The Role of Cell Cycle Machinery in Ischemic Neuronal Death

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Thesis submitted to the

Faculty of Graduate and Postdoctoral Studies

in partial fulfillment of the requirements

for the Doctorate in Philosophy degree in Neuroscience

Graduate program in Neuroscience

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Abstract

Ischemic stroke occurs as a result of a lack or severe reduction of blood supply to the brain. Presently therapeutic interventions are limited and there is a need to develop new and efficacious stroke treatments. To this end, a great deal of research effort has been devoted to studying the potential molecular mechanisms involved in ischemic neuronal death. Correlative evidence demonstrated a paradoxical activation of the cell cycle machinery in ischemic neurons. The levels and activity of key cell cycle regulators including cyclin D1, Cdk2 and Cdk4 are upregulated following ischemic insults. However, the functional relevance of these various signals following ischemic injury was unclear. Accordingly, the research described in this thesis address the functional relevance of the activation of the cell cycle machinery in ischemic neuronal death.

The data indicate that the inhibition of Cdk4 protects neurons from ischemia-induced delayed death, whereas abrogation of Cdk5 activity prevents excitotoxicity-induced damage *in vitro* and *in vivo*. Examination of upstream activators of mitotic-Cdks showed that Cdc25A is a critical mediator of delayed ischemic neuronal death. Investigation of the potential molecular mechanism by which cell cycle regulators induced neuronal death revealed perturbations in the levels and activity of key downstream targets of Cdk4. The retinoblastoma protein family members, pRb and p130 are increasingly phosphorylated following ischemic stresses. Importantly, p130 and E2F4 proteins are drastically reduced following ischemic insults. Additionally, E2F1 association with promoters of pro-apoptotic genes are induced while that of E2F4 is reduced. These changes appear to be important

determinants in ischemic neuronal death. Cumulatively, the data supports the activation of the cell cycle machinery as a pathogenic signal contributing to ischemic neuronal death.

The development of neuroprotectant strategies for stroke has been hampered in part by its complex pathophysiology. Previous research indicated that flavopiridol, a general CDK-inhibitor, is unable to provide sustained neuroprotection beyond one week following cerebral ischemia. The potential benefit of combining flavopiridol with another neuroprotectant, minocycline, was explored. The data indicate that while this approach provided histological protection 10 weeks after insult, the protected neurons are not functional due to progressive dendritic degeneration. This evidence indicates that targeting cell cycle pathways in stroke while important must be combined with other therapeutic modalities to fully treat stroke-induced damage.

Dedication

For:

My sister Amen,
For her courage and being my inspiration

And

My grandmother Noghadoteki,
For the woman that I am today

"We know very little, and yet it is astonishing that we know so much, and still more astonishing that so little knowledge can give us so much power"

-Bertrand Russell

Acknowledgements

My heartfelt thanks to my thesis supervisor Dr. David S. Park without whom I simply would not have embarked on this journey in science. Thank you for giving me an incredible opportunity to be a part of a wonderful lab and grow my scientific 'wings'. I am truly grateful for the scientific guidance and wisdom you have imparted to me over the years but above all thank you for being a great mentor and a wonderful human being. Finally, my long residence at the "Park lab" has yielded me certain privileges; I think I am one of the very few people in the lab who knows what the "S" stands for!

To the members of my thesis advisory committee, Dr. Steffany Bennett, Dr. Hsiao-Huei Chen and Dr. Charlie Thompson: thank you for all your insightful comments and suggestions and for providing letters of support on my behalf over the years.

I have had the privilege of working with and amongst incredible people over the years in the Park lab, with many of whom I have shared authorship. The body of work presented in this thesis would not have come to fruition without the scientific input, technical help and suggestion from many people. My sincere thanks to Juliet Rashidian with whom I shared first authorship of my first manuscript, Hossein Aleyasin and Mario Rios my teachers in the lab, Yi Zhang for all his work on the E2F4 manuscript, Zohreh, Aweis and Maryam (summer students), Carmen, Steve Callaghan, Jason MacLaurin (Slack lab) for all their technical help and suggestions. Thanks to Dianbo, Alvin, Haque, Isabella, Micheal O'Hare and to everyone else in the "Park lab", past and present, not mentioned here for all their help over the years. I especially want to thank Yasmilde, Elizabeth and Dr. Patrice Smith for their editorship of this thesis.

To Petro Verkhogliad and Gladys Akhigbe, my super elite support club and team of special advisors, you are simply the best! Thank you for all your love, support, and enthusiasm and, for keeping me level headed all these years.

To my grandmother, the greatest woman I know, thank you for all your parables and words of wisdom. To Mary Omoiyekemwen and Grace Akhigbe, thank you for your selfless devotion and teaching me the value of hard work. To Iyobo, Evelyn and Festus, thank you for all your words of encouragement. To the rest of my family and friends: thank you for all your unconditional love and understanding over the years and, for allowing me to be the absentee friend, daughter, granddaughter, niece, cousin, sister and aunty without ever wavering in your excitement when I do show up!

Thanks to the Heart and Stroke Foundation of Canada for their doctoral research award and the QEIIGSST for providing me with salary support to perform this research.

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List of Abbreviations

-/-	Gene knockout
+/+	Gene wild-type
2VO	2-vessel occlusion
4VO	4-vessel occlusion
Aβ	Amyloid beta
Ab	Antibody
ACSF	Artificial Cerebrospinal Fluid
AD	Alzheimer's disease
AIF	Apoptosis Inducing Factor
ALS	Amyotrophic Lateral Sclerosis
AMPA	amino-3-hydroxy-5-methyl-4-propionate
ANOVA	Analysis of Variance
APAF	Apoptosis Activation Factor 1
APE	Apurinic/apyrimidinic Endonuclease
ATP	Adenosine triphosphate
AV	Adenovirus
BBB	Blood Brain Barrier
Bcl2	B-cell lymphoma 2
BER	Base Excision Repair
CA1	Cornu Ammonis 1
CAD	Caspase Activated DNAse
CAK	Cdk Activating Kinase
CAT	Catalase
CBF	Cerebral Blood Flow
Cdc25	Cell division cycle 25
Cdk	Cyclin dependent kinase

CGN	Cerebellar Granule Neuron
CIP	Cdk Interacting Protein
CKI	Cdk Inhibitor
CMV	Cytomegalovirus
CNS	Central Nervous System
COX	Cycloxygenase
CTD	C-terminal domain
D	Aspartic acid
DAMPs	danger/damage associated molecular patterns
DED	Death Effector Domain
DN	Dominant Negative
DNA	Deoxyribonucleic Acid
DREAM	DP, Rb, E2F And MuvB
E2F	E2 promoter binding factor
ECM	Extra Cellular Matrix
EEG	Electroencephalogram
Endo G	Endonuclease G
ETC	Electron Transport Chain
FADD	Fas-Associated Death Domain
fEPSP	field Excitatory Postsynaptic Potentials
g	gram
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GSK-3β	Glycogen Synthase Kinase -3β
H&E	Hematoxylin and Eosin
HD	Huntington's Disease
HDAC	Histone Deacetylase

HMGB1	High-Mobility Group Box-1
hrs	hours
HSP	Heat Shock Protein
ICAM-1	Intracellular Adhesion Molecule-1
ICH	Intracerebral hemorrhage
ICV	Intracerebral ventricular
IL	Interleukin
INK4	Inhibitor of Cdk4
iNOS	inducible Nitric Oxide Synthase
IRBP	Interstitial Retinol-Binding Protein
K	Lysine
KIP	Kinase Inhibitory Protein
MAP-2	Microtubule Associated Protein 2
MCAO	Middle Cerebral Artery Occlusion
MMPs	Matrix metalloproteinases
MOMP	Mitochondrial Outer membrane permeabilization
mRNA	messenger RNA
Myb	Myeloblastosis
N	Sample size
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCLK	Neuronal Cdc2-Like Kinase
NGF	Nerve growth factor
NMDA	N-methyl D-aspartic acid
NOS	Nitric Oxide Synthase
OGD	Oxygen Glucose Deprivation
OGG1	7, 8-Dihydro-8-oxoguanine DNA glycosylase
P	Proline

P	Probability
PAR	Poly (ADP-ribose)
PARP	Poly ADP-ribose polymerase
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PLA2	Phospholipase A2
PNS	Peripheral Nervous System
Prx	Peroxiredoxin
PSTAIRE	Proline Serine Threonine Alanine Isoleucine Arginine Glutamic acid
PTP	Permeability Transition Pore
R	Arginine
rAAV	recombinant Adeno-Associated Virus
Rb	Retinoblastoma
RNA	Ribonucleic acid
RNAPII	RNA polymerase II
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
S	Serine
SAH	Subarachnoid hemorrhage
SD	Standard Deviation
SEM	Standard Error of the Mean
shRNA	short hairpin RNA
siRNA	small interfering RNA
SOD	Superoxide Dismutase
SV40 Tag	Simian Virus 40 large T-antigen
T	Threonine

TIA	Transient ischemic attack
TLR	Toll-like Receptors
TNF	Tumor Necrosis Factor
TNFR1	Tumor necrosis factor receptor 1
tPA	tissue-type Plasminogen Activator
TRADD	Tumor necrosis receptor-1-associated death domain
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
UNG	Uracile-DNA glycosylase
VCAM-1	vascular cell adhesion molecule-1
XRCC1	X-ray repair cross-complementing group 1
Y	Tyrosine

List of Manuscripts

I. Rashidian J**, Iyirhiaro G,** Aleyasin H, Rios M, Vincent I, Callaghan S, Bland RJ, Slack RS, During MJ, Park DS. (2005) Multiple cyclin-dependent kinases signals are critical mediators of ischemia/hypoxic neuronal death in vitro and in vivo. ***Proceedings of the National Academy of Science USA, 102(39): 14080-5***

II. **Iyirhiaro GO,** Brust TB, Rashian J, Galehdar Z, Phillips M, Slack RS, MacVicar BA, Park DS. (2008) Delayed combinatorial treatment with flavopiridol land minocycline provides longer term protection for neuronal soma but not dendrites following global ischemia. ***Journal of Neurochemistry, 105(3): 703-13.***

III. **Iyirhiaro GO**, Estey C, Callaghan SM, During MJ, Slack RS and Park DS. Cdc25A is a critical mediator of ischemic neuronal death in vitro and in vivo. ***Manuscript in preparation***.

IV. **Iyirhiaro GO**, Zhang Y, Estey C, O'Hare MJ, Safarpour F, Parsanejad M, Wang S, Abdel-Messih E, Callaghan SM, During MJ, Slack RS, and Park DS. Regulation of ischemic neuronal death by E2F4/p130 complexes. ***In revision for The Journal of Biological Chemistry, 2013***.

Appended Article:

V. Biwas SC, Zhang Y, **Iyirhiaro G,** Willett RT, Rodriguez Gonzalez Y, Cregan SP, Slack RS, Park DS, Greene LA. (2010) Sertad1 plays an essential role in developmental and pathological neuron death. ***Journal of Neuroscience, 30(11):3973-82.***

Thesis Format

This thesis is presented as a collection of manuscripts, in accordance with the guidelines set forth by the Department of Cellular and Molecular Medicine, Neuroscience program; Faculty of Medicine, University of Ottawa. It is composed of four research manuscripts preceded by an introductory section and followed by a general discussion of the thesis findings.

Chapter one is a general introduction to stroke and the cyclin dependent kinase (CDK) family. It provides a review of CDK biology, their known roles and evolving functions. It reviews the relevant scientific research and rationale for the work presented in the subsequent chapters (chapters 2, 3, 4 and 5).

Chapter 2 presents a manuscript entitled "Multiple cyclin-dependent kinases signals are critical mediators of ischemia/hypoxic neuronal death *in vitro* and *in vivo."* This manuscript is presented as published in the Proceedings of the National Academy of Sciences of the United States of America (PNAS) in 2005.

Chapter 3 is a manuscript entitled "Cdc25A is a critical mediator of ischemic neuronal death *in vitro* and *in vivo*". This manuscript is in preparation for submission.

Chapter 4 presents a manuscript entitled "Regulation of ischemic neuronal death by E2F4/p130 complexes". This manuscript is in revision for The Journal of Biological Chemistry.

Chapter 5 is a manuscript entitled "Delayed combinatorial treatment with flavopiridol and minocycline provides longer term protection for neuronal soma but not dendrites

following global ischemia". This manuscript was published in the Journal of Neurochemistry and is presented as published.

Chapter 6 presents a synopsis and general discussion of the major thesis research findings, their implications and future considerations.

CHAPTER 1

General Introduction

1.1. ETIOLOGY AND EPIDEMIOLOGY OF STROKE

Stroke is the loss of brain function caused by a transient or permanent interruption of blood supply to the brain (Lo et al., 2003, Jeyaseelan et al., 2008, Deb et al., 2010). This results in a period of ischemia in the affected brain tissue that is characterized by oxygen and glucose deprivation. Stroke can occur as a result of a ruptured or occluded blood vessel and, in certain cases cardiac arrest (Dirnagl et al., 1999, Lo et al., 2003). In the case of acute stroke, clinical symptoms include weakness of the face, arm or leg or one side of the body, sudden confusion, inability to speak or understand, sudden difficulty walking, dizziness and sudden severe headache (Rosamond et al., 2007, Rosamond et al., 2008).

Although anyone can suffer a stroke, the risk of experiencing one is influenced by a number of factors, some of which can be modified by medication or a change in life style. These include diabetes, hypertension, atherosclerosis, atrial fibrillation, smoking and obesity (Lo et al., 2003, Roger et al., 2011). Other factors associated with an increased risk of stroke are gender, race, age and prior cerebrovascular events (Lo et al., 2003, Romero et al., 2008, Roger et al., 2011). These risk factors are inherent in the individual and cannot be modified (Table 1.1).

Table 1.1. Modifiable and non-modifiable risk factors associated with stroke.

Non Modifiable Risk Factors	Modifiable Risk Factors	
	Medical	Lifestyle
Age	Diabetes	Smoking
Gender	Hypertension	Obesity
Race/ethnicity	Atherosclerosis	Stress
Prior stroke or TIA[1]	High cholesterol	Alcohol use
	Atrial Fibrillation (AF)[2]	Physical Inactivity

[1] Transient ischemic attack or silent stroke
[2] Cardiac abnormality characterized by irregular heart beat

Presently, stroke is one of the leading causes of death worldwide, accounting for ~9% of all deaths (Donnan et al., 2008). In Canada, stroke is the third leading cause of death and accounts for ~6% of all death (www.heartandstroke.com). In addition, more individuals, (5-10 fold) experience milder or covert strokes, without overt or immediate signs of damage (Vermeer et al., 2003, Vermeer et al., 2007). Individuals who experience acute stroke often suffer some form of impairment or permanent disability. It is estimated that 15-30% of individuals who experience a stroke are permanently disabled, 25% die and 50-70% recover functional independence (Jeyaseelan et al., 2008).

The associative cost of treating stroke-related injuries is tremendous and exerts a financial strain on the health care system in Canada and elsewhere. It is estimated that stroke consumes up to 4% of direct health care costs in industrialized nations (Donnan et al., 2008). The cumulative economic cost of treating strokes patients, including lost wages and productivity, in Canada is estimated to be ~$3.6 billion per year (Public Health Agency of Canada, www.phac-aspc.gc.ca). In the United States, the associated price tag for treating stroke is $53.9 billion dollars annually (Roger et al., 2011). These figures are expected to rise as the baby boomer population ages.

1.1.1. SUBTYPES OF STROKE

Stroke can be broadly divided into two types, ischemic and hemorrhagic stroke (Figure 1.1). 87% of all strokes are ischemic and 13% are hemorrhagic (Doyle et al., 2008). Both types of strokes can be sub-typed based on the nature of the occlusion or hemorrhage. Ischemic stroke can be embolic, thrombotic or transient. Likewise, hemorrhagic stroke can be intracerebral or subarachnoid (Figure 1.1) (Donnan et al., 2008, Doyle et al., 2008, Jeyaseelan et al., 2008, Roger et al., 2011).

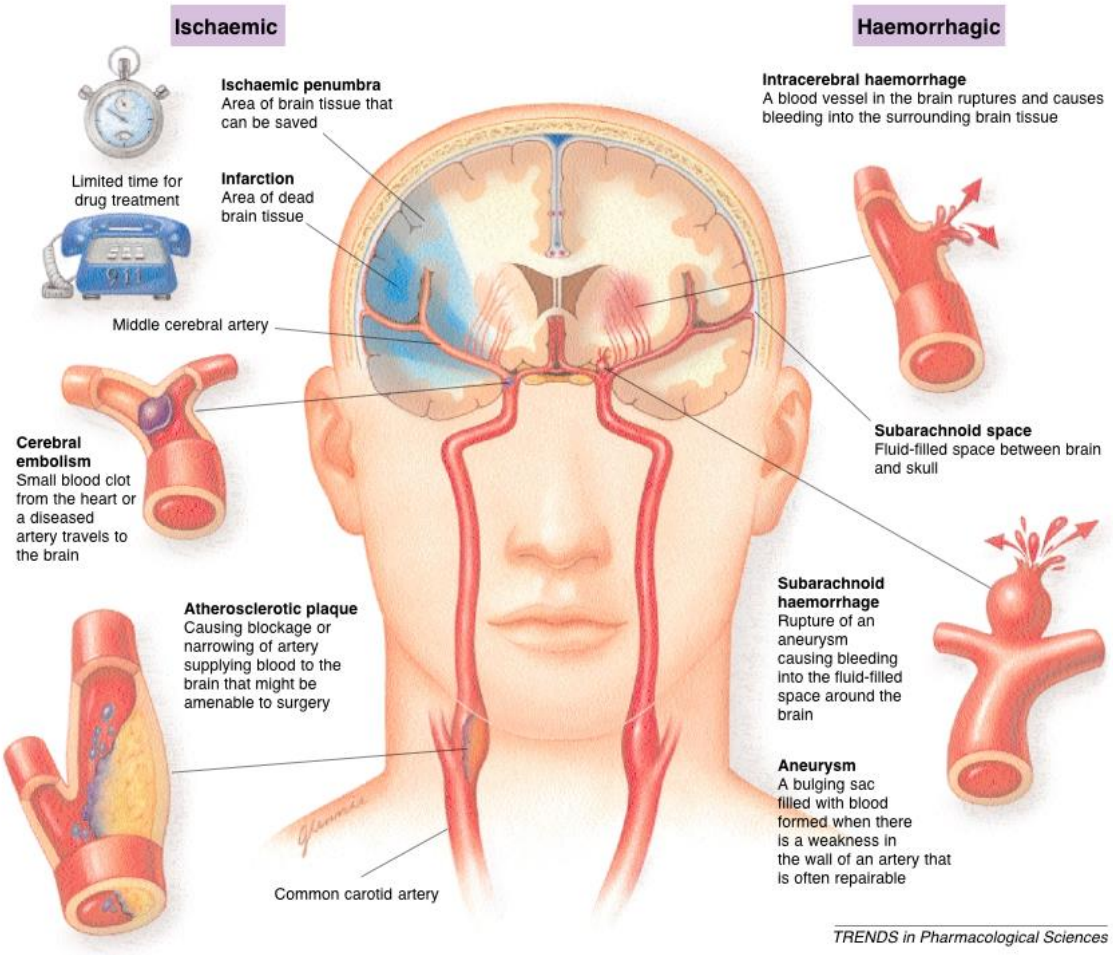
Ischaemic
Haemorrhagic
Ischaemic penumbra
Area of brain tissue that can be saved
Limited time for drug treatment
Infarction
Area of dead brain tissue
Middle cerebral artery
Cerebral embolism
Small blood clot from the heart or a diseased artery travels to the brain
Atherosclerotic plaque
Causing blockage or narrowing of artery supplying blood to the brain that might be amenable to surgery
Common carotid artery
Intracerebral haemorrhage
A blood vessel in the brain ruptures and causes bleeding into the surrounding brain tissue
Subarachnoid space
Fluid-filled space between brain and skull
Subarachnoid haemorrhage
Rupture of an aneurysm causing bleeding into the fluid-filled space around the brain
Aneurysm
A bulging sac filled with blood formed when there is a weakness in the wall of an artery that is often repairable
TRENDS in Pharmacological Sciences

Figure 1.1

Figure 1.1. Main types of stroke. There are two main types of strokes, ischemic and hemorrhagic strokes. *Adapted from (Richard Green et al., 2003) with permission.*

1.1.1.i. Embolic stroke

This type of stroke occurs when a blood clot (embolus) formed elsewhere in the body, usually in the heart, blocks an artery in the brain (Jeyaseelan et al., 2008). Atrial Fibrillation, a heart disorder characterized by irregular heart rhythm is a common cause of emboli and a risk factor for embolic stroke (Jeyaseelan et al., 2008).

1.1.1.ii. Thrombotic stroke

Thrombotic stroke occurs when fat deposits or blood clot forms within an artery of the brain and blocks blood supply to the brain (Jeyaseelan et al., 2008). This can sometimes lead to embolic stroke when the thrombus is dislodged and blocks an artery elsewhere in the brain. A common cause of thrombotic stroke is arteriosclerosis (Jeyaseelan et al., 2008).

1.1.1.iii. Transient ischemic stroke (TIA)

Transient ischemic stroke or mini-stroke is characterized by a brief episode of focal neurologic dysfunction without acute infarction (Easton et al., 2009). The symptoms are similar to that experienced with acute stroke. This type of stroke usually resolves quickly and without medical intervention. TIA increases the risk of a more severe stroke. Approximately 15% of all acute strokes are preceded by TIA (Roger et al., 2011). Among individuals who experience TIA, 3-10% will have a stroke within 2 days and 9-17% within 3 months of the initial attack (Easton et al., 2009, Roger et al., 2011). Thus, TIA is generally regarded as a warning sign for a more acute stroke.

1.1.1.iv. Intracerebral hemorrhage (ICH)

Intracerebral hemorrhage refers to a stroke caused by a ruptured brain artery resulting in spillage of blood into the surrounding tissue (Jeyaseelan et al., 2008). ICH accounts for

~10% of all strokes and is commonly caused by high blood pressure, vascular malformations, use of blood thinning medication and trauma (Rosamond et al., 2007, Donnan et al., 2008, Jeyaseelan et al., 2008, Roger et al., 2011).

1.1.1.v. Subarachnoid hemorrhage (SAH)

Subarachnoid hemorrhage is caused by a ruptured blood vessel near the brain surface resulting in the spillage of blood into the subarachnoid space (Donnan et al., 2008). It is commonly caused by a ruptured aneurysm and accounts for ~3% of all strokes (Rosamond et al., 2007, Donnan et al., 2008, Jeyaseelan et al., 2008, Roger et al., 2011).

1.1.1.vi. Covert stroke (silent infarct)

This type of stroke occurs without obvious clinical symptoms associated with acute stroke or TIA. It is caused by the occlusion or rupture of small perforating arteries in the sub-cortical or deep structures of the brain (Vermeer et al., 2007). Silent infarcts are associated with an increased risk of a subsequent stroke and, are more prevalent than acute cerebral infarctions (Vermeer et al., 2007, Gioia et al., 2012, Markus, 2012).

1.2. CLINICAL TREATMENT OF STROKE

Presently, the clinical treatment of acute stroke is limited primarily to the use of recombinant tissue type plasminogen activator (r-tPA), a thrombolytic and endovascular surgeries. r-tPA is currently the single most effective treatment for acute ischemic stroke (Donnan et al., 2008). Tissue plasminogen activator (tPA) is a naturally occurring serine protease produced in the brain (Wang et al., 2004b). It is found on neurons, glial and endothelial cells. It is known to participate in a wide range of physiological processes, including neuronal migration and growth, synaptic remodeling, learning and memory,

degradation of extra cellular matrix (ECM) and the breakdown of blood clots (fibrinolysis) (Kaur et al., 2004, Wang et al., 2004b). The latter underlies its use as a thrombolytic in the treatment of ischemic stroke. tPA cleaves the plasminogen zymogen into an active enzyme, plasmin, a protease that catalyzes the breakdown of fibrin (Kaur et al., 2004, Wang et al., 2004b).

In spite of its effectiveness, tPA is administered in only about 5-8% of stroke cases (Donnan et al., 2008). This clinical under usage is influenced by a number of factors. First, tPA can only be used in cases of ischemic strokes. Second, the established therapeutic window for tPA is 3.5 hours from the onset of stroke (Albers et al., 2002, Hacke et al., 2004). Beyond this time, its effectiveness decreases and becomes confounded by serious adverse effects. The majority of stroke patients presents at the hospital well beyond this prescribed time and are unable to benefit from treatment with tPA (Evenson et al., 2009, Kaneko et al., 2011). According to a recent report by the Canadian Stroke Network, only 35% of patients in Canada presented at the hospital within 3.5 hours of the onset of stroke. (www.canadianstrokenetwork.ca). Lastly, treatment with r-tPA is associated with an increased risk of intracerebral hemorrhage (Hacke et al., 2004), observed in about 6-13% of those treated (Albers et al., 2002, Hacke et al., 2004). Thus, it is contraindicated for use in patients with risk factors associated with ICH, such as hypertension.

In addition to the caveats already mentioned, there is preclinical evidence to suggest that the use of t-PA may exacerbate brain injury in cases of severe occlusion. Data from a murine model of photochemical-induced thrombotic stroke showed that, although t-PA null mice exhibited greater brain damage with mild ischemic insult, they were protected from a more severe ischemia, when compared with their wild type littermate controls (Nagai et al.,

2002). This deleterious effect of t-PA is thought to be due at least in part to its pleiotropic effects outside of the vascular unit (Kaur et al., 2004). t-PA has been shown to plays a role in glutamate-induced excitotoxicity (Tsirka et al., 1995) and to cleave the NMDAR subunit, NR1, increasing calcium influx (Wang et al., 2004b). Thus, in cases of severe ischemia, where there are likely to be damage to blood vessels and the blood brain barrier (BBB), t-PA may produce neurotoxic effects when exposed to the surrounding brain tissue.

The limitations and concerns associated with the use of tPA have generated a need for new, safe and efficacious treatment avenues to mitigate the impact of stroke. However, such undertaking requires a better understanding of the complex pathological signals and events that are activated in stroke. In this regard, major research efforts have been made to identify molecular perpetrators of ischemic neuronal death. Emerging research data implicate cell cycle molecules as potential ischemic neuronal death perpetrators. Accordingly, this thesis investigates the activation of the cell cycle machinery as a potential pathogenic signal contributing to ischemic neuronal death.

A great deal of the work presented in this thesis employs experimental animal stroke models. Much of what is known about the pathophysiology of stroke has been derived primarily from animal stroke models. Therefore, the following section will briefly describe some of the more commonly used experimental models, including those used in this thesis.

1.3. EXPERIMENTAL ANIMAL MODELS OF CEREBRAL ISCHEMIA

A number of experimental models have been developed to study ischemic stroke *in vivo*. While both large and small animals have been employed, the vast majority of cerebral ischemia is modeled in small rodents, in particular, the mouse, gerbil and rat (Traystman,

2003). The relative ease of variable control, surgical manipulations, coupled with the low associative cost of acquisition and maintenance makes the rodent model an indispensable tool in ischemic stroke research (Traystman, 2003). Cerebral ischemic models can be broadly divided into two main categories based on whether they produce global or focal brain lesions.

1.3.1. GLOBAL CEREBRAL ISCHEMIA

Global cerebral ischemia involves the near or complete blockade of blood flow to the entire brain (Traystman, 2003). This type of insult typically produces wide spread injury to vulnerable neuronal populations, including the neocortex, striatum and the hippocampus (Lipton, 1999, Traystman, 2003). Global ischemia can be achieved using several methods including decapitation, cardiac arrest, neck tourniquet, two-vessel occlusion (2VO) with/without hypotension and four-vessel occlusion (4VO). The former three techniques produce complete global ischemia. The latter three methods are generally considered to be incomplete global ischemia and represent the most commonly used techniques. The distinction between complete and incomplete global ischemia is based on whether or not there is residual blood supply to the brain (Ginsberg and Busto, 1989, Lipton, 1999, Traystman, 2003).

1.3.1.i. Two vessel-occlusion (2VO) model

The 2VO method involves the bilateral occlusion of the two common carotid arteries. This procedure is more commonly used to produce transient forebrain ischemia in the gerbil, which lacks posterior communicating arteries (Traystman, 2003). Cerebral blood flow (CBF) is reduced to almost zero in most animals. CBF in the cortex is <1% and ~4% of control

values in the hippocampus with electroencephalogram (EEG) failure occurring within 20 seconds of insult (Lipton, 1999). A five minute occlusion time is sufficient to produce injury and death of hippocampal CA1 neurons. This model involves a relatively simple surgical procedure but can produce variable result since not all stroke attempts are successful. Additionally, gerbils are susceptible to seizures, which may confound the results (Traystman, 2003).

1.3.1.ii. Two vessel-occlusion (2VO) with hypotension model

The 2VO with hypotension model is similar to what is described above for the gerbil. This procedure is typically performed in the rat and involves bilateral occlusion of the two common carotids arteries coupled with systemic hypotension. Bleeding or hemorrhage and pharmacologic agent are used in order to achieve arterial blood pressure of ~50mmHg (Lipton, 1999, Traystman, 2003). CBF is reduced to <1% in the cortex and ~4% of control values in the hippocampus (Lipton, 1999). EEG becomes isoelectric within 15 to 25 seconds of ischemia. Like the gerbil 2VO model, this method also produces damage in the hippocampus, neocortex and caudoputamen (Ginsberg and Busto, 1989, Lipton, 1999). Although this method is surgically less challenging than the 4VO model (described below), it does have some drawbacks. This includes the need for anesthesia and hypotension with drugs, which may confound data interpretation. Some discrepancy in CBF reduction and pathologic outcome is observed between animals in this model (Ginsberg and Busto, 1989, Traystman, 2003).

1.3.1.iii. Four vessel-occlusion (4VO) model

The 4VO model is more commonly used to produce reversible forebrain ischemia in rats. This procedure is performed in two stages, typically over 2 days. The first stage

involves the placement of ligature loosely around the two common carotid arteries and electro-cauterization of both vertebral arteries. The second stage involves the transient occlusion of both common carotid arteries in the awakened rat to produce ischemia (Ginsberg and Busto, 1989, Traystman, 2003). This procedure can reliably reduce blood flow to <3% of control values in the hippocampus, striatum and neocortex (Traystman, 2003) with an isoelectric EEG within 30 to 40 seconds (Lipton, 1999). Occlusion durations of 6 to 10 minutes, when performed at 36-37^{o}C, results in the death of CA1 neuron that is near complete in 3 days (Lipton, 1999). This model has been validated to produce consistent and reproducible neuropathology. Additionally it can be performed in the awakened rat. A major drawback of the 4VO model is that it is surgically challenging to perform and is successful in about 50 to 75% of animals (Ginsberg and Busto, 1989, Lipton, 1999, Traystman, 2003).

1.3.2. FOCAL CEREBRAL ISCHEMIA

In contrast to global ischemia, focal cerebral ischemia involves the transient or permanent occlusion of blood flow to a specific region of the brain. In this case, only the region of the brain supplied by the occluded vessel becomes ischemic. Focal ischemia can be accomplished via multiple techniques including vessel occlusion, most often the middle cerebral artery (MCA), photothrombosis, thrombi injection (thromboebolic model) and direct application or injection of endothelin-1 (Ginsberg and Busto, 1989, Lipton, 1999, Traystman, 2003).

1.3.2.i. Middle cerebral artery occlusion (MCAO) model

Generally, the MCAO model involves the surgical insertion and advancement of a coated or uncoated monofilament into the internal carotid artery, until it blocks blood flow in

the MCA. This produces infarction in the MCA territory, (frontoparietal cortex and lateral caudoputamen), a region most often lesioned in human strokes. The MCAO model represents one of the most commonly used models of focal cerebral ischemia in both rats and mice, primarily because of its useful approximation of human hemispheric infarction (Traystman, 2003, Durukan and Tatlisumak, 2007). However, lesion reproducibility in this model depends on the shape, diameter, insertion length and coating of the suture. Major drawbacks with this model include the risk of subarachnoid hemorrhage due to vessel rupture, hyperthermia due to hypothalamic injury and inadequate MCAO (Durukan and Tatlisumak, 2007).

1.3.2.ii. Photothrombosis model

The photothrombosis model involves the injection of a photoactive dye (rose Bengal or erythrosine B) followed by irradiation using a light beam of a specific wavelength. The photothrombosis model is less invasive than the MCAO and can typically be used to produce lesions anywhere within the cortical region of the brain (Durukan and Tatlisumak, 2007). However, a major disadvantage of this model is that the infarction produced typically lacks a penumbra, due to vasogenic edema and blood brain barrier (BBB) breakdown. This model also produces end-arterial occlusive lesions that are resistant to therapeutic strategies reliant on enhancement of collateral perfusion (Traystman, 2003, Durukan and Tatlisumak, 2007).

1.3.2.iii. Thromboembolic model

The thromboembolic model of cerebral ischemia often involves the injection of thrombi or non-clot material such as microspheres into extracranial arteries to reach and cause an occlusion of more distal intracranial vessels (Durukan and Tatlisumak, 2007). This technique often produces multifocal lesions. Since the majority of strokes are

thromboembolic in nature, this model is thus considered to closely mimic the human condition. However, this method produces variable results depending on the composition and formation of emboli (Traystman, 2003, Durukan and Tatlisumak, 2007).

1.3.2.iv. Endothelin-1 model

Endothelin-1 is a potent vasoconstrictor and works through receptors present in the vascular endothelium (Durukan and Tatlisumak, 2007). It can be applied topically anywhere on the cortex or injected to produce infarction in the desired area of the brain including the MCA. As with photothrombosis, this model is minimally invasive, when compared with MCAO. This model, however, is dose dependent. In addition, endothelin-1 may induce astrocytosis and facilitate axonal sprouting (Traystman, 2003, Durukan and Tatlisumak, 2007). Thus, this model may not be appropriate in experiments evaluating neural repair.

As evident from the preceding text, each of the different stroke models briefly described has its inherent strengths and weaknesses. Nevertheless, these models have helped to generate a wealth of information regarding the pathophysiology of ischemic stroke. For our studies, we have employed the 4VO and the endothelin-1 models of global and focal ischemia respectively. These models serve as a platform with which to address mechanistically the physiologic relevance of our *in vitro* observations and to determine the role of cell cycle machinery in ischemic neuronal death.

1.4. PATHOPHYSIOLOGY OF ISCHEMIC NEURONAL DEATH

The mechanism(s) of neuronal death following cerebral ischemia is complex and may be determined by multiple parameters such as location, duration and severity of insult. For example, neuronal death in the ischemic core, the region most severely affected by the lack

of blood flow, occurs within minutes to a few hours and, is marked primarily by necrotic and excitotoxic cell death (Mergenthaler et al., 2004, Durukan and Tatlisumak, 2007). This early and rapid death in the ischemic core is mediated mainly by ionic imbalance and excessive intracellular calcium. However, in the ischemic penumbra, an area surrounding the core of the infarct, cell death occurs in a delayed apoptotic-like manner that can take several hours to days depending on the severity of injury (Mergenthaler et al., 2004). The penumbra is, typically, less severely affected by the initial ischemic insult owing to residual perfusion by collateral blood vessels. However, if blood supply is not restored within hours, the ischemic penumbra progresses to infarction and becomes part of the ischemic core (Durukan and Tatlisumak, 2007). The ischemic penumbra present early following insult can later account for up to 50% of the infarct volume (Mergenthaler et al., 2004). Although the cells within the ischemic penumbra are functionally marginalized, they are salvageable and potentially amenable to neuroprotectant strategies. The factors and signaling pathways resulting in rapid and delayed neuronal death in stroke are complex and are not fully understood. However, our knowledge of some of these events has been significantly advanced by research using experimental stroke models.

The brain utilizes aerobic metabolism of glucose as its primary source of energy. Consequently, it relies extensively on constant and adequate blood supply in order to meet its metabolic needs. This includes energy required for the maintenance of ionic gradients, synaptic activity and biosynthesis. Accordingly, CBF is regulated to meet the brain's metabolic requirements. Typically, CBF in the adult is ~50ml /100g of brain tissue/min. However, during cerebral ischemia, blood flow in the ischemic core can drop to below 10% of its normal level (Markus, 2004, Moustafa and Baron, 2008, Deb et al., 2010). The severe

drop in CBF results in oxygen and glucose deprivation (OGD) to the affected brain tissue. Adenosine triphosphate (ATP), the brain's primary energy source, is depleted within minutes of insult causing an energy crisis (Durukan and Tatlisumak, 2007). Oxygen deprivation causes a switch from oxidative respiration to anaerobic glycolysis leading acidosis, which is thought to interfere with protein synthesis and contribute to the formation of free radicals (Mergenthaler et al., 2004, Manzanero et al., 2013).

Severe reduction in cellular ATP causes dysfunction of energy dependent processes, including the maintenance of ion pumps, resulting in ionic imbalance (Durukan and Tatlisumak, 2007). Consequently, there is an influx of Na^+ and efflux of K^+ causing depolarization and activation of voltage-gated calcium channels. Extracellular signaling molecules, including the excitatory neurotransmitter glutamate, are released from synaptic terminals. Impairment of active presynaptic and astrocytic reuptake causes accumulation of extracellular glutamate. This leads to persistent activation of glutamate receptors including the N-Methyl D Aspartate (NMDA), a-amino-3-hydroxy-5-methyl-4-propionate (AMPA) and metabotropic glutamate receptors (Mergenthaler et al., 2004, Durukan and Tatlisumak, 2007, Brouns and De Deyn, 2009). Activation of the glutamate receptors, in particular the NMDAR leads to an increase in intracellular Ca^{2+}, triggering an excitotoxic cascade. Additionally, activation of the AMPAR causes increase in intracellular Na^+ and Cl^- ions. Together with passive influx of water, these events lead to disturbances in ion homeostasis, intracellular calcium overload, mitochondrial damage, edema and necrosis (Kristian and Siesjo, 1998, Mergenthaler et al., 2004, Brouns and De Deyn, 2009).

In addition to necrosis, which predominates in the ischemic core, neuronal injury in the peri-infarcted/ penumbra region is precipitated by excessive activation of many different, and likely related, signals that coalesce in delayed death. Some of these include the

activation of Ca^{2+} dependent proteases such as calpains (calcium-dependent cysteine proteases), phospholipase A2 (PLA2), and nitric oxide synthase (NOS). These enzymes, once activated, contribute to the activation of other signals, degradation of structural proteins, generation of free radicals, oxidative damage and cell death (Figure 1.2) (Dirnagl et al., 1999, Durukan and Tatlisumak, 2007). Activation of calpains, for example, can lead to the cleavage of cytoskeletal elements including spectrin, microtubule-associated proteins and neurofilaments causing further damage to cell integrity and dysregulation in ion homeostasis (Bevers and Neumar, 2008). Alternatively, calpains may facilitate neuronal apoptosis via the activation of the pro-apoptotic members of the B-cell lymphoma 2 (Bcl2) family, caspases and apoptosis inducing factor (AIF) (Bevers and Neumar, 2008, Broughton et al., 2009). Indeed, apoptosis is believed to play an important role in ischemic neuronal death. In this regard, elements of both the cell intrinsic and extrinsic apoptotic programs participate in ischemic neuronal death (discussed below).

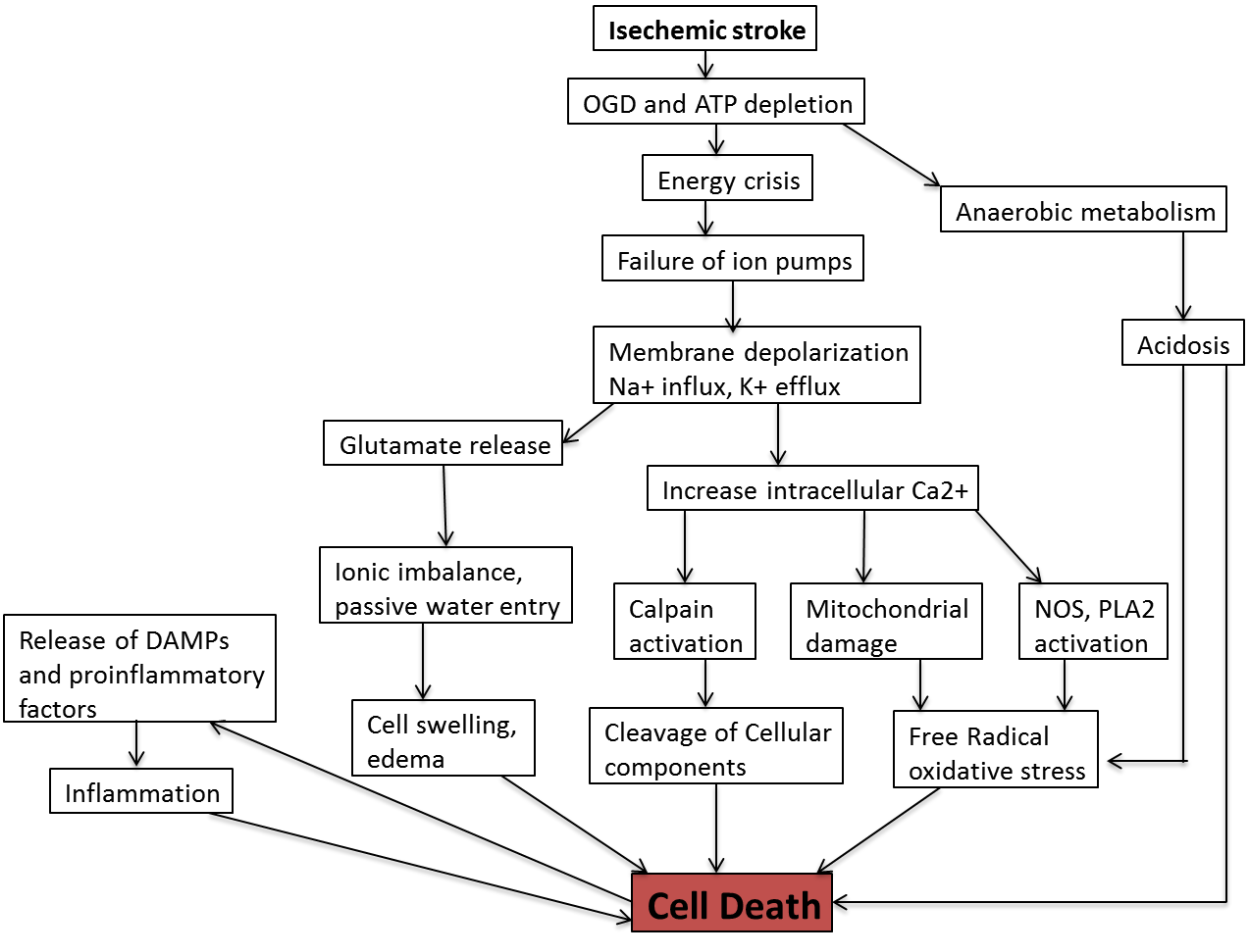
Isechemic stroke
OGD and ATP depletion
Energy crisis
Anaerobic metabolism
Failure of ion pumps
Acidosis
Membrane depolarization
Na+ influx, K+ efflux
Glutamate release
Increase intracellular Ca2+
Ionic imbalance,
passive water entry
Calpain
activation
Mitochondrial
damage
NOS, PLA2
activation
Release of DAMPs
and proinflammatory
factors
Cell swelling,
edema
Cleavage of Cellular
components
Free Radical
oxidative stress
Inflammation
Cell Death

Figure 1.2

Figure 1.2. General overview of events during cerebral ischemic injury.

Ischemic brain damage is precipitated by oxygen and glucose deprivation (OGD) and leads to the pathologic activation of multiple pathways that culminate in cell death.

1.4.1. APOPTOSIS PATHWAYS AND ISCHEMIC NEURONAL DEATH

1.4.1.i. Intrinsic apoptosis pathway

The cell intrinsic apoptosis program consists of a caspase-dependent and -independent signaling cascades which lead to death. The former involves mitochondrial outer membrane permeabilization (MOMP) and the release of cyctochrome C. Once released, cyctochrome C forms a complex, known as the apoptosome, with apoptosis activation factor 1 (APAF-1), pro-caspase-9 and ATP (Mergenthaler et al., 2004, Sugawara et al., 2004). Caspase-9 is an apical/ initiator caspase, a subgroup that also includes caspase-2, -8 and -10. Upon activation, caspase-9 mediates the activation of downstream effector/executioner caspases, which includes caspase-3, -6 and -7. The effector caspases orchestrate the degradation and neat dismantling of various cellular components. These include the degradation of cytoskeletal proteins such as actin, fondrin, gelsolin, nuclear lamins, the activation of caspase activated DNAse (CAD) and the inhibition of the DNA repair enzyme, poly ADP-ribose polymerase (PARP), resulting in apoptosis (Sugawara et al., 2004, Broughton et al., 2009).

The caspase-independent apoptosis pathway, mainly involves the translocation of AIF and endonuclease G (Endo G) from the mitochondrial intermembrane space to the nucleus. Once released, AIF and Endo G mediate the degradation of DNA, nuclear fragmentation and cell death (Figure 1.3) (Broughton et al., 2009).

The release of apoptogenic factors from the mitochondria is facilitated by the actions of the pro-apoptotic Bcl2 family members. These include Bax, Bak, Bad, Bim, Bid, Puma and Noxa which regulate apoptosis by promoting or antagonizing the release of pro-apoptotic factors such as cytochrome c from the mitochondria. Bax and Bak promote

apoptosis by directly forming channels in the mitochondrial outer membrane (Mergenthaler et al., 2004, Sugawara et al., 2004). The other pro-apoptotic Bcl2 proteins regulate cell death by antagonizing the actions of anti-apoptotic family members, which include Bcl-2, Bcl-XL and Bcl-W. The anti-apoptotic Bcl-2 members promote cell survival by interacting and antagonizing Bax/Bak, blocking mitochondrial release of cytochrome c (Mergenthaler et al., 2004, Sugawara et al., 2004).

1.4.1.ii. Extrinsic apoptosis pathway

The extrinsic apoptosis pathway commences with the binding of death-inducing ligands to their respective death receptors (DRs) present on the surface of the cell membrane. This triggers an intracellular signaling cascade that culminates in the activation of pro-caspase-8 and downstream executioner caspases. Examples include the binding of the Fas ligand or tumor necrosis factor (TNF) to their respective receptors, FasR and TNFR1 on the cell surface. This triggers receptor oligomerization and aggregation of their intracellular death domains (DD), which act in the recruitment of adaptor proteins such as fas-associated death domain (FADD) and tumor necrosis receptor-1-associated death domain (TRADD). The adaptor proteins recruit pro-caspase-8 via their death effector domain (DED) resulting in its subsequent oligomerization and auto-activation (Doyle et al., 2008, Broughton et al., 2009). Once activated, caspase-8 activates downstream executioner caspases including caspase-3 and caspase-7, engaging apoptosis. Alternatively, activated caspase-8 can facilitate a crosstalk with the intrinsic apoptotic program by cleaving Bid. The proteolytically cleaved form of Bid (tBid) translocates to the mitochondria where it induces Bax/Bak oligomerization, cytochrome C release, caspase-9 activation and apoptosis (Figure 1.3) (Sugawara et al., 2004, Broughton et al., 2009).

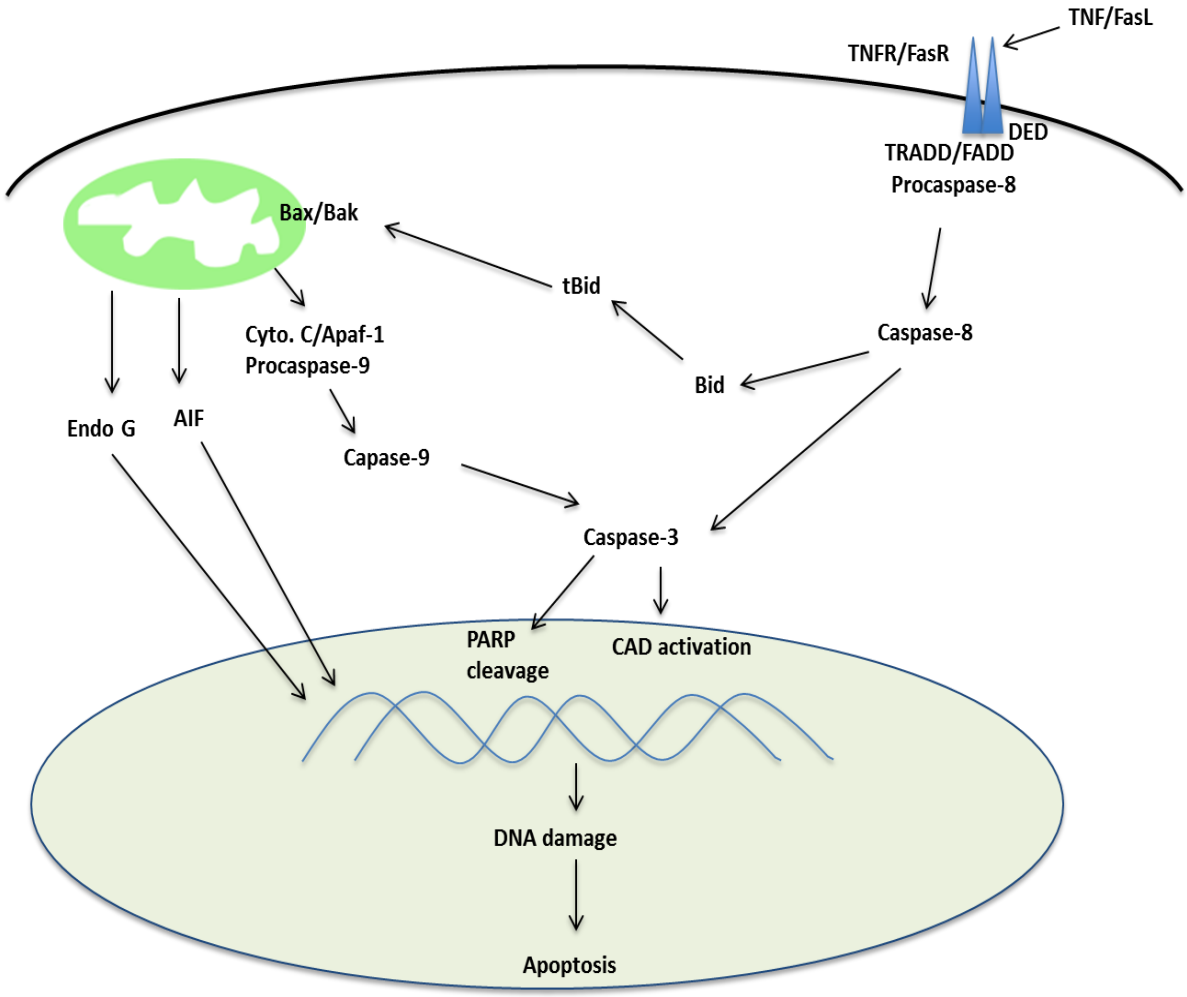
TNF/FasL
TNFR/FasR
DED
TRADD/FADD
Procaspase-8
Bax/Bak
tBid
Cyto. C/Apaf-1
Procaspase-9
Caspase-8
Bid
Endo G
AIF
Capase-9
Caspase-3
PARP
cleavage
CAD activation
DNA damage
Apoptosis

Figure 1.3

Figure 1.3. Overview of the main components of the intrinsic and extrinsic apoptosis pathways. Components of both pathways are involved in mediating ischemic neuronal death.

1.4.1.iii. Apoptosis in ischemic neuronal death

The involvement of both the intrinsic and extrinsic apoptosis pathways in ischemic neuronal death has been demonstrated in models of stroke. With regard to the intrinsic apoptosis program, increased expression and activation of pro-apoptotic proteins are detected in ischemic brains. An increase in Bax mRNA and protein is detected following global ischemia (Krajewski et al., 1995, Chen et al., 1996). Bid, a factor in both the intrinsic and extrinsic apoptosis pathways, is cleaved following oxygen glucose deprivation (OGD) (Plesnila et al., 2001) and focal ischemia (Plesnila et al., 2001, Yin et al., 2002). Notably, inhibition of pro-apoptotic or over-expression of anti-apoptotic Bcl-2 family members is protective in models of ischemia. Bid deficiency is protective in both OGD (Plesnila et al., 2001) and focal ischemia models of stroke (Plesnila et al., 2001, Yin et al., 2002). Similarly, over-expression of the anti-apoptotic factors, Bcl-2 (Martinou et al., 1994, Linnik et al., 1995, Zhao et al., 2003) and Bcl-XL (Wiessner et al., 1999, Kilic et al., 2002b) reduces brain infarct following ischemia. Likewise, intraperitoneal (Cao et al., 2002) or intravenous (Kilic et al., 2002a) administration of Bcl-XL is also protective following focal ischemia induced by MCAO.

The release of apoptogenic factors, involved in both caspase-dependent and independent apoptosis, from the mitochondria is also detected in models of stroke. Cyctochrome C is released following global (Perez-Pinzon et al., 1999, Sugawara et al., 1999) and focal (Fujimura et al., 1998) cerebral ischemia. Similarly, nuclear translocation of AIF is detected following glutamate exposure and OGD in cultured neurons as well as transient MCAO in mice (Culmsee et al., 2005). In addition, Harlequin mutant mice, which

express 80% less AIF than wild type animals exhibits smaller infarcts following MCAO (Culmsee et al., 2005).

Finally, the activation of caspases, including caspase-1, -3, and -9, has been shown following cerebral ischemia (Zhang et al., 2003, Mergenthaler et al., 2004). Caspase-1 belongs to a subclass of caspases that includes caspase-4, -5, -11, -12 and -14, and is involved in cytokine maturation and inflammation. Caspase-1 is thought to act upstream of the mitochondria, as an apical caspase, through Bid activation and has been shown to induce apoptosis following OGD in vitro and MCAO (Zhang et al., 2003). In accordance with its observed role in apoptosis, mice null for caspase-1 (Schielke et al., 1998) or expressing a dominant negative mutant form (Friedlander et al., 1997) exhibit reduced brain damage following MCAO. Pharmacologic inhibition of caspase-1 has also been shown to be protective following ischemia (Ross et al., 2007).

Caspase-3 (Namura et al., 1998, Ni et al., 1998, Luo et al., 2002) and caspase-9 (Cao et al., 2002, Mouw et al., 2002) are also activated following global and focal cerebral ischemia. Mice null for caspase-3 have smaller infarcts following ischemia (Le et al., 2002). Additionally, the general inhibition of caspases (Hara et al., 1997, Endres et al., 1998, Inoue et al., 2004) or the select inhibition of caspase-1 (Ross et al., 2007), -3 (Hatip-Al-Khatib et al., 2004) and -9 (Cao et al., 2002) have been shown to reduce ischemic brain damage.

The extrinsic apoptosis signaling pathway is also engaged in cerebral ischemia. Fas, FasL and FADD are upregulated following global ischemia in rats (Jin et al., 2001a). Similarly, Fas and FasL are induced following focal ischemia and mice expressing mutant Fas exhibit smaller infarcts compared to wild-type control animals (Rosenbaum et al., 2000). TNFα, another component of the extrinsic apoptosis pathway, is released following OGD of

cortical neurons and induces the activation of caspase-8 and -3 in a TNFαR1 dependent manner (Badiola et al., 2009).

The activation of both the intrinsic and extrinsic apoptosis machinery can also be extended to human strokes. Activation of caspase-3 and PARP-1 has been demonstrated in the *post mortem* brains of human stroke patients (Love et al., 2000a, Love et al., 2000b, Sairanen et al., 2009). Similarly, over-expression of Fas and FasL has been observed in the *post-mortem* brain tissues obtained from stroke patients (Sairanen et al., 2006).

1.4.2. OXIDATIVE STRESS

The studies described in the preceding section highlight the involvement of the apoptosis machinery in ischemic neuronal death. The activation of cell death signals in the ischemic brain is influenced by factors such as oxidative stress, DNA damage and inflammation. With regard to oxidative stress, an increase in free radicals including reactive oxygen and nitrogen species (ROS and RNS respectively) is believed to be a significant contributor to ischemic neuronal death.

Free radicals are produced at basal levels, under physiologic condition, and can function as signaling molecules in some biological processes (Olmez and Ozyurt, 2012). They are produced as a result of normal mitochondrial respiration or as by products from various metabolic processes. However, because of their reactive nature and propensity to cause damage to cellular components such as proteins, lipids and nucleic acids, the levels of free radicals are kept in check by various antioxidant systems. These include superoxide dismutase (SOD), catalase (CAT), peroxiredoxin (Prx) and glutathione peroxidases (Olmez and Ozyurt, 2012). Under certain pathologic conditions, such as ischemia, the levels of free

radical species exceed the capacity of the cell's antioxidant systems resulting in oxidative stress. This can occur as a result of an increase in the level of free radicals and/or a decrease in the cell antioxidant capacity. During ischemia and following reperfusion, there is a drastic increase in free radical production that is unmatched by the cell antioxidant mechanisms (Lo et al., 2003, Olmez and Ozyurt, 2012, Manzanero et al., 2013). This increase in free radicals is facilitated by the activation of Ca^{2+} dependent proteases, phospholipase A2, NOS, mitochondrial damage and inflammation (Brouns and De Deyn, 2009). For example, the activation of Ca^{2+} dependent proteases causes the conversion of xanthine dehydrogenase to xanthine oxidase (Warner et al., 2004, Kumar and Dogra, 2008). This enzyme, in turn, catalyzes the metabolism of hypoxanthine to uric acid resulting in the production of H_2O_2 and the superoxide anion ($^{\cdot}O_2^-$) (Warner et al., 2004). Similarly, activation of PLA2 results in the liberation of arachidonic acid from membrane phospholipids. Arachidonic acid is metabolized by cycloxygenase (COX) resulting in the production of prostaglandins, pro-inflammatory factors and $^{\cdot}O_2^-$. (Warner et al., 2004, Kumar and Dogra, 2008). Likewise, the activation of calcium-dependent isoform of NOS results in the increased production of nitric oxide ($NO^{\cdot}$), a reactive RNS, from L-arginine (Wang et al., 2007, Olmez and Ozyurt, 2012).

Free radicals are also generated as a consequence of mitochondrial respiration and damage. The mitochondria are generally regarded as a major contributor of ROS following ischemia. Leakage of electrons from the mitochondrial electron transport chain (ETC) leads to the reduction of molecular O_2 to the superoxide anion. Another major source of ROS is the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase or NOX which catalyzes the oxidation of NADPH, producing superoxide anions (Brouns and De Deyn, 2009, Manzanero et al., 2013). Other free radicals, such as the highly reactive peroxynitrite, may be formed

when a superoxide anion reacts with $NO^{\cdot}$. Similarly hydroxide anions are produced from H_2O_2 in the presence of Cu^{+} and Fe^{2+} (Figure 1.4) (Lo et al., 2003).

Inflammation is another major contributor to oxidative stress in the ischemic brain. Activated glia, including microglia, macrophages and leukocytes produce a number of pro-inflammatory mediators including iNOS and COX-2, which contribute to the generation of ROS (Wang et al., 2007).

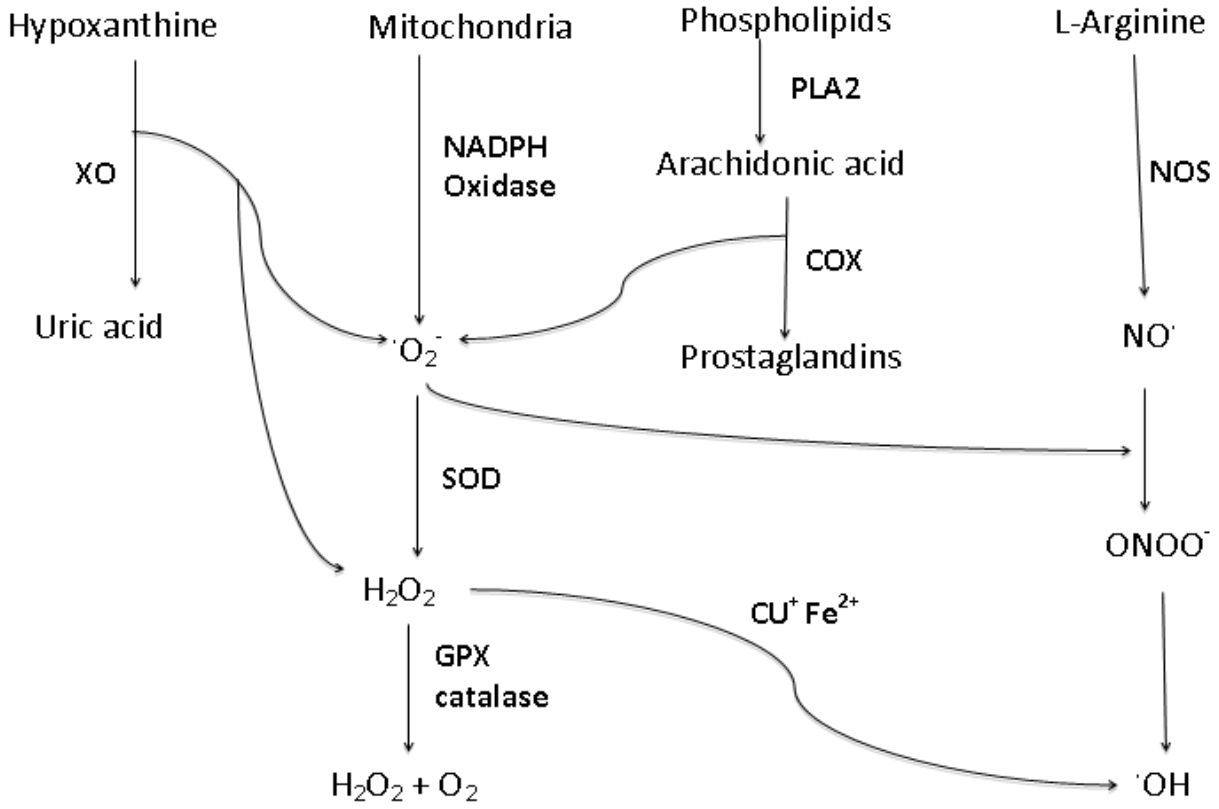
Hypoxanthine
Mitochondria
Phospholipids
L-Arginine
PLA2
XO
NADPH
Oxidase
Arachidonic acid
NOS
COX
Uric acid
$O_2^{\cdot-}$
Prostaglandins
NO·
SOD
ONOO⁻
H_2O_2
CU^+ Fe^{2+}
GPX
catalase
$H_2O_2 + O_2$
·OH

Figure 1.4

Figure 1.4. Free radicals are produced by multiple sources during cerebral ischemic injury. Superoxide anions ($^{\cdot}O_2^-$) are generated by the mitochondria, xanthine oxidase (OX), NADPH oxidase and cyclooxygenase (COX). Reactive nitrogen species are generated by nitric oxide synthase (NOS) and the reaction of nitric oxide ($NO^{\cdot}$) with superoxide anions from other pathways to form peroxynitrite ($ONOO^-$). Hydroxy radicals ($^{\cdot}OH$) are generated in the presence of copper (Cu^+) and iron (Fe^{2+}) from hydrogen peroxide (H_2O_2).

Several lines of evidence support the involvement of free radicals in the pathophysiology of cerebral ischemic damage. Increase in free radicals is observed in animal models of stroke (Chan et al., 1998, Kawase et al., 1999b, Kim et al., 2000, Kim et al., 2002). Similarly, enzymes involved in the generation of free radicals, such as COX-2 are induced following global (Ohtsuki et al., 1996, Koistinaho et al., 1999, Sasaki et al., 2004) and focal ischemia (Collaco-Moraes et al., 1996, Nogawa et al., 1997, Ahmad et al., 2009). Induction of COX-2 is also observed in *post mortem* brain tissues of stroke patients who died as a result of an infarction of the MCA territory (Iadecola et al., 1999). Importantly, COX-2 deficiency (Iadecola et al., 2001, Sasaki et al., 2004) or its pharmacologic inhibition (Nogawa et al., 1997, Dore et al., 2003) attenuates neuronal damage in animal models of cerebral ischemia. Conversely, transgenic mice constitutively over-expressing COX-2 exhibit larger infarcts following focal ischemia (Dore et al., 2003).

In line with the role for oxidative stress in stroke, deficiency in the antioxidants, copper zinc (CuZn)-SOD (SOD1) and manganese (Mn)SOD (SOD2), have been shown to exacerbate cerebral ischemic injury (Kondo et al., 1997, Kawase et al., 1999b, Kim et al., 2002). SOD1 and SOD2 are isoforms of SOD, primarily located in the cytoplasm and mitochondria respectively. Both enzymes catalyze the conversion of superoxide anions to the milder oxidant H_2O_2. In contrast, over-expression of SOD1 in the rodent attenuates neuronal injury following global (Murakami et al., 1997, Chan et al., 1998) and focal (Kinouchi et al., 1991, Kim et al., 2001a) cerebral ischemia. Similarly, the use of pharmacologic free radical scavengers has also been shown to attenuate DNA fragmentation, caspase-3 activation and ischemic lesions induced by photothrombosis (Kim et al., 2000).

The consequence of oxidative stress is damage to cellular components including lipids, proteins and DNA. Free radical-induced damage to proteins, lipids and DNA are

observed in brains subjected to ischemic insults (Fukuyama et al., 1998, Takizawa et al., 1999, Kim et al., 2001a). Elevated levels of nitrotyrosine, a marker of peroxynitrite-induced protein damage, are observed for up to 48 hours following focal ischemia in rats (Fukuyama et al., 1998, Takizawa et al., 1999). Likewise, oxidative damage to DNA is observed in animal models of cerebral ischemia (Jin et al., 1999a, Cui et al., 2000, Huang et al., 2000, Kim et al., 2001a, Lan et al., 2003, Liu et al., 2011)

1.4.3. DNA DAMAGE

With regard to DNA damage, studies have shown that it is a potent inducer of neuronal death (Park et al., 1997a, Park et al., 1998a, Park et al., 2000a) and an important factor in the pathophysiology of a number of neurodegenerative conditions including Parkinson's disease (PD) (Shimura-Miura et al., 1999, Zhang et al., 1999, Yamaguchi et al., 2006, Nakabeppu et al., 2007), Alzheimer's disease (AD) (Nunomura et al., 2001), Amyotrophic lateral sclerosis (ALS) (Kikuchi et al., 2002) and stroke (Endres et al., 2004, Liu et al., 2011).

The involvement of DNA damage in ischemic neuronal death is supported by a number of observations. As already mentioned, an increase in DNA damage is observed in multiple models of stroke (Nagayama et al., 2000, Lan et al., 2003, Liu et al., 2011). Indeed, oxidative damage to DNA is observed early in the ischemic brain, prior to evidence of apoptosis and cell death (Chen et al., 1997, Cui et al., 2000, Huang et al., 2000). A number of proteins involved in DNA damage repair are downregulated in ischemic tissue undergoing cell death (Fujimura et al., 1998, Fujimura et al., 1999b, Kawase et al., 1999b, Kim et al., 2001a). For example, the levels and nuclear immunoreactivity of apurinic/apyrimidinic

endonuclease (APE/Ref-1), X-ray repair cross-complementing group 1 (XRCC1), proteins involved in the DNA base excision repair pathway, are persistently decreased in global (Kawase et al., 1999a) and focal (Fujimura et al., 1999c) models of cerebral ischemia. Similarly the Ku proteins (Ku70 and Ku86), involved in the non-homologous end joining (NHEJ) DNA repair pathway, are also decreased following cerebral ischemia (Kim et al., 2001b). The decline in these proteins was found to be concomitant with an increase in DNA damage and to precede cell death (Kim et al., 2001b). This suggests that accumulation of DNA damage coupled with the decline in repair mechanisms may be a contributing factor in ischemic neuronal death.

A number of studies have also shown the upregulation of enzymes involved in DNA damage repair. 7, 8-Dihydro-8-oxoguanine DNA glycosylase (OGG1) (Lin et al., 2000, Liu et al., 2011), Uracile-DNA glycosylase (UNG) (Endres et al., 2004) and polymerase β (Lan et al., 2003), enzymes involved in DNA base excision repair (BER) are induced following focal ischemia. Interestingly, these DNA repair proteins appeared to be induced in neuronal populations that survived ischemia (Lan et al., 2003), suggesting that having an efficient DNA repair is associated with ischemic resistance and survival. In support of this, mice deficient in certain DNA damage repair enzymes, including OGG1 (Liu et al., 2011) and UNG (Endres et al., 2004), have been shown to exhibit greater brain infarcts when subjected to focal ischemia compared with wildtype controls.

The accumulation of DNA damage in the ischemic brain may facilitate neuronal death by activating PARP and p53. PARP is activated in response to DNA damage and utilizes nicotinamide adenine dinucleotide (NAD+) as a substrate in the synthesis of long polymers of poly (ADP-ribose) or PAR (Doyle et al., 2008). This causes depletion of cellular

NAD+ and its dependent processes, including the generation of ATP, resulting in energy failure and cell death (Sugawara et al., 2004, Doyle et al., 2008, Broughton et al., 2009). In support of this, PARP activation and NAD depletion are observed following ischemic insults (Endres et al., 1997, Iwashita et al., 2004). Furthermore, genetic deletion (Endres et al., 1997, Goto et al., 2002) or pharmacologic inhibition of PARP (Lo et al., 1998, Iwashita et al., 2004) has largely proven to be protective in models of cerebral ischemia.

Similarly, p53 induction and activation is observed in models of stroke (Hong et al., 2010). Once activated, p53 promotes the expression of pro-apoptotic proteins such as Puma, Noxa, Bax and the FasR, resulting in apoptosis signaling (Broughton et al., 2009, Hong et al., 2010). Alternatively, p53 may induce apoptosis, independent of its transcriptional activities by interacting and forming an inhibitory complex with the mitochondrial anti-apoptotic proteins. p53 has been shown to translocate and interact with Bcl-XL in the mitochondria following global ischemia (Endo et al., 2006). This interaction, along with the release of cytochrome C and CA1 neuron death, is attenuated by pharmacologic inhibition of p53 (Endo et al., 2006). Interestingly, mitochondrial p53 accumulation is also linked to necrotic cell death following oxidative stress and cerebral ischemic injury. A recent study by Vaseva and colleagues showed that p53 accumulation in the mitochondria facilitated permeability transition pore (PTP) opening and necrosis by physically interacting with cyclophilin D (Vaseva et al., 2012). Thus, accumulation of DNA damage in the ischemic brain can trigger both necrotic and apoptotic neuronal cell death.

1.4.4. Inflammatory Response and Cerebral Ischemic Damage

Inflammation is considered to be an important factor contributing to secondary tissue damage and the pathophysiology of stroke. An inflammatory cascade is triggered post-

ischemia by the release of inflammatory factors and molecules, collectively known as danger/damage associated molecular patterns (DAMPs) from injured or necrotic cells (Iadecola and Anrather, 2011). DAMPs released under ischemic conditions include the high-mobility group box-1 (HMGB1), a nuclear DNA binding protein, heat shock protein (HSP60), β-amyloid (Aβ) and ATP (Iadecola and Anrather, 2011). DAMPs activate receptors such as the Toll-like Receptors (TLR) present on glia (microglia and astrocytes), endothelial cells, oligodendrocytes and neurons (Iadecola and Anrather, 2011). ROS and other pro-inflammatory mediators are also released from injured tissue triggering glia and endothelial cell activation (Doyle et al., 2008, Jin et al., 2010b, Iadecola and Anrather, 2011). Activated glial cells release a number of pro-inflammatory cytokines including IL-1B, IL-6 and TNF-a, chemokines such as MCP-1, MIP1a and ROS (Jin et al., 2010b, Iadecola and Anrather, 2011). These factors induce the expression of adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) on the vascular endothelium and L-selectin, LFA-1 (CD11a/CD18), Mac-1(CD11b/CD18) on circulating leukocytes (Deb et al., 2010, Jin et al., 2010b, Iadecola and Anrather, 2011). The expression of these molecules facilitates the adhesion of leukocytes with the endothelium and their trans-endothelial migration into the brain. This leads to the accumulation of leukocytes, including neutrophil granulocytes, monocytes/macrophages and lymphocytes in the brain and the amplification of inflammatory responses (Jin et al., 2010b). The recruited immune cells produce more cytokines, chemokines, ROS and matrix metalloproteinases (MMPs). This causes further activation of resident cells and infiltration of immune cells into the brain, resulting in the disruption of the blood brain barrier (BBB),

brain edema and neuronal death (Figure 1.5) (Wang et al., 2007, Jin et al., 2010b, Iadecola and Anrather, 2011).

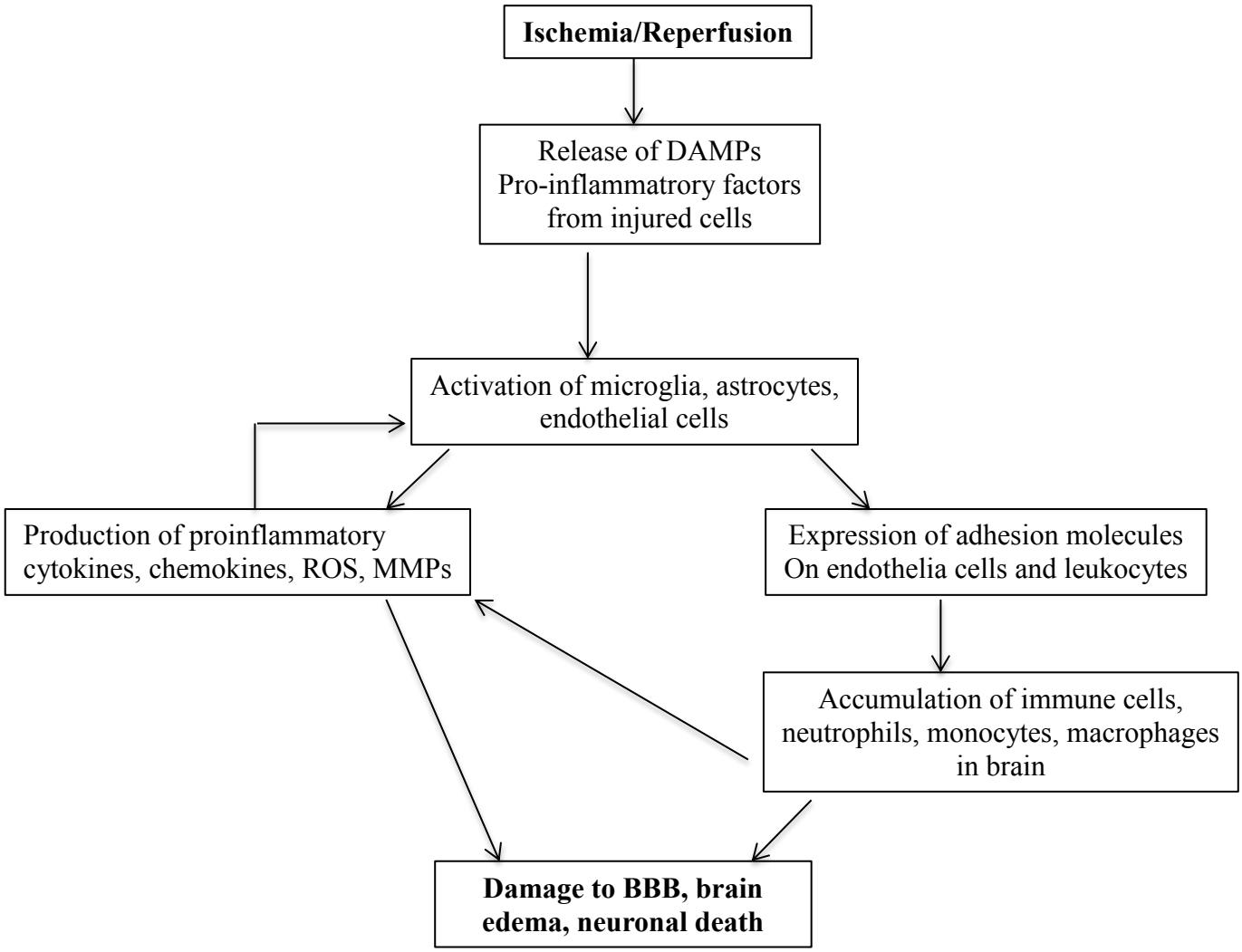
Ischemia/Reperfusion
Release of DAMPs
Pro-inflammatrory factors
from injured cells
Activation of microglia, astrocytes,
endothelial cells
Production of proinflammatory
cytokines, chemokines, ROS, MMPs
Expression of adhesion molecules
On endothelia cells and leukocytes
Accumulation of immune cells,
neutrophils, monocytes, macrophages
in brain
Damage to BBB, brain
edema, neuronal death

Figure 1.5

Figure 1.5. Overview of inflammatory response processes following cerebral ischemia/ reperfusion.

Although inflammation may facilitate tissue repair through the removal of cellular debris and induction of growth/neurotrophic factors, the general perception is that inflammation exacerbates tissue damage following cerebral ischemia (Deb et al., 2010, Iadecola and Anrather, 2011). In support of this, genetic and pharmacologic approaches that inhibit or dampen inflammatory responses have been shown to attenuate ischemic brain damage (Yrjanheikki et al., 1998, Yrjanheikki et al., 1999, Deb et al., 2010, Jin et al., 2010a, Yoon et al., 2013).

Inflammation may perpetuate and exacerbate cerebral ischemic tissue damage by promoting micro-vascular occlusion (Brouns and De Deyn, 2009). The induction of adhesion molecules promotes the accumulation of immune cells such as neutrophils in the microvasculature. This can cause microvascular occlusion and further damage to the brain tissue. In line with this, strategies targeting the inhibition of adhesion molecules and immune cell infiltration are protective following cerebral ischemia (Chopp et al., 1994, Zhang et al., 1995, Kitagawa et al., 1998, Soriano et al., 1999).

Additional tissue damage may be caused by the noxious molecules secreted by activated glial and immune cells, resulting in the death of neurons and damage to the BBB (Brouns and De Deyn, 2009). Activated immune cells are known to produce ROS and, pro-inflammatory cytokines such as MMPs and TNF-α which can be deleterious to neurons (Wang et al., 2007, Deb et al., 2010, Jin et al., 2010b). As previously discussed, ROS can damage DNA, lipids and proteins resulting in cell death. Similarly, TNF-α can directly activate the cell extrinsic apoptosis pathway. Likewise, the release of MMPs can lead to the breakdown of the BBB, resulting in the leakage of blood components into the brain and edema. The activation of MMPs, including MMP-2 and MMP-9, has been linked to increase

BBB permeability following stroke (Rosenberg et al., 1996, Rosenberg et al., 1998, Fujimura et al., 1999a, Gasche et al., 1999, Rosenberg, 2002, Brouns and De Deyn, 2009).

1.4.5. ISCHEMIC DEATH SIGNALS

The preceding sections highlighted some of the pathological processes and signals contributing to cerebral ischemic damage. A plethora of potential death mediators have been suggested to participate in ischemic neuronal damage. Although a great deal is now known about some of these molecular signals, not all have been thoroughly investigated. One such signal involves the activation of cyclin-dependent kinases (Cdks) and in particular those involved in the regulation of cell division.

Prior to the studies presented in this dissertation, a body of correlative evidence suggested the involvement of Cdks in ischemic neuronal injury. However, evidence for their functional involvement as mediators of ischemic neuronal death remained to be delineated. Moreover, it was unclear which member of the Cdk family could function as an ischemic death mediator. Accordingly, the studies presented in this thesis (chapters 2, 3 and 4) were designed to address this gap in knowledge and to further our understanding by investigating the molecular mechanism by which mitotic-Cdks are activated and signals cell death in cerebral ischemia. In addition, in chapter 5, the potential benefit of a Cdk inhibition-based combinatorial treatment strategy is evaluated in a global cerebral ischemia model.

The remainder of this chapter is dedicated to the review of Cdks, their more prominent roles, and their implication in ischemic neuronal death.

1.5. CYCLIN-DEPENDENT KINASES (CDKS)

The mammalian cyclin-dependent kinases are a family of structurally related serine/threonine kinases with homology to the yeast (*Saccharomyces pombe*) cell division cycle 2 (Cdc2) and Cdc28 in the *Saccharomyces cerevisiae* (Malumbres and Barbacid, 2005, Lolli, 2010). Cdks catalyze the transfer of ϒ-phosphate of ATP to protein substrates. They typically catalyze the phosphorylation of serine (S) or threonine (T) in the following context: [S/T*]PX[K/R], where [S/T*] designates the phosphorylated serine or threonine, P is proline, X is any amino acid, K is lysine and R is arginine (Brown et al., 1999, Ubersax et al., 2003).

The first mammalian Cdk, Cdk1 was identified based on its ability to complement the yeast cell division mutant. Since this first discovery, the mammalian Cdk family has grown to include twenty (Cdk1-20, Table 1.2) related proteins (Malumbres and Barbacid, 2005, Gopinathan et al., 2011). Cdks have been reported to play a role in diverse cellular function ranging from cell cycle regulation, basal gene transcription, cell differentiation, neuronal development and synaptic transmission (Gopinathan et al., 2011) (See Table 1.2).

Table 1.2.

Cdk	Synonym	Cyclin/activating partner	Cyclin binding motif	Function
Cdk1	Cdc2, Cdc28	A1, A2, B1, B2	PSTAIRE	Cell cycle (G2-M)
Cdk2		A1, A2, E1, E2	PSTAIRE	Cell cycle (G1-S)
Cdk3		C, E1, E2, A1, A2	PSTAIRE	Cell cycle (G0-G1-S)
Cdk4	PSK-J3	D1, D2, D3	PISTVRE	Cell cycle (G1-S)
Cdk5		p35, p39	PSSALRE	Senescence, postmitotic neurons
Cdk6		D1, D2, D3	PLSTIRE	Cell cycle (G1-S)
Cdk7	MO15, CAK, STK1	H	NRTALRE	Cdk activating kinase, transcription
Cdk8	K35	C	SMSACRE	Transcription
Cdk9	CdcL4	T1, T2, K	PITALRE	Transcription
Cdk10		Unknown	PISSLRE	Transcription, cell cycle (G2-M)
Cdk11	Cdc2L1, Cdc2L2	L1, L2	PITSLRE	Transcription, cell cycle (M)
Cdk12	CRK7, CrkRS, CD2L7	L1, L2	PITAIRE	Transcription and RNA splicing
Cdk13	CHED, Cdc2L5	L1, L2	PITAIRE	Cholinergic signaling, and hematopoietic cell proliferation, transcription, RNA splicing
Cdk14	PFTAIRE1, Pftk1	D3, Y	PFTAIRE	Testis and brain-specific,G1-Sphase transition, Wnt signaling in mitosis
Cdk15	PFTAIRE2	Unknown	PFTAIRE	Unknown
Cdk16	PCTAIRE1, Pctk1, Crk5	Unknown	PCTAIRE	Cell cycle (S-G2), neurite out-growth
Cdk17	PCTAIRE2, Pctk2	Unknown	PCTAIRE	Neuron-specific
Cdk18	PCTAIRE3, Pctk3	K	PCTAIRE	Brain-specific, transcription
Cdk19	CDC2L6, CDKL8	C	SMSACRE	Transcriptional regulation,G0-S phase transition
Cdk20	CCRK,CAKp42, p42 CDCH	Unknown	PNQALRE	Cell cycle regulation

Table 1.2. **Known mammalian Cdks, their activating partners and functions.**

Adapted and modified with permission (Malumbres and Barbacid, 2005, Gopinathan et al., 2011).

In some cases, the cellular function(s) of some Cdk family members are still in their infancy and evolving, others like Cdk15 remain unknown.

In general, the active Cdk is a heterodimeric complex composed of regulatory (cyclin) and catalytic (Cdk) subunits. Cyclins, named for their oscillating pattern of expression during the cell cycle, are a large family of related proteins that share a conserved stretch of 100 amino acid sequence known as the cyclin box (Morgan, 1997). The monomeric Cdk is inactive and requires the binding of a cyclin to function. (Morgan, 1997). However, not all Cdks bind a cyclin for function or have a direct role in the cell cycle (Malumbres and Barbacid, 2005, Gopinathan et al., 2011).

1.5.1. STRUCTURE OF CDKS

The structural information on Cdks is derived primarily from the detailed analysis of the human Cdk2 crystal structure. Cdk2 is considered to be representative of the Cdk family. Studies on the structure of Cdk2 revealed that Cdks are composed of a small amino terminal, composed of five anti-parallel β-strands and a single α-helix and a large mainly α-helical carboxy-terminal lobe, within which lies the ATP binding/active-site clef (De Bondt et al., 1993, Morgan, 1995, Bartova et al., 2004). The protein substrate binding site is located at the entrance of the clef but is blocked in the monomeric Cdk2 by a large flexible loop (the T-loop) arising from the carboxy-terminal lobe (De Bondt et al., 1993). The T-loop contains several amino acid residues that prevent access to the ϒ-phosphate of ATP. A short conserved helical segment (αL12) in the T-loop displaces the N-terminal helix, known as the PSTAIRE or C-helix, and prevents activation (Morgan, 1995, 1997, Albers et al., 2002). The PSTAIRE motif is important for cyclin binding and is conserved among Cdks. However,

there are variations in the sequence among Cdks (Table 1.2) (Gopinathan et al., 2011). The overall conformation of the monomeric inactive Cdk is such that the substrate binding site is inaccessible and key residues in the active site are incorrectly oriented preventing kinase reaction. This feature is thought to distinguish Cdks from other serine/threonine kinases, in that it necessitates extensive structural changes for activity. These changes are conferred by the binding of cyclin and phosphorylation of Cdk on activating threonine in the T-loop (Morgan, 1997).

Another important feature of Cdks is the presence of a glycine-rich loop known as the G-loop at the ATP binding site near the amino terminus. The G-loop contains two inhibitory sites: a semi conserved (threonine) T14 and (tyrosine) Y15 (Cdk2: 11-GEG**TY**G) that results in the loss of kinase activity when phosphorylated (Hanks and Quinn, 1991, De Bondt et al., 1993).

1.5.2. REGULATION OF CDKS

Most of what is known about how Cdks are regulated stems from the analysis of crystal structures and biochemical studies on cyclinA/Cdk2 complexes. The activity of Cdks is intricately regulated by three main mechanisms: binding of cyclins, phosphorylation and dephosphorylation of key amino acid residues, and by the interactions with endogenous protein inhibitors.

1.5.2.i. Activation

In general the activation of Cdks is mediated by a) the binding of a cognate cyclin b) the phosphorylation of conserved threonine in the activating T-loop and c) Cdc25 mediated dephosphorylation of inhibitory threonine and/or tyrosine in the glycine rich G-loop.

1.5.2.I.A. BINDING OF CYCLINS AND OTHER PROTEIN ACTIVATORS

The monomeric Cdk requires extensive structural modification for catalytic activity. This occurs primarily with the binding of a cognate cyclin. The binding of cyclin causes realignment of the Cdk active site and the movement of the T-loop relieving the blockade of the catalytic cleft. This helps to facilitate the binding of both ATP and the protein substrate. It also exposes the activating threonine (Thr.160 on Cdk2) making it a better substrate for phosphorylation (Jeffrey et al., 1995, Morris et al., 2002, Stevenson et al., 2002, Bartova et al., 2004). The binding of cyclin, for the most part confers a basal level of catalytic activity on Cdks. A noted exception to this is the cyclin D/Cdk4 complexes, which require phosphorylation on T172 for activity (Day et al., 2009, Takaki et al., 2009). In addition, the assembly and nuclear localization of cyclin D/Cdk4 complexes appears to require the presence of the Cdk interacting protein/Kinase inhibitory protein (CIP/KIP) family of proteins (endogenous Cdk inhibitors). For example, expression of CIP/KIP proteins (p21, p27 and p57) has been shown to promote the formation of cyclin D/Cdk4 complexes (LaBaer et al., 1997). In contrast, deficiency in p21 and p27 results in the lack of detectable amounts of cyclin D/Cdk4 complexes (Cheng et al., 1999). This additional regulatory step, at present, is thought to be unique to cyclin D/Cdk4 complexes.

The requirement of a cognate cyclin is not universal to all Cdk members. At least one Cdk member, Cdk5, is known to dispense with the obligate requirement of a cyclin for activation. Cdk5 activity is regulated by the binding of p35 and p39 (Malumbres and Barbacid, 2005).

In addition to activation by cyclins and in the case of Cdk5, p35/p39, a new class of proteins that can activate Cdks, in particular Cdk1, Cdk2 and Cdk5 has been reported

(Dinarina et al., 2005, Nebreda, 2006). This new class of proteins called Speedy or RINGO (for Rapid Inducer of G2/M progression in Oocytes) was first identified in *Xenopus laevis* as regulators of the meiotic cell cycle. The Speedy/RINGO proteins lack primary sequence homology with cyclins and five members, Speedy/Ringo A1 &A2, B, C, D & E, of this family have been identified in mammals (Ferby et al., 1999, Lenormand et al., 1999, Gopinathan et al., 2011). The human Speedy/RINGO A1 (spy1) facilitates Cdk2-mediated entry into S-phase in somatic cells (Porter et al., 2002). An interesting feature of this new class of Cdk activators is their ability to fully activate Cdk1 and Cdk2 in the absence of activating loop phosphorylation (Cheng et al., 2005). Additionally, Speedy/RINGO A1 can interact with both Cdk2 and its endogenous inhibitor to overcome $p27^{kip1}$-mediated block of S-phase in somatic cells (Porter et al., 2003). Thus far, Speedy/RINGO is known to only interact with and activate Cdk1, Cdk2 and Cdk5 but not Cdk4. However, another Cdk interacting protein, Trip-Br1 (also known as $p34^{SEI1}$) is known to bind and regulate Cdk4 activity (Sugimoto et al., 1999, Li et al., 2004a). Similar to the Speedy/RINGO A1 interaction with Cdk2, Trip-Br1 renders cyclin D/Cdk4 complexes resistant to the inhibitory effect of the Cdk inhibitor $p16^{INK4a}$ (Sugimoto et al., 1999, Li et al., 2004a).

1.5.2.I.B. ACTIVATING PHOSPHORYLATION BY THE CYCLIN-DEPENDENT KINASE ACTIVATING KINASE (CAK)

The phosphorylation of a conserved threonine (Cdk2-T160, Cdk1-T161, Cdk4-T172, Cdk6-T177 and Cdk7-T176) in the activating T-loop is another step in Cdk activation that is necessary to bring about its full catalytic activity. This process optimizes ATP alignment and the stability of Cdk substrate binding (De Bondt et al., 1993, Jeffrey et al., 1995, Schulze-Gahmen et al., 1995, Morgan, 1997, Bartova et al., 2004). The phosphorylation of the

activating threonine is catalyzed by cyclin-dependent kinase activating kinase (CAK), composed of Cdk7/cyclin H and MAT1. CAK-mediated threonine phosphorylation of Cdk1 and Cdk2 complexes results in drastic increase in Cdk catalytic activity (80-300 fold) (Desai et al., 1992, Gu et al., 1992, Connell-Crowley et al., 1993, Morgan, 1997). However, there is evidence to suggest that this general mechanism of CAK-dependent activation may not apply equally to all Cdks. Variations in phosphorylation mediated activation/enhancement of Cdk catalytic activity have been noted. For example, although Cdk5 catalytic activity is drastically increased by phosphorylation of Ser159 (equivalent to T160 on Cdk2), CAK is unable to phosphorylate or further activate Cdk5/p35 complexes (Poon et al., 1997, Sharma et al., 1999). The identity of the Cdk5 activating kinase *in vivo* is presently unknown. However, casein kinase I (CKI) can phosphorylate Cdk5 *in vitro* and may be a potential candidate (Sharma et al., 1999).

Cdk9 is another member of the Cdk family that appears to employ a CAK-independent activation mechanism. The Cdk9/cyclinT complex has been shown to undergo autophosphorylation of T186 for its activation (Baumli et al., 2008). Similarly, Cdk7/cyclin H has been shown to employ both CAK-dependent and independent activation mechanisms. Association of the co-factor MAT1 with Cdk7/cyclin H results in full Cdk7 catalytic activity in the absence of T170 phosphorylation by CAK (Fisher et al., 1995).

1.5.2.I.C. DEPHOSPHORYLATION OF INHIBITORY RESIDUES IN THE G-LOOP BY CDC25 PHOSPHATASES

A key step in Cdk activation appears to be the dephosphorylation of the inhibitory threonine and tyrosine residues in the G-loop (T14 and Y15 - Cdk1 & Cdk2, T17-Cdk4, T24-Cdk6). Phosphorylation of T14 and Y15 on Cdk2 causes steric hindrance that disrupts the

binding of substrates at the catalytic site (Endicott et al., 1999, Johnson and Lewis, 2001, Bartova et al., 2004). Dephosphorylation of these residues on mitotic Cdks is carried out by the Cdc25 dual specificity phosphatases.

The mammalian Cdc25 family is composed of three isoforms, Cdc25 A, B and C. The Cdc25 phosphatases are thought to act cooperatively to regulate normal cell division by performing the unique task of dephosphorylating threonine and tyrosine residues on mitotic Cdks. This task appears to be crucial to Cdk activation, at least among the core mitotic-Cdks (Cdk1, 2, 4 & 6). For example, when phosphorylated at T14/Y15, the T-loop phosphorylated cyclin/Cdk complex is still inactive (Sebastian et al., 1993, Rudolph et al., 2001). This suggests that Y15 /T14 exert a dominant effect over the T160 phosphorylated cyclin/Cdk complex. Thus Cdc25-mediated dephosphorylation is essential for the activation of mitotic-cyclin/Cdk complexes. In contrast, the phosphorylation of Y15 on Cdk5 appears to have a kinase activating, rather than inhibitory, effect on its activity (Zukerberg et al., 2000).

1.5.2.ii. Inhibition of Cdks

The activity of the Cdk complexes is negatively regulated via two main mechanisms. a) Through inhibitory phosphorylation of threonine/tyrosine in the G-loop. b) Via interaction with endogenous protein inhibitors.

1.5.2.II.A. INHIBITORY PHOSPHORYLATION

As already stated, Cdk activity is inhibited by the phosphorylation of conserved threonine and/or tyrosine residues in the G-loop. This phosphorylation of T14 and Y15 on Cdk1 and Cdk2 is catalyzed by Wee1 and Myt1 kinases (Obaya and Sedivy, 2002). However, the identity of the kinase(s) that catalyzes the equivalent site on other Cdks is

presently unknown. The phosphorylation of the G-loop threonine and tyrosine residues is an important mechanism of Cdk inactivation since it has the ability to render Cdks inactive even when bound to a cognate cyclin and phosphorylated on activating sites (Sebastian et al., 1993, Rudolph et al., 2001, Bartova et al., 2004).

1.5.2.II.B. BINDING OF ENDOGENOUS CYCLIN DEPENDENT KINASE INHIBITORS (CKIS)

Cyclin/Cdk complexes are also subject to negative regulation by two classes of cyclin dependent kinase inhibitors (CKIs), the CIP/KIP and the inhibitor of Cdk4 (INK4) family of CKIs. The CIP/KIP family is composed of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. This family of CKI broadly inhibits cyclin/Cdk complexes, including cyclin D, cyclin E and cyclin A-dependent kinases (Sherr and Roberts, 1999). The INK4 family is composed of p15, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$ proteins. The INK4 family specifically inhibits cyclin D containing complexes (cyclin D/Cdk4/Cdk6) (Coleman et al., 1997).

1.6. BIOLOGICAL FUNCTION OF CDKS

1.6.1. CDKS AND CELL CYCLE REGULATION

One the most prominent function of Cdks is in the regulation of the cell cycle. The cell cycle is a tightly regulated, ordered series of events that commence with the precise duplication of the cell genomic material and culminate in the production of two genetically identical daughter cells (Gopinathan et al., 2011). This is an integral part of development in multicellular organisms and as such must be strictly regulated in order to avoid genetic and developmental defects. The complete molecular details involved in cell division and regulation are still evolving and are likely to be complex. However, the task of cell cycle regulation is performed primarily by members of the Cdk family, in particular the mitotic-

Cdks. Presently, the core mitotic, or cell cycle Cdks include Cdks 1, 2, 4 and 6 and their cognate cyclins. Together these proteins form the mitotic engines that regulate the unidirectional progression of the cell cycle (Malumbres, 2011), (Figure 1.6).

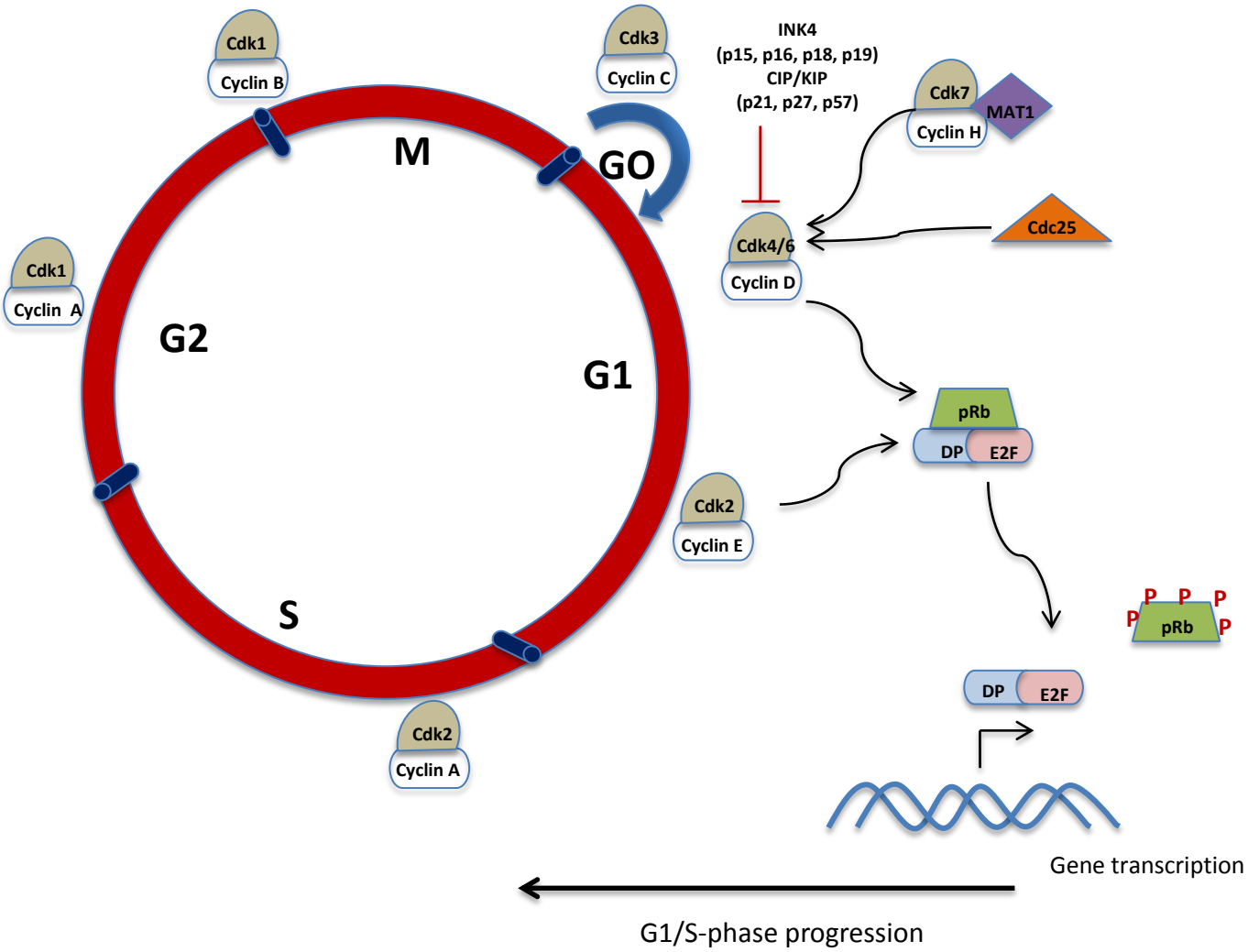
Cdk1
Cyclin B
Cdk3
Cyclin C
INK4
(p15, p16, p18, p19)
CIP/KIP
(p21, p27, p57)
Cdk7
MAT1
Cyclin H
M
GO
Cdk1
Cyclin A
G2
G1
Cdk4/6
Cyclin D
Cdc25
pRb
DP
E2F
Cdk2
Cyclin E
S
P
pRb
DP
E2F
Cdk2
Cyclin A
Gene transcription
G1/S-phase progression

Figure 1.6

Figure 1.6. General representation of the mammalian cell cycle.

The cell cycle is divided into four phases**,** a synthesis or S-phase and a mitotic or M-phase, each of which is preceded by a Gap/growth phase, designated G1 and G2 respectively. Each phase of the cell cycle is regulated by different cyclin/Cdk complexes. Cdk complexes are in turn regulated by positive and negative regulators and target members of the retinoblastoma protein.

1.6.1.i. Cell cycle regulation and the core mitotic Cdks

In the current scheme, the cell cycle is divided into two major periods of activity: a synthesis phase (S-phase) and a mitotic phase (M-phase) with two intervening gaps (Gap1 or G1 and Gap2 or G2). During the S-phase, the genomic content of the cell is duplicated. This is followed by mitosis, or the M-phase, a period where the newly duplicated genome is equally divided between the two daughter cells. Both the synthesis and mitotic phases are preceded by G1 and G2, respectively (Harper and Adams, 2001). G1 and G2, also referred to as the growth phases, are periods marked by high biosynthesis of proteins and enzymes required for the subsequent phases. These include the production of proteins required for DNA replication in the S-phase and microtubules in the M-phase (Figure 1.6) (Harper and Adams, 2001).

The phases of the cell cycle are regulated by different cyclin/Cdk complexes. Each Cdk bind a subset of cyclins and is sequentially and periodically activated during the different phases. During G1, the D-type cyclins are synthesized in response to mitogenic stimuli and form a complex with Cdk4/6 (Sherr and Roberts, 1999, Obaya and Sedivy, 2002, Malumbres, 2011). This complex is activated by CAK phosphorylation of T172 and the dephosphorylation of T17 by Cdc25. Activated Cdk4/6 complexes phosphorylate members of the retinoblastoma (Rb) tumor suppressor proteins, including pRb (p105), p107 and p130 (Malumbres and Barbacid, 2005, Gopinathan et al., 2011). Typically, hypo-phosphorylated retinoblastoma proteins associate with the E2F/DP transcription factors and inhibit their ability to activate target genes. Phosphorylation of Rb proteins by Cdk4/6 is thought to partially relieve this inhibition resulting in activation of E2F/DP complexes. Activated E2F/DP mediates the transcription of genes required for cell cycle progression. This includes the transcription of cyclin E, which accumulates and subsequently forms a complex with

Cdk2 in mid to late G1 (Dyson, 1998, Sherr and Roberts, 1999, Obaya and Sedivy, 2002). The cyclin E/Cdk2 complex is initially held inactive by its association with $p27^{KIP1}$. However, p27 becomes sequestered by the accumulating cyclin D/Cdk4 complexes, as part of their assembly process. This results in the activation of cyclin E/Cdk2 complexes, which then contributes to pRb phosphorylation. The hyper-phosphorylation of pRb results in more E2F/DP mediated transcription of target genes which includes cyclin A, cyclin B and Cdk1. Cyclin E/Cdk2 phosphorylates $p27^{KIP1}$, targeting it for ubiquitin mediated proteasome degradation and thus ensuring activation of more Cdk2 complexes (Harper and Adams, 2001). Cyclin E/Cdk2 also phosphorylates NPAT, a protein involved in the activation of histone gene transcription. At the G1/S-phase boundary, cyclin A becomes associated with Cdk2 and Cdk1. The activated cyclin A/Cdk2 complex phosphorylates proteins important for DNA replication and progression from S-phase. This includes, the phosphorylation of Cdc6, a protein required for the initiation of DNA replication. Phosphorylation of Cdc6 by cyclin A/Cdk2 causes its export out of the nucleus and thus helps to prevent re-replication of DNA (Obaya and Sedivy, 2002, Malumbres and Barbacid, 2005). This is important since DNA must only be replicated once per cell cycle. Another substrate of cyclin A/Cdk2 is HIRA, a protein that represses the transcription of histones. Phosphorylation relieves its repressor activity, resulting in histone transcription. Cyclin A/Cdk2 also phosphorylates E2F1, causing its inactivation and dissociation from DNA (Obaya and Sedivy, 2002, Malumbres and Barbacid, 2005). The G2/M-phase of the cell cycle is primarily regulated by the cyclin B/Cdk1 complex. The activation of cyclin B/Cdk1 is thought to trigger entry into M-phase and the phosphorylation of a number of substrates important in mitosis. These include structural and regulatory proteins such as microtubule–associated protein 4 (MAP4), dynein, tau, nuclear lamins and histones. The cyclin B/Cdk1 complex also phosphorylates cyclin B

resulting in its degradation and exit from mitosis. (Figure 1.6) (Obaya and Sedivy, 2002, Malumbres and Barbacid, 2005).

1.6.1.ii. Non-core mitotic Cdks and the cell cycle

In addition to the basic and generally accepted model discussed above, there is evidence to support the involvement of other Cdk family members in the regulation of the cell cycle. Cyclin C/Cdk3 complexes are activated during the G0-G1-phase of the cell cycle and contribute to pRb phosphorylation. Cyclin C/Cdk3 mediated pRb phosphorylation at S807/811 has been shown to be required for G0 exit and G1-phase cell cycle entry (Ren and Rollins, 2004). Expression of S807/S811 mutant pRb, that cannot be phosphorylated was shown to cause G0/G1-phase cell cycle arrest (Ren and Rollins, 2004), suggesting a role for cyclin C/Cdk3 in the regulation of G0/G1-phase regulation. Similarly, Cdk14 and Cdk20 are thought to contribute to G1-S-phase cell cycle regulation (Ng et al., 2007, Shu et al., 2007). siRNA mediated knockdown of Cdk14 causes G1 cell cycle arrest, while ectopic expression promotes proliferation. Cdk14 can also phosphorylate pRb (Shu et al., 2007). Cdk20 knockdown has also been shown to arrest cells in G1/S-phase. Its levels are elevated in high-grade glioblastoma multiforme (Ng et al., 2007).

As with the G0/G1/S-phase, the involvement of additional Cdk family members in G2-M-phase regulation has been suggested. In particular, Cdk10 and Cdk11 are implicated in G2/M-phase cell cycle regulation (Li et al., 1995, Li et al., 2004b). Expression of a dominant negative mutant or Cdk10 anti-sense has been shown to arrest cells at the G2/M-phase of the cell cycle (Li et al., 1995). Similarly, Cdk11 deficiency has been shown to lead to multiple mitotic defects (Li et al., 2004b). Cdk11 null mice die embryonically at the blastocyst stage due to mitotic arrest of the blastomere (Li et al., 2004b). Additionally,

siRNA mediated knockdown of Cdk11 leads to mitotic arrest caused by abnormalities in centrosome maturation, spindle structure and function, as well as, sister chromatid cohesion (Petretti et al., 2006, Hu et al., 2007, Malumbres, 2011). Overall Cdks are considered important molecular engines that regulate proper cell division.

1.6.1.iii. The atypical Cdk5 and the cell cycle

1.6.1.iiiA. Cdk5 and cell cycle progression

Cdk5, historically considered an atypical Cdk, may also be involved in cell cycle regulation. This recently discovered role of Cdk5 is still evolving, but adds a new dimension to the vast repertoire of functions already ascribed to this Cdk family member. Cdk5 was first identified based on its sequence homology to human Cdk1. It bears an ~60% sequence identity to human Cdk1 and Cdk2 and was initially called neuronal Cdc2-like kinase (NCLK), primarily because its expression and activity were enriched in nervous tissues (Hellmich et al., 1992, Lew et al., 1992a, Lew et al., 1992b, Meyerson et al., 1992). Cdk5 was considered unique among Cdks because initial studies revealed that unlike the prototypic Cdk, it was not activated by a cyclin and does not require an activating phosphorylation for activity. Another unusual feature of Cdk5 was that it did not appear to have a role in cell cycle regulation. In fact, earlier studies on Cdk5 pointed to a predominantly neuronal function (Hellmich et al., 1992, Lew et al., 1992a, Lew et al., 1992b). Recent studies have not only demonstrated that Cdk5 can bind and be activated by cyclins, but also that its activity is not limited to tissues of the nervous system. Cdk5 has been shown to be activated by at least two cyclins, cyclin G and cyclin I. The latter is considered an atypical cyclin because its levels do not appear to oscillate during the cell cycle (Brinkkoetter et al., 2009).

Although Cdk5 activity is discernibly high in neurons, its activity has been detected in non-neuronal cells including pancreatic β-cells, endothelial cells, adipocytes, epithelial cells, monocytes, myocytes and leukocytes among others (Arif, 2012). Accordingly, the roles of Cdk5 have grown to include: extra-neuronal functions that include angiogenesis, insulin secretion, hematopeitic cell differentiation, regeneration, wound healing, adhesion and migration, senescence, gene expression and apoptosis (Mao and Hinds, 2010, Arif, 2012). Interestingly, one of the proposed extra-neuronal functions of Cdk5 is in the regulation of the cell cycle. This proposed new role is contrary to the earlier held view of Cdk5 and is supported in part by studies in cancer biology. Cdk5 has been shown to be upregulated in several forms of cancers: including lung cancer, colorectal cancer, head/neck cancer, ovarian cancer, lymphoma, myeloma and sarcoma amongst others (Levacque et al., 2012). Importantly, the cyclin G1/Cdk5 complex had been shown to contribute to cell proliferation in lung cancer, by phosphorylating and stabilizing c-Myc resulting in the induction of cyclin B1 (Seo et al., 2008). Cdk5 has also been reported to be associated with the midbody structure in HeLa cells. The midbody structure is associated with the completion of cytokinesis (Lee et al., 2010). Interestingly, Cdk5 null fibroblasts are defective in cytokinesis, resulting in aneuploidy (Lee et al., 2010). These reports suggest, at least in part, that Cdk5 may play a role in the regulation of the cell cycle and proliferation.

1.6.1.IIIB. CDK5 AND NEURONAL CELL CYCLE SUPPRESSION.

While the above reports suggest a role for Cdk5 in cell proliferation, other reports implicates Cdk5 in the suppression of the cell cycle and the maintenance of a post-mitotic state in neurons. Initial indications of a potential cell cycle suppressive role for Cdk5 came from studies of Cdk5-/- mice. Analysis of these mice showed that Cdk5 deficiency leads to

the loss of neuronal cell cycle control and failure to differentiate *in vitro* and *in vivo* (Cicero and Herrup, 2005). Cdk5-/- neurons continued to express immature neuronal and cell proliferation phenotype that was rescued by the re-expression of Cdk5 (Cicero and Herrup, 2005). This suggested a Cdk5 specific effect on cell cycle suppression.

Subsequent studies into the potential mechanism by which Cdk5 exerted a repressive effect on cell cycle in post-mitotic neurons revealed that it acts by forming a complex with E2F1 in the nucleus (Zhang et al., 2010b). Cdk5 binding to E2F1 in the nucleus was found to displace its transcriptional co-activator DP1, preventing the activation of cell cycle genes (Zhang et al., 2010b). In this scheme, the nuclear retention of Cdk5 was found to be a key determinant in the suppression of cell cycle in neurons (Zhang et al., 2008). The localization of Cdk5 to the nucleus was facilitated by its interaction with p27. Molecular manipulations that prevented this interaction lead to the loss of nuclear Cdk5 (Zhang et al., 2010a). Once in the nucleus, Cdk5 forms a p35-dependent complex with E2F1 (Zhang et al., 2010b). In these studies, the loss of nuclear Cdk5, stimulated by treatment with the β-Amyloid peptides, promoted cell cycle re-entry of neurons (Zhang et al., 2008, Zhang et al., 2010a). These studies suggest that Cdk5 is required for cell cycle suppression in the post-mitotic neuron. Intriguingly, this cell cycle suppression function of Cdk5 was found to be independent of its kinase activity (Zhang et al., 2010b).

1.6.2. CDKS AND GENE TRANSCRIPTION.

Cdks also play a notable role in the regulation of gene transcription and RNA processing. A number of Cdks including Cdk7-11 and Cdk19 are associated with transcriptional control. Others including Cdk11-13 have been linked to RNA processing. The CAK enzyme formed by Cdk7/cyclin H/MAT1 is a subunit of the general transcription factor

TFIIH, involved in promoter clearance and progression of transcription from the pre-initiation to the initiation stage (Lolli and Johnson, 2005). Cdk7 phosphorylates the C-terminal domain (CTD) of RNA polymerase II (RNAPII). Interestingly, free CAK (not associated with TFIIH) is unable to phosphorylate the CTD of RNAPII. Similarly, CAK associated with TFIIH is ineffective at phosphorylating Cdk2. Thus, CAK plays two distinct roles in the regulation of the cell cycle and gene transcription (Lolli and Johnson, 2005).

Cdk8/cyclin C and Cdk9/cyclin T are also involved in the regulation of transcription. Cdk8/cyclin C forms a component of the RNA polymerase II holoenzyme complex. Cdk8/cyclin C phosphorylates the CTD of RNAPII, as well as cyclin H in TFIIH, inhibiting its kinase activity and gene transcription (Akoulitchev et al., 2000).

The positive transcription elongation factor P-TEFb is composed of Cdk9 in association with cyclin T or cyclin K. P-TEFb phosphorylates the CTD of RNAPII, the negative elongation factors, DRB Sensitivity Inducing Factor (DSIF) and Negative Elongation Factor (NELF), to promote the transition of elongation from abortive to productive transcription (Marshall and Price, 1995, Loyer et al., 2005, Peterlin and Price, 2006).

Cdk10 has been shown to interact with and inhibit the transactivation of the Ets2 transcription factor. Ets2 is a member of the Ets family of transcription factors and a regulator of Cdk1 expression (Kasten and Giordano, 2001, Malumbres and Barbacid, 2005). Similarly, Cdk11/cyclin L has been proposed to be involved in transcriptional regulation. An isoform of Cdk11, $Cdk11^{P110}$ interacts with RNAPII and the transcription factors TFIIF and TFIIS (Loyer et al., 2005). Importantly, antibody directed against the catalytic domain of $Cdk11^{P110}$ reduces the production of RNA transcripts from TATA-like and GC-rich promoters (Loyer et al., 2005).

Lastly, Cdk19 is a component of the mediator complex, a multi-protein transcription co-activator (Sato et al., 2004, Gopinathan et al., 2011). In addition to their involvement with transcriptional control, Cdks -11, -12 and -13 are known to interact with splicing factors and are thus implicated in RNA processing as well (Berke et al., 2001, Ko et al., 2001b, Dickinson et al., 2002, Berro et al., 2008, Gopinathan et al., 2011).

