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GRADE / DEGREE

Department of Neuroscience

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Role of cyclin-dependent kinases in stroke models of neuronal injury

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Role of cyclin-dependent kinases in stroke models of neuronal injury

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This thesis is submitted as a partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Neuroscience

University of Ottawa

September 2008

Ottawa, Ontario, Canada

Your file Votre référence
ISBN: 978-0-494-59527-5
Our file Notre référence
ISBN: 978-0-494-59527-5

Abstract

Ischemic stroke results from a transient or permanent reduction in cerebral blood flow. In spite of much research in trying to develop intervention strategies, most clinical trials have reported disappointing outcomes. These failures demonstrate that developing effective therapeutic treatments requires a more complete understanding of intracellular signals that lead to neuronal death. The understanding of stroke is made more difficult by the fact that neurons in different regions of brain may die through distinct mechanisms including rapid and delayed cell death pathways. The signaling pathways regulating these mechanisms, however, are not fully defined. Previous studies had suggested that inappropriate activation of cyclin-dependent kinases (CDKs) might be potential regulators of ischemic damage. Although correlative evidence had increasingly implicated upregulation of multiple CDKs signals in neuronal death models, the relevance of these signals was not known. Accordingly, this thesis research explored the functional role of G1-phase CDKs (Cdk2, Cdk4) and non-cell cycle Cdk5 in different models of neuronal death evoked by ischemia. The data indicates that these CDKs act preferentially to regulate damage in distinct models. While Cdk4 is essential in apoptotic and delayed type of death, Cdk5 is critical for excitotoxic death, *in vitro*. In adult and mature neuronal models (*in vivo*), these two CDKs also contributed differently. In global ischemia induced by 10min global ischemia, Cdk4 is a mediator of death signaling. Cdk5, however, plays the main role in 5min global ischemia and in a model of focal ischemia.

To further decipher the significance of these signals, we investigated downstream effectors of Cdk4 and Cdk5. Our data shows that ischemia induces phosphorylation of Rb by Cdk4. This study suggests that Rb likely plays a functional role in this pathway.

Consistent with this notion, expression of a constitutively active Rb is protective.

Studying Cdk5 targets, however, is more complicated. Cdk5 is localized in different compartments of the cell and is suggested to have both pro-death and pro-survival functions. Accordingly, we initially localized its activity in stroke models. This research shows that Cdk5 function is context-dependent. While cytoplasmic Cdk5 signal increases in both models of global and focal ischemia, nuclear Cdk5 is essential only in the focal model. We further demonstrate that peroxidase Prx2 is a cytoplasmic target for Cdk5. Phosphorylation and inactivation of this enzyme by Cdk5 is a critical signal leading to stroke damage. Moreover, our data implicates the survival factor MEF2D, as a nuclear target for Cdk5 in focal ischemia. Nevertheless, the significance of this signal in ischemic death pathway is not clear at present, as attempts to increase MEF2 activity did not appear to affect stroke damage

Taken together, our results both indicate the functional significance of the cell cycle Cdk4 and neuronal Cdk5 signals as well as identify the pathways and circumstances by which they regulate ischemic damage.

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- Delayed combinational treatment with flavopiridol and minocycline provides longer term protection for neuronal soma but not dendrites following global ischemia

Acknowledgments

This thesis owes its existence to the help, support, and inspiration of many people. In the first place, I would like to express my sincere appreciation and gratitude to my supervisor, Dr. David Park, for his guidance, encouragement and patient support through the years. Thank you for providing me such an excellent opportunity to join your lab and perform this research.

Thank you to the members of my thesis advisory committee, Dr. Steffany Bennett, Dr. William Staines and Dr. Luc Sabourin. I am very grateful for their insightful comments and support.

I have been honored to work with so many wonderful individuals over the years. I would like to express my sincere thanks to all my friends and colleagues in the "Park lab" for being there and helping me through all stages of this research. Many heartfelt thanks to Dianbo Qu for his invaluable guidance. Thank you to Grace Iyirhiaro for helping with a significant part of the first paper. I would like to appreciate Patrice Smith for sharing her experience and knowledge with me. I would also like to extend my sincere gratitude to Hossein Aleyasin for being such an exceptional friend and teacher through the years and being there for me in all tough moments. Thank to Maxime Rousseaux and Maryam Phillips for their help and assistance. I am especially grateful to Steve Callaghan for being helpful and supportive for several years. I am truly thankful for all the help I received from Matthew Mount, Michael O'Hare, Zohreh Galehdar, Mohammad Parsanejad, Isabella Irrcher, Arman Lira, Yasmilde Rodriguez, Yi Zhang, Elizabeth Keramaris, and everyone else in the "Park lab".

Thank you to the administrative assistants of the Neuroscience program, Paul-Andre David and Cellular and Molecular Medicine, Donna Hooper and Sylvie Deblois, for their help.

I wish to express my appreciation to my family, especially to my parents, whose enormous love and support give me courage and strength to continue. Special thanks to Ramtin for all his encouragements and efforts to make me believe in myself.

I would also like to convey thanks to the Heart and Stroke Foundation of Canada for providing me financial support to perform this research.

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*. ***Proceeding of the National Academy of Science USA***, 102(39): 14080-5

II. **Rashidian J**, Rousseaux MW, Venderova K, Qu D, Callaghan SM, Phillips M, Bland RJ, During MJ, Mao Z, Slack RS, Park DS. (2008) Differential roles of nuclear and cytoplasmic Cdk5 following stroke: targeting of Prx2 in ischemic injury. ***(Submitted)***

Appended Articles:

III. Qu D, **Rashidian J**, Mount MP, Aleyasin H, Parsanejad M, Lira A, Haque E, Zhang Y, Callaghan S, Daigle M, Rousseaux MW, Slack RS, Albert PR,Vincent I, Woulfe JM, Park DS. (2007) Role of Cdk5-mediated phosphorylation of Prx2 in MPTP toxicity and Parkinson's disease. ***Neuron***, 55(1): 37-52.

IV. Iyirhiaro G, Brust TB, **Rashidian J**, Galehdar Z, Phillips M, Slack RS, Mac Vicar B, Park DS (2008) Delayed combinational treatment with flavopiridol and minocycline provides longer term protection for neuronal soma but not dendrites following global ischemia. ***Journal of Neurochemistry.*** 105(3): 703-13.

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List of Abbreviations

-/-	Gene knockout
4VO	4 vessels occlusion
AAV	Adeno-Associated Virus
Ab	Antibody
AD	Alzheimer's Disease
AIF	Apoptosis Inducing Factor
ALS	Amyotrophic Lateral Sclerosis
AMPA	α amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ANOVA	Analysis of Variance
Apaf1	Apoptotic protease activating factor-1
ATP	adenosine-5'-triphosphate
AV	Adenovirus
BSA	Bovine Serum Albumine
CA1	Cornu Ammonis 1
CAD	Caspase Activated DNAse
CAK	Cdk Activating Kinase
CBF	Cerebral Blood Flow
Cdc2	Cell division cycle 2
Cdc25	Cell division cycle 25
CDK	Cyclin-Dependent Kinase
CDKI	CDK Inhibitor
CGN	Cerebellar Granule Neurons

CMV	Cytomegalovirus
CNS	Central Nervous System
Cont	Contralateral to treatment
COX	Cyclooxygenase
CV	Cresyl Violet
Cys	Cysteine
d	day
D	Aspartic acid
DD	Death Domain
DN	Dominant Negative
DS	Down's Syndrome
eNOS	endothelia Nitric Oxide Synthase
FADD	Fas-Associated Death Domain
g	gram
GSK-3β	Glycogen Synthase Kinase -3β
GFP	Green Fluorescent Protein
h	hour
HD	Huntington's Disease
IAP	Inhibitor of Apoptosis Protein
ICAM-1	Intercellular Adhesion Molecule-1
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase
Ipsi	Ipsilateral to treatment

JNK3	c-Jun N-terminal Kinase 3
KA	Kainic Acid
KO	Gene knockout
μCi	micro curi
μl	micro litter
mM	milli Mole
μM	micro Mole
MCAO	Middle Cerebral Artery Occlusion
MEF2	Myocyte Enhancer Factor-2
min	minute
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRC	Mitochondrial Respiratory Chain
mRNA	messenger RNA
MWM	Morris Water Maze
n	sample size
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NC	Negative Control
NES	Nuclear Exclusion Signal
NF-κB	Nuclear Factor-κB
NLS	Nuclear Localizing Signal
nNOS	neuronal Nitric Oxide Synthase
NOS	Nitric Oxide Synthase
NMDA	N-methyl D-aspartic acid

NPC	Niemann-Pick type C
$O_2^{\cdot-}$	Superoxide anion
$OH^{\cdot}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
P	Probability
P	Phospho
ρM	pico Mole
PARP	Poly (ADP-Ribose) Polymerase
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PSTAIRE	Proline-Serine-Threonine-Alanine-Isoleucine-Arginine-Glutamic acid
Prx	Peroxiredoxin
R	Arginine
rAAV	recombinant Adeno-Associated Virus
Rb	Retinoblastoma
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
Ser	Serine

SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
Smac	Second mitochondria-derived activator of caspase
SOD	Superoxide Dismutase
Thr	Threonine
TIA	Transient Ischemic Attack
TNF	Tumor Necrosis Factor
tPA	tissue-Type Plasminogen Activatior
TRADD	TNF Receptor-Associated Death Domain
Tyr	Tyrosine
vs	versus
WT	Wild Type

Thesis format

This thesis has been prepared with the format conformed to the guidelines set forth by the Department of Cellular and Molecular Medicine, Neuroscience program. It has been presented as a collection of 4 chapters, including an introduction followed by two manuscripts and a general discussion as it relates to our current knowledge in the field.

Chapter 1, as an introductory section, presents a brief summary of stroke and the processes governing neuronal death following an ischemic attack. I have also presented a brief overview about cell cycle and its regulatory elements, with emphasis on CDKs and their role in neuronal death. In addition, Cdk5, as a non-cell cycle CDK, has been introduced to reader and its importance in stroke has also been outlined. Finally, this chapter refers to the existed problems in this field and provides the objectives of the present work to answer these problems in order to extend the current knowledge about the signaling pathways in neuronal death.

Chapter 2 presents an article entitled: "Multiple cyclin-dependent kinases signals are critical mediators of ischemia/hypoxic neuronal death, *in vitro* and *in vivo*". This paper, for the first time, provided functional evidence for contribution of cell cycle Cdk4 and non-cell cycle Cdk5 in adult models of ischemia and also explored their diverse roles in different neuronal death pathways. This article was published in the *Proceedings of the National Academy of Sciences USA (2005), 102(39): 14080-5.*

Chapter 3 presents a manuscript entitled: "Differential roles of nuclear and cytoplasmic Cdk5 following stroke: targeting of Prx2 in ischemic injury". This manuscript

differentiates roles of Cdk5 in different contexts and has been submitted for publication (2008).

Since all the data has been discussed in detail in each manuscript, I have presented an overview of our major findings in chapter 4. The significance of these findings in our current knowledge in this field for developing more effective therapeutic strategies has also been discussed.

CHAPTER 1

General Introduction

1.1. Stroke

Stroke is the result of a transient or permanent interruption of blood supply to the brain, which then causes permanent damage. This occurs primarily when a blood vessel carrying oxygen and nutrients to the brain is either ruptured or blocked. In some cases, cardiac arrest can cause stroke. Oxygen and glucose deprivation trigger several cascades of cellular signals that ultimately lead to neuronal damage in the affected regions or the whole brain. On average, every 10 minutes someone in Canada has a stroke (www.canadianstrokenetwork.ca). According to another report, a stroke occurs every 40 seconds in the United States (Donnan et al., 2008). Stroke may cause permanent neurological damage, complications and disability and even death. Stroke is the second most common cause of death (9% of deaths) in the world (Donnan et al., 2008). For those patients who survive, stroke has adverse impacts on the life style of these individuals. Many stroke survivors and their families face huge challenges, suffering physically, mentally, socially and financially. In industrial countries, stroke consumes more than 4% of direct health-care costs which includes hospital care, physician services, drugs and research (Donnan et al., 2008). In Canada, this cost is estimated about $2.7 billion annually (www.canadianstrokenetwork.ca). The estimated direct and indirect cost for stroke in 2008 in USA is $65.5 billion (Rosamond et al., 2008).

Subtypes of stroke

Stroke can be classified into three classes: ischemic stroke, hemorrhagic stroke and transient ischemic attack.

1. Ischemic. Ischemic stroke counts for more than 80% of the cases (Thrift et al., 2001). In this type of stroke, a blood vessel is blocked for different reasons. These reasons include

thrombosis (occlusion of the vessel by blood clot formed in brain), embolism (blood clot formed somewhere else other than brain such as in heart and traveled through the blood stream) and systemic hypertension (such as in shock). A thrombotic stroke, also referred as cerebral thrombosis, occurs in almost 50% of all stroke cases. Although these are the most common causes of ischemic stroke, there are many other possible causes. For example, traumatic injury to the blood vessels of the neck, or disorders of blood clotting may also cause ischemic stroke (Jeyaseelan et al., 2008).

2. Hemorrhagic. Hemorrhagic ischemia counts for 13% of the stroke cases. In this type of ischemia, a fragile blood vessel ruptures and bleeds into the surrounding area in the brain. There are two types of hemorrhagic ischemia: a) intracerebral hemorrhage occurs when an artery ruptures within the brain and b) subarachnoid hemorrhage occurs when an artery ruptures outside of the brain and the skull fills with blood (Jeyaseelan et al., 2008).

3. Transient ischemic attack (TIA). This type is also known as mini-stroke. In TIA, the blood flow is temporary interrupted in the brain and symptoms disappear within minutes to hours. Since stroke causes permanent damage, TIA, by definition, may not be classified as stroke. The growing evidence, however, shows that patients who have had a TIA are highly at risk of having a severe attack with permanent brain injury. Studies have revealed that about 10% of these patients will have a stroke within 3 months following TIA (Jagoda and Chan, 2008).

1.2. Tissue-type plasminogen activator: a therapeutic agent

In ischemic stroke, the blood clot must be removed quickly to avoid damage to brain. Recombinant tissue-type plasminogen activatior (tPA) is one of the most effective treatments being used since 1995 [The National Institute of Neurological Disorders and Stroke (1995)]. Endogenous tPA is a secreted protease whose activity in blood turns inactive plasminogen into a broad spectrum active protease called plasmin. Plasmin breakdowns fibrin meshes and dissolves blood clots (Gravanis and Tsirka, 2008). The half-life of tPA is very short and is inactivated within 5-10 minutes in the body. Exogenous administrated recombinant tPA works through the same mechanism and helps endogenous enzyme to catalyze this thrombolytic reaction. Although the effectiveness of recombinant tPA has been confirmed (Gravanis and Tsirka, 2008) , it has been used only in 3-8% of the ischemic stroke cases. The reasons for this are several. First; there is a narrow time frame window for receiving tPA after stroke attack. Restoration of the blood is effective only if tissue is still alive. The most effective time to receive tPA is within 3 hours following the onset of ischemia and administration of the drug within 3-5 hours after attack shows moderate benefit (Clark et al., 1999). Therefore, patient care within the first 3 hours after an ischemic event is highly critical. Second; administration of tPA may have the risk of intracerebral hemorrhage, although this only happens in 6-7% of patients (Gardell, 1993). Administration of tPA may change the coagulation/fibrinolysis balance towards the fibrinolysis and disrupts some microscopic vessels (Collen et al., 1986). Third; tPA is not efficient enough to dissolve big clots. It takes longer time for tPA to dissolve bigger clots. Since it is removed from blood circulation quickly, it is likely to be less effective for larger blockages [The IMS investigators (2004)]. Fourth; tPA might have

neurotoxic effects, although this potential problem is controversial. For example, tPA cleaves NR1 subunit of NMDA receptors and increases calcium influx to the cell (Nicole et al., 2001). tPA is also able to mediate degradation of key proteins of the extracellular matrix (Chen and Strickland, 1997). It has also been shown that tPA exacerbates edema probably by activation of microglia and infilteration of inflammatory cells (Siao and Tsirka, 2002). Disruption of the blood brain barrier is another mechanism for tPA to exacerbate edema in brain (Thiex et al., 2003; Yepes et al., 2003). In spite of the limitations and side effects mentioned above, administration of tPA is the only current therapeutic strategy for stroke yet available. To increase tPA efficacy and reduce its side effects , significant research has been directed to discover alternative and new targets to use in combination with tPA.

1.3. Animal models of stroke

Several animal models have been widely used for studying pathogenesis of stroke and discovering therapeutic strategies. Most of these experimental models consist of ischemic stroke induced in rodent or non-rodent animals. Based on the type of injury induced in the animals these models can be divided into three categories: global, focal, and multifocal cerebral ischemia. In global ischemia cerebral blood flow is stopped (complete global ischemia) or reduced to a level that is enough to hamper metabolism (incomplete global ischemia) in the whole brain. Global ischemia is induced by different techniques such as cardiac arrest, bilateral common carotid artery occlusion, 4-vessel occlusion, and 2-vessel occlusion plus hypotension (Graham et al., 2004). In focal ischemia blood flow is restricted in a specific region of the brain. It is induced through different methods such as middle cerebral artery occlusion (MCAO) or injection of

vasoconstrictors to a target region (discussed below). In multifocal ischemia, blood clots are being formed using different techniques and ischemia is induced in several sites of the brain (Graham et al., 2004). In the present study, we have used two models of 4-vessel occlusion and injection of endothelin-1 to induce global and focal ischemia, respectively. These two models are discussed further below.

- *Global ischemia induced by 4-vessel occlusion (4VO).*

As stated above, in global ischemia blood flow is reduced in all or most parts of the brain and causes neuronal injury in selective vulnerable regions. The 4VO model of global ischemia is a two-stage procedure. In the first stage, two common carotid arteries are loosely looped and the vertebral arteries are electrocauterized via the first cervical vertebra. On the second day, the common carotid arteries are occluded temporarily (5-30 minutes) while the animal is awake (Pulsinelli and Brierley, 1979). This technique is successful in approximately 50 to 75% of the animals, but the effects of ischemia are variable between rat strains (Pulsinelli and Buchan, 1988). During occlusion blood flow is reduced to less than 3% of control in neocortex, striatum, and hippocampus, and reperfusion causes a strong cerebral hyperemia within 5 to 15 min (Pulsinelli et al., 1982a). There is a selective vulnerability within regions of brain to this model. Neocortex, striatum, hippocampus, thalamus and cerebellum are damaged in 4VO with the highest vulnerability in CA1 region of hippocampus (Pulsinelli et al., 1982b).

- *Focal ischemia induced by endothelin-1*

Focal ischemia is represented as a reduction of blood flow in specific region(s) of the brain. One of the animal models being used to investigate the pathophysiology and mechanisms involved in focal ischemia is administration of endothelin-1. Endothelin-1 is

a 21-amino acid peptide with a powerful vasoconstrictive effect (Yanagisawa et al., 1988). Endothelin-1 works through receptors located on vascular endothelium. In rat, endothelin receptors are present on smooth muscles of arteries in different regions such as cerebral cortex and therefore, endothelin-1 can act on the arteries in this region. It has been shown that topical application of endothelin-1 to the exposed middle cerebral artery can cause up to 80% reduction in blood flow (Robinson et al., 1991). Endothelin-1 has also been injected adjacent to MCA (Sharkey et al., 1993) or onto the cortical surface (Fuxe et al., 1997). In another study, direct injection of endothelin-1 into the striatum has been reported to decrease blood flow 4 minutes after injection and produce a 60% reduction within 20 minutes in rats (Fuxe et al., 1992). Although endothelin-1 has not been shown to be directly neurotoxic, it may cause excessive release of glutamate and therefore, exacerbates neuronal death (Sasaki et al., 1997). This method, compared to other methods of focal ischemia such as MCAO, is less invasive and mortality is low. In addition, the procedure is easier and could be induced in any desired region. It should be noted that this method is highly dose dependent.

1.4. Pathophysiology of stroke

Multiple factors, including severity and duration of insult as well as vulnerability of the neurons, determine the fate of brain cells following cerebral ischemia. The brain has high levels of oxygen and glucose consumption and is exclusively dependent on oxidative phosphorylation for energy production. Accordingly, reduction in cerebral blood flow (CBF) supplying these elements will affect the integrity and function of neurons. Depending upon the degree of oxygen and glucose reduction, different pathophysiological events could happen in the restricted area. Normal CBF is around 50-

60 ml/100gr/min. During ischemia, it is reduced in a pattern such that the lowest flow (<10) is at the center of ischemia and is gradiently increased through the surrounding region until it reaches a normal level (>50) (Moustafa and Baron, 2008). In cerebral ischemia the center of infarction is known as the core. In the core, a massive blood flow reduction causes complete breakdown in cellular metabolism, energy production and ionic homeostasis lead to cell death within minutes. Damage to the core is irreversible (Astrup et al., 1981). The penumbra is the region surrounding the core. This region is hypoperfused by collateral vessels and has a maintained metabolism and structure, though non-functional (Sharp et al., 2000). Blood flow in the penumbra is too low to mantain neuronal electrical activity (Astrup et al., 1981).

Cerebral ischemia triggers multiple pathways that likely occur in an overlapping manner. Very briefly, restricted blood flow in the ischemic region impairs oxygen and glucose delivery and ATP generation by mitochondria (Martin et al., 1994). Energy impairment causes neuronal and glial membrane depolarization, activation of Ca^{2+} channels and release of excitatory amino acids, such as glutamate, into the extracellular space. This, in turn, induces an excitotoxic form of cell death. Meanwhile other energy-dependent processes, such as glutamate uptake are hampered. This exacerbates glutamate accumulation, activates glutamate channels and causes Ca^{2+} influx. Ca^{2+} activates various enzymes that cause tissue damage, such as endonucleases, proteases, phospholipases, cyclooxygenases (COXs) and nitric oxide synthase (NOS) (White et al., 2000). These enzymes generate free radicals and cause lipids and protein peroxidation and membrane damage. In addition to the cell membrane, inner membranes of mitochondria are also disrupted. Respiratory chain function and production of ATP are therefore hampered.

Moreover, an increase in mitochondrial permeability transition results in mitochondrial swelling (Kristian and Siesjo, 1998). Finally, cytochrome *c* is released and apoptosis can proceed given the appropriate signaling conditions (Dirnagl et al., 1999; Hou and MacManus, 2002; Lo et al., 2003; Carbonell and Rama, 2007).

Ca^{2+} influx and free radicals also trigger the inflammation pathways through induction of injured brain cells to produce several inflammatory mediators. These mediators cause infiltration of multiple types of inflammatory cells to the ischemic area and propagate the injury (Dirnagl et al., 1999; Lo et al., 2003; Carbonell and Rama, 2007).

Eventually, all these events lead to neuronal damage through several pathways including excitotoxicity/ionic imbalance, oxidative/nitrosative stress, apoptosis and inflammation, dependent upon the severity of ischemia. These mechanisms are likely interrelated (Figure 1.1).

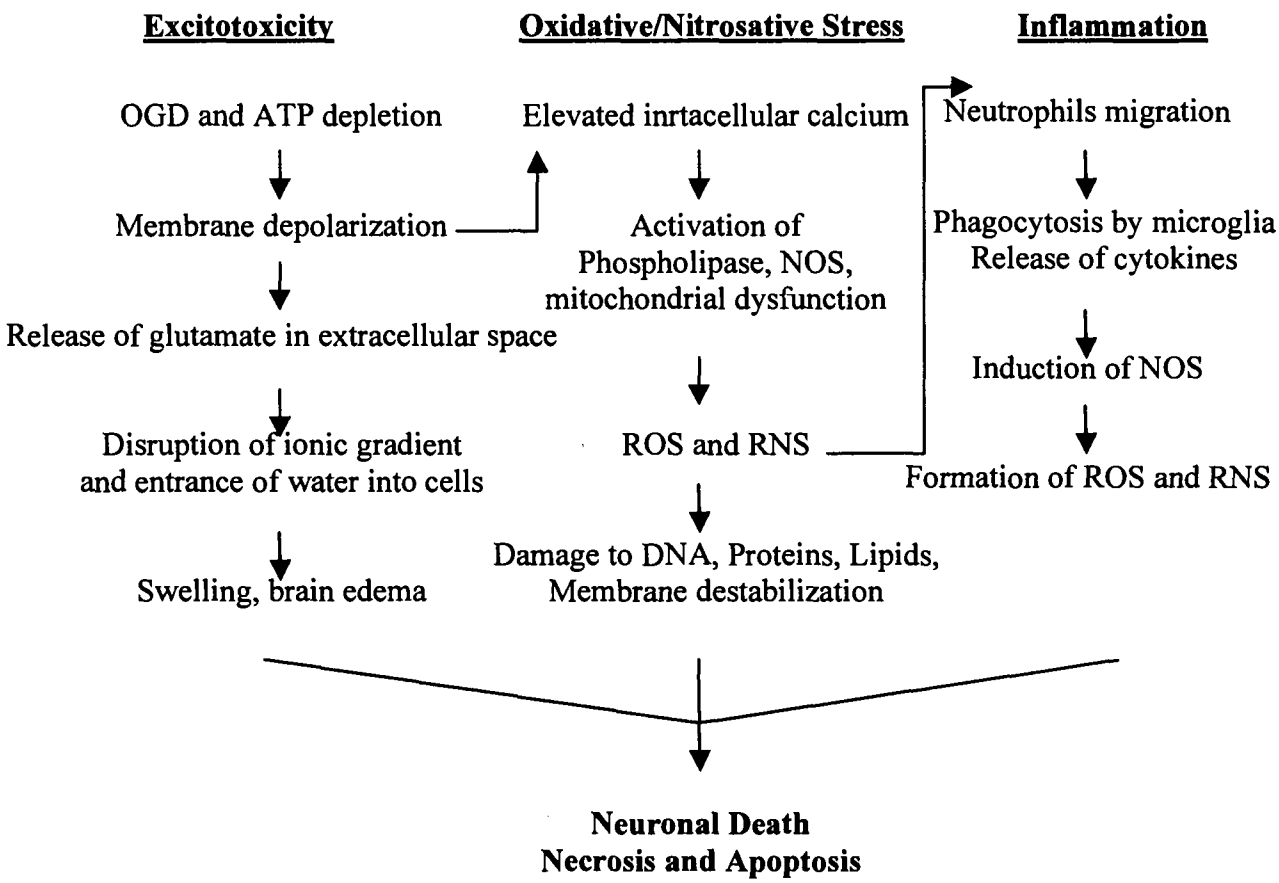
Excitotoxicity
Oxidative/Nitrosative Stress
Inflammation
OGD and ATP depletion
Elevated inrtacellular calcium
Neutrophils migration
Membrane depolarization
Activation of
Phospholipase, NOS,
mitochondrial dysfunction
Phagocytosis by microglia
Release of cytokines
Release of glutamate in extracellular space
Induction of NOS
Disruption of ionic gradient
and entrance of water into cells
ROS and RNS
Formation of ROS and RNS
Damage to DNA, Proteins, Lipids,
Membrane destabilization
Swelling, brain edema
Neuronal Death
Necrosis and Apoptosis

Figure 1.1

Figure 1.1. An overview of different mechanisms involved in ischemic neuronal death. Multiple pathways including excitotoxicity, oxidative/nitrosative stress and inflammation are triggered following energy failure, ionic disturbances and membrane depolarization in focal ischemia. Details are outlined in the text.

1.4.1. *Excitotoxicity and ionic imbalance*

As implicated above, energy deficit is the starting point for ischemic signaling pathways. The human brain requiers 20% of total oxygen consumption to generate ATP and mentain ionic gradients (Doyle et al., 2008). It has been estimated that Na^+/K^+ ATPase pump located in the plasma membrane consumes 70% of the energy generated in the brain (Mehta et al., 2007). This pump keeps a high intracellular K^+ concentration and the low intracellular Na^+ concentration required for the propagation of action potentials in cells. After ischemia and mitochondrial inhibition of ATP synthesis, ATP is being consumed within a few minutes and the function of Na^+/K^+ ATPase is hampered. This leads to influx of Na^+ and efflux of K^+ through the plasma membrane as it gets depolarized (Dirnagl et al., 1999). Energy deficit also inhibits the Ca^{2+} ATPase pump, which maintains the very low concentrations of Ca^{2+} inside the cell. During ischemia, intracellular Ca^{2+} concentrations elevate to 50–100 μM and activate several Ca^{2+} dependent enzymes such as proteases, lipases and DNases (Doyle et al., 2008). Activation of these enzymes, as well as lack of ATP for re-synthesis of cellular components causes a rapid cell death in the ischemic core.

Membrane depolarization also leads to release of excitatory neurotransmitter glutamate into the extracellular space, as well as impairing astrocytic glutamate uptake (Takahashi et al., 1997; Budd, 1998; Mori et al., 2004; Giffard and Swanson, 2005). Glutamate is the most abundant excitatory neurotransmitter in nervous system. It is involved in synaptic plasticity and cognitive functions through ionotropic (NMDA, AMPA and kainate receptors) and metabotropic receptors (Meldrum, 2000). In the physiological conditions, extracellular glutamate concentration is in the micromolar

range, whereas the cytosolic concentration is in milimolar range (Fillenz, 1995). This gradient is maintained by Na^+-dependent glutamate transporters located on presynaptic and postsynaptic membranes (Fillenz, 1995). Following ischemia and membrane depolarization, Na^+ accumulates inside the cells and causes reversal of glutamate transporters and allows glutamate to exit cells. Increase in the levels of extracellular glutamate leads to activation of NMDA and AMPA receptors. Opening of NMDA receptors causes further membrane depolarization, more permeablization of these receptors to Na^+ and K^+ and greater Ca^{2+} influx (excitotoxicity) (Olney, 1969). Eexcess amount of extracellular glutamate increases permeablization of AMPA and kainate receptors to Na^+, $K^+$and Cl^- (Choi, 1990). AMPA receptors are not normally Ca^{2+} permeable due to presence of their GluR2 subunit; however, this subunit is reduced after ischemia increasing the Ca^{2+} permeability of these receptors as well (Choi, 1990; Liu et al., 2006; Peng et al., 2006). It has been shown that blocking NMDA and AMPA receptors provide neuroprotection in models of ischemia (Lipton, 2006; Montero et al., 2007). Metabotropic glutamate (mGlu) receptors also contribute to excitotoxicity. These receptors modulate excitatory synaptic transmission and enhance the induction and progression of excitotoxic neuronal death (Bruno et al., 2001).

Acidosis, a hallmark of anaerobic metabolism of ischemia, further exacerbates Ca^{2+} overload. Acidosis activates Na^+-selective acid-sensing ion channels (ASICs). ASICs are permeable to Ca^{2+} (Simon, 2006; Isaev et al., 2008). This phenomenon is glutamate-independent and is not inhibited by NMDA antagonists.

Overall, the massive disruption in ionic gradient and passive entrance of water causes cell swelling, brain edema and necrosis. Necrosis happens mostly in the core

(Choi and Rothman, 1990; Green and Reed, 1998). In the regions where necrosis is less prominent, excitotoxicity leads to apoptosis (Yuan and Yankner, 2000; Graham and Chen, 2001) and/or inflammation (Mergenthaler et al., 2004).

1.4.2. *Oxidative/nitrosative stress*

The brain is very sensitive to free radicals due to high contents of unsaturated fatty acids (Candelario-Jalil et al., 2001). These radicals are normally produced at basal levels and their generation and elimination rates are equal. In this case, they function as intracellular messengers (Bredt and Snyder, 1994; Rhee, 1999). However, several types of stresses including cerebral ischemia, and particularly reperfusion, induce massive generation of free radicals and thereby, impose oxidative/nitrosative stress (Chan, 2001; Nita et al., 2001). Free radicals are highly reactive and cause damage to proteins, lipids, DNA and finally lead to neuronal death. These radicals form two groups: reactive oxygen species (ROS) and reactive nitrogen species (RNS).

- *ROS*

Oxygen-based free radicals are highly reactive species. There are different sources of ROS in brain:

1. Mitochondrial. The major source of ROS is the mitochondria. The mitochondrial respiratory chain (MRC) consumes the oxygen used by cell for electron transfer and ATP synthesis during the oxidative phosphorylation process (Moro et al., 2005). It has been shown that electrons can leak from the MRC (Adam-Vizi, 2005). These leaky electrons reduce O_2 to superoxide anion ($O_2^{\cdot-}$). Superoxide anion is the primary ROS generated by MRC and is highly reactive. This anion has a very short half-life and is catalyzed to hydrogen peroxide (H_2O_2) by mitochondrial superoxide dismutase (Mn-SOD or SOD2)

or cytosolic/mitochondrial superoxide dismutase (Cu,Zn-SOD or SOD1) (Moro et al., 2005). Hydrogen peroxide, by itself, is a mild oxidant and at basal levels functions as an intracellular messenger (Rhee, 1999). In contrast, H_2O_2 is able to generate hydroxyl radical ($OH^{\cdot}$) in the presence of metal ions such as Fe^{2+} or Cu^{+} (Giulivi et al., 1995). $OH^{\cdot}$ is the most reactive ROS and cannot be eliminated by enzymatic reaction. Therefore, extra amount of H_2O_2 has to be removed by cytoprotective enzymes such as GSH, PRX etc. to avoid excessive production of $OH^{\cdot}$.

2. Enzymatic.

a) During ischemia, elevated concentrations of intracellular Ca^{2+} activate phospholipase A_2. This enzyme releases fatty acid arachidonic acid from the cell membrane. Cyclooxygenase-2 (COX-2) then adds two molecules of O_2 to this acid to produce prostaglandin PGG2. PGG2 releases a $O_2^{\cdot-}$ and rapidly peroxidized to PGH2 (Moro et al., 2005).

b) Other enzymes producing $O_2^{\cdot-}$ are xanthine oxidase and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase. Degradation of adenine nucleotides during ischemia increases hypoxanthine which is then oxidized by xanthine oxidase and forms $O_2^{\cdot}$. NADPH is oxidized by NADPH oxidase and again releases $O_2^{\cdot-}$ (Moro et al., 2005).

There are several lines of evidence demonstrating that ROS are produced during ischemia. Studies have shown production of superoxide and hydroxyl radicals, as well as induction of COX-2 and SOD following stroke in animal models (Ohtsuki et al., 1996; Piantadosi and Zhang, 1996; Planas et al., 1999; Fukui et al., 2002; Kim et al., 2002). Also, pharmacological inhibition of COX-2 has proven to be protective in global

ischemia model (Nakayama et al., 1998). More importantly, SOD1 and SOD2 deficiency has been shown to exacerbate neuronal injury in global and focal ischemia, respectively (Kawase et al., 1999; Kim et al., 2002).

- *RNS*

Like oxygen-based free radicals, nitrogen-based radicals also contribute to neuronal death. Nitric oxide (NO°) is one of the RNS, which increases in ischemia models (Malinski et al., 1993; Sato et al., 1993; Tominaga et al., 1994; Zhang et al., 1995). Importantly, it has been shown that the levels of NO° metabolites in cerebrospinal fluid and serum in stroke patients are higher than in control individuals (Shibata et al., 1996; Krupinski et al., 1998; El Kossi and Zakhary, 2000). NO° is produced enzymatically from L-arginine by isoforms of NO synthase (NOS) including eNOS (endothelial), nNOS (neuronal), iNOS (inducible) or alternatively, nonenzymatically at low pH (during ischemia) (Zweier et al., 1999; Moro et al., 2005). Although most of the brain cells are able to produce several isoforms of NOS, commonly accepted distribution of these enzymes is that nNOS is produced by neurons, iNOS by glial cells and eNOS by endothelial cells (Knowles and Moncada, 1994).

nNOS and eNOS are constitutively expressed in the brain and produce basic levels of NO°. Upon ischemic insult when intracellular calcium is elevated, NOS levels and activities are increased and produce high amounts of NO° (Moro et al., 2005). Importantly, several studies have shown an increase in the activity and level of nNOS after focal ischemia (Kader et al., 1993; Zhang et al., 1994) and hypoxia (Matsuoka et al., 1997). It has been shown that eNOS levels and activity is also increased in focal ischemia (Zhang et al., 1993b; Veltkamp et al., 2002). nNOS and eNOS levels and activities are

modulated through different mechanisms at the transcription, post transcription (mRNA stability) and posttranslational levels or by NO° itself (Moro et al., 2005).

iNOS expression and activity is calcium-independent and is induced by inflammatory responses following ischemia. As will be discussed later on, inflammation is another cascade of reactions that occurs after ischemic injury and contributes to an irreversible injury. Cytokines, adhesion molecules and iNOS are produced by endothelial cells, glial cells and leukocytes (Mehta et al., 2007). Several studies have demonstrated an increase in the level and activity of iNOS in different models of ischemia, *in vitro* and *in vivo* (Endoh et al., 1994; Iadecola et al., 1995; Cardenas et al., 1998; Cardenas et al., 2000). iNOS expression is basally regulated at transcriptional level. Cytokines released after ischemia, or activation of NMDA receptors by glutamate have been reported to activate some transcription factors, such as NF-κB, and induce iNOS (Xie et al., 1994; Cardenas et al., 2000).

One of the NO° derivatives is peroxynitrite ($ONOO^{-}$). $ONOO^{-}$ is a strong oxidant and nitrating agent and is rapidly formed through reaction of NO° with $O_2^{\cdot -}$ (Radi et al., 2002).

Nitrogen free radicals induce cell death by variety of different mechanisms:

a) ATP depletion through inhibition of mitochondrial respiratory complexes, glycolysis pathway, crebs cycle, uncoupling oxidative phosphorylation in mitochondria (Cleeter et al., 1994; Welter et al., 1996; Castro et al., 1998; Sharpe and Cooper, 1998; Souza and Radi, 1998; Brown and Borutaite, 2002; Yamamoto et al., 2002).

b) Triggering apoptotic pathways through releasing cytochrome *c* or translocation of Bax (Shidoji et al., 1999; Smaili et al., 2001).

Sources of ROS and NOS in ischemia have been summarized in figure 1.2.

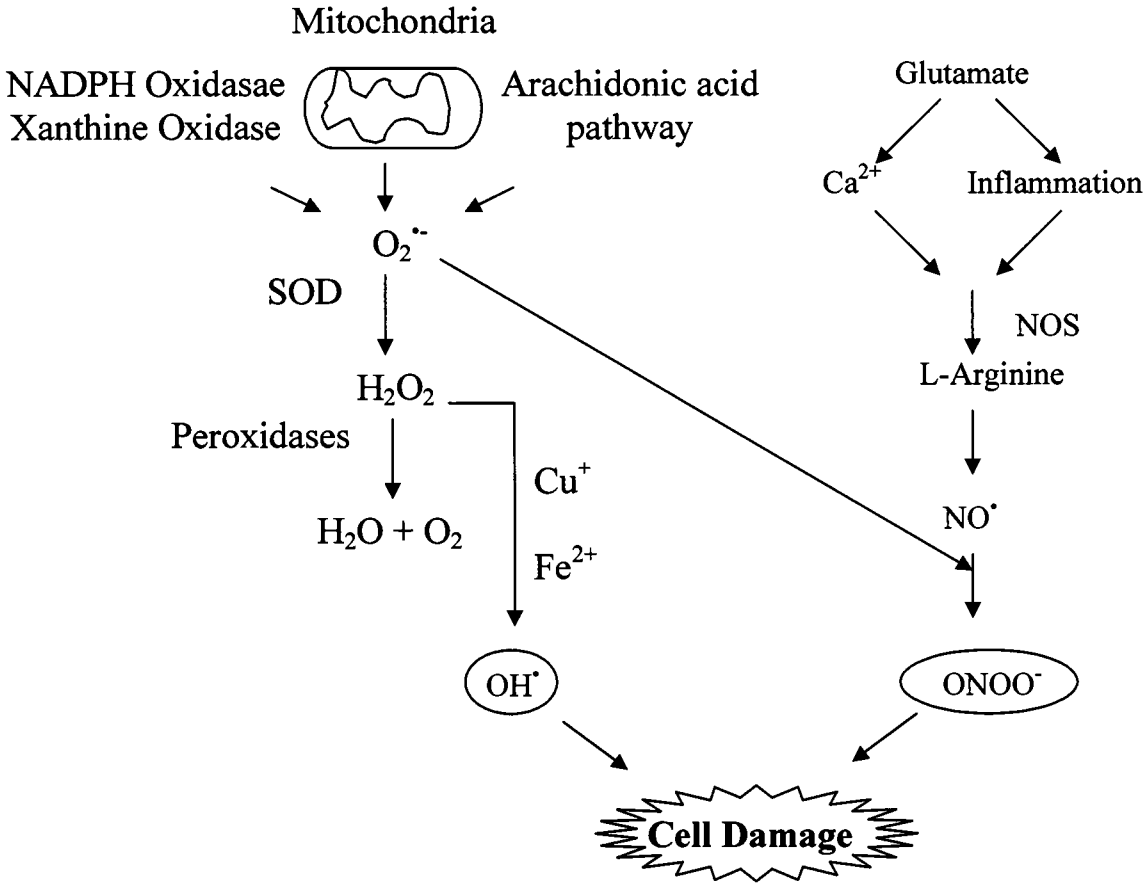
Mitochondria
NADPH Oxidasae
Xanthine Oxidase
Arachidonic acid
pathway
Glutamate
Ca2+
Inflammation
O2•-
SOD
NOS
L-Arginine
H2O2
Peroxidases
Cu+
H2O + O2
NO•
Fe2+
OH•
ONOO-
Cell Damage

Figure 1.2

Figure 1.2. Major sources of free radicals generated in stroke. Superoxide anion ($O_2^{\cdot -}$) is the primary ROS generated and ultimately produces highly reactive hydroxyl radical ($OH^{\cdot}$). $O_2^{\cdot -}$, alternatively, can react with nitric oxide ($NO^{\cdot}$) produced in other pathways, to generate peroxynitrite ($ONOO^-$). $ONOO^-$ is a strong oxidant and nitrating agent. ROS and RNS induce neuronal death through different mechanisms (explained in text).

1.4.3. *Inflammation*

Ischemia is also associated with inflammation. Immune system attack starts a few hours after onset of ischemia and initiates a cascade of inflammatory events that propagate brain injury (Dirnagl et al., 1999). The major elements involved in inflammation following ischemia are:

1. Cell adhesion molecules (e.g. integrins, selectins, immunoglobulins). These molecules facilitate neurotrophil migration by promoting cell-to-cell interaction. Upregulation of these molecules by endothelial cells has been shown after ischemia in animals (Huang et al., 2000) and human (Shyu et al., 1997). Importantly, inhibition of these adhesin molecules, such as intercellular adhesion molecule-1 (ICAM-1) and selectin has been protective in stroke models (Mocco et al., 2002; Vemuganti et al., 2004).

2. Cytokines (IL-1, IL-6, TNF-α). IL-1 functions by releasing arachidonic acid and inducing NOS. As a matter of fact, IL-1 is increased in neurons, microglia and astrocytes following ischemia (Zhang et al., 1998) and its receptor antagonist reduces damage (Relton et al., 1996). Similarly, IL-6 and TNF-α messages and proteins are upregulated in animal models, as well as in stroke patients (Liu et al., 1994; Wang et al., 1995; Zaremba et al., 2001). Cytokines also promote expression of adhesion molecules (Mehta et al., 2007).

3. iNOS and COX-2. As discussed earlier, these two enzymes generate free radicals and their activities are increased in ischemia (Iadecola et al., 1997; Yokota et al., 2004). Moreover, inhibition of these two enzymes is protective (Iadecola et al., 2001; Sugimoto and Iadecola, 2003).

4. In addition to above mediators, activated microglial cells produce a number of other neurotoxins, including glutamate, quinolinic acid and extracellular proteases, which further increase microglial activation and inflammatory responses in stroke (Tikka et al., 2001).

1.5. Characteristics of neuronal death in cerebral ischemia

Ischemic infarct is a heterogeneous region within which cells die through diverse pathways. In this region cells can die through different mechanisms, depending on multiple factors, including severity, duration of insult and energy level. Characterization of these pathways is crucial to design effective therapeutic strategies.

1.5.1. *Necrosis*

As stated above, energy breakdown, disruption of ionic ingredient, and water entry into cells change their volume. This ultimately ruptures plasma membrane and induces leakage of cell contents into surrounding tissue and necrosis. DNA fragmentation is a late event happening by serine proteases in necrosis (Dong et al., 1997). Necrosis is characterized by swelling cytoplasmic organelles and disrupted plasma membrane. There is no systemic mechanism for death in necrosis, as there is in other pathways. This excitotoxiciy-mediated type of death happens within minutes to hours after blood flow blockage (Dirnagl et al., 1999).

1.5.2. *Apoptosis*

In global ischemia apoptosis is actively involved in neuronal death (Chen et al., 1996; Chen et al., 1998; Sugawara et al., 1999). In focal ischemia, apoptosis is more predominant in the penumbra where energy production is not completely impaired and cell integrity is maintained (MacManus et al., 1994; Li et al., 1995; Charriaut-Marlangue

et al., 1996). This event involves synthesis of variety of proteins. Apoptosis in ischemia is triggered by several signals such as excessive free radicals (Chan, 2001), DNA damage (Chopp et al., 1996), death-receptor ligation (Martin-Villalba et al., 1999), p53 induction (MacManus and Linnik, 1997), growth factor deprivation, TNF production and cytochrome *c* release from mitochondria (Mehta et al., 2007). Apoptosis is activated through following mechanisms:

1.5.2.1. *Mitochondria-mediated apoptosis*

Mitochondria play a very important role in the regulation of apoptosis. These organelles maintain a variety of proteins in their intermembrane space. These proteins are released into cytoplasm and/or nucleus upon different stimuli and promote apoptotic pathways. This pathway is triggered by different stimuli such as DNA damage, oncogenes, or growth factor deprivation. The following proteins are the major players of this pathway:

- Cytochrome *c*. Cytochrome *c* is a component of mitochondrial electron transfer system and is located in intermembrane space. When cytochrome *c* is released it binds to a cytoplasmic protein called Apaf1 (apoptotic protease activating factor-1)(Liu et al., 1996b; Zou et al., 1997). In this oligomer, cytochrome *c* increases Apaf1 affinity to ATP (Jiang and Wang, 2000). Binding of ATP renders these oligomers to polymerize and make a multimeric molecule called apoptosome (Zou et al., 1999). Subsequently, molecules of procaspase-9 (caspase-9 precursor) are recruited to the apoptosome and cleaved to active capase-9. Furthermore, caspase-9 cleaves and activates downstream targets such as caspases-3, 6 and 7 (Rodriguez and Lazebnik, 1999). These executioner caspases later on, activate, cleave and degrade many fundamental and structural proteins

such as, endonucleases, lamin, spectrin, PARP, and cause plasma and nuclear membrane degradation, DNA fragmentation, nuclear condensation and breaking down the cellular integrity (Hengartner, 2000; Wang, 2001). Several lines of evidence have demonstrated cytochrome *c* release from mitochondria (Fujimura et al., 1998; Perez-Pinzon et al., 1999; Sugawara et al., 1999) and activation of caspases-9 (Krajewski et al., 1999) and-3 (Chen et al., 1998) in different models of focal and global ischemia.

- Smac/Diablo (Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with a low isoelectric point). Smac/Diablo is a nuclear-encoded mitochondrial protein. Smac contains a mitochondrial targeting sequence that is removed when it is translocated from cytoplasm to mitochondrial intermembrane space (Du et al., 2000). This protein, concurrent with cytochrome *c* is released into the cytosol upon apoptosis (Du et al., 2000). Smac is an inhibitor of IAP (Inhibitor of Apoptosis Protein) (Verhagen et al., 2000). IAPs are a group of proteins known to bind to caspases and inhibit their activities (Deveraux et al., 1999). Upon apoptosis stimuli, active form of Smac released into cytosol binds to IAPs and competitively blocks their inhibitory effect on caspases-3, 7 and 9 (Sun et al., 1999; Chai et al., 2001; Srinivasula et al., 2001). A recent study has shown that Smac is released into cytoplasm following global ischemia (Sugawara et al., 2002).

- AIF (Apoptosis Inducing Factor). AIF is an oxidoreductase protein and is located in mitochondrial intermembrane space (Susin et al., 1999). Following apoptotic stimuli, AIF is released and translocated to nucleus and causes chromatin condensation and DNA fragmentation (Susin et al., 1999). Nuclear translocation of AIF has been shown in focal cerebral ischemia (Plesnila et al., 2004).

- Endonuclease G. This enzyme is necessary for mitochondrial DNA replication by removing RNA primers (Tiranti et al., 1995). Endonuclease G is coded by nucleus and at least a part of it is located in the mitochondrial intermembrane space. Following apoptotic stresses endonuclease G is released from mitochondria and causes nuclear fragmentation (Wang, 2001).

- Bcl-2 family proteins. Bcl-2 family proteins regulate mitochondrial outer membrane permeabilization (Borner, 2003) and can be either pro-apoptotic (such as Bax, Bad, Bak, Bid, Noxa, Bik) or anti-apoptotic (such as Bcl-2, Bcl-xL, Bcl-w) (Mehta et al., 2007). These proteins can be further divided based on their structure. The pro-apoptotic group binds mitochondrial outermembrane, as well as endoplasmic reticulum and nuclear envelope. The anti-apoptotic group sequesters Bax, Bad, and other pro-apoptotic Bcl-2 proteins and maintains mitochondrial integrity (Eskes et al., 2000; Chipuk and Green, 2008). Following apoptotic stimuli, such as stroke, some of the pro-apoptotic proteins are translocated to the mitochondria. For example, Bax and Bad, which are sequestered in cytosol, and Bim, which is located in microtubules, move to mitochondrial outermembrane (Chen et al., 1996; Gillardon et al., 1996; Cao et al., 2001). These Bcl-2 pro-apototic proteins accumulate and make channels in mitochondrial membrane and lead to release of cytochrome *c* into cytosol (Adams and Cory, 2001; Antonsson, 2001; Kaufmann and Hengartner, 2001). As described in more detail above, cytochrome *c* promotes formation of the apoptosome and the consequent caspases cascade activation. There is growing evidence suggesting modulation and involvement of Bcl-2 proteins in ischemia. Studies have shown that overexpression of Bcl-2 is protective in stroke models (Zhao et al., 2003) and prevented cytochrome *c* release and caspase-3 activitation. It has

also been shown that administration of Bcl-xL is protective in focal ischemia (Cao et al., 2002b). Moreover, high immunoreactivity of Bax has been reported in cerebral ischemia (Krajewski et al., 1995). More importantly, Bid deficient mice are resistant to ischemia and caspase-3 activation was attenuated in these mice (Yin et al., 2002).

1.5.2.2. *Death receptors-mediated apoptosis*

Apoptosis can be preceded by activation of specific death receptors such as FAS and TNF receptors (Nagata, 1997). These receptors contain an intracellular death domain (DD). Binding of ligands to the receptors recruits some specific adapter molecules such as, TNF receptor-associated death domain (TRADD) or Fas-associated death domain (FADD) to the DD. This homotrimerization triggers some death inducing signals inside the cells (Mehta et al., 2007). One of these signals is the binding of procaspase-8 to adapter and its auto-proteolysis to active caspase-8. Then, active caspase-8 activates caspse-3 to initiate cascade of caspases. This signaling pathway has been shown in a model of global ischemia (Jin et al., 2001).

1.5.2.3. *Crosstalk between two pathways*

In some circumstances these two pathways, mitochondrial- and death receptors-mediated apoptosis, interact with each other through a pro-apoptotic protein Bid. Bid is cleaved to a truncated form (tBid) by caspase-8 and translocated to mitochondria where cytochrome *c* is released (Li et al., 1998). The role of Bid has been shown in MCAO (Plesnila et al., 2002). It has been also shown that Bid-/- mice are more resistant to ischemia (Plesnila et al., 2001).

1.5.2.4. *Role of caspases in two pathways*

As noted above, caspases are major players in apoptotic death in both mitochondria- and death receptors-mediated pathways. Caspases are a family of cysteine aspartate-specific proteases, which are expressed in almost all cell types as inactive forms. Upon various stimuli, caspases are proteolytically cleaved and form heterotetrameric (2 small subunits and 2 large subunits) active enzymes. These enzymes could be divided into two subfamilies as a) those of the IL-1 converting enzyme family that involved in induction of inflammation (1,4,5,11,12,14) and b) those that are involved in apoptosis (2,3,6,7,8,9,10). The latter group can be divided into two subgroups: initiator caspases (2, 8, 9, 10) and effector caspases (3,6,7) (Earnshaw et al., 1999). In several neurodegenerative disorders, including stroke, effector caspases are activated and target numerous proteins including cytoskeletal proteins such as actin, fodrin, gelsolin, as well as nuclear proteins such as lamins, DNA repair proteins (PARP) and the inhibitor of CAD (caspase activated DNAse) and lead to cell death (Love, 2003a). Caspase-3 is the most abundant caspase in brain and several studies have demonstrated increase in its activity following both focal and global ischemia in animal models (Namura et al., 1998; Ni et al., 1998; Cao et al., 2002a) and ischemic human brain (Rami et al., 2003). Moreover, caspse inhibitors have been shown to be protective in stroke models (Mouw et al., 2002; Inoue et al., 2004). Importantly, studies have shown that caspase-3 deficient mice are more resistant to focal ischemia (Friedlander et al., 1997).

The mitochondria-mediated and death receptors-mediated apoptosis pathways and their interaction have been schematically shown in figure 1.3.

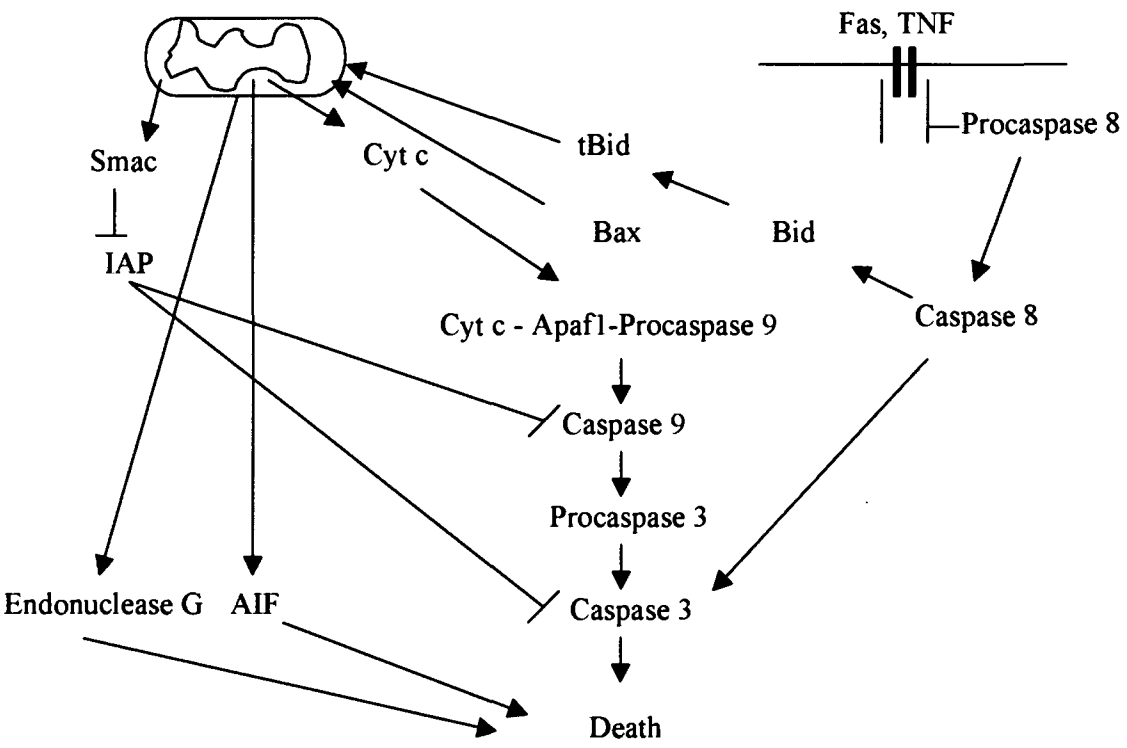
Mitochondrial pathway
Death receptors pathway
Fas, TNF
Procaspase 8
Smac
Cyt c
tBid
Bax
Bid
IAP
Cyt c - Apaf1-Procaspase 9
Caspase 8
Caspase 9
Procaspase 3
Endonuclease G
AIF
Caspase 3
Death

Figure 1.3

Figure 1.3. Mitochondrial-mediated and death receptors-mediated apoptosis and cross talk between these two pathways.

1.6. Complexity of signaling pathways regulating stroke

According to what has been described so far, it has been realized that the mechanisms involved in ischemic neuronal death are complex and analyses of these mechanisms is complicated by the observations that neurons in variant regions of an infarct area may die through distinct mechanisms. As stated before, a relatively rapid excitatory death occurs in the core within minutes to a few hours after stroke (Astrup et al., 1981). Alternatively, the neurons in penumbra experience less intense ischemia and display a more delayed type of death with characteristics of apoptosis happening within days (Dirnagl et al., 1999). The signaling pathways, which regulate rapid and delayed ischemic death are not fully defined. However, there is a growing body of evidence highlighting the importance of CDK family of kinases. This occurs through at least two mechanisms. First, we propose that the cell cycle machinery is critical in controlling neuronal death. Indeed, it has been suggested that inappropriate activation of some of the cell cycle elements, including cyclin-dependent kinases (CDKs), might initiate a cascade of signals leading to death. The hypothesis, compatible with this notion is that activation of cell cycle CDKs, in the context of post-mitotic neurons, signals death rather than cell cycle progression.

A second hypothesis, as it relates to CDKs, states that deregulated Cdk5 activity (an activity critical for brain development) can also induce neuronal damage. In this case, one model states that calpain proteases cleave the Cdk5 activator to a smaller and more stable form, which changes Cdk5 localization and converts it into a death inducer (Patrick et al., 1999).

This research has been designed to test the above hypotheses and results are presented in the next two chapters. The main theme of data presented in chapter 2 is to illustrate how reactivation of cell cycle CDKs and upregulation of Cdk5 is a critical step in stroke models and to describe how they signal differently in distinct models of ischemic neuronal death. To further decipher their signals, we have focused on the CDK targets in both chapters 2 and 3. Accordingly, in the following sections of the present chapter the role of CDKs in mammalian cell cycle will be briefly reviewed and the evidence implicating CDKs and other cell cycle regulatory elements in neuronal ischemic death will be outlined. In a separate section Cdk5, as a non-cell cycle CDK, and its significant role in stroke models will be discussed in detail.

1.7. Cyclin-dependent kinases (CDKs)

CDKs are a large and growing family of proline-directed serine/threonine kinases which are recognized by a conserved sequence of PSTAIRE within their catalytic subunit (Morgan, 1997). The typical phosphorylation sequence for CDKs is [S/T*]PX[K/R], where S/T* indicates the phosphorylated serine or threonine, X is any amino acid and K/R is the basic amino acid lysine (K) or arginine (R) (Songyang et al., 1996). The catalytic activity of these kinases is highly dependent on binding to another group of cell cycle regulators named cyclins (Pines, 1993b). At least 13 CDKs and a considerable number of cyclins (A-T) have been identified so far. Although CDKs are best recognized for their regulatory function in cell cycle (Cdk1, Cdk2, Cdk4/6), some are known for their role in other biological processes including transcriptional control such as Cdk7, Cdk8, Cdk9 (Serizawa et al., 1995; Gold and Rice, 1998; Akoulitchev et al., 2000) and neuronal function (Cdk5) (Dhavan and Tsai, 2001).

1.7.1. *CDKs structure.*

CDK family members are small proteins (~34-40kD). Studying tertiary structure of Cdk2 has revealed major structural features of this family. Cdk2 contains a small N-terminal lobe and a larger C-terminal lobe. ATP fits in a cleft between the lobes and protein substrate binds at the entrance of the cleft. Conserved PSTAIRE sequence is located within N-terminal lobe. Two features restrain Cdk2 to catalyze a kinase reaction: a) A large and flexible loop, called T-loop, in C-terminal lobe which covers and blocks substrate binding site at the cleft. b) Some misdirected key amino acids within ATP phosphate binding site (Morgan, 1997). As will be discussed below, different mechanisms overcome these obstacles and enable CDK for its proper function.

1.7.2. *Regulation of CDKs activity*

Activation

- Cyclins. Partial activation of a CDK requires binding to a cyclin (Cdk5 is an exception). Association of cyclin with CDK through different regions, especially through PSTAIRE sequence, increases CDK kinase activity by several mechanisms. This binding changes conformation of active site, reorientation of residues that interact with the phosphates of ATP and allows ATP binding. This leads to correct positioning for phosphotransfer process. Moreover, this conformational alteration facilitates phosphorylation of CDK by other activators (see below) (Morgan, 1997).

- Phosphorylation. Full activation of CDKs requires phosphorylation of a conserved threonine residue proximal to ATP-binding site (Thr160 in Cdk2) located in T-Loop (Gu et al., 1992). Cdk activating kinase (CAK) catalyzes this reaction. CAK is a complex of Cdk7/cyclin H combined with an assembly factor Mat1 (Kaldis, 1999). This phosphorylation flattens the T-loop and changes the conformation of protein-substrate binding site and improves substrate binding (Morgan, 1997).

- Dephosphorylation. Phosphorylation of Tyr15 and Thr14 (see below) inhibits CDKs activity. This effect is in tun removed by Cdc25 family phosphatases (Sebastian et al., 1993). Members of Cdc25 (A, B, and C) are themselves activated by cyclin/CDK complexes.

Inhibition

- CDK inhibitors (CKIs). Endogenous CKIs have been divided into two families (Sherr and Roberts, 1999):

a) Ink4 family including p16, p15, p18, and p19. This family specifically binds to cyclin binding site on Cdk4/6 and abolishes formation of cyclin D/Cdk4/6 complexes (Coleman et al., 1997).

b) Cip/Kip family including p21, p27, and p57. These inhibitors are able to block activity of all CDKs by binding to preformed cyclin/cdk complexes (Sherr and Roberts, 1999). This group can bind to both cyclin and CDK (Morgan, 1997).

- Phosphorylation. As stated earlier, phosphoryaltion of CDKs on Tyr15 (Cdk1) and Thr14 (Cdk2) inhibit CDKs activity. In case of Cdk4/6, phospho-Tyr17 is inhibitory. Wee1 and Myt1 are two enzymes responsible for this phosphorylation (Obaya and Sedivy, 2002).

1.7.3. *Cell cycle-related CDKs*

Cell cycle is a tightly regulated process that governs cell proliferation and growth. During this process cells are transfered from a non-dividing and post-mitotic stage (G0) to a proliferation stage. Typically, each cell cycle is divided into four phases; two phases are associated with DNA synthesis (S) and mitosis (M), each separated by two gaps (G1 and G2) (Figure 1.4). Progression of these stages is timely controlled by CDKs (Morgan, 1997). As shown in figure 1.4, Cdk1, Cdk2, Cdk4/6, and recently Cdk3, are the known regulators of cell cycle so far (Morgan, 1997; Ren and Rollins, 2004). Cell cycle CDKs are continuously expressed but their activity is controlled during each phase. Cyclins are expressed upon different cellular stimuli (Obaya and Sedivy, 2002). For example, cyclin D (D1, D2, D3) is upregulated by growth factors stimuli and assembles with Cdk4/6 at early G1 phase (Aktas et al., 1997). Cyclin E/Cdk2 is formed at the late stages of G1 (Dulic et al., 1992; Koff et al., 1992). A major substrate for G1 CDKs is retinoblastoma

tumour suppressor protein (Rb) and its related pocket proteins, p107 and p130 (Beijersbergen and Bernards, 1996). Rb at its hypophosphorylated status binds to and inhibits activity of transcription factors known as E2F-DP complexes. During G1/S transition, Cdk4/6 and then Cdk2 sequentially phosphorylate Rb at different sites (Lundberg and Weinberg, 1998). Once Rb is phosphorylated by Cdk4/6, the repression is partially removed from E2F-DP and, therefore, some genes such as cyclin E are transcribed. Next, cyclin E/Cdk2 complexes, formed in mid to late G1 phase, completely phosphorylate and inactivate Rb and therefore release E2F-DP. Full activation of E2F-DP leads to transcription of several genes important for entering S phase such as cyclin A, cyclin B, Cdk1 and proteins required for DNA replication (Dyson, 1998). Interestingly, E2F genes are also E2F-DP targets (Obaya and Sedivy, 2002). Moreover, phosphorylation of p27 by cyclin E/Cdk2 leads to its degradation (Sheaff et al., 1997). These events render additional activity of E2F-DP. Cyclins D and E undergo proteosomal degradation at early S phase (Singer et al., 1999; Germain et al., 2000). Expression of cyclin A and formation of cyclin A/Cdk2 is necessary for transition of G1/S. Progression of S phase and DNA replication requires inactivation of E2F-DP complexes (Lukas et al., 1996). This is partly mediated by cyclinA/Cdk2 activity. Phosphorylation of E2F-DP heterodimer by this complex blocks its DNA binding activity (Xu et al., 1994). During S and G2 phases cyclins A and B levels are increased and they make a complex with Cdk1. At the mitosis (M) stage cylinB/Cdk1 is translocated into nucleus and mitosis begins (Ford and Pardee, 1998). Cyclins A and B are phosphorylated by their partner CDKs and subjected to degradation (Stewart et al., 1994). With regards to Cdk3, there is recent evidence associating this CDK to cell cycle. It has been suggested that cyclin C/Cdk3

complex promotes G0/G1 transition through phosphorylation of Rb (Ren and Rollins, 2004). As it has been noticed, proper progression of cell cycle requires orderly activation, and inactivation of CDKs with their partners.

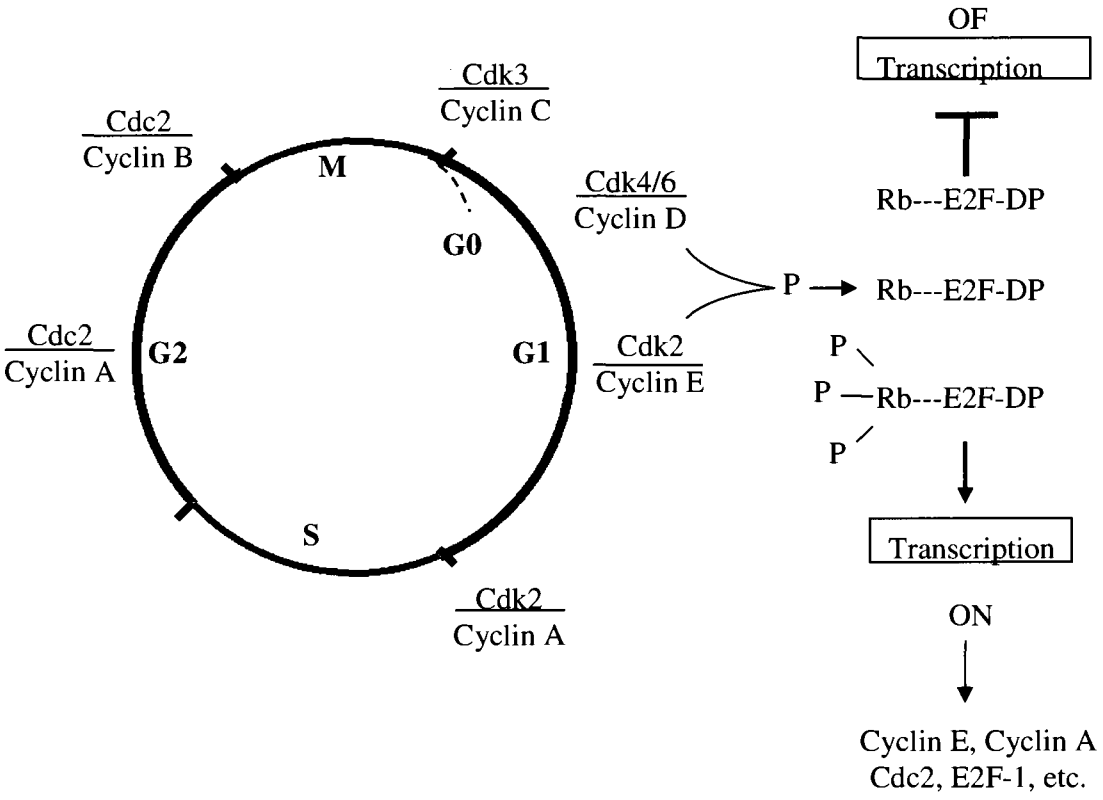
OF
Transcription
Rb---E2F-DP
Cdk3
Cyclin C
Cdc2
Cyclin B
M
G0
Cdk4/6
Cyclin D
P
Rb---E2F-DP
Cdc2
Cyclin A
G2
G1
Cdk2
Cyclin E
P
P
Rb---E2F-DP
P
S
Transcription
Cdk2
Cyclin A
ON
Cyclin E, Cyclin A
Cdc2, E2F-1, etc.

Figure 1.4

Figure 1.4. A schematic representation of mammalian cell cycle. Cell cycle is regulated by different complexes of CDKs with their activating partners, cyclins. Rb is sequentially phosphorylated by G1 phase CDKs resulting in release of E2F-DP transcription factors and regulating of the genes required for S phase entry.

1.7.3.1. *Cell cycle-related CDKs and ischemic neuronal death*

Growing evidence suggests that one of the mechanisms controlling neuronal death might be re-entry of neurons into cell cycle. Neurons are highly differentiated, post-mitotic cells whose cell cycle machinery is downregulated under normal conditions. Cdk4 and Cdk2 activities decline in differentiated neurons and, consequently, phosphorylated Rb decreases, resulting in greater inhibition of E2F-DP activity (Kranenburg et al., 1995). However, numerous studies have revealed that cell cycle machinery is re-activated in neurons in response to several stresses. For instance re-activation of cell cycle has been shown to be required for neuronal death induced by DNA damage (Park et al., 1997b), ß-amyloid exposure (Giovanni et al., 2000), growth factor withdrawal (Freeman et al., 1994; Gao and Zelenka, 1995) as well as in neurodegenerative disorders such as Alzheimer's disease (AD) (Herrup and Arendt, 2002; Yang et al., 2003), Parkinson's disease (PD) (El-Khodor et al., 2003), amyotrophic lateral sclerosis (ALS) (Nguyen et al., 2003), Niemann-Pick type C (NPC) (Zhang et al., 2004) and stroke. With regards to the latter, multiple lines of evidence, both *in vitro* and *in vivo*, suggest an essential role for cell cycle regulators in ischemic neuronal injury. For instance, upregulation of Cdk2 activity and cyclin D/E levels have been observed in cultured neurons following oxygen glucose deprivation (OGD) and kainic acid (KA) exposure (Katchanov et al., 2001; Verdaguer et al., 2002). Moreover, phosphorylation of Rb and increase in E2F level has been demonstrated in *in vitro* models of ischemia (Gendron et al., 2001; Verdaguer et al., 2002; Smith et al., 2003b). Furthermore, in some functional studies pharmacological CDK inhibitors such as flavopiridol, olomoucine and

roscovitine protected cultured neurons from ischemic insults (Katchanov et al., 2001; Giardina and Beart, 2002; Verdaguer et al., 2003; Verdaguer et al., 2004).

The implication of cell cycle elements in stroke are made more significant in the more relevant situation; where ischemia occurs in fully mature neurons. In this situation, where there is little or any cell cycle machinery present, deregulation of these elements might lead to death. Supporting this notion are several correlative studies, which have shown deregulation of G1/S-CDKs activity and G1/S-cyclins levels following different *in vivo* models of ischemia. For instance, upregulation of cyclin D1 has been demonstrated in MCAO model of focal ischemia (Guegan et al., 1997; Osuga et al., 2000; Katchanov et al., 2001; Wen et al., 2005), systemic injection of KA (Liu et al., 1996a; Timsit et al., 1999; Ino and Chiba, 2001) and global ischemia (Timsit et al., 1999; Small et al., 2001; Kato et al., 2003). According to this research, aberrant expression/activity of Cdk4 has been implicated in focal ischemia (Hayashi et al., 1999b; Osuga et al., 2000), KA injection (Ino and Chiba, 2001), and global ischemia (Kato et al., 2003). Although most of the studies have implicated cyclin D/Cdk4 in ischemia, deregulation of Cdk2 and cyclin A has been also shown in ischemic neuronal loss (Li et al., 1997). Along with the above evidence, some other intriguing observations have shown that endogenous CDK inhibitors are downregulated in dying neurons (Katchanov et al., 2001; Kuan et al., 2004) while surviving neurons have upregulated these proteins (van Lookeren Campagne and Gill, 1998). In adult rodent models, some functional studies have shown that inhibition of CDKs activity is protective. For example, pharmachological inhibitors of cyclin D1/Cdk4 have been neuroprotective in focal ischemia (Wen et al., 2005). More importantly, we

have reported that flavopiridol has attenuated CDK-Rb pathway and reduced E2F level in focal and global ischemia (Osuga et al., 2000; Wang et al., 2002).

Although these studies emphasize that cell cycle CDKs might play an essential role in ischemia, they are not conclusive. These studies are based on some correlative observations as well as using non-specific inhibitors. Consequently, the present research was designed to functionally relate cell cycle signals to neuronal death.

- *Retinoblastoma tumor suppressor protein (Rb): a target for cell cycle CDKs in stroke*

Rb is the coordinator of cell fate, life or death. In the present work we have focused on Rb as the main target for cell cycle CDKs to define mechanisms that mediate neuronal ischemic death. Rb belongs to pocket proteins family and regulates several cellular functions including G1/S transition and survival (Dasgupta et al., 2006). As stated above, Rb regulates cell cycle transition through binding to E2F-DP complexes and repressing transcription. The transcriptional repression is mediated through two mechanisms. a) Rb binds to E2F transactive domain and inhibits its interaction with transcriptional machinery (Helin et al., 1993). b) The formed Rb/E2F-DP complex, next, binds to the E2F responsive promoters to recruit various chromatin remodeling enzymes to the same site. These enzymes, such as histone deacetylases and histone methylases, interact with chromatin and silence transcription (Frolov and Dyson, 2004). The ability of Rb to bind to E2F and chromatin remodeling enzymes is regulated by sequential CDKs phosphorylation (Harbour et al., 1999). Rb contains at least 16 CDK Pro-directed Ser/Thr phosphorylation sites (Delston and Harbour, 2006).

Rb emerges its antiapoptotic role through modulation of E2F, or alternatively, through other mechanisms independent of E2F.

1. E2F-dependent antiapoptotic role of Rb. Studying Rb-null mice has revealed that antiapototic function of Rb, at least partially, is through repression of E2F activity. These mice have massive apoptosis in different tissues including nervous system and embryos die at mid-late gestation (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Interestingly in Rb/E2F1 double mutants apoptosis has been largely suppressed in nervous system, indicating that apart of the survival role of Rb is attributed through repression of E2F1; although these mice die due to defect in other tissues (Tsai et al., 1998). Several pro-apoptotic promoters have been identified as E2F targets like Apaf1 (Moroni et al., 2001), caspases (Nahle et al., 2002) and p73 (Lissy et al., 2000). Accordingly, repression of E2F by Rb inhibits transcription of these pro-apoptotic genes.
2. E2F-independent antiapoptotic role of Rb. Rb can exert its antiapoptotic function through interaction with targets other than E2Fs. For example, Rb inhibits turnover of BCL-x_L, an antiapoptotic member of Bcl-2 family proteins (Deverman et al., 2002). Another study has shown that Rb physically interacts with and inhibits JNK stress-activated protein kinase and protects cells from UV-induced apoptosis (Shim et al., 2000).

A major question of interest is how inactivation of Rb in some contexts leads to cell cycle progression and in distinct cases causes cell death. Multiple models have been proposed in this regard. Some studies have proposed the "promoter-specific regulation" model. According to this model, phosphorylation of Rb by cyclin-CDK releases Rb repression from S-phase entry promoters but does not release the repression from apoptotic genes. Degradation of Rb by caspases is necessary for removing repression from apoptotic promoters and cell death (Chau and Wang, 2003). A second potential

mechanism to explain how Rb dictates cells to proliferate or die is being explained by "context-dependent" model. This model proposes that phosphorylation of Rb by kinases, like CDKs, activates both groups of S-phase and apoptotic genes. Nevertheless, other parallel activities, in combination with Rb inactivation, define cell fate. In this scenario, mitogenic stimuli activate some survival signals, such as AKT and NF-κB. These survival factors, in turn, antagonize pro-apoptotic proteins and enable cells to proliferate (Chau and Wang, 2003). Another possible mechanism has been explained by Dasgupta and collegues. According to them, mitogenic or apoptotic signals activate different kinases. These kinases phosphorylate Rb at distinct sites and, thereby, different promoters will be activated (Dasgupta et al., 2006).

In summary, the molecular mechanisms by which Rb regulates cell division or apoptosis are not fully established yet. While most of the studies are focused on Rb-mediated transcriptional modulation, this tumor suppressor factor might regulate apoptosis by mechanisms other than gene regulation (Ma et al., 2003).

1.7.4. *Cyclin-dependent kinase 5 (Cdk5)*

Cdk5, also known as neuronal Cdc2-like kinase (NCLK), is a member of CDKs family which phosphorylates Ser/Thr residues immediately preceding a proline residue. In contrast to other CDKs, Cdk5 does not require a cyclin to be activated. Unlike the classic CDKs, there is no solid evidence for direct role of Cdk5 in cell cycle progression. However, emerging evidence suggesting that Cdk5 might control cell cycle, at least indirectly. It has been shown that Cdk5 can bind to cyclins D1, D3 and E (Xiong et al., 1992; Zhang et al., 1993a; Miyajima et al., 1995), but it is not activated by these interactions. Cdk5 is also able to phosphorylate Rb (Honma et al., 1997; Hamdane et al., 2005). Some recent findings indicated that Cdk5 is necessary for neuronal cell cycle arrest (Cicero and Herrup, 2005). According to this study, Cdk5 is necessary to suppress neuronal cell cycle. It probably accomplishes this by competing with Cdk4/6 for binding to cyclins or through a specific phosphorylation of Rb that stabilizes Rb/E2F complex or alternatively, by phosphorylation of RNA polymerase (Cicero and Herrup, 2005). Importantly, Zhang and collegues have shown that nuclear localization of Cdk5 is essential for neurons to remain in postmitotic status (Zhang et al., 2008).

Cdk5 is ubiquitously expressed, however, its activity is mainly restricted to postmitotic neurons since its activators, p35 and p39, are predominantly expressed in these cells (Tsai et al., 1994; Tang et al., 1995). P35 contains a myristoylation signal motif at the N-terminal region that targets Cdk5 to membranous compartments of the cell. P35 itself is a target for Cdk5 and its level is regulated by ubiquitination (Saito et al., 1998; Patrick et al., 1999). Phosphorylation by Cdk5 targets p35 to proteosomal degradation (Saito et al., 1998; Patrick et al., 1999).

Cdk5 is a key regulator of neuronal morphology and functions and its role in neurodevelopment is well established. Initial evidence suggesting the importance of Cdk5 in neuronal development came from the study of Cdk5 knockout (KO) mice. Disruption of the Cdk5 gene causes a severe impairment in neuronal layering especially in cerebral cortex (Ohshima et al., 1996; Gilmore et al., 1998). This deficiency is lethal, resulting in perinatal death. Expression of reconstituted Cdk5, regulated by a p35 promoter, rescues these mice (Tanaka et al., 2001). P35 KO mice display similar deficits in neuronal lamination but abnormalities are less severe. These animals survive, although they are more susceptible to seizures (Chae et al., 1997; Hallows et al., 2003). Interestingly, p39 KO mice show no phenotypic brain abnormalities but p35/p39 double mutants have similar phenotype observed in Cdk5 deficient mice (Ko et al., 2001). This indicates p35 as the major activator of Cdk5.

Besides activation by p35 and p39, there are other posttranslational mechanisms that regulate Cdk5 activity. Similar to other CDKs, phosphorylation of Thr14 downregulates Cdk5 activity (Matsuura and Wang, 1996). Phosphorylation of Tyr15, however, is stimulatory (Zukerberg et al., 2000). Like other CDKs, Ser159 on Cdk5, an equivalent residue for Thr160 on Cdk2, is phosphorylated by Cdk7, but the importance of this phophorylation is not clear yet. While one study showed phosphorylation of Ser159 was stimulatory (Rosales et al., 2003), others have reported that it is not necessary (Poon et al., 1997) or even it inhibits Cdk5 activity (Tarricone et al., 2001).

In addition to development, Cdk5 has been implicated in numerous neuronal biological functions such as neuronal migration (Ohshima et al., 1999), axonal guidance (Kwon et al., 1999), cell adhesion (Kwon et al., 2000), axonal transport (Julien and

Mushynski, 1998), dopamine signaling (Bibb et al., 1999), synaptic transmission and plasticity (Li et al., 2001), drug addiction (Bibb et al., 2001), and pain signaling (Pareek et al., 2006).

1.7.4.1. *Cdk5 as a pro-survival mediator*

In addition to its role in normal brain development, Cdk5 is also believed to be critical for survival. In support of this recent evidence indicates that Cdk5 is capable of protecting neurons through regulation of c-Jun N-terminal kinase 3 (JNK3) pathway. Cdk5 attenuates neuronal death by phosphorylation of JNK3 in a UV irradiation model (Li et al., 2002). This process leads to downregulation of phospho c-Jun and suppression of apoptosis. The anti-apoptotic role of Cdk5 is supported by the observation that showed Cdk5-deficient cortical neurons increased JNK3 activity, c-Jun phosphorylation and consequently sensitivity to apoptotic stimuli (Li et al., 2002). The second proposed mechanism by which Cdk5 has a pro-survival role is through regulation of PI3K/Akt kinase signaling pathway (Li et al., 2003). This pathway signals for survival in neurons and many other cell types. Cdk5 promotes neuronal survival through phosphorylation of the neuregulin receptors (ErbB2/ErbB3), which then activate the PI3K/Akt pathway. A third potential mechanism for Cdk5 to contribute in cell survival is through Bcl-2. A recent study revealed that Cdk5 is activated in BDNF-induced differentiated neuron-like cells and inhibits the apoptotic death through ERK-mediated upregulation of Bcl-2 (Wang et al., 2006). It has also been suggested that Cdk5 prevents apoptosis through modulation of MAPK signaling pathway (Zheng et al., 2007). Moreover, a very recent study demonstrated that phosphorylation of Bcl-2 at Ser-70 by Cdk5 is necessary for its neuoroprotective effect (Cheung et al., 2008).

1.7.4.2. *Cdk5 as a pro-death mediator*

Despite the critical role of Cdk5 in development/survival, emerging evidence has also implicated its essential role in neuronal death. Initially, the pathogenic face of Cdk5 was unmasked by introducing p25, a potent activator of Cdk5. Accordingly, p35 is cleaved to p25 by calpain, a calcium-dependent cysteine protease, under some pathological conditions. Several *in vitro* studies have reported cleavage of p35 to p25 in neuronal-death inducing conditions (Patrick et al., 1999; Kusakawa et al., 2000; Tseng et al., 2002). P25 is 208-residue carboxy-terminal fragment of p35 and is more stable and over activates Cdk5. P25 changes Cdk5 cellular localization, as this cleaved form no longer contains the myristolation domain that targets it to the membrane. This, presumably, alters Cdk5 substrates too. Importantly, Cdk5 and p25 levels are altered in different pathogenic states. Upregulation of p25 and deregulation of Cdk5 has been implicated in several neurodegenerative diseases:

- Alzheimer's disease (AD). Accumulation of p25, upregulation of Cdk5 activity, and phosphorylation of tau, a microtubule-associated protein, has been reported in post-mortem brains of AD (Patrick et al., 1999; Tseng et al., 2002).

- Parkinson's disease (PD). It has been found that Cdk5 and p35 are colocalized in Lewy bodies in post-mortem brains of PD patients (Nakamura et al., 1997). We have recently shown induction of p25 and deregulation of Cdk5 in an MPTP mouse model of PD (Smith et al., 2003a).

- Amyotrophic Lateral Sclerosis (ALS). In transgenic mouse model of ALS, Cdk5 activity and p25/p35 ratio is increased in spinal cord neurons and tau is phosphorylated (Nguyen et al., 2001; Nguyen et al., 2003).

- Niemann Pick type C (NPC). Deregulation of Cdk5 and accumulation of p25 has been associated with development of NPC in a mouse model of this neurodegenerative disease (Bu et al., 2002; Zhang et al., 2004).

- Ischemia. In a model of focal ischemia (MCAO), Cdk5 and p35/25 immunoreactivity is enhanced (Hayashi et al., 1999a). It has been shown that there is a link between p25 and neuronal death in global ischemia (Wang et al., 2003). P25 is produced following global ischemia and deregulated Cdk5 induces its death signal through phosphoryaltion of NMDA receptors (Wang et al., 2003).

1.7.4.3. *Controversies over Cdk5/p25 role in neuropathogenesis*

Although the above evidence suggests a central role for Cdk5/p25 in neurodegenerative diseases, this notion is still controversial. There is a general concept that formation of p25 turns physiologically normal Cdk5 to a pathogenic Cdk5 molecule. There are some reports, though, questioning this notion:

- In spite of the observations demonstrating elevation of p25 levels in post-mortem brains of AD patient (Patrick et al., 2001; Tseng et al., 2002), some other groups have suggested that p25 level does not change (Tandon et al., 2003) and is even lower (Yoo and Lubec, 2001) compared to controls. Likewise, Takashima *et al.* argue that p25 may not be directly involved in AD pathology since they could not show hyperphosphorylation of tau nor neuronal death in p25 overexpressing mice (Takashima et al., 2001). Similarly, Bian et al. did not show genesis of neurofibrillary tangles, one of the pathological hallmarks of AD, in p25 transgenic mice (Bian et al., 2002).

- The role of Cdk5/p25 in pathogenesis of ALS is controversial too. Although initial observations suggested a potential role for Cdk5/p25 in pathogenesis of ALS (Nguyen et

al., 2001; Nguyen et al., 2003), Takahashi et al. showed that this motor neuron disease could be developed and progressed with the same pathological features as control in p35 null mice (where p25 can not be produced) (Takahashi and Kulkarni, 2004).

- The role of Cdk5/25 in developing NPC is not clear yet. Similar to ALS, soon after early studies introduced Cdk5/p25 in pathogenesis of this disease, Hallow et al. showed that p35/p25 was not necessary for neuronal death in NPC (Takahashi and Kulkarni, 2004). This disease was developed and progresses with the same pathological features (tau phosphorylation and lesion formation) in mouse model lacking p35/p25. However, they did not rule out the p35-independent Cdk5 activity, concerning that Cdk5 may negatively regulate some other kinases which phosphorylate tau. So, Cdk5 might be indirectly involved in the pathogenesis of this disease.

Accordingly, while increase in p25 level has been essential in some paradigms, simply association of Cdk5 pathogenesis with p25 may not be relevant in all cases. As the above evidence implies the role of p25, at least in a direct manner, seems to be dismissible in distinct models.

1.7.4.4. *Cell localization and Cdk5 pathogenesis*

Several studies have demonstrated that Cdk5 is localized in both nuclear and cytoplasmic compartments of the cell (Ino and Chiba, 1996; Nguyen et al., 2001; Qu et al., 2002; O'Hare et al., 2005). Recent evidence suggests that cell localization might be important in dual functions of Cdk5 in survival or death. For instance we have recently shown that in a DNA damaging apoptosis model induced by camtothecin, inhibition of cytoplasmic Cdk5 sensitizes cultured cortical neurons and CGNs to death (O'Hare et al., 2005). In contrast, activation of nuclear Cdk5, in an *in vitro* model of excitotoxicity

promotes death (Gong et al., 2003; O'Hare et al., 2005). This suggests that cytoplasmic activity is normal while nuclear activity is pathogenic. However, Cdk5 is known to phosphorylate tau, a cytoplasmic protein, and induce death (Cruz and Tsai, 2004). These observations propose that the nature of Cdk5 signal is complex and it may depend on multiple factors including localization and consequently, its targets. With regards to this project, understanding of how and where Cdk5 is activated is critical to elucidate signaling pathways contributing in stroke pathogenesis. Therefore, a part of this work was designed to differentiate Cdk5 activity in cytoplasm and nucleus in models of ischemia and to determine its targets in these compartments.

1.7.4.5. *Cdk5 targets in neuronal death*

Although the list of physiological substrates is growing very rapidly (Dhavan and Tsai, 2001; Shelton and Johnson, 2004) there are few studies demonstrating pathological targets for Cdk5. As implicated above, Cdk5 is localized in both nucleus and cytoplasm (Ino and Chiba, 1996; Nguyen et al., 2001; Qu et al., 2002; O'Hare et al., 2005). Accordingly, we have focused on both possible nuclear and cytoplasmic substrates for Cdk5 in ischemia models. We focused on MEF2D as a nuclear target and Prx2, as a cytoplasmic candidate.

- *Myocyte Enhancer Factor-2D (MEF2D)*

MEF2D belongs to MEF2 family of transcription factors known to control important cellular functions such as proliferation, differentiation and survival (Heidenreich and Linseman, 2004). The MEF2 isoforms (A-D) have the highest expression in muscles and neurons of central nervous system (Potthoff and Olson, 2007). The mechanisms regulating MEF2s activity are not completely understood but recent

evidence indicates that phosphorylation plays an important role. Phosphorylation of MEF2s can result in activation or inhibition of their activities, depending on the phosphorylated site (Heidenreich and Linseman, 2004). In this regard, initial reports suggested that phosphorylation of MEF2s might be associated with neuronal death (Mao and Wiedmann, 1999). Further studies identified Cdk5 as a possible regulator of phosphorylation and inactivation of MEF2s in neurotoxicity *in vitro* (Gong et al., 2003). According to this observation Cdk5 phosphorylates MEF2A, C and D on distinct sites and inactivates their functions.

Importance of MEF2D in neuronal damage. According to *in vitro* evidence, MEF2D is direct target for Cdk5. It is phosphorylated at Ser444 and inactivated by Cdk5 following neurotoxicity induced by oxidative stress and glutamate (Gong et al., 2003). The same group showed that this phosphorylation promotes MEF2D degradation by caspase-3 (Tang et al., 2005). More importantly, it has been previously shown in our lab that in an animal model of PD phosphorylation of MEF2D on Ser444 and its subsequent degradation is one of the mechanisms induced by Cdk5 in dopaminergic neuronal loss (Smith et al., 2006). Given our observations that nuclear Cdk5 activity is elevated in response to focal ischemia, we studied the phosphorylation of MEF2D by Cdk5 and its importance as a signaling pathway involved in ischemic neuronal death.

- *Peroxiredoxin 2 (Prx2)*

Prx2 belongs to the peroxiredoxins family whose members are involved in variety of cellular functions including cell cycle progression, apoptosis and more importantly, cellular antioxidant defense (Immenschuh and Baumgart-Vogt, 2005). Six isoforms of Prxs have been identified in mammalian cells which are divided in to three major subgroups: 1)

2-Cys Prx (Prx1, 2, 3 and 4) contains both N-terminal and C-terminal conserved cysteins. 2) Atypical 2-Cys Prx (Prx5) contains only N-terminal conserved cystein but requires another non-conserved cystein for catalytic activity. 3) 1-Cys Prx (Prx6) contains only one N-terminal conserved cystein and that is enough for catalytic activity. Prx1, 2 and 6 are cytosolic, while Prx3 is located in mitochondria. Prx4 is localized in endoplasmic reticulum, as well as secreted in extracellular space. Finally, Prx5 is present in peroxisomes and mitochondria (Rhee et al., 2005). Prxs are a part of cellular defense against oxidative stresses through enzymatic degradation of hydrogen peroxide and peroxynitrite.

Hydrogen peroxide reductase activity. Functionally, the N-terminal Cys is oxidized by peroxides to a sulfenic acid derivative (Cys-SOH) which then forms an intermolecular (in 2-Cys Prxs) or an intramolecular (in atypical 2-Cys Prx) disulfide (S-S). The intermolecular S-S is then reduced by thioredoxin (Trx) together with Trx reductase (TrxR) and NADPH. In this reaction H_2O_2 is converted to H_2O (Figure 1.5).

Peroxynitrite reductase activity. Like H_2O_2, peroxynintrite oxidizes the –SH on Prxs, which then leads to formation of sulfenic acid and nitrite. Similarly, the S-S is formed in the presence of a thiol group (Radi et al., 1991). It has been shown that mammalian Prx3, 5 and 6 have peroxynitrite reductase activity (Peshenko and Shichi, 2001; Dubuisson et al., 2004).

Regulation of Prxs activity. Different posttranslational mechanisms regulate Prxs activity. Phosphorylation, oligomerization, proteolysis and overoxidation are some of these regulatory mechanisms. In regards to phosphoryaltion, it has been shown that phosphorylation of Prx1, 2, 3 and 4 by a number of CDKs, including Cdk1 inhibits their activities (Chang et al., 2002). It has been shown that Prxs are able to make multimers in

the certain conditions and this oligomerization increases their activity (Wood et al., 2002). Additionally, specific proteolysis of C-terminus of the Prxs protects them against overoxidation (Koo et al., 2002). Compared to full-length protein, the C-terminal truncated form is more resistant to oxidative stresses, although this modification makes it more susceptible to phosphoryaltion. It has also been shown that calpain cleaves Prx2 *in vitro* (Schroder et al., 1998).

Importance of Prx2 in neuronal damage. Prxs are widely expressed antioxidant enzymes (Rhee et al., 2005). Oxidative stress is one of the mechanisms involved in pathogenesis of a number of neurodegenerative diseases (Halliwell, 2006). As such, the role of Prxs as an antioxidant defense molecules is highly important. According to evidence, Prx2 levels are significantly increased in neurodegenerative disorders such as PD, AD, Down's syndrome (DS) and Huntington disease (HD) (Kim et al., 2001; Krapfenbauer et al., 2003; Sorolla et al., 2008). We have recently shown that Prx2 interacts with Cdk5/p35 and is phosphorylated on Thr89 by this complex (Qu et al., 2007). Phosphorylaton of Prx2 results in reduction of peroxidase activity and neuronal death *in vitro* and in an animal model of PD. More importantly, we showed that phospho-Prx2 increases in brains from PD patients (Qu et al., 2007). A recent study has suggested nitrosylation and inactivation of Prx2 as another mechanism for oxidative stress induced in PD (Fang et al., 2007). Given that oxidative stress is one of the important mechanisms involved in ischemia we studied the phosphorylation of Prx2 by Cdk5 and its importance as a signaling pathway involved in ischemic neuronal death. Since Prx2 is located in cytoplasm and our results implicated that activity of Cdk5 is elevated in cytoplasm in

both models of focal and global ischemia, this antioxidant was our candidate to be studied as cytoplasmic Cdk5 target.

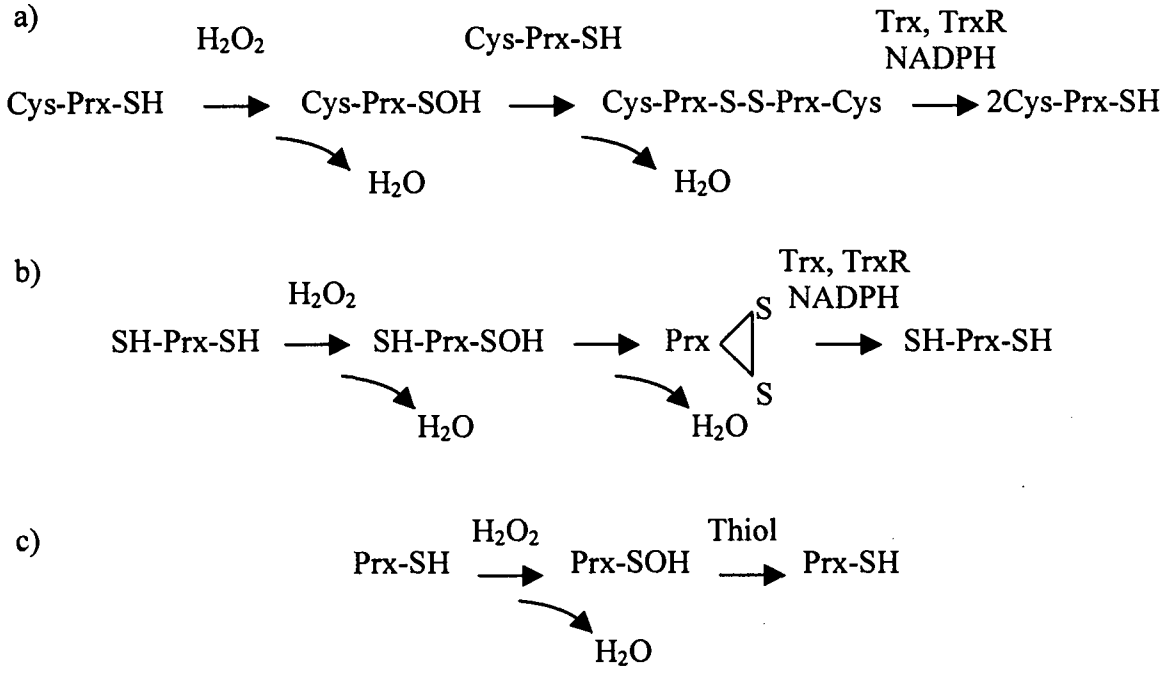
a)
H_2O_2
Cys-Prx-SH
Trx, TrxR
NADPH
Cys-Prx-SH
Cys-Prx-SOH
Cys-Prx-S-S-Prx-Cys
2Cys-Prx-SH
H_2O
H_2O
b)
Trx, TrxR
NADPH
H_2O_2
S
SH-Prx-SH
SH-Prx-SOH
Prx
SH-Prx-SH
S
H_2O
H_2O
c)
H_2O_2
Thiol
Prx-SH
Prx-SOH
Prx-SH
H_2O

Figure 1.5

Figure 1.5. The mechanisms of peroxidase reaction in three subgroups of Prxs: a) 2-Cys Prx, b) Atypical 2-Cys Prx, c) 1-Cys Prx.

1.8. Statement of research problem, rationale and objectives

Previous findings suggested that cell cycle CDKs and Cdk5 might play an important role in neurodegeneration paradigms like stroke. However, the relevance of such signals in neuronal injury was unknown because:

- This data was correlative in nature (showing that cell cycle CDK pathways or Cdk5 activity/levels were upregulated in neurons following stroke).
- The observations relied on general CDK pharmacological inhibitors, such as flavopiridol which are now known to also inhibit non-CDK related kinases such as GSK-3β.
- Lack of an adult model since most of the data was based on the observations carried on *in vitro*, using embryonic or postnatal neuronal cultures whose cell cycle signals have not been completely downregulated.

Accordingly, several important issues remained unanswered:

a) Which CDKs are both necessary and sufficient for ischemic death?

b) Do cell cycle CDKs and /or Cdk5 act differentially in ischemic models of excitotoxic versus more delayed apoptotic-like death?

c) By which mechanism do CDKs activation potentially regulate stroke-induced damage? In another word, what is (are) downstream target (s) for CDKs in stroke?

Consequently, this proposal is designed to examine above questions and to propose following objectives:

Objective 1. Determine whether cell cycle-related CDKs, Cdk2 and Cdk4, as well as non-cell cycle Cdk5, are critical in different models of ischemic death *in vitro* and *in vivo*.

Objective 2. Determine downstream target for cell cycle CDKs-mediated signal in stroke, *in vitro* and *in vivo*.

These two objectives have been addressed in Chapter 2, where viral expression of kinase-dead dominant negative mutants of the Cdk2, Cdk4 and Cdk5 have specifically inhibited their activities and have provided a powerful tool to explore the importance of individual CDKs in stroke models. In this chapter, for the first time, the functional significance of CDKs-mediated signals has been examined in adult models of stroke. Importantly, we have shown that distinct CDKs contribute in different mechanisms of ischemic death.

Objective 3. Determine how/where Cdk5 induces its pro-death signal in stroke models. This objective has been explored in Chapter 3 where we have demonstrated that the nature of Cdk5-mediated death signal is context-dependent. We have shown that multiple pathways depend upon cellular localization, regulate Cdk5-mediated death signals.

Taken together, this project was designed to elucidate pathogenic role of CDKs in stroke-mediated neuronal death, to identify CDKs downstream targets in this model and to evaluate whether inhibition of these signals would provide protection against the insult.

CHAPTER 2

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

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

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Statement of author contribution

This manuscript examines the role of Cdk2, Cdk4 and Cdk5 in different models of neuronal death induced by stroke, *in vitro* and *in vivo*. It also introduces retinoblastoma protein (Rb) as a target for Cdk4 in these models.

The experiments presented in this manuscript were predominantly carried out by J Rashidian, with assistance from G Iyirhiaro (co-first author) for *in vitro* part. All *in vivo* experiments, including viruses (AAV) injections, global ischemia induction, endothelin-1 injection, brain extractions, immunoblotting, immunohistochemistry, and behavioral analyses were performed by J Rashidian. The *in vitro* experiments performed with virally infected CGN cultures, as well as hypoxic excitotoxicity on p35 CGNs were carried out by J Rashidian. G Iyirhiaro performed experiments with cyclin D1 and DNCdk4 CGNs, as well as glutamate exposure on p35 CGNs. H Aleyasin generated DNCdk4 transgenic mice. P35 mice were provided by I. Vincent lab. All types of AV used in this research were prepared by S Callaghan. AAVs were made by MJ During and R Bland. M Rios (a former Master's student) trained me when I joined the lab. All figures and text for this manuscript were prepared by J Rashidian with guidance and editorial assistance from Dr. David Park.

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. Barabara Vanderhyden for production of transgenic mice, Dr. Jean-Pierre Julien for providing NSE 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.

Introdauction

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 ischemic death 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 noncyclin 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., 2000).

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 breedings 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 16–18 h in the presence of the NMDA blocker, MK801 (10 μM, 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 24 h after reoxygenation, then stained with Hoescht 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 5 h and then reoxygenated for 1 h. 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 50 μM for 70 min and then washed off with conditioned medium and incubated for 2 h. This was performed in the presence or absence of MK801 (10 μM). 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 80–100 g. 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. rAAV1 was diluted by mixing 2 μl of virus stock (10^{10} genomes per microliter) with 1 μl of 20% mannitol in PBS and was administered by a pump (Harvard infusion pump, Harvard Apparatus) into the hippocampus (from bregma: -3.6 mm anterioposterior, ± 2.1 mm lateral, -2.75 mm deep) or striatum (from bregma: +0.9 mm anterioposterior, +2.8 mm lateral, -5.8 mm deep)

over a 30-min period, as described (Wang et al., 2002).

Global ischemia model. Hippocampal rAAV1-injected rats, weighing 180–220 g, 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, and quantified, as described (Wang et al., 2002).

Focal ischemia model (Endothelin-1 injection). Striatal rAAV1-injected rats were subjected to endothelin injection. Endothelin-1 (400 pM; Calbiochem) was dissolved in 1 μl of H_2O and injected over a period of 3 min 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. Catherine's, ON, Canada) by using the method described by Swanson et al. (Swanson et al., 1990).

Immunohistochemistry. Coronal sections (14 μm) 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 cyclin D1 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 Cdk5 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, 1993a). 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 Cdk5 appears to play a minimal functional role under these conditions.

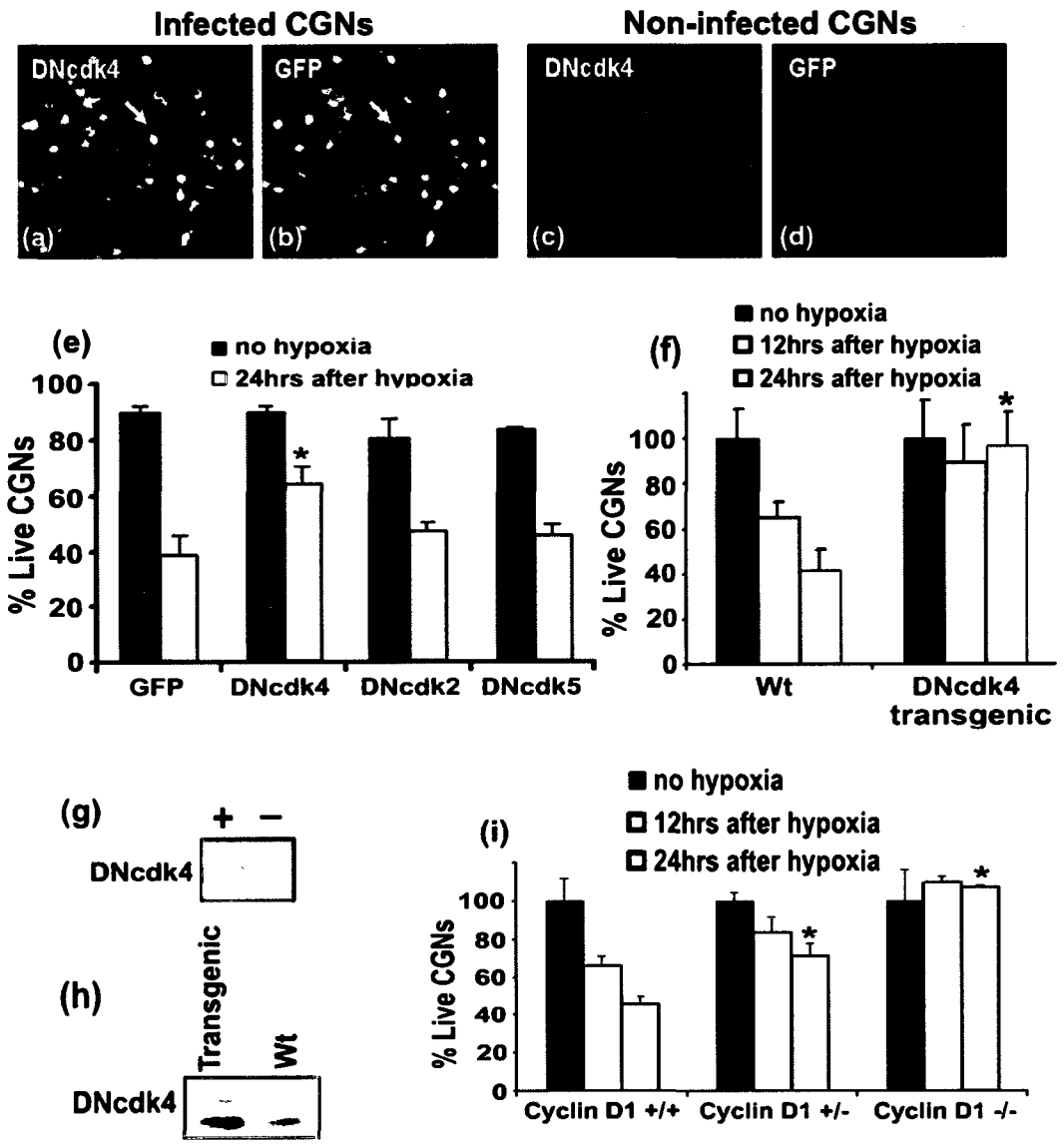

Infected CGNs
Non-infected CGNs
DNcdk4
GFP
DNcdk4
GFP
(a)
(b)
(c)
(d)
(e)
no hypoxia
24hrs after hypoxia
% Live CGNs
100
80
60
40
20
0
*
GFP
DNcdk4
DNcdk2
DNcdk5
(f)
no hypoxia
12hrs after hypoxia
24hrs after hypoxia
% Live CGNs
100
80
60
40
20
0
*
Wt
DNcdk4 transgenic
(g)
+
−
DNcdk4
(h)
Transgenic
Wt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

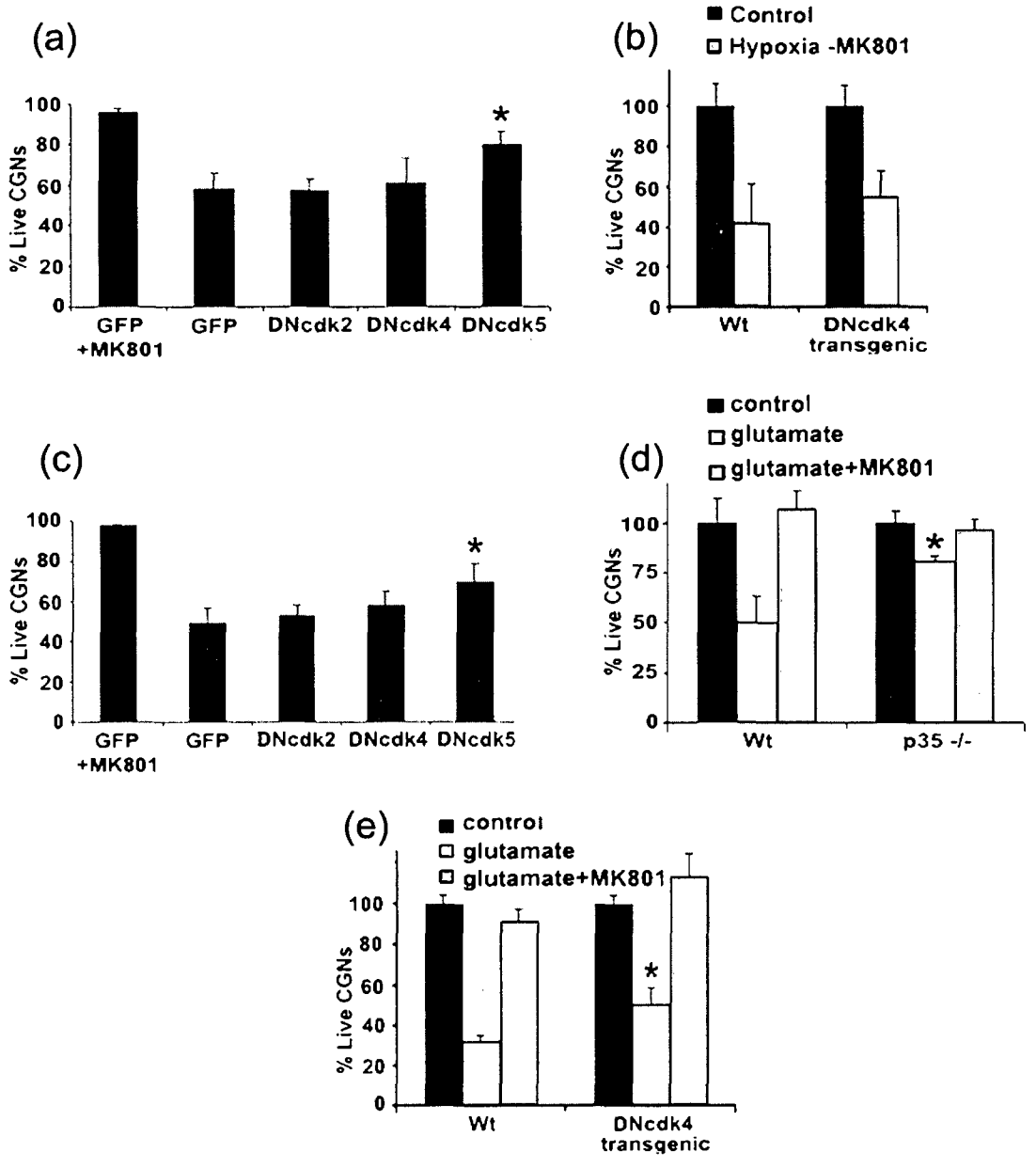

(a)
% Live CGNs
100
80
60
40
20
0
*
GFP +MK801
GFP
DNcdk2
DNcdk4
DNcdk5
(b)
Control
Hypoxia -MK801
% Live CGNs
100
80
60
40
20
0
Wt
DNcdk4 transgenic
(c)
% Live CGNs
100
80
60
40
20
0
*
GFP +MK801
GFP
DNcdk2
DNcdk4
DNcdk5
(d)
control
glutamate
glutamate+MK801
% Live CGNs
100
75
50
25
0
*
Wt
p35 -/-
(e)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

Figure 2.2

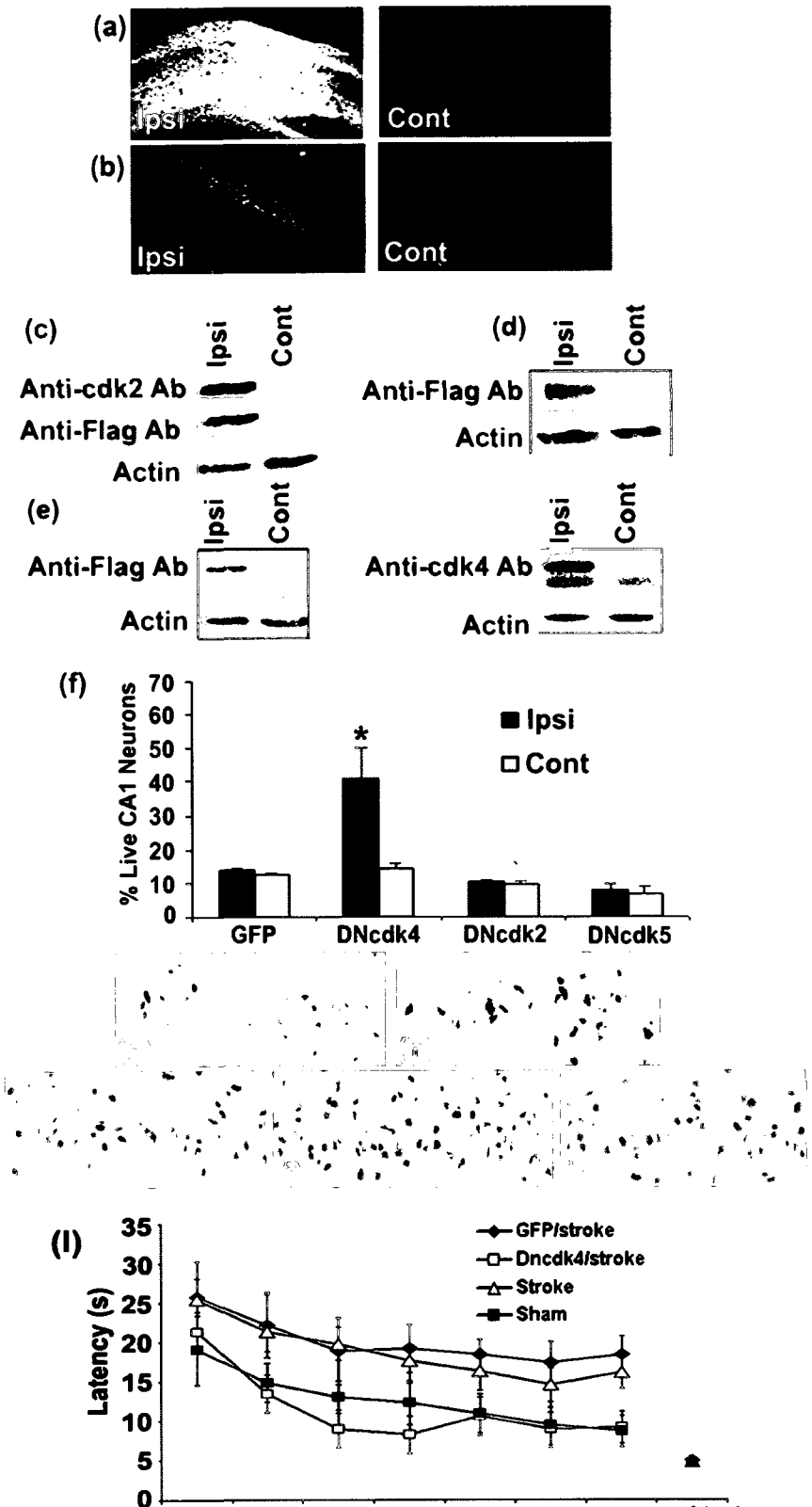
Figure 2.2. Cdk5/p35 is more involved in excitotoxic ischemia than Cdk4/cyclin D1. (a) AV-infected CGNs expressing DNCdk2/4/5 or GFP alone were subjected to 5-h hypoxia and 1- to 2-h reoxygenation in the absence of MK801 (n = 4). (b) CGNs from DNcdk4 transgenic mice or WT littermate controls subjected 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)
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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
(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

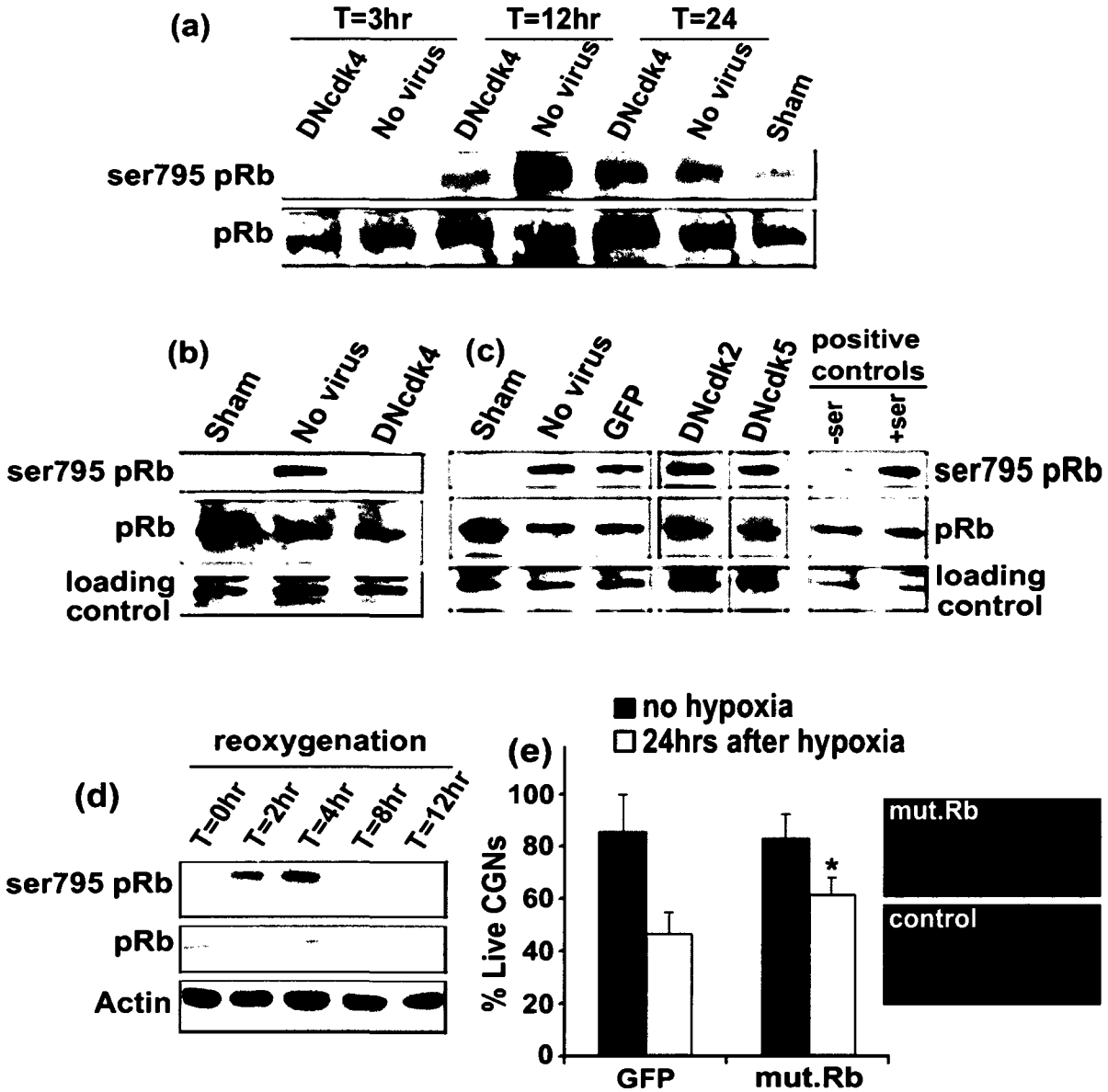
Figure 2.3. DNCdk4 expression but not DNCdk2 or -5 provides significant protection from 10-min 4VO-induced delayed neuronal death *in vivo*. (a–e) Expressions of flag-tagged-DNCDKs constructs, as well as GFP (Ipsi, ipsilateral injected hemisphere; Cont, contralateral noninjected hemisphere). Expression 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.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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

Fgure 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 nondamaged 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 (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.

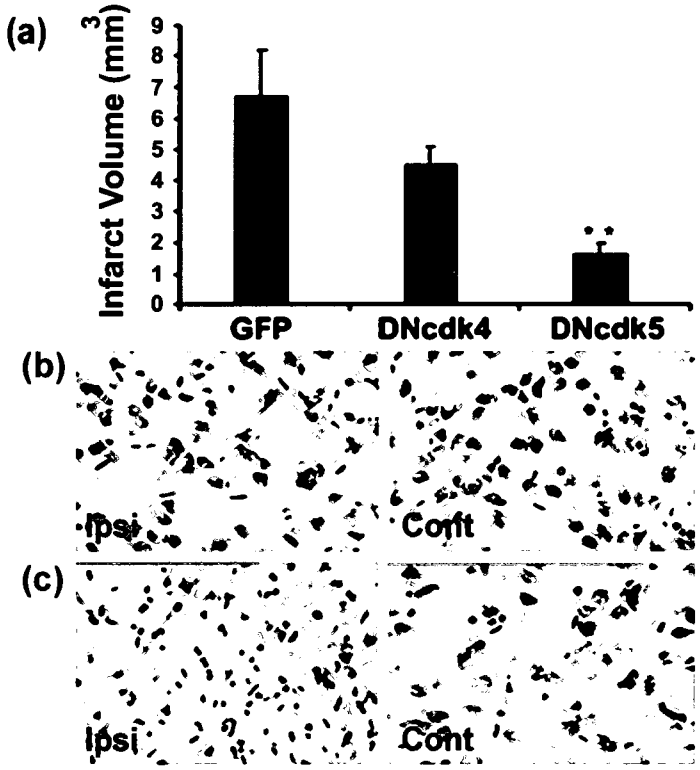
(a)
Infarct Volume (mm3)
9
8
7
6
5
4
3
2
1
0
GFP
DNcdk4
DNcdk5
(b)
Ipsi
Cont
(c)
Ipsi
Cont

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 blocks 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 (Giovanni et al., 2000). 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., 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.

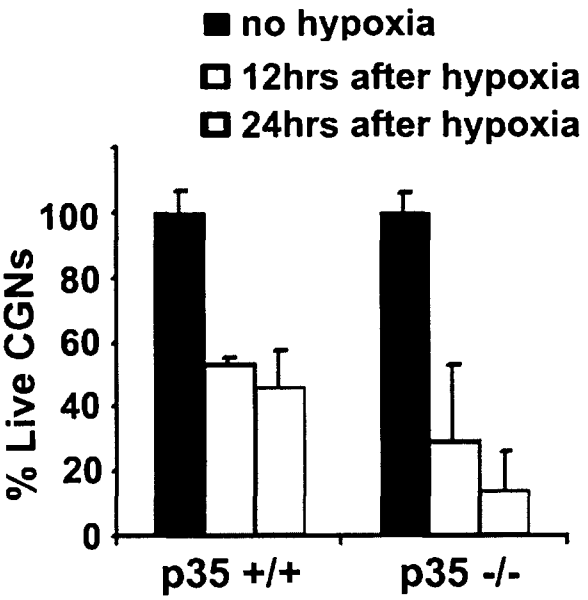
no hypoxia
12hrs after hypoxia
24hrs after hypoxia
% Live CGNs
100
80
60
40
20
0
p35 +/+
p35 -/-

Figure 2.6

Figure 2.6 (supporting information). CGNs from p35 deficient mice are not resistant to hypoxia in the presence of MK801 (n=3).

Refrences

Arundine M, Tymianski M (2003) Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. Cell Calcium 34:325-337.

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

Briones TL, Therrien B (2000) Behavioral effects of transient cerebral ischemia. Biol Res Nurs 1:276-286.

Connell-Crowley L, Harper JW, Goodrich DW (1997) Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. Mol Biol Cell 8:287-301.

Copani A, Uberti D, Sortino MA, Bruno V, Nicoletti F, Memo M (2001) Activation of cell-cycle-associated proteins in neuronal death: a mandatory or dispensable path? Trends Neurosci 24:25-31.

De Azevedo WF, Jr., Mueller-Dieckmann HJ, Schulze-Gahmen U, Worland PJ, Sausville E, Kim SH (1996) Structural basis for specificity and potency of a flavonoid inhibitor of human CDK2, a cell cycle kinase. Proc Natl Acad Sci U S A 93:2735-2740.

Dhavan R, Tsai LH (2001) A decade of CDK5. Nat Rev Mol Cell Biol 2:749-759.

Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 22:391-397.

Ekholm SV, Reed SI (2000) Regulation of G(1) cyclin-dependent kinases in the mammalian cell cycle. Curr Opin Cell Biol 12:676-684.

Giovanni A, Keramaris E, Morris EJ, Hou ST, O'Hare M, Dyson N, Robertson GS, Slack RS, Park DS (2000) E2F1 mediates death of B-amyloid-treated cortical neurons in a manner independent of p53 and dependent on Bax and caspase 3. J Biol Chem 275:11553-11560.

Gold MO, Rice AP (1998) Targeting of CDK8 to a promoter-proximal RNA element demonstrates catalysis-dependent activation of gene expression. Nucleic Acids Res 26:3784-3788.

Gong X, Tang X, Wiedmann M, Wang X, Peng J, Zheng D, Blair LA, Marshall J, Mao Z (2003) Cdk5-mediated inhibition of the protective effects of transcription factor MEF2 in neurotoxicity-induced apoptosis. Neuron 38:33-46.

Hallows JL, Chen K, DePinho RA, Vincent I (2003) Decreased cyclin-dependent kinase 5 (cdk5) activity is accompanied by redistribution of cdk5 and cytoskeletal proteins and increased cytoskeletal protein phosphorylation in p35 null mice. J Neurosci 23:10633-10644.

He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B (1998) A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A 95:2509-2514.

Katchanov J, Harms C, Gertz K, Hauck L, Waeber C, Hirt L, Priller J, von Harsdorf R, Bruck W, Hortnagl H, Dirnagl U, Bhide PG, Endres M (2001) Mild cerebral ischemia induces loss of cyclin-dependent kinase inhibitors and activation of cell cycle machinery before delayed neuronal cell death. J Neurosci 21:5045-5053.

Leclerc S, Garnier M, Hoessel R, Marko D, Bibb JA, Snyder GL, Greengard P, Biernat J, Wu YZ, Mandelkow EM, Eisenbrand G, Meijer L (2001) Indirubins inhibit

glycogen synthase kinase-3 beta and CDK5/p25, two protein kinases involved in abnormal tau phosphorylation in Alzheimer's disease. A property common to most cyclin-dependent kinase inhibitors? J Biol Chem 276:251-260.

Liu DX, Greene LA (2001) Regulation of neuronal survival and death by E2F-dependent gene repression and derepression. Neuron 32:425-438.

MacManus JP, Jian M, Preston E, Rasquinha I, Webster J, Zurakowski B (2003) Absence of the transcription factor E2F1 attenuates brain injury and improves behavior after focal ischemia in mice. J Cereb Blood Flow Metab 23:1020-1028.

Markgraf CG, Velayo NL, Johnson MP, McCarty DR, Medhi S, Koehl JR, Chmielewski PA, Linnik MD (1998) Six-hour window of opportunity for calpain inhibition in focal cerebral ischemia in rats. Stroke 29:152-158.

Morris EJ, Dyson NJ (2001) Retinoblastoma protein partners. Adv Cancer Res 82:1-54.

Murase K, Kato H, Kogure K (1993) Limited but evident protective effects of MK-801 and pentobarbital on neuronal damage following forebrain ischemia in the gerbil under normothermic conditions. Neurosci Lett 149:229-232.

O'Hare MJ, Hou ST, Morris EJ, Cregan SP, Xu Q, Slack RS, Park DS (2000) Induction and modulation of cerebellar granule neuron death by E2F-1. J Biol Chem 275:25358-25364.

Osuga H, Osuga S, Wang F, Fetni R, Hogan MJ, Slack RS, Hakim AM, Ikeda JE, Park DS (2000) Cyclin-dependent kinases as a therapeutic target for stroke. Proc Natl Acad Sci U S A 97:10254-10259.

Park DS, Levine B, Ferrari G, Greene LA (1997) Cyclin dependent kinase inhibitors and dominant negative cyclin dependent kinase 4 and 6 promote survival of NGF-deprived sympathetic neurons. J Neurosci 17:8975-8983.

Park DS, Morris EJ, Bremner R, Keramaris E, Padmanabhan J, Rosenbaum M, Shelanski ML, Geller HM, Greene LA (2000) Involvement of retinoblastoma family members and E2F/DP complexes in the death of neurons evoked by DNA damage. J Neurosci 20:3104-3114.

Pines J (1993a) Cyclins and their associated cyclin-dependent kinases in the human cell cycle. Biochem Soc Trans 21:921-925.

Smith PD, Crocker SJ, Jackson-Lewis V, Jordan-Sciutto KL, Hayley S, Mount MP, O'Hare MJ, Callaghan S, Slack RS, Przedborski S, Anisman H, Park DS (2003) Cyclin-dependent kinase 5 is a mediator of dopaminergic neuron loss in a mouse model of Parkinson's disease. Proc Natl Acad Sci U S A 100:13650-13655.

Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR (1990) A semiautomated method for measuring brain infarct volume. J Cereb Blood Flow Metab 10:290-293.

Timsit S, Rivera S, Ouaghi P, Guischard F, Tremblay E, Ben-Ari Y, Khrestchatisky M (1999) Increased cyclin D1 in vulnerable neurons in the hippocampus after ischaemia and epilepsy: a modulator of in vivo programmed cell death? Eur J Neurosci 11:263-278.

van den Heuvel S, Harlow E (1993) Distinct roles for cyclin-dependent kinases in cell cycle control. Science 262:2050-2054.

Wang F, Corbett D, Osuga H, Osuga S, Ikeda JE, Slack RS, Hogan MJ, Hakim AM, Park DS (2002) Inhibition of cyclin-dependent kinases improves CA1 neuronal survival and behavioral performance after global ischemia in the rat. J Cereb Blood Flow Metab 22:171-182.

Wang J, Liu S, Fu Y, Wang JH, Lu Y (2003) Cdk5 activation induces hippocampal CA1 cell death by directly phosphorylating NMDA receptors. Nat Neurosci 6:1039-1047.

Zolotukhin S, Potter M, Zolotukhin I, Sakai Y, Loiler S, Fraites TJ, Jr., Chiodo VA, Phillipsberg T, Muzyczka N, Hauswirth WW, Flotte TR, Byrne BJ, Snyder RO (2002) Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. Methods 28:158-167.

CHAPTER 3

Differential roles of nuclear and cytoplasmic cdk5 following stroke: targeting of Prx2 in ischemic injury

Submitted (2008)

Rashidian J, Rousseaux MW, Venderova K, Qu D, Callaghan SM, Phillips M, Bland RJ, During MJ, Mao Z, Slack RS, Park DS

Statement of author contribution

This manuscript determines differential roles of Cdk5 in two models of focal and global ischemia. It also introduces Prx2 and MEF2D as targets for Cdk5 in these models.

The experiments presented in this manuscript were mainly carried out by J Rashidian, with assistance from M Phillips (a summer student) and MW Rousseaux (a Ph.D student). All *in vitro* and most of the *in vivo* experiments, including viruses (AAV) injections, global ischemia induction, endothelin-1 injection, brain extractions and immunoblotting were performed by J Rashidian. M Phillips assisted with cutting some of the rat brains and survival counting. MW Rousseaux performed a part of the experiments done with the p35 mice, including induction of focal ischemia and analysis of the brains for pospho-Prx2. K Venderova (a post doctoral fellow) took care of the p35 mice colony. D Qu (a post doctoral fellow) provided a number of reagents including all forms of Prx2 constructs, which were used to generate AV and AVV, as well as anti phospho-Prx2 antibody. He also supported the project with his advices. Z Mao provided Ser444MEF2D construct, which was used to generate AV, as well as anti phospho-MEF2D antibody. All types of AV used in this research were prepared by S Callaghan. AAVs were made by MJ During and R Bland. All figures (except the figure and graph demonstrating phospho-Prx2 in p35 mice) and text for this manuscript were prepared by J Rashidian with guidance and editorial supervision from Dr. David Park.

Differential roles of nuclear and cytoplasmic Cdk5 following stroke: targeting of Prx2 in ischemic injury

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Key words: Cyclin-dependent kinases, Stroke, Neuronal death

Acknowledgements:

We thank Dr.Inez Vincent for providing P35 deficient mice. 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 Stroke Recovery (DSP) and National Institutes of Health.

Abstract

Recent evidence suggests that abnormal activation of cyclin-dependent kinase 5 (Cdk5) is a critical pro-death signal in models of stroke. However, the mechanism(s) by which this occurs is unclear. Complicating the role of Cdk5 is the growing observations that Cdk5 can exist in multiple cellular regions and possess both pro-survival or pro-death characteristics. Accordingly, we determined where cdk5 was activated in models of ischemia and how manipulation of Cdk5 in differing compartments may affect neuronal death. Here, we show a critical function for cytoplasmic cdk5 in both focal and global models of stroke *in vivo*. Cdk5 is activated in the cytoplasm and expression of DNCdk5 localized to the cytoplasm is protective. Importantly, we also demonstrate the antioxidant enzyme Prx2 as a critical cytoplasmic target of Cdk5. In contrast, the potential role of Cdk5 in the nucleus is context-dependent. In models of focal ischemia, Cdk5 is activated and functionally relevant while there is no evidence for activation following global ischemia. Importantly, MEF2D, a previously described nuclear target of Cdk5 *in vitro* is also phosphorylated by Cdk5. However, MEF2D expression does not block death suggesting the presence of other relevant nuclear targets for Cdk5. Taken together our results indicate the existence of multiple pathways which regulate Cdk5-mediated death in multiple cell compartments depending upon context.

Introduction

Stroke results from a transient or permanent reduction in blood flow to the brain. The mechanisms involved in ischemic neuronal death are not fully defined. In this regard, accumulating evidence has associated cyclin-dependent kinases (CDKs) to stroke damage (Rashidian et al., 2007). CDKs are best recognized for their role in regulating cell cycle progression (Pines, 1993b). However, they are now noted for their roles in other biological processes (Gold and Rice, 1998). Specifically, some specialized members are predominately active in the nervous system. Cdk5, a Pro directed Ser/Thr kinase, is one such member. Cdk5 does not utilize cyclins to promote activation. Instead, it utilizes p35 and p39 which are predominantly expressed in CNS (Dhavan and Tsai, 2001). Moreover, in contrast to other CDKs, there is no functional evidence for involvement of Cdk5 in classical cell cycle regulation. Instead, cdk5 is considered a key element in neuronal development and function (Dhavan and Tsai, 2001; Li et al., 2001; Johansson et al., 2005). Cdk5 is also believed to be critical for neuronal survival following exposure to apoptotic stimuli (Li et al., 2002; Li et al., 2003; Wang et al., 2006).

Interestingly, in contrast to above evidence implicating the role of Cdk5 in survival/development, there is increasing evidence suggesting pathogenic role of Cdk5 in neurodegenerative disorders such as Parkinson's disease (PD) (Smith et al., 2003a), Alzheimer's disease (AD) (Cruz and Tsai, 2004), amyotrophic lateral sclerosis (ALS) (Nguyen and Julien, 2003) , Niemann-Pick type C (NPC) (Bu et al., 2002) and stroke (Wang et al., 2003; Rashidian et al., 2005). The pathogenic role of Cdk5 is proposed to be unmasked by calpain-mediated cleavage of the p35 activator to a more stable active p25 form which is mislocalized to the nucleus under some neurotoxic conditions (Patrick

et al., 1999; Kusakawa et al., 2000; O'Hare et al., 2005). P35 contains a myristoylation motif and associates with membranous compartments of the cell, while p25, lacks this motif and localizes Cdk5 to soluble fractions (Kusakawa et al., 2000). Moreover, several studies have demonstrated that Cdk5 can be both nuclear and cytoplasmic (Ino and Chiba, 1996; O'Hare et al., 2005; Fu et al., 2006). Recent evidence suggests that cell localization might be important for its pro-survival/pro-death role. We have recently shown that inhibition of cytoplasmic Cdk5 sensitizes neurons to DNA damage, suggesting a pro-survival role of cytoplasmic Cdk5 under normal conditions (O'Hare et al., 2005). In contrast, nuclear Cdk5, in an *in vitro* model of excitotoxicity is death promoting, through inhibition of the survival properties of MEF2D (O'Hare et al., 2005; Tang et al., 2005). This would suggest that cytoplasmic activity is normal while nuclear activity is pathogenic. However, this simplistic view is likely incorrect. For example, Cdk5 can phosphorylate tau in the cytoplasm, a presumptive pro-death stress (Cruz and Tsai, 2004) . These observations suggest that Cdk5 activity is much more complex. Moreover, the role of Cdk5 localization in adult models of injury, particularly in stroke is unclear. Understanding of where and how Cdk5 is activated and contributes to pathogenesis is critical, particularly in light of its pro-survival function(s).

We have previously shown that Cdk5 is critical for ischemia-induced death *in vivo* and *in vitro* (Rashidian et al., 2005). However, the mechanisms by which Cdk5 promotes death, its localization, or its downstream targets (cytoplasmic or nuclear) following stroke are unknown. Here, we provide evidence that Cdk5 can promote death in different ways. Cytoplasmic Cdk5 activity is essential for death in multiple paradigms of ischemia (focal and global) *in vivo*. Importantly, we also identify the peroxidase, Prx2

as a common critical cytoplasmic target in this pathway. The role of nuclear Cdk5 activity, however, is more context-dependent and is only relevant following focal ischemia.

Materials and Methods

Animals. All animal experiments were approved by the University of Ottawa Animal Care Committee and conformed to the guidelines set forth by the Animal Care Council of Canada and Canadian Institutes of Health Research.

Viral construction. Recombinant adeno-associated virus (AAV) vectors were constructed by subcloning cDNA sequences of DNCdk5-Flag (Gong et al., 2003; Smith et al., 2003a), WtCdk5-NLS(NES)-GFP, DNCdk5-NLS(NES)-GFP (O'Hare et al., 2005) and Prx2-Flag and its mutants (T89A, T89E) in the AM/CBA-pl-WPRE-bGH plasmid. Then viruses were generated as previously described (Zolotukhin et al., 2002). The adenoviruses (AV) expressing Prx2, Prx2T89A, Prx2T89E and MEF2D-S444, which expressed GFP by a separate promoter were engineered as described before (He et al., 1998).

P35 knock out Mice. P35-/- mice were generated by breeding heterozygote p35 mutants and genotyped as described before (Hallows et al., 2003).

Cell culture. CGN cultures were prepared from 7-9 day postnatal mice as previously described (Rashidian et al., 2005).

Glutamate model. 5-day plated CGNs were infected with AV, as described before (Rashidian et al., 2005). On day 7, cultures were exposed to 50μM glutamate for 60min, in the presence or absence of MK801, and then washed and incubated for 2h. Survival was evaluated as described previously (Rashidian et al., 2005).

Viral injection. Male Wistar rats weighting 80-100 g were injected with AAV (3μl; $2x10^{10}$ genomes in 20%mannitol) 2 weeks prior to induction of ischemia. AAV was administered into the hippocampus (from bregma: -3.6mm anterioposterior, +2.1mm

lateral, -2.75mm deep; in 4VO model) or striatum (from bregma: +0.9mm anteriorposterior, +2.8mm lateral, -5.8mm deep; in endothelin model) as described before (Rashidian et al., 2005). AV were delivered into striatum (3μl; 10^7 particles/μl), using above coordinates one week prior to ischemia as has described (Smith et al., 2003a).

Global ischemia. 5-min 4 vessels occlusion (4VO) was induced as described before (Rashidian et al., 2005) and brains were collected and stained for hematoxylin and eosin 4 days after surgery. Cell counts from the left and right hippocampi were averaged and expressed as counts/mm for CA1.

Focal ischemia. 400ρM/μl endothelin-1 (Calbiochem) was injected into striatum (1μl in rats, 2x1μl in mice) as described before (Rashidian et al., 2005). Brains were collected 4 days after injection and 14μm-coronal sections of striatum were stained with cresyl violet (CV). The infarct volume was measured using the method described (Swanson et al., 1990).

Immunohistochemistry. 14μm-coronal sections at the level of striatum were collected as freefloating in 0.01MPBS. Sections were permeablized and blocked in buffer A (50mM Tris, pH7.5+100mM NaCl+0.3%TritonX-100) plus 3%BSA overnight. Next, sections were washed in buffer A and incubated in anti-p89Prdx2 Ab (1:100) coincubated with a blocking peptide for specificity at 4°C overnight. Then slides were visualized using Alexa Fluor 488 anti-rabbit IgG (1:2000; Invitrogen).

Western blot analyses. To detect expression of Prx2 and its variants, MEF2D and pS444MEF2D in brain a 2-mm punch was obtained and analyzed by western blot using anti-Prdx2 (1:500), anti-pT89Prx2 (1:500) (Qu et al., 2007), anti-MEF2D (1:2000, BD

Transduction Lab) and anti-pS444MEF2D (1:500) (Gong et al., 2003) Abs. B-actin (Sigma) was used for loading control.

Cdk5 kinase assay. 100μg proteins from hippocampus or striatum were incubated overnight with anti-Cdk5 Ab (2μg of Ab/sample; Santa Cruz). 30μl of 50% slurry of Protein A-Sepharose (Sigma) were then added to immunoprecipitates, and samples were incubated for 2h at 4°C. The immune complexes were then pelleted and washed in PBS and incubated with 2μg of histone H1 and 1μCi [^{32}P] ATP at 30°C for 30min. Kinase activity was determined by SDS-PAGE and autoradiography.

Extraction of nuclear and cytoplasmic proteins. Hippocampal CA1 or striatal proteins were obtained using a 2mm punch and nuclear and cytoplamic proteins were extracted as described before (Wang et al., 2002).

Results

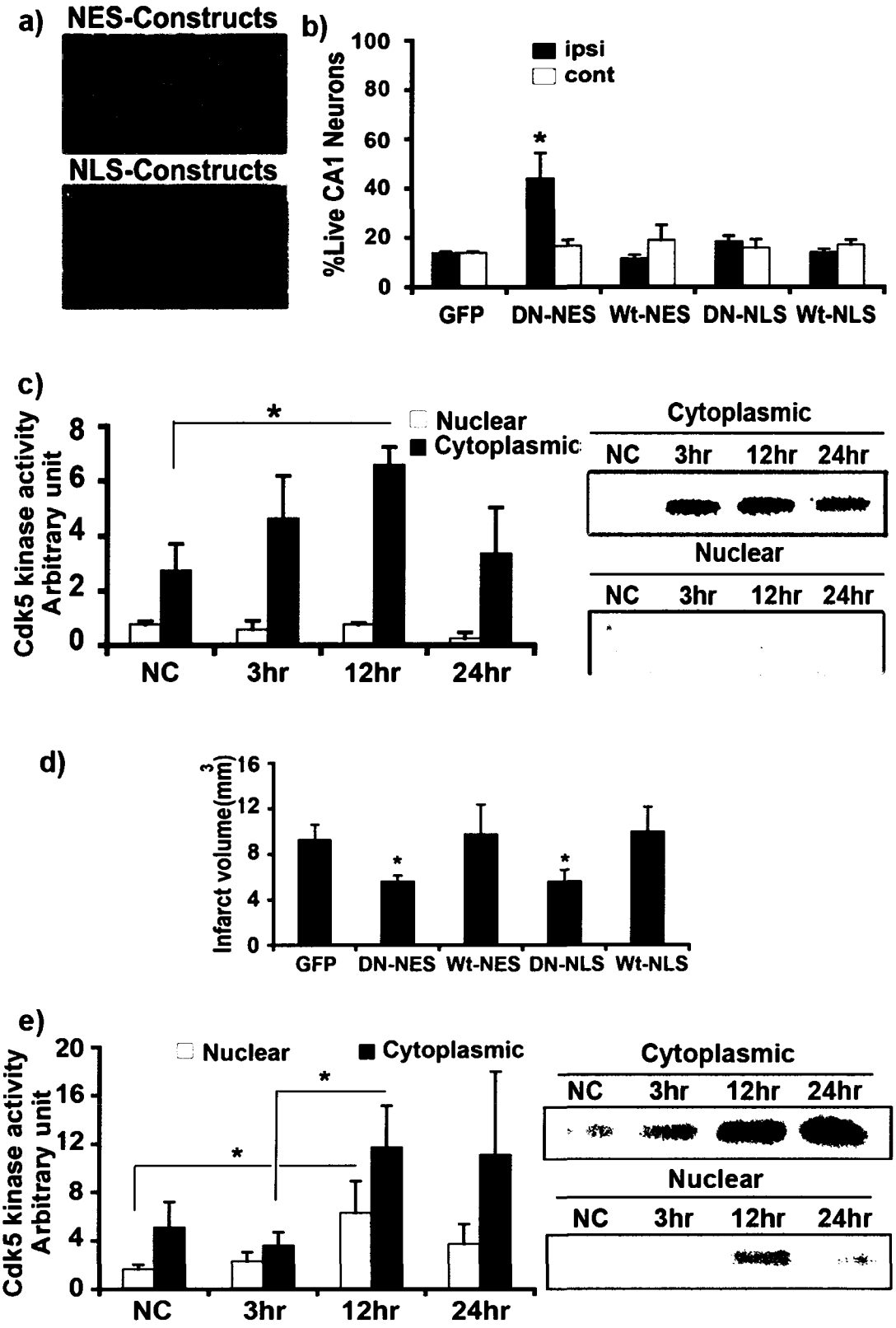
Differential role of cytoplasmic and nuclear Cdk5 in multiple models of stroke

We have previously provided evidence that Cdk5 plays an essential role following both 5-min global ischemia and in focal models of stroke induced by exposure to the vasoconstrictor endothelin (Rashidian et al., 2005) (Figure 3.6; supporting information). However, the mechanism(s) by which Cdk5 might promote ischemic death is unknown. Recent evidence, at least in culture models, suggests that cell localization might play an important role for Cdk5 as a pro-survival/pro-death mediator. Accordingly, as a first step in deciphering the mechanism by which Cdk5 promotes death *in vivo*, we examined whether nuclear or cytoplasmic Cdk5 was essential for death. To determine this, we constructed and utilized recombinant adenoviral associated vectors (AAV) expressing a wild type (Wt) or kinase dead dominant negative (DN) mutant of Cdk5 fused to GFP (for visualization) as well as either a nuclear exclusion signal (NES) for expression excusively in the cytoplasm or a nuclear localization signal (NLS) for targeting exclusively to the nucleus. We have shown previously that these fusion constructs target exclusively to either cytoplasm or nucleus (O'Hare et al., 2005). For the global model, recombinant AAV vectors carrying these constructs or GFP, as control, were injected unilaterally into hippocampus 2 weeks prior to induction of 5min-4VO. Expression of constructs was confirmed by visualizing GFP (Figure 3.1a). Survival of CA1 neurons was assessed 4 days after 4VO. Neuronal counts for the CA1 region showed significant increase in survival in the DNCdk5-NES-injected side compared with non-injected side (44% survival vs 16%; Figure 3.1b). In comparison, overexpression of all other constructs, including DNCdk5-NLS, did not result in any significant survival (Figure 3.1b). These

results suggest us that activation of cytoplasmic, but not nuclear, Cdk5 leads to neuronal death following 4VO. To confirm that cytoplasmic Cdk5 activation may be more critical in transmitting the death signal, we assessed Cdk5 kinase activity in both the cytoplasm and nucleus after 4VO. Cdk5 was immunoprecipitated from nuclear and cytoplasmic extracts of CA1 at different time points following 4VO and was then subjected to *in vitro* kinase assay using histone H1 as substrate. As Figure 3.1c indicates, Cdk5 activity was dramatically increased 12hs after reperfusion (2.5 folds) in cytoplasm, while nuclear samples did not show significant increase in activity. This suggesting that abnormal activation of cytoplasmic Cdk5 has an essential role in neuronal death in 4VO.

The above results were quite surprising to us given previous results from our lab (O'Hare et al., 2005), as well as others (Gong et al., 2003) that, at least in *in vitro* cultured neurons, nuclear Cdk5 is critical for excitoxic death. This suggested that perhaps, the role of Cdk5 *in vivo* in the adult may differ from that of immature neurons grown in the dish. However, to confirm this, we also performed similar protection experiments as described above in another adult model, a focal model induced by endothelin-1. Recombinant AAV vectors carrying the DN or Wt constructs described above or GFP, as control, were injected into the striatum 2 weeks prior to injection of endothelin-1. Regions of striatum with infarct were distinguished by CV staining 4 days after injection, as described before (Rashidian et al., 2005). In contrast to the 4VO model, there was a very significant decrease (40%) in damage in both DNCdk5-NLS and DNCdk5-NES expressing brains, compared to GFP-expressing ones (Figure 3.1d). Overexpression of WtCdk5-NES in cytoplasm or WtCdk5-NLS in nucleus, however, was not protective (Figure 3.1d). This indicates that the participation of nuclear and

cytoplasmic Cdk5 differs in various models of stroke. In a focal model, both nuclear and cytoplasmic Cdk5 signal for death in excitotoxic type of death. We also assessed Cdk5 kinase activity in the cytoplasm and nucleus after injection of endothelin-1 similar to that performed after global insult. As Figure 3.1e indicates, Cdk5 activity dramatically increased 12hs after injection compare to negative controls in both cytoplasm and nucleus. These results suggest that both nuclear and cytoplasmic Cdk5 are likely mediators of excitotoxic neuronal death in focal models of ischemia where death is likely more rapid. However, in the global model where death is more delayed, cytoplasmic Cdk5 appears to be the sole mediator of death.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NES-Constructs
NLS-Constructs
b)
%Live CA1 Neurons
100
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60
40
20
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ipsi
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GFP
DN-NES
Wt-NES
DN-NLS
Wt-NLS
c)
Cdk5 kinase activity
Arbitrary unit
8
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2
0
*
Nuclear
Cytoplasmic
NC
3hr
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NC 3hr 12hr 24hr
Nuclear
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d)
Infarct volume(mm3)
16
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GFP
DN-NES
Wt-NES
DN-NLS
Wt-NLS
e)
Cdk5 kinase activity
Arbitrary unit
20
16
12
8
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Nuclear
Cytoplasmic
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NC
3hr
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Cytoplasmic
NC 3hr 12hr 24hr
Nuclear
NC 3hr 12hr 24hr

Figure 3.1

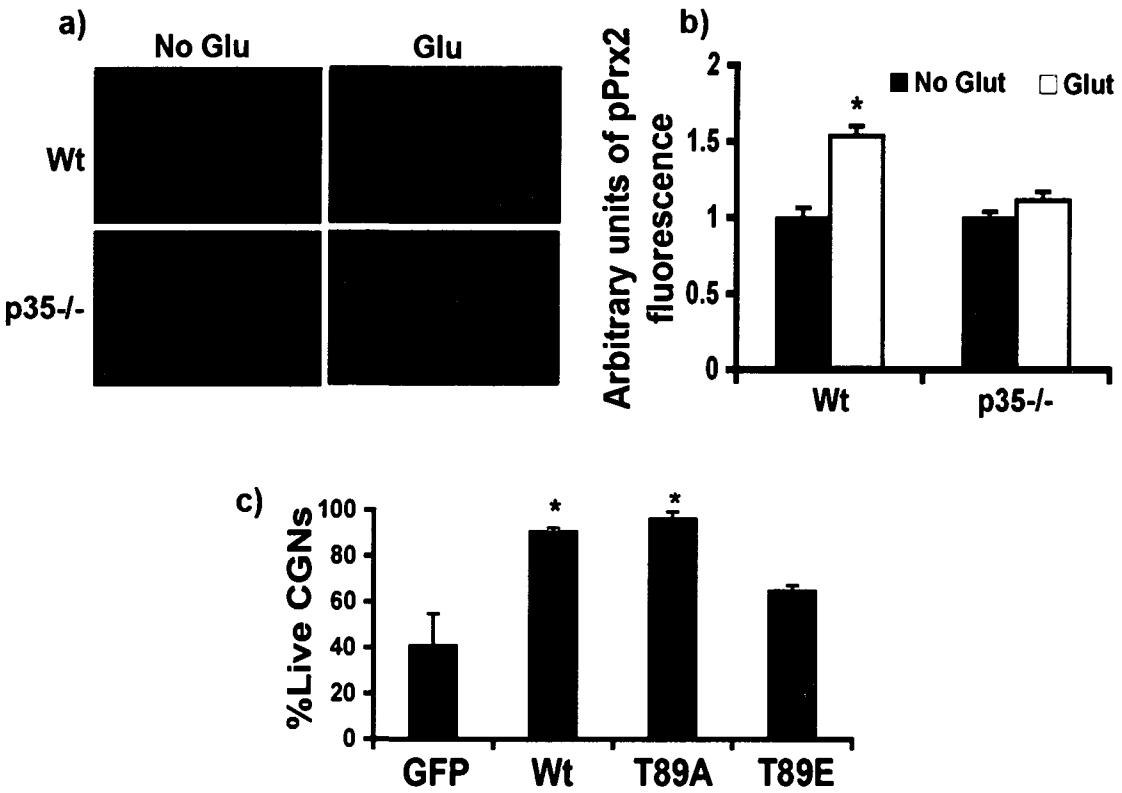
Figure 3.1. Cytoplasmic Cdk5 is mediator of neuronal death following global ischemia, while in focal ischemia both nuclear and cytoplasmic Cdk5 signal death. (a) Expression of GFP-fused DN(Wt)Cdk5-NLS in the nuclei and DN(Wt)Cdk5-NES in the cytoplasm of neurons by AAV in brains was detected using GFP fluorescence. (b) Quantification of surviving CA1 neurons expressing GFP (n=3), DNCdk5-NES (n=5), WtCdk5-NES (n=5), DNCdk5-NLS (n=5) and WtCdk5-NLS (n=5) 4 days after 4VO. (c) Cdk5 kinase assay on nuclear and cytoplasmic proteins from hippocampus using histone H1 as substrate at the different time points after 4VO. The graph presents densitometry values from n=4 experiments. (d) Infarct volume of focal ischemic brains expressing GFP (n=7), DNCdk5-NES (n=7), WtCdk5-NES (n=8), DNCdk5-NLS (n=7) and WtCdk5-NLS (n=7) 4 days after injection of endothelin-1. (e) Cdk5 kinase assay on nuclear and cytoplasmic proteins from striatum using histone H1 as substrate at the time courses after injection of endothelin-1. The graph presents densitometry values from n=5 experiments. "NC" represents non-stroked control animals; ipsi is injected and cont is non-injected side. Data is presented as mean ±SEM (*ttest, $p<0.05$).

Prx2 is a substrate for cytoplasmic Cdk5 in ischemic death

Our results above indicate that in both focal and global models of ischemia, cytoplasmic Cdk5 is a relevant death modulator. We next determined what cytoplasmic target for Cdk5 might be important in ischemic damage. We have recently reported that peroxiredoxin 2 (Prx2), a cytoplasmic peroxidase, is a Cdk5 target (Qu et al., 2007). This antioxidant enzyme is phosphorylated at Thr89 and inactivated by Ccdk5. Therefore, we examined whether Prx2 may act as a downstream target for cytoplasmic Cdk5.

To do this, we first examined the importance of Prx2 in *in vitro* model of death induced by glutamate. We have previously reported that Cdk5 is essential for excitotoxic neuronal death induced by glutamate in CGNs (Rashidian et al., 2005). Therefore, we were interested to determine whether Prx2 and its phosphorylation may play a role in this model. We first evaluated the effect of glutamate on Thr89 phosphorylation utilizing the phospho-specific Ab p-T89 in CGN cultures from p35-/- mice. The increase in pT89Prx2 was observed in cultures by immunofluorescence and was quantified over a number of neurons by image analyses (Figure 3.2a). Quantification indicated a maximum of 50% increase in pT89Prx2 signal in glutamate treated Wt CGNs compare to non-treated controls. In contrast, p35-/- cultures showed only 10% statistically non-significant increase upon glutamate treatment compare to controls (Figure 3.2b). These results indicate that Prx2 is phosphorylated by Cdk5 upon glutamate exposure. To determine whether this phosphorylation is relevant in this paradigm, we infected CGNs with AV expressing WtPrx2, a mutant of Prx2 which cannot be phosphorylated at the Thr89 (Prx2T89A), or a phospho-mimicing form which we have shown to be less active (Prx2T89E), prior to glutamate treatment. As shown in Figure 3.2c, neurons exposed to

glutamate expressing WtPrx2 and Prx2T89A showed about 90% survival vs. 40% survival in GFP-expressing controls. Expression of Prx2T89E showed dramatically less protection than the other Prx2 constructs. Together, this data suggests that phosphorylation of Prx2 on Thr89 by Cdk5 in glutamate model triggers death pathways in CGN cultures.


a)
No Glu
Glu
Wt
p35-/-
b)
Arbitrary units of pPrx2 fluorescence
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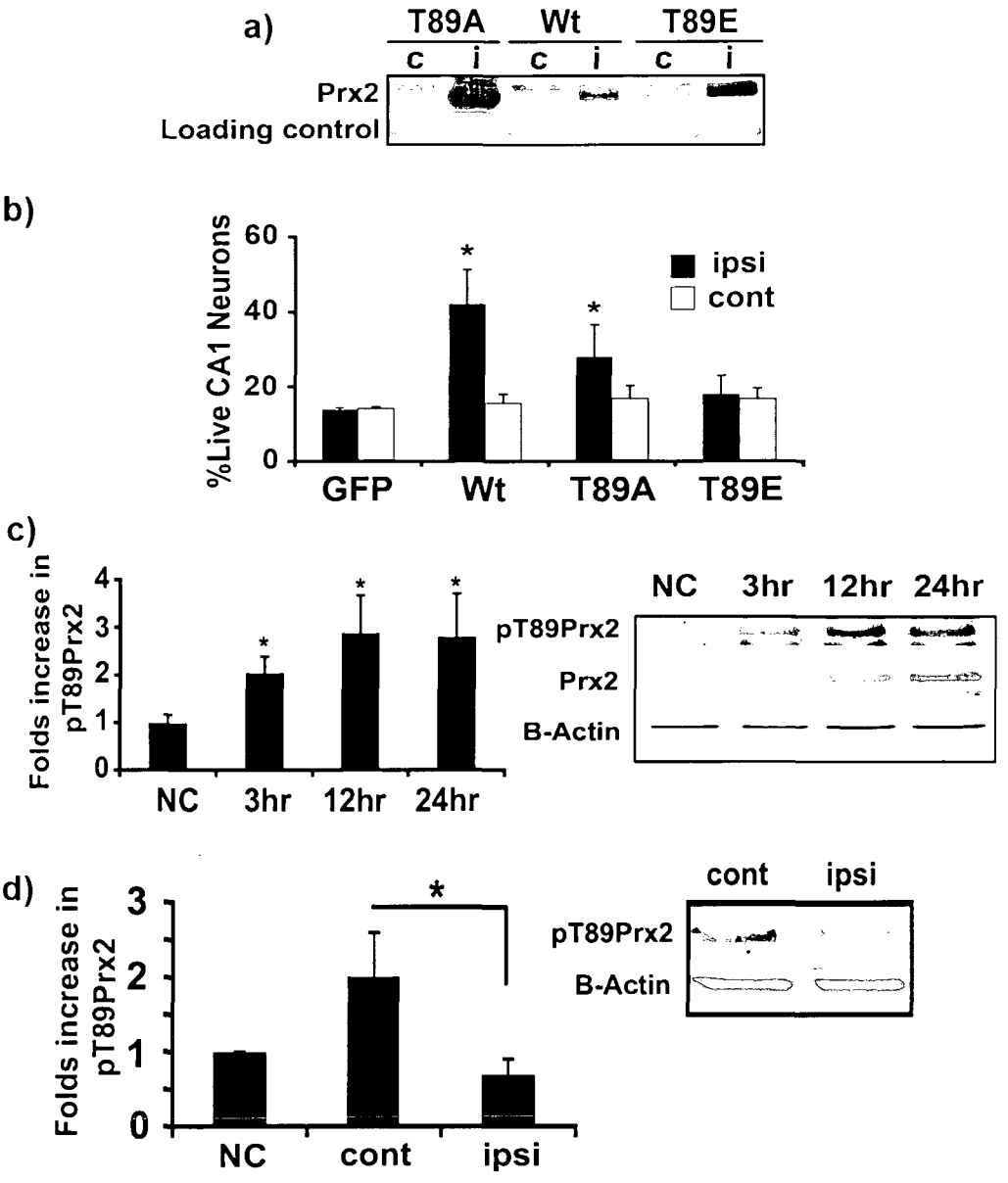
1.5
1
0.5
0
No Glut
Glut
*
Wt
p35-/-
c)
%Live CGNs
100
80
60
40
20
0
*
*
GFP
Wt
T89A
T89E

Figure 3.2

Figure 3.2. Prx2 is phosphorylated at Thr89 by Cdk5 in glutamate model and causes neuronal death. (a) Phosphorylation of Prx2 is attenuated in p35 deficient CGNs compare to cultures from wild type (Wt) littermates following glutamate excitotoxicity. Cultures were fixed with 4% PFA after glutamate exposure and immunostained for pT89Prx2. (b) Quantification of surviving CGNs after glutamate exposure. CGNs from each mouse (Wt n=7; p35-/- n=4) were plated on 3 wells and intensity of fluorescent signal in soma of neurons was measured by image analyses from 60 neurons in 4 random fields/well. (c) Overexpression of Prx2 protects CGNs against glutamate. 5-day plated CGNs were infected with AV expressing Wt, phosphorylation resistant (T89A) and phospho-mimicing (T89E) mutants of Prx2 or only GFP as control and exposed to glutamate 2 days after infection. Fixed cultures were then counted for live GFP expressing neurons. Graph is representative of n=3 experiments. Data is presented as mean ±SEM (*ttest, $p<0.05$).

To confirm this *in vivo*, we next examined whether Prx2 may be important in the global model of ischemia. We generated AAV expressing WtPrx2, Prx2T89A, and Prx2T89E, and targeted these constructs to the hippocampus prior to 4VO. Expression of these constructs was confirmed by Western blot (Figure 3.3a). Evaluation of CA1 neurons showed that expression of WtPrx2 and Prx2T89A significantly protected this region from ischemia (42% and 28% alive, respectively) in comparison to expression of Prx2T89E (18% alive) or GFP (14% alive) (Figure 3.3b). The above evidence indicates that Prx2 may be important for ischemic damage. To show a connection between Prx2 and Cdk5, we determined whether Prx2 is phosphorylated on Thr89 following ischemia in a Cdk5-dependent manner. We first analyzed for total Prx2 and pT89Prx2 by Western blot at different time points following insult. As Figure 3.3c shows, both total Prx2 and pT89Prx2 levels were increased at 3hs, with maximum levels at 24hs after reperfusion. Moreover, the specific activity of phosphorylation (pT89Prx2 to total Prx2) also showed 2.8 fold increase compared to control animals. To ensure that this phosphorylation is Cdk5-dependent, DNCdk5 was expressed unilaterally in hippocampus prior to 4VO. As demonstrated in Figure 3.3d, the increase in pT89Prx2 depends on Cdk5 activity since expression of DNCdk5 attenuated phosphorylation. Taken together, the above data suggests that Prx2 potentially is cytoplasmic Cdk5 target and is phosphorylated and inactivated in a model of global ischemia since restoration of this antioxidant protects CA1 neurons in this model.


a)
T89A
Wt
T89E
c
i
c
i
c
i
Prx2
Loading control
b)
%Live CA1 Neurons
60
40
20
0
*
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ipsi
cont
GFP
Wt
T89A
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c)
Folds increase in pT89Prx2
4
3
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NC
3hr
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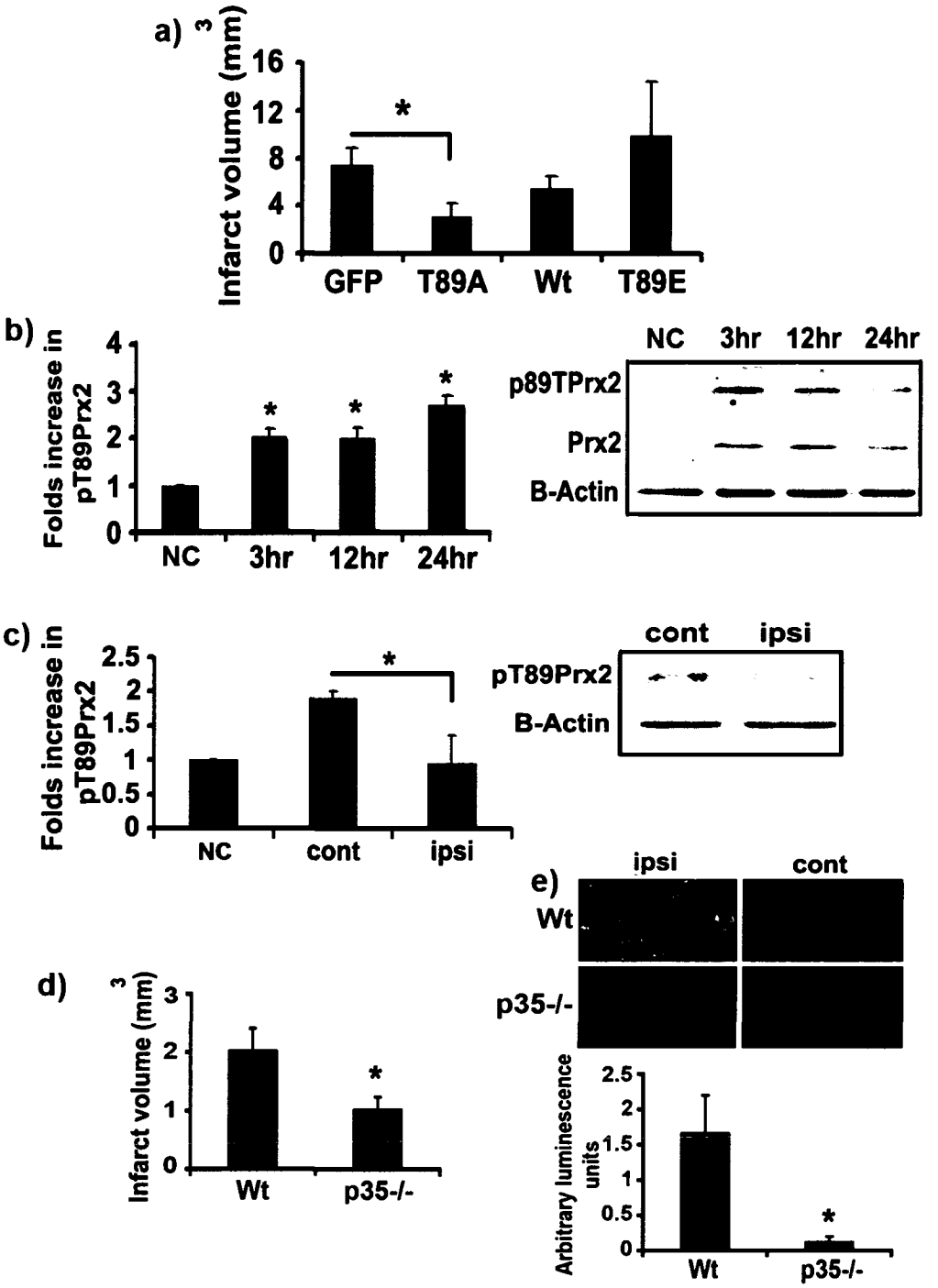
24hr
pT89Prx2
Prx2
B-Actin
d)
Folds increase in pT89Prx2
3
2
1
0
*
NC
cont
ipsi
cont
ipsi
pT89Prx2
B-Actin

Figure 3.3

Figure 3.3. Prx2 is phosphorylated by Cdk5 in global ischemia and leads to neuronal death. (a) AAV-mediated overexpression of Wt, T89A, and T89E mutants of Prx2 in hippocampus was detected by western blot using anti-Prx2 Ab (i, injected side; c, non-injected side). (b) Quantification of surviving CA1 neurons expressing GFP (n=3), Wt (n=9), T89A (n=11) or T89E (n=5) after 4VO. (c) Analysis of pPrx2 level in hippocampus at time points after 4VO by western blot. The membrane is representative of n=6 experiments and graph presents densitometry values of pPrx2 relative to Prx2. (d) Inhibition of Cdk5 attenuates pPrx2. Rats were unilaterally injected with AAV expressing DNCdk5 into hippocampus and subjected to 4VO. Total proteins from both sides of the hippocampus were analyzed for pPrx2 by western blot at 24hs after 4VO. The membrane is representative of n=3 experiments and graph presents densitometry values of pPrx2 relative to Prx2. "NC" represents non-stroked control animals. Data is presented as mean ±SEM (*ttest, $p<0.05$).

Similar results with Prx2 were also observed in the focal model of ischemia. We virally overexpressed WtPrx2, mutants Prx2T89A and Prx2T89E, and GFP in striatum before injection of endothelin-1. As Figure 3.4a shows, expression of Prx2T89A, the phosphorylation resistant mutant, provided significant protection (58% smaller infarct) from ischemia in comparison to expression of GFP. In contrast, expression of Prx2T89E, the phospho-mimicking mutant, did not offered any protection. Interestingly, unlike in the global model, we did not see significant protection by WtPrx2. This suggests that perhaps other death signals might be increased in the focal model compared to the global model such as those events in the nucleus. To determine whether Prx2 is phosphorylated in this model, striatal protein samples were analyzed for total Prx2 and pT89Prx2 by Western blot. As Figure 3.4b shows, both total Prx2 and pT89Prx2 levels were increased. Similar to the global model, the level of pT89Prx2 relative to total Prx2 was elevated 2.7 fold at 24hs after stroke. To test whether phosphorylation of Prx2 was Cdk5-mediated; we examined the effects of Cdk5 inhibition in two ways. First, we expressed DNCdk5 and measured its affect on Prx2 phosphorylation. As shown in Figure 3.4c, such expression attenuated phosphorylation. Second, we also examined the effects of p35 deficiency. We first measured the extent of damage induced by endothelin-1. As figure 3.4d demonstrates, p35-/- mice displayed significantly smaller infarct (50%) compare to Wt mice. Next, we carried out immunofluorescent analysis on the coronal sections obtained at the level of striatum. In p35-/- sections, the increase in pT89Prx2 signal observed upon ischemia was significantly reduced compare to the ones obtained from Wt (Figure 3.4e). Thus, p35 deficiency effectively prevents Prx2 phosphoryation and reduces damage following stroke insult. Taken together, the above data suggests that Prx2 is a

critical cytoplasmic Cdk5 target, which is phosphorylated and inactivated following focal ischemic injury.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Folds increase in pT89Prx2
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p89TPrx2
Prx2
B-Actin
c)
Folds increase in pT89Prx2
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ipsi
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ipsi
pT89Prx2
B-Actin
d)
Infarct volume (mm3)
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p35-/-
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Wt
p35-/-
Arbitrary luminescence units
2.5
2
1.5
1
0.5
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Wt
p35-/-

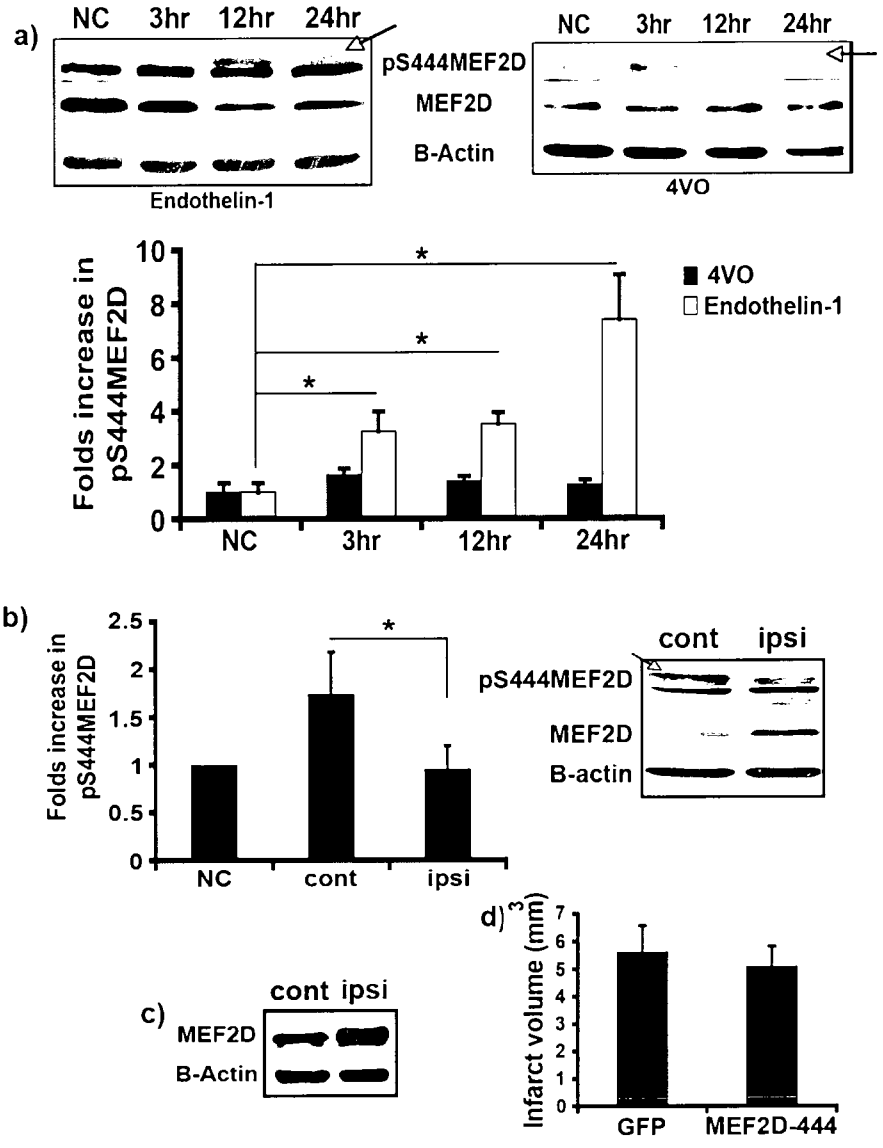
Figure 3.4

Figure 3.4. Prx2 is phosphorylated by Cdk5 in focal ischemia and causes neuronal death. (a) Quantification of infarct volume in brains overexpressing GFP (n=5), Wt (n=7), as well as T89A (n=5) and T89E (n=4) forms of Prx2 following injection of endothelin-1. (b) Analysis of pPrx2 level in striatum at the time points following endothelin injection by western blot. The membrane is representative of n=3 experiments and graph presents densitometry values of pPrx2 relative to Prx2. (c) Inhibition of Cdk5 attenuates pPrx2. Rats were unilaterally injected with AAV expressing DNCdk5 and then endothelin-1 into both sides of striatum. Total proteins from both sides of the striatum were analyzed for pPrx2 by western blot at 24hs after ischemia. The membrane is representative of n=3 experiments and graph presents densitometry values of pPrx2 to Prx2. ipsi, virus injected side; cont, non-injecte side. "NC" represents non-stroked control animals. (d) P35 deficient mice are resistant to focal ischemia. P35-/- (n=5) or Wt (n=7) mice were injected with endothelin-1 in striatum and the infarct volume was measured. (e) Increased pPrx2 signal is attenuated in p35 deficient mice in focal ischemia. Sections from mice brains (Wt n=5; p35-/- n=3) at striatum level were probed with anti-pT89Prx2 Ab and rabbit Alexa Fluor 488 Ab (green) as secondary Ab. Relative amount of luminescence was assessed by normalizing the average intensity of signal emitted (20 counts/brain) in the endothelin-injected side to that of the non-injeted side. Data is presented as mean ± SEM (*ttest, $p<0.05$).

MEF2 is a substrate for nuclear Cdk5 in ischemic death

In the focal model of ischemia, nuclear Cdk5, in addition to cytoplasmic, appears to play a role in the death process. To further examine the mechanisms by which nuclear Cdk5 promotes neuronal death, we investigated the survival promoting transcription factor MEF2D. Previous evidence indicates that MEF2D is a direct target for Cdk5-mediated neurotoxicity, at least in cultured neurons. Cdk5 has been reported to phosphorylate MEF2D on Ser444 which leads to its inactivation (Gong et al., 2003). Accordingly, we next determined whether this nuclear target plays an important role in excitotoxicity induced by endothelin-1. We first performed Western blot analysis on protein samples extracted from endothelin-injected striatum using a specific anti-pS444MEF2D Ab. As shown in Figure 3.5a, MEF2D is phosphorylated at Ser444 following ischemia with the maximum levels at 24hs. This result indicates that MEF2D plays an essential role in excitotoxicity. In contrast, the level of pS444MEF2D does not change following global ischemia. This is consistent with the notion that nuclear Cdk5 activity is not induced with the latter insult. To confirm that the phosphorylation observed following focal insult depends on Cdk5 activity, we examined the effect of inhibition of Cdk5 by expression of DNCdk5. As Figure 3.5b shows, expression of DNCdk5 attenuated pS444MEF2D. To further decipher the role of phosphorylation of MEF2D in our model, we virally expressed a mutant form of MEF2D which can not be phosphorylated at Ser444 (MEF2D-444), as well as GFP in striatum and subjected these rats to focal ischemia. As our data shows (Figure 3.5d) the phosphorylation resistant MEF2D did not protect.

Overall, these results suggesting that in an *in vivo* model of excitotoxic type of neuronal death cytoplasmic Prx2 and nuclear MEF2D, in nucleus, are targeted and modulated by Cdk5. Targeting Prx2 leads to loss of striatal neurons but the role of MEF2D in this model, unlike *in vitro* (Tang et al., 2005), is unclear.


a)
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MEF2D
B-Actin
Endothelin-1
4VO
Folds increase in pS444MEF2D
4VO
Endothelin-1
b)
cont
ipsi
c)
d)
Infarct volume (mm3)
GFP
MEF2D-444

Figure 3.5

Figure 3.5. MEF2D is phosphorylated at Ser444 by Cdk5 in focal ischemia. (a) Analysis of pMEF2D level at the time points following endothelin injection or 4VO. Total proteins extracted from striatum (focal ischemia) or hippocampus (4VO) were subjected to western blot utilizing anti-pS444MEF2D and then MEF2D Abs. The membrane is representative of n=3 experiments and graph presents densitometry values of pMEF2D relative to MEF2D. (b) Inhibition of Cdk5 attenuates pMEF2D in focal ischemia. Rats were unilaterally injected with AAV expressing DNCdk5 into striatum and 14 days later endothelin-1 was injected into both sides. Proteins from both sides of the striatum were analyzed for pMEF2D by western blot at 24hs after ischemia. The membrane is representative of n=3 experiments and graph presents densitometry values of pMEF2D relative to MEF2D. (c) Expression of AV mediated MEF2D-444 in striatum was detected by western blot. (d) Quantification of infarct volume in brains ovrexpressing GFP (n=3) and MEF2D-444 (n=4) following focal ischemia. Data is presented as mean ± SEM (*ttest, $p<0.05$).

Discussion

Although Cdk5 has received considerable attention as a potential mediator of neuronal damage, evidence for its functional importance in adult models of injury has only recently begun to emerge (Smith et al., 2003a; Wang et al., 2003). Specifically, we have recently shown that Cdk5 modulates ischemic death pathways in 5-min 4VO and following focal ischemia induced by endothelin-1 (Rashidian et al., 2005). However, the mechanism by which Cdk5 regulates this type of damage is not completely clear. The situation is made much more complex by the observations that Cdk5 complexes have both normal and pathogenic functions and can potentially be localized to multiple cellular compartments including membranes, cytoplasm, and nucleus. To better explore this issue, we a) examined which compartment (cytoplasmic versus nuclear) Cdk5 may play a functional role in multiple and distinct models of ischemic neuronal death and b) examined potential Cdk5 targets which may impact on this survival. Presently, we provide evidence for a common critical pro-death role for cytoplasmic Cdk5 in multiple models of ischemia *in vivo* through phosphorylation of the antioxidant enzyme Prx2. However, the role of nuclear Cdk5 appears more context-dependent.

Differential roles of Cdk5 in cytoplasm and nucleus

Cdk5 displays two opposing and seemingly contradictory functions. It is a key regulator of neuronal morphology and its activity is essential during development (Dhavan and Tsai, 2001) . In contrast, several lines of evidence have suggested that it could also be essential in neuronal death. The manner by which a key regulator of proper neuronal function can also possess a pathogenic face has been of considerable interest.

While several possibilities might account for this contradiction, cell localization may be critical In this regard; Cdk5 activity was initially thought to be predominately localized membranous compartments. Presumably, these membrane bound Ccdk5 complexes fulfilled the "normal" function of Cdk5. In support of this, we have shown that inhibition of cytoplasmic Cdk5 actually promotes death following DNA damage *in vitro* (O'Hare et al., 2005) . In contrast, a pathogenic form of Cdk5 was proposed to occur through calpain-mediated cleavage of the activator to a shorter more stable form. The hyperactivity of this complex was presumed to be deleterious. This cleavage also deletes the myristoylation sequence allowing for Cdk5 to be abnormally in the nucleus (Patrick et al., 1999; Kusakawa et al., 2000). In support of this, a recent report indicated that nuclear Cdk5 activity increases in response to glutamate and that a nuclear target of Cdk5, MEF2, is functionally relevant for excitotoxic death, at least *in vitro* (Tang et al., 2005). These observations have lead to a simplistic model by which normal Cdk5 activity is membrane bound while pathogenic Cdk5 is nuclear. However, the situation is likely much more complex. For example, recent reports have suggested that Cdk5/p35 complex can normally be soluble (Sato et al., 2007), and that p35 can even be specifically imported into nucleus (Fu et al., 2006) . In addition, whether the simplistic model described above holds true in all cases, particularly following adult injury is unclear. Indeed, one important possibility is that Cdk5 complexes may also play a role in death/survival in the cytoplasm. Surprisingly, our results indicate that in a global ischemia, cytoplasmic Cdk5 appears to play a crucial role in promoting death. This contrasts with our previous findings that cytoplasmic Cdk5 functions to promote survival following DNA damage (O'Hare et al., 2005) or that nuclear Cdk5 activity is critical in

promoting excitotoxic death (O'Hare et al., 2005; Tang et al., 2005). These latter studies were performed *in vitro* and suggest that the function of Cdk5 is critically context dependent and may differ in the adult. This data is also critical in that it shows that Cdk5 does not necessarily promote death in the nucleus. Indeed, under select conditions (i.e. global ischemia) we could not detect robust nuclear Cdk5 activation. In models of focal ischemia, on contrast, activation and requirement of Cdk5 activity is found in both nuclear and cytoplasmic fractions. The reasons for these differences are unclear, but likely are mediated through the specifics of the signaling environment for each ischemic model. It is clear that the activation of Cdk5 is not solely mediated through formation of p25 in either global or focal model (data not shown). Indeed, we have noticed that p25 is formed in both fractions, but that there is no correlation between the timing of the formation of p25 and the induction of Cdk5 activity (data not shown). This indicates that other signals are more critical in determining the extent of Cdk5 activation.

Cytoplasmic target for Cdk5 in stroke

How does Cdk5 induce its death signal within cytoplasm? Presently, we describe a new cytoplasmic target for Cdk5 in stroke, the antioxidant enzyme Prx2. Oxidative stress is a critical mediator of death in stroke and management of ROS has been shown to improve stroke in a number of stroke contexts (Margaill et al., 2005). Prx2, a cytoplasmic antioxidant enzyme with peroxidase activity, is abundantly expressed in neurons (Jin et al., 2005) and likely a major candidate in the defense of the neurons to oxidative stress. We have recently provided evidence that Prx2 is a substrate for Cdk5 (Qu et al., 2007). We have shown that Prx2 can be phosphorylated by Cdk5 on Thr89 and that this modification leads to its inactivation (Qu et al., 2007). Presently, we demonstrate

upregulation of phosphorylation of Thr89 following focal and global ischemia in a manner dependent upon Cdk5. We also show that modulation of Prx2 levels modulates this death providing evidence for a functional importance to this activity. Interestingly, Prx2 has been implicated in other neurodegenerative situations. For example, Prx2 was found co-aggregated with GPx1 and SOD-1 in neuronal Lewy body-like hyaline inclusions of spinal cord of familial ALS (Kato et al., 2004). We have also shown that Prx2 is phosphorylated in PD (Qu et al., 2007). Therefore, modulation of Prx2 levels may be a common and critical factor in the management of free radical stress so critical for death regulation.

In addition to Prx2, there is likely other cytoplasmic targets of Cdk5 in stroke. Potential targets include tau (Patrick et al., 1999; Cruz and Tsai, 2004). For example, tau has been proposed to be phosphorylated at Ser199/202 by Cdk5 following transient forebrain ischemia (Morioka et al., 2006).They supported this observation by use of a relatively non-specific CDK inhibitor olomoucine, however, and it is unclear whether other CDKs may account for this phosphorylation activity. In addition, the actually role of tau phosphorlyation in this case, is unclear. A second target also includes the NMDA receptor NR2A subunit. Cdk5 is reported to phosphorylate this subunit leading to increased calcium influx and neuronal death molecular cascades (Li et al., 2001). Understanding of whether/how these multiple targets interact will be of interest in future studies.

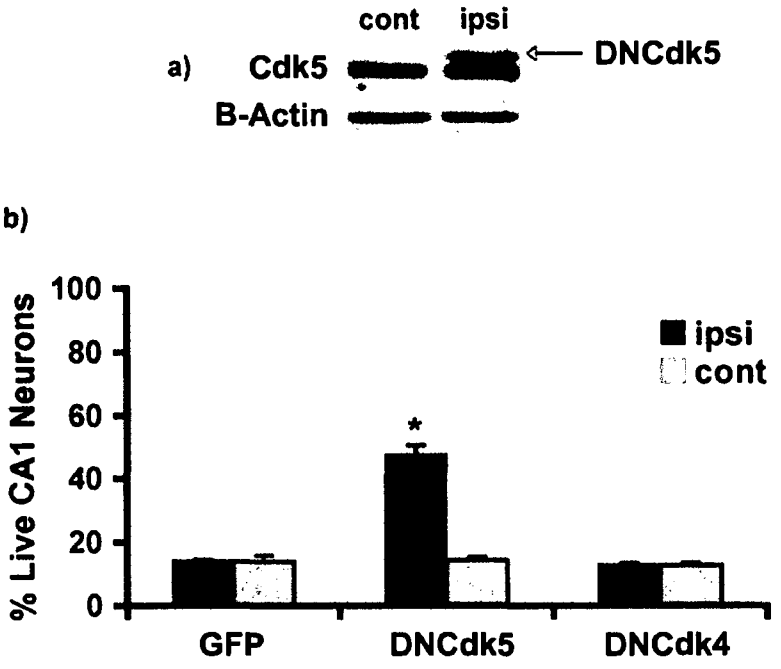
Nuclear target for Cdk5 in stroke

In addition to its role in the cytoplasm, Cdk5 may also have additional roles in the nucleus. In fact, several targets which are predominately nuclear have been described.

These include the tumor suppressor p53 and the transcription factor MEF2D. For example, under select apoptotic conditions Cdk5 has been shown to increase p53 levels (Lee et al., 2007). Cdk5 has also been shown in inactivate survival factors such as MEF2D. In this case, Cdk5 phosphorylates MEF2D on Ser444 suppressing its transcriptional activity. This Ser444 phosphorylation has been shown to occur *in vitro* models of death following peroxide and excitotoxicity exposure in a manner dependent on Cdk5 (Gong et al., 2003; Tang et al., 2005). We have also shown it to occur in adult models of injury in dopaminergic neurons following MPTP exposure (Smith et al., 2006). In both cases, the case for the importance of MEF2D was made since modulation of its activity appeared to regulate death. Presently, we show that MEF2D is also phosphorylated on Ser444 in a focal ischemic model where nuclear Cdk5 activity is observed but not following global insult where nuclear Cdk5 activity is not detected. However, the importance of MEF2D phosphorylation by Cdk5 in stroke is unclear. Expression of MEF2D did not appear to have a protective role like it was observed in other models of death described. While it is always possible that an insufficient amount of exogenous MEF2D activity was achieved. Our results also suggest the possibility that modulation of MEF2D by itself is insufficient to account for the death promoting effects of MEF2D in the nucleus. In this regard, it will be interesting to examine other potential nuclear targets of cdk5 such as p53 which has also been implicated in stroke, albeit in a complex fashion (Crumrine et al., 1994).

In summary, we have described the critical nature of cytoplasmic Cdk5-Prx2 activity in modulating ischemic neuronal death. These results also indicate the complex

nature of Cdk5 signalling in neuronal injury, suggesting that the nuclear Cdk5 activity is not necessarily required for death.


a)
cont
ipsi
Cdk5
DNCdk5
B-Actin
b)
% Live CA1 Neurons
100
80
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40
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0
ipsi
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GFP
DNCdk5
DNCdk4

Figure 3.6

Figure 3.6 (supporting information). Inhibition of Cdk5 provides significant protection from 5min-4VO. (a) Expression of AAV-mediated expression of flag-tagged DNCdk5 in hippocampus shown by western blot using anti-Cdk5 antibody. ipsi: injected side, cont: non-injected side. b) Quantification of surviving CA1 neurons expressing GFP (n=3), DNCdk5 (n=5) and DNCdk4 (n=3) four days after 4VO. ipsi is injected side, cont is non-injected side. Data is presented as mean ±SEM (*ttest, $p<0.05$).

References

Bu B, Li J, Davies P, Vincent I (2002) Deregulation of cdk5, hyperphosphorylation, and cytoskeletal pathology in the Niemann-Pick type C murine model. J Neurosci 22:6515-6525.

Crumrine RC, Thomas AL, Morgan PF (1994) Attenuation of p53 expression protects against focal ischemic damage in transgenic mice. J Cereb Blood Flow Metab 14:887-891.

Cruz JC, Tsai LH (2004) Cdk5 deregulation in the pathogenesis of Alzheimer's disease. Trends Mol Med 10:452-458.

Dhavan R, Tsai LH (2001) A decade of CDK5. Nat Rev Mol Cell Biol 2:749-759.

Fu X, Choi YK, Qu D, Yu Y, Cheung NS, Qi RZ (2006) Identification of nuclear import mechanisms for the neuronal Cdk5 activator. J Biol Chem 281:39014-39021.

Gold MO, Rice AP (1998) Targeting of CDK8 to a promoter-proximal RNA element demonstrates catalysis-dependent activation of gene expression. Nucleic Acids Res 26:3784-3788.

Gong X, Tang X, Wiedmann M, Wang X, Peng J, Zheng D, Blair LA, Marshall J, Mao Z (2003) Cdk5-mediated inhibition of the protective effects of transcription factor MEF2 in neurotoxicity-induced apoptosis. Neuron 38:33-46.

Hallows JL, Chen K, DePinho RA, Vincent I (2003) Decreased cyclin-dependent kinase 5 (cdk5) activity is accompanied by redistribution of cdk5 and cytoskeletal

proteins and increased cytoskeletal protein phosphorylation in p35 null mice. J Neurosci 23:10633-10644.

He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B (1998) A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A 95:2509-2514.

Ino H, Chiba T (1996) Intracellular localization of cyclin-dependent kinase 5 (CDK5) in mouse neuron: CDK5 is located in both nucleus and cytoplasm. Brain Res 732:179-185.

Jin MH, Lee YH, Kim JM, Sun HN, Moon EY, Shong MH, Kim SU, Lee SH, Lee TH, Yu DY, Lee DS (2005) Characterization of neural cell types expressing peroxiredoxins in mouse brain. Neurosci Lett 381:252-257.

Johansson JU, Lilja L, Chen XL, Higashida H, Meister B, Noda M, Zhong ZG, Yokoyama S, Berggren PO, Bark C (2005) Cyclin-dependent kinase 5 activators p35 and p39 facilitate formation of functional synapses. Brain Res Mol Brain Res 138:215-227.

Kato S, Saeki Y, Aoki M, Nagai M, Ishigaki A, Itoyama Y, Kato M, Asayama K, Awaya A, Hirano A, Ohama E (2004) Histological evidence of redox system breakdown caused by superoxide dismutase 1 (SOD1) aggregation is common to SOD1-mutated motor neurons in humans and animal models. Acta Neuropathol 107:149-158.

Kusakawa G, Saito T, Onuki R, Ishiguro K, Kishimoto T, Hisanaga S (2000) Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25. J Biol Chem 275:17166-17172.

Lee JH, Kim HS, Lee SJ, Kim KT (2007) Stabilization and activation of p53 induced by Cdk5 contributes to neuronal cell death. J Cell Sci 120:2259-2271.

Li BS, Zhang L, Takahashi S, Ma W, Jaffe H, Kulkarni AB, Pant HC (2002) Cyclin-dependent kinase 5 prevents neuronal apoptosis by negative regulation of c-Jun N-terminal kinase 3. Embo J 21:324-333.

Li BS, Ma W, Jaffe H, Zheng Y, Takahashi S, Zhang L, Kulkarni AB, Pant HC (2003) Cyclin-dependent kinase-5 is involved in neuregulin-dependent activation of phosphatidylinositol 3-kinase and Akt activity mediating neuronal survival. J Biol Chem 278:35702-35709.

Li BS, Sun MK, Zhang L, Takahashi S, Ma W, Vinade L, Kulkarni AB, Brady RO, Pant HC (2001) Regulation of NMDA receptors by cyclin-dependent kinase-5. Proc Natl Acad Sci U S A 98:12742-12747.

Margaill I, Plotkine M, Lerouet D (2005) Antioxidant strategies in the treatment of stroke. Free Radic Biol Med 39:429-443.

Morioka M, Kawano T, Yano S, Kai Y, Tsuiki H, Yoshinaga Y, Matsumoto J, Maeda T, Hamada J, Yamamoto H, Fukunaga K, Kuratsu J (2006) Hyperphosphorylation at serine 199/202 of tau factor in the gerbil hippocampus after transient forebrain ischemia. Biochem Biophys Res Commun 347:273-278.

Nguyen MD, Julien JP (2003) Cyclin-dependent kinase 5 in amyotrophic lateral sclerosis. Neurosignals 12:215-220.

O'Hare MJ, Kushwaha N, Zhang Y, Aleyasin H, Callaghan SM, Slack RS, Albert PR, Vincent I, Park DS (2005) Differential roles of nuclear and cytoplasmic cyclin-

dependent kinase 5 in apoptotic and excitotoxic neuronal death. J Neurosci 25:8954-8966.

Patrick GN, Zukerberg L, Nikolic M, de la Monte S, Dikkes P, Tsai LH (1999) Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature 402:615-622.

Pines J (1993) Cyclins and cyclin-dependent kinases: take your partners. Trends Biochem Sci 18:195-197.

Qu D, Rashidian J, Mount MP, Aleyasin H, Parsanejad M, Lira A, Haque E, Zhang Y, Callaghan S, Daigle M, Rousseaux MW, Slack RS, Albert PR, Vincent I, Woulfe JM, Park DS (2007) Role of Cdk5-mediated phosphorylation of Prx2 in MPTP toxicity and Parkinson's disease. Neuron 55:37-52.

Rashidian J, Iyirhiaro GO, Park DS (2007) Cell cycle machinery and stroke. Biochim Biophys Acta 1772:484-493.

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. Proc Natl Acad Sci U S A 102:14080-14085.

Sato K, Zhu YS, Saito T, Yotsumoto K, Asada A, Hasegawa M, Hisanaga S (2007) Regulation of membrane association and kinase activity of Cdk5-p35 by phosphorylation of p35. J Neurosci Res 85:3071-3078.

Smith PD, Mount MP, Shree R, Callaghan S, Slack RS, Anisman H, Vincent I, Wang X, Mao Z, Park DS (2006) Calpain-regulated p35/cdk5 plays a central role in

dopaminergic neuron death through modulation of the transcription factor myocyte enhancer factor 2. J Neurosci 26:440-447.

Smith PD, Crocker SJ, Jackson-Lewis V, Jordan-Sciutto KL, Hayley S, Mount MP, O'Hare MJ, Callaghan S, Slack RS, Przedborski S, Anisman H, Park DS (2003) Cyclin-dependent kinase 5 is a mediator of dopaminergic neuron loss in a mouse model of Parkinson's disease. Proc Natl Acad Sci U S A 100:13650-13655.

Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR (1990) A semiautomated method for measuring brain infarct volume. J Cereb Blood Flow Metab 10:290-293.

Tang X, Wang X, Gong X, Tong M, Park D, Xia Z, Mao Z (2005) Cyclin-dependent kinase 5 mediates neurotoxin-induced degradation of the transcription factor myocyte enhancer factor 2. J Neurosci 25:4823-4834.

Wang CX, Song JH, Song DK, Yong VW, Shuaib A, Hao C (2006) Cyclin-dependent kinase-5 prevents neuronal apoptosis through ERK-mediated upregulation of Bcl-2. Cell Death Differ 13:1203-1212.

Wang F, Corbett D, Osuga H, Osuga S, Ikeda JE, Slack RS, Hogan MJ, Hakim AM, Park DS (2002) Inhibition of cyclin-dependent kinases improves CA1 neuronal survival and behavioral performance after global ischemia in the rat. J Cereb Blood Flow Metab 22:171-182.

Wang J, Liu S, Fu Y, Wang JH, Lu Y (2003) Cdk5 activation induces hippocampal CA1 cell death by directly phosphorylating NMDA receptors. Nat Neurosci 6:1039-1047.

Zolotukhin S, Potter M, Zolotukhin I, Sakai Y, Loiler S, Fraites TJ, Jr., Chiodo VA, Phillipsberg T, Muzyczka N, Hauswirth WW, Flotte TR, Byrne BJ, Snyder RO (2002) Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. Methods 28:158-167.

CHAPTER 4

General Discussion

4.1. Summary

Although there has been much efforts made in attempting to improve clinical stroke outcomes, it is still the third most leading cause of death and disability world widely. To date, the most effective treatment is restoration of blood flow using tPA, but only in very restricted situations with limited therapeutic windows. One of the major obstacles in designing and improving effective therapeutic strategies is our incomplete understanding of mechanisms involved in ischemic neuronal death. The complexity of neuronal death following stroke suggests that death signaling pathways must be studied in different contexts that prevail in the brain following an ischemic attack. This may lead to more focused efforts to discover novel targets and minimize the stroke damage. This thesis was principally focused on studying the role of CDKs, including Cdk2, Cdk4 and Cdk5 in different models of stroke. Several studies have recently suggested dysfunction of these factors in neuronal injury but none of them have been conclusive. Accordingly, in this thesis research we explored the contribution of specific cell cycle and a non-cell cycle CDKs in neurodegeneration evoked by stroke in distinct delayed and apoptotic paradigms. More importantly, we showed that how diminishing these signals improves outcomes following stroke. Since a more comprehensive discussion of results has been presented as part of each manuscript, I will only provide an overview of the major findings and their significance in the field of our knowledge. Finally, I will very briefly discuss about implication of our results in developing more effective therapeutic approaches.

4.2. Major Findings

4.2.1 *Neuronal Death Induced by CDKs in Stroke*

Re-activation of cell cycle elements such as CDKs following injury, in the context of post-mitotic neurons, has been a remarkable finding. However, the significance of these signals in promoting damage and consequently, the importance of inhibition of the signals in neuroprotection were not fully defined. The use of pharmacological reagents and non-specific inhibitors suggested a potential role for CDKs in neuronal injury such as stroke but did not provide conclusive evidence. This research was performed to specifically evaluate the role of G1 phase cell cycle CDKs, Cdk2 and Cdk4, as well as a related member of this family, Cdk5, in stroke models. For the first time, results presented in chapter 2, provide functional evidence to show that upregulation of Cdk4 and Cdk5 signals contribute to the stroke. We showed that specific inhibition of these CDKs protected insulted neurons. This data is further supported by the observation that inhibition of Cdk4 signal in the global model of ischemia not only promoted survival of hippocampal CA1 neurons but also improved behavioral outcomes. The significance of these findings relies on the fact that they are obtained in fully matured adult neurons where there is likely very little if any cell cycle machinery present under basal conditions.

Another major finding in chapter 2 is that our data represents a model in which these two CDKs are activated preferentially in different models of stroke. In *in vitro* rapid excitotoxic model Cdk5 signals for death while Cdk4 is essential in delayed and apoptotic type of death. Similarly, these CDKs act differently in distinct models of

ischemia, *in vivo*. In death induced by focal model of stroke, Cdk5 is the main player while in global model, depending on the duration of insult, one of these CDKs is activated. According to our data, Cdk4 is involved in 10 minutes of occlusion while Cdk5 is important in 5 minutes insult. The latter data suggests that shorter insult time is probably associated with NMDA receptors modulation. This interpretation is consistent with following two observations. First, an NMDA receptors antagonist, MK-801, is protective in shorter global ischemia in the gerbil but not following a more prolonged insult (Murase et al., 1993). Second, phosphorylation of NMDA receptors by Cdk5 in 5 minutes global ischemia increases their current (Wang et al., 2003). This differential contribution of CDKs in distinct models is highly important since, as it was discussed in chapter 1, different pathways of neuronal death are triggered following a stroke attack. These observations implicate that CDKs are not activated randomly but instead, multiple CDKs specifically signal these pathways in an ischemic region.

4.2.2 *Cell Cycle Signal- Focus on Cdk4*

While our results substantiated an essential role for Cdk4 and Cdk5 in stroke-induced neuronal death, further experiments were conducted to identify downstream effectors of these signals. Pertaining to cell cycle Cdk4, our evidence implicates Rb as a downstream mediator of delayed and apoptotic death pathway. We showed that a constitutively active form of Rb rescued neurons in delayed death induced by hypoxia *in vitro*. Interestingly, as stated in chapter 1, Rb is a physiological target for G1 phase CDKs. Indeed, phosphorylation of Rb is a gateway for transition from G1 to S phase and regulation of the genes required for cell cycle moving forward (Dyson, 1998). Therefore,

cell cycle re-entry appears to be a common pathway used in proliferating cells and dying neurons, except that the cycle is likely aborted in the insulted neurons.

Although cell cycle is likely terminated at G1/S checkpoint, where E2F-DP complexes regulate apoptotic genes in dying neurons, some groups have suprisingly reported S phase entry. For example, induction of DNA synthesis and expression of a DNA synthesis marker (PCNA: Proliferating Cell Nuclear Antigen) has been shown in several models of focal and global ischemia (Li et al., 1997; Tomasevic et al., 1998; Kato et al., 2003; Wen et al., 2005). Incorporation of BrdU in apoptotic neurons has also been observed in these cells (Kuan et al., 2004; Wen et al., 2005). Interestingly, these injured neurons with elevated G1 phase CDKs and presenting S phase markers are also co-localized with apoptotic markers (TUNEL positive: Terminal Transferase dUTP Nick End Labeling assay detecting fragmented DNA). This suggests that the cell cycle has been triggered in these cells and they have entered S phase to resume DNA synthesis but do not complete the cycle. As evidence shows, they undergo apoptotic death rather than replication. Up to this date, there is no *in vivo* evidence for G2 entry in ischemic neurons. Although expression of Cdk1, as an E2F target and a G2 marker, has been observed in other apoptotic neuronal death model *in vitro* (Konishi and Bonni, 2003). In this model, lowering potassium concentration induces apoptosis through upregulation of Cdk1 by E2F1.

Although it is not pointed out in chapter 2, to further identify Cdk4-Rb signal in global ischemia we investigated the role of DP1 and Cdk1 in this pathway. We demonstratedn that DP1 in complex with E2F is actively involved in this pathway since a dominant interfering form of DP1 improved neuronal survival following insult.

Nevertheless, induction of Cdk1, as a target for E2F-DP, does not seem to be necessary for inducing death. This finding is important because it shows that apoptosis occurs through different mechanism in our model compare to what Konishi and Bonni showed. Taken together, investigation of the responsive apoptotic genes following global ischemia might be an important area of future research in the stroke field.

4.2.3. *Non-Cell Cycle Signal-Focus on Cdk5*

The functional importance of Cdk5 in adult models of neuronal injury has recently begun to emerge (Smith et al., 2003a; Wang et al., 2003). As discussed earlier, our research presented in chapter 2 provided a model in which Cdk5 is activated preferentially in some selected models of stroke. These models are endothelin 1-induced focal ischemia, 5min-induced global ischemia, as well as excitotoxicity-induced by glutamate or hypoxia. One of the main challenges about Cdk5 role, particularly in stroke, is lack of clarity in death-inducing mechanisms. The fact that Cdk5 has been reported to be both normal and pathogenic and can potentially be localized to multiple cellular compartments makes this situation more complicated. Presently, we found that Cdk5 signal is activated differently in distinct models of stroke. While cytoplasmic Cdk5 is essential in ischemic death in both focal and global models used in here, nuclear Cdk5 seems to be crucial only in the focal model. Although not presented in chapter 3, our unpublished data did not show any correlation between the level of p25 and Cdk5 activity in these two models. In our observations, p25 increased in both nucleus and cytoplasm. The important point here is that, in spite of elevated nuclear p25 in global model, we did not see increase in Cdk5 activity in this fraction. These results contradict with the original proposed model for Cdk5 activity (Patrick et al., 1999; Kusakawa et al., 2000). Based on

this model, p35-mediated membranous Cdk5 is considered as a normal complex with physiological functions. Cdk5/p25, however, is localized in soluble fractions of cell and signals for death, especially in the nucleus.

In summary our data indicates that, Cdk5 pro-death activity is context-dependent. We propose that both nuclear and cytoplasmic Cdk5 can potentially be pathogenic or physiologic and this is not solely p25-dependent. The reason for this is not clear for us. Most likely, other factors including signaling environment (type of insult, type of cells, timing, other activators, etc) specifies the location and the nature (either pathogenic or physiologic) of signals. To support this, although we noticed a pro-death role for Cdk5 in CGN cultures insulted with glutamate or excitotoxic hypoxia (chapter 2), Panickar and colleges reported a pro-survival role for Cdk5 in oxygen and glucose deprivation model (Panickar et al., 2008). They demonstrated that overexpression of wild type Cdk5 protected septal neuronal cultures in this model, while a dominant negative form gave little or no protection (Panickar et al., 2008). More importantly, it has been shown in our lab that inhibition of cytoplasmic Cdk5 in DNA damaging model sensitizes neurons to the insult (O'Hare et al., 2005). These observations are in agreement with the concept that the nature of Cdk5 activity is context-dependent and whether or not Cdk5 acts to promote or suppress death is dependent on the signaling environment. The idea that Cdk5 pathogenesis may not be entirely p25-depenent has also been supported by several observations. In a DNA damaging model, apoptotic signals are triggered when neuronal cultures lose the supporting p35 messages in cytoplasm, long before p25 is generated in nucleus (O'Hare et al., 2005). This model also shows a clear survival activity for Cdk5 in cytoplasm. In another intriguing study, p25 is not associated with neurodegeneration

induced by β-amyloid in Drosophila neurons (Lin et al., 2007). In Drosophila, p35 is not cleaved to p25 because the consensus cleavage site for calpain is absent. Interestingly in this model, Cdk5 translocates from the cell membrane to the perinuclear region, which again is a p25-independent phenomenon (Lin et al., 2007).

In the light of localization of Cdk5 activity in cytoplasm in both focal and global ischemia, we identified Prx2 as an effector of this signal in both models. We have suggested that manipulation and inactivation of Prx2 is one of the possible mechanisms for Cdk5 to signal for death in ischemia. This finding is particularly relevant. Oxidative stress is one of the mechanisms involved in pathogenesis of a number of neurodegenerative diseases including stroke (Kim et al., 2002; Halliwell, 2006). Importantly, Prx2 levels are significantly increased in a number of neurodegenerative disorders (Kim et al., 2001; Krapfenbauer et al., 2003; Sorolla et al., 2008). Pertaining to this research, we have recently demonstrated phosphorylation and inactivation of Prx2 by Cdk5 in the models of PD, *in vitro* and *in vivo*. More importantly, we showed that phospho-Prx2 increases in the brains from PD patients (Qu et al., 2007). It is important to point out that Prx2 is also thought to have some chaperone activity, as well as peroxidase activity (Jang et al., 2004; Moon et al., 2005). Upon exposure to oxidative stress, Prx2 reversibly forms a high molecular weight structure with an efficient chaperone function. Removal of stress, however, reverses these chaperone molecules to low molecular weight peroxidases (Jang et al., 2004; Moon et al., 2005). The functional importance of these high molecular chaperones is presumably to protect cells against protein unfolding or aggregation due to oxidative stress (Jang et al., 2004). The existence of this dual function for Prx2 in the present study is not known. Whether or not these

chaperone molecules are formed here and whether it is relevant as a defense mechanism in stroke will be of further interests. Finally, it would be interesting to investigate whether Cdk5 regulates other Prx members in stroke models. For instance, changes in the expression of Prx3 have been reported previously in an excitotoxic model of hippocampus injury (Hattori et al., 2003) and also transient forebrain ischemia (Hwang et al., 2005). As described in chapter 1, Prx3 is located in mitochondria and its amino acids sequence contains a potential motif for Cdk5 phosphorylation. Given that Cdk5 and p35 have been detected in mitochondrial fraction of cortical neurons (Cheung et al., 2008) it would be intriguing to study Prx3 as another cytoplasmic target for Cdk5 in stroke models.

In our attempt to investigate nuclear effectors of Cdk5 in the focal ischemia, we studied whether the survival factor MEF2D could be the relevant nuclear target. As presented in chapter 3, we found that Cdk5 phosphorylates MEF2D on Se444 following focal insult. It has been suggested that phosphorylation and inactivation of MEF2D by Cdk5 plays a central role in dopaminergic neuronal loss in animal model of PD (Smith et al., 2006). The relevance of this mechanism in stroke, however, is not clear. Surprisingly, expression of a phosphorylation resistant form of MEF2D did not offer protection. While it is possible that an insufficient amount of exogenous MEF2D activity was achieved, it will be interesting to examine other members of MEF2 family. MEF2A and MEF2C are also phosphorylated and inactivated by Cdk5 on Ser408 and Ser388, respectively (Gong et al., 2003; Tang et al., 2005). Therefore, examining these two transcription factors might elucidate the mechanism by which nuclear Cdk5 regulate neuronal death in focal ischemia.

Because of the lack of protection observed with MEF2 expression, it will be also interesting to examine other potential nuclear targets such as p53. P53 plays an important role in tumor suppression by inducing apoptosis (Vousden and Lane, 2007). It has been shown that p53 expression increases in damaged neurons following focal ischemia (Chopp et al., 1992) and mice lacking p53 are resistant to ischemic damage (Crumrine et al., 1994). Importantly, several studies have demonstrated that p53 is a substrate for Cdk5. It has been reported that Cdk5 phosphorylates and stabilizes p53 in different *in vitro* models of neuronal damage (Zhang et al., 2002; Lee et al., 2007; Lee et al., 2008). Studying p53 as a target for Cdk5 to induce neuronal death, though, will not be straightforward. Although p53 is well known as a regulator of pro-apoptotic genes, emerging evidence suggests that it also contributes in pro-survival pathways by activation of several genes whose products are anti-apoptotic (Janicke et al., 2008).

4.2.4. *Crosstalk Between Cell Cycle and Non-Cell Cycle Signals*

An important question that arises from the present research is whether there is any interaction between death pathways mediated by cell cycle-Cdk4 and Cdk5. The rationale behind this question is that while Cdk5 is not a classic cell cycle regulator, it can potentially interact with known cell cycle elements. For instance, Cdk5 is able to bind to cyclins D1, D3 and E (Xiong et al., 1992; Zhang et al., 1993a; Miyajima et al., 1995) but no resulting activity has been reported. Other evidence has suggested that Cdk5 might regulate Rb activity with the same manner as cell cycle-CDKs. Evidence shows that inducible Cdk5/p25 can bind to Rb and phosphorylate this protein, *in vitro* (Honma et al., 1997; Hamdane et al., 2005). In fact, Lee et al. have detected Cdk5-Rb complexes in

embryonic mouse brain homogenates (Lee et al., 1997). It is important to note that the role of E2F1, the effector of Rb, in focal ischemia has been shown previously (MacManus et al., 2003). In present research, we reported that Cdk5 is more essential for neuronal death in focal ischemia. Accordingly, it would be interesting to examine if Rb is phosphorylated by Cdk5 in the endothelin model. Given our observation that the functional role of the Cdk5 nuclear target MEF2D is unclear in stroke, studying Rb as a potential nuclear substrate for Cdk5 signal will be intriguing.

Another possible interaction between Cdk4 and Cdk5 has been reported in an animal model of ALS (Nguyen et al., 2003). According to this study, both Cdk4 and Cdk5 are deregulated in a mouse model of ALS. They also detected phospho-Rb immunoprecipitated with Cdk4 but not with Cdk5. Interestingly, they showed that expression of neurofilament H, which acts as a phosphorylation sink for Cdk5, decreases Cdk4 and phospho-Rb levels (Nguyen et al., 2003). Accordingly, they proposed that Cdk5 activity might be upstream of Cdk4 deregulation in this model. We reported in chapter 2 that Cdk5 activity is more critical in focal ischemia model and inhibition of its signal significantly reduced the damage (Figure 2.5). Analysis of results also shows a trend in case of Cdk4 inhibition. Although not significant, blocking Cdk4 signal resulted in a smaller damage (Figure 2.5). Therefore, it would be interesting to investigate whether there is any interaction between these two CDKs in this model. Assessing Cdk4 activity or/and levels before and after blocking Cdk5 might reveal existence of such an interaction.

4.3. Relevance of CDKs Signals in Stroke Patients

A major question of interest in here is that how this experimental *in vitro* and *in vivo* evidence apply to the human. Unfortunately, there is little known about aberrant deregulation of cell cycle, and particularly CDKs, in stroke cases in human. However, a recent investigation performed on patients who died after cardiac arrest or focal brain infarction showed that the levels of cyclin D1, Cdk2 and Cdk4 are elevated in their brains (Love, 2003b). Cdk5 deregulation has also been demonstrated in human brain after stroke. In a study conducted on human post-mortem stroke brain, increased expression of Cdk5, p35 and p25 was shown in stroke-affected regions. Interestingly, translocation of Cdk5 and p35 and colocalization of them in nucleus was also observed in most of the cases (Mitsios et al., 2007). This evidence suggests that results obtained from experimental studies may corroborate with the human condition

4.4. Concluding remarks

Due to complexities attributed to neuronal death following stroke, designing combinational therapy strategies appears to be a necessity in this field. Modulating different pathways at specific target, location and time to reduce the damage is the message of this research. In conclusion, our results indicate for the first time that both Cdk4 and Cdk5 are critical in stroke. Moreover, we have defined potentially critical downstream targets including the antioxidant enzyme Prx2. These results not only highlight the importance of multiple CDK members in differing stroke contexts but it also sheds light on the sophisticated nature of stroke signaling. It is this sophistication that has made therapeutic strategies so diffcult to design. We believe that while CDK s may not be the only target required to promote stroke recovery, it may be a critical factor in this strategy.

APPENDICES

Appendix 1: Refrences Cited

(1995) Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. N Engl J Med 333:1581-1587.

(2004) Combined intravenous and intra-arterial recanalization for acute ischemic stroke: the Interventional Management of Stroke Study. Stroke 35:904-911.

Adam-Vizi V (2005) Production of reactive oxygen species in brain mitochondria: contribution by electron transport chain and non-electron transport chain sources. Antioxid Redox Signal 7:1140-1149.

Adams JM, Cory S (2001) Life-or-death decisions by the Bcl-2 protein family. Trends Biochem Sci 26:61-66.

Akoulitchev S, Chuikov S, Reinberg D (2000) TFIIH is negatively regulated by cdk8-containing mediator complexes. Nature 407:102-106.

Aktas H, Cai H, Cooper GM (1997) Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the Cdk inhibitor p27KIP1. Mol Cell Biol 17:3850-3857.

Antonsson B (2001) Bax and other pro-apoptotic Bcl-2 family "killer-proteins" and their victim the mitochondrion. Cell Tissue Res 306:347-361.

Arundine M, Tymianski M (2003) Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. Cell Calcium 34:325-337.

Astrup J, Siesjo BK, Symon L (1981) Thresholds in cerebral ischemia - the ischemic penumbra. Stroke 12:723-725.

Beijersbergen RL, Bernards R (1996) Cell cycle regulation by the retinoblastoma family of growth inhibitory proteins. Biochim Biophys Acta 1287:103-120.

Bian F, Nath R, Sobocinski G, Booher RN, Lipinski WJ, Callahan MJ, Pack A, Wang KK, Walker LC (2002) Axonopathy, tau abnormalities, and dyskinesia, but no neurofibrillary tangles in p25-transgenic mice. J Comp Neurol 446:257-266.

Bibb JA, Chen J, Taylor JR, Svenningsson P, Nishi A, Snyder GL, Yan Z, Sagawa ZK, Ouimet CC, Nairn AC, Nestler EJ, Greengard P (2001) Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. Nature 410:376-380.

Bibb JA, Snyder GL, Nishi A, Yan Z, Meijer L, Fienberg AA, Tsai LH, Kwon YT, Girault JA, Czernik AJ, Huganir RL, Hemmings HC, Jr., Nairn AC, Greengard P (1999) Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signalling in neurons. Nature 402:669-671.

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

Borner C (2003) The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions. Mol Immunol 39:615-647.

Bredt DS, Snyder SH (1994) Nitric oxide: a physiologic messenger molecule. Annu Rev Biochem 63:175-195.

Briones TL, Therrien B (2000) Behavioral effects of transient cerebral ischemia. Biol Res Nurs 1:276-286.

Brown GC, Borutaite V (2002) Nitric oxide inhibition of mitochondrial respiration and its role in cell death. Free Radic Biol Med 33:1440-1450.

Bruno V, Battaglia G, Copani A, D'Onofrio M, Di Iorio P, De Blasi A, Melchiorri D, Flor PJ, Nicoletti F (2001) Metabotropic glutamate receptor subtypes as targets for neuroprotective drugs. J Cereb Blood Flow Metab 21:1013-1033.

Bu B, Li J, Davies P, Vincent I (2002) Deregulation of cdk5, hyperphosphorylation, and cytoskeletal pathology in the Niemann-Pick type C murine model. J Neurosci 22:6515-6525.

Budd SL (1998) Mechanisms of neuronal damage in brain hypoxia/ischemia: focus on the role of mitochondrial calcium accumulation. Pharmacol Ther 80:203-229.

Candelario-Jalil E, Mhadu NH, Al-Dalain SM, Martinez G, Leon OS (2001) Time course of oxidative damage in different brain regions following transient cerebral ischemia in gerbils. Neurosci Res 41:233-241.

Cao G, Luo Y, Nagayama T, Pei W, Stetler RA, Graham SH, Chen J (2002a) Cloning and characterization of rat caspase-9: implications for a role in mediating caspase-3 activation and hippocampal cell death after transient cerebral ischemia. J Cereb Blood Flow Metab 22:534-546.

Cao G, Minami M, Pei W, Yan C, Chen D, O'Horo C, Graham SH, Chen J (2001) Intracellular Bax translocation after transient cerebral ischemia: implications for a role of the mitochondrial apoptotic signaling pathway in ischemic neuronal death. J Cereb Blood Flow Metab 21:321-333.

Cao G, Pei W, Ge H, Liang Q, Luo Y, Sharp FR, Lu A, Ran R, Graham SH, Chen J (2002b) In Vivo Delivery of a Bcl-xL Fusion Protein Containing the TAT Protein

Transduction Domain Protects against Ischemic Brain Injury and Neuronal Apoptosis. J Neurosci 22:5423-5431.

Carbonell T, Rama R (2007) Iron, oxidative stress and early neurological deterioration in ischemic stroke. Curr Med Chem 14:857-874.

Cardenas A, De Alba J, Moro MA, Leza JC, Lorenzo P, Lizasoain I (1998) Protective effect of N-(3-(aminomethyl)benzyl) acetamidine, an inducible nitric oxide synthase inhibitor, in brain slices exposed to oxygen-glucose deprivation. Eur J Pharmacol 354:161-165.

Cardenas A, Moro MA, Hurtado O, Leza JC, Lorenzo P, Castrillo A, Bodelon OG, Bosca L, Lizasoain I (2000) Implication of glutamate in the expression of inducible nitric oxide synthase after oxygen and glucose deprivation in rat forebrain slices. J Neurochem 74:2041-2048.

Castro LA, Robalinho RL, Cayota A, Meneghini R, Radi R (1998) Nitric oxide and peroxynitrite-dependent aconitase inactivation and iron-regulatory protein-1 activation in mammalian fibroblasts. Arch Biochem Biophys 359:215-224.

Chae T, Kwon YT, Bronson R, Dikkes P, Li E, Tsai LH (1997) Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality. Neuron 18:29-42.

Chai J, Shiozaki E, Srinivasula SM, Wu Q, Datta P, Alnemri ES, Shi Y (2001) Structural basis of caspase-7 inhibition by XIAP. Cell 104:769-780.

Chan PH (2001) Reactive oxygen radicals in signaling and damage in the ischemic brain. J Cereb Blood Flow Metab 21:2-14.

Chang TS, Jeong W, Choi SY, Yu S, Kang SW, Rhee SG (2002) Regulation of peroxiredoxin I activity by Cdc2-mediated phosphorylation. J Biol Chem 277:25370-25376.

Charriaut-Marlangue C, Margaill I, Represa A, Popovici T, Plotkine M, Ben-Ari Y (1996) Apoptosis and necrosis after reversible focal ischemia: an in situ DNA fragmentation analysis. J Cereb Blood Flow Metab 16:186-194.

Chau BN, Wang JY (2003) Coordinated regulation of life and death by RB. Nat Rev Cancer 3:130-138.

Chen J, Nagayama T, Jin K, Stetler RA, Zhu RL, Graham SH, Simon RP (1998) Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. J Neurosci 18:4914-4928.

Chen J, Zhu RL, Nakayama M, Kawaguchi K, Jin K, Stetler RA, Simon RP, Graham SH (1996) Expression of the apoptosis-effector gene, Bax, is up-regulated in vulnerable hippocampal CA1 neurons following global ischemia. J Neurochem 67:64-71.

Chen ZL, Strickland S (1997) Neuronal death in the hippocampus is promoted by plasmin-catalyzed degradation of laminin. Cell 91:917-925.

Cheung ZH, Gong K, Ip NY (2008) Cyclin-dependent kinase 5 supports neuronal survival through phosphorylation of Bcl-2. J Neurosci 28:4872-4877.

Chipuk JE, Green DR (2008) How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? Trends Cell Biol 18:157-164.

Choi DW (1990) Possible mechanisms limiting N-methyl-D-aspartate receptor overactivation and the therapeutic efficacy of N-methyl-D-aspartate antagonists. Stroke 21:III20-22.

Choi DW, Rothman SM (1990) The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. Annu Rev Neurosci 13:171-182.

Chopp M, Li Y, Zhang ZG, Freytag SO (1992) p53 expression in brain after middle cerebral artery occlusion in the rat. Biochem Biophys Res Commun 182:1201-1207.

Chopp M, Chan PH, Hsu CY, Cheung ME, Jacobs TP (1996) DNA damage and repair in central nervous system injury: National Institute of Neurological Disorders and Stroke Workshop Summary. Stroke 27:363-369.

Cicero S, Herrup K (2005) Cyclin-dependent kinase 5 is essential for neuronal cell cycle arrest and differentiation. J Neurosci 25:9658-9668.

Clark WM, Wissman S, Albers GW, Jhamandas JH, Madden KP, Hamilton S (1999) Recombinant tissue-type plasminogen activator (Alteplase) for ischemic stroke 3 to 5 hours after symptom onset. The ATLANTIS Study: a randomized controlled trial. Alteplase Thrombolysis for Acute Noninterventional Therapy in Ischemic Stroke. Jama 282:2019-2026.

Clarke AR, Maandag ER, van Roon M, van der Lugt NM, van der Valk M, Hooper ML, Berns A, te Riele H (1992) Requirement for a functional Rb-1 gene in murine development. Nature 359:328-330.

Cleeter MW, Cooper JM, Darley-Usmar VM, Moncada S, Schapira AH (1994) Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the

mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. FEBS Lett 345:50-54.

Coleman KG, Wautlet BS, Morrissey D, Mulheron J, Sedman SA, Brinkley P, Price S, Webster KR (1997) Identification of CDK4 sequences involved in cyclin D1 and p16 binding. J Biol Chem 272:18869-18874.

Collen D, Bounameaux H, De Cock F, Lijnen HR, Verstraete M (1986) Analysis of coagulation and fibrinolysis during intravenous infusion of recombinant human tissue-type plasminogen activator in patients with acute myocardial infarction. Circulation 73:511-517.

Connell-Crowley L, Harper JW, Goodrich DW (1997) Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. Mol Biol Cell 8:287-301.

Copani A, Uberti D, Sortino MA, Bruno V, Nicoletti F, Memo M (2001) Activation of cell-cycle-associated proteins in neuronal death: a mandatory or dispensable path? Trends Neurosci 24:25-31.

Crumrine RC, Thomas AL, Morgan PF (1994) Attenuation of p53 expression protects against focal ischemic damage in transgenic mice. J Cereb Blood Flow Metab 14:887-891.

Cruz JC, Tsai LH (2004) Cdk5 deregulation in the pathogenesis of Alzheimer's disease. Trends Mol Med 10:452-458.

Dasgupta P, Padmanabhan J, Chellappan S (2006) Rb function in the apoptosis and senescence of non-neuronal and neuronal cells: role in oncogenesis. Curr Mol Med 6:719-729.

De Azevedo WF, Jr., Mueller-Dieckmann HJ, Schulze-Gahmen U, Worland PJ, Sausville E, Kim SH (1996) Structural basis for specificity and potency of a flavonoid inhibitor of human CDK2, a cell cycle kinase. Proc Natl Acad Sci U S A 93:2735-2740.

Delston RB, Harbour JW (2006) Rb at the interface between cell cycle and apoptotic decisions. Curr Mol Med 6:713-718.

Deveraux QL, Stennicke HR, Salvesen GS, Reed JC (1999) Endogenous inhibitors of caspases. J Clin Immunol 19:388-398.

Deverman BE, Cook BL, Manson SR, Niederhoff RA, Langer EM, Rosova I, Kulans LA, Fu X, Weinberg JS, Heinecke JW, Roth KA, Weintraub SJ (2002) Bcl-xL deamidation is a critical switch in the regulation of the response to DNA damage. Cell 111:51-62.

Dhavan R, Tsai LH (2001) A decade of CDK5. Nat Rev Mol Cell Biol 2:749-759.

Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 22:391-397.

Dong Z, Saikumar P, Weinberg JM, Venkatachalam MA (1997) Internucleosomal DNA cleavage triggered by plasma membrane damage during necrotic cell death. Involvement of serine but not cysteine proteases. Am J Pathol 151:1205-1213.

Donnan GA, Fisher M, Macleod M, Davis SM (2008) Stroke. Lancet 371:1612-1623.

Doyle KP, Simon RP, Stenzel-Poore MP (2008) Mechanisms of ischemic brain damage. Neuropharmacology 55:310-318.

Du C, Fang M, Li Y, Li L, Wang X (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 102:33-42.

Dubuisson M, Vander Stricht D, Clippe A, Etienne F, Nauser T, Kissner R, Koppenol WH, Rees JF, Knoops B (2004) Human peroxiredoxin 5 is a peroxynitrite reductase. FEBS Lett 571:161-165.

Dulic V, Lees E, Reed SI (1992) Association of human cyclin E with a periodic G1-S phase protein kinase. Science 257:1958-1961.

Dyson N (1998) The regulation of E2F by pRB-family proteins. Genes Dev 12:2245-2262.

Earnshaw WC, Martins LM, Kaufmann SH (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 68:383-424.

Ekholm SV, Reed SI (2000) Regulation of G(1) cyclin-dependent kinases in the mammalian cell cycle. Curr Opin Cell Biol 12:676-684.

El Kossi MM, Zakhary MM (2000) Oxidative stress in the context of acute cerebrovascular stroke. Stroke 31:1889-1892.

El-Khodor BF, Oo TF, Kholodilov N, Burke RE (2003) Ectopic expression of cell cycle markers in models of induced programmed cell death in dopamine neurons of the rat substantia nigra pars compacta. Exp Neurol 179:17-27.

Endoh M, Maiese K, Wagner J (1994) Expression of the inducible form of nitric oxide synthase by reactive astrocytes after transient global ischemia. Brain Res 651:92-100.

Eskes R, Desagher S, Antonsson B, Martinou JC (2000) Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol Cell Biol 20:929-935.

Fang J, Nakamura T, Cho DH, Gu Z, Lipton SA (2007) S-nitrosylation of peroxiredoxin 2 promotes oxidative stress-induced neuronal cell death in Parkinson's disease. Proc Natl Acad Sci U S A 104:18742-18747.

Fillenz M (1995) Physiological release of excitatory amino acids. Behav Brain Res 71:51-67.

Ford HL, Pardee AB (1998) The S phase: beginning, middle, and end: a perspective. J Cell Biochem Suppl 30-31:1-7.

Freeman RS, Estus S, Johnson EM, Jr. (1994) Analysis of cell cycle-related gene expression in postmitotic neurons: selective induction of Cyclin D1 during programmed cell death. Neuron 12:343-355.

Friedlander RM, Gagliardini V, Hara H, Fink KB, Li W, MacDonald G, Fishman MC, Greenberg AH, Moskowitz MA, Yuan J (1997) Expression of a dominant negative mutant of interleukin-1 beta converting enzyme in transgenic mice prevents neuronal cell death induced by trophic factor withdrawal and ischemic brain injury. J Exp Med 185:933-940.

Frolov MV, Dyson NJ (2004) Molecular mechanisms of E2F-dependent activation and pRB-mediated repression. J Cell Sci 117:2173-2181.

Fu X, Choi YK, Qu D, Yu Y, Cheung NS, Qi RZ (2006) Identification of nuclear import mechanisms for the neuronal Cdk5 activator. J Biol Chem 281:39014-39021.

Fujimura M, Morita-Fujimura Y, Murakami K, Kawase M, Chan PH (1998) Cytosolic redistribution of cytochrome c after transient focal cerebral ischemia in rats. J Cereb Blood Flow Metab 18:1239-1247.

Fukui S, Ookawara T, Nawashiro H, Suzuki K, Shima K (2002) Post-ischemic transcriptional and translational responses of EC-SOD in mouse brain and serum. Free Radic Biol Med 32:289-298.

Fuxe K, Bjelke B, Andbjer B, Grahn H, Rimondini R, Agnati LF (1997) Endothelin-1 induced lesions of the frontoparietal cortex of the rat. A possible model of focal cortical ischemia. Neuroreport 8:2623-2629.

Fuxe K, Kurosawa N, Cintra A, Hallstrom A, Goiny M, Rosen L, Agnati LF, Ungerstedt U (1992) Involvement of local ischemia in endothelin-1 induced lesions of the neostriatum of the anaesthetized rat. Exp Brain Res 88:131-139.

Gao CY, Zelenka PS (1995) Induction of cyclin B and H1 kinase activity in apoptotic PC12 cells. Exp Cell Res 219:612-618.

Gardell SJ (1993) The search for the ideal thrombolytic agent: maximize the benefit and minimize the risk. Toxicol Pathol 21:190-198.

Gendron TF, Mealing GA, Paris J, Lou A, Edwards A, Hou ST, MacManus JP, Hakim AM, Morley P (2001) Attenuation of neurotoxicity in cortical cultures and hippocampal slices from E2F1 knockout mice. J Neurochem 78:316-324.

Germain D, Russell A, Thompson A, Hendley J (2000) Ubiquitination of free cyclin D1 is independent of phosphorylation on threonine 286. J Biol Chem 275:12074-12079.

Giardina SF, Beart PM (2002) Kainate receptor-mediated apoptosis in primary cultures of cerebellar granule cells is attenuated by mitogen-activated protein and cyclin-dependent kinase inhibitors. Br J Pharmacol 135:1733-1742.

Giffard RG, Swanson RA (2005) Ischemia-induced programmed cell death in astrocytes. Glia 50:299-306.

Gillardon F, Lenz C, Waschke KF, Krajewski S, Reed JC, Zimmermann M, Kuschinsky W (1996) Altered expression of Bcl-2, Bcl-X, Bax, and c-Fos colocalizes with DNA fragmentation and ischemic cell damage following middle cerebral artery occlusion in rats. Brain Res Mol Brain Res 40:254-260.

Gilmore EC, Ohshima T, Goffinet AM, Kulkarni AB, Herrup K (1998) Cyclin-dependent kinase 5-deficient mice demonstrate novel developmental arrest in cerebral cortex. J Neurosci 18:6370-6377.

Giovanni A, Keramaris E, Morris EJ, Hou ST, O'Hare M, Dyson N, Robertson GS, Slack RS, Park DS (2000) E2F1 mediates death of B-amyloid-treated cortical neurons in a manner independent of p53 and dependent on Bax and caspase 3. J Biol Chem 275:11553-11560.

Giulivi C, Boveris A, Cadenas E (1995) Hydroxyl radical generation during mitochondrial electron transfer and the formation of 8-hydroxydesoxyguanosine in mitochondrial DNA. Arch Biochem Biophys 316:909-916.

Gold MO, Rice AP (1998) Targeting of CDK8 to a promoter-proximal RNA element demonstrates catalysis-dependent activation of gene expression. Nucleic Acids Res 26:3784-3788.

Gong X, Tang X, Wiedmann M, Wang X, Peng J, Zheng D, Blair LA, Marshall J, Mao Z (2003) Cdk5-mediated inhibition of the protective effects of transcription factor MEF2 in neurotoxicity-induced apoptosis. Neuron 38:33-46.

Graham SH, Chen J (2001) Programmed cell death in cerebral ischemia. J Cereb Blood Flow Metab 21:99-109.

Graham SM, McCullough LD, Murphy SJ (2004) Animal models of ischemic stroke: balancing experimental aims and animal care. Comp Med 54:486-496.

Gravanis I, Tsirka SE (2008) Tissue-type plasminogen activator as a therapeutic target in stroke. Expert Opin Ther Targets 12:159-170.

Green DR, Reed JC (1998) Mitochondria and apoptosis. Science 281:1309-1312.

Gu Y, Rosenblatt J, Morgan DO (1992) Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15. Embo J 11:3995-4005.

Guegan C, Levy V, David JP, Ajchenbaum-Cymbalista F, Sola B (1997) c-Jun and cyclin D1 proteins as mediators of neuronal death after a focal ischaemic insult. Neuroreport 8:1003-1007.

Halliwell B (2006) Oxidative stress and neurodegeneration: where are we now? J Neurochem 97:1634-1658.

Hallows JL, Chen K, DePinho RA, Vincent I (2003) Decreased cyclin-dependent kinase 5 (cdk5) activity is accompanied by redistribution of cdk5 and cytoskeletal proteins and increased cytoskeletal protein phosphorylation in p35 null mice. J Neurosci 23:10633-10644.

Hamdane M, Bretteville A, Sambo AV, Schindowski K, Begard S, Delacourte A, Bertrand P, Buee L (2005) p25/Cdk5-mediated retinoblastoma phosphorylation is an early event in neuronal cell death. J Cell Sci 118:1291-1298.

Harbour JW, Luo RX, Dei Santi A, Postigo AA, Dean DC (1999) Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. Cell 98:859-869.

Hattori F, Murayama N, Noshita T, Oikawa S (2003) Mitochondrial peroxiredoxin-3 protects hippocampal neurons from excitotoxic injury in vivo. J Neurochem 86:860-868.

Hayashi T, Warita H, Abe K, Itoyama Y (1999a) Expression of cyclin-dependent kinase 5 and its activator p35 in rat brain after middle cerebral artery occlusion. Neurosci Lett 265:37-40.

Hayashi T, Sakurai M, Abe K, Itoyama Y (1999b) DNA fragmentation precedes aberrant expression of cell cycle-related protein in rat brain after MCA occlusion. Neurol Res 21:695-698.

He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B (1998) A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A 95:2509-2514.

Heidenreich KA, Linseman DA (2004) Myocyte enhancer factor-2 transcription factors in neuronal differentiation and survival. Mol Neurobiol 29:155-166.

Helin K, Harlow E, Fattaey A (1993) Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. Mol Cell Biol 13:6501-6508.

Hengartner MO (2000) The biochemistry of apoptosis. Nature 407:770-776.

Herrup K, Arendt T (2002) Re-expression of cell cycle proteins induces neuronal cell death during Alzheimer's disease. J Alzheimers Dis 4:243-247.

Honma N, Hosono Y, Kishimoto T, Hisanaga S (1997) Phosphorylation of retinoblastoma protein at apoptotic cell death in rat neuroblastoma B50 cells. Neurosci Lett 235:45-48.

Hou ST, MacManus JP (2002) Molecular mechanisms of cerebral ischemia-induced neuronal death. Int Rev Cytol 221:93-148.

Huang J, Choudhri TF, Winfree CJ, McTaggart RA, Kiss S, Mocco J, Kim LJ, Protopsaltis TS, Zhang Y, Pinsky DJ, Connolly ES, Jr. (2000) Postischemic cerebrovascular E-selectin expression mediates tissue injury in murine stroke. Stroke 31:3047-3053.

Hwang IK, Hua L, Yoo KY, Kim DW, Kang TC, Choi SY, Won MH, Kim DH (2005) Antioxidant-like protein 1 is altered in non-pyramidal cells and expressed in astrocytes in the gerbil hippocampal CA1 region after transient forebrain ischemia. Brain Res 1062:111-119.

Iadecola C, Zhang F, Xu S, Casey R, Ross ME (1995) Inducible nitric oxide synthase gene expression in brain following cerebral ischemia. J Cereb Blood Flow Metab 15:378-384.

Iadecola C, Zhang F, Casey R, Nagayama M, Ross ME (1997) Delayed reduction of ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene. J Neurosci 17:9157-9164.

Iadecola C, Niwa K, Nogawa S, Zhao X, Nagayama M, Araki E, Morham S, Ross ME (2001) Reduced susceptibility to ischemic brain injury and N-methyl-D-aspartate-

mediated neurotoxicity in cyclooxygenase-2-deficient mice. Proc Natl Acad Sci U S A 98:1294-1299.

Immenschuh S, Baumgart-Vogt E (2005) Peroxiredoxins, oxidative stress, and cell proliferation. Antioxid Redox Signal 7:768-777.

Ino H, Chiba T (1996) Intracellular localization of cyclin-dependent kinase 5 (CDK5) in mouse neuron: CDK5 is located in both nucleus and cytoplasm. Brain Res 732:179-185.

Ino H, Chiba T (2001) Cyclin-dependent kinase 4 and cyclin D1 are required for excitotoxin-induced neuronal cell death in vivo. J Neurosci 21:6086-6094.

Inoue S, Drummond JC, Davis DP, Cole DJ, Patel PM (2004) Combination of isoflurane and caspase inhibition reduces cerebral injury in rats subjected to focal cerebral ischemia. Anesthesiology 101:75-81.

Isaev NK, Stelmashook EV, Plotnikov EY, Khryapenkova TG, Lozier ER, Doludin YV, Silachev DN, Zorov DB (2008) Role of acidosis, NMDA receptors, and acid-sensitive ion channel 1a (ASIC1a) in neuronal death induced by ischemia. Biochemistry (Mosc) 73:1171-1175.

Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA, Weinberg RA (1992) Effects of an Rb mutation in the mouse. Nature 359:295-300.

Jagoda A, Chan YF (2008) Transient ischemic attack overview: defining the challenges for improving outcomes. Ann Emerg Med 52:S3-6.

Jang HH, Lee KO, Chi YH, Jung BG, Park SK, Park JH, Lee JR, Lee SS, Moon JC, Yun JW, Choi YO, Kim WY, Kang JS, Cheong GW, Yun DJ, Rhee SG, Cho MJ, Lee SY (2004) Two enzymes in one; two yeast peroxiredoxins display oxidative

stress-dependent switching from a peroxidase to a molecular chaperone function. Cell 117:625-635.

Janicke RU, Sohn D, Schulze-Osthoff K (2008) The dark side of a tumor suppressor: anti-apoptotic p53. Cell Death Differ 15:959-976.

Jeyaseelan K, Lim KY, Armugam A (2008) Neuroprotectants in stroke therapy. Expert Opin Pharmacother 9:887-900.

Jiang X, Wang X (2000) Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. J Biol Chem 275:31199-31203.

Jin K, Graham SH, Mao X, Nagayama T, Simon RP, Greenberg DA (2001) Fas (CD95) may mediate delayed cell death in hippocampal CA1 sector after global cerebral ischemia. J Cereb Blood Flow Metab 21:1411-1421.

Jin MH, Lee YH, Kim JM, Sun HN, Moon EY, Shong MH, Kim SU, Lee SH, Lee TH, Yu DY, Lee DS (2005) Characterization of neural cell types expressing peroxiredoxins in mouse brain. Neurosci Lett 381:252-257.

Johansson JU, Lilja L, Chen XL, Higashida H, Meister B, Noda M, Zhong ZG, Yokoyama S, Berggren PO, Bark C (2005) Cyclin-dependent kinase 5 activators p35 and p39 facilitate formation of functional synapses. Brain Res Mol Brain Res 138:215-227.

Julien JP, Mushynski WE (1998) Neurofilaments in health and disease. Prog Nucleic Acid Res Mol Biol 61:1-23.

Kader A, Frazzini VI, Solomon RA, Trifiletti RR (1993) Nitric oxide production during focal cerebral ischemia in rats. Stroke 24:1709-1716.

Kaldis P (1999) The cdk-activating kinase (CAK): from yeast to mammals. Cell Mol Life Sci 55:284-296.

Katchanov J, Harms C, Gertz K, Hauck L, Waeber C, Hirt L, Priller J, von Harsdorf R, Bruck W, Hortnagl H, Dirnagl U, Bhide PG, Endres M (2001) Mild cerebral ischemia induces loss of cyclin-dependent kinase inhibitors and activation of cell cycle machinery before delayed neuronal cell death. J Neurosci 21:5045-5053.

Kato H, Takahashi A, Itoyama Y (2003) Cell cycle protein expression in proliferating microglia and astrocytes following transient global cerebral ischemia in the rat. Brain Res Bull 60:215-221.

Kato S, Saeki Y, Aoki M, Nagai M, Ishigaki A, Itoyama Y, Kato M, Asayama K, Awaya A, Hirano A, Ohama E (2004) Histological evidence of redox system breakdown caused by superoxide dismutase 1 (SOD1) aggregation is common to SOD1-mutated motor neurons in humans and animal models. Acta Neuropathol 107:149-158.

Kaufmann SH, Hengartner MO (2001) Programmed cell death: alive and well in the new millennium. Trends Cell Biol 11:526-534.

Kawase M, Murakami K, Fujimura M, Morita-Fujimura Y, Gasche Y, Kondo T, Scott RW, Chan PH (1999) Exacerbation of delayed cell injury after transient global ischemia in mutant mice with CuZn superoxide dismutase deficiency. Stroke 30:1962-1968.

Kim GW, Kondo T, Noshita N, Chan PH (2002) Manganese superoxide dismutase deficiency exacerbates cerebral infarction after focal cerebral

ischemia/reperfusion in mice: implications for the production and role of superoxide radicals. Stroke 33:809-815.

Kim SH, Fountoulakis M, Cairns N, Lubec G (2001) Protein levels of human peroxiredoxin subtypes in brains of patients with Alzheimer's disease and Down syndrome. J Neural Transm Suppl:223-235.

Knowles RG, Moncada S (1994) Nitric oxide synthases in mammals. Biochem J 298 (Pt 2):249-258.

Ko J, Humbert S, Bronson RT, Takahashi S, Kulkarni AB, Li E, Tsai LH (2001) p35 and p39 are essential for cyclin-dependent kinase 5 function during neurodevelopment. J Neurosci 21:6758-6771.

Koff A, Giordano A, Desai D, Yamashita K, Harper JW, Elledge S, Nishimoto T, Morgan DO, Franza BR, Roberts JM (1992) Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. Science 257:1689-1694.

Konishi Y, Bonni A (2003) The E2F-Cdc2 cell-cycle pathway specifically mediates activity deprivation-induced apoptosis of postmitotic neurons. J Neurosci 23:1649-1658.

Koo KH, Lee S, Jeong SY, Kim ET, Kim HJ, Kim K, Song K, Chae HZ (2002) Regulation of thioredoxin peroxidase activity by C-terminal truncation. Arch Biochem Biophys 397:312-318.

Krajewski S, Mai JK, Krajewska M, Sikorska M, Mossakowski MJ, Reed JC (1995) Upregulation of bax protein levels in neurons following cerebral ischemia. J Neurosci 15:6364-6376.

Krajewski S, Krajewska M, Ellerby LM, Welsh K, Xie Z, Deveraux QL, Salvesen GS, Bredesen DE, Rosenthal RE, Fiskum G, Reed JC (1999) Release of caspase-9 from mitochondria during neuronal apoptosis and cerebral ischemia. Proc Natl Acad Sci U S A 96:5752-5757.

Kranenburg O, Scharnhorst V, Van der Eb AJ, Zantema A (1995) Inhibition of cyclin-dependent kinase activity triggers neuronal differentiation of mouse neuroblastoma cells. J Cell Biol 131:227-234.

Krapfenbauer K, Engidawork E, Cairns N, Fountoulakis M, Lubec G (2003) Aberrant expression of peroxiredoxin subtypes in neurodegenerative disorders. Brain Res 967:152-160.

Kristian T, Siesjo BK (1998) Calcium in ischemic cell death. Stroke 29:705-718.

Krupinski J, Vodovotz Y, Li C, Slowik A, Beevers D, Flanders KC, Lip G, Kumar P, Szczudlik A (1998) Inducible nitric oxide production and expression of transforming growth factor-beta1 in serum and CSF after cerebral ischaemic stroke in man. Nitric Oxide 2:442-453.

Kuan CY, Schloemer AJ, Lu A, Burns KA, Weng WL, Williams MT, Strauss KI, Vorhees CV, Flavell RA, Davis RJ, Sharp FR, Rakic P (2004) Hypoxia-ischemia induces DNA synthesis without cell proliferation in dying neurons in adult rodent brain. J Neurosci 24:10763-10772.

Kusakawa G, Saito T, Onuki R, Ishiguro K, Kishimoto T, Hisanaga S (2000) Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25. J Biol Chem 275:17166-17172.

Kwon YT, Tsai LH, Crandall JE (1999) Callosal axon guidance defects in p35(-/-) mice. J Comp Neurol 415:218-229.

Kwon YT, Gupta A, Zhou Y, Nikolic M, Tsai LH (2000) Regulation of N-cadherin-mediated adhesion by the p35-Cdk5 kinase. Curr Biol 10:363-372.

Leclerc S, Garnier M, Hoessel R, Marko D, Bibb JA, Snyder GL, Greengard P, Biernat J, Wu YZ, Mandelkow EM, Eisenbrand G, Meijer L (2001) Indirubins inhibit glycogen synthase kinase-3 beta and CDK5/p25, two protein kinases involved in abnormal tau phosphorylation in Alzheimer's disease. A property common to most cyclin-dependent kinase inhibitors? J Biol Chem 276:251-260.

Lee EY, Chang CY, Hu N, Wang YC, Lai CC, Herrup K, Lee WH, Bradley A (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature 359:288-294.

Lee JH, Kim HS, Lee SJ, Kim KT (2007) Stabilization and activation of p53 induced by Cdk5 contributes to neuronal cell death. J Cell Sci 120:2259-2271.

Lee JH, Jeong MW, Kim W, Choi YH, Kim KT (2008) Cooperative roles of c-Abl and Cdk5 in regulation of p53 in response to oxidative stress. J Biol Chem 283:19826-19835.

Lee KY, Helbing CC, Choi KS, Johnston RN, Wang JH (1997) Neuronal Cdc2-like kinase (Nclk) binds and phosphorylates the retinoblastoma protein. J Biol Chem 272:5622-5626.

Li BS, Zhang L, Takahashi S, Ma W, Jaffe H, Kulkarni AB, Pant HC (2002) Cyclin-dependent kinase 5 prevents neuronal apoptosis by negative regulation of c-Jun N-terminal kinase 3. Embo J 21:324-333.

Li BS, Ma W, Jaffe H, Zheng Y, Takahashi S, Zhang L, Kulkarni AB, Pant HC (2003) Cyclin-dependent kinase-5 is involved in neuregulin-dependent activation of phosphatidylinositol 3-kinase and Akt activity mediating neuronal survival. J Biol Chem 278:35702-35709.

Li BS, Sun MK, Zhang L, Takahashi S, Ma W, Vinade L, Kulkarni AB, Brady RO, Pant HC (2001) Regulation of NMDA receptors by cyclin-dependent kinase-5. Proc Natl Acad Sci U S A 98:12742-12747.

Li H, Zhu H, Xu CJ, Yuan J (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94:491-501.

Li Y, Chopp M, Jiang N, Zaloga C (1995) In situ detection of DNA fragmentation after focal cerebral ischemia in mice. Brain Res Mol Brain Res 28:164-168.

Li Y, Chopp M, Powers C, Jiang N (1997) Apoptosis and protein expression after focal cerebral ischemia in rat. Brain Res 765:301-312.

Lin H, Lin TY, Juang JL (2007) Abl deregulates Cdk5 kinase activity and subcellular localization in Drosophila neurodegeneration. Cell Death Differ 14:607-615.

Lipton SA (2006) NMDA receptors, glial cells, and clinical medicine. Neuron 50:9-11.

Lissy NA, Davis PK, Irwin M, Kaelin WG, Dowdy SF (2000) A common E2F-1 and p73 pathway mediates cell death induced by TCR activation. Nature 407:642-645.

Liu B, Liao M, Mielke JG, Ning K, Chen Y, Li L, El-Hayek YH, Gomez E, Zukin RS, Fehlings MG, Wan Q (2006) Ischemic insults direct glutamate receptor subunit 2-lacking AMPA receptors to synaptic sites. J Neurosci 26:5309-5319.

Liu DX, Greene LA (2001) Regulation of neuronal survival and death by E2F-dependent gene repression and derepression. Neuron 32:425-438.

Liu T, Clark RK, McDonnell PC, Young PR, White RF, Barone FC, Feuerstein GZ (1994) Tumor necrosis factor-alpha expression in ischemic neurons. Stroke 25:1481-1488.

Liu W, Bi X, Tocco G, Baudry M, Schreiber SS (1996a) Increased expression of cyclin D1 in the adult rat brain following kainic acid treatment. Neuroreport 7:2785-2789.

Liu X, Kim CN, Yang J, Jemmerson R, Wang X (1996b) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86:147-157.

Lo EH, Dalkara T, Moskowitz MA (2003) Mechanisms, challenges and opportunities in stroke. Nat Rev Neurosci 4:399-415.

Love S (2003a) Apoptosis and brain ischaemia. Prog Neuropsychopharmacol Biol Psychiatry 27:267-282.

Love S (2003b) Neuronal expression of cell cycle-related proteins after brain ischaemia in man. Neurosci Lett 353:29-32.

Lukas J, Petersen BO, Holm K, Bartek J, Helin K (1996) Deregulated expression of E2F family members induces S-phase entry and overcomes p16INK4A-mediated growth suppression. Mol Cell Biol 16:1047-1057.

Lundberg AS, Weinberg RA (1998) Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol Cell Biol 18:753-761.

Ma D, Zhou P, Harbour JW (2003) Distinct mechanisms for regulating the tumor suppressor and antiapoptotic functions of Rb. J Biol Chem 278:19358-19366.

MacManus JP, Linnik MD (1997) Gene expression induced by cerebral ischemia: an apoptotic perspective. J Cereb Blood Flow Metab 17:815-832.

MacManus JP, Hill IE, Huang ZG, Rasquinha I, Xue D, Buchan AM (1994) DNA damage consistent with apoptosis in transient focal ischaemic neocortex. Neuroreport 5:493-496.

MacManus JP, Jian M, Preston E, Rasquinha I, Webster J, Zurakowski B (2003) Absence of the transcription factor E2F1 attenuates brain injury and improves behavior after focal ischemia in mice. J Cereb Blood Flow Metab 23:1020-1028.

Malinski T, Bailey F, Zhang ZG, Chopp M (1993) Nitric oxide measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion. J Cereb Blood Flow Metab 13:355-358.

Mao Z, Wiedmann M (1999) Calcineurin enhances MEF2 DNA binding activity in calcium-dependent survival of cerebellar granule neurons. J Biol Chem 274:31102-31107.

Margaill I, Plotkine M, Lerouet D (2005) Antioxidant strategies in the treatment of stroke. Free Radic Biol Med 39:429-443.

Markgraf CG, Velayo NL, Johnson MP, McCarty DR, Medhi S, Koehl JR, Chmielewski PA, Linnik MD (1998) Six-hour window of opportunity for calpain inhibition in focal cerebral ischemia in rats. Stroke 29:152-158.

Martin RL, Lloyd HG, Cowan AI (1994) The early events of oxygen and glucose deprivation: setting the scene for neuronal death? Trends Neurosci 17:251-257.

Martin-Villalba A, Herr I, Jeremias I, Hahne M, Brandt R, Vogel J, Schenkel J, Herdegen T, Debatin KM (1999) CD95 ligand (Fas-L/APO-1L) and tumor necrosis factor-

related apoptosis-inducing ligand mediate ischemia-induced apoptosis in neurons. J Neurosci 19:3809-3817.

Matsuoka Y, Kitamura Y, Tooyama I, Kimura H, Taniguchi T (1997) In vivo hypoxia-induced neuronal damage with an enhancement of neuronal nitric oxide synthase immunoreactivity in hippocampus. Exp Neurol 146:57-66.

Matsuura I, Wang JH (1996) Demonstration of cyclin-dependent kinase inhibitory serine/threonine kinase in bovine thymus. J Biol Chem 271:5443-5450.

Mehta SL, Manhas N, Raghubir R (2007) Molecular targets in cerebral ischemia for developing novel therapeutics. Brain Res Rev 54:34-66.

Meldrum BS (2000) Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J Nutr 130:1007S-1015S.

Mergenthaler P, Dirnagl U, Meisel A (2004) Pathophysiology of stroke: lessons from animal models. Metab Brain Dis 19:151-167.

Mitsios N, Pennucci R, Krupinski J, Sanfeliu C, Gaffney J, Kumar P, Kumar S, Juan-Babot O, Slevin M (2007) Expression of cyclin-dependent kinase 5 mRNA and protein in the human brain following acute ischemic stroke. Brain Pathol 17:11-23.

Miyajima M, Nornes HO, Neuman T (1995) Cyclin E is expressed in neurons and forms complexes with cdk5. Neuroreport 6:1130-1132.

Mocco J, Choudhri T, Huang J, Harfeldt E, Efros L, Klingbeil C, Vexler V, Hall W, Zhang Y, Mack W, Popilskis S, Pinsky DJ, Connolly ES, Jr. (2002) HuEP5C7 as a humanized monoclonal anti-E/P-selectin neurovascular protective strategy in a

blinded placebo-controlled trial of nonhuman primate stroke. Circ Res 91:907-914.

Montero M, Nielsen M, Ronn LC, Moller A, Noraberg J, Zimmer J (2007) Neuroprotective effects of the AMPA antagonist PNQX in oxygen-glucose deprivation in mouse hippocampal slice cultures and global cerebral ischemia in gerbils. Brain Res 1177:124-135.

Moon JC, Hah YS, Kim WY, Jung BG, Jang HH, Lee JR, Kim SY, Lee YM, Jeon MG, Kim CW, Cho MJ, Lee SY (2005) Oxidative stress-dependent structural and functional switching of a human 2-Cys peroxiredoxin isotype II that enhances HeLa cell resistance to H2O2-induced cell death. J Biol Chem 280:28775-28784.

Morgan DO (1997) Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol 13:261-291.

Mori T, Tateishi N, Kagamiishi Y, Shimoda T, Satoh S, Ono S, Katsube N, Asano T (2004) Attenuation of a delayed increase in the extracellular glutamate level in the peri-infarct area following focal cerebral ischemia by a novel agent ONO-2506. Neurochem Int 45:381-387.

Morioka M, Kawano T, Yano S, Kai Y, Tsuiki H, Yoshinaga Y, Matsumoto J, Maeda T, Hamada J, Yamamoto H, Fukunaga K, Kuratsu J (2006) Hyperphosphorylation at serine 199/202 of tau factor in the gerbil hippocampus after transient forebrain ischemia. Biochem Biophys Res Commun 347:273-278.

Moro MA, Almeida A, Bolanos JP, Lizasoain I (2005) Mitochondrial respiratory chain and free radical generation in stroke. Free Radic Biol Med 39:1291-1304.

Moroni MC, Hickman ES, Lazzerini Denchi E, Caprara G, Colli E, Cecconi F, Muller H, Helin K (2001) Apaf-1 is a transcriptional target for E2F and p53. Nat Cell Biol 3:552-558.

Morris EJ, Dyson NJ (2001) Retinoblastoma protein partners. Adv Cancer Res 82:1-54.

Moustafa RR, Baron JC (2008) Pathophysiology of ischaemic stroke: insights from imaging, and implications for therapy and drug discovery. Br J Pharmacol 153 Suppl 1:S44-54.

Mouw G, Zechel JL, Zhou Y, Lust WD, Selman WR, Ratcheson RA (2002) Caspase-9 inhibition after focal cerebral ischemia improves outcome following reversible focal ischemia. Metab Brain Dis 17:143-151.

Murase K, Kato H, Kogure K (1993) Limited but evident protective effects of MK-801 and pentobarbital on neuronal damage following forebrain ischemia in the gerbil under normothermic conditions. Neurosci Lett 149:229-232.

Nagata S (1997) Apoptosis by death factor. Cell 88:355-365.

Nahle Z, Polakoff J, Davuluri RV, McCurrach ME, Jacobson MD, Narita M, Zhang MQ, Lazebnik Y, Bar-Sagi D, Lowe SW (2002) Direct coupling of the cell cycle and cell death machinery by E2F. Nat Cell Biol 4:859-864.

Nakamura S, Kawamoto Y, Nakano S, Akiguchi I, Kimura J (1997) p35nck5a and cyclin-dependent kinase 5 colocalize in Lewy bodies of brains with Parkinson's disease. Acta Neuropathol 94:153-157.

Nakayama M, Uchimura K, Zhu RL, Nagayama T, Rose ME, Stetler RA, Isakson PC, Chen J, Graham SH (1998) Cyclooxygenase-2 inhibition prevents delayed death

of CA1 hippocampal neurons following global ischemia. Proc Natl Acad Sci U S A 95:10954-10959.

Namura S, Zhu J, Fink K, Endres M, Srinivasan A, Tomaselli KJ, Yuan J, Moskowitz MA (1998) Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. J Neurosci 18:3659-3668.

Nguyen MD, Julien JP (2003) Cyclin-dependent kinase 5 in amyotrophic lateral sclerosis. Neurosignals 12:215-220.

Nguyen MD, Lariviere RC, Julien JP (2001) Deregulation of Cdk5 in a mouse model of ALS: toxicity alleviated by perikaryal neurofilament inclusions. Neuron 30:135-147.

Nguyen MD, Boudreau M, Kriz J, Couillard-Despres S, Kaplan DR, Julien JP (2003) Cell cycle regulators in the neuronal death pathway of amyotrophic lateral sclerosis caused by mutant superoxide dismutase 1. J Neurosci 23:2131-2140.

Ni B, Wu X, Su Y, Stephenson D, Smalstig EB, Clemens J, Paul SM (1998) Transient global forebrain ischemia induces a prolonged expression of the caspase-3 mRNA in rat hippocampal CA1 pyramidal neurons. J Cereb Blood Flow Metab 18:248-256.

Nicole O, Docagne F, Ali C, Margaill I, Carmeliet P, MacKenzie ET, Vivien D, Buisson A (2001) The proteolytic activity of tissue-plasminogen activator enhances NMDA receptor-mediated signaling. Nat Med 7:59-64.

Nita DA, Nita V, Spulber S, Moldovan M, Popa DP, Zagrean AM, Zagrean L (2001) Oxidative damage following cerebral ischemia depends on reperfusion - a biochemical study in rat. J Cell Mol Med 5:163-170.

O'Hare MJ, Hou ST, Morris EJ, Cregan SP, Xu Q, Slack RS, Park DS (2000) Induction and modulation of cerebellar granule neuron death by E2F-1. J Biol Chem 275:25358-25364.

O'Hare MJ, Kushwaha N, Zhang Y, Aleyasin H, Callaghan SM, Slack RS, Albert PR, Vincent I, Park DS (2005) Differential roles of nuclear and cytoplasmic cyclin-dependent kinase 5 in apoptotic and excitotoxic neuronal death. J Neurosci 25:8954-8966.

Obaya AJ, Sedivy JM (2002) Regulation of cyclin-Cdk activity in mammalian cells. Cell Mol Life Sci 59:126-142.

Ohshima T, Gilmore EC, Longenecker G, Jacobowitz DM, Brady RO, Herrup K, Kulkarni AB (1999) Migration defects of cdk5(-/-) neurons in the developing cerebellum is cell autonomous. J Neurosci 19:6017-6026.

Ohshima T, Ward JM, Huh CG, Longenecker G, Veeranna, Pant HC, Brady RO, Martin LJ, Kulkarni AB (1996) Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. Proc Natl Acad Sci U S A 93:11173-11178.

Ohtsuki T, Kitagawa K, Yamagata K, Mandai K, Mabuchi T, Matsushita K, Yanagihara T, Matsumoto M (1996) Induction of cyclooxygenase-2 mRNA in gerbil hippocampal neurons after transient forebrain ischemia. Brain Res 736:353-356.

Olney JW (1969) Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. Science 164:719-721.

Osuga H, Osuga S, Wang F, Fetni R, Hogan MJ, Slack RS, Hakim AM, Ikeda JE, Park DS (2000) Cyclin-dependent kinases as a therapeutic target for stroke. Proc Natl Acad Sci U S A 97:10254-10259.

Panickar KS, Nonner D, White MG, Barrett JN (2008) Overexpression of Cdk5 or non-phosphorylatable retinoblastoma protein protects septal neurons from oxygen-glucose deprivation. Neurochem Res 33:1852-1858.

Pareek TK, Keller J, Kesavapany S, Pant HC, Iadarola MJ, Brady RO, Kulkarni AB (2006) Cyclin-dependent kinase 5 activity regulates pain signaling. Proc Natl Acad Sci U S A 103:791-796.

Park DS, Levine B, Ferrari G, Greene LA (1997a) Cyclin dependent kinase inhibitors and dominant negative cyclin dependent kinase 4 and 6 promote survival of NGF-deprived sympathetic neurons. J Neurosci 17:8975-8983.

Park DS, Morris EJ, Greene LA, Geller HM (1997b) G1/S cell cycle blockers and inhibitors of cyclin-dependent kinases suppress camptothecin-induced neuronal apoptosis. J Neurosci 17:1256-1270.

Park DS, Morris EJ, Bremner R, Keramaris E, Padmanabhan J, Rosenbaum M, Shelanski ML, Geller HM, Greene LA (2000) Involvement of retinoblastoma family members and E2F/DP complexes in the death of neurons evoked by DNA damage. J Neurosci 20:3104-3114.

Patrick GN, Zukerberg L, Nikolic M, de la Monte S, Dikkes P, Tsai LH (1999) Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature 402:615-622.

Patrick GN, Zukerberg L, Nikolic M, de La Monte S, Dikkes P, Tsai LH (2001) reply: Neurobiologyp25 protein in neurodegeneration. Nature 411:764-765.

Peng PL, Zhong X, Tu W, Soundarapandian MM, Molner P, Zhu D, Lau L, Liu S, Liu F, Lu Y (2006) ADAR2-dependent RNA editing of AMPA receptor subunit GluR2 determines vulnerability of neurons in forebrain ischemia. Neuron 49:719-733.

Perez-Pinzon MA, Xu GP, Born J, Lorenzo J, Busto R, Rosenthal M, Sick TJ (1999) Cytochrome C is released from mitochondria into the cytosol after cerebral anoxia or ischemia. J Cereb Blood Flow Metab 19:39-43.

Peshenko IV, Shichi H (2001) Oxidation of active center cysteine of bovine 1-Cys peroxiredoxin to the cysteine sulfenic acid form by peroxide and peroxynitrite. Free Radic Biol Med 31:292-303.

Piantadosi CA, Zhang J (1996) Mitochondrial generation of reactive oxygen species after brain ischemia in the rat. Stroke 27:327-331; discussion 332.

Pines J (1993a) Cyclins and their associated cyclin-dependent kinases in the human cell cycle. Biochem Soc Trans 21:921-925.

Pines J (1993b) Cyclins and cyclin-dependent kinases: take your partners. Trends Biochem Sci 18:195-197.

Planas AM, Soriano MA, Justicia C, Rodriguez-Farre E (1999) Induction of cyclooxygenase-2 in the rat brain after a mild episode of focal ischemia without tissue inflammation or neural cell damage. Neurosci Lett 275:141-144.

Plesnila N, Zinkel S, Amin-Hanjani S, Qiu J, Korsmeyer SJ, Moskowitz MA (2002) Function of BID -- a molecule of the bcl-2 family -- in ischemic cell death in the brain. Eur Surg Res 34:37-41.

Plesnila N, Zhu C, Culmsee C, Groger M, Moskowitz MA, Blomgren K (2004) Nuclear translocation of apoptosis-inducing factor after focal cerebral ischemia. J Cereb Blood Flow Metab 24:458-466.

Plesnila N, Zinkel S, Le DA, Amin-Hanjani S, Wu Y, Qiu J, Chiarugi A, Thomas SS, Kohane DS, Korsmeyer SJ, Moskowitz MA (2001) BID mediates neuronal cell death after oxygen/ glucose deprivation and focal cerebral ischemia. Proc Natl Acad Sci U S A 98:15318-15323.

Poon RY, Lew J, Hunter T (1997) Identification of functional domains in the neuronal Cdk5 activator protein. J Biol Chem 272:5703-5708.

Potthoff MJ, Olson EN (2007) MEF2: a central regulator of diverse developmental programs. Development 134:4131-4140.

Pulsinelli WA, Brierley JB (1979) A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke 10:267-272.

Pulsinelli WA, Buchan AM (1988) The four-vessel occlusion rat model: method for complete occlusion of vertebral arteries and control of collateral circulation. Stroke 19:913-914.

Pulsinelli WA, Levy DE, Duffy TE (1982a) Regional cerebral blood flow and glucose metabolism following transient forebrain ischemia. Ann Neurol 11:499-502.

Pulsinelli WA, Brierley JB, Plum F (1982b) Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann Neurol 11:491-498.

Qu D, Li Q, Lim HY, Cheung NS, Li R, Wang JH, Qi RZ (2002) The protein SET binds the neuronal Cdk5 activator p35nck5a and modulates Cdk5/p35nck5a activity. J Biol Chem 277:7324-7332.

Qu D, Rashidian J, Mount MP, Aleyasin H, Parsanejad M, Lira A, Haque E, Zhang Y, Callaghan S, Daigle M, Rousseaux MW, Slack RS, Albert PR, Vincent I, Woulfe JM, Park DS (2007) Role of Cdk5-mediated phosphorylation of Prx2 in MPTP toxicity and Parkinson's disease. Neuron 55:37-52.

Radi R, Beckman JS, Bush KM, Freeman BA (1991) Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. J Biol Chem 266:4244-4250.

Radi R, Cassina A, Hodara R, Quijano C, Castro L (2002) Peroxynitrite reactions and formation in mitochondria. Free Radic Biol Med 33:1451-1464.

Rami A, Sims J, Botez G, Winckler J (2003) Spatial resolution of phospholipid scramblase 1 (PLSCR1), caspase-3 activation and DNA-fragmentation in the human hippocampus after cerebral ischemia. Neurochem Int 43:79-87.

Rashidian J, Iyirhiaro GO, Park DS (2007) Cell cycle machinery and stroke. Biochim Biophys Acta 1772:484-493.

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. Proc Natl Acad Sci U S A 102:14080-14085.

Relton JK, Martin D, Thompson RC, Russell DA (1996) Peripheral administration of Interleukin-1 Receptor antagonist inhibits brain damage after focal cerebral ischemia in the rat. Exp Neurol 138:206-213.

Ren S, Rollins BJ (2004) Cyclin C/cdk3 promotes Rb-dependent G0 exit. Cell 117:239-251.

Rhee SG (1999) Redox signaling: hydrogen peroxide as intracellular messenger. Exp Mol Med 31:53-59.

Rhee SG, Chae HZ, Kim K (2005) Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. Free Radic Biol Med 38:1543-1552.

Robinson MJ, Macrae IM, Todd M, Reid JL, McCulloch J (1991) Reduction in local cerebral blood flow induced by endothelin-1 applied topically to the middle cerebral artery in the rat. J Cardiovasc Pharmacol 17 Suppl 7:S354-357.

Rodriguez J, Lazebnik Y (1999) Caspase-9 and APAF-1 form an active holoenzyme. Genes Dev 13:3179-3184.

Rosales J, Han B, Lee KY (2003) Cdk7 functions as a cdk5 activating kinase in brain. Cell Physiol Biochem 13:285-296.

Rosamond W, Flegal K, Furie K, Go A, Greenlund K, Haase N, Hailpern SM, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell C, Roger V, Sorlie P, Steinberger J, Thom T, Wilson M, Hong Y (2008) Heart disease and stroke statistics--2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 117:e25-146.

Saito T, Ishiguro K, Onuki R, Nagai Y, Kishimoto T, Hisanaga S (1998) Okadaic acid-stimulated degradation of p35, an activator of CDK5, by proteasome in cultured neurons. Biochem Biophys Res Commun 252:775-778.

Sasaki Y, Takimoto M, Oda K, Fruh T, Takai M, Okada T, Hori S (1997) Endothelin evokes efflux of glutamate in cultures of rat astrocytes. J Neurochem 68:2194-2200.

Sato K, Zhu YS, Saito T, Yotsumoto K, Asada A, Hasegawa M, Hisanaga S (2007) Regulation of membrane association and kinase activity of Cdk5-p35 by phosphorylation of p35. J Neurosci Res 85:3071-3078.

Sato S, Tominaga T, Ohnishi T, Ohnishi ST (1993) EPR spin-trapping study of nitric oxide formation during bilateral carotid occlusion in the rat. Biochim Biophys Acta 1181:195-197.

Schroder E, Willis AC, Ponting CP (1998) Porcine natural-killer-enhancing factor-B: oligomerisation and identification as a calpain substrate in vitro. Biochim Biophys Acta 1383:279-291.

Sebastian B, Kakizuka A, Hunter T (1993) Cdc25M2 activation of cyclin-dependent kinases by dephosphorylation of threonine-14 and tyrosine-15. Proc Natl Acad Sci U S A 90:3521-3524.

Serizawa H, Makela TP, Conaway JW, Conaway RC, Weinberg RA, Young RA (1995) Association of Cdk-activating kinase subunits with transcription factor TFIIH. Nature 374:280-282.

Sharkey J, Ritchie IM, Kelly PA (1993) Perivascular microapplication of endothelin-1: a new model of focal cerebral ischaemia in the rat. J Cereb Blood Flow Metab 13:865-871.

Sharp FR, Lu A, Tang Y, Millhorn DE (2000) Multiple molecular penumbras after focal cerebral ischemia. J Cereb Blood Flow Metab 20:1011-1032.

Sharpe MA, Cooper CE (1998) Interaction of peroxynitrite with mitochondrial cytochrome oxidase. Catalytic production of nitric oxide and irreversible inhibition of enzyme activity. J Biol Chem 273:30961-30972.

Sheaff RJ, Groudine M, Gordon M, Roberts JM, Clurman BE (1997) Cyclin E-CDK2 is a regulator of p27Kip1. Genes Dev 11:1464-1478.

Shelton SB, Johnson GV (2004) Cyclin-dependent kinase-5 in neurodegeneration. J Neurochem 88:1313-1326.

Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 13:1501-1512.

Shibata M, Araki N, Hamada J, Sasaki T, Shimazu K, Fukuuchi Y (1996) Brain nitrite production during global ischemia and reperfusion: an in vivo microdialysis study. Brain Res 734:86-90.

Shidoji Y, Hayashi K, Komura S, Ohishi N, Yagi K (1999) Loss of molecular interaction between cytochrome c and cardiolipin due to lipid peroxidation. Biochem Biophys Res Commun 264:343-347.

Shim J, Park HS, Kim MJ, Park J, Park E, Cho SG, Eom SJ, Lee HW, Joe CO, Choi EJ (2000) Rb protein down-regulates the stress-activated signals through inhibiting c-Jun N-terminal kinase/stress-activated protein kinase. J Biol Chem 275:14107-14111.

Shyu KG, Chang H, Lin CC (1997) Serum levels of intercellular adhesion molecule-1 and E-selectin in patients with acute ischaemic stroke. J Neurol 244:90-93.

Siao CJ, Tsirka SE (2002) Tissue plasminogen activator mediates microglial activation via its finger domain through annexin II. J Neurosci 22:3352-3358.

Simon RP (2006) Acidotoxicity trumps excitotoxicity in ischemic brain. Arch Neurol 63:1368-1371.

Singer JD, Gurian-West M, Clurman B, Roberts JM (1999) Cullin-3 targets cyclin E for ubiquitination and controls S phase in mammalian cells. Genes Dev 13:2375-2387.

Smaili SS, Hsu YT, Sanders KM, Russell JT, Youle RJ (2001) Bax translocation to mitochondria subsequent to a rapid loss of mitochondrial membrane potential. Cell Death Differ 8:909-920.

Small DL, Monette R, Fournier MC, Zurakowski B, Fiander H, Morley P (2001) Characterization of cyclin D1 expression in a rat global model of cerebral ischemia. Brain Res 900:26-37.

Smith PD, Mount MP, Shree R, Callaghan S, Slack RS, Anisman H, Vincent I, Wang X, Mao Z, Park DS (2006) Calpain-regulated p35/cdk5 plays a central role in dopaminergic neuron death through modulation of the transcription factor myocyte enhancer factor 2. J Neurosci 26:440-447.

Smith PD, Crocker SJ, Jackson-Lewis V, Jordan-Sciutto KL, Hayley S, Mount MP, O'Hare MJ, Callaghan S, Slack RS, Przedborski S, Anisman H, Park DS (2003a) Cyclin-dependent kinase 5 is a mediator of dopaminergic neuron loss in a mouse model of Parkinson's disease. Proc Natl Acad Sci U S A 100:13650-13655.

Smith RA, Walker T, Xie X, Hou ST (2003b) Involvement of the transcription factor E2F1/Rb in kainic acid-induced death of murine cerebellar granule cells. Brain Res Mol Brain Res 116:70-79.

Songyang Z, Lu KP, Kwon YT, Tsai LH, Filhol O, Cochet C, Brickey DA, Soderling TR, Bartleson C, Graves DJ, DeMaggio AJ, Hoekstra MF, Blenis J, Hunter T, Cantley LC (1996) A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, phosphorylase kinase, calmodulin-dependent kinase II, CDK5, and Erk1. Mol Cell Biol 16:6486-6493.

Sorolla MA, Reverter-Branchat G, Tamarit J, Ferrer I, Ros J, Cabiscol E (2008) Proteomic and oxidative stress analysis in human brain samples of Huntington disease. Free Radic Biol Med 45:667-678.

Souza JM, Radi R (1998) Glyceraldehyde-3-phosphate dehydrogenase inactivation by peroxynitrite. Arch Biochem Biophys 360:187-194.

Srinivasula SM, Hegde R, Saleh A, Datta P, Shiozaki E, Chai J, Lee RA, Robbins PD, Fernandes-Alnemri T, Shi Y, Alnemri ES (2001) A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. Nature 410:112-116.

Stewart E, Kobayashi H, Harrison D, Hunt T (1994) Destruction of Xenopus cyclins A and B2, but not B1, requires binding to p34cdc2. Embo J 13:584-594.

Sugawara T, Fujimura M, Morita-Fujimura Y, Kawase M, Chan PH (1999) Mitochondrial release of cytochrome c corresponds to the selective vulnerability of hippocampal CA1 neurons in rats after transient global cerebral ischemia. J Neurosci 19:RC39.

Sugawara T, Noshita N, Lewen A, Gasche Y, Ferrand-Drake M, Fujimura M, Morita-Fujimura Y, Chan PH (2002) Overexpression of copper/zinc superoxide

dismutase in transgenic rats protects vulnerable neurons against ischemic damage by blocking the mitochondrial pathway of caspase activation. J Neurosci 22:209-217.

Sugimoto K, Iadecola C (2003) Delayed effect of administration of COX-2 inhibitor in mice with acute cerebral ischemia. Brain Res 960:273-276.

Sun C, Cai M, Gunasekera AH, Meadows RP, Wang H, Chen J, Zhang H, Wu W, Xu N, Ng SC, Fesik SW (1999) NMR structure and mutagenesis of the inhibitor-of-apoptosis protein XIAP. Nature 401:818-822.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441-446.

Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR (1990) A semiautomated method for measuring brain infarct volume. J Cereb Blood Flow Metab 10:290-293.

Takahashi M, Billups B, Rossi D, Sarantis M, Hamann M, Attwell D (1997) The role of glutamate transporters in glutamate homeostasis in the brain. J Exp Biol 200:401-409.

Takahashi S, Kulkarni AB (2004) Mutant superoxide dismutase 1 causes motor neuron degeneration independent of cyclin-dependent kinase 5 activation by p35 or p25. J Neurochem 88:1295-1304.

Takashima A, Murayama M, Yasutake K, Takahashi H, Yokoyama M, Ishiguro K (2001) Involvement of cyclin dependent kinase5 activator p25 on tau phosphorylation in mouse brain. Neurosci Lett 306:37-40.

Tanaka T, Veeranna, Ohshima T, Rajan P, Amin ND, Cho A, Sreenath T, Pant HC, Brady RO, Kulkarni AB (2001) Neuronal cyclin-dependent kinase 5 activity is critical for survival. J Neurosci 21:550-558.

Tandon A, Yu H, Wang L, Rogaeva E, Sato C, Chishti MA, Kawarai T, Hasegawa H, Chen F, Davies P, Fraser PE, Westaway D, St George-Hyslop PH (2003) Brain levels of CDK5 activator p25 are not increased in Alzheimer's or other neurodegenerative diseases with neurofibrillary tangles. J Neurochem 86:572-581.

Tang D, Yeung J, Lee KY, Matsushita M, Matsui H, Tomizawa K, Hatase O, Wang JH (1995) An isoform of the neuronal cyclin-dependent kinase 5 (Cdk5) activator. J Biol Chem 270:26897-26903.

Tang X, Wang X, Gong X, Tong M, Park D, Xia Z, Mao Z (2005) Cyclin-dependent kinase 5 mediates neurotoxin-induced degradation of the transcription factor myocyte enhancer factor 2. J Neurosci 25:4823-4834.

Tarricone C, Dhavan R, Peng J, Areces LB, Tsai LH, Musacchio A (2001) Structure and regulation of the CDK5-p25(nck5a) complex. Mol Cell 8:657-669.

Thiex R, Kuker W, Muller HD, Rohde I, Schroder JM, Gilsbach JM, Rohde V (2003) The long-term effect of recombinant tissue-plasminogen-activator (rt-PA) on edema formation in a large-animal model of intracerebral hemorrhage. Neurol Res 25:254-262.

Thrift AG, Dewey HM, Macdonell RA, McNeil JJ, Donnan GA (2001) Incidence of the major stroke subtypes: initial findings from the North East Melbourne stroke incidence study (NEMESIS). Stroke 32:1732-1738.

Tikka T, Fiebich BL, Goldsteins G, Keinanen R, Koistinaho J (2001) Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. J Neurosci 21:2580-2588.

Timsit S, Rivera S, Ouaghi P, Guischard F, Tremblay E, Ben-Ari Y, Khrestchatisky M (1999) Increased cyclin D1 in vulnerable neurons in the hippocampus after ischaemia and epilepsy: a modulator of in vivo programmed cell death? Eur J Neurosci 11:263-278.

Tiranti V, Rossi E, Ruiz-Carrillo A, Rossi G, Rocchi M, DiDonato S, Zuffardi O, Zeviani M (1995) Chromosomal localization of mitochondrial transcription factor A (TCF6), single-stranded DNA-binding protein (SSBP), and endonuclease G (ENDOG), three human housekeeping genes involved in mitochondrial biogenesis. Genomics 25:559-564.

Tomasevic G, Kamme F, Wieloch T (1998) Changes in proliferating cell nuclear antigen, a protein involved in DNA repair, in vulnerable hippocampal neurons following global cerebral ischemia. Brain Res Mol Brain Res 60:168-176.

Tominaga T, Sato S, Ohnishi T, Ohnishi ST (1994) Electron paramagnetic resonance (EPR) detection of nitric oxide produced during forebrain ischemia of the rat. J Cereb Blood Flow Metab 14:715-722.

Tsai KY, Hu Y, Macleod KF, Crowley D, Yamasaki L, Jacks T (1998) Mutation of E2f-1 suppresses apoptosis and inappropriate S phase entry and extends survival of Rb-deficient mouse embryos. Mol Cell 2:293-304.

Tsai LH, Delalle I, Caviness VS, Jr., Chae T, Harlow E (1994) p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. Nature 371:419-423.

Tseng HC, Zhou Y, Shen Y, Tsai LH (2002) A survey of Cdk5 activator p35 and p25 levels in Alzheimer's disease brains. FEBS Lett 523:58-62.

van den Heuvel S, Harlow E (1993) Distinct roles for cyclin-dependent kinases in cell cycle control. Science 262:2050-2054.

van Lookeren Campagne M, Gill R (1998) Increased expression of cyclin G1 and p21WAF1/CIP1 in neurons following transient forebrain ischemia: comparison with early DNA damage. J Neurosci Res 53:279-296.

Veltkamp R, Rajapakse N, Robins G, Puskar M, Shimizu K, Busija D (2002) Transient focal ischemia increases endothelial nitric oxide synthase in cerebral blood vessels. Stroke 33:2704-2710.

Vemuganti R, Dempsey RJ, Bowen KK (2004) Inhibition of intercellular adhesion molecule-1 protein expression by antisense oligonucleotides is neuroprotective after transient middle cerebral artery occlusion in rat. Stroke 35:179-184.

Verdaguer E, Jimenez A, Canudas AM, Jorda EG, Sureda FX, Pallas M, Camins A (2004) Inhibition of cell cycle pathway by flavopiridol promotes survival of cerebellar granule cells after an excitotoxic treatment. J Pharmacol Exp Ther 308:609-616.

Verdaguer E, Jorda EG, Stranges A, Canudas AM, Jimenez A, Sureda FX, Pallas M, Camins A (2003) Inhibition of CDKs: a strategy for preventing kainic acid-induced apoptosis in neurons. Ann N Y Acad Sci 1010:671-674.

Verdaguer E, Garcia-Jorda E, Canudas AM, Dominguez E, Jimenez A, Pubill D, Escubedo E, Pallas JC, Camins A (2002) Kainic acid-induced apoptosis in cerebellar granule neurons: an attempt at cell cycle re-entry. Neuroreport 13:413-416.

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 102:43-53.

Vousden KH, Lane DP (2007) p53 in health and disease. Nat Rev Mol Cell Biol 8:275-283.

Wang CX, Song JH, Song DK, Yong VW, Shuaib A, Hao C (2006) Cyclin-dependent kinase-5 prevents neuronal apoptosis through ERK-mediated upregulation of Bcl-2. Cell Death Differ 13:1203-1212.

Wang F, Corbett D, Osuga H, Osuga S, Ikeda JE, Slack RS, Hogan MJ, Hakim AM, Park DS (2002) Inhibition of cyclin-dependent kinases improves CA1 neuronal survival and behavioral performance after global ischemia in the rat. J Cereb Blood Flow Metab 22:171-182.

Wang J, Liu S, Fu Y, Wang JH, Lu Y (2003) Cdk5 activation induces hippocampal CA1 cell death by directly phosphorylating NMDA receptors. Nat Neurosci 6:1039-1047.

Wang X (2001) The expanding role of mitochondria in apoptosis. Genes Dev 15:2922-2933.

Wang X, Yue TL, Young PR, Barone FC, Feuerstein GZ (1995) Expression of interleukin-6, c-fos, and zif268 mRNAs in rat ischemic cortex. J Cereb Blood Flow Metab 15:166-171.

Welter R, Yu L, Yu CA (1996) The effects of nitric oxide on electron transport complexes. Arch Biochem Biophys 331:9-14.

Wen Y, Yang S, Liu R, Simpkins JW (2005) Cell-cycle regulators are involved in transient cerebral ischemia induced neuronal apoptosis in female rats. FEBS Lett 579:4591-4599.

White BC, Sullivan JM, DeGracia DJ, O'Neil BJ, Neumar RW, Grossman LI, Rafols JA, Krause GS (2000) Brain ischemia and reperfusion: molecular mechanisms of neuronal injury. J Neurol Sci 179:1-33.

Wood ZA, Poole LB, Hantgan RR, Karplus PA (2002) Dimers to doughnuts: redox-sensitive oligomerization of 2-cysteine peroxiredoxins. Biochemistry 41:5493-5504.

Xie QW, Kashiwabara Y, Nathan C (1994) Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J Biol Chem 269:4705-4708.

Xiong Y, Zhang H, Beach D (1992) D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell 71:505-514.

Xu M, Sheppard KA, Peng CY, Yee AS, Piwnica-Worms H (1994) Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. Mol Cell Biol 14:8420-8431.

Yamamoto T, Maruyama W, Kato Y, Yi H, Shamoto-Nagai M, Tanaka M, Sato Y, Naoi M (2002) Selective nitration of mitochondrial complex I by peroxynitrite: involvement in mitochondria dysfunction and cell death of dopaminergic SH-SY5Y cells. J Neural Transm 109:1-13.

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332:411-415.

Yang Y, Mufson EJ, Herrup K (2003) Neuronal cell death is preceded by cell cycle events at all stages of Alzheimer's disease. J Neurosci 23:2557-2563.

Yepes M, Sandkvist M, Moore EG, Bugge TH, Strickland DK, Lawrence DA (2003) Tissue-type plasminogen activator induces opening of the blood-brain barrier via the LDL receptor-related protein. J Clin Invest 112:1533-1540.

Yin XM, Luo Y, Cao G, Bai L, Pei W, Kuharsky DK, Chen J (2002) Bid-mediated mitochondrial pathway is critical to ischemic neuronal apoptosis and focal cerebral ischemia. J Biol Chem 277:42074-42081.

Yokota C, Kaji T, Kuge Y, Inoue H, Tamaki N, Minematsu K (2004) Temporal and topographic profiles of cyclooxygenase-2 expression during 24 h of focal brain ishemia in rats. Neurosci Lett 357:219-222.

Yoo BC, Lubec G (2001) p25 protein in neurodegeneration. Nature 411:763-764; discussion 764-765.

Yuan J, Yankner BA (2000) Apoptosis in the nervous system. Nature 407:802-809.

Zaremba J, Skrobanski P, Losy J (2001) Tumour necrosis factor-alpha is increased in the cerebrospinal fluid and serum of ischaemic stroke patients and correlates with the volume of evolving brain infarct. Biomed Pharmacother 55:258-263.

Zhang H, Xiong Y, Beach D (1993a) Proliferating cell nuclear antigen and p21 are components of multiple cell cycle kinase complexes. Mol Biol Cell 4:897-906.

Zhang J, Krishnamurthy PK, Johnson GV (2002) Cdk5 phosphorylates p53 and regulates its activity. J Neurochem 81:307-313.

Zhang J, Cicero SA, Wang L, Romito-Digiacomo RR, Yang Y, Herrup K (2008) Nuclear localization of Cdk5 is a key determinant in the postmitotic state of neurons. Proc Natl Acad Sci U S A 105:8772-8777.

Zhang M, Li J, Chakrabarty P, Bu B, Vincent I (2004) Cyclin-dependent kinase inhibitors attenuate protein hyperphosphorylation, cytoskeletal lesion formation, and motor defects in Niemann-Pick Type C mice. Am J Pathol 165:843-853.

Zhang Z, Chopp M, Goussev A, Powers C (1998) Cerebral vessels express interleukin 1beta after focal cerebral ischemia. Brain Res 784:210-217.

Zhang ZG, Chopp M, Bailey F, Malinski T (1995) Nitric oxide changes in the rat brain after transient middle cerebral artery occlusion. J Neurol Sci 128:22-27.

Zhang ZG, Chopp M, Zaloga C, Pollock JS, Forstermann U (1993b) Cerebral endothelial nitric oxide synthase expression after focal cerebral ischemia in rats. Stroke 24:2016-2021; discussion 2021-2012.

Zhang ZG, Chopp M, Gautam S, Zaloga C, Zhang RL, Schmidt HH, Pollock JS, Forstermann U (1994) Upregulation of neuronal nitric oxide synthase and mRNA,

and selective sparing of nitric oxide synthase-containing neurons after focal cerebral ischemia in rat. Brain Res 654:85-95.

Zhao H, Yenari MA, Cheng D, Sapolsky RM, Steinberg GK (2003) Bcl-2 overexpression protects against neuron loss within the ischemic margin following experimental stroke and inhibits cytochrome c translocation and caspase-3 activity. J Neurochem 85:1026-1036.

Zheng YL, Li BS, Kanungo J, Kesavapany S, Amin N, Grant P, Pant HC (2007) Cdk5 Modulation of mitogen-activated protein kinase signaling regulates neuronal survival. Mol Biol Cell 18:404-413.

Zolotukhin S, Potter M, Zolotukhin I, Sakai Y, Loiler S, Fraites TJ, Jr., Chiodo VA, Phillipsberg T, Muzyczka N, Hauswirth WW, Flotte TR, Byrne BJ, Snyder RO (2002) Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. Methods 28:158-167.

Zou H, Li Y, Liu X, Wang X (1999) An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J Biol Chem 274:11549-11556.

Zou H, Henzel WJ, Liu X, Lutschg A, Wang X (1997) Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90:405-413.

Zukerberg LR, Patrick GN, Nikolic M, Humbert S, Wu CL, Lanier LM, Gertler FB, Vidal M, Van Etten RA, Tsai LH (2000) Cables links Cdk5 and c-Abl and facilitates Cdk5 tyrosine phosphorylation, kinase upregulation, and neurite outgrowth. Neuron 26:633-646.

Zweier JL, Samouilov A, Kuppusamy P (1999) Non-enzymatic nitric oxide synthesis in biological systems. Biochim Biophys Acta 1411:250-262.

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Appendix 3: Additional Publications

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Role of Cdk5-Mediated Phosphorylation of Prx2 in MPTP Toxicity and Parkinson's Disease

Dianbo Qu,[1] Juliet Rashidian,[1,3] Matthew P. Mount,[1,3] Hossein Aleyasin,[1,3] Mohammad Parsanejad,[1] Arman Lira,[1] Emdadul Haque,[1] Yi Zhang,[1] Steve Callaghan,[1] Mireille Daigle,[1] Maxime W.C. Rousseaux,[1] Ruth S. Slack,[1] Paul R. Albert,[1] Inez Vincent,[2] John M. Woulfe,[1] and David S. Park[1,*]
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DOI 10.1016/j.neuron.2007.05.033

SUMMARY

We reported previously that calpain-mediated Cdk5 activation is critical for mitochondrial toxin-induced dopaminergic death. Here, we report a target that mediates this loss. Prx2, an antioxidant enzyme, binds Cdk5/p35. Prx2 is phosphorylated at T89 in neurons treated with MPP^+ and/or MPTP in animals in a calpain/Cdk5/p35-dependent manner. This phosphorylation reduces Prx2 peroxidase activity. Consistent with this, $p35^{-/-}$ neurons show reduced oxidative stress upon MPP^+ treatment. Expression of Prx2 and Prx2T89A, but not the phosphorylation mimic Prx2T89E, protects cultured and adult neurons following mitochondrial insult. Finally, downregulation of Prx2 increases oxidative stress and sensitivity to MPP^+. We propose a mechanistic model by which mitochondrial toxin leads to calpain-mediated Cdk5 activation, reduced Prx2 activity, and decreased capacity to eliminate ROS. Importantly, increased Prx2 phosphorylation also occurs in nigral neurons from postmortem tissue from Parkinson's disease patients when compared to control, suggesting the relevance of this pathway in the human condition.

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by motor symptoms including tremor, muscle rigidity, paucity of voluntary movements, and postural instability (Hoehn and Yahr, 1967; Lang and Lozano, 1998). The pathological hallmarks of PD are the loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNc) and formation of Lewy bodies (Braak et al., 2003). The pathogenic process in PD is not clearly understood. A small portion (less than 10%) of PD patients have familial forms of the disease, and several PD genes have been identified (Abou-Sleiman et al., 2006). However, the vast majority of patients have idiopathic forms of PD. Parkinsonism can be induced in humans by exposure to the mitochondrial complex 1 toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This DAergic toxin results in parkinsonism symptoms indistinguishable from those of Parkinson's disease (Burns et al., 1985; Langston et al., 1983). Experimentally, it induces specific loss of DAergic neurons in the SNc (Burns et al., 1983) and produces a profound reduction of striatal dopamine levels with little alteration in other catecholamine neurotransmitter systems (Jonsson et al., 1986). Degeneration is a consequence of conversion of MPTP by glia to its toxic metabolite MPP^+ followed by specific uptake by DAergic neurons and presumed targeting of the mitochondria (Lang and Lozano, 1998).

The cellular consequences of mitochondrial dysfunction as induced by agents such as MPTP are numerous and include poor calcium homeostasis and oxidative stress (Wang and Yuen, 1994). How this dysregulation occurs and the consequence/management of these stresses are not fully understood. Recently, and consistent with improper calcium management, we reported that calpains are activated and required for MPTP-induced death in adult mice in vivo (Crocker et al., 2003). Calpains are conserved cysteine proteases regulated by calcium and possessing diverse biological function (Sorimachi et al., 1997). This was consistent with earlier reports of elevated calpain levels in postmortem PD patients (Mouatt-Prigent et al., 1996). We also showed that calpains were more activated in PD patients than in control individuals (Crocker et al., 2003). Importantly, calpain inhibition not only limited DAergic loss but also improved animal behavior following toxin treatment (Crocker et al., 2003).

What are the possible downstream targets of calpain activation? We recently provided evidence that the activator of Cdk5, p35, may be critical (Smith et al., 2003, 2006). Cdk5, not thought to be central to the core cell-cycle machinery, has been implicated in brain function including neuronal development, neuritic outgrowth, and neurotransmitter (dopamine) signaling (Dhavan and Tsai, 2001). Cdk5 activity is regulated by its activating partners, p35 and p39 (Dhavan and Tsai, 2001). While important for brain

development, recent evidence has shown that inappropriate activation of the Cdk5/p35 signal may lead to neuronal death through pathogenic activation of calpains, which proteolytically cleave p35 to a more active p25 form (Dhavan and Tsai, 2001) at least in cultured systems. However, its functional role in adult degeneration in vivo as well as in PD was unknown. To this end, we found that Cdk5 plays an essential role in DAergic loss in vivo (Smith et al., 2003, 2006). For example, we observed that MPTP induced Cdk5 activation and that inhibition of such activity with DN Cdk5 expression, Cdk inhibitors, or p35 deficiency attenuated DAergic death and behavioral deficits associated with MPTP treatment (Smith et al., 2003, 2006). This observation is made more significant by observations of increased p35 in postmortem PD brains (Nakamura et al., 1997). It is also entirely consistent with our observations that calpains are activated, are required for death, and mediate p35 to p25 cleavage in the MPTP model of PD (Smith et al., 2003). In support of this, we showed that inhibition of calpain led to reduced p35 to p25 conversion and Cdk5 activation (Smith et al., 2006). This suggested to us a model by which deregulated calcium leads to calpain activation, inappropriate Cdk5 activity, and DAergic cell death (Smith et al., 2006). However, the mechanism(s) by which Cdk5 promotes DAergic loss is still unknown. To address this, we presently performed mass spectrometry-based interactomics to identify Cdk5-interacting proteins. We identified an intriguing target that has direct implications for the way in which cells handle oxidative stress, linking Cdk5 activity with oxidative stress, a common theme in PD.

Oxidative stress is thought to be a critical mediator of damage in PD (Przedborski, 2005). Reactive oxygen species (ROS) has been observed in the SNc of PD patients and animal models of PD (Przedborski, 2005). Importantly, neurons in general have high levels of ROS. Therefore, systems to handle such stress are of paramount importance. Peroxidases are a key in this management system. Three types of peroxidases, peroxiredoxins (Prxs), catalase, and glutathione peroxide (GPx), function to eliminate H_2O_2 in mammalian cells (Rhee et al., 2005). In mammalian cells, six isoforms of Prx were identified. Although Prxs as a family are relatively ubiquitous, there is some specificity in regards to cell type and subcellular localization. For instance, Prx1 is distributed in the cytoplasm of oligodendrocytes and microglia, while Prx2 is located in the cytoplasm of neurons, and Prx3 is localized in the mitochondria of neurons (Jin et al., 2005). Whether and how Prxs participate in neuronal damage as well as how they are regulated have yet to be examined.

Presently, we report that Prx2 directly associates with the Cdk5 kinase complex through p35. In addition, Cdk5 phosphorylates Prx2 at T89 resulting in reduction of Prx2 peroxidase activity and neuronal death in MPP^+-treated cells in vitro and the MPTP mouse model of PD in vivo. Prx2 activity is functionally relevant since modulation of Prx2 activity is protective. Importantly, $p35^{-/-}$ neurons have reduced ROS and improved survival consistent with the above findings. These findings provide a mechanistic link of how a mitochondrial damaging agent, through calpain-mediated Cdk5 activation and downregulation of an important antioxidant enzyme, can increase oxidative load leading ultimately to death.

RESULTS

Identification of Prx2 as a Cdk5-Interacting Protein

We have shown that Cdk5 plays an essential role in loss of DAergic neurons in the MPTP mouse model of PD (Smith et al., 2003, 2006). It has also been demonstrated that Cdk5/p35 exists as macromolecular complexes in brain extracts, implying that Cdk5 associates with different proteins and functions in different signaling pathways (Lee et al., 1996). To identify these complexes, we initially utilized bacterially expressed GST-p10, a C-terminal truncated form of p35 containing a 98 amino acid residue N terminus fused with GST, as a bait to isolate p35-interacting proteins from mouse brain extracts. The interacting proteins were eluted with 1 M NaCl from GST-immobilized GSH beads. The eluted proteins were visualized by Coomassie blue staining after separation by SDS-PAGE (Figure 1A). The visualized specific bands were subjected to protein identification by tandem mass spectrometry. One of three specifically isolated proteins was found to be Prx2 for which three identified peptides matches were found (Figure 1B).

Prx2 is an antioxidative enzyme with peroxidase activity. Importantly, it also contains a conserved motif optimal for Cdks. Because of these reasons and the potential importance of ROS in PD, we chose this target for our study. To confirm the interaction between p35 and Prx2, an in vitro binding assay was carried out utilizing bacterially expressed proteins. GST-Prx2 or GST alone was incubated with His-tagged p35 and subjected to SDS-PAGE and western blot analyses using p35 antibody. A specific interaction was observed only with GST-Prx2 (Figure 1C). Similarly, a reverse binding experiment was performed where GST control or GST-Cdk5 was incubated with His-Prx2 alone or with both His-p35 and His-Prx2. Specific interaction was only observed with GST-Cdk5, His-p35, and His-Prx2 coincubation (Figure 1C). Finally, we also examined whether we could detect interaction through a means independent of bacterially expressed proteins by utilizing the yeast two-hybrid interaction assay. Consistent with our previous results, we could also detect interaction between p10 and Prx2 (Figure S1 available with this article online). These results indicate that Prx2 specifically interacted with the Cdk5/p35 complex through its association with p35.

To test whether endogenous Prx2 may exist in a complex with Cdk5/p35 in vivo, immunoprecipitation was performed using control IgG, p35 (C-19), and Cdk5 (C-8) antibodies on brain extracts (Figure 1D). The complexes were then analyzed by SDS-PAGE and Western blot analyses using a Prx2 antibody. Both immunoprecipitates using either Cdk5 or p35 antibody showed an associated

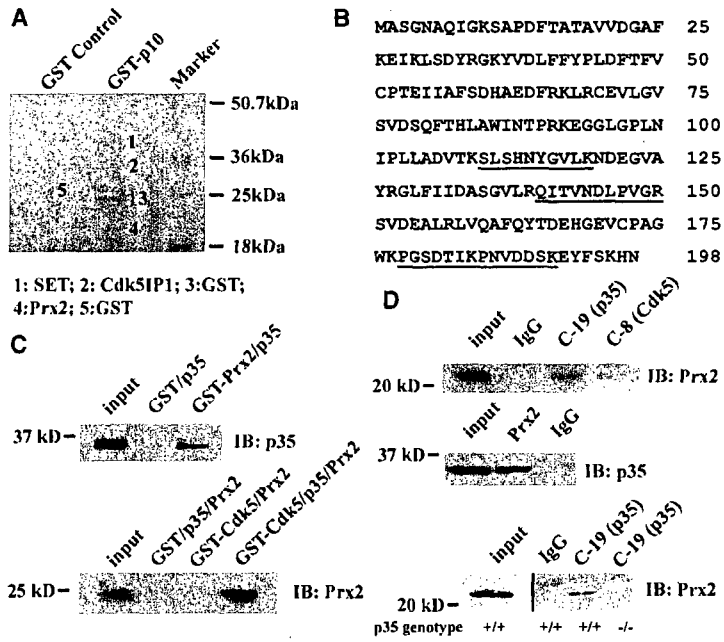

Figure 1. Identification of Prx2 as Cdk5/p35 Interactor

(A) Isolation of Cdk5-interacting proteins by affinity purification. Fifty micrograms of bacterially expressed GST and GST-p10 as bait was immobilized on GSH beads and coincubated with 1 mg of mouse brain homogenate at 4°C for 3 hr. The eluted proteins were precipitated by TCA after elution by 1 M NaCl. The precipitates were separated by 10% of SDS-PAGE then visualized by colloidal Coomassie Blue staining.

(B) Identification of Prx2 by a tandem mass spectrometry. The specific bands from GST-p10 were excised from the stained SDS-PAGE for mass spectrometric sequencing. Three underlined peptides were sequenced by the mass spectrometry and matched to mouse Prx2.

(C) Confirmation of the interaction between Prx2 and Cdk5/p35. Two micrograms of bacterially expressed GST and GST-Prx2 was incubated with 10 μg of His-p35 at 4°C for 2 hr. The GST-tagged proteins were retrieved with GSH beads. The proteins were subjected to SDS-PAGE and detected using C-19 for p35 by western blots (top panel). Similar to the above description, GST was incubated with His-p35 and His-Prx2 or GST-Cdk5 was incubated with His-Prx2 alone or with both His-p35 and His-Prx2 at 4°C for 2 hr. The bound proteins were separated by SDS-PAGE and detected by western blot using anti-Prx2 antibody after retrieval of GST-fused proteins by GSH-Sepharose (bottom panel).

(D) Association of Prx2 with Cdk5/p35 in vivo. (Top panel) Control IgG, C-19 for p35, and C-8 for Cdk5 were incubated with 500 μg of mouse brain lysate. Antibodies were isolated by IP beads and the coupled proteins were subjected to SDS-PAGE followed by anti-Prx2 western blot. (Middle panel) Control IgG or anti-Prx2 (Abcam) as indicated was incubated with mouse brain lysate. Following immunoprecipitation, samples were subjected to western blot analyses using anti-p35 antibody. (Bottom panel) Control IgG or anti-p35 were incubated with WT (+/+) or p35-deficient (−/−) mouse brain lysate as indicated. Following immunoprecipitation, samples were subjected to western blot analyses using anti-Prx2 antibody. Note that all lanes are from the same gel. However, because of the high intensity of the input-positive control signal, exposure time was reduced for this lane.

Prx2 signal by western blot while the control IgG did not. The reverse interaction assay where immunoprecipitation with Prx2 preceded western blot analyses for p35 was also performed using brain extracts. Consistent with the previous pulldown, a specific interaction between Prx2 and p35 was also observed (Figure 1D). Finally, to further confirm the specificity of the interaction assay, we also performed a p35 immunoprecipitation using p35 wild-type (WT) or knockout brain extracts followed by western blot analyses utilizing Prx2 antibody. A positive interaction was only observed with WT brain extract and not with p35-deficient brains. Taken together, this indicates that endogenous Prx2 associates with Cdk5/p35 in vivo.

Prx2 Is a Substrate of Cdk5 and Its Peroxidase Activity Is Regulated through Phosphorylation by Cdk5

The consensus sequence of Cdk5 phosphorylation is Pro (P)-directed Ser (S) or Thr (T) surrounded in the +3 position by basic amino acids, Arg (R), Lys (K), or His (H) (Songyang et al., 1996). There is a potential motif in Prx2 containing Pro-directed Thr, T^{89}PRK, optimal for Cdk5 phosphorylation. To investigate whether Prx2 is a substrate of Cdk5, Prx2 and Prx2T89A, a Prx2 mutant in which nonphosphorylatable Ala (A) replaced Thr (T), were subjected to in vitro kinase assay with Cdk5 alone, Cdk5/p35, or Cdk5/p25 active complexes. Incubation of bacterially expressed Prx2 or Prx2T89A with Cdk5 alone did not result in any radiolabel signal, indicating that the activating binding partner of Cdk5 was required for phosphorylation. The recombinant Prx2 was phosphorylated when either p35 or p25 was present along with Cdk5. In contrast, the recombinant Prx2T89A showed almost no detectable phosphorylation (Figure 2A). This indicates that both Cdk5/p35 and Cdk5/p25 can phosphorylate Prx2 and that almost all the phosphorylation occurs on the T89 residue.

To assess the effects of Cdk5-mediated Prx2 phosphorylation on peroxidase activity, WT Prx2 was purified from bacteria. It was then incubated with GST-Cdk5 and GST-p25 purified from bacteria. Afterwards, phospho-Prx2 was separated from nonphosphorylated Prx2 by Q-column. Equal amounts of phosphorylated and nonphosphorylated Prx2 were then assessed for peroxidase activity by monitoring the H_2O_2-dependent oxidation of NADPH in the presence of thioredoxin (Trx) and Trx reductase. The peroxidase activity of phospho-Prx2 was 37% of that of nonphosphorylated Prx2 (Figure 2B). To confirm isolation and phosphorylation of Prx2 as just described,

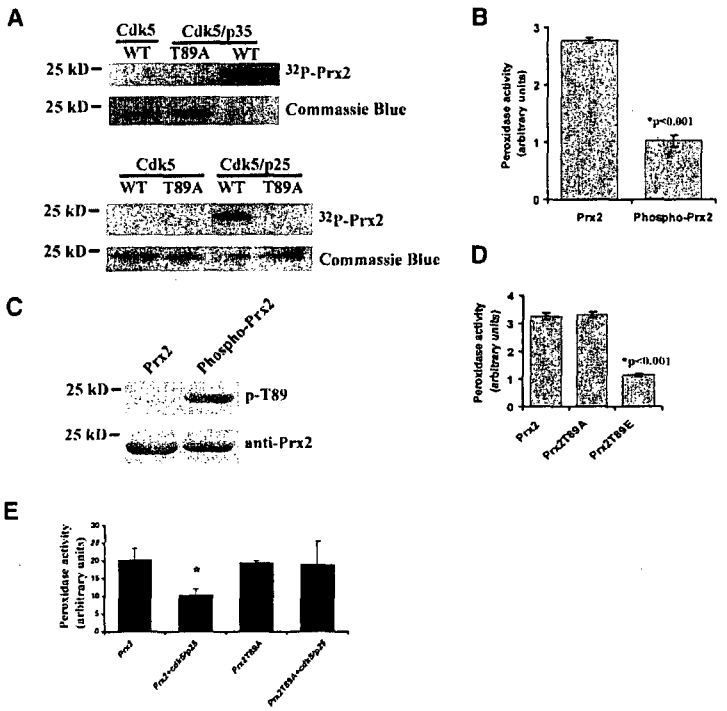

Figure 2. Prx2 Is an In Vitro Substrate of Cdk5, and Peroxidase Activity of Prx2 Is Modulated through Phosphorylation

(A) Prx2 is a substrate of Cdk5/p35 and Cdk5/p25. One microgram of purified His-Prx2 or His-PrxT89A was incubated with 50 ng of purified GST-Cdk5, GST-Cdk5/GST-p35, or GST-Cdk5/GST-p25 and 1 μCi of [γ-^{32}P]ATP at 30°C for 30 min. The proteins were separated by SDS-PAGE for autoradiography.

(B) Peroxidase activity of Prx2 is reduced by phosphorylation. Two hundred micrograms of His-Prx2 was incubated with 10 μg of GST-Cdk5/GST-p25 and 10 μM ATP at 30°C for 6 hr. The reaction mixture was separated by Q-column to isolate phospho-Prx2 from Prx2 after clearing GST-tagged Cdk5 and p25 by GSH-Sepharose. 0.5 μg of Prx2 or phospho-Prx2 was used for the peroxidase assay at 30°C for 10 min. The consumption of NADPH was measured at 340 nm wavelength by spectrophotometer. The data are the mean ± SEM (n = 3).

(C) Determination of purity of phospho-Prx2 and confirmation of specificity of p-T89 for phospho-Prx2. One microgram of Prx2 or purified phospho-Prx2 as isolated in (B) was probed by western blot analyses using the p-T89 antibody or a pan-Prx2 antibody.

(D) Peroxidase activity of Prx2T89E is lower than Prx2. 0.5 μg of purified His-Prx2, His-Prx2T89A, or His-PrxT89E was assayed for peroxidase activity as described above in (B). The data is the mean ± SEM (n = 4).

(E) Peroxidase activity of Prx2T89A is not affected by Cdk5 phosphorylation. Ten micrograms of purified His-Prx2 or His-Prx2T89A was incubated with or without GST-Cdk5/GST-p25 (3 μg each) overnight at 30°C in the presence of 2 mM DTT. Following dialyses, peroxidase activity was measured as described in (B). The data are the mean ± SEM (n=3).

we generated a phospho-antibody specific for phosphorylated T89 of Prx2. The phospho-T89 (p-T89) antibody recognized phosphorylated Prx2 but not the nonphosphorylated form isolated by Q-column upon western blot analyses (Figure 2C). A pan-Prx2 antibody was also isolated and shows that total Prx2 levels were approximately equal between nonphosphorylated and phosphorylated forms (Figure 2C).

The above results indicate that Cdk5 phosphorylation of Prx2 results in downregulation of peroxidase activity. To confirm this and ascertain that this is due to phosphorylation at T89, we isolated bacterially expressed Prx2, the Prx2T89A mutant, and a Prx2T89E mutant designed to mimic phosphorylation. Equal amounts of proteins were assayed for peroxidase activity. Importantly, the mutant Prx2T89E resulted in a 66% reduction in peroxidase activity compared to Prx2. The mutant Prx2T89A on the other hand was not significantly different from WT (Figure 2D). Importantly, we would predict that if Cdk5-mediated phosphorylation of Prx2 at T89 leads to downregulation of peroxidase activity, the Prx2T89A mutant should not be responsive to Cdk5 phosphorylation. Consistent with this notion, phosphorylation of recombinant WT Prx2 but not the Prx2T89A mutant by Cdk5 in vitro led to reduced peroxidase activity (Figure 2E). Finally, we also observed that phosphatase treatment of WT Prx2 previously phosphorylated by Cdk5 reversed the decrease in peroxidase (see Figure S2). Taken together, our data, at least in vitro, indicate that Cdk5 phosphorylates Prx2 at T89, which results in reduced Prx2 activity.

Prx2 Plays a Protective Role in Cortical Neurons Insulted by Neurotoxin MPP$^+$

We next determined whether Prx2 and its phosphorylation may play a role in neuronal death induced via mitochondrial stress by evaluating the effects of MPP$^+$, the active metabolite of MPTP, on death of cultured cortical neurons. It is important to note that these cultures are completely neuronal as evaluated by MAP2 staining (see Figure S3). We first evaluated the effect of MPP$^+$ on T89 phosphorylation utilizing the phospho-specific antibody p-T89 for phosphorylated Prx2 at T89. Analysis of an MPP$^+$ time course by western blot indicated a maximal increase in phospho-Prx2 signal at 24 hr (Figure 3A). This coincided with the peak in Cdk5 kinase activity measured under the same conditions (Figure 3B). In the latter assay, immunoprecipitated Cdk5 from neurons treated by MPP$^+$ at different time points was subjected to a kinase assay using histone H1 as a substrate. The increase in Prx2 phosphorylation at T89 was also observed in cortical neurons analyzed by immunofluorescence using the same phospho-T89 antibody after MPP$^+$ treatment (Figures 3C, 3D,

and 3E). There was a notable increase in phospho-labeling upon MPP^+ stress in the soma and neurites. This increase of fluorescence was quantified over a number of neurons by image analyses, showing a 90% increase in neurites and 62% increase in the soma. Blocking peptide treatment was used as a control for specificity and shows the required loss of fluorescent signal. Importantly, levels of Prx2 did not dramatically change upon MPP^+ treatment. This was observed both with western blot (Figure 3A) and upon immunofluorescent analyses (Figure 3C). Similar observations of increased phosphorylation of Prx2 were also observed in midbrain cultures containing dopamine neurons exposed to MPP^+ (see Figure S4). In addition, we also observed Prx2 phosphorylation when midbrain neurons were treated with another mitochondrial toxin, rotenone (Greenamyre et al., 2003) (see Figure S5).

We next determined whether a reduction in Prx2 activity accompanied the increase in T89 phosphorylation by analyzing a time course following MPP^+ treatment of cortical neurons. Importantly, Prx2 activity decreased at 24 hr, opposite to that of T89 Prx2 phosphorylation and Cdk5 activation (Figure 3F). These results suggest that Prx2 peroxidase activity is regulated by T89 phosphorylation in cultured neurons following mitochondrial insult.

To evaluate whether T89 phosphorylation of Prx2 plays a role in neuronal death induced by MPP^+, cortical neurons were infected with virus expressing Prx2, Prx2T89A, and Prx2T89E. The viability of the infected neurons was assessed by evaluating nuclear integrity after exposing cultures to MPP^+ for 48 hr. Expression of Prx2 and Prx2T89A significantly protected neurons from death in comparison to that of GFP and Prx2T89E (Figure 3G). Conversely, we also evaluated whether downregulation of Prx2 might sensitize neuronal cultures to MPP^+ treatment. We designed three different siRNA sequences to Prx2. The siRNA sequences 1 and 2 showed the most significant reduction in Prx2 levels (Figure 3H). These siRNA sequences sensitized the neuronal cultures to the toxic effects of MPP^+ treatment (Figure 3H). Finally, we also examined for ROS under these conditions by 2′, 7′-Dichlororescein diacetate (DCF) staining. As shown in Figure 3I, both siRNA sequences 1 and 2 significantly increased the number of DCF-positive neurons. Taken together, our results suggest a model by which a decrease of Prx2 peroxidase activity mediated through T89 phosphorylation after MPP^+ insult enhances oxidative stress, resulting in neuronal death.

T89 Phosphorylation of Prx2 Is Mediated by Cdk5 in Neurons after MPP^+ Treatment

To investigate whether T89 of Prx2 is phosphorylated in neurons by Cdk5, lysates from WT and $p35^{-/-}$ neurons treated with MPP^+ were analyzed by western blot. Absence of p35 led to a significant decrease of phosphorylation of Prx2 at T89 (Figure 4A). It is important to note, however, that this reduction was not absolute, suggesting that, at least in the present in vitro paradigm, other activators of Cdk5 such as p39 might also be present. It might also be due to the actions of other kinases. Prx2 levels were not observed to vary between WT and $p35^{-/-}$ neurons with or without MPP^+ treatment. To further confirm this reduction in phospho-Prx2 signal with p35 deficiency, we carried out immunofluorescent analyses. In $p35^{-/-}$ neurons, the increase in phospho-T89 signal observed upon MPP^+ treatment in WT neurons was significantly reduced (Figure 4B). Consistent with this observation, similar inhibition of Prx2 phosphorylation was observed with treatment of the Cdk inhibitor Roscovitine, but not with the GSK3 inhibitor lithium (see Figure S6). These data indicate that the phosphorylation of Prx2 at T89 in neurons following MPP^+ insult is significantly dependent upon Cdk5 activity. Next, we examined peroxidase activity of Prx2 in WT and $p35^{-/-}$ neurons following MPP^+ treatment. The data clearly showed that Prx2 peroxidase activity was decreased following MPP^+ treatment in WT neurons. However, this reduction did not occur in $p35^{-/-}$ neurons (Figure 4C). This indicates that peroxidase activity of Prx2 is regulated via phosphorylation by Cdk5. These data also suggested that $p35^{-/-}$ neurons should show reduced oxidative stress and resistance to MPP^+-induced neuronal death. To test this, ROS levels were measured utilizing DCF. ROS was significantly increased in WT neurons upon MPP^+ exposure as measured by both average intensity (54% increase) (Figures 4E and 4F) and number of DCF-positive neurons (34% increase) (data not shown). In contrast, $p35^{-/-}$ neurons showed reduced ROS levels upon MPP^+ treatment (35% decrease average density, Figure 4F; 25% decrease total number of cells, data not shown) when compared to WT littermate controls. $p35^{-/-}$ neurons were also substantially protected from MPP^+-induced death when compared to WT littermate control neurons (Figure 4D). Finally, based upon our previous observations that calpain-mediated activation of Cdk5 is important for neuronal death (Smith et al., 2006), we would predict that calpain inhibition should also block Prx2 phosphorylation. Consistent with this, cotreatment of MPP^+-exposed neuronal cultures with the calpain inhibitor PD150606 (Sedarous et al., 2003; Wang et al., 1996) led to decreased Prx2 signal as measured by immunofluorescence analyses (Figures 4G and S7). Taken together, our data indicate that Cdk5 kinase activity has a critical role in the reduction of Prx2 peroxidase activity through phosphorylation of Prx2 at T89 following MPP^+ insult and that this is a contributing factor to ROS increase and the ensuing neuronal death.

Prx2 Prevents the Loss of DAergic Neurons in the SNc in MPTP Mouse Model of PD

The phosphorylation of Prx2 at T89 by Cdk5 has a functional role in neuronal death in the MPP^+-induced cell death model. These data led us to further investigate the effects of Prx2 on neuronal death in an in vivo mouse model of PD. We employed the MPTP mouse model of PD to assess roles of Prx2 in the loss of DAergic neurons. We first determined the effects of expression of WT Prx2 and its mutants, Prx2T89A and Prx2T89E, on survival of DAergic neurons following MPTP administration. These

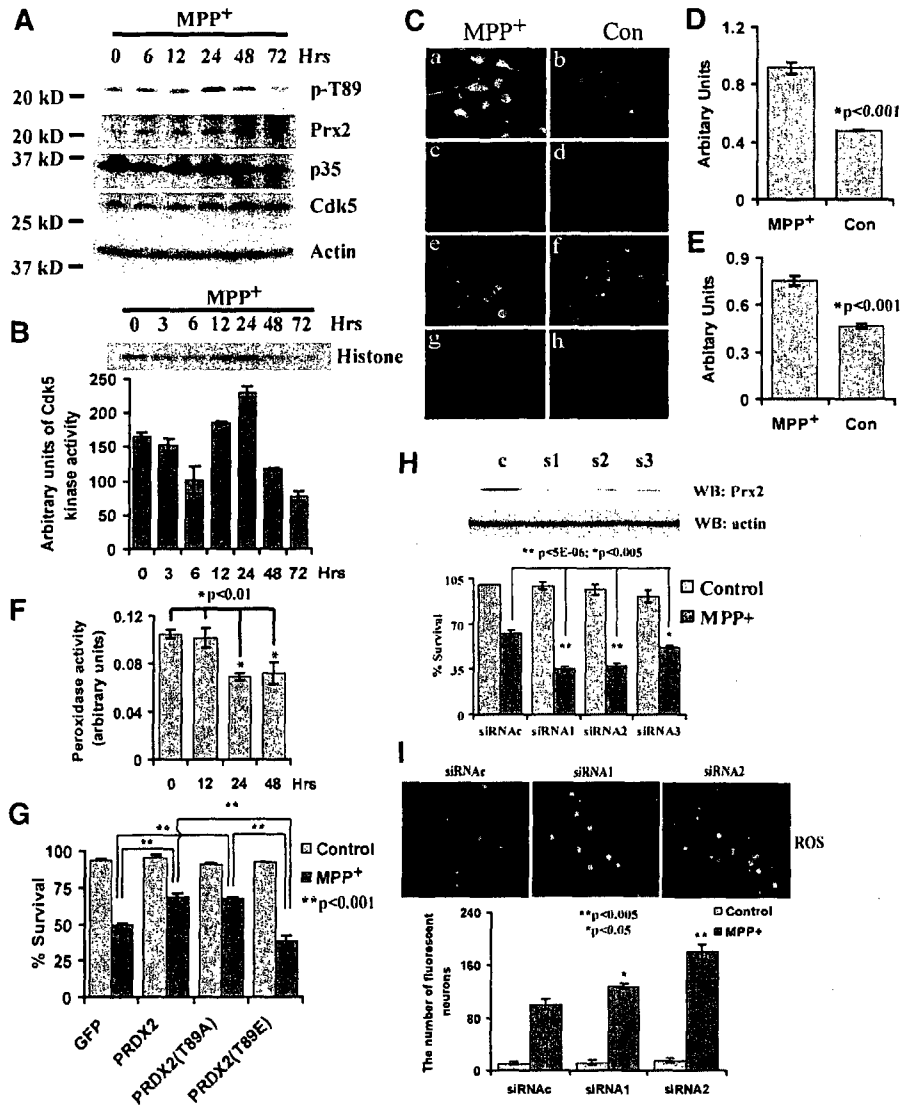

Figure 3. Reduction of Prx2 Peroxidase Activity and Associated Phosphorylation at T89 in Neurons after MPP+ Treatment

(A) Increase of phospho-Prx2 after MPP+ insult. Cultured cortical neurons were treated with 20 μM MPP+. The treated neurons were harvested at different time points, 6, 12, 24, 48, or 72 hr after MPP+ treatment. Forty micrograms of cell lysate was analyzed by western blot analyses utilizing p-T89, anti-Prx2, C-19 for p35, C-8 for Cdk5, or anti-β-actin antibodies. Similar results were observed in three independent experiments.

(B) Increase of Cdk5 kinase activity in neurons after MPP+ treatment. Neurons were treated as above described. Cdk5 was isolated from 50 μg of cell lysate by immunoprecipitation using the C-8 antibody and was incubated with 0.5 μCi of [γ-^{32}P]ATP at 30°C for 30 min using histone H1 as a substrate. The proteins were separated by SDS-PAGE for autoradiography. The density of autoradiographic bands was normalized from three experiments and is presented as mean ± SEM.

(C) Increase of phospho-Prx2 in neurons by immunofluorescent staining. After 24 hr treatment by MPP+, neurons were fixed by 4% paraformaldehyde. (a–d) Neurons were incubated with p-T89 antibody and coincubated with the appropriate phosphorylated peptide sequence used to produce the antibody (c and d) or the control nonphosphorylated peptide sequence (a and b). (e–h) Neurons were fixed as above and stained with our pan-Prx2 antibody without (e and f) or with (g and h) a quenching peptide used to produce the antibody as control. The images were captured by fluorescent microscopy.

(D and E) Distribution of phospho-Prx2 in neurons treated by MPP+. The fluorescent signal in soma (D) or dendrites (E) was measured from 200 neurons through image analysis. The data are the mean ± SEM.

(F) Downregulation of Prx2 peroxidase activity after MPP+ treatment. Prx2 was isolated from cultured neurons treated with MPP+ for the indicated times using a monoclonal anti-Prx2 antibody obtained from Abcam. Peroxidase activity was measured as described above. The data are the mean ± SEM (n = 3).

constructs were targeted unilaterally to the SNc DAergic neurons using an adenoviral-mediated gene delivery approach as we have previously performed (Crocker et al., 2001, 2003; Kalia et al., 2004; Kim et al., 2005; Smith et al., 2003). Protein expression of adenoviral Prx2 and its mutant was verified by western blot analysis (Figure 5B). We found that expression of Prx2 and Prx2T89A resulted in significant DAergic neuroprotection following MPTP treatment when compared with the contralateral untreated side or with GFP-injected control animals as analyzed by counting the number of tyrosine hydroxylase (TH) positive neurons (Figures 5A and 5B). In contrast, expression of Prx2T89E showed similar effects to GFP controls (Figures 5A and 5B). As an independent assessment of survival, we also examined for the number of neurons in the SNc region by cresyl violet staining. This also ensures that loss of neurons by MPTP treatment is not simply due to loss of TH expression. Our cresyl violet analysis was similar to that of TH assessment (Figure 5C). These data indicate that Prx2 can modulate DAergic neuron survival in the SNc following MPTP administration and suggest that the T89 regulatory site may be important in this DAergic cell death process.

Cdk5-Mediated Phosphorylation of Prx2 at T89 Plays a Pivotal Role in DAergic Neuron Damage by Regulation of Prx2 Peroxidase Activity in an MPTP Mouse Model of PD

The above in vivo evidence only demonstrates that Prx2 could potentially be important in the MPTP model. To further support this, we examined whether endogenous Prx2 may be modulated at T89 following MPTP treatment. Accordingly, the SNc extracts obtained from mice treated with MPTP for various times were subject to western blot analyses. As shown in Figure 6A, Prx2 phosphorylation increased following MPTP treatment, reaching the highest level of phosphorylation at 3 days following injected MPTP. To determine whether the phosphorylation of Prx2 at T89 is mediated by Cdk5, the SNc lysates from $p35^{-/-}$ mice or WT littermate controls were treated with MPTP and analyzed 3 days post-treatment. The level of phosphorylated Prx2 in $p35^{-/-}$ mice was significantly less than that in WT mice after MPTP treatment (Figure 6B). To further confirm the increase in the level of phosphorylated Prx2 in DAergic neurons in SNc of MPTP-treated mice, we assessed Cdk5-phosphorylated Prx2 by immunofluorescence analyses. Phosphorylated Prx2 was clearly observed in DAergic neurons in the SNc of WT mice treated with MPTP but not substantially observed in that of $p35^{-/-}$ mice (Figure 6C). We quantified the average fluorescent signal from TH-positive neurons by image analyses. As shown in Figure 6D, the fluorescent signal of phospho-Prx2 in WT SNc increased approximately 30%–50% in comparison to untreated WT controls or treated and untreated $p35^{-/-}$ mice.

To examine whether the peroxidase activity of Prx2 is affected in the MPTP mouse model of PD, Prx2 was isolated from $p35^{-/-}$ SNc or WT littermate controls with and without MPTP treatment and assayed for activity. Prx2 isolated from WT mice treated by MPTP showed a significant decrease in peroxidase activity in comparison to untreated WT controls. In contrast, $p35^{-/-}$ animals did not show this reduction following MPTP treatment (Figure 6E). Finally, we have previously shown that calpains are central for Cdk5 activation (Smith et al., 2006). Consistent with this, adenoviral-mediated expression of the calpain inhibitor calpastatin blocks increase in phospho-Prx2 following MPTP treatment in vivo (Figure S8). Taken together, these data indicate that calpain-mediated Cdk5 activation mediates phosphorylation and reduction of Prx2 activity in an in vivo model of PD and that this activity plays an important role in the death of DAergic neurons.

Relevance to Human PD

While the above evidence strongly implicates the importance of Prx2 in the MPTP model of PD, we directly examined its potential relevance to human PD. Accordingly, we first examined the phospho-Prx2 signal in human PD postmortem samples and controls. The equivalent T89 site is also present in human Prx2. As shown in Figure 7, nigral DAergic neurons from human midbrain PD and control samples were clearly detected by the presence of neuromelanin, granular brown pigmented regions detectable even without staining (see arrowheads in Figures 7A and 7B). When stained using phospho-Prx2 antibody and DAB visualization, little or no signal was detected in the perikarya of dopamine neurons from control midbrain samples. However, significant staining (black color) was observed in soma of dopamine neurons from PD patients (see arrow, Figure 7A). Staining in the region of neurons that resemble dopamine neurons in size and location but that did not contain neuromelanin was also observed in

(G) Prx2 protects neuron form death after MPP^+ treatment. Cortical neurons were infected with virus expressing GFP alone or along with Prx2, Prx2T89A, or Prx2T89E and cultured for 3 days. The cells were exposed to MPP^+ for 48 hr and neuronal survival was evaluated by assessing nuclear integrity of GFP-positive neurons. The data are the mean ± SEM (n = 3). Similar results were obtained when equivalent constructs were transfected (data not shown).

(H) Downregulation of Prx2 by siRNA oligonucleotide treatment sensitizes cortical neurons to MPP^+ treatment. Three independent siRNA sequences (s1/siRNA1, s2/siRNA2, s3/siRNA3) and a control sequence (c/siRNAc) were evaluated for their ability to downregulate endogenous Prx2 levels in cortical neurons as described in Experimental Procedures (top panel). (Bottom graph) Transfected cultures were then assayed for survival following 48 hr MPP^+ treatment by MTT assay. The data are the mean ± SEM (n = 3).

(I) Downregulation of Prx2 by siRNA oligonucleotide treatment increases ROS levels following MPP^+ treatment. (Top panel) Representative DCF fluorescence in control (siRNAc) or siRNA1 or siRNA2 oligonucleotide-treated cultures after treatment with 20 μM MPP^+ for 24 hr under a fluorescent microscope. (Bottom graph) Quantification of the number of DCF fluorescence-positive cells in cells treated as above. Random fields were analyzed for the number of DCF-positive neurons. The data are the mean ± SEM (n = 4).

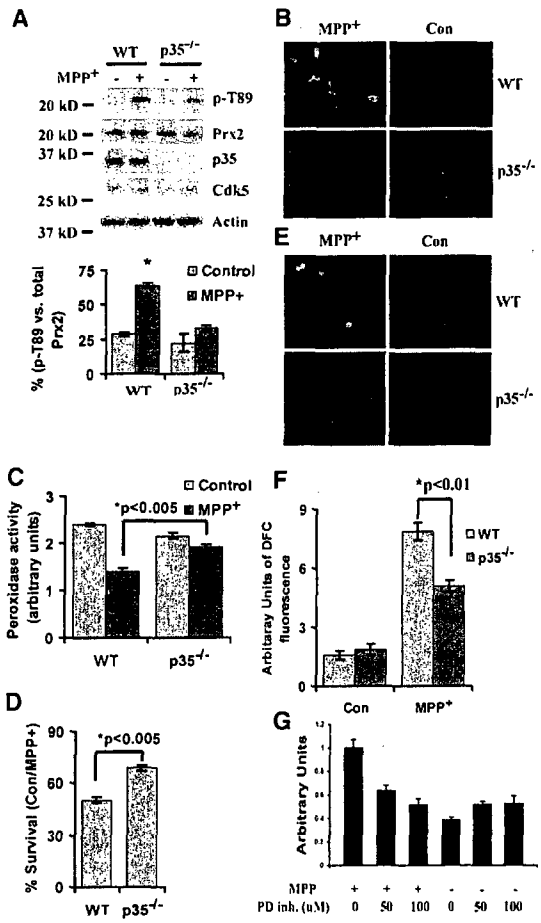

Figure 4. Prx2 Is a Substrate of Cdk5 in Neurons Treated with MPP+

(A and B) Cdk5 phosphorylates Prx2 in neurons treated by MPP+. (A) Neurons from WT or p35$^{-/-}$ embryos were treated with MPP+ insult for 24 hr. The cell lysates were subjected to SDS-PAGE and p-T89 western blot analyses. The membranes were then stripped and reprobed with anti-Prx2, C-19 for p35, C-8 for Cdk5, or anti-β-actin. The bottom panel shows densitometric values of phospho-Prx2 relative to Prx (p-T89/total Prx2*100). Each value is the mean ± SEM (n = 3). (B) Likewise, cultures as indicated were subjected to immunofluorescent staining utilizing the p-T89 antibody. Similar results were obtained in three independent experiments.

(C) Phosphorylation of Prx2 at T89 by Cdk5 reduces peroxidase activity. Cortical cultures from WT or p35$^{-/-}$ embryos were treated with MPP+ for 24 hr as described above. Peroxidase activity assay was carried out also as described above. The data are the mean ± SEM (n = 3).

(D) p35$^{-/-}$ neurons are resistant to MPP+-induced death. Neurons from WT or p35$^{-/-}$ embryos were exposed to MPP+ for 48 hr. The viability of neurons was measured by MTT assay. The survival percentage was obtained by comparing value from the MPP+-treated neurons to that of the nontreated neurons in either p35$^{-/-}$ or WT neuronal cultures. The data are presented as mean ± SEM (n = 3).

(E and F) The role of Cdk5/p35 in MPP+-induced ROS. (E) Representative DCF fluorescence in WT and p35$^{-/-}$ neurons after treatment with 20 μM MPP+ for 24 hr under a fluorescent microscope. (F) Quantification of DCF fluorescence signal in WT and p35$^{-/-}$ neurons either treated or untreated with MPP+. Random fields were analyzed for average fluorescence intensity. The data are the mean ± SEM (n = 4).

(G) Calpain inhibitors block increase in MPP+-induced phospho-Prx2 signal. Cortical neuronal cultures were untreated or treated with 20 μM MPP+ and/or the calpain inhibitor PD150606, as indicated. Cultures were fixed and stained for phospho-Prx2 and Hoechst. For representative pictures, please see Figure S7. The fluorescent signal in soma was measured by image analyses from 45 neurons in 3 random fields. The data are presented as mean ± SEM.

PD samples. Staining of neuritic processes was observed in both control and PD samples. The number of phospho-Prx2-positive dopamine neurons was then quantified from five PD and six control samples. As shown in Figure 7C, a significant increase in phospho-Prx2-positive neurons was observed in PD patient samples when compared to controls.

Finally, we examined whether familial PD genes may impact Prx2 phosphorylation. The PD gene, *dj-1*, has also been linked to management of ROS (Bonifati et al., 2003; Kim et al., 2005). Interestingly, DJ-1 expression blocked Prx2 phosphorylation in neurons treated with MPP+ (Figure S6C). Modulation of another PD gene, *pink1* (Valente et al., 2004), however, did not affect Prx2 phosphorylation, suggesting some specificity in the way PD genes impact Prx2 phosphorylation (Figure S6D). Taken together, our human patient data as well as that with DJ-1 further support the importance of Prx2 in PD.

DISCUSSION

ROS are generated as a result of normal metabolism (Adam-Vizi, 2005). However, generation of excessive oxidative load beyond a cell's homeostatic capacity can be deleterious. Mitochondrial dysfunction and excess ROS have been strongly implicated in the pathogenesis of PD (Jenner, 1998; Przedborski, 2005). However, how these events are initiated, are regulated, and interact to promote neuronal death is not completely clear. Recently, we demonstrated that calpain-mediated Cdk5 activation plays an essential role in DAergic loss in the MPTP model of PD (Crocker et al., 2003; Smith et al., 2003, 2006). These findings were important since they provided a plausible link between the actions of a mitochondrial damaging agent (MPTP) and activation of a pathogenic calcium-dependent process (calpain activation) consistent with known deregulation of calcium homeostasis in PD. However, the manner by which Cdk5 regulates downstream pathogenic events was not completely known. Presently, we identified a novel Cdk5 target, Prx2, an antioxidant enzyme with peroxidase activity (Rhee et al., 2005). We provide evidence that Prx2 is a physiological substrate of Cdk5. Cdk5 activation downregulates Prx2 peroxidase activity in PD models of death both in culture and in animals. Modulation of Prx2 activity also regulates neuronal loss. These data provide a mechanistic link of how the mitochondrial damaging agent MPTP leads to nigral loss by Cdk5 activation, phosphorylation/inactivation of an

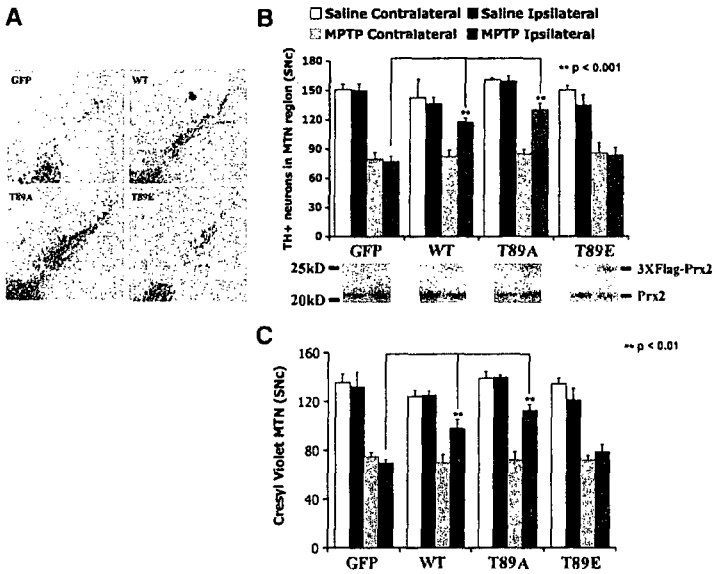

Figure 5. Prx2 Protects DAergic Loss following MPTP Administration

(A) The adenoviruses (2 μl, 1 × 10^7 particles per μl) expressing Prx2, Prx2T89A, and Prx2T89E were injected directly into the striatum of animals 7 days before initiation of MPTP treatment. A GFP-expressing virus was used as a control. Brains were sectioned into 14 μm slices for TH DAB staining. Representative pictures of the ipsilateral side of animals injected with the indicated virus and treated with MPTP were shown.

(B) Quantification of the number of DAergic neurons from ipsilateral or controlateral for the indicated treatment groups are shown. The data are presented as mean ± SEM (n = 4–5/group). Expression of Prx2, Prx2T89A, and Prx2T89E in the SNc extracts was confirmed by western blot analyses using a pan-antibody for Prx2.

(C) Quantitation of neurons in the SNc region by cresyl violet staining. The data are presented as mean ± SEM (n = 4/group).

important antioxidant enzyme, and consequent increase in oxidative load (see Figure 8). The observation of increased Prx2 phosphorylation in human PD tissue as well as modulation by DJ-1 also indicates the potential importance of this pathway in human PD.

Prx2 Interacts with Cdk5/p35 Complexes and Is a Substrate of Cdk5

Our results demonstrate that Cdk5/p35 interacts with Prx2. Prx2 is a member of the Prx family that contains at least six members. The identification of the Prx2 form as an interacting partner is particularly relevant since Prx2 is localized to neurons, including the DAergic neurons of the SNc (Jin et al., 2005; Sarafian et al., 1998). This is, in turn, consistent with known DAergic functions of Cdk5, particularly in models of PD as reported previously (Smith et al., 2003, 2006). In contrast, Prx1, also localized to the cytoplasm, is distributed in oligodendrocytes and microglia (Jin et al., 2005). These results, particularly in vivo, point to a neuron-specific Cdk5-Prx pathway of ROS management rather than a non-cell-autonomous mode of action regulated by other brain cell types such as glia. It is important to point out that this does not exclude the potential importance of other Prx members in neuronal loss. For example, Prx3, a mitochondrially localized enzyme (Watabe et al., 1994), also has potential Cdk5 sites. It will be interesting to determine whether this member might also play a Cdk5-dependent role in mitochondrial stress-induced death.

Our initial results indicated that the N-terminal portion of p35 was sufficient to bind to Prx2. However, it is important to note that both p35 and p25 can efficiently phosphorylate Prx2, at least in vitro. This suggests that stable binding to a Cdk5/p35 complex per se mediated by the p10 fragment is not required for efficient phosphorylation. We speculate that the p10 portion may be an important regulatory domain that regulates how efficiently p35 or Cdk5/p25 complexes may phosphorylate Prx2. Careful analyses will have to be performed to further study this interesting observation. Nonetheless, our results indicate not only that both Cdk5 complexes phosphorylate Prx2 on T89, but also that this modification significantly downregulates its activity.

Under basal conditions, p35 is abundantly localized to the inner cellular membrane, through a myristoylation anchor (Patrick et al., 1999). Appropriate activation of this form of Cdk5/p35 is the presumptive "normal" activity of this complex. However, p35 can be converted to a pathogenic p25 form by calpain-mediated cleavage (Lee et al., 2000; Smith et al., 2003, 2006). This results in a more stable active Cdk5 activator as well as the potential to be mislocalized to the nucleus (Gong et al., 2003; O'Hare et al., 2005). One suggested nuclear target of the Cdk5/p25 complex is Mef2, which we and others have shown is important in models of oxidative stress in vitro (Gong et al., 2003) and following MPTP in vivo (Smith et al., 2006).

Cytoplasmic Cdk5 activity might also be pathogenic. For example, a portion of p25 could also be localized to the cytoplasm, and cytoplasmic targets such as tau have been previously proposed for Cdk5 particularly in models of Alzheimer's disease (Patrick et al., 1999). In this regard, we have identified an important cytoplasmic target of Cdk5 that could be regulated by either Cdk5/p35 or Cdk5/p25 complexes. The observation that Cdk5/p25 complexes efficiently phosphorylate Prx2 on T89, however, is consistent with a pathogenic role of this complex.

Cdk5-Mediated Prx2 Downregulation in PD and Oxidative Stress

The identification of Prx2 as a target of Cdk5 is particularly relevant since DAergic neurons are thought to be

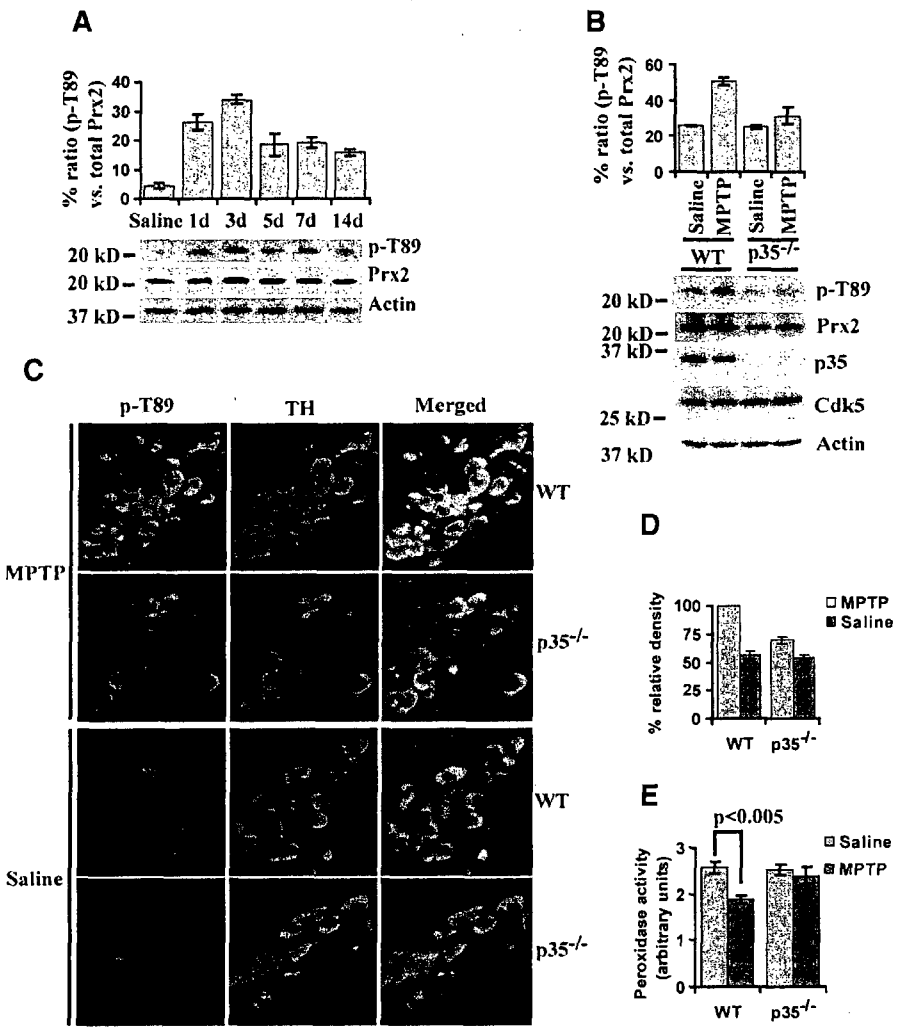

Figure 6. Peroxidase Activity of Prx2 Is Regulated in an In Vivo MPTP Mouse Model of PD

(A) Phosphorylation of Prx2 at T89 is increased after MPTP administration. The SNc extracts were obtained from animals treated with MPTP for the indicated times. (Bottom panel) The SNc lysates were subjected to SDS-PAGE and western blot probed as indicated using p-T89, anti-Prx2, and anti-β-actin antibodies. The top panel shows densitometric values of phospho-Prx2 relative to Prx (p-T89/total Prx2*100). Each value is the mean ± SEM (n = 3).

(B) Prx2 is a substrate of Cdk5 complexes in DAergic neurons from MPTP-administrated mice. (Bottom panel) The SNc lysates from WT or $p35^{-/-}$ mice 3 days following MPTP or saline administration were analyzed by western blot analyses using p-T89, anti-Prx2, anti-p35 (C-19), anti-Cdk5 (C-8), and anti-β-actin antibodies. The top panel shows densitometric values of phospho-Prx2 relative to Prx (p-T89/total Prx2*100). Each value is the mean ± SEM (n = 3).

(C) Increased phospho-Prx2 is colocalized with TH-positive neurons. The sections from WT or $p35^{-/-}$ mice were analyzed 3 days following MPTP or saline treatment. Sections were double-stained using p-T89 antibody (green) and anti-TH monoclonal (red) antibody for 24 hr at 4°C. The sections were incubated with Alex-488-conjugated antibody specific for rabbit IgG and Alex-594-conjugated antibody for mouse IgG for 3 hr at room temperature. The sections were visualized by fluorescent microscopy.

(D) The fluorescent signals from the p-T89 labeling (C) were quantified densitometrically by imaging analysis. Three sets of animals (n = 1 animal/treatment group/set) were individually stained and analyzed by densitometric analyses. 40–50 TH-positive neurons for each animal (over 3–6 slides/animal) were measured for p-T89 signal. This value was then averaged. Within each set of animals, the average value for each treatment group was normalized to the WT MPTP value. The values were then averaged for all three sets of animals for an n = 3 (mean ± SEM).

(E) Downregulation of peroxidase activity of Prx2 is mediated by Cdk5 in mice administrated by MPTP. Prx2 was isolated from 50 μg of SNc lysates from WT or $p35^{-/-}$ mice obtained 3 days following treatment with saline, or MPTP was analyzed for peroxidase activity. The data are presented as mean ± SEM (n = 3).

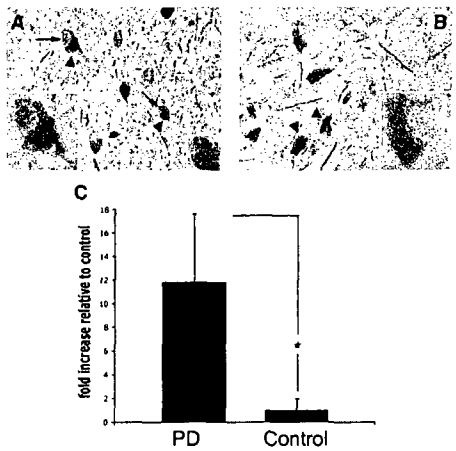

Figure 7. Prx2 Phosphorylation in Human PD
Phosphorylation of Prx2 is increased in human PD. Human substantia nigra obtained from (A) PD and (B) control individuals. Sections were immunostained using p-T89 Prx2 antibody and visualized by DAB staining. Neuromelanin pigment indicative of dopamine nigral neurons is present as punctate brown staining (arrowheads) while the phospho-Prx2 signal shows as black staining in the soma (arrows). Two examples of phospho-Prx2 positive (A) and negative (B) neurons are labeled with arrows/arrowheads. (C) Quantitation of phospho-Prx2-positive neurons in PD (n = 5) and control (n = 6) individuals are shown (mean ± SEM). *p < 0.05 (Student's t test).

particularly susceptible to oxidative stress (Smythies and Galzigna, 1998). There are several lines of evidence that support a link between ROS and PD. For example, ROS levels are very high in PD patients (Jenner, 1998; Przedborski, 2005). Numerous enzymes that produce ROS have been implicated as critical in in vivo models of PD (Przedborski, 2005). Damaging ROS has also been shown to occur in animals following exposure to mitochondrial poisons (Ara et al., 1998; Schapira, 2001). Importantly, the mitochondria, a major source of oxidative stress, participates in PD pathogenesis (Przedborski, 2005). Consistent with this notion, familial forms of PD have been associated with mitochondrial dysfunction. Indeed, the familial PD gene *dj-1* is thought to possess direct antioxidant functions (Bonifati et al., 2003; Canet-Aviles et al., 2004; Dawson and Dawson, 2003; Kim et al., 2005; Martinat et al., 2004; Shendelman et al., 2004).

Our evidence suggests that regulation of Prx2 is important in a toxin model of PD. For example, alteration of Prx2 levels modulates death both in vitro and in vivo following MPP^+/MPTP. It must be noted, however, that there are limitations to relating the in vivo MPTP model to PD and caution must be observed in making any direct comparisons to the human condition. For example, the relatively acute toxic nature of the MPTP model might not reflect accurately what occurs in the idiopathic PD. Accordingly, to support our MPTP data, we also report that phosphorylation of Prx2 also occurs in the nigral region of PD patients. This is consistent with the relevance of our findings to the human condition. However, standard and important caveats to interpreting any postmortem data apply here as well.

Using the MPTP model as an important first step in understanding the nigral degenerative process, we have identified how Prx2 is modulated to promote death following exposure to this mitochondrial toxin. We have shown previously that Cdk5 is hyperactivated and plays a major functional role in dopamine loss in the MPTP model (Smith et al., 2003). It is likely that Cdk5 acts to modify several downstream targets. For example, we had also previously shown that Cdk5 targets the nuclear transcription factor and survival factor Mef2 on a site known to suppress its activity (Smith et al., 2006). However, cytoplasmic targets may also be critical. We believe that Prx2 is one such important cytoplasmic factor. This is supported by our data showing that Prx2 is phosphorylated at T89 both in vitro and in vivo following MPP^+/MPTP and that this is associated with a decrease in peroxidase activity. In support of

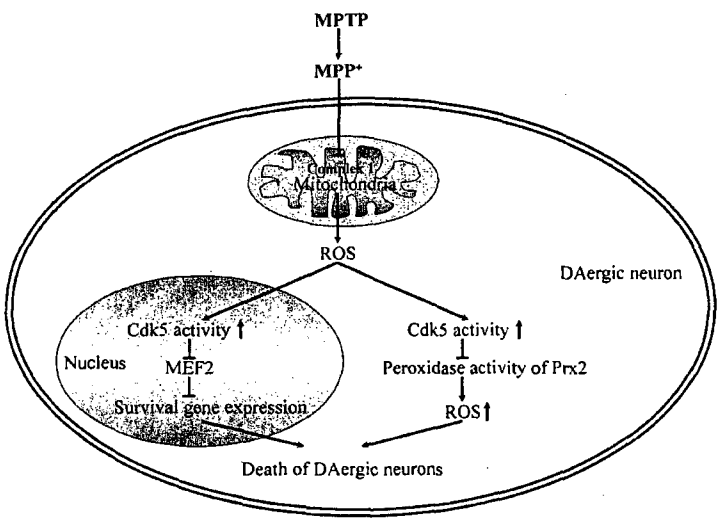

Figure 8. Model of Cdk5-Mediated DAergic Loss in MPTP Mouse Model of PD
Cdk5 kinase activity is activated by mitochondrial stress induced by MPP^+, a metabolite of MPTP. Activated Cdk5 regulates DAergic loss through phosphorylation of cytoplasmic substrate Prx2 to inhibit its antioxidative ability and phosphorylation of nuclear target Mef2 to inhibit Mef2 prosurvival function.

this, a mutant mimicking constitutively phosphorylated Prx2T89E does not protect neurons from mitochondrial insult, whereas WT or a mutant lacking the T89 phosphorylation site effectively promotes survival. Most importantly, Prx2 phosphorylation is dependent on the Cdk5 complex since p35-deficient animals, which are resistant to death induced by MPP^+ or MPTP, have reduced Prx2 phosphorylation and Prx2 peroxidase activity. It is important to highlight that, in addition to peroxidase activity, Prx2 is also thought to possess some chaperone activity at least in cell lines (Moon et al., 2005). The relevance of this in the present context is not completely known. However, we have determined that the higher molecular weight complexes of Prx2 indicative of its chaperone activity do not change following MPP^+ insult, suggesting that its chaperone activity may not be relevant in this model (D.Q. and D.S.P., unpublished results). Finally, as mentioned previously, whether other members of Prx may be important in nigral degeneration is unknown. Intriguingly, Prx1 has been shown to be phosphorylated by cell-cycle Cdk members (Yang et al., 2002). The latter has been also implicated in neuronal death (Bu et al., 2002; Busser et al., 1998; McShea et al., 1997; Nguyen et al., 2003; Osuga et al., 2000; Rashidian et al., 2005; Rideout et al., 2003; Wang et al., 2002; Zhang et al., 2004). Therefore, whether/how other Prx members are regulated by Cdk members will be of further interest.

In summary, we have uncovered an important mechanism by which calpain-mediated Cdk5 activation regulates DAergic neurodegeneration in an MPTP model of PD via downregulation of Prx2 peroxidase activity. We propose that this loss significantly enhances the ROS environment and leads to DAergic neuron loss (Figure 8). This central pathway in addition to other pathways mediated by additional calpain or Cdk5 targets ultimately lead to nigral degeneration in response to MPTP (Figure 8). These findings are particularly relevant to human PD since both deregulated Cdk5 and increased ROS have been shown in the human PD condition (Jenner, 1998; Nakamura et al., 1997; Przedborski, 2005). Furthermore, we presently, show that phosphorylated Prx2 is increased in human PD patients and that Prx2 phosphorylation is also modified by *dj-1*, a known PD gene (Bonifati et al., 2003). How the latter links to Prx2 phosphorylation will be of great interest in future studies. Taken together, our findings suggest that strategies to modulate Prx2 activity serve as beneficial targets for treatment of PD. This is of particular importance since Cdk5 is thought to have normal beneficial roles in neurons (Li et al., 2002) and modulating a relevant downstream target rather than Cdk5 directly may be a better therapeutic strategy with regard to this pathway.

EXPERIMENTAL PROCEDURES

Animals

Eight-week-old male C57BL/6 mice (22–28 g; Charles River Laboratories, USA) were used for MPTP experiments. All animal experiments conformed to the guidelines set forth by the Canadian Council for the Use and Care of Animals in Research (CCAC) and the Canadian Institutes for Health Research (CIHR) and had approval from the University of Ottawa Animal Care Committee.

Antibodies

The following antibodies were utilized: Tyrosine hydroxylase (TH) (Immunostar, USA), C-8 for Cdk5 (Santa Cruz, USA), C-19 for p35 (Santa Cruz, USA), β-Actin (monoclonal, Sigma, Canada), Prx2 (monoclonal, Abcam, UK), and Alex-labeled secondary antibodies (Invitrogen, Canada). Prx2 and phospho-Prx2T89 polyclonal antibodies were generated and initially purified from rabbit using standard protocols from Biogenes (Berlin, Germany) by immunization with carrier protein-conjugated phosphopeptide, LAWINpTPRKEGGLG. The phospho-Prx2 antibody p-T89 was obtained by first purifying the serum using the phosphorylated peptide. The pan-Prx2 antibody was obtained using the nonphosphorylated peptide. The phospho-specific antibody was further purified by adsorbing onto bacterially expressed and purified GST-Prx2 to remove any remaining crossreactivity to nonphosphorylated Prx2. MAP2 was obtained from Santa Cruz (H-300; 1:300).

Isolation of p35-Binding Proteins

The assay was carried out as previously described (Qu et al., 2002).

Mass Spectrometry

The specific bands on GST-p10 lane were subjected for protein identification by a tandem mass spectrometry as previously described (Shevchenko et al., 1996; Wilm et al., 1996).

Fusion Proteins

All GST and His fusion proteins were expressed in *E. coli* and affinity purified using GSH-beads and Ni-NTA Agarose (QIAGEN Inc, Canada) as per manufacturer's instruction.

In Vitro Binding Assay

The binding assay was performed as previously described (Qu et al., 2002).

Yeast Two-Hybrid

Plasmid construction: Plasmids were constructed using standard subcloning procedures. Briefly, an NcoI/SalI digest of p10 and an NcoI/BamHI digest of Prx2 were subcloned into NcoI/XhoI-digested pAS2-1 (Clontech, Canada) and Nco/BamHI-digested pACT2 (Clontech), respectively. Yeast two-hybrid screening (Clontech) pAS2-p10 and pACT2-Prx were transformed into Y187 and AH109 strains by the LiAc method (Ito et al., 1993) and plated onto SD-Trp^- and SD-Leu^- plates, respectively, as previously described (Mao et al., 2004). Plates were incubated for 5 days at 30°C. Resultant colonies were mated and selected on SD-$Leu^-Trp^-His^-$ for 3–7 days at 30°C.

Immunoprecipitation

Samples were harvested in lysis buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.2% Triton X-100) supplemented with protease inhibitors. Immunoprecipitations (IPs) were performed through incubation of antibodies with lysates overnight followed by incubation with anti-rabbit or anti-mouse Ig IP beads (eBiosciences, USA) at 4°C for 1 hr. The beads were washed three times by lysis buffer without protease inhibitors.

Neuronal Cultures

The primary culture of mouse cortical neurons was carried out as described previously (Fortin et al., 2001; Xiang et al., 1996). Alternatively, for midbrain neuