP4 and ICP0 might be expressed in neurons compared to following footpad infection as a greater virus dose would be anticipated to reach the neuron, resulting in promoter repression (Palmer *et al.* 2000). Footpad injection of replication-incompetent vector 1764 27^{-}4^{-}pR19lacZ (see figure 3.3) at high titre resulted in low-level gene delivery to the DRG. This probably indicated the requirement for replication for the virus to reach the sensory terminals. High titre sciatic nerve injection, on the other hand, resulted in high levels of gene delivery, which were sustained for at least 1 month.

Based on these results we tested the transduction of motor neurons following footpad injection of 1764pR20.9 and after sciatic nerve injection of 1764pR20.5 and 1764 27^{-}4^{-} pR19lacZ.

Table 3.1. Summary of results reported by Palmer *et al* (2000) in the adult mouse DRG. Levels of gene delivery are expressed as +, signifying low-level gene delivery, to ++++ indicating high-level gene delivery.

		3 days	2 weeks	2 months
1764pR20.9	Footpad	+++	++	++
	Sciatic nerve	+	++	++
1764pR20.5	Footpad	++	+	+
	Sciatic nerve	++++	+	+
1764 27^{-}4^{-}pR19lacZ	Footpad	+	+	+
	Sciatic nerve	+++	+++	+++

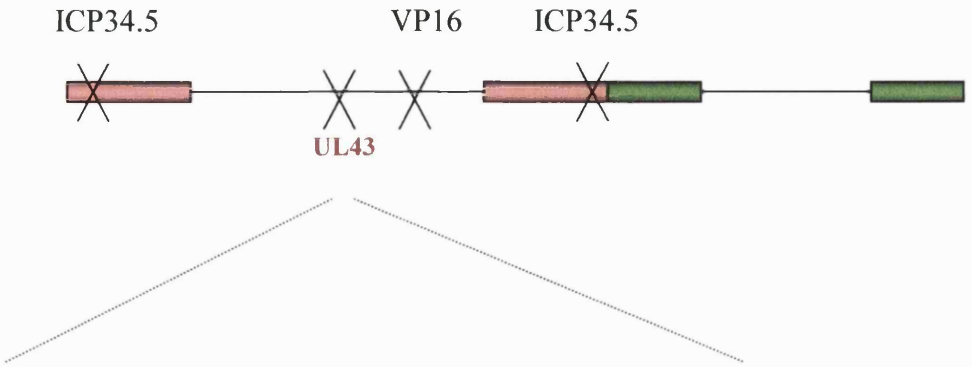
3.3. Materials and Methods

3.3.1. Vectors and promoter constructs used

Vectors tested were based on wild type HSV-1 strain 17+ (Brown *et al.* 1973). Vector 1764 (see figure 3.2 (Coffin *et al.* 1996)) refers to 17+ deleted for both copies of ICP34.5 (MacLean *et al.* 1991) and with the *in*1814 mutation in VP16 (Ace *et al.* 1989). Vector 1764 $27^{-}4^{-}$ (Thomas *et al.* 1999) refers to the 1764 backbone deleted for ICP27 (Howard *et al.* 1998) and in both copies of ICP4 (see figure 3.3).

Promoter constructs pR20.5 (Thomas *et al.* 1999) and pR20.9 (Palmer *et al.* 2000) were inserted into the 1764 backbone in the UL43 gene at the unique *Nsi*I site (nt 94911). These vectors were termed 1764pR20.5 and 1764pR20.9 respectively (Palmer *et al.* 2000). The pR19lacZ cassette (see figure 3.3) was recombined into the endogenous LAT regions of the replication-incompetent vector backbone between the two *Bst*XI sites (nt 120220 and 120408) (Palmer *et al.* 2000). The resulting virus is referred to as 1764 $27^{-}4^{-}$pR19lacZ (Palmer *et al.* 2000). The LAT P2 region refers to nt 118866 to 120219 (Palmer *et al.* 2000). LAP1, the TATA box-containing promoter represented in figures 3.2 and 3.3, refers to the promoter identified by (Goins *et al.* 1994). In figure 3.1 it refers to the *Dde*I to *Sty*I region (nt 118181 to 118878) and in figure 3.2 to the *Not*I to *Sty*I region (nt 118439 to 118878). HSV nucleotide numbers refer to GenBank file HE1CG.

. **1764**



. **pR20.5**

. **pR20.9**

Figure 3.2- Replication competent vector backbone and expression cassettes. The 1764 vector backbone is deleted for ICP34.5 and has an inactivated VP16. Cassettes pR20.5 and pR20.9 are flanked by unique sites for the blunt-cutting enzyme *Srf*I allowing excision and insertion into the *Nsi*I site of the nonessential gene UL43. RSV, Rous sarcoma virus promoter. MMLV LTR, Moloney murine leukemia virus promoter. GFP, Green fluorescent protein. LacZ, β-galactosidase. CMV, cytomegalovirus promoter. LAP1 and LAT P2, HSV latency associated promoters.

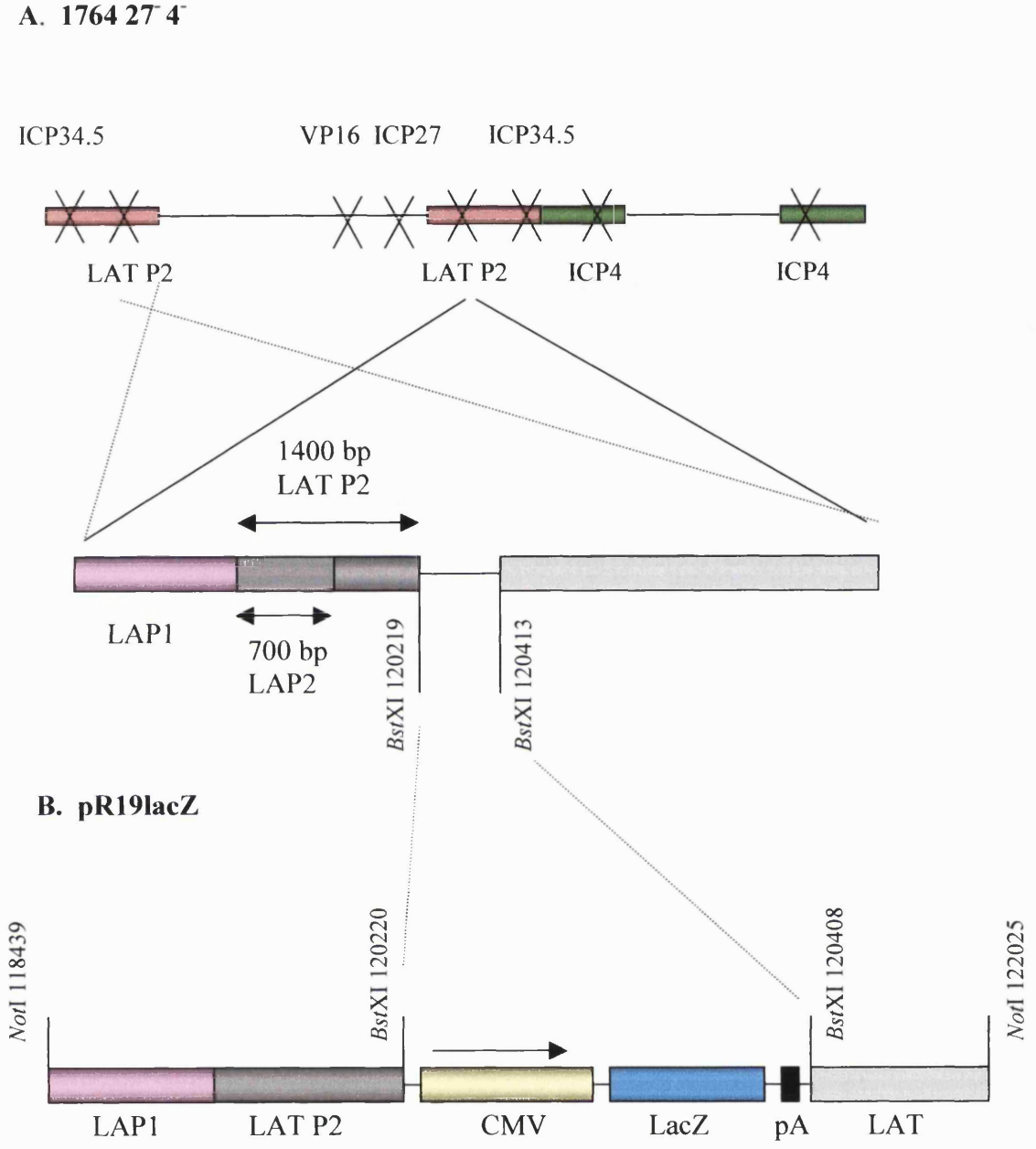


Figure 3.3- Replication incompetent vector backbone and transgene casette. **A.** 1764 27-4- vector backbone showing insertion site in the LAT region (between the two *Bst*XI sites). **B.** Structure of plasmid pR19lacZ.

3.3.2. Peripheral administration of HSV-1 vectors in the adult mouse

Surgery and tissue preparation was performed as described in sections 2.2.5, 2.2.5.5.1 and 2.2.5.5.2.

3.3.2.1. Gene delivery using 1764pR20.9

3-week-old Balb/c mice were injected in the footpad (see section 2.2.5.2.1) with 25 µl of 1764pR20.9 at a titre of $1x10^8$ pfu/ml. Animals were sacrificed 2 days and 2 weeks after virus inoculation (3 animals per timepoint).

3.3.2.2. Gene delivery using 1764pR20.5 or 1764 27-4-pR19lacZ

3-week-old Balb/c mice were injected in the sciatic nerve (see section 2.2.5.2.2) with:
(a) 2-5 µl of 1764pR20.5 at a titre of $1x10^7$ pfu/ml. Animals were sacrificed 2 days and 1 week later (3 animals per timepoint).
(b) 2-5 µl of 1764 27-4-pR19lacZ at a titre of $2x10^8$ pfu/ml. Animals were sacrificed 2 days, 1 week and 1 month later (3 animals per timepoint).

3.3.2.3. Gene delivery using 1764pR20.9 in Balb/c and SCID (severe combined immunodeficient) mice

3-week-old mice were injected in the footpad with 25 µl of 1764pR20.9 at a titre of $1x10^8$ pfu/ml. Animals were sacrificed 2 days, 2 weeks, 3 weeks, 1 month and 2 months after virus inoculation (3 Balb/c and 3 SCID mice per timepoint).

3.3.3. Peripheral administration of HSV-1 vectors in the adult rat

Surgery and tissue preparation was performed as described in sections 2.2.5, 2.2.5.5.1 and 2.2.5.5.2.

3.3.3.1. Gene delivery using 1764pR20.9

Adult Lewis rats were injected in the footpad (see section 2.2.5.2.1) with 25 µl of 1764pR20.9 at a titre of $3x10^8$ pfu/ml. Animals were sacrificed 2 days and 2 weeks after virus inoculation (3 animals per timepoint).

3.3.3.2. Gene delivery using 1764pR20.5 or 1764 27-4-pR19lacZ

Adult rats were injected in the sciatic nerve (see section 2.2.5.2.2) with:
(a) 2-5 μl of 1764pR20.5 at a titre of $1x10^8$ pfu/ml. Animals were sacrificed 2 days and 2 weeks later (3 animals per timepoint).
(b) 2-5 μl of 1764 27-4-pR19lacZ at a titre of $2x10^8$ pfu/ml. Animals were sacrificed 2 days and 2 weeks later (3 animals per timepoint).

3.3.4. Peripheral administration of HSV-1 vectors in the neonate rat

Surgery and tissue preparation was performed as described in sections 2.2.5, 2.2.5.5.1 and 2.2.5.5.2.

3.3.4.1. Gene delivery using 1764pR20.9

P2 neonate rats were injected in the footpad (see section 2.2.5.2.1) with 25 μl of 1764pR20.9 at a titre of $3x10^8$ pfu/ml. Animals were sacrificed 2 days and 1 week after virus inoculation (3 animals per timepoint).

P2 neonate rats were injected in the tibialis anterior in three different places with a total of 25 μl of 1764pR20.9 at a titre of $3x10^8$ pfu/ml. Animals were sacrificed 2 days after virus inoculation.

3.3.4.2. Gene delivery using 1764pR20.5 or 1764 27-4-pR19lacZ

P2 neonate rats were injected in the sciatic nerve (see section 2.2.5.2.2) with:
(a) 2-5 μl of 1764pR20.5 at a titre of $1x10^8$ pfu/ml. Animals were sacrificed 2 days and 1 week later (3 animals per timepoint).
(b) 2-5 μl of 1764 27-4-pR19lacZ at a titre of $2x10^8$ pfu/ml. Animals were sacrificed 2 days and 1 week later (3 animals per timepoint).

3.4. Results

3.4.1. High-level short-term gene delivery to adult mouse motor neurons following peripheral inoculation of replication-competent vectors

Replication-competent vectors 1764pR20.9 and 1764pR20.5 were tested for their ability to mediate gene delivery to adult mouse motor neurons following peripheral administration. Footpad administration of 1764pR20.9 and sciatic nerve inoculation of 1764pR20.5 resulted in high levels of gene delivery to motor neurons 2 days postinoculation (see figures 3.4 and 3.5). X-Gal staining was observed in motor neurons located in the L3-L5 region of the spinal cord, as expected since these neurons project through the sciatic nerve and innervate the footpad. High levels of transgene expression were obtained as shown by the X-Gal staining of axonal processes in addition to the soma (see figures 3.4 and 3.5). Gene delivery was limited to motor neurons ipsilateral to the injection site with no transgene expression being detected in the contralateral side, other regions of the spinal cord or the brain stem. Although these vectors are replication competent, the observed lack of trans-synaptic spread to other neurons or other cell types suggests the viruses are not replicating after reaching the motor neuron.

3.4.2. Sciatic nerve inoculation does not increase the efficiency of motor neuron transduction

Sciatic nerve inoculation might be expected to result in higher transduction efficiency than footpad injection as the vector has direct access to the axon. In the case of gene delivery to the DRG, sciatic nerve injection of 1764pR20.5 (see figure 3.5) has been shown to give much higher levels of gene delivery than footpad inoculation (Palmer *et al.* 2000). However, gene delivery to motor neurons after injection of 1764pR19lacZ (the 1764 backbone (figure 3.2) with the pR19lacZ cassette (figure 3.3)) was identical using either the footpad or the sciatic nerve route (Palmer *et al.* 2000). In accordance with these results, we show that sciatic nerve injection of 1764pR20.5 gives slightly less efficient transgene delivery (see figure 3.5) than footpad injection of 1764pR20.9 (see figure 3.4). It appears, therefore, that in this particular animal model,

the 3-week-old Balb/c female mouse, HSV-1 infection of motor neurons is equally or more efficient at the neuromuscular junction when compared to the axon.

3.4.3. Reduction in gene expression after motor neuron transduction with replication–competent vectors

Footpad injection of 1764pR20.9 results in high-levels of motor neuron transduction but pathological changes such as extensive mononuclear cell infiltration can be seen (see figure 3.4). Although latent gene expression is observed in the DRG (see figure 3.7) no transgene expression is observed in motor neurons 2 weeks after vector inoculation (see figure 3.4). In addition, in the L3-L5 levels of spinal cord ipsilateral to the injection, nuclear debris and extensive mononuclear infiltration indicative of an inflammatory response can be observed. Cell infiltrations identical to those reported here were described in lumbar anterior spinal cord of adult MF1 mice injected into the muscle with wild-type HSV-1 strain SC16 (Wharton *et al.* 1995). Similarly, Keir *et al* reported inflammatory infiltrates surrounding virally infected motor neurons as soon as 3 days post intramuscular injection (Keir *et al.* 1995). Sciatic nerve injection of 1764pR20.5 did not induce such pathological changes 2 days after injection, but at 1 week post inoculation, mononuclear infiltrates can be seen surrounding the virally infected cells and the number of motor neurons expressing β-galactosidase is greatly reduced (see figure 3.5). The decrease in transgene expression in both situations using the replication-competent backbone 1764, taken together with the local inflammation observed, seems indicative of a host immune response directed either to the virus or to the transgene. In order to test the involvement of the immune response, an experiment was designed using 1764pR20.9 (the virus which elicited the strongest inflammatory response) in either Balb/c or SCID mice (see section 3.4.5).

3.4.4. Long-term gene delivery to adult mouse motor neurons following sciatic nerve injection with a replication-incompetent vector

As mentioned in sections 3.1 and 3.2, our laboratory reported stable long-term gene expression in the adult mouse DRG following sciatic nerve injection of the replication-incompetent vector 1764 27-4-pR19lacZ (Palmer *et al.* 2000). Following injection of this vector in the sciatic nerve of adult Balb/c mice (see section 3.3.2.2), long-term β-

galactosidase expression was also achieved in the lumbar motor neurons (see figure 3.6). Stable gene expression is shown by similar efficiencies of transduction 2 days, 1 week and 1 month after vector inoculation. The efficiency of transduction of sensory neurons in the DRG (see figure 3.6) is in accordance with that reported by Palmer *et al* (Palmer *et al.* 2000). Unlike the results obtained with replication-competent vectors 1764pR20.9 and 1764pR20.5 (see 3.4.3), no pathological changes were observed. Furthermore, the motor neurons appeared healthy and identical to those on the side contralateral to the injection. High levels of transgene expression were obtained as demonstrated by the intense X-Gal staining of the motor neuron soma and in some cases, the neurites. At 1 week postinoculation, a punctate pattern of X-gal staining was observed in some of the motor neurons. Dobson *et al* also reported this phenomenon in both acutely and latently peripherally infected adult mice motor neurons of the hypoglossal nucleus (Dobson *et al.* 1990). These authors also used a replication-deficient vector, in this case deleted for both copies of ICP4 and with MMLV LTR driving *lacZ* in the ICP4 locus in a position antisense to ICP4 (Dobson *et al.* 1990). Goins *et al* also reported a "speckled" staining in adult mice sensory neurons of the trigeminal ganglia seven days postinoculation with a vector containing *lacZ* under LAP2 inserted in the gC locus (Goins *et al.* 1994). In a manner similar to the situation seen in the DRG, we show here that the LAT P2 region allows gene expression from a heterologous promoter (CMV) during HSV-1 latency in motor neurons.

3.4.5. Involvement of the host immune response in the reduction of transgene expression following peripheral inoculation with 1764pR20.9

Peripheral inoculation of replication-competent vectors based on the 1764 backbone resulted in short-term gene expression associated with pathological changes (see 3.4.3). In order to test the possible involvement of the host immune response, 1764pR20.9 was injected into the footpad of Balb/c and SCID mice (see section 3.3.2.3 and figures 3.7 and 3.8). SCID (severe combined immunodeficient) mice have a recessive mutation in an enzyme required for the differentiation of lymphocytes (Janeway C.A. and Travers P. 1997). As a result, they lack B- and T-cells and exhibit only a non-adaptive immune response. Comparative analysis of DRG at different time points (see figure 3.7) showed increased efficiency in gene delivery to DRG of SCID mice when compared to Balb/c mice. This became obvious from 3 weeks after virus inoculation. In Balb/c animals,

latent *lacZ* expression can be observed but there is an obvious decrease in the numbers of neurons expressing the transgene. In SCID mice however, the number of neurons expressing the transgene slightly decreases at 2-3 weeks but remains relatively constant from then on. This experiment shows that the decrease in transgene expression observed in the DRG following footpad injection of 1764pR20.9, is likely due to an adaptive host-mediated immune response.

In the spinal cord 2 days after virus injection, mononuclear infiltration is considerably reduced in SCID mice as compared to Balb/c mice (see figure 3.8). In both cases mononuclear cells can be seen surrounding and engulfing neurons, suggestive of a neuronophagy process. At 2 weeks postinoculation however, whilst extensive inflammation is observed in the Balb/c spinal cord, none can be seen in the SCID animals. In addition, transgene expression is maintained until 2 weeks (to at least some extent) in SCID mice when no transgene expression can be observed in Balb/c animals. At 3 weeks after virus inoculation, no transgene expression is observed in either Balb/c or SCID mice. Analysis of L3-L5 levels of anterior spinal cord reveals extensive motor neuron loss in Balb/c animals. In SCID animals, this is not observed and without careful orientation of the spinal cords it is not possible to detect which is the side ipsilateral to injection.

Of particular interest here is the study by McMenamin *et al* on the immune response in Balb/c mice after direct brain inoculation with a 1716lacZ vector (17+ deleted for both copies of ICP34.5 (MacLean *et al.* 1991) with *lacZ* under LAP1 in UL43 (McMenamin *et al.* 1998; Coffin *et al.* 1996) . At three days after injection, a non-specific immune response was seen as macrophages densely clustered around the site of injection (McMenamin *et al.* 1998). At one week postinoculation, massive accumulation of macrophages was seen and the immune response was of a more specific nature with infiltration of T-cells (McMenamin *et al.* 1998). The early cell infiltrations we observe at 2 days could thus be macrophages as SCID mice still have a non-adaptive (innate) immune response. This also correlates with our observations of neuronophagy-like behaviour from the infiltrated cells. The fact that we don't see any cellular infiltrates at 2 weeks postinoculation in the SCID mice is also corroborated by results from McMenamin *et al*, suggesting these are mainly T-cells and T-cell activated macrophages (and thus non-present in SCID animals). In order to identify with

certainty our infiltrates, one would have to perform immunohistochemistry for the appropriate cell markers. In the experiment by McMenamin *et al*, *lacZ* expression was maximal at 2 days decreasing until one week (McMenamin *et al.* 1998). When Coffin *et al* directly injected the brains of Balb/c mice with 1716lacZ and of 1764lacZ (the 1764 backbone with *lacZ* under LAP1 in UL43), transgene expression was detected for 2-5 days and decreased until 15 days postinoculation. The brief expression in both cases is likely due to the use of LAP1 as the promoter to drive transgene expression as it has been shown to lack a long-term capability, both in its natural position (Margolis *et al.* 1993; Dobson *et al.* 1989) and ectopically (see section 1.3.3.6) (Dobson *et al.* 1995; Margolis *et al.* 1992). Given the extensive immune response seen (McMenamin *et al.* 1998), the possible additional effect of the immune response in both studies (McMenamin *et al.* 1998; Coffin *et al.* 1996) cannot be eliminated. In our experiment it is not clear what mechanism is involved in the reduction in transgene expression in motor neurons. In SCID animals, a less dramatic decrease in transgene expression was observed between 2 days and 2 weeks, but nonetheless a very significant decrease was still observed (unlike in the DRG where transgene levels were maintained). This indicates that the adaptive immune response can account for some of the decrease observed in transgene expression, but other factors are also likely to be involved. These could be the non-specific or innate immune response (macrophage infiltrations seen at early stages) and/or vector motor neuron cytotoxicity and/or promoter-shutdown. In the case of the latter, this would reflect differences in promoter activity between motor neurons and sensory neurons as in the DRG, promoter shutdown of 1764pR20.9 does not seem to occur. This is shown in SCID mice, where levels of transgene expression are maintained over time (see figure 3.7), and also by work in our laboratory in which X-Gal detection was performed in parallel to *in situ* hybridisation for the LATs (Palmer *et al.* 2000). The latter showed that the number of neurons in the DRG expressing β-galactosidase one month after injection of 1764pR20.9 was very similar to the number of neurons expressing LAT mRNA (Palmer *et al.* 2000).

3.4.6. Results with 1764pR20.9 and 1764pR20.5 following peripheral inoculation in the adult rat

Following inoculation of 1764pR20.9 into the footpad of adult rats (see section 3.3.3.1), no gene delivery was observed in the DRG or in spinal motor neurons 2 days (see figure

3.9) and 2 weeks after inoculation (results not shown). Identical results were obtained after sciatic nerve inoculation of 1764pR20.5 (see section 3.3.3.2 and figure 3.9). The lack of gene delivery observed in the adult rat with the replication-competent vectors contrasts with the situation observed in adult Balb/c mice (see above). However, these are different animals which may have different susceptibilities to HSV-1 infection. Indeed, even among strains of the same animal species, variation in susceptibility has been seen (Lopez 1975) and the non-adaptive immune response has been shown to play a key role (Simmons *et al.* 1992). In the Lewis rat, it may be the case where the non-adaptive immune response to the replication-competent virus prior to retrograde transport to the neuronal cell body prevents efficient gene transfer being observed.

3.4.7. Gene delivery to the adult rat following sciatic nerve inoculation with 1764 27^{-}4^{-}pR19lacZ

Replication-incompetent vector 1764 27^{-}4^{-}pR19lacZ was injected into the sciatic nerve of adult rats (see section 3.3.3.2). β-galactosidase was detected in sensory neurons of the DRG and in motor neurons of the lumbar spinal cord (see figure 3.10) 2 days and 2 weeks after inoculation. The efficiency of gene delivery to both motor neurons and DRG was low, but was maintained for at least 2 weeks. Although longer time points were not tested, one might expect long term gene expression in the motor neurons as our laboratory has reported stable *lacZ* expression for at least one month following direct spinal cord injection with the same virus (Lilley *et al.* 2001). A virus with the 1764 backbone and the pR19lacZ cassette was also tested by sciatic nerve injection where no gene delivery was observed (results not shown). Thus, the lack of gene delivery seen with the replication competent viruses above seems to be a consequence of their level of disablement rather than promoter shutdown. The fact that the severely disabled vector could give gene delivery might indicate that this virus is less immunogenic as it lacks several essential HSV-1 genes which will reduce overall HSV gene expression in neurons. The less disabled 1764 viruses on the other hand can express immediate-early gene products after inoculation at high titres and these might trigger an immune response leading to elimination of infected cells.

3.4.8. Short-term multiple gene delivery to neonate rat DRG following footpad inoculation with replication-competent vector 1764pR20.9

In contrast to the situation observed in the adult rat (see section 3.4.6), footpad inoculation of 1764pR20.9 in neonate rats (see section 3.3.4.1) mediated high levels of transgene delivery to the DRG (see figure 3.11). The vector mediated multiple gene delivery as seen in figure 3.11 with the DRG expressing both GFP and *lacZ*. As for all cases following virus inoculation in either the footpad or the sciatic nerve which result in gene delivery to the DRG: (i) X-Gal staining was detected in DRG L3, L4 and L5 (ii) no X-gal staining was observed in the contralateral DRG and (iii) DRG L4 had the most transduced neurons (see lumbosacral vertebral column in figure 3.11). At 1 week post injection, a massive decrease in transgene expression in the DRG was observed (see figure 3.11). The fact that 1764pR20.9 can mediate gene delivery to the DRG in the neonate but not in the adult rat is potentially suggestive of an immune involvement as this might eliminate the virus or infected cells in the adult animal but might be less efficient at doing so in the immunologically immature neonate animal. The amount of motor neuron transduction observed was extremely low and only observed at 2 days post inoculation (see figure 3.11).

3.4.9. Lack of gene delivery to motor neurons following footpad injection of 1764pR20.9 is not due to a lack of motor terminals

A possible hypothesis to explain the low levels of motor neuron transduction obtained following neonate rat injection, could be the poor availability of motor neuron terminals. To test this, we injected the same amount of 1764pR20.9 vector at 3 different sites of the tibialis anterior muscle. At 2 days after the injection, the number of transduced motor neurons was in each case identical to that observed after footpad injection and thereby ruled out the hypothesis of that there was a lack of sufficient motor terminals in the neonate footpad (see figure 3.11 C).

Interestingly, although the footpad is the site of choice for vector injection for uptake by sensory terminals, equally extensive gene delivery to the DRG was observed following intramuscular injection as following footpad injection.

3.4.10. High-level short term gene delivery to neonate rat motor neurons after sciatic nerve inoculation of 1764pR20.5

In contrast to the situation in the adult rat, neonate rat sciatic nerve inoculation of 1764pR20.5 (see section 3.3.4.2) resulted in highly efficient gene delivery to motor neurons (see figure 3.12). A large percentage of motor neurons from spinal regions L3 to L5 were transduced and intense X-gal staining could be observed in individual motor neurons. Sensory neuron transduction was also observed, but at low levels. Similarly to the situation observed in the neonate DRG after footpad inoculation, transgene expression decreased dramatically by 1 week after vector injection. In addition, at 1 week post inoculation, extensive motor neuron loss could be observed in the spinal cord side ipsilateral to the injection (see figure 3.12). Dying motor neurons could be observed with fragmented soma and diffused cytoplasm. Neonate animals are immunologically immature and as expected, no obvious signs of an immune response were observed. Neonate motor neurons, however, are extremely sensitive to injury (Snider *et al.* 1992). Thus, one explanation for the motor neuron loss observed might be that the replication-competent vector based on the 1764 backbone is cytotoxic to the neonatal motor neuron. Based on these results, the decrease in transgene expression observed in the DRG after footpad injection of 1764pR20.9 in neonate animals could be due to sensory neuron loss due to vector cytotoxicity. In order to further investigate vector cytotoxicity in the DRG further histological analysis is required.

3.4.11. Gene delivery mediated by replication-incompetent vector 1764 27^{-}4^{-} pR19lacZ

Sciatic nerve injection of 1764 27^{-}4^{-}pR19lacZ in the neonate rat resulted in gene delivery to the DRG and motor neurons (see figure 3.13) 2 days after vector inoculation. Surprisingly, as opposed to the stable transgene expression observed with this virus in both adult rat and mouse (see figures 3.6 and 3.10), numbers of motor neurons expressing β-galactosidase decreased significantly by one week after injection. In the case of the DRG, some transgene expressing neurons could be detected but in the spinal cord none could be found. The reasons for decreased transgene expression could be due to one or more of the following: promoter-shutdown, a host immune response and/or vector cytotoxicity. However, the same vector has been shown to drive expression in

adult rat motor neurons for at least 2 weeks after sciatic nerve injection (see section 3.4.7) and by Lilley *et al* for 1 month after direct spinal cord inoculation (Lilley *et al.* 2001). Promoter shutdown could occur differently in the neonate than in the adult as neonatal neurons are developing immature neurons. The host immune response is probably not involved as neonate animals are immunologically immature and no cell infiltrates indicative of an inflammatory process were detected. Although 1764 27^{-}4^{-} pR19lacZ is a replication-incompetent vector that does not express significant amounts of any immediate early gene, vector cytotoxicity is a possibility which may be associated with inoculation at high titer. A high-titre stock of the virus will inevitably have carry-over material from the complementing cell line which might contain complementing cell-derived toxic viral proteins which might cause cytotoxicity. Another possibility is associated with the likely high viral particle to pfu ratio of such a highly disabled virus. Indeed, Yao *et al* showed that defective viral particles can package ICP0 and ICP4 into particles, which would be toxic (Yao and Courtney 1992). However, even with these possibilities, close analysis of the spinal cord at 1 week postinoculation with 1764 27^{-}4^{-}pR19lacZ showed no motor neuron death, as opposed to with replication-competent vector 1764pR20.5 where significant death was observed. However, as there is a significant difference in the numbers of motor neurons transduced with each virus (1764 27^{-}4^{-}pR19lacZ does not transduce many motor neurons), if they were to be dying their detection would be extremely difficult.

3.4.12. The sciatic nerve injection technique does not cause cell death in the neonate rat

A possible explanation for motor neuron death following sciatic nerve inoculation of 1764pR20.5 or 1764 27^{-}4^{-}pR19lacZ (see 3.4.9), would be the injection technique itself. Indeed, given the great sensitivity of neonate motor neurons to axotomy, the motor neuron death observed could be due to axonal injury caused by the injection technique and not by the virus itself. To test this, PBS was injected into the sciatic nerve of neonate rats similarly to when a virus was injected (using a pulled glass capillary, 3-5 µl of PBS was injected). One week after the PBS injection, no cell death could be detected. It can therefore be concluded that the motor neuron death observed is caused by the injected virus rather than the injection technique itself.

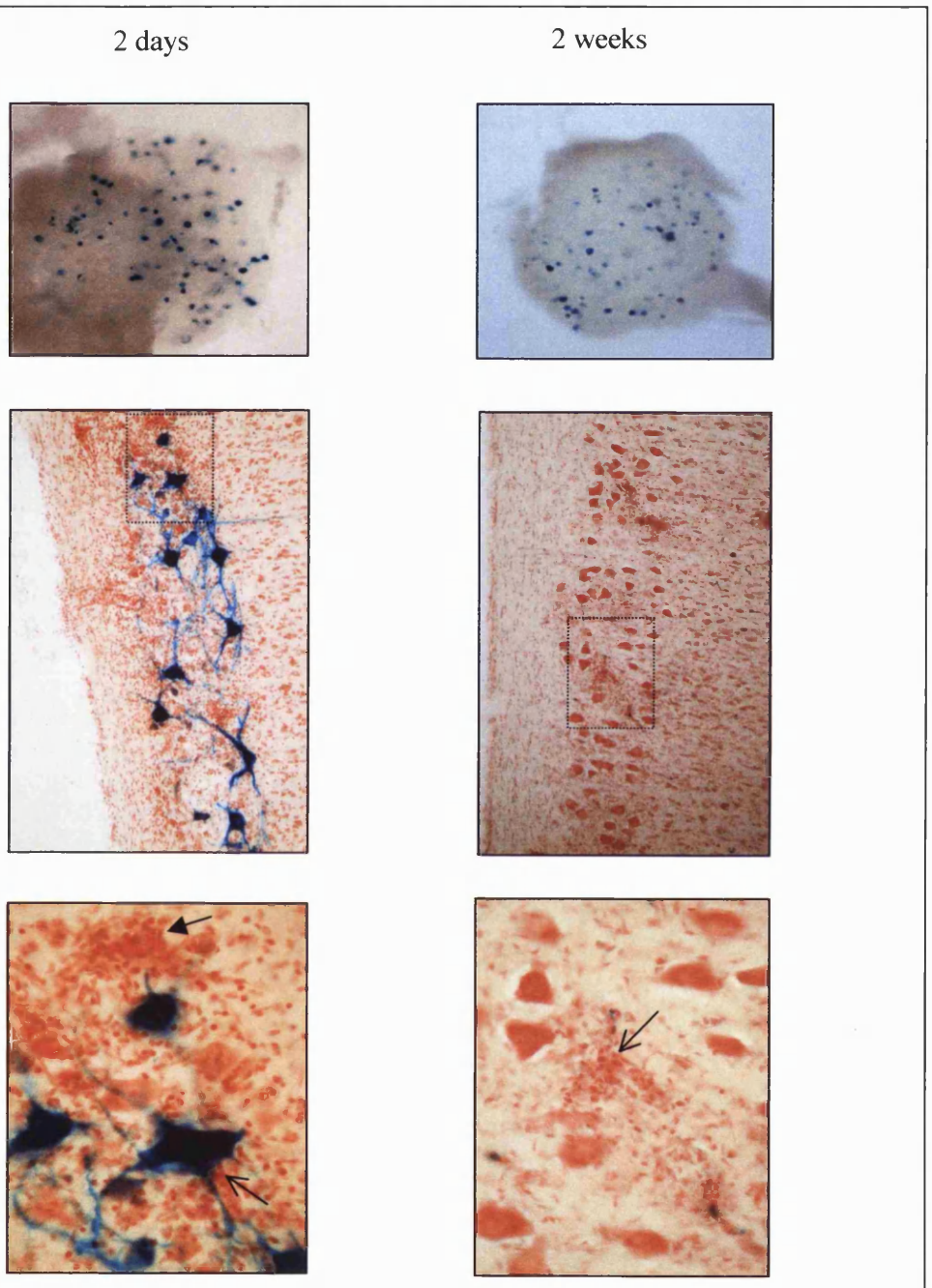


Figure 3.4. *In vivo* gene delivery following footpad inoculation of 1764pR20.9 into 3-week-old Balb/c mice. DRG and spinal cord were removed 2 days and 2 weeks postinoculation. Dotted squares show areas enlarged below. Open arrows show mononuclear cell infiltrates surrounding a transduced cell (at 2 days) and concentrating around cellular debris (at 2 weeks). The closed arrow shows the cellular infiltates engulfing a motor neuron.

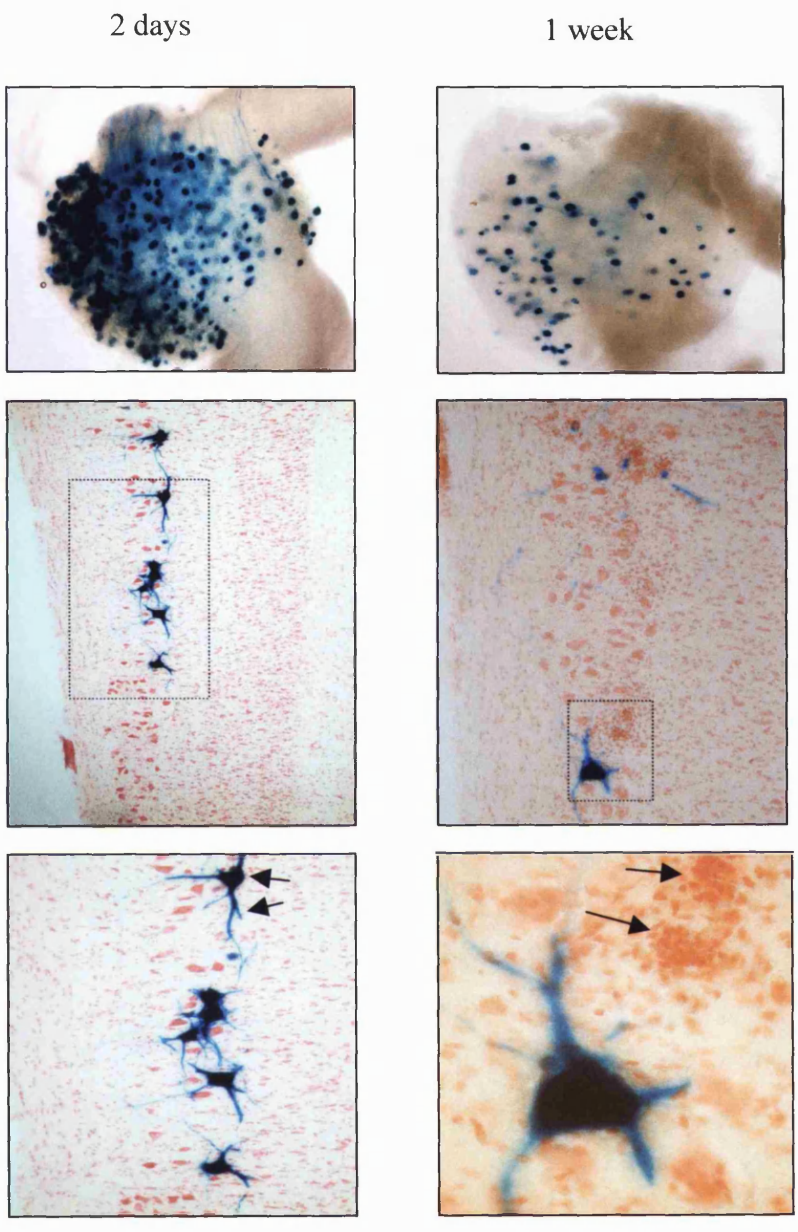


Figure 3.5. *In vivo* gene delivery following sciatic nerve injection of 1764pR20.5 into 3-week-old Balb/c mice. DRG and spinal cord were removed and stained with X-Gal, 2 days and 1 week after virus injection. The dotted squares show the areas seen at a higher magnification. At 2 days postinoculation, X-Gal staining can be seen in the motor neuron soma and neurites (indicated with arrows). At 1 week postinoculation, motor neurons are suurrounded by cellular infiltrates and cellular debris (indicated with arrows).

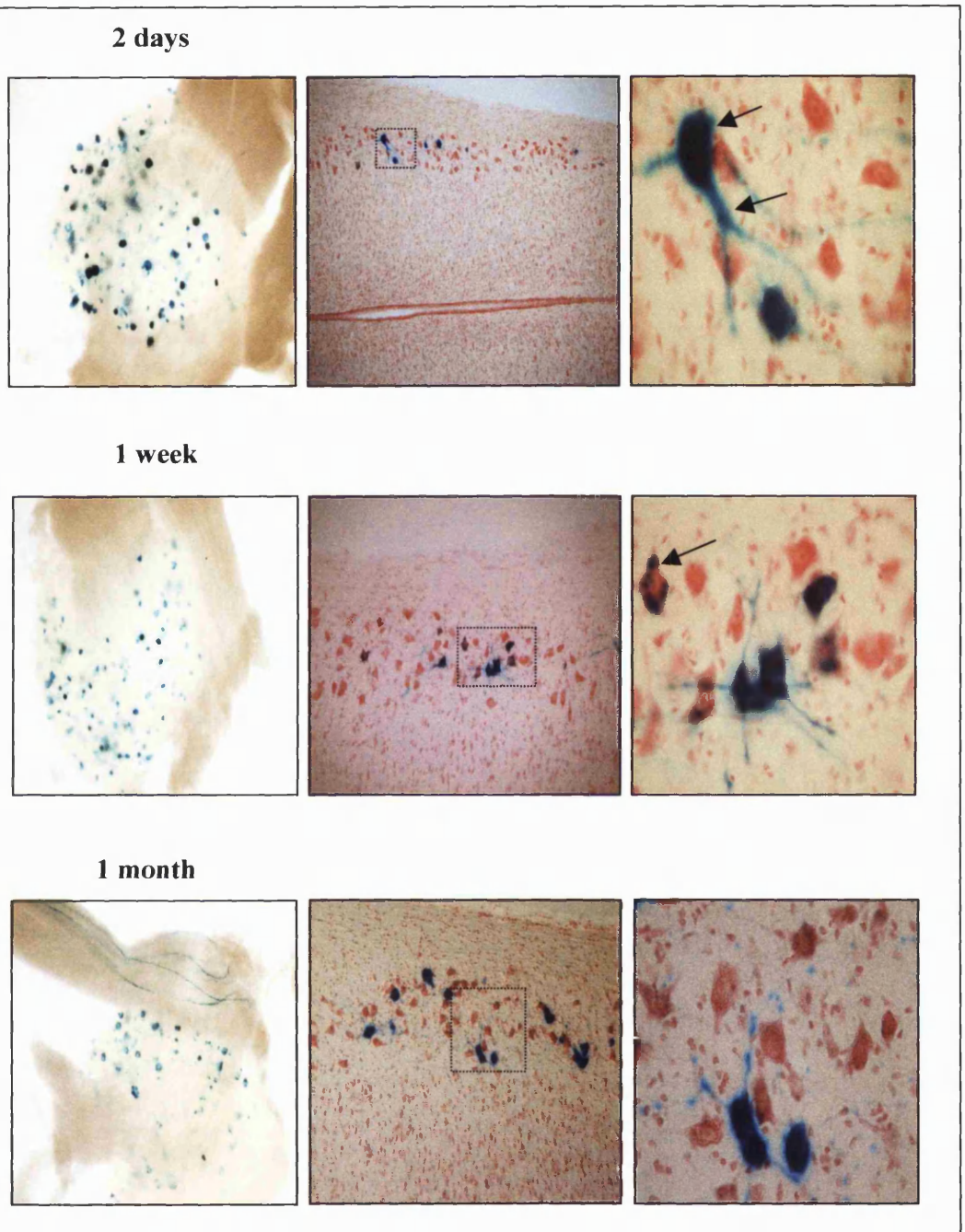


Figure 3.6. *In vivo* gene delivery following sciatic nerve inoculation of 1764 27^{-}4^{-} pR19lacZ into 3-week-old Balb/c mice. DRG and spinal cords were removed and processed for β-galactosidase expression, 2 days, 1 week and 1 month after inoculation. The dotted squares show the areas seen at a higher magnification. Arrows show X-Gal staining in the soma and in the neurites (at 2 days) and speckled X-Gal staining pattern (at 1 week).

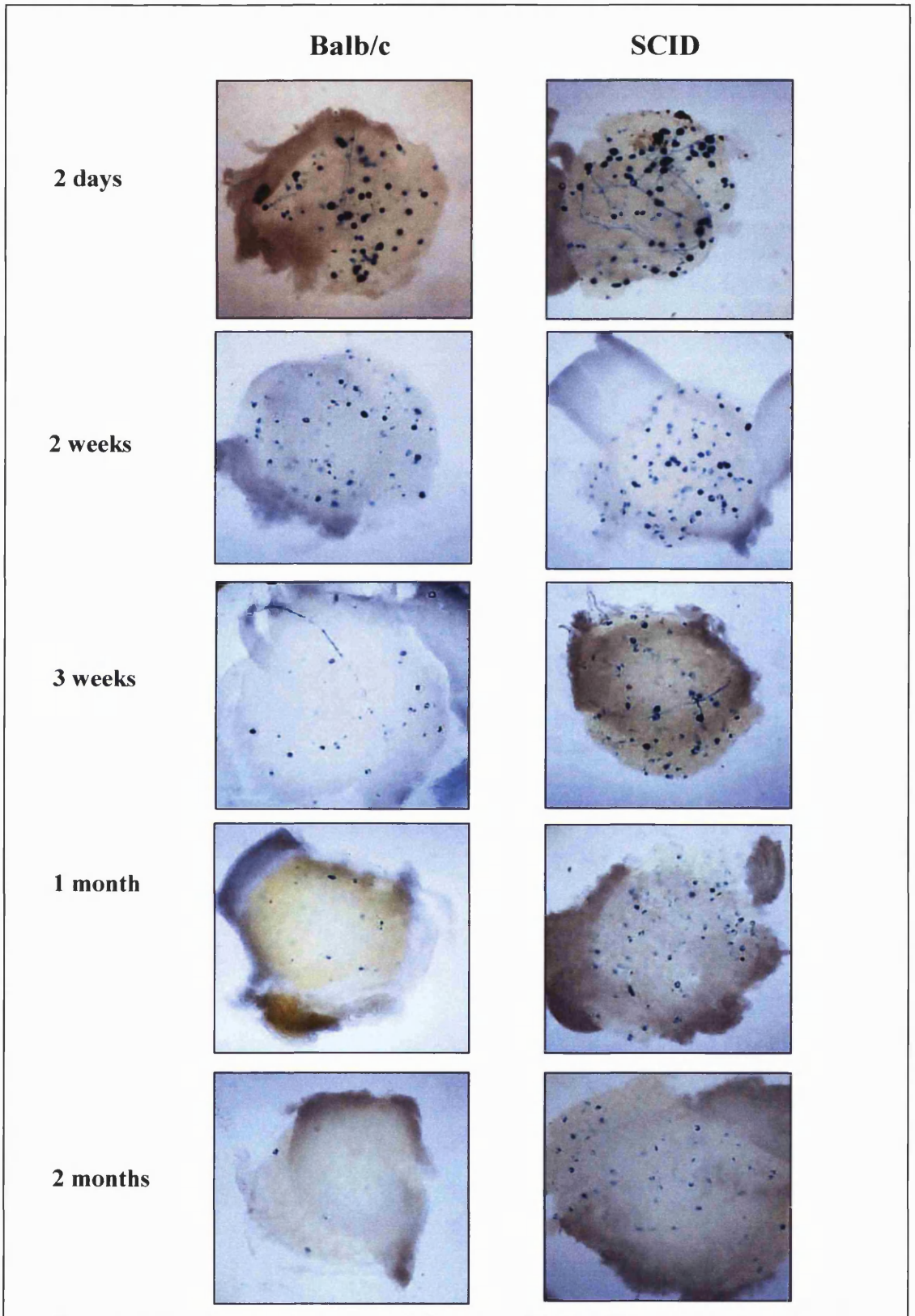


Figure 3.7. Comparative gene delivery to DRG after footpad inoculation of 1764pR20.9 in either Balb/c or SCID mice. DRG were removed and stained with X-Gal, 2 days, 2 weeks, 3 weeks, 1 month and 2 months later.

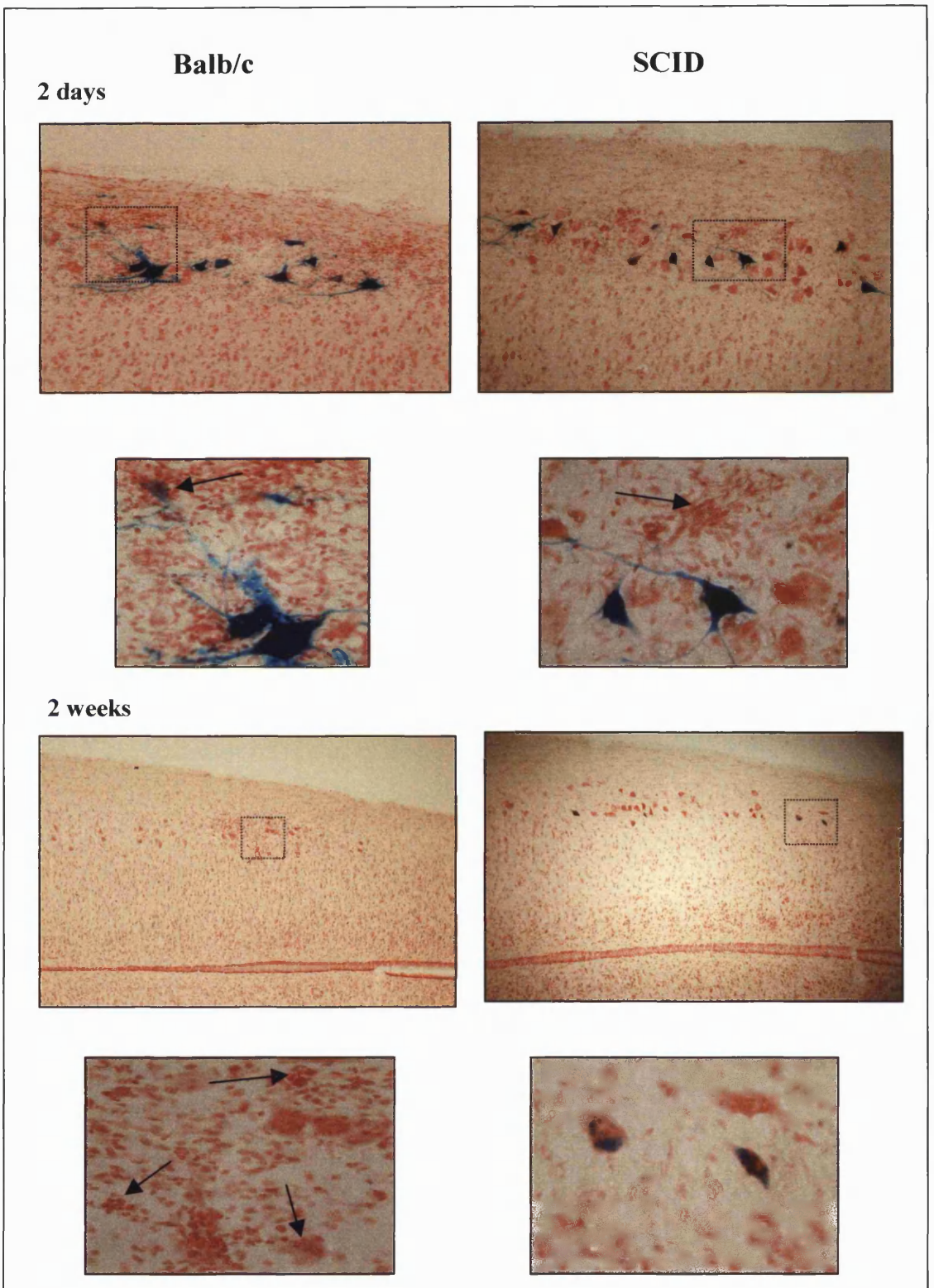


Figure 3.8- See legend on following page

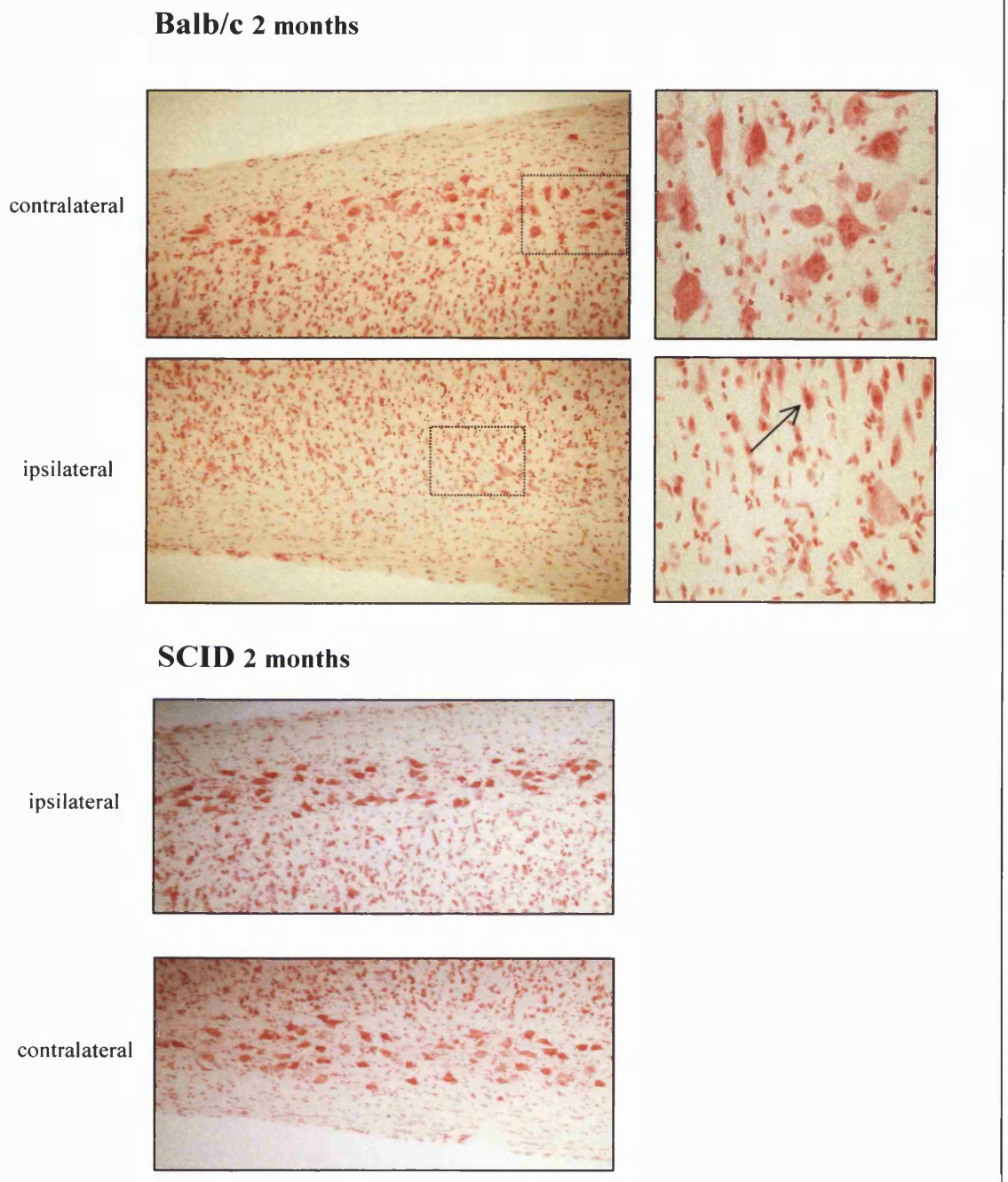


Figure 3.8. Comparative gene delivery to motor neurons after footpad inoculation of 1764pR20.9 into either Balb/c or SCID mice. Cell infiltrates in the spinal cord ipsilateral to the injection can be observed (closed arrows) in both Balb/c (at 2 days and 2 weeks) and SCID mice (only at 2 days). At 3 weeks postinoculation, extensive motor neuron loss can be observed in the anterior spinal cord ipsilateral to the injection in Balb/c animals (results shown for 2 months following virus injection). Shrunken neurons can also be observed (open arrow). In SCID mice, at 2 months postinoculation the side ipsilateral to the injection is indistinguishable from the contralateral side.

Figure 3.9. DRG and spinal cord of adult rats 2 days postinoculation with **A.** 1764pR20.5 in the sciatic nerve **B.** 1764pR20.9 in the footpad.

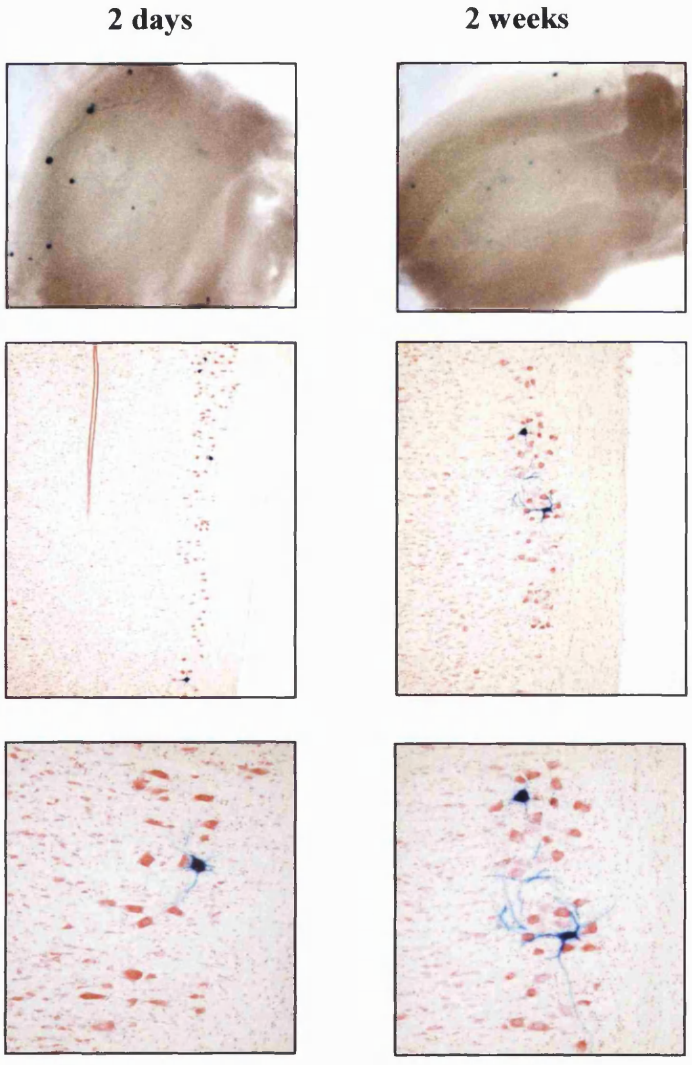


Figure 3.10. Gene delivery to adult rat DRG and spinal cord after sciatic nerve inoculation of replication-incompetent vector 1764 27-4-pR19lacZ. Animals were sacrificed 2 days and 2 weeks after vector administration.

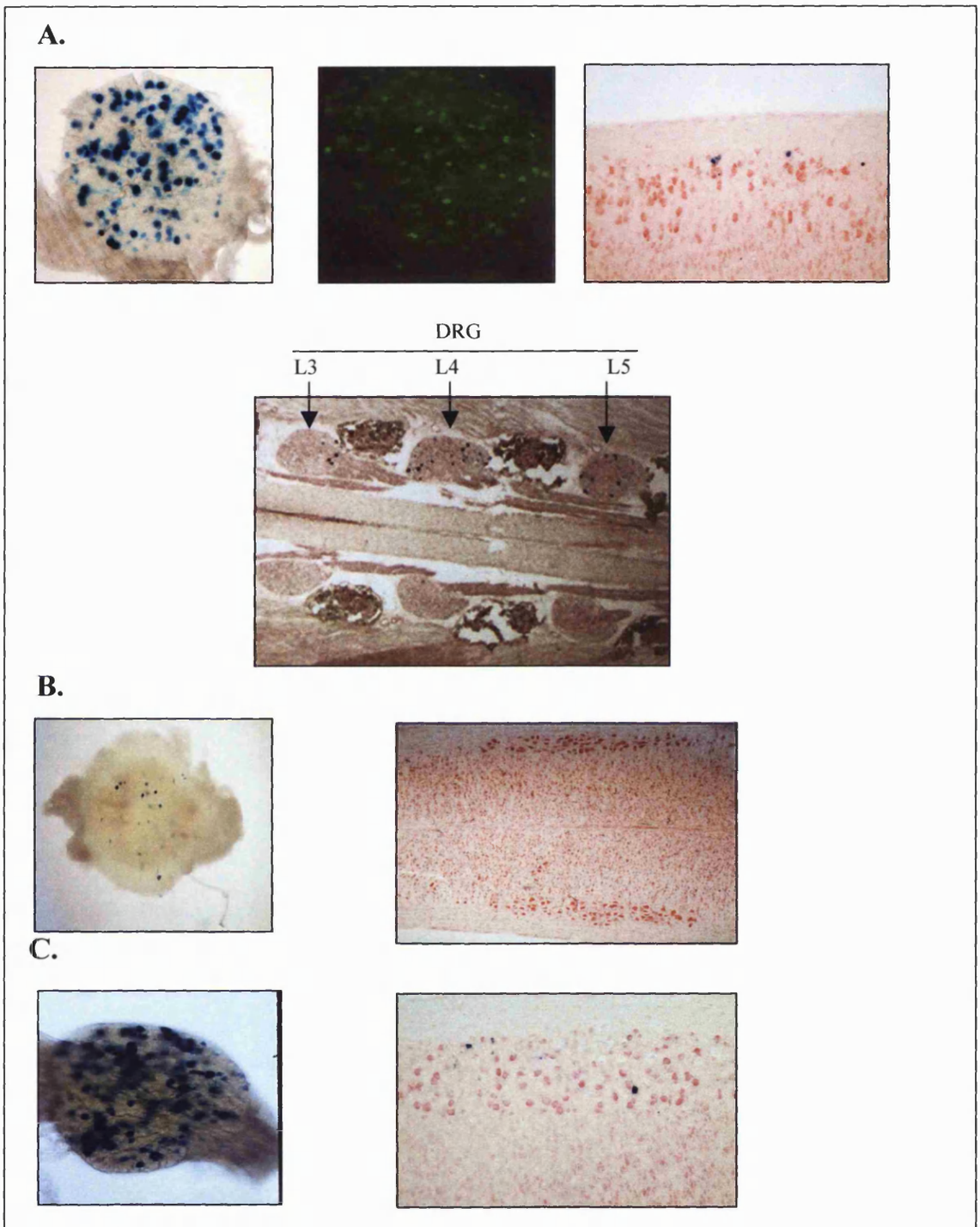


Figure 3.11. Short-term multiple gene delivery after neonate rat footpad injection with 1764pR20.9 **A-** 2 days postinoculation, DRG neurons express both β-galactosidase and GFP. Section through the lumbosacral vertebral column shows DRG L3, L4 and L5 ipsilateral and contralateral to the site of injection. Few transduced motor neurons can be seen in the anterior spinal cord. **B-** 2 weeks posinoculation, DRG and spinal cord. **C-** 2 days post intramuscular inoculation of the 1764pR20.9 vector, DRG and spinal cord.