1.6.3. CDKS AND NEURONAL FUNCTION.

The most prominent Cdk with neuronal function is Cdk5, and as already discussed, Cdk5 activity was first detected in neurons and more recently in other cell types (Arif, 2012). Some of the earliest indications of Cdk5 neuronal functions came from observations in cortical neuronal cultures, demonstrating a requirement for Cdk5/p35 kinase activity for neurite outgrowth (Nikolic et al., 1996). In these early experiments, Nikolic and colleagues showed that the expression of dominant negative Cdk5 inhibited neurite outgrowth. This phenotype was rescued by the co-expression of the wildtype protein, thus demonstrating a Cdk5 kinase-specific function in neurite outgrowth (Nikolic et al., 1996). Subsequent studies in the Cdk5, p35 and p39 knock-out mice would later underscore the importance of Cdk5 in CNS cytoarchitecture and corticogenesis. Mice null for Cdk5 were shown to exhibit dysfunction in neuronal layering of various brain structures including the cortex, hippocampus, olfactory bulb and the cerebellum, resulting in death *in utero* or within 12 hours of birth (Ohshima et al., 1996). Similar to the Cdk5 null mice, p35 deficient animals were also shown to display severe defects in cortical lamination, with mild dysfunction in the hippocampus and dentate gyrus (Chae et al., 1997). Unlike the Cdk5 mice, p35 mice are viable but have an increased susceptibility to seizures (Chae et al., 1997). This milder phenotype of p35 mice is attributed to the presence of another Cdk5 activator, p39 in

neurons. Indeed, p39 null mice are grossly normal and have no apparent neuronal dysfunction (Ko et al., 2001a). However, the compound p35/p39 double null mice exhibit a neuronal phenotype that is indistinguishable from that of Cdk5 null animals (Ko et al., 2001a). Cumulatively, these observations suggested an important role for Cdk5 activity in neuronal development.

Further studies in the Cdk5 null (Gilmore et al., 1998) and conditional knock-out (Hirasawa et al., 2004, Hirota et al., 2007, Jessberger et al., 2008) mice have revealed that it is important in neuronal migration. Conditional deletion of Cdk5 in perinatal mice results in migration defects in the affected neuronal population (Hirasawa et al., 2004). Similar observations have been made in migrating neuroblasts and newly generated adult hippocampal neurons in the conditional Cdk5 knock-out (Hirota et al., 2007) and knock-down brains respectively (Jessberger et al., 2008). In addition to these reports, a role for Cdk5 has been described in a wide variety of neuronal processes, including synaptic transmission and plasticity (Li et al., 2001, Hawasli et al., 2007, Hawasli et al., 2009), neurogenesis (Lagace et al., 2008), dopamine signaling and drug addiction (Bibb et al., 1999, Bibb et al., 2001, Takahashi et al., 2005, Benavides et al., 2007), axonal guidance (Kwon et al., 1999), neuronal differentiation (Li et al., 2007, Zheng et al., 2010), neuronal cell cycle suppression (Cicero and Herrup, 2005) and neuronal survival (Li et al., 2002, Li et al., 2003, Cheung and Ip, 2004, Zheng et al., 2007, Cheung et al., 2008). A role for Cdk5 has also been described in a number of neuropathological conditions. This will be discussed further in a subsequent section of this thesis.

A number of the recently identified Cdks, including Cdk14, Cdk16, Cdk17 and Cdk18 have been proposed to have neuronal functions. Very little is known about these new Cdk members at the present time. It is interesting to note that these Cdks, similar to Cdk5,

exhibit high expression in the brain. Cdk14 has been suggested to play a role in neuronal differentiation and function (Besset et al., 1998, Yang and Chen, 2001). However, studies into its neuronal functions are lacking. Cdk16 is implicated in the development of dendrites (Cole, 2009). Its suppression has been shown to inhibit dendritic outgrowth during development and maintenance at later stages (Fu et al., 2011). In contrast, expression of Cdk16 has been shown to induce neurite outgrowth in Neuro-2A cells (Graeser et al., 2002).

The protein expression of Cdk17 is restricted to brain tissue and is highest in pyramidal cells of the cerebral cortex. However, its possible neuronal functions are also presently unknown (Hirose et al., 1997, Cole, 2009). Cdk18 is upregulated in *post mortem* human Alzheimer's disease brains and may be indirectly involved in Tau hyperphosphorylation at Thr231 and Ser235 (Herskovits and Davies, 2006). Co-transfection of Cdk18 and Tau in cell cultures was found to result in the hyperphosphorylation of Tau. Interestingly, expression of kinase-dead mutants of Cdk18 were found to exert a similar effects on Tau phosphorylation (Herskovits and Davies, 2006). The implications of Cdk18 upregulation in AD brain and its physiologic relevance in the non-diseased brain are not well understood.

1.7. CDKS AND NEURONAL DEATH

In addition to their involvement in the already described physiologic processes, a new, more pathogenic, role for Cdks is emerging. A role for Cdks has been described in numerous neuronal death paradigms *in vitro* (Freeman et al., 1994, Park et al., 1997b, Copani et al., 1999, Rideout et al., 2003, Schwartz et al., 2007) and *in vivo* (Patrick et al., 1999, Nguyen et al., 2001, Bu et al., 2002, Smith et al., 2003a, Luo et al., 2005) including models

of stroke (Osuga et al., 2000, Wang et al., 2002). The latter forms the basis of this thesis and will therefore be discussed in depth in subsequent sections.

1.7.1. CELL CYCLE MACHINERY DURING NEURONAL DIFFERENTIATION

A long held view is that terminally differentiated neurons are post-mitotic and do not divide. As neurons differentiate and become matured, a key step in the differentiation process is a permanent exit from the cell cycle. The cell cycle machinery is actively downregulated (Kranenburg et al., 1995), degraded (Azuma-Hara et al., 1999), sequestered or re-distributed to different cellular compartments (Sumrejkanchanakij et al., 2003, Sumrejkanchanakij et al., 2006). For example, upon the induction of differentiation, key cell cycle proteins are downregulated in N1E-115 neuroblastoma (Kranenburg et al., 1995), PC12 (Dobashi et al., 1995) and cortical progenitor cells (Sumrejkanchanakij et al., 2003) *in vitro*. During differentiation, the protein levels of cyclin D1, cyclin A, Cdk2, Cdk1, Cdk4, E2f1 and proliferating cell nuclear antigen (PCNA) are reduced (Dobashi et al., 1995, Kranenburg et al., 1995, Azuma-Hara et al., 1999, Sumrejkanchanakij et al., 2003, Sumrejkanchanakij et al., 2006). In neuroblastoma cells, the kinase activity of Cdk2 and Cdk4 are diminished resulting in an increase in hypophosphorylated pRb and the appearance of pRb/E2F complexes upon differentiation (Kranenburg et al., 1995). Although the levels of some cell cycle proteins remain detectable in differentiated neurons, studies indicate that these proteins are re-distributed to a different cellular compartment or become associated with inhibiting proteins (Kranenburg et al., 1995, Sumrejkanchanakij et al., 2003, Sumrejkanchanakij et al., 2006). For example, cyclin D1 is localized to both the nucleus and cytoplasm of undifferentiated neuroblastoma (Sumrejkanchanakij et al., 2006) and cortical progenitor (Sumrejkanchanakij et al., 2003) cells. However, upon differentiation cyclin D1

becomes predominantly localized to the cytoplasm (Sumrejkanchanakij et al., 2003, Sumrejkanchanakij et al., 2006). Interestingly, ectopically expressed cyclin D1 also localizes to the cytoplasm in post-mitotic neurons (Sumrejkanchanakij et al., 2003), suggesting that nuclear exclusion of cyclin D1 is important in the maintenance of a post-mitotic state. In a similar fashion, Cdk4 is re-localized to the cytoplasm in differentiated neurons (Sumrejkanchanakij et al., 2003) and becomes associated with the endogenous CKI $p27^{KIP1}$ (Kranenburg et al., 1995, Sumrejkanchanakij et al., 2003). Recent studies suggest that the E2F1 cell cycle promoting activity is also held in check in terminally differentiated neurons, at least in part through its association with Cdk5/p35 (Zhang et al., 2010b) (discussed in section 1.6.1.iiib). The cumulative effect of these cellular activities is the active repression of the cell cycle machinery and the maintenance of a post-mitotic state.

Factors that hinder cell cycle suppression have been shown to inhibit or delay neuronal differentiation. Over-expression of Cdc2 or Cdk2 inhibits nerve growth factor (NGF) induced differentiation of PC12 (Dobashi et al., 1995, Dobashi et al., 2000). Conversely, factors that promote cell cycle repression induce neuronal differentiation. Pharmacologic or anti-sense mediated inhibition of Cdc2 and Cdk2 induces neuronal differentiation of PC12 (Dobashi et al., 2000). Furthermore, over-expression of the CKI $p57^{KIP2}$ in E14.5 to E15.5 mice was shown to promote cell cycle exit and neuronal differentiation of cortical progenitor cells (Tury et al., 2011). Thus the silencing of the cell cycle machinery is an important component of neuronal differentiation.

1.7.2. Cell Cycle Machinery in Matured Neuron and Non-pathologic Neuronal Death

In the matured, terminally differentiated neuron, the cell cycle machinery must also be suppressed. Factors hindering this process result in cell cycle re-entry and death instead of proliferation. The best evidence of this comes from studies of forced cell cycle re-entry in matured neurons, using simian virus 40 large T-antigen (SV40 T-ag). SV40 Tag is a powerful oncogene that induces tumorigenic transformation in proliferating cells by binding the retinoblastoma tumor suppressor proteins (DeCaprio et al., 1988). This subverts the need for Cdk mediated inactivation of pRb, resulting in the activation of E2F, cell cycle progression and proliferation. When cell cycle induction is forced in different neuronal populations using the SV40 T-ag, it causes cell death instead of proliferation (al-Ubaidi et al., 1992b, Hammang et al., 1993). In contrast, if cell cycle induction is forced at a developmental point prior to terminal mitosis and differentiation, tumors instead of neuronal death are observed (al-Ubaidi et al., 1992a, Howes et al., 1994). Al-Ubaidi *et al* showed that postnatal expression of the simian virus large T-ag driven by the opsin promoter in the rod photoreceptors led to their degeneration instead of hyperplasia or tumors (al-Ubaidi et al., 1992b). However, when the opsin promoter is replaced by the regulatory element, interstitial retinol-binding protein (IRBP) driving embryonic expression of T-ag, transgenic mice developed retinal and brain tumors (al-Ubaidi et al., 1992a). This suggests an incompatibility of cell cycle activation in matured terminally differentiated neurons. This notion is supported by a number of other key observations including those by Fedderson *et al*, which showed that SV40 T-ag driven cell cycle activation in post-mitotic cerebellar Purkinje cells led to neuronal loss and ataxia in mice (Feddersen et al., 1992). This was accompanied by BrdU (bromo-deoxyuridine) incorporation and increase in TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining, markers for cell cycle progression and apoptosis respectively, in post-mitotic Purkinje neurons (Feddersen et al., 1995). This

neuronal loss in the SV40 T-ag transgenic mice was shown to be pRb dependent. In contrast to mice expressing wild type T-ag, the expression of a mutant T-ag that lacked the ability to bind pRb, failed to induce the death of Purkinje cells (Feddersen et al., 1995). Consistent with these observations, Rb germ-line knock-out mice were shown to exhibit ectopic mitosis in normally post-mitotic neuronal populations and widespread death in the CNS (Clarke et al., 1992, Jacks et al., 1992, Lee et al., 1992). These mice also displayed defects in neurogenesis, hematopoiesis and died before embryonic day 16 (E16) (Clarke et al., 1992, Jacks et al., 1992, Lee et al., 1992). Cell death in the CNS of the Rb-/- mouse was later shown to be p53 dependent, while the ectopic proliferation phenotype was attributed to an increase in E2F1 activity (Macleod et al., 1996). Accordingly, E2F1 deficiency was shown to partially rescue cell proliferation defects and apoptosis in the Rb/E2F1 double mutant embryos (Tsai et al., 1998). Likewise, E2F3 mutation was also shown to rescue ectopic proliferation and p53-dependent apoptosis in the Rb-/- mouse (Ziebold et al., 2001). In contrast, over-expression of E2F1 was found to accelerate T-ag-induced ataxia and Purkinje cell loss in T-ag/E2F1 double transgenic mice (Athanasiou et al., 1998). Collectively, these studies suggested that: 1) The induction of cell cycle machinery in post-mitotic neurons leads to death and 2) that the loss of cell cycle control in differentiating neurons leads to death that is mediated in parts via pRb inactivation. This latter point was later challenged by the studies on conditional pRb knock-out mice.

Studies in the conditional Rb-/- mice later revealed that most of the phenotypes observed in the whole embryo Rb-/- mice were due to defects in placental development and erythropoiesis (de Bruin et al., 2003, Wu et al., 2003). Indeed, when the placental defect is corrected, Rb-/- mutant mice survived until birth and showed normal nervous system and hematopoietic development. Although these mice displayed ectopic cell division in the CNS,

they lacked the massive apoptotic phenotype seen in the whole embryo Rb-/- mice. These findings were corroborated by other studies employing chimeric Rb mice(Lipinski et al., 2001, de Bruin et al., 2003, Wu et al., 2003) and conditional deletion of Rb in different neuronal populations (Ferguson et al., 2002, MacPherson et al., 2003, Wu et al., 2003). These observations in the conditional Rb knock-out mice suggested that ectopic cell cycle activation, per se, does not cause neuronal death, at least in the context of neuronal development and differentiation. However, studies employing other approaches *in vitro* continued to show that expression of various cell cycle proteins in matured, neuronal cultures led to cell death and not proliferation (Azuma-Hara et al., 1999, O'Hare et al., 2000, Sumrejkanchanakij et al., 2003).

1.7.3. THE CELL CYCLE MACHINERY AND PATHOLOGIC NEURONAL DEATH.

The studies described above provided evidence that cycle suppression in the post-mitotic neuron is essential to its survival. A more pathogenic view of cell cycle activation emerged when it was shown that under certain stress conditions post-mitotic neurons upregulated cell cycle proteins. Numerous studies reported the upregulation of various components of the cell cycle machinery, in dying neurons exposed to different stressors. Cyclin D1 message was selectively induced in sympathetic neurons undergoing NGF withdrawal-induced death (Freeman et al., 1994). Likewise, cyclin D1 and Cdk4 protein levels, as well as kinase activity, increased following serum withdrawal of PC12 (Dobashi et al., 1998) and terminally differentiated neuroblastoma cells (Kranenburg et al., 1996). Similar observations were made in other models of neuronal death, induced by KCl withdrawal (Padmanabhan et al., 1999, Martin-Romero et al., 2000), genotoxic (Park et al., 1997b, Park et al., 2000a) and excitotoxic stimuli (Park et al., 2000b). These reports were

also extended to other cell culture models of neuronal death, induced by β-amyloid toxicity (Copani et al., 1999), proteosomal inhibition (Rideout et al., 2003) and oxidative stress (Schwartz et al., 2007). Additionally, the protein levels of the Cdk inhibitor $p27^{KIP1}$ were found to be downregulated following KCl withdrawal (Padmanabhan et al., 1999, Martin-Romero et al., 2000). The total level of pRb was found to be diminished, while an increase was observed in the phosphorylated form (Padmanabhan et al., 1999). Collectively, these observations suggested a potential association between cell cycle activation and pathologic neuronal death *in vitro*. Further credence was lent to these observations when various studies reported evidence of cell cycle activation in neurons undergoing pathologic death *in vivo*. Cell cycle activation has been described in animal models of AD (Park et al., 2007), PD (Hoglinger et al., 2007), Huntington's disease (HD) (Pelegri et al., 2008), ALS (Nguyen et al., 2003) and stroke (Katchanov et al., 2001). Importantly, the expression of cell cycle proteins have also been described in the *post mortem* human brain tissues from these diseases (Ranganathan et al., 2001, Jordan-Sciutto et al., 2003, Pelegri et al., 2008), including stroke (Love, 2003). The evidence implicating cell cycle machinery in ischemic neuronal death is discussed in detail in the following section.

1.8. MITOTIC CDKS IN ISCHEMIC NEURONAL DEATH

With regard to stroke, several lines of *in vitro* and *in vivo* evidence suggested a potential role for cell cycle machinery in ischemic neuronal death. As with the studies described in the preceding section, the upregulation of cell cycle proteins was observed in various *in vitro* and *in vivo* models of ischemic neuronal death. Increase in cyclin D1 protein and activation of Cdk2 were observed following OGD of cortical neurons *in vitro* (Katchanov et al., 2001). Similarly, increase in cyclin D1, cyclin E and Cdk2 levels were

reported in CGNs exposed to kainic acid (Verdaguer et al., 2002). In addition to these reports, the loss of the Cdk inhibitors, p27 and p21, were also reported following OGD (Katchanov et al., 2001) and hypoxia (Bossenmeyer-Pourie et al., 2002) in primary neuronal cultures *in vitro*.

These observations have also been recapitulated in various animal models of ischemic neuronal death *in vivo*. Induction of cyclin D1 protein levels have been reported in focal (Guegan et al., 1997, Li et al., 1997c, Li et al., 1998, Osuga et al., 2000, Katchanov et al., 2001, Wen et al., 2005), global (Li et al., 1997a, Timsit et al., 1999) and toxin-induced (Liu et al., 1996, Timsit et al., 1999, Park et al., 2000b, Ino and Chiba, 2001) models of ischemia in the rodent. Similarly, increases in Cdk4 protein levels have been reported following focal ischemia (Li et al., 1997c, Osuga et al., 2000) and kainic acid-induced toxicity (Ino and Chiba, 2001) in the rat. Induction of Cdk2 protein levels and activity has been observed following hypoxia/ischemia (Kuan et al., 2004) and MCAO (Li et al., 1997b, Katchanov et al., 2001). Interestingly, elevated levels of cyclin D1, Cdk2 and Cdk4 proteins have been demonstrated in the *post mortem* brain tissues of patients who died as a result of cardiac arrest or focal ischemia (Love, 2003).

While the preponderance of the evidence indicated that the G1/S-phase cell cycle proteins are aberrantly induced in cerebral ischemia, the expression of other regulators such as cyclin H (a component of the CAK enzyme), have also been reported (Jin et al., 1999b). Interestingly, endogenous Cdk inhibitors appear to be downregulated following ischemic insults. $p16^{INK4A}$ and $p27^{KIP1}$ are downregulated following mild focal ischemia (Katchanov et al., 2001) and hypoxia/ischemia insult (Kuan et al., 2004). In contrast, $P21^{WAF1/CIP1}$ mRNA levels are upregulated in surviving neurons following ischemic insults (van Lookeren

Campagne and Gill, 1998a, b, Tomasevic et al., 1999), suggesting possible compensation to cell cycle activation in these cells.

Collectively, these observations suggest the possible induction of the cell cycle in ischemic neurons. However, the expression of cell cycle proteins alone may not necessarily reflect bona fide cell cycle activation or progression. In this regard, increased incorporation of BrdU, the upregulation of Ki67 and PCNA expression have been reported in models of ischemia, *in vitro* (Bossenmeyer-Pourie et al., 1999, Bossenmeyer-Pourie et al., 2002, Verdaguer et al., 2002, Verdaguer et al., 2004) and *in vivo* (Tomasevic et al., 1998, Kuan et al., 2004, Wen et al., 2005). While these observations strongly indicate cell cycle activation, an important question is whether this signals death in ischemic neurons. To this end, a number of studies demonstrated that pharmacologic blockade of the cell cycle protected neurons from ischemia-induced death *in vitro* (Bossenmeyer-Pourie et al., 1999, Katchanov et al., 2001) and *in vivo* (Osuga et al., 2000, Wang et al., 2002). Treatment with the general Cdk inhibitor olomoucine prevented hypoxia (Bossenmeyer-Pourie et al., 1999) and OGD induced death of neurons *in vitro* (Katchanov et al., 2001). Similarly, infusion of flavopiridol, another Cdk inhibitor attenuated neuronal death induced by focal (Osuga et al., 2000) and global cerebral ischemia (Wang et al., 2002). Taken together, these studies suggested a potential involvement of cell cycle activation in ischemic neuronal death. Under this context, it was hypothesized that the inappropriate activation of the cell cycle machinery in adult neurons following ischemic insult leads to death. While this hypothesis presented a fascinating notion, the supporting evidence was grounded mainly in correlative observations and the use of non-specific, pharmacologic Cdk inhibitors. Functional evidence supporting the involvement of the cell cycle machinery in ischemic neuronal death was lacking.

1.9. CDK5 IN ISCHEMIC NEURONAL DEATH

Although the use of pharmacological Cdk inhibitors provided some evidence for the involvement of cell cycle machinery in ischemic neuronal damage, it also presented some concerns. Cdk inhibitors, such as flavopiridol, lack specificity and can inhibit multiple Cdks Flavopiridol is known to inhibit multiple mitotic Cdks (Cdk1, 2, 4 & 6), transcriptional Cdks (Cdk7 and Cdk9), and the neuronal Cdk5 (Blagosklonny, 2004, Newcomb, 2004). Additionally, flavopiridol is known to modulate other cellular signals including NFκB (Newcomb, 2004), an important factor in neuronal death and survival (Aleyasin et al., 2004). These concerns were made significant by studies implicating Cdk5 in many forms of neuronal death including DNA damage, excitotoxicity (O'Hare et al., 2005), oxidative stress (Zambrano et al., 2004), and Aβ toxicity *in vitro* (Liu et al., 2004b). Cdk5 is also implicated in animal models of neuropathology including ALS (Nguyen et al., 2001), Alzheimer's disease (Patrick et al., 1999), Parkinson's disease (Smith et al., 2003a), Huntington's disease (Luo et al., 2005) and Niemann-Pick disease (Bu et al., 2002). Importantly, Cdk5 is implicated in models of ischemic neuronal death (Nath et al., 2000, Wang et al., 2003, Weishaupt et al., 2003).

With regard to cerebral ischemia, increase in Cdk5 protein levels and that of its activator p35, have been reported following MCAO (Hayashi et al., 1999). Similarly, induction of Cdk5 kinase activity is observed in the post-decapitation model of global ischemia (Green et al., 1997). The conversion of p35 to p25, a more stable Cdk5 activator, under pathologic stress conditions is well documented (Patrick et al., 1999, Nguyen et al., 2001, Bu et al., 2002). Although controversial, the accumulation of p25 is believed to be pathogenic because it promotes constitutive activation and dysregulation of Cdk5 (Patrick et

al., 1999). In line with this view, increased levels of p25 and Cdk5 activity are observed following focal (Nath et al., 2000, Weishaupt et al., 2003) and global ischemia (Wang et al., 2003), as well as glutamate exposure of cortical neurons *in vitro* (Lee et al., 2000). Taken together, these observations implicate Cdk5 in the ischemic neuronal death. Accordingly, it has been hypothesized under this context that the dysregulation of Cdk5, a neuronal Cdk, rather than cell cycle regulators, leads to ischemic neuronal death.

1.10. STATEMENT OF RESEARCH PROBLEM, RATIONALE, OBJECTIVES AND HYPOTHESIS

It is difficult to ascertain, from the evidence presented in the preceding sections, whether mitotic-Cdks are functionally sufficient to mediate ischemic neuronal death. First, the evidence for their involvement is mainly correlative. Importantly, the pleiotropic nature of drugs such as flavopiridol, prevents the unambiguous determination of which Cdk family member is important in mediating ischemic neuronal death. Specifically, it is unclear from these studies, whether the attenuation of ischemic neuronal death results from the inhibition of mitotic-Cdks or Cdk5. Second, if mitotic-Cdks are involved in ischemic neuronal death, it is unclear how they are activated. As discussed, an essential part of mitotic-Cdk activation is the dephosphorylation of the inhibitory tyrosine and threonine by Cdc25. However there is no evidence for the involvement of Cdc25 in cerebral ischemic damage. Third, if cell cycle regulators are involved in ischemic neuronal damage, the molecular mechanism by which they signal death and their downstream effectors are unclear.

Accordingly, the studies presented in this thesis were designed with the goal of elucidating the role of the cell cycle machinery in ischemic neuronal death. ***It is my hypothesis that cell cycle signal(s), activated following ischemic insult, functionally contribute to ischemic neuronal death.*** To this end a number of primary research objectives have been established. They are as follows:

Objective 1:

Determine the role of the mitotic Cdk2, Cdk4 and the neuronal, Cdk5 in ischemic neuronal death. This objective is presented in chapter 2 of the thesis.

Objective 2:

Determine the potential involvement of upstream activators of mitotic-Cdks in ischemic neuronal death. This objective is presented in chapter 3.

Objective 3:

Determine the potential downstream effectors of cell cycle-induced death signaling in ischemic neurons. This objective is presented in chapters 2 and 4.

Objective 4:

Evaluate the potential benefit of a treatment strategy that combines Cdk inhibition with the inhibition of inflammatory processes. This objective is presented in chapter 5.

CHAPTER 2

Multiple cyclin-dependent kinases signals are critical mediators of ischemia/hypoxic neuronal death in vitro and in vivo.

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Proceedings of the National Academy of Sciences USA, (2005), 102(39): 14080-5

STATEMENT OF AUTHOR CONTRIBUTION

In this first manuscript we investigate the functional role of the cell cycle regulators cyclin D1, cdk2, cdk4 as well as the neuronal cdk5 in neuronal death induced by ischemic stresses. Using multiple ischemic models in vitro and in vivo, this manuscript defines a context for the different CDK family members.

This manuscript was published as co-first author with J Rashidian, a then senior Ph.D student in the lab. G Iyirhiaro conducted and generated all the results in figures 2.1f, 2.1g, 2.1h, 2.1i, 2.2b, 2.2d, 2.2e and 2.4d. J Rashidian generated the results in all other figures and wrote the manuscript with assistance from G Iyirhiaro. H Aleyasin generated the DNCdk4 transgenic mice. M Rios provided technical expertise and training. The p35 mice were generously provided by I Vincent's lab. S Callaghan generated all the AV used in this manuscript. AAVs used in this manuscript were prepared and provided by RJ Bland and MJ During. RS Slack provided technical support and review of the manuscript. DS Park provided scientific oversight and editorial guidance on the entire project and manuscript.

Multiple cyclin-dependent kinases signals are critical mediators of ischemia/hypoxic neuronal death *in vitro* and *in vivo*

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Key Words: Hypoxia, Stroke

Acknowledgements:

We thank Dr. Barbara Vanderhyden for production of transgenic mice, Dr. Jean-Pierre Julien for providing NES promoter and Michael O'Hare for reading of the manuscript. This work was supported by grants from Canadian Institutes of Health Research, Heart and Stroke Foundation of Ontario, the Canadian Stroke Network, The Centre for Brain Recovery, (DSP) and National Institute of Health (NIH AG12721 to IV).

ABSTRACT

The mechanisms involving neuronal death after ischemic/hypoxic insult are complex, involving both rapid (excitotoxic) and delayed (apoptotic-like) processes. Recent evidence suggests that cell cycle regulators such as cyclin-dependent kinases are abnormally activated in neuropathological conditions, including stroke. However, the function of this activation is unclear. Here, we provide evidence that inhibition of the cell cycle regulator, Cdk4, and its activator, cyclin D1, plays critical roles in the delayed death component of ischemic/hypoxic stress by regulating the tumor suppressor retinoblastoma protein. In contrast, the excitotoxic component of ischemia/hypoxia is predominately regulated by Cdk5 and its activator p35, components of a cyclin-dependent kinase complex associated with neuronal development. Hence, our data both characterize the functional significance of the cell cycle Cdk4 and neuronal Cdk5 signals as well as define the pathways and circumstances by which they act to control ischemic/hypoxic damage.

INTRODUCTION

The mechanisms involved in ischemic neuronal death are complex and depend upon multiple factors, including severity and duration of insult. In the core of the infarct, a relatively rapid excitatory death occurs within minutes to a few hours (Dirnagl et al., 1999) . This type of neuronal death occurs after energy failure and Ca^{2+} overload. Numerous Ca^{2+}-mediated enzymes such as calpains are activated and participate in the neuronal loss. The region surrounding this core infarct area, the penumbra, experiences less intense ischemia and displays a more delayed type of cell death with characteristics of apoptosis (Dirnagl et al., 1999). The signaling pathways that regulate both rapid and delayed ischemia are not fully defined.

Cyclin-dependent kinases (CDKs) are a large group of Ser/Thr kinases that are best characterized for their role in cell cycle progression. In this regard, distinct kinase members, along with their cognate cyclin-activating partners, regulate different phases of the cell cycle. Of relevance to the present work, cyclin D/Cdk4 and cyclin E/Cdk2 complexes regulate G_1/S transition, partly by phosphorylating and inactivating the tumor suppressor retinoblastoma protein (Rb). Consequently, Rb is released from the transcription factor, E2F. E2F then activates genes required for S phase progression (Ekholm and Reed, 2000).

In addition to this crucial role in cell cycle regulation, CDK members have also been implicated in other fundamental biological processes, including transcription and neuronal function (Gold and Rice, 1998). As an example of the latter, Cdk5 is selectively active in neurons and, together with its non-cyclin activators, p35 and p39, regulates numerous neuronal processes (Dhavan and Tsai, 2001).

Growing evidence suggests that multiple CDK members may also participate in neuronal death. In general, two important hypotheses have emerged. The first describes a paradoxical situation by which inappropriate activation of cell cycle-related CDKs in terminally differentiated neurons leads to death instead of proliferation (Copani et al., 2001). In support of this, correlative evidence demonstrating activation/up-regulation of cell cycle components has been reported in a number of neuronal death paradigms, including stroke. For instance, increased cyclin D1 expression, down-regulation of p^{16ink4}, and phosphorylation of Rb have been reported in multiple in vivo stroke paradigms (Timsit et al., 1999, Osuga et al., 2000, Katchanov et al., 2001, Wang et al., 2002). However, no studies have yet conclusively shown that cell cycle CDKs are critical functionally for neuronal death in adult models of injury. The question of whether inappropriate cell cycle signals are required for death in neuronal injury or whether they may be an epiphenomenon of diseased neurons remains unresolved.

A second hypothesis proposes that deregulated Cdk5 activity can also induce neuronal damage. In this case, one model states that calpain proteases cleave the p35 to a smaller more stable and mislocalized p25 form. This, in turn, converts Cdk5 into a death inducer. Such inappropriate activation of Cdk5 has been reported in neuronal death induced by a variety of insults, including stroke. Pertinent to the latter, Wang et al. (Wang et al., 2003) showed that accumulation of p25 after transient forebrain ischemia activates Cdk5 and induces CA1 cell death.

Interestingly, we have shown that administration of flavopiridol, a general CDK inhibitor, is protective in both focal (Osuga et al., 2000) and global (Wang et al., 2002)

ischemia. However, flavopiridol inhibits both cell cycle CDKs and Cdk5 (De Azevedo et al., 1996, Smith et al., 2003a) as well as non-CDK-related kinases such as GSK-3β (Leclerc et al., 2001). Accordingly, the role of specific CDKs in stroke-induced damage remains unknown.

Taken together, the above observations highlight the following questions: (i) Are specific cell cycle CDKs important in an adult in vivo model of neuronal death such as stroke? (ii) If so, how might these CDKs regulate death after ischemic/hypoxic/excitotoxic insult? and (iii) Under what conditions do cell cycle CDKs or Cdk5 participate in neuronal death after ischemia/hypoxia/excitotoxicity? To answer these questions, we have examined the role of cell cycle CDKs and Cdk5 in ischemic/hypoxic models of delayed and excitotoxic death both in vitro and in vivo.

MATERIALS AND METHODS

Viral Construction. Recombinant adeno-associated virus (rAAV1) vectors were constructed by subcloning cDNA sequences (XbaI fragment) of DNCdk2, 4 (van den Heuvel and Harlow, 1993, Park et al., 1997a) and 5 (Gong et al., 2003, Smith et al., 2003a) into the SpeI sites of the AM/CBA-pl-WPRE-bGH plasmid. The virus was then generated and purified as described (Zolotukhin et al., 2002). For adenovirus (AV) construction, the same sequences were subcloned into the pAdTrack vector under a cytomegalovirus (CMV) promoter. The construct also contains a second CMV promoter that separately controls expression of GFP. The construct was then used to generate recombinant AV, as described(He et al., 1998). The AV containing the ΔK11 Rb mutant was generated, as described (Park et al., 2000a).

Transgenic Mice/Knockouts. All animal experiments conformed to the guidelines set forth by the Canadian Council for the Use and Care of Animals in Research and the Canadian Institutes for Health Research.

Dominant Negative Cdk4 Transgenic Mice. Mice expressing DNCdk4 were generated by using a fusion construct composed of a full length human Cdk4 harboring a D158R mutation (see Supporting Text 2.1).

Cyclin D1 Null Mice. Cyclin D1 heterozygous breeding pairs were commercially obtained from The Jackson Laboratory on a mixed C57BL/6 × 129S2 background.

P35 Null Mice. P35 null mice have been characterized by Hallows et al. (Hallows et al., 2003). Pups from heterozygous breeding were screened by PCR as described.

Cell Culture. Cerebellar granule neuron (CGN) cultures were prepared from 7- to 9-day postnatal mice, as described (O'Hare et al., 2000).

Hypoxia. Hypoxia was induced by using a humidified environmental chamber (Coy Laboratory Products, Ann Arbor, MI) set at 37°C, 1% O_2, and 5% CO_2. Five-day plated CGNs were infected with recombinant AV expressing DNCdk2/4/5, ΔK11 Rb mutant, or GFP by itself as control with a multiplicity of infection of 40. For a more delayed model of death, cultures were incubated in the chamber on day 7 for 16–18h in the presence of the NMDA blocker, MK801 (10μM, Research Biochemicals, Natick, MA) and then reoxygenated at 37°C. Control plates contained MK801 but were not exposed to hypoxia. All cultures were fixed (4% paraformaldehyde) at times 12 and 24h after reoxygenation, then stained with Hoechst 33342 (Sigma), and GFP-positive cells were evaluated for nuclear integrity [analyses of dominant negative CDKs (DNCDKs)]. Nuclei from dying neurons showed severe condensation or fragmentation. For analyses of the effects of the ΔK11 Rb mutant, cultures were first fixed and analyzed for Rb overexpression by using anti-Rb Ab (BD PharMingen). Because this vector did not express GFP, Rb-positive neurons were evaluated for survival as above. Random fields of infected neurons were evaluated for live vs. dead neurons. Data are presented as percentage live/dead ± SEM.

For a more excitotoxic death paradigm, infected cultures were incubated in the hypoxic chamber in the absence of MK801 for 5h and then reoxygenated for 1h. Cultures were then fixed as above and stained for Hoechst. The total number of live GFP-positive neurons per well was evaluated and compared with the number of GFP-positive live neurons in control non hypoxia-induced wells. This analysis was performed for each virus.

Expression of the DNCDKs was confirmed by anti-Cdk2/4/5 Abs (Santa Cruz Biotechnology).

Alternatively, neurons from transgenic mice (see above) were used instead of viruses. Both delayed and excitotoxic models were performed, as described above, and cultures were evaluated by lysing the neurons in each well with a lysis buffer that disrupts cells but leaves healthy nuclei intact. Nuclei that displayed characteristics of blebbing and disruption of nuclear membrane were excluded (O'Hare et al., 2000). Data are expressed relative to untreated controls ± SEM.

Glutamate Model of Neuronal Death. Five-day plated CGNs were infected with AV, carrying DNCdk2/4/5 or GFP by itself as control, as described above. On day 7, glutamate was added to the wells to a final concentration of 50μM for 70min and then washed off with conditioned medium and incubated for 2h. This was performed in the presence or absence of MK801 (10μM). Survival was evaluated as described above for the hypoxia (-MK801) death model.

Viral Injection in Vivo. All in vivo studies were performed in male Wistar rats weighing 80–100g. DNCDKs or GFP control were unilaterally (survival studies) or bilaterally (behavioral studies) delivered by injecting rAAV1 vector 2 weeks before induction of global ischemia or injection of endothelin-1. rAAV1 was diluted by mixing 2μl of virus stock (10^{10} genomes per microliter) with 1μl of 20% mannitol in PBS and was administered by a pump (Harvard infusion pump, Harvard Apparatus) into the hippocampus (from bregma: -3.6mm anterioposterior, ± 2.1mm lateral, -2.75mm deep) or striatum (from bregma: +0.9mm anterioposterior, +2.8mm lateral, -5.8mm deep) over a 30-min period, as described (Wang et

al., 2002).

Global Ischemia Model. Hippocampal rAAV1-injected rats, weighing 180–220g, were induced via transient global ischemia [four-vessel occlusion (4VO)], as described (Wang et al., 2002). Brains were collected 4 days after 4VO surgery, sectioned, stained for hematoxylin/eosin (He et al., 1998), and quantified, as described (Wang et al., 2002).

Focal Ischemia Model (Endothelin Injection). Striatal rAAV1-injected rats were subjected to endothelin injection. Endothelin-1 (400pM; Calbiochem) was dissolved in 1μl of H_2O and injected over a period of 3min into the viral-injected striatal region, as described (Biernaskie and Corbett, 2001). Brains were collected 4 days after injection, and coronal sections of the striatum were collected as described (Smith et al., 2003a) and stained with cresyl violet. The infarct volume was measured on each slice by a microcomputer-based image display system (Imaging Research, St. Catharine's, ON, Canada) by using the method described by Swanson et al. (Swanson et al., 1990).

Immunohistochemistry. Coronal sections (14μm) were obtained at the level of middorsal hippocampus or striatum from global or focal ischemia-induced brains, respectively (Smith et al., 2003a). Expression of GFP was shown by using GFP fluorescence, and expression of DNCdk2 was analyzed by using anti-Cdk2 Ab (Santa Cruz Biotechnology).

Western Blot Analyses. For analyses of DNCDKs expression in the hippocampus or striatum, a 2-mm punch was obtained and analyzed by Western blot, as described (Smith et al., 2003a). Membranes were probed with anti-Cdk2/4/5 (Santa Cruz Biotechnology) or anti-flag (Sigma) Abs. Actin was used as loading control (Sigma). Rb phosphorylation was

determined in vivo from nuclear proteins extracted from hippocampal extracts, as described (Wang et al., 2002), by using antiphospho Rb-Ser-795 or –Ser-807/811 Abs (Cell Signaling Technology, Beverly, MA), or anti-Rb Abs (BD PharMingen). For Western blot analyses using cultured neurons, CGNs were harvested at the appropriate times by methods previously described (O'Hare et al., 2000).

Morris Water Maze Test. In this test, animals are screened for their ability to find a hidden platform in a pool of milky water by using fixed visual clues, as described (Wang et al., 2002) (see Supporting Text 2.2).

RESULTS

Cdk4 and CyclinD1 as Mediators of Delayed Neuronal Death Induced by Nonexcitotoxic Hypoxic Insult in Vitro.

To test the importance of individual CDKs in delayed models of ischemic death, we used an in vitro model of death where neuronal loss occurs in the presence of the NMDA blocker MK801 (Figure 2.1). CGNs were infected with a GFP-containing AV expressing kinase-dead dominant negative mutants of the G_1-related CDKs (DNCdk2/4), the neuronal CDK, DNCdk5, or an empty viral control. Cultures were then subjected to hypoxia in the presence of MK801, and GFP-expressing neurons were assessed for nuclear integrity. Dead cells displayed condensed and/or fragmented nuclei, whereas healthy nuclei were intact and did not show any signs of condensation. As shown in Figure 2.1e, neurons exposed to hypoxia expressing DNCdk4 showed 65% survival vs. 39% survival in GFP-expressing controls. Expression of DNCdk2 and DNCdk5, however, did not show any significant protection when compared with GFP expression alone. These data indicate that Cdk4 plays an important role in delayed ischemic death, whereas the role of Cdk2 or -5 is less central.

To confirm that Cdk4 may be a critical mediator of delayed death, we generated transgenic mice expressing flag-tagged DNCdk4 under a neuron-specific enolase promoter. PCR analyses showed incorporation of the transgene and expression the DNCdk4 construct in a variety of regions, including in CGNs (Figure 2.1g and 2.1h). These mice were grossly normal and did not display any major identifiable abnormalities in brain development (data not shown). Consistent with the viral data described above, CGNs from DNcdk4 transgenic mice were more resistant to hypoxia and showed 96% survival vs. 41% survival in WT

controls, in the presence of MK801, after 24 h of reoxygenation (Figure 2.1f). The expression of DNCdk4 was confirmed in these neuronal cultures by Western blot. Taken together, these results suggest the importance of Cdk4 in hypoxia-induced delayed death and suggest that the protective effects observed were not due to a viral delivery artifact.

Cyclin D proteins are required activators of Cdk4 (Pines, 1993). To further confirm that Cdk4 plays an important role in delayed hypoxic death, we cultured CGNs from cyclin D1-deficient mice and littermate controls. As shown in Figure 2.1i, cyclin D1-deficient mice were much more resistant to hypoxia in the presence of MK801 than controls. In contrast, and consistent with the lack of protective effects of DNcdk5 in this model, neurons cultured from p35-deficient animals were not resistant to delayed death (+MK801) induced by hypoxia (Figure 2.6, supporting information). Taken together, the above results suggest that Cdk4 activity is functionally important in delayed death, because inhibition of Cdk4 or deletion of its activator cyclin D1 is protective. In contrast, Cdk2 or -5 appears to play a minimal functional role under these conditions.

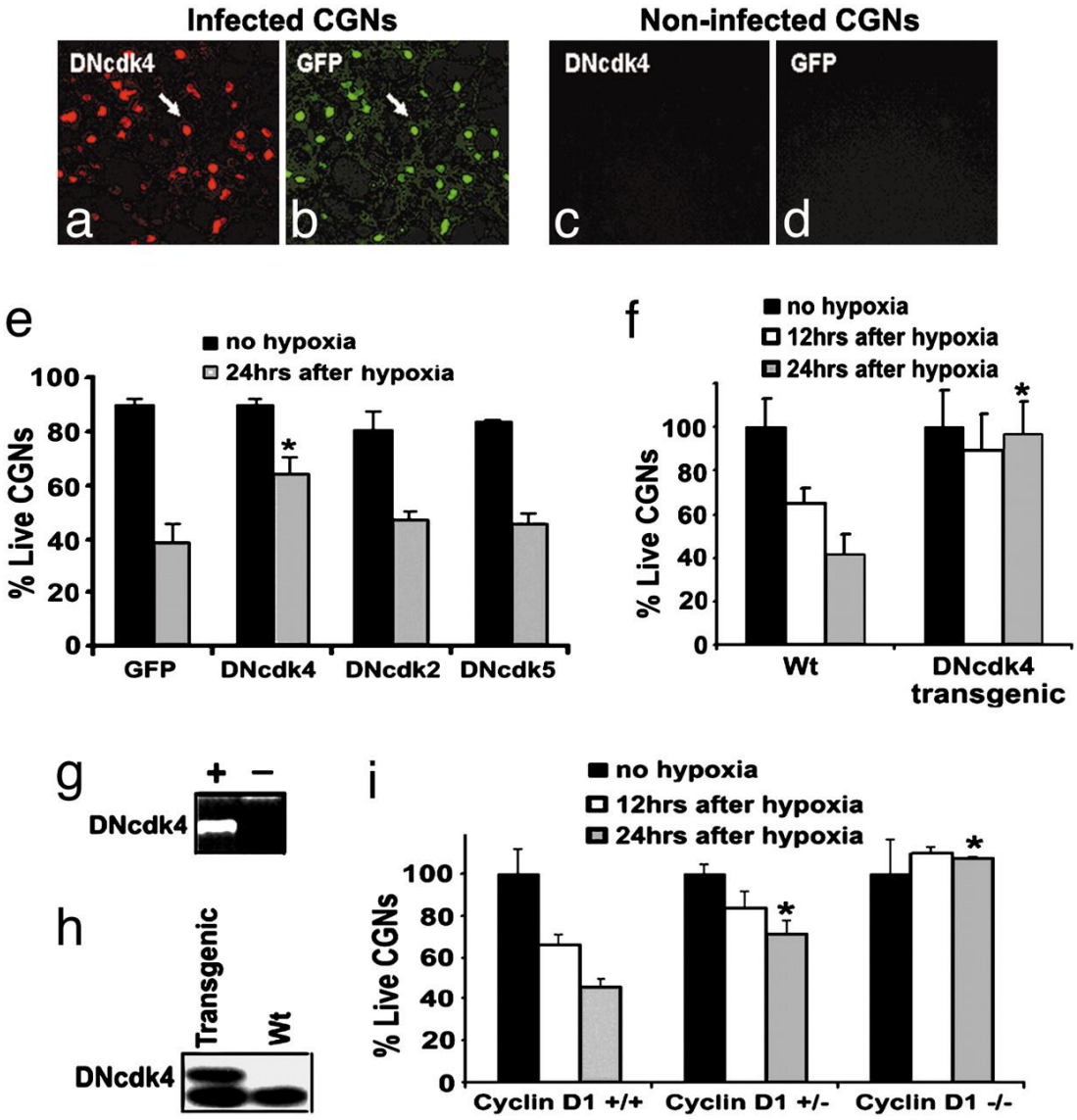
Infected CGNs
Non-infected CGNs
DNcdk4
GFP
DNcdk4
GFP
a
b
c
d
e
no hypoxia
24hrs after hypoxia
% Live CGNs
100
80
60
40
20
0
*
GFP
DNcdk4
DNcdk2
DNcdk5
f
no hypoxia
12hrs after hypoxia
24hrs after hypoxia
% Live CGNs
100
80
60
40
20
0
*
Wt
DNcdk4 transgenic
g
+
–
DNcdk4
h
Transgenic
Wt
DNcdk4
i
no hypoxia
12hrs after hypoxia
24hrs after hypoxia
% Live CGNs
100
80
60
40
20
0
*
*
Cyclin D1 +/+
Cyclin D1 +/-
Cyclin D1 -/-

Figure 2.1

Figure 2.1. Delayed ischemic neuronal death in vitro is mediated by Cdk4 and cyclin D1. (a–d) Expression of DNCDKs in CGN cultures. Coexpression of (a) DNCdk4 was detected by using an anti-Cdk4 Ab, whereas expression of (b) GFP was detected by using fluorescence in the same culture. DNCdk2/5-infected cells showed similar results (data not shown). (c and d) Immunofluorescence of noninfected cultures visualized as in a and b as negative controls. (e) Quantitation of survival after 16–18 h of hypoxia followed by 24 h of reoxygenation, in the presence of MK801 (n = 3). (f) CGNs from transgenic mice expressing DNCdk4 are resistant to hypoxia in the presence of MK801. Experiments were performed as in e (n = 3). (g) PCR for the presence of the DNCdk4 transgene in (+) transgenic mice and (-) littermate controls. (h) Western blot showing expression of DNCdk4 in CGNs from transgenic mice compared with WT controls using an anti-Cdk4 Ab. (i) CGNs from cyclin D1-deficient mice are resistant to hypoxia in the presence of MK801, as described in e (n = 3). The data are mean ± SEM. * denotes significance ($P < 0.05$, t test)

Cdk5 as Mediator of Excitotoxic Neuronal Death in Vitro.

We next determined whether cell cycle CDKs and/or Cdk5 participate in more rapid excitotoxic death. To test this, we examined whether DNCdk2/4/5 is protective in models of hypoxia where death is induced in the absence of MK801 (Figure 2.2a and 2.2b). Alternatively, we also examined whether direct glutamate-induced death depends upon these CDKs (Figure 2.2c-e). In contrast to the delayed hypoxic model described above, Cdk5 appears to play a predominant role in excitotoxic death when compared with Cdk4 and Cdk2. As shown in Figure 2.2a, viral-mediated DNCdk5 expression blocked death induced by hypoxia (-MK801). DNcdk5-expressing cells showed significantly more survival compared with GFP-expressing controls in this model. The lack of protection by DNCdk4 was also confirmed by using CGNs from DNCdk4 transgenic mice (Figure 2.2b). Similar results were obtained by using a direct model of excitotoxicity by glutamate exposure (Figure 2.2c), where DNCdk5 was more efficient in promoting survival than DNcdk4 (70% survival in DNCdk5 expressing neurons vs. 49% survival in GFP-expressing controls). Because p35 is an important activator of Cdk5, we also asked whether CGNs from p35-deficient mice were resistant to excitotoxic death. As shown in Figure 2.2d, CGNs cultured from p35-deficient mice were significantly more resistant to glutamate-induced death when compared with littermate controls (81% vs. 50%). Similar results were obtained with p35 heterozygous neurons after hypoxia (-MK801) when compared with WT controls (data not shown). Moreover, consistent with the weak protective effects of DNcdk4, cyclin D1 deficiency was not protective after glutamate exposure when compared with littermate controls (data not shown). Interestingly, neurons from DNCdk4 transgenic mice were slightly resistant to glutamate exposure when compared with littermate controls (Figure 2.2e), suggesting that in select cases of excitotoxicity, Cdk4 may also have a role. However, this role is minor in

comparison with that of Cdk5. Taken together, these results suggest that in excitotoxic death, Cdk5 plays a central role, whereas the cell cycle CDK, Cdk4, is more significant in delayed modes of death.

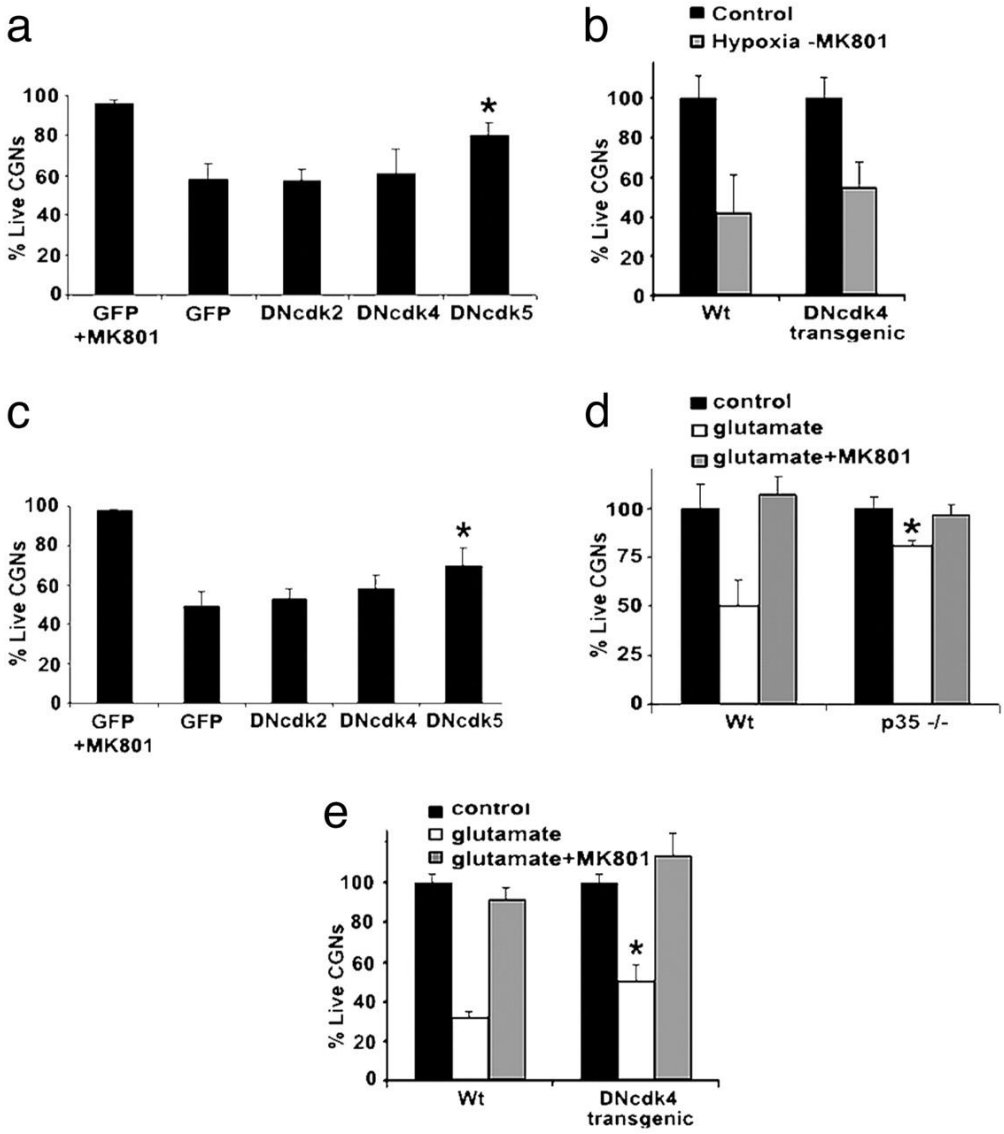
a
% Live CGNs
100
80
60
40
20
0
*
GFP +MK801
GFP
DNcdk2
DNcdk4
DNcdk5
b
Control
Hypoxia -MK801
% Live CGNs
Wt
DNcdk4 transgenic
c
% Live CGNs
GFP +MK801
GFP
DNcdk2
DNcdk4
DNcdk5
d
control
glutamate
glutamate+MK801
% Live CGNs
100
75
50
25
0
Wt
p35 -/-
e
control
glutamate
glutamate+MK801
% Live CGNs
Wt
DNcdk4 transgenic

Figure 2.2

Figure 2.2. Cdk5/p35 is more involved in excitotoxic ischemia than Cdk4/cyclin D1. (a) AV-infected CGNs expressing DNCdk2/4/5 or GFP alone were subjected to 5-h hypoxia and 1- to 2-h reoxygenation in the absence of MK801 (n = 4). (b) CGNs from DNcdk4 transgenic mice or WT littermate controls subjected to hypoxia as in a (n = 3). (c) AV-infected CGNs expressing DNCdk2/4/5 or GFP alone subjected to glutamate (50 μM; n = 4). (d) CGNs from p35-deficient mice are resistant to glutamate-induced death (n = 3). (e) CGNs from DNCdk4 transgenic mice subjected to glutamate (n = 3). * denotes significance ($P < 0.05$, t test). The data are mean ± SEM.

Cdk4 as Mediator of Delayed Death in a Global Model of Stroke.

We next asked whether Cdk4 played a role in ischemic delayed death in adult models of injury. This question is significant, because no clear indication of a functional role of Cdk4 in adult injury has previously been demonstrated. To examine this question, we used a 10-min transient forebrain 4VO model of delayed ischemia where CA1 neurons die with a protracted (>24 h) time course after reperfusion. We have shown (Wang et al., 2002) that the Cdk4/Rb pathway is activated in this model. Recombinant rAAV1 vectors expressing DNCdk2/4/5 or GFP were injected unilaterally into the hippocampus 2 weeks before 10-min 4VO insult to allow for expression of the constructs. Expression of the constructs was confirmed by immunohistochemistry and Western blot (Figure 2.3a-e). Survival of CA1 neurons was assessed 4 days after induction of 4VO. Normal CA1 neurons are characterized by round soma and clear intact nuclei by hematoxylin/eosin analyses (Figure 2.3g), whereas dying neurons appeared shrunken with pyknotic nuclei (Figure 2.3i). Neuronal counts of CA1 region showed a dramatic increase in survival in the DNCdk4-injected hemisphere compared with noninjected hemisphere (Figure 2.3f). In comparison, DNCdk2- and -5-injected rats did not result in any significant survival in the CA1 region. No changes in neuronal numbers in the CA1 region were detected with GFP-treated animals (Figure 2.3f). These results are consistent with the previously described in vitro data and indicate that Cdk4 and neither Cdk2 nor -5 plays a critical role in delayed death in vivo.

We next asked whether protection by DNCdk4 might lead to improved behavioral outcomes. It has been shown that damage to the CA1 region results in impaired spatial learning and memory (Briones and Therrien, 2000). Accordingly, we used the Morris water maze test to test whether DNCdk4-injected rats had improved memory function. Animals

were injected bilaterally with DNCdk4 or GFP control and were subjected to either sham or 4VO surgery. As shown in Figure 2.3l, GFP-injected/stroked rats consistently spent almost twice as much time finding the platform (escape latency) when compared with DNcdk4-expressing animals. To ensure that any differences in latency time were not due to motor or visual deficits, a cued test was performed at the end of the test, and no difference was observed between groups.

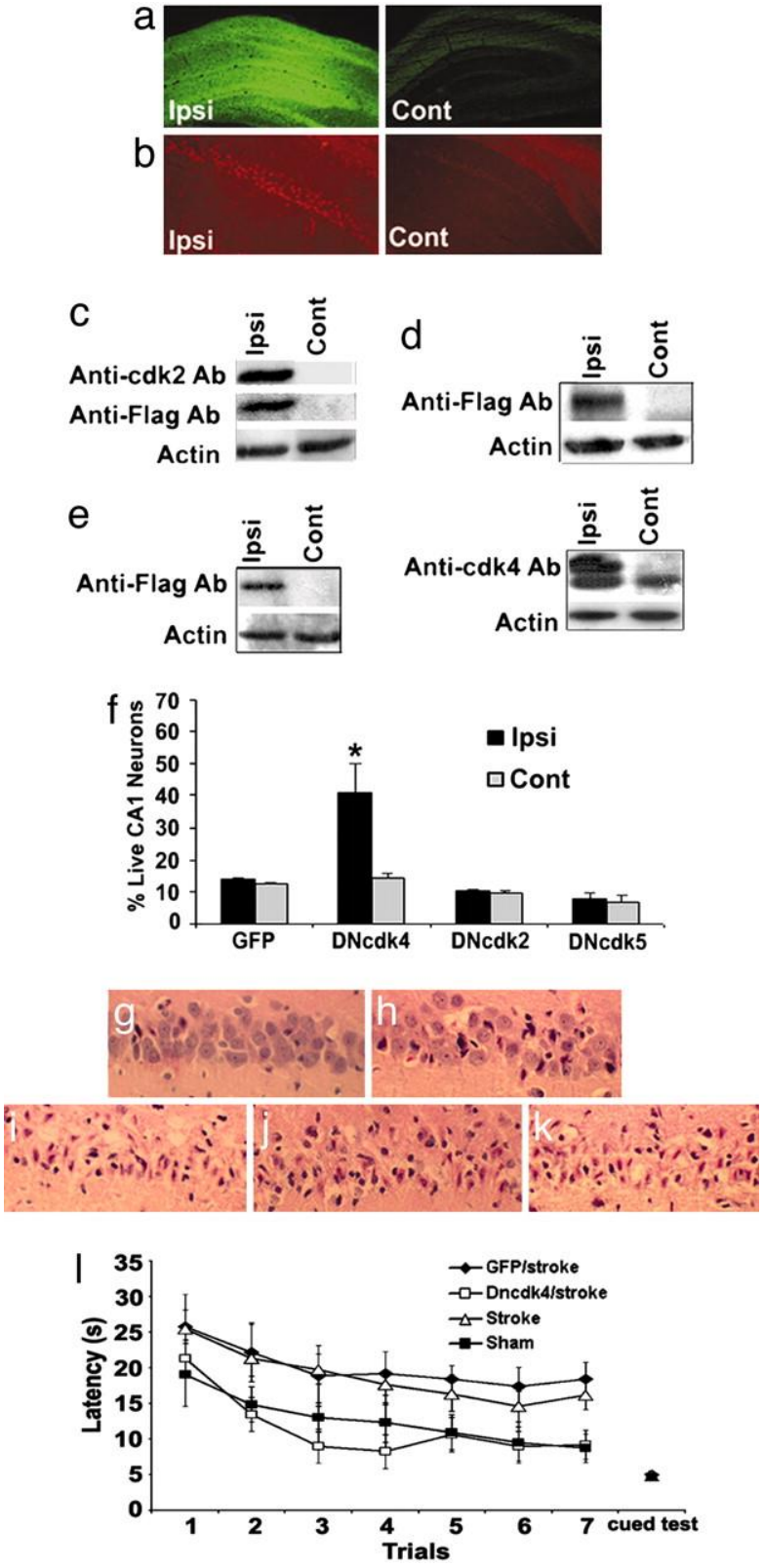
a
Ipsi
Cont
b
Ipsi
Cont
c
Ipsi
Cont
Anti-cdk2 Ab
Anti-Flag Ab
Actin
d
Ipsi
Cont
Anti-Flag Ab
Actin
e
Ipsi
Cont
Anti-Flag Ab
Actin
Ipsi
Cont
Anti-cdk4 Ab
Actin
f
% Live CA1 Neurons
70
60
50
40
30
20
10
0
*
Ipsi
Cont
GFP
DNcdk4
DNcdk2
DNcdk5
g
h
i
j
k
l
Latency (s)
35
30
25
20
15
10
5
0
GFP/stroke
Dncdk4/stroke
Stroke
Sham
1
2
3
4
5
6
7
cued test
Trials

Figure 2.3

Figure 2.3. DNCdk4 expression but not DNCdk2 or -5 provides significant protection from 10-min 4VO-induced delayed neuronal death in vivo. (a–e) Expressions of flag-tagged-DNCDKs constructs, as well as GFP (Ipsi, ipsilateral injected hemisphere; Cont, contralateral noninjected hemisphere). Expression of (a) GFP and (b) DNCdk2 shown using GFP fluorescence and anti-Cdk2 Ab, respectively. (c–e) Western blot of hippocampi injected with virus expressing (c) DNCdk2, (d) DNCdk5, and (e) DNCdk4 using anti-flag or -CDK Abs; actin loading control. (f) Quantitation of surviving CA1 neurons expressing GFP (n = 3), DNCdk4 (n = 7), DNcdk2 (n = 8), or DNCdk5 (n = 4). Survival assessments were performed 4 days after 4VO. Data are presented as mean ± SEM. * denotes significance ($P < 0.05$, t test Ipsi vs. Contra). (g–k) Representative sections from the CA1 region from animals treated with (g) sham, (h) DNCdk4-injected + 4VO, (i) GFP-injected + 4VO, (j) DNCdk2-injected + 4VO, and (k) DNCdk5-injected + 4VO. Sections are stained for hematoxylin/eosin. (l) Improved escape latency in the Morris water maze test (MWM) test in rats expressing DNCdk4 in hippocampus and subjected to 10-min 4VO. Rats injected bilaterally with DNCdk4 and stroked (n = 8), GFP and stroked (n = 7), noninjected and stroked (n = 6), and sham (n = 7) rats were subjected to the MWM test. The data are presented as mean ± SEM. There was a significant difference ($P < 0.01$, ANOVA) between DNCdk4- and GFP-expressing stroked animals during the testing periods but not with the cued test

The Role of Rb in Delayed Ischemic Death.

What are the mechanisms by which Cdk4 may signal death? Previous reports have indicated that Rb is phosphorylated efficiently on Ser-795 by Cdk4 (Connell-Crowley et al., 1997). Accordingly, we examined whether Rb may act as a downstream mediator of Cdk4 after 10 min of 4VO. As shown in Figure 2.4a, a dramatic increase in Ser-795 Rb phosphorylation was observed 12 h after reperfusion. No phosphorylation of Rb on Ser-807/-811 sites was observed (data not shown), suggesting some selectivity in Rb phosphorylation. As shown in Figure 2.4a-c, the increase in Ser-795 Rb phosphorylation after ischemia depends upon Cdk4 activity. DNCdk4-expressing animals showed reduced Rb phosphorylation, as determined by Western blot analyses of CA1 extracts. In contrast, DNCdk5 or -2 expression failed to attenuate the increased Ser-795 phospho Rb signal. This indicates that Cdk4 is a mediator of Ser-795 phosphorylation.

Rb phosphorylation could activate a number of potentially proapoptotic responses such as E2F, JNKs, and NF-κB (Morris and Dyson, 2001). Accordingly, we examined whether expression of a mutant Rb with several phosphorylation sites removed (including Ser-795) might be protective in ischemic injury. Because multiple phosphorylation sites are removed, it might be expected to act as a constitutively active form of Rb. Unfortunately, we could not obtain Rb expression in rAAV1, perhaps due to size limitations of the constructs used. However, we could express the active Rb by using AV for testing in vitro. As shown in Figure 2.4d and similar to our in vivo results, Rb becomes phosphorylated in vitro after reoxygenation in our hypoxia (+MK801) model of delayed injury. As shown in Figure 2.4e, expression of constitutively active Rb was significantly protective after hypoxic insult when

compared with the GFP control. CGNs exposed to hypoxia (+MK801) expressing mutant Rb showed 74% survival vs. 51% survival in GFP-expressing controls after 24 h of reoxygenation. Taken together with the results above, we propose that Cdk4 may transduce the delayed hypoxic death signal, at least in part through phosphorylation of Rb.

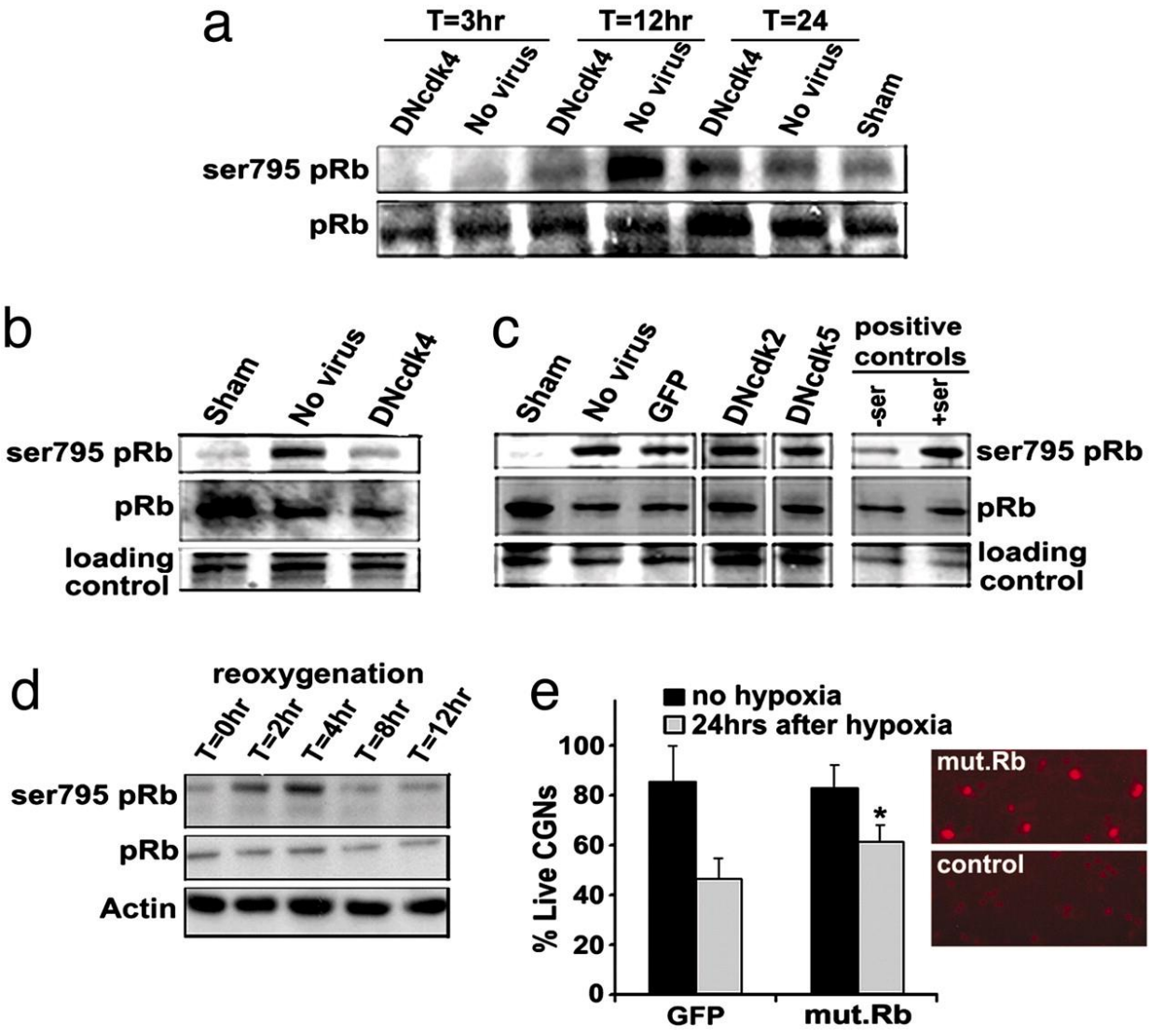
a
T=3hr
T=12hr
T=24
DNcdk4
No virus
DNcdk4
No virus
DNcdk4
No virus
Sham
ser795 pRb
pRb
b
Sham
No virus
DNcdk4
ser795 pRb
pRb
loading control
c
Sham
No virus
GFP
DNcdk2
DNcdk5
positive controls
-ser
+ser
ser795 pRb
pRb
loading control
d
reoxygenation
T=0hr
T=2hr
T=4hr
T=8hr
T=12hr
ser795 pRb
pRb
Actin
e
no hypoxia
24hrs after hypoxia
% Live CGNs
100
80
60
40
20
0
*
GFP
mut.Rb
mut.Rb
control

Figure 2.4

Figure 2.4. Phosphorylation of Rb on Ser-795 is diminished by DNCdk4 expression. (a) Time course of phosphorylation of Rb on Ser-795 and effects of DNCdk4 expression. Animals were injected unilaterally with DNCdk4-expressing virus. At the indicated times after 4VO, ipsilateral (with virus) or contralateral (no virus) nuclear hippocampi proteins were extracted and analyzed for Ser-795 phosphorylation by Western blot. Total Rb was used as loading control. (b and c) Comparison of the effects of DNCdk2/4/5 and GFP on Ser-795 Rb phosphorylation 12 h after ischemia. Positive control refers to nuclear hippocampal proteins extracted from noninjected animal O(-Ser) and 12 h (+Ser) after reperfusion. All lanes in c are from the same Western blot; Coomassie blue staining as loading control. (d) Time course of phosphorylation of Rb on Ser-795 in vitro. CGNs were subjected to 16 h of hypoxia, in the presence of MK801, followed by up to 12 h of reoxygenation; actin was used as loading control. (e) Expression of mutant Rb provides significant protection from hypoxia-induced delayed neuronal death in vitro. Quantitation of survival after 16–18 h of hypoxia followed by 24-h reoxygenation, in the presence of MK801. The data are mean ± SEM (n = 3). * denotes significance ($P < 0.05$, t test hypoxia GFP vs. hypoxia mut.Rb)

Cdk5 as Mediator of Focal Stroke in Vivo.

Although the above results indicate that Cdk4 may be more important in delayed ischemic death present in the global model of stroke, we next asked whether, then, cdk5 may be more effective in a focal model of stroke where more rapid excitotoxic forms of death may predominate. Endothelin-1 is a powerful and long-lasting vasoconstrictive peptide that has been used to induce focal stroke (Biernaskie and Corbett, 2001). Accordingly, we injected endothelin-1 directly into the striatum. Recombinant rAAV1 vectors expressing DNCdk4/5 or GFP were injected unilaterally into the striatum, where robust expression was observed (data not shown). Regions of striatum with infarct could be distinguished from non-damaged normal regions by cresyl violet staining. Damaged regions displayed readily detectable shrunken compacted dying neurons 4 days after endothelin treatment. Measurement of infarct volume showed a very significant decrease in the damaged region in DNCdk5-expressing brains, compared with GFP or DNCdk4-expressing brains (Figure 2.5a). In contrast, inhibition of Cdk4 was less protective against endothelin-1-induced lesion than DNCdk5 (Figure 2.5a). Animals injected with virus (GFP, DNCdk4/5) did not show damage in the absence of endothelin-1 (data not shown). This indicates that Cdk5 and not Cdk4 plays a critical role in the excitotoxic type of death in a focal model of ischemia in vivo and is consistent with the in vitro results.

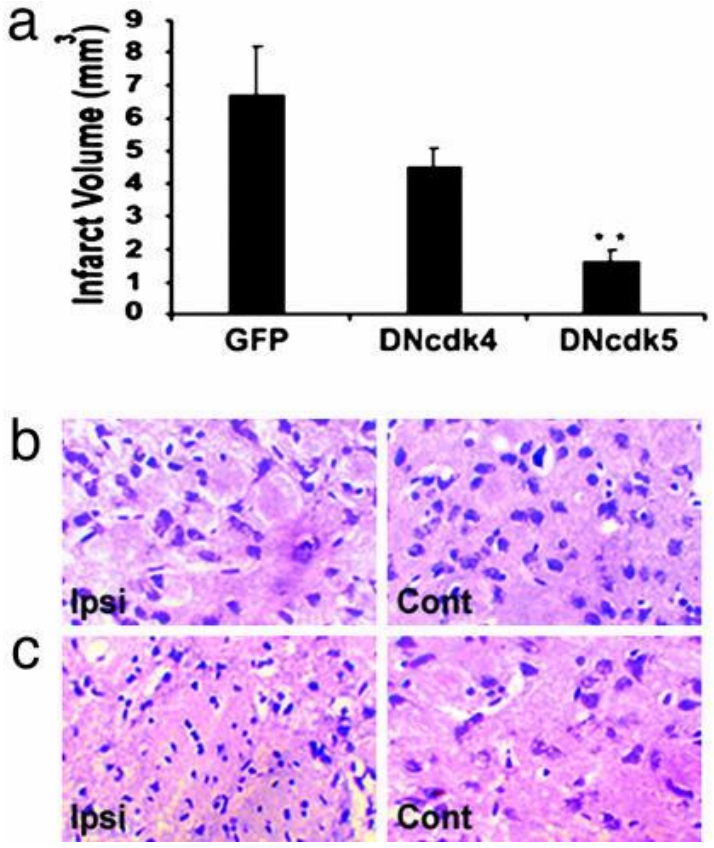
a
Infarct Volume (mm3)
9
8
7
6
5
4
3
2
1
0
GFP
DNcdk4
DNcdk5
b
Ipsi
Cont
c
Ipsi
Cont

Figure 2.5

Figure 2.5. DNCdk5 but not DNCdk4 expression provides significant protection from endothelin-induced excitotoxic neuronal death in vivo. (a) Infarct volume of focal ischemic brains expressing GFP (n = 4), DNCdk4 (n = 4), or DNCdk5 (n = 4) measured 4 days after endothelin injection. Data are presented as mean ± SEM. * denotes significance ($P < 0.01$, DNCdk5- vs. GFP-expressing brains). There was also a significant difference ($P < 0.05$) between DNCdk4- and DNCdk5- but not between GFP- and DNCdk4-expressing brains ($P > 0.05$; ANOVA, Newman–Keuls Multiple Comparison Test). (b and c) Representative sections of the striatum from animals treated with (b) DNCdk5 or (c) GFP followed by endothelin injection. Sections are stained with cresyl violet

DISCUSSION

Although the potential role of the cell cycle in neuronal death has been hypothesized, clear functional data indicating the relevance of such a signal in neuronal injury in vivo have not been reported. In the present investigation, we used both in vitro and in vivo paradigms of ischemia/hypoxia to explore the role of cell cycle signaling in neuronal death. Our results are significant, because they (i) provide clear evidence of the importance of cell cycle CDKs and (ii) define the conditions under which distinct CDK members participate in stroke-induced death signaling. Our results serve to resolve an outstanding question of whether cell cycle CDKs or neuronal CDKs such as Cdk5 are important in neuronal death.

Because both rapid/excitotoxic as well as delayed mechanisms of death (and spectra in between) appear in any single brain exposed to loss of blood flow, we explored whether Cdk5 and/or Cdk4 may (i) participate in ischemic processes and (ii) act differentially in models of excitotoxic vs. more delayed apoptotic-like death. Our data point to a model by which Cdk5 acts preferentially to regulate excitotoxic damage, whereas Cdk4 is involved in pathways of ischemic/hypoxic injury where excitotoxic mechanisms are not the primary mode of death. The evidence for this can be summarized as follows. First, in ischemic models of NMDA receptor-independent delayed death in vitro, DNCdk4 expression as well as cyclin D1 deficiency robustly block death, whereas DNCdk5 expression or p35 deficiency is not protective. In contrast, DNCdk5 expression and/or p35 deficiency are protective in *in vitro* models of excitotoxic damage. In these latter excitotoxic paradigms, DNCk4 is less protective when compared with DNCdk5, and cyclin D1-deficient neurons fail to show resistance to death. These results also help to resolve a persistent controversy over which CDKs (cell cycle or Cdk5) may be important in neuronal death. We provide evidence that

both pathways are of importance, but their significance likely depends upon the type of death insult and the differential initiating death pathways. These in vitro results are also consistent with our data in vivo. For example, in a global model of stroke, where more delayed modes of death are thought to predominate, Cdk4 appears to play a significant role. In contrast, in more severe focal models of stroke, where death is thought to be more rapid and excitotoxic, Cdk5 appears to play a more important role.

How might Cdk4 mediate a death signal? In both mild focal ischemia and a global model of stroke, Rb is phosphorylated on a known Cdk4 site, Ser-795. Our data show that ischemia-induced elevated Rb phosphorylation depends upon Cdk4. Moreover, it is likely that Rb plays a functional role, because expression of a constitutively active Rb, which cannot be phosphorylated on Ser-795 is protective, at least in vitro. However, it must be stressed that definitive evidence that Cdk4 acts solely through Rb has yet to be presented. The downstream effectors of Rb-mediated death in stroke are not completely clear. However, recent reports have indicated that E2F1, a well characterized Rb target, is important in neuronal death (Liu and Greene, 2001). For example, E2F1 expression kills neurons in vitro, and E2F1-deficient neurons are resistant to potassium deprivation (O'Hare et al., 2000) and β-amyloid exposure (Park et al., 2000b). Interestingly, E2F1-deficient mice are also resistant to mild focal ischemia (MacManus et al., 2003), again suggesting that Cdk4/Rb pathway may be more significant in situations with more delayed ischemic death. Finally, it is important to mention that, because Cdk4 and Cdk6 have potentially overlapping functions, the role of Cdk6 cannot be excluded.

Unlike with Cdk4, we propose that Cdk5 is more relevant in acute excitotoxic death

than delayed ischemic injury. Numerous reports have indicated that increased intracellular Ca^{2+} is a critical proximal event in excitotoxicity (Arundine and Tymianski, 2003). Reports using in vitro systems have also established that Ca^{2+}-activated proteases, calpains, cleave p35 to a more stable and mislocalized p25, from which mediate the pathogenic effects of Cdk5 (Dhavan and Tsai, 2001). Accordingly, the participation of Cdk5 in excitotoxicity is concordant with the deregulated Ca^{2+}, the prime effectors of excitotoxic damage. The link between excitotoxicity, calpains, and Cdk5 is strengthened by reports that calpain inhibition is also protective in focal models of stroke (Markgraf et al., 1998). Numerous substrates of Cdk5 have been reported. Of these candidates, two are particularly intriguing with regard to neuronal death. A recent report has indicated that MEF2 is phosphorylated and inactivated by a p25/Cdk5 complex, which is mislocalized from the cytoplasm to the nucleus (Gong et al., 2003). The importance of this mechanism in adult models of ischemic injury is presently unknown and should be clarified in future studies. An alternative potential mechanism involves Cdk5-mediated phosphorylation of the NMDA receptor 2A subunit at Ser-1232 (Wang et al., 2003). This phosphorylation is although to potentiate the activity of the NMDA receptor. The description of the NMDA receptor subunit as a Cdk5 substrate is consistent with our hypothesis that Cdk5 is functionally more relevant in excitotoxic mechanisms of death. Similar to Wang et al. (Wang et al., 2003), we have also shown that DNCdk5 expression is protective, with a shorter 5-min ischemic global insult (data not shown). This is likely due to the fact that in the global model, shorter insult times lead to more MK801-responsive death pathways, as has been reported (Murase et al., 1993).

CONCLUSION

We have shown that cell cycle CDKs and Cdk5 modulate distinct ischemic death pathways.

Because both excitotoxic and delayed pathways are critical in mediating stroke damage, strategies designed to inhibit multiple CDK members may be an important and effective therapeutic strategy.

SUPPORTING TEXT

2.1. The construct was tagged at the C terminus with a 9-aa flag sequence and was driven by a neuron-specific enolase promoter. This construct was used to generate founder lines following a standard pronuclei microinjection protocol for generating transgenic mice (transgenic core facility, University of Ottawa, Ottawa). DNCdk4 genotyping protocol was performed by using 5'-GAT GTG GAG TGT TGG CTG TAT CT-3' (DNcdk4 5') and 5'-CAT TTG TCA TCA TCG TCC TTG TAG-3' (DNCdk4 3') to amplify the DNCdk4 (351-bp) transgene. PCR conditions for this amplification were 94°C for 1.5 min (one cycle), 94°C for 20 sec, 60°C for 30 sec (-0.5°C per cycle), 72°C for 35 sec (12 cycles), 94°C for 20 sec, 55°C for 30 sec, 72°C for 35 sec (25 cycles), and 72°C for 2 min. DNCdk4 expression was further confirmed by performing Western blot with anti-Cdk4 Ab (Santa Cruz Biotechnology) on cerebellar granule neuron (CGN) extracts harvested from individual animals.

2.2. The pool was divided into four quadrants; starting and platform locations were randomly chosen and remained fixed for each training/testing cycle. On the first day, animals were trained to find the platform by using visual clues. Once the animals found the platform, they were allowed to sit for 10 sec and were then placed in the home cage for 60 sec. If the animals failed to find the platform in 60 sec, they were then manually placed on it. On the second day (test day), rats were assessed for finding the platform (latency time). Animals

received seven training or testing trials per day. Each training/testing cycle was repeated every 3 days, with the platform placed in a different location. A total of six cycles were performed. All of the clues and room setting remained unchanged, and the water temperature was maintained at 22°C for the duration of the study. On the last day of testing, a cued test was performed by using a black-colored platform raised above the water surface.

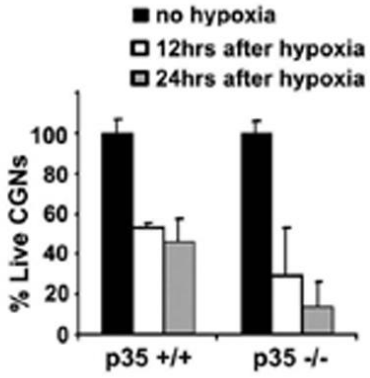
no hypoxia
12hrs after hypoxia
24hrs after hypoxia
% Live CGNs
100
80
60
40
20
0
p35 +/+
p35 -/-

Figure 2.6

Figure 2.6. Cerebellar granule neurons (CGNs) from p35-deficient mice are not resistant to hypoxia in the presence of MK801 (n = 3).

CHAPTER 3

Cdc25A is a critical mediator of ischemic neuronal death *in vitro* and *in vivo*.

Grace O. Iyirhiaro, Carmen Estey, Steve M. Callaghan, Matthew J. During, Ruth S. Slack, and David S. Park.

Manuscript in preparation.

STATEMENT OF AUTHOR CONTRIBUTION

This manuscript investigates the potential involvement of the Cdc25 dual specificity phosphatase family (upstream activators of mitotic-Cdks) in ischemic neuronal death. Each individual Cdc25 family member is examined using three different ischemic cell death models *in vitro* and one adult model of cerebral ischemia *in vivo*.

G.O Iyirhiaro designed and performed all of the experiments contained in this manuscript. Animal surgeries were performed by G.O Iyirhiaro with technical assistance from C. Estey (lab technician). C. Estey captured all the immunofluorescence images for the Cdc25A with hypoxia and glutamate studies and blinded the experiments for evaluation of neuronal survival. Dr. M.J During produced the original AAV vector. The vector was modified to include the H1 expression cassette by S. Callaghan. G.O Iyirhiaro produced all of the final shRNA containing vectors and adeno-associated viruses used in the experiments. Dr. R.S Slack contributed reagents. The manuscript and figures were prepared by G.O Iyirhiaro with scientific and editorial guidance from Dr. D.S Park.

Cdc25A is a critical mediator of ischemic neuronal death *in vitro* and *in vivo*.

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K1H 8M5

ABSTRACT

Dysregulation of cell cycle machinery is implicated in a number of neuronal death contexts including stroke. Increasing evidence suggests that cyclin-dependent kinases (Cdks) are inappropriately activated in mature neurons under ischemic stress conditions. We previously demonstrated a functional role for cyclin D1/Cdk4/pRb pathway in delayed neuronal death induced by ischemia. However, the molecular signal(s) leading to cyclin D/Cdk4/pRb activation following ischemic insult is presently not clear. Here, we investigate the cell division cycle 25 (Cdc25) dual specificity phosphatases as potential upstream regulators of ischemic neuronal death and Cdk4 activation. We show that a pharmacologic inhibitor of Cdc25 family members (A, B & C) protects neurons from hypoxia-induced delayed death. Furthermore, we show that the protection is associated with a therapeutic window of 2 hours. The major contributor to the death process appears to be Cdc25A. shRNA mediated knockdown of Cdc25A protects neurons in a delayed model of hypoxia-induced death *in vitro*. Similar results were observed *in vivo* following global ischemia. In contrast, shRNA to Cdc25B or C or neurons singly or doubly deficient for Cdc25B/C were not significantly protective. Finally, we show that Cdc25A activity, but not levels, is upregulated in the hippocampus following global ischemic insult *in vivo* and that shRNA to Cdc25A blocks Ser795 pRb phosphorylation. Overall, our results indicate that Cdc25A plays an important role in delayed neuronal death mediated by ischemia.

INTRODUCTION

The regulation of ischemic neuronal death is complex and involves the activation of a plethora of death signals. One such pathogenic signal involves the activation of cyclin-dependent kinases (Cdks). These are a family of proline directed Serine/Threonine kinases that play important roles in a number of biological processes including cell division, transcription, neuronal development and death (Pines, 1995, Dhavan and Tsai, 2001, Gopinathan et al., 2011, Herrup, 2012). With respect to the latter, the involvement of Cdks has been reported in a number of pathologic stress models including DNA damage (Park et al., 1997a, Park et al., 1998a, Park et al., 1998b, Park et al., 2000a), oxidative stress (Zambrano et al., 2004), excitotoxicity (O'Hare et al., 2005) and Aβ toxicity (Giovanni et al., 2000, Liu et al., 2004b). Importantly, Cdks are also implicated in a number of neurodegenerative conditions including Parkinson's disease (PD)(Jordan-Sciutto et al., 2003, Smith et al., 2003a), Alzheimer's disease (AD) (Hernandez-Ortega et al., 2011, Moh et al., 2011), Amyotrophic Lateral Sclerosis (ALS) (Nguyen and Julien, 2003) and Stroke (Osuga et al., 2000, Rashidian et al., 2007). Of relevance to the present study is the evidence demonstrating the involvement of core cell cycle proteins, in particular, cyclin D/Cdk4 in ischemic neuronal death (Guegan et al., 1997, Katchanov et al., 2001, Rashidian et al., 2005).

Cdk4 is a core cell cycle protein involved in the regulation of the G1/S-phase transition in proliferating cells. For example, during the G1/S-phase Cdk4 associates with cyclin D and is activated via phosphorylation by cyclin-dependent kinase activating kinase (CAK) (Pines, 1995). It has been proposed that Cdk4 is also positively regulated by a mandatory dephosphorylation of an inhibitory tyrosine within the ATP binding site by members of the Cdc25 dual specificity phosphatases (Pines, 1995). Once activated, Cdk4

phosphorylates the retinoblastoma tumor suppressor protein (pRb) leading to its inactivation and the release of the E2F transcription factors (Pines, 1995). Together with its co-activator Dp1, E2F1 mediates transcription of genes required for the progression to the S-phase (Pines, 1995).

In neurons, empirical evidence supports the role of Cdk4 as death mediator under ischemic stresses. First, cyclin D1 and Cdk4 levels and activity are increased in models of cerebral ischemia including oxygen glucose deprivation (OGD) (Katchanov et al., 2001), focal (Katchanov et al., 2001, Wen et al., 2005, Cai et al., 2009) and global (Timsit et al., 1999, Small et al., 2001) ischemia. In addition, an increase in cyclin D1 immunoreactivity is observed in human stroke brains (Love, 2003). Second, pharmacologic inhibition of CDKs is protective in focal (Osuga et al., 2000) and global (Wang et al., 2002) ischemic models. Importantly, expression of kinase dead Cdk4 is protective against delayed neuronal death induced by hypoxia and global ischemia (Rashidian et al., 2005).

Critical downstream targets of Cdk4 have also been implicated in neuronal death. For example, we have shown that the retinoblastoma protein (pRb) is increasingly phosphorylated following hypoxia/reoxygenation and global ischemic insults (Rashidian et al., 2005). This ischemia-induced pRb phosphorylation is attenuated by dominant negative Cdk4 expression (Rashidian et al., 2005). Similarly, E2F1 levels are induced in models of cerebral ischemia (Osuga et al., 2000, Gendron et al., 2001, Hwang et al., 2006) and its deficiency has been shown to be protective (MacManus et al., 1999, Gendron et al., 2001, MacManus et al., 2003). While these studies have helped to elucidate the downstream effectors of Cdk4, very little is known of the upstream signal(s) leading to its activation

following ischemic stress. Here, we provide evidence for the first time for the central role of Cdc25A in delayed ischemic death both *in vitro* and *in vivo*.

METHODS

Viral construction. Recombinant adeno-associated virus (rAAV) vectors expressing EGFP and shRNA to Cdc25A, B and C were constructed by subcloning the p-Silencer H1 expression cassette (Ambion) into the KpnI sites of a modified AM/CBA-EGFP-pl-WPRE-bGH plasmid. Briefly, the AM/CBA-EGFP-pl-WPRE-bGH plasmid was digested with BamHI and HindIII, and then blunted removing the MCS (multiple cloning sites) region. The EGFP fragment from pEGFP-N2 (Clontech) replaced the MCS to produce AM/CBA-EGFP-pl-WPRE-bGH. The re-engineered AM/CBA-EGFP-pl-WPRE-bGH vector was re-digested with KpnI and blunted. The pSilencer 3.0 HI vector (Ambion) was digested with PVUII and the expression cassette containing the H1 promoter, BamHI and HindIII sites was subcloned into the blunted AM/CBA-EGFP-pl-WPRE-bGH vector. shRNA targeting Cdc25A, shRNA#1, 5'-GCCGATACCCATATGAATA-3' (Ambion, S139728), shRNA #2, 5'-GAAATATATTTCTCCAGAA-3' (Ambion, S139729); Cdc25B 5'-AGAGAGTGATTTAAAGGAT-3' (Ambion, S139731), and Cdc25C 5'-CCATTACTACAGTTCCAAA-3' (Ambion, S158198) or pSilencer control #1 shRNA 5'-AGTACTGCTTACGATACGG-3'(Ambion) were sub-cloned into BamHI and HindIII sites within the HI expression cassette in the new AAV vector (AM/CBA-EGFP-HI-pl-WPRE-bGH). The final rAAV vector contained two separate promoters, CBA (chicken beta actin) and HI that drove the expression of GFP and shRNA respectively. The vectors containing shRNA to Cdc25A/B/C were used to produce rAAVs as previously described (Zolotukhin et al., 2002). Viral efficiency was tracked using GFP expression.

Cdc25B and C null mice. Cdc25 B and C null mice have been previously described

(Ferguson et al., 2005) and were obtained from Dr. Piwnica-Worms (Department of Cell Biology and Physiology, Washington University School of Medicine). Mice singly or doubly heterozygous for Cdc25B and C were bred and genotyped by PCR using primers 5'-GACTGTAGGGACAGGGTCCA-3', 5'-GCAGCCTGCTACAAAGTTCC-3', 5'-TAGTGACCACCGTGGACTGA-3', 5'-GGGAGGA TTGGGAAGACAAT-3' for Cdc25B and 5'-GGTTCCTTGGATTCATCTGGAC-3', 5'-CCCT ACCATGAGTGCAGGGCACC-3', 5'-CCTCGTGCTTTACGGTATCGCC-3' for Cdc25C.

Cell culture and treatments. Primary cerebellar granule neuron (CGN) cultures were harvested from 7-9 days postnatal CD1 (Charles River, Quebec) or Cdc25B and/or C single or double knockout mice as previously described (O'Hare et al., 2000). CGN cultures were treated with Cdc25 inhibitor NSC95397 [2,3-bis-(2-hydroxyethylsulfanyl)-1,4-naphtho-quinone], generously provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). 10mM Stock solution of NSC95397 prepared in dimethylsulfoxide (DMSO) and stored at -80^{o}C was diluted in complete CGN media immediately before use. Alternatively, CGNs were infected with rAAV expressing shRNA to Cdc25A/B/C or scrambled shRNA as control at IFU of 50 or 200. All CGN cultures were subjected to hypoxia or glutamate insult after one week in culture.

Hypoxia. Hypoxia was conducted as previously described (Rashidian et al., 2005) using a humidified environmental hypoxia chamber (Coy Laboratory Products, Ann Arbor, MI) set at 37°C, 1% O_2, 5% CO_2 and balanced with N_2. Gas levels were electronically monitored and maintained at set values. Delayed and excitotoxic hypoxic death models were carried out as previously described (Rashidian et al., 2005). Briefly, delayed hypoxic death was induced by incubating CGN cultures in hypoxia chamber for 18 hours in the presence of the 10uM NMDA blocker, MK801 (Research Biochemicals, Natick, MA) and then reoxygenated at 37^{o}C. Control cultures were incubated at 37^{o}C and were not subjected to hypoxia (Rashidian et al., 2005). Alternatively, excitotoxic hypoxic death was induced by incubating CGN cultures in the hypoxia chamber in the absence of MK801 for 5 hours and reoxygenated for 1 hour at 37^{o}C. Non-virally infected cultures were lysed at various times after hypoxia using mildly disruptive lysis buffer as previously described (Rashidian et al., 2005). Nuclei of healthy cells remained intact and were counted, while nuclei of unhealthy cells showing disrupted nuclear membrane and characteristics of blebbing and were excluded.

Virally infected cells were fixed with 1:1 4% paraformaldehyde and lana's fixative and stained with Hoechst 33342 (Sigma). Neuronal survival was evaluated by examining nuclear integrity of GFP positive cells in random fields as previously described (O'Hare et al., 2005). Dying neurons exhibited severe nuclear condensation or fragmentation. For delayed death alive neurons were scored over total GFP positive cells. For excitotoxic death total alive GFP from five random fields were counted and presented as percent survival $\pm$ SEM.

Glutamate exposure. CGNs were transiently exposed, for 70 minutes, to 50μM glutamate in presence or absence of MK801 and treated as previously described (Rashidian et al., 2005). In brief, cultures were washed with conditioned media after 70 minutes and incubated for 1 hour at 37^{o}C. Neuronal survival was examined and scored as described above.

Viral injections *in vivo*. All animal experiments were performed in accordance with the guidelines set forth by the Canadian Council for the Use and Care of Animals in Research (CCAC) with approval from the University of Ottawa Animal Care Committee. Male Wistar rats weighing 80-125g were unilaterally injected with rAAV expressing shRNA to Cdc25A/B/C or control shRNA and GFP two weeks prior to global cerebral ischemia as previously described (Rashidian et al., 2005) with the following modification: 2μl of rAAV (10^{11} IFU per microliter) with 1μl of 20% mannitol in PBS was stereotaxically delivered over 30 minutes to the hippocampus (from bregma: -3.6mm anterioposterior, $\pm$2.1mm lateral and -2.75mm deep) using Harvard infusion pump (Harvard Apparatus).

Global cerebral ischemia. rAAV injected and non-injected male Wistar rats were subjected to the four vessel occlusion (4VO) method of transient global cerebral ischemia for 10 minutes as previously described (Wang et al., 2002, Iyirhiaro et al., 2008). Rats were sacrificed four days following global ischemia and assessed for CA1 neuron survival. Brains were extracted, sectioned and stained with Hematoxylin/Eosin (H&E). Neuronal survival was evaluated as previously described (Wang et al., 2002, Iyirhiaro et al., 2008).

Immunohistochemistry. Deparafinization and heat-mediated antigen retrieval using citrate buffer (50mM, pH7.6) was performed on rat brain sections as previously described (Iyirhiaro et al., 2008). Sections were permeablized for 10 minutes with 0.3% Triton X-100 and

blocked in 10% normal goat serum (Jackson Immuno Research) diluted in 2% bovine serum albumin (Fisher Scientific) in 0.01M Phosphate buffered saline (PBS) for 1 hour at room temperature. This was followed by an overnight incubation at 4^{o}C with mouse monoclonal anti-GFP antibody (1:200, Abcam). The sections were subsequently incubated with Alexa 488 goat anti-mouse secondary antibody (1:200, Jackson) for 1 hour at room temperature and stained with Hoechst.

Phosphatase assay. Cdc25A phosphatase activity was assayed by monitoring the hydrolysis of 4-nitrophenol phosphate (pNPP) (Sigma) as previously described (Zhang et al., 2006). Hippocampal tissues were extracted at designated times following 4VO and homogenized in immunoprecipitation (IP) buffer (50mM HEPES pH 7.5, 150mM NaCl, 1mM EDTA, 2.5mM EGTA, 1mM DTT, 0.1% Tween 20 and protease inhibitor cocktail (Roche)). 100ug of total protein from hippocampal lysate was incubated with 2ug of rabbit polyclonal anti Cdc25A (Upstate Biotechnology) overnight at 4^{o}C. No antibody was added to control lysates. The antigen-antibody complex was captured with protein A-Sepharose beads (Sigma) overnight at 4^{o}C. The bead-antigen-antibody complex was subsequently incubated with phosphatase reaction buffer containing 50mM Tris, pH8.0, 50mM NaCl, 1mM EDTA, 1mM DTT and 1mM pNPP for 4 hours at 37^{o}C. The reaction was stopped by the addition of 5N NaOH. Cdc25A phosphatase activity was monitored by measuring the absorbance of *p*-nitrophenol at 405nm.

Western blot analysis. At the designated times following global ischemia, nuclear proteins were extracted from hippocampal brain lysates as previously described (Wang et al., 2002). For Cdc25 expression and down-regulation studies, whole lysates from the hippocampus were used. Samples were run on SDS-polyacrylamide gels and transferred onto PVDF

membrane (Millipore). Membranes were probed with the following antibodies: anti-Cdc25A (1:1000, Upstate, Santa Cruz Biotechnology), anti-Cdc25B (1:1000, Cell Signaling Technology), anti-Cdc25C (1:1000, Abbiotec), anti-pRb Ser795 (1:500, Cell Signaling Technology and Imgenex), anti-pRb (1:1000, BD Pharmingen) and anti β-actin (1:5000, Sigma).

Statistical analysis. Multiple comparisons were analyzed using ANOVA, with *post hoc test*. Where appropriate, the Student's *t*-test was used for two group comparisons.

RESULTS

Inhibition of Cdc25 protects neurons from delayed death mediated by hypoxia.

The Cdc25 dual specificity family of phosphatases is composed of three family members (Cdc25A, Cdc25B and Cdc25C) that are thought to share a similar function in regulating the cell cycle (Ray et al., 2007a). NSC95397 is a drug known to inhibit all three Cdc25 members (Lazo et al., 2002). Accordingly, we first determined whether this inhibitor would block death of cultured primary neurons exposed to ischemic stress. Since NSC95397 has been shown to exhibit some cytotoxic properties in dividing cell lines (Han et al., 2004), we first evaluated its cytotoxicity in our primary neuronal cultures. CGNs were treated with varying concentrations of NSC95397 up to 1.5μM and examined for neuronal survival 24 hours later. As shown in figure 3.1A, no cytotoxicity was observed when cultures were treated with up to 0.5μM of NSC95397. However, significant cell death is observed when neurons are treated with 1μM (~68% survival) and 1.5μM (~2% survival) of NSC95397 ($p<0.01$ and 0.005, respectively) (Figure 3.1A & B). We therefore chose to test the survival properties of the inhibitor at 0.5μM or lower concentrations.

To test whether Cdc25 is required for delayed neuronal death, we treated CGN cultures with up to 0.5μM NSC95397 prior to insult and subjected them to hypoxia for 18 hours in the presence of MK801 as previously described (Rashidian et al., 2005). In this model, neuronal death occurs in a delayed manner following reoxygenation but not during hypoxia. We have shown previously that this form of death is reliant on Cdk4 (Rashidian et al., 2005). Neuronal survival was examined at 1, 2 and 4 days following hypoxia. As shown in figure 3.1C & D, CGN cultures treated with the Cdc25 inhibitor were significantly more

resistant to hypoxia induced delayed neuronal death (Figure 3.1C & D). Treatment with the Cdc25 inhibitor provided significant protection against delayed neuronal death at all concentrations tested 24 hours following hypoxia (Figure 3.1D). Remarkably, neurons treated with the Cdc25 inhibitor remained significantly protected from delayed death four days after insult. Neuronal survival was reduced to 20% in the no drug treatment control cultures 4 days after hypoxia. In contrast neuronal survival of 49% (at 0.1μM), 68% (at 0.2μM, $p<0.05$), 69% (at 0.3μM, $p<0.05$), 81% (at 0.4μM, $p<0.01$) and 82% (at 0.5μM, $p<0.005$) were observed in the Cdc25 inhibitor treated cultures (Figure 3.1D).

We next determined whether the Cdc25 inhibitor could be protective following initiation of hypoxic insult (a more clinically relevant situation). To this end, CGNs were treated with 0.5μM of the Cdc25 inhibitor either immediately or up to 4 hours after hypoxia. As shown in figure 3.1E, post treatment of CGNs with the Cdc25 inhibitor provided significant protection when administered immediately or up to 2 hours following hypoxia (49% vs 74% at 2 hours, $p<0.05$) (Figure 3.1E). This indicates a significant therapeutic window for Cdc25 action following ischemic insult.

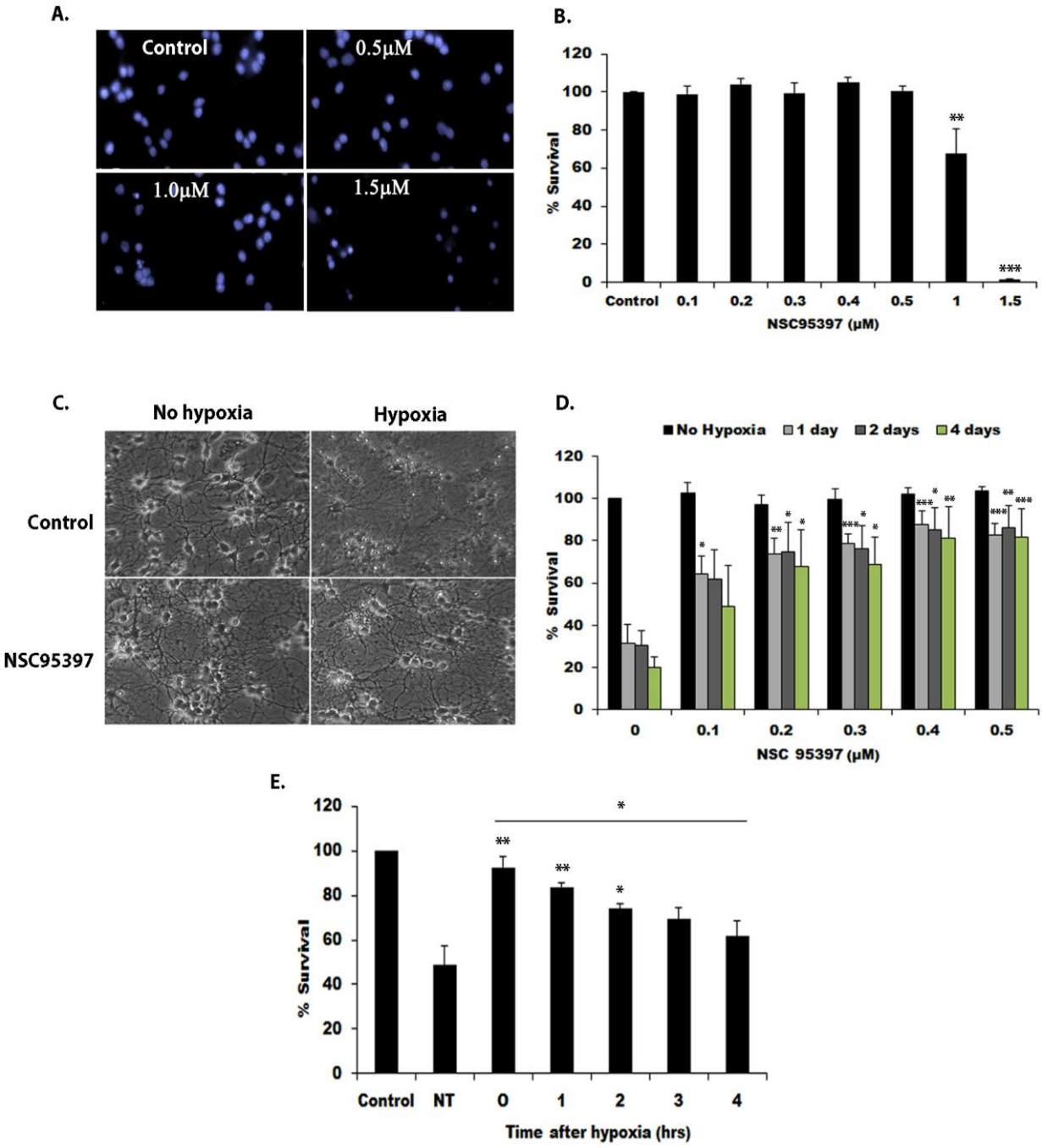
A.
Control
0.5μM
1.0μM
1.5μM
B.
% Survival
120
100
80
60
40
20
0
**
***
Control
0.1
0.2
0.3
0.4
0.5
1
1.5
NSC95397 (μM)
C.
No hypoxia
Hypoxia
Control
NSC95397
D.
No Hypoxia
1 day
2 days
4 days
% Survival
120
100
80
60
40
20
0
0
0.1
0.2
0.3
0.4
0.5
NSC 95397 (μM)
E.
% Survival
120
100
80
60
40
20
0
*
**
**
*
Control
NT
0
1
2
3
4
Time after hypoxia (hrs)

Figure 3.1

Figure 3.1. Treatment with the Cdc25 phosphatase inhibitor (NSC95397) protects neurons from delayed cell death induced by hypoxia. (A) CGNs were treated with different concentrations of Cdc25 inhibitor NSC95397, fixed and stained with hoechst 24 hours later and (B) lysed and evaluated for survival in the absence of insult 24 hours later. (C & D) CGNs treated with varying concentration of NSC95397 were subjected to 18 hours of hypoxia in the presence of MK801, (C) photomicrograph taken 24 hours after hypoxia and (D) survival was assessed at 1, 2 and 4 days after hypoxia. (E) CGNs were treated with 0.5µM NSC95397 immediately (0hr) and up to 4 hours following 18 hours hypoxia in the presence of MK801. Survival was assessed 24 hours following hypoxia. Results are expressed as % of control + SEM. *Denotes statistical significance $p<0.05$, ** $p<0.01$, ***$p<0.005$ compare to (A) no drug control, (D) hypoxia treated, no drug control, (E) No treatment (NT). $N\geq3$.

Cdc25A but not Cdc25B or C is required for delayed death by hypoxia.

Although the above results demonstrate the importance of Cdc25 in delayed neuronal death, it was unclear whether all three or a single member of this phosphatase family is required for death. To this end, we sought to determine which Cdc25 member (s) is/are important in mediating ischemic neuronal death. We first asked whether the inhibition of Cdc25A alone could protect neurons from delayed neuronal death mediated by hypoxia. Although mice singly or doubly null for Cdc25B or C are viable, mice null for Cdc25A die very early in development, thus precluding the use of Cdc25A null mice in our studies (Ray et al., 2007b, Lee et al., 2009). Accordingly, we generated a recombinant adeno-associated virus (rAAV) both expressing shRNA to Cdc25A and GFP from a separate promoter. CGN cultures were infected one day after plating with AAV expressing Cdc25A-shRNA (Figure 3.2A) or control-shRNA. Infection was monitored by assessing GFP expression. Cultures were subjected to hypoxia/reoxygenation in presence of MK801 as described above. Neuronal survival was examined 24 hours later by scoring the number of alive GFP over total GFP positive cells and expressed as percent survival. As shown in figure 3.2B, neurons expressing Cdc25A shRNA were significantly more resistant to delayed death compare to those treated with control shRNA. Neuronal survival was 87% in the Cdc25A knockdown cultures compare with 64% in the control shRNA expressing cells ($p<0.01$) (Figure 3.2B). This result suggests that down-regulation of Cdc25A alone can provide resistance against delayed neuronal death. Next, we asked whether deficiency in one or both of Cdc25B and/or Cdc25C could provide similar protection against delayed death. In this case, we were able to utilize neurons from mice either singly or doubly deficient for Cdc25B or C. The results showed that there is no significant difference in survival between wildtype neurons and those

deficient for Cdc25B (Figure 3.2C), Cdc25C (Figure 3.2D), or Cdc25B/C combined (Figure 3.2E). Taken together, these results suggest that Cdc25A plays a critical and distinct role in delayed neuronal death with little or no contribution from Cdc25B and Cdc25C.

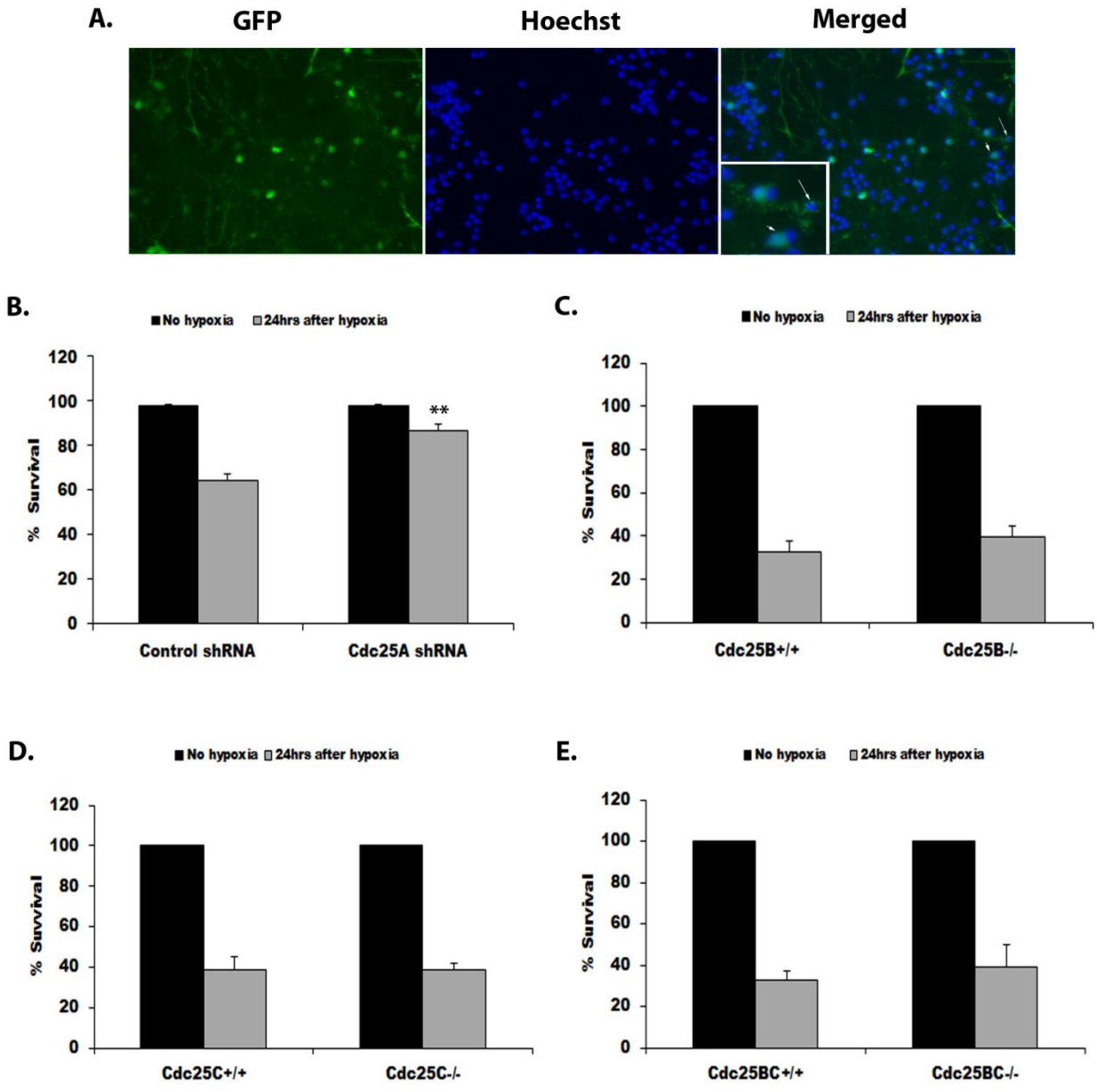
A.
GFP
Hoechst
Merged
B.
No hypoxia
24hrs after hypoxia
% Survival
120
100
80
60
40
20
0
**
Control shRNA
Cdc25A shRNA
C.
No hypoxia
24hrs after hypoxia
% Survival
120
100
80
60
40
20
0
Cdc25B+/+
Cdc25B-/-
D.
No hypoxia
24hrs after hypoxia
% Survival
120
100
80
60
40
20
0
Cdc25C+/+
Cdc25C-/-
E.
No hypoxia
24hrs after hypoxia
% Survival
120
100
80
60
40
20
0
Cdc25BC+/+
Cdc25BC-/-

Figure 3.2

Figure 3.2. Cdc25A knockdown but not Cdc25B/C deficiencies protect neurons from delayed cell death induced by hypoxia (A & B) CGNs were infected with adeno-associated virus (AAV) expressing Cdc25A or control shRNA and subjected to 18 hours hypoxia in the presence of MK801, (A) stained with anti-GFP antibody and hoechst 24 hours after hypoxia and (B) assessed for survival by scoring alive GFP+ neurons over total infected cells. (C-E) CGNs from transgenic mice null for (C) Cdc25B (D) Cdc25C (E) or doubly null for Cdc25B & C were subjected to hypoxia as in (A) and assessed for survival 24 hour later. Cell viability was assessed by nuclei integrity. Results are expressed as % of control + SEM. **Denotes statistical significance $p<0.01$, $N\geq3$.

Cdc25A knockdown exacerbates excitotoxic neuron death by hypoxia and glutamate.

Ischemic neuronal death is composed of both delayed and excitotoxic death signals. While our previous evidence suggests that hypoxia induced delayed death involves Cdc25A, the potential role of Cdc25 members in excitotoxic death was unknown. To this end, we examined the role of Cdc25 in neuronal death induced by excitotoxic insults. CGNs were first treated with NSC95397 and subjected to hypoxia in the absence of the NMDA blocker MK801 for 5 hours and examined an hour later for survival. As shown in figure 3.3A, the general Cdc25 inhibitor did not protect neurons from excitotoxic cell death induced by hypoxia regardless of the drug concentration (Figure 3.3A). This result suggests that, in contrast to delayed neuronal death, Cdc25 signal is not required for excitotoxic cell death induced by hypoxia. To confirm this result, we evaluated individual Cdc25 members in excitotoxic cell death induced by hypoxia. CGN cultures infected with AAV expressing Cdc25A-shRNA or control-shRNA were subjected to hypoxia without MK801 as already described. Cdc25A knockdown did not protect and surprisingly even sensitized CGNs to excitotoxic death induced by hypoxia (Figure 3.3B). In contrast to the results obtained with Cdc25A knockdown, neurons deficient in either Cdc25B (Figure 3.3C) or Cdc25B & C (Figure 3.3E) showed a protective trend (although not significant) against excitotoxic-hypoxia. Interestingly, Cdc25C deficiency alone resulted in significant protective effects against excitotoxicity induced by hypoxia (49% in Cdc25C+/+ vs 71% in Cdc25C-/-, $p<0.05$), (Figure 3.3D).

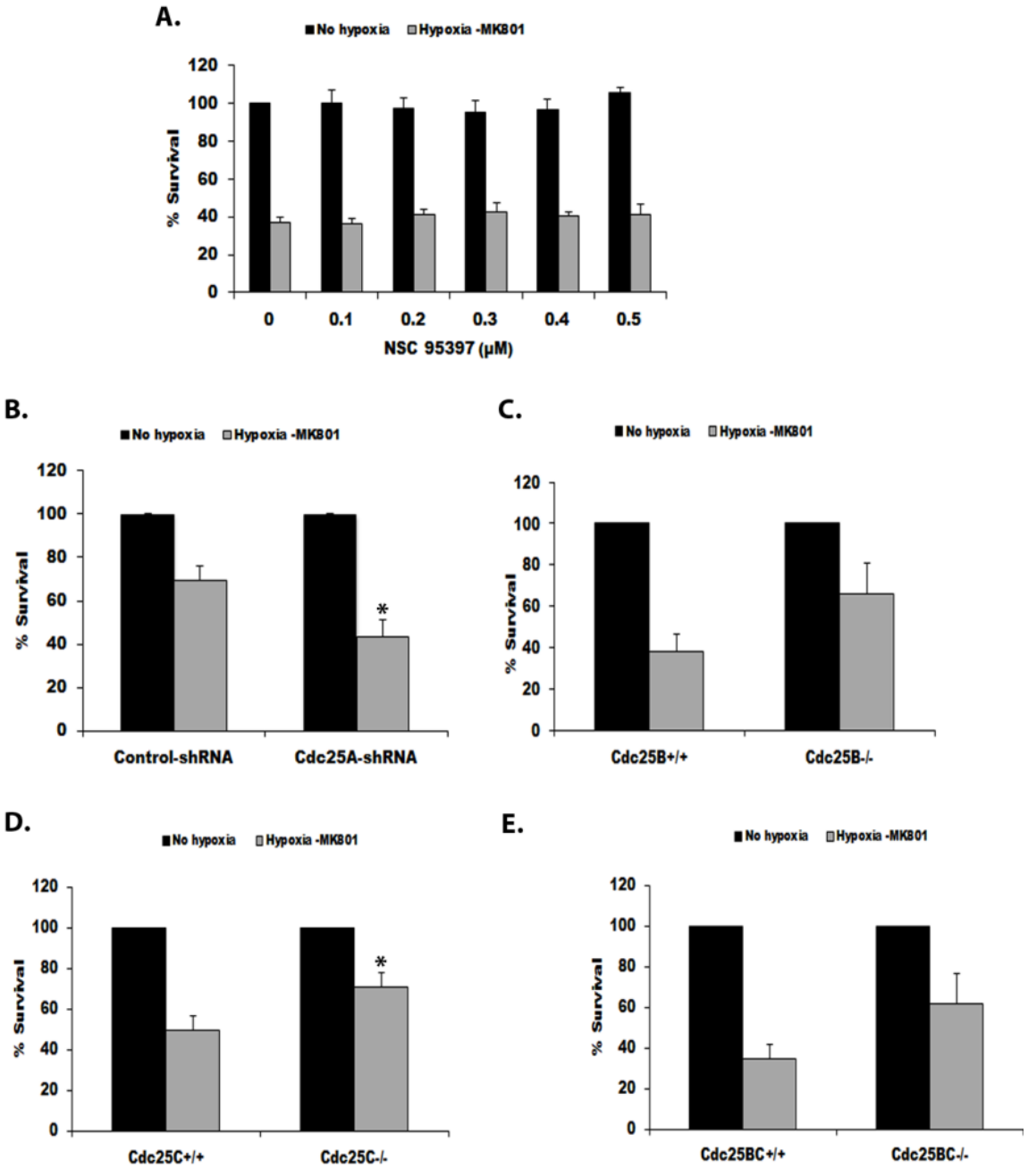
A.
No hypoxia
Hypoxia -MK801
% Survival
120
100
80
60
40
20
0
0
0.1
0.2
0.3
0.4
0.5
NSC 95397 (μM)
B.
No hypoxia
Hypoxia -MK801
% Survival
Control-shRNA
Cdc25A-shRNA
*
C.
No hypoxia
Hypoxia -MK801
% Survival
Cdc25B+/+
Cdc25B-/-
D.
No hypoxia
Hypoxia -MK801
% Survival
Cdc25C+/+
Cdc25C-/-
*
E.
No hypoxia
Hypoxia -MK801
% Survival
Cdc25BC+/+
Cdc25BC-/-

Figure 3.3

Figure 3.3. Cdc25A knockdown sensitizes CGNs to excitotoxic death mediated by hypoxia. (A) CGNs treated with Cdc25 inhibitor NSC95397 were subjected to 5 hypoxia and 1 hour reoxygenation in the absence of MK801 and examined for survival. (B-E) CGNs (B) infected with AAV expressing Cdc25A or control shRNA, (C) from Cdc25B null mice, (D) Cdc25C null mice, and (E) doubly null for Cdc25B and C were similarly subjected to hypoxia without MK801 as in (A). Cell viability was assessed by nuclei integrity using mild lysis buffer (A, C-E) or following immunofluorescence staining with anti-GFP antibody and hoechst. Results are expressed as % of control + SEM. *Denotes statistical significance $p<0.05$, $N\geq3$.

To further investigate the role of Cdc25 in excitotoxic cell death, CGNs treated with the Cdc25 inhibitor NSC95397 were transiently exposed to 50μM glutamate for 70 minutes as previously described (Rashidian et al., 2005). As shown in figure 3.4A, Cdc25 inhibition did not protect neurons from glutamate-induced excitotoxicity even at the highest drug concentration (42% vs 30% survival in the 0μM and 0.5μM drug treated cultures respectively) (Figure 3.4A). However, identical to the results observed with hypoxia-induced excitotoxicity, Cdc25A knockdown sensitized neurons to glutamate induced death (69% vs 43%; $p<0.05$). Neurons deficient for Cdc25B (Figure 3.4C) or Cdc25C (Figure 3.4D) displayed no difference in survival when compared with wildtype controls. In contrast, deficiency in both Cdc25B and C resulted in a slight but significant protection against glutamate exposure (38% vs 56%, $p<0.05$). Taken together, these results suggest that the combined Cdc25s do not play a central role in excitotoxic death. However, there are subtle effects with individual Cdc25 members that will be further discussed below.

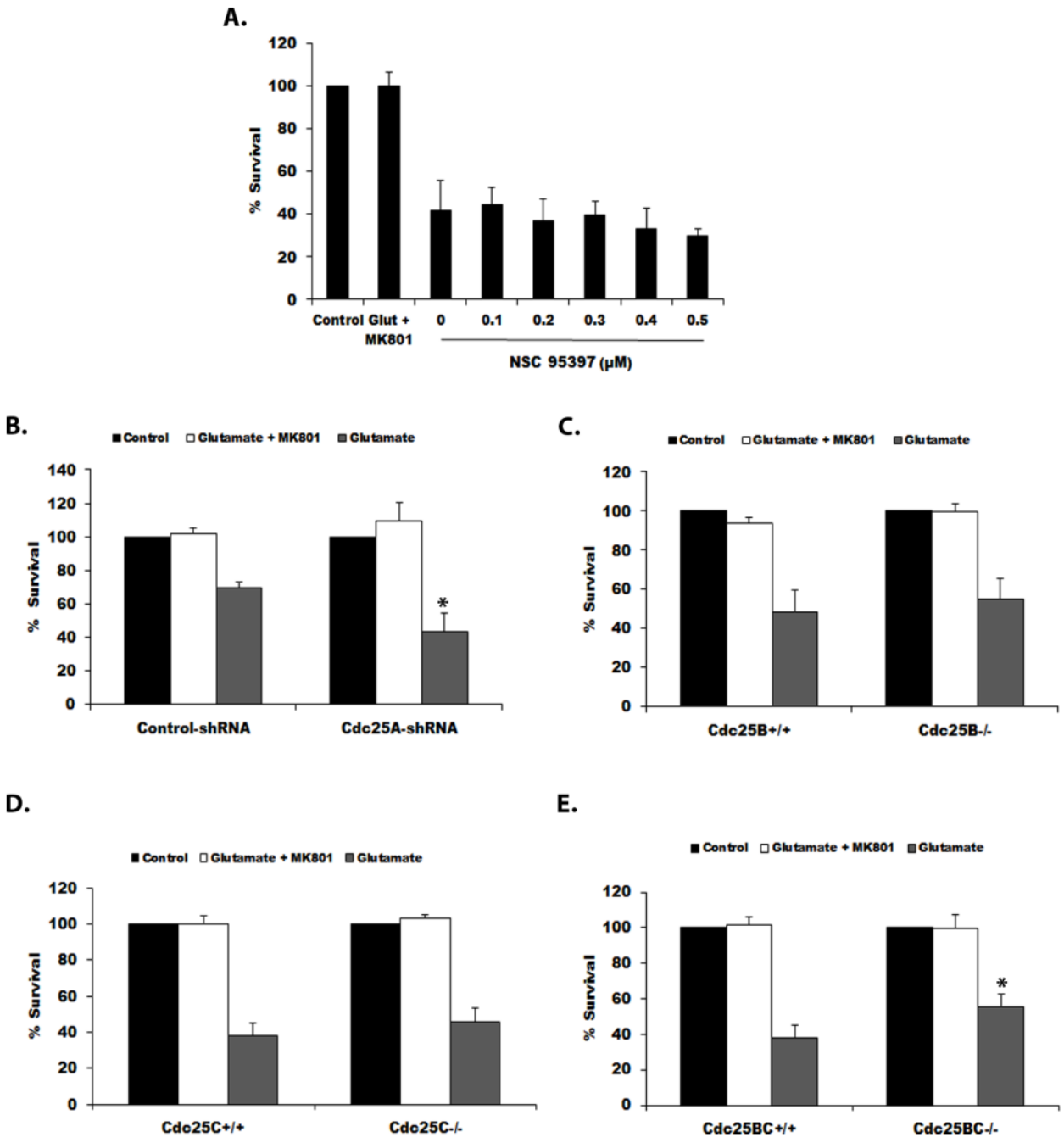
A.
% Survival
120
100
80
60
40
20
0
Control
Glut + MK801
0
0.1
0.2
0.3
0.4
0.5
NSC 95397 (μM)
B.
Control
Glutamate + MK801
Glutamate
% Survival
140
120
100
80
60
40
20
0
*
Control-shRNA
Cdc25A-shRNA
C.
Control
Glutamate + MK801
Glutamate
% Survival
120
100
80
60
40
20
0
Cdc25B+/+
Cdc25B-/-
D.
Control
Glutamate + MK801
Glutamate
% Survival
120
100
80
60
40
20
0
Cdc25C+/+
Cdc25C-/-
E.
Control
Glutamate + MK801
Glutamate
% Survival
120
100
80
60
40
20
0
*
Cdc25BC+/+
Cdc25BC-/-

Figure 3.4

Figure 3.4. Cdc25A knockdown sensitizes CGNs to excitotoxic death mediated by glutamate. (A) CGNs treated with the Cdc25 inhibitor were subjected to 50μM transient glutamate exposure for 70 minutes and assessed for survival. (B-E) CGNs (B) infected AAV expressing Cdc25A or control shRNA, (C) null for Cdc25B, (D) null for Cdc25C, and (E) doubly null for Cdc25B & C were similarly treated as in (A). Cell survival was assessed by nuclei integrity using mild lysis buffer (A, C-E) or following immunofluorescence staining with anti-GFP antibody and hoechst. Results are expressed as % of control + SEM. *Denotes statistical significance $p<0.05$, $N\geq3$.

Cdc25A knockdown protects CA1 neurons from global ischemia.

The evidence above clearly indicates a role for Cdc25A in delayed ischemic death *in vitro* and a mixed effect of Cdc25 members with excitotoxicity. We next asked whether Cdc25 members might play a physiological role in the adult model of global cerebral ischemia. In this model, there appears to be both components of delayed and also excitotoxic death. Since the global ischemia model is not easily reproducible in mice, we therefore switched to the rat system. We generated AAVs expressing shRNAs for all three individual Cdc25 members. Rats were injected unilaterally with AAV expressing shRNA to Cdc25A, Cdc25B or Cdc25C and GFP. As control, a separate group of rats were injected with AAV expressing control shRNA. Four-vessel occlusion (4VO) was induced two weeks following AAV injection as previously described (Rashidian et al., 2005). Rats were sacrificed 4 days following global ischemia. Efficient viral delivery and infection was tracked by staining for GFP expression in the rat hippocampus (Figure 3.5A). Western blot analysis for Cdc25A (Figure 3.5H), Cdc25B (Figure 3.5I) and Cdc25C (Figue 3.5J) demonstrated efficient knockdown of Cdc25A, B and C respectively. As shown in figure 3.5K, AAV injection on its own or Cdc25A, B or C knockdown alone, did not cause neuronal death in the absence of insult when compared to the no virus control (Figure 3.5B-C, & 3.5K, white bars). However, upon ischemic insult neuronal survival in the hippocampal CA1 decreased drastically to (13%) and (10%) in the no virus and AAV-shRNA-control groups respectively (Figure 3.5K, grey bars). Neuronal survival was significantly improved in the Cdc25A knockdown (Figure 3.5E & K) compare with control-shRNA group (Figure 3.5D & K); (10% vs 51%, in the control and Cdc25A knockdown respectively, $p<0.05$). No difference in neuronal survival was observed in the Cdc25B (Figure 3.5F & K) and Cdc25C (Figure 3.5G & K) groups as

compared to control-shRNA. These results are in agreement with our *in vitro* finding demonstrating a role for Cdc25A in delayed neuronal death.

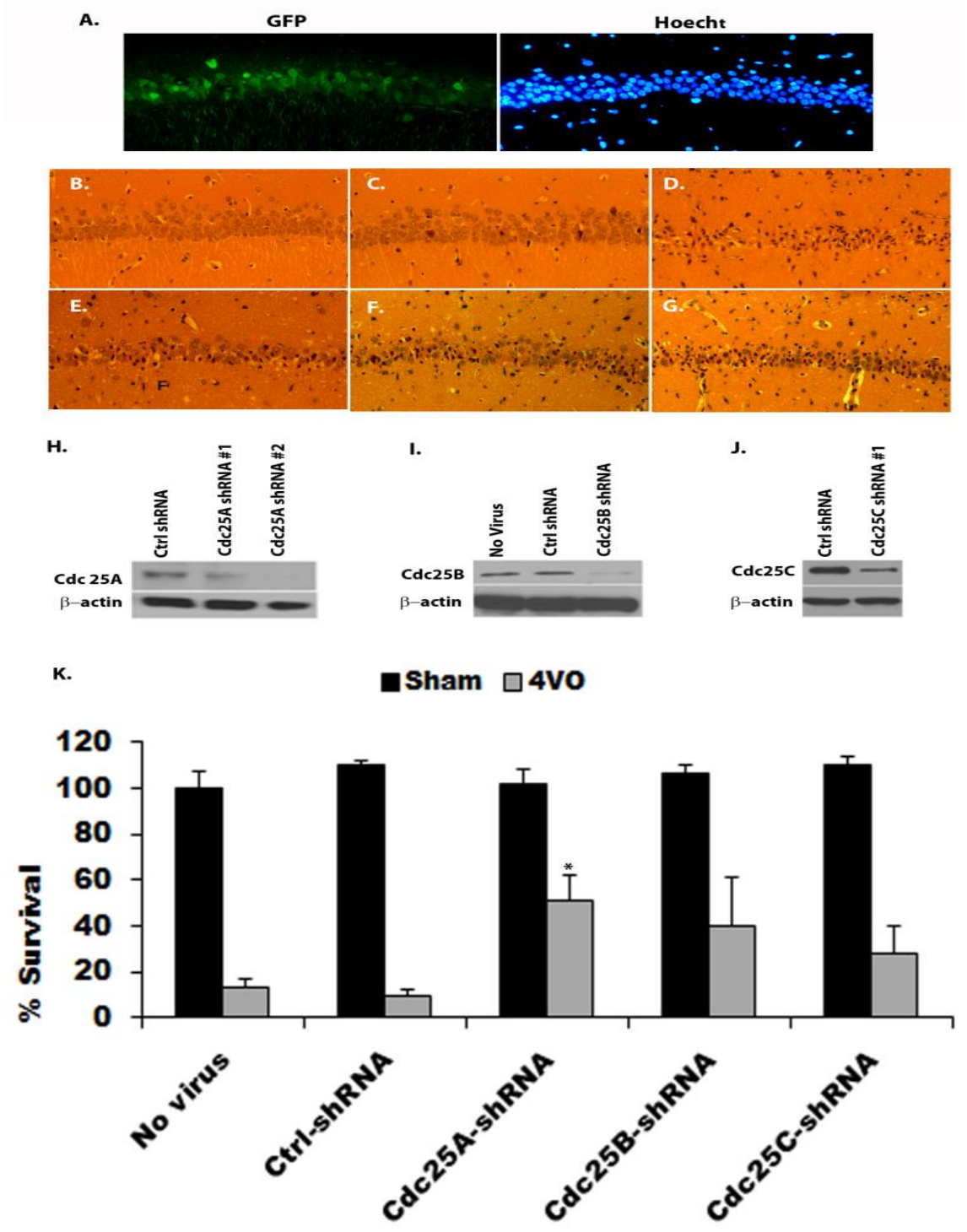
A.
GFP
Hoecht
B.
C.
D.
E.
F.
G.
H.
Ctrl shRNA
Cdc25A shRNA #1
Cdc25A shRNA #2
Cdc 25A
β–actin
I.
No Virus
Ctrl shRNA
Cdc25B shRNA
Cdc25B
β–actin
J.
Ctrl shRNA
Cdc25C shRNA #1
Cdc25C
β–actin
K.
Sham
4VO
% Survival
120
100
80
60
40
20
0
*
No virus
Ctrl-shRNA
Cdc25A-shRNA
Cdc25B-shRNA
Cdc25C-shRNA

Figure 3.5

Figure 3.5. Cdc25A knockdown protects rat hippocampal CA1 neurons from delayed death mediated by global cerebral ischemia. (A) Immunohistochemistry of GFP expression in hippocampal CA1 neurons in rats injected with AAV expressing control (ctrl)-shRNA. (B-G) Representative hematoxylin and eosin stained sections of hippocampal CA1 neurons of (B) non-AAV injected sham (C) ctrl-shRNA sham (D) ctrl-shRNA + 4VO (E) Cdc25A-shRNA + 4VO (F) Cdc25B-shRNA + 4VO (G) Cdc25C-shRNA + 4VO animals. (H-J) Western blot analysis showing (H) Cdc25A, (I) Cdc25B and (J) Cdc25C knockdown in rats injected with AAV-shRNA to Cdc25A, B and C respectively. (K) Quantitation of surviving CA1 neurons four days after 10 minutes 4VO in non-injected and AAV injected rats. Data are expressed as + SEM, * Denotes statistical significance $p<0.05$ compared to shRNA control, $N\geq3$.

Cdc25A phosphatase activity is induced following ischemic insults.

Cdc25A levels and activity has been shown to change under neuro-pathologic stress conditions including AD and DNA damage (Ding et al., 2000, Zhang et al., 2006). In addition, the preponderance of the evidence above, both *in vitro* and *in vivo* indicates the critical nature of Cdc25A in ischemia-induced delayed death. Accordingly, we focused our efforts on examining the levels and activity of Cdc25A following global ischemia. As shown in figure 3.6A, Cdc25A levels did not significantly change up to 12 hours after global ischemic insult *in vivo*. Cdc25A activity increased 8 hours following global ischemia (Figure 3.6C). Similarly, Cdc25A activity was also induced 2 and 8 hours following hypoxia of CGN cultures *in vitro* (Figure 3.6D). Taken together, these results suggest that Cdc25A activity is induced following ischemic insult.

Cdc25A knockdown attenuates pRb phosphorylation following global ischemia.

We have shown that pRb is increasingly phosphorylated at Ser795, a Cdk4 targeted site, following ischemic insult (Rashidian et al., 2005). Cdk4 has been shown to mediate neuronal death by modulating the activity of downstream pRb/E2F (Rashidian et al., 2005). Thus, we asked whether Cdc25A signals death via Cdk4 by examining pRb phosphorylation at Ser795 following global ischemia. As shown in figure 3.6E, pRb is induced 12 hours following 4VO. This ischemia-induced pRb phosphorylation is attenuated by the expression of Cdc25A shRNA (Figure 3.6E). Thus, our result indicates that downregulation of Cdc25A inhibits the activation of Cdk4 mediated downstream target, pRb/E2F, a pathway previously shown to be critical in stroke.

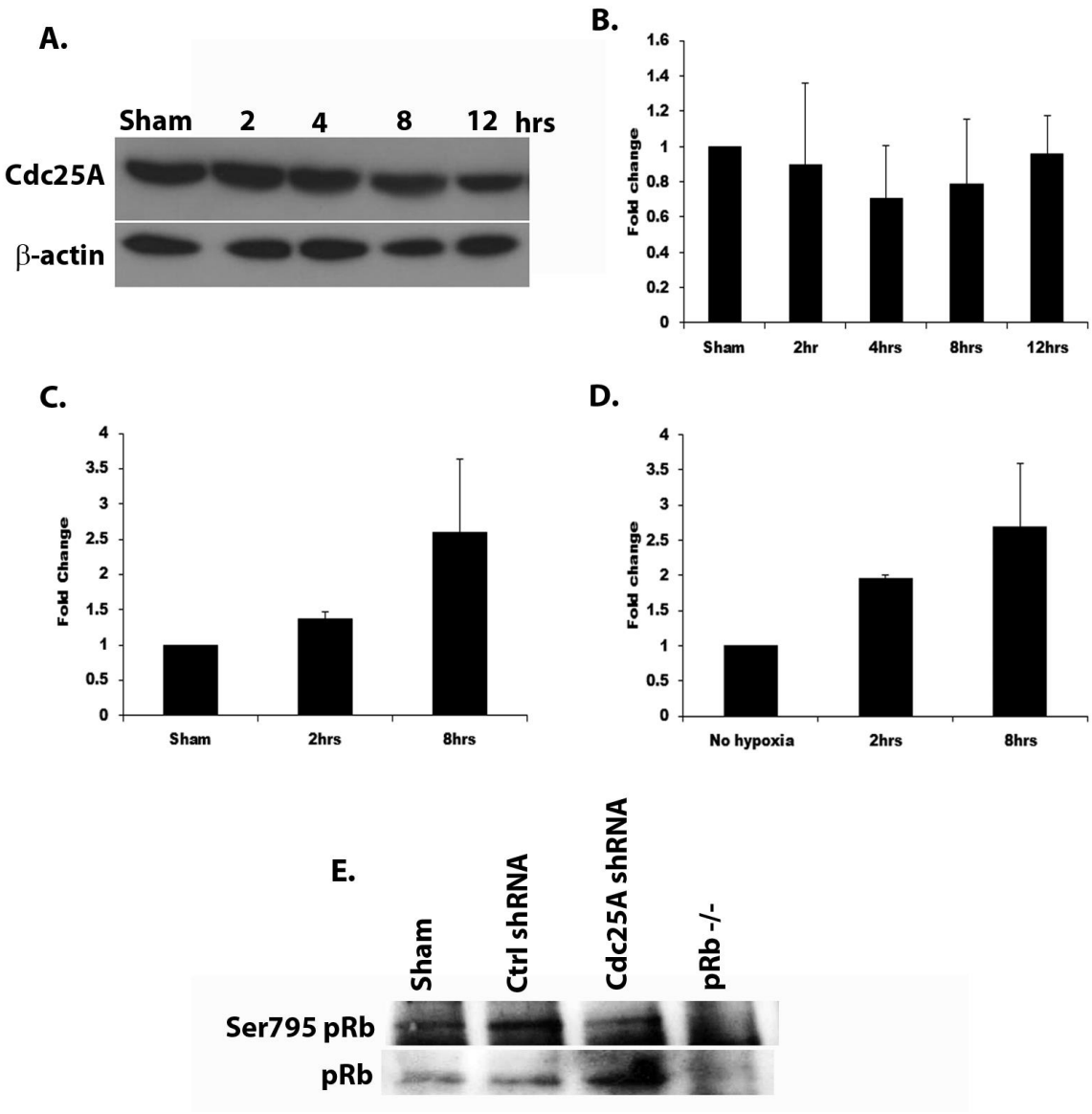
A.
Sham
2
4
8
12 hrs
Cdc25A
β-actin
B.
Fold change
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Sham
2hr
4hrs
8hrs
12hrs
C.
Fold Change
4
3.5
3
2.5
2
1.5
1
0.5
0
Sham
2hrs
8hrs
D.
Fold change
4
3.5
3
2.5
2
1.5
1
0.5
0
No hypoxia
2hrs
8hrs
E.
Sham
Ctrl shRNA
Cdc25A shRNA
pRb -/-
Ser795 pRb
pRb

Figure 3.6

Figure 3.6. Cdc25A activity is increased following ischemic insults. (A) Western blot analysis time course of Cdc25A protein levels following global ischemia (B) Densitometry of Cdc25A protein levels as in (A), N=2. (C &D) Cdc25A phosphatase activity is increased following (C) 4VO in rats (N=2) and (D) hypoxia in CGN *in vitro,* (N=2). Cdc25A protein was immunoprecipitated following insult and subjected to phosphatase assay using pNPP as substrate. Cdc25A activity was assayed by measuring the liberation of pNPP substrate at OD410. Results are expressed as fold over control + SD. (E) Western blot analysis of hippocampal nuclear lysate from rats injected with Cdc25A or control shRNA and subjected to 10 minutes 4VO, followed by reperfusion for 12 hours. Blots were probed with anti-pRb Ser795 and anti-pRb antibodies.

DISCUSSION

The importance of cyclin D/Cdk4/pRb in ischemic neuronal death signaling has previously been demonstrated (Rashidian et al., 2005). However, very little is known of the proximal signals that activate Cdk4 in the context of cerebral ischemic insults. In the present study, we have investigated the Cdc25s as potential upstream regulator(s) of Cdk4 associated death signal in ischemia. Accordingly, we used three different *in vitro* and one *in vivo* model to examine whether the CDC25s are involved in ischemic neuronal death. Our data using the pharmacologic inhibitor NSC95397 suggest that Cdc25s in general are critical for delayed but less so in excitotoxic death in two different *in vitro* models. This inhibition paradigm is noteworthy on at least two counts. First, treatment with the Cdc25 inhibitor prevented neuronal death for up to 4 days following hypoxic insult without reduction in the level of protection over time. Second, protection by Cdc25 inhibition was associated with a significant therapeutic window (2 hours).

A closer examination of whether all three or a single member of the Cdc25 phosphatase family is required for ischemic neuronal death presents a more complex picture. The loss of Cdc25A is protective in delayed ischemic death. In contrast, its loss sensitizes neurons to excitotoxicity. Cdc25B/C is less relevant in delayed death but appear to play a pro-death role only in select conditions of excitotoxicity. For example, where glutamate mediated mechanisms predominates, single or compound deficiency of Cdc25B/C weakly promoted protection. Under conditions where glutamate exposure is the direct mode of insult, neither Cdc25B nor C appears to play a critical role, although their compound deficiency was marginally but significantly protective. In the global model of cerebral ischemia, where again multiple signals likely exist (Abe et al., 1995, Nitatori et al., 1995,

Nakamura et al., 1999, Won et al., 2001, Pagnussat et al., 2007), Cdc25A deficiency is protective while Cdc25B/C deficiencies only show a trend towards protection. These latter observations are consistent with a more delayed nature of death in the 10 minutes four vessel occlusion model of death *in vivo*. Overall, our results suggest that Cdc25A plays a critical role in delayed ischemic neuronal death while other Cdc25 members may participate in excitotoxicity under select contexts.

How might Cdc25A promote cell death? One way in which Cdc25A may mediate neuronal death is through Cdk4. In multiple neuronal death paradigms, activation of Cdk4 is associated with an increase in Ser795 pRb phosphorylation (Park et al., 2000a, Rashidian et al., 2005, Zhang et al., 2006). We show, in the present study, that pRb phosphorylation at Ser795 phosphorylation is attenuated upon downregulation of Cdc25A in the global ischemia model. This suggests that Cdc25A may signal death following ischemia through Cdk4 activation. In agreement with this, expression of dominant negative Cdk4 is protective against delayed death induced by ischemic insults and is associated with attenuation of Ser795 pRb phosphorylation (Rashidian et al., 2005). Induction of Cdk4 activity and pRb phosphorylation has been reported in other neuronal death models including DNA damage (Park et al., 2000a, Zhang et al., 2006). Interestingly, we have shown that Cdc25A is also required for neuronal death induced by DNA damage (Zhang et al., 2006) suggesting that Cdc25A mediated activation may be a common mechanism whereby cell cycle signals are activated in delayed neuronal death.

Taken together, our results show that Cdc25A plays a central role in delayed neuronal death induced by ischemia. Importantly, our data support a model whereby Cdc25A activity is induced and leads to Cdk4 activation following cerebral ischemia. Consequently, pRb is

phosphorylated, leading to cell death. In other neuronal death paradigms, such as those induced by NGF deprivation (Biswas et al., 2005) and β-amyloid (Biswas et al., 2007), activation of Cdk4 is associated with induction of more downstream death effectors such as Myb and Bim. The relevance of these factors in Cdk4 signaling in the context of ischemic neuronal death is presently not clear and is a subject for future studies.

ACKNOWLEDGEMENTS:

We thank Dr. Piwnica-Worms, Washington University School of Medicine for generously providing us with Cdc25B/C transgenic mice. This work was supported by grants from the Heart and Stroke Foundation of Canada (HSFC), the Heart and Stroke Foundation of Ontario (HSFO), the Canadian Institutes of Health Research (CIHR), the Center for Stroke Recovery (CSR), the Neuroscience Canada/Krembil Foundation (DSP); DSP is a HSFO scholar; GOI is a HSFC Doctoral Research Award and the Queen Elizabeth II Graduate Scholarships in Science and Technology recipient.

CHAPTER 4

Regulation of ischemic neuronal death by E2F4/p130 complexes.

Grace O. Iyirhiaro, Yi Zhang, Carmen Estey, Michael J. O'Hare, Farzaneh Safarpour, Mohammad Parsanejad, Suzi Wang, Elizabeth Abdel-Messih, Steve M. Callaghan, Matthew J. During, Ruth S. Slack, and David S. Park.

The Journal of Biological Chemistry 2013, (In Revision).

STATEMENT OF AUTHOR CONTRIBUTION

This manuscript examines the role of E2F1 and E2F4 (downstream effectors of mitotic Cdks) in ischemic neuronal death. The relative contribution of select activating and repressive E2F members to neuronal death is examined using both DNA damage and ischemic models *in vitro*. Changes in p130/E2F4 as well as E2F target genes in response to ischemic insult are also examined in this manuscript

G.O Iyirhiaro designed and performed the experiments contained in Figures 4.3, 4.5, 4.6 and 4.7. The experiments contained in figure 4.1 were carried out by Dr. M.J O'Hare. Experiments contained in figures 4.2 and 4.4 were performed by Yi Zhang. Densitometry analysis contained in figures 4.4B and D was performed by G.O Iyirhiaro. C. Estey (lab technician) assisted with 4VO surgeries and performed immunofluorescence staining on rat brain sections. E. Abdel-Messih, F. Safarpour, M. Parsanejad and S. Wang provided technical assistance. S.M Callaghan generated the viruses used in the experiments and managed the E2F4 transgenic mouse colony, including genotyping. Dr. M.J During generated the AAV vector used to produced virus. Dr. R.S Slack contributed reagents. The manuscript was written and prepared by G.O Iyirhiaro with scientific and editorial guidance from Dr. D.S Park.

Regulation of ischemic neuronal death by E2F4/p130 complexes.

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Running title: *E2F4/p130 complexes in hypoxia/ischemia in neurons*

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Dept. of Cellular and Molecular Medicine, Neuroscience,

University of Ottawa, 451 Smyth Road

Ottawa, ON, Canada

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ABSTRACT

Inappropriate activation of cell cycle proteins, in particular cyclin D/Cdk4, is implicated in neuronal death induced by various pathologic stresses including DNA damage and ischemia. Key targets of Cdk4 in proliferating cells include members of the E2F transcription factors which mediate the expression of cell cycle proteins as well as death inducing genes. However, the presence of multiple E2F family members complicates our understanding of their role in death. Presently, we focused on whether E2F4, an E2F member believed to exhibit crucial control over the maintenance of a differentiated state of neurons, may be critical in ischemic neuronal death. We observed that in contrast to E2F1 and 3 which sensitize to death, E2F4 plays a crucial protective role in neuronal death evoke by DNA damage, hypoxia and global ischemic insult both *in vitro* and *in vivo*. E2F4 occupies promoter regions of pro-apoptotic factors such as B-Myb under basal conditions. Following stress exposure, E2F4/p130 complexes are rapidly lost along with the presence of E2F4 at E2F containing B-Myb promoter sites. In contrast, E2F1 presence at B-Myb sites increases with stress. Furthermore, B-Myb and C-Myb expression increases with ischemic insult. Taken together, we propose a model by which E2F4 plays a protective role in neurons from ischemic insult by forming repressive complexes which prevent pro-death factors such as B-Myb from being expressed.

INTRODUCTION

Inappropriate activation of cell cycle machinery is implicated in a number of neuronal death models induced by NGF deprivation (Park et al., 1997a), excitotoxicity (Park et al., 2000b), oxidative stress (Schwartz et al., 2007), DNA damage (Park et al., 1997b) and stroke (Osuga et al., 2000, Gendron et al., 2001, Wang et al., 2002, Rashidian et al., 2005). For example, increase in cyclin D1 levels and its associated kinase activity is observed following DNA damage (an upstream mediator of stroke damage) and ischemic insult (Osuga et al., 2000, Katchanov et al., 2001, Rashidian et al., 2005, Wen et al., 2005). In addition, treatment with pharmacologic cyclin-dependent kinase (Cdk) inhibitors such as flavopiridol protects neurons from both DNA damage and ischemia induced cell death (Park et al., 1997b, Osuga et al., 2000, Wang et al., 2002). Expression of a dominant negative form of Cdk4, an important regulator of the G1/S-phase of the cell cycle, is protective following DNA damage and global cerebral ischemia (Park et al., 1998a, Rashidian et al., 2005). Together, these studies reveal a crucial role for Cdk4 in neuronal death induced by DNA damage and ischemic insult. However, the downstream effector(s) of cell cycle re-activation in neurons under ischemic stress remains unclear. In this regard, members of the E2F transcription factors may play a pivotal role.

The E2Fs consist of eight related family members, generally classified as activators (E2F1, 2, and 3a) or repressors (E2F3b, 4-8) on the basis of their ability to promote or repress gene transcription (Iaquinta and Lees, 2007). The activity of E2Fs (E2F1-5) is regulated by their association with the pocket proteins which include retinoblastoma protein (pRb), p107 and p130 (Dimova and Dyson, 2005, Iaquinta and Lees, 2007). E2F association with pocket proteins can promote or repress the expression of its targets which include genes

involved in cell cycle progression, DNA damage and apoptosis (Dimova and Dyson, 2005, Iaquinta and Lees, 2007). For example, in dividing cells, pRb association with E2F prevents the expression of genes required for DNA synthesis and cell cycle progression. However, phosphorylation of pRb by Cdk4/6 disrupts its association with E2F and results in transactivation of its target genes. In addition to transactivation, E2F complexes can also repress gene function. For example, E2F4/p130 can recruit chromatin modification factors such as HDACs to promoters of target genes, to form an active repression complex (Dyson, 1998, Frolov and Dyson, 2004, Liu et al., 2004a, Dimova and Dyson, 2005). In this scheme, phosphorylation of p130 by Cdks disrupts its association with E2F4 resulting in derepression of target gene. Thus E2F can function as effectors of Cdk signaling via the transactivation or derepression of target genes.

The involvement of E2F and in particular E2F1 in cell cycle induced death of neurons under pathologic stress is suggested by a number of key observations. First, phosphorylation of pRb, a preferred partner for activating E2Fs such as E2F1, is observed in neuronal death models including DNA damage and ischemia (Osuga et al., 2000, Park et al., 2000a, Wang et al., 2002). Second, expression of kinase dead Cdk4 and treatment with flavopiridol attenuates pRb phosphorylation following DNA damage and ischemia (Osuga et al., 2000, Park et al., 2000a, Rashidian et al., 2005). Importantly, expression of mutant pRB or dominant negative DP-1, a binding partner to E2Fs, prevents neuronal death following DNA damage and/or hypoxia (Park et al., 2000a, Rashidian et al., 2005). Finally, E2F1 deficiency is protective following ischemic insult both *in vitro* and *in vivo* (MacManus et al., 1999, Gendron et al., 2001, MacManus et al., 2003). These observations suggest that cell cycle reactivation in neuron under pathologic stress signals death through pRb inactivation and activation or de-repression of E2F1 target genes.

In addition, to E2F1 and pRb, other E2Fs may also be important in neuronal death. E2F4/p130 complexes are present basally in post-mitotic neurons suggesting that they may act to repress genes important in neuronal survival/function (Greene et al., 2004, Greene et al., 2007). Interestingly, in the absence of insult, siRNA mediated down-regulation of p130 or E2F4 induced apoptosis of cortical neurons (Liu et al., 2005). In addition, expression of wild type or phosphorylation resistant p130 is protective following NGF deprivation of PC12 neurons (Liu et al., 2005). Whether p130/E2F4 complexes play a role in neuronal death *in vivo* is unclear. In the present study we examined the potential involvement of E2F4 in neuronal death induced by ischemic stress and DNA damage. The results of our study demonstrate an important role for E2F4 in the survival of neurons which contrasts to the role of activating E2Fs such as E2F1/3.

EXPERIMENTAL PROCEDURES

Viral construction. Plasmids containing human E2F4 cDNA sequence was sub-cloned into the SpeI sites of the AM/CBA-pI-WPRE-bGH vector and used to generate recombinant adeno-associated virus (rAAV) as previously described (Zolotukhin et al., 2002). Plasmids containing Bmyb-luciferase with wild type E2F site and a mutant contruct harboring a mutation that abolishes E2F binding have previously been reported (Liu and Greene, 2001). The plasmids were subcloned into AAV vector for efficient delivery into primary neurons. Adenoviruses expressing E2F1 have already been described (O'Hare et al., 2000).

Transgenic mice. E2F1 (Field et al., 1996, McClellan et al., 2007), E2F3 (Leone et al., 2001, McClellan et al., 2007) and E2F4 (Humbert et al., 2000) deficient mice have been previously described. Knockout mice were generated from heterozygous breeding pairs and genotyped by PCR using already published primers (Field et al., 1996, Humbert et al., 2000). E2F1 null mice were genotyped using the following primers: 5'-GGATATGATTCTTGGACTTCTTGG-3'; 5'-CTAAATCTGACCACCAAACGC-3' and 5'-CAAGTGCCAGCGGGGCTGCTAAAG-3'

Cell cultures and treatments. Primary cerebellar granule neurons (CGNs) and cortical neuronal cultures were established as previously described (O'Hare et al., 2000, O'Hare et al., 2005) from CD1 (Charles River, Quebec) or E2F transgenic mice. CGNs were transfected with E2F4 siRNA cocktail or control siRNA (Santa Cruz) using lipofectamine 2000 (Gonzalez et al., 2008) 5 days after plating. Alternatively CGNs were infected with adenovirus expressing E2F1, E2F4 or GFP at the time of plating at MOI 50. Cortical

neuronal cultures were co-transfected with E2F1, E2F3 or E2F4 along with GFP containing plasmids using calcium phosphate method 3 days after plating as previously described (Xia et al., 1996, Gonzalez et al., 2008). Cortical neurons were treated with 10μM camptothecin (Sigma, St. Louis, MO, USA) 3-4 days after plating and were examined for survival at indicated times as previously described (Gonzalez et al., 2008). Cortical cultures from transgenic mice and CGNs were lysed at designated times after insult using a mildly disruptive lysis buffer and evaluated as previously described (Rashidian et al., 2005). Neurons co-transfected with E2F and GFP plasmids were fixed and stained with Hoechst 33342 (Sigma). Viability was assessed by nuclear integrity in GFP positive cells in random fields as previously described (O'Hare et al., 2005).

Hypoxia. CGN cultures were subjected to hypoxia at 1% O_2, and 5% CO_2 balanced with N_2 in a humidified hypoxia chamber (Coy Laboratory Products, Ann Arbor, MI) after one week in culture as previously described (Rashidian et al., 2005). Hypoxia was induced in the presence of 10μM MK801 (Research Biochemicals, Natick, MA), NMDA channel blocker, for 18 hours followed by reoxygenation at normoxia for 24 hours for survival studies. Alternatively, CGN cultures were subjected to varying duration of hypoxia and reoxygenation for biochemical studies. Control cultures were maintained in a humidified incubator at 37^{o}C and were not treated with hypoxia.

Viral Injection. Animal experiments were carried out in accordance with the Canadian Council for the Use and Care of Animals in Research (CCAC) guidelines, with approval from the University of Ottawa Animal Care Committee. Intra-hippocampal recombinant adeno-associated viral (rAAV) injections in rat have been previously described (Rashidian et

al., 2005). Briefly, male Wistar rats (80-125g) were unilaterally injected with rAAV carrying EGFP control or E2F4 (10^{10} genome per microliter) two weeks before four vessel occlusion insult. 2μl of rAAV diluted in PBS was with 1μl of mannitol (20%) was stereotaxically injected using a Harvard infusion pump (Harvard Apparatus) into the hippocampus (from bregma: -3.6mm anterioposterior, $\pm$2.1mm lateral and -2.75mm deep).

Global cerebral Ischemia. Four vessel occlusion (4VO) method of global ischemia was induced as previously described for 10 minutes (Wang et al., 2002, Iyirhiaro et al., 2008). Four days following ischemia rats were perfused, sacrificed and brains were extracted, sectioned, stained for histological assessment. Neuronal viability of cells in the hippocampal CA1 was assessed as previously described (Wang et al., 2002, Iyirhiaro et al., 2008).

Immunohistochemistry. Antigen retrieval and deparafinization was carried out on brain sections as previously described (Iyirhiaro et al., 2008). Following permeabilization with 0.3% triton X-100 for 10 minutes, rat brain sections were blocked in 10% normal goat serum (Jackson Immuno Research) diluted in 2% bovine serum albumin (Fisher Scientific) in 0.01M phosphate buffered saline (PBS) for an hour at room temperature. Sections were incubated with mouse monoclonal anti-GFP (Abcam) or anti-E2F4 antibodies (Abcam, 1:200) overnight at 4^{o}C. For visualization, sections were incubated with Alexa 488 goat anti-mouse (1:200) secondary antibody (Jackson Immuno Research) for 1 hour at room temperature. Neuronal nuclei were stained with hoechst 33342 (Sigma).

Western blotting. Cell and hippocampal tissue samples were collected at designated times after insult and homogenized in solubilization buffer (0.0625M Tris, 2.5mM EDTA, 2.5mM EGTA, 10% glycerol, 2% SDS, 0.001% bromophenol blue and 5% β-mercaptoethanol) (O'Hare et al., 2000). Samples were ran on SDS-polyacrylamide gels and transferred onto PVDF membrane (Milipore). Membranes were probed with the following antibodies: anti-

E2F4 (Abcam), anti-p130 (BD Transduction) anti-phospho p130 (Santa Cruz) and anti β-actin (Sigma). Densitometry was performed on western blots using Image J. software and normalized to loading control. The results were expressed as fold change over sham operated animal in the 4VO time course and over no hypoxia control for hypoxia/reoxygenation experiments.

Semiquantitative reverse transcription-PCR. At the indicated times following 4VO, total RNA was extracted from hippocampal tissues using QIAcube (Qiagen) following manufacturer protocol. 100ng of total RNA was used for cDNA synthesis and targeted gene amplification using SuperScript One-Step RT-PCR kit (Invitrogen). cDNA synthesis and amplification was conducted using the following conditions: 42 ^{o}C for 45 min, 94^{o}C for 2 minutes, followed by cycles of 94 ^{o}C for 1 min, Tm for 30 s, and 72 ^{o}C for 1 min.The rat MYB genes were amplified with the following primers: 5'-GGCTGCCGTGGCTACTACTTCTAA-3' and 5'-CGCGCCGTTTCTTCTGTCG-3' for B-Myb at a Tm of 59 ^{o}C for 35 cycles; 5'-ATGCCCTGGAAGTGAACAAC-3' and 5'-CAGCTTTTGTAAGCGGGTTC-3' for C-Myb at Tm of 54 ^{o}C for 35 cycles. Expression of GAPDH mRNA was used as a standard for loading control. GAPDH was amplified using the following primers: 5'-ATCCGTTGTGGATCTGACATGC-3' and 5'-TGTCATTGAGAGCAATGCCAGC-3 at a Tm of 52 ^{o}C for 28 cycles'. Densitometry was performed on results and normalized to loading control. The results were expressed as fold change over sham operated controls.

ChIP assay. CGNs treated with/without hypoxia and reoxygenation were subjected to chromatin immunoprecipitation (ChIP) assay as previous described (Zhang et al., 2010c). The mouse B-Myb promoter region was amplified using the following primers 5'-CCTCCTCCTTCTCCTCCTTC-3' and 5-CACTATACCCGTGCGCTTCT-3'. PCR products were resolved on an agarose gel. Densitometry was performed on results and expressed as fold change over no hypoxia control.

Luciferase assay. One day after plating, CGNs were infected with wide-type or mutant luciferase viruses along with AAV-β-galactosidase as an internal control. After hypoxia-reoxygenation, cells were lysed in buffer provided in the Promega Luciferase System (Promega). Luciferase assay was performed according to the manufacturer's instructions. Relative luciferase activities were obtained by normalizing the luciferase activity against β-galactosidase activity. Results were presented as fold increase in reference to control values.

RESULTS.

E2F family members differentially regulate neuronal death mediated by genotoxic stress.

Aberrant activation of neuronal cell cycle induced by pathologic stress such as DNA damage and ischemic insult is thought to contribute to cell death through regulation of E2F members (Osuga et al., 2000, Park et al., 2000a, Zhang et al., 2010c). However, the relative contribution of different E2F members in neuronal death is not clear. To begin to examine the involvement of different E2F members, we focused initially on the effect of select activating E2Fs (E2F1, E2F3) and the repressive E2F4 members in neuronal death. Primary cortical neurons null for these E2F members were treated with the DNA damaging agent camptothecin and evaluated for survival. Neurons null for E2F1 (Figure 4.1A) and E2F3 (Figure 4.1B) were more resistant to DNA damage induced death when compared to those from their littermate controls. E2F1-/- neurons were significantly more resistant to DNA induced death at 8 hours (59% survival, $p<0.01$) and 12 hours (23% survival, $p<0.05$) compared with wild type control (43% and 13% survival at 8 and 12 hours respectively). Similarly, neuronal survival in the E2F3-/- (59% and 27%) was greater than that observed for the wild type (43% and 20%), $p<0.01$ and $p<0.05$ at 8 and 12 hours respectively. In contrast, E2F4 deficiency resulted in sensitization to DNA damage induced death at 12 hours (21% survival) compared with wild type control (44%, $p<0.01$; Figure 4.1C).

We next tested the effect of expression of E2F1, E2F3 and E2F4 on neuronal death induced by DNA damage. As shown in figure 4.1D-F, expression of E2F1 (Figure 4.1D) and E2F3 (Figure 4.1E) significantly ($p<0.01$) reduced neuronal survival compared with neurons transfected with vector only. In contrast, over-expression of E2F4 resulted in an increase in

neuronal survival (46% in vector and 65% in E2F4 expressing neurons, $p<0.01$). Together, these results demonstrate a pro-apoptotic role for E2F1 and E2F3 and a pro-survival role for E2F4 in neuronal death induced by DNA damage.

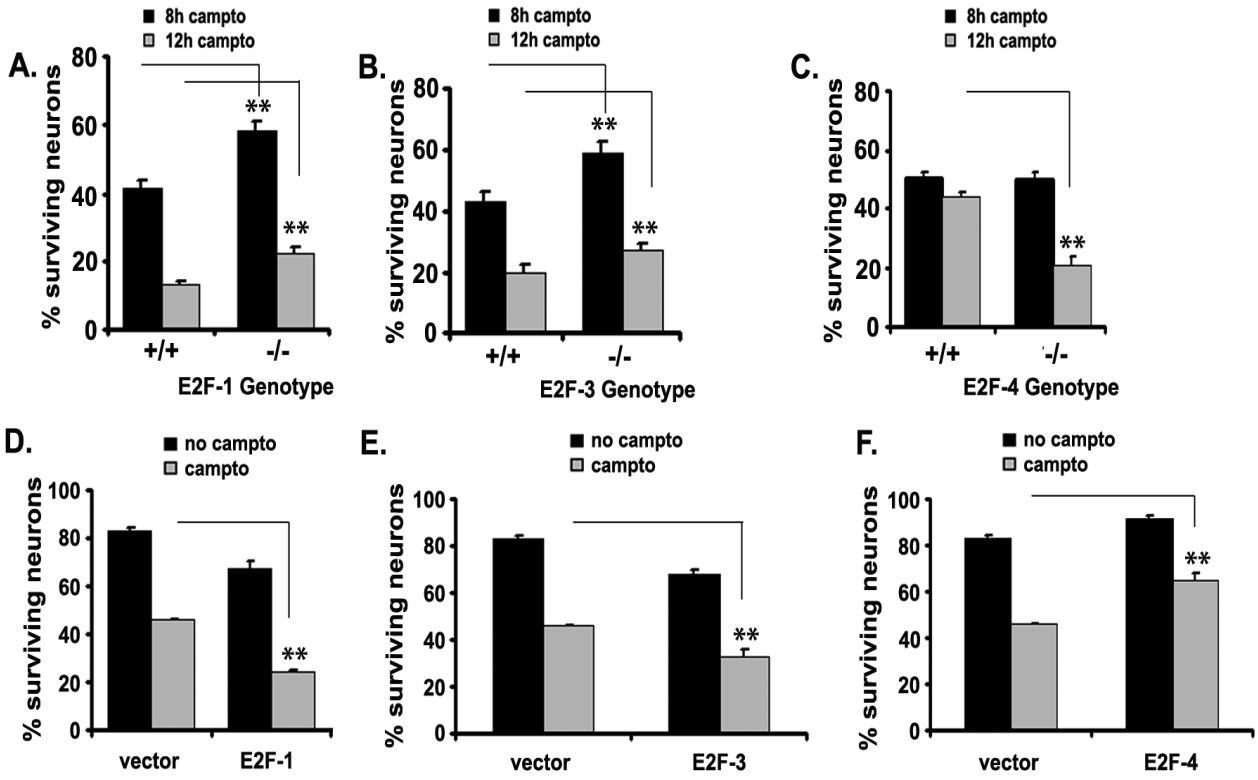
A.
8h campto
12h campto
% surviving neurons
80
60
40
20
0
**
**
+/+
-/-
E2F-1 Genotype
B.
8h campto
12h campto
% surviving neurons
80
60
40
20
0
**
**
+/+
-/-
E2F-3 Genotype
C.
8h campto
12h campto
% surviving neurons
80
60
40
20
0
**
+/+
-/-
E2F-4 Genotype
D.
no campto
campto
% surviving neurons
100
80
60
40
20
0
**
vector
E2F-1
E.
no campto
campto
% surviving neurons
100
80
60
40
20
0
**
vector
E2F-3
F.
no campto
campto
% surviving neurons
100
80
60
40
20
0
**
vector
E2F-4

Figure 4.1

FIGURE 4.1. E2F family members play differential role in neuronal death induced by genotoxic stress. A, E2F1 B, E2F3 and C, E2F4 wild type and knock-out neurons were treated with 10μM camptothecin and evaluated for survival at the indicated times. D, E2F-1 E, E2F-3 and F, E2F-4 containing plasmid or vector control were transfected in cortical neuronal cultures and treated with camptothecin. Survival was assessed 24 hours later. Results are expressed as % of control + SEM. $P<0.05$, $^{**}p<0.01$.

E2F1 and E2F4 have opposing effect on neuronal survival following hypoxia/reoxygenation.

We next determined the effect of E2Fs in a more physiologically relevant model of ischemic neuronal death *in vitro* with focus on E2F1 and 4. To this end, primary cerebellar granule neurons (CGNs) infected with adenovirus expressing E2F1, E2F4 or GFP were subjected to 18 hours of hypoxia in the presence of MK801 followed by reoxygenation for 24 hours. Neurons treated in this fashion die in a delayed manner dependent upon cell cycle activation (Rashidian et al., 2005). Consistent with our observation with DNA damage, expression of E2F1 (Figure 4.2A) significantly reduced neuronal survival compared to GFP control (24% survival in E2F1 vs 45% in GFP expressing neurons, $p<0.01$). In contrast, expression of E2F4 was significantly protective following hypoxia/reoxygenation compared with GFP control (65% survival in E2F4 vs 45% in the GFP expressing neurons, $p<0.01$) (Figure 4.2A).

Loss of function studies was consistent with the observations above (Figure 4.2, C and D). In this regard, we focused on the role of E2F4 since loss of function studies with E2F1 following ischemic insult has previously been shown to promote survival (MacManus et al., 1999, Gendron et al., 2001, MacManus et al., 2003, Smith et al., 2003b). CGNs were transfected with siRNA to E2F4 or control siRNA and subjected to hypoxia and reoxygenation. E2F4 knock-down in transfected culture was verified by western blot analysis (Figure 4.2B). We observed sensitization of neurons to death induced by hypoxia/reoxygenation when E2F4 was transiently knocked-down (3% survival) compared to siRNA control (29% survival), $p<0.001$. Interestingly, neurons transfected with E2F4 siRNA were remarkably more vulnerable to neuronal death even in the absence of any insult (19% survival in E2F4 knock-down vs 84% in siRNA-control cultures, $p<0.001$) (Figure

4.2C). CGNs from E2F4-/- and E2F4+/+ mice were also similarly subjected to hypoxia/reoxygenation. E2F4-/- neurons also showed increased sensitivity to hypoxia-induced neuronal death (23% survival in E2F4 null vs 58% in wild type neurons, $p<0.01$) (Figure 4.2D). These results suggest that unlike E2F1, E2F4 plays a pro-survival role in neuronal death induced by hypoxia.

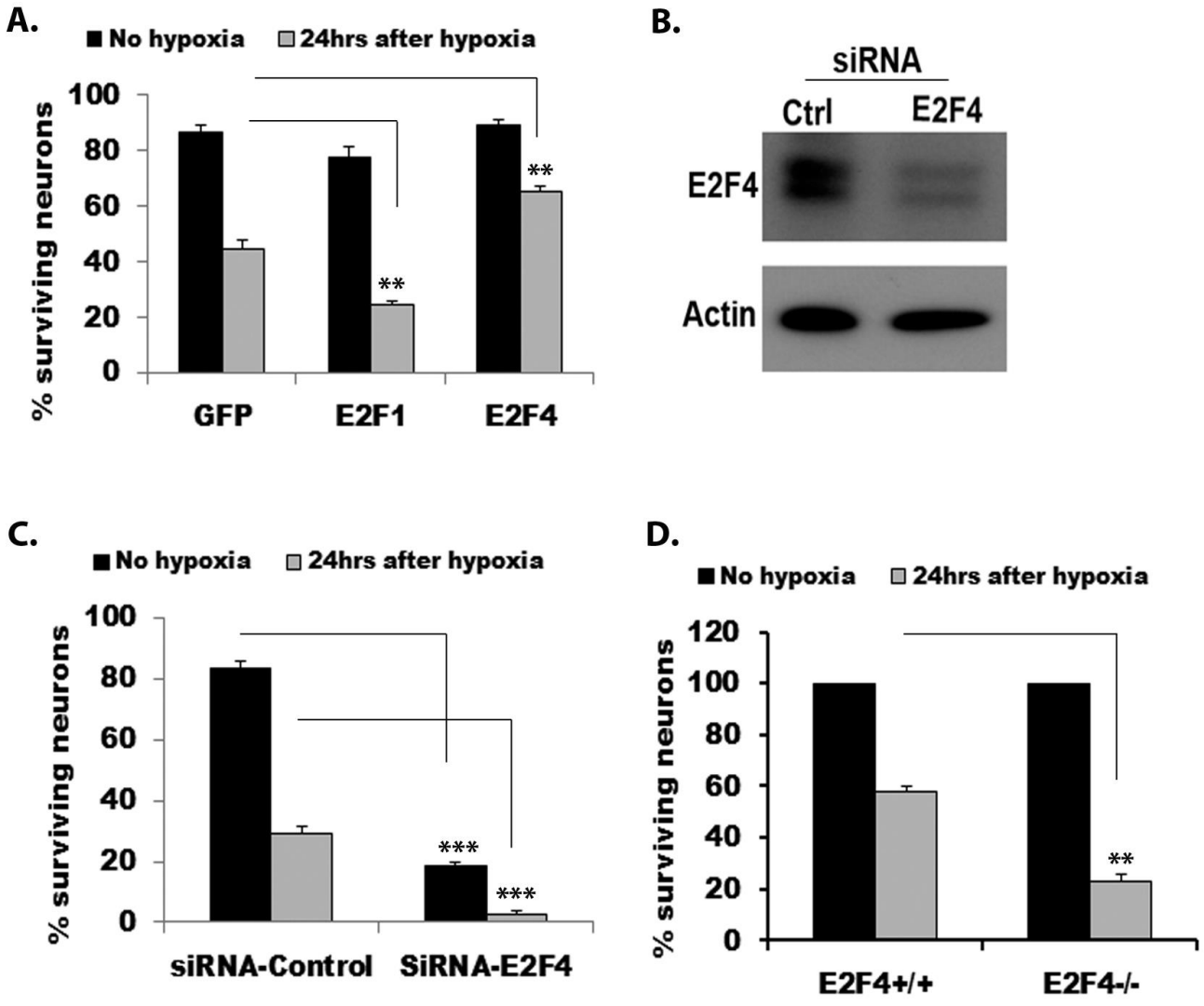
A.
No hypoxia
24hrs after hypoxia
% surviving neurons
100
80
60
40
20
0
**
**
GFP
E2F1
E2F4
B.
siRNA
Ctrl
E2F4
E2F4
Actin
C.
No hypoxia
24hrs after hypoxia
% surviving neurons
100
80
60
40
20
0
***
***
siRNA-Control
SiRNA-E2F4
D.
No hypoxia
24hrs after hypoxia
% surviving neurons
120
100
80
60
40
20
0
**
E2F4+/+
E2F4-/-

Figure 4.2

FIGURE 4.2. E2F1 and E2F4 have opposing roles in neuronal death induced by hypoxia.

