The Effect of Brn3a and Zhangfei on the Nerve Growth Factor Receptor, trkA.

A Thesis submitted to the College of Graduate Studies and
Research in Partial Fulfillment of the Requirement for the Degree
of Doctor of Philosophy in the Department of Veterinary Microbiology,
University of Saskatchewan, Saskatoon

By
Ximena Paola Valderrama Linares

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts therefore for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of Department of Veterinary Microbiology, WCVM
University of Saskatchewan
52 Campus Drive
Saskatoon, Saskatchewan S7N 5B4, Canada

ABSTRACT

Herpes simplex viruses (HSV) establish latent infections in sensory neurons of their host and are maintained in this state by little understood mechanisms that, at least in part, are regulated by signalling through nerve growth factor (NGF) and its receptor tropomyosin related kinase, trkA. Previous studies have demonstrated that Zhangfei is a transcriptional factor that is expressed in differentiated neurons and is thought to influence HSV replication and latency. Zhangfei, like the HSV *trans*-activator VP16 and Luman, binds the ubiquitous nuclear protein host cell factor (HCF) inhibiting the ability of VP16 and Luman to initiate HSV replication.

Recently, Brn3a, another neuronal factor thought to influence HSV latency and reactivation was found to possess an HCF-binding domain and could potentially require HCF for activity. The neuronal POU IV domain protein, Brn3a, among its many regulatory functions has been described as an enhancer of the NGF receptor trkA, during development in mouse. I therefore investigated the possible link between Brn3a, TrkA, NGF signaling, HCF, Zhangfei and HSV-1 latency and reactivation. I hypothesized that Zhangfei would also suppress the ability of Brn3a to activate the expression of TrkA and that this would have an impact on NGF-TrkA signaling and, consequently on HSV-1 reactivation from latency.

My first study determined which Brn3a/trkA promoter interactions were important for trkA transcription. I constructed a plasmid that contains 1043 base pairs of genomic sequences that extend from 30 nucleotides upstream of trkA coding region. In contrast to previous data, a short 190 bp region that lies proximal to the trkA initiation codon was sufficient for Brn3a *trans*-activation in NGF-differentiated PC12, Vero and human medulloblastoma cells. At least two portions of the 190 bp fragment bind to Brn3a. In addition, Brn3a increased

endogenous levels of trkA transcripts in PC12 cells and initiated trkA expression in medulloblastoma cells, which normally do not express trkA.

The second step was to determine the effects of HCF and Zhangfei association with Brn3a on trkA *trans*-activation. I found that Brn3a required HCF for activating the trkA promoter and that Zhangfei has a suppressive effect over Brn3a-trkA activation in non-neuronal cells. In sympathetic neuron-like NGF-treated PC12 cells, Zhangfei did not suppress the ability of Brn3a to activate the TrkA promoter, however, Zhangfei was able capable of inducing the expression of TrkA in the absence of Brn3a. Both Brn3a and Zhangfei induced the expression of endogenous trkA in PC12 cells.

Since Vero and PC12 cells are not from human origin I wanted to examine the ability of Zhangfei to induce trkA transcription in medulloblastoma cells, that because of its tumor nature do not express trkA. TrkA transfections in these cells have shown to drive them to cell arrest or apoptosis. Since Zhangfei is not express in medulloblastoma tumors I then used ONS-76 medulloblastoma cells as a model to determine Zhangfei's envolvement in the NGF-trkA signaling pathway.

I show herein that in ONS-76 medulloblastoma cells resveratrol, an inducer of apoptosis and differentiation, increased the expression of Zhangfei and trkA as well as Early Growth Response Gene 1 (Egr1), a gene normally activated by NGF-trkA signalling. ONS-76 cells stop growing soon after treatment with resveratrol and a portion of the cell undergo apoptosis. While the induction of Zhangfei in resveratrol-treated cells was modest albeit consistent, the infection of actively growing medulloblastoma cells with an adenovirus vector expressing Zhangfei mimicked the effects of resveratrol. Zhangfei activated the expression of trkA and Egr1 and caused these cells to display markers of apoptosis. The phosphorylation of Erk1, an intermediate kinase in the NGF-trkA signaling critical for differentiation, was observed in Zhangfei

infected cells, supporting the hypothesis that Zhangfei is a mediator of trkA-NGF signaling in theses cells leading either to differentiation or apoptosis. Binding of HCF by Zhangfei did not appear to be required for this effect as a mutant of Zhangfei incapable of binding HCF was also able to induce the expression of trkA and Egr1.

In in vivo and in vitro models of HSV-1 latency, the virus reactivates when NGF supply to the neuron is interrupted. Based on the above evidence Zhangfei, in HSV-1 latently infected neurons, would have the ability to prolong a state of latency by inducing trkA expression allowing the activation of NGF-trkA signaling pathway. Since NGF is produced by many cell types it is possible that reactivation is triggered not by a decrease in NGF but by a down-regulation of TrkA expression.Therefore, if Zhangfei expression is suppress the trkA signaling could be interrupted or shifted towards apoptosis signaling, this would allow neuronal HCF-binding proteins like Luman, which can activate HSV IE expression, to initiate HSV IE expression and subsequently viral replication.

ACKNOWLEDGEMENTS

I would like to express my most deepest appreciation to my supervisor professor Dr. Vikram Misra, who showed me that science is solved with simple questions. He provided me with optimism, encouragement and free discussion throughout my program and with his unforgettable daily smile and greeting. I express my gratitude to Dr. Valerie Verge for her valuable advice, time and always so enthusiastic to discuss my research work. Appreciation is extended to the other members of my committee for their time, effort and guidance: Dr. Deborah Haines, Bernhard Juurlink, Dr. David Schreyer and Robert Dunn.

A special thank you to Dr. Norman Rawlings for believing in me, for his support and guidance in the initial stages of my Ph.D. program. I would like to thank Dr. John Gordon for his valuable advice and constant encouragement to pursue an academic career. To my lab members, Philipe, Oksana and specially to Noreen for her technical assistance and friendship. To the graduate students and staff of the Department of Veterinary Microbiology for good conversation, laughter, support and encouragement in my everyday life. To Brian, Vivian, Sharon and Lipin from GMP, for your technical assistance and provision of materials during my research. To Aasma and Pat for their patience, unconditional friendship, for those life comforting conversations and for your support in the highs and lows during my program. You all have certainly made me a better caring and understanding person.

A thank you to my friends and colleagues I had the opportunity to share the years I lived in Saskatoon and supported all my energy driven crazy ideas. To all the people I shared a smile, a coffee, a chat, a story, or just a simple day a thank you. I will certainly treasure each and every moment. My eternal thank you to my husband Marcelo and my three little men, Sebastian, Vicente and Jose, for understanding long hours of work, their unconditional love and patience and overall for being my family.

DEDICATION

I dedicate this research work to my beloved men, Marcelo, Sebastian, Vicente and Jose, who have given me the strength and love during these years. And to my grandmother Hilda Salot who has inspired my life and my great-grandmother Claudia Gassaniga for being my Guardian Angel.

Table of Contents

PERMISSION TO USE i
ABSTRACT ii
ACKNOWLEDGEMENTS v
DEDICATION vi
TABLE OF CONTENTS vii
LIST OF FIGURES xi
LIST OF TABLES xiii
LIST OF ABREVIATIONS xiv

1. INTRODUCTION 1
1.1. Herpes simplex virus 1
1.1.1. Lytic infection 1
1.1.1.1. Initiation of the lytic cycle by VP16, Host Cell Factor and Oct1 2
1.1.1.2. Virion protein 16 (VP16) 2
1.1.1.3. Host Cell Factor (HCF) 3
1.1.2. Latent infection 4
1.1.2.1. Establishment and maintenance of latency 5
1.1.2.1.1. The role of NGF-trkA signaling in the maintenance of Latency 8
1.1.2.2. Reactivation from latency 9
1.1.2.2.1. The role of HCF in reactivation 9
1.1.3. Host proteins that mimic VP16 in its interactions with HCF 10
1.1.3.1. Luman 10
1.1.3.2. Zhangfei 11
1.2. Signaling transduction in neurons in response to NGF 12
1.2.1. Nerve Growth Factor and its receptors 12
1.2.1.1. The role of trkA in neuronal differentiation, survival and development 13

1.2.1.2. Signal transduction by NGF-trkA 14
1.2.1.3. Signal transduction by NGF-trkA in neuronal tumor cells 16
1.2.1.4. The activation of trkA transcription 16
1.2.2. Brn3a, a POU domain containing transcription factor 17
1.2.2.1. Brn3a regulatory roles 17
1.2.2.2. Brn3a and trkA transcription 18
1.3. Rationale, Hypothesis and Objectives 20
1.4. References 22

2. NOVEL BRN3A CIS-ACTING SEQUENCES MEDIATE TRANSCRIPTION OF HUMAN trkA IN NEURONS. 34
2.1. Abstract 35
2.2. Introduction 36
2.3. Materials and Methods 38
2.3.1. Cell Culture 38
2.3.2. Collagen Coated plates 38
2.3.3. Plasmids 38
2.3.4. Transfections and CAT-reporter assay 39
2.3.5. Chloramphenicol acetyl transferase (CAT) assays 40
2.3.6. RNA preparation and Real Time PCR (QPCR) analysis 40
2.3.7. Electrophoretic mobility shift assay (EMSA) 41
2.3.8. Purification of GST-Brn3a fusion protein 42
2.3.9. Antibodies and Immunoblot 43
2.4. Results 44
2.4.1. Brn3a can activate trkA proximal promoter sequences 44
2.4.2. Effect of NGF induced differentiation and exogenous Brn3a on the expression of endogenous trkA in PC12 cells 46
2.4.3. Activation of trkA in medulloblastoma cells 47
2.5. Discussion 49
2.6. Acknowledgements 52

2.7. References 53

3. ZHANGFEI, A NOVEL REGULATOR OF THE HUMAN NERVE GROWTH FACTOR RECEPTOR, trkA. 70
3.1. Abstract 71
3.2. Introduction 72
3.3. Materials and Methods 76
3.3.1. Cell Culture 76
3.3.2. Plasmids 76
3.3.3. Transfections 77
3.3.4. Chloramphenicol acetyl transferase (CAT) assays 77
3.3.5. Antibodies, immunoblot and immunoprecipitation 78
3.3.6. Quantitative real-time PCR (QPCR) analysis 78
3.3.7. Suppression of Zhangfei expression using siRNA 79
3.4. Results 80
3.4.1. Brn3a requires nuclear HCF for efficient activation of the trkA promoter 80
3.4.2. Zhangfei suppresses the ability of Brn3a to activate the trkA promoter in non-neuronal cells 81
3.4.3. The effect of Zhangfei is cell-type and promoter specific 82
3.4.4. Zhangfei activates endogenous expression of trkA in PC12 cells 83
3.5. Discussion 84
3.6. Acknowledgements 87
3.7. References 88

4. INDUCTION OF THE NEURONAL RECEPTOR TYROSINE KINASE, trkA, IN MEDULLOBLASTOMA CELLS BY RESVERATROL AND THE HCF-BINDING PROTEIN ZHANGFEI. 102
4.1. Abstract 103
4.2. Introduction 105

4.3. Materials and Methods 110
4.3.1. Cell Culture 110
4.3.2. Immunoblots 111
4.3.3. Adenovirus vectors expressing Zhangfei 111
4.3.4. RNA preparation and Real Time PCR (QPCR) analysis 111
4.3.5. Apoptotic cells 112
4.4. Results 113
4.4.1. Differentiated medulloblastoma cells show trkA-mediated signalling 113
4.4.2. Expression of Zhangfei in growing ONS-76 cells leads to suppression of growth, induction of trkA and trkA-NGF pathway 114
4.4.3. Zhangfei does not require binding to HCF to induce trkA and Egr1 expression 115
4.4.4. Zhangfei induces the phosphorylation of Erk 115
4.4.5. Resveratrol as well as Zhangfei triggers apoptosis in ONS-76 cells 116
4.4.6. Unlike resveratrol, Zhangfei does not suppress the growth of diploid human fibroblast 116
4.4.7. Resveratrol and Zhangfei inhibit the growth of another medulloblastoma cell-line 117
4.5. Discussion 118
4.6. Acknowledgements 121
4.7. References 122

5. GENERAL DISCUSION AND CONCLUSIONS 138
5.1. References 146

List of Figures

Figure 1.1	Nerve Growth Factor signaling through the tyrosine kinase, trkA.	15
Figure 2.1	The trkA promoter.	57
Figure 2.2	Brn3a activates the trkA promoter in Vero cells.	58
Figure 2.3	Sequences proximal to TrKA coding sequences required for Brn3a activated transcription in Vero cells.	59
Figure 2.4	Brn3a binds directly to trkA proximal promoter sequences.	61
Figure 2.5	Brn3a binding to overlapping oligonucleotides representing the proximal trkA promoter.	63
Figure 2.6	Transcription of trkA and Brn3a in NGF treated PC12 cells.	65
Figure 2.7	The trkA proximal promoter (pTrkA+190) is activated in PC12 cells.	67
Figure 2.8	Brn3a increases trkA transcript levels in medulloblastoma cells.	69
Figure 3.1	Some proteins with HCF binding motifs.	93
Figure 3.2	Brn3a requires HCF for efficient activation of the trkA promoter.	95
Figure 3.3	Zhangfei suppresses the ability of Brn3a to activate the trkA promoter in non-neuronal cells.	97
Figure 3.4	The effect of Zhangfei is dependant on the promoter and cell-type	99
Figure 3.5	Brn3a and Zhangfei activate endogenous trkA in PC12 cells.	.
Figure 3.6	Model for the role of Zhangfei in unstressed and stressed neurons.	100
Figure 4.1	Resveratrol suppresses the growth of ONS-76 medulloblastoma cells.	101
Figure 4.2	Treatment with resveratrol leads to an increase in transcripts for Zhangfei, trkA, Egr1 and NGF.	128

Figure 4.3 Ectopic expression of Zhangfei in growing ONS-76 cells leads to the suppression of their growth and expression of trkA and Egr1. 130
Figure 4.4 Zhangfei leads to the phosphorylation of Erk1 in ONS-76 cells 132
Figure 4.5 Resveratrol and ectopic Zhangfei induce apoptosis in ONS-76 cells. 133
Figure 4.6 Effect of resveratrol or Zhangfei on growth of human diploid fibroblasts (MRC5) and medulloblastoma cells (UW228). 135
Figure 4.7 Potential role for Zhangfei in the establishment and maintenance of HSV latency, a model. 137

List of Tables

Table 2.1.	Genomic human TrkA promoter constructs.	39
Table 2.2.	Overlapping oligonucleotides corresponding to 190 bp of the TrkA proximal promoter sequence used for electromobility shift assay.	42

List of Abbreviations

ATF4	Activation transcription factor 4
β-gal	β-Galactosidase
bp	base pair
b-Zip	basic leucine zipper
Brn3a	brain specific homeobox/POU domain protein 3A
cAMP	cyclic adenosine monophosphate
CAT	choramphenicol acetyltransferase
CMV	cytomegalovirus
c-Myc	cell proliferation mediator
CRE	cAMP response element
CREB	cAMP response element binding protein
D-MEM	dulbeco's modified eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DRG	dorsal root ganglia
EDTA	ethylenediaminetetraacetic acid
Egr1	early growth response protein 1
ELISA	enzyme-linked Immunosorbant Assay
EMSA	electrophoretic Mobility Shift Assay
ER	endoplasmic Reticulum
ERK	Extracelullar signal-regulated kinase
GABP	GA-binding protein
GAP-43	Growth associated protein-43
GST	glutathione-s-transferase
Ham's F12	Ham's F12- nutrient mixture medium
HBM	HCF-binding motif
HCF	Host cell factor
HIPK2	Homedomain interacting protein kinase 2
HSV	Herpes simplex virus

HSV-1	Herpes simplex virus type 1
IE	Immediate Early
IFN-γ	interferon - gamma
kDa	kilo Dalton
KIf7	Kruppel like factor 7
Krox20	Homolog to EGR2 in humans
L	late
LAT	Latency associated transcript
MAPK	Mitogen associated protein kinase
MEK1	methyl ethyl ketone 1
Miz-1	Myc-interacting protein
mRNA	messenger ribonucleic acid
NCS	Newborn Calf Serum
NF-kB	Transcription factor 65
NGF	Nerve Growth Factor
Oct1	Octamer-binding transcription factor 1
OD	Optical Density
ONS-76	Human medulloblastoma cells
PAGE	Polyacrylamide Gel Electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PC12	rat pheochromocytoma cells
PLC	phospholipase C
Pen/Strep	penicillin and streptomycin
P13-K	phosphatidylinositol 3-kinase
PMSF	phenylmethyl-sulfonyl fluoride
QPCR	quantitative PCR
RNA	ribonucleic acid
SDS	sodium Dodecyl Sulfate
siRNA	small interfering RNA
SNAP25	Synaptosomal-associated 25 kDa protein

SnRNP	small nuclear ribonucleo protein
SSEA3	Stage specific embryonic antigen 3
Sp1	trans-acting transcription factor 1
TG	Trigeminal ganglion
TrkA	Tropomyosin related kinase A
UPR	unfolded protein response
UPRE	Unfolded protein response element
VP16	Virion protein 16
Xbp1	Tax-responsive element-binding protein 5

1. INTRODUCTION

1.1 Herpes Simplex virus

Herpes Simplex virus type 1 (HSV-1) causes recurrent lesions on epithelial surfaces (reviewed in (Roizman, 2001, Efstathiou and Preston, 2005). The virus uses two complementary strategies to avoid immune surveillance while maintaining itself in its primary host and continuing the chain of infection to other hosts. Initial infection of epithelial cells by HSV leads to active replication of the virus. Destruction of adjoining cells and the response of the host to infection lead to characteristic “cold sores”. The virus also infects sensory neurons innervating the site of viral replication. The viral genome is delivered by retrograde axonal transport to the neuronal cell body in sensory ganglia where it establishes a latent infection. Periodic reactivation from latency, in response to a variety of stressors, leads to replication of the latent virus in the neuron. Anterograde axonal transport of virus to epithelial surfaces and replication in epithelial cells causes recurrent lesions and potential dissemination to other hosts.

1.1.1 Lytic infection

Expression of viral genes during lytic infection in epithelial cells is temporally regulated so that the approximately 80 viral genes involved can be categorized as Immediate Early (IE or α), Early (E or β) and Late (L or γ) depending on when in the replicative cycle they are expressed. Since the efficient expression of E and L genes depends on IE gene products, these regulatory proteins are crucial for the progression of the lytic cycle.

1.1.1.1 Initiation of the lytic cycle by VP16, Host Cell Factor and Oct-1

Initiation of the transcription of the IE genes is induced by the assembly of a multi-protein complex made up of virion protein 16 (VP16) and two cellular proteins: Oct-1 and Host Cell Factor (HCF). Oct-1 is a Pit-1, Oct-1, Unc-86 (POU) homology domain containing transcription factor that is involved in histone, immunoglobulin and small nuclear RNA gene expression. HCF regulates cellular proliferation and may have other roles as well. VP16 is synthesized in the late stages of lytic infection and is incorporated into the tegument of the virion. Upon subsequent infection of cells by the virus, VP16 is released into the cell where it combines with HCF. HCF promotes the nuclear translocation of VP16 (LaBoissiere et al., 1999) and acts as a co-activator of VP16 (Luciano and Wilson, 2002). The VP16-HCF heterodimer then recognizes the POU domain of Oct-1 bound to TAATGARAT (where R is a purine) motifs present in multiple copies in the promoters of all HSV IE genes. The activation domain of VP16 interacts with several components of the transcription machinery leading to the expression of IE genes. Immediate Early proteins subsequently regulate the expression of the E and L genes (reviewed in Roizman, 2001).

1.1.1.2 Virion Protein 16 (VP16)

VP16 is an essential structural component of HSV-1 and is also a potent activator of viral IE gene expression. Recruitment of VP16 to the DNA binding complex and its ability to activate transcription are distinct activities located in two discrete domains (Triezenberg et al., 1988, Greaves and O'Hare, 1989). The amino-terminal portion of the protein contains structural elements that interact with HCF and Oct-1. The HCF binding motif (HBM) of VP16 is a four amino acid domain, EHTY, which as D/EHxY (where x can be any of several amino acids) is conserved in several other viral and cellular HCF binding proteins. The acidic carboxy-terminal portion of VP16 is required for transcriptional activation since truncations or insertions within the intact protein

abolished activation without detectable effect on complex formation (Greaves and O'Hare, 1989, Werstuck and Capone, 1989, Regier et al., 1993).

1.1.1.3 Host Cell Factor (HCF)

Host Cell Factor is a chromatin-associated protein that is present in most cells and its structure is conserved in the metazoa. The mature form of HCF is derived from a 2035 amino acid precursor, which is processed by internal cleavage at a number of specific HCF repeats to give rise to polypeptides with molecular masses that range from 110 to 150 kDa. After cleavage, most of the amino- and carboxy-terminal portions of the protein remain stably associated by non-covalent interactions (Wilson et al., 1993, Kristie et al., 1995). The amino terminus of the protein is made up of six related repeats of a motif of about 50 amino acids located within residues 1-380 (Kristie et al., 1995, Hughes et al., 1999). This Kelch repeat (named after a *Drosophila* protein) forms the beta-propeller structure required for its interaction with VP16. In addition, HCF also contains an activation domain in its carboxyl terminus that contributes to VP16 trans-activation but is not required for complex formation.

Although HCF was initially discovered as a component of the VP16-induced transcriptional complex it is important for many cellular processes including cell-cycle progression (Goto et al., 1997), cytokinesis (Julien and Herr, 2003), chromatin structure modification and RNA splicing (Ajuh et al., 2002).

A single amino acid substitution in the HCF Kelch domain in a hamster cell line results in a temperature sensitive phenotype (tsBN67). At the non-permissive temperature the cells cease proliferation after a few cell divisions and arrest in the G_o/G_1 phase (Goto et al., 1997) implying that HCF is required for cell cycle progression (Wysocka et al., 2001, Reilly et al., 2002). Consistent with this role, HCF also interacts with other proteins that have cell cycle-regulatory roles. The cell cycle regulatory factor Myc-interacting protein (Miz-1) causes cell cycle arrest at G_1, and is antagonized by direct interaction with the

cell proliferation mediator c-Myc. HCF functions in a manner similar to c-Myc in modulating Miz-1 function (Piluso et al., 2002).

HCF is associated with spliceosomes and in tsBN67 cells, at the non-permissive temperature, it fails to associate with splicing snRNPs causing inefficient spliceosome assembly and defective splicing. The arrest of these cells in the G_0/G_1 phase of the cells cycle at the non-permissive temperature suggets a connection between the involvement of HCF in splicing and cell-cycle progression (Ajuh et al., 2002).

Several cellular proteins, like VP16, interact with the Kelch domain of HCF. These proteins all contain the four amino acid HBM (D/EHxY) present in VP16. These proteins include, Luman/LZIP/CREB3 (Freiman and Herr, 1997, Lu et al., 1997) and Zhangfei (Lu and Misra, 2000b), E2F4 (Luciano and Wilson, 2003, Knez et al., 2006) and Krox20 (Luciano and Wilson, 2003). Like VP16 these proteins require HCF for at least some of their functions. Several other potential cellular HCF-binding proteins, including the neuronal transcription factor Brn3a, possess an HBM (Luciano and Wilson, 2003) but their actual binding to HCF has not been confirmed. More recently, proteins like GA-binding protein (GABP) (Vogel and Kristie, 2000), Sp1 (Gunther et al., 2000), a protein phosphatase (Ajuh et al., 2000), the nuclear receptor co-regulatory factor PGC-1 (Lin et al., 2002) and the cell cycle regulatory factor Myc-interacting protein (Miz-1) (Piluso et al., 2002) have also been shown to associate to HCF. These proteins do not possess the D/EHxY HBM and associate with parts of HCF other than the Kelch domain.

1.1.2. Latent infection

HSV-1 infects endings of sensory neurons innervating the epithelial site of primary infection. The process involves fusion of the viral envelope with the axon plasma membrane (Antinone et al., 2006). While the viral envelope and its embedded glycoproteins are not required for transport of the virion to the

neuronal cell body, they are required for infection of the neuron. The glycoprotein E, for instance, has been shown to be essential for targeting the virus to the axon initial segment (Wang et al., 2005a). The HSV-1 nucleocapsid and tegument are transported to the neuronal cell body in sensory ganglia by cellular transport motors traveling along microtubules (Bearer et al., 2000). Several proteins of the tegument and capsid interact with components of the dynein motor complex in yeast two-hybrid or in *in vitro* assays (Kamal et al., 2000, Chuang et al., 2001, Douglas et al., 2004, Antinone et al., 2006). Of these, the capsid protein VP26 interacts at the axon tips with dynein light chains (Douglas et al., 2004) suggesting an important role for VP26 in loading the virion onto dynein. However, more recent studies (Dohner et al., 2006), have shown that VP26 is dispensable for transport. Studies using a reconstituted capsid transport system that allows the tracking of fluorescent virions from which various components of the virion have stripped, suggest that proteins of the inner tegument, VP1-3 and UL37 play a key role in transport (Wolfstein et al., 2006). The outer tegument, comprising VP13/14, VP22, vhs and US11, is probably removed during the initial stages following de-envelopment. Interestingly, VP16, which is regarded as a component of the outer tegument, remains associated with the inner tegument (Wolfstein et al., 2006). The tenacious association of VP16 with the capsid is consistent with the observations of Bearer et al (Bearer et al., 2000) who demonstrated retrograde axonal transport of GFP-VP16 containing HSV virions. These authors also found VP16 to be associated with capsids despite detergent treatment.

1.1.2.1 Establishment and maintenance of latency

Following infection of the neuron, the HSV genome is released into the nucleus and may either enter the lytic cycle or is directed to a latent state (reviewed in (Preston, 2000)). While reasons for the suppression of the lytic cycle leading to latency have not been clearly established, several lines of evidence suggest that a block in IE gene expression leads to the establishment of latency (reviewed in (Preston, 2000, Efstathiou and Preston, 2005). This

failure to induce IE genes is thought to be the result, in part, of the virus inability to assemble the VP16-HCF-Oct-1 complex necessary during lytic replication for the activation of IE gene promoters. There are several potential reasons for this: the absence or inability of VP16 to reach the neuronal nucleus during axonal transport, low levels of Oct-1 (reviewed in Valyi-Nagy et al. 2006), cytoplasmic sequestering of HCF in sensory neurons (Kristie et al., 1999) and the presence in sensory neurons of other members of the POU family, such as Oct-2, that can compete with Oct-1 for binding to TAATGARAT elements (Lillycrop et al., 1991 , Lillycrop et al., 1994).

In contrast to lytic replication in epithelial cells the viral genome in latently infected neurons is almost completely quiescent. During latency viral proteins are not detected, nevertheless a proportion of neurons in sensory ganglia harboring latent HSV express a family of viral RNAs, called the latency-associated transcripts (LATs) (Lachmann, 2003), these are encoded by the segment of HSV genome that is complementary to the IE gene ICP0 (Lachmann, 2003). While LAT contain open codon reading frames no proteins derived from these sequences have been reliably detected in neurons.

HSV-1 LAT is believed to enhance the establishment and maintenance of latency either by repressing lytic gene expression or by protecting the infected neuron from death. Infection of neuronal cell clones that stably express LAT results in greatly reduced levels of transcripts for HSV-1 IE genes like ICP0, ICP4 and ICP27 (Garber et al., 1997, Peng et al., 2005). As well, LAT deleted HSV strains establish latency in fewer neurons in the mouse and rabbit models of HSV neuropathogenesis (Thompson and Sawtell, 1997).

Prevention of apoptosis by LAT has been mapped to two regions of the gene. These regions are able to interfere with caspase-9 induced apoptosis and thereby can influence the number of latently infected neurons (Jin et al., 2003, Kent et al., 2003). Recently, LAT was shown to code for a microRNA (miRNA)

with an anti-apoptotic role (Gupta et al., 2006). The LAT miRNA accumulates in cells transiently transfected with the LAT gene fragment or infected with a wild-type strain of HSV-1. miR-LAT exerts its anti-apoptotic effect by downregulating Transforming Growth Factor β, a potent inhibitor of cell growth and an inducer of apoptosis, and SMAD3, which regulates transcription of target genes that have important roles in diverse cellular processes.

In contrast to lytic replication, during latent infection the HSV genome is assembled into nucleosomal structures. Nucleosomal chromatin-mediated eukaryotic genes activation or repression is regulated by histone modification. The LAT gene promotes the assembly of heterochromatin on viral lytic gene promoters during latency (Wang et al., 2005b, Amelio et al., 2006a). The only gene that is associated with hypoacetylated active chromatin during latency is LAT and the LAT promoter is insulated from the hyperacetylated lytic cycle genes (Amelio et al., 2006b).

During the establishment of latency in both *in vivo* and in tissue culture systems, the ends of the viral genome become joined, and there is strong evidence that the latent genome persists as a nonreplicating circular episome (Jackson and DeLuca, 2003, Strang and Stow, 2005).

While viral proteins are not consistently detected in LAT-expressing neurons when biochemical techniques are used, sensory ganglia harbouring latent HSV contain long lasting CD8+T cells specific for a dominant epitope of the HSV glycoprotein B (Khanna et al., 2003). These cells produce γ-interferon and other cytokines that can suppress viral replication. These observations have prompted a hypothesis that the latent HSV genome is not silent but that IE and E viral proteins are produced at low levels. These proteins, especially gB, stimulate CD8+ T cells which, via γ-interferon maintain latency by suppressing viral replication (Khanna et al., 2004).

1.1.2.1.1 The role of NGF-trkA signaling in the maintenance of latency

When sensory ganglia from mice latently infected with HSV are removed from the animal and cultured with cells permissive to HSV replication, latent virus is reactivated (Hill et al., 1997). Reactivation can be prevented if the explanted ganglia are maintained in medium containing the neurotrophin, Nerve Growth Factor (NGF) and the effect of NGF can be reversed by treatment with specific antibodies (Wilcox and Johnson, 1988). In addition, latent virus can be maintained in NGF-treated primate and rodent neurons (Wilcox et al., 1990) and in rat pheochromocytoma (PC12) cells (Block et al., 1994). In these cells, HSV can be reactivated by NGF-deprivation or treatment with antibodies against either NGF or the NGF receptor, the tropomyosin related kinase (trkA). Virus can also be reactivated in these cells by treatment with chemicals such as hydroxydopamine and colchicine (Wilcox et al., 1990) that either destroy the NGF-responsive sympathetic nerve terminal or block the ability of the NGF-trkA signaling endosomes to be retrogradely transported, thereby interfering with the ability of NGF to signal in the affected neurons. These observations suggest that expression of HSV lytic cycle genes in latently infected neurons may be suppressed through the action of NGF and trkA (Huang et al., 1999, Huang and Reichardt, 2003).

Neurons in sensory ganglia are a diverse population of cells. Approximately 40-50% mature sensory neurons express trkA and are dependant on NGF for function (Verge et al., 1992, Yang et al., 2000). Although all sensory ganglia cells are capable of supporting productive HSV infection, some populations are more permissive to latent infection than others. Three subpopulations of sensory neurons have been associated with HSV latency in *in vitro* or *in vivo* studies (Wilcox and Johnson, 1988, Laycock et al., 1994, Yang et al., 2000, Jones, 2003): trkA-expressing neurons that transduce nociceptive information, those that express the developmentally regulated globoseries glycoconjugate SSEA3 and neurons that contain antigen recognized by the monoclonal antibodies A5 and KH10. These populations are not mutually

exclusive. The A5 immunoreactive neurons are most likely to result in latent viral infection and more than 70% of neurons harbouring latent HSV express trkA and respond to NGF (Yang et al., 2000).

There is evidence that indicates that the levels of NGF supplied by the innervated target cells can mediate NGF-dependent effects on HSV-1 latency. NGF-ligand interaction and signal transduction could also be involved in the ability of HSV to sense changes that favor reactivation (Grimes et al., 1997) and interruption of NGF-trkA signalling by suppression of trkA expression might have the same consequence as NGF deprivation (Verge et al., 1992).

1.1.2.2 Reactivation from latency

In humans many stimuli such as physical or emotional stress, hyperthermia, exposure to UV light, menstruation, physical trauma (reviewed (Roizman, 2001) surgical manipulation of the trigeminal root ganglion (Tenser, 1998) and critical illness with prolonged intubation (Bruynseels et al., 2003) have been implicated as triggers for the reactivation of latent HSV. Similar stimuli can induce reactivation in animal models of HSV pathogenesis (Sawtell and Thompson, 1992). While many stimuli or physiological conditions are recognized as inducers of HSV reactivation, the biochemical mechanisms that link the stimuli to molecular events in the neuron that lead to the viral replication are poorly defined.

1.1.2.2.1 The role of HCF in reactivation

Although there is little evidence to support this concept, it is logical to assume that the mechanism for the initiation of the viral replicative cycle in the neuron during reactivation from latency is similar to the mechanism that initiates the replicative process in epithelial cells. The three initiating components for IE gene expression in epithelial cells are VP16, HCF and Oct-1. Latently infected neurons contain no detectable VP16 and while Oct-1 is important for IE expression it is not essential for IE induction (Nogueira et al., 2004). HCF, in

contrast, does appear to be important for IE expression both in epithelial cells and neurons (Narayanan et al., 2005) and may be a critical component of the switch from latency to viral replication. In addition to facilitating VP16-mediated IE gene induction it also mediates activation by GA binding protein (GABP) (Vogel and Kristie, 2000), and Sp1 (Gunther et al., 2000), two cellular factors with response elements in the promoters of IE genes. Depletion of HCF in cells suppresses IE gene activation by VP16 as well as by GABP and Sp1 (Narayanan et al., 2005).

While HCF is a nuclear protein in most cells it is located in the cytoplasm of sensory neurons in unstressed mice and stress as well as stimuli that induce reactivation in the mouse model of latency induce translocation of the protein to the nucleus (Kristie et al., 1999).

1.1.3 Host proteins that mimic VP16 in its interaction with HCF

Since viral proteins, including the virion associated IE gene activator VP16, are absent from latently infected neurons it is likely that cellular factors lead to the activation of IE gene expression and subsequently to virus replication in the neuron. And, since HCF appears to be required for reactivation (see above) it is likely that neuronal HCF-binding proteins substitute for VP16 in the initiation of viral lytic gene expression. Our laboratory has identified two basic leucine zipper (b-Zip) containing transcription factors, Luman and Zhangfei, that, like VP16 bind HCF and appear to play a role in HSV latency and reactivation (Akhova et al., 2005).

1.1.3.1 Luman

Luman (also called L-Zip or CREB3), was initially identified in a yeast two-hybrid selection system as a cellular HCF-binding protein (Freiman and Herr, 1997, Lu et al., 1997). Luman is a type II endoplasmic reticulum (ER) imbedded b-Zip protein and a member of the CREB/ATF family of transcription factors (Lu and Misra, 2000a). Luman is released from the ER by regulated

proteolytic cleavage to relocate to the nucleus where the protein can bind to cyclic AMP response elements (CRE) and unfolded protein response elements (UPRE) and activate transcription from promoters containing them (Lu and Misra, 2000a, Raggo et al., 2002, Misra et al., 2005). These include CRE in promoters of the HSV IE gene ICP0. In addition, Luman, by inducing the expression of Herp, is able to promote cell survival against ER stress-induced apoptosis (Liang et al., 2006).

Luman interacts with HCF through the tetrapeptide DHTY which is homologous to the EHAY binding sequence of HCF with VP16. While HCF is present in the nucleus of most cells, it colocalizes with Luman in the cytoplasm of neurons (Lu et al., 1997). While transcripts for Luman are present in many tissues, the protein has only been identified in neurons of the trigeminal ganglion where it associates, in the cytoplasm, with HCF (Lu et al., 1997).

1.1.3.2 Zhangfei

Zhangfei was also discovered as a binding partner for HCF in a yeast two-hybrid screen of a human cDNA library (Lu and Misra, 2000b). It possesses an HBM and binds HCF both *in vitro* and *in vivo*. Zhangfei suppresses the activity of both Luman (Misra et al., 2005) and VP16 (Akhova et al., 2005) to activate transcription. A mutant of Zhangfei that cannot bind HCF was impaired in its ability to suppress Luman and VP16 suggesting that HCF is required for the effect. The mechanism by which Zhangfei exerts its effect is not known.

Zhangfei is a b-Zip domain containing protein although its basic region lacks a crucial asparagine residue required in other b-Zip transcription factors for binding DNA. Perhaps as a consequence of this, it does not, as a homo-dimer, associate with known binding sites for b-Zip factors and neither does it in *in vitro* assays activate promoters containing these elements (Lu and Misra, 2000b). Recently, however, Zhangfei was shown to dimerize with the b-Zip transcription factor ATF4 and enhance its binding to the CRE (Hogan et al.,

2006). This suggests a role for Zhangfei in the activation of the MEK1-ATF4 signaling pathway. Zhangfei can also dimerize with the b-Zip protein Xbp1 (Chauhan, unpublished). Unlike ATF4-Zhangfei dimers, the association of Zhangfei with Xbp-1 leads to the suppression of its ability to activate gene expression.

Zhangfei and Luman have incompatible b-Zip domains (Vinson et al., 2002) and do not associate with each other through these motifs. These proteins do not appear to bind each other if they are co-synthesized along with HCF *in vitro* (Misra et al., 2005). This suggests that if suppression of Luman by Zhangfei requires that the two proteins associate with each other, cellular proteins must act as a bridge.

Zhangfei suppresses the ability of VP16 to activate the expression of the IE protein ICP0 and, consequently, in Zhangfei expressing cells all temporal classes of HSV proteins (IE, E and L) and progeny virus are reduced (Akhova et al., 2005). As with Luman, the association between Zhangfei and VP16 can not be shown although Zhangfei appears to have a profound effect on the ability of VP16 to form a multiprotein complex involving HCF and Oct-1 on TAATGARAT elements on IE promoters.

While Zhangfei mRNA is present in several human tissues and cultured cell-lines of epithelial and fibroblastic origin, the protein can only be detected in neurons in sensory ganglia and in the cerebrum (Akhova et al., 2005).

1.2 Signal transduction in neurons in response to NGF

1.2.1 Nerve Growth Factor and its receptors.

Neurotrophins enhance the viability and differentiation of specific neurons of the peripheral and central nervous system (Kaplan and Miller, 2000). They

exert their effect by binding to two classes of cell surface receptors: members of the tropomyosin related kinase (trk) receptor family (trkA, trkB, trkC), known also as high affinity receptors, and the low affinity p75 neurotrophin receptor, a member of the tumor necrosis factor (TNF) receptor family.

Nerve Growth Factor is one of four members of the neutrophin family and its cognate receptors are trkA and p75. These two receptors can augment or oppose each other. Both are found in most NGF responsive sensory, sympathetic and forebrain cholinergic neurons (Parada et al., 1992, Greene and Kaplan, 1995, Kaplan and Miller, 2000, Huang and Reichardt, 2003). The p75 receptor can transmit positive and negative signals regulating survival and death events. trkA receptors transmit positive signals such as enhanced survival, growth and differentiation of sensory nociceptive and sympathetic neurons (Tusher et al., 2001, Ma et al., 2003). p75 can upregulate survival signals when NGF secretion is low by enhancing the formation of high affinity binding sites between NGF and trkA (Mahadeo et al., 1994). NGF has also been shown to be necessary for the maintenance of HSV latency in sensory neurons (see section 1.1.2.1.1) and its expression pattern may play a critical role in the development of specific neuronal cell types (Fedtsova and Turner, 1995, Xiang et al., 1996, Artinger et al., 1998, Chiarugi et al., 2002).

1.2.1.1 The role of NGF-trkA in differentiation, survival and development

In the peripheral nervous system trkA is expressed primarily in small to medium diameter nociceptive sensory neurons of trigeminal ganglia, dorsal root ganglia and in sympathetic neurons (Martin-Zanca et al., 1990, Crowley et al., 1994). In the central nervous system trkA is found primarily in basal forebrain cholinergic neurons (reviewed in (Lei and Parada, 2007)). trkA-NGF interactions in these cells control cell fate, differentiation, survival, proliferation and axonal growth. trkA expression in nociceptive neurons and some sympathetic neurons continues after birth and signaling through trkA is thought to be important for the survival of sympathetic neurons and maintenance of the

differentiated phenotype in both these neuronal populations. Absence of trkA during development of neurons, results in loss of approximately 75% of neurons. Studies in trkA knock-out mice have shown insensitivity to heat and pain and greatly reduced numbers of small to medium diameter neurons in trigeminal and dorsal root ganglia (Fagan et al., 1996). Mutations in the trkA receptor or its ligand NGF also lead to the loss of neurons in the trigeminal ganglion due to apoptotic death (Huang et al., 1999). In addition, NGF delivered to sensory neurons at the site of injury associated with diseases such as Type 2 diabetes, is being examined as a therapeutic modality (Sasaki et al., 2004, Walwyn et al., 2006).

1.2.1.2 Signal transduction of NGF-trkA

The activation of trkA is critical for initiating NGF signal transduction (Huang and Reichardt, 2003). NGF binds to trkA molecules causing their dimerization and autophosphorylation of tyrosine residues in the Trk cytoplasmic domain (Zhou et al., 1994) (Fig.1). trkA phosphorylated residues (Y^{490}, Y^{674}, Y^{675}, Y^{751}, and Y^{785}) serve as anchors for binding and activating downstream signalling molecules that coordinate neuronal survival and differentiation (Kaplan and Miller, 2000, Yuan and Yankner, 2000). These include the Ras-Erk, PI3 kinase-Akt and PLCγ-MEK1/2 pathways. Tyrosine residues at positions 490 and 785 appear to be important for activating pathways in sensory neurons (Yuan and Yankner, 2000, Huang and Reichardt, 2003). Phosphotyrosine 490 interacts mainly with Shc, which provides mechanisms for the activation of the Ras-Erk dependent gene transcription and neurite growth pathway. Ras also activates RhoA, which in turn inhibits $p21^{CIP}$–induced cell arrest. Phosphotyrosine 751 interacts with the PI3-Akt kinase for cell survival. This pathway is mediated either by stimulation of pro-survival factors Bcl-xL and NF-κB or by the inhibition of apoptosis through the Caspase cascade (Kaplan and Miller, 2000, Yuan and Yankner, 2000, Shimoke et al., 2005). Phosphotyrosine 785 interacts with PLCγ and like the phosphotyrosine 490-Shc mediated signalling it also activates MEK1/2 induced cell growth. MEK1/2 is,

however, phosphorylated by Protein kinase C (PKC). NGF can stimulate cell survival by preventing apoptosis induced by a variety of stimuli. These include inhibition, by activation of the PI3K pathway (Shimoke et al., 2004, Shimoke et al., 2005) of ER stress induced apoptosis (inactivation of caspase-3, -9, –12).

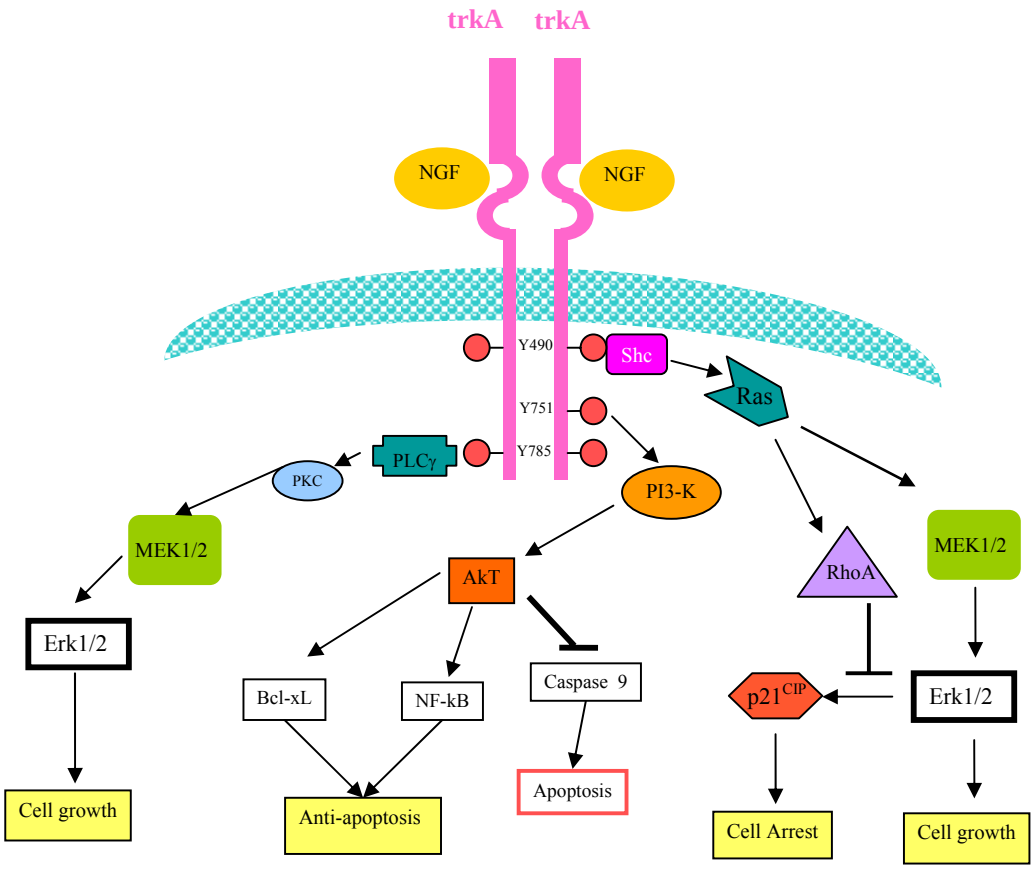


Figure 1.1. Nerve growth factor signaling through the receptor trkA. Representation of trkA signaling pathways found in neuronal cells of different origin. Signal transduction causes phosphorylation of downstream proteins that will induce (→) or suppress (⊥) gene expression.

1.2.1.3 Signal transduction by NGF-trkA in neuronal tumor cells

In addition to the role of NGF-trkA signalling in preserving sensory neurons, this pathway may be important in regulating uncontrolled cell division in neuroblastomas and medulloblastomas, two childhood neuronal cancers. Neuroblastomas are derived from sympathetic neuroblasts of the peripheral nervous system and medulloblastomas are embryonic cerebellar neoplasms of the central nervous system resembling neuro-epithelial tumors. The presence of trkA in neuroblastomas is associated with favorable outcomes in patients and in medulloblastomas trkA expression correlates with increased rates of apoptosis (Tajima et al., 1998, Eberhart et al., 2001, Ohta et al., 2006). Moreover, induction of trkA expression in tumor cells devoid of trkA makes them responsive to NGF. NGF-trkA signaling in these cells activates MAPK and induces cellular immediate early genes (Egr-1, c-Fos). This leads to either their differentiation (Eggert et al., 2000) or to apoptosis through the activation of a novel Ras and/or Raf signaling pathway (Muragaki et al., 1997, Chou et al., 2000). These observations indicate that the response to trkA activation by NGF is cell type specific.

1.2.1.4 The activation of trkA transcription

The 5' non-coding region of the trkA gene in mouse and human has been characterized (Parada et al., 1992, Park et al., 1998). The transcription start site is located 70/71 bp and 30-50 bp upstream of the ATG translation initiation codon in mouse and human respectively. trkA promoter mutants tested in PC12 and N2a cell lines showed that the main regulatory region for trkA transcription in mouse is located in a 150 bp segment immediately upstream of the transcriptional start site (Parada et al., 1992). In the human a 138 bp segment lying 30 bp upstream of the trkA transcriptional start site region is the main activator of transcription in neuroblastoma cell lines (SH-SY5Y, LA-N-6, IMR32) (Park et al., 1998). In both species these regulatory regions form multiple binding complexes indicating the presence of several regulatory elements.

In spite of several transcription factors being associated with the regulation of trkA transcription (e.g. Brn3a, Klf7, SP1, Runx1, HIPK2) (reviewed in (Lei and Parada, 2007)) and there being compelling evidence that trkA is regulated by many physiological and pathological conditions, the molecular mechanisms that regulate trkA expression and the cell type specific signaling pathways that follow activation have not been identified.

1.2.2 Brn3a, POU domain containing transcription factor

Brn3a is a member of the POU IV (or Brn-3) subfamily of POU transcription factors. These factors are classified according to the similarity of their common DNA binding POU domain. The Brn3 subfamily is comprised of Brn3a, Brn3b and Brn3c. They have the ability to bind and activate or repress transcription through their affinity to octamer promoter sequences (Xiang et al., 1995, Gruber et al., 1997, Ma et al., 2003). Brn3a has also been described as a potential HCF binding protein (Luciano and Wilson, 2003). The POU domain consists of an amino-terminal POU-specific domain (POU IV Box) and a POU homedomain at the C-terminus separated by a short flexible linker region, both are highly conserved and contribute to site recognition (Gerrero et al., 1993). The structural bipartite POU domain has inherent conformational flexibility conferring functional diversity to this class of transcription factors (Phillips and Luisi, 2000).

1.2.2.1 Brn3a regulatory roles

Brn3a is consistently expressed in a subset of sensory neurons of the developing and adult central nervous system as well as in the peripheral nervous system (Gerrero et al., 1993, Xiang et al., 1996). In the peripheral nervous system Brn3a is expressed in dividing precursor cells of trigeminal sensory ganglia. In the central nervous system, Brn3a appears in the very early stages of neuron differentiation in midbrain, hindbrain and spinal cord (Fedtsova and Turner, 1995, McEvilly et al., 1996). Presence of Brn3a at specific stages of

development has shown to be an adequate marker for sensory precursors and sensory neuronal cells.

Brn3a has the ability to modify gene transcription by binding directly to promoters or indirectly by interacting with other cellular proteins that have promoter interactions. The activation of anti-apoptotic (Bcl-2 and Bcl-xL (Smith et al., 1998)), differentiation (alpha-internexin (Budhrammahadeo et al., 1995), neurofilament (Smith et al., 1997) and synaptic genes (SNAP25 (Lakin et al., 1995)) is mediated by direct binding of Brn3a to promoter sequences. The interaction of Brn3a with other proteins during gene activation is complex. Its activating or suppressing effects are dependant on the specific binding protein, the specific target gene and the cell type (Hudson et al., 2005). The protein p53 is an example of Brn3a differential regulation of target genes. In *in vitro* transfection assays in the ND7 neuronal cell line, known to express Brn3a, p53 activates transcription of two genes associated with apoptosis; Noxa and Bax. Noxa is induced by stress related stimuli and Bax is present during naturally occurring apoptosis in neurons of the TG and DRG during development. When p53 and Brn3a are co-expressed, cell survival increases as Brn3a suppresses the binding of p53 to the promoter of NOXA and Bax apoptotic genes (Hudson et al., 2005). In contrast, Brn3a also cooperates with p53 to activate the transcription of cyclin dependant kinase p21 increasing its endogenous levels and favoring cell cycle arrest (Hudson et al., 2005). This differential regulatory effect of Brn3a on p53 trans-activation of genes suggests the means by which Brn3a controls survival and /or differentiation of neuronal cells.

1.2.2.2 Brn3a and trkA transcription

Brn3a inactivation causes widespread losses of nociceptive neurons (Huang et al., 2001) and a significant attenuation of trkA gene expression in sensory ganglia (Huang et al., 1999). Recently Brn3a has been described as an enhancer of trkA transcription thereby contributing to NGF signaling (Ma et al., 2003). Most neurons expressing trkA receptors during early stages of

embryonic development are not affected by the absence of Brn3a, however after week 12.5 of gestation in mice, absence of Brn3a triggers a total loss of trkA receptors and abrupt apoptosis of cells. Only 30% of neurons survive after birth, these lack trkA expression and their survival is not dependent on NGF (Huang et al., 1999, Ma et al., 2003). While Brn3a does not appear to be required for the initiation of trkA expression, it is required for trkA sustained expression and protection of sensory neurons from apoptosis (Ma et al., 2003). Absence of Brn3a does not reduce the expression of p75, another NGF receptor, indicating the Brn3a specifically promotes the expression of the trkA receptor (Huang et al., 1999). However, following withdrawal of NGF, over-expression of Brn3a enhances survival of sensory neurons but not sympathetic neurons (Huang et al., 1999, Ensor et al., 2001) indicating that in sensory neurons, Brn3a promotion of survival is only partially dependant on trkA signaling pathway.

The activation of trkA transcription by Brn3a was described by Ma and others (Ma et al., 2000). In these studies a 457 bp segment of genomic DNA lying upstream from mouse trkA coding sequences that specifies appropriate trkA expression during embryogenesis in sympathetic neurons and neurons of the TG and DRG was defined (Ma et al., 2000). Within this region several potential binding motifs were identified including two sites that associate with Brn3a (Ma et al., 2003) and appear to be unique to the trkA promoter. These binding sites T(A/T)ATT described for mouse (Ma et al., 2000, Ma et al., 2003), are also present in the human trkA promoter sequence (Ma et al., 2003). Although rich in A and T residues this motif differs from the consensus Brn3a binding domain described by Xiang et al (Xiang et al., 1995). This consensus sequence (A/G)CTCATTAA(T/C) is recognized by all members of the Brn-3 POU family in ganglion cells of the vertebrate retina. Subsequently Gruber and others (Gruber et al., 1997) described an octamer binding element AT(A/T)A(T/A)T(A/T)AT that has a higher affinity (1000 fold) for Brn3a than the consensus described by Xiang for the POU family. *In vitro* analysis indicates

that the binding motif T(A/T)ATT has a lower affinity for Brn3a binding than these consensus octamers and is required mainly to enhance expression of trkA in sensory neurons (Ma et al., 2003). The information related to the relevance of Brn3a on the transcription regulation of trkA is controversial. Recently, Lei and others (Lei et al., 2006) showed that the minimal enhancer sequence (457bp) in the trkA promoter is not activated by Brn3a alone in sympathetic neuron-like cells (PC12) (Lei and Parada, 2007) and that Brn3a transcription activation is only possible with the cooperation of the zinc finger protein Klf7 (Lei et al., 2006).

1.3 Rationale, Hypothesis and Objectives

The main goal of our research group is to determine how HSV-1 "senses" stress in the peripheral nervous system and responds by reactivating from latency. We have identified two HCF-binding neuronal proteins – Luman and Zhangfei - that may play a role in this process. Zhangfei, in an HCF-dependant manner, suppress the activity of other HCF-binding proteins, including VP16, and inhibits HSV-1 replication. Recently, Brn3a, another neuronal factor thought to influence HSV latency and reactivation was found to possess an HCF-binding domain. The objective of the work described in this thesis was to explore interactions between Brn3a, HCF and Zhangfei in the regulation of HSV-1 latency and reactivation.

Based on the following observations:

- NGF is required for the maintenance of HSV latency and deprivation of NGF leads to reactivation.
- HSV establishes latency in neurons that have high affinity receptors (trkA) for NGF.
- Brn3a regulates the expression of the trkA gene.
- Brn3a has an HCF binding motif that can potentially bind HCF.

- Zhangfei suppresses the activation of HCF binding protein VP16 and Luman in a HCF dependent manner.
- Zhangfei is present in neurons.

I hypothesized that:

Brn3a binds HCF and Zhangfei suppresses the ability of Brn3a to induce the expression of trkA thereby interrupting the NGF/trkA signalling pathway in sensory neurons.

My objectives were to:

.

1) To confirm that Brn3a activates the trkA promoter and identify Brn3a cis-acting DNA sequences involved in trkA transcription.

2) To determine if Brn3a binds HCF and whether this is required for the trans-activation of trkA.

3) To determine the effect of Zhangfei on Brn3a trans-activation of the trkA promoter.

In the course of my studies I found that in neuronal cells Zhangfei, instead of suppressing the ability of Brn3a to activate the trkA promoter, actually activated it. I therefore explored this observation further and determined whether Zhangfei could be used to induce trkA expression and restore NGF responsiveness in medulloblastoma cells that normally do not express trkA.

1.4 References

Ajuh, P., Chusainow, J., Ryder, U. and Lamond, A. I., 2002. A novel function for human factor C1 (HCF-1), a host protein required for herpes simplex virus infection, in pre-mRNA splicing. Embo J. 21, 6590-6602.

Ajuh, P. M., Browne, G. J., Hawkes, N. A., Cohen, P. T., Roberts, S. G. and Lamond, A. I., 2000. Association of a protein phosphatase 1 activity with the human factor C1 (HCF) complex. Nucleic Acids Res. 28, 678-686.

Akhova, O., Bainbridge, M. and Misra, V., 2005. The neuronal host cell factor-binding protein Zhangfei inhibits herpes simplex virus replication. J Virol. 79, 14708-14718.

Amelio, A. L., Giordani, N. V., Kubat, N. J., O'Neil J, E. and Bloom, D. C., 2006a. Deacetylation of the herpes simplex virus type 1 latency-associated transcript (LAT) enhancer and a decrease in LAT abundance precede an increase in ICP0 transcriptional permissiveness at early times postexplant. J Virol. 80, 2063-2068.

Amelio, A. L., McAnany, P. K. and Bloom, D. C., 2006b. A chromatin insulator-like element in the herpes simplex virus type 1 latency-associated transcript region binds CCCTC-binding factor and displays enhancer-blocking and silencing activities. J Virol. 80, 2358-2368.

Antinone, S. E., Shubeita, G. T., Coller, K. E., Lee, J. I., Haverlock-Moyns, S., Gross, S. P. and Smith, G. A., 2006. The Herpesvirus capsid surface protein, VP26, and the majority of the tegument proteins are dispensable for capsid transport toward the nucleus. J Virol. 80, 5494-5498.

Artinger, K. B., Fedtsova, N., Rhee, J. M., Bronner-Fraser, M. and Turner, E., 1998. Placodal origin of Brn-3-expressing cranial sensory neurons. J Neurobiol. 36, 572-585.

Bearer, E. L., Breakefield, X. O., Schuback, D., Reese, T. S. and LaVail, J. H., 2000. Retrograde axonal transport of herpes simplex virus: evidence for a single mechanism and a role for tegument. Proc Natl Acad Sci U S A. 97, 8146-8150.

Block, T., Barney, S., Masonis, J., Maggioncalda, J., Valyi-Nagy, T. and Fraser, N. W., 1994. Long term herpes simplex virus type 1 infection of nerve growth factor-treated PC12 cells. J Gen Virol. 75 (Pt 9), 2481-2487.

Bruynseels, P., Jorens, P. G., Demey, H. E., Goossens, H., Pattyn, S. R., Elseviers, M. M., Weyler, J., Bossaert, L. L., Mentens, Y. and Ieven, M.,

2003. Herpes simplex virus in the respiratory tract of critical care patients: a prospective study. Lancet. 362, 1536-1541.

Budhrammahadeo, V., Morris, P. J., Lakin, N. D., Theil, T., Ching, G. Y., Lillycrop, K. A., Moroy, T., Liem, R. and Latchman, D. S., 1995. Activation of the alpha-internexin promoter by the brn-3a transcription factor is dependent on the n-terminal region of the protein. Journal of Biological Chemistry. 270, 2853-2858.

Chiarugi, V., Del Rosso, M. and Magnelli, L., 2002. Brn-3a, a neuronal transcription factor of the POU gene family: indications for its involvement in cancer and angiogenesis. Mol Biotechnol. 22, 123-127.

Chou, T. T., Trojanowski, J. Q. and Lee, V. M., 2000. A novel apoptotic pathway induced by nerve growth factor-mediated TrkA activation in medulloblastoma. J Biol Chem. 275, 565-570.

Chuang, J. Z., Milner, T. A. and Sung, C. H., 2001. Subunit heterogeneity of cytoplasmic dynein: Differential expression of 14 kDa dynein light chains in rat hippocampus. J Neurosci. 21, 5501-5512.

Crowley, C., Spencer, S. D., Nishimura, M. C., Chen, K. S., Pitts-Meek, S., Armanini, M. P., Ling, L. H., McMahon, S. B., Shelton, D. L., Levinson, A. D. and et al., 1994. Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell. 76, 1001-1011.

Dohner, K., Radtke, K., Schmidt, S. and Sodeik, B., 2006. Eclipse phase of herpes simplex virus type 1 infection: Efficient dynein-mediated capsid transport without the small capsid protein VP26. J Virol. 80, 8211-8224.

Douglas, M. W., Diefenbach, R. J., Homa, F. L., Miranda-Saksena, M., Rixon, F. J., Vittone, V., Byth, K. and Cunningham, A. L., 2004. Herpes simplex virus type 1 capsid protein VP26 interacts with dynein light chains RP3 and Tctex1 and plays a role in retrograde cellular transport. J Biol Chem. 279, 28522-28530.

Eberhart, C. G., Kaufman, W. E., Tihan, T. and Burger, P. C., 2001. Apoptosis, neuronal maturation, and neurotrophin expression within medulloblastoma nodules. J Neuropathol Exp Neurol. 60, 462-469.

Efstathiou, S. and Preston, C. M., 2005. Towards an understanding of the molecular basis of herpes simplex virus latency. Virus Res. 111, 108-119.

Eggert, A., Ikegaki, N., Liu, X., Chou, T. T., Lee, V. M., Trojanowski, J. Q. and Brodeur, G. M., 2000. Molecular dissection of TrkA signal transduction

pathways mediating differentiation in human neuroblastoma cells. Oncogene. 19, 2043-2051.

Ensor, E., Smith, M. D. and Latchman, D. S., 2001. The BRN-3A transcription factor protects sensory but not sympathetic neurons from programmed cell death/apoptosis. J Biol Chem. 276, 5204-5212.

Fagan, A. M., Zhang, H., Landis, S., Smeyne, R. J., Silos-Santiago, I. and Barbacid, M., 1996. TrkA, but not TrkC, receptors are essential for survival of sympathetic neurons in vivo. J Neurosci. 16, 6208-6218.

Fedtsova, N. G. and Turner, E. E., 1995. Brn-3.0 expression identifies early post-mitotic CNS neurons and sensory neural precursors. Mech Dev. 53, 291-304.

Freiman, R. N. and Herr, W., 1997. Viral mimicry: common mode of association with HCF by VP16 and the cellular protein LZIP. Genes Dev. 11, 3122-3127.

Garber, D. A., Schaffer, P. A. and Knipe, D. M., 1997. The LAT-associated function reduces productive cycle gene expression during acute infection of murine sensory neurons with herpes simplex virus type 1. J Virol. 71, 5885-5893.

Gerrero, M. R., McEvilly, R. J., Turner, E., Lin, C. R., O'Connell, S., Jenne, K. J., Hobbs, M. V. and Rosenfeld, M. G., 1993. Brn-3.0: a POU-domain protein expressed in the sensory, immune, and endocrine systems that functions on elements distinct from known octamer motifs. Proc Natl Acad Sci U S A. 90, 10841-10845.

Goto, H., Motomura, S., Wilson, A. C., Freiman, R. N., Nakabeppu, Y., Fukushima, K., Fujishima, M., Herr, W. and Nishimoto, T., 1997. A single-point mutation in HCF causes temperature-sensitive cell-cycle arrest and disrupts VP16 function. Genes Dev. 11, 726-737.

Greaves, R. F. and O'Hare, P., 1989 Separation of requirements for protein-DNA complex assembly from those for functional activity in the herpes simplex virus regulatory protein Vmw65. . J Virol 63 1641-1650.

Greene, L. A. and Kaplan, D. R., 1995. Early events in neurotrophin signalling via Trk and p75 receptors. Curr Opin Neurobiol. 5, 579-587.

Grimes, M. L., Beattie, E. and Mobley, W. C., 1997. A signaling organelle containing the nerve growth factor-activated receptor tyrosine kinase, TrkA. Proc Natl Acad Sci U S A. 94, 9909-9914.

Gruber, C. A., Rhee, J. M., Gleiberman, A. and Turner, E. E., 1997. POU domain factors of the Brn-3 class recognize functional DNA elements which are distinctive, symmetrical, and highly conserved in evolution. Mol Cell Biol. 17, 2391-2400.

Gunther, M., Laithier, M. and Brison, O., 2000. A set of proteins interacting with transcription factor Sp1 identified in a two-hybrid screening. Mol Cell Biochem. 210, 131-142.

Gupta, A., Gartner, J. J., Sethupathy, P., Hatzigeorgiou, A. G. and Fraser, N. W., 2006. Anti-apoptotic function of a microRNA encoded by the HSV-1 latency-associated transcript. Nature. 442, 82-85.

Hill, J. M., Garza, H. H. J., Helmy, M. F., Cook, S. D., Osborne, P. A., Johnson, E. M. J., Thompson, H. W., Green, L. C., O'Callaghan, R. J. and Gebhardt, B. M., 1997. Nerve growth factor antibody stimulates reactivation of ocular herpes simplex virus type 1 in latently infected rabbits. Journal of Neurovirology. 3, 206-211.

Hogan, M. R., Cockram, G. P. and Lu, R., 2006. Cooperative interaction of Zhangfei and ATF4 in transactivation of the cyclic AMP response element. FEBS Lett. 580, 58-62.

Huang, E. J., Liu, W., Fritzsch, B., Bianchi, L. M., Reichardt, L. F. and Xiang, M., 2001. Brn3a is a transcriptional regulator of soma size, target field innervation and axon pathfinding of inner ear sensory neurons. Development. 128, 2421-2432.

Huang, E. J. and Reichardt, L. F., 2003. Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem. 72, 609-642.

Huang, E. J., Zang, K., Schmidt, A., Saulys, A., Xiang, M. and Reichardt, L. F., 1999. POU domain factor Brn-3a controls the differentiation and survival of trigeminal neurons by regulating Trk receptor expression. Development. 126, 2869-2882.

Hudson, C. D., Morris, P. J., Latchman, D. S. and Budhram-Mahadeo, V. S., 2005. Brn-3a transcription factor blocks p53-mediated activation of proapoptotic target genes Noxa and Bax in vitro and in vivo to determine cell fate. J Biol Chem. 280, 11851-11858.

Hughes, T. A., La Boissiere, S. and O'Hare, P., 1999. Analysis of functional domains of the host cell factor involved in VP16 complex formation. J Biol Chem. 274, 16437-16443.

Jackson, S. A. and DeLuca, N. A., 2003. Relationship of herpes simplex virus genome configuration to productive and persistent infections. Proc Natl Acad Sci U S A. 100, 7871-7876.

Jin, L., Peng, W., Perng, G. C., Brick, D. J., Nesburn, A. B., Jones, C. and Wechsler, S. L., 2003. Identification of herpes simplex virus type 1 latency-associated transcript sequences that both inhibit apoptosis and enhance the spontaneous reactivation phenotype. J Virol. 77, 6556-6561.

Jones, C., 2003. Herpes simplex virus type 1 and bovine herpesvirus 1 latency. Clin Microbiol Rev. 16, 79-95.

Julien, E. and Herr, W., 2003. Proteolytic processing is necessary to separate and ensure proper cell growth and cytokinesis functions of HCF-1. Embo J. 22, 2360-2369.

Kamal, A., Stokin, G. B., Yang, Z., Xia, C. H. and Goldstein, L. S., 2000. Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I. Neuron. 28, 449-459.

Kaplan, D. R. and Miller, F. D., 2000. Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol. 10, 381-391.

Kent, J. R., Kang, W., Miller, C. G. and Fraser, N. W., 2003. Herpes simplex virus latency-associated transcript gene function. J Neurovirol. 9, 285-290.

Khanna, K. M., Bonneau, R. H., Kinchington, P. R. and Hendricks, R. L., 2003. Herpes simplex virus-specific memory CD8+ T cells are selectively activated and retained in latently infected sensory ganglia. Immunity. 18, 593-603.

Khanna, K. M., Lepisto, A. J., Decman, V. and Hendricks, R. L., 2004. Immune control of herpes simplex virus during latency. Curr Opin Immunol. 16, 463-469.

Knez, J., Piluso, D., Bilan, P. and Capone, J. P., 2006. Host Cell Factor-1 and E2F4 Interact Via Multiple Determinants in Each Protein. Mol Cell Biochem. 288, 79-90.

Kristie, T. M., Pomerantz, J. L., Twomey, T. C., Parent, S. A. and Sharp, P. A., 1995. The cellular c1 factor of the herpes simplex virus enhancer complex is a family of polypeptides. Journal of Biological Chemistry. 270, 4387-4394.

Kristie, T. M., Vogel, J. L. and Sears, A. E., 1999. Nuclear localization of the C1 factor (host cell factor) in sensory neurons correlates with reactivation of

herpes simplex virus from latency. Proc Natl Acad Sci U S A. 96, 1229-1233.

LaBoissiere, S., Hughes, T. and O'Hare, P., 1999. HCF-dependent nuclear import of VP16. Embo J. 18, 480-489.

Lachmann, R., 2003. Herpes simplex virus latency. Expert Rev Mol Med. 2003, 1-14.

Lakin, N. D., Morris, P. J., Theil, T., Sato, T. N., Moroy, T., Wilson, M. C. and Latchman, D. S., 1995. Regulation of neurite outgrowth and SNAP-25 gene expression by the Brn-3a transcription factor. J Biol Chem. 270, 15858-15863.

Laycock, K. A., Brady, R. H., Lee, S. F., Osborne, P. A., Johnson, E. M. and Pepose, J. S., 1994. The role of nerve growth factor in modulating herpes simplex virus reactivation in vivo. Graefes Archive For Clinical and Experimental Ophthalmology. 232, 421-425.

Lei, L. and Parada, L. F., 2007. Transcriptional regulation of Trk family neurotrophin receptors. Cell Mol Life Sci. 64, 522-532.

Lei, L., Zhou, J., Lin, L. and Parada, L. F., 2006. Brn3a and Klf7 cooperate to control TrkA expression in sensory neurons. Dev Biol.

Liang, G., Audas, T. E., Li, Y., Cockram, G. P., Dean, J. D., Martyn, A. C., Kokame, K. and Lu, R., 2006. Luman/CREB3 induces transcription of the endoplasmic reticulum stress response protein, Herp, through an ERSE-II element. Mol Cell Biol.

Lillycrop, K. A., Dawson, S. J., Estridge, J. K., Gerster, T., Matthias, P. and Latchman, D. S., 1994. Repression of a herpes simplex virus immediate-early promoter by the oct-2 transcription factor is dependent on an inhibitory region at the n terminus of the protein. Molecular & Cellular Biology. 14, 7633-7642.

Lillycrop, K. A., Dent, C. L., Wheatley, S. C., Beech, M. N., Ninkina, N. N., Wood, J. N. and Latchman, D. S., 1991 The octamer binding protein Oct-2 represses HSV immediate early gene in cell lines derived from letently infectable sensory neurons. . Neuron 7 381-390.

Lin, J., Puigserver, P., Donovan, J., Tarr, P. and Spiegelman, B. M., 2002. Peroxisome proliferator-activated receptor gamma coactivator 1beta (PGC-1beta), a novel PGC-1-related transcription coactivator associated with host cell factor. J Biol Chem. 277, 1645-1648.

Lu, R. and Misra, V., 2000a. Potential role for Luman, the cellular homologue of herpes simplex virus VP16 (alpha gene trans-inducing factor), in herpesvirus latency. J Virol. 74, 934-943.

Lu, R. and Misra, V., 2000b. Zhangfei: a second cellular protein interacts with herpes simplex virus accessory factor HCF in a manner similar to Luman and VP16. Nucleic Acids Res. 28, 2446-2454.

Lu, R., Yang, P., O'Hare, P. and Misra, V., 1997. Luman, a new member of the CREB/ATF family, binds to herpes simplex virus VP16-associated host cellular factor. Mol Cell Biol. 17, 5117-5126.

Luciano, R. L. and Wilson, A. C., 2002. An activation domain in the C-terminal subunit of HCF-1 is important for transactivation by VP16 and LZIP. Proc Natl Acad Sci U S A. 99, 13403-13408.

Luciano, R. L. and Wilson, A. C., 2003. HCF-1 functions as a coactivator for the zinc finger protein Krox20. J Biol Chem. 278, 51116-51124.

Ma, L., Lei, L., Eng, S. R., Turner, E. and Parada, L. F., 2003. Brn3a regulation of TrkA/NGF receptor expression in developing sensory neurons. Development. 130, 3525-3534.

Ma, L., Merenmies, J. and Parada, L. F., 2000. Molecular characterization of the TrkA/NGF receptor minimal enhancer reveals regulation by multiple cis elements to drive embryonic neuron expression. Development. 127, 3777-3788.

Mahadeo, D., Kaplan, L., Chao, M. V. and Hempstead, B. L., 1994. High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. Implications for multi-subunit polypeptide receptors. J Biol Chem. 269, 6884-6891.

Martin-Zanca, D., Barbacid, M. and Parada, L. F., 1990. Expression of the trk proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development. Genes Dev. 4, 683-694.

McEvilly, R. J., Erkman, L., Luo, L., Sawchenko, P. E., Ryan, A. F. and Rosenfeld, M. G., 1996. Requirement for Brn-3.0 in differentiation and survival of sensory and motor neurons. Nature. 384, 574-577.

Misra, V., Rapin, N., Akhova, O., Bainbridge, M. and Korchinski, P., 2005. Zhangfei is a potent and specific inhibitor of the host cell factor-binding transcription factor Luman. J Biol Chem. 280, 15257-15266.

Muragaki, Y., Chou, T. T., Kaplan, D. R., Trojanowski, J. Q. and Lee, V. M., 1997. Nerve growth factor induces apoptosis in human medulloblastoma cell lines that express TrkA receptors. J Neurosci. 17, 530-542.

Narayanan, A., Nogueira, M. L., Ruyechan, W. T. and Kristie, T. M., 2005. Combinatorial transcription of herpes simplex virus and varicella zoster virus immediate early genes is strictly determined by the cellular coactivator HCF-1. J Biol Chem. 280, 1369-1375.

Nogueira, M. L., Wang, V. E., Tantin, D., Sharp, P. A. and Kristie, T. M., 2004. Herpes simplex virus infections are arrested in Oct-1-deficient cells. Proc Natl Acad Sci U S A. 101, 1473-1478.

Ohta, T., Watanabe, T., Katayama, Y., Kurihara, J., Yoshino, A., Nishimoto, H. and Kishimoto, H., 2006. TrkA expression is associated with an elevated level of apoptosis in classic medulloblastomas. Neuropathology. 26, 170-177.

Parada, L. F., Tsoulfas, P., Tessarollo, L., Blair, J., Reid, S. W. and Soppet, D., 1992. The Trk family of tyrosine kinases: receptors for NGF-related neurotrophins. Cold Spring Harb Symp Quant Biol. 57, 43-51.

Park, K. C., Shimizu, K. and Hayakawa, T., 1998. Interferon yield and MHC antigen expression of human medulloblastoma cells and its suppression during dibutyryl cyclic AMP-induced differentiation: do medulloblastoma cells derive from bipotent neuronal and glial progenitors? Cell Mol Neurobiol. 18, 497-507.

Peng, W., Henderson, G., Inman, M., BenMohamed, L., Perng, G. C., Wechsler, S. L. and Jones, C., 2005. The locus encompassing the latency-associated transcript of herpes simplex virus type 1 interferes with and delays interferon expression in productively infected neuroblastoma cells and trigeminal Ganglia of acutely infected mice. J Virol. 79, 6162-6171.

Phillips, K. and Luisi, B., 2000. The virtuoso of versatility: POU proteins that flex to fit. J Mol Biol. 302, 1023-1039.

Piluso, D., Bilan, P. and Capone, J. P., 2002. Host cell factor-1 interacts with and antagonizes transactivation by the cell cycle regulatory factor Miz-1. J Biol Chem. 277, 46799-46808.

Preston, C. M., 2000. Repression of viral transcription during herpes simplex virus latency. J Gen Virol. 81 Pt 1, 1-19.

Raggo, C., Rapin, N., Stirling, J., Gobeil, P., Smith-Windsor, E., O'Hare, P. and Misra, V., 2002. Luman, the cellular counterpart of herpes simplex virus VP16, is processed by regulated intramembrane proteolysis. Mol Cell Biol. 22, 5639-5649.

Regier, J. L., Shen, F. and Triezenberg, S. J., 1993. Pattern of aromatic and hydrophobic amino acids critical for one of two subdomains of the VP16 transcriptional activator. Proc Natl Acad Sci U S A. 90, 883-887.

Reilly, P. T., Wysocka, J. and Herr, W., 2002. Inactivation of the retinoblastoma protein family can bypass the HCF-1 defect in tsBN67 cell proliferation and cytokinesis. Mol Cell Biol. 22, 6767-6778.

Roizman, B. S., A. E., 2001. Herpes simplex viruses and their replication. In: Knipe, D. M. H., P.M (Ed.), Fields Virology, vol.2. Lippincott Willaims and Wilkins, pp. 2399-2460.

Sasaki, K., Chancellor, M. B., Goins, W. F., Phelan, M. W., Glorioso, J. C., de Groat, W. C. and Yoshimura, N., 2004. Gene therapy using replication-defective herpes simplex virus vectors expressing nerve growth factor in a rat model of diabetic cystopathy. Diabetes. 53, 2723-2730.

Sawtell, N. M. and Thompson, R. L., 1992. Herpes simplex virus type 1 latency-associated transcription unit promotes anatomical site-dependent establishment and reactivation from latency. J Virol. 66, 2157-2169.

Shimoke, K., Amano, H., Kishi, S., Uchida, H., Kudo, M. and Ikeuchi, T., 2004. Nerve growth factor attenuates endoplasmic reticulum stress-mediated apoptosis via suppression of caspase-12 activity. J Biochem (Tokyo). 135, 439-446.

Shimoke, K., Kishi, S., Utsumi, T., Shimamura, Y., Sasaya, H., Oikawa, T., Uesato, S. and Ikeuchi, T., 2005. NGF-induced phosphatidylinositol 3-kinase signaling pathway prevents thapsigargin-triggered ER stress-mediated apoptosis in PC12 cells. Neurosci Lett. 389, 124-128.

Smith, M. D., Dawson, S. J., Boxer, L. M. and Latchman, D. S., 1998. The N-terminal domain unique to the long form of the Brn-3a transcription factor is essential to protect neuronal cells from apoptosis and for the activation of Bbcl-2 gene expression. Nucleic Acids Res. 26, 4100-4107.

Smith, M. D., Morris, P. J., Dawson, S. J., Schwartz, M. L., Schlaepfer, W. W. and Latchman, D. S., 1997. Coordinate induction of the three neurofilament genes by the Brn-3a transcription factor. J Biol Chem. 272, 21325-21333.

Strang, B. L. and Stow, N. D., 2005. Circularization of the herpes simplex virus type 1 genome upon lytic infection. J Virol. 79, 12487-12494.

Tajima, Y., Molina, R. P., Jr., Rorke, L. B., Kaplan, D. R., Radeke, M., Feinstein, S. C., Lee, V. M. and Trojanowski, J. Q., 1998. Neurotrophins and neuronal versus glial differentiation in medulloblastomas and other pediatric brain tumors. Acta Neuropathol (Berl). 95, 325-332.

Tenser, R. B., 1998. Trigeminal neuralgia: mechanisms of treatment. Neurology. 51, 17-19.

Thompson, R. L. and Sawtell, N. M., 1997. The herpes simplex virus type 1 latency-associated transcript gene regulates the establishment of latency. J Virol. 71, 5432-5440.

Triezenberg, S. J., LaMarco, K. L. and McKnight, S. L., 1988. Evidence of DNA:protein interactions that mediate HSV-1 immediate early gene activation by VP16. Genes and Development. 2, 730-742.

Tusher, V. G., Tibshirani, R. and Chu, G., 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A. 98, 5116-5121.

Verge, V. M., Merlio, J. P., Grondin, J., Ernfors, P., Persson, H., Riopelle, R. J., Hokfelt, T. and Richardson, P. M., 1992. Colocalization of NGF binding sites, trk mRNA, and low-affinity NGF receptor mRNA in primary sensory neurons: responses to injury and infusion of NGF. J Neurosci. 12, 4011-4022.

Vinson, C., Myakishev, M., Acharya, A., Mir, A. A., Moll, J. R. and Bonovich, M., 2002. Classification of human B-ZIP proteins based on dimerization properties. Mol Cell Biol. 22, 6321-6335.

Vogel, J. L. and Kristie, T. M., 2000. The novel coactivator C1 (HCF) coordinates multiprotein enhancer formation and mediates transcription activation by GABP. Embo J. 19, 683-690.

Walwyn, W. M., Matsuka, Y., Arai, D., Bloom, D. C., Lam, H., Tran, C., Spigelman, I. and Maidment, N. T., 2006. HSV-1-mediated NGF delivery delays nociceptive deficits in a genetic model of diabetic neuropathy. Exp Neurol. 198, 260-270.

Wang, F., Tang, W., McGraw, H. M., Bennett, J., Enquist, L. W. and Friedman, H. M., 2005a. Herpes simplex virus type 1 glycoprotein e is required for axonal localization of capsid, tegument, and membrane glycoproteins. J Virol. 79, 13362-13372.

Wang, Q. Y., Zhou, C., Johnson, K. E., Colgrove, R. C., Coen, D. M. and Knipe, D. M., 2005b. Herpesviral latency-associated transcript gene promotes assembly of heterochromatin on viral lytic-gene promoters in latent infection. Proc Natl Acad Sci U S A. 102, 16055-16059.

Werstuck, G. and Capone, J. P., 1989 Identification of a domain of the herpes simplex virus trans- activator Vmw65 required for protein-DNA complex formation through the use of protein A fusion proteins. . J Virol 63 5509-5513.

Wilcox, C. and Johnson, E., 1988. Characterization of nerve growth factor-dependent herpes simplex virus latency in neurons in vitro. J Virol. 62, 393-399.

Wilcox, C. L., Smith, R. L., Freed, C. R. and Johnson, E. M. J., 1990. Nerve growth factor-dependence of herpes simplex virus latency in peripheral sympathetic and sensory neurons in vitro. Journal of Neuroscience. 10, 1268-1275.

Wilson, A. C., LaMarco, K., Peterson, M. G. and Herr, W., 1993. The VP16 accessory protein HCF is a family of polypeptides processed from a large precursor protein. Cell. 74, 115-125.

Wolfstein, A., Nagel, C. H., Radtke, K., Dohner, K., Allan, V. J. and Sodeik, B., 2006. The inner tegument promotes herpes simplex virus capsid motility along microtubules in vitro. Traffic. 7, 227-237.

Wysocka, J., Reilly, P. T. and Herr, W., 2001. Loss of HCF-1-chromatin association precedes temperature-induced growth arrest of tsBN67 cells. Mol Cell Biol. 21, 3820-3829.

Xiang, M., Gan, L., Zhou, L., Klein, W. H. and Nathans, J., 1996. Targeted deletion of the mouse POU domain gene Brn-3a causes selective loss of neurons in the brainstem and trigeminal ganglion, uncoordinated limb movement, and impaired suckling. Proc Natl Acad Sci U S A. 93, 11950-11955.

Xiang, M., Zhou, L., Macke, J. P., Yoshioka, T., Hendry, S. H., Eddy, R. L., Shows, T. B. and Nathans, J., 1995. The Brn-3 family of POU-domain factors: primary structure, binding specificity, and expression in subsets of retinal ganglion cells and somatosensory neurons. J Neurosci. 15, 4762-4785.

Yang, L., Voytek, C. C. and Margolis, T. P., 2000. Immunohistochemical analysis of primary sensory neurons latently infected with herpes simplex

virus type 1. J Virol. 74, 209-217.

Yuan, J. and Yankner, B. A., 2000. Apoptosis in the nervous system. Nature. 407, 802-809.

Zhou, J., Holtzman, D. M., Weiner, R. I. and Mobley, W. C., 1994. Expression of TrkA confers neuron-like responsiveness to nerve growth factor on an immortalized hypothalamic cell line. Proc Natl Acad Sci U S A. 91, 3824-3828.

2. NOVEL BRN3A CIS-ACTING SEQUENCES MEDIATE TRANSCRIPTION OF HUMAN trkA IN NEURONS.

Ximena Valderrama and Vikram Misra*

Department of Veterinary Microbiology, Western College of Veterinary Medicine, 52 Campus Road, University of Saskatchewan, Saskatoon, Saskatchewan, S7N5B4, CANADA

*Phone number: 1+ -306-966-7218
Fax: 1+ - 306-966-7244
e-mail: vikram.misra@usask.ca

Key words: Brn3a, trkA, nerve growth factor, medulloblastomas, PC12 cells.

To be submitted to the Journal of Neurochemistry

2.1 Abstract

trkA, the receptor tropomyosin related kinase for nerve growth factor (NGF), is critical not only for the correct spatial and temporal development of sensory neurons during embryogenesis but also for the survival of sensory neurons, the differentiation and apoptosis of neuronal tumors and suppression of latent herpes simplex virus genomes. While the regulation of the expression of trkA is a complex process, the transcription factor Brn3a is known to play an important role as an enhancer of trkA transcription during development in the mouse. Despite considerable information on the regulation of trkA during embryogenesis, the mechanisms by which the expression of trkA is regulated in differentiated neurons, or the factors that influence its expression in tumor cells, have not been identified.

Since NGF-trkA signalling appears to be important for a variety of neuronal functions in differentiated neurons and may play a role in inducing the apoptosis or differentiation of neuronal tumors, we initiated studies to determine whether Brn3a/trkA promoter interactions may be important in these cells. We constructed a plasmid that contains 1043 base pairs of genomic sequences that extend to 30 nucleotides upstream of trkA coding region. In contrast to previous data, a short 190 bp region that lies proximal to the trkA initiation codon was sufficient for Brn3a responsiveness in Vero cells. This region was also sufficient for Brn3a *trans*-activation in NGF-differentiated PC12 cells. At least two portions of the 190 bp fragment bind to Brn3a with an affinity high enough to be detected in electromobility shift assays. In addition, Brn3a increased levels of endogenous trkA transcripts in PC12 cells and initiated trkA expression in medulloblastoma cells, which normally do not express trkA.

2.2 Introduction

The receptor tropomyosin related kinase trkA is the cognate high-affinity receptor for nerve growth factor (NGF) (Huang and Reichardt, 2003, Parada et al., 1992). In the developing mouse embryo, trkA is expressed in neural crest derived neurons destined to become sensory neurons with cell bodies in trigeminal and dorsal root ganglia (Eberhart et al., 2001, Sasaki et al., 2004). It is also present in sympathetic neurons. trkA-NGF interactions in these developing cells control cell fate, differentiation, survival, proliferation, axonal growth and target innervation (Bibel and Barde, 2000, Huang et al., 1999).

In addition to the role of NGF-trkA signalling in preserving sensory neurons, this pathway may also be important in regulating uncontrolled cell division in neuroblastomas and medulloblastomas, two childhood neuronal cancers. Thus, the presence of trkA in neuroblastomas correlates with favourable outcomes in patients (Eberhart et al., 2001, Ohta et al., 2006, Tajima et al.,1998). In addition, restoration of trkA expression in medulloblastoma tumor cells devoid of trkA makes them responsive to NGF and leads to their differentiation or apoptosis (Chou et al., 2000, Eggert et al., 2000, Muragaki et al., 1997).

While the regulation of the expression of trkA is likely complex, the transcription factor Brn3a is thought to play an important role. Brn3a is a POU homeodomain containing transcription factor (Guerrero et al., 1993) that is expressed in sensory neurons (Artinger et al., 1998, Fedtsova and Turner, 1995) and the expression of trkA is greatly reduced in Brn3a -/- mice (Huang et al., 1999). While it does not appear to be required for the initiation of trkA expression, Brn3a is required for its sustained expression and for the protection of sensory neurons from apoptosis (Ma et al., 2003).

Ma et al. (2003) defined a 457 base pair (bp) segment of genomic DNA lying upstream from mouse trkA coding sequences that specify appropriate trkA expression during embryogenesis in neurons of the trigeminal and dorsal root ganglia (Ma et al., 2000). They identified several motifs within this region that bind neuronal proteins. These include two sites for association with Brn3a (Ma et al., 2003). Since NGF-trkA signalling appears to be important for a variety of neuronal functions in differentiated neurons, and may play a role in inducing the apoptosis or differentiation of neuronal tumors, we initiated studies to determine whether other Brn3a/trkA promoter interactions may be important in these cells.

In this article we show that in contrast to *cis*-acting sequences identified as being required for correct spatial expression of the trkA promoter during development of the mouse, in differentiated PC12 cells and medulloblastoma cells sequences that lie immediately adjacent to the human trkA promoter play an important role in activation by Brn3a. In addition, exogenous Brn3a increased expression of endogenous trkA in differentiated PC12 cells and restored trkA expression in human medulloblastoma cells that normally do not express trkA.

2.3 Materials and Methods

2.3.1 Cell Culture

Cells were grown in 75-cm^2 tissue culture flasks. African green monkey kidney (Vero) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% newborn calf serum and 1% penicillin-streptomycin. Rat pheochromocytoma (PC12) cells (provided by D. D. Mousseau, University of Saskatchewan) were maintained in Complete Medium (CM) containing RPMI 1640 supplemented with 10% horse serum (heat treated to inactivate complement), 5% foetal bovine serum, 2 mM glutamine and 1% penicillin-streptomycin. PC12 cells were plated at a density of 2 x 10^6 in 6 well collagen coated plates. To differentiate PC12 cells they were "fasted" in low serum media, RPMI 1640 supplemented with 1% of foetal bovine serum and 1% penicillin-streptomycin, overnight before treatment with 50 ng/ml of nerve growth factor (NGF, Cederlane Laboratories, Hornby, Ontario, Canada). NGF was reconstituted in 0.02% acetic acid in fetal bovine serum before dilution. Human medulloblastoma cells, ONS-76 (Yamada et al., 1989), were obtained from Michael Taylor (University of Toronto) and grown in DMEM with 10% foetal bovine serum and 1% penicillin-streptomycin.

2.3.2 Collagen coated plates

Six well plates were incubated at room temperature for 1 hour with 2 mls of a 0.02N acetic acid solution containing 50 µg/ml (5 µg/cm^2) rat tail collagen (VWR CACB354236). Plates were then rinsed with 0.1N sterile PBS and used immediately.

2.3.3 Plasmids.

The plasmid pRK-55, coding for human Brn3a, was a gift from Mengqing Xiang (Robert Wood Johnson Medical School). A 1043 bp portion of the trkA promoter region was amplified using PCR from HeLa cell genomic DNA (-1073 to -30 from the trkA initiator codon). The set of primers shown in figure 1B,

which were used to amplify the 1043bp fragment, included a 5' KpnI restriction enzyme site and a 3' XhoI site to facilitate cloning. The amplified 1043 bp fragment was then cloned using a TOPO cloning kit (Invitrogen). After confirming the sequence of the cloned DNA, it was transferred to pCAT3basic (Clontech), a chloramphenicol acetyltransferase (CAT) reporter plasmid. The resulting plasmid was named ptrkA1043. Constructs generated from ptrkA1043 are shown in Table 2.1.

Constructs	Deletion from 1043bp segment	Size of insert remaining fragment
ptrkA338	BssHII-XhoI	378
ptrkA470	KpnI-XmaIII / ApaI-XhoI	453
ptrkA+190	KpnI-ApaI	175
ptrkA-190	ApaI-XhoI	868
ptrkAM12(-/-)	SmaI-SmaI / BssHII-BssHII	650
ptrkAM1	BssHII-BssHII	824
ptrkAM2	SmaI-SmaI	869

Table 2.1. Genomic human trkA promoter constructs. Mutant constructs were generated from plasmid ptrkA1043. Sequences were deleted by restriction endonuclease digestion and recloned by ligating complementary ends or by rendering the ends blunt and then ligating them. The size of the remaining fragments represent only genomic sequence.

2.3.4 Transfections and CAT-reporter assay

Vero and PC12 cells were transfected using the calcium phosphate method as described previously (Chen and Okayama, 1988) with plasmids, purified through CsCl gradients. Vero cells were plated at a density of $1x10^6$ cells in 6 well plates, transfected the next day with 6.5 µg of DNA and harvested 48 hr later. PC12 cells were seeded at a density of 2×10^6 per well (collagen coated 6 well plates) in CM and incubated overnight at 37°C in a 10% CO_2 incubator. The next day, medium was replaced with 1.5 ml/ well RPMI 1640 + 10% normal calf serum +1% penicillin-streptomycin + 2mM L-glutamine and incubated for at least one hour at 37 °C in 5% CO_2 incubator. DNA concentrations for transfection were 2µg of reporter plasmid, 1µg pCMVBGal

and 1.5 µg of pRK-Brn3a DNA/well. After transfection, cells were incubated for 5 hours in 5% CO_2 and then treated with glycerol as follows: cells were rinsed with pre-warmed RPMI and then 0.5 ml of pre-warmed 25% glycerol in 2X BES was added. After 45 sec, cells were rinsed twice with RPMI 1640 and 2 ml of PC12 CM. Low serum medium (1% foetal bovine serum) was added instead if cells are going to be treated with NGF. The plates were returned to the 10% CO_2 incubator and harvested 48 hr later. To transfect ONS-76 cells, they were plated at a density of $2X10^5$ cells per well of a 6-well plate. The next day, 1.5 µg of DNA and 6 µl of Lipofectamine 2000 (Invitrogen) were mixed and added to the cells overlayed by 2 ml of OptiMEM (Invitrogen) with 10% fetal serum and no antibiotics. The cells were analyzed after overnight incubation.

2.3.5 Chloramphenicol acetyl transferase (CAT) assays

For chloramphenicol acetyl transferase (CAT) assays 250 ng or 1 µg of pCMVBGal, a plasmid specifying beta-galactosidase, was added to transfection mixtures in Vero and PC12 cells respectively. Lysates were assayed for beta-galactosidase (Tajima et al., 1998) and for CAT using an enzyme-linked immunosorbent assay kit (Roche Applied Science). CAT values were adjusted for transfection efficiency using beta-galactosidase values. In figures showing the results of the CAT assays, each data point is the average of replicate transfections with the bar representing the range. The data are representative of at least two, and usually three, independent experiments that gave the same results.

2.3.6 RNA preparation and Real time PCR (QPCR) analysis

Total RNA was extracted from cells in 6 well tissue culture plates using Trizol (Invitrogen) as suggested by the manufacturer and dissolved in 20 µl of DEPC-treated water. Next, 5 µg of total RNA was used for first strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen). To detect and quantitate trkA and other transcripts we used Brilliant SYBR Green QPCR Master Mix (Stratagene). Samples were amplified in a Mx3005XP QPCR

thermocycler (Stratagene) using the following thermocycle conditions: samples were heated once at 95 °C for 10 min, 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 1 min. Data were analyzed using the thermocycler-associated software. For each sample, threshold values for the assayed transcripts (trkA, Brn3a and Erg1) were normalized for total input RNA concentrations using cycle threshold values for transcripts for the "normalizer" house-keeping gene, GAPDH. The normalized values in each experiment were compared to a "calibrator" sample to determine the relative increase in the amount of a transcript. Results were analyzed for significant difference using Student T-TEST. The primer sets for transcript amplification were used at a final concentration of 40 nM. The sequences of the primers were as follows Brn3a-F: 5'-tggcgtccatctgcgactc-3'; Brn3a-R: 5'- ctcaggttgttcattttctc-3';trkAcd-F: 5' - gagggcaaaggctctggactcca-3'; trkAcd-R: 5'- agactccgaagcgcacgatg-3': Gapdh-F: 5'-gcctcctgcaccaccaactg; Gapdh-R: 5'- gggccatccacagtcttctgg. Following amplification the melting curves for the products were generated to ensure that the product represented a homogenous species. In addition, the PCR products were analyzed by electrophoresis to ensure that a product of the predicted size had been generated. The expected sizes of amplified products were 300 bp for Brn3a, 300 bp for trkA, and 130 bp for GAPDH. Amplified products were visualized by gel electrophoresis and when first optimizing the reactions the sequence of the PCR products was determined.

2.3.7 Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides (Table 2.2) were labeled with ^{32}P and 1 µl of probe containing 10,000 to 40,000 cpm were incubated in 5X gel shift buffer containing 20 mM HEPES-KOH, 0.5 mM DTT, 50 mM KCL and 5 mM $MgCl_2$. The 5 x gel-shift buffer was mixed with 4% Ficoll 400, and 1 µl of (1µg/µl) poly (dI-dC) (Roche), and 1 µl (1.5 µg) purified GST-Brn3a or GST protein. Finally, water was added to a total volume of 20 µl. The mix was incubated on ice for 40 minutes. For competition assays, 100X of unlabeled double-stranded oligonucleotides were mixed with radiolabeled probes. Electrophoresis was

done using 6% non-denaturing polyacrylamide gels in 0.5 x TBE buffer at 4°C at a constant voltage (150 V) for 3 hours. Gels were then dried and either autoradiographed on Kodak X-OMATR film or using a Typhoon Trio (GE Healthcare) multi-purpose scanner.

Oligo name	Sequence 5' to 3'	Size (nt)
1-F	Cctaacaggggaggggcagagggggggcgtcagagagt	40
1-R	Actctctgacgccccccctctgccccctcccctgttagg	40
24-F	Agggggggcgtcagagagtaggaagcgggtggagaagag	40
24-R	Ctcttctccacccgcttcctactctctgacgcccccccct	40
40-F	Aggaagcgggtggagaagaggggcaaggcggggccgggcg	40
40-R	Cgcccggccccgccttgcccctcttctccacccgcttcct	40
60-F	Gggcaaggcggggccgggcgggggccgctggctccgccct	40
60-R	Agggcggagccagcggcccccgcccggccccgccttgccc	40
80-F	Ggggccgctggctccgcccttcctggcggctgggtcttt	40
80-R	Aaagacccagccgccaggaaagggcggagccagcggcccc	40
100-F	Ttcctggcggctgggtctttaacaccgcccagcg**c**acatg	40
100-R	Catgtgcgctgggcggtgttaaagacccagccgccaggaa	40
120-F	Aacaccgcccagcgcacatgtcgggggaggcctggcagct	40
120-R	Agctgccaggcctcccccgacatgtgcgctgggcggtgtt	40
140-F	Tcgggggaggcctggcagctgcagctgggagcgcacagacgg ctg	45
140-R	Cagccgtctgtgcgctcccagctgcagctgccaggcctcccccga	45
HM1-F	Tctaagagatctattaatttcttcacgaataaatcgatgc	40
HM1-R	Caagagcatcgatttattcgtgaagaaattaatagatct	39
HM2-F	Gttctacctaacttactacaagtgacatgctcactcccctaggc	44
HM2-R	Cgcgtgcctaggggagtgagcatgtcacttggagtaagttaggt	44

Table 2.2. Overlapping oligonucleotides corresponding to 190 bp of the trkA proximal promoter sequence used for electromobility shift assay.

2.3.8 Purification of GST-Brn3a fusion protein

The GST-Brn3a (-13) fusion protein does not include the first 13 amino acids of the Brn3a coding sequence. These amino acids have been shown not to affect Brn3a's biological activity. *Escherichia coli* BL21 competent cells were transformed with a plasmid expressing GST-Brn3a. 500 ml of cell culture at OD_{600}= 0.6 was induced to express the fusion protein with 1 mM isopropylthio-b-

D-galactoside for 4 hours. The cell pellet was resuspended in lysis buffer (Tris, 0.01M, pH 7.5; EDTA, 0.001M, sodium chloride, 0.15M; 15 TritonX-100, Protease Inhibitor Cocktail [Roche Molecular Biochemicals]; 0.25 mg egg-white lysozyme) and disrupted by sonication on ice. The supernatant was purified using Glutathione-Sepharose 4B beads (GE Healthcare). Cells transformed with pGEX-KG, the control plasmid expressing only GST, were processed simultaneously.

2.3.9 Antibodies and Immunoblot

The fusion protein GST-Brn3a (-13) was produced in *Escherichia coli* BL21 (DE3) (Novagen) and purified by using glutathione-Sepharose beads (Amersham Pharmacia) as described previously (Park et al., 1998). Antibodies were produced at the University of Saskatchewan Animal Resources Centre by immunizing rabbits with about 150 µg of protein in Freund's complete adjuvant as described previously (Muragaki et al., 1997). The anti-Brn3a serum specifically detects Brn3a in Western blots of in vitro-synthesized Brn3a (TnT, Promega) and lysates of transfected mammalian cells (data not shown). Procedures for immunoblotting were as previously described (Akhova et al., 2005).

2.4 Results

The 457 bp segment of DNA identified by Ma et al. (2000) as sufficient for appropriate trkA expression during mouse enbryogenesis contains binding sites for many transcription factors. Within this segment the authors identified two sites for the binding of Brn3a (Ma et al., 2003). Since NGF-trkA signalling appears to be important for a variety of neuronal functions in differentiated neurons and may play a role in inducing the apoptosis or differentiation of neuronal tumors, we initiated studies to determine whether other Brn3a/trkA promoter interactions may be important in these cells.

Using PCR we amplified a 1043 bp region of DNA that lies upstream from the coding sequences for human trkA. This fragment contains regions homologous to the 457 bp DNA segment important in mice, including the putative Brn3a-binding motifs (Fig 2.1B). An analysis of the sequence of the amplified fragment showed that it was similar to the corresponding portion of the mouse genome (66%) with almost perfect sequence identity for the putative Brn3a-binding sites (Fig 2.1C). To assess the ability of the DNA fragment to function as a promoter, we cloned it upstream of the coding sequences of the reporter protein CAT in pCAT3Basic. We refer to this construct as ptrkA1043.

2.4.1 Brn3a can activate trkA proximal promoter sequences

To determine if the trkA upstream sequences could respond to Brn3a, we transfected Vero cells with ptrkA1043 and varying amounts of a plasmid that expresses Brn3a. For our preliminary experiments we chose Vero cells because these Green Monkey kidney cells, which have a fibroblastic phenotype, express no Brn3a or trkA (results not shown) and would be unlikely to express other neuron specific transcriptional factors. Figure 2.2 shows that Brn3a activated expression of CAT over a hundred fold in a dose-dependent manner. To determine if the two putative Brn3a sites or other regions of the DNA fragment were important for activation, we constructed a series of reporter plasmids from

which various portions of the fragment had been deleted (Fig 2.3). While deletion of the proximal motif, M2 (ptrkAM1, Fig 2.3) suppressed activation by about 50%, deletion of the distal site, M1 (ptrkAM2, Fig 2.3), or simultaneous deletion of both sites (ptrkAM1/2(-/-)) had relatively little effect. It is puzzling that while deleting sequences that include M1 and M2 (ptrkAM12(-/-)) has no effect on activation, deletion of M1 or M2 by itself have less activation than both of them together. I am not sure of the events affecting theses deletions. The most profound effect was caused by the deletion of the 190 bp segment that lies 30 bp upstream from the coding sequences of trkA (see constructs ptrkA-190, ptrkA470 and ptrkA338). A construct that contained only these sequences had almost the same level of activity as the entire 1043 bp fragment (see construct ptrkA+190). Interestingly, this 190 bp segment does not contain sequences homologous to the 457 bp region found to be important during development in mice.

To determine if the 190 bp fragment could bind Brn3a, we examined interactions between the DNA fragments and purified recombinant fusion protein made up of GST and Brn3a. In electromobility shift assays, while purified GST alone had no effect on the mobility of the fragment (not shown), GST-Brn3a retarded the mobility of the radioactively labeled fragment to reveal two bands (C1 and C2 in lane 2, Fig 2.4A). An excess of unlabelled fragment inhibited the formation of these complexes (lane 3) and inclusion of antiserum against GST-Brn3a retarded their mobility further (lane 4). These results show that the 190 bp fragment contained one and possibly more sites for binding Brn3a.

To identify which portion of the 190 bp fragment bound Brn3a, we repeated the electromobility shift assays with overlapping 40 bp double-stranded oligonucleotides spanning the 190 bp fragment (Fig 2.5A). We also included oligonucleotides representing HM1 and HM2, human sequences containing homologues of the putative mouse Brn3a binding sites (M1 and M2) described earlier by Ma et al. (2000). HM1 specifically bound Brn3a (Fig 2.5B

lane 2) while there was no specific binding for HM2. Four of the oligonucleotides representing the 190 bp fragment appeared to form complexes with Brn3a. A gel showing analysis of oligonucleotides 40 and 60 is shown in figure 5B. Using a phosphor-imager and image analysis software we calculated the amount of radioactivity in the retarded bands (Brn3a-DNA complexes, * in Figure 2.5B) from the various oligonucleotides expressed as a percentage of unbound radioactivity (Fig 2.5C). Our results suggest that Brn3a binds to at least two regions of the 190 bp fragment (denoted by asterisks in Fig 2.5A).

2.4.2 Effect of NGF induced differentiation and exogenous Brn3a on the expression of endogenous trkA in PC12 cells

To determine if our observations in Vero cells for Brn3a activation of the 190 bp fragment were valid for neuronal cells, we examined PC12 cells. These are rat pheochromocytoma cells that differentiate into sympathetic neuron-like cells when treated with NGF and low concentrations of serum. They have been used extensively as an *in vitro* model to study ligand-receptor interactions and cellular differentiation in response to NGF (Greene and Tischler, 1976, Schubert and Whitlock, 1977). We first measured the level of trkA and Brn3a transcripts in PC12 cells over six days of NGF treatment. The levels of trkA transcripts in these cells increased (Fig 2.6A, B day 0, 4, 6 in lanes 1, 2, and 3, respectively) in response to NGF while levels of Brn3a decreased (Fig 2.6A). We confirmed progressive differentiation of cells by the detection of increasing levels of neurofilament, a marker for differentiation (Fig 2.6C day 0, 4, 6 in lanes 1, 2, and 3, respectively). We next transfected NGF treated and mock-treated PC12 cells with a plasmid expressing Brn3a. Forty-eight hours after treatment, NGF treated cells (+) expressing exogenous Brn3a (when compared with NGF-treated cells transfected with an empty vector, pcDNA) showed a 25-fold increase in the level of trkA transcripts (Fig 2.6D). There was no such increase in mock-treated cells. NGF treated cells expressing Brn3a are designated as (+) and all transfected with an empty vector as (-) in figure 6D.

We next examined PC12 cells to determine if the 190 bp proximal trkA promoter was active in these cells. NGF treated or mock-treated cells were transfected with the empty reporter plasmid (pCAT3basic) or ptrkA+190. The 190 bp fragment was active in both NGF-treated and mock-treated PC12 cells (Fig 2.7A), indicating that in these cells the 190 bp fragment has a relatively high level of basal activity. This is in contrast to Vero cells, where in the absence of activators such as exogenous Brn3a, the 190 bp fragment has almost no activity when compared with the empty reporter plasmid, pCAT3basic (not shown). Despite high the levels of basal activity in NGF-treated PC12 cells, ptrkA+190 was activated almost eight fold by exogenous Brn3a (Fig 2.7B). When PC12 cells were transfected with a plasmid lacking the proximal promoter (trkA-190), no activity was observed in the presence or absence of NGF, indicating that trkA+190 is responsible for trkA gene activation by Brn3a (Fig 2.7C).

2.4.3 Activation of trkA in medulloblastoma cells

Medulloblastomas are childhood neuronal cancers that do not normally express trkA. However, if medulloblastoma cells are induced to express trkA they become responsive to NGF-trkA signalling (Chou et al., 2000, Fedtsova and Turner, 1995, Sacristan et al., 1999) and this is associated with a favourable prognosis. We examined the ONS-76 medulloblastoma cell line, which was derived from a large cerebellar tumor in a two-year old girl (Yamada et al., 1989). The cells express both glial and neuronal markers and can be induced to differentiate into neuron-like cells by treatment with dibutryl cyclic AMP (Xiang et al., 1995, Lakin et al., 1995). ONS-76 cells were transfected with a plasmid expressing Brn3a or an empty vector (pcDNA) and examined for trkA transcripts. Cells transfected with pcDNA contained low or undetectable levels of Brn3a transcripts and low levels of trkA. Transfection with a plasmid expressing Brn3a caused a dramatic increase in the levels of trkA transcripts in these cells (Fig 2.8A). We next determined if the trkA proximal promoter (Trk+190) was active in these cells. The reporter plasmid was activated 2 fold in response to exogenous Brn3a ($P \leq 0.007$) (Fig 2.8 B,C) and the promoter

lacking the 190bp of the proximal promoter (ptrkA-190) failed to activate transcription of the reporter plasmid.

2.5 Discussion

Nerve growth factor and its high-affinity receptor trkA play important roles in cell fate determination, neuronal survival, differentiation and growth (Bibel and Barde, 2000, Crowley et al., 1994, Huang and Reichardt, 2003, Martin-Zanca et al., 1990). trkA is normally expressed in small to mediuml-diameter nociceptive sensory neurons in dorsal root and trigeminal ganglia and also in sympathetic neurons (Huang and Reichardt, 2003, Huang et al., 1999, Parada et al., 1992). It is also required for the survival and function of sensory neurons after birth, for the suppression of the growth of neuronal tumors (Chou et al., 2000, Eberhart et al., 2001, Eggert et al., 2000, Muragaki et al., 1997, Ohta et al., 2005, Tajima et al.,1998 Liu et al, 1996, Martin-Zanca et al., 1990) and in the maintenance of Herpes simplex virus latency (Hill et al., 1997, Kriesel, 1999, Laycock et al., 1994, Wilcox et al., 1990). Despite extensive information on trkA regulation during embryogenesis, little is known about the mechanisms that regulate trkA expression in differentiated neurons or neuronal tumors.

Ma et al. (2000) identified a 457 bp region of DNA as the minimal enhancer required for the proper spatial and temporal expression of trkA in trigeminal, dorsal root and sympathetic ganglia in mice during development (Ma et al., 2000). In addition to two binding sites for Brn3a, this enhancer contains binding sites for several other transcription factors. While their studies indicated that mutation of each Brn3a-binding site alone had a relatively minor effect on trkA function, simultaneous mutation of both significantly reduced trkA expression indicating that although the two sites are functionally redundant, they are important for maintaining trkA enhancer function.

To determine how Brn3a might influence trkA expression in differentiated neurons, we examined a 1043 bp DNA segment of human genomic DNA that included the region corresponding to the 457 bp region identified by Ma as the developmental minimal enhancer. We initially examined the ability of Brn3a to

stimulate transcription from the 1043 bp DNA fragment in non-neuronal Vero cells. Since these cells do not express trkA, we could use them as a “black box” to eliminate effects by neuron-specific transcription factors and concentrate on the effects of exogenously supplied Brn3a alone. While the 1043 bp fragment did indeed respond to Brn3a, the two putative Brn3a sites appeared to not be required for its activity.

We found that Brn3a was not able to bind to the 3’ binding motif (M2, HM2 in Fig 2.5) in the minimal enhancer. While Brn3a did bind the 5’ motif (M1, HM1 in Fig 2.5), this motif did not contribute to reporter gene activation in either a non-neuronal cell line (Vero, Fig 2.3) or in sympathetic neuron-like cells (PC12, results not shown). These data are consistent with Ma et al’s (2000) *in vivo* studies in which expression of trkA was not completely suppressed when either site was mutated (Ma et al., 2003). In addition, our results are also supported by those of Lei and others (Lei et al., 2006) that showed that the minimal enhancer is not active in sympathetic neuron-like cells. These authors found that in PC12 cells the trkA minimal enhancer could not be activated by exogenous Brn3a unless the cells were also transfected to co-express the transcription factor Klf7. This indicates that *in vivo* binding of Brn3a to these sites in the minimal enhancer identified by Ma et al may require its association with other proteins.

In contrast to previous data, we found that a short 190 bp region that lies proximal to the trkA coding sequence and does not overlap the 457 bp minimal developmental enhancer, was sufficient for trkA promoter activation by Brn3a in Vero cells (Fig 2.3). This region was also sufficient for Brn3a responsiveness in NGF-differentiated PC12 cells (Fig 2.7). At least two portions of the 190 bp fragment bind to Brn3a with an affinity high enough to be detected in electromobility shift assays (Fig 2.5).

Sacristan et al. (1999) and Chang et al (1998) defined the site at which transcription of the trkA gene begins (TSS in Fig 2.4B) and the region of the trkA promoter that is sufficient to direct accurate transcription in trkA. This region encompasses 150 bp upstream of the transcription start site in the mouse and 138 bp upstream of the start of translation for trkA in humans. These regions are a part of the 190 bp identified by us as important for Brn3a responsiveness. In addition, in our study exogenous Brn3a was only able to activate the transcription of endogenous trkA in the sympathetic-like neuron PC12 (Fig 2.8) and not in Vero cells suggesting that this activation is tissue specific and requires other cell type specific factors. These data are also consistent with those of Sacristan et al (1999) and Chang et al (1998) who found that neuron-specific proteins bound to regions of the trkA proximal promoter. An analisis of the proximal promoters revealed the presence of multiple recognition binding sites for known transcription factors, including ATF, Egr1, Sp1, AP2 and p53. Many of them have been associated with either cell differentiation of neuronal gene expression.

The 190 bp proximal promoter sequence of the human trkA promoter has a high level of homology (82%) with mouse and rat sequences but it also contains 34 additional nucleotides (Fig 2.4B). The region does not contain the putative motifs for Brn3a found in the trkA minimal enhancer described by Ma et al. (2000). However, the 190 bp sequence does have a motif with some similarities to Brn3a-DNA binding sequences identified by Xiang et al. (1995) and Gruber et al. (1997) (Fig 2.4C). These authors defined two consensus DNA sequences for Brn3a binding. The sequences are associated with the activation of anti-apoptotic genes (Bcl-2 and Bcl-xL (Smith et al., 1998)), genes linked to differentiation (alpha-internexin (Budhram-Mahadeo et al., 1995), neurofilament (Smith et al., 1997) and synaptic genes (SNAP25 (Lakin et al., 1995). The potential Brn3a binding sequence in the proximal trkA promoter is 40 nt upstream of the transcription start site for trkA. It differs by only 2 nt out of 11 nt from the one described by Xiang et al. (1995). Moreover, we showed that

recombinant Brn3a, in the absence of other cellular proteins, binds to a fragment containing this potential binding sequence (Fig 2.5) ruling out the requirement for other proteins to interact with Brn3a for transcription activation of the trkA proximal promoter.

Since nociceptive neurons depend on trkA and NGF during development and trkA is active in differentiated sensory neurons, it is likely that Brn3a has a distinctive role in NGF-trkA signalling in these cells. In addition, Brn3a null mice not only have aggressive apoptosis of sensory neurons but also have reduced trkA transcription.

Because our results showed that Brn3a activates trkA expression, restoration of which has been shown to inhibit cell-division in medulloblastomas, we determined if Brn3a could induce trkA expression in these cells. As expected, Brn3a was able to increase trkA transcription in ONS-76 medulloblastoma cells (Fig 2.8) and the 190 bp fragment activated reporter gene expression. Our results suggest that pharmacological or gene therapy procedures that induce Brn3a may lead to regression of these tumors.

2.6 Acknowledgements

This work was supported by a Discovery Grant to VM from the Natural Sciences and Engineering Research Council of Canada. XV was supported partially by the NSERC Discovery Grant and partially by a University of Saskatchewan graduate scholarship.

2.7 References

Akhova, O., Bainbridge, M. and Misra, V.,2005. The neuronal host cell factor-binding protein Zhangfei inhibits herpes simplex virus replication. J. Virol. 79, 14708-14718.

Artinger, K. B., Fedtsova, N., Rhee, J. M., Bronner-Fraser, M. and Turner E., 1998. Placodal origin of Brn-3-expressing cranial sensory neurons. J. Neurobiol. 36, 572-585.

Bibel, M. and Barde, Y. A., 2000. Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. Genes & Dev. 14, 2919-2937.

Budhram-Mahadeo, V., Morris, P. J., Lakin, N. D., Theil, T., Ching, G. Y., Lillycrop, K. A., Möröy, T., Liem, R. K. and Latchman, D. S., 1995. Activation of the alpha-internexin promoter by the Brn-3a transcription factor is dependent on the N-terminal region of the protein. J. Biol. Chem. 270, 2853–2858.

Chang, B. B., Persegiev, S. P., de Diego, J. G., Sacristan, M. P., Zanca, D. M. and Kilpatrick L., 1998. Proximal promoter sequences mediate cell-specific and elevated expression of the favorable prognosis marker trkA in Human neuroblastoma cells. J. Biol. Chem. 273, 39-44.

Chen, C. A. and Okayama, H., 1988. Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. Bio Techniques 6, 632-638.

Chou, T. T., Trojanowski, J. Q. and Lee, V. M., 2000. A novel apoptotic pathway induced by nerve growth factor-mediated trkA activation in medulloblastoma. J. Biol. Chem. 275, 565-570.

Crowley, C., Spencer, S. D., Nishimura, M. C., Chen, K. S., Pitts-Meek, S., Armanini, M. P., Ling, L. H., McMahon, S. B., Shelton, D. L. and Levinson, A. D., 1994. Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76, 1001-1011.

Eberhart, C. G., Kaufman, W. E., Tihan, T. and Burger, P. C., 2001. Apoptosis, neuronal maturation, and neurotrophin expression within medulloblastoma nodules. J. Neuropathol. Exp. Neurol. 60, 462-469.

Eggert, A., Ikegaki, N., Liu, X., Chou, T. T., Lee, V. M., Trojanowski, J. Q. and Brodeur, G. M., 2000. Molecular dissection of trkA signal transduction pathways mediating differentiation in human neuroblastoma cells. Oncogene 19, 2043-2051.

Fedtsova, N. G. and Turner, E. E., 1995. Brn-3.0 expression identifies early post-mitotic CNS neurons and sensory neural precursors. Mech. Dev. 53, 291-304.

Gerrero, M. R., McEvilly, R. J., Turner, E., Lin, C. R., O'Connell, S., Jenne, K. J., Hobbs, M. V. and Rosenfeld, M. G., 1993. Brn-3.0: a POU-domain protein expressed in the sensory, immune, and endocrine systems that functions on elements distinct from known octamer motifs. Proc. Natl Acad. Sci. USA 90, 10841-10845.

Greene, L. A. and Tischler, A. S., 1976. Establishment of a Noradrenergic Clonal Line of Rat Adrenal Pheochromocytoma Cells which Respond to Nerve Growth Factor Proc. Natl Acad. Sci. U S A 73, 2424-2428.

Gruber, C. A., Rhee, J. M., Gleiberman, A. and Turner, E. E., 1997. POU domain factors of the Brn-3 class recognize functional DNA elements which are distinctive, symmetrical, and highly conserved in evolution. Mol. Cell Biol. 17, 2391-2400.

Hill, J. M., Garza, H. H. J., Helmy, M. F., Cook, S. D., Osborne, P. A., Johnson, E. M. J., Thompson, H. W., Green, L. C., O'Callaghan, R. J. and Gebhardt, B. M., 1997. Nerve growth factor antibody stimulates reactivation of ocular herpes simplex virus type 1 in latently infected rabbits. J. Neuro. Viro. 3, 206-211.

Huang, E. J. and Reichardt, L. F., 2003. Trk receptors: roles in neuronal signal transduction. Annu. Rev. Biochem. 72, 609-642.

Huang, E. J., Zang, K., Schmidt, A., Saulys, A., Xiang, M. and Reichardt, L. F., 1999. POU domain factor Brn-3a controls the differentiation and survival of trigeminal neurons by regulating Trk receptor expression. Development 126, 2869-2882.

Kriesel, J. D., 1999. Reactivation of herpes simplex virus: the role of cytokines and intracellular factors. Curr. Opin. Infect. Dis. 12, 235-238.

Lakin, N. D., Morris, P. J., Theil, T., Sato, T. N., Möröy, T., Wilson, M. C. and Latchman, D. S., 1995. Regulation of neurite outgrowth and SNAP-25 gene expression by the Brn-3a transcription factor. J. Biol. Chem. 270, 15858–15863.

Laycock, K. A., Brady, R. H., Lee, S. F., Osborne, P. A., Johnson, E. M. and Pepose, J. S., 1994. The role of nerve growth factor in modulating herpes simplex virus reactivation in vivo. Graefes Arch. Clin. Exp. Ophthalmol. 232, 421-425.

Lei, L., Zhou, J., Lin, L. and Parada, L. F., 2006. Brn3a and Klf7 cooperate to control trkA expression in sensory neurons. Dev. Biol. 300, 758-769.

Liu, Y. Z., Dawson, S. J. and Latchman, D. S., 1996. Alternative splicing of the Brn-3a and Brn-3b transcription factor RNAs is regulated in neuronal cells. J. Mol. Neurosci. 7, 77–85.

Ma, L., Lei, L., Eng S. R., Turner, E. and Parada, L. F., 2003. Brn3a regulation of trkA/NGF receptorexpression indeveloping sensory neurons. Development 130, 3525-3534.

Ma, L., Merenmies, J. and Parada, L. F., 2000. Molecular characterization of the trkA/NGF receptor minimal enhancer reveals regulation by multiple cis elements to drive embryonic neuron expression. Development 127, 3777-3788.

Martin-Zanca, D., Barbacid, M. and Parada, L. F., 1990. Expression of the trk proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development. Genes & Dev. 4, 683-694.

Muragaki, Y., Chou, T. T., Kaplan, D. R., Trojanowski, J. Q. and Lee, V. M., 1997. Nerve growth factor induces apoptosis in human medulloblastoma cell lines that express trkA receptors. J. Neurosci. 17, 530-542.

Ohta, T., Watanabe, T., Katayama, Y., Kurihara, J., Yoshino, A., Nishimoto, H. and Kishimoto, H., 2006. trkA expression is associated with an elevated level of apoptosis in classic medulloblastomas. Neuropathology 26, 170-177.

Parada, L. F., Tsoulfas, P., Tessarollo, L., Blair, J., Reid, S. W. and Soppet, D., 1992. The Trk family of tyrosine kinases: receptors for NGF-related neurotrophins. Cold Spring Harb. Symp. Quant. Biol. 57, 43-51.

Park, K. C., Shimizu, K. and Hayakawa, T., 1998. Interferon yield and MHC antigen expression of human medulloblastoma cells and its suppression during dibutyryl cyclic AMP-induced differentiation: do medulloblastoma cells derive from bipotent neuronal and glial progenitors? Cell. Mol. Neurobiol. 18, 497-507.

Sacristán, M. P., de Diego, J. G., Bonilla, M. and Martín-Zanca, D., 1999. Molecular cloning and characterization of the 5' region of the mouse *trkA* proto-oncogene. Oncogene 18, 5836-5842.

Sasaki, K., Chancellor, M. B., Goins, W. F., Phelan, M. W., Glorioso, J. C., de Groat, W. C. and Yoshimura, N., 2004. Gene therapy using replication-defective herpes simplex virus vectors expressing nerve growth factor in a rat model of diabetic cystopathy. Diabetes 53, 2723-2730.

Schubert, D. and Whitlock, C., 1977. Alteration of Cellular Adhesion by Nerve Growth Factor Proc. Natl Acad. Sci. U S A 74, 4055-405.

Smith, M. D., Dawson, S. J., Boxer, L. M. and Latchman, D. S., 1998. The N-terminal domain unique to the long form of the Brn-3a transcription factor is essential to protect neuronal cells from apoptosis and for the activation of Bcl-2 gene expression. Nucleic Acids. Res. 15, 4100–4107.

Smith, M. D., Morris, P. J., Dawson, S. J., Schwartz, M. L., Schlaepfer, W. W. and Latchman, D. S., 1997. Coordinate induction of the three neurofilament genes by the Brn-3a transcription factor. J. Biol. Chem. 272, 21325–21333.

Tajima, Y., Molina, Jr. R. P., Rorke, L. B., Kaplan, D. R., Radeke, M., Feinstein, S. C., Lee, V. M. and Trojanowski, J. Q., 1998. Neurotrophins and neuronal versus glial differentiation in medulloblastomas and other pediatric brain tumors. Acta Neuropathol. (Berl) 95, 325-332.

.Wilcox, C. L., Smith, R. L., Freed, C. R. and Johnson, E. M. J., 1990. Nerve growth factor-dependence of herpes simplex virus latency in peripheral sympathetic and sensory neurons in vitro. J. Neurosci. 10, 1268-1275.

Xiang, M., Zhou, L., Macke, J. P., Yoshioka, T., Hendry, S. H., Eddy, R. L., Shows, T. B. and Nathans, J., 1995. The Brn-3 family of POU-domain factors: primary structure, binding specificity, and expression in subsets of retinal ganglion cells and somatosensory neurons. J. Neurosci. 15, 4762-4785.

Yamada, M., Shimizu, K., Tamura, K., Okamoto, Y., Matsui, Y., Moriuchi, S., Park, K., Mabuchi, E., Yamamoto, K. and Hayakawa, T., 1989. [Establishment and biological characterization of human medulloblastoma cell lines]. No To Shinkei 41, 695-702.

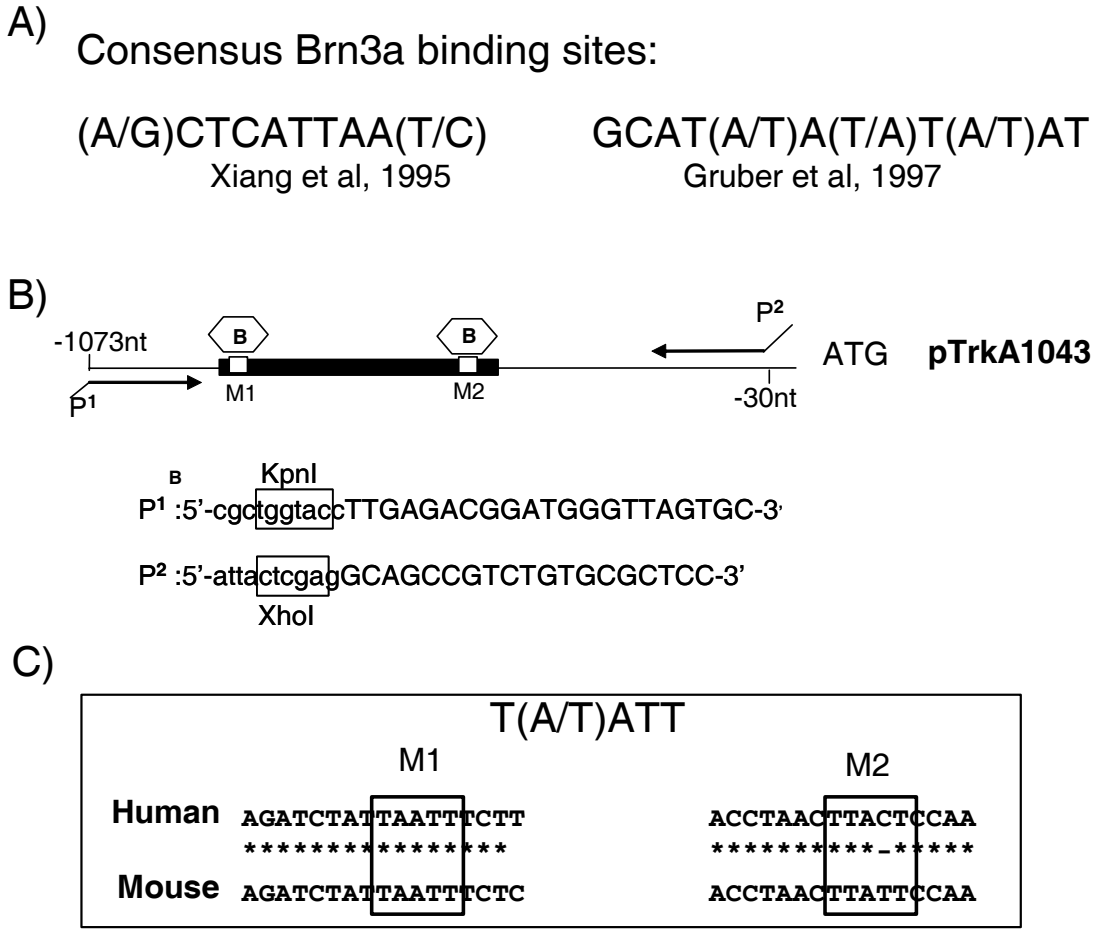


Figure 2.1. The trkA promoter. A) Consensus Brn3a binding sites (Gruber et al., 1997, Xiang et al., 1995) **B)** A schematic representation of the human trkA-genomic fragment showing the location of the two putative Brn3a (B) binding motifs (M1, M2) identified in the mouse trkA minimal enhancer (Ma et al.,2000, 2003). The 457 bp segment required for appropriate expression during embryogenesis is represented as a solid box. The sequence and location of primers P1 and P2 used to amplify human trkA is shown. Nucleotides representing sites for the restriction endonucleases KpnI and XhoI were added to the 5' ends of P1 and P2 to facilitate cloning. The amplified fragment extends from 30 nucleotides (-30) upstream of the initiator codon for the trkA protein to -1073 nucleotides. The 1043 bp PCR product obtained with P1 and P2 and DNA from human cells was cloned into pCAT3basic. The resulting reporter plasmid was called ptrkA1043. **C)** The putative Brn3a core-binding sequences in the minimal enhancer of human and mouse trkA. Asterisks (*) indicate identity.

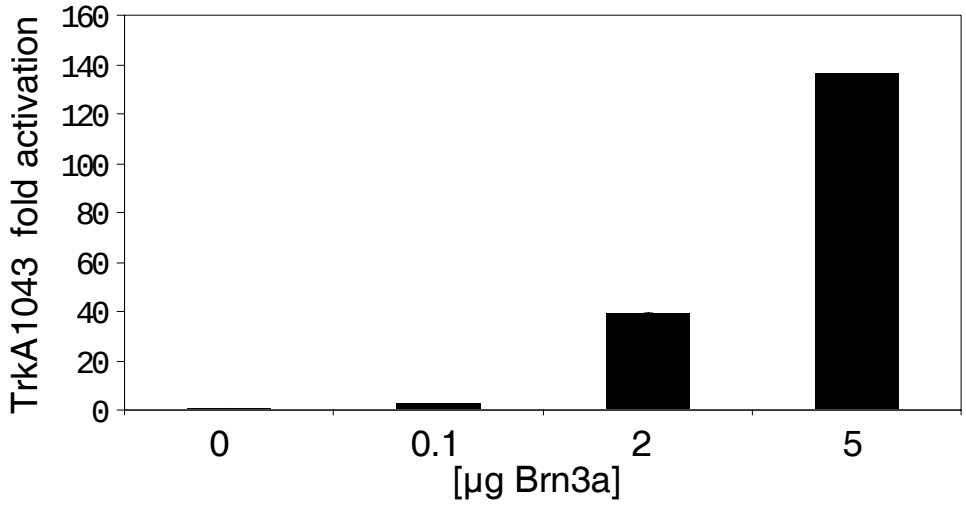


Figure 2.2. Brn3a activates the trkA promoter in Vero cells. Vero (African Green Monkey kidney) cells were transfected with ptrkA1043, a reporter plasmid with the human trkA upstream sequences linked to the coding sequences for chloramphenicol acetyl transferase (CAT) and increasing amounts of a plasmid specifying human Brn3a. Each reaction contained equal amounts of a plasmid coding for beta-galactosidase and the total DNA concentration of each reaction was adjusted to 5 µg with pcDNA. Cells were harvest 48 hr after transfection and CAT and beta-galactosidase activities measured. The CAT activity in each sample was adjusted for transfection efficiency using beta-galactosidase values. The CAT activity of samples without Brn3a expressing plasmid (0) was taken as 1. The results are from a representative experiment. The values are the mean of replicates in the experiment.

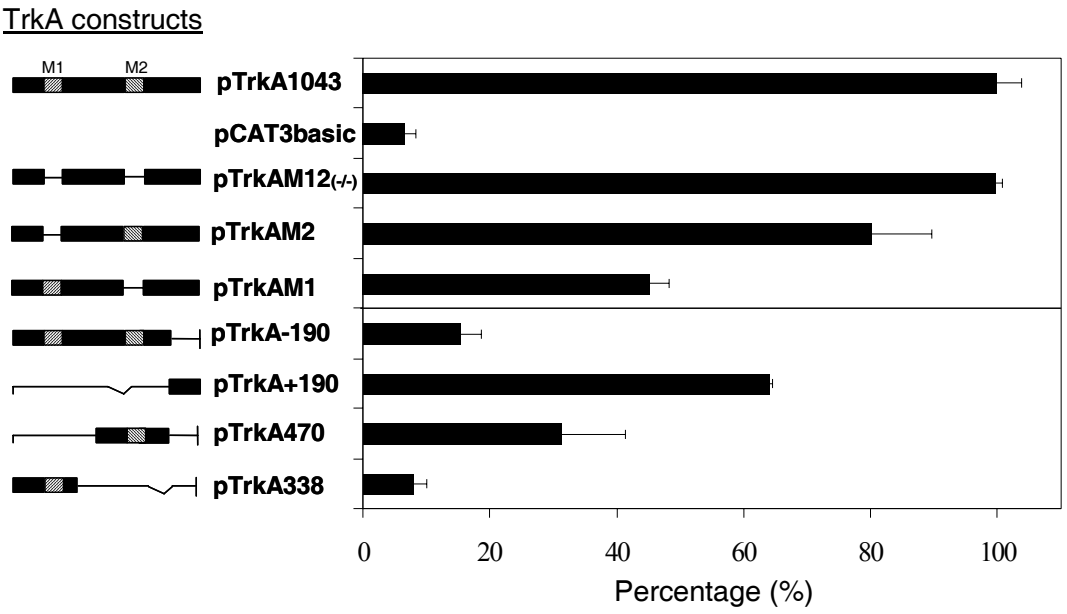
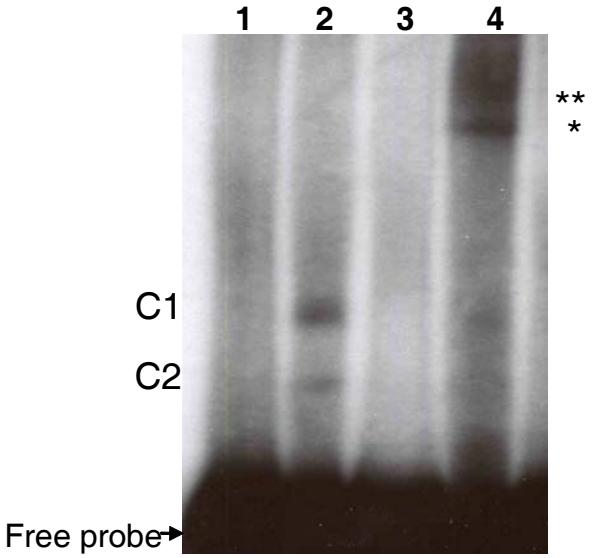


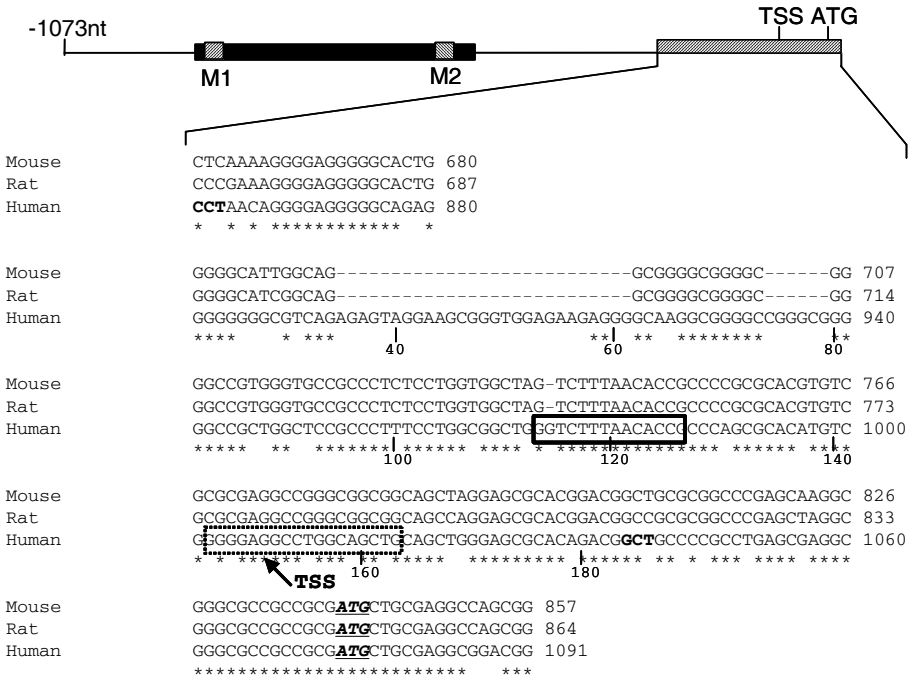
Figure 2.3. Sequences proximal to trkA coding sequences required for Brn3a activated transcription in Vero cells. trkA genomic fragments were generated by restriction enzyme digestion and re-ligation of the fragment. The striped boxes represent the putative Brn3a binding motifs (M1, M2). Horizontal lines represent sequences deleted from original clone. The different reporter constructs (0.25 µg) or pCAT3basic were introduced into Vero cells together with 3 µg of a plasmid specifying Brn3a. A plasmid expressing beta-galactosidase was included in the reactions to assess transfection efficiency. Cells were harvested 48 hr after transfection and CAT and beta-galactosidase measured. CAT activity of the samples is expressed relative to the activity of plasmid ptrkA1043 (100%). Values are the means of those obtained from two independent experiments, each analyzed in duplicate. Bars indicate the standard deviation from the mean.

Figure 2.4. Brn3a binds directly to trkA proximal promoter sequences. **A)** Electromobility shift assays were performed using 1.5 µg of purified GST-Brn3a fusion protein and at least 5 ng of ^{32}P-labeled double stranded oligonucleotides representing the 190 bp trkA proximal sequences. Lane 1 contains probe alone, lane 2- probe + GST-Brn3a , lane 3- probe + GST-Brn3a + 100 fold excess of unlabelled competitor, lane 4- probe + GST-Brn3a + Anti-Brn3a serum. Samples were analyzed on a 6% non-denaturing polyacrylamide gel after incubating reaction for 40 min on ice. C1 and C2 represent complexes formed with Brn3a and the labelled oligonucleotide. Asterisks (* and **) note the location of bands with decreased electrophoretic mobility in the presence of antibodies against Brn3a. **B)** Sequence homology of the trkA 5' proximal promoter region. Nucleotide sequence homology between species is indicated by asterisks (*), and sequences that are missing in the mouse and rat trkA gene are indicated by dashes (-). Potential Brn3a binding site depicted by the bold box and transcription start site (TSS) depicted by a thin lined box. Start codon ATG is shown in italics and underlined. **C)** Potential binding site for Brn3a similar to consensus binding sequences in other promoters (Smith et al.,1997, Hill et al., 1997), identical nucleotides at corresponding positions are indicated by asterisks (*).

A)



B)



C)

Brn3a binding motifs	Xiang et al, 1995
Consensus Brn3a	GCTCATTAAC *_**_*****
Motif TrkA190	5′GGTCTTTAACACC- 3′

Figure 2.4

Figure 2.5. Brn3a binding to overlapping oligonucleotides representing the proximal trkA promoter. **A)** Oligonucleotides representing the trkA proximal promoter including 132 bp upstream of the site where transcription begins (TSS) and 38 bp downstream from it. Probes were 40 bp long (except probe 140, which was 45 bp) and overlapped by 20 bp. Regions that bound to Brn3a (based on results in Fig 2.5C) are indicated by asterisks (*). **B)** A representative gel. HM1 and HM2 are oligonucleotides representing the human M1 and M2 motifs. Electromobility shift assays were performed using 1.5 µg of purified GST-Brn3a fusion protein (except lanes 1) and at least 5 ng of ^{32}P-labeled probes representing sequences within the trkA proximal promoter. Competition was performed with an excess of the corresponding unlabeled oligonucleotides (lane 3) and GST protein was used to show protein specificity (lane 4). Samples were separated on a 6% nondenaturing polyacrylamide gel after incubation for 40 min on ice. **C)** The proportion of each probe bound by GST-Brn3a. The radioactivity in analytical gel was detected using a Typhoon Trio scanner and radioactivity in the protein-probe complex and unbound probe was calculated using ImageQuant software. Binding to the various probes is expressed as a percentage of unbound probes. Human Brn3a only bound to huM1 and to probes 40, 100, 120, and 140. Sequence segments for Brn3a binding in the proximal trkA promoter are indicated in (A) by asterisks.

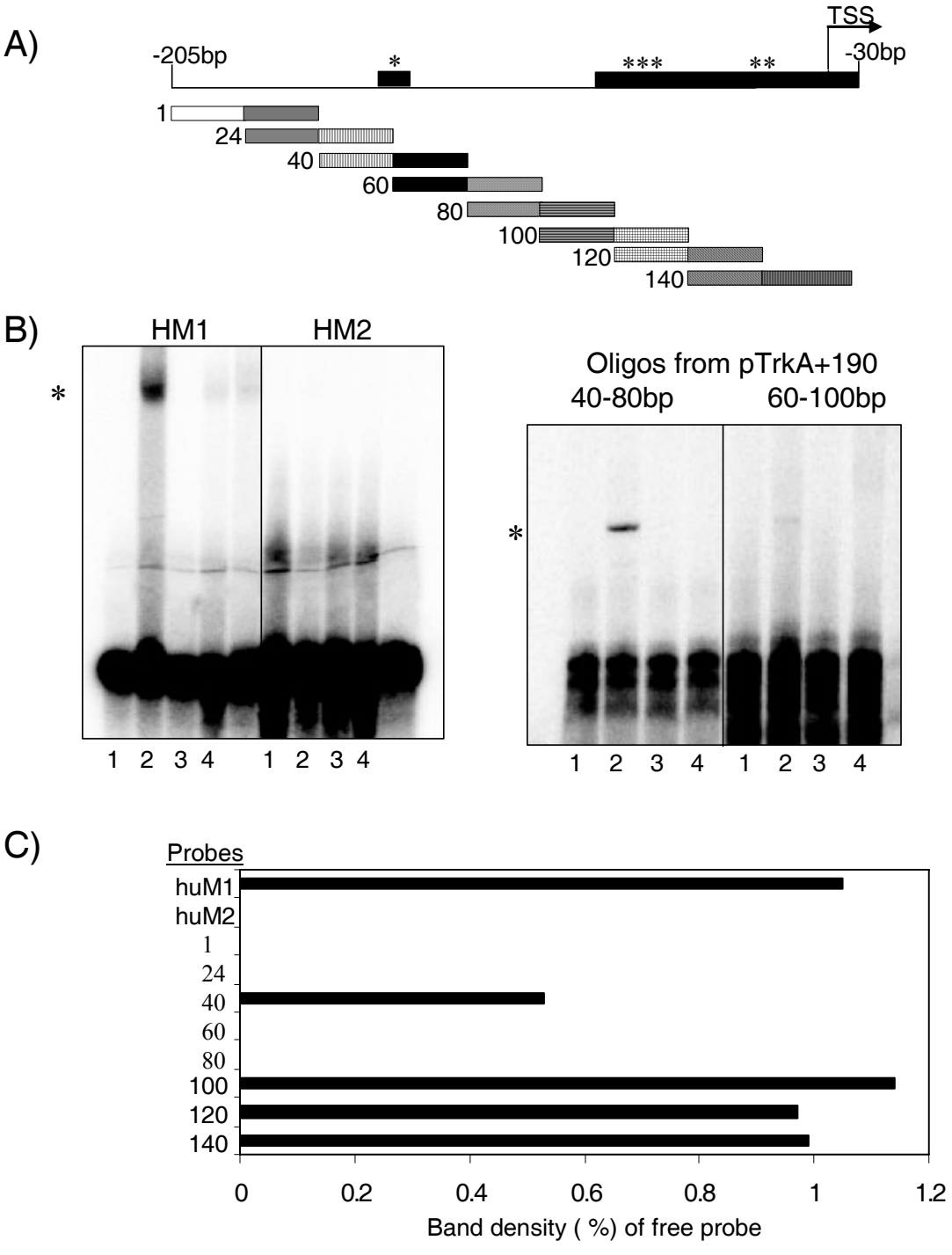
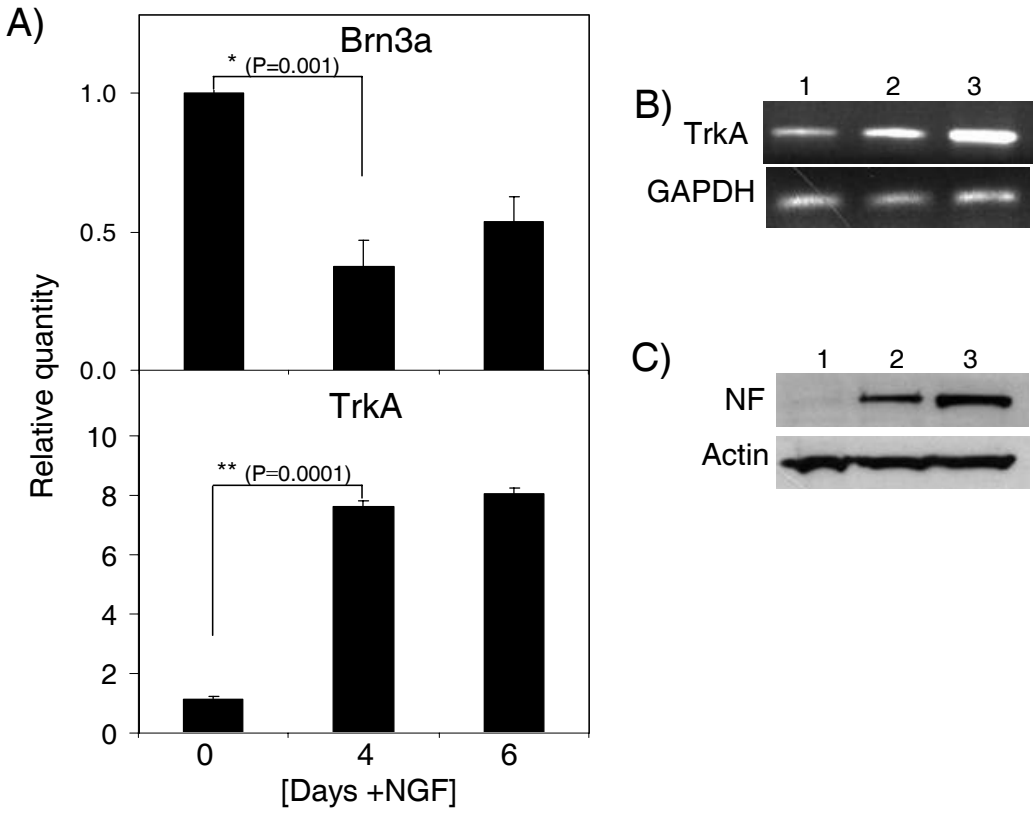

A)
TSS
-205bp
-30bp
*
***
**
1
24
40
60
80
100
120
140
B)
HM1
HM2
*
1 2 3 4 1 2 3 4
Oligos from pTrkA+190
40-80bp
60-100bp
*
1 2 3 4 1 2 3 4
C)
Probes
huM1
huM2
1
24
40
60
80
100
120
140
0
0.2
0.4
0.6
0.8
1
1.2
Band density (%) of free probe

Figure 2.5

Figure 2.6. Transcription of trkA and Brn3a in NGF treated PC12 cells. A) The expression of endogenous trkA and Brn3a transcripts in PC12 cells treated with NGF (50 ng/ml) for 6 days was determined by quantitative real time PCR. Level of transcripts is expressed as relative to the day NGF was added (0), which is regarded as "1". **B**) PCR amplification of trkA and GAPDH day 0, 4 and 6 after NGF treatment (lanes 1, 2, and 3 respectively). **C**) Western blots of PC12 (70 ug protein per lane) at day 0, 4 and 6 (lanes 1, 2, 3 respectively) after NGF treatment, showing neurofilament (NF) and beta-actin. **D)** PC 12 cells were transfected with pcDNA3 or a plasmid expressing Brn3a (1.5 µg) and relative amounts of trkA transcripts measured in NGF treated and untreated cells. Cells were harvested 48 hr after NGF treatment. Values represent averages of three independent experiments, each done in duplicate. Bars are standard errors of means. Statistical differences of (P) are denoted by asterisks (*).


A)
Brn3a
* (P=0.001)
1.0
0.5
0.0
Relative quantity
TrkA
** (P=0.0001)
10
8
6
4
2
0
0
4
6
[Days +NGF]
B)
1
2
3
TrkA
GAPDH
C)
1
2
3
NF
Actin

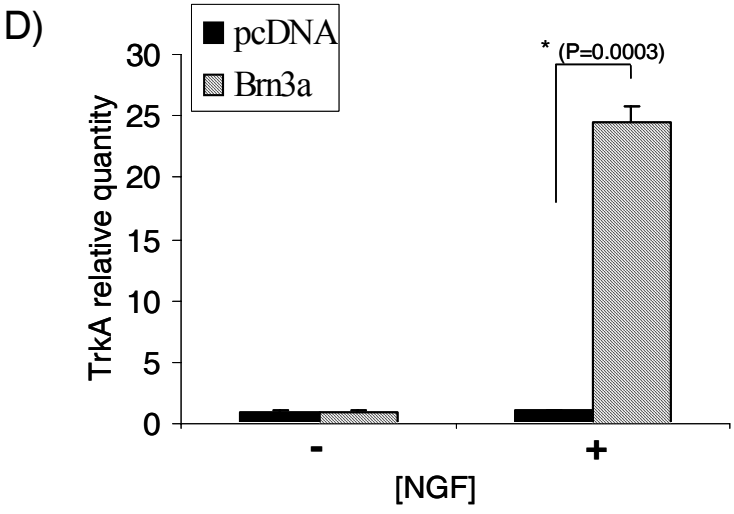

D)
pcDNA
Brn3a
* (P=0.0003)
TrkA relative quantity
30
25
20
15
10
5
0
-
+
[NGF]

Figure 2.6

Figure 2.7. The trkA proximal promoter (ptrkA+190) is activated in PC12 cells. A) PC12 cell were transfected with either pCAT3basic or a reporter plasmid containing 190 bp of the trkA proximal promoter (ptrkA+190). They were then treated for 48 hr with NGF (50 ng/ml). **B)** Brn3a activation of the trkA promoter in PC12 cells. PC12 cells were treated with NGF (50ng/ml) for 48 hours and then transfected with ptrkA+190 or pCAT3basic. The transfection mixtures also contained increasing amounts of a plasmid specifying Brn3a. **C)** Cells were transfected either with ptrkA+190 or ptrkA-190 and transfected with Brn3a expression plasmid.

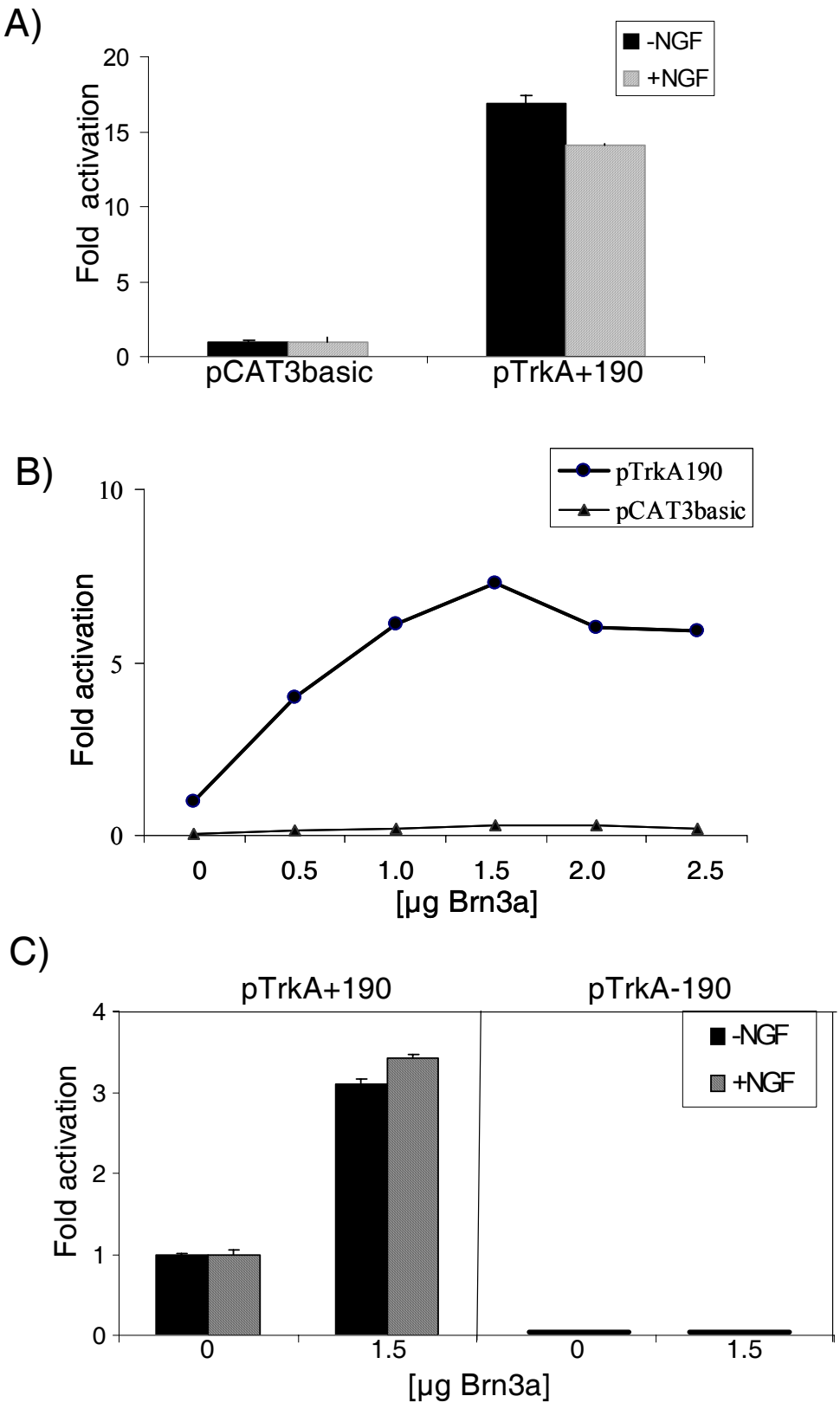

A)
-NGF
+NGF
Fold activation
20
15
10
5
0
pCAT3basic
pTrkA+190
B)
pTrkA190
pCAT3basic
Fold activation
10
5
0
0
0.5
1.0
1.5
2.0
2.5
[µg Brn3a]
C)
pTrkA+190
pTrkA-190
-NGF
+NGF
Fold activation
4
3
2
1
0
0
1.5
0
1.5
[µg Brn3a]

Figure 2.7

Figure 2.8. Brn3a increases trkA transcript levels in medulloblastoma cells.A) ONS-76 medulloblastoma cells were transfected with a plasmid expressing Brn3a; trkA transcripts were measured 48 hr later. Values represent averages of two independent experiments, each done in duplicate. Bars are standard errors of mean. Statistical differences (with P values shown) are denoted by asterisks (*). **B)** Cells were transfected with 0, 250 ng or 750 ng of a plasmid expressing Brn3a together with 300 ng of ptrkA+190 or ptrkA-190, CAT expression was measured 24 hr later. **C)** Western blots of samples in 'B' probed for Brn3a or beta-actin.

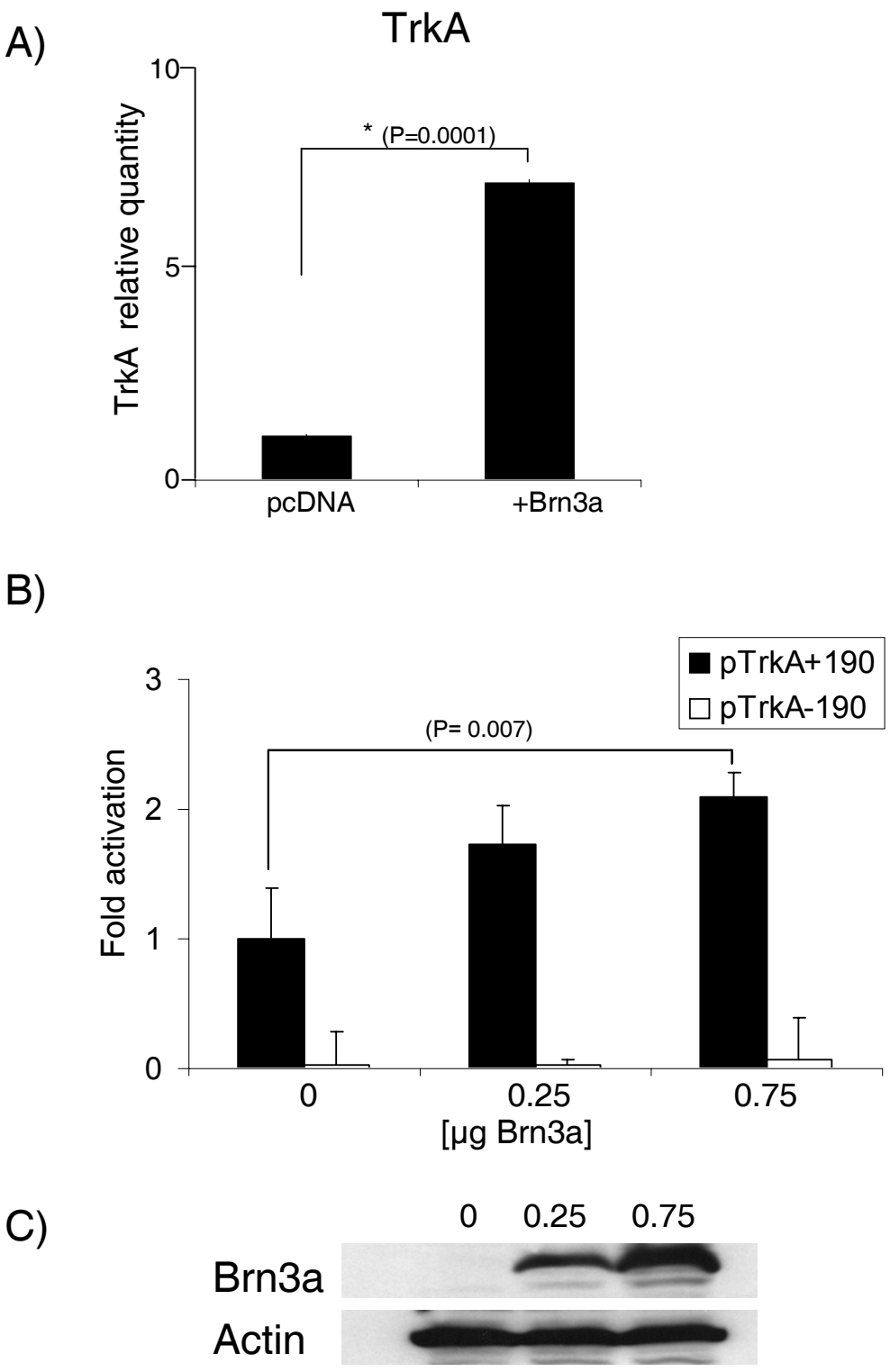

A)
TrkA
TrkA relative quantity
10
5
0
* (P=0.0001)
pcDNA
+Brn3a
B)
pTrkA+190
pTrkA-190
Fold activation
3
2
1
0
(P= 0.007)
0
0.25
0.75
[µg Brn3a]
C)
0
0.25
0.75
Brn3a
Actin

Figure 2.8

3. ZHANGFEI, A NOVEL REGULATOR OF THE HUMAN NERVE GROWTH FACTOR RECEPTOR, trkA.

Ximena Valderrama and Vikram Misra*
Department of Veterinary Microbiology, Western College of Veterinary Medicine, 52 Campus Road, University of Saskatchewan, Saskatoon, Saskatchewan, S7N5B4, CANADA

*Phone number: 1+ -306-966-7218
Fax: 1+ - 306-966-7244
e-mail: vikram.misra@usask.ca

Key words: Brn3a, trkA, nerve growth factor, PC12 cells, Zhangfei, HCF, host cell factor, herpes simplex virus

3.1 Abstract

The replication of herpes simplex virus (HSV) in epithelial cells and during reactivation from latency in sensory neurons, depends on a ubiquitous cellular protein called Host Cell Factor (HCF). The HSV *trans*-activator, VP16, which initiates the viral replicative cycle, binds HCF as do some other cellular proteins. Of these, the neuronal transcription factor Zhangfei suppresses the ability of VP16 to initiate the replicative cycle. It also suppresses Luman, another cellular transcription factor that binds HCF. Interactions of Nerve Growth Factor (NGF) and its receptor tropomyosin related kinase (trkA) appear to be critical for maintaining HSV latency. Since the neuronal transcription factor Brn3a, which regulates trkA expression, has a motif for binding HCF, we investigated if Zhangfei had an effect on its activity. We found that while Brn3a required HCF for activating the trkA promoter and Zhangfei suppressed its activity in non-neuronal cells, in neuron-like NGF-treated PC12 cells, both Brn3a and Zhangfei activated the trkA promoter and induced the expression of endogenous trkA.

3.2 Introduction

Herpes Simplex virus type 1 (HSV-1) causes recurrent lesions on epithelial surfaces. The virus uses two complimentary strategies to avoid immune surveillance while maintaining itself in its primary host and continuing the chain of infection to other hosts. Initial infection of epithelial cells by HSV-1 leads to active replication of the virus. The virus also infects sensory neurons innervating the site of viral replication. The viral genome is delivered by retrograde axonal transport to the neuronal cell body in sensory ganglia where it establishes a latent infection. Periodic reactivation from latency, in response to a variety of stressors, leads to replication of the latent virus in the neuron, transport of virus to epithelial surfaces were, viral replication causes recurrent lesions and potential dissemination to other hosts (reviewed in (Roizman, 2001).

Expression of viral genes during lytic infection in epithelial cells is temporally regulated so that the approximately 80 viral genes involved can be categorized as Immediate Early (IE or α), Early (E or β) or Late (L or γ) depending on when in the replicative cycle they are expressed. Since the efficient expression of E and L genes depends on IE gene products, these regulatory proteins are crucial for the progression of the lytic cycle.

Initiation of the transcription of the IE genes is induced by the assembly of a multi-protein complex made up of the HSV-1 virion protein 16 (VP16) and two cellular proteins: Oct-1 and Host Cell Factor (HCF). VP16 recognizes HFC through its HCF binding motif (HBM), a four amino acid domain – EHAY – that, as the consensus sequence, D/EHxY, is conserved in several cellular and viral HCF-binding proteins. The VP16-HCF heterodimer then recognizes the POU domain of Oct1 bound to TAATGARAT (R is a purine) motifs present in multiple copies in the promoters of all HSV IE genes. The activation domain of VP16 interacts with several components of the transcription machinery leading to the

expression of IE genes. Immediate early proteins subsequently regulate the expression of the E and L genes (Jones, 2003).

Following infection of sensory neurons innervating the epithelial site of viral replication, the HSV-1 virion is transported along axonal microtubules to the neuronal cell body. This process requires proteins present in the viral tegument (Bearer and Satpute-Krishnan, 2002, Luxton et al., 2005), a structure that surrounds the viral nucleocapsid. The tegument comprises, among other viral proteins, VP16. Although the molecular mechanisms that suppress the expression of viral lytic cycle genes, as a prelude to the establishment of a latent infection in the neuron, have not been defined, they most likely involve the blocking IE gene expression (reviewed in (Preston, 2000, Efstathiou and Preston, 2005).

During latency no viral proteins can be detected consistently in the neuron and the viral genome is silent with the exception of the latency associated transcripts which have an anti-apoptotic role (Thompson and Sawtell, 2001, Jin et al., 2003, Gupta et al., 2006). A variety of stimuli have been shown to result in the reactivation of the latent viral genome. Among these is the disruption of Nerve Growth Factor (NGF) induced signaling in the neuron (Wilcox and Johnson, 1988, Wilcox et al., 1990, Block et al., 1994, Laycock et al., 1994, Hill et al., 1997, Jordan et al., 1998). NGF is a neurotropin that plays an important role in the differentiation and survival of sensory neurons (Bibel and Barde, 2000, Huang and Reichardt, 2003). Its high affinity receptor in neurons is the tropomyosin related kinase (trkA) (Parada et al., 1992, Huang and Reichardt, 2003).

Since the latently infected neuron contains no detectable VP16 during viral reactivation, the induction of HSV IE gene expression, and subsequently the viral lytic cycle, must be mediated by cellular proteins. In addition, since HCF appears to be critical for the induction of HSV-1 IE gene expression and,

consequently, the induction of the lytic cycle both in epithelial cells and neurons (Kristie et al., 1999, Khurana and Kristie, 2004, Narayanan et al., 2005) it is conceivable that these viral IE gene-inducing cellular proteins require and bind HCF. To examine this possibility our laboratory and others have identified two neuronal proteins – Luman (Freiman and Herr, 1997, Lu et al., 1997) and Zhangfei (Lu and Misra, 2000b) – that bind HCF and require it for their activity. Luman is a ER-anchored basic leucine zipper (b-Zip) protein. When released from the ER by regulated proteolysis Luman is a potent transcription activator of promoters that contain cyclic AMP and unfolded protein response elements (Lu et al., 1997, Raggo et al., 2002, Liang et al., 2006). These elements include those in the promoters of the HSV-1 IE gene ICP0 and the latency associated transcripts (Lu et al., 1997). Zhangfei is also a b-Zip protein. In contrast to Luman, it acts as a suppressor and reduces gene activation by both Luman (Misra et al., 2005) and VP16 (Akhova et al., 2005). Its effect on VP16 leads to a profound decrease in viral replication in HSV-1 infected cells that express Zhangfei (Akhova et al., 2005).

Among the cellular proteins that possess an HBM (although its ability to bind HCF has not been established) is another neuronal transcription factor called Brn3a (Fig 3.1). This factor has been shown to be important in regulating the expression of trkA during differentiation of sensory neurons in the developing mouse embryo (Ma et al., 2000, Ma et al., 2003). We have recently identified *cis*-acting domains in the proximal trkA promoter that bind Brn3a and shown that exogenous Brn3a induces the expression of trkA in NGF-differentiated PC12 cells and in medulloblastoma cells that do not normally express trkA (Valderrama, manuscript submitted, Chapter 2 in this thesis). In this article we examine Brn3a–Zhangfei interactions to determine if Zhangfei can suppress the ability of Brn3a to activate the trkA promoter. We discovered that while Brn3a requires HCF for activity and that Zhangfei suppresses its activity in non-neuronal cells, however, in neuron-like NGF-differentiated PC12

cells, Zhangfei not only did not suppress Brn3a, it was capable of activating the expression of trkA in the absence of Brn3a.

3.3 Materials and Methods

3.3.1 Cells Culture.

Vero cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen), with 10% newborn calf serum and 1% penicillin-streptomycin. Rat pheochromocytoma (PC12) cells (provided by D. D. Mousseau, University of Saskatchewan) were maintained in Complete Medium (CM) containing RPMI 1640, 10% horse serum (heat treated to inactivate complement), 5% fetal bovine serum, 2 mM glutamine and 1% penicillin-streptomycin. PC12 cells were differentiated with nerve growth factor (NGF, 50ng/ml) and preincubated overnight in RPMI 1640 supplemented with 1% of fetal bovine serum and 1% penicillin-streptomycin.

3.3.2 Plasmids.

The plasmid pRK-55, coding for human Brn3a, was a gift from Mengqing Xiang (Robert Wood Johnson Medical School). Using PCR from HeLa cell genomic DNA a 1043 bp portion of the trkA promoter region was amplified (-1073 to -30 from the trkA initiator codon) and cloned into the chloramphenicol acetyltransferase (CAT) reporter plasmid pCAT3basic (Clontech). The resulting plasmid was named ptrkA1043.

The construction of ptrkA1043 and ptrkA+190, chloramphenicol acetyl transferase (CAT) reporter plasmids with sequences corresponding to the 3' of the human trkA promoter have been previously described (Valderrama and Misra, submitted, Chapter 2 this thesis). These reporter plasmids contain 1043 and 190 base pairs of sequence upstream from the start of transcription of the human trkA gene, respectively. Both reporters are activated by Brn3a. The reporter pCAT3BATF6 has 5 copies of the unfolded protein response element linked to coding sequences for CAT. Plasmids expressing Zhangfei (Lu and Misra, 2000b) and Luman (Raggo et al., 2002, Misra et al., 2005) have also been described previously.

3.3.3 Transfections.

Vero and PC12 cells were transfected using the calcium phosphate method as described previously (Chen and Okayama, 1988). Vero cells were plated at a density of $1x10^6$ cells, transfected the next day and harvested 48 hr later. PC12 cells were seeded at a density of 2×10^6 per well (collagen coated) in CM and incubated overnight at 37°C in a 10% CO_2 incubator. The next day, medium was replaced with RPMI 1640 + 10% normal calf serum +1% penicillin-streptomycin + 2mM L-glutamine and incubated for at least one hour at 37 °C in 5% CO_2 incubator. DNA concentrations for transfection were 2µg of reporter plasmid, 1.5 µg pCMVBGal and 1.5µg of pRK-Brn3a or Luman (pcLuS221Op, which expresses the truncated constitutively active form of Luman) and 2.5µg of Zhangfei or Zhangfei mutant with an altered HBM (pcZFY224A, which cannot bind HCF) DNA/well. After 5 hours in 5% CO_2 incubation cells were glycerol shocked as follows: cells were rinsed with RPMI and then incubated for 45 sec with 0.5 ml of pre-warmed 25% glycerol in 2X BES was added. Cells were rinsed with RPMI 1640 and incubated in 10% CO_2 with 2 ml of PC12 CM or low serum medium (1% foetal bovine serum) if cells were treated with NGF. Cells were harvested 48 hr later for analysis.

3.3.4 Chloramphenicol acetyl transferase (CAT) assays.

For chloramphenicol acetyl transferase (CAT) assays 250ng or 1µg of pCMVBGal, a plasmid specifying beta-galactosidase, was added to transfection mixtures in Vero and PC12 cells respectively. Lysates were assayed for beta-galactosidase and for CAT using an enzyme-linked immunosorbent assay kit (Roche Applied Science). CAT values were adjusted for transfection efficiency using beta-galactosidase values. In figures expressing data as CAT results each data point is the average of replicate transfections. with the bar representing the range. The data are representative of at least two, and usually three, independent experiments.

3.3.5 Antibodies and Immunoblot and immunoprecipiation.

Antibodies against Brn3a, Luman and Zhangfei were produced at the University of Saskatchewan Animal Resources Centre by immunizing rabbits with about 150µg of protein in Freund's complete adjuvant as described previously (Misra et al., 1995). The anti-Brn3a serum specifically detects Brn3a in immuno-blots of *in vitro*-synthesized Brn3a (TnT, Promega) and lysates mammalian cells transfected to express Brn3a (data not shown). Procedures for immunoblotting and *in vitro* translation of control plasmids and HCF (pSL7) plasmid were as previously described (LaBoissiere et al., 1999). For immunoprecipitation, GST-pulldown assays were S^{35}-labelled proteins (Lu and Misra, 2000a), and biotinylation proteins (Suzuki et al., 2004). In brief, ^{35}S-labeled HCF N-C and biotin-labeled Luman, Luman (DHTY78AGTA) or Brn3a were synthesized using a TnT system (Promega). 35S-HCF was precipitated with GST-Luman, GST-Luman GST-Luman (DHTY78AGTA) or GST-Brn3a produced in E. coli, or biotinylated proteins linked to avidin beads. The precipitates were washed extensively and analyzed by SDS-PAGE and autoradiography.

3.3.6 Quantitative real-time PCR (QPCR).

Total RNA was extracted with Trizol (Invitrogen) as suggested by the manufacturer. First strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen) was used for QPCR. Brilliant SYBR Green QPCR Master Mix (Stratagene) was used to quantitate transcripts of interest. The concentration of transcripts in each sample was normalized to a relative amount of GAPDH transcripts expressed as cycle threshold (C_t). Samples were amplified in a Mx3005XP QPCR thermocycler (Stratagene) using the following thermocycle conditions: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 60 °C for 1 min and 72°C for 1 min. Data were analyzed using the thermocycler-associated software. The primer sets for transcript amplification were used at a final concentration of 40 nM. The sequences of the primers were as follows trkAcd-F: 5'-gagggcaaaggctctggactcca-3'; trkAcd-R: 5'-agactccgaagcgcacgatg-3': Gapdh-

F: 5’-gcctcctgcaccaccaactg; Gapdh-R: 5’-gggccatccacagtcttctgg-3.

3.3.7 Suppression of Zhangfei expression using siRNA.

Several portions of the Zhangfei coding regions were assessed for their ability as, siRNA, to suppress Zhangfei protein synthesis. Double stranded oligonucleotides representing a 5’ overhanging BspI site, 22 Zhangfei-coding nucleotides in the sense orientation, a nine nucleotide loop, 22 complementary nucleotides, five T residues as a polymerase III terminator and an overhanging XbaI site were substituted for green fluorescent protein coding sequences in plasmid mU6pro (Yu et al., 2002). The construct mU6ZF14 was the most effective in suppressing Zhangfei protein synthesis. This was designed to target the Zhangfei coding sequence GGAGCTGCGGGCCGAGAAT . It suppressed Zhangfei protein expression, as determined in immunoblots, by over 80% and completely suppressed the ability of Zhangfei to inhibit the ability of Luman to activate a UPRE-containing promoter. Control siRNA, RAD9 coding sequences, cloned in pmU6pro had no effect on Zhangfei expression.

3.4 Results

3.4.1 Brn3a requires nuclear HCF for efficient activation of the trkA promoter.

Luciano and Wilson (Luciano and Wilson, 2003) identified almost fifty viral and cellular proteins that posses the D/EHxY HBM. The proteins with HBM included Brn3a (Fig 3.1). To determine if Brn3a requires HCF for activity, we exploited the natural ability of ER-anchored full-length Luman to sequester HCF in the cytoplasm (Misra et al., 2005) as a means of depleting cells of nuclear HCF. Briefly, we used a mutant of full-length Luman (Lu N160G), which does not bind Luman response elements in promoters and is therefore transcriptionally inactive. This mutant, however, retains HCF in the endoplasmic reticulum (ER) and has the ability to severely inhibit the activity of transcription factors that require HCF while having no effect on factors that are HCF-independent (Misra et al., 2005). We compared the activity of Brn3a in the presence of another mutant, Luman DHTY78AGTA. This mutant has an altered HBM and does not sequester HCF. In cells expressing this mutant most of the HCF is nuclear. We cotransfected cells with a plasmid expressing Brn3a and ptrkA1043, a CAT reporter plasmid with 1043 bp of sequence that lies upstream from the start of transcription of the human trkA gene, as well as plasmids specifying Luman DHTY78AGTA or Luman N160G. We found that while Luman N160G reduced levels of activation by more than 50%, Luman DHTY78AGTA had no effect (Fig 3.2A,B). This suggested that like other HCF-binding proteins, Brn3a requires HCF for efficient activation of the trkA promoter.

We then determined if Brn3a was able to bind directly to HCF. We constructed and purified GST-linked Brn3a and synthesized biotinylated Brn3a and examined the ability of these proteins to bind radiolabelled HCF. As a positive control we used Luman, which binds HCF, and as a negative control we included a mutant of Luman (DHTY78AGTA) that does not bind HCF. HCF bound to the proteins was precipitated with glutathione beads (for GST-fusion

proteins) or avidin coated magnetic beads (biotinylated proteins). In both binding assays Brn3a did not appear to bind HCF (Fig. 3.2C). This suggests that although Brn3a requires HCF for activating the trkA promoter, the *in vitro* synthesized proteins do not form dimers that are stable enough to be recovered in "pull-down" assays.

3.4.2 Zhangfei suppresses the ability of Brn3a to activate the trkA promoter in non-neuronal cells.

Since Zhangfei can suppress the activity of at least two other HCF-binding transcription factors Luman and VP16 (Misra et al., 2003, Misra et al., 2005) we examined the ability of Brn3a to activate the 190 bp trkA minimal promoter (ptrkA+190) in the presence of increasing amounts of a plasmid specifying Zhangfei. We performed these experiments in non-neuronal Vero cells that do not express Brn3a or other neuronal factors to examine the interactions of Brn3a and Zhangfei without the confounding effects of other neuronal factors. Figure 3.3A shows that Zhangfei suppressed the activity of Brn3a in a dose dependent manner.

To determine if Zhangfei was directly responsible for the suppression, we examined the ability of Zhangfei to suppress Brn3a in the presence or absence of a plasmid specifying siRNA against Zhangfei. This plasmid completely abrogates the ability of Zhangfei to suppress Luman, while a plasmid expressing siRNA against Rad9, a non-specific siRNA, has no effect *(results not shown)*. These siRNAs had a very similar effect on the ability of Zhangfei to suppress the activity of Brn3a (Fig 3.3B). In the presence of siRNA Zhangfei, the effect of Zhangfei on Brn3a was eliminated. The combination of Brn3a and Zhangfei and siRNA Zhangfei activated the promoter to a greater extent than Brn3a alone. Real time PCR of the transcripts involved revealed that Vero cells express relative amount of endogenous Zhangfei transcripts. The effect of the siRNA Zhangfei not only abrogated ectopic Zhangfei but also endogenous resulting in a higher activation of the trkA promoter by Brn3a. These results are consistent

with our observations on the effects of Zhangfei on the other HCF-binding proteins, VP16 and Luman (Misra et al., 2003, Misra et al., 2005).

To determimine if HCF binding by Zhangfei was required for Brn3a suppression, we compared the ability of Zhangfei or its HBM domain mutant (ZF Y224A), which does not bind HCF, to suppress Brn3a activation. In contrast to Zhangfei, its HBM mutant did not suppress Brn3a activation of either ptrkA1043 (Fig 3.3C) or the shorter promoter construct (ptrkA+190, data not shown).

3.4.3 The effect of Zhangfei is cell-type and promoter specific.

We next examined the effect of Zhangfei on Brn3a in rat pheochromocytoma cells (PC12) treated with NGF. When PC12 cells are treated with NGF, they differentiate into neuron-like cells which have used extensively as surrogates for neurons in various studies on neuronal function and HSV-1 latency (Wilcox and Johnson, 1987, Wilcox and Johnson, 1988, Wilcox et al., 1990, Smith et al., 1992, Colgin et al., 2001). NGF-treated cells were transfected with ptrkA+190 and plasmids specifying either Brn3a, Zhangfei or a combination of the plasmids. Figure 3.4A shows that in contrast to Vero cells (Fig 3.4B, also see Fig 3.3), in PC12 cells Zhangfei did not suppress the ability of Brn3a to activate the minimal trkA promoter. Zhangfei, in PC12 cells, in contrast to the effects in Vero cells, activated the trkA promoter even in the absence of Brn3a.

Our results suggested that the effect of Zhangfei on the trkA promoter was cell-type specific. To determine if the effect of Zhangfei was promoter and target transcription factor specific as well, we examined the effect of Zhangfei on the ability of Luman to activate a promoter with unfolded protein response elements (pCAT3BATF6). For this experiment we used Luman S221Op, a truncated mutant of Luman that is not anchored in the ER and is constitutively active (Raggo et al., 2002). As reported earlier, in Vero cells Luman S221Op activated the unfolded protein response elements-containing promoter several

hundred fold and Zhangfei completed abrogated this activity (Fig 3.4D). While the activity of Luman S221Op was not as profound in PC12 cells (Fig 3.4C) as in Vero cells (Fig 3.4D), Zhangfei had a suppressive effect (Fig 3.4C).

The ability of Zhangfei to activate the trkA promoter (Fig 3.4A) in PC12 was surprising since it does not do so in Vero cells. To determine if HCF is required for Zhangfei to activate trkA, we examined Zhangfei Y224A (Fig 3.4E) and discovered that the mutant was as effective in activating the minimal trkA promoter as the wild-type protein. This suggests that while Zhangfei can activate the trkA promoter in PC12 cells, it does not require HCF to do so.

3.4.4 Zhangfei activates endogenous expression of trkA in PC12 cells.

We have previously shown (Valderrrama and Misra, submitted, Chapter 2 in this thesis) that not only does Brn3a activate the trkA promoter, expression of exogenous Brn3a in NGF-treated PC12 cells and in medulloblastoma cells leads to an increase in endogenous trkA transcripts. To determine if Zhangfei, like Brn3a, could enhance endogenous trkA expression in NGF-differentiated PC12 cells, we transfected these cells with plasmids expressing Brn3a and Zhangfei, either alone or in combination. When compared to trkA transcript levels in untransfected PC12 cells, both Brn3a and Zhangfei enhanced trkA RNA levels between twenty and thirty fold (Fig 3.5). While Brn3a and Zhangfei did not appear to act synergistically, cells expressing both proteins had significantly higher levels of transcripts than either of the two proteins on their own.

3.5 Discussion

Evidence from cultured neuronal cells as well as experimental animal models for HSV-1 latency and reactivation suggest an important role for NGF-trkA signaling in the establishment and maintenance of viral latency (Wilcox and Johnson, 1988, Laycock et al., 1994, Hill et al., 1997, Kriesel, 1999). Recent data showing that neuronal cell-death caused by pseudorabies virus, which is closely related to HSV-1, is modulated by Brn3a, a transcription factor known to activate trkA expression, support these observations (Geenen et al., 2007). In addition, HCF is critical for the initiation of the replicative cycle of HSV-1 and other alphaherpesviruses such as varicella-zoster virus and pseudorabies virus (Kristie et al., 1999, Khurana and Kristie, 2004, Narayanan et al., 2005). The recent observation that Brn3a possesses a HBM and could potentially require HCF for activity prompted us to investigate a possible link between Brn3a, trkA, NGF signaling, HCF and HSV-1 latency and reactivation. Based on our previous observations that Zhangfei has a suppressive effect on Luman (Misra et al., 2005) and VP16 (Akhova et al., 2005), two other HCF-binding proteins influence HSV-1 gene expression, we hypothesized that Zhangfei would also suppress the ability of Brn3a to activate the expression of trkA and that this would have an impact on NGF-trkA signaling and, consequently on HSV-1 reactivation from latency. Our results showed that Zhangfei did not suppress the ability of Brn3a to activate the trkA promoter, and in contrary to our expectations induced the expression of trkA in the absence of Brn3a.

We studied whether Brn3a requires HCF for its transcription activation function and if so, if this required direct interaction between the proteins. We found (Fig 3.2A), that Brn3a required HCF for transcription activation of the trkA promoter. However, Brn3a did not bind to HCF *in vitro* (Fig 3.2C). These results suggest that either Brn3a requires conformational changes to bind to HCF or HCF dependent activation requires the presence of other proteins found in the cellular environment. Brn3a dependency on HCF for the *trans*-activation of trkA

in non-neuronal cells suggests that it was possible that Zhangfei could regulate Brn3a's function. As with Luman and VP16, Zhangfei suppressed Brn3a transactivation of trkA in an HCF dependant manner in Vero cells. In contrast, in PC12 cells, a sympathetic-like neuronal model, Zhangfei had no repressive effects on Brn3a trans-activation function. Instead, Zhangfei activated the trkA minimal promoter (ptrkA+190) and increased trkA endogenous transcription without the requirement to bind to HCF.

Previous studies (Akhova et al., 2005, Misra et al., 2005) and the results described here suggest that Zhangfei, which does not appear to bind known response elements for b-Zip transcription factors on its own, can either suppress or activate other factors. Zhangfei is one of four viral and human b-Zip factors that lack an asparagine residue in their basic domain. In other b-Zip factors this residue has been shown to be critical for binding to DNA response elements. The other factors that lack the asparagine residue are CCAAT/enhancer binding protein homologous protein (CHOP) (Ubeda et al., 1996, Ubeda et al., 1999), the human T-cell leukemia virus b-Zip factor (HBZ) (Gaudray et al., 2002) and the Kaposi sarcoma herpesvirus b-Zip protein (K-Bzip) (Izumiya et al., 2003a, Izumiya et al., 2003b, Liao et al., 2003). CHOP has gene activating as well as suppressing roles depending on its dimmerization partner and the nature of the cell expressing the proteins. As a dimmer of the b-Zip factors C/EBPα and β, CHOP blocks conversion of 3T3L1 cells to adipocytes (Batchvarova et al., 1995) and by suppressing osteocalcin gene transcription in osteoblasts prevents osteoblast differentiation (Shirakawa et al., 2006). In contrast, as a partner of C/EBPβ in some cells CHOP activates the expression of carbonic anhydrase (Sok et al., 1999). CHOP also acts as a positive regulator of gene expression in association witn non-C/EBP proteins such as ATF3 (Chen et al., 1996), JunD, c-Jun and c-Fos (Ubeda et al., 1999, Ubeda and Habener, 2003).

Some of the functions of Zhangfei, such as the suppressive effects on Luman and VP16 and the effect on Brn3a in non-neuronal cells, are dependent

on binding HCF. A mutant of Zhangfei that does not bind HCF is inefficient in this role. In contrast, the effect of Zhangfei on the activation of trkA in neuronal cells is independent of HCF. Based on these observations we propose a hypothetical role for Zhangfei in the establishment of HSV-1 latency as well as its reactivation from this state (Fig 3.6).

In unstressed neurons the normal function of Zhangfei is to activate trkA expression. Drawing parallels with CHOP, Zhangfei probably accomplishes this as a partner of another b-Zip protein such as ATF4 (17). trkA expression in these cells promotes NGF-trkA signaling. In unstressed neurons HCF is held in the cytoplasm (Kristie et al., 1999), possibly in association with ER-anchored Luman (Lu and Misra, 2000a). Since some Luman is constitutively released from the ER and untimely gene activation by Luman might be detrimental to the cell (Raggo et al., 2002) the role of Zhangfei is to suppress Luman's activity in an HCF-dependant manner. Zhangfei also, again in an HCF-dependant manner, suppresses the VP16-induced expression of viral IE genes in herpes simplex virions delivered to the neuronal cell body, thereby promoting the establishment of latency. The suppression of Luman, which can also activate IE genes, aids in this.

In response to stress we hypothesize that two simultaneous events occur. The expression of Zhangfei ceases, leading to decrease in trkA and, consequently, NGF-trkA signaling. This primes the neuron for HSV-1 reactivation (Wilcox and Johnson, 1988, Wilcox et al., 1990, Block et al., 1994, Laycock et al., 1994, Hill et al., 1997, Jordan et al., 1998). Stress also increases the release of Luman and HCF from the ER and their translocation to the nucleus where they further facilitate reactivation by activating the expression of IE genes.

3.6 Acknowledgements:.

This work was supported by a Discovery Grant to VM from the Natural Sciences and Engineering Research Council of Canada. XV was supported partially by the Discovery grant and partially by a University of Saskatchewan graduate scholarship.

3.7 Reference:

Akhova, O., Bainbridge, M. and Misra, V., 2005. The neuronal host cell factor-binding protein Zhangfei inhibits herpes simplex virus replication. J Virol. 79, 14708-14718.

Batchvarova, N., Wang, X. Z. and Ron, D., 1995. Inhibition of adipogenesis by the stress-induced protein CHOP (Gadd153). Embo J. 14, 4654-4661.

Bearer, E. L. and Satpute-Krishnan, P., 2002. The role of the cytoskeleton in the life cycle of viruses and intracellular bacteria: tracks, motors, and polymerization machines. Curr Drug Targets Infect Disord. 2, 247-264.

Bibel, M. and Barde, Y. A., 2000. Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. Genes Dev. 14, 2919-2937.

Block, T., Barney, S., Masonis, J., Maggioncalda, J., Valyi-Nagy, T. and Fraser, N. W., 1994. Long term herpes simplex virus type 1 infection of nerve growth factor-treated PC12 cells. J Gen Virol. 75 (Pt 9), 2481-2487.

Chen, B. P., Wolfgang, C. D. and Hai, T., 1996. Analysis of ATF3, a transcription factor induced by physiological stresses and modulated by gadd153/Chop10. Mol Cell Biol. 16, 1157-1168.

Chen, C. A. and Okayama, H., 1988. Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. Biotechniques. 6, 632-638.

Colgin, M. A., Smith, R. L. and Wilcox, C. L., 2001. Inducible Cyclic AMP Early Repressor Produces Reactivation of Latent Herpes Simplex Virus Type 1 in Neurons In Vitro. J Virol. 75, 2912-2920.

Efstathiou, S. and Preston, C. M., 2005. Towards an understanding of the molecular basis of herpes simplex virus latency. Virus Res. 111, 108-119.

Freiman, R. N. and Herr, W., 1997. Viral mimicry: common mode of association with HCF by VP16 and the cellular protein LZIP. Genes Dev. 11, 3122-3127.

Gaudray, G., Gachon, F., Basbous, J., Biard-Piechaczyk, M., Devaux, C. and Mesnard, J. M., 2002. The complementary strand of the human T-cell leukemia virus type 1 RNA genome encodes a bZIP transcription factor that down-regulates viral transcription. J Virol. 76, 12813-12822.

Geenen, K., Nauwynck, H. J., De Regge, N., Braeckmans, K. and Favoreel, H. W., 2007. Brn-3a suppresses pseudorabies virus-induced cell death in

sensory neurons. J Gen Virol. 88, 743-747.

Gupta, A., Gartner, J. J., Sethupathy, P., Hatzigeorgiou, A. G. and Fraser, N. W., 2006. Anti-apoptotic function of a microRNA encoded by the HSV-1 latency-associated transcript. Nature. 442, 82-85.

Hill, J. M., Garza, H. H. J., Helmy, M. F., Cook, S. D., Osborne, P. A., Johnson, E. M. J., Thompson, H. W., Green, L. C., O'Callaghan, R. J. and Gebhardt, B. M., 1997. Nerve growth factor antibody stimulates reactivation of ocular herpes simplex virus type 1 in latently infected rabbits. Journal of Neurovirology. 3, 206-211.

Huang, E. J. and Reichardt, L. F., 2003. Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem. 72, 609-642.

Izumiya, Y., Lin, S. F., Ellison, T., Chen, L. Y., Izumiya, C., Luciw, P. and Kung, H. J., 2003a. Kaposi's sarcoma-associated herpesvirus K-bZIP is a coregulator of K-Rta: physical association and promoter-dependent transcriptional repression. J Virol. 77, 1441-1451.

Izumiya, Y., Lin, S. F., Ellison, T. J., Levy, A. M., Mayeur, G. L., Izumiya, C. and Kung, H. J., 2003b. Cell cycle regulation by Kaposi's sarcoma-associated herpesvirus K-bZIP: direct interaction with cyclin-CDK2 and induction of G1 growth arrest. J Virol. 77, 9652-9661.

Jin, L., Peng, W., Perng, G. C., Brick, D. J., Nesburn, A. B., Jones, C. and Wechsler, S. L., 2003. Identification of herpes simplex virus type 1 latency-associated transcript sequences that both inhibit apoptosis and enhance the spontaneous reactivation phenotype. J Virol. 77, 6556-6561.

Jones, C., 2003. Herpes simplex virus type 1 and bovine herpesvirus 1 latency. Clin Microbiol Rev. 16, 79-95.

Jordan, R., Pepe, J. and Schaffer, P. A., 1998. Characterization of a nerve growth factor-inducible cellular activity that enhances herpes simplex virus type 1 gene expression and replication of an ICP0 null mutant in cells of neural lineage. Journal of Virology. 72, 5373-5382.

Khurana, B. and Kristie, T. M., 2004. A Protein Sequestering System Reveals Control of Cellular Programs by the Transcriptional Coactivator HCF-1. J Biol Chem. 279, 33673-33683.

Kriesel, J. D., 1999. Reactivation of herpes simplex virus: the role of cytokines and intracellular factors. Curr Opin Infect Dis. 12, 235-238.

Kristie, T. M., Vogel, J. L. and Sears, A. E., 1999. Nuclear localization of the C1 factor (host cell factor) in sensory neurons correlates with reactivation of herpes simplex virus from latency. Proc Natl Acad Sci U S A. 96, 1229-1233.

LaBoissiere, S., Hughes, T. and O'Hare, P., 1999. HCF-dependent nuclear import of VP16. Embo J. 18, 480-489.

Laycock, K. A., Brady, R. H., Lee, S. F., Osborne, P. A., Johnson, E. M. and Pepose, J. S., 1994. The role of nerve growth factor in modulating herpes simplex virus reactivation in vivo. Graefes Archive For Clinical and Experimental Ophthalmology. 232, 421-425.

Liang, G., Audas, T. E., Li, Y., Cockram, G. P., Dean, J. D., Martyn, A. C., Kokame, K. and Lu, R., 2006. Luman/CREB3 induces transcription of the endoplasmic reticulum stress response protein, Herp, through an ERSE-II element. Mol Cell Biol.

Liao, W., Tang, Y., Lin, S. F., Kung, H. J. and Giam, C. Z., 2003. K-bZIP of Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 (KSHV/HHV-8) binds KSHV/HHV-8 Rta and represses Rta-mediated transactivation. J Virol. 77, 3809-3815.

Lu, R. and Misra, V., 2000a. Potential role for Luman, the cellular homologue of herpes simplex virus VP16 (alpha gene trans-inducing factor), in herpesvirus latency. J Virol. 74, 934-943.