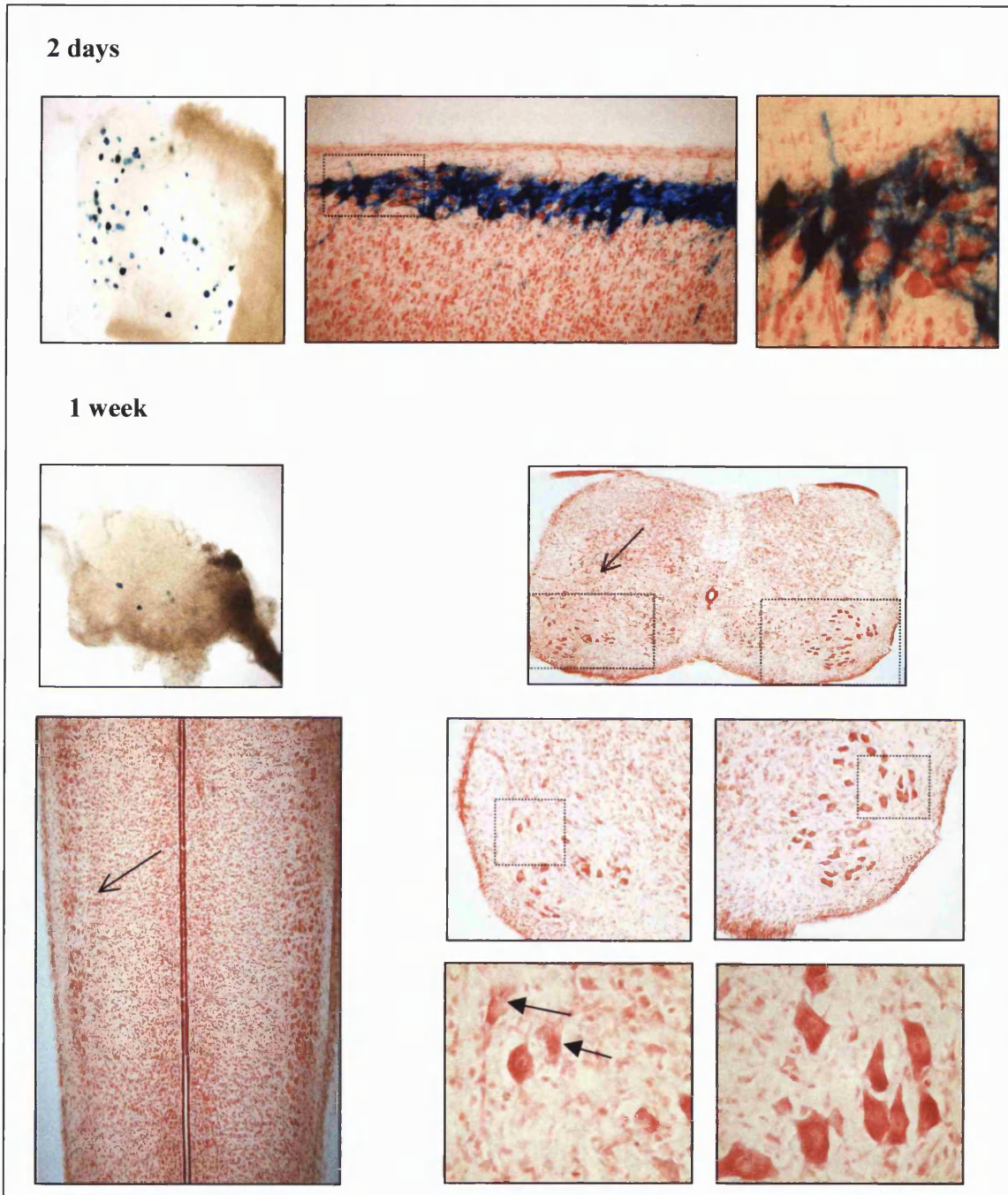
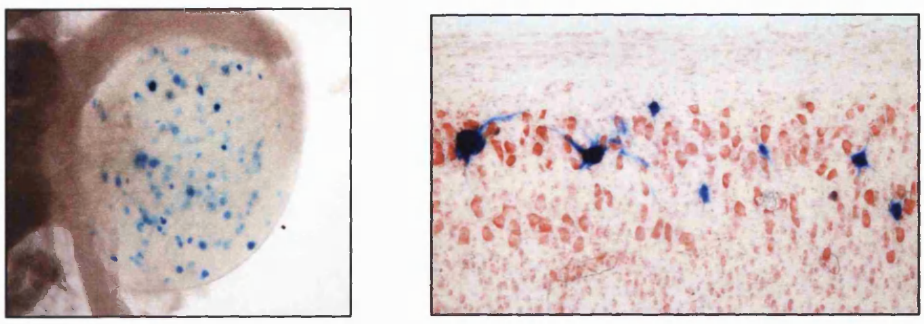


Figure 3.12. Gene delivery to neonate rat DRG and motor neurons following sciatic nerve injection of 1764pR20.5. DRG and spinal cords were removed 2 days and 1 week postinoculation. Motor neuron loss can be observed at 1 week post inoculation in both longitudinal and transverse spinal cord sections. The side ipsilateral to virus injection is noted with an open arrow. Dying neurons with diffuse cytoplasm and fragmented soma are noted with a closed arrow. Dotted squares show areas seen at a higher magnification.

2 days



1 week

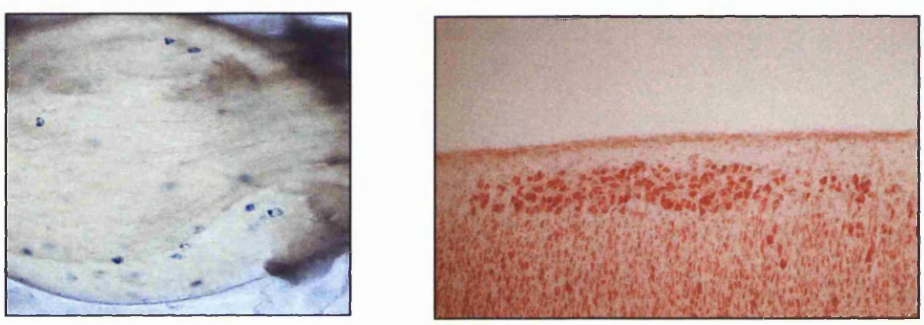


Figure 3.13. Gene delivery to neonate rat DRG and motor neurons after sciatic nerve injection of 1764 27-4-pR19lacZ. DRG and anterior spinal cords are shown for 2 days and 1 week after virus injection.

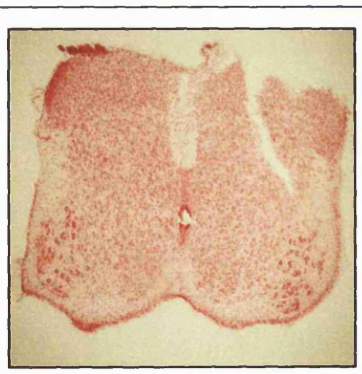


Figure 3.14. Spinal cord of neonate rat, 1 week after PBS sciatic nerve injection. No neuronal death is detected in the side ipsilateral to the injection (left hand side).

3.5. Discussion

The potential for disabled HSV-1 vectors to mediate gene delivery to the PNS has been extensively explored. The ability of these vectors to mediate gene delivery to motor neurons following the same peripheral route of inoculation, however, has not been thoroughly investigated. We aimed to test different HSV vectors for their ability to mediate safe, long-term gene delivery to motor neurons. A summary of the results is given in table 3.2. Given the plethora of animal models used in studies on motor neuron rescue, we tested the vectors in adult mice (where there is the possibility of their use in transgenic models of motor neuron disease), adult rats (for possible use in ventral root avulsion models) and in neonate rats (for potential use in axotomy models). We tested HSV-1 mutants based on the 1764 backbone (deleted for ICP34.5 and with an inactivating mutation in VP16). These are disabled by mutations to prevent pathogenicity but the mutations do not completely block viral replication (Palmer *et al.* 2000). In addition, a vector further disabled for ICP4 and ICP27 which is incapable of replication on any cell (other than complementing cell lines providing ICP4 and ICP27), was tested. Different promoter constructs were used, all including latency promoter elements in order to determine if these elements can provide long-term expression in motor neurons.

3.5.1. Mice

In the adult mouse, we found that high-titre sciatic nerve inoculation of the replication-incompetent vector 1764 27-4-pR19lacZ gave long term gene delivery to motor neurons. In addition, gene expression levels remained stable up to at least one month. Here, the CMV promoter is inserted just downstream of LAT P2, and we show that in the motor neuron, as in DRG neurons, this results in latent gene expression. To our knowledge, this is the first example of HSV-1 vector-mediated long-term gene delivery to motor neurons following peripheral inoculation.

Dobson *et al*, showed that an HSV-1 vector deleted for ICP4 was capable of delivering the *lacZ* gene to motor neurons in the hypoglossal nucleus following inoculation in the tongue, but transgene expression was transient (Dobson *et al.* 1990).

The highest levels of gene delivery observed in the adult mouse, albeit in the short-term, were after footpad injection with the replication-competent vector 1764pR20.9. Replication-competent vectors were generally found to mediate only short-term gene delivery to motor neurons. Here, cell infiltrates could be observed on the side of the injection (after 2 days for 1764pR20.9 and after one week for 1764pR20.5) and surrounding transduced cells, suggesting the involvement of the host immune response. Given that the replication-incompetent virus did not induce cell infiltration or pathological changes, it can be assumed that the immune response is acting against virus proteins produced by the replication-competent viruses rather than to β-galactosidase or to structural components of the virus. ICP27, for example, has been shown to be a major target for cytotoxic T lymphocytes in the Balb/c mouse (Banks *et al.* 1993). Similar results to those reported here in the spinal cord, have previously been reported following brain inoculation of 1716lacZ, a minimally disabled virus deleted for ICP34.5 and with *lacZ* under LAP1 promoter control inserted in UL43 (McMenamin *et al.* 1998). Here, macrophage infiltration followed by a later adaptive immune response with T-cell and T-cell activated macrophage infiltrations, were observed. We analysed the contribution of the adaptive immune response to the decrease in transgene expression observed, using SCID mice. In the DRG it appears that the adaptive immune response is responsible for the decrease observed in transgene expression as gene expression is maintained at high levels in SCID rather than in normal mice. The clearance of HSV-1 from the DRG by the host immune response does not necessarily imply neuronal destruction as CD8+ T cells have been shown to have a non-cytolytic role in the control of HSV-1 infection, clearing the virus whilst sparing the neurons (Simmons *et al.* 1992). In motor neurons, on the other hand, the host adaptive immune response is responsible for neuronal death as shown in the SCID versus BALB/c experiment described in section 3.4.5. Here it was shown that the motor neuron death seen in Balb/c mice after vector inoculation was not observed in SCID mice. However, the host adaptive immune response is only partially responsible for the decrease in transgene expression observed in the motor neuron, as despite the fact that no cell death was observed, transgene expression did still decrease in SCID mice. Other factors which could be responsible for the reduction in transgene expression could be the non-adaptive immune response, vector cytotoxicity, and/or promoter shutdown.

Unlike DRG neurons, motor neurons lie within the central nervous system, are surrounded by glial cells instead of the neural crest derived satellite cells of the DRG, and are within the blood-brain barrier. Furthermore, HSV-1 naturally establishes latency in DRG. It is therefore not necessarily surprising that there are differences in the effects of the vectors in the two cell types. It is for example also known that the outcome of HSV-1 productive infection in these two cell types is different. Wharton *et al* showed that following intramuscular injection of wild type HSV-1 in adult mice, sensory neurons of the DRG survived, with only limited productive viral infection, whereas in the anterior spinal cord, extensive necrosis and virus spread was observed (Wharton *et al.* 1995). Different transcription factor environments are also found in different cell types and levels of Oct proteins have been show to vary within the CNS (Stokoya *et al* 1992). Indeed, levels of Oct-2 and Oct-1 have been shown to be crucial for either the establishment of HSV latency or permissiveness to productive infection (see section 1.3.3.4.2) (Lillycrop *et al.* 1994). The replication-incompetent vector used here lacking ICP4 and ICP27 could establish latency in the motor neuron (shown by long-term transgene expression in a few cells in adult mice using the pR19lacZ promoter cassette). It would be of interest to also test the pR19lacZ cassette in a 1764 backbone in a SCID animal to determine whether the pR20.9 promoter used behaves differently in motor neurons. Interestingly, others have also reported differences between ganglionic sensory neurons and motor neurons in efficiencies of gene delivery with the same vector. Studies using an HSV-1 vector with a LAP1-MMLV-LTR *lacZ* construct in the gC locus showed long term transgene expression in the DRG (Lokensgard *et al.* 1994) but not in motor neurons following intramuscular injection (Keir *et al.* 1995). Dobson *et al* found that after inoculation of the tongue with an ICP4 deleted virus, long term expression was obtained in the sensory neuron in the trigeminal ganglia but not in the motor neurons of the hypoglossal nucleus (Dobson *et al.* 1990). The authors suggest the motor neuron could be modulating gene expression following stress or trauma leading to promoter shut down (Dobson *et al.* 1990). The same could be happening in our case, with the motor neuron modulating gene expression in a way not observed in the DRG.

In conclusion, in the adult mouse replication-competent vectors, although capable of transgene delivery to motor neurons after either footpad (1764pR20.9) or sciatic nerve (1764pR20.5) inoculation, are not capable of driving long-term gene expression. This is

partially due to the host adaptive immune response. Using a vector incapable of replication, long-term *lacZ* expression in motor neurons was obtained, albeit in only a small number of cells.

3.5.2. Rats

Lewis rats were much less susceptible to productive HSV-1 infection than Balb/c mice. This susceptibility has been suggested to be due to a difference in the host non-adaptive immune system (Simmons *et al.* 1992). Indeed, the replication-incompetent vector was better for gene delivery as compared to the replication-competent viruses, the opposite situation to in the mouse. Certain gene products expressed by the replication-competent viruses, such as ICP0, ICP4 and ICP27 might be triggering the immune response and resulting in virus clearance before reaching the neuronal soma. Stable gene delivery was possible with 1764 27^{-}4^{-}pR19lacZ up to 2 weeks. The involvement of the immune response in limiting infection in the adult rat with replication-competent vectors was also confirmed by experiments in neonate animals. Here, neuronal infection with the replication-competent vectors was possible, possibly due to the immature immune system of these animals. Interestingly, many adenovirus studies have used neonate rats exploiting their immature immune system (Gravel *et al.* 1997; Baumgartner and Shine 1998; Gimenez *et al.* 1997). Indeed, first generation adenoviruses have been reported to maintain low-level transcription from the viral genome (Yang *et al.* 1994), and in the adult animal immune responses result in decrease of transgene expression due to destruction of virus-infected cells (Yang *et al.* 1995). In our case, using HSV-1 vectors in the neonate rat, footpad inoculation of 1764pR20.9 gave high-levels of transgene expression in the DRG and sciatic nerve injection of 1764pR20.5 gave very high levels of gene expression in motor neurons. The latter was from all the animal models used, the highest gene delivery we observed to motor neurons following peripheral administration. The replication-incompetent vector 1764 27^{-}4^{-} pR19lacZ was also capable of gene delivery to the DRG and to the motor neuron after sciatic nerve inoculation but with lower efficiency when compared with 1764pR20.9 or 1764pR20.5, respectively. For all cases, gene delivery was of a short-term nature, rapidly decreasing between 2 days and 1 week. In the case of 1764pR20.5, extensive motor neuron death could be observed in the spinal cord ipsilateral to vector injection. We believe the replication-competent vector is cytotoxic to the fragile developing motor neurons.

Indeed, both sensory and motor neurons are very sensitive to damage in the first few days of postnatal life (Snider *et al.* 1992). Infection with replication-competent virus might be tolerated by an adult animal but in the case of the neonate, neurons might not tolerate the stress and/or viral-induced trauma. In the case of the decrease in transgene expression observed with 1764pR20.9 in the DRG, cell death might be occurring or on the other hand, given the differences between motor neurons and ganglionic sensory neurons mentioned above, promoter shutdown could be occurring as neuronal gene expression changes in response to trauma.

Perhaps the most surprising result with HSV in the neonate was the lack of stable gene expression obtained with replication-incompetent vector 1764 27-4-pR19lacZ. However, although no neuronal death could be observed in the spinal cords of animals inoculated with this vector, this does not necessarily mean none is occurring as only a small number of motor neurons were initially transduced, and thus detection of the death of only a small number of cells is difficult. Cytotoxicity with the replication-incompetent vector would not be expected, as it is incapable of replication and cannot express immediate-early genes. Cytotoxicity of the replication-incompetent virus could however be associated with the high titre injection used, or with either cytotoxic material carried over from the complementing cells, or defective viral particles carrying ICP0 and/or ICP4 (Yao and Courtney 1992). Although we would expect the same to be the case with injections in adult animals, the adult motor neuron is probably more resistant to toxic insults.

This chapter has defined parameters allowing disabled HSV-1 vector- mediated gene delivery to motor neurons following peripheral inoculation. Considering these parameters, we have defined viruses suitable for this purpose. It was shown to be possible to achieve stable long-term gene expression in adult mouse motor neurons exploring the LAT region of the HSV genome. This vector could be of use in a transgenic animal model for motor neuron degeneration, particularly as it can be applied to a well-defined area. Indeed, in some transgenic models some motor neuron pools are known to be particularly vulnerable, such as the facial nucleus in *pmn* mice (Holtmann *et al.* 1999). We have also defined vectors of potential use in a neonate animal axotomy model, where neurons die shortly after trauma. These vectors could be used to deliver potentially therapeutic factors to the injured neonate motor neuron. The peripheral

inoculation of HSV-1 vectors offers a serious advantage over direct inoculation in that it allows targeted gene delivery to the neurons projecting to the site of injection. One might therefore test a therapeutic factor without the interference from production of the factor by neighbouring cells. In some cases, low-level gene delivery might be required, for instance in the study of autocrine factors.

Table 3.2. Summary of results obtained testing different attenuated HSV-1 vectors for their capability to mediate gene delivery to motor neurons following peripheral administration. A scoring from + to ++++++ was given ranging from poor to high levels of gene delivery.

Virus	Animal species	Age	Injection route	Timepoint	Results				Comments
					DRG		Motor neurons		
					SCID	Balb/c	SCID	Balb/c	
1764pR20.9 (C)*	Mouse	Adult	Footpad	2 days	+++	+++	++++	++++	Cellular infiltrates in the spinal cord were present at 2 days in Balb/c and SCID animals. Motor neuron loss was observed in Balb/c animals.
				2 weeks	+++	++	-	+	
				1 month	+++	+	-	-	
				2 months	+++	+	-	-	
1764pR20.5 (C)*			Sciatic nerve	2 days	+++++		+++		Cellular infiltrates and cellular debris was observed.
				1 week	++		+		
1764 4-27- pR19lacZ (I)*			Sciatic nerve	2 days	+++		++		No pathological changes were observed.
				1 week	+++		++		
				1 month	+++		++		
1764pR20.9 (C)*	Rat	Adult	Footpad	2 days	-		-		
				2 weeks	-		-		
1764pR20.5 (C)*			Sciatic nerve	2 days	-		-		
				2 weeks	-		-		
1764 4-27- pR19lacZ (I)*			Sciatic nerve	2 days	+		+		No pathological changes were observed.
				2 weeks	+		+		
1764pR20.9 (C)*	Rat	Neonate	Footpad	2 days	++++		+		
				1 week	+		-		
			Tibialis anterior	2 days	+++		+		
1764pR20.5 (C)*			Sciatic nerve	2 days	+++		+++++		Motor neuron loss was observed.
				1 weeks	+		-		
1764 4-27- pR19lacZ (I)*			Sciatic nerve	2 days	+++		+		
				1 week	+		-		

* C and I refer to the replication status of the virus, *i.e.* replication competent (C) or incompetent (I), for reference see sections 3.2 and 3.3.

Chapter 4

Construction of HSV-1 vectors to deliver Reg-2 or CNTF

Chapter 4 – Construction of HSV-1 vectors to deliver Reg-2 or CNTF

4.1. Introduction

In vitro, Reg-2 has been shown to promote motor neuron survival (Nishimune *et al.* 2000). *In vivo*, it appears likely therefore that Reg-2 is a neurotrophic factor for motor neurons (see section 1.4.4.4). Indeed, in the adult rat, Reg-2 is only produced in regenerating motor neurons and in a subset of regenerating sensory neurons. Blocking of Reg-2 by the addition of an anti-Reg-2 polyclonal antiserum results in the reduced regeneration of Reg-2-containing axons (Livesey *et al.* 1997). In the neonate rat, Reg-2 is usually expressed until postnatal day 5, with axotomy, Reg-2 levels drop (Livesey *et al.* 1997) and motor neuron death occurs (Snider *et al.* 1992). In order to establish if Reg-2 is in fact a neurotrophic factor for motor neurons it is necessary to demonstrate survival effects in a model of neuronal death either by the addition of purified protein or by genetic means.

The previous chapter tested the ability of disabled HSV-1 vectors to deliver genes to motor neurons following peripheral administration (see Chapter 3). In order to exploit these vectors for studies on motor neuron survival, the length and level of transgene expression obtained with the different vectors needs to be considered. For example, while long-term transgene delivery in the adult mouse was possible using the replication-incompetent virus 1764 27-4-pR19lacZ (see section 3.4.4), the number of motor neurons transduced were very low. Thus, an experiment on *e.g.* a transgenic animal model would be unlikely to be successful with this virus. With the replication-competent vector 1764pR20.9, high level short-term gene expression was obtained following footpad inoculation in the adult mouse. In this case, although the number of transduced motor neurons was satisfactory, gene expression was short lived (less than 2 weeks). This vector therefore also could not be used in a transgenic mouse model with any great expectation of success, as motor neurons undergo a progressive degenerative process over a few weeks in the transgenic models available, probably requiring longer term expression than is possible with this vector (Ikeda *et al.* 1995; Bordet *et al.* 1999; Haase *et al.* 1997). In the neonate rat, nerve axotomy

results in rapid neuronal death (Sendtner *et al.* 1990; Snider *et al.* 1992). Thus, 1764pR20.5 might be used to deliver potential trophic factors to motor neurons where it might be expected results would be observed. 1764pR20.9 gave high-level short-term expression in neonate DRG. Similarly therefore, this vector could be used in an axotomy model for the delivery of potential trophic factors to DRG neurons which also die soon after axotomy (Schmalbruch 1987; Yip *et al.* 1984). Thus, 1764pR20.5 and 1764pR20.9 are promising for use in neonate axotomy models of neuronal death.

As discussed, Reg-2 has not yet been demonstrated as a neurotrophic factor *in vivo*, although several lines of evidence suggest this to be the case. This chapter aims to construct vectors to deliver Reg-2 to motor neurons in order to test for neurotrophic properties *in vivo*. As a positive control, a vector was also constructed to express CNTF, a factor well-known for its neurotrophic properties in motor neurons (Masu *et al.* 1993; Sendtner *et al.* 1992; Arakawa *et al.* 1990; Sendtner *et al.* 1990). It has also been shown in cultured motor neurons that Reg-2 is required for CNTF to have survival promoting effects (Nishimune *et al.* 2000).

Considering the results with 1764pR20.9 in the neonate DRG and that Reg-2 is expressed by a subset of regenerating sensory neurons as well as motor neurons (Livesey *et al.* 1997), the role of Reg-2 as a trophic factor in neonate DRG will also be investigated.

4.2. Materials and Methods

4.2.1. Administration of vectors

4 P2 Lewis rats were injected with 2-5 µl of $1x10^8$ of 1764pR20.5 in the sciatic nerve. Axotomy was performed as described in 2.2.5.4. 2 days later the animals were sacrificed and tissues processed as in 2.2.5.5.

3 P2 Lewis rats were injected in the footpad with 25 µl of $3x10^8$ pfu/ml of 1764pR20.9. The following day, the sciatic nerve ipsilateral to the side injected was axotomised as

described in 2.2.5.4. 2 days later the animals were sacrificed and tissues processed as in 2.2.5.5.

4.2.2. Construction of viral vectors expressing Reg-2 and CNTF

4.2.2.1. Reg-2

pGEX-4T-1Reg-2 was obtained from Prof. S. P. Hunt, Anatomy Department, UCL. In this plasmid, the Reg-2 sequences were amplified using PCR primers with restriction enzyme sites to facilitate cloning at later stages (*Xba*I and *Xho*I) (see section 2.2.1.13 and figure 4.1). The PCR product was then cloned into pBluescript II SK (Stratagene) between the *Xba*I and *Xho*I sites creating pBluescript II SK Reg-2 (see sections in 2.2.1). Reg-2 was sequenced using T7 and T3 primers to confirm that no errors had occurred during the PCR (see section 2.2.1.11.1). In order to allow insertion into the viral genome by homologous recombination, Reg-2 was then subcloned into the shuttle plasmids (either pR20.5 or pR20.9) flanked by UL43 sequences (see 2.2.1) allowing insertion at the *Nsi*I (nt 94911) site of UL43. Here Reg-2 was removed from pBluescript II SK Reg-2 as an *Xba*I/*Xho*I fragment and inserted into pGEM-5 pR20.5/UL43 (available in the laboratory) replacing GFP between *Hind*III and *Xho*I giving pGEM-5 pR20.5 Reg-2/UL43 (see figure 4.2). The same *Xba*I/*Xho*I fragment containing Reg-2 was cloned into pR20.9 giving pGEM-5 pR20.9 Reg-2/UL43 (see figure 4.2).

For co-transfection with 1764 viral DNA (see 2.2.3 and example in figure 4.3), pGEM-5 pR20.9 Reg-2/UL43 and pGEM-5 pR20.5 Reg-2/UL43 were digested with *Sca*I (see 2.2.1). *Sca*I cuts once in pGEM-5 and once near the end of the flanking region. *LacZ*-expressing plaques were selected until a pure stock of *lacZ*- expressing virus was obtained giving the viruses 1764pR20.5/Reg-2 and 1764pR20.9/Reg-2.

In order to confirm the genomic structure of the viruses, Southern blots were performed on viral DNA isolated from purified plaques (see section 2.2.1.12). The purified DNA was digested with *Xba*I and *Xho*I and probed with a 520 bp (Reg-2) *Xba*I/*Xho*I fragment from pBluescript II SK Reg-2.

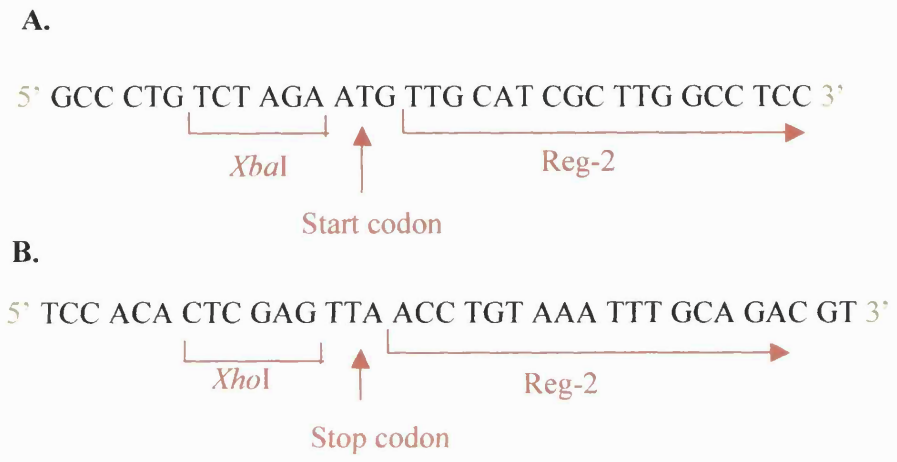


Figure 4.1. Primers designed to amplify Reg-2. **A.** Reg2F and **B.** Reg2B. Enzyme restriction sites added and start and stop codons are indicated. In the case of Reg2B, the sequences indicated are complementary and reversed.

4.2.2.2. CNTF

pNGF-CNTF Chim5 was obtained as a gift from Prof. M. Sendtner, University of Wurzburg, Germany. This plasmid contains a NGF leader sequence-CNTF genomic DNA construct, which includes the whole coding regions of exon I and II and a 1kb intron, cloned 3' of a complementary DNA fragment coding for the first 20 amino acids of mouse prepro-nerve growth factor that include the whole signal peptide sequence (Sendtner *et al.* 1992). This construct allows the secretion of CNTF (Sendtner *et al.* 1992). NGF-CNTF was removed from pNGF-CNTF Chim5 using *Hind*III and *Xba*I and was inserted into pGEM-5 pR20.5/UL43 and pGEM-5 pR20.9/UL43 replacing *lacZ* between the two *Eco*RI sites (see section 2.2.1). The ligation of blunt-ended *Eco*RI and *Xba*I sites resulted in the regeneration of an *Eco*RI and a *Xba*I restriction site. The plasmids generated were named pGEM-5 pR20.9/NGF-CNTF and pGEM-5 pR20.5/NGF-CNTF (see figure 4.2). The latter could be linearised with *Ssp*I, as it cuts once in the pGEM-5 backbone. pGEM-5 pR20.9/NGF-CNTF on the other hand, could not be linearised and so circularised plasmid was used in the co-transfections with viral DNA. Constructs containing NGF-CNTF

replacing *lacZ* were then inserted into the UL43 locus of purified genomic 1764 viral DNA by homologous recombination (see 2.2.3 and see figure 4.3 using 1764pR20.5/NGF-CNTF as an example). When using pGEM-5 pR20.9/NGF-CNTF, homologous recombination occurred at a lower efficiency due to the use of circularised plasmid DNA. GFP-expressing plaques were selected until pure and the constructed viruses named 1764pR20.5/NGF-CNTF and 1764pR20.9/NGF-CNTF.

The genomic structure of 1764pR20.5/NGF-CNTF and 1764pR20.9/NGF-CNTF was confirmed by Southern blotting (see section 2.2.1.12). Purified viral DNA was digested with *Xba*I and probed with an *Xmn*I/*Xba*I fragment from NGF-CNTF Chim5.

4.2.3. Confirmation of Reg-2 expression

4.2.3.1. Immunohistochemistry

1764pR20.5/Reg-2, 1764pR20.5, 1764pR20.9/Reg-2 and 1764pR20.9 were each stereotaxically injected into the adult rat striatum as described in section 2.2.5.2.3. (2 animals/virus; 5 µl of 5 $x10^7$ pfu/ml). Animals were sacrificed two days later, the brains removed and processed for Reg-2 immunoreactivity as described in section 2.2.4.2.2. For each animal a few sections were stained with X-Gal to confirm the injections had been successful. In animals injected with 1764pR20.5/Reg-2 or 1764pR20.9/Reg-2, a few sections were lightly stained with X-Gal and also processed for immunohistochemistry.

1764pR20.9 and 1764pR20.9/Reg-2 were injected into adult mouse footpad (2 animals/virus; 25µl of 1 $x10^8$ pfu/ml). 2 days later the mice were sacrificed, the spinal cords removed and processed for Reg-2 immunoreactivity (see 2.2.4.2.2). For each animal a few sections were stained with X-Gal to confirm the injections had been successful.

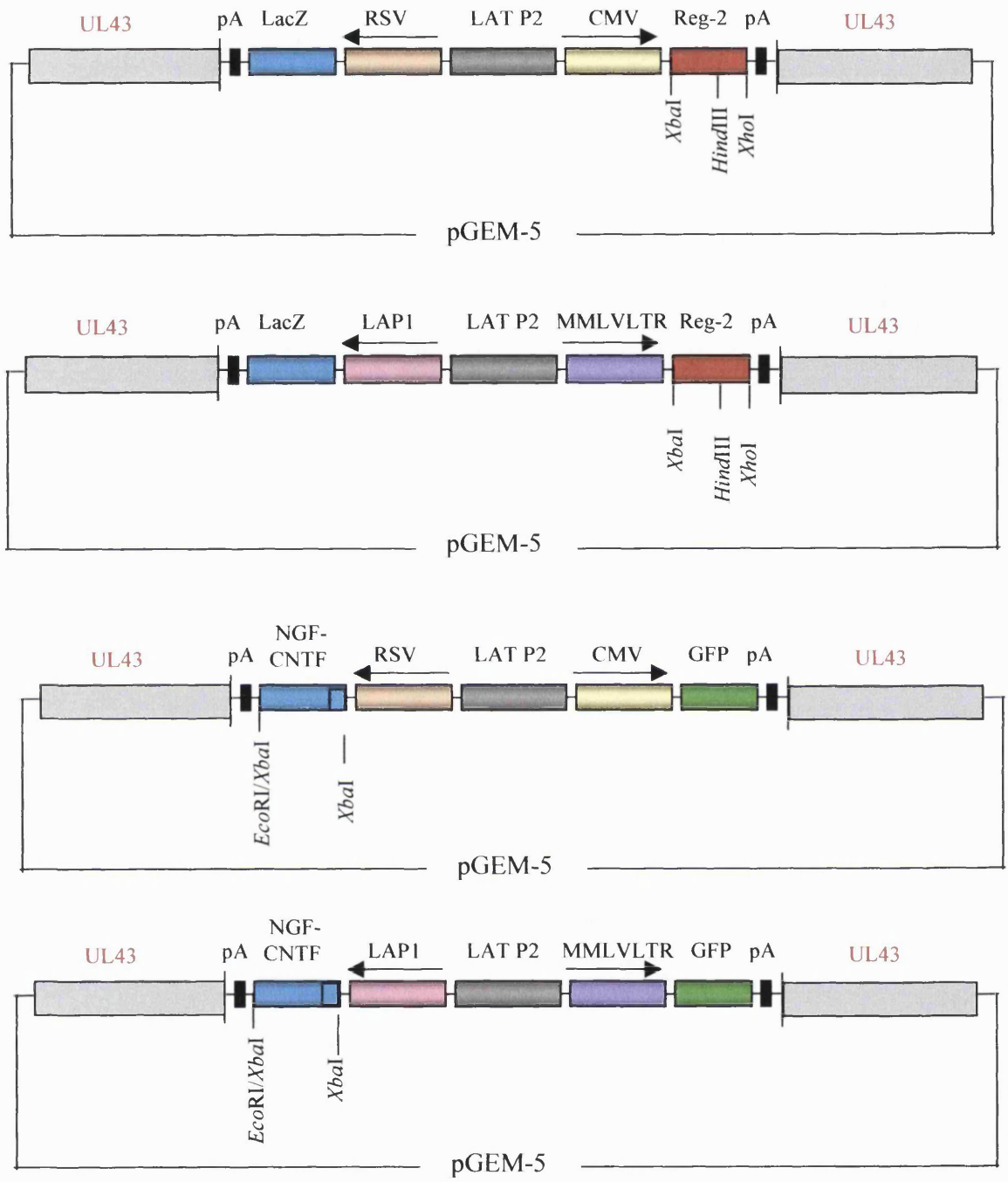


Figure 4.2. The plasmid constructs allowing insertion into 1764. From top to bottom, pGEM-5 pR20.5/Reg-2, pGEM-5 pR20.9/Reg-2, pGEM-5 pR20.5/NGF-CNTF and pGEM-5 pR20.9/NGF-CNTF. The promoter constructs are inserted in the non-essential viral gene UL43 at the *Nsi*I site nt 94911. The UL43 flanking regions are from *Bam*HI nt 919619 to nt 94911 and from nt 94912 to *Eco*RI nt 96751. Important restriction sites are noted in the diagrams. RSV, Rous sarcoma virus promoter. MMLV LTR, Moloney murine leukemia virus promoter. GFP, Green fluorescent protein. LacZ, β-galactosidase. CMV, cytomegalovirus promoter. LAP1 and LAT P2, HSV latency associated promoters.

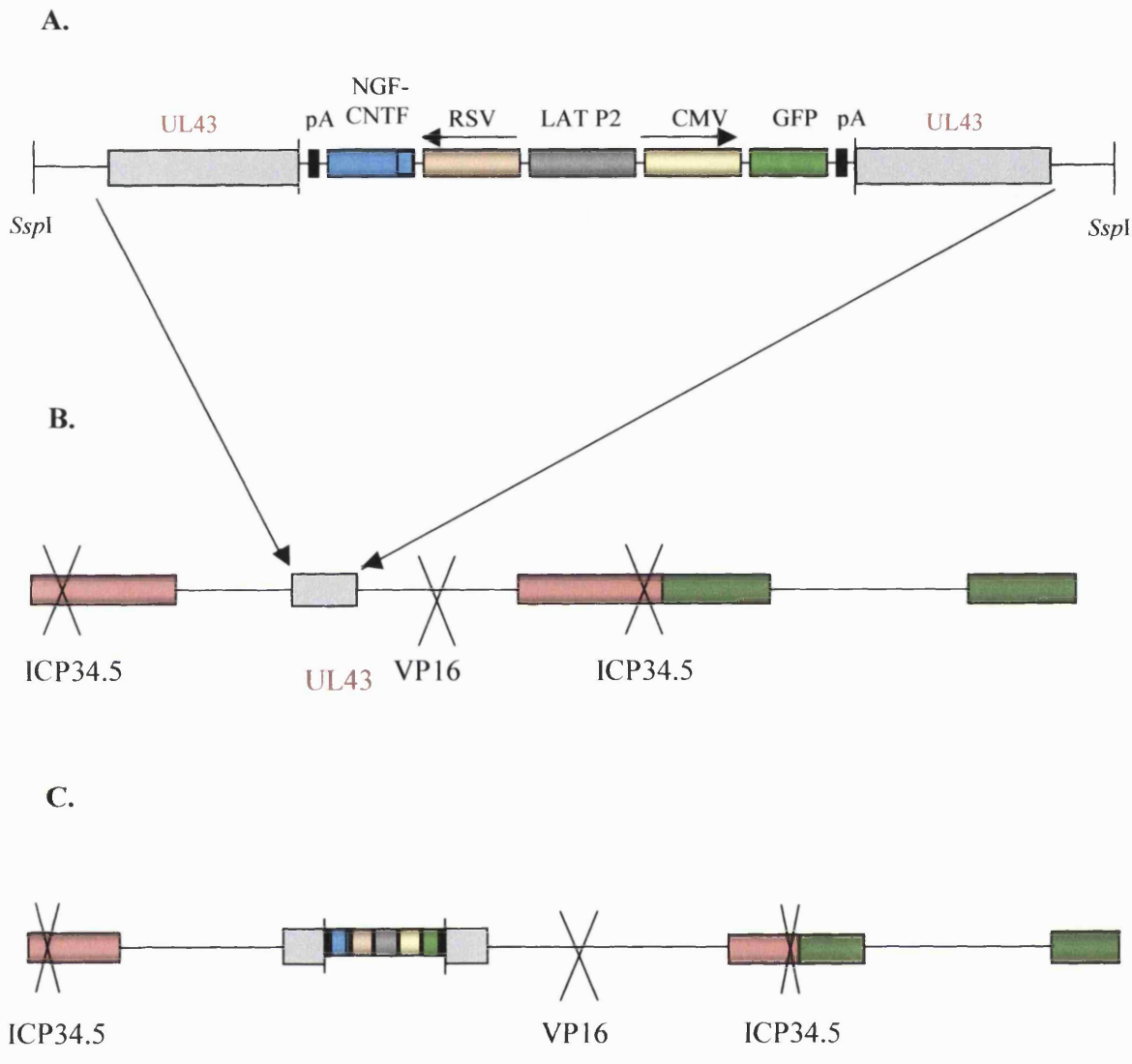


Figure 4.3. Construction of 1764pR20.5/NGF-CNTF. **A.** Plasmid pGEM-5 pR20.5/NGF-CNTF linearised with *Ssp*I. **B.** Structure of purified 1764 viral DNA. The DNA construct flanked by the UL43 regions enters the virus into the UL43 gene by homologous recombination, generating a new virus **C.** 1764pR20.5/NGF-CNTF.

4.2.4. Confirmation of CNTF expression and activity

4.2.4.1. Western blotting

BHK cells were infected with 1764pR20.5 or 1764pR20.5/NGF-CNTF at an m.o.i of 0.01 and left until complete c.p.e. was observed. Protein was extracted from the cells and western blotting performed as described in section 2.2.4.1. As a positive control, rat recombinant CNTF (Promega) was used at a final concentration of 0.05 mg/ml.

4.2.4.2. Cell proliferation assay

TF-1.CN5a.1 (ATCC* CRL-2512) cells express the alpha subunit of human ciliary neurotrophic factor receptor and proliferate in response to CNTF at picograms/ml concentrations (Kitamura *et al.* 1989). A proliferation assay was performed on these cells using supernatants from BHK cells infected with 1764pR20.5/NGF-CNTF. A well of a 6-well plate of BHK cells was infected at an m.o.i of 0.01 and left until complete cytopathic effect (c.p.e.) was observed (approximately two days). Cells were washed twice with HBSS, overlaid with 0.5 ml of SFM and left overnight. The supernatant was then taken and cellular debris removed by centrifugation (4 min at 3,500 rpm). The supernatant was then removed and further spun for one hour at 12,000 rpm at 4° C in order to pellet any virus. The supernatant was then kept at –20°C until use and a sample titred in order to confirm no virus was left in the media. The proliferation assay also included supernatant from 1764pR20.5 infected cells treated as described for 1764pR20.5/NGF-CNTF. $3x10^4$ TF-1.CN5a.1 cells were used/treatment. 200 µl of media were used/treatment and were performed in triplicate. Cells were treated with 5, 10 or 20 µl of virally conditioned supernatant, with CNTF (Sigma) at a final concentration of 2 ng/ml, or received no treatment. 2 and 6 days later the cells were counted using a haemocytometer.

*ATCC® is a registered trademark of the American Type Culture Collection

4.3. Results

4.3.1. Vectors 1764pR20.9 and 1764pR20.5 can give gene delivery following axotomy

1764pR20.5 and 1764pR20.9 were tested for gene delivery following axotomy in neonate rats, which was not tested in Chapter 3. Figure 4.4 shows that both vectors were successful at giving gene delivery. Footpad injection of 1764pR20.9 with nerve axotomy did not however result in any increased gene delivery to motor neurons. This was expected given the virus is delivered to the footpad and the sciatic nerve is transected at mid-thigh level and so although there is exposure of the motor axon there is no virus in the surrounding area to infect it. With the sciatic nerve injection of 1764pR20.5, there was an obvious increase in gene delivery to the DRG (compare figure 4.4 B. and figure 3.12), likely due to the increased exposure of the axon to the virus. Gene delivery to motor neurons following sciatic nerve injection however, did not increase efficiency of gene delivery, possibly because the efficiency was already nearly maximal. In the spinal cord some low-level transynaptic viral spread could be observed (see figure 4.4), indicating some viral replication was occurring. Interestingly, X-Gal staining could be observed in the sciatic nerve and in the L4 ventral root (see figure 4.4). This probably corresponds to axonal staining as X-Gal stained processes can be observed in the whole-mount DRG. Such intense staining is likely to be the result of very high-level transgene expression in the neuron.

4.3.2. 1764pR20.5/Reg-2 and 1764pR209/Reg-2 express Reg-2

Following the construction of viruses 1764pR20.5/Reg-2 and 1764pR20.9/Reg-2 (see section 4.2.2.1), a Southern blot was performed to confirm their new genomic structure. Both vectors were probed for the entire Reg-2 gene (520 bp) (see section 4.2.2.1). Vectors 1764pR20.9 and 1764pR20.5 were used as negative controls and plasmids pGEM-5 pR20.9/Reg-2 and pGEM-5 pR20.5/Reg-2 were used as positive controls. From the analysis of figure 4.5 A and B, we can see that for both cases, not all purified plaques contained Reg-2 cDNA. Interestingly, all plaques did contain at least half of the promoter cassette construct as the plaques were purified on the basis of GFP-expression.

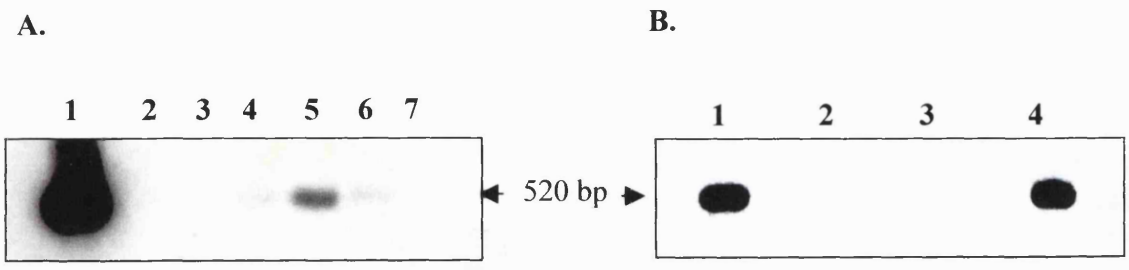


Figure 4.5. Detection of Reg-2 by Southern blot **A.** 1764pR20.5/Reg-2. Lanes 1- pBluescript II SK Reg-2. 2- 1764pR20.5. 3-7- purified plaques. **B.** 1764pR20.9/Reg-2. Lanes 1- pBluescript II SK Reg-2. 2- 1764pR20.9. 3-4- purified plaques.

This problem has been previously encountered in the laboratory when inserting similar cassettes containing the LAT P2 element into the ICP27 or ICP4 locus (C. Lilley, personal communication). It appears to result from the homologous recombination between the endogenous LAT P2 in the LAT region and the LAT P2 in the promotor cassette introduced (pR20.5 and pR20.9, in our case). Such homologous recombination between LAT P2 elements within the same genome or between different viral genomes, could result in the deletion of half of the expression cassette (knocking out Reg-2), one half of the intended flanking regions (UL43) and the viral genome in between the homologous elements (this would include VP16 and ICP27). Such an aberrant recombinational event would be expected to produce recombinants lacking ICP27, and be replication incompetent. The selective pressure for ICP27 should therefore prevent such recombinants being purified. However, to minimise any recombinational instability preventative measures were used. The selected viruses were serially passaged as little as possible: (i) the original selected plaque was grown to a small master stock and whenever a new virus stock was required, this master stock was used (ii) high-titre cell-associated-virus was never used to propagate virus.

Having selected purified plaques positive for Reg-2 by Southern blotting, 1764pR20.5/Reg-2 and 1764pR20.9/Reg-2 were grown to high–titre and tested for Reg-2 expression. The adult rat brain provided a platform in which to test the viruses as the rat brain does not usually express Reg-2 (Livesey *et al.* 1997) (Prof. S. P. Hunt, personal communication), and as 1764 viruses can give gene delivery to the rat brain in the short term (McMenamin *et al.* 1998). 1764pR20.5/Reg-2 and 1764pR20.9/Reg-2 were injected in the adult rat brain and immunohistochemistry for Reg-2 expression performed, 2 days later. Co-localisation of β-galactosidase and Reg-2 expression was observed in cortical neurons (figure 4.6 and 4.7) for both 1764pR20.5/Reg-2 and 1764pR20.9/Reg-2. Indeed, when injected into the adult rat striatum, replication-competent viruses give transgene delivery at the site of the injection and at high levels in projecting neurons in the cortex . Illustrating this point, figure 4.6 also shows Reg-2 immunoreactivity in the cortex (shown for 1764pR20.5/Reg-2 and 1764pR20.9/Reg-2) as well as the striatum (shown for 1764pR20.9/Reg-2). As expected, no Reg-2 immunoreactivity was ever detected in rat brains injected with 1764pR20.5 or 1764pR20.9 (data not shown).

Chapter 3 demonstrated that 1764pR20.9 could give high-level short-term gene expression in adult mouse spinal motor neurons (see section 3.4.1). 1764pR20.9/Reg-2 was therefore injected into the footpad of adult mice and 2 days later Reg-2 immunoreactivity in lumbar spinal cord sections assessed (see section 2.2.4.2.2). The antibody used was raised against Reg-2 and mice are known to express a homologue of rat Reg-2, RegIIIβ. There was therefore the possibility that crossreactivity could occur. However, no Reg-2 was detected in the spinal cords of naïve mice, or mice injected with 1764pR20.9 (data not shown). This could be due to either the lack of peripheral damage or to differences between homologue epitopes recognised by the antibody. Reg-2 expression could be detected in spinal motor neurons of mice injected with 1764pR20.9/Reg-2 (see figure 4.8). β-galactosidase expression confirmed the presence of the vector in the spinal cords (see figure 4.8).

4.3.3. CNTF expression using 1764pR20.5/NGF-CNTF and 1764pR20.9/NGF-CNTF

As a positive control in experiments testing any neurotrophic properties of Reg-2, CNTF was chosen. CNTF lacks a leader sequence but still manages to reach motor neuron targets from the Schwann cells in which it is produced (Stockli *et al.* 1989). It has been suggested that CNTF is a lesion factor, a non-secreted protein located in the Schwann cell cytosol which upon peripheral nerve injury is released in minute amounts potent enough to exert a biological action on motor neurons (Masu *et al.* 1993; Sendtner *et al.* 1990; Stockli *et al.* 1989). Although the work described here would deliver CNTF directly to motor neurons, CNTF initiates a cascade of events by binding to a receptor on the cell membrane and thus requires to be secreted for activity. The secretion of CNTF would also allow activity on other non-transduced, injured motor neurons. Furthermore, as Reg-2 is a secreted factor it was appropriate to use a similarly secreted factor as a control. The CNTF construct has previously been used to prevent the degeneration of motor neurons in *pmn* mice (Sendtner *et al.* 1992) and to promote the survival of neonate motor neurons after axotomy (Gravel *et al.* 1997).

The genomic structures of the viruses 1764pR20.5/NGF-CNTF and 1764pR20.9/NGF-CNTF were confirmed by Southern blotting (see figure 4.9). Viral DNA from the purified plaques was digested such that probing with an 860 bp CNTF fragment from NGF-CNTF Chim5 results in the labelling of the NGF-CNTF gene, released as a 1.8 kb fragment.

In figure 4.9, it can be observed that not all purified plaques contain NGF-CNTF, similarly to the case with Reg-2. As discussed in 4.3.1, this is may be due to a recombinational instability due to the insertion of the LAT P2 element.

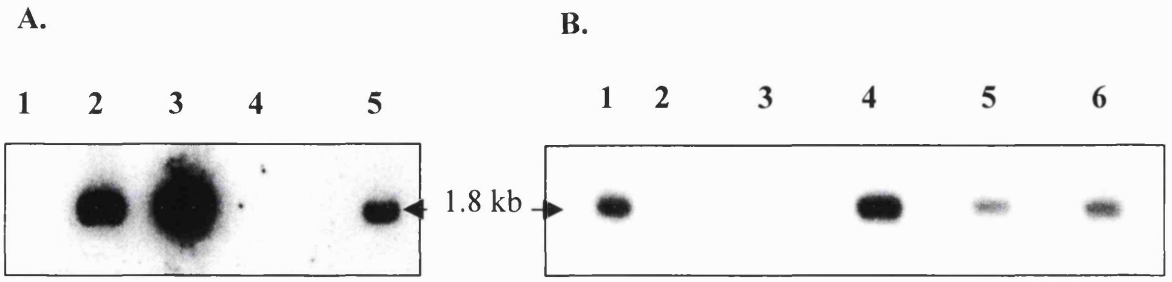


Figure 4.9. Detection of NGF-CNTF by Southern blot. **A.** 1764pR20.5 NGF-CNTF. Lanes 3- pGEM-5 pR20.5/NGF-CNTF. 4- 1764pR20.5. 1,2 and 5- purified plaques. **B.** 1764pR20.9 NGF-CNTF. Lanes 1- pGEM-5 pR20.9/NGF-CNTF. 2- 1764pR20.9. 3-6 - purified plaques.

Two methods were used to confirm CNTF expression, western blot and a cell proliferation assay. It was not possible to confirm expression in 1764pR20.9/NGF-CNTF, as both methods used required expression of CNTF in BHK cells *in vitro*. Following *in vitro* infection of BHK cells with 1764pR20.9, although GFP fluorescence can be easily detected, very low-levels of *lacZ* expression are detected (Palmer *et al.* 2000). *In vivo* however, strong *lacZ* expression can be observed (see chapter 3) (Palmer *et al.* 2000). It has been proposed that a repressing viral protein is responsible for the lack of *lacZ* expression detected when the virus is replicating. Indeed, ICP4 and ICP0 have been shown to repress the LAP2 promoter (Goins *et al.* 1994). Given that no repression is observed when using only the LAP1 or the MMLV LTR promoter or LAP1-MMLV LTR chimeric promoters, the repression observed during replication must result from the participation of LAT P2 sequences. Considering that GFP expression can be detected when the virus is replicating one must assume the replication-dependent repression of LAT P2 must affect somehow the LAP1 promoter. Given these problems in obtaining CNTF expression in a replicating virus context, it was concluded from the Southern blot results that 1764pR20.9/NGF-CNTF should express CNTF *in vivo*.

The western blot performed on cells infected with the 1764pR20.5/NGF-CNTF virus (see figure 4.10), detected two bands of similar size. These are likely to correspond to two

forms of the protein present in the cell, a larger species before leader sequence cleavage and a smaller species after leader sequence cleavage. Indeed, the smaller protein detected was the same apparent size as the commercially available recombinant CNTF protein (26 kDa).

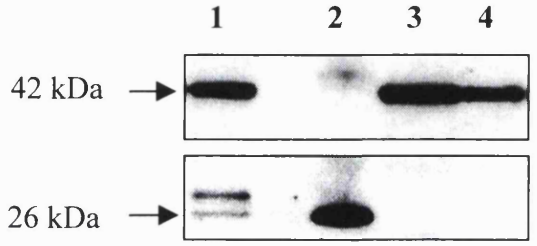
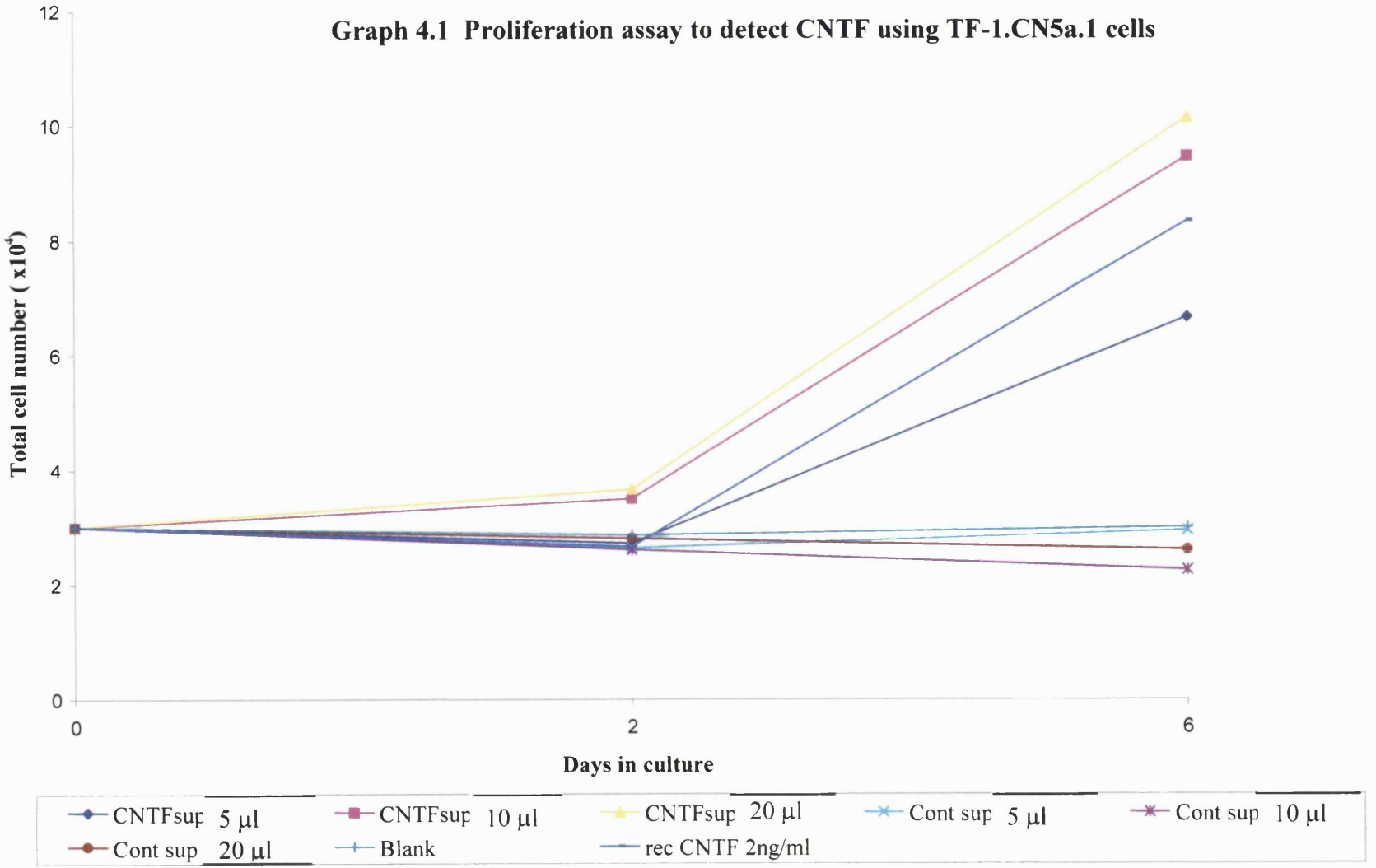


Figure 4.10. Detection of CNTF by western blot. Lanes 1- 1764pR20.5/NGF-CNTF infected cells. 2- rat recombinant CNTF. 3- 1764pR20.5 infected cells. 4- mock infected cells. The superior panel shows probing of the membrane for actin as a control for amount of protein loaded. The lower panel shows probing of the membrane for CNTF.

Having confirmed the protein is expressed and the signal peptide cleaved, cell proliferation assays with the supernatant of cells infected with the 1764pR20.5/NGF-CNTF virus were performed to test if it was secreted and biologically active. As shown in graph 4.1, TF-1. CN5a.1 cells proliferated in response to supernatant from cells infected with 1764pR20.5/NGF-CNTF. In addition, this response increased with increasing amounts of conditioned supernatant. That there is a substantial amount of CNTF secreted by cells infected with the 1764pR20.5/NGF-CNTF virus is indicated by the proliferation response to 10 or 20µl of conditioned media which is superior to the response using recombinant CNTF at a final concentration of 2 ng/ml.