A, Primary CGN cultures were infected with adenovirus expressing E2F1, E2F4 or GFP control and then treated with hypoxia followed by reoxygenation for 24 hours. B, western blot showing E2F4 knock-down in CGN cultures treated with E2F4 siRNA. C, CGNs transfected with E2F4 or control siRNA and D, CGNs from E2F4 KO and wild type mice were similarly subjected to hypoxia and reoxygenation as in (A). Neuronal survivals were evaluated 24 hours after hypoxia. Results are expressed as % of control + SEM. **$p<0.01$, ***$p<0.001$.

E2F4 and p130 proteins are reduced following hypoxia/reoxygenation in vitro.

E2F4 is known to form repressive complexes with p130 in neurons which may be important in neuronal survival (Liu et al., 2005). Thus, we asked whether there were perturbations in total E2F4 and p130 protein levels following hypoxia/reoxygenation insult. CGN cultures were subjected to varying duration of hypoxia or 18 hours of hypoxia followed by varying reoxygenation times. Total proteins was harvested at the indicated times and analysed by western blot (Figure 4.3, A and C). Blots were probed with antibody against E2F4 (Figure 4.3A) or p130 (Figure 4.3C). E2F4 (Figure 4.3, A and B) protein levels were significantly ($p<0.05$) diminished immediately following hypoxia and remained reduced for up to 8 hours following reoxygenation when compared to untreated cultures. While, there was a slight re-induction of E2F4 levels at 2 hours reoxygenation, it did not reach the levels of untreated controls. Similar, to E2F4, p130 (Figure 4.3, C and D) protein levels were also drastically reduced ($p<0.05$) immediately following hypoxia with a brief re-induction at 4 hours reoxygenation. However, levels appeared to diminish again thereafter. The reason for this induction is unclear at the moment.

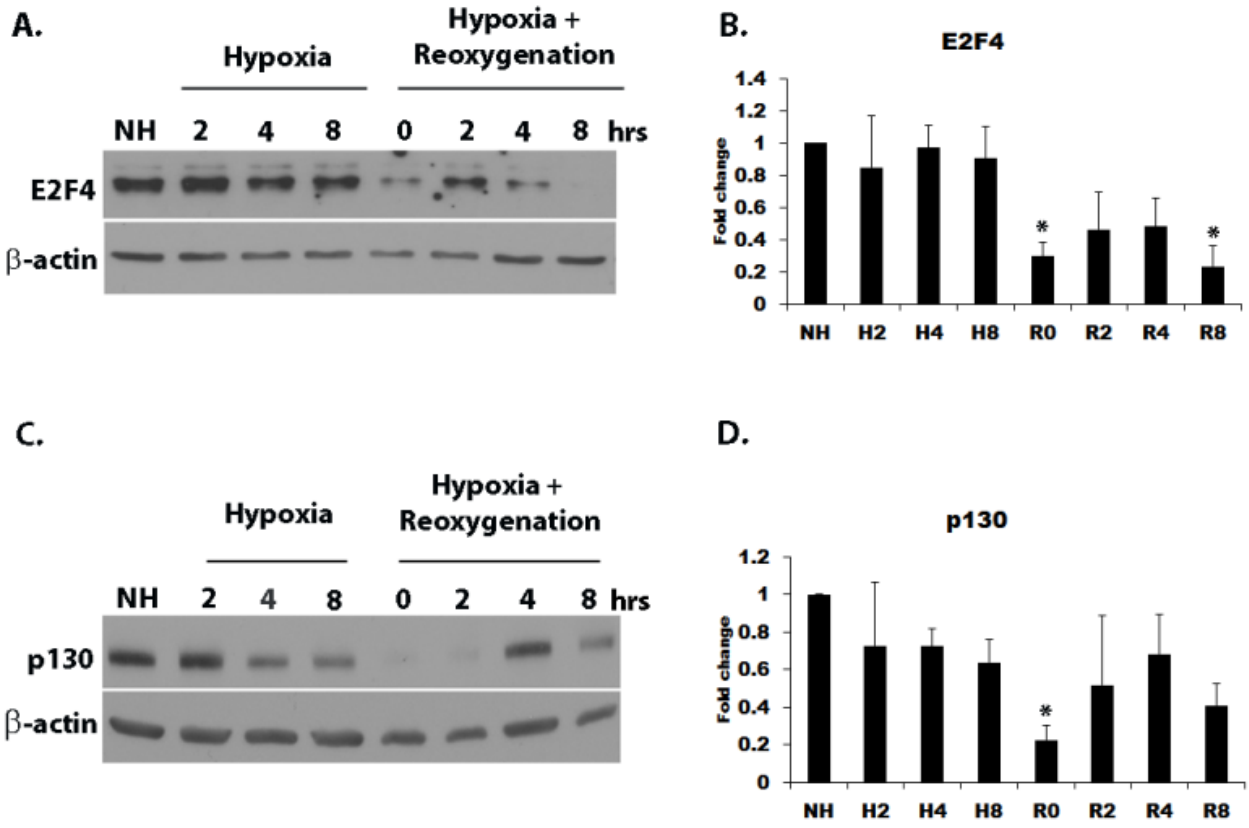
A.
Hypoxia
Hypoxia + Reoxygenation
NH 2 4 8 0 2 4 8 hrs
E2F4
β-actin
B.
E2F4
Fold change
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NH H2 H4 H8 R0 R2 R4 R8
C.
Hypoxia
Hypoxia + Reoxygenation
NH 2 4 8 0 2 4 8 hrs
p130
β-actin
D.
p130
Fold change
1.2
1
0.8
0.6
0.4
0.2
0
NH H2 H4 H8 R0 R2 R4 R8

Figure 4.3

FIGURE 4.3. p130 and E2F4 protein levels are down-regulated following hypoxia/reoxygenation of CGNS in culture. A, Western blot showing a loss of E2F4 protein in CGNs subjected to varying duration of hypoxia with or without reoxygenation. B, Densitomery of E2F4 protein levels as in (A). C, Western blot showing loss of p130 following hypoxia with or without reoxgenation. D, Densitometry of p130 protein levels as in (C). $N \geq 3$, $^{*}p < 0.05$.

Hypoxia/reoxygenation induces a concomitant reduction of E2F4 and induction of E2F1 binding at the B-Myb promoter.

The previous evidence indicates that p130 is and then lost following ischemia. We next determined how E2F members such as E2F1 or 4 may differ in binding to known E2F sites. In this regard, we focused on B-Myb, a pro-death factor previously described for neurons and regulated by E2Fs (Liu et al., 2004a, Liu et al., 2005). We examined the relative promoter occupancy of both E2F1 and E2F4 following hypoxia/reoxygenation *in vitro*. Chromatin immunoprecipitation (ChIP) was carried out on lysates from CGN cultures treated with 18 hours hypoxia and 16 reoxygenation using E2F1 and E2F4 specific antibodies. E2F1 and E2F4 associated chromatin was subjected to PCR using B-Myb promoter specific primers. As shown in figure 4.4A, endogenous E2F4 occupancy at the B-Myb promoter was present in the untreated no hypoxia samples under basal conditions. This occupancy decreased with hypoxia/reoxygenation (Figure 4.4, A and B). This contrasts with E2F1 where occupancy is low under basal conditions and increases with hypoxic stress (Figure 4.4, A and B). Consistent with the loss of E2F4, ChIP analysis performed using p130 specific antibody also showed that it is lost from the B-Myb promoter following hypoxia/reoxygenation (Figure 4.4C). This result indicates that E2F1 and E2F4 may mutually regulate B-Myb in an opposing manner to increase B-Myb expression and promote death. To further investigate this possibility, we next examined for changes in E2F mediated activity at the B-Myb promoter. To this end, CGNs were infected with AAV expressing E2F reporter constructs, B-Myb-promoter-luciferase containing wild type or mutated E2F sites as control, and β-galactosidase one day after plating. The cells were treated with hypoxia and varying duration of reoxygenation after week in culture. Luciferase and β-galactosidase analysis showed that E2F activity at the B-Myb promoter is significantly (2.5 folds, $p<0.05$)

induced immediately following hypoxia and remains elevated during reoxygenation (Figure 4.4E). Because E2F4 is known to form repressive complexes and our results indicates that it is lost following hypoxic stress, we next investigated the effects of its deficiency on overall E2F activity at B-Myb promoter. E2F4 wild type and knock-out CGNs were similarly infected with AAVs expressing the same E2F reporter constructs as above and luciferase activity was measured following 18 hours of hypoxia and 4 hours of reoxygenation (Figure 4.4F). Consistent with our earlier results, luciferase activity increased two folds ($p<0.01$) following hypoxia/rexoygenation in the E2F4 wild type CGNs (Figure 4.4F). In the E2F4 null CGNs, the basal luciferase activity was elevated (2 folds, $p<0.05$) compared with E2F4 wild type cultures and was not further increased with hypoxia/reoxygenation (Figure 4.4F). This result indicates that E2F4 is important in the basal suppression of B-Myb.

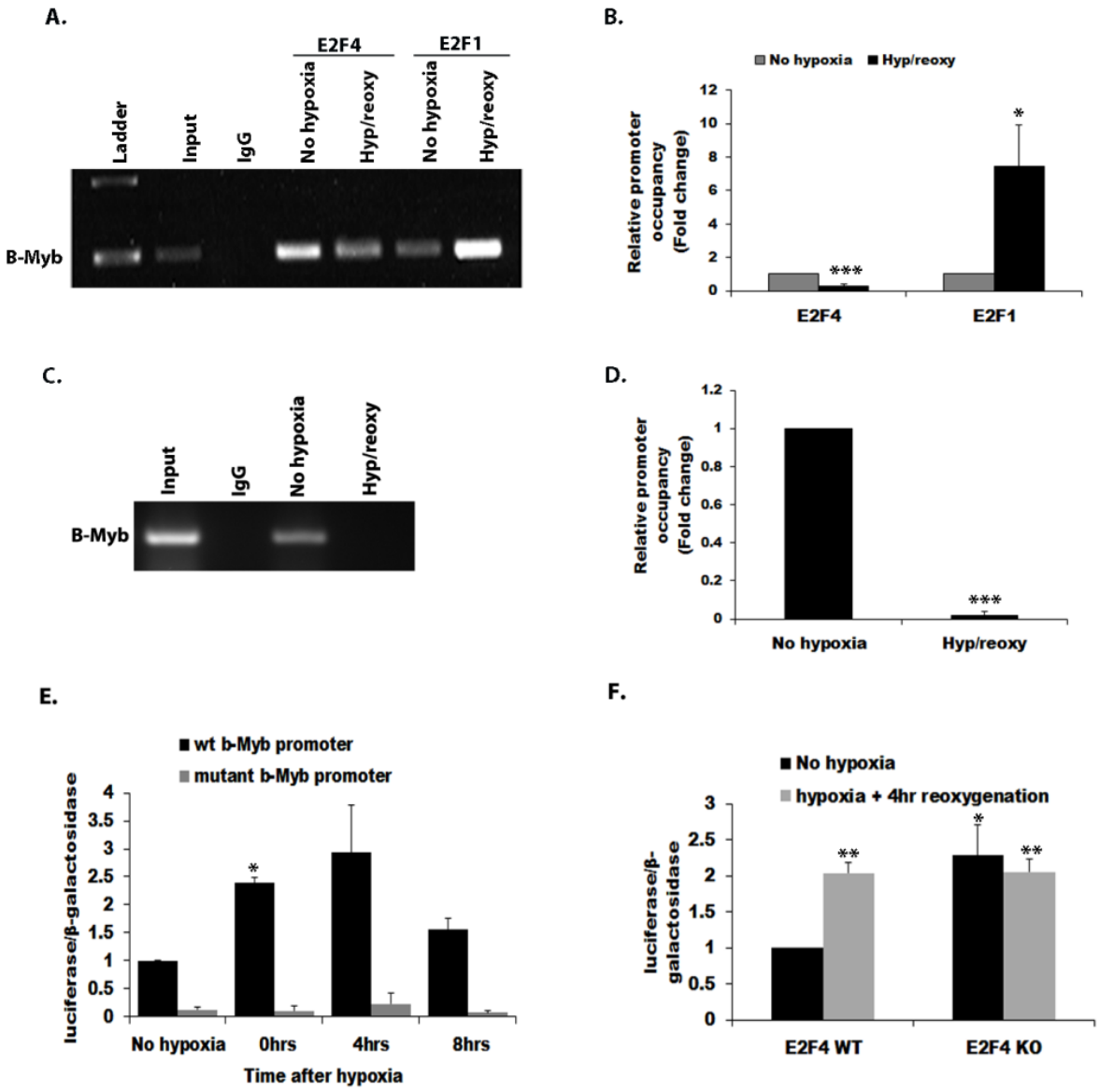
A.
E2F4
E2F1
Ladder
Input
IgG
No hypoxia
Hyp/reoxy
No hypoxia
Hyp/reoxy
B-Myb
B.
No hypoxia
Hyp/reoxy
Relative promoter occupancy (Fold change)
12
10
8
6
4
2
0
*
***
E2F4
E2F1
C.
Input
IgG
No hypoxia
Hyp/reoxy
B-Myb
D.
Relative promoter occupancy (Fold change)
1.2
1
0.8
0.6
0.4
0.2
0
***
No hypoxia
Hyp/reoxy
E.
wt b-Myb promoter
mutant b-Myb promoter
luciferase/β-galactosidase
4
3.5
3
2.5
2
1.5
1
0.5
0
*
No hypoxia
0hrs
4hrs
8hrs
Time after hypoxia
F.
No hypoxia
hypoxia + 4hr reoxygenation
luciferase/β-galactosidase
3
2.5
2
1.5
1
0.5
0
**
*
**
E2F4 WT
E2F4 KO

Figure 4.4

FIGURE 4.4. Reduction of E2F4 and induction of E2F1 binding at the B-Myb promoter following hypoxia and reoxygenation. A, ChIP was conducted with E2F4 and E2F1 antibody on CGNs treated with hypoxia for 18 hours followed by reoxygenation for 16 hours. B, Densitometry was performed on B-Myb signal following ChIP as shown in (A). C, p130 is lost at the B-Myb promoter following hypoxia and reoxygenation as in (A). ChIP was performed with p130 antibody. D, Densitometry of B-Myb signal following ChIP as shown in (C). E, E2F activity increases after hypoxia and reoxygenation in CGN. Cells were infected with AAV expressing B-Myb-promoter-luciferase with wild type E2F or mutated E2F site and β-galactosidase. Cells were treated with 18 hours hypoxia and luciferase activity and β-galactosidase were measured at the indicated times. Data represents values of luciferase/β-galactosidase activity. F, Luciferase assay was conducted in E2F4 wild type and knockout CGNs treated as in (E). Bars represent the mean ±SEM, $N \geq 3$, *$p<0.05$, **$p<0.01$, *** $p<0.005$.

E2F4 expression is protective following transient cerebral ischemia in vivo.

To further investigate the role of E2F4 under ischemic conditions, we next examined its effects in an *in vivo* model of stroke induced by global ischemia. AAV expressing E2F4 or GFP control were unilaterally injected into the hippocampal CA1 region two weeks prior to insult, as previously described (Rashidian et al., 2005). Global ischemia was induced for 10 minutes using the four vessel occlusion method as previously described (Wang et al., 2002, Iyirhiaro et al., 2008). E2F4 over-expression and efficiency of viral delivery was verified by immunohistochemistry staining of CA1 neurons (Figure 4.5A) and confirmed by western blot performed on hippocampal protein lysate from AAV injected rats (Figure 4.5D). Analysis of CA1 neurons four days following global ischemia showed significantly more live CA1 neurons (55% survival) in E2F4 expressing rats compared to those expressing GFP (3%, $p<0.05$) (Figure 4.5, B and C). GFP or E2F4 over-expression alone had no effect on neuronal viability in the absence of insult (Figure 4.5C).

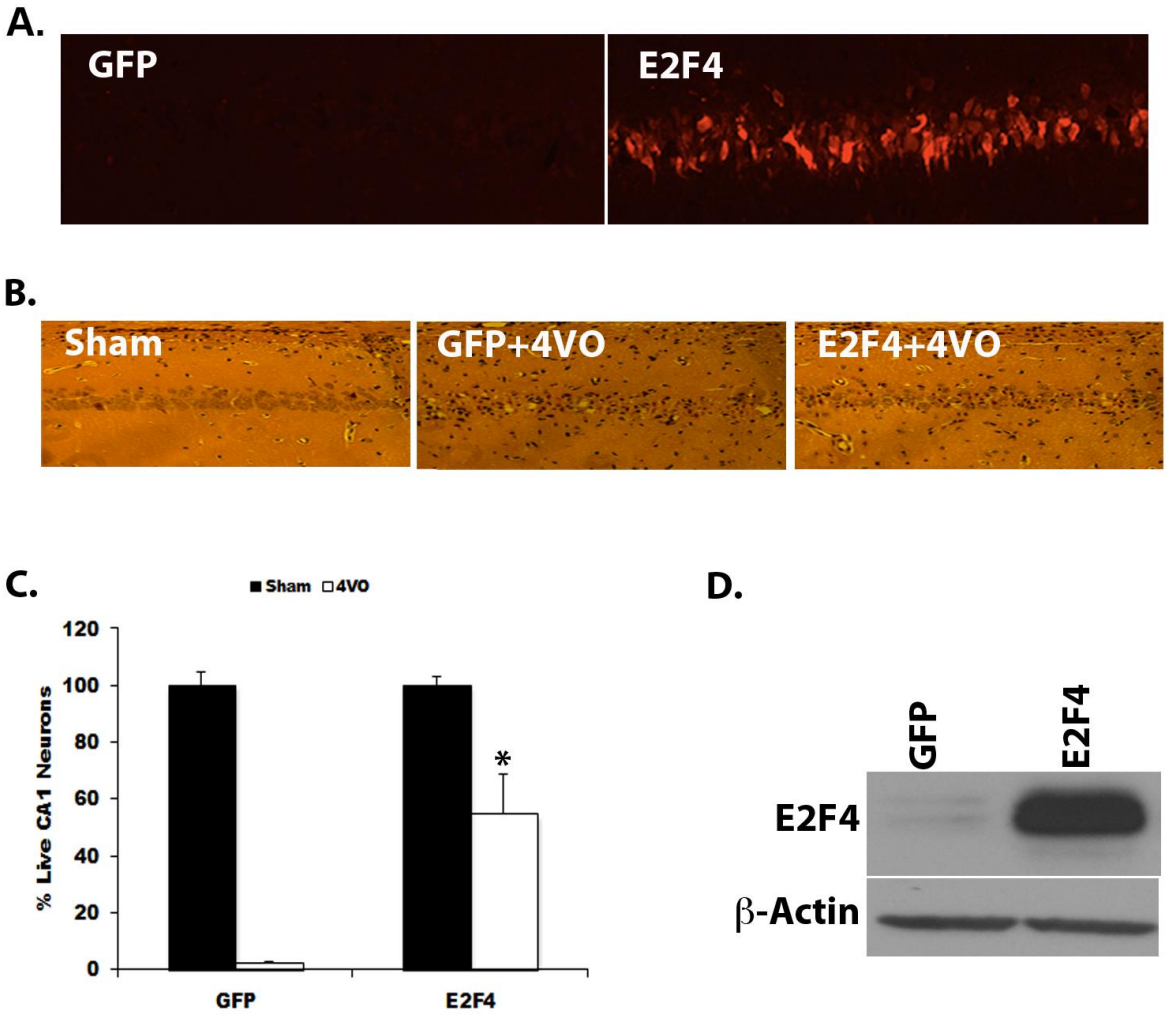
A.
GFP
E2F4
B.
Sham
GFP+4VO
E2F4+4VO
C.
Sham
4VO
% Live CA1 Neurons
120
100
80
60
40
20
0
*
GFP
E2F4
D.
GFP
E2F4
E2F4
β-Actin

Figure 4.5

FIGURE 4.5. E2F4 expression protects CA1 neurons from global cerebral ischemia. A, immunofluorescence staining showing E2F4 overexpression in the hippocampal CA1 neurons of rats injected with AAV expressing E2F4. B, H&E stained sections of CA1 neurons in sham and 4VO operated rats, injected with GFP or E2F4 expressing AAV. C, Quantitation of live CA1 neurons following 4VO in GFP and E2F4 injected rats. $N \geq 4$ per group, data is % + SEM. * Denotes significance at $p<0.05$ compared with GFP control. D, Western blot analysis of E2F4 expression in the hippocampus of GFP and E2F4 injected rats.

E2F4 and p130 proteins are diminished following global ischemia.

We also asked whether perturbations in E2F4 and p130 levels occurred *in vivo* following cerebral ischemia. To examine for these changes, rats were subjected to 10 minutes 4VO and sacrificed at various times following reperfusion, as described in the experimental procedures. Hippocampal lysates were extracted and subjected to western blot analysis (Figure 4.6A). Blots were probed with antibody against p130, E2F4 and actin for loading control. p130 levels were slightly (although not significantly) increased at early time points following ischemia, but declined precipitously 24 hours following reperfusion (Figure 4.6, A and B). Similarly, E2F4 protein level was dramatically reduced 24hrs following ischemia (Figure 4.6, A and C). The biological function of p130 (Dyson, 1998, Hansen et al., 2001), as well as its stability and downregulation (Tedesco et al., 2002) is known to be regulated by Cdk-mediated phosphorylation. p130 has been shown to contain multiple Cdk phosphorylation sites, including Ser952 (Hansen et al., 2001). We therefore examined p130 Ser952 phosphorylation following ischemia *in vivo*. Cdk-mediated phosphorylation of Ser952 was increased following cerebral ischemia (Figure 4.6, D and E). These results indicate that p130 is phosphorylated at early time points following ischemic insult and that, both p130 and E2F4 proteins are subsequently lost in the death process. These results are similar to those observed with hypoxia/reoxygenation *in vitro*.

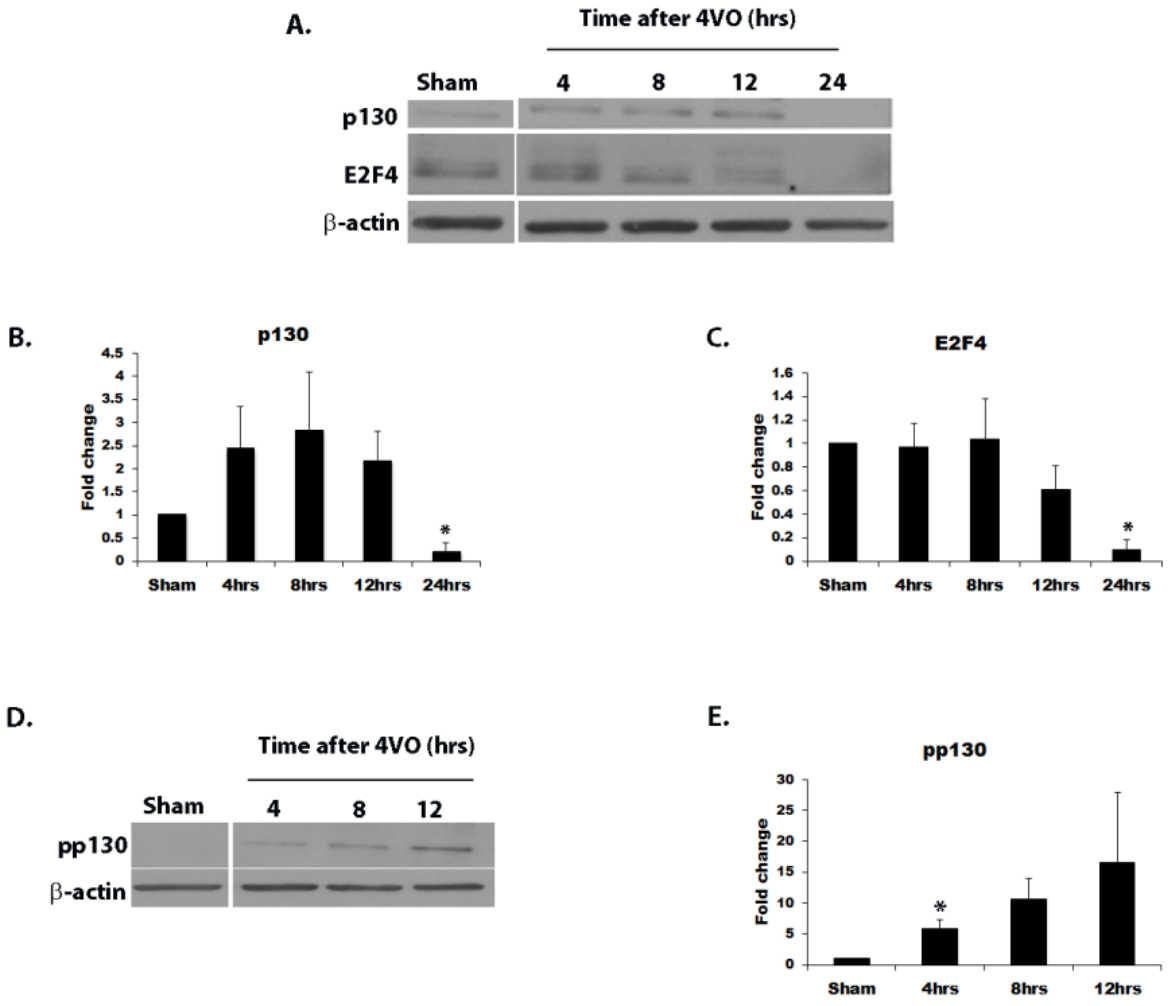
A.
Time after 4VO (hrs)
Sham
4
8
12
24
p130
E2F4
β-actin
B.
p130
Fold change
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Sham
4hrs
8hrs
12hrs
24hrs
C.
E2F4
Fold change
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Sham
4hrs
8hrs
12hrs
24hrs
D.
Time after 4VO (hrs)
Sham
4
8
12
pp130
β-actin
E.
pp130
Fold change
30
25
20
15
10
5
0
*
Sham
4hrs
8hrs
12hrs

Figure 4.6

FIGURE 4.6. p130 and E2F4 protein levels are decreased following global cerebral ischemia in rats. A, Western blots of p130 and E2F4 expression following 4VO in rats. Animals were subjected to 4VO and sacrificed at the indicated times. Hippocampal tissue was extracted and subjected to western blot analysis. B,C, Densitometry of western blot shown in (A). D, Western blot of p130 phosphorylation following 4VO. Rats were treated as in (A) and subjected to analysis using antibody directed at p130 phosphorylated at Ser952. E) Densitometry of phospho p130-Ser952. $N \geq 3$, * denotes significance $p < 0.05$ compared with sham.

B- and C-Myb transcripts are induced following global ischemia in vivo.

Because our results showed that E2F activity is increased at the B-Myb promoter *in vitro*, we examined for potential changes in its transcript levels as well as that of the related C-Myb following ischemic insult *in vivo*. We observed that both B-Myb (Figure 4.7, A and B) and C-Myb (Figure 4.7, C and D) mRNA transcript levels are increased 3 fold ($p<0.05$) 24 hours following global ischemia.

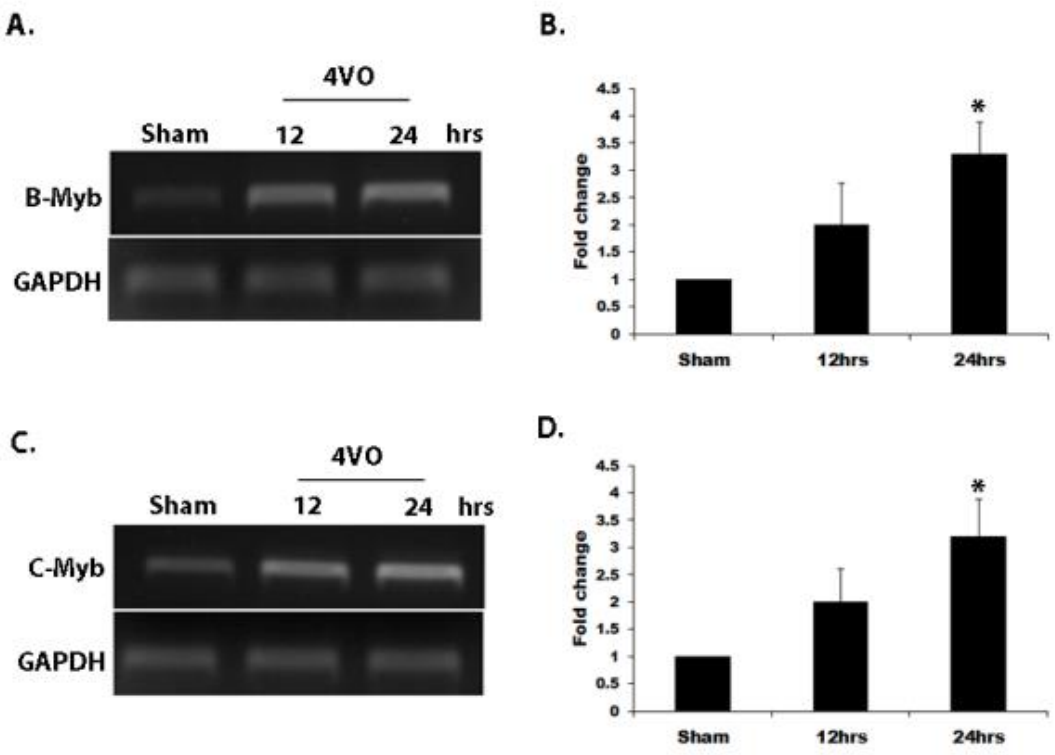
A.
4VO
Sham
12
24
hrs
B-Myb
GAPDH
B.
Fold change
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Sham
12hrs
24hrs
C.
4VO
Sham
12
24
hrs
C-Myb
GAPDH
D.
Fold change
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Sham
12hrs
24hrs

Figure 4.7

FIGURE 4.7. B- and C-Myb mRNA transcript levels are increased following global cerebral ischemia. A, Semi-quantitative RT-PCR of B-Myb message following global ischemia. Rats were subjected to 10 minutes 4VO followed by 12 and 24 hours of reperfusion. Hippocampal tissues were extracted and analyzed by semi-quantitative RT-PCR. GAPDH is shown as control. B, Densitometry of B-Myb levels. Data is expressed as fold over sham operated control. C, Semi-quantitative RT-PCR of C-Myb message following 4VO as described in (A). F, Densitometry of C-Myb mRNA levels. Bars represent the mean ±SEM, $N \geq 3$, $^{*}p < 0.05$.

DISCUSSION

Cell cycle induced death of neuron has been described in numerous cell death paradigms (Park et al., 1997a, Greene et al., 2007, Rashidian et al., 2007, Hernandez-Ortega et al., 2011, Moh et al., 2011). However, the downstream effectors of this pathway in the context of pathologic neuronal death are not fully understood, particularly in the context of ischemic damage. In this regard, a growing body of evidence suggested that death following ischemic insult is mediated via pRb/E2F1.

In line with this, we found that E2F1 deficiency was protective while its over-expression exacerbated death induced by DNA damage and hypoxia/reoxygenation. Our data is consistent with a pro-apoptotic role previously demonstrated for E2F1 in other contexts including death induced by staurosporine (Hou et al., 2000), β-amyloid (Giovanni et al., 2000), potassium deprivation (O'Hare et al., 2000, Konishi and Bonni, 2003, Yuan et al., 2011), kainic acid (Smith et al., 2003b), OGD (Gendron et al., 2001) and ischemia (Giovanni et al., 2000, Hou et al., 2000, O'Hare et al., 2000, Gendron et al., 2001, Smith et al., 2003b). Similar to E2F1, E2F3, another member of the activating E2F family, is pro-apoptotic. Over-expression of E2F3 promoted cell death in response to camptothecin treatment while its downregulation was protective. This is in line with a previous report demonstrating a protective role for E2F3 deficiency in the developing CNS in response to DNA damage (Martinez et al., 2010).

The role of the repressive E2F4 contrasts to that of activating E2F members by promoting survival under conditions of ischemic stress. This is supported by several findings in our present study; acute downregulation of E2F4 in CGNs using siRNA caused death in the absence of insult. Hypoxia/reoxygenation resulted in death that was exacerbated with

E2F4 deficiency and knock-down in culture. In contrast, we found that over-expression of E2F4 protected neurons from death induced by hypoxia/reoxygenation *in vitro* and global cerebral ischemia *in vivo*. The data presented here as well as others (MacManus et al., 1999, Gendron et al., 2001, MacManus et al., 2003) demonstrate that in the context of cerebral ischemic damage, the activity of both the activating and repressive E2Fs is an important determinant of neuronal survival and death.

The differential role of activating and repressive E2Fs in neuronal survival is interesting. While the exact reasoning behind this role is not clear, there are some intriguing hypotheses generated by our present data. First, we showed that p130/E2F4 levels are present basally but are reduced following ischemic insult. Second, we show that E2F4 binding to the E2F site on the B-Myb promoter is reduced following hypoxia. This suggests a model whereby p130/E2F4 basally exists to form active repressive complexes but is lost after death-inducing insult. These repressive complexes may be critical for neuronal survival. In support of this, p130 predominantly occupies E2F sites in cultured neurons (Liu et al., 2005) and has been shown along with E2F4 to participate in the mammalian DREAM complex (Litovchick et al., 2007). Additionally, p130 levels are high in neurons (Baldi et al., 1997, Kusek et al., 2001) and its loss has been suggested to promote death (LeCouter et al., 1998). In particular, p130 deficiency leads to strain-dependent lethality in null mice (LeCouter et al., 1998) and its downregulation in cultured neurons promotes apoptosis (Liu et al., 2005).

In this scenario, we suggest that E2F4 is protective because it would facilitate the formation of repressive complexes which are lost following ischemic insult. In contrast to E2F4, we propose that E2F1 acts to promote death by directly activating pro-death genes. This is supported in the present study by the observation that E2F1 is pro-death and its occupancy on death-inducing genes is increased following hypoxic insult. Therefore, we

propose a model by which E2F4 repressive complexes which are present at target promoters are lost, allowing for E2F1 transactivating activity to increase. Both would then contribute to increase in expression of targets genes such as Myb leading to death. Indeed we show that targets such as B-Myb and C-Myb are increased following ischemia *in vivo*. It will be important to fully test this hypothesis in the future**.**

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ACKNOWLEDGMENTS

We thank Yasmilde Rodriguez Gonzalez for the critical reading of the manuscript.

FOOTNOTES

***** This work was supported by grants from the Heart and Stroke Foundation of Canada (HSFC), the Heart and Stroke Foundation of Ontario (HSFO), the Canadian Institutes of Health Research (CIHR), the Center for Stroke Recovery (CSR), the Neuroscience Canada/Krembil Foundation (DSP).

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[4]The abbreviations used are: Cdk, cyclin-dependent kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AAV, adeno-associated virus; 4VO, four vessel occlusion; CGN, cerebellar granule neuron; GFP, green fluorescent protein; CA1 cornu ammonis 1, ChIP, chromatin immunoprecipitation.

CHAPTER 5

Combinatorial Treatment With Flavopiridol And Minocycline Provides Longer Term Histological but Not Functional Protection Following Global Ischemia.

Iyirhiaro GO, Brust TB, Rashidian J, Galehdar Z, Osman A, Phillips M, Slack RS, MacVicar BA and Park DS

Journal of Neurochemistry, *(2008), 105(3): 703-13*

STATEMENT OF AUTHOR CONTRIBUTION

This final manuscript explores the short and long-term therapeutic potential of a combinatorial treatment strategy that include the general inhibition of CDKs and inflammatory processes. Here Flavopiridol, a pan CDK inhibitor is combined with Minocycline, a drug with anti-inflammatory properties in a post-ischemia treatment strategy in the rat 4VO model.

All experiments in this manuscript were performed by GO Iyirhiaro with the following exceptions: T Brust performed the electrophysiology experiments presented in figure 5.5 in the laboratory of Dr. BA MacVicar. J.Rashidian, and Z Galehdar provided some technical assistance during the 4VO surgeries. A Osman and M Phillips (summer students) assisted with some brain sectioning and staining. Dr. RS Slack provided technical support and review of the manuscript. All text and figures in this manuscript (except Figure 5.5 and associated method) were written and prepared by GO Iyirhiaro with editorial guidance from Dr. DS Park.

Combinatorial Treatment With Flavopiridol And Minocycline Provides longer Term Histological but Not Functional Protection Following Global Ischemia.

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Keywords: CDKs, flavopiridol, minocycline, cerebral ischemia,

Running Title: Functional protection and ischemia

ACKNOWLEDGEMENTS:

We thank Sung Yuh and Catherine Millar for their technical assistance and Hossein Aleyasin for the review of our manuscript. This work was supported by grants from Heart and Stroke Foundation of Canada, Heart and Stroke Foundation of Ontario, Canadian Institutes of Health Research, Center for Stroke Recovery, and the Canadian Stroke Network (DSP); Heart and Stroke Foundation of Canada Doctoral Research Award (GOI).

ABSTRACT

We previously reported that delayed administration of the general cyclin-dependent kinase inhibitor flavopiridol following global ischemia provided transient neuroprotection and improved behavioral performance. However, it failed to provide longer term protection. In the present study, we investigate the ability of delayed flavopiridol in combination with delayed minocycline, another neuroprotectant to provide sustained protection following global ischemia. We report that a delayed combinatorial treatment of flavopiridol and minocycline provides synergistic protection both 2 and 10 weeks following ischemia. However, protected neurons in the hippocampal CA1 are synaptically impaired as assessed by electrophysiological field potential recordings. This is likely because of the presence of degenerated processes in the CA1 even with combinatorial therapy. This indicates that while we have addressed one important pre-clinical parameter by dramatically improving long-term neuronal survival with delayed combinatorial therapy, the issue of synaptic preservation of protected neurons still exists. These results also highlight the important observation that protection does not always lead to proper function.

INTRODUCTION

Cyclin-dependent kinases (CDKs) are a growing family of kinases with prominent roles in the regulation of the eukaryotic cell cycle, transcriptional regulation, and neuronal development. In addition to these conventional roles, a new and more pathogenic role for CDKs is emerging. For example, a role for CDKs has been described in numerous paradigms of neuronal death including models of Parkinson's disease and cerebral ischemia (Osuga et al., 2000, Wang et al., 2002, Smith et al., 2003a, Shelton and Johnson, 2004) .

Several lines of evidence suggest a role for CDKs as mediators of ischemic injury. For example, increased cyclin D1 and activation of Cdk2 is observed following oxygen glucose deprivation (Katchanov et al., 2001). Neurons expressing dominant negative (DN) Cdk4 or derived from cyclin D1 null mutants are resistant to hypoxic injury (Rashidian et al., 2005). Further supporting the role of CDKs is our observations that pRb, a downstream target for CDKs, is increasingly phosphorylated following hypoxia/reoxygenation (Rashidian et al., 2005). These data can also be extended *in vivo*. For example, increased cyclin D1 activity has been reported in focal and global models of ischemia in the rodent (Osuga et al., 2000, Wang et al., 2002). Finally, we have shown that virally delivered DNCdk4 can protect CA1 neurons from global ischemia and that pRb phosphorylation is increased following global ischemia (Wang et al., 2002, Rashidian et al., 2005).

The neuronal CDK, Cdk5 also appears to play a role in ischemic injury. For example, increased levels of a cleaved and more pathogenic form of p35, the activator of Cdk5, are observed following both focal and global ischemia. DNCdk5 has been shown to inhibit glutamate and hypoxia mediated excitotoxicity *in vitro* (Rashidian et al., 2005). Likewise,

neurons derived from p35 null mice are resistant to glutamate-induced death (Rashidian et al., 2005) and inhibition of Cdk5 is protective in an *in vivo* model of ischemia (Weishaupt et al., 2003, Shelton and Johnson, 2004, Rashidian et al., 2005).

Taken together, this evidence establishes a crucial role for CDKs as mediators of ischemic death. Can CDKs be utilized as therapeutic target for ischemic injury? Studies using pharmacological CDK inhibitors have demonstrated neuroprotection in multiple animal models of stroke (Osuga et al., 2000, Katchanov et al., 2001, Wang et al., 2002). For example, we previously showed that single dose administration of flavopiridol 4h following global ischemia protected CA1 neurons and resulted in improved behavioural performance 7–9 days following reperfusion. However, this protection was not sustained at 28 days post-ischemia (Wang et al., 2002). This observation suggests that while CDK inhibition can act to block neuron intrinsic mechanisms of death and may be beneficial in the treatment of ischemic injury, it alone is insufficient to fulfill all the pre-clinical criteria required of an effective neuroprotectant (Wang et al., 2002, Rashidian et al., 2005). In support of this, we have also shown that sustained inhibition of death pathways in the continual presence of a chronic stressor can unmask alternative pathways of death. For example, inhibition of caspases in cortical neurons treated with DNA damaging agent provides only transient protection and is associated with a more protracted non-apoptotic death (Stefanis et al., 1999). In this regard, it is very difficult to attain long-lasting sustained neuroprotection while neurons are exposed to chronic extrinsic stresses (Wang et al., 2002). We hypothesized that this may also be the case in the global model of ischemia where chronic stresses such as inflammation are known to be activated (Stoll et al., 1998, Yrjanheikki et al., 1998). Indeed studies utilizing anti-inflammatory agents have reported therapeutic benefits in models of

cerebral ischemia (Yrjanheikki et al., 1998, Yrjanheikki et al., 1999, Hewlett and Corbett, 2006, Peeling et al., 2006). For example, Yrjanheikki *et al.* showed that treatment with minocycline, a tetracycline derivative reduces signs of inflammation in the brain and protects neurons following global ischemia (Yrjanheikki et al., 1998). Minocycline is a multi-target drug that exhibits anti-inflammatory properties and can inhibit intrinsic cell death process such as the release of cytochrome *c*, caspases, and inducible nitric oxide synthase (Yrjanheikki et al., 1998, Zhu et al., 2002). We hypothesized that combinatorial strategies including CDK inhibition might be more effective in providing enduring protection. Presently, we investigated the benefit of a combinatorial treatment regimen of delayed flavopiridol and delayed minocycline administration that targets both CDKs and inflammation in the rat global ischemia model. We show that this delayed combination provides synergistic protection against death which is more enduring than the delayed administration of either drug alone. However, we also show that these protected neurons are not normally functional, suggesting that other processes must be modified before long-lasting functional protection can be attained.

MATERIALS AND METHODS

All experiments conformed to the guidelines set forth by the Canadian Council for the Use and Care of Animals in Research (CCAC) with approval from the University of Ottawa Animal Care Committee.

Global ischemia. Global ischemia was performed on male Wistar rats (180–220 g; Charles River, Saint-Constant, Quebec, Canada) using the four vessel occlusion (4VO) method for 10 min (Wang et al., 2002). All surgical procedures were performed under 2–2.5% halothane carried in 1% oxygen delivered by a face mask. All animals were allowed to breathe spontaneously during all surgical procedures and were allowed unrestricted access to food and water before and after global ischemia. To facilitate global ischemia, the common carotid arteries were exposed through a ventral midline neck incision and loosely looped with silk suture. The vertebral arteries were exposed through a dorsal neck incision and cauterized at the level of the first vertebra. All incisions were closed with surgical clips and rats were allowed to recover anesthesia and returned to their home cage. The following day, rats were again anesthetized a ligature was passed through the neck ventral to the cervical and paravertebral muscles but dorsal to the trachea, esophagus, carotids arteries, and external jugular veins. Upon recovery from anesthesia (assessed by a pain response to tail-pinching), rats were quickly occluded for 10 min with the aid of the suture placed the previous day and aneurysm clips. Ischemic rats displayed loss of responsiveness within 10–15s of occlusion, running behavior, and loss of righting reflexes. The ligature surrounding the paravertebral musculature was then tightened to prevent the opening of collateral blood flow. At the end of the 10min occlusion period, the aneurysm clips clamping the carotids arteries and the ligatures surrounding the paravertebral musculature and carotid arteries were removed. Core

body temperature was measured using rectal thermometer and was maintained between 36.5°C and 37.5°C for all surgical procedures. Rats were allowed to recover in a temperature-controlled incubator at 37°C and thereafter on heating pads maintained at 37°C for 24 h. Ischemic rats displayed loss of responsiveness within 10–15 s of occlusion, running behavior and loss of righting reflexes. Rats that did not remain unresponsive during and at least 10 min following reperfusion or developed seizures or pulmonary edema were excluded from further studies.

Lateral intracerebral ventricular infusion. Four hours following global ischemia, rats were infused with a single dose of 5 μL flavopiridol (500 μmol/L) (a gift from Peter J. Worland) or vehicle as described previously (Wang et al., 2002). Flavopiridol was first dissolved in complete dimethyl sulfoxide (DMSO). The concentrated solution was then diluted 100-fold with artificial CSF (ACSF) to a final concentration of 500 μmol/L. Five microliter ACSF containing 1% DMSO was used as vehicle in place of flavopiridol treatment as control. Flavopiridol administration was previously shown not to affect core body temperature even 24 h after treatment as shown by telemetry measurements (Wang et al., 2002).

Intraperitoneal injection of minocycline. Twenty-four hour following global ischemia rats were injected intraperitoneally with 0.5 mL minocycline (Sigma, St. Louis, MO, USA) dissolved in water. Rats were injected twice a day at 45 mg/kg on day 1 and 22.5 mg/kg for additional an 13 days. For short-term histological studies, rats were killed on day 15. For the long-term histological and functional studies, rats were killed 8–10 weeks following global ischemia. Minocycline administration was previously shown not to affect post-operative core

body temperature 24 h following ischemia (Yrjanheikki et al., 1998, Yrjanheikki et al., 1999).

Histology and CA1 cell survival. At the designated times following global ischemia, rats were anesthetized with sodium pentobarbital and perfused with 0.9% saline solution followed by 4%*p*-formaldehyde buffered with 0.1 mol/L phosphate (pH 7.4). The perfused rat brains were extracted and stored in 10% formalin (Fisher Scientific, Ottawa, Canada) for 7 days and then embedded in paraffin. Coronal sections (7μm) at the level of the hippocampus were obtained, deparaffinized, and stained for hematoxylin and eosin. Alternatively, brains were stored in 4%*p*-formaldehyde overnight and cryopreserved in 10% sucrose solution containing 0.2% sodium azide. Fourteen micrometer coronal sections of hippocampi were then obtained from these brains with aid of a cryostat and stored between −20°C and −80°C until analysis. Bilateral counts of morphologically live cells in the mid-CA1 subregion (bregma −3.60 to −4.5) of the hippocampus were counted and expressed as counts per millimeter. At least two bilateral counts per animal were made. The final data are presented as percentage of sham control ± SEM where appropriate.

Blood gas analysis. For blood gas (pCO_2, pO_2, pH, and HCO_3^-) analysis, global ischemia was induced in a separate subset of rats as already described above. Arterial blood samples were collected in the anesthetized animals by heart puncture in 1mL heparinized syringe (Sarstedt, Montreal, Canada) an hour following reperfusion, flavopiridol infusion and in the sham operated rats. Blood gases were measured using a blood gas analyzer (Stat Profile pHOx; Nova Biomedical, Mississauga, Canada). Arterial blood gases were measured an hour following sham or global ischemia surgery and an hour following flavopiridol infusion. Arterial pH, pCO_2, pO_2, and HCO_3^- did not significantly differ between sham operated and

untreated 4VO rats. Similarly, infusion of flavopiridol in ischemic rats did not result in significant changes in any of these parameters (Table 5.1).

Table 5.1. Summary of selected physiological parameters 1 hour after reperfusion or flavopiridol infusion.

Variables	Sham operated	4VO	4VO + Flavopiridol (500µM)
pH (mmHg)	7.38±0.04	7.33±0.01	7.37±0.02
pCO_2 (mmHg)	64.3±8.39	70.28±7.88	59.07±2.99
pO_2 (mmHg)	36±10.39	49.6±3.71	44.43± 5.73
HCO_3^- (mm/L)	38 ±3.50	36.45± 3.02	33.33± 1.35

Mean±SEM. N=4 for sham, N= 3 for 4VO and 4VO+Flavopiridol.

Immunohistochemistry. Frozen rat brain sections were thawed, rinsed with 0.01 mol/L phosphate-buffered saline (PBS), and incubated with monoclonal anti-CD11b (OX-42) (1 : 200; Serotec, Raleigh, NC, USA), anti-CD68 (ED1) (1 : 100; Serotec), and glial fibrillary acidic protein (GFAP) (1 : 200; Chemicon, Temecula, CA, USA) diluted in 0.01 mol/L PBS with 0.3 Triton X-100 overnight at 4°C in order to detect microglia cells and astrocytes, respectively. Cy-3 conjugated donkey anti-mouse IgG antibody (1 : 200; Jackson, West Grove, PA, USA) or Alexa 488 or Alexa 594 (1 : 300) were used for visualization of immunolabeling. Alternatively, paraffin embedded rat brain sections were deparafinized with xylenes and rehydrated in 100%, 95%, and 85% ethyl alcohol. Heat-mediated antigen retrieval steps using citrate buffer (50 mmol/L, pH 7.6) were performed on sections. Briefly, deparafinized sections were heated for 2–3 min at high in the microwave and allowed to cool for 3 min at 21°C (this procedure was performed five times and the sections were allowed to cool for 20 min at 21°C). Following antigen retrieval, sections were rinsed with 0.01 mol/L PBS for 5 min and then endogenous peroxidase activity was blocked with 0.3% H_2O_2/0.01 mol/L PBS. Non-specific binding sites were blocked with normal donkey serum (1 : 75) in 3% bovine serum albumin/0.01 mol/L PBS. Sections were then incubated with monoclonal anti-microtubule-associated protein 2 (MAP-2) (1 : 250; Sigma) or rabbit monoclonal anti-synaptophysin (1 : 250; Abcam, Cambridge, MA, USA) overnight at 21°C. Finally, sections were incubated with Avidin-Biotin Complex (Vector Labs, Burlington, Canada) for 1 h at 21°C and developed with 3,3′-diaminobenzidine/$NiCl_2$/H_2O_2 reaction.

Quantification of immunohistochemistry. To quantify immune cells in the CA1, digital images of CD11b, CD68, and GFAP stained sections (bregma −3.60 to −4.5) were acquired using Axioskop 2 Mot microscope (Zeiss, Toronto, Canada), QICAM Fast mono 12 bit digital camera (Q-Imaging, Surrey, Canada), and Northern Eclipse software (Empix Imaging Inc., Mississauga, Canada) under 20× objective. Images were captured such that the CA1 region was centered in the field. Images were captured as gray scale and then pseudo-colored where appropriate using Northern eclipse. Cells positive for each appropriate staining were then counted over the entire field and expressed as counts per millimeter of CA1 ± SEM. Images of synaptophysin and MAP-2 were similarly captured as above but under the 40× objective. To quantify the number of processes, a box, 400 × 50 pixels in area was demarcated using the image program. This box was placed approximately 50–80 pixels ventral to the cell bodies in the CA1. The number of processes in the selected region was counted and expressed as number of processes ± SEM. At least two bilateral counts per animal were made. Alternatively, the total number of CA1 neurons in the field was evaluated along with the number of processes similar to that described above. The number of processes was then expressed as dendrites/neuron.

Hippocampal slice preparation. A separate group of rats underwent 4VO and combined treatments as already described above and were subjected to electrophysiology at 8–10 weeks following global ischemia. Briefly, rats were anesthetized with halothane, subjected to intracardial perfusion with ice-cold ACSF (see below), and decapitated according to protocols approved by the UBC committee on animal care. Brains were rapidly extracted and placed into ice-cold oxygenated dissection medium containing the following (in mmol/L): 87 NaCl, 2.5 KCl, 2 NaH_2PO_4, 7 $MgCl_2$, 25 $NaHCO_3$, 0.5 $CaCl_2$, 25 d-glucose,

and 75 sucrose. Hippocampal slices (400-μm thick) were cut using a vibrating tissue slicer (VT1000S; Leica, Nussloch, Germany) and maintained for 1–5 h at 24°C in ACSF containing (in mmol/L): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 26 $NaHCO_3$, 2.5 $CaCl_2$, and 10 d-glucose, and aerated with 95% O_2/5% CO_2. For electrophysiological recordings, slices were transferred to a submerged recording chamber and allowed to equilibrate for at least 1 h. The bath solution was perfused with aerated ACSF at a rate of 1.5–2 mL/min.

Electrophysiology. Field excitatory post-synaptic potentials (fEPSPs) were evoked by orthodromic stimulation of the Schaffer collateral pathway using a bipolar tungsten-stimulating electrode. Glass micropipettes filled with ACSF (resistance 1–3 MΩ) were used to measure CA1 fEPSPs in *stratum radiatum*. fEPSP signals were amplified 1000 times with an AC amplifier, band-pass filtered at 0.1–100 Hz, digitized at 10 kHz using a Digidata 1320A interface board (Axon Instruments, Foster City, CA, USA), and transferred to a computer for analysis. Data were analyzed using Clampfit 9.0 (Axon Instruments). Baseline synaptic responses were established by evoking fEPSPs every 30 s (0.03 Hz) for at least 20 min. Input–Output curves were generated by systematically increasing the voltage delivered by the stimulating electrode (4–10 V in increments of 1 V), and measuring the resulting fEPSP slope. The mean normalized fEPSP slope was plotted as a function of time with error bars representing the SEM. Statistical significance was assessed using a Student's *t*-test ($p < 0.05$).

Statistical analysis. Multiple comparisons were analyzed using anova and Tukey's test as *post hoc*. Where appropriate Student's *t*-test was used for two group comparisons.

RESULTS

Combined treatment with flavopiridol and minocycline (short-term)

To evaluate the potential benefit of a combinatorial treatment strategy targeting CDKs and inflammation, we combined flavopiridol and minocycline in a treatment regimen as described in Materials and Methods. We treated rats with flavopiridol or vehicle 4 h following 10 min of 4VO and/or minocycline or saline starting at 24 h post-ischemia twice a day for 2 weeks. Control rats underwent sham 4VO and were infused with vehicle intracerebral ventricular and received saline treatment for 2 weeks. Rats were killed on day 15 following global ischemia and analysis of live CA1 neurons was carried out using hematoxylin and eosin staining. Live CA1 neurons were assessed as cells with clearly intact nuclei and a round soma. Quantification of live cells in the hippocampal CA1 showed a dramatically greater increase in the number of neurons surviving 2 weeks following global ischemia in rats receiving both flavopiridol and minocycline treatment (Figure 5.1b) than rats receiving either minocycline (Figure 5.1d) or flavopiridol alone (Figure 5.1c) or vehicles + saline treatment (Figure 5.1e) Eighty-eight percent of cells survived in the CA1 of rats treated with flavopiridol + minocycline compare with 31%, 23%, and 9% survival in rats receiving flavopiridol alone, minocycline alone, and saline treatment, respectively, when compared with sham 4VO control animals (Figure 5.1a). All ischemic groups showed a significant reduction ($p < 0.001$) in the number of live cells in the CA1 when compared with the Sham control group except for the group receiving both flavopiridol and minocycline. We also observed that flavopiridol treatment alone did not significantly protect CA1 neurons 2 weeks following ischemia (Figure 5.1c). This is in accordance with previous observations

(Wang et al., 2002). Thus, our results suggest that synergistic protection of CA1 neurons can be obtained by utilizing both CDK inhibitor and minocycline.

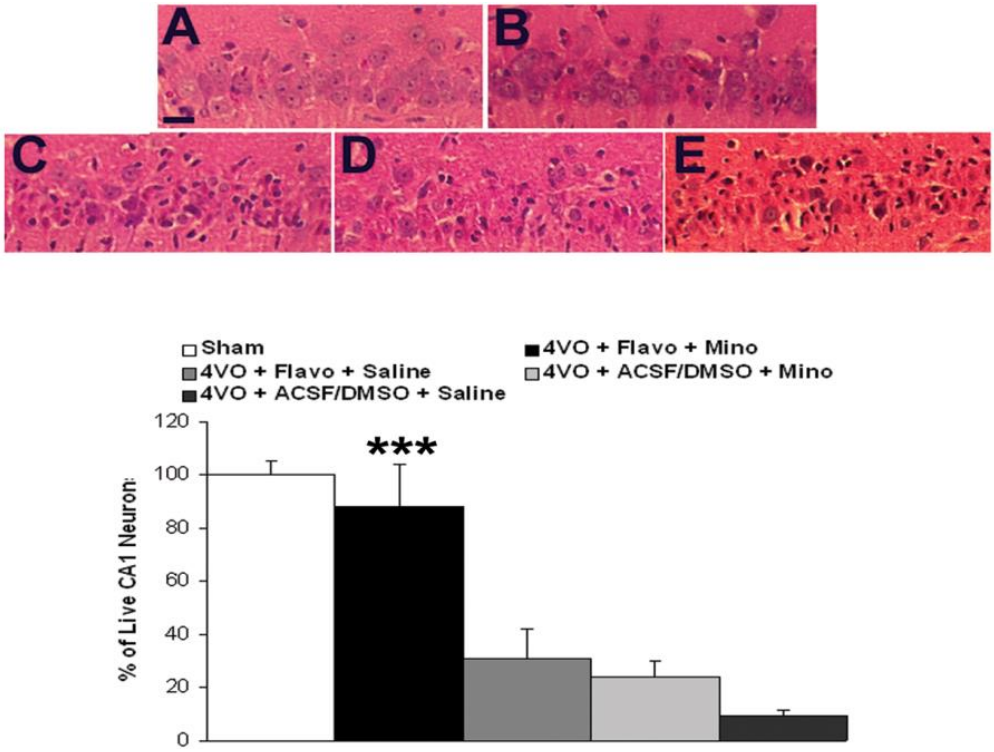

A
B
C
D
E
Sham
4VO + Flavo + Saline
4VO + ACSF/DMSO + Saline
4VO + Flavo + Mino
4VO + ACSF/DMSO + Mino
% of Live CA1 Neuron
120
100
80
60
40
20
0
***

Figure 5.1

Figure 5.1. Combined treatment of flavopiridol and minocycline provides synergistic protection neurons 2 weeks following 10-mins 4VO. (a–e) Hematoxylin and eosin stained representative sections of CA1 of (a) sham control, $n = 5$, (b) 4VO + flavopiridol + minocycline, $n = 5$, (c) 4VO + flavopiridol + saline, $n = 4$, (d) 4VO + ACSF/DMSO vehicle + minocycline, $n = 6$, and (e) 4VO + ACSF/DMSO vehicle + saline treated rats, $n = 5$. Images were captured under 20× objective. (f) Quantification of CA1 surviving neurons. Data are expressed as percentage of sham control ± SEM. ***denotes significance ($p < 0.001$ vs. 4VO + saline control). Scale bar = 25 μm.

Effects of Flavo/minocycline on inflammatory processes following global stroke

Because minocycline is known to modulate inflammatory reactions in the brain, we evaluated these processes by examining microgliosis as well as astrogliosis following ischemia in our treatment groups. The presence of microglia was assessed using the CD11b antibody. Our result showed no CD11b staining in our sham control rats (Figure 5.2a) which is in sharp contrast to a robust microgliosis seen in the untreated ischemic rats both at 2 and 5 days (Figure 5.2b and f) following ischemia. The number of CD11b expressing microglia was increased significantly at 5 days, 95.7 ± 15.5 (Figure 5.2f) following global ischemia compared with 41.3 ± 5.3 at 2 days ($p < 0.02$). Treatment of ischemia-induced rats with minocycline alone (Figure 5.2d) or minocycline + flavopiridol (Figure 5.2e) blocked increases in microglia. Surprisingly, flavopiridol also inhibited microglia CD11b expression (Figure 5.2c). We also evaluated the potential presence of immune cells expressing CD68 in the brain. CD68 is commonly expressed on monocytes, macrophages, and microglia. We observed that CD68+ cells were present at 2 and 5 days following ischemia (Figure 5.2h-l) but not in the sham operated (Figure 5.2g) rats. No significant difference was observed in the number of CD68+ cells in the CA1 regardless of treatment in the ischemic group at 2 days (Figure 5.2l). In contrast, treatment with either flavopiridol or minocycline alone or together resulted in significant reduction ($p < 0.05$) in the number of CD68+ cells in the CA1 at 5 days following ischemia (Figure 5.2h-l) compared with untreated ischemic (4VO + ACSF/DMSO + saline) rats.

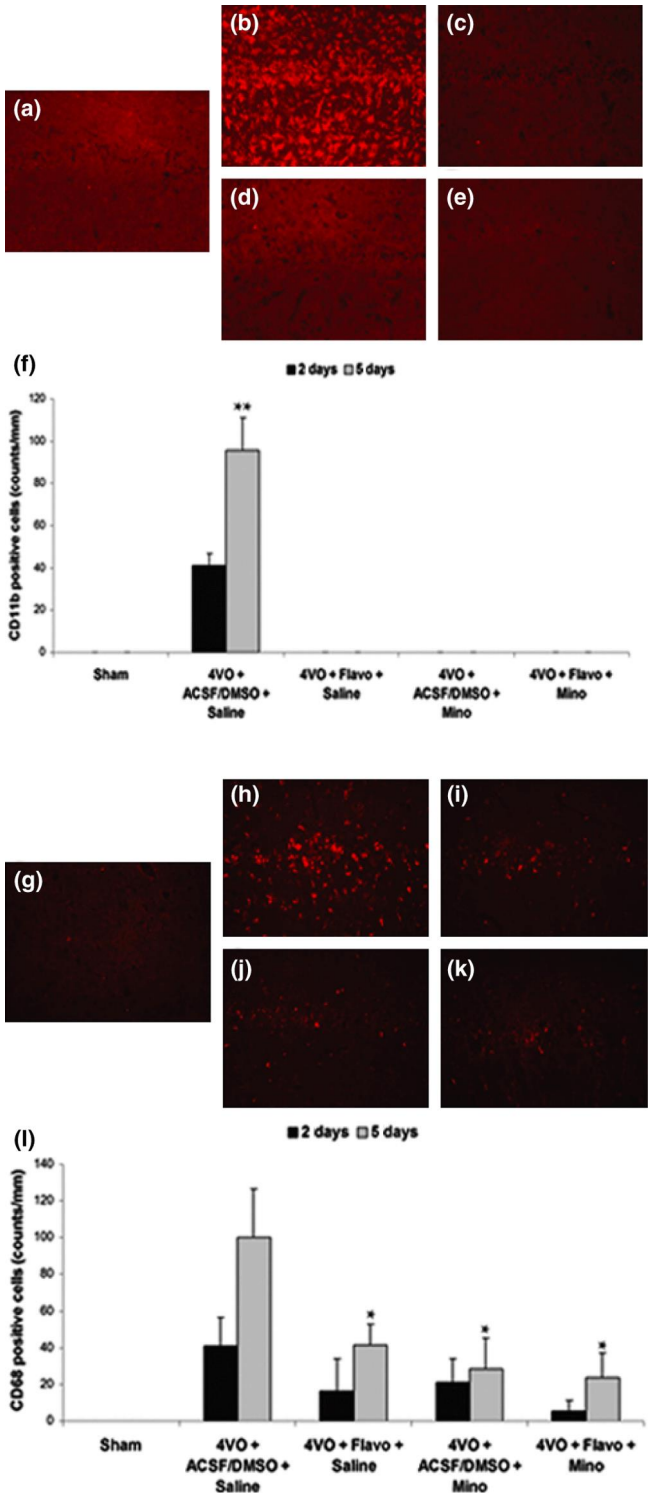
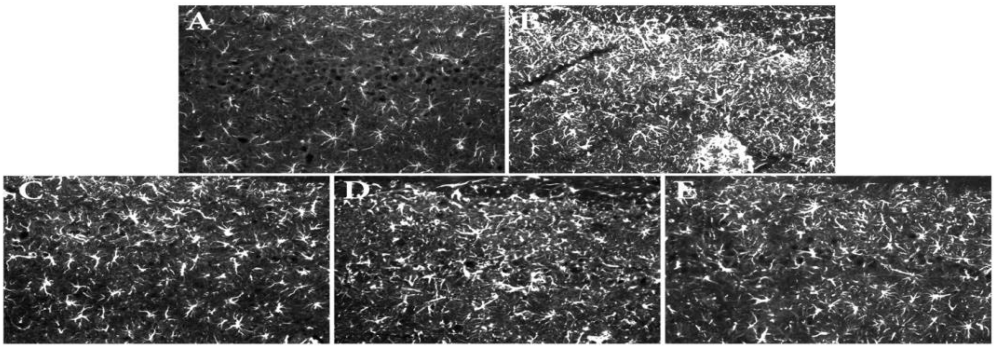

(a)
(b)
(c)
(d)
(e)
(f)
2 days
5 days
CD11b positive cells (counts/mm)
120
100
80
60
40
20
0
**
Sham
4VO + ACSF/DMSO + Saline
4VO + Flavo + Saline
4VO + ACSF/DMSO + Mino
4VO + Flavo + Mino
(g)
(h)
(i)
(j)
(k)
(l)
2 days
5 days
CD68 positive cells (counts/mm)
140
120
100
80
60
40
20
0
*
*
*
Sham
4VO + ACSF/DMSO + Saline
4VO + Flavo + Saline
4VO + ACSF/DMSO + Mino
4VO + Flavo + Mino

Figure 5.2

Figure 5.2. Induction of CD11b and CD68 in the rat hippocampus 5 days following global ischemia. Photomicrographs of microglial CD11b expression using OX-42 antibody (a–e) and CD68 expression using ED1 antibody (g–k). (a and g) sham control, $n = 3$, (b and h) 4VO + ACSF/DMSO vehicle + saline, $n = 3$, (c and i) 4VO + flavopiridol + saline, $n = 5$, (d and j) 4VO + ACSF/DMSO vehicle + minocycline, $n = 3$, and (e and k) 4VO + flavopiridol + minocycline treated rats, $n = 3$. (f and l) Quantification of CD11b and CD68 positive cells in the CA1 at 2 and 5 days following 4VO, $n = 3$ per group at 2 and 5 days except for 4VO + flavopiridol + saline, $n = 5$. Data are expressed as counts per millimeter of CA1 ± SEM. **denotes significance ($p < 0.02$ vs. 4VO + saline control at 2 days) and *$p < 0.05$ vs. 4VO + saline control at 5 days.

Finally, we evaluated astrogliosis 2 and 5 days following global ischemia using antibody directed against GFAP. At 2 days, the number of GFAP+ cells in the CA1 was similar in all groups including sham. In contrast, the number of GFAP+ cells in the CA1 at 5 days increased significantly ($p < 0.01$) in the non-treated (4VO + ACSF/DMSO + saline) group when compared with sham (Figure 5.3a vs. b, and f). However, flavopiridol treatment alone or in combination with minocycline appeared to reduce astrogliosis at 5 days compared with the non-treated ischemic group, $p < 0.05$ and $p < 0.01$, respectively (Figure 5.3c and e vs. b, and f). Minocycline alone also appeared to reduce astrogliosis (Figure 5.3d and f). Taken together, our results indicate that different aspects of the inflammatory/immune/astrogliosis response are modulated by flavopiridol or minocycline. The implications of this are discussed further below.


A
B
C
D
E

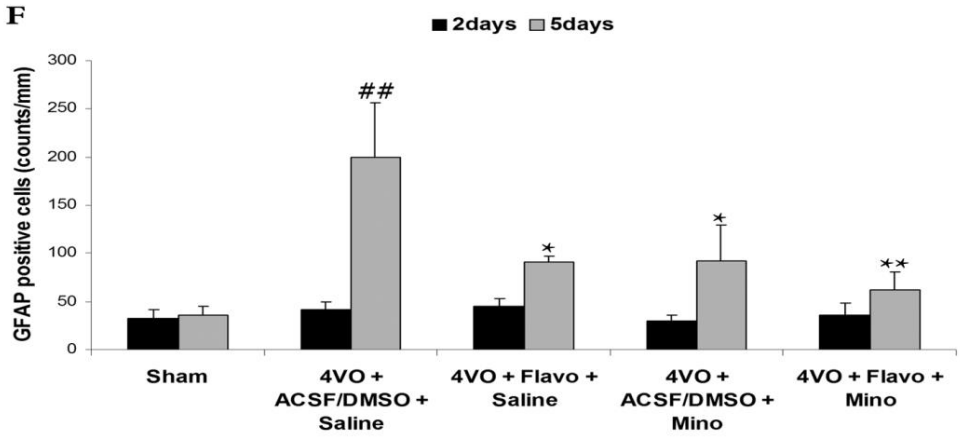

F
2days
5days
GFAP positive cells (counts/mm)
300
250
200
150
100
50
0
##
*
*
**
Sham
4VO + ACSF/DMSO + Saline
4VO + Flavo + Saline
4VO + ACSF/DMSO + Mino
4VO + Flavo + Mino

Figure 5.3

Figure 5.3. Photomicrographs of GFAP stained rat brain sections 5 days following global ischemia. (a) Sham control, $n = 4$, (b) 4VO + ACSF/DMSO vehicle + saline, $n = 3$, (c) 4VO + flavopiridol + saline, $n = 5$, (d) 4VO + ACSF/DMSO vehicle + minocycline, $n = 3$, and (e) 4VO + flavopiridol + minocycline treated rats, $n = 3$. (f) Quantification of GFAP positive cells in the CA1 at 2 and 5 days following ischemia. $n = 3$ per group at 2 days and as described above at 5 days. Data are expressed as counts per millimeter of CA1 ± SEM. *denotes significance $p < 0.05$, **$p < 0.01$ vs. 4VO + saline control at 5 days; and $^{\#\#}p < 0.01$ vs. sham control at 5 days.

Combinatorial treatment of flavopiridol and minocycline (long-term)

The end goal of any treatment strategy for stroke is long-term sustained functional protection. Accordingly, we investigated the potential for long-term functional benefit in our combinatorial drug strategy following global ischemia. Rats were treated as previously described above for our short-term study but were killed 10 weeks following global ischemia. All ischemic rats irrespective of treatment showed a significant loss of CA1 neurons in the hippocampi compared with the sham operated control rats ($p < 0.05$). Consistent with our results at 2 weeks, a greater degree of neurons were spared in the hippocampi of flavopiridol + minocycline (43%) treated ischemic rats compared with the vehicle + saline control ischemic rats (9%), or those ischemic rats singly treated with flavopiridol or minocycline alone (7% and 7%) (Figure 5.4b vs. c-e). However, there was a dramatic reduction in the level of neuroprotection observed in the flavopiridol + minocycline treated ischemic rats at 10 weeks compared with that obtained at 2 weeks following ischemia (43% vs. 88% at 10 and 2 weeks, respectively; $p < 0.05$). In contrast to the result obtained at 2 weeks, there was no difference between flavopiridol treated rats and saline treated ischemic rats at 10 weeks following global ischemia. This result is consistent with our previous observation that protection by flavopiridol alone is not sustained at 28 days post-ischemia (Wang et al., 2002).

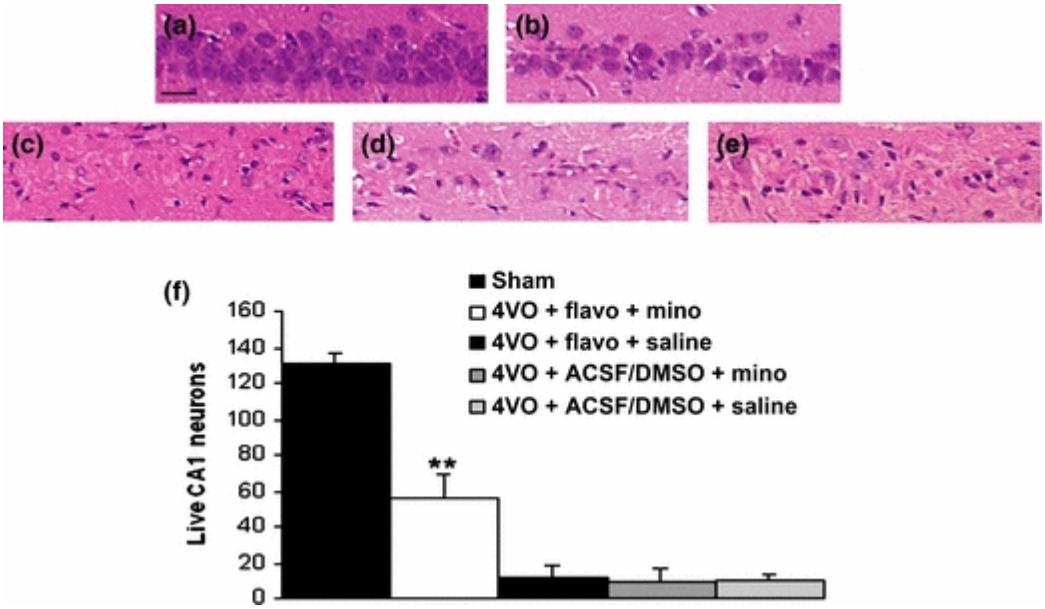

(a)
(b)
(c)
(d)
(e)
(f)
Sham
4VO + flavo + mino
4VO + flavo + saline
4VO + ACSF/DMSO + mino
4VO + ACSF/DMSO + saline
Live CA1 neurons
160
140
120
100
80
60
40
20
0
**

Figure 5.4

Figure 5.4. Combinatorial treatment of flavopiridol and minocycline protects CA1 neurons 10 weeks following global ischemia. (a–e) Hematoxylin and eosin stained representative sections of CA1 of (a) sham control, $n = 9$, (b) 4VO + flavopiridol + minocycline, $n = 4$, (c) 4VO + flavopiridol + saline, $n = 5$, (d) 4VO + ACSF/DMSO vehicle + minocycline, $n = 4$, and (e) 4VO + ACSF/DMSO vehicle + saline treated rats, $n = 4$. Images were captured under 20× objective. (f) Quantification of surviving CA1 neurons 10 weeks following 4VO. *n* is as described above. Data are expressed as percentage of sham control ± SEM. **denotes significance ($p < 0.01$ vs. 4VO + saline control). Scale bar = 25 μm.

Flavopiridol and minocycline treatment does not confer protection of processes

To assess synaptic function in our long-term treated rats, a separate group of rats were treated with flavopiridol + minocycline or saline as already described in the Materials and Methods. Rat hippocampal slices were collected and subjected to electrophysiological analyses. The Shaffer-collateral pathway was stimulated at intensities ranging from 4 to 10 V. fEPSPs were recorded in area CA1 of the hippocampus. The mean fEPSP slope was plotted as a function of stimulus intensity. Although the threshold of activation was similar for all conditions, the input–output curve generated for both untreated ischemic animals and flavopiridol + minocycline treated ischemic animals was significantly depressed compared with sham control animals (Figure 5.5) at stimulus intensities ranging from 6 to 10 V ($p < 0.05$). These results confirm that synaptic impairment can be readily detected in rats subjected to 4VO, as the magnitude of synaptic responses generated by a given stimulation intensity (from 6 to 10 V) was significantly attenuated in ischemic rats. However, there was no significant difference between untreated ischemic animals and flavopiridol + minocycline treated ischemic animals. Our result here thus suggests that although the combined treatment of flavopiridol and minocycline can protect CA1 neurons even at 10 weeks following global ischemia, the protected neurons are synaptically impaired.

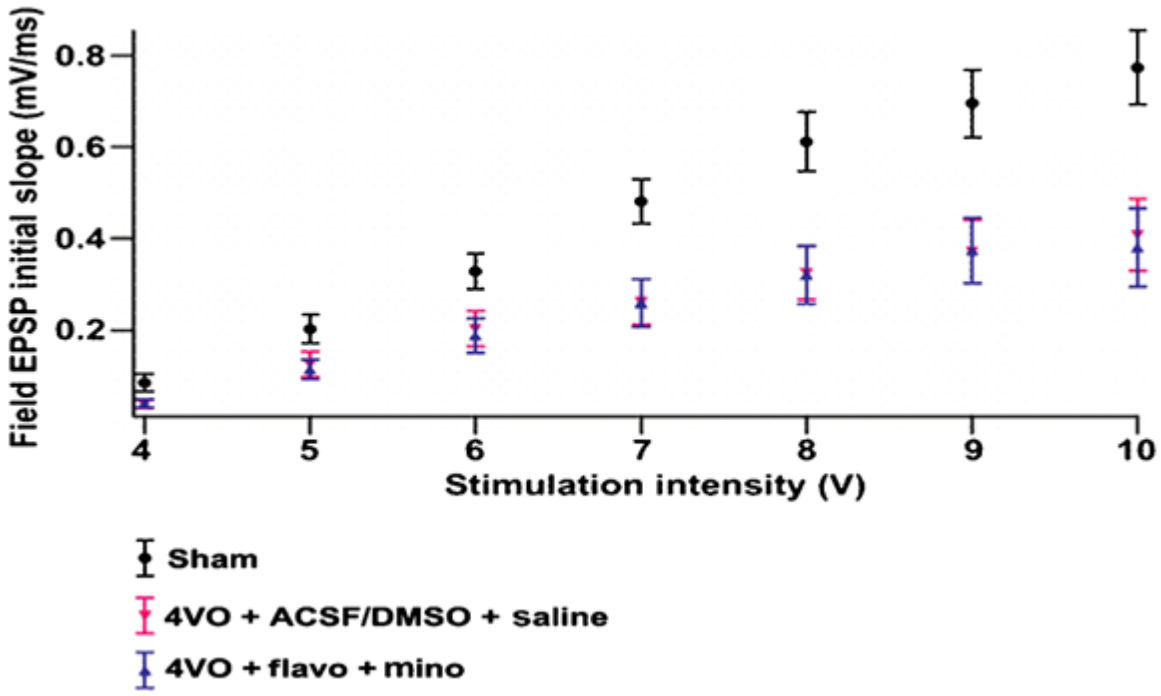

Field EPSP initial slope (mV/ms)
0.8
0.6
0.4
0.2
4
5
6
7
8
9
10
Stimulation intensity (V)
Sham
4VO + ACSF/DMSO + saline
4VO + flavo + mino

Figure 5.5

Figure 5.5. CA1 neurons protected by combined treatment of flavopiridol and minocycline are synaptically impaired. Electrophysiology recording of CA1 neurons 10 weeks following global ischemia. The Shaffer-collateral pathway was stimulated at intensities ranging from 4 to 10 V. Field excitatory post-synaptic potentials (fEPSPs) were recorded in the area of the CA1. The mean fEPSP slope is plotted as a function of stimulus intensity. $n = 5$, 6, and 8 for Sham, 4VO + ACSF/DMSO vehicle + saline, and 4VO + Flavo + Mino, respectively.

To probe the potential nature of the synaptic impairment of spared CA1 neurons in our treatment regimen, we evaluated the integrity of their processes both at 2 and 10 weeks following global ischemia. To this end, we immunostained for the pre-synaptic marker synaptophysin and MAP-2. Our result show that while the neuronal soma in the CA1 appeared to have been protected following our combined treatment regimen both at two and at 10 weeks, the dendrites of these neurons were not spared (Figure 5.6c and d) as is evident from the loss of synaptophysin staining when compared with sham control (Figure 5.6a). Synaptophysin stained dendrites were significantly reduced in the CA1 both at 2 weeks ($p < 0.01$ and $p < 0.05$) and at 10 weeks ($p < 0.001$ and $p < 0.05$) post-ischemia (Figure 5.6e and f, respectively). Generally, more synaptophysin stained processes were observed in the combined treatment group at 2 weeks than at 10 weeks (Figure 5.6c vs. 5.6d). However, the difference between these two time points was not significant (Figure 5.6e and f).

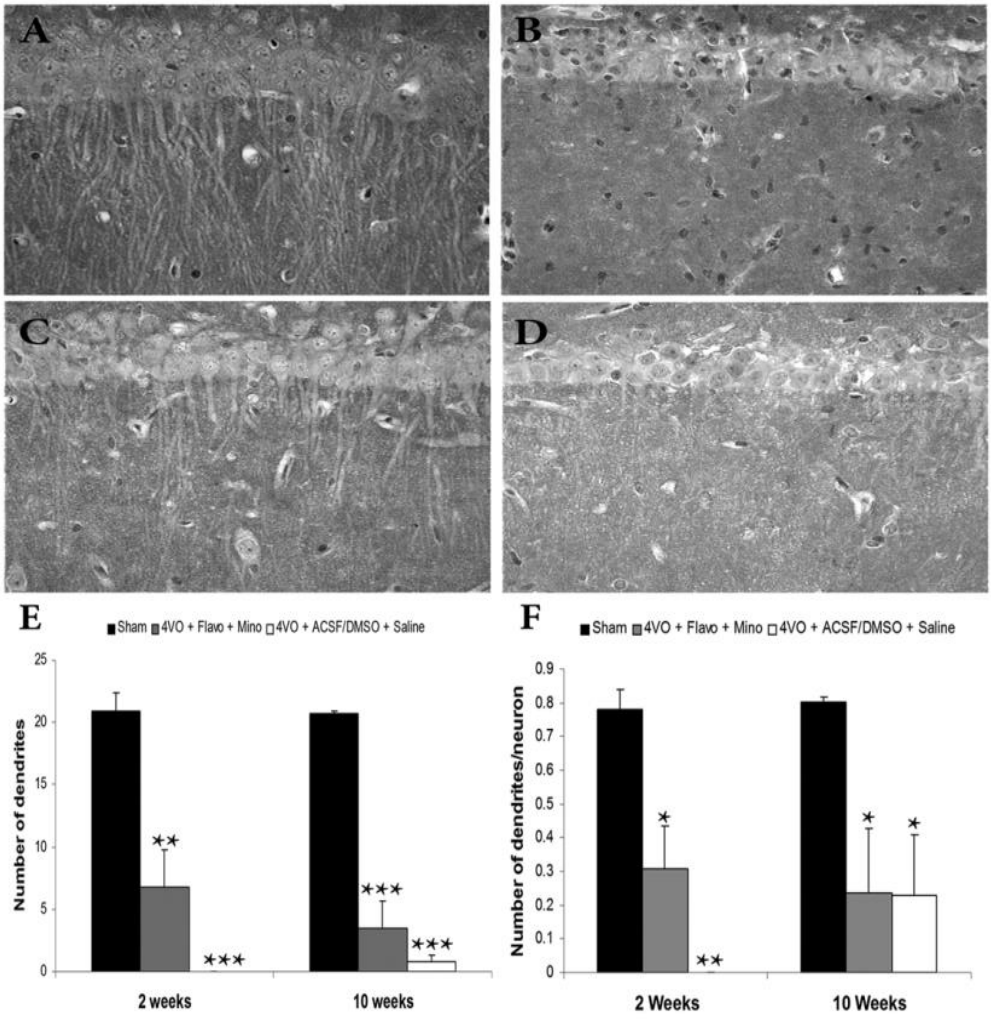

A
B
C
D
E
Sham 4VO + Flavo + Mino 4VO + ACSF/DMSO + Saline
Number of dendrites
25
20
15
10
5
0
**
***
***
***
2 weeks
10 weeks
F
Sham 4VO + Flavo + Mino 4VO + ACSF/DMSO + Saline
Number of dendrites/neuron
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
**
*
*
2 Weeks
10 Weeks

Figure 5.6

Figure 5.6. Synaptophysin marker staining showed degenerating processes in animals treated with combination flavopiridol and minocycline. Representative photomicrographs of synaptophysin stained sections (a) sham control, $n = 3$, (b) 4VO + ACSF/DMSO vehicle + saline control, $n = 3$, (c) 4VO + flavopiridol + minocycline at 2 weeks, $n = 4$, and (d) 4VO + flavopiridol + minocycline at 10 weeks, $n = 3$. Images were captured under 40× objective (e and f) Quantification of synaptophysin stained dendrites in the CA1, n is as described above. *denotes significance $p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (vs. Sham control at the same time point).

Similarly, a decline in MAP-2 immunoreactivity was observed in the combined treatment group both at 2 weeks ($p < 0.001$) and at 10 weeks ($p < 0.01$) when compared with sham control (Figure 5.7c and d vs. a). The decline in MAP-2 staining was reminiscent of the results obtained with synaptophysin immunostaining, in that more MAP-2 staining is observed at 2 weeks in comparison with the 10 weeks group (Figure 5.7e and f). Taken together, our data indicate that while the combinatorial strategy provides protection for neuronal soma, synaptic function is impaired likely because of degenerating processes.

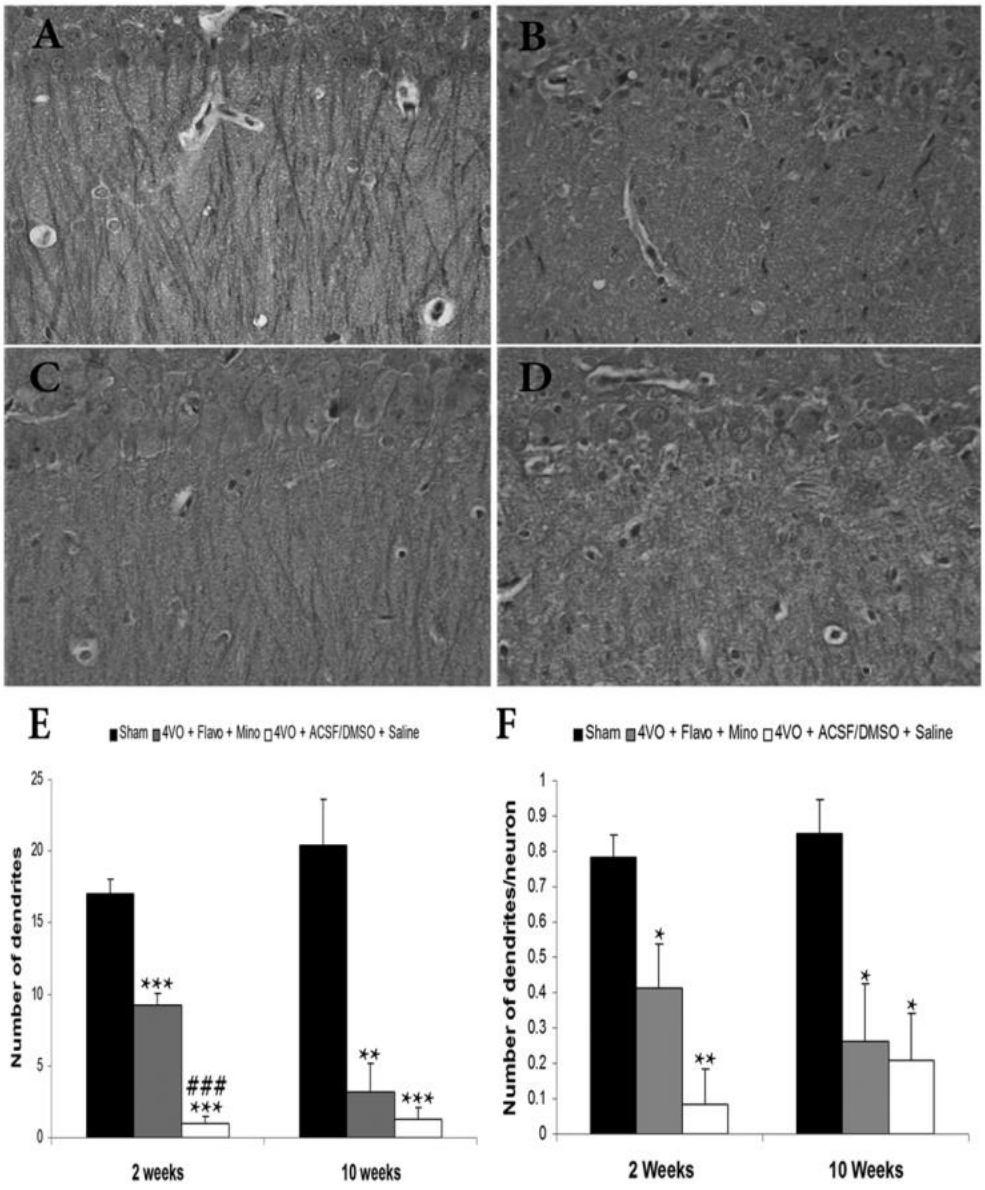

A
B
C
D
E
Sham 4VO + Flavo + Mino 4VO + ACSF/DMSO + Saline
Number of dendrites
25
20
15
10
5
0
***
###
***
**
***
2 weeks
10 weeks
F
Sham 4VO + Flavo + Mino 4VO + ACSF/DMSO + Saline
Number of dendrites/neuron
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
**
*
*
2 Weeks
10 Weeks

Figure 5.7

Figure 5.7. MAP-2 marker staining showed degenerating processes in animals treated with combination flavopiridol and minocycline. Representative photomicrographs of MAP-2 stained sections. (a) Sham control, $n = 3$, (b) 4VO + ACSF/DMSO vehicle + saline control, $n = 3$, (c) 4VO + flavopiridol + minocycline at 2 weeks post-ischemia, $n = 4$, and (d) 4VO + flavopiridol + minocycline at 10 weeks post-ischemia, $n = 3$. Images were captured under 20× objective. (e and f) Quantification of MAP-2 stained processes in the CA1, n is as described above. *denotes significance $p < 0.05$; **$p < 0.01$, ***$p < 0.001$ (vs. Sham control at the same time point); and $^{###}p < 0.001$ (vs. 4VO + Flavo + Mino at 2 weeks).

DISCUSSION

We showed recently that multiple CDK members act to mediate ischemic neuronal death (Rashidian et al., 2005). This suggested that CDKs might be important therapeutic targets for stroke. Indeed, we have previously shown that the administration of the general CDK inhibitor can protect against focal stroke. In addition, it can also transiently protect against global ischemia-induced neuronal death and behavioral deficit even when administered 4 hrs post-ischemia (Wang et al., 2002). These findings indicate that CDK inhibition fulfills almost all of the pre-clinical requirements of a potentially effective clinical target. However, stroke is a multi-faceted condition involving a myriad of potential death mediators including the activation of multiple CDK members and inflammatory pathways. Thus, potential therapeutic benefits realized by the continuing blockade of intracellular death signaling may be hampered by alternate death mediating pathways. Indeed this is true for CDK inhibition. Accordingly, in the present study, we tested the hypothesis that a combinatorial treatment strategy targeting CDKs and inflammation may be more effective in providing enduring neuroprotection. To this end, we evaluated the potential short- and long-term benefits of two known neuroprotectants, flavopiridol and minocycline, in a combined treatment regimen following global ischemia.

Minocycline is a second generation tetracycline derivative that has been shown to have anti-inflammatory properties separate from its antimicrobial actions (Yrjanheikki et al., 1998, Blum et al., 2004). Minocycline has a remarkable ability to cross the blood–brain barrier and has been shown to confer neuroprotection in multiple models of neurological disorders, particularly those with inflammation disorder component such as amyotrophic lateral sclerosis (ALS), multiple sclerosis, Huntington's disease, and stroke (Yrjanheikki et

al., 1998, Chen et al., 2000, Zhu et al., 2002, Giuliani et al., 2005). Minocycline is a broad-spectrum neuroprotectant that exerts its protective effects through several mechanisms. In addition to inhibiting microgliosis, minocycline also attenuates proapoptotic processes in neurons. For example, minocycline has been shown to inhibit the release of cytochrome *c*, caspase 1, caspase 3, and inducible nitric oxide synthase(Yrjanheikki et al., 1998, Zhu et al., 2002), and inhibit p38 mitogen-activated protein kinase activation (Du et al., 2001, Tikka et al., 2001). Thus, neuroprotection associated with early administration of minocycline following ischemic insult is likely due at least in part to its direct effects on neuronal death processes.

In the present study, our choice to administer minocycline starting at 24 h post-ischemia was based on our observation that inflammation as assessed by microglial CD11b staining occurred in a delayed manner following global ischemia. Indeed, we did not observe CD11b staining (a marker for microglia) at earlier time points (6 and 12 h) following ischemia (Iyirhiaro G. O. and Park D. S., unpublished data, 2005). Thus, we reasoned that by delaying the administration of minocycline, we could inhibit inflammatory markers in the brain without affecting some of the earlier death processes described above. For this reason, we did not examine administration of minocycline immediately following stroke. Our results show that in spite of this rather delayed administration of minocycline starting at 24 h post-ischemia, microglial CD11b expression was potently inhibited when assessed at 2 and 5 days as well as later time points (10 and 14 and 70 days; data not shown) following ischemia. In addition, while repeated injections of minocycline have been reported to contribute to inconsistent absorption when administered intraperitoneally (Fagan et al., 2004), the lack of CD11b staining at these time points suggests that appropriate absorption and efficacy was

achieved in the brains of treated animals in our study. Although neither flavopiridol nor minocycline alone was capable at providing longer term protection by themselves, a combination of both drugs provided protection for neuronal soma both at 2 and 10 weeks. What is the mechanism by which this synergistic protection is conferred? Interestingly, flavopiridol appears to inhibit microgliosis similar to minocycline. This is consistent with other reports in models of traumatic brain injury (TBI) suggesting that flavopiridol can inhibit microgliosis (Di Giovanni et al., 2005). However in this case and our own experiments, it is unclear whether reduced microglial CD11b expression is a primary effect of flavopiridol treatment or simply the result of reduced damage. In our evaluation of CD68+ cells and astrogliosis following ischemia, there was a general trend towards a greater reduction in the rats treated with flavopiridol and minocycline than either drug by itself. However, no significant difference was observed between the combined flavopiridol + minocycline treated group when compared with flavopiridol or minocycline treated rats. This may be because of individual variation between animals observed in our experiments. There was a general trend for either flavopiridol or minocycline treatment by themselves to reduce inflammatory processes in our study although the mechanism by which this occurs is not explored in the present study. Regardless of the exact mechanism, however, it is likely that flavopiridol and minocycline are inhibiting multiple signaling pathways which cannot be inhibited by either drug alone. We do not believe that enduring neuronal protection is due simply to a prolonged dosing schedule. Indeed, we have previously shown that prolonged treatment of animals with flavopiridol had no effect on long-term protection (Wang et al., 2002).

Finally, the best indicator of the efficacy of any neuroprotectant strategy is the achievement of protection of synaptic connectivity. In our model, although the combinatorial treatment of flavopiridol and minocycline provided remarkable long-term protection for neuronal soma, electrophysiological analysis demonstrated that these neurons are synaptically impaired. Indeed, our examination of the integrity of processes of protected CA1 neurons using antibodies to synaptophysin and MAP-2 shows degeneration that is particularly more pronounced at 10 weeks following global ischemia. This result suggests that over the course of time the processes of the protected neurons have degenerated. Thus, the lack of function of protected CA1 neurons in this study can be directly attributed to lack of dendritic preservation. This may also potentially explain the later cell drop off in our study. Indeed, we observed a significant reduction in the number of protected neurons at 10 weeks when compared with almost complete protection at 2 weeks following global ischemia. It is interesting to note however that the synaptic impairment observed in our study may not necessarily translate into an overt cognitive behavioral deficit in the long-term. We have previously reported cognitive deficit in the stroke animals when the Morris Water Maze task is administered 1 week following ischemia (Wang et al., 2002). To test more enduring effects, we did examine long-term behavioral deficits that were associated with single or combinatorial flavopiridol + minocycline therapy. However, we were unable to detect cognitive deficit in any of our ischemic groups (including vehicle + 4VO) when compared with sham control (Iyirhiaro G. O. and Park D. S., unpublished data, 2005). This most likely reflects adaptation and or compensation in the brain circuitry. Alternatively, it may reflect the sensitivity of the test paradigm used in detecting deficits in the long-term. Behavioral differences have been known to dissipate with time following stroke because of spontaneous

remapping and recovery. Thus, our behavior paradigm may not have been challenging enough to detect behavioral difference in the long-term.

There are two other reasons why this combinatorial strategy may not have promoted long-term synaptic improvements. First it is possible that the use of a broad spectrum mitotic inhibitor such as flavopiridol could potential inhibit endogenous brain repair mechanism such as ischemia-induced neurogenesis. Indeed, stroke-induced neurogenesis has been demonstrated in models of cerebral ischemia, including human stroke (Liu et al., 1998, Jin et al., 2001b, Zhang et al., 2001, Jin et al., 2006). Furthermore, neurogenesis and agents that promote neurogenesis such as erythropoietin and vascular endothelial growth factor are associated with improved neurological function (Sun et al., 2003, Wang et al., 2004a, Thored et al., 2006). Further study is needed to address this issue. Second, use of minocycline for 2 weeks in our paradigm may prevent some of the benefits of inflammation. Indeed inflammation may play a dual role in the ischemic brain. It can mediate the removal of debris from dead or dying cells and facilitate recovery (Lucas et al., 2006, Wang et al., 2007). Furthermore, microglia cells have been demonstrated, at least *in vitro* to produce neuroprotective factors such as neurotrophin 3, nerve growth factor, basic fibroblast growth factor, brain-derived neurotrophic factor, and plasminogen (Kim and de Vellis, 2005). Thus, chronic treatment with anti-inflammatory agent as carried out in the present study may suppress the normally beneficial function of inflammation. However, persistent inflammation following ischemia can also exacerbate tissue damage through the recruitment of inflammatory cells and production of cytotoxic agents (Wang et al., 2007). Accordingly, studies, including our own, targeting components of the inflammatory reaction have

demonstrated protection in models of cerebral ischemia (Yrjanheikki et al., 1998, Yrjanheikki et al., 1999, Lucas et al., 2006, Wang et al., 2007).

In summary, our result using standard histological methods show that we can provide enduring protection for neuronal soma using both CDKs and an inflammation inhibition strategy but our electrophysiological data suggest that we must still address the issue of preserving neuronal processes and maintaining synaptic integrity. These findings highlight the importance of using both histological and functional measures in assessing future neuroprotection strategies.

CHAPTER 6

General Discussion

6.1. SUMMARY

A great deal of progress has been made towards our understanding of the pathophysiological processes that lead to neuronal death in stroke. However, the complexity of signals and molecular interplay that ensues following an ischemic event is still being unraveled. The goal of stroke research is to better understand these processes in the hope of identifying targets that will be amenable to new and efficacious therapeutic strategies. Much of the work presented in this thesis identifies cell cycle signals as critical mediators of ischemic neuronal death. Chapter 5 of the thesis explores the integration of Cdks as potential therapeutic targets for treatment of cerebral ischemic injury. A thorough discussion of results has already been presented as part of each of the thesis manuscripts. As such, the key findings presented in the thesis as well as their implications will be briefly summarized in the subsequent sections.

6.2. OVERVIEW OF MAJOR FINDINGS

Prior to this thesis work, it was unclear whether the upregulation of cell cycle machinery in ischemic neurons was an artifact or causative of cell death. Correlative data implicating cell cycle molecules was obscured by evidence of Cdk5 involvement in ischemic neuronal death. This was confounded by the non-specific nature of pharmacologic agents used in studies examining the role of Cdks in stroke-related injuries. In addition, data potentially implicating the cell cycle machinery had been obtained using immature neurons in cell cultures. Consequently, the physiological relevance of cell cycle signal in ischemic neuronal death was unclear. The undertaking of this thesis was fuelled by the lack of appropriate studies directly testing the functional relevance of cell cycle signals in ischemic

neuronal death. The data presented in this thesis provides a pathway by which cell cycle signals are activated following cerebral ischemia and lead to neuronal death. This is supported by a number of key findings in this thesis.

6.2.1. CDK4 MEDIATES DELAYED ISCHEMIC NEURONAL DEATH.

Using multiple systems and relevant adult ischemic models, we provided functional evidence, for the first time, in chapter 2, that the mitotic Cdk4 is a mediator of delayed ischemic neuronal death. In contrast, we found that the neuronal Cdk5 was more relevant in the context of excitotoxic neuronal death. It is interesting to note that, although previously published correlative data also implicated Cdk2 in ischemic neuronal death (Katchanov et al., 2001, Verdaguer et al., 2002, Love, 2003, Kuan et al., 2004), this Cdk was not relevant in our models. There are two possibilities for this observation. First, it is possible that select cell cycle signals are activated in different ischemic paradigms. Second, it is likely that Cdk4 engages the cell death machinery early prior to Cdk2 activation in the cell cycle. This would explain why inhibition of Cdk4, but not Cdk2, was protective following global ischemia.

6.2.2. CDC25A IS ACTIVATED AND CONTRIBUTES TO DELAYED NEURONAL DEATH

In chapter 3, it was shown that delayed ischemic neuronal death involves upstream activators of the mitotic-Cdks, in this case, Cdk4/6. The data demonstrates that inhibition of Cdc25 phosphatase is remarkably protective against delayed ischemic neuronal death. Furthermore, among the three Cdc25s, Cdc25A was critical for ischemia-induced delayed death. As discussed in the introductory section of this thesis, Cdc25 phosphatase activity is crucial to the activation of mitotic-Cdks, but not Cdk5. Thus, the implication of the results in chapter 3 is that activation of mitotic-Cdks and in our paradigms Cdk4/6 is required for

ischemic neuronal death. Another important implication of the data presented in chapter 3 is that it demonstrates that Cdk activation in adult neurons under pathologic stress such as cerebral ischemia occurs in ways similar to those observed in proliferating cells. However, unlike in mitotic cells where Cdc25 activity leads to proliferation, in neurons it signals death. Collectively, the data in chapters 2 and 3 demonstrate the involvement of cell cycle machinery in ischemic neuronal death.

6.2.3. DOWNSTREAM TARGETS OF CDC25/CDK4 PATHWAY ARE ACTIVATED AND LEADS TO DEATH.

A critical question addressed in this thesis, is how the activation of cell cycle signals death in ischemic neurons. This question is an interesting and challenging one. In one context, the same signal activated in the right type of cell (mitotic cell) leads to proliferation and in another (in adult neuron) causes death. Major targets for activated G1/S Cdks in proliferating cells include members of the retinoblastoma proteins (pRb, p107 & p130). These proteins through their interaction with various factors including members of the E2F transcription factors, chromatin-remodeling complexes acts as gate keepers of cell proliferation, differentiation, life and death (Dimova and Dyson, 2005, Du and Pogoriler, 2006, Macaluso et al., 2006, Talluri and Dick, 2012). The data presented in chapters 2 and 4 shows that in ischemic neurons these proteins are also targets of Cdk4. They serve as proximal death initiating signals, at least in part through the activation/de-repression of death inducing genes. The data presented as part of chapter 2 demonstrated that pRb is increasingly phosphorylated by Cdk4 in response to ischemic insults *in vitro* and *in vivo*. Importantly, we showed that expression of a phosphorylation resistant mutant form of pRb is protective against hypoxia-induced neuronal death. Interestingly, I showed in chapter 4 that another

member of the retinoblastoma protein family, p130 is also increasingly phosphorylated following ischemic stress. Importantly I provided evidence that both p130 and E2F4 proteins are lost following ischemic stress. A seminal finding in chapter 4 is the discovery that both the activating E2F1 and the repressive E2F4 act cooperatively to differentially regulate the death inducing factor B-Myb in response to ischemic stress.

6.3. IMPORTANT CONSIDERATIONS AND FUTURE DIRECTIONS.

6.3.1. POTENTIAL FOR CDK5 INVOLVEMENT IN PATHOGENIC CELL CYCLE RE-ENTRY IN NEURONS

The work presented in chapter 2 of this thesis demonstrated a differential activation of Cdk4 and Cdk5 in ischemic damage. While we have shown that Cdk4 signal prevails under conditions of delayed ischemic death and Cdk5 in excitotoxicity; the possibility of a cross talk between Cdk5 dysregulation and neuronal cell cycle re-entry was recently proposed (Kim et al., 2008, Zhang et al., 2010b, Zhang and Herrup, 2011, Chang et al., 2012). Indeed it has been suggested that Cdk5 may act upstream of cell cycle re-entry in neurons. As discussed in the introduction section of this thesis, loss of nuclear Cdk5 is associated with neuronal cell cycle re-entry, at least in the context of Aβ induced toxicity (Zhang and Herrup, 2008, Zhang et al., 2010a). One line of evidence showed that Cdk5 prevented the expression of cell cycle genes through its interaction with E2F1 and displacement of DP1 (Zhang et al., 2010b, Zhang and Herrup, 2011). Interestingly, this function of Cdk5 was reported to be independent of its kinase activity (Zhang and Herrup, 2008).

A second line of evidence, showed that dysregulation of p25/Cdk5 led to aberrant expression of cell cycle proteins and apoptosis in a p25 inducible transgenic mouse model of Alzheimer's disease (Kim et al., 2008). This was shown to occur via Cdk5-mediated inhibition of HDAC1 activity leading to expression of cell cycle genes (Kim et al., 2008).

A third line of evidence proposed that Cdk5 acts upstream of Cdk4 by activating Cdc25 (Chang et al., 2012). Chang et al. showed in the context of Aβ induced neurotoxicity that Cdk5 phosphorylates all three members of the Cdc25 family increasing their phosphatase activity and activation of Cdk1, Cdk2 and Cdk4 (Chang et al., 2012).

While these reports were not rigorously investigated in this thesis, they would predict that inhibition of Cdk5 or over-expression of DNCdk5 would be protective in models where Cdk4 activation is implicated. Thus it would be expected that inhibition of Cdk5 activity would be protective following 10 minutes global ischemia in the studies presented in chapter 2. However, we did not find this to be the case in our studies. Nonetheless, an interesting possibility exist that under certain contexts there may be crosstalk between Cdk4 and Cdk5. For example, I found that expression of DNCdk4 was partially protective in glutamate induced death. Similarly, Cdc25C and compound Cdc25B&C deficiencies were protective following excitotoxic death induced by hypoxia and glutamate respectively. These are models in which we showed that inhibition of Cdk5 is protective. It will be interesting in the future to fully test whether dysregulation of Cdk5 in these models also leads to Cdk4 activation.

6.3.2. POSSIBLE INVOLVEMENT OF TRIP-BR1 IN THE PATHOGENIC ACTIVATION OF CDK4/PRB/E2F PATHWAY.

As briefly discussed in chapter 1, Cdk4 activity can be modulated by at least one other interacting protein, Trip-Br1. Interaction of Trip-Br1 with Cdk4 not only renders it resistant to the inhibitory effects of $p16^{INK4A}$ but also stimulates pRb phosphorylation (Sugimoto et al., 1999). Interestingly, Trip-Br1 can also interact with E2F1/DP1 to stimulate gene transcription (Hsu et al., 2001). Published work from Dr. Park's laboratory has shown that this Cdk4 interacting protein is upregulated in at least three neuronal death paradigms induced by DNA damage, NGF withdrawal and Aβ. Downregulation of Trip-Br1 attenuates pRb phoshorylation following NGF withdrawal (Biswas et al., 2010). Although not included in this thesis, I have data showing that this Cdk4 interacting proteins is also induced following global ischemia. Additionally, I have data showing that down-regulation of Trip-Br1 provides protection from ischemic neuronal death induced by 10 minutes 4VO and hypoxia/reoxygenation insult *in vitro* (Iyirhiaro et al, unpublished data). Given the ability of this protein to stimulate Cdk4 activity, it would be interesting to examine whether Trip-Br1 contributes to Cdk4 activation as well as downstream induction of E2F1 activity following ischemic insult. For example it would be intriguing to test whether Cdk4 kinase activity is altered following downregulation or over-expression of Trip-Br1. We showed in chapter 4 that E2F1 association with the promoter of the pro-apoptotic B-Myb gene is induced following ischemia. It would also be interesting to test if Trip-Br1 also directly contributes to the induction of E2F1 transcriptional activity following ischemic insults.

6.4. A CELL CYCLE INDUCED CELL DEATH PATHWAY FOR ISCHEMIC NEURONS

Overall, the work presented in chapters 2, 3 and 4 describes a pathway by which cell cycle signals are activated by ischemic insult and contribute to cell death. In this scheme,

Cdc25 phosphatase activity is increased resulting in the activation of G1/S-phase Cdks. Once activated, the G1/S-phase Cdks, in particular Cdk4/6, phosphorylate members of the retinoblastoma proteins including pRb and p130. Phosphorylation of these proteins disrupts their interaction with their E2F partners. Consequently p130/E2F4 repressor complexes are lost, allowing for the transcription of E2F1/DP dependent death inducing genes such as Myb (Figure 6.1).

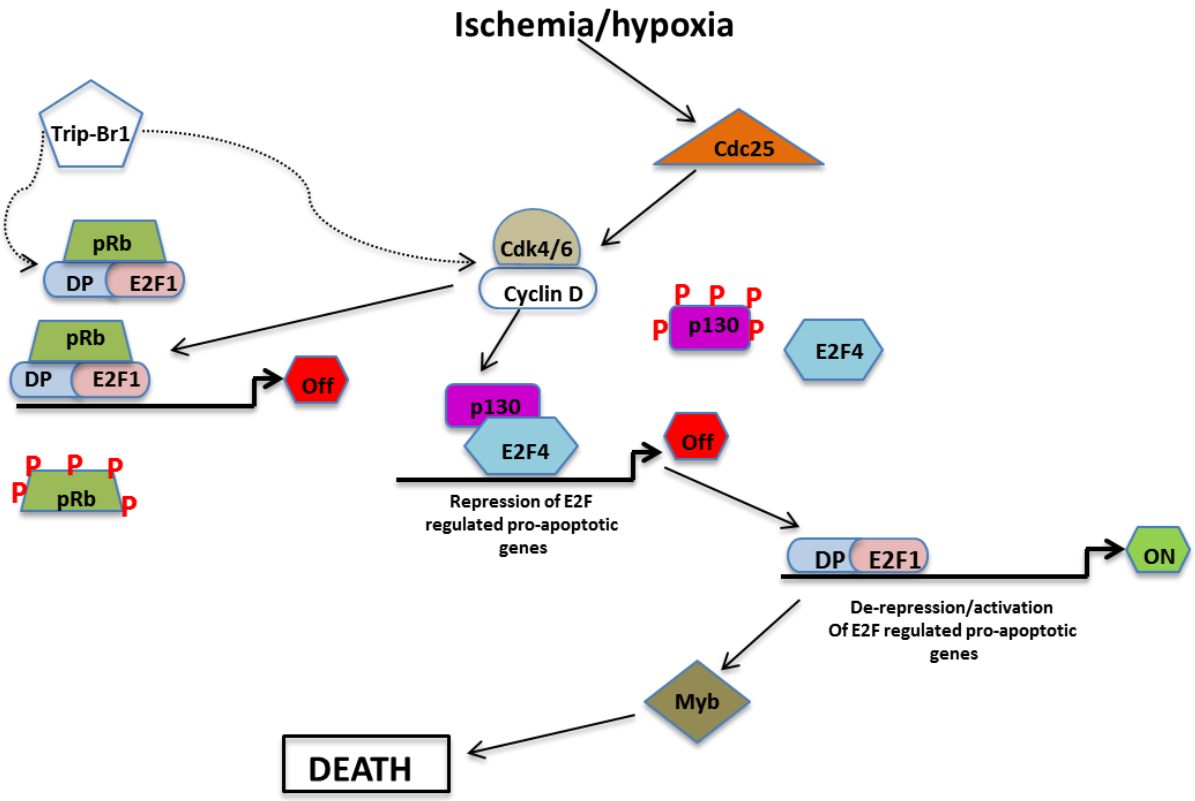

Ischemia/hypoxia
Trip-Br1
Cdc25
pRb
DP
E2F1
Cdk4/6
Cyclin D
P P P
P p130 P
E2F4
pRb
DP
E2F1
Off
p130
E2F4
Off
P P P
P pRb P
Repression of E2F
regulated pro-apoptotic
genes
DP
E2F1
ON
De-repression/activation
Of E2F regulated pro-apoptotic
genes
Myb
DEATH

Figure 6.1

Figure 6.1. Schematic representation of proposed cycle cell induced neuronal death pathway following ischemic/hypoxic insult. In response ischemic/hypoxic stress, Cdc25 activity increases resulting in the activation of Cdk4/6. Consequently, the retinoblastoma proteins pRb and p130 becomes hyper-phosphorylated interfering with their ability to bind and repress E2F regulated death-inducing genes such as Myb. As a result p130/E2F4 complexes, the major repressive E2F complex are lost. E2F1/DP association with the Myb promoter increases resulting in Myb induction and cell death. Alternatively, there is a possibility that in addition to the pathway described by the work presented in this thesis (solid arrows); that increase in Cdk4 and/or E2F1/DP activity is facilitated by Trip-Br1 following ischemic stress (dotted arrows). This possibility remains to be fully tested (dotted arrows).

6.5. MOVING BEYOND THE BENCH TO BEDSIDE WITH CDK- INHIBITION BASED STRATEGY.

A major goal of stroke research is to identify targets that will be amenable to therapeutic strategies and ultimately to minimize the devastating impact of cerebral ischemic damage. In this regard, we have identified Cdks as mediators of ischemic neuronal death. However a number of parameters must be met before any neuroprotectant strategy, including Cdk inhibition can progress from the bench to the bedside. These include

I. The ability to achieve therapeutic dosage without toxicity or adverse effects
II. Long therapeutic window.
III. Must demonstrate efficacy in multiple models of stroke.
IV. Demonstrate efficacy as a monotherapy.
V. Demonstrate long-term histologic and functional protection.

6.5.1. CDK-INHIBITION BASED STRATEGY AS POTENTIAL TREATMENT FOR CEREBRAL ISCHEMIA

Previous studies from Dr. Park's laboratory have shown that the pan-Cdk inhibitor flavopiridol meets all but the last of these criteria. In particular, Wang et al. showed that one of the caveats of using flavopiridol as a mono therapeutic agent is that it does not provide long-term protection. Indeed, flavopiridol administered to rats four hours after global ischemia provided better histologic and behavioural outcomes compared to non-treated animals one week following insult. However, it failed to provide protection three weeks after insult (Wang et al., 2002).

The work presented in chapter 5 of this thesis pushed the boundary of Cdk-inhibition strategy with flavopiridol by employing a combinatorial treatment approach. The studies in chapter 5 showed that administration of flavopiridol and minocycline provided long-term (10 weeks) histological protection for neurons following global cerebral ischemia. A drawback in these studies was that in spite of the long-term preservation of neuronal cell bodies, neuronal processes continued to degenerate. The preserved neurons lacked function. These results suggest that additional factors must be addressed in order to attain long-term functional neuroprotection with this strategy. Overall, the work presented in chapter 5 demonstrates, as a proof of concept, that therapeutic approaches targeting multiple pathologic signals activated in stroke may be more beneficial than a single therapy strategy. Importantly, it emphasizes the need for a better understanding of biological processes important to the proper functioning of neurons.

Stroke is a complex pathology that involves the activation of many pathologic processes, any number of which is sufficient to trigger neuronal dysfunction and death. Employing a multi-target approach may provide an avenue of achieving efficacious treatment for cerebral ischemia-related injuries. However, a number of caveats must be addressed before such an approach becomes clinically viable. These include the following, in addition to criteria already discussed above:

I. The “on target” specificity of each drug
II. The “off target” effect of each drug
III. The potential of drug interaction and adverse effects
IV. Drug delivery and dosing regimen.

6.6. CONCLUSION

On the whole the results presented in this thesis emphasize the importance of cell cycle suppression for the survival of adult neurons under ischemic stress conditions. As discussed, cell cycle re-entry is a feature of a number of neuropathological conditions. Thus it would be prudent in the future to further our understanding of the mechanism(s) responsible for the loss of cell cycle suppression in neurons. Such endeavors may likely reveal new and amenable drug targets that could prove beneficial for the treatment of stroke-related injuries and possibly other neurological conditions.

Appendix I:

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Authors: Biswas SC, Zhang Y, **Iyirhiaro G**, Willett RT, Rodriguez Gonzalez Y, Cregan SP, Slack RS, Park DS, Greene LA.

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Appendix III:

Additional Publication

Sertad1 Plays an Essential Role in Developmental and Pathological Neuron Death.

Subhas C. Biswas, Yi Zhang, **Grace Iyirhiaro**, Ryan T. Willett, Yasmilde Rodriguez Gonzalez, Sean P. Cregan, Ruth S. Slack, David S. Park, and Lloyd A. Greene.

Published in: ***The Journal of Neuroscience*** *(2010), 30(11):3973-3982*

Author contribution

This study represents a collaborative effort between the laboratories of Dr. David S. Park and Dr. Lloyd A. Greene. My contribution to this study was examining changes in Sertad1 protein levels in cortical neuronal cultures following DNA damage insult resulting in the generation of figure 1B.

Neurobiology of Disease

Sertad1 Plays an Essential Role in Developmental and Pathological Neuron Death

Subhas C. Biswas,[1]* Yi Zhang,[3]* Grace Iyirhiaro,[3] Ryan T. Willett,[2] Yasmilde Rodriguez Gonzalez,[3] Sean P. Cregan,[4] Ruth S. Slack,[3] David S. Park,[3,5] and Lloyd A. Greene[1]

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Developmental and pathological death of neurons requires activation of a defined pathway of cell cycle proteins. However, it is unclear how this pathway is regulated and whether it is relevant *in vivo*. A screen for transcripts robustly induced in cultured neurons by DNA damage identified Sertad1, a Cdk4 (cyclin-dependent kinase 4) activator. Sertad1 is also induced in neurons by nerve growth factor (NGF) deprivation and Aβ (β-amyloid). RNA interference-mediated downregulation of Sertad1 protects neurons in all three death models. Studies of NGF withdrawal indicate that Sertad1 is required to initiate the apoptotic cell cycle pathway since its knockdown blocks subsequent pathway events. Finally, we find that Sertad1 expression is required for developmental neuronal death in the cerebral cortex. Sertad1 thus appears to be essential for neuron death in trophic support deprivation *in vitro* and *in vivo* and in models of DNA damage and Alzheimer's disease. It may therefore be a suitable target for therapeutic intervention.

Introduction

Neuronal loss by apoptosis is a physiological process during development (Oppenheim, 1991) and a pathological hallmark of many neurodegenerative disorders such as Alzheimer's disease (AD) and of additional insults to the nervous system such as DNA damage (Park et al., 1997a). There are striking and mutually informative similarities between the molecular mechanisms that govern neuron death under these various conditions (Greene et al., 2004, 2007). However, the molecular events, particularly those that initiate death of neurons during development and disease/injury, are incompletely understood.

One major focus regarding the mechanisms of developmental and disease-associated neuron death has been the aberrant activation of cell cycle-related proteins (Becker and Bonni, 2004; Greene et al., 2004, 2007; Herrup et al., 2004). Past studies have indicated a sequential and multistep pathway that is activated by various apoptotic insults including nerve growth factor (NGF) deprivation, DNA damage, and β-amyloid (Aβ) exposure and that is required for neuron death. The first described step is rapid activation of the G_1/S kinase cyclin-dependent kinase 4 (Cdk4). This in turn hyperphosphorylates members of the Rb family, leading to dissociation of complexes comprised of Rb family members and E2F transcription factors. Ultimately, these events lead to induction of proapoptotic genes such as Bim and to activation of the core apoptotic machinery (Greene et al., 2007).

An important and currently unresolved issue about the apoptotic cell cycle pathway is how Cdk4 is activated in neurons by apoptotic stimuli. Understanding this will not only further elaborate how apoptotic stimuli lead to neuron death but may also identify additional molecular targets for therapeutic intervention. In addition, it must be recognized that the majority of evidence that causally links the steps in the apoptotic cell cycle pathway has been generated by *in vitro* studies. Thus, it remains important to demonstrate that the elements that make up this pathway are relevant *in vivo*.

The protein Sertad1, also known as p34(SEI-1) or Trip-Br1, has been implicated as a regulator of Cdk4 activity. Sertad1 was first identified as an antagonist of p16 INK4a that facilitates the formation and activation of cyclin D–Cdk4 complexes (Sugimoto et al., 1999). Additional studies revealed that it directly binds and activates Cdk4 in a concentration-dependent manner (Li et al., 2004). Functions in addition to regulation of Cdk4 have been described for Sertad1 including stimulation of the transcriptional activities of E2F1 (Hsu et al., 2001) and p53 (Watanabe-Fukunaga et al., 2005). Sertad1 was also additionally reported to exhibit antiapoptotic activity by stabilizing X-linked inhibitor of apoptosis protein (XIAP) in cancer cells (Hong et al., 2009).

In a screen for genes regulated in neurons after DNA damage, we identified Sertad1 transcripts as being robustly induced. Ac-

Received Dec. 29, 2009; accepted Jan. 25, 2010.

This work was supported in part by grants from the National Institute of Neurological Disorders and Stroke (L.A.G.), Alzheimer's Disease Research Center/Taub pilot grant (Columbia University; S.C.B.), Indian Institute of Chemical Biology (S.C.B.), Canadian Institute of Health Research (R.S.S., D.S.P.), Heart and Stroke Foundation Ontario (R.S.S., D.S.P.), and World Class University Program through National Research Foundation of Korea Grant R31-2008-000-20004-0 (D.S.P.). We thank Janet Peterson for technical assistance. We also thank Dr. Julio Pozueta for providing us dodecamer β-amyloid.

*S.C.B. and Y.Z. contributed equally to this work.

cordingly, we examined the potential role of Sertad1 in neuron death induced by apoptotic stimuli relevant both to normal development and to neurodegeneration. We find that Sertad1 is required for neuronal apoptosis both *in vitro* and *in vivo*. Furthermore, our findings indicate that Sertad1 is essential for initiating the Cdk4-dependent cascade of cell cycle events in neuronal cells that follow from trophic factor deprivation.

Materials and Methods

Materials. Platinum TaqDNA polymerase, V5 antibody, green fluorescent protein (GFP) antibody, and Lipofectamine 2000 were from Invitrogen; anti-human NGF antiserum and anti-β-actin antibody were from Sigma-Aldrich; anti-Sertad1 antibody and Bim antibody were from Abcam; anti-ERK1, phospho-Rb, and C-Myb antibodies were from Santa Cruz Biotechnology; Zsgreen antibody was from Clontech; and the *In Situ* Cell Death Detection kit, TMR red was from Roche Applied Science. pSIREN vector was from BD Biosciences. Human recombinant NGF was a kind gift from Genentech. Camptothecin was obtained from Sigma-Aldrich. Bis-isopropylthiomethyl-K-252a (CEP11004) was obtained from Cephalon. E2F-1 and control short interfering RNAs (siRNAs) and E2F-1 antibody were purchased from Santa Cruz Biotechnology. p53 mice were genotyped according to published protocols (Aleyasin et al., 2004).

Cell culture. PC12 cells were cultured and neuronally differentiated as previously described (Greene and Tischler, 1976). For NGF deprivation, after a week of NGF treatment, the cultures were washed with NGF-free medium twice, and anti-NGF antibody (1:100) was added. Control cells were washed with serum-free medium and maintained in RPMI 1640 medium supplied with NGF without serum. Neonatal rat superior cervical ganglion sympathetic (SCG) neurons were cultured as previously described (Park et al., 1998). HEK293 cells were cultured in DMEM with 10% fetal bovine serum. Embryonic rat and mouse cortical neurons were cultured as previously described (Park et al., 1998).

Microarray. Total RNA was extracted from cortical neuron cultures using Trizol reagent according to the manufacturer's instructions (Invitrogen). RNA was sent to the Ottawa Genomics Innovation Centre Microarray Facility for processing and expression analysis using the Affymetrix Mouse 430 array (Affymetrix). Probe signals were scaled and normalized according to standard facility procedures.

Semiquantitative reverse transcriptase-PCR. Total RNA was extracted using TriPure isolation reagent (Roche Applied Science). Fifty nanograms of total RNA were used for cDNA synthesis and gene amplification reactions using SuperScript One-Step RT-PCR kit (Invitrogen). cDNA synthesis was performed at 48°C for 45 min, followed by a 2 min initial denaturation step at 94°C. This was followed by 30 cycles (Sertad1) or 25 cycles (S12) at 94°C for 30 s, melting temperature (Tm) 60°C for 30 s, and 72°C for 1 min. Targeting primers were as follows: 5′-CGCAAGCGGGAGGAGGAGAC-3′ and 5′-AGGGGCTGGGGGCTGGATGG-3′ for Sertad1, 5′-GGAAGGCATAGCTGCTGG-3′ and 5′-CCTCGATGACATCCTTGG-3′ for S12. Transcript levels were normalized against S12 signals, and results were reported as times fold increase in reference to untreated control values. Data are presented as mean ± SEM of three independent experiments.

Reverse transcription-quantitative PCR. Each sample of total RNA was isolated from cultured neurons by using TRI reagent (Molecular Research Center). cDNA was transcribed from total RNA with Superscript RT II (Invitrogen). The primers used for PCR amplification of rat Sertad1 were 5′-GCCTCCTGGAAGATCTCAGTC-3′ and 5′-CATTCTCAGGGACAGGTTTGA-3′. The primers for α-tubulin were 5′-ATGAGGCCATCTATGACATC-3′ and 5′-TCCACAAACTGGATGGTAC-3′. Equal amounts of cDNA template were used for each PCR analysis of Sertad1 or α-tubulin. Quantitative PCR was performed using a Cepheid SmartCycler following the manufacturer's specifications. α-Tubulin was used for Sertad1 transcript normalization. cDNA was added to a 25 μl volume reaction mix containing OmniMix HS master mix (Cepheid) and SYBR Green I (Invitrogen) together with appropriate primers at 0.2 μM each. Analyses of growth curves of real-time fluorescence and of melting curves were performed as described previously (Troy et al., 2000).

Table 1. Sertad1 upregulation in gene chip analysis

	Untreated	Camptothecin[a]	Camptothecin/untreated signal ratio	*p* value
Sertad1	135.8	6264.2	46.1	0.000167
Noxa	542.8	2141.6	3.95	0.000273
Puma	1124.2	3640.7	3.24	0.00013
Tubb3[b]	22,718.3	21,207.1	0.93	NS[c]

Microarray analysis of RNA extracted from cortical neurons after campothecin (10 M) treatment for 8 h or with vehicle (0.1% DMSO). Fold change represents the ratio of signal in campothecin-treated neurons relative to vehicle-treated neurons for each probe set.

[a]Cortical neurons were treated with camptothecin for 8 h.

[b]Tubb3 is β3-tubulin.

[c]Not significant.

Western immunoblotting. Neuronal PC12 cells were lysed and protein was analyzed by Western immunoblotting as described previously (Biswas and Greene, 2002). For mouse cortical neurons, Sertad1 was detected using a chicken IgY antibody against Sertad1 (1:1000; Genway). Goat anti-chicken HRP (1:3000) was used as secondary antibody.

Plasmids. Rat Sertad1 was generated by reverse transcription (RT)-PCR of PC12 cDNA. The primers for the amplification were 5′-AGGATGCTGAGCAAAGGTCT-3′ and 5′-GCGCCCAGGTCCTGGTGGCC-3′. The PCR product was gel purified and cloned into pCDNA3.1 vector (Invitrogen), and then verified by sequencing. Sertad1 was also subcloned into pCMS-EGFP vector (Clontech) by using primers 5′-GATCTCGAGACCATGCTGAGCAAAGGTCTG-3′ and 5′-CTAGTCGACCTAGCGCCCAGGTCCTGGTGG-3′.

Preparation of short hairpin RNA. Sertad1 short hairpin RNAs (shRNAs) were prepared in the pSIREN vector by using BD Knockout RNAi Systems according to the manufacturer's instructions (BD Biosciences) based on the following sequences: 5′-CCGTGGCTTCTAGCTCTCT-3′ (#2), 5′-GCTCCACCACAGCCTTCGG-3′ (#3), 5′-CCAGACCTCCGACACCTGG-3′ (#4), 5′-GATCTCAGTCATATTGAGG-3′ (#5). pSIREN-shRNA-RAND-Zsgreen was as described previously (Sproul et al., 2009). For *in utero* electroporation (see below), GFP constructs of Sertad1 shRNA and control shRNA were prepared by subcloning the shRNA expression cassette from pSIREN vector into pCMS-EGFP backbone sequence. The (CMV promoter)-MCS sequence in pCMS-EGFP was substituted with the (U6 promoter-shRNA) sequence from pSIREN-RetroQ-zsGreen by subcloning with BglII and EcoRI restriction enzymes. The control shRNA is an inactive mutant of the primary siRNA knockdown construct for GATA2: 5′-GCACCTGATGTCTTCTTCAACC-3′.

Transfections. DNA was prepared with Plasmid Maxi kits (QIAGEN). Neuronal PC12 cells were cotransfected with 0.5 μg of plasmid pCDNA-V5, pCDNA-Sertad1-V5, pCMS-EGFP, pCMS-Sertad1-EGFP, pSIREN-shRNA-Sertad1-Zsgreen (#2, #3, #4, or #5), pSIREN-shRNA-Rand-Zsgreen, or pSIREN-shRNA-Luc-Zsgreen in 500 μl of serum-free medium per well in 24-well dishes using Lipofectamine 2000. Six hours later, medium with Lipofectamine 2000 was replaced with fresh complete medium. HEK293 cells were transfected as previously described (Xu et al., 2001). E2F-1 siRNA were transfected as previously described (Zhang et al., 2006).

Sertad1 shRNAs and viruses. Mouse Sertad1-specific shRNA oligos (Applied Biosystems) were cloned into pSilencer3.0-H1 vector (Applied Biosystems). The shRNA fragments containing the H1 promoter were subcloned into the pAdTrack vector. shRNA adenoviruses and DN-c-Jun adenoviruses were constructed as previously described (He et al., 1998).

Preparation of amyloid. Lyophilized, HPLC-purified $A\beta_{1-42}$ was purchased from American Peptide, and dodecamer $A\beta_{1-42}$ was prepared as described previously (Barghorn et al., 2005). Briefly, $A\beta_{1-42}$ was reconstituted in 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to 1 mM, and HFIP was removed by evaporation in a Speed Vac, and then resuspended to 5 mM in anhydrous DMSO. This stock was then diluted with PBS to a final concentration of 400 μM. SDS was added to a final concentration of

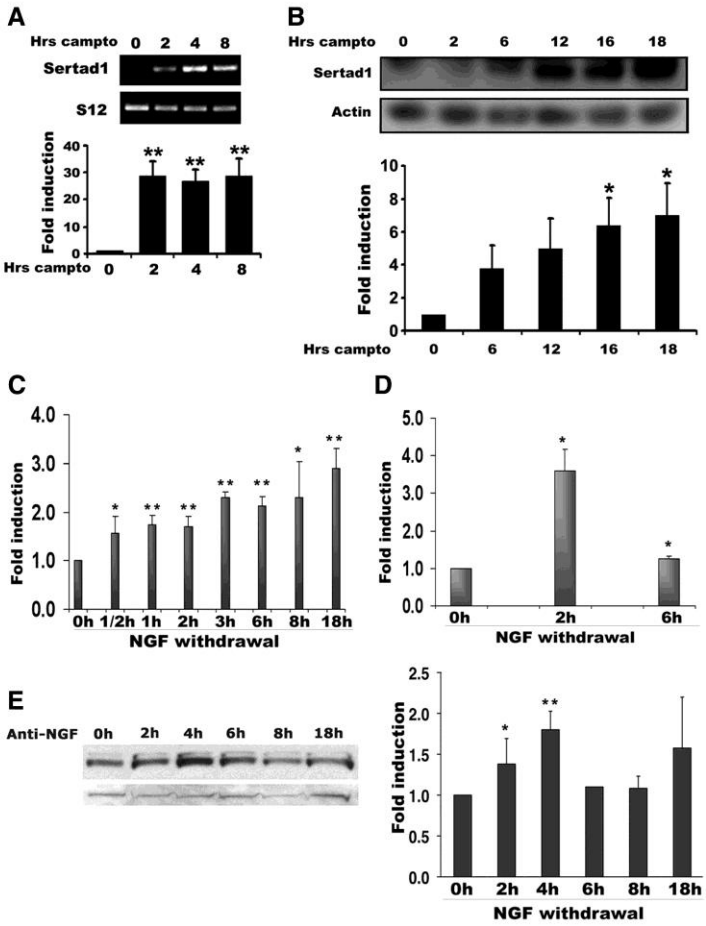

Figure 1. Sertad1 mRNA and protein levels are upregulated after DNA damage and NGF deprivation. ***A***, ***B***, Total mRNA or whole-cell lysates were extracted from cortical neurons treated with camptothecin (10 μM) for the indicated times and subjected to RT-PCR (***A***, top) or Western blot (***B***, top), respectively. Densitometry of all signals was performed using NIH ImageJ software and Sertad1 signals were normalized to S12 (***A***, bottom) or actin (***B***, bottom). Data are presented as fold increase relative to untreated control. Each bar represents the mean ± SEM from four independent experiments (one-way ANOVA/Tukey's least significant difference test; significance comparisons with untreated controls, $*p < 0.05$, $**p < 0.01$). ***C***, ***D***, Sertad1 mRNA levels are increased in response to NGF deprivation. Neuronal PC12 cells (***C***) and sympathetic neurons (***D***) were subjected to NGF deprivation for the indicated times, and total RNA was isolated, reverse transcribed, and amplified by PCR using specific primers for Sertad1 and α-tubulin. Data represent means ± SEM of three experiments (neuronal PC12 cells) or two experiments (SCG neurons), each performed with three replicate cultures. The asterisks denote statistically significant differences from 0 h control: $*p < 0.05$; $**p < 0.001$. ***E***, NGF withdrawal elevates Sertad1 protein levels. Neuronal PC12 cells were subjected to NGF withdrawal for the indicated times, and cell proteins were subjected to Western immunoblotting using enhanced chemiluminescence for the detection of Sertad1 and ERK 1 (loading control). The right panel shows quantification of Sertad1 signals, normalized against ERK1 expression. Data represent means ± SEM of five experiments (except 6 h which is a single experiment). The asterisks denote statistically significant differences from 0 h control: $*p < 0.05$; $**p < 0.001$.

0.2% and the resulting solution was incubated at 37°C for 18–24 h. The preparation was diluted again with PBS to a final concentration of 100 μM and incubated at 37°C for 18–24 h.

In utero *electroporation.* Sprague Dawley rats (Charles River Laboratories) were housed, cared for, and electroporated under the guidelines established by Columbia University Medical Center Institutional Animal Care and Use Committee. Timed pregnant rats [embryonic day 16 (E16)] were anesthetized with ketamine/xylazine (100/10 mixture; 0.1 mg/g body weight, i.p.). The uterine horns were exposed, and the left lateral ventricles of embryos were injected with DNA constructs of a control shRNA or Sertad1 shRNA (1–5 μg/μl) and Fast Green (2 mg/ml; Sigma-Aldrich) by using pulled glass capillaries (Sutter Instrument). Electroporation was accomplished with a BTX electro square electroporator, model ECM830 (BTX). The head of each embryo was held between tweezer-type circular electrodes (Harvard Apparatus) across the uterus wall, and five electrical pulses (amplitude, 50 V; duration, 50 ms; intervals, 100 ms) were delivered. Brains from postnatal pups of 5 d of age were fixed in 4% paraformaldehyde (PFA) by cardiac perfusion.

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling assay and immunohistochemistry. Rat pups were anesthetized and perfused transcardially with 4% PFA in PBS, pH 7.4. After perfusion, brains were dissected out from the skull and postfixed overnight in fresh fixative. Then brains were washed with PBS, pH 7.4, and cryoprotected in 30% sucrose phosphate buffer. They were frozen with OCT and dry ice and sectioned (20 μm thick) in the coronal plane with a cryostat. To visualize nuclei with DNA cleavage, residues of fluorescein-labeled nucleotides were catalytically added to DNA fragments by terminal deoxynucleotidyl transferase (TdT). Briefly, sections were fixed in fresh 4% PFA/PBS at room temperature for 20 min, washed in PBS three times for 5 min, permeabilized with proteinase K for 5 min on ice, and incubated with nucleotide mix and TdT (*In Situ* Cell Death Detection kit, TMR red) at 37°C for 1 h. Apoptotic cells exhibit strong, nuclear red fluorescence. To visualize GFP-positive cells, sections were then immunostained with rabbit anti-GFP antibody (1:1000; Invitrogen) in 3% nonimmune goat serum overnight at 4°C, followed by secondary labeling with goat anti-rabbit antibody (1:4000; Alexa Fluor 488; Invitrogen) for 1 h.

Survival assays. Neuronal PC12 cells, sympathetic neurons, or cortical neurons were transfected with either pCMS-Sertad1-EGFP, pCMS-EGFP, pSIREN-Sertad1-shRNA, pSIREN-Luc-shRNA, or a Random pSIREN-ZsGreen, and then 48 h later deprived of NGF (in case of neuronal PC12 cells and sympathetic neurons) or treated with 1.25 μM dodecamer Aβ (in case of cortical neurons). The numbers of surviving transfected (green) cells per well were assessed just after treatment and at 24 and 48 h after NGF deprivation or Aβ exposure as described previously (Biswas et al., 2007). Data represent means ± SEM of three experiments performed in triplicate.

Immunostaining. Neuronal PC12 cells were transfected as described above with appropriate constructs of shRNA. Forty-eight hours later, cells were subjected to NGF withdrawal for 18 h and then immunostained as described by Angelastro et al. (2003). Briefly, PC12 cells were fixed with 4% paraformaldehyde for 10 min. After three washes with PBS, cells were blocked in 3% nonimmune goat serum for 2 h. The cultures were immunolabeled with rabbit anti-Bim (1:1000; Abcam) antibody, rabbit C-Myb antibody (1:500; Santa Cruz Biotechnology), or rabbit p-Rb antibody (1:100; Santa

Cruz Biotechnology) in 3% nonimmune goat serum overnight at 4°C, followed by secondary labeling with goat anti-rabbit antibody (1:1000; Alexa Fluor 568; Invitrogen) for 1 h. For Sertad1 knockdown experiment, the cultures were immunostained with anti-Sertad1 antibody (1:200; Genway) followed by secondary labeling with goat anti-chicken antibody (1:500; Invitrogen) for 1 h.

Results

Sertad1 is rapidly induced in response to DNA damage and NGF deprivation

In an initial microarray screen for genes induced in cortical neurons by treatment with the DNA-damaging agent camptothecin (8 h), we identified Sertad1 as a candidate regulated gene. Induction was 46-fold over controls (Table 1). In the same screen, we detected increases in the Puma and Noxa genes as expected and previously reported (Aleyasin et al., 2004; Cregan et al., 2004). In contrast, β-tubulin did not change (Table 1). To verify the array data, we performed a semiquantitative reverse transcriptase-PCR assay. Consistent with the array data, the RT-PCR results demonstrated a robust (~27-fold) increase of Sertad1 message as early as 2 h after camptothecin exposure. This increase persisted even at 8 h after camptothecin treatment (Fig. 1*A*). A similar magnitude of increase was obtained by quantitative PCR (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Next, we asked whether the DNA damage-induced elevation in Sertad1 message is associated with an increase in protein level. As revealed by Western blotting (Fig. 1*B*), Sertad1 protein expression was significantly induced by 6 h (~3.2-fold) of camptothecin exposure and gradually increased until 18 h (~7.2-fold). Under the conditions of our experiments, neuronal death first becomes apparent by 8–10 h of camptothecin exposure and 50% of neurons die within 16–20 h. Thus, Sertad1 induction is observed early and before overt signs of death.

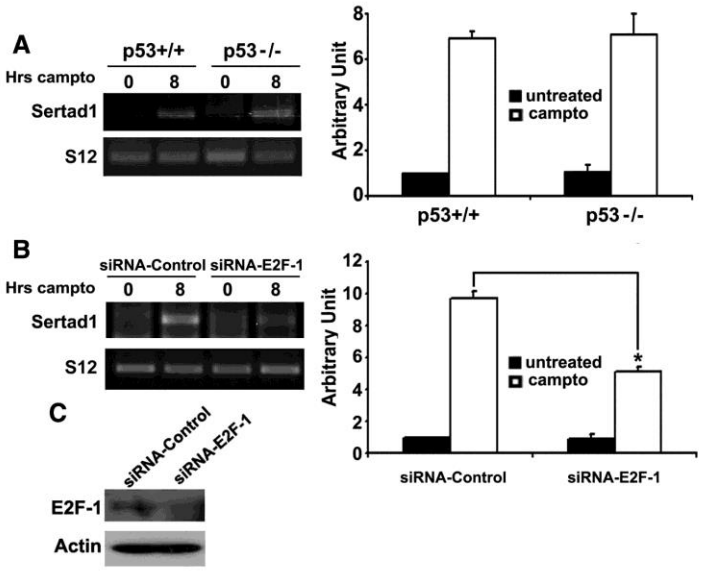

Figure 2. ***A***, p53 deficiency does not inhibit camptothecin-induced upregulation of Sertad1. Cortical neurons from $p53^{+/+}$ and $p53^{-/-}$ mice were treated with and without camptothecin for 8 h. Left, Sertad1 levels were then analyzed by RT-PCR. S12 was shown as a control for equal input. Right, Densitometric analysis of Sertad1 signals normalized against S12 signals. ***B***, E2F-1 downregulation partially blocks camptothecin-induced upregulation of Sertad1. Cortical neurons were transfected with E2F-1 and control siRNA and were treated with and without camptothecin for 8 h. Left, Sertad1 levels were then analyzed by RT-PCR. S12 was shown as a control for equal input. Right, Densitometric analysis of Sertad1 signals normalized against S12 signals. Significance comparisons with untreated controls: $*p < 0.05$. Error bars indicate SEM. ***C***, Western blot analyses showing downregulation of E2F-1 in cells treated with E2F-1 siRNA compared with control siRNA. Actin is provided as loading control.

To identify upstream signals that mediate upregulation of Sertad1 mRNA in response to DNA damage, we tested three potential candidates: p53, E2F1, and c-Jun N-terminal kinases (JNKs). As shown in Figure 2*A*, germ line deficiency of p53 does not affect camptothecin-induced upregulation of Sertad1 message in cultured cortical neurons. In contrast, siRNA-mediated knockdown of E2F-1 blocks Sertad1 mRNA upregulation by ~50% (Fig. 2*B*). Finally, neither JNK inhibitor CEP11004 nor dominant-negative c-Jun expression affect camptothecin-induced Sertad1 upregulation (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). Together, these findings indicate that E2F1, but not p53 or the JNK pathway at least partially regulates Sertad mRNA expression after DNA damage.

Because neuronal death caused by DNA damage shares with death evoked by trophic factor deprivation a reliance on cell cycle proteins (Park et al., 1996, 1997a,b; Liu and Greene, 2001; Zhang et al., 2006; Gonzalez et al., 2008), we also investigated whether Sertad1 expression is regulated in response to NGF withdrawal. For this purpose, we used neuronally differentiated PC12 cells and primary cultures of SCG neurons. Both cell types undergo apoptosis (evident starting at ~18 h) in response to NGF deprivation (Rukenstein et al., 1991; Xu et al., 2001). Similar to DNA damage, a time course performed by quantitative PCR revealed that Sertad1 mRNA levels were elevated in neuronal PC12 cells as early as one-half an hour after NGF deprivation and were consistently and significantly increased in SCG neurons after 2 h of such treatment (Fig. 1*C*,*D*). Sertad1 protein expression was also elevated by 2–4 h in response to NGF deprivation, and this change was similar in magnitude (by approximately twofold) to that of mRNA (Fig. 1*E*). Thus, both Sertad1 mRNA and protein expression are induced in response to DNA damage and trophic factor withdrawal, although this response is more modest in the case of NGF deprivation.

Sertad1 overexpression is not sufficient to trigger neuron death but enhances death in response to NGF deprivation

Next, we examined whether elevated expression of Sertad1 is sufficient in itself to trigger neuron death in the presence of trophic support. Expression of Sertad1 alone did not induce death of neuronal PC12 cells (Fig. 3*A*), cortical neurons (supplemental Fig. S3, available at www.jneurosci.org as supplemental material), or cerebellar granule neurons (data not shown). However, overexpression of Sertad1 significantly enhanced the level of neuronal cell death that occurs in response to NGF deprivation (Fig. 3*B*).

Sertad1 is required for neuron death induced by NGF deprivation and DNA damage

Because of the observed upregulation of Sertad1 in multiple neuronal death paradigms and the sensitization to death with Sertad1

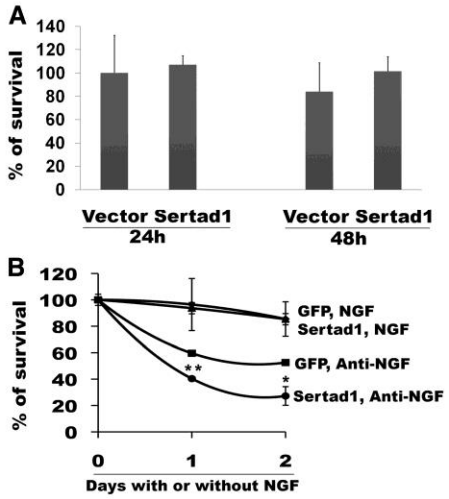

Figure 3. Overexpression of Sertad1 is not sufficient to cause death but enhances death in response to NGF withdrawal. ***A***, Neuronal PC12 cells were transfected with pCMS-EGFP or pCMS-Sertad1-EGFP. At 24 and 48 h after transfection, surviving transfected (green) cells were counted. Data represent mean of a representative experiment performed with three replicate cultures per point. Similar results were obtained in an independent experiment. ***B***, Neuronal PC12 cells were transfected with pCMS-EGFP or pCMS-Sertad1-EGFP. After 48 h, they were maintained with or deprived of NGF and surviving transfected cells were counted at 24 and 48 h. Data represent mean ± SEM of a representative experiment performed with three replicate cultures per point. Similar results were obtained in an independent experiment. The asterisks denote statistically significant differences from control (GFP) in absence of NGF: $*p < 0.05$; $**p < 0.01$.

overexpression, we next examined whether neuron death in our systems requires Sertad1. We first studied the role of Sertad1 in NGF deprivation. To achieve this, we prepared several shRNAs specifically targeted to rat Sertad1 and identified three that substantially reduced expression of the overexpressed protein (Fig. 4*A*). Each of the effective shRNAs significantly protected neuronal PC12 cells from death induced by NGF withdrawal (Fig. 4*B*,*C*). Moreover, most of the Sertad1 shRNA-expressing cells preserved overall neuron morphology even after 2 d of NGF deprivation (Fig. 4*B*). This is reminiscent of the preservation of neuronal morphology after NGF deprivation that is achieved with small molecule Cdk inhibitors (Park et al., 1996, 1997b) or Cdk4 shRNAs (Biswas et al., 2005). Similar experiments with SCG neurons also showed that downregulation of Sertad1 by two independent shRNAs significantly blocks death and preserves overall neuron morphology after NGF deprivation (Fig. 4*D*) (data not shown).

Similarly, we used a shRNA knockdown strategy to assess whether Sertad1 also plays a required role in neuronal death after DNA damage. In this case, we used adenoviruses to deliver shRNAs to cultured mouse cortical neurons. We screened two potential shRNA sequences directed against mouse Sertad1 to knockdown endogenous Sertad1. As shown in Figure 5, *A* and *B*, infection with both Sertad1 shRNA viruses, but not a control virus, significantly reduced the Sertad1 immunostaining signal (54 and 33%, respectively) after camptothecin treatment. Survival assays in response to camptothecin treatment were performed using these two Sertad1 shRNAs along with a control shRNA virus. As shown in Figure 5*C*, expression of the two different Sertad1 shRNAs resulted in significant protection from camptothecin at times up to 48 h. This finding is consistent with our observations in the NGF deprivation model and indicates that Sertad1 plays a required role in the neuronal death process.

Sertad1 is induced and required for neuron death in a model of Alzheimer's disease

We additionally examined the importance of Sertad1 in a culture model directly relevant to neurodegeneration. A substantial body of evidence has implicated cell cycle molecules in neuron death associated with Alzheimer's disease (Herrup et al., 2004; Greene et al., 2007; Zhu et al., 2007; Copani et al., 2008). Moreover, there are striking similarities between the molecular mechanisms of neuron death induced by NGF deprivation, DNA damage, and Aβ exposure (Greene et al., 2007), and recent findings support a mechanism in which neuron death triggered by NGF deprivation is mediated by elevated production and release of Aβ (Matrone et al., 2008a,b). We therefore examined whether Sertad1 is induced and is necessary for death of cultured cortical neurons induced by exposure to aggregated Aβ. Sertad1 transcripts were increased by nearly fourfold within 3 h of Aβ treatment (Fig. 6*A*). Downregulation of Sertad1 by shRNA significantly protected the cortical neurons from Aβ induced death (Fig. 6*B*). Moreover, Sertad1 shRNA-expressing cells showed preservation of overall neuron morphology in presence of aggregated Aβ (Fig. 6*C*). Together, these findings indicate that Sertad1 is induced and plays a required role in *in vitro* paradigms of both development and pathological neuron death.

Sertad1 is required for developmental death of cortical neurons *in vivo*

The above evidence indicates the importance of Sertad1 in *in vitro* models of neuronal death. However, its involvement *in vivo* is unknown. Critically, this uncertainty extends generally to whether the cell cycle pathways may be of importance in developmental death *in vivo*. Because limiting levels of target-derived trophic support appear to regulate neuron death *in vivo*, our *in vitro* data led us to test whether Sertad1 is essential for developmental neuron death in the early postnatal cerebral cortex. We used *in utero* electroporation to deliver DNA encoding Sertad1 shRNA and GFP or a control DNA expressing random shRNA and GFP into the left lateral ventricles of E16 rat embryonic brains. DNA delivered in this manner is taken up by ventricular zone progenitor cells that subsequently differentiate and migrate toward the pial surface. Because maximum cortical neuron death occurs during the first week of development (Spreafico et al., 1995), we killed the DNA-electroporated rat pups on postnatal day 5 (P5) and then analyzed their fixed cortices by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assays coupled with immunohistochemistry for GFP to assess the proportions of transfected neurons undergoing death. Past studies have established that TUNEL staining faithfully reports apoptotic neurons in developing brain as judged by nuclear morphology and caspase 3 activation (Sophou et al., 2006). Transfected, morphologically identified neurons were mostly present in cortical layers II, III, and IV of the electroporated side of the brain (Fig. 7*A*; supplemental Fig. S4, available at www.jneurosci.org as supplemental material). Counts of TUNEL-positive cells indicated that ~14 per 1000 transfected neurons ($1.4 \pm 0.13\%$; $N = 3$) were apoptotic in these layers of the cerebral cortex for those animals electroporated with DNA expressing control shRNA (Fig. 7*A*,*B*). This is consistent with a past study that reported ~15 apoptotic (TUNEL-positive)

nuclei per 1000 neurons in the same cortical layers of normal P5 brains (Spreafico et al., 1995). In contrast, we found an average of only 6 apoptotic nuclei per 1000 Sertad1 shRNA-expressing neurons ($0.6 \pm 0.03\%$; $N = 3$) in the same three layers of the cerebral cortex (Fig. 7*B*,*C*). There were no evident effects of the Sertad1 shRNA on the migration or morphology of the transfected neurons. Together, our findings indicate that Sertad1 plays an essential role in neuron death during cortical development *in vivo*.

Sertad1 is required for Rb phosphorylation and consequent myb and Bim induction in response to NGF deprivation

Our past studies (Park et al., 1997b; Liu and Greene, 2001; Liu et al., 2004, 2005; Biswas et al., 2005, 2007; Greene et al., 2007) have established a sequential pathway for neuron death caused by NGF deprivation and Aβ exposure in which (1) activated Cdk4 phosphorylates members of Rb family of transcription-regulating proteins; (2) such phosphorylation leads to dissociation of repressor complexes containing Rb family members, E2F transcription factor proteins, and chromatin modifiers; (3) there is consequent derepression and elevated expression of E2F-responsive genes including the transcription factors B- and C-Myb; and (4) the induced mybs activate transcription of the gene encoding the proapoptotic BH3-only protein Bim. If Sertad1 acts as anticipated at the proximal end of this pathway by binding and activating Cdk4, we would predict that the downstream responses such as Rb phosphorylation, Myb induction, and Bim induction should be blocked by Sertad1 downregulation. We first assessed the effects of shRNA-mediated Sertad1 knockdown on Rb phosphorylation after NGF deprivation. As shown in Figure 8*A* and consistent with our past findings, NGF deprivation (18 h) raised the proportion of cells with a high level of phospho-Rb immunostaining from ~8 to 65%. Transfection with Sertad1 shRNA reduced this to an average of 12.5%. Similar findings were achieved for expression of C-Myb and its downstream target Bim (Fig. 8*B*,*C*). In each case, shRNAs targeted to Sertad1 strongly suppressed the large increases in the proportions of transfected cells that showed high staining for C-Myb and Bim after NGF deprivation. Together, these results support the conclusion that Sertad1 is required for the activation of a neuronal apoptotic pathway in response to NGF deprivation that depends on Cdk-mediated Rb family phosphorylation and consequent induction of the E2F-responsive gene Myb and its proapoptotic target Bim.

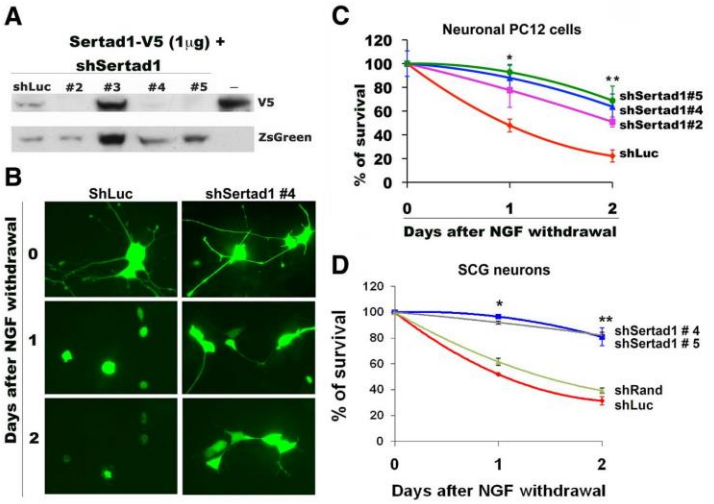

Figure 4. shRNAs targeted to Sertad1 specifically block gene expression and neuron death in response to NGF deprivation. ***A***, Sertad1 shRNAs suppress Sertad1 expression. HEK293 cells were cotransfected with pCDNA-Sertad1-V5 along with pSIREN-Luc (Luciferase)-Zsgreen shRNA or pSIREN-Sertad1-Zsgreen shRNAs. After 48 h, cells were lysed and subjected to Western immunoblotting using enhanced chemiluminescence for the detection of V5 and Zsgreen. ***B***, Sertad1 knockdown prevents neuronal degeneration and death after NGF deprivation. Neuronal PC12 cells were transfected with pSIREN-Random-Zsgreen shRNA (control shRNA) or pSIREN-Sertad1#5-ZsGreen shRNA (Sertad1 shRNA), maintained for 48 h, and then washed twice with RMPI 1640 medium and maintained with or without NGF. Representative images of transfected cells were taken in each case before and after 24 and 48 h of treatment. ***C***, ***D***, Sertad1 shRNAs provide protection against death evoked by NGF deprivation of neuronal PC12 cells or SCG neurons. Neuronal cells were transfected with pSIREN-Luc-Zsgreen shRNA (shLuc), pSIREN-Sertad1-ZsGreen shRNA (shRnad), or pSIREN-Sertad1-ZsGreen shRNAs (shSertad1#2, shSertad1#4, and shSertad1#5), maintained for 48 h, and then deprived of NGF as indicated. Numbers of surviving transfected (green) cells were counted just before NGF deprivation and after 24 and 48 h of NGF deprivation. Data are from one of three independent experiments, each with comparable results and are shown as means ± SEM, performed in triplicates. The asterisks denote statistically significant differences from control (shLuc or shRand): $*p < 0.05$; $**p < 0.001$.

Discussion

Cell cycle proteins have been implicated as required elements in the mechanisms of postmitotic neuron death, both during normal development and in response to injury, stroke, and a range of neurodegenerative diseases (Smith et al., 2004; Neve and McPhie, 2006; Greene et al., 2007; Nunomura et al., 2007; Rashidian et al., 2007). However, the proximal events that initiate the involvement of cell cycle proteins, particularly the activation of Cdk4, have been unclear. Here, we performed experiments that now implicate the cell cycle regulatory protein Sertad1 in three different paradigms of neuron death: NGF deprivation, DNA damage, and Aβ exposure. We find that Sertad1 expression is rapidly elevated in cultured neurons in all three apoptotic paradigms and that downregulation of Sertad1 by shRNA is protective in each case. We further show that downregulation of Sertad1 inhibits the increase in Rb phosphorylation and consequent induction of Myb and Bim that mediate neuron death caused by NGF deprivation. These findings thus place Sertad1 proximal to the other defined events in the Cdk4-dependent apoptotic cell cycle pathway that is triggered by loss of trophic support.

The steps that comprise the apoptotic cell cycle pathway described here have been determined mostly by *in vitro* experimentation. We used *in vivo* electroporation to extend our studies to the developing cortex and found that, in this instance also, Sertad1 plays an essential role in normally occurring neuronal death. Such findings thus implicate not only Sertad1 but also events downstream of it in developmental neuron death.

Sertad1 has been found to directly bind and activate Cdk4 as well as to render active Cdk4–cyclinD1 complexes resistant to inhibition by p16(INK4a) (Sugimoto et al., 1999). Such actions are entirely consistent with our findings that knockdown of Sertad1 protects neurons in our three death paradigms, each of

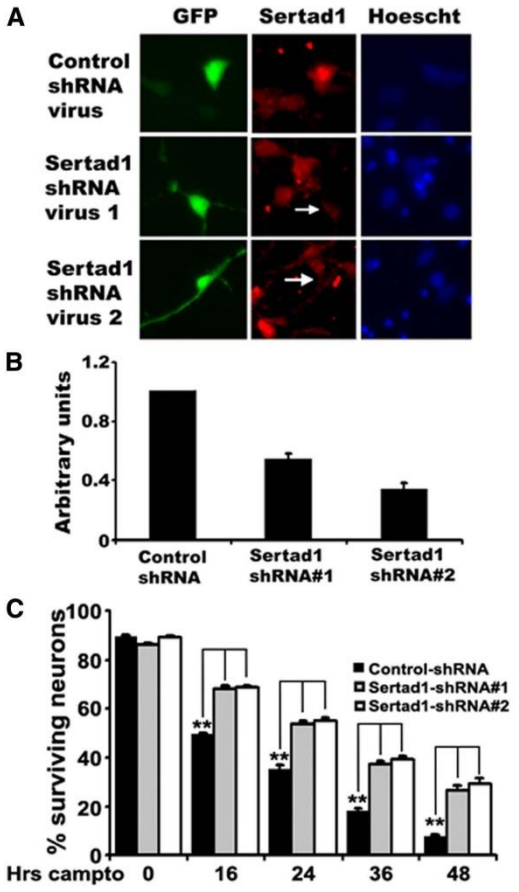

Figure 5. Sertad1 downregulation by adenoviral shRNAs protects cortical neurons from DNA damage-evoked cell death. ***A***, ***B***, shRNA-mediated knockdown of Sertad1 in cortical neurons. Cortical neurons were infected with two adenoviral shRNAs targeting mouse Sertad1 gene or a control virus at the time of plating, and 24 h after infection cells were treated with camptothecin for 16 h to induce Sertad1 expression. Cells were fixed, and immunostaining was performed using Sertad1 chicken IgY antibody. ***A***, Both Sertad1 shRNA viruses repress the upregulation of Sertad1 in cortical neurons treated with camptothecin (arrows indicate Sertad1 shRNA-expressing cells). ***B***, Quantification of fluorescence intensity of Sertad1-immunostained neurons infected with control and Sertad1 shRNA viruses. Values are mean ± SEM ($n = 30–35$). ***C***, Expression of two Sertad1 shRNAs diminishes death of primary cultured cortical neurons treated with camptothecin. Cortical neurons were infected with control and Sertad1 shRNA viruses (multiplicity of infection, 10) at the time of plating. Two days after plating, cells were treated with 10 μM camptothecin for 16, 24, 36, and 48 h, fixed, and stained with Hoechst 33258 (0.25 μg/ml). GFP-positive cells were counted based on nuclear integrity. Significance comparisons with camptothecin-treated controls: $^{**}p < 0.01$. Data represent the mean ± SEM from three independent experiments.

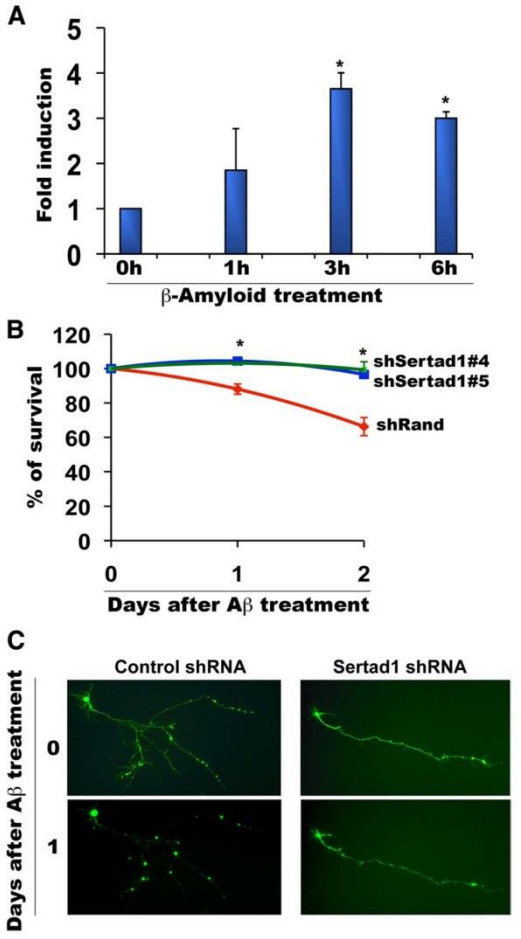

Figure 6. Sertad1 is induced and is required for degeneration of cultured cortical neurons in response to Aβ treatment. ***A***, Sertad1 mRNA levels are increased in response to Aβ. Cortical neurons were treated with 1.25 μM dodecamer Aβ for the indicated times and total RNA was isolated, reverse transcribed, and amplified by PCR using Sertad1- and α-tubulin-specific primers. Data represent means ± SEM of three independent experiments. The asterisks denote statistically significant differences from control (0 h): $^{*}p < 0.01$. ***B***, ***C***, Sertad1 knockdown prevents neuronal degeneration and death after Aβ treatment. Cortical neurons were transfected with pSIREN-Random-shRNA-Zsgreen (shRand) or pSIREN-Sertad1-shRNA-ZsGreen (shSertad1-#4, #5), maintained for 48 h and then treated with 1.25 μM dodecamer Aβ. Numbers of surviving transfected (green) cells were counted just before treatment and after 24 and 48 h with or without treatment. Data are from one of three independent experiments, each with comparable results, and are shown as means ± SEM, performed in triplicates. The asterisks denote statistically significant differences from control (shRand): $^{*}p < 0.01$. Representative images of transfected neurons for pSIREN-Random-Zsgreen shRNA (control ShRNA) and pSIREN-Sertad1#5-ZsGreen shRNA (Sertad1 shRNA) were taken before and after 24 h of treatment.

which requires Cdk4 activation (Greene et al., 2004, 2007). Our studies of the effects of Sertad1 knockdown on cell cycle events triggered by NGF deprivation also support the idea that the capacity of Sertad1 to activate Cdk4 is crucial to its death-promoting actions in neurons. For example, this mechanism would explain why Sertad1 knockdown blocks hyperphosphorylation of Rb, a major cellular target of Cdk4 as well as its inhibition of the subsequent induction of Myb and of Bim. In contrast, other known functions of Sertad1 seem less likely to account for its role in neuron death caused by NGF withdrawal. For instance, Sertad1 binds the E2F1 partner DP-1, thereby enhancing the transactivation of DP-1/E2F1 complexes (Hsu et al., 2001). However, our past findings showed that gene de-repression rather than transactivation is required for neuron death triggered by

NGF deprivation (Liu and Greene, 2001). Such a mechanism would also not account for our observations that Sertad1 knockdown blocks Rb hyperphosphorylation and induction of Myb (which is repressed by E2F complexes) and Bim. Another potentially relevant action of Sertad1 is its capacity to stimulate p53 transcriptional activity (Watanabe-Fukunaga et al., 2005). However, the major proapoptotic targets of p53, Noxa and Puma, do not appear to be either induced or required for death in response to NGF deprivation (S. C. Biswas and L. A. Greene, unpublished observations), and it is unclear that p53 plays a major role in death associated with this paradigm (Sadoul et al., 1996). In addition to activating Cdk4, Sertad1 was recently reported to stabilize XIAP and thus disinhibit caspases in cancer cells (Hong et al., 2009). Such an action of Sertad1, if occurred in neurons, would have an antiapoptotic effect and does not seem to be consistent with the role found here of being proapoptotic in response to NGF deprivation, DNA damage, and Aβ. However, in our death paradigms, either camptothecin or Aβ evokes a series of death pathways that are both caspase-dependent and caspase-independent (Stefanis et al., 1999; Selznick et al., 2000; Cregan et al., 2002). Even if Sertad1 confers antiapoptotic properties, delayed neuronal death still occurs in a caspase-independent manner in these paradigms.

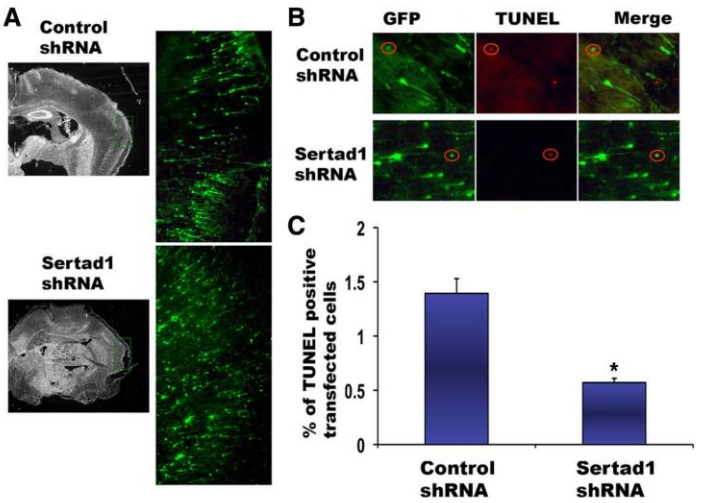

Figure 7. shRNAs targeted to Sertad1 block cortical neuron death *in vivo* during development. ***A***, Distribution of transfected neurons in cortex of the electroporated brain. GFP-expressing DNA constructs of control shRNA or Sertad1 shRNA #4 were electroporated into the left lateral ventricles of E16 rat embryos, which were subsequently harvested at P5. The brains were fixed and subjected to staining for eGFP expression. Morphologically identified eGFP-expressing neurons are mostly present in cortical layers II, III, and IV of the electroporated side of the brain in each case. ***B***, Location of TUNEL-positive eGFP-expressing neurons in cortices of brains electroporated with control and Sertad1 shRNA. Note that TUNEL-positive eGFP-expressing neurons are encircled in each case. ***C***, Sertad1 shRNA inhibits developmental cell death of cortical neurons. The cortices of animals electroporated with control and Sertad1 shRNAs were evaluated for proportions of transfected cells (eGFP positive) that show TUNEL staining. Data represent means (SEM of 3 animals, each from an independent electroporation). Approximately 2000–3000 cells were evaluated per animal. The asterisks denote statistically significant differences between the two mean values: $^*p < 0.01$.

Although Cdk4 activity is essential for neuron death induced by DNA damage, this paradigm of death differs from that caused by trophic factor withdrawal in that it requires both E2F1 and p53 as well as induction of Puma (Park et al., 2000; Wyttenbach and Tolkovsky, 2006). This raises the possibility that, in addition to its role in activating Cdk4, the capacities of Sertad to activate E2F1 and p53 may be relevant to neuronal death triggered by DNA damage. Additionally, we found that E2F1 at least partially mediates death induced by camptothecin.

Although Sertad1 overexpression enhanced neuronal death caused by NGF deprivation, it was not sufficient to promote neuron death when overexpressed in healthy neurons. This observation indicates that Sertad1 is necessary, but not sufficient for neuron death and that additional events are required to initiate the death pathway. This could include, for instance, modification of Sertad1 triggered by apoptotic stimuli, induction of additional proteins essential for Cdk4 activation, or promotion of Sertad1-independent events such as phosphorylation/dephosphorylation of Cdk4 that are needed for its full activation. A past study has shown that activation of Cdc25A is required for Cdk4 activation and neuron death in response to DNA damage (Zhang et al., 2006), raising the possibility for its involvement at least in the case of this death paradigm.

Past studies have described strong parallels between the mechanisms by which neurons die in response to NGF deprivation and Aβ treatment (Greene et al., 2007). This also appears to be the case for the vulnerable populations of neurons in AD that coexpress elevated levels of Cdk4, hyperphosphorylated Rb, and Bim protein (Biswas et al., 2007). A unifying explanation has recently been provided by the observations that neurotrophic factor deprivation causes the enhanced formation and release of Aβ, which then interacts with cells to trigger an apoptotic pathway (Matrone et al., 2008a,b). In this light, it is significant that Sertad1, as in the case of trophic deprivation, is also required for neuron death triggered by exposure to aggregated Aβ. Such findings further support a common pathway for the two apoptotic stimuli and raise the possibility that Sertad1 may be a therapeutic target for amelioration of neuron death and degeneration in AD. In addition to AD, activation of cell cycle proteins has also been implicated in a number of neurodegenerative diseases and nervous system insults associated with neuron death (Becker and Bonni, 2004; Greene et al., 2004, 2007; Herrup et al., 2004). For example, Sertad1 was among those genes that were induced in a cellular model of Parkinson's disease (Ryu et al., 2005), raising the possibility of its involvement at least in this disorder and perhaps in others.

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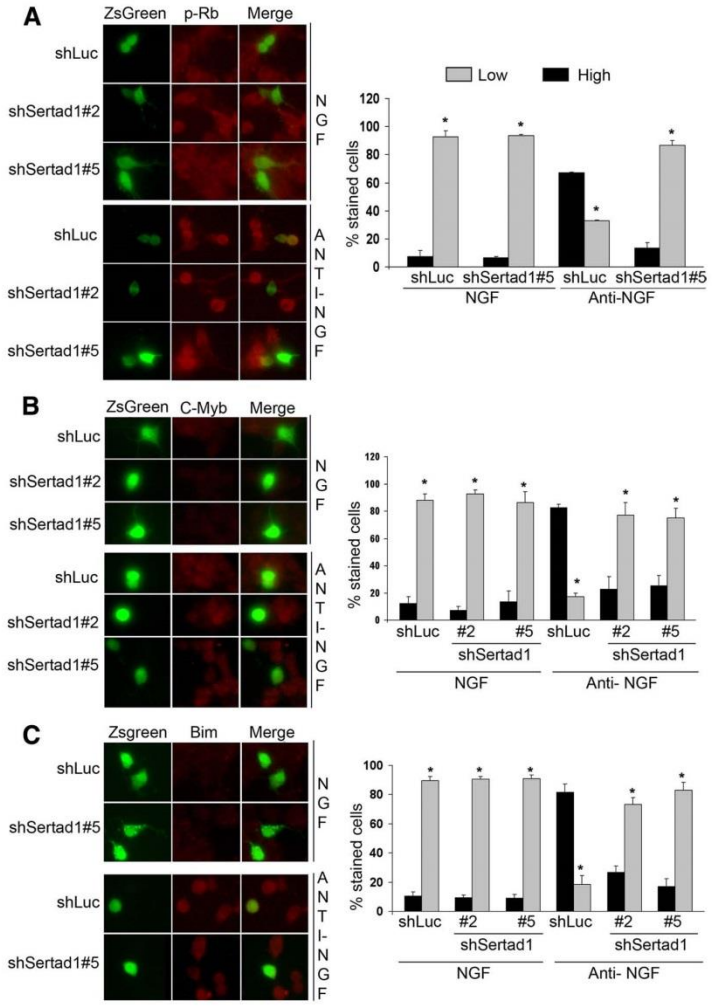

Figure 8. shRNAs targeted to Sertad1 block Rb phosphorylation and repress upregulation of endogenous Myb and Bim in PC12 cells subjected to NGF deprivation. ***A–C***, Left panels show neuronal PC12 cells that were transfected with the indicated constructs, maintained for 48 h, and then deprived of NGF for 18 h after which they were immunostained with antibodies against phospho-Rb (p-Rb), C-Myb, or Bim. The percentage of stained cells shown in the right panels pertain to the proportions of transfected cells (as indicated by Zs-Green expression) that show p-Rb, C-Myb, or Bim staining at either high (more than that of vast majority of NGF-maintained cells) or low (equal or less than that of vast majority of NGF-maintained cells) levels. Data represent means (SEM of 3 independent experiments, each performed in triplicate). Approximately 50 cells were evaluated per culture. The asterisks denote statistically significant differences between low-staining cells and high-staining cells: $*p < 0.001$.

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