Lu, R. and Misra, V., 2000b. Zhangfei: a second cellular protein interacts with herpes simplex virus accessory factor HCF in a manner similar to Luman and VP16. Nucleic Acids Res. 28, 2446-2454.

Lu, R., Yang, P., O'Hare, P. and Misra, V., 1997. Luman, a new member of the CREB/ATF family, binds to herpes simplex virus VP16-associated host cellular factor. Mol Cell Biol. 17, 5117-5126.

Luciano, R. L. and Wilson, A. C., 2003. HCF-1 functions as a coactivator for the zinc finger protein Krox20. J Biol Chem. 278, 51116-51124.

Luxton, G. W., Haverlock, S., Coller, K. E., Antinone, S. E., Pincetic, A. and Smith, G. A., 2005. Targeting of herpesvirus capsid transport in axons is coupled to association with specific sets of tegument proteins. Proc Natl Acad Sci U S A. 102, 5832-5837.

Ma, L., Lei, L., Eng, S. R., Turner, E. and Parada, L. F., 2003. Brn3a regulation of TrkA/NGF receptor expression in developing sensory neurons. Development. 130, 3525-3534.

Ma, L., Merenmies, J. and Parada, L. F., 2000. Molecular characterization of the TrkA/NGF receptor minimal enhancer reveals regulation by multiple cis elements to drive embryonic neuron expression. Development. 127, 3777-3788.

Misra, V., Rapin, N. and Akhova, O., 2003. ZhangFei is a potent and specific inhibitor of two other HCF binding transcription factors, Luman and herpes simplex virus VP16. submitted.

Misra, V., Rapin, N., Akhova, O., Bainbridge, M. and Korchinski, P., 2005. Zhangfei is a potent and specific inhibitor of the host cell factor-binding transcription factor Luman. J Biol Chem. 280, 15257-15266.

Misra, V., Walker, S., Hayes, S. and O'Hare, P., 1995. The bovine herpesvirus alpha gene trans-inducing factor activates transcription by mechanisms different from those of its herpes simplex virus type 1 counterpart VP16. JVirol. 69, 5209-5216.

Narayanan, A., Nogueira, M. L., Ruyechan, W. T. and Kristie, T. M., 2005. Combinatorial transcription of herpes simplex virus and varicella zoster virus immediate early genes is strictly determined by the cellular coactivator HCF-1. J Biol Chem. 280, 1369-1375.

Parada, L. F., Tsoulfas, P., Tessarollo, L., Blair, J., Reid, S. W. and Soppet, D., 1992. The Trk family of tyrosine kinases: receptors for NGF-related neurotrophins. Cold Spring Harb Symp Quant Biol. 57, 43-51.

Preston, C. M., 2000. Repression of viral transcription during herpes simplex virus latency. J Gen Virol. 81 Pt 1, 1-19.

Raggo, C., Rapin, N., Stirling, J., Gobeil, P., Smith-Windsor, E., O'Hare, P. and Misra, V., 2002. Luman, the cellular counterpart of herpes simplex virus VP16, is processed by regulated intramembrane proteolysis. Mol Cell Biol. 22, 5639-5649.

Roizman, B. S., A. E., 2001. Herpes simplex viruses and their replication. In: Knipe, D. M. H., P.M (Ed.), Fields Virology, vol.2. Lippincott Willaims and Wilkins, pp. 2399-2460.

Shirakawa, K., Maeda, S., Gotoh, T., Hayashi, M., Shinomiya, K., Ehata, S., Nishimura, R., Mori, M., Onozaki, K., Hayashi, H., Uematsu, S., Akira, S., Ogata, E., Miyazono, K. and Imamura, T., 2006. CCAAT/enhancer-binding protein homologous protein (CHOP) regulates osteoblast differentiation. Mol Cell Biol. 26, 6105-6116.

Smith, R. L., Pizer, L. I., Johnson, E. M. J. and Wilcox, C. L., 1992. Activation of second-messenger pathways reactivates latent herpes simplex virus in neuronal cultures. Virology. 188, 311-318.

Sok, J., Wang, X. Z., Batchvarova, N., Kuroda, M., Harding, H. and Ron, D., 1999. CHOP-Dependent stress-inducible expression of a novel form of carbonic anhydrase VI. Mol Cell Biol. 19, 495-504.

Suzuki, H., Ogawa, C., Usui, K. and Hayashizaki, Y., 2004. In vitro pull-down assay without expression constructs. Biotechniques. 37, 918, 920.

Thompson, R. L. and Sawtell, N. M., 2001. Herpes Simplex Virus Type 1 Latency-Associated Transcript Gene Promotes Neuronal Survival. J Virol. 75, 6660-6675.

Ubeda, M. and Habener, J. F., 2003. CHOP transcription factor phosphorylation by casein kinase 2 inhibits transcriptional activation. J Biol Chem. 278, 40514-40520.

Ubeda, M., Vallejo, M. and Habener, J. F., 1999. CHOP enhancement of gene transcription by interactions with Jun/Fos AP-1 complex proteins. Mol Cell Biol. 19, 7589-7599.

Ubeda, M., Wang, X. Z., Zinszner, H., Wu, I., Habener, J. F. and Ron, D., 1996. Stress-induced binding of the transcriptional factor CHOP to a novel DNA control element. Molecular And Cellular Biology. 16, 1479-1489.

Wilcox, C. and Johnson, E., 1987. Nerve growth factor deprivation results in the reactivation of latent herpes simplex virus in vitro. J Virol. 61, 2311-2315.

Wilcox, C. and Johnson, E., 1988. Characterization of nerve growth factor-dependent herpes simplex virus latency in neurons in vitro. J Virol. 62, 393-399.

Wilcox, C. L., Smith, R. L., Freed, C. R. and Johnson, E. M. J., 1990. Nerve growth factor-dependence of herpes simplex virus latency in peripheral sympathetic and sensory neurons in vitro. Journal of Neuroscience. 10, 1268-1275.

Yu, J. Y., DeRuiter, S. L. and Turner, D. L., 2002. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc Natl Acad Sci U S A. 99, 6047-6052.

Protein	amino acid number	HBM	Accession number
HSV-VP16	362	EHAY	P06492
Luman	78	DHTY	AAB69652
Zhangfei	221	DHDY	Q9NS37
Krox20	162	DHLY	P11161
E2F4	389	DHDY	Q16254
Brn3a	19	EHKY	Q01851
Consensus		**D/EHxY**	

Figure 3.1. Some proteins with HCF binding motifs. The location of the first amino acid of the motif in the proteins is indicated as are their accession numbers. The consensus Host Cell Factor binding motif (HBM) is shown (where D=aspartic acid, E=glutamic acid, H=histadine, x=any amino acid and Y=tyrosine) All the proteins listed with the exception of Brn3a have been shown to bind HCF and require it for activity.

Figure 3.2. Brn3a requires HCF for efficient activation of the TrkA promoter. **A)** Brn3a requirement for HCF was assessed by co-expressing Brn3a in Vero cells with an ER-associated mutant of Luman (Lu N160G) that possesses no transcriptional activity alone but retains HCF in the cytoplasm. As a control Brn3a was either expressed alone or with an ER-associated mutant of Luman with an altered HBM (Lu DHTY:AGTA), which does not retain HCF in the cytoplasm. Neither Lu N160G nor Lu DHTY:AGTA activate the trkA promoter. All transfections contained the reporter plasmid ptrkA1043. **B)** Immunoblot of cell lysates in 'A' screened for Brn3a, Luman or beta-actin **C)** *In vitro* binding of radioactive (^{35}S) HCF to Brn3a as assessed by GST or biotin-avidin precipitation. Lane 1 - input HCF alone, Lane 2 – HCF + GST or biotinylated Luman, Lane 3 – HCF + GST or biotinylated Luman (DHTY78AGTA) (mutated HBM), Lane 4 – HCF + GST or biotinylated Brn3a.

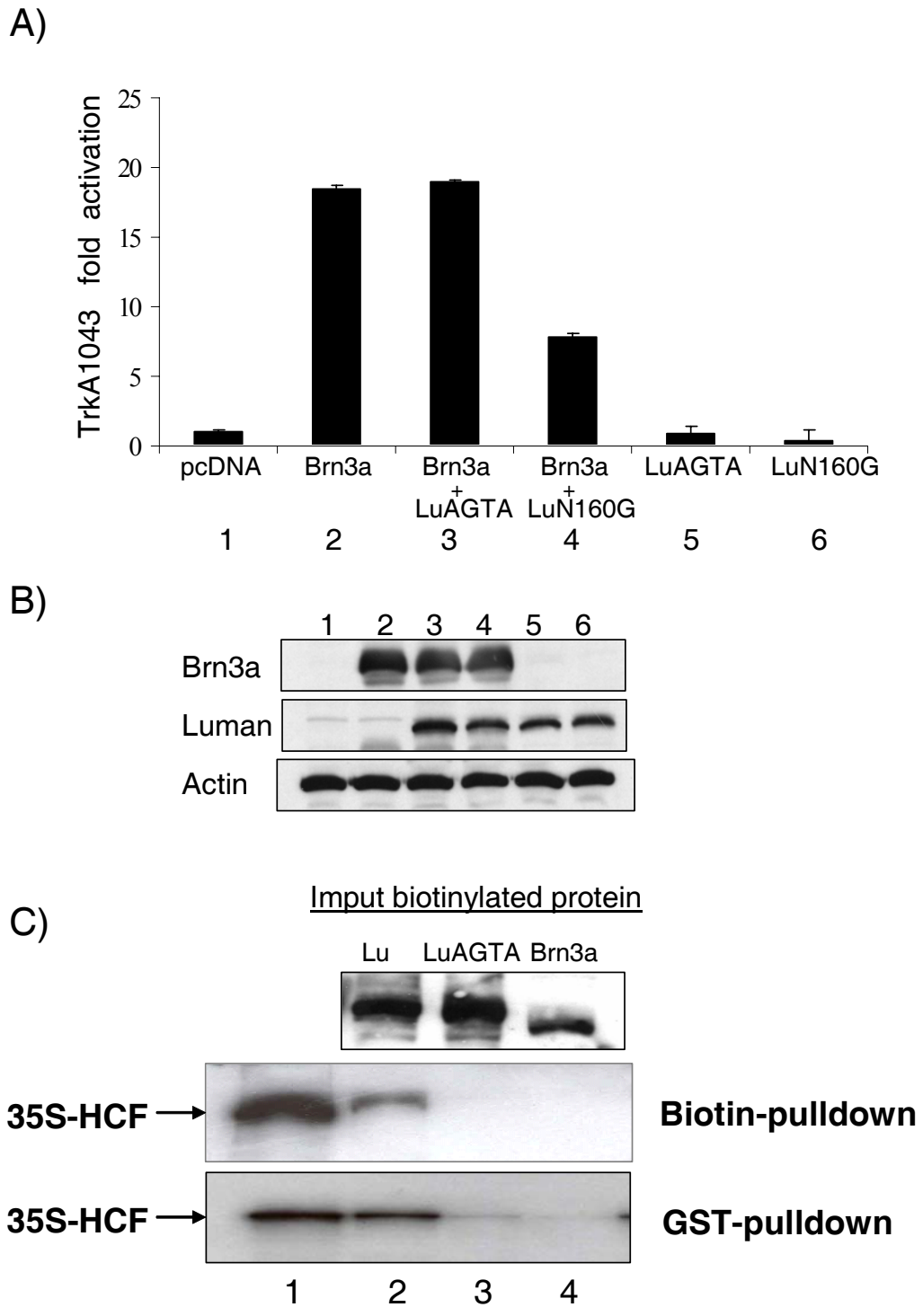

A)
TrkA1043 fold activation
25
20
15
10
5
0
pcDNA
Brn3a
Brn3a + LuAGTA
Brn3a + LuN160G
LuAGTA
LuN160G
1
2
3
4
5
6
B)
1 2 3 4 5 6
Brn3a
Luman
Actin
C)
Imput biotinylated protein
Lu
LuAGTA
Brn3a
35S-HCF
Biotin-pulldown
35S-HCF
GST-pulldown
1
2
3
4

Figure 3.2

Figure 3.3. Zhangfei suppresses the ability of Brn3a to activate the TrkA promoter in non-neuronal cells. A) Vero cells were transfected with the trkA promoter reporter ptrkA+190, a plasmid that specifies Brn3a and varying amounts (0-3 µg) of a plasmid that expresses Zhangfei. The first bar (ptrkA+190) represents CAT activity of the reporter without Brn3a and is regarded as 1. Total DNA in each reaction was adjusted to 6 µg with the empty vector pcDNA3. **B)** siRNA against Zhangfei inhibits its suppressive effect on Brn3a. Vero cells were transfected with the trkA reporter and pcDNA, a plasmid specifying Brn3a, and a Zhangfei expressing plasmid. One batch of cells also received a plasmid expressing siRNA ZF, the other a plasmid expressing an irrelevant siRNA (Rad9). **C)** Zhangfei must bind HCF to suppress Brn3a activity. Vero cells were transfected with a trkA promoter reporter (ptrkA1043), a plasmid expressing Brn3a and varying amounts of a plasmid expressing either Zhangfei or a mutant with a HBM that does not bind HCF (ZFY224A). CAT activity was measured 48 hr after transfection.

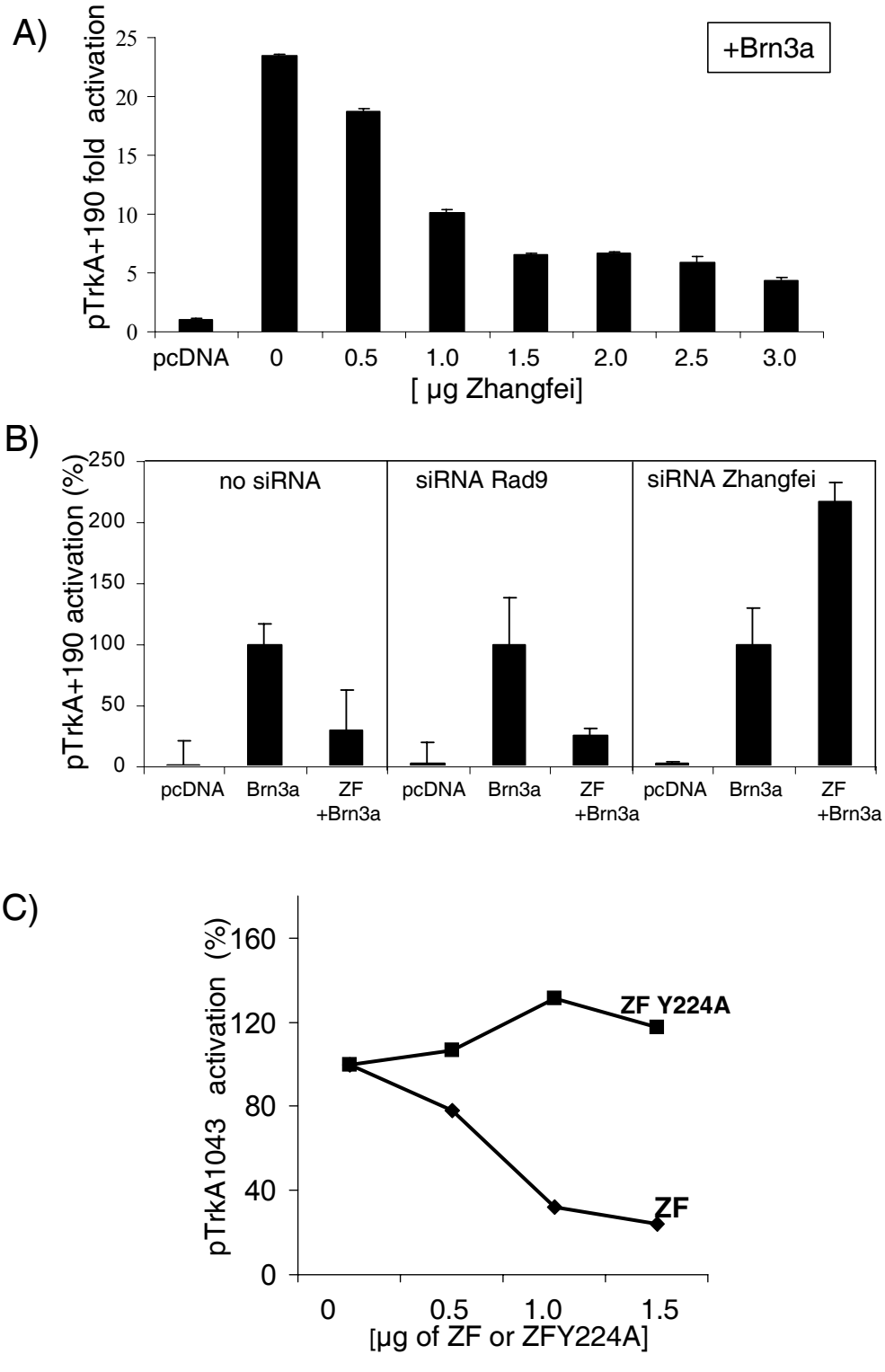

A)
+Brn3a
pTrkA+190 fold activation
25
20
15
10
5
0
pcDNA
0
0.5
1.0
1.5
2.0
2.5
3.0
[µg Zhangfei]
B)
pTrkA+190 activation (%)
250
200
150
100
50
0
no siRNA
siRNA Rad9
siRNA Zhangfei
pcDNA
Brn3a
ZF +Brn3a
C)
pTrkA1043 activation (%)
160
120
80
40
0
ZF Y224A
ZF
0
0.5
1.0
1.5
[µg of ZF or ZFY224A]

Figure 3.3

Figure 3.4. The effect of Zhangfei is dependant on the promoter and cell-type. A) PC12 cells were transfected with the trkA reporter, ptrkA+190, and pcDNA3, or plasmids expressing Brn3a alone, Zhangfei alone or both Brn3a and Zhangfei. After overnight incubation cells were treated with NGF and assayed for CAT 48 hr later. **B)** Vero cells were transfected with the trkA reporter and pcDNA, a plasmid specifying Brn3a, and a Zhangfei expressing plasmid. **C)** PC12 cells were transfected with a reporter with Luman-responsive UPRE binding sites (pCATB3-ATF6) and pcDNA3, or plasmids expressing Luman alone, Zhangfei alone or both Luman and Zhangfei. After overnight incubation cells were treated with NGF and assayed for CAT 48 hr later. **D)** Vero cells were transfected with pCATB3-ATF6 and pcDNA, a plasmid specifying Luman , and a Zhangfei expressing plasmid. **E)** Cells were transfected with ptrkA+190, and pcDNA3, or plasmids expressing Zhangfei or and mutant ZhangfeiY224A (mutated HBM).

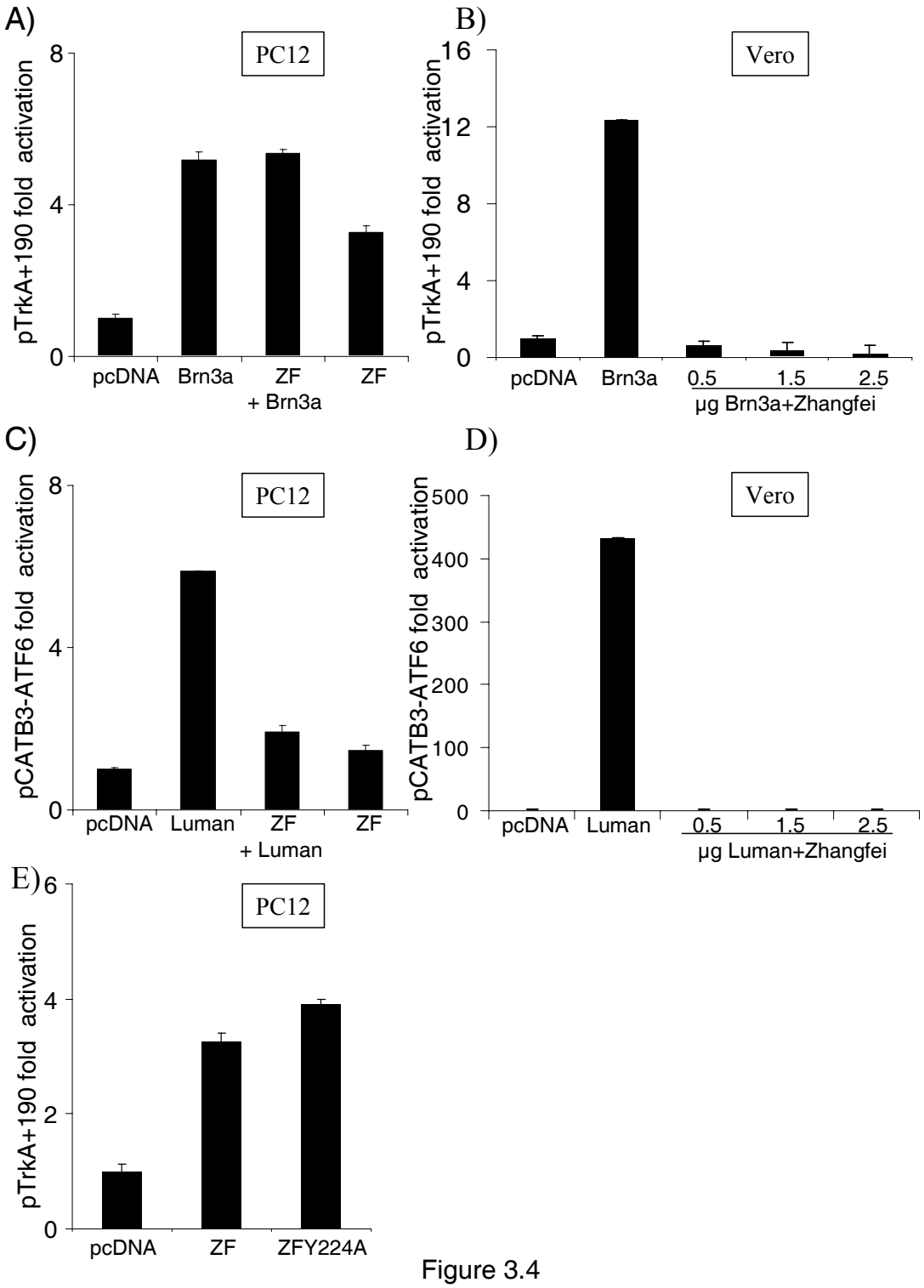

A)
PC12
pTrkA+190 fold activation
8
4
0
pcDNA
Brn3a
ZF
+ Brn3a
ZF
B)
Vero
pTrkA+190 fold activation
16
12
8
4
0
pcDNA
Brn3a
0.5
1.5
2.5
µg Brn3a+Zhangfei
C)
PC12
pCATB3-ATF6 fold activation
8
4
0
pcDNA
Luman
ZF
+ Luman
ZF
D)
Vero
pCATB3-ATF6 fold activation
500
400
300
200
100
0
pcDNA
Luman
0.5
1.5
2.5
µg Luman+Zhangfei
E)
PC12
pTrkA+190 fold activation
6
4
2
0
pcDNA
ZF
ZFY224A

Figure 3.4

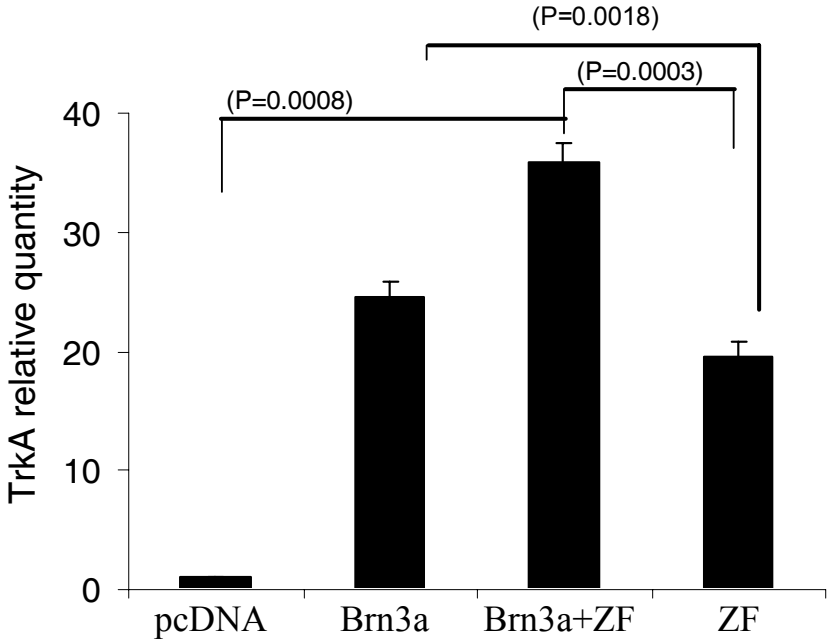


Figure 3.5. Brn3a and Zhangfei activate endogenous TrkA in PC12 cells. Cells were transfected with plasmids expressing pcDNA, Brn3a, Brn3a+Zhangfei or Zhangfei. Forty-eight hr after NGF treatment RNA from the cells was assayed for trkA transcripts using quantitative real-time PCR. Values are normalized to GAPDH transcripts in each sample and the value for the sample with no Brn3a or Zhangfei was designated as 1. Bars are standard errors of means.

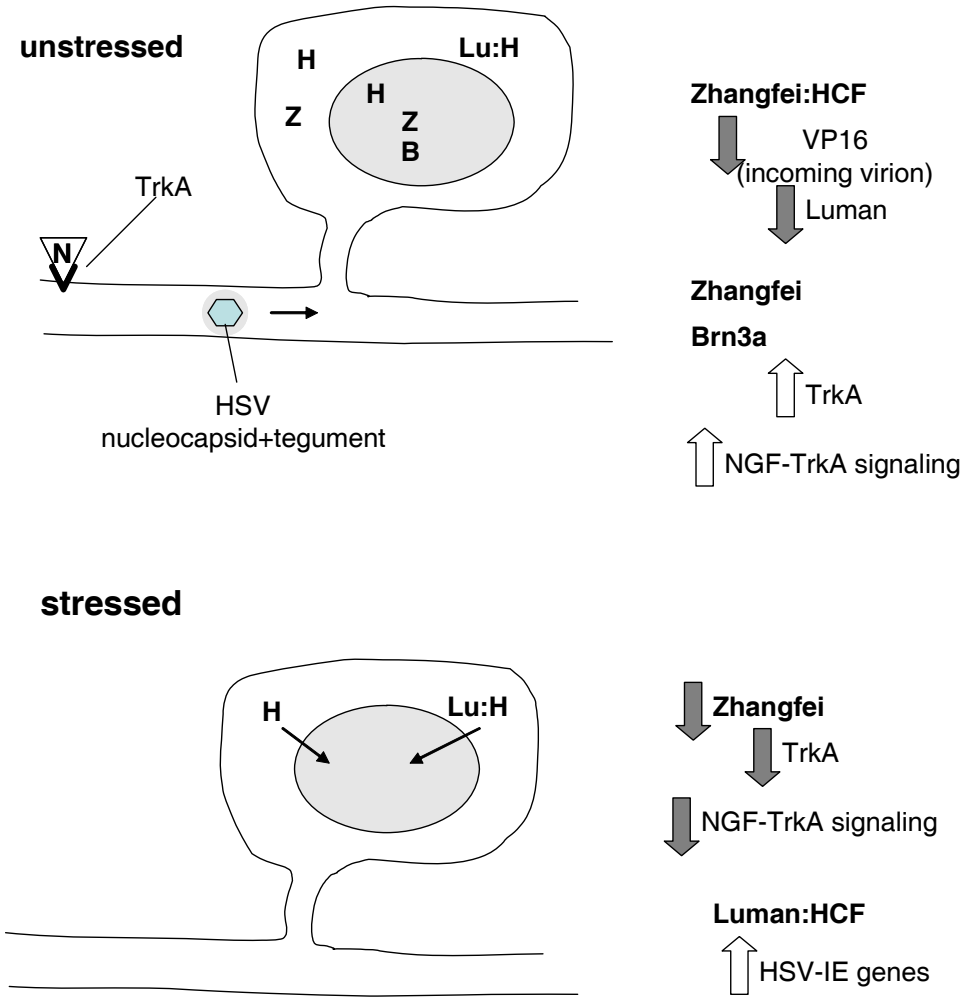


Figure 3.6. Model for the role of Zhangfei in unstressed and stressed neurons. N=NGF, trkA=NGF receptor tyrosine kinase, H=HCF, Lu=Luman, B=Brn3a. In unstressed neurons Luman and associated HCF is anchored in the ER. Zhangfei, in an HCF-independent manner, along with Brn3a activates the expression of trkA. trkA enables NGF-mediated signaling which create suppresses HSV-1 reactivation in latently infected neurons. Zhangfei also suppresses HSV-1 lytic cycle by suppressing VP16 associated with incoming viral nucleocapsids with associated VP16 (tegument). Zhangfei also suppresses any Luman that might be released from the ER thereby further blocking the HSV-1 lytic cycle. In response to stress the expression of Zhangfei is suppressed thereby decreasing trkA expression and, consequently, suppressing NGF signaling and triggering HSV-1 reactivation. A reduction in Zhangfei also allows proteolytically activated Luman to induce the expression of IE genes and hence the HSV-1 lytic cycle.

4. INDUCTION OF THE NEURONAL RECEPTOR TROPOMYOSIN RELATED KINASE, trkA, IN MEDULLOBLASTOMA CELLS BY RESVERATROL AND THE HCF-BINDING PROTEIN ZHANGFEI.

Ximena Valderrama[1], Noreen Rapin[1], Valerie M.K.Verge[2] and Vikram Misra[1]*
[1]Department of Veterinary Microbiology and [2]Department of Anatomy and Cell Biology and Cameco MS Neuroscience Research Center, University of Saskatchewan, Saskatoon, Saskatchewan, S7N5B4, CANADA

*Phone number: 1+ -306-966-7218
Fax: 1+ - 306-966-7244
e-mail: vikram.misra@usask.ca

Key words: trkA, nerve growth factor, medulloblastomas, Zhangfei, HCF, host cell factor, herpes simplex virus

4.1 Abstract

Herpes simplex viruses (HSV) establish latent infections in the sensory neurons of their hosts and are maintained in this state by little understood mechanisms that, at least in part, are regulated by signalling through nerve growth factor (NGF) and its receptor tyrosine kinase, trkA. NGF-trkA interactions influence other neuronal functions as well, including the correct spatial and temporal development of sensory neurons during embryogenesis, the survival of sensory neurons and the differentiation and apoptosis of neuronal tumors. Zhangfei is a transcriptional factor that is expressed in differentiated neurons and is thought to influence HSV replication and latency. Zhangfei, like the HSV transactivator VP16, binds the ubiquitous nuclear protein host cell factor (HCF). Zhangfei inhibits the ability of VP16 to initiate HSV replication and may play a role in the establishment of viral latency. Since we were unable to detect Zhangfei in neuronal tumor cells we hypothesized that ectopic expression of the protein in medulloblastoma cells may induce differentiation. We show that in ONS-76 medulloblastoma cells resveratrol, an inducer of apoptosis and differentiation, increased the expression of Zhangfei and trkA as well as Early Growth Response Gene 1 (Egr1), a gene normally activated by NGF-trkA signalling. ONS-76 cells stopped growing soon after treatment with resveratrol. A portion of the cells underwent apoptosis while others extended long interconnecting processes and remained viable for more than thirty days. While the induction of Zhangfei in resveratrol-treated cells was modest albeit consistent, the infection of actively growing medulloblastoma cells with an adenovirus vector expressing Zhangfei mimicked the effects of resveratrol. Zhangfei activated the expression of trkA and Egr1 and caused these cells to display markers of apoptosis. Binding of HCF by Zhangfei did not appear to be required for this effect as a mutant of Zhangfei incapable of binding HCF also induced the expression of trkA and Egr1. UW228, another medulloblastoma cell-line was also susceptible to the suppressive effects of resveratrol and Zhangfei. In contrast, while resveratrol suppressed the growth of human diploid

fibroblasts (MRC5), Zhangfei did not appear to have an effect on these cells for three days after infection.

4.2 Introduction

The tropomyosin related kinase (trkA), is the main high-affinity receptor for nerve growth factor (NGF) (Parada et al., 1992, Huang and Reichardt, 2003). In the developing mouse embryo, trkA is expressed in neural crest derived neurons destined to become sensory neurons with cell bodies in trigeminal and dorsal root ganglia (Martin-Zanca et al., 1990, Crowley et al., 1994). It is also present in sympathetic neurons. trkA-NGF interactions in neuronal cells control cell fate, differentiation, survival, proliferation, axonal growth and target innervation (Bibel and Barde, 2000, Huang and Reichardt, 2003). The expression of trkA in pain-sensing nociceptive neurons and some sympathetic neurons continues after birth and signalling through them is thought to be important for the maintenance of differentiated phenotype and survival of these cells. Consistent with this is the ability of NGF to reverse injury-induced alterations in sensory neuron phenotype (Verge et al., 1992, Verge et al., 1995) and its ability to mitigate aspects of diabetic neuropathy (Sasaki et al., 2004, Walwyn et al., 2006). trkA expressing neurons are also the site of herpes simplex virus (HSV) latency and deprivation of cells harbouring latent HSV of NGF triggers reactivation of virus (Wilcox et al., 1990, Laycock et al., 1994, Hill et al., 1997, Kriesel, 1999).

Beyond the role of NGF-trkA signalling in maintaining sensory neurons, this pathway may also be important in regulating uncontrolled cell division in neuroblastomas and medulloblastomas, two childhood neuronal cancers. Thus the presence of trkA in neuroblastomas and medulloblastomas correlates with increased rates of apoptosis in tumor cells and favourable outcomes in patients (Tajima et al., 1998, Eberhart et al., 2001, Ohta et al., 2006). In addition, restoration of trkA expression in tumor cells devoid of trkA makes them responsive to NGF and leads to their differentiation or apoptosis (Muragaki et al., 1997, Chou et al., 2000, Eggert et al., 2000).

Herpes simplex virus type 1 (HSV-1) uses two complimentary strategies to avoid immune surveillance and to maintain itself in its host. Infection begins with virus replication and the lysis of infected epithelial cells. During lytic replication, the virus infects sensory nerves and establishes a latent infection in neuronal nuclei located in sensory ganglia (reviewed in (Efstathiou and Preston, 2005)). The virus is thought to be almost completely quiescent during latent infection. Only one viral transcript and no readily detectible viral proteins are expressed.

A variety of physiological and psychological stressors reactivate the latent viral genome to reenter the lytic phase. Following reactivation, the virus replicates in neurons and travels down the axons of sensory neurons to replicate again in epithelial cells causing a recrudescent lesion (reviewed in (Roizman, 2001)). However, the molecular mechanisms responsible for reactivation are not well understood. Neurons latently infected in culture, and explanted ganglia from mice with latent HSV, require NGF for the maintenance of the latent state and depletion of NGF induces the virus to enter the productive cycle (Wilcox and Johnson, 1987, Wilcox and Johnson, 1988, Wilcox et al., 1990). Similarly, increase in intracellular cAMP by the treatment of latently infected neurons with forskolin leads to reactivation (Smith et al., 1992). Colgin and others (Colgin et al., 2001) have reported that levels of inducible cAMP Early Repressor, an inhibitory transcriptional regulator of the cAMP response element (CRE) binding family of proteins, are elevated in response to both NGF depletion and elevation in intracellular levels of cAMP. NGF depletion and increase in cAMP levels may also derepress the expression of productive cycle genes. The withdrawal of NGF as well as treatment with trichostatin-A, a drug that inhibits histone deacetylases, activates lytic cycle genes (Arthur et al., 2001, Danaher et al., 2005). Since trichostatin-A treatment of neurons induces cellular factors that block NGF signaling (Sano and Kitajima, 1996) this suggests a role for histone remodeling in reactivation. Stimulation of the vanilloid/capsaicin receptor (VR-1), which is a calcium channel, by capsaicin,

heat or stimulators of PKC or PKA, leads to reactivation in a cell culture model (Hunsperger and Wilcox, 2003a). This implicates VR-1 as a common pathway for several of the reactivating stimuli. The induction of caspase 3 in latently infected neurons also causes reactivation (Hunsperger and Wilcox, 2003b).

During lytic infection of epithelial cells by HSV-1 the expression of viral genes is temporally regulated and the approximately 80 viral genes involved can be described as Immediate Early (IE), Early (E) or Late (L) depending upon the order of their expression. Initiation of the transcription of the IE genes is induced by the assembly of a multi-protein complex made up of the virion protein VP16 and the two cellular proteins: Oct-1 and host cellular factor (HCF) (reviewed in (Wysocka and Herr, 2003)).

HCF is a ubiquitously expressed chromatin-associated protein. The mature form of HCF is derived from a 2035 amino acid precursor which is processed by internal proteolytical cleavage to amino and carboxyl terminal portions that are held together by no-covalent interactions (Wilson et al., 1993, Kristie et al., 1995). Although HCF was initially discovered as a component of the VP16-induced transcriptional complex it is important for many cellular processes including cell-cycle progression and cytokinesis (Julien and Herr, 2003), chromatin structure modification and RNA splicing (Ajuh et al., 2002, Wysocka et al., 2003). In addition to these cellular roles, HCF participates in the regulation of the IE gene expression of HSV-1, varicella-zoster virus (Narayanan et al., 2005) and possibly that of other alpha herpesviruses as well. VP16 binds to the amino-terminal component of HCF, which possesses a □-propeller-like structure formed by six Kelch repeats (Wilson et al., 1997, Hughes et al., 1999).

Several cellular proteins, which like VP16, interact with the amino-terminus of HCF share the HCF, binding motif (HBM) D/EHxY. These include, E2F4 (Luciano and Wilson, 2003, Knez et al., 2006), Krox20 (Luciano and Wilson, 2003), Luman/LZIP/CREB3 (Lu et al., 1997, Lu et al., 1998, Lu and

Misra, 2000a) and Zhangfei (Lu and Misra, 2000b). The motif is present in other proteins as well, but HCF binding by these proteins has not been examined (Luciano and Wilson, 2003).

Zhangfei has the characteristics of a basic leucine-zipper (b-Zip) containing protein. However, unlike other b-Zip proteins Zhangfei does not bind any of the known cognate sequences for b-Zip proteins nor does it activate promoters containing these sequences (Lu and Misra, 2000b, Hogan et al., 2006). Zhangfei has a profoundly repressive effect upon HCF-dependant transcription activation by Luman (Misra et al., 2005) and VP16 (Akhova et al., 2005). HCF binding by both Zhangfei and its targets is required for efficient suppression by Zhangfei. Interestingly, while Zhangfei and Luman have incompatible b-Zip domains and do not interact directly, Zhangfei does have the capacity to dimerize with other factors such as Xbp1, and ATF4 through their b-Zip domains (Newman and Keating, 2003). The effect of these interactions can lead to suppression of gene expression, with Xbp1 (Chauhan et al manuscript submitted) or lead to gene activation, with ATF4 (Hogan et al., 2006).

Since Zhangfei was not detected in neuronal tumor cells we hypothesised that ectopic expression of the protein in medulloblastoma cells may induce them to differentiate. We show that in medulloblastoma cells resveratrol, an inducer of apoptosis and differentiation in these cells, increased the expression of Zhangfei as well as trkA and Early Growth Response Gene 1 (Egr1), a gene normally activated by NGF-trkA signalling. Medulloblastoma cells stopped growing soon after treatment with resveratrol. A portion of the cells underwent apoptosis while others extended long processes and remained viable for more than thirty days. While the induction of Zhangfei in resveratrol-treated cells was modest, albeit consistent, the ectopic expression Zhangfei in actively growing medulloblastoma cells mimicked the effects of resveratrol. Zhangfei activated the expression of trkA and EgR1 and caused these cells to display markers of apoptosis. Binding

of HCF by Zhangfei did not appear to be required for this effect as a mutant incapable of binding HCF also induced the expression of trkA and Egr1.

4.3 Materials and Methods

4.3.1 Cell Culture

Human medulloblastoma cells (ONS-76, (Yamada et al., 1989) and UW228 (Keles et al., 1995)) were obtained from Michael Taylor (University of Toronto) and grown in Dulbecco's modified Eagle's medium (D-MEM) with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin. For some experiments medulloblastoma cells were plated at a density of 2 X 10^4 cells per cm^2. After incubating overnight the medium was replaced with D-MEM containing 1% FBS and antibiotics and 100μM resveratrol (Sigma). Stock solutions of resveratrol were made up in dimethyl sulphoxide (DMSO) DMSO and control cultures received an equivalent amount of DMSO. For long term-cultures of ONS-76 cells in resveratrol half the medium was replaced every other day. Rat pheochromocytoma (PC12) cells (provided by D. D. Mousseau, University of Saskatchewan) were maintained in Complete Medium containing RPMI 1640 supplemented with 10% horse heat inactivated serum, 5% of foetal bovine serum, 2 mM glutamine and 1% penicillin-streptomycin. PC12 cells were cultured at a density of 2 x 10^6 in 6 well collagen coated plates. To differentiate PC12 cells they were "fasted" in low serum media, RPMI 1640 supplemented with 1% foetal bovine serum and 1% penicillin-streptomycin, overnight before treatment with nerve growth factor (50 ng/ml). Human diploid fibroblasts, MRC-5 cells (American Type Tissue Culture Collection), were grown in D-MEM containing 10% FBS and antibiotics

To determine the growth rate cells were plated at a density of 2 X 10^4 cells per cm^2 in wells of six-well culture dishes. At daily intervals cells in the culture supernatant as well as adherent cells recovered by typsinization and collected in phosphate buffered saline containing 10% FBS were pelleted and resuspended in 0.5 ml PBS containing 0.08% trypan blue. Trypan blue containing and excluding cells were counted separately in a haemocytometer in triplicate samples.

4.3.2 Immunoblots

Proteins in cell lysates were separated by SDS-polyacrylamide gel electrophoresis and probed by immunoblotting as described earlier. Rabbit polyclonal antibodies against Erk1/2 (Map kinase p44/42) that detect total Erk1/2 or phosphorylated Erk1/2 (Cederlane/Cell Signaling, Hornby, Ontario, Canada) specific for human Erk1 wee used to probe the immunoblots.

4.3.3 Adenovirus vectors expressing Zhangfei

The construction, growth and purification of adenovirus vectors (Adeno-X Expression System, Clontech) expressing Zhangfei or beta-galactosidase has been described (Misra et al., 2005). ONS-76 cells in 6 well plates were infected at a multiplicity of infection of 10 plaque-forming units of Adeno-Zhangfei or Adeno-LacZ per cell in 0.2 ml of OptiMEM, or were mock-infected. After 1 hr at 37^{o}C, 2 ml of medium containing 10% serum were added. The next day the medium was replaced with medium containing 1% serum. Cells were photographed in a phase-contrast microscope or harvested for RNA extraction 48 hr later.

4.3.4 RNA preparation and Real time PCR (QPCR) analysis.

Total RNA was extracted from cells in 6 well tissue culture plates using Trizol (Invitrogen) as suggested by the manufacturer and dissolved in 20 µl of DEPC-treated water. Next, 5 µg of total RNA was used for first strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen). To detect and quantitate trkA and other transcripts we used Brilliant SYBR Green QPCR Master Mix (Stratagene). Samples were amplified in an Mx3005XP QPCR thermocycler (Stratagene) using the following thermocycle conditions: one cycle at 95 °C for 10 min, 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 1 min. Data were analyzed using the thermocycler-associated software. For each sample cycle threshold values for the assayed transcripts (trkA, NGF, Zhangfei and Egr1) were normalized for total input RNA concentrations using cycle threshold values for transcripts for the “normalizer” house-keeping gene,

GAPDH. The normalized values in each experiment were compared to a "calibrator" sample to determine the relative increase in the amount of the transcript. Results were analyzed for significant differences using the Student T-TEST. The primer sets for transcript amplification were used at a final concentration of 40 nM. The sequences of the primers were as follows:
NGF-F: 5' – caggactcacaggagcaagc; NGF-R: 5' – gccttcctgctgagcacaca; trkAcd-F: 5' - gagggcaaaggctctggactcca-3'; trkAcd-R: 5'- agactccgaagcgcacgatg-3': ZF-F: 5'- ctgaccacctccctcttcag-3'; ZF-R: 5'- cagaactccaccgacacctt-3'; Gapdh-F: 5'-gcctcctgcaccaccaactg; Gapdh-R: 5'- gggccatccacagtcttctgg; Egr1-F: 5' - gcacccaccagtggcaac-3' and Egr1-R: 5'- 'gggatcatgggaacctgg-3. Following amplification, the melting curves for the products were generated to ensure that the product represented a homogenous species. In addition, the PCR products were analyzed by electrophoresis to ensure that a product of the predicted size had been generated. The expected sizes of amplified products were 300 bp for Egr1, 150 bp for Zhangfei, 300 bp for trkA ,130 bp for GAPDH and 300 bp for NGF.

4.3.5 Apoptotic cells

To estimate the number of apoptotic cells in a culture the proportion of cells expressing active caspase 3 or external phosphatidylserine were determined using the Active Caspase-3 PE MAb Apoptosis kit and the Annexin V-FITC Apoptosis Detection Kit. Both kits were purchased from BD Biosciences Pharmingen. The techniques used were as suggested by the manufacturer. Fluorescent cells were analyzed by flow cytometry on a Beckman-Coulter Epics XL flow-cytometer and analyzed using EXPO32 software. Figures were generated using Flow Jo 8.1.

4.4 Results

Resveratrol or 3,5,4'-trihydroxy-*trans*-stilbene, is a phytoalexin found in many plant products including red wine. Resveratrol results in differentiation and apoptosis of neuroblastomas and medulloblastomas (Tredici et al., 1999, Nicolini et al., 2003, Wang et al., 2003, Chen et al., 2004, Zhang et al., 2006). ONS-76 is a human medulloblastoma cell line derived from a cerebellar tumor in a 2-year-old girl (Yamada et al., 1989). The cells exhibit the characteristics of both neuronal and glial cells (Park et al., 1998). We found that treating ONS-76 medulloblastoma cells with 100 μM resveratrol caused an immediate cessation of their growth with no increase in viable cells in the 24 hr after treatment (Fig 1). Cultures after 24 hours of resveratrol treatment contained few trypan blue staining cells suggesting that the lack of increase in cell numbers was not due to cell death. While there was a gradual decrease in cell numbers over a period of time most cells became elongated with processes that appeared to reach out to other cells (Fig 4.1C). The cells remained healthy up until at least 30 days after which they were fixed in methanol.

4.4.1 Differentiated medulloblastoma cells show trkA-mediated signalling.

Since the restoration of trkA signalling in medulloblastomas leads to their apoptosis or differentiation, we questioned whether the reverse was true: could the induced differentiation of these cells lead to trkA expression and if so, did Zhangfei played a role in this? Medulloblastoma cells were induced to differentiate with resveratrol (Zhang et al., 2006). Zhangfei, trkA, NGF, Egr1 and GAPDH transcript levels in resveratrol-differentiated cells and growing cells (control cells were treated with an equivalent amount of DMSO, the solvent for resveratrol) were measured using qRT-PCR. Egr1 is one of the genes activated as a result of NGF-trkA signalling (Harada et al., 2001).

Differentiation of ONS-76 cells was accompanied by a modest but significant increase in Zhangfei (1.4 fold), trkA (4 fold), Egr1 (7 fold) and NGF (11 fold)

transcripts (Fig 4.2A). These results suggested that resveratrol induces NGF-trkA signalling in ONS-76 cells. trkA signalling requires its ligand NGF. Although we did not add NGF to the cultures, the qRT-PCR results indicated that ONS-76 cells express endogenous NGF and that its level increases when the cells are treated with resveratrol. To confirm that the resveratrol-treated cells contain NGF, thereby allowing autocrine stimulation of the induced trkA receptor, we examined the ability of lysates from resveratrol or DMSO-treated cells to induce neurite formation in rat PC12 cells. These cells can be induced to differentiate into sympathetic neuron-like cells if treated with NGF. One of the characteristics of differentiated PC12 cells is the sprouting of neurite-like processes (Fig 4.2C, top panel). High concentrations of lysates from ONS-76 cells were toxic for PC12 cells. PC12 cells treated with lysate from resveratrol-treated ONS-76 cells diluted 40 fold showed some neurite-like processes (Fig 4.2C, lower panel, arrows). Lysates of growing ONS-76 cells also induced some neurites in PC12 cells although these were less obvious than in cells treated with an equivalent concentration of lysate from resveratrol-treated ONS-76 cells (not shown). Concentrations of resveratrol greater than 25 μM were toxic to PC12 cells. No neurites were seen in PC12 cells treated with 25μM of resveratrol.

4.4.2 Expression of Zhangfei in growing ONS-76 cells leads to suppression of growth, induction of trkA and the trkA-NGF pathway.

There appeared to be a progressive increase in the magnitude of transcripts for Zhangfei, trkA and Egr1 in response to resveratrol (Fig 4.2 A). One interpretation of these results is that Zhangfei plays a role in the induction of trkA expression, which then leads to an increase in Egr1 expression. To study this possibility, we infected ONS-76 cells with adenovirus vectors expressing either Zhangfei (Ad-ZF) or the irrelevant bacterial protein, beta galactosidase (Ad-LacZ). Cells were also mock-infected (MI). Cells were counted at the time of infection and then for the next four days (Fig 4.3A). Infection with adenovirus expressing Lac-Z had no effect on the growth of ONS-76 cells. In contrast cells infected with adenovirus expressing Zhangfei stopped growing by 2 days

following infection. At this time (Fig 4.3D) cells infected with adeno-LacZ were crowded while, cells infected with adenovirus expressing Zhangfei appeared to have died (bright, refractory cells in Fig 4.3D) or were spread out. Many of these cells had long processes. When these cells were examined for Zhangfei, trkA and Egr1 transcripts, we found that compared to mock-infected and adeno-beta galactosidase expressing cells the cells infected with adeno-Zhangfei showed a five-fold increase in trkA transcripts and an almost thirty-fold increase in Egr1 transcripts. As expected, adeno-ZF infected cells had 2000 fold more Zhangfei transcripts than MI or adeno-LacZ infected cells (not shown). These results suggest that in ONS-76 medulloblastoma cells the expression of Zhangfei on its own can lead to an enhancement of trkA signalling.

4.4.3 Zhangfei does not require binding to HCF to induce trkA and Egr1 expression

Zhangfei binds HCF (Lu and Misra, 2000b) and a mutant with a non-functional HBM does not suppress the activity of Luman and VP16 (Akhova et al., 2005, Misra et al., 2005) suggesting that at least some of the functions of Zhangfei require HCF. To determine if HCF binding was needed for Zhangfei to activate trkA and Egr1 expression, we compared the level of transcripts for these two genes in cells infected with adenovirus vectors expressing beta galactosidase, Zhangfei or Zhangfei (Y224A). The latter is a mutant in which the tyrosine residue in the HBM ($DHDY_{224}$) has been changed to alanine (DHDA). Figure 4.3C shows that, if anything, Zhangfei Y224A was more effective at activating trkA and Egr1 than wild-type Zhangfei. This might be caused by a damping effect of HCF on Zhangfei which has more affinity for binding to HCF.

4.4.4 Zhangfei induces the phosphorylation of Erk.

Since the transcription of Egr1, an immediate early gene in the NGF-trkA pathway, is enhanced through the phosphorylation of extracellular signal-regulated kinase (Erk1/2) (Harada et al., 2001) we examined ONS-76 cells treated with either DMSO or resveratrol, or cells infected with either adeno-beta

galactosidase or adeo-Zhangfei for Erk1/2 and phosphorylated Erk1. An antibody against Erk1/2 detected the p42 form of Erk1 in all four samples (Fig 4.4, top panel). A less intense band for p44 Erk2 was also detected. Only cells infected with adeno-Zhangfei contained detectable phospho-Erk1 (Fig 4.4, lower panel).

4.4.5 Resveratrol as well as Zhangfei trigger apoptosis in ONS-76 cells.

Since resveratrol induces some medulloblastoma cells to enter apoptosis (Wang et al., 2003, Zhang et al., 2006) we determined if expression of Zhangfei in these cells would do so as well. ONS-76 cells were treated with either DMSO or resveratrol or infected with either adeno-LacZ or adeno-ZF. Twenty-four hours following infection cells were analyzed for two markers of apoptosis, expression of activated caspase 3 or the detection by annexin V of phosphatidylserine on the surface of the plasma membrane. Resveratrol caused an increase in the number of apoptotic cells in the culture, 20 fold as measured by caspase 3 and 3 fold as determined by annexin V. Zhangfei caused a similar increase in apoptotic cells 11 fold as measure by caspase 3 and 4.5 fold by annexin V (Fig 4.5).

4.4.6 Unlike resveratrol Zhangfei does not suppress the growth of diploid human fibroblasts.

While resveratrol has been shown to have anti-tumor and anti-oxidant activity it is thought to be relatively non-toxic. One would therefore expect it to have no effect on the growth rate of normal cells. While it is not possible to examine the growth of normal neurons, we examined the effects of resveratrol and Zhangfei on MRC5 cells, a human diploid cell line. MRC5 cells are untransformed human lung fibroblasts derived from the normal lung tissue of a 14-week-old male foetus (Jacobs et al., 1970). This cell line is considered to be a normal diploid human cell-line with a 46, XY karyotype occurring in over 70% of cells. MRC5 cells undergo senescence after 40-50 doublings. MRC5 cells were either treated with DMSO or resveratrol or infected with adeno-LacZ or

adeno-ZF. Surprisenly, resveratrol inhibited the growth of MRC5 cells as efficiently as it did ONS-76 cells (Fig 4.6A). In contrast, the growth rates of Zhangfei expressing cells (over three days) were not different from cells treated with DMSO or infected with adeno-LacZ, nor was microscopic morphology different from normal MRC5 cells.

4.4.7 Resveratrol and Zhangfei inhibit the growth of another medulloblastoma cell-line

To determine if resveratrol and Zhangfei affected the growth of other medulloblastoma cell lines we examine UW228 cells. This cell line was derived from a posterior fossa medulloblastoma and the cells are positive for neurofilaments and synaptophysin (Keles et al., 1995). As with ONS-76 cells, resveratrol and Zhangfei suppressed the growth of UW228 cell (Fig 4.6B) indicating that the effects were not restricted to one medulloblastoma cell line.

4.5 Discussion

Zhangfei suppresses the initiation of HSV-1 gene expression in cells normally permissive to viral expression (Akhova et al., 2005). We have reported its presence in mature sensory neurons (Akhova et al., 2005), which are the site of HSV-1 latency, suggesting that Zhangfei may be involved in the suppression of viral gene expression during the establishment of HSV-1 latent infection in these cells. We have also detected Zhangfei in pyramidal neurons. We did not detect Zhangfei in actively growing cells of medulloblastoma cell lines and in cells in the trigeminal ganglia of the developing early mouse embryo. In these cells the protein was not apparent until 14 days of gestation, after which it increased in expression (Nazarali, personal communication). These observations led us to speculate that Zhangfei may be involved in the differentiation of neurons. In this study we examined the possibility that ectopic expression of Zhangfei in medulloblastoma cells induces signalling pathways that may lead to their differentiation. To test this hypothesis we examined ONS-76 cells induced to undergo differentiation with resveratrol. Since medulloblastoma cells can also be induced to differentiate if they are made to express trkA, we also examined these cells for trkA, NGF and Egr1 transcripts. In PC12 cells treated with NGF the phosphorylation of Erk1/2 leads to the activation of Egr1, (Harada et al., 2001, Levkovitz and Baraban, 2002) which is an immediate early response gene in the NGF-trkA signalling pathway. Egr1 then by activating p35 and CdK5 stimulates neural outgrowth and the withdrawal of the cells from the cell-cycle. The phosphorylation of Erk1/2 is also critical for differentiation of embryonic stem cells into neurons (Li et al., 2006). We found that transcripts for Zhangfei as well as the other genes tested were elevated in resveratrol differentiated cells when compared with undifferentiated cells (Fig 4.2). By infecting growing ONS-76 cells with an adenovirus vector expressing Zhangfei we showed that Zhangfei could lead to an increase in the expression of trkA and Egr1 (Fig 4.3). Our observation that Erk1 was phosphorylated in these cells (Fig 4.4) supports our hypothesis that Zhangfei is

a mediator of trkA-NGF signalling in these cells leading to either their differentiation or apoptosis.

Nerve growth factor-trkA signalling is important for the development and differentiation of nociceptive sensory neurons and sympathetic neurons during development of the mouse (Martin-Zanca et al., 1990, Crowley et al., 1994, Bibel and Barde, 2000, Huang and Reichardt, 2003). It is also required for the survival and function of sensory neurons after birth and plays a role in regulating the development of neuronal tumors (Muragaki et al., 1997, Tajima et al., 1998, Chou et al., 2000, Eggert et al., 2000, Eberhart et al., 2001, Ohta et al., 2006) as well as in the maintenance of HSV-1 latency (Wilcox et al., 1990, Laycock et al., 1994, Hill et al., 1997, Kriesel, 1999). While there is information on transcription factors that regulate trkA expression during embryonic development, the mechanisms by which trkA expression in neuronal tumors is regulated is not known.

Zhangfei suppresses the activity of other HCF-binding proteins such as Luman and VP16. While both Zhangfei and Luman are b-Zip proteins, they appear to have incompatible interacting domains (Vinson et al., 2002, Newman and Keating, 2003) and do not heterodimerize (Lu et al., 1997, Liang et al., 2006). However, since both bind HCF it is possible that their interactions involve HCF and, perhaps, other associated proteins. VP16 is not a b-Zip protein and interactions between it and Zhangfei probably involve HCF as well. While Zhangfei and Luman do not dimerize through their b-Zip domains Zhangfei does interact with other proteins through their b-Zip regions. The results of these interactions can activate or suppress transcription of target genes. Thus, Zhangfei and Xbp1 (Chauhan personal communication) have a suppressive effect and Zhangfei as a heterodimer with ATF4 (Hogan et al., 2006) activates promoters containing unfolded protein response elements. Although it is not known whether these b-Zip-mediated interactions of Zhangfei require HCF, it is possible that they do not. Zhangfei may therefore, in a HCF-independent

manner, activate transcription in neurons by dimerizing with b-Zip proteins such as ATF4.

Interestingly, we found that while both resveratrol and Zhangfei suppressed the growth of at least two medulloblastoma cell-lines they differed in their effects on human diploid cells considered to be relatively normal in their growth characteristics. While resveratrol prevented these cells from growing, Zhangfei had no apparent effect three days after treatment. Since we infected these cells with an adenovirus vector that expressed Zhangfei from the very active cytomegalovirus immediate early promoter they produced large amounts of protein. The cells did eventually die but this was probably because of toxic effects of large amounts of Zhangfei protein. We have yet to examine the effects of long-term expression of physiological levels of the protein in normal cells.

In our model (Figure 4.7) for the involvement of Zhangfei in HSV latency, Zhangfei acts to promote HSV latency by suppressing VP16. It also acts to maintain latency by stimulating trkA expression and the NGF-trkA signalling pathway. *In vitro* models of HSV latency, the virus reactivates when latently infected cells are deprived of NGF. In animal models when sectioning of the trigeminal nerve interrupts NGF-trkA signalling, or when infected neurons are cultured with HSV permissive cells in the absence of NGF, the latent virus reactivates. Since NGF is produced by many cell types and is upregulated during inflammation (which paradoxically has been linked to reactivation) it is possible that reactivation is triggered not by a decrease in NGF but by a down-regulation of trkA expression. We hypothesize that this is brought about by stress-related suppression or decrease in expression in neurons of Zhangfei. This would allow neuronal HCF-binding proteins such as Luman, which can activate HSV IE expression, to initiate HSV IE expression and subsequently viral replication.

4.6 Acknowledgements: a Discovery Grant to VM from the Natural Sciences and Engineering Research Council of Canada supported this work. XV was supported partially by the NSERC Discovery Grant and partially by a University of Saskatchewan graduate scholarship.

4.7 References:

Ajuh, P., Chusainow, J., Ryder, U. and Lamond, A. I., 2002. A novel function for human factor C1 (HCF-1), a host protein required for herpes simplex virus infection, in pre-mRNA splicing. Embo J. 21, 6590-6602.

Akhova, O., Bainbridge, M. and Misra, V., 2005. The neuronal host cell factor-binding protein Zhangfei inhibits herpes simplex virus replication. J Virol. 79, 14708-14718.

Arthur, J. L., Scarpini, C. G., Connor, V., Lachmann, R. H., Tolkovsky, A. M. and Efstathiou, S., 2001. Herpes Simplex Virus Type 1 Promoter Activity during Latency Establishment, Maintenance, and Reactivation in Primary Dorsal Root Neurons In Vitro. J Virol. 75, 3885-3895.

Bibel, M. and Barde, Y. A., 2000. Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. Genes Dev. 14, 2919-2937.

Chen, Y., Tseng, S. H., Lai, H. S. and Chen, W. J., 2004. Resveratrol-induced cellular apoptosis and cell cycle arrest in neuroblastoma cells and antitumor effects on neuroblastoma in mice. Surgery. 136, 57-66.

Chou, T. T., Trojanowski, J. Q. and Lee, V. M., 2000. A novel apoptotic pathway induced by nerve growth factor-mediated TrkA activation in medulloblastoma. J Biol Chem. 275, 565-570.

Colgin, M. A., Smith, R. L. and Wilcox, C. L., 2001. Inducible Cyclic AMP Early Repressor Produces Reactivation of Latent Herpes Simplex Virus Type 1 in Neurons In Vitro. J Virol. 75, 2912-2920.

Crowley, C., Spencer, S. D., Nishimura, M. C., Chen, K. S., Pitts-Meek, S., Armanini, M. P., Ling, L. H., McMahon, S. B., Shelton, D. L., Levinson, A. D. and et al., 1994. Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell. 76, 1001-1011.

Danaher, R. J., Jacob, R. J., Steiner, M. R., Allen, W. R., Hill, J. M. and Miller, C. S., 2005. Histone deacetylase inhibitors induce reactivation of herpes simplex virus type 1 in a latency-associated transcript-independent manner in neuronal cells. J Neurovirol. 11, 306-317.

Eberhart, C. G., Kaufman, W. E., Tihan, T. and Burger, P. C., 2001. Apoptosis, neuronal maturation, and neurotrophin expression within medulloblastoma nodules. J Neuropathol Exp Neurol. 60, 462-469.

Efstathiou, S. and Preston, C. M., 2005. Towards an understanding of the molecular basis of herpes simplex virus latency. Virus Res. 111, 108-119.

Eggert, A., Ikegaki, N., Liu, X., Chou, T. T., Lee, V. M., Trojanowski, J. Q. and Brodeur, G. M., 2000. Molecular dissection of TrkA signal transduction pathways mediating differentiation in human neuroblastoma cells. Oncogene. 19, 2043-2051.

Harada, T., Morooka, T., Ogawa, S. and Nishida, E., 2001. ERK induces p35, a neuron-specific activator of Cdk5, through induction of Egr1. Nat Cell Biol. 3, 453-459.

Hill, J. M., Garza, H. H. J., Helmy, M. F., Cook, S. D., Osborne, P. A., Johnson, E. M. J., Thompson, H. W., Green, L. C., O'Callaghan, R. J. and Gebhardt, B. M., 1997. Nerve growth factor antibody stimulates reactivation of ocular herpes simplex virus type 1 in latently infected rabbits. Journal of Neurovirology. 3, 206-211.

Hogan, M. R., Cockram, G. P. and Lu, R., 2006. Cooperative interaction of Zhangfei and ATF4 in transactivation of the cyclic AMP response element. FEBS Lett. 580, 58-62.

Huang, E. J. and Reichardt, L. F., 2003. Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem. 72, 609-642.

Hughes, T. A., La Boissiere, S. and O'Hare, P., 1999. Analysis of functional domains of the host cell factor involved in VP16 complex formation. J Biol Chem. 274, 16437-16443.

Hunsperger, E. A. and Wilcox, C. L., 2003a. Capsaicin-induced reactivation of latent herpes simplex virus type 1 in sensory neurons in culture. J Gen Virol. 84, 1071-1078.

Hunsperger, E. A. and Wilcox, C. L., 2003b. Caspase-3-dependent reactivation of latent herpes simplex virus type 1 in sensory neuronal cultures. J Neurovirol. 9, 390-398.

Jacobs, J. P., Jones, C. M. and Baille, J. P., 1970. Characteristics of a human diploid cell designated MRC-5. Nature. 227, 168-170.

Julien, E. and Herr, W., 2003. Proteolytic processing is necessary to separate and ensure proper cell growth and cytokinesis functions of HCF-1. Embo J. 22, 2360-2369.

Keles, G. E., Berger, M. S., Srinivasan, J., Kolstoe, D. D., Bobola, M. S. and Silber, J. R., 1995. Establishment and characterization of four human

medulloblastoma-derived cell lines. Oncol Res. 7, 493-503.

Knez, J., Piluso, D., Bilan, P. and Capone, J. P., 2006. Host Cell Factor-1 and E2F4 Interact Via Multiple Determinants in Each Protein. Mol Cell Biochem. 288, 79-90.

Kriesel, J. D., 1999. Reactivation of herpes simplex virus: the role of cytokines and intracellular factors. Curr Opin Infect Dis. 12, 235-238.

Kristie, T. M., Pomerantz, J. L., Twomey, T. C., Parent, S. A. and Sharp, P. A., 1995. The cellular c1 factor of the herpes simplex virus enhancer complex is a family of polypeptides. Journal of Biological Chemistry. 270, 4387-4394.

Laycock, K. A., Brady, R. H., Lee, S. F., Osborne, P. A., Johnson, E. M. and Pepose, J. S., 1994. The role of nerve growth factor in modulating herpes simplex virus reactivation in vivo. Graefes Archive For Clinical and Experimental Ophthalmology. 232, 421-425.

Levkovitz, Y. and Baraban, J. M., 2002. A dominant negative Egr inhibitor blocks nerve growth factor-induced neurite outgrowth by suppressing c-Jun activation: role of an Egr/c-Jun complex. J Neurosci. 22, 3845-3854.

Li, Z., Theus, M. H. and Wei, L., 2006. Role of ERK 1/2 signaling in neuronal differentiation of cultured embryonic stem cells. Dev Growth Differ. 48, 513-523.

Liang, G., Audas, T. E., Li, Y., Cockram, G. P., Dean, J. D., Martyn, A. C., Kokame, K. and Lu, R., 2006. Luman/CREB3 induces transcription of the endoplasmic reticulum stress response protein, Herp, through an ERSE-II element. Mol Cell Biol.

Lu, R. and Misra, V., 2000a. Potential role for Luman, the cellular homologue of herpes simplex virus VP16 (alpha gene trans-inducing factor), in herpesvirus latency. J Virol. 74, 934-943.

Lu, R. and Misra, V., 2000b. Zhangfei: a second cellular protein interacts with herpes simplex virus accessory factor HCF in a manner similar to Luman and VP16. Nucleic Acids Res. 28, 2446-2454.

Lu, R., Yang, P., O'Hare, P. and Misra, V., 1997. Luman, a new member of the CREB/ATF family, binds to herpes simplex virus VP16-associated host cellular factor. Mol Cell Biol. 17, 5117-5126.

Lu, R., Yang, P., Padmakumar, S. and Misra, V., 1998. The herpesvirus transactivator VP16 mimics a human basic domain leucine zipper protein,

Luman, in its interaction with HCF. J Virol. 72, 6291-6297.

Luciano, R. L. and Wilson, A. C., 2003. HCF-1 functions as a coactivator for the zinc finger protein Krox20. J Biol Chem. 278, 51116-51124.

Martin-Zanca, D., Barbacid, M. and Parada, L. F., 1990. Expression of the trk proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development. Genes Dev. 4, 683-694.

Misra, V., Rapin, N., Akhova, O., Bainbridge, M. and Korchinski, P., 2005. Zhangfei is a potent and specific inhibitor of the host cell factor-binding transcription factor Luman. J Biol Chem. 280, 15257-15266.

Muragaki, Y., Chou, T. T., Kaplan, D. R., Trojanowski, J. Q. and Lee, V. M., 1997. Nerve growth factor induces apoptosis in human medulloblastoma cell lines that express TrkA receptors. J Neurosci. 17, 530-542.

Narayanan, A., Nogueira, M. L., Ruyechan, W. T. and Kristie, T. M., 2005. Combinatorial transcription of herpes simplex virus and varicella zoster virus immediate early genes is strictly determined by the cellular coactivator HCF-1. J Biol Chem. 280, 1369-1375.

Newman, J. R. and Keating, A. E., 2003. Comprehensive identification of human bZIP interactions with coiled-coil arrays. Science. 300, 2097-2101.

Nicolini, G., Rigolio, R., Scuteri, A., Miloso, M., Saccomanno, D., Cavaletti, G. and Tredici, G., 2003. Effect of trans-resveratrol on signal transduction pathways involved in paclitaxel-induced apoptosis in human neuroblastoma SH-SY5Y cells. Neurochem Int. 42, 419-429.

Ohta, T., Watanabe, T., Katayama, Y., Kurihara, J., Yoshino, A., Nishimoto, H. and Kishimoto, H., 2006. TrkA expression is associated with an elevated level of apoptosis in classic medulloblastomas. Neuropathology. 26, 170-177.

Parada, L. F., Tsoulfas, P., Tessarollo, L., Blair, J., Reid, S. W. and Soppet, D., 1992. The Trk family of tyrosine kinases: receptors for NGF-related neurotrophins. Cold Spring Harb Symp Quant Biol. 57, 43-51.

Park, K. C., Shimizu, K. and Hayakawa, T., 1998. Interferon yield and MHC antigen expression of human medulloblastoma cells and its suppression during dibutyryl cyclic AMP-induced differentiation: do medulloblastoma cells derive from bipotent neuronal and glial progenitors? Cell Mol Neurobiol. 18, 497-507.

Roizman, B. S., A. E., 2001. Herpes simplex viruses and their replication. In: Knipe, D. M. H., P.M (Ed.), Fields Virology, vol.2. Lippincott Willaims and Wilkins, pp. 2399-2460.

Sano, M. and Kitajima, S., 1996. Inhibition of the nerve growth factor-induced outgrowth of neurites by trichostatin A requires protein synthesis de novo in PC12D cells. Brain Res. 742, 195-202.

Sasaki, K., Chancellor, M. B., Goins, W. F., Phelan, M. W., Glorioso, J. C., de Groat, W. C. and Yoshimura, N., 2004. Gene therapy using replication-defective herpes simplex virus vectors expressing nerve growth factor in a rat model of diabetic cystopathy. Diabetes. 53, 2723-2730.

Smith, R. L., Pizer, L. I., Johnson, E. M. J. and Wilcox, C. L., 1992. Activation of second-messenger pathways reactivates latent herpes simplex virus in neuronal cultures. Virology. 188, 311-318.

Tajima, Y., Molina, R. P., Jr., Rorke, L. B., Kaplan, D. R., Radeke, M., Feinstein, S. C., Lee, V. M. and Trojanowski, J. Q., 1998. Neurotrophins and neuronal versus glial differentiation in medulloblastomas and other pediatric brain tumors. Acta Neuropathol (Berl). 95, 325-332.

Tredici, G., Miloso, M., Nicolini, G., Galbiati, S., Cavaletti, G. and Bertelli, A., 1999. Resveratrol, map kinases and neuronal cells: might wine be a neuroprotectant? Drugs Exp Clin Res. 25, 99-103.

Verge, V. M., Merlio, J. P., Grondin, J., Ernfors, P., Persson, H., Riopelle, R. J., Hokfelt, T. and Richardson, P. M., 1992. Colocalization of NGF binding sites, trk mRNA, and low-affinity NGF receptor mRNA in primary sensory neurons: responses to injury and infusion of NGF. J Neurosci. 12, 4011-4022.

Verge, V. M., Richardson, P. M., Wiesenfeld-Hallin, Z. and Hokfelt, T., 1995. Differential influence of nerve growth factor on neuropeptide expression in vivo: a novel role in peptide suppression in adult sensory neurons. J Neurosci. 15, 2081-2096.

Vinson, C., Myakishev, M., Acharya, A., Mir, A. A., Moll, J. R. and Bonovich, M., 2002. Classification of human B-ZIP proteins based on dimerization properties. Mol Cell Biol. 22, 6321-6335.

Walwyn, W. M., Matsuka, Y., Arai, D., Bloom, D. C., Lam, H., Tran, C., Spigelman, I. and Maidment, N. T., 2006. HSV-1-mediated NGF delivery delays nociceptive deficits in a genetic model of diabetic neuropathy. Exp Neurol. 198, 260-270.

Wang, Q., Li, H., Wang, X. W., Wu, D. C., Chen, X. Y. and Liu, J., 2003. Resveratrol promotes differentiation and induces Fas-independent apoptosis of human medulloblastoma cells. Neurosci Lett. 351, 83-86.

Wilcox, C. and Johnson, E., 1987. Nerve growth factor deprivation results in the reactivation of latent herpes simplex virus in vitro. J Virol. 61, 2311-2315.

Wilcox, C. and Johnson, E., 1988. Characterization of nerve growth factor-dependent herpes simplex virus latency in neurons in vitro. J Virol. 62, 393-399.

Wilcox, C. L., Smith, R. L., Freed, C. R. and Johnson, E. M. J., 1990. Nerve growth factor-dependence of herpes simplex virus latency in peripheral sympathetic and sensory neurons in vitro. Journal of Neuroscience. 10, 1268-1275.

Wilson, A. C., Freiman, R. N., Goto, H., Nishimoto, T. and Herr, W., 1997. VP16 targets an amino-terminal domain of HCF involved in cell cycle progression. Mol Cell Biol. 17, 6139-6146.

Wilson, A. C., LaMarco, K., Peterson, M. G. and Herr, W., 1993. The VP16 accessory protein HCF is a family of polypeptides processed from a large precursor protein. Cell. 74, 115-125.

Wysocka, J. and Herr, W., 2003. The herpes simplex virus VP16-induced complex: the makings of a regulatory switch. Trends Biochem Sci. 28, 294-304.

Wysocka, J., Myers, M. P., Laherty, C. D., Eisenman, R. N. and Herr, W., 2003. Human Sin3 deacetylase and trithorax-related Set1/Ash2 histone H3-K4 methyltransferase are tethered together selectively by the cell-proliferation factor HCF-1. Genes Dev. 17, 896-911.

Yamada, M., Shimizu, K., Tamura, K., Okamoto, Y., Matsui, Y., Moriuchi, S., Park, K., Mabuchi, E., Yamamoto, K., Hayakawa, T. and et al., 1989. [Establishment and biological characterization of human medulloblastoma cell lines]. No To Shinkei. 41, 695-702.

Zhang, P., Li, H., Wu, M. L., Chen, X. Y., Kong, Q. Y., Wang, X. W., Sun, Y., Wen, S. and Liu, J., 2006. c-Myc downregulation: a critical molecular event in resveratrol-induced cell cycle arrest and apoptosis of human medulloblastoma cells. J Neurooncol. 80, 123-131.

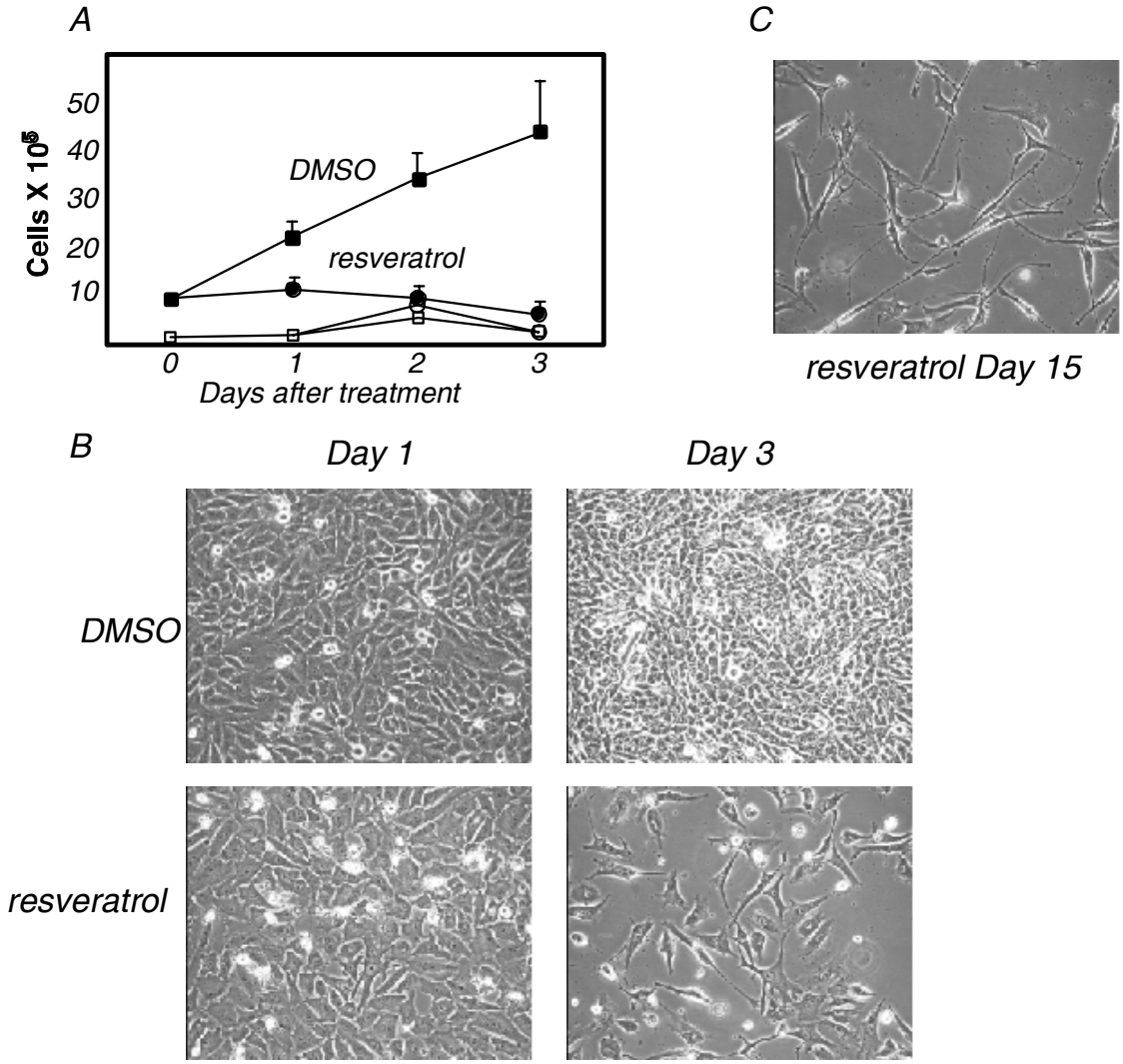


Figure 4.1. Resveratrol suppresses the growth of ONS-76 medulloblastoma cells. A) ONS-76 cells were plated in 6 well culture dishes in medium containing 10% FBS. After overnight incubation the cells were treated with either medium containing 1% FBS and 100 μM resveratrol (squares) or medium containing DMSO (circles). At this time and every day for the next three days viable (solid symbols) and trypan blue staining cells (open symbols) were counted in triplicate well. Bars indicate standard deviation from the mean. **B**) phase-contrast micrographs of DMSO and resveratrol-treated cells at day 1 and three after beginning of treatment. **C**) Photomicrograph of resveratrol-treated cells 15 days after treatment.

Figure 4.2. Treatment with resveratrol leads to an increase in transcripts for Zhangfei, TrkA, Egr1 and NGF. **A**) RNA was purified from cultures of ONS-76 cells treated with either DMSO or resveratrol and assayed for Zhangfei, trkA, Egr1, NGF and GAPDH transcripts by qRT-PCR. Bars represent standard deviations from the mean. For Zhangfei the histogram represent the mean of quadruplicate samples, for trkA and Egr1 the values are from duplicate samples. Bars represent standard deviation from the mean values from independent experiments, each in duplicate (5 experiments for Zhangfei, 4 for trkA and 3 for Egr1 and NGF). **B**) To determine if resveratrol-treated cells contained NGF, lysates of DMSO or resveratrol treated ONS-76 cells were added to PC12 cells. As a control PC12 cells received no treatment (middle panel) or were treated with NGF (top panel). Forty-eight hours later cells were photographed. Arrows indicate neurite-like projections sprouting from PC12 cells in response to NGF or lysate from resveratrol-treated ONS-76 cells.

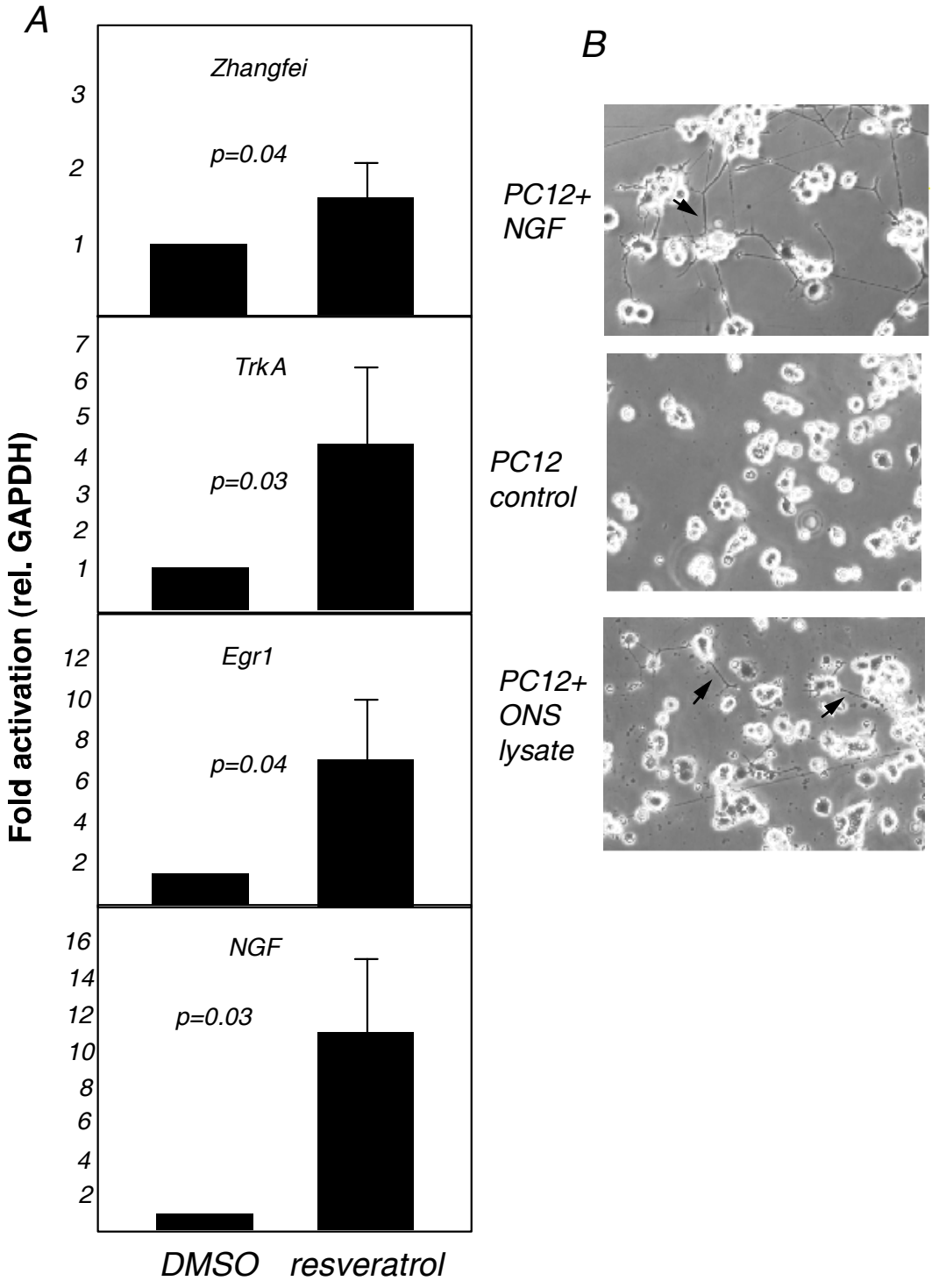

A
B
Zhangfei
p=0.04
TrkA
p=0.03
Egr1
p=0.04
NGF
p=0.03
Fold activation (rel. GAPDH)
DMSO
resveratrol
PC12+ NGF
PC12 control
PC12+ ONS lysate

Figure 4.2

Figure 4.3. Ectopic expression of Zhangfei in growing ONS-76 cells leads to the suppression of their growth and expression of TrkA and Egr1. ONS-76 cells were either mock-infected (MI) or infected with adenovirus vectors expressing beta-galactosidase (Ad-LacZ) or Zhangfei (Ad-ZF). **A**) Viable cells in triplicate samples were counted at the time of infection and for four days following infection. **B**) RNA extracted from infected cells was extracted 48 hr after infection and assayed for Zhangfei, trkA, Egr1 and GAPDH. Bars represent the mean of triplicate samples. **C**) RNA was extracted from ONS cells infected with adenovirus vectors expressing beta-galactosidase, Zhangfei or Zhangfei (Y224A), a mutant that does not bind HCF. Transcripts for trkA and Egr1 and GAPDH were assayed. **D**) Micrographs (20X) of ONS-76 cells infected with either adeno-LacZ or adeno-ZF. Cells were photographed 48 hr after infection.

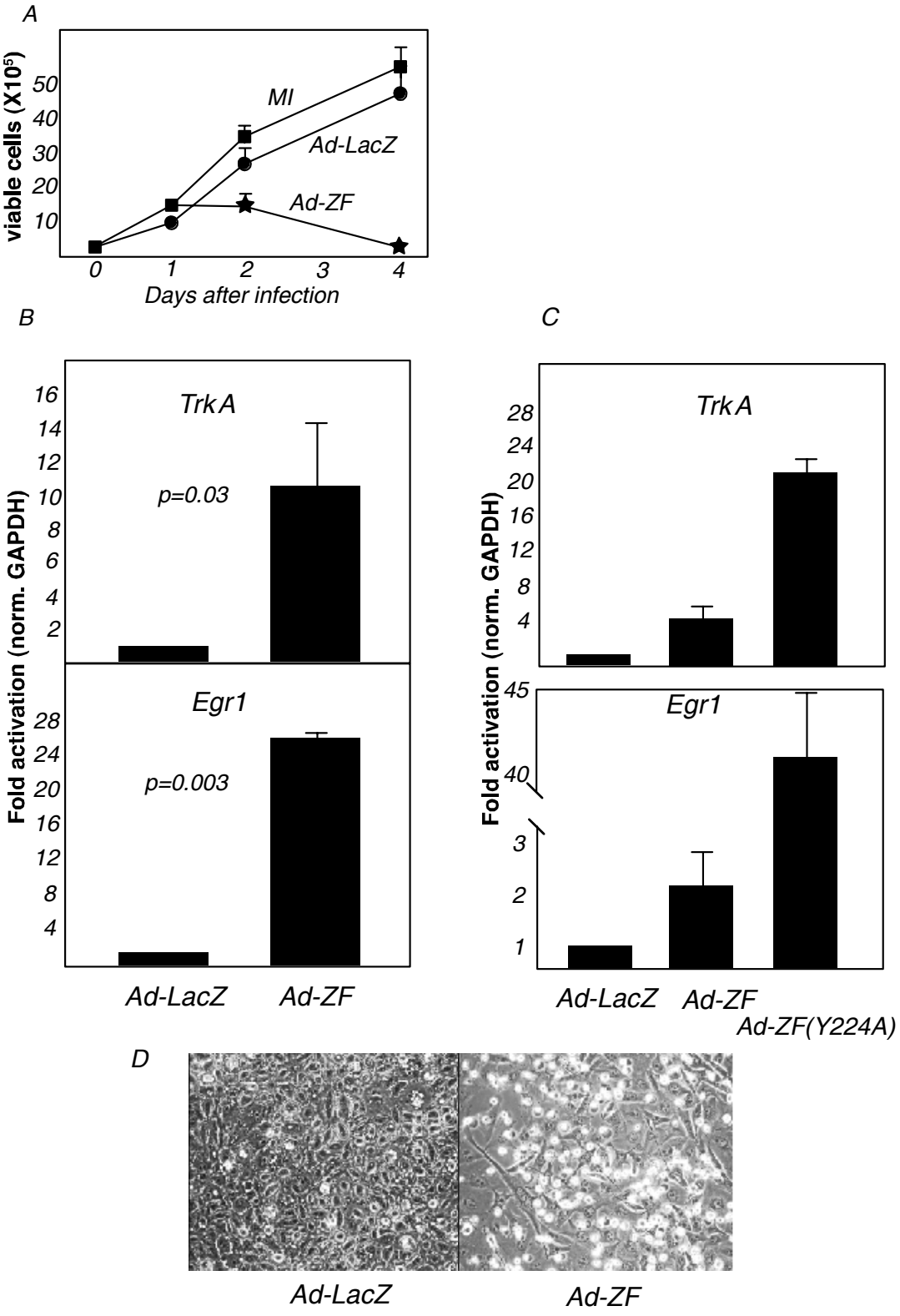

A
viable cells (X10⁵)
50
40
30
20
10
MI
Ad-LacZ
Ad-ZF
0
1
2
3
4
Days after infection
B
Fold activation (norm. GAPDH)
TrkA
p=0.03
Egr1
p=0.003
Ad-LacZ
Ad-ZF
C
TrkA
Egr1
Ad-LacZ
Ad-ZF
Ad-ZF(Y224A)
D
Ad-LacZ
Ad-ZF

Figure 4.3

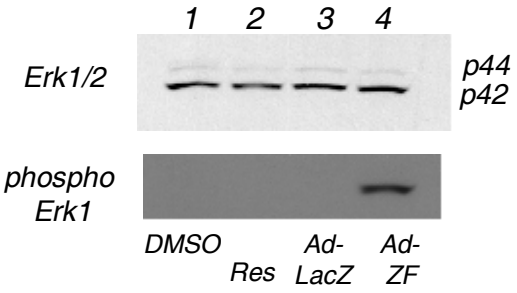


Figure 4.4. Zhangfei leads to the phosphorylation of Erk1 in ONS-76 cells. ONS-76 cells were treated with either DMSO or 100 μM resveratrol or were infected with adenovirus vectors expressing beta-galactosidase (Ad-LacZ) or Zhangfei (Ad-ZF). Proteins in cell lysates were separated by SDS-PAGE and probed with antibodies against Erk1/2 (top panel). The bound antibodies were then stripped and the blot reprobed with antibodies specific for phosphorylated Erk1 (lower panel).

Figure 4.5. Resveratrol and ectopic Zhangfei induce apoptosis in ONS-76 cells. ONS-76 cells were treated with either DMSO or resveratrol or were infected with adenovirus vectors expressing either beta-galactosidase or Zhangfei. Twenty four hours after treatment or infection, cultures were analyzed for apoptotic cells by examining cells for Caspase 3 or Annexin V. Numbers in the figures represent percent of cells expressing these markers of apoptosis.

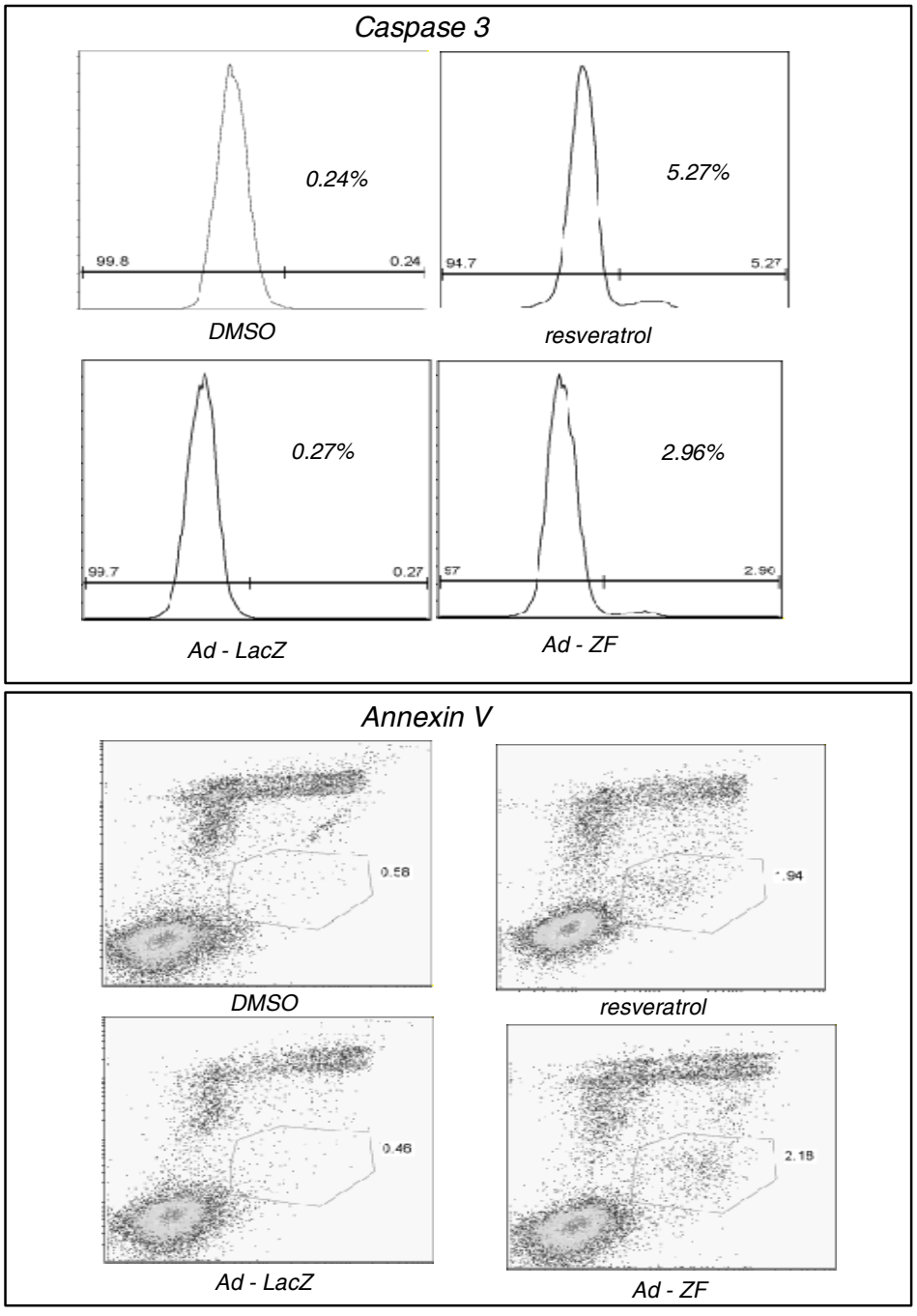

Caspase 3
0.24%
99.8
0.24
DMSO
5.27%
94.7
5.27
resveratrol
0.27%
99.7
0.27
Ad - LacZ
2.96%
97
2.96
Ad - ZF
Annexin V
0.58
DMSO
.94
resveratrol
0.46
Ad - LacZ
2.18
Ad - ZF

Figure 4.5

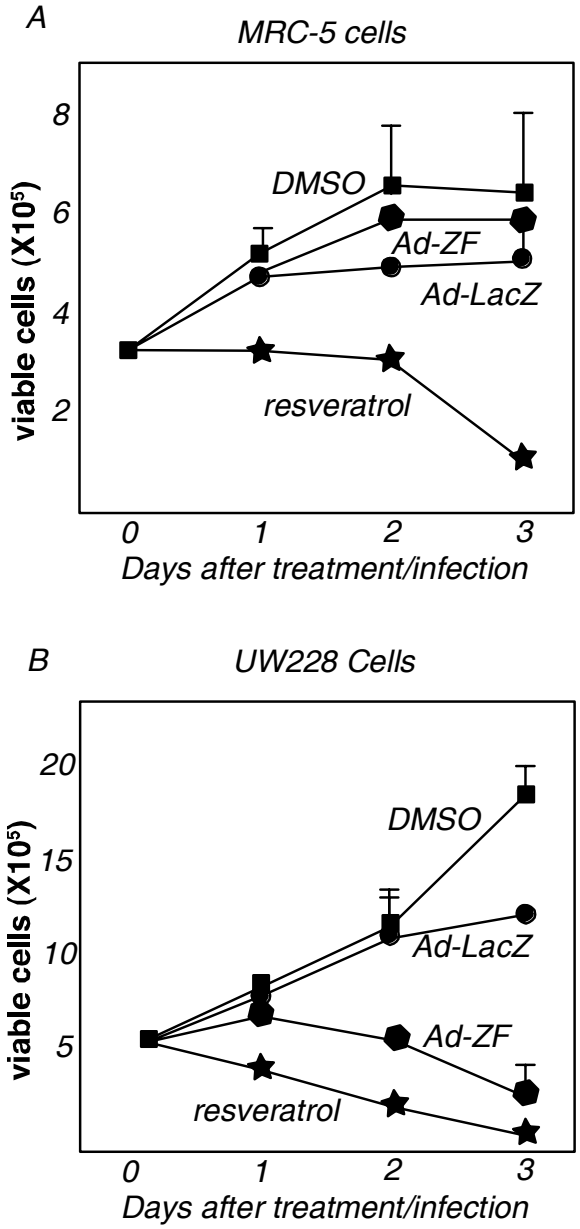


Figure 4.6. Effect of resveratrol or Zhangfei on growth of human diploid fibroblasts (MRC5) and medulloblastoma cells (UW228). MRC5 (**A**) or UW228 (**B**) cells were treated with either DMSO or resveratrol or were infected with adenovirus vectors expressing either beta-galactosidase (Lac-Z) or Zhangfei (ZF). Viable cells were counted, daily for three days after treatment or infection.

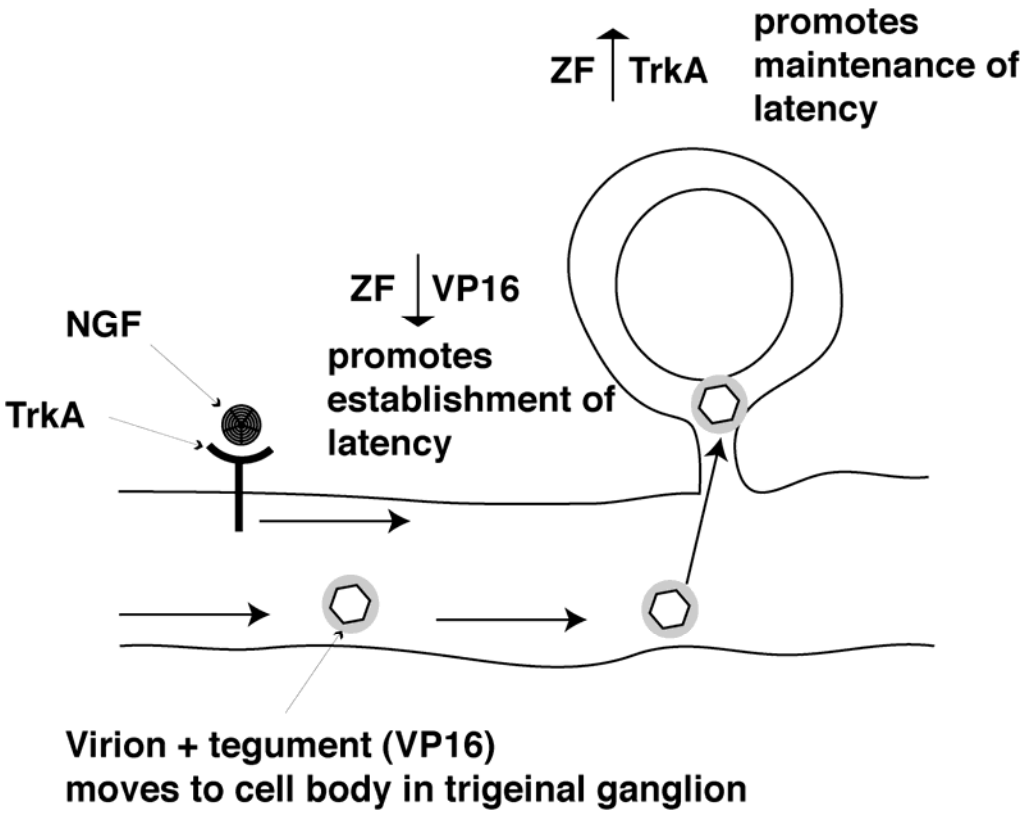


Figure 4.7. Potential role for Zhangfei in the establishment and maintenance of HSV latency, a model: A herpes simplex virion including its tegument (grey zone surrounding the hexagon representing the nucleocapsid) travels by retrograde transport along microtubules to the neuronal cell body. Here, Zhangfei suppresses the ability of VP16 to initiate viral Immediate Early gene expression and, consequently, the lytic cycle. In the uninfected neuron Zhangfei induces the expression of trkA, which is transported to cell membranes. Interaction of NGF with cell-membrane associated TrKA initiates signalling pathways that maintain homeostasis and in neurons with latent HSV-1 induce conditions that help to maintain the HSV genome in a latent state.

5. GENERAL DISCUSSION AND CONCLUSIONS

Nerve growth factor is a critical neutrophin required during development for survival, proliferation and differentiation of neurons (Parada et al., 1992, Greene and Kaplan, 1995, Kaplan and Miller, 2000, Huang and Reichardt, 2003). Nerve growth factor (NGF) actions are attributed mainly to the activation of its receptor tropomyosin related kinase trkA, generally expressed in neurons of the peripheral and central nervous system (Huang and Reichardt, 2003). NGF-trkA binding allows autophosphrylation of its cytoplasmic tail (C-terminal) activating a number of signaling pathways that regulate gene expression. In adults, NGF is no longer required for survival and is believed to play a role in the maintenance of homeostasis of neurons or in sprouting of neurons after tissue injury (reviewed in (Lei and Parada, 2007)). But more importantly, NGF is thought to mediate the inflammatory and immune response after tissue injury (damage) or by recognizing other noxious stimuli. The trkA neutrophin receptor and the downstream intracellular signaling pathways mediate sensitizing actions of NGF in mature nociceptive neurons.

NGF, like many other factors is produced by and acts on several types of cells. TrkA receptors are usually located at the peripheral termini of sensory neurons and are activated by NGF released by mast cells, macrophages, keratinocytes and T cells located near the site of tissue trauma, by cell in the shin and muscle and by glial cells neighboring the body of the sensory neuron. It has been proposed that HSV-1 latency is sensitive to NGF signal transduction (Hill et al., 1997) and that these latently infected medium and small diameter sensory neurons would express the TrkA (Yang et al., 2000).

Initial infection by HSV-1 is generally associated with lesions in and around the mouth, characterized by a group of small blisters or watery vesicles on the skin or on mucous membranes (virus replication - lytic phase). These lesions are preceded by tingling and burning in the skin area. The vesicles break and the skin appears normal within 6 to 10 days after the onset of the lesion (virus enters latency in sensory neurons). Lesions may often reappear (reactivation of the virus from latency) at the same site for many years and may be precipitated by any one of a number of factors, such as sunburn, upper-respiratory and gastrointestinal tract infections, fevers, emotional stress, or anxiety.

The aim of this study was to contribute to the understanding of the mechanisms, in sensory nociceptive neurons, that link the NGF/TrkA signaling cascade with the maintenance of HSV latency and reactivation from it. Based on the observations that NGF is normally upregulated in target cells upon sensing noxious stimulus (chemical or emotional stress, injury, fever among others) and that HSV reactivates *in vitro* when deprived of NGF, it would be plausible to hypothesize that reactivation of HSV is triggered by a downregulation of the TrkA receptor, disrupting NGF signaling cascade, rather than by a decrease in the availability of NGF.

To date most of the information related to HSV infection has addressed the molecular biology of the virus life cycle (lytic and latent state) in epithelial cells and in neurons *in vitro* and *in vivo* (reviewed in (Roizman, 2001, Efstathiou and Preston, 2005). Although, there is detailed information available on the host cellular changes associated with HSV infection, it has been difficult to define direct roles for host proteins in cellular mechanisms associated with latency and reactivation. Moreover, the specific mechanisms by which the latent virus senses stress have not been clearly defined.

Nevertheless, the chromatin associated protein Host cell factor 1 (HCF-1) is an essential for initiating the viral replicative cycle both in epithelial cells and in neurons (Luciano and Wilson, 2002). In epithelial cells it does so in association with the virion transactivator, VP16. While VP16 is responsible for lytic virus replication in epithelial cells it has been suggested that in neurons the process of reactivation from latency is regulated by a cellular factor that would replace VP16 function. VP16 is produced in the late phase of replication and only the viral genome is available at the time of reactivation in the sensory neuron. A search for cellular proteins that also require HCF for their cellular function and could be associated to HSV life cycle, led to the discovery of the proteins Luman and Zhangfei. Luman is able to activate transcription of HSV IE in a manner similar to VP16 (Lu and Misra, 2000a) and Zhangfei is able to suppress transcription activation of genes by these proteins in an HCF dependant manner *in vitro* (Misra et al., 2005).

Not only VP16 is absent during HSV latency in sensory neurons (reviewed in Valyi-Nagy et al. 2006) , HCF is located mainly in the cytoplasm in these cells making it largely unavailable for HCF dependant nuclear function (Kristie et al., 1999). Knowing that under stress signals HCF is able to translocate to the nucleus making it available for proteins that depend on its association for function, it is believed that HCF would be the most appropriate and critical switch from viral latency to reactivation.

Based on the above previous findings it was essential to further understand the role of host proteins, in the context of triggering changes in neuron signaling pathways that have been associated to HSV reactivation from latency. Since Zhangfei has shown to be a suppressor of viral and host proteins in an HCF dependant manner, it seemed likely that it would depend on HCF nuclear translocation to modify or alter transcription activation of genes modifying neuronal signaling and possibly HSV latency.

The specific objective of this study was to contribute to the understanding of how Zhangfei regulates gene expression in the context of HSV-1 latency and reactivation. I demonstrate herein the ability of Zhangfei to regulate transcription activation of promoters in a cell type and promoter specific manner.

Brn3a, a marker for neuronal development and differentiation, was selected in the search for other HCF binding proteins that could be regulated by Zhangfei and could also be associated with neuronal cellular processes. Brn3a has been extensively studied in the context of development in the mouse. Others have shown that Brn3a acts ti enhance trkA transcription by binding to 2 motifs in a 457 bp segment of the promoter. While attempting to confirm these results in the human I identified novel *cis*-acting domains in the proximal trkA promoter that bind Brn3a and showed that exogenous Brn3a could induce the expression of trkA in NGF-differentiated PC12 cells and in medulloblastoma cells that do not normally express trkA (Chapter 2). In addition, the Brn3a enhancer motifs found to be active during mouse development did not appear to activate the human trkA promoter indicating that not only is trkA transcription differentially regulated in a cell type specific manner, but that it is most likely that Brn3a has a distinctive role in NGF-trkA signaling during development and during differentiation of nociceptive neurons.

Brn3a's *trans*-activation of TrkA requires HCF for activity, Zhangfei, known for its HCF dependant regulation of gene transactivation, suppressed Brn3a's activity in non-neuronal cells while in neuron-like NGF-differentiated PC12 cells Zhangfei did not suppress Brn3a. Surprisingly, Zhangfei was capable of activating the expression of trkA in the absence of Brn3a and without the requirement of HCF in these differentiated cells. This evidence supports the hypothesis that Zhangfei like Brn3a also differentially regulates gene transcription in a cell type specific manner. In addition, my observation that in PC12 cells Zhangfei suppresses Luman while activating Brn3a, suggests that

Zhangfei has the ability to regulate transcription in a promoter specific manner as well (Chapter 3).

The HCF dependency of Zhangfei to regulate Luman's trans-activation of genes in PC12 cells suggests that this process is triggered in neurons upon stress when HCF is allowed to translocate to the nucleus. On the contrary, transcription activation of trkA by Zhangfei can occur with or without nuclear HCF indicating that either this is a regular cellular activation process (unstressed) or that Zhangfei upregulates trkA in order to restore normal NGF-TrkA signaling after noxious stimuli. More importantly, these findings indicate that Zhangfei plays an important role in the activation and/or maintenance of the NGF/trkA signaling cascade.

TrkA induction has been widely studied during development and a series of suppressors and activators have been suggested for mouse in *in vitro* models. In general, there little information on the human TrkA promoter activation, mainly because of the lack of appropriate human cell models to study it. To demonstrate that Zhangfei was able to induce trkA gene transcription and had an effect on the NGF-trkA signaling pathway I used human medulloblastomas (which do not normally express TrkA). Zhangfei was able to induce TrkA transcription, activate phosphorylation of the intermediate kinase Erk1 allowing the transcription activation of the IE gene Egr1 (Chapter 4). This confirms Zhangfei's involvement in NGF signal transduction through the activation of trkA.

Activation of the Erk1 pathway by TrkA phosphorylation has been implicated in hyperalgesia and in the sensitization of sensory neurons. In the context of HSV-1, reactivation has been associated with neuronal sensitization to temperature (fever), immune response (infection), hormone imbalance (emotional distress) and cytotoxicity (food intoxication). It is unknown how the

virus senses these changes and which are the molecular thresholds considered by the virus to allow itself to reactivate.

This study has made an important contribution to the understanding of the possible role of Zhangfei on the NGF-TrkA signaling pathway and its association with HSV latency and reactivation. Finding an appropriate *in vitro* cellular human model for establishment of HSV latency would allow to further study Zhangfei's association with the virus life cycle. Although Zhangfei is unable to bind DNA as a homodimer it has been demonstrate herein its ability to activate gene transcription. Several proteins have been suggested as heterodimer partners for Zhangfei. Further studies should identify Zhangfei's partner involved in the activation of TrkA and if this activation is by direct or indirect binding to the proximal promoter. Discovering the specific protein partner would also shed information on the timing of Zhangfei's participation in NGF signal transduction.

General Conclusions:

1. Novel Brn3a cis-acting sequences that are important for trkA trans-activation.
2. Zhangfei is able to influence NGF-trkA signaling and consequently may influence HSV latency and reactivation.
3. It may be possible to control the growth of medulloblastomas by inducing the expression of Zhangfei

The specific conclusions of this thesis are:

Chapter 2: Brn3a transactivation of the trkA promoter

- Brn3a binding to the previously reported 5' binding motif in the trkA promoter in the 457 bp minimal developmental enhancer, does not

contribute to reporter gene activation in the non-neuronal Vero cell line nor in sympathetic neuron-like cells, PC12.

- A short 190 bp region that lies proximal to the trkA coding sequence and does not overlap the 457 bp minimal developmental enhancer, is sufficient for Brn3a activation of the trkA promoter in non-neuronal as well as in neuronal cells.
- Recombinant Brn3a, in the absence of other cellular proteins, binds directly to at least two portions of the 190 bp fragment.
- Brn3a requires HCF for transcription activation of the trkA promoter in Vero cells. However, Brn3a does not bind to HCF *in vitro*.
- Exogenous Brn3a activates the transcription of endogenous trkA in the sympathetic-like neuron PC12 and ONS76 medulloblastoma cells but not in Vero cells suggesting that this activation is tissue specific.

Chapter 3: Zhangfei suppression of Brn3a transactivation of the trkA promoter

- As with Luman and VP16, Zhangfei suppresses Brn3a transactivation of trkA in an HCF dependant manner in Vero cells. In contrast, in PC12 cells Zhangfei has no repressive effects on Brn3a trans-activation function.
- In PC12 cells Zhangfei activates the trkA minimal promoter and increases trkA endogenous transcription independent of HCF. In addition, Zhangfei suppresses Luman in these cells suggesting that Zhangfei's role on gene transcription is cell-type as well as promoter specific.

Chapter 4: Zhangfei activation of endogenous trkA in Medulloblastomas.

- Resveratrol differentiated cells have elevated levels of Zhangfei, trkA and Egr1 compared with undifferentiated cells.
- Infection of growing ONS-76 cells with an adenovirus vector expressing Zhangfei leads to the activation of trkA and Egr1 and may be responsible for the initiation of trkA-NGF signaling in these cells leading to either their differentiation or apoptosis.

- Resveratrol and Zhangfei differ in their effects on human diploid cells considered to be relatively normal in their growth characteristics. Resveratrol prevents these cells from growing while Zhangfei has no apparent effect supporting the hypothesis that Zhangfei cellular effects are cell type specific.

Based on these observations I propose a hypothetical role for Zhangfei in the establishment of HSV-1 latency. In this model Zhangfei acts to promote HSV latency by suppressing VP16 and Luman induced expression of viral IE genes from herpesvirus genome delivered to the neuronal cell body. Zhangfei also acts to maintain latency by stimulating trkA expression and the NGF-trkA signaling pathway, probably accomplishing this as a partner of another b-Zip protein.

Since NGF is produced by many cell types it is possible that reactivation is triggered not by a decrease in NGF but by a down-regulation of trkA expression. I hypothesize that this is brought about by stress related suppression or decrease in expression in neurons of Zhangfei. This would allow neuronal HCF-binding proteins such as Luman, which can activate HSV IE expression, to initiate HSV IE expression and subsequently viral replication.

5.1 References

Efstathiou, S. and Preston, C. M., 2005. Towards an understanding of the molecular basis of herpes simplex virus latency. Virus Res. 111, 108-119.

Greene, L. A. and Kaplan, D. R., 1995. Early events in neurotrophin signalling via Trk and p75 receptors. Curr Opin Neurobiol. 5, 579-587.

Hill, J. M., Garza, H. H. J., Helmy, M. F., Cook, S. D., Osborne, P. A., Johnson, E. M. J., Thompson, H. W., Green, L. C., O'Callaghan, R. J. and Gebhardt, B. M., 1997. Nerve growth factor antibody stimulates reactivation of ocular herpes simplex virus type 1 in latently infected rabbits. Journal of Neurovirology. 3, 206-211.

Huang, E. J. and Reichardt, L. F., 2003. Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem. 72, 609-642.

Kaplan, D. R. and Miller, F. D., 2000. Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol. 10, 381-391.

Kristie, T. M., Pomerantz, J. L., Twomey, T. C., Parent, S. A. and Sharp, P. A., 1995. The cellular c1 factor of the herpes simplex virus enhancer complex is a family of polypeptides. Journal of Biological Chemistry. 270, 4387-4394.

Lei, L. and Parada, L. F., 2007. Transcriptional regulation of Trk family neurotrophin receptors. Cell Mol Life Sci. 64, 522-532.

Lu, R. and Misra, V., 2000a. Potential role for Luman, the cellular homologue of herpes simplex virus VP16 (alpha gene trans-inducing factor), in herpesvirus latency. J Virol. 74, 934-943.

Luciano, R. L. and Wilson, A. C., 2002. An activation domain in the C-terminal subunit of HCF-1 is important for transactivation by VP16 and LZIP. Proc Natl Acad Sci U S A. 99, 13403-13408.

Misra, V., Rapin, N., Akhova, O., Bainbridge, M. and Korchinski, P., 2005. Zhangfei is a potent and specific inhibitor of the host cell factor-binding transcription factor Luman. J Biol Chem. 280, 15257-15266.

Parada, L. F., Tsoulfas, P., Tessarollo, L., Blair, J., Reid, S. W. and Soppet, D., 1992. The Trk family of tyrosine kinases: receptors for NGF-related neurotrophins. Cold Spring Harb Symp Quant Biol. 57, 43-51.

Roizman, B. S., A. E., 2001. Herpes simplex viruses and their replication. In: Knipe, D. M. H., P.M (Ed.), Fields Virology, vol.2. Lippincott Willaims and Wilkins, pp. 2399-2460.

Yang, L., Voytek, C. C. and Margolis, T. P., 2000. Immunohistochemical analysis of primary sensory neurons latently infected with herpes simplex virus type 1. J Virol. 74, 209-217.