Graph 4.1 Proliferation assay to detect CNTF using TF-1.CN5a.1 cells



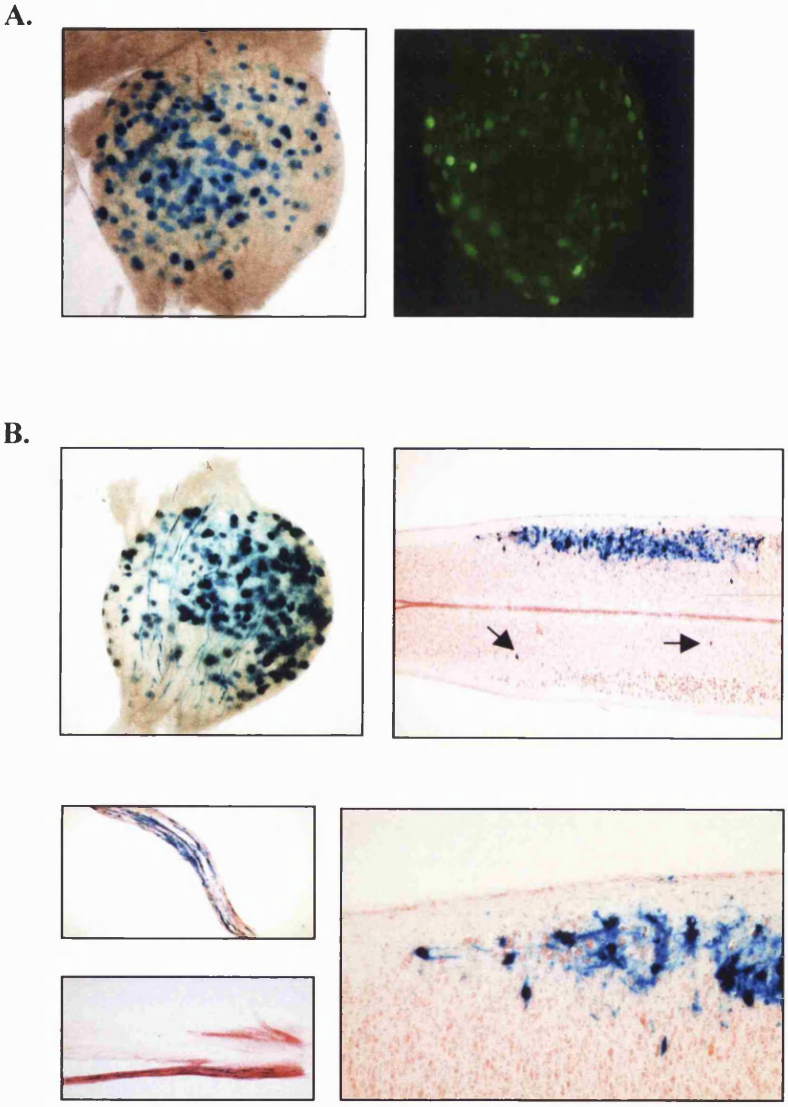


Figure 4.4. HSV-1 mediated gene delivery following sciatic nerve axotomy in the neonate rat. **A.** neonate rat DRG 3 days after footpad inoculation of 1764pR20.9 and 2 days after axotomy. **B.** neonate rat DRG and spinal cord 2 days after sciatic nerve injection of 1764pR20.5 and nerve axotomy. Some evidence of trans synaptic spread is seen (noted by arrows). X-Gal staining of axons can be seen in the sciatic nerve and in the ventral root.

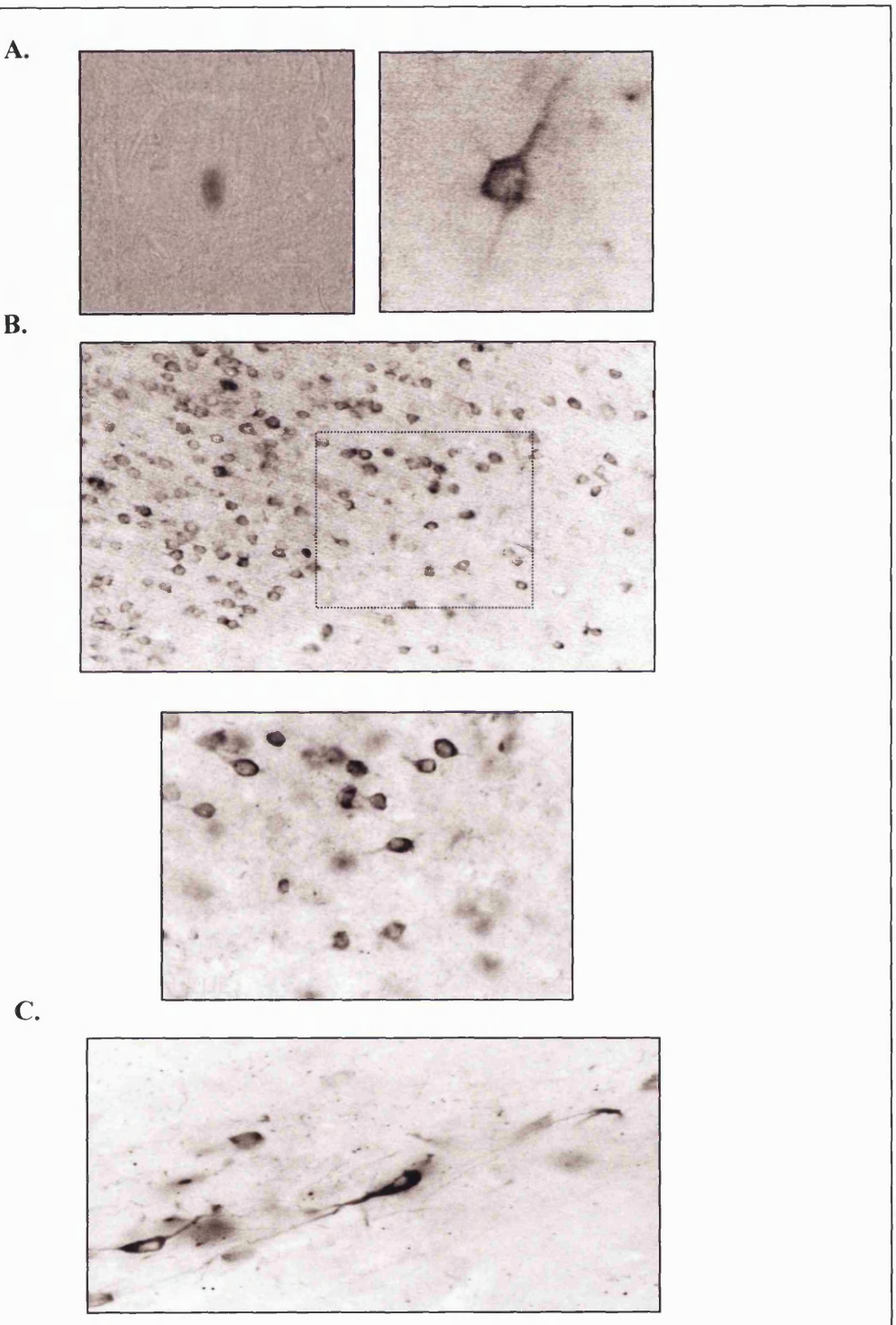


Figure 4.6. Confirmation of Reg-2 expression by 1764pR20.5/Reg-2. Reg-2 immunoreactivity can be seen in the adult rat brain following striatal injection of the vector. **A.** Co-localisation of X-Gal staining (left panel) and Reg-2 detection (right panel) in an infected cortical neuron. **B.** Extensive Reg-2 immunoreactivity can be observed in the cortex. The dotted square shows the area seen below at a higher magnification. **C.** This panel shows Reg-2-expressing neurons of the substantia nigra.

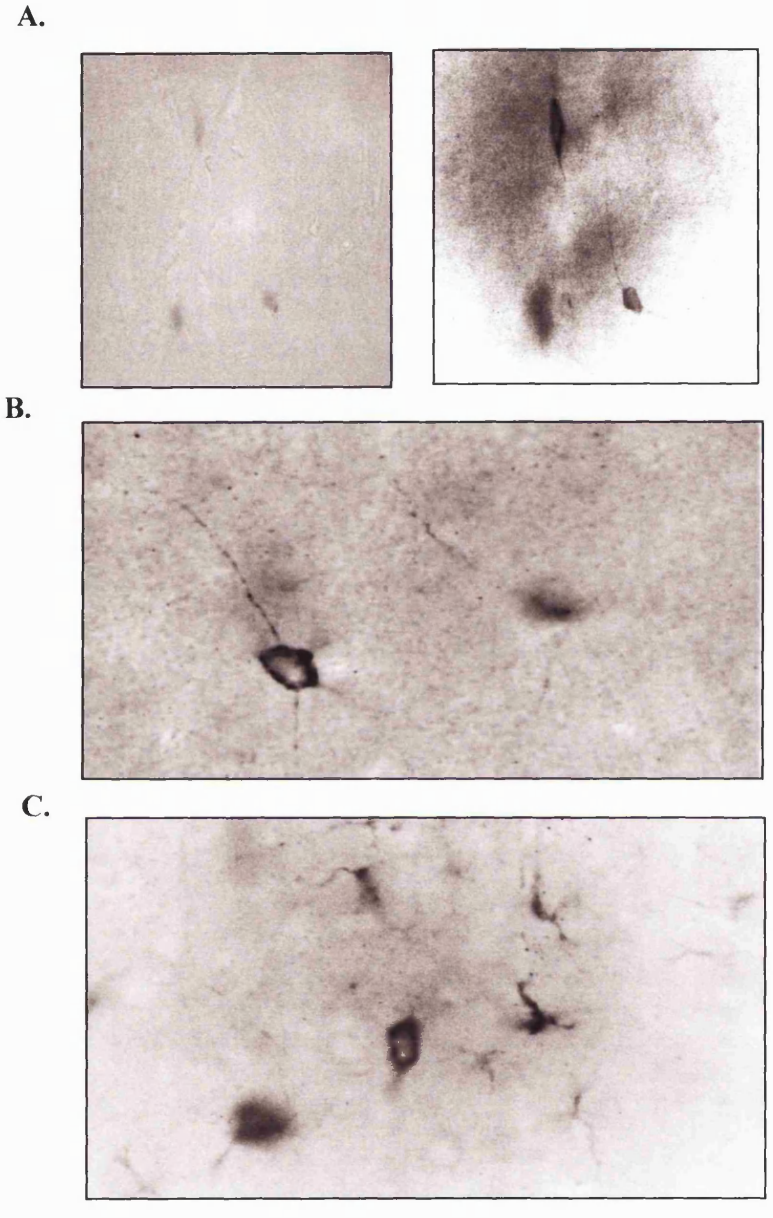


Figure 4.7. Confirmation of Reg-2 expression by 1764pR20.9/.Reg-2. Reg-2 immunoreactivity can be seen in the adult rat brain following injection of the vector 1764pR20.9/Reg-2. **A.** Co-localisation of X-Gal staining (left panel) and Reg-2 detection (right panel) in infected cortical neurons is shown. **B.** Reg-2-expressing cortical neurons. **C.** Reg-2-expressing striatal neurons.

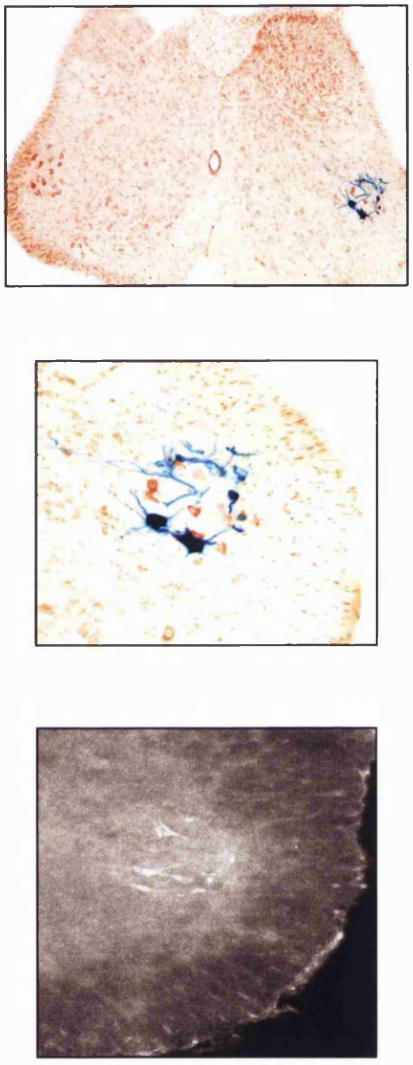


Figure 4.8. Reg-2 expression following footpad injection of 1764pR20.9/Reg-2 in the adult mouse. *LacZ* detection in spinal motor neurons is shown in the two upper panels. In the lower panel, Reg-2 immunoreactivity in motor neurons in a different section of the same animal is shown.

4.4. Discussion

This chapter reports a working model for the use of disabled HSV-1 vectors in the testing of potential factors for neurotrophic activities. The well established model of neonate axotomy here provides a platform in which novel trophic factors could be tested for promoting neuronal survival. Factors found to have a neurotrophic activity in such a model could be of use in the development of therapies for motor neuron and other degenerative diseases, as has previously been the case for factors such as CNTF and BDNF (Ochs *et al.* 2000; Sendtner *et al.* 1992; Sendtner *et al.* 1990; Miller *et al.* 1996). Having developed a working model which allows gene delivery in a situation of motor neuron death, vectors were modified to express genes of interest. Reg-2, appears to be a promising factor in motor neuron survival as it is only produced when motor neurons are regenerating and causes Schwann cells to proliferate. Reg-2 is thus unique in that it is produced by the motor neuron, possibly to prepare the peripheral nerve environment for regeneration. Reg-2 has also been shown to promote motor neuron survival in culture (Nishimune *et al.* 2000). This chapter demonstrated that the vectors produced could deliver Reg-2 to the adult rat brain, an area in which the protein is not usually expressed. In addition, it was also shown that 1764pR20.9/Reg-2 could deliver Reg-2 to adult mouse motor neurons. Furthermore, in both adult rat brain and in adult mouse motor neurons, Reg-2 expressed from the vectors was in the cell cytosol (see figures 4.6 and 4.7) as is the case when it is expressed naturally. As a control, vectors were also constructed to express CNTF. Here a construct which allows secretion from the cell (Sendtner *et al.* 1990) was used and it was confirmed that CNTF was indeed secreted from the virally infected cells as demonstrated using a cell proliferation assay.

Chapter 5

Delivery of Reg-2 to spinal motor neurons following axotomy

Chapter 5 – Delivery of Reg-2 to spinal motor neurons following axotomy

5.1. Introduction

Motor neuron cell death occurs during embryonic development and also under pathological conditions in postnatal life, such as after axotomy in the neonate animal and in neurodegenerative disorders (reviewed by (Sendtner *et al.* 2000). In the neonatal animal, motor neurons are exquisitely sensitive to axotomy, with 90% undergoing apoptosis within one week after sciatic nerve cut at P1 (Greensmith and Vrbova 1996). The extensive motor neuron death observed in the neonate illustrates the dependence of these cells on peripherally derived factors (reviewed by (Sendtner *et al.* 2000)). Given the rapid and reproducible cell death obtained, axotomy of developing neurons has become a well-established, reliable bioassay for testing putative neurotrophic factors (Sendtner *et al.* 2000). Since the first report in 1990 using CNTF, a variety of neurotrophic factors have been found to promote motor neuron survival under these conditions (Sendtner *et al.* 1990). These have included BDNF, GDNF, NT-4/5, LIF and CT-1, amongst others (Cheema *et al.* 1994; Oppenheim *et al.* 1995; Schmalbruch and Rosenthal 1995; Sendtner *et al.* 1992). The survival effect afforded by these factors has however, been found to be transient declining substantially after one or two weeks (Schmalbruch and Rosenthal 1995; Vejsada *et al.* 1994; Vejsada *et al.* 1995; Vejsada *et al.* 1998). The transient rescue observed might reflect the dependence of motor neurons on a combination of neurotrophic factors rather than any individual factor alone. Indeed, combinations of trophic factors have in some cases shown to have better survival-promoting effects as compared to each factor alone (*e.g.* BDNF and CNTF in the *wobbler* mouse (Mitsumoto *et al.* 1994), NT-3 and CNTF in the *pmn* mouse (Haase *et al.* 1997), BDNF and CNTF (Gravel *et al.* 1997) or BDNF and GDNF on axotomised motor neurons (Vejsada *et al.* 1998). The transient survival effects might also be related to the mode of administration of the neurotrophic factors. These have usually been delivered systemically or directly to the nerve stump (Ikeda *et al.* 1996; Vejsada *et al.* 1995). The lack of targeting and short half-life of neurotrophic factors by such routes could lead to the transient effects. In addition, serious side effects might be expected from systemic high concentrations of some of the factors tested. Indeed, such side effects were observed in clinical trials with the systemic administration of high

concentrations of CNTF required such that a therapeutic concentration reached the target site (Miller *et al.* 1996). Ideally, the continuous localised release of the optimal combination of neurotrophic factors might be anticipated to lead to the long-term sustained rescue of motor neurons. As a result, virus-mediated gene therapy has been explored as a means of achieving targeted continuous neurotrophic factor expression to promote motor neuron survival effects (Baumgartner and Shine 1998; Bordet *et al.* 1999; Gravel *et al.* 1997; Haase *et al.* 1997). First-generation adenoviruses have been used following axotomy to deliver GDNF (Baumgartner and Shine 1998), BDNF and/or CNTF (Gravel *et al.* 1997) and have lead to prolonged motor neuron survival as opposed to the effect observed with conventional repeated delivery.

We have shown that an HSV-1 vector lacking the neurovirulence factor ICP34.5 and the transactivator of immediate-early gene expression VP16, is retrogradely transported by motor axons and can transduce motor neurons following peripheral administration in the neonate rat (see chapter 3). This vector, termed 1764 (Coffin *et al.* 1996), has also been successfully tested following axotomy for gene delivery to motor neurons (see chapter 4). In order to test the putative neurotrophic effects of Reg-2 *in vivo*, we have constructed a virus capable of gene delivery and expression of Reg-2 in neurons (see chapter 4). Using quantification of spinal motor neuron survival following sciatic nerve axotomy in the neonate rat, we aimed to use the modified HSV-1 virus to test for any rescue effects of Reg-2. In parallel, we aimed to test CNTF via HSV-1 mediated gene delivery as a positive control.

5.2. Materials and Methods

5.2.1. Viral mediated Reg-2 delivery following axotomy

P2 neonate rats were injected in the sciatic nerve with 2-5 µl of $1x10^8$ pfu/ml of either 1764pR20.5 or 1764pR20.5/Reg-2 (see section 2.2.5.2.2). The sciatic nerve was then axotomised (see section 2.2.5.4) and three days later, the animals were sacrificed and the spinal cords were dissected and processed for Reg-2 immunohistochemistry (see section 2.2.4.2.2). Two animals were used per virus.

5.2.2. Testing of Reg-2 for neurotrophic properties following axotomy

P2 neonate rats were injected in the sciatic nerve with one of the following: 1764pR20.5, 1764pR20.5/Reg-2, 1764pR20.5/NGF-CNTF or vehicle (serum free media) (see 2.2.5.2). The injections were followed by sciatic nerve axotomy and retrograde neuronal labelling with fluororuby (prepared as previously described in (Richmond *et al.* 1994) (see 2.2.5.4). One group of neonates underwent only nerve axotomy and labelling. These animals were terminated 60-70 hours later. All other animals were terminated 7 days later and the spinal cords were dissected and processed as described below.

5.2.2.1. Histology and data evaluation

In order to determine the number of surviving motor neurons, the lumbar spinal cords were serially sectioned at 30 μm and mounted with antifade mounting medium (see section 2.2.5.3). Fluororuby-labelled sciatic motor neurons (found in spinal cord segments L3 to L6) were visualised under a Leica fluorescent microscope and the profiles of labelled cells were counted on each transverse section (~ 90 sections per cord) as previously described (Pennica *et al.* 1996; Vejsada *et al.* 1998). No correction factor was applied to the counts. Different treatments were directly compared, typically with n = 4-6 animals per treatment. For each group, the results obtained were pooled and the means and ± standard deviation reported. For comparison between groups, cell counts were submitted to an unpaired Student's *t*-test. In all comparisons, the level of significance was set at 99% ($p < 0.01$) unless stated otherwise.

5.2.3. Analysis of 1764pR20.5 cytotoxicity following axotomy

P2 neonate rats were treated in three possible ways (i) injection into the sciatic nerve with 2-5 μl of $1x10^8$ pfu/ml of 1764pR20.5 (ii) sciatic nerve injection as described in (i) but followed by sciatic nerve axotomy (iii) sciatic nerve axotomy. One week later the animals were sacrificed and the spinal cords dissected and serially sectioned at 30 μm as described in 2.2.5.5.2. Sections were counterstained with neutral red as described in 2.2.5.5.2. Three animals were used per treatment.

5.3. Results

5.3.1. Detection of HSV-1 mediated Reg-2 expression following axotomy

During rat development, Reg-2 is expressed from embryonic day 15 until postnatal day 5 (Livesey *et al.* 1997). Shortly after sciatic nerve axotomy in the neonate rat, constitutive Reg-2 protein levels become significantly lower than the levels observed in uninjured motor neurons (Livesey *et al.* 1997). Given that the rapid downregulation of Reg-2 expression after neonate axotomy precedes neuronal death (Livesey *et al.* 1997), the 1764pR20.5/Reg-2 vector was constructed in order to artificially up-regulate the levels of Reg-2 in the axotomised motor neuron to assess any effects on neuronal survival. Although this vector has already been shown to mediate Reg-2 expression in transduced cells of the adult rat brain (see chapter 4), an experiment was devised to test the delivery of Reg-2 in axotomised motor neurons. The timecourse of this experiment (animals were sacrificed three days post viral administration) was chosen considering that transgene expression mediated by 1764pR20.5 lasts approximately one week in the neonate rat motor neuron, decreasing rapidly after 2 days (see chapter 3, section 3.4.10). Furthermore, we decided to perform the axotomy at P2 in order to induce Reg-2 downregulation and so that the animals would be terminated at age P5, an age at which we expect low levels of constitutive Reg-2 (Livesey *et al.* 1997) thus facilitating the comparison between virally mediated Reg-2 expression in the axotomised side and the constitutive Reg-2 levels in the nonlesioned side. As seen in figure 5.1, in the side contralateral to axotomy, low levels of Reg-2 are detected as expected for an animal at age P5. In axotomised motor neurons infected with the control vector 1764pR20.5, Reg-2 levels are markedly reduced when compared to the nonlesioned side. In comparison, axotomised motor neurons infected with the vector 1764pR20.5/Reg-2 give high levels of Reg-2 expression. Thus, the disabled HSV-1 vector has achieved up-regulation of Reg-2 levels observed in neonate rat motor neurons following sciatic nerve axotomy.

5.3.2. Neuroprotective effect of Reg-2 and CNTF on axotomised spinal motor neurons following targeted transduction with a minimally disabled HSV-1 vector

Studies using the axotomy model usually involve either the facial or the sciatic nerve (Gravel *et al.* 1997; Ikeda *et al.* 1996; Pennica *et al.* 1996; Sendtner *et al.* 1992; Vejsada *et al.* 1995; Vejsada *et al.* 1998). When using the facial nerve, given that all axons projecting to this nerve are motor axons belonging to neurons located in a well-defined anatomical structure (the facial nucleus), simple cell staining without previous retrograde tracing is sufficient to allow motor neuron counting (Baumgartner and Shine 1997; Casanovas *et al.* 1996; Gravel *et al.* 1997; Sendtner *et al.* 1992). In the case of the sciatic nerve, motor neurons projecting to this nerve constitute only a certain percentage (approximately 50%) of the lumbar motor neurons (Swett *et al.* 1986). Furthermore, the proximal and distal ends of the sciatic motor neuron column are not well-defined and in the case of the distal end, even variable between spinal segments L5 and L6 (Schmalbruch 1986; Schmalbruch and Rosenthal 1995). As result, in studies involving sciatic nerve axotomy some caution is necessary when evaluating cell survival. A simple counting of Nissl-stained material as used in the facial nucleus, is not appropriate as two sources of error are possible: the false identification of boundaries of the sciatic motor column and false positive or negative identification of cells as motor neurons (see review by (Fritzsch and Sonntag 1991)). The latter is of particular importance in a scenario in which motor neurons are undergoing apoptosis and as result appear shrunken and atrophied (Dubois-Dauphin *et al.* 1994; Greensmith and Vrbova 1996). In this case, cell counting based on traditional cell staining is complicated as counting these small cells might lead to false positives and the non-inclusion of these cells in the counts may lead to underestimates of motor neuron numbers. In order to avoid these problems, motor neurons projecting to the sciatic nerve can be retrogradely labelled (Casanovas *et al.* 1996; Pennica *et al.* 1996; Vejsada *et al.* 1995; Vejsada *et al.* 1998). Indeed, " ...single labelling of motor neurons through their cut axon is a reliable procedure to estimate numbers of neurons projecting to a given target..." (Fritzch and Sonntag, 1991). Several studies have reported the use of the fluorescent compound fluorogold (Pennica *et al.* 1996; Vejsada *et al.* 1995; Vejsada *et al.* 1998). Due to some difficulty in obtaining this tracer, dextran conjugated to rhodamine (Fluororuby (Molecular Probes)) was used in our study, based on studies reporting the comparison of labelling efficacy between different tracers (Fritzsch and

Sonntag 1991; Novikova *et al.* 1997; Richmond *et al.* 1994). Fluororuby, like fluorogold and other fluorescent tracers, has the obvious advantage of visibility under the fluorescent microscope without further need for histochemical or immunohistochemical processing. Fluororuby and fluorogold have been shown to retrogradely label motor neurons with the same efficiency one week after administration at the nerve stump and have also been shown to be non-toxic to neurons (Novikova *et al.* 1997). In addition, fluororuby has also been demonstrated to be very effective when applied to the nerve stump but not following intramuscular injection (Fritzsch and Sonntag 1991; Richmond *et al.* 1994). This is ideal for the purposes described here, as we are only interested in labelling the sciatic nerve neurons. In the case of leakage of tracer into the surrounding musculature, no tracer should be taken up (Richmond *et al.* 1994).

Experiments assessing motor neuron survival following axotomy and viral transduction were based on two studies evaluating sciatic motor neuron survival following administration of neurotrophic factors to the nerve stump (Pennica *et al.* 1996; Vejsada *et al.* 1998). Postnatal age 2 rats were injected in the sciatic nerve with 1764pR20.5/Reg-2 followed by axotomy and retrograde labelling. One week later, the surviving fluorescently labelled motor neurons were counted on the operated side. In a similar fashion, vector 1764pR20.5/NGF-CNTF was used as a positive control. The vector 1764pR20.5 and vehicle (serum free medium) were used as negative controls (see 5.2.2 and 5.2.2.1). The maximum number of sciatic motor neurons labelled in the neonate rat was determined by axotomy and retrograde labelling for 60-70 hours (Pennica *et al.* 1996; Vejsada *et al.* 1998). This precise timepoint was chosen, as lower motor neuron counts due to incomplete labelling or motor neuron degeneration could occur with shorter or longer survival times (Vejsada *et al.* 1998). In our case, 1417± 172 (n=4) labelled motor neurons were counted (see figure 5.2A), similarly to the motor neurons counted by others (1385 ± 166) following the same protocol (Vejsada *et al.* 1998). We assumed this number to represent 100% cell survival to determine motor neuron treatment effects for each group.

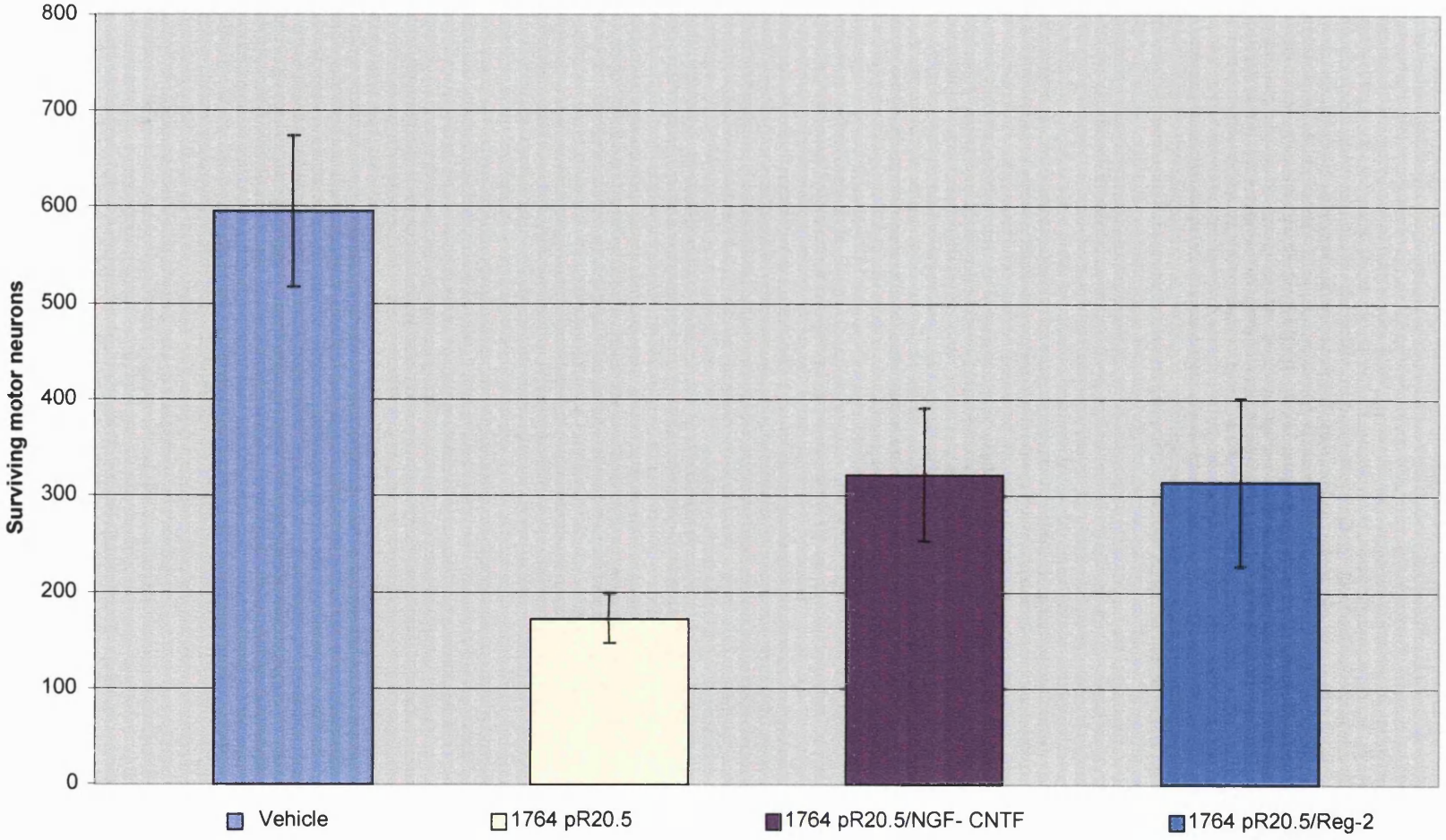
The results obtained with the different treatments are shown in table 5.1 and represented graphically in chart 5.1. One week after sciatic nerve axotomy at postnatal age P2, 58% of sciatic motor neurons had died with 596 ± 78 (n=5) surviving motor neurons counted.

This figure is similar to that reported in the literature in the same conditions (Pennica *et al.* 1996; Vejsada *et al.* 1998). As expected, degenerating motor neurons could be observed in the ventral horn of the spinal cords (see figure 5.2). Following treatment with 1764pR20.5, there were 172 ± 25 surviving motor neurons, 30% less than those observed after vehicle injection and axotomy. Extensive cellular debris could be observed where fluororuby labelled motor neurons had died (see figure 5.2). Thus, the vector 1764pR20.5 appeared to kill cells which had not died yet of axotomy-induced injury. Indeed, in chapter 3, section 3.5.10, the rapid decrease in transgene expression from 2 days to one week following injection of the same vector in the non-axotomised neonate rat was attributed to the motor neuron death observed. Considering this, there are two possible explanations for the extensive cell death observed. Firstly, one could consider that all dead cells (88%) had been transduced by the virus with the surviving motor neurons being those that had not been infected. Secondly, if one assumed that the virus had not transduced every dead cell but only a smaller percentage, axotomy could account for killing a proportion of the neurons not virally infected. In some cases, there would be neurons which were both virally transduced and committed to die due to axotomy-induced damage. As a positive control, 1764pR20.5/NGF-CNTF was constructed as CNTF is a well-established neurotrophic factor. We thus expect to demonstrate CNTF's neurotrophic properties when delivered via a 1764 backbone replication competent HSV-1 vector. Following motor neuron infection with 1764pR20.5/NGF-CNTF and nerve axotomy, 323 ± 69 (n=6, $p<0.01$, Student's t-test comparing with results for 1764pR20.5) motor neurons survived. Although this still corresponds to a higher cell death than that observed with vehicle, nearly twice as many cells survived as compared to following infection with 1764pR20.5 and nerve axotomy. Similarly, infection of motor neurons with 1764pR20.5/Reg-2 previous to nerve axotomy resulted in 315 ± 88 (n=6, $p<0.05$, Student's t-test comparing with results for 1764pR20.5) surviving motor neurons. There is no statistically significant difference between the results obtained for 1764pR20.5/NGF-CNTF and for 1764pR20.5/Reg-2. In both cases, spinal cords treated with 1764pR20.5/NGF-CNTF or 1764pR20.5/Reg-2 appeared to have less cellular debris than those treated with control vector 1764pR20.5 (see figure 5.2-C-E). In our working model, it thus appears that CNTF and Reg-2 are acting in a neuroprotective manner, protecting against the combined effects of nerve axotomy and viral infection.

Table 5.1. Rescue effects of different treatments in the neonatal axotomy paradigm.

Treatment	Number of motor neurons counted	% of surviving sciatic motor neurons	% of sciatic motor neuron loss
Vehicle	596 ± 78	42	58
1764pR20.5	172 ± 25	12	88
1764pR20.5/NGF-CNTF	323 ± 69	22	78
1764pR20.5/Reg-2	315 ± 88	23	77

Chart 5.1. Motor neuron survival following neonate sciatic nerve axotomy: HSV-1 mediated delivery of Reg-2 and NGF-CNTF



5.3.3. Motor neuron infection with 1764pR20.5 following axotomy results in more cell death than after virus administration or axotomy alone

Having established that Reg-2 has a neuroprotective effect on injured motor neurons, we thought it was necessary to further investigate the nature of the cell death caused by the vector 1764pR20.5 following axotomy. Thus, we compared cell death after (i) sciatic nerve axotomy (ii) sciatic nerve injection of 1764pR20.5 and (iii) both sciatic nerve injection of 1764pR20.5 and nerve axotomy. All surgery was performed on animals of age P2 and survival time was one week (see 5.2.3). As can be seen in figure 5.3, neuronal death is obvious one week following sciatic nerve inoculation with vector 1764pR20.5. Similarly, a significant amount of motor neuron degeneration can be seen in the spinal cords of axotomised animals. The crude histological analysis of lumbar spinal cord sections for each group of animals revealed the extent of motor neuron death to be relatively similar. Interestingly, when animals were injected with 1764pR20.5 and axotomised, a much more extensive neuronal death was observed (see figure 5.3). Thus, the hypothesis in 5.3.2 that the motor neuron loss observed in the axotomy paradigm in presence of vector 1764pR20.5 was exclusively due to virus transduction, is incorrect. It appears that the second hypothesis may be correct and that there are three groups of motor neurons dying due to the different insults (see figure 5.4). One group of non-virally infected cells which are committed to die due to axotomy, another group of cells which although axotomised die due to virus cytotoxicity and a further group in which cells which are virally transduced are also killed by the axotomy-induced damage. As result, the neuronal death observed cannot be said to be the additive result of axotomy and virally induced death, as both types of insult will overlap in a number of neurons (an example is given in figure 5.4). The total motor neuron death observed in an axotomised animal infected with a 1764-based virus is therefore given by Equation 1,

Equation 1. Total motor neuron death = a + b - c, where

- a is the total number of motor neurons that die within a week of axotomy.
- b is the total number of motor neurons that die due to virus cytotoxicity.
- c is the number of motor neurons that die due to both axotomy and virus cytotoxicity.

In figure 5.4, a, b and c are represented schematically.

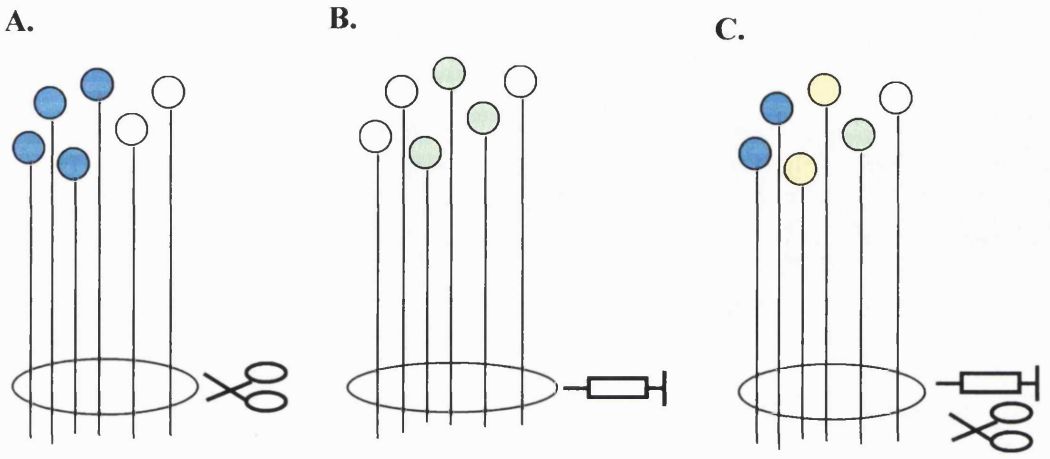
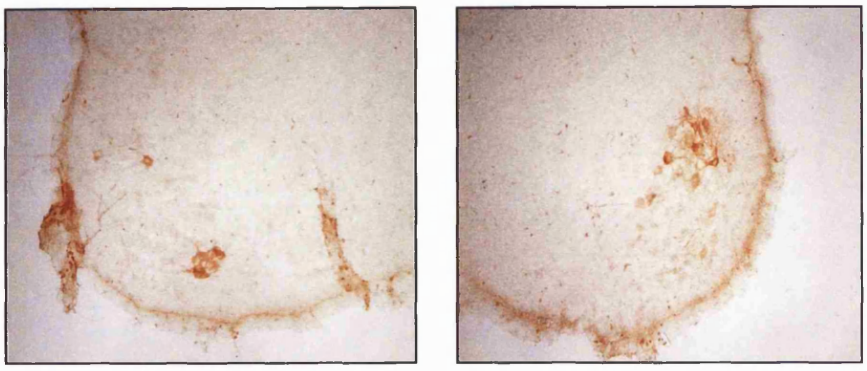


Figure 5.4. Schematic representation of neuronal death occurring following axotomy and/or 1764pR20.5 administration. **A.** After nerve axotomy, a certain percentage of motor neurons die within a week, seen in blue (a=4). **B.** After injection of 1764pR20.5, transduced motor neurons die within a week, seen in green (b=3). **C.** 1764pR20.5 administration within an axotomy paradigm results in some cells affected by both the axotomy and virus cytotoxicity, seen in yellow (c=2). Replacing the values in equation 1, the total motor neuron death observed in **C** is thus $a + b - c = 4 + 3 - 2 = 5$.

A.



B.

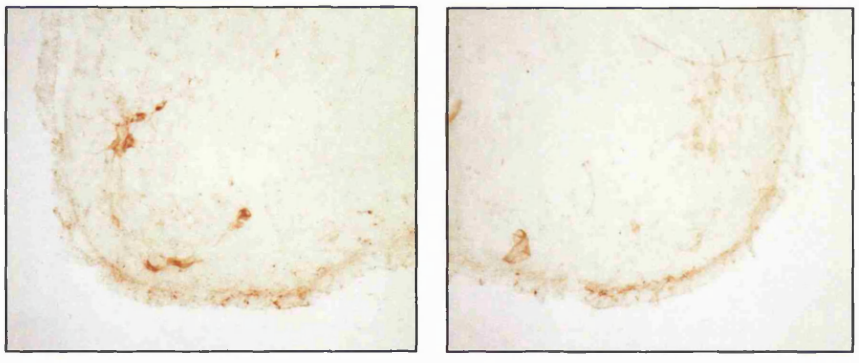
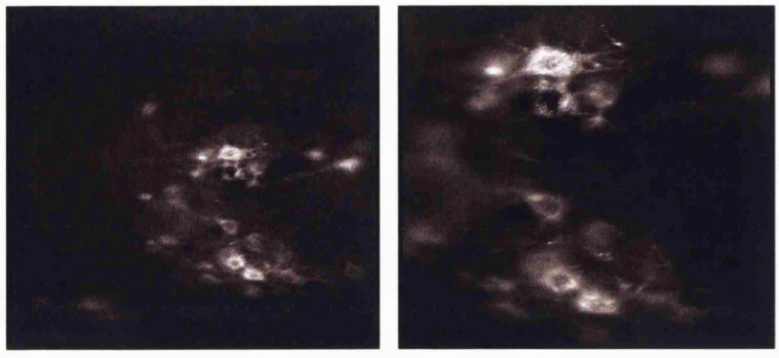
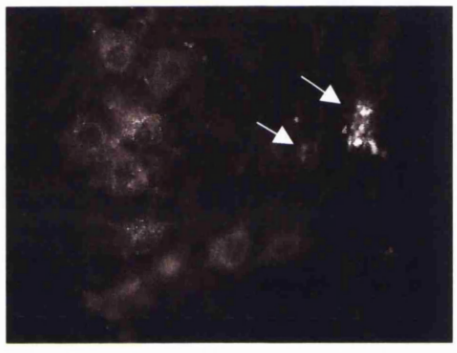


Figure 5.1. Reg-2 immunohistochemistry following virus injection and sciatic nerve axotomy in the neonate rat. **A.** 1764pR20.5/Reg-2. **B.** 1764pR20.5. In both **A** and **B**, the images on the left are contralateral to the axotomised side. Note the down-regulation of Reg-2 following axotomy in animals injected with control virus and the high levels of Reg-2 expression in axotomised animals injected with 1764 pR20.5/Reg-2.

A.



B.



C.

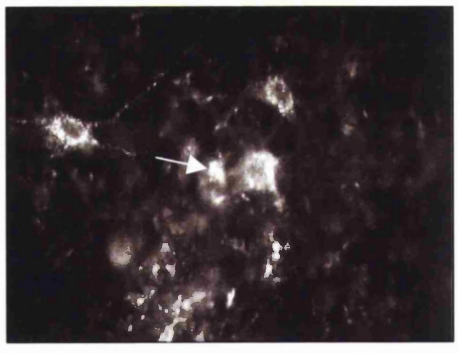
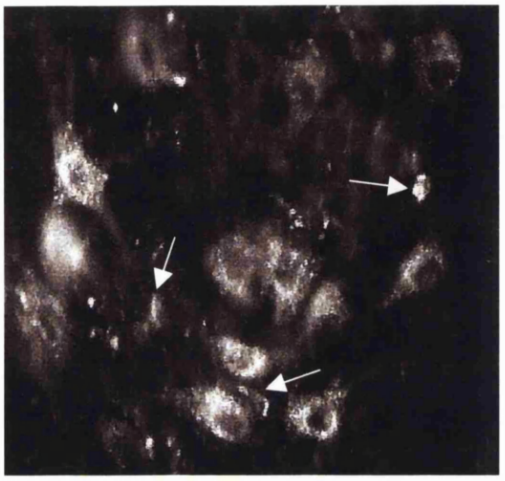


Figure 5.2. Retrograde labelling of sciatic motor neurons. **A.** Sciatic motor neurons 60-70 hrs after nerve axotomy. **B.** Labelled motor neurons one week after axotomy with nerve inoculation of vehicle. **C.** Sciatic motor neurons one week following sciatic nerve injection with vector 1764pR20.5 and nerve axotomy. Extensive cellular debris can be observed. Arrows indicate cellular death.

D.



E.

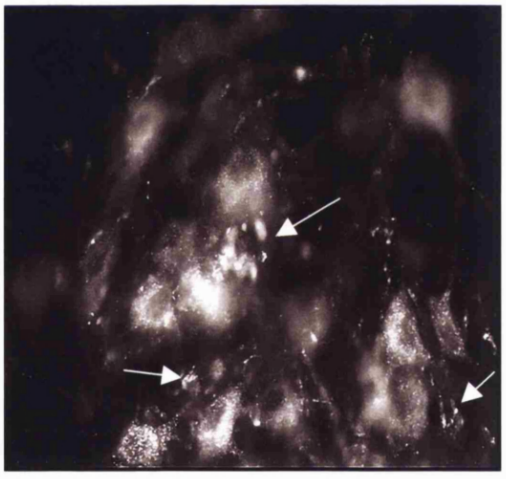


Figure 5.2. (continuation) – Retrograde labelling of sciatic motor neurons. **D.** Labelled motor neurons one week after sciatic nerve injection of 1764 pR20.5/NGF-CNTF and axotomy. **E.** Sciatic motor neurons one week after inoculation with 1764pR20.5/Reg-2 and axotomy. The arrows point out degenerated motor neurons.

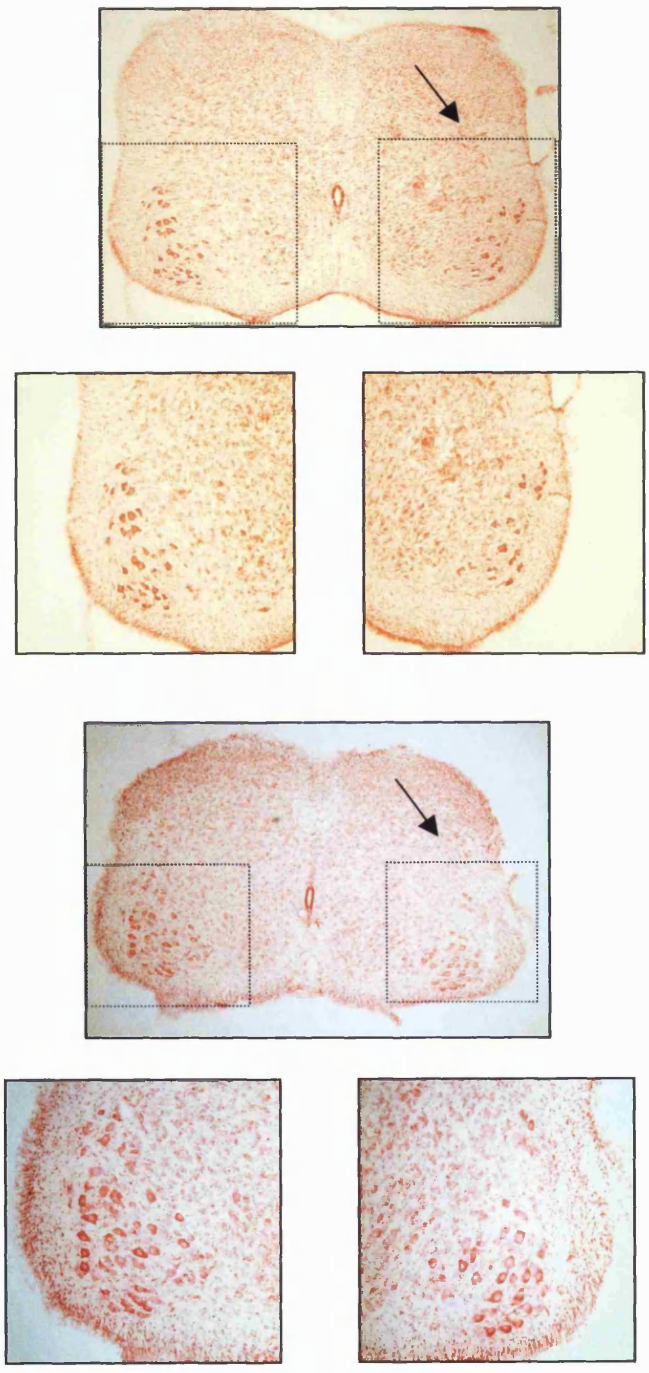


Figure 5.3. Cellular death after virus inoculation and/or axotomy. **A.** Sciatic nerve inoculation of 1764pR20.5. **B.** Sciatic nerve axotomy. The closed arrows indicate the injected or axotomised side. The dotted squares show areas seen below at a higher magnification.

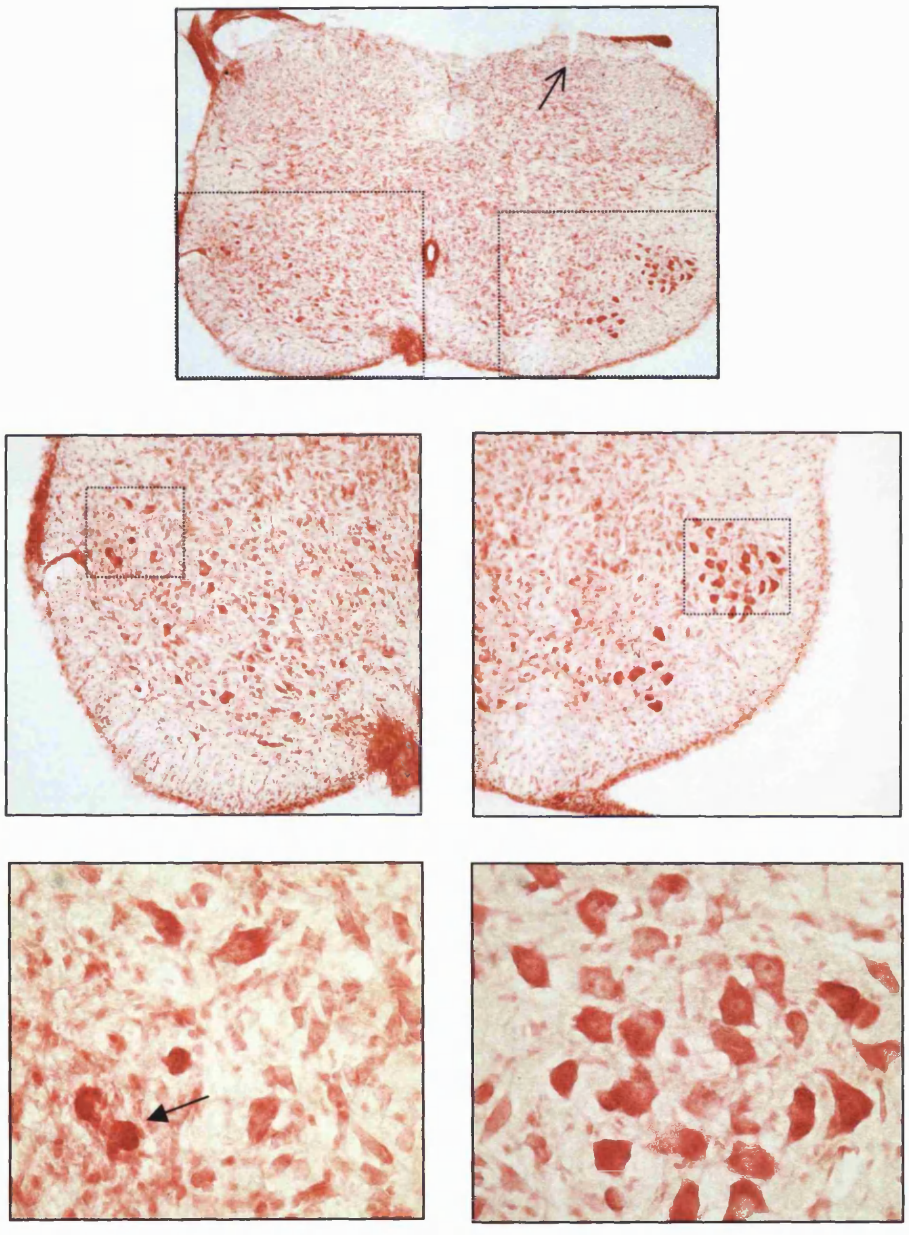


Figure 5.3. (continuation) Cellular death after sciatic nerve inoculation of 1764pR20.5 and nerve axotomy. The open arrow indicates a cut in the dorsal horn contralateral to the injected and axotomised side. The dotted squares show the areas seen below at a higher magnification. The closed arrow indicates an atrophied motor neuron.

5.4. Discussion

We have demonstrated here that a minimally disabled HSV-1 vector can mediate expression of Reg-2 in motor neurons following nerve axotomy in the neonate rat. Furthermore, we have shown that HSV-1 mediated gene delivery of Reg-2 or CNTF in the axotomy paradigm results in the partial prevention of motor neuron death which accompanies both axotomy and viral infection.

Reg-2 has been shown to promote embryonic motor neuron survival in culture in an autocrine/paracrine manner (see section 1.4.4.4) (Nishimune *et al.* 2000). Thus, we tested whether the genetic modification of neonate rat sciatic motor neurons to produce Reg-2 through its normal secretory pathway would protect them and their uninfected neighbours from axotomy-induced death. For this purpose we used a replication-defective HSV-1 vector modified to deliver the Reg-2 gene under the control of the CMV promoter and the LAT P2 enhancer, injected at the time of axotomy. We demonstrated that the transduced motor neurons expressed high-levels of Reg-2 unlike their control counterparts in which Reg-2 is down-regulated following axotomy (see 5.3.1).

In an experiment to test the rescue capabilities of Reg-2 on axotomised motor neurons we included a CNTF- expressing HSV-1 vector as a positive control as CNTF has been shown to rescue spinal motor neurons following sciatic nerve axotomy (Ikeda *et al.* 1996; Vejsada *et al.* 1995). We demonstrated that following axotomy, delivery of either CNTF or Reg-2 resulted in a similar rescue effect on a percentage of axotomised motor neurons (approximately double the number of control cells counted). The interpretation of this rescue effect is complicated by the fact that there is a significant difference between the vector 1764pR20.5 and the vehicle control groups (12% versus 42% motor neuron survival, respectively). Interestingly, in a study using first-generation adenovirus to deliver neurotrophic factors to the neonate motor neuron following axotomy, a similar result was reported (Baumgartner and Shine 1998). In this case, one week after axotomy at P3, the percentage of surviving sciatic motor neurons after injection with viral vector Adv.RSV- βgal was also significantly less than in the vehicle control group (14% versus 35%) (Baumgartner and Shine 1998). As there was no cell death observed when the vector was injected in the absence of axotomy, the authors

concluded that viral transduction had accelerated the rate of motor neuron death (Baumgartner and Shine 1998). In our case, although we also see a difference between control vector and vehicle groups, we demonstrated that viral transduction *per se* caused cell death (see 5.3.3). In addition, neuronal death observed after control vector injection and axotomy was more extensive than that previously observed. Hence, the cell death observed in our case is not due to an accelerated death rate because of viral transduction, but due to a mixture of factors: virus cytotoxicity and/or axotomy. As a result, one cannot specify that Reg-2 or CNTF protect the neurons against axotomy-induced damage. One must conclude that Reg-2 and CNTF exert neuroprotective efffects against virus cytotoxicity and/or axotomy. A plethora of possible scenarios arises. On one hand, previous indications of neurotrophism for both factors taken together with the fact that they are secreted, leads to the possibility of action on a population of motor neurons not virally infected but which would otherwise die due to the axotomy. On the other hand, the same rescue effects might be applicable to prevent motor neuron death from virus induced damage. It is promising that Reg-2's rescue effects are similar to those of CNTF, but because each factor might be acting on differently injured motor neurons one cannot directly compare their neurotrophic activities. A repeat of the experiment in the absence of axotomy would provide information as to whether the factors in question are able to rescue motor neurons infected by the virus. Unfortunately, due to time restraints this has not been possible.

The testing of Reg-2 in a viral backbone following axotomy has proved to be somewhat more complicated than first anticipated as the timing of axotomy and of virus injection has proved to be crucial. We have shown that as the rat matures it becomes less susceptible to infection by a 1764 backbone replication-competent HSV-1 vector (see chapter 3). Even if motor neuron transduction were to be achieved after injection at P10 and if the transduction of these more mature motor neurons did not result in cell cytotoxicity, there would still be a major caveat. Although motor neurons would still die after axotomy, albeit a much lower percentage than that observed at P2, at this age Reg-2 levels are up-regulated in more than 95% of axotomised motor neurons (Livesey *et al.* 1997). Hence, the testing of Reg-2 as a neurotrophic factor is compromised as it is imperative that it is tested at an early postnatal age (up to P5) when motor neurons are very sensitive to damage, be it interruption of trophic derived support or infection with a vector.

Chapter 6

HSV-1 mediated delivery of Reg-2 to the DRG following axotomy

Chapter 6 – HSV-1 mediated delivery of Reg-2 to the DRG following axotomy

6.1. Introduction

Reg-2 is not normally expressed in the adult rat but within 24 hours of sciatic nerve crush or transection, all motor neurons and approximately 12-15% of L4-L5 DRG neurons express Reg-2 (Livesey *et al.* 1997; Davis *et al.* 2002). After peripheral nerve injury, Reg-2 expression in the DRG follows a dynamic pattern changing between different sensory neuron populations (Davis *et al.* 2002). Indeed, by 24 hours after sciatic nerve axotomy in the adult rat, more than 95% of Reg-2-expressing sensory neurons are small diameter, predominantly nociceptive sensory neurons. By 7 days post axotomy however, Reg-2 expression has completely shifted to large-diameter neurons, a proportion of which co-express galanin (Davis *et al.* 2002). Interestingly, different forms of nerve insult result in different patterns of Reg-2 expression in DRG sensory neurons (Davis *et al.* 2002). Inflammation, for instance, induces the rapid up-regulation of Reg-2 expression in small-diameter neurons without any switching to other cellular populations. Considering Reg-2 has been shown to be required for the effects of the neuropoietic cytokines of the LIF/CNTF family on developing motor neurons, it has been suggested that the expression of Reg-2 in the small diameter nociceptive neurons could be related to a role as an inflammatory mediator (Nishimune *et al.* 2000; Davis *et al.* 2002).

In the neonate animal, sensory DRG neurons as well as motor neurons are dependent on peripherally derived factors and as result are vulnerable to peripheral nerve injury. Sciatic nerve axotomy results in the loss of approximately 40-50% of the sensory neurons in the L4-L5 DRG after 7 days (Yip *et al.* 1984). In this scenario, neurons have been shown to die by an apoptotic mechanism (Whiteside *et al.* 1998) and to be temporarily rescued by the administration of NGF, GDNF, LIF or CNTF (Lo *et al.* 1995; Yip *et al.* 1984; Cheema *et al.* 1994; Matheson *et al.* 1997).

Reg-2 expression in neonatal motor neurons is down regulated following sciatic nerve axotomy (Livesey *et al.* 1997). Although it is known that a subset of neonate DRG sensory neurons expresses Reg-2 up to P5, the aim is to investigate Reg-2 expression following sciatic nerve axotomy. In addition, the effect of HSV-1 mediated Reg-2 gene delivery to the neonatal DRG following nerve axotomy will be tested. CNTF will be used as a positive control.

6.2 Materials and Methods

6.2.1 Reg-2 expression in the neonate rat DRG following sciatic nerve axotomy

Unilateral sciatic nerve transection was carried out on P1 Lewis rats as described in 2.2.5.1. The animals were sacrificed 24 hours, 5 and 21 days later. Ipsilateral and contralateral L4 and L5 DRG were dissected and processed for Reg-2 immunohistochemistry as described in 2.2.4.2. The same sections were then processed for immunoreactivity of IB4 or N52.

In order to compare numbers of Reg-2 expressing cells in the ipsilateral versus the contralateral ganglia, following detection of Reg-2 by imunofluorescence, random fields of view at a magnification of 10X were counted for Reg-2-expressing profiles.

For assessing the co-localisation of Reg-2 with IB4 or N52 positive cells, random fields of view (minimum of 20) at a magnification of 10X were counted for Reg-2-expressing profiles and for double labelling.

6.2.2 HSV-1 mediated Reg-2 delivery following axotomy

P1 neonate rats were injected in the footpad with one of the following: 1764pR20.9, 1764 pR20.9/Reg-2, 1764pR20.9/NGF-CNTF or vehicle (serum free media) (see 2.2.5.2). The following day, the sciatic nerve on the same side as the footpad injection was axotomised and neurons retrogradely labelled with fluororuby (prepared as described in (Richmond *et al.* 1994) (see 2.2.5.4). One group of neonate animals didn't receive any treatment and only

underwent nerve axotomy and labelling. This group was terminated 60 hours later. All other animals were terminated 7 days later and the DRG were dissected and processed as described below.

6.2.2.1 Histology and data evaluation

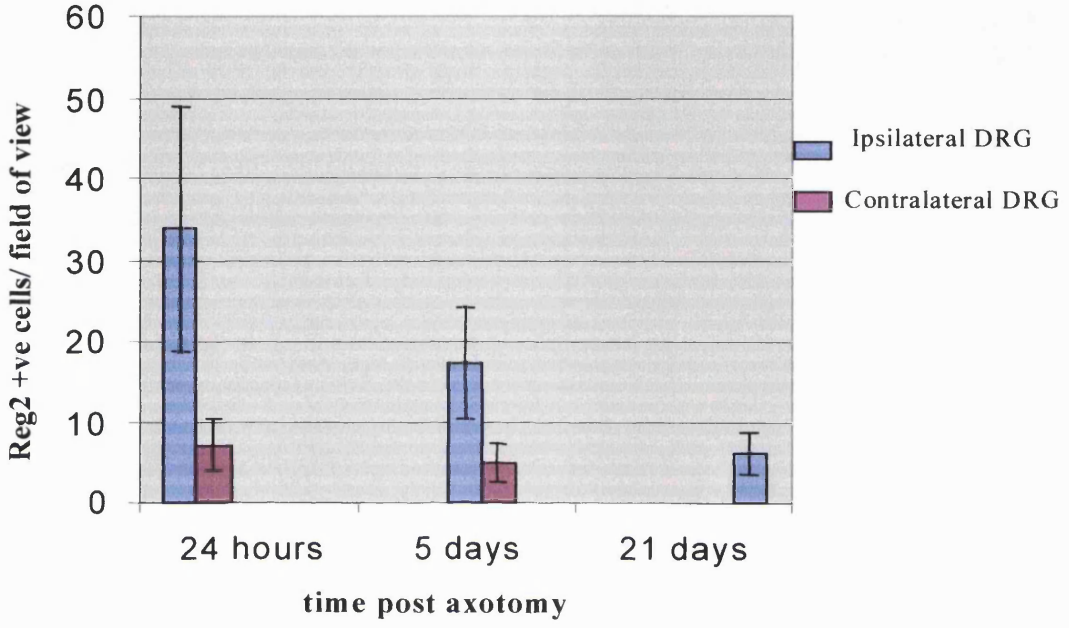
In order to determine the number of surviving axotomised sensory DRG neurons, L4 and L5 DRG were serially sectioned at 14 μm and mounted with antifade mounting medium (see section 2.2.5.3). Fluororuby-labelled sciatic sensory neurons were visualised under a Leica fluorescent microscope and the profiles of labelled cells were counted on every fourth section. No correction factor was applied to the counts. Different treatments were directly compared, typically with n = 3-4 animals per treatment. For each group, the results obtained were pooled and the means and ± standard deviation reported. For comparison between groups, raw cell counts were submitted to an unpaired Student's *t*-test. In all comparisons, the level of significance was set at 95% ($p < 0.05$).

6.3 Results

6.3.1 Reg-2 expression is rapidly up-regulated following sciatic nerve axotomy in the neonate

L4 and L5 DRG were assessed for Reg-2 immunoreactivity at different timepoints after sciatic nerve axotomy in the neonate rat. The results obtained can be seen in figure 6.1 and plotted in graph 6.1. We found that Reg-2 expression was up-regulated in sensory neurons in L4 and L5 ganglia within 24 hours of nerve transection. Furthermore, Reg-2-expressing cells were still present at 21 days post axotomy. Similarly to the situation observed in the contralateral DRG, the number of Reg-2 expressing cells in L4 and L5 DRG on the side ipsilateral to nerve transection was found to decrease with time. At 21 days post axotomy, no Reg-2 expressing cells could ever be detected in the DRG contralateral to nerve cut, as expected since Reg-2 expression in the DRG is reported to last up to P5 (Livesey *et al.* 1997).

Graph 6.1. Reg-2 expression in the neonate rat DRG following sciatic nerve axotomy



6.3.2 A dynamic pattern of Reg-2 expression in the DRG following axotomy in the neonate

The pattern of Reg-2 expression in the adult rat DRG has been shown to vary depending on the type of nerve injury (Davis *et al.* 2002). We investigated whether following nerve axotomy in the neonate animal, the up-regulation of Reg-2 expression followed a dynamic pattern. At the same timepoints as described in the previous section, Reg-2 immunoreactivity was assessed in the IB4 and in the N52 positive population (see table 6.1 and figure 6.2). In the non-axotomised sensory DRG neuron, at both 24 hours and at 5 days, ~90% of Reg-2-expressing cells co-localised with the IB4 binding population (see figure 6.2 A, B, E and F). 24 hours after axotomy, 60% of Reg-2 expressing cells were IB4 positive and 40% were N52 positive (see figure 6.2 C and D). Interestingly, at 5 days post nerve transection, only a very small proportion of Reg-2 expressing cells were IB4 positive

(11%) (no fluorescent micrographs could be taken due to very light IB4 staining) with the majority of Reg-2 expressing cells now belonging to the N52 population (80%) (see figure 6.2 G). At 21 days post axotomy, Reg-2 expressing cells seemed to co-localise equally with the IB4 or N52 population (25% and 20% respectively). It is interesting to note that a large proportion of Reg-2 expressing cells did not co-localise with neither the IB4 nor the N52 neuronal population (see figure 6.2 H and I). In the sections used for the detection of Reg-2 and IB4 or N52, the non-colocalising Reg-2 expressing cells appeared to be of small to medium size.

Table 6.1. Pattern of Reg-2 expression following sciatic nerve axotomy in the neonate rat: localisation in the IB4 or N52 immunoreactive neuronal populations

Time post axotomy	Reg-2 expression co-localisation	% found in ipsilateral L4+L5	% found in contralateral L4+L5
24 hours	**IB4**	60	87
	N52	40	8
5 days	**IB4**	11	90
	N52	80	8
21 days	**IB4**	25	No Reg-2 expression
	N52	20	No Reg-2 expression

6.3.3 Reg-2 does not have a neuroprotective effect on axotomised sensory neurons of the DRG following transduction with a disabled HSV-1 vector

To assess whether Reg-2 could exert any neuroprotective effects on injured sensory neurons, we modified an HSV-1 vector, which we have shown is capable of mediating transgene expression in the neonate rat DRG following axotomy, to express Reg-2 (see chapter 4). In order to test our system, we also modified the same vector to express CNTF, as it has been shown to rescue neonatal sensory neurons following sciatic nerve axotomy (Lo *et al.* 1995). Studies examining the survival effects of putative neurotrophic factors on

sensory neurons in the DRG following sciatic nerve axotomy have usually involved the comparison between total neuronal counts on the ipsilateral and contralateral ganglia (Lo *et al.* 1995; Cheema *et al.* 1994; Matheson *et al.* 1997). These total counts have been based on the serial sectioning of the DRG and the application of a correction factor for split nucleoli to the final counts (the Abercrombie correction factor) (Abercrombie M 1946). Although the Abercrombie method has undoubtedly been widely used, it does have flaws in the estimation of mean object height and is biased by "lost caps" (see review by (Hedreen 1998)). Other methods now used to estimate total neuronal counts in the DRG include the optical disector and the physical disector (see (Groves *et al.* 1997)). In any case, stereology in the DRG still remains controversial as to the most accurate method available. When choosing any counting method it is vital to first decide what the results are wanted for. If the counts are to be compared between different experimental groups, then in effect no correction factor is required presuming the experiments are conducted identically as the error present should be the same in all groups. If the results obtained however are to be compared with other studies, than it is essential that the same methods (and correction factors by consequence) be applied.

In our case, we aimed to compare the neurotrophic effects upon sensory neuron transduction with different factor-expressing viruses. We decided to use fluororuby to label all the axotomised sciatic sensory neurons since these are only a proportion of the total number of sensory neurons in L4-L6 DRG (Schmalbruch 1987). Indeed, a similar retrograde tracer, fluorogold, has previously been shown to effectively label sensory neurons projecting to the axotomised sciatic nerve (Zhou *et al.* 1999). As for the experiment in motor neurons (see chapter 5), an initial number of sciatic sensory neurons was determined, in order to express the effects of different treatments as a percentage of this initial number. The number of labelled sensory neurons was determined by serially sectioning the DRG, and counting fluororuby profiles in every fourth section. The total count obtained was multiplied by four and no correction factor was applied given all the experiments were conducted following the exact same protocol, and should thus have the same error. Indeed, as the results are expressed in terms of proportion, any biases introduced in the counting should be common to both procedures and cancelled out when

the proportion is calculated. In addition, to decrease double counting, thick sections of tissue were cut (14 μm). In a study on sensory neuron loss in the DRG after sciatic nerve axotomy in the neonate rat, it was reported that the extent of cell loss in individual ganglia varied indicating that L4, L5 and L6 varied in their contributions to the nerve (Schmalbruch 1987). The authors concluded that studies quantifying the effect of different treatments on the number of primary sensory neurons after sciatic nerve axotomy should analyse more than one of the ganglia contributing to the nerve (Schmalbruch 1987). As result, although several studies using the sciatic nerve axotomy model have examined survival effects on one DRG, either L4 or L5 (Lo *et al.* 1995; Cheema *et al.* 1994; Matheson *et al.* 1997), we decided to analyse both L4 and L5. The results for this experiment can be seen in table 6.2 and plotted in graph 6.2.

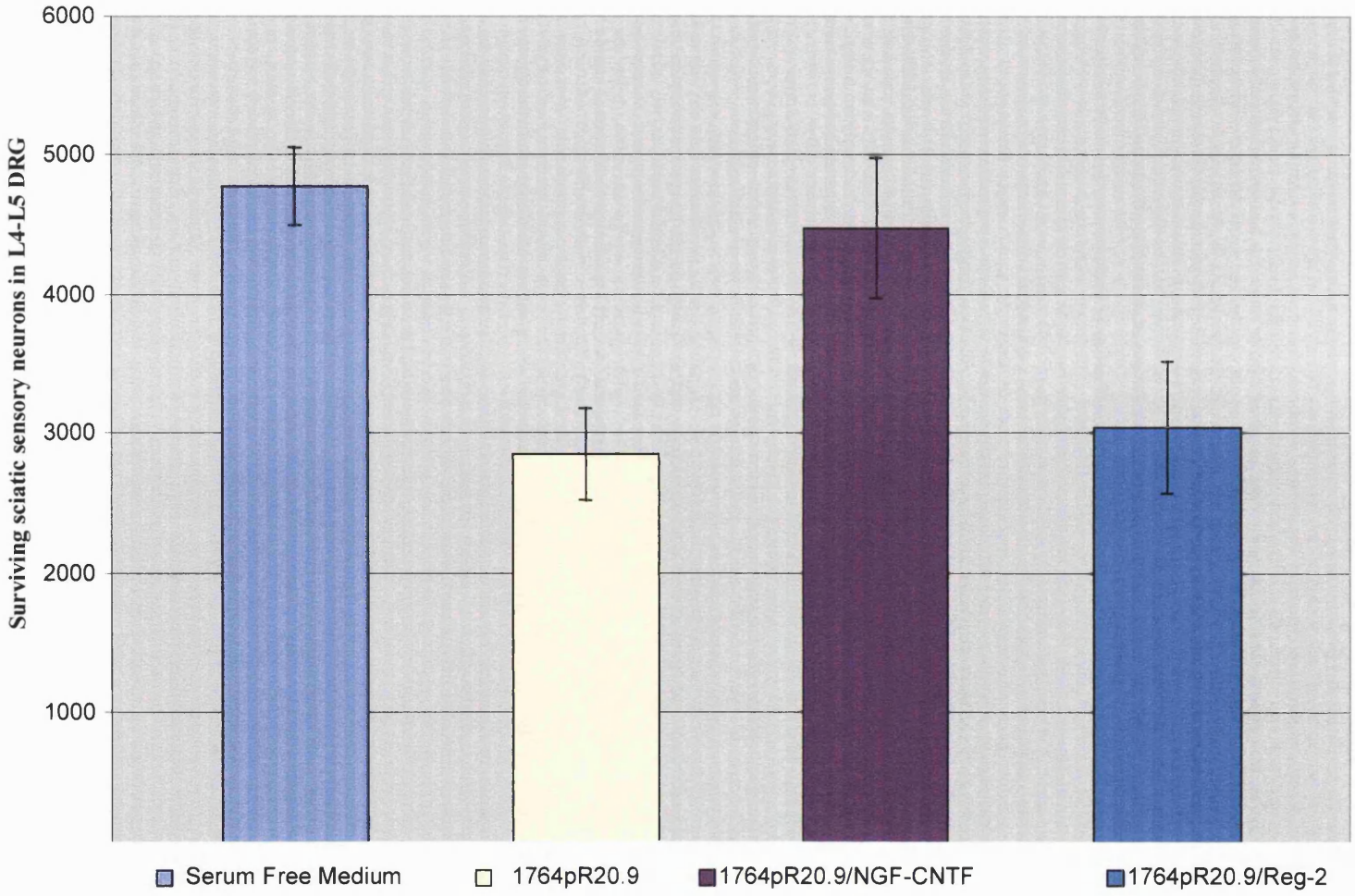
The initial estimate of sciatic sensory neurons in the L4 and L5 DRG following the labelling of the axonal stump and a survival time of 60 hours, was of 6094 ± 218 (n = 3) neurons. One week after injection of vehicle and axotomy, the number of surviving sciatic sensory neurons in the L4-L5 DRG was of 4778 ± 283 (n=3). This corresponded to a significant loss of 21% of initially counted neurons. Other studies using different methods to evaluate cell survival have inferred a greater cellular loss (Yip *et al.* 1984; Cheema *et al.* 1994) but since these have used different methods to estimate neuronal populations, one cannot directly compare results. These studies however are relevant in that they concluded that a significant number of sensory neurons had been lost soon after the axotomy (Yip *et al.* 1984; Cheema *et al.* 1994). Therefore, our initial sciatic sensory neuron number may be an underestimate, as some neurons could have already died 60 hours post axotomy. Even if this were to be the case, we still tested the rescue effects of different factors as we were aware that in the event a factor were to rescue 100% of the sensory population lost after axotomy, the number of surviving neurons would probably be higher than that initially estimated. One week after nerve axotomy and transduction of sensory neurons in L4-L5 DRG with control vector 1764pR20.9, 2848 ± 332 (n=3) surviving sciatic sensory neurons were counted. This was 32% less ($p<0.05$) than that observed for vehicle injection leading us to conclude the viral vector used had a toxic effect in the neonate primary sensory afferents. This toxicity had already been suggested in chapter 3 as the reason for the rapid

decrease in transgene expression observed between 2 days and 1 week after vector injection. To test our model, we used the same vector modified to express CNTF. One week following injection of the vector 1764pR20.9/NGF-CNTF and nerve axotomy, 4474 ± 506 (n=4) surviving sciatic sensory neurons in L4-L5 DRG were counted. This was significantly higher than the number of neurons surviving axotomy and injection with control vector. Indeed, 1626 neurons that would have died due to vector toxicity and/or nerve axotomy were rescued. Furthermore, there was no significant difference between 1764pR20.9/NGF-CNTF and vehicle. Having established that our model could confirm the neurotrophic properties of CNTF, 1764pR20.9/Reg-2 was tested. Under the same experimental conditions, 3043 ± 469 (n=4) fluororuby labelled neurons were counted in L4-L5 DRG. Unlike the results obtained when CNTF was delivered to the axotomised sensory neuron, there was no significant difference between the result obtained for 1764pR20.9/Reg-2 and that obtained when sensory neurons were transduced with control vector 1764pR20.9. In our model it thus appears that Reg-2 does not have a neuroprotective effect on injured primary sensory neurons.

Table 6.2. Rescue effects of different treatments on L4-L5 sciatic sensory neurons following nerve axotomy in the neonate rat.

Treatment	Number of surviving sciatic sensory neurons in L4-L5	% of cell loss
Vehicle	4778	21%
1764pR20.9	2848	53%
1764pR20.9/NGF/CNTF	4474	26%
1764pR20.9/Reg-2	3043	50%

Chart 6.2. Sciatic sensory neuron survival in L4-L5 DRG following nerve axotomy in the neonate: HSV-1 mediated delivery of Reg-2 and CNTF



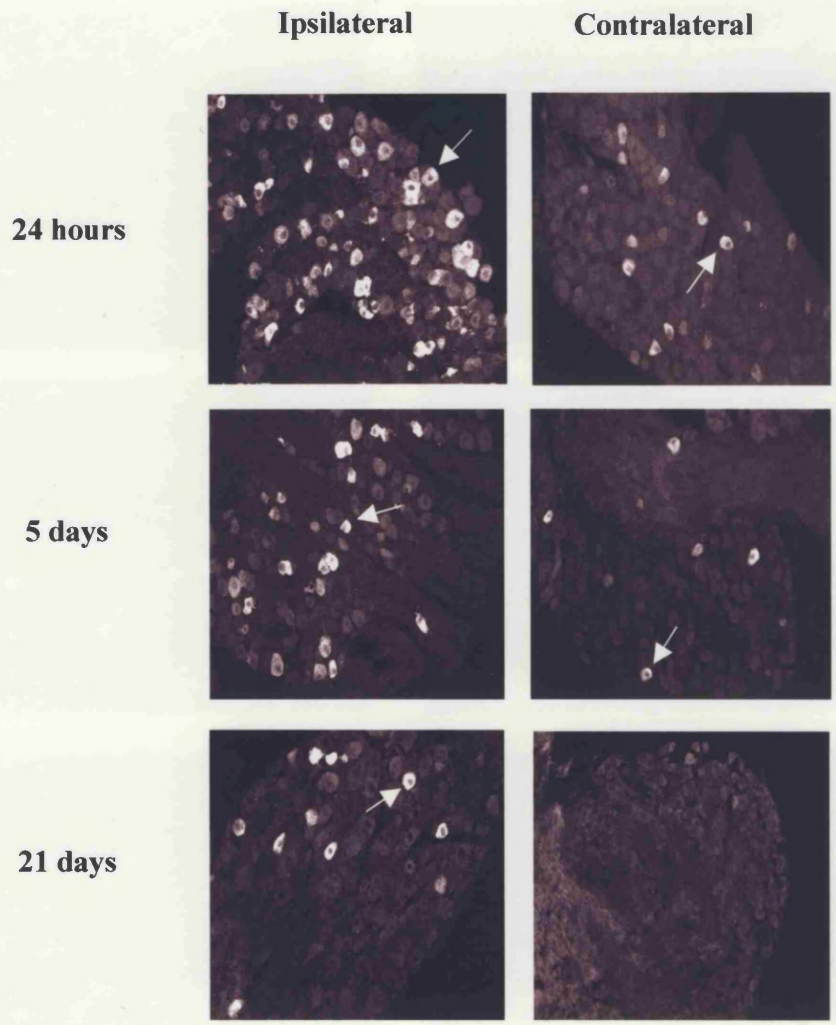


Figure 6.1. Reg-2 expression in the neonate rat DRG following axotomy. Images of Reg-2 expression detected by immunofluorescence are shown. At different times post sciatic nerve transection, a representative section of ipsilateral and contralateral DRG can be observed. Arrows show immunoreactive neurons. Note the massive up-regulation in Reg-2 expression in the ipsilateral DRG lasting at least 21 days.

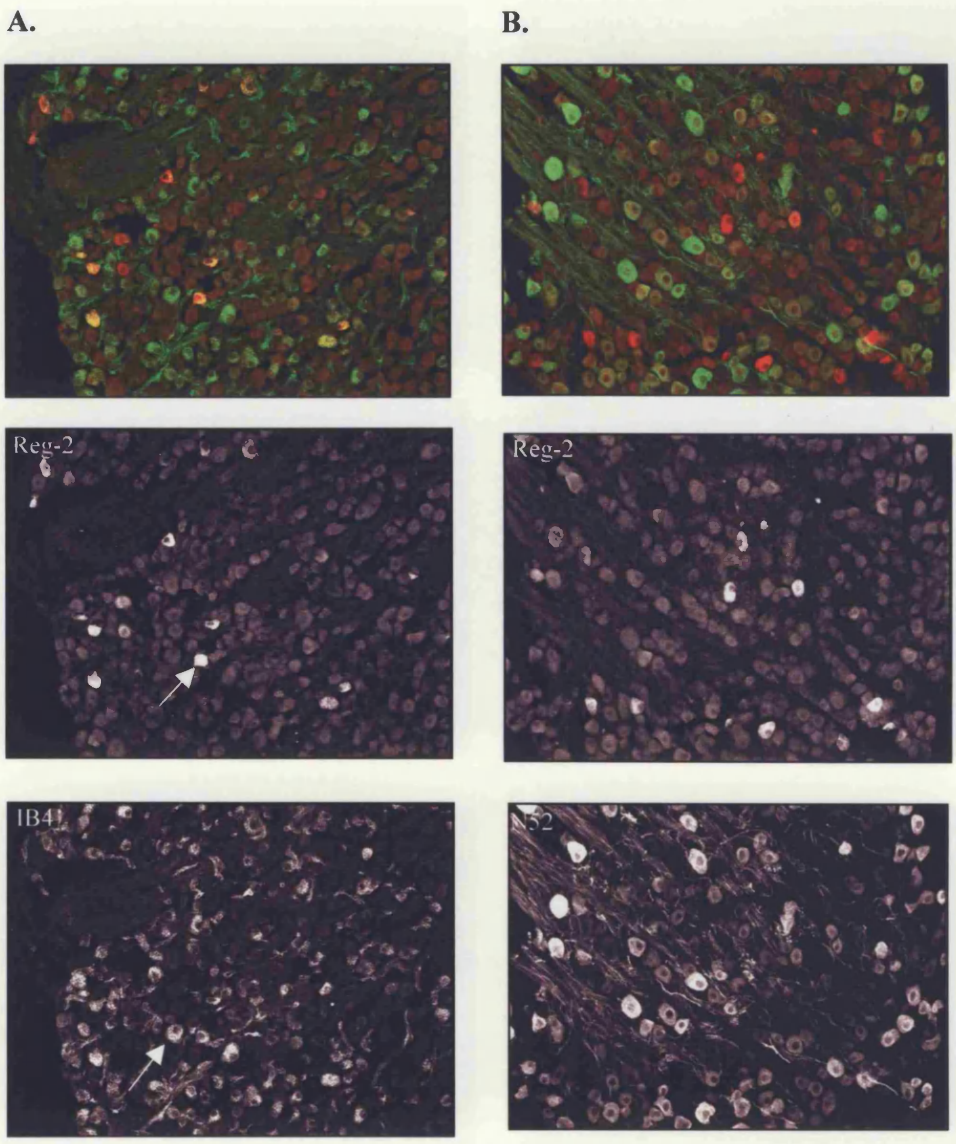


Figure 6.2. Pattern of Reg-2 expression in the neonate rat DRG (contralateral to sciatic nerve axotomy, 24 hour timepoint). Co-localisation with either IB4 (**A**) or N52 (**B**) is shown. Co-localisation can be observed in the cases where red and green merges resulting in a yellow coloured cell. Note how a large proportion of Reg-2 expressing cells co-localises with the IB4 binding population. The arrows point out an IB4 positive Reg-2 expressing cell.

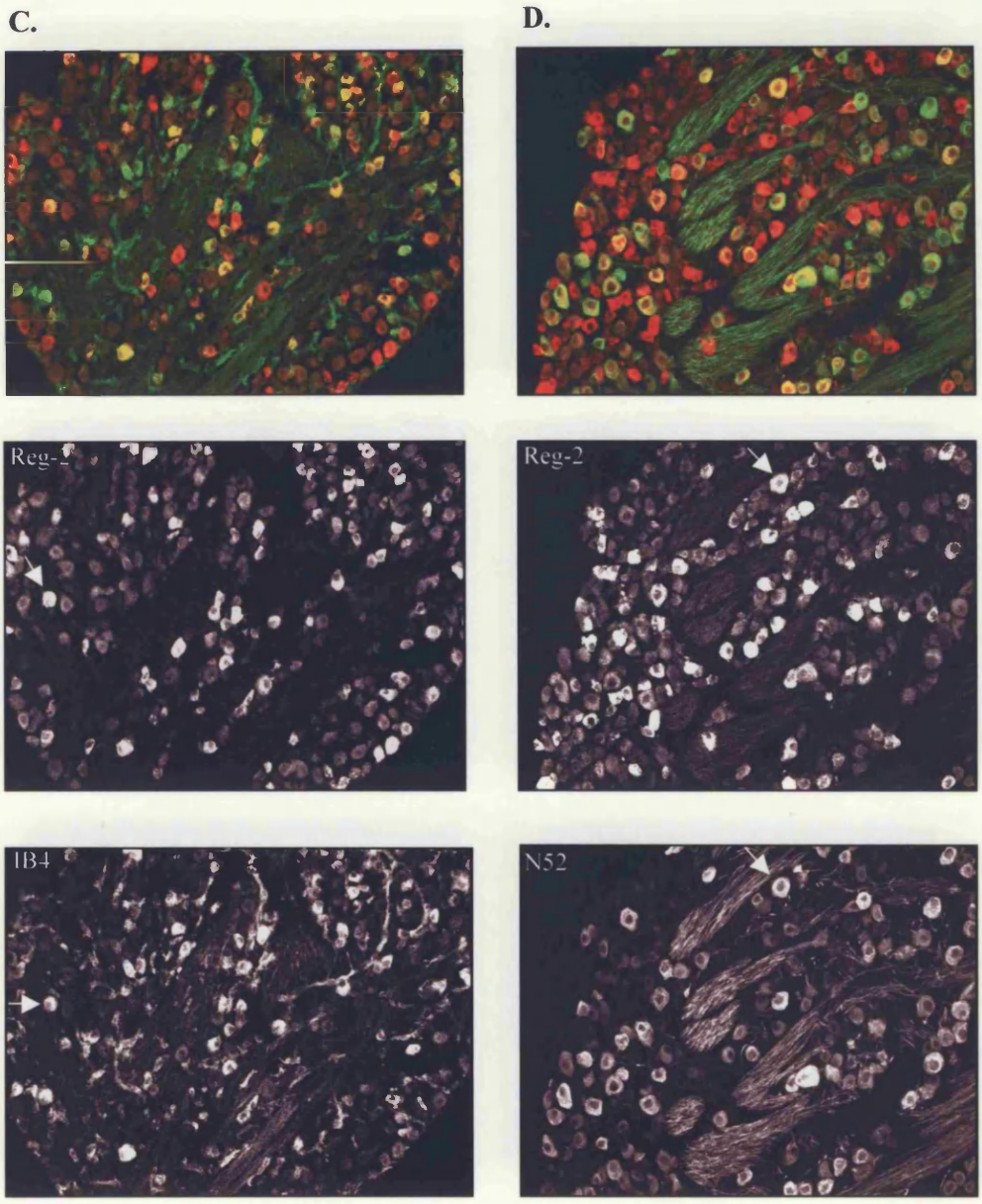


Figure 6.2 (continuation) Pattern of Reg-2 expression in the neonate rat DRG, 24 hours after sciatic nerve axotomy. Co-localisation with either IB4 (**C**) or N52 (**D**) is shown. Note that many Reg-2 expressing cells co-localise with IB4 or N52. The arrows point out examples of co-localisation.

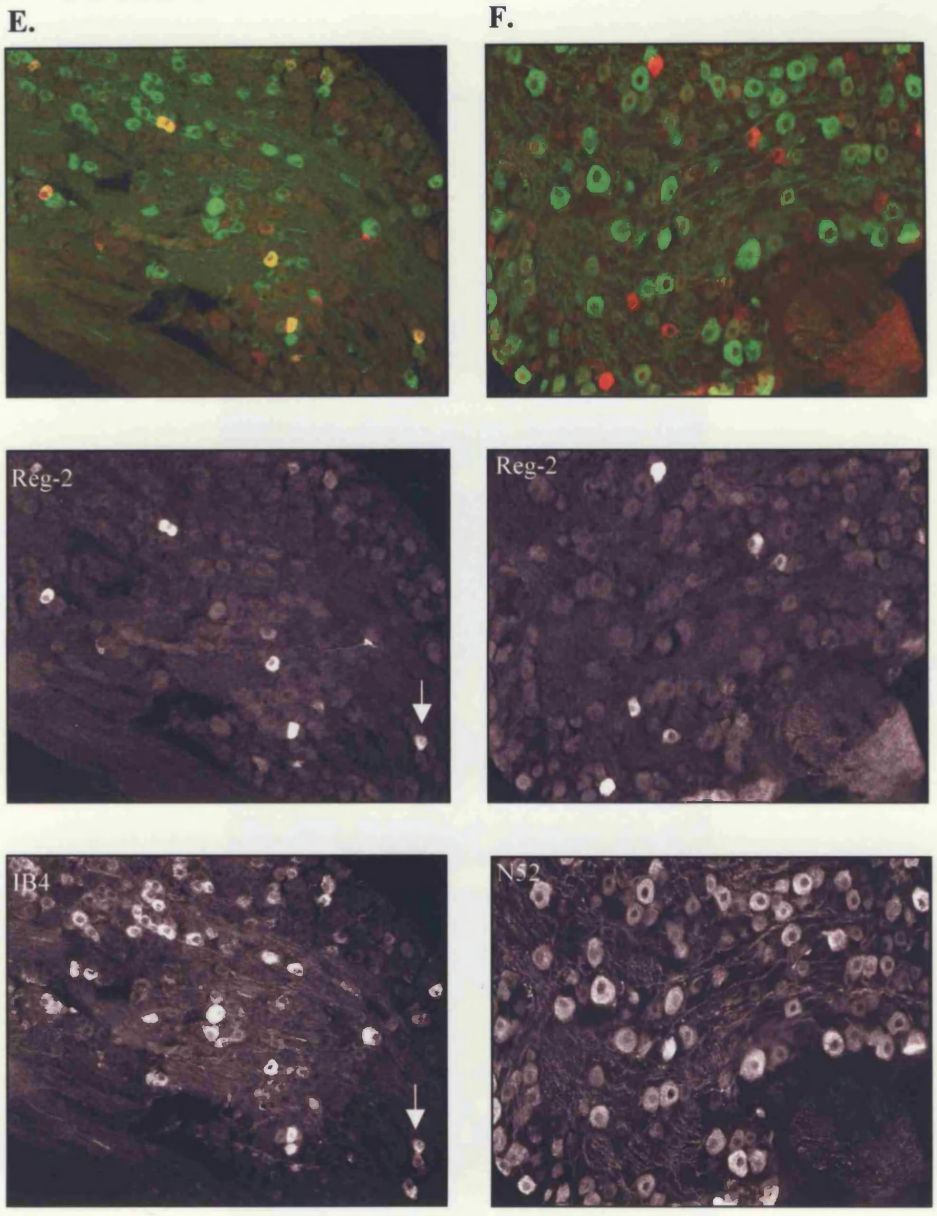


Figure 6.2 (continuation) Pattern of Reg-2 expression in the neonate rat DRG (contralateral to sciatic nerve axotomy, 5 day timepoint). Co-localisation with IB4 (**E**) or N52 (**F**) is shown. Note how all Reg-2 expressing profiles are also IB4 positive (arrows show one example).

G.

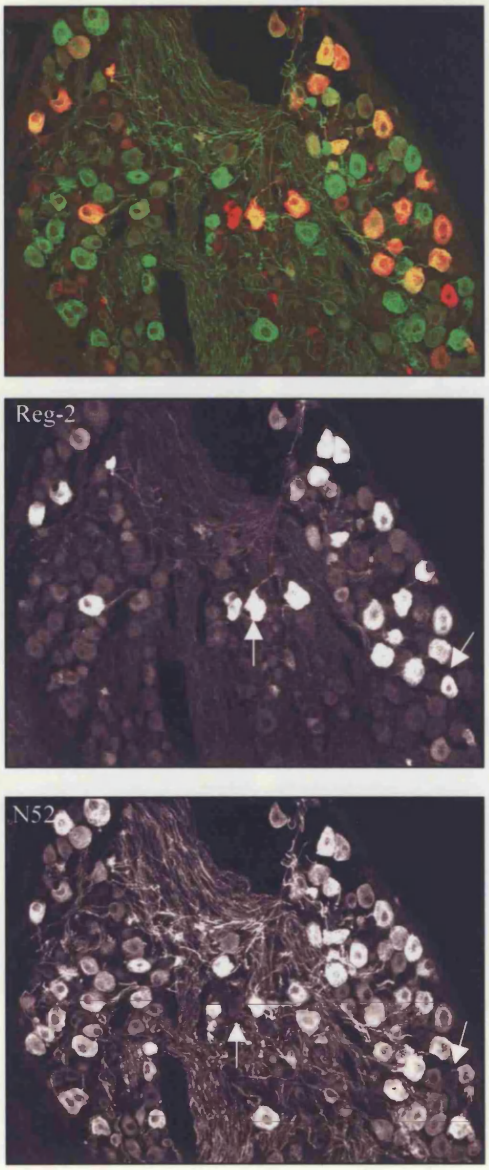


Figure 6.2 (continuation) Pattern of Reg-2 expression in the neonate rat DRG, 5 days after sciatic nerve axotomy. The vast majority of Reg-2-expressing cells are also N52 positive. Note the shift in the size of Reg-2 expressing cells from the 24 hour timepoint (in figure 6.3, C and D). The arrows point out the Reg-2 expressing cells which are not N52 positive.

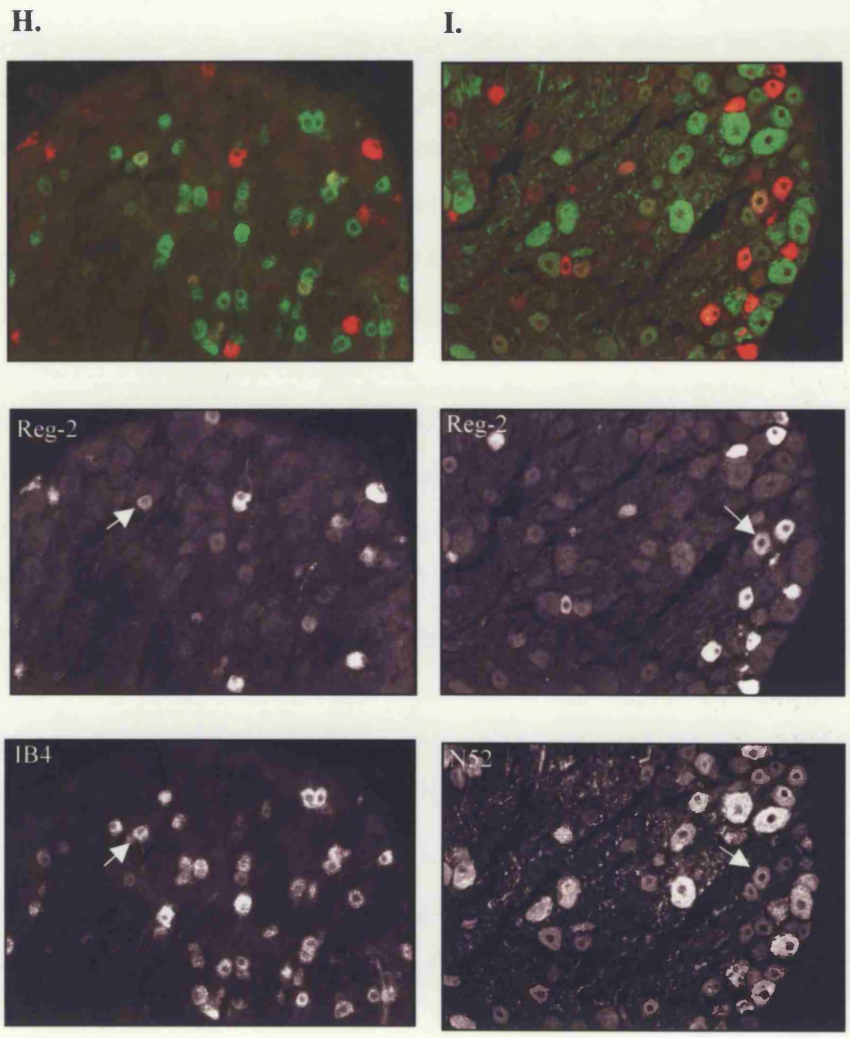


Figure 6.2 (continuation) Pattern of Reg-2 expression in the neonate rat DRG, 21 days following sciatic nerve axotomy. Co-localisation of Reg-2 expression with IB4 (**H**) or N52 (**I**) is shown. Note that for each case only a very small proportion of co-localising cells can be found (pointed out by arrows).

6.4. Discussion

We have shown that following sciatic nerve axotomy in the neonate rat, Reg-2 expression in sensory neurons in the L4-L5 DRG is rapidly up-regulated and it appears to follow a dynamic pattern changing between biochemically distinct sensory neuron pouplations. At 24 hours, a large proportion of Reg-2 expressing cells are small-diameter neurons whereas at 5 days post-axotomy Reg-2 expression has switched to a larger sized population. When Reg-2 was delivered to L4-L5 DRG neurons via an HSV-1 minimally disabled vector it failed to demonstrate a neuroprotective effect.

Reg-2 is known to be expressed in a subset of sensory neurons during development from embryonic day 15 up to postnatal day 5 and in the adult following sciatic nerve transection, crush or peripherally induced inflammation (Livesey *et al.* 1997; Davis *et al.* 2002). The sensory neuron populations expressing Reg-2 are of importance in understanding the role of this factor in the DRG. Based on size and biochemical markers, DRG sensory neurons can be classified into subpopulations (reviewed by (Alvares and Fitzgerald 1999). Small diameter neurons which comprise ~70% of the DRG neuronal population, have unmyelinated axons and are considered nociceptive in function. These can be further divided into two groups, peptide expressing and non-peptide expressing. The former express calcitonin-related gene product (CGRP) and are responsive to nerve growth factor (NGF) whereas the latter express cell surface glycoconjugates recognised by the lectin IB4 and are responsive to GDNF. The IB4 binding population comprises ~ 30% of the total DRG neuronal population. Interestingly, this group of cells switches from dependence on NGF in embryonic life to GDNF in postnatal life (Molliver *et al.* 1997). Indeed, in the two weeks following birth, IB4 neurons down-regulate expression of the NGF receptor tyrosine kinase (TrkA) whilst also up-regulating the expression of the GDNF receptor tyrosine kinase, RET (Molliver *et al.* 1997). The large diameter neuron population have myelinated axons and are considered mechanoreceptive and proprioceptive in function. These cells can be identified using an antibody directed to high molecular weight neurofilament protein, such as N52.

In the neonate rat DRG, following sciatic nerve transection, Reg-2 expression is up-regulated within 24 hours. This result was unexpected, given that in motor neurons, Reg-2 is rapidly down-regulated in the same experimental conditions (Livesey *et al.* 1997). Although Reg-2 expression in developing sensory neurons has been shown to be dependent on cytokines of the LIF/CNTF family acting through a receptor complex containing LIFR (Livesey *et al.* 1997), the precise factor that controls Reg-2 expression in the DRG is still unknown. Our results suggest it could be negatively regulated by a peripherally derived factor, the removal of which would result in the observed up-regulation of Reg-2 expression. Alternatively, Reg-2 expression might be positively regulated by injury-induced factors in the DRG.

Reg-2 expression in the neonate primary sensory afferents co-localises mainly (~90%) with the IB4 neuronal population. The pattern of Reg-2 expression observed following sciatic nerve axotomy was similar to that reported for the adult DRG following sciatic nerve injury (Davis *et al.* 2002). We observed a switch in the neuronal population expressing Reg-2, from mainly small nociceptive sensory neurons to large non-nociceptive neurons. Davis *et al* observed that following sciatic nerve transection in the adult rat, within 24 hours >95% of Reg-2 expressing sensory neurons were IB4 positive and by 5 days post-axotomy the Reg-2 expressing profiles had switched to the larger diameter sensory neuron population (Davis *et al.* 2002). Following sciatic nerve crush, similar results were reported except that only a proportion (~50%) of the Reg-2 expressing neurons were IB4 positive at 24 hours (Davis *et al.* 2002). Interestingly, our results are more similar to those obtained following sciatic nerve crush as 24 hours post axotomy in the neonate rat only a proportion of Reg-2 expressing cells were IB4 positive (60%). Similarly to the situation observed in the adult rat, following the complete shift towards the large sized neuronal population observed at 5 days, by 21 days there were few large sized neurons expressing Reg-2. In fact, at this timepoint, Reg-2 profiles appeared to be small to medium in size. There are several possibilities concerning the role of Reg-2 in the sensory neuron. On the one hand, the up-regulation of Reg-2 expression in a situation where ~50% of DRG sensory neurons die (Yip *et al.* 1984), is not suggestive of a neuroprotective role of this factor. On the other hand, the fact that at 21 days post axotomy there are Reg-2 expressing cells which have obviously

escaped the apoptotic fate of other axotomised sensory neurons, suggests that Reg-2 may be involved in protecting those cells. The role of Reg-2 in the DRG, might be a local one in addition to the one already suggested at the site of nerve regeneration stimulating Schwann cell division (Livesey *et al.* 1997). As proposed in the adult, the expression of Reg-2 by the nociceptive IB4 sensory neuron population suggests a role as an inflammatory mediator (considering it is exclusively expressed by this population after peripheral inflammation in the adult rat) (Davis *et al.* 2002). The reason for the shift from small to large cells is unknown. The adult studies showed that Reg-2 expression in the large cells co-localised in part with galanin and NPY, which are factors up regulated in response to nerve injury (Davis *et al.* 2002). Reg-2 has been shown to exert an autocrine/paracrine survival effect on motor neurons (Nishimune *et al.* 2000). Reg-2 produced by the IB4 population may act in a paracrine fashion on large diameter cells causing them to up-regulate Reg-2. The rapid up-regulation of Reg-2 expression following sciatic nerve transection besides acting locally in the DRG might also be delivered to the site of injury in an attempt to aid regeneration by causing Schwann cell proliferation.

Given the possibility of Reg-2 acting as a survival factor in the injured sensory neuron, we tested the effect of further up-regulating the expression of Reg-2 using a modified HSV-1 vector. From the counts of surviving axotomised neurons we established that the control vector 1764pR20.9 was having a toxic effect on the neonatal sensory neurons. This is in all identical to the situation observed in the neonatal rat motor neurons following infection with a vector of the same backbone, 1764 pR20.5 (see previous chapter). Nevertheless, in our working model we confirmed the previously reported neurotrophic properties of CNTF (Lo *et al.* 1995). Remarkably, the neuroprotective effects following transduction with the virus containing NGF-CNTF (the construct for secretable CNTF) were such that there was no difference from the animals injected with vehicle. In other words, 27% of neurons that would otherwise die when axotomised and transduced with control vector 1764pR20.9, were protected. In contrast, Reg-2 did not have any survival mediating effects when sensory neurons were transduced with 1764pR20.9/Reg-2. Thus, in this particular working model Reg-2 cannot protect neonate sensory DRG neurons against axotomy and/or viral toxicity.

Reg-2 in the neonate DRG does not appear to have a survival role contrarily to the initially suggested by the expression of Reg-2 at 21 days post axotomy. Thus, in the DRG, Reg-2 might act as an inflammatory mediator (given its expression by the nociceptive IB4 population) and even as a regenerative factor. Reg-2 *per se*, however, does not mediate survival effects in the DRG.

Chapter 7

General discussion

Chapter 7- Discussion

At a time when the human genome project is expanding our knowledge of gene sequences, the discrepancy between the level of sequence information and the level of knowledge of the function of the encoded proteins is becoming increasingly apparent. As a result, the understanding of the function of proteins encoded by the thousands of genes in the human genome is one of the current challenges of biological research.

Protein function can be partly inferred from sequence homology to other characterised proteins, cellular localisation and pattern of expression, structure and post-translational modifications. The ultimate proof of protein function, however, tends to come from experiments which involve the use of antibody blocking, the study of factors affecting the role of the protein in question, and/or genetic manipulation. The *in vivo* manipulation of gene expression, in particular, offers a strategy which enables the analysis of the function of a protein within the context of a living organism. Genetic manipulation can be carried out by gene overexpression (using transgenic mice or gene transfer techniques) or by the disruption of gene expression (using knockout animals or antisense technology). In the case of gene transfer, viral vectors have been explored as vehicles to mediate gene delivery to somatic post-mitotic cells of animals of any postnatal age.

Reg-2 is a secreted protein that belongs to the Reg gene family and is expressed in developing sensory and motor neurons (Livesey *et al.* 1997). Reg-2 expression is dependent on cytokines of the LIF/CNTF family and may be an obligatory intermediate in the survival pathway of these cytokines in cultured developing motor neurons (Nishimune *et al.* 2000). In the adult rat, Reg-2 is expressed by all regenerating motor neurons and by a subset of regenerating DRG neurons (Livesey *et al.* 1997). In cultured developing motor neurons, the addition of recombinant Reg-2 to the media results in increased cell survival. As a result, Reg-2 has been referred to as a neurotrophic factor for motor neurons (Nishimune *et al.* 2000). However, proof of the role of Reg-2 as a neurotrophic factor would ultimately require the demonstration of this fact by genetic manipulation.

Herpes simplex virus is a promising vector for gene delivery to the nervous system. It is naturally neurotropic, infecting ganglionic sensory neurons where it can remain latent in the nucleus for the lifetime of the host. The propensity of HSV to enter a latent state provides a means by which long-term gene delivery might be achieved. Experimental infections with wild type virus have shown that HSV-1 can also infect motor neurons (Ugolini 1992). The ability to undergo retrograde axonal transport to reach the cell nucleus offers the possibility for gene delivery to motor neurons and/or primary sensory afferents following peripheral administration. For these reasons our laboratory has chosen HSV-1 as a vector for gene delivery in the nervous system.

The work presented in this thesis aimed to investigate the neurotrophic properties of Reg-2 using HSV-1 vectors to mediate the up-regulation of Reg-2 expression in an animal model of neuronal death.

Work in chapter 3 assessed a number of attenuated HSV-1 vectors (with different combinations of essential and non-essential gene deletions) *in vivo* for their ability to give gene delivery to motor and sensory neurons after peripheral administration. This resulted in the identification of optimal vectors for gene delivery to motor neurons and to DRG sensory neurons in an experimental frame appropriate for use in a model of neuronal death. Chapter 4 was concerned with the use of the selected vectors in a model system of neuronal death, as well as with the modification of the vectors for use in experiments to test the neuroprotective effects of Reg-2. In chapter 5, a vector expressing Reg-2 was tested for its capacity to mediate motor neuron survival in a neonate axotomy model of cell death. Chapter 6 analysed the pattern of Reg-2 expression in the neonate animal DRG following nerve axotomy. Reg-2 expression was up regulated using an attenuated HSV-1 vector in the neonate axotomy model, and the survival-promoting effects of Reg-2 in the DRG were analysed.

Results in chapter 3 indicated that gene delivery to motor neurons and to DRG sensory neurons following peripheral administration of HSV-1 vectors depended on the age and the animal species used. The same vectors were tested in rats and mice at different ages. There were three possible models in which the vectors could be used (1) transgenic mice

(2) ventral root avulsion in the adult rat and (3) neonate rat nerve axotomy. Two types of vector were tested, one replication competent (1764), deficient for ICP34.5 and for VP16 and one replication incompetent, identical to 1764 but further deleted for essential genes ICP4 and ICP27. The expression cassettes used were either located in the endogenous LAT region (pR19lacZ, in the replication incompetent vector) or in the nonessential gene UL43 (pR20.5 and pR20.9, in the replication competent vector).

In the adult mouse, replication competent vectors mediated high-level gene expression in motor neurons but only in the short-term, despite longer lasting gene expression in the DRG. Within two weeks of viral administration, cell infiltrates indicative of an inflammatory response were present in the spinal cords. Extensive cellular debris could be observed and no transgene expressing motor neurons were present. The vector that elicited the strongest inflammatory reaction, 1764pR20.9 was tested in mice lacking B and T lymphocytes (SCID mice) to assess the contribution of the adaptive immune response to the decrease in transgene expression observed. In the DRG of SCID mice stable gene expression continued throughout latency with no drop-off in expression. Thus in the DRG sensory neuron, the decrease in gene expression after transduction with the replication competent vector 1764pR20.9 was very likely due to viral clearance by the adaptive immune response. Although some transgene expressing motor neurons were present at two weeks in the spinal cords of the SCID mice, none were observed at later time points and so it can be deduced that the adaptive immune response was at the most only partly responsible for the decrease in transgene expression observed. Other factors involved in limiting transgene expression in the adult mouse motor neuron might be promoter shutdown, vector cytotoxicity, or the innate immune response (discussed below). The spinal motor neuron and the DRG sensory neuron are different cell types and hence the length of gene expression following transduction with a disabled HSV-1 vector does not necessarily correlate in both cells. It should of course be kept in mind that HSV-1 is naturally neurotrophic for the primary sensory afferent.

Sciatic nerve inoculation of the replication incompetent vector in the adult mouse at high titres resulted in gene expression in motor neurons at both early and late times after

infection. This is the first report of long-term sustained gene expression in motor neurons following peripheral administration with a disabled HSV-1 virus. Similarly to the situation observed in the DRG, it appears that the LAT P2 region was capable of conferring long-term properties onto the heterologous CMV promoter. This vector is deficient for IE gene expression and the only gene expressed at significant levels in non-complementing cells is the IE/E gene ICP6 (Lilley *et al.* 2001), previously shown to be non-cytotoxic in fibroblasts at least (Johnson *et al.* 1994). Indeed, we showed that the replication-incompetent vector was non-cytotoxic to adult mouse motor neurons *in vivo* and did not trigger a host-mediated immune response as no pathological changes were detected in the spinal cords. This is an important factor in the interpretation of the results described with replication competent vectors in the same animal model. The host-mediated immune response against the 1764 vectors is thus not directed against the structural components of the virus or to its *lacZ* transgene. It seems therefore that replication competent HSV vectors trigger the adult mouse immune response due to *de novo* HSV gene expression. In order to investigate the possibility of promoter shutdown in the motor neuron, SCID mice should be tested for motor neuron transduction with a 1764 vector containing the pR19lacZ expression cassette shown to drive long-term gene expression when in a replication incompetent background.

In chapter 3 results were also presented indicating the lesser susceptibility of the Lewis rat to infection with HSV-1 vectors in comparison to Balb/c mice. It has been suggested that the non-adaptive immune response is important in the resistance of animals to infection by HSV-1 (Simmons *et al.* 1992). Indeed, this could be the case in the Lewis rat as in the adult animal only the replication incompetent vector was capable of mediating gene delivery to either DRG or motor neurons. The replication competent vectors may have been destroyed by the non-adaptive immune system before even reaching the axon terminals. The replication incompetent vector, deficient in the expression of IE genes and thus potentially immunogenic gene products might possess a stealth-like quality with regard to the immune system of the adult Lewis rat. This vector was capable of driving transgene expression for at least two weeks without a decrease in expression. This result is in accordance with studies in our laboratory in which the same vector was directly injected into the adult rat spinal cord and long-term expression was observed in the transduced

motor neurons ((Lilley *et al.* 2001), F. Groutsi, unpublished observations). Further evidence of an involvement of the adult rat immune sytem in limiting HSV-1 infection was provided by the results obtained in the neonate rat and discussed below.

In the neonate rat, the same replication competent vectors that could not mediate gene delivery in the adult rat were able to promote transgene expression. Indeed, sciatic nerve inoculation of 1764pR20.5 mediated very high-level motor neuron transduction. The other replication competent vector tested, 1764pR20.9, mediated high-level sensory neuron transduction after footpad injection. These results further suggested the involvement of the immune response in limiting HSV-1 vector mediated gene delivery in the adult rat, as the immunologically immature neonate animals would lack such a response. Thus, following the peripheral inoculation of HSV-1 disabled vectors in the adult, mouse or rat, the host immune response appears to be involved to different extents in limiting transgene expression. In order to circumvent this response, vectors that are replication incompetent and impaired for IE gene expression can be used or alternatively, neonate or immunocompromised animals can be used.

The gene transfer mediated by the replication competent vectors in the neonate rat was of a temporary nature, lasting less than one week. The rapid decrease in transgene expression observed was attributed to vector cytotoxicity as the spinal cords of animals one week after injection with vector 1764pR20.5 revealed extensive cell loss on the side ipsilateral to vector injection. Indeed, neonatal motor neurons and DRG sensory neurons are more vulnerable to damage than their adult counterparts as they are still in the end-stage of development (Oppenheim 1991). Even with the replication incompetent vector, gene delivery to DRG and motor neurons was short-term. This was unexpected, as the same vector in the adult rat gave stable gene expression for at least two weeks after peripheral inoculation. The short-term gene delivery observed may be the result of cytotoxicity, not from the virus itself as it is severely impaired for any IE gene expression, but rather from a carry-over of material from the complementing cells in which the virus was grown on. Although this would also be the case in the adult animal, the neonatal neuron is more susceptible to damage. Alternatively, following virus-induced stress, it is conceivable that

the neonatal neuron may alter gene expression in such a way that causes promoter shutdown. Thus when neonate animals are used, the potential cytotoxicity of HSV vectors might be exacerbated as neonate neurons are particularly sensitive to toxic insults.

Based on the information generated in chapter 3 concerning the gene delivery capabilities of each vector in different animals, the most suitable vectors to up-regulate Reg-2 in a model of neuronal death were chosen. It was decided to use 1764pR20.5 and 1764pR20.9 for the delivery of Reg-2 to the axotomised neonate rat motor neuron and DRG sensory neuron respectively. Firstly, both vectors were capable of transduction of a large proportion of neurons. Secondly, although gene expression was only maintained for less than one week, sciatic nerve axotomy in the neonate rat results in the death of ~60% of spinal motor neurons (Pennica *et al.* 1996; Vejsada *et al.* 1998) and 40-50% of DRG sensory neurons (Yip *et al.* 1984) within one week. In chapter 4, it was found that both vectors were capable of mediating gene expression in the neonate rat axotomy model.

A problem in the use of these vectors in the neonate rat is the cytotoxicity caused by the vectors themselves. In the event that the up-regulation of Reg-2 expression were not to protect neurons from axotomy-induced death, it was necessary to ensure that the chosen vectors (1764pR20.5 and 1764pR20.9) could demonstrate the neurotrophic properties of a well-established factor. As result, in addition to Reg-2 expressing vectors a CNTF expressing vector was constructed. Indeed, CNTF has been shown to promote motor neuron (Sendtner *et al.* 1990) and DRG sensory neuron (Lo *et al.* 1995) survival after neonate sciatic nerve axotomy. *In vivo* Reg-2 expression mediated by vectors 1764pR20.5 and 1764pR20.9 was confirmed by immunohistochemistry. In the case of CNTF, expression was confirmed *in vitro*.

Chapter 5 described the testing of the neurotrophic properties of Reg-2 in the neonate rat motor neuron following axotomy. Following sciatic nerve axotomy in the neonate rat, motor neurons have been shown to downregulate Reg-2 expression (Livesey *et al.* 1997). Chapter 5 demonstrated that following transduction with 1764pR20.5/Reg-2, axotomised motor neurons expressed high-levels of Reg-2 as detected by immunohistochemistry.

When assessing motor neuron survival after sciatic nerve axotomy, it was also confirmed that the replication competent vector 1764pR20.5 was cytotoxic. In a scenario of axotomy and infection with the 1764pR20.5 vector, motor neurons died due to the axotomy and/or the virus-induced damage. Vectors expressing either Reg-2 or CNTF were capable of rescuing ~10% of motor neurons that would otherwise die due to axotomy and/or virus cytotoxicity. There was no significant difference between the motor neuron survival obtained for Reg-2 and for CNTF. Given that the motor neurons are in this case dying due to an interplay of factors, it was not possible to correlate the effects of Reg-2 with those of CNTF directly, as each factor could protect against different insults, either against the axotomy, the virus induced cytotoxicity or both. In order to further define the neuroprotective effects of Reg-2 in comparison to CNTF, the experiment should be repeated in the absence of axotomy to determine whether Reg-2 and/or CNTF are able to protect against the damage induced by the virus. Nevertheless, using a disabled HSV vector it was possible to confirm the neuroprotective effects of CNTF on the developing rat motor neurons. Furthermore, the HSV-1 mediated up-regulation of Reg-2 expression also had a neuroprotective effect on the damaged neonatal rat motor neuron adding to the evidence that Reg-2 can act as a neurotrophic factor for motor neurons (see (Nishimune *et al.* 2000; Livesey *et al.* 1997)). Here it was suggested that the neuroprotective role of Reg-2 *in vivo* may involve the same survival pathways shown to mediate its neurotrophic properties *in vitro* (the phosphatidylinositol-3-kinase/Aky kinase and/or the NF-κB pathway) (Nishimune *et al.* 2000).

Chapter 6 studied the role of Reg-2 in the DRG sensory neuron. Although it is well known that Reg-2 is downregulated in neonatal rat motor neurons following nerve axotomy, the situation concerning Reg-2 expression in the DRG under the same circumstances was unknown. Chapter 6 surprisingly showed that in DRG sensory neurons Reg-2 expression is up-regulated following sciatic nerve axotomy. The implications of this result are significant. As this is the opposite of what is observed in the motor neuron, the function of Reg-2 in DRG neurons might be different to motor neurons. Furthermore, questions are raised concerning the mechanism of regulation of Reg-2 expression. In neonate DRG sensory neurons Reg-2 might be negatively regulated by a target derived factor in contrast

to the suggested positive regulation of Reg-2 in the neonate rat motor neuron. In the future, it would be of interest to establish if the sensory neurons expressing Reg-2 in the DRG are those which have or have not been axotomised given that only a percentage of neurons in the L4 and L5 DRG project to the sciatic nerve.

Given that Reg-2 expression in the adult rat DRG after peripheral nerve injury follows a dynamic pattern, the pattern of Reg-2 expression in the neonate rat DRG was also investigated. Here it was found that in the resting neonatal DRG, the vast majority of Reg-2 expressing sensory neurons (~90%) belonged to the small diameter IB4 binding population. After sciatic nerve axotomy, the pattern of Reg-2 expression was similar to that observed in the adult DRG, switching between different sensory neuron populations. At 24 hours post axotomy, 60% of Reg-2 expressing cells were small sized IB4 positive sensory neurons whereas at 5 days post axotomy 80% of Reg-2 expressing cells were large sized sensory neurons. By 21 days post axotomy, Reg-2 expressing cells appeared medium in size and only a small proportion co-localised with either IB4 or N52. In order to infer any results concerning the autocrine/paracrine effects of Reg-2 in the neonatal axotomised DRG sensory neuron it would be of interest to repeat the experiment with labelling of the sciatic motor neurons using a tracer. In this way, whether Reg-2 expressed by the IB4 population was acting on the large sized neuronal population in a paracrine fashion, would be determined. In the adult rat some evidence of paracrine effects were observed after sciatic nerve axotomy. Davis et al showed that 5% of Reg-2 expressing cells at 5 days post axotomy (neurons of large diameter) did not co-localise with tracer and were thus uninjured sensory neurons not of sciatic origin (Davis *et al.* 2002).

In the case that Reg-2 were to have a survival effect on a proportion of the DRG sensory neurons (maybe the ones observed expressing Reg-2 at 21 days after sciatic nerve axotomy), the effect of further up-regulation of Reg-2 in the DRG following axotomy was tested using a disabled HSV-1 vector. As occurs when using a vector of the same backbone in motor neurons, 1764pR20.9 was shown to be cytotoxic to the neonatal DRG sensory neurons. Nevertheless, when the vector was modified to deliver CNTF, significantly less sensory neurons died when assessed one week later. The vector modified to express Reg-2,

on the other hand, was unable to rescue any sensory neurons. Therefore, Reg-2 does not appear to exert any significant neuroprotective effects on damaged neonatal DRG sensory neurons.

The significance of the cellular switching, the up-regulation of Reg-2 following injury, and the probable lack of survival effects of Reg-2 on DRG sensory neurons is not perfectly clear at this point. DRG sensory neurons and the spinal motor neurons are very different cell types, of different developmental origin, located within different environments, and of different function. Hence, it is feasible that Reg-2 may not have the same function or effect in both cell types. Reg-2 might for example function as a neurotrophic factor for motor neurons but not for DRG sensory neurons. However, as the pathways shown to be required by Reg-2 in the *in vitro* developing motor neuron do exist in the DRG sensory neuron, this is somewhat surprising. The fact that the Reg-2 receptor is as yet unknown may explain this apparent anomaly. Indeed, it could be that although many DRG sensory neurons have the capacity to express Reg-2, only a small proportion of the neurons express the receptor. A similar situation was observed with IL-6 in the neonate rat DRG (Their *et al.* 1999). Although IL-6 was expressed and secreted by neonate rat DRG sensory neurons, recombinant IL-6 only promoted neuronal survival when its receptor IL-6R was also added to the DRG cultures (Their *et al.* 1999). The neonate rat DRG sensory neuron did express the IL-6 receptor but at levels too low to allow trophic effects of IL-6 (Their *et al.* 1999). For Reg-2, a similar situation could also be the case. If Reg-2 *per se* does not actually have a role in neonate sensory neuron survival, it might be that it is simply involved in pathways responding to other factors, be it a "lesion" factor pathway or of an inflammatory response nature (as suggested by Davis *et al* in the adult rat) (Davis *et al.* 2002).

The results presented in this thesis have identified disabled HSV-1 vectors capable of gene delivery to spinal ganglia and motor neurons following peripheral administration. Furthermore, these HSV-1 vectors were exploited to investigate the putative neurotrophic properties of the Schwann cell mitogen, Reg-2. HSV-1 vectors were genetically modified to mediate the up-regulation of Reg-2 expression in a neuronal death context to assess for neuronal survival. Reg-2 appeared to be neuroprotective in the developing motor neuron

but not in the DRG sensory neuron. Further information concerning the role of Reg-2 *in vivo* will doubtless come from the recently obtained knockout animal.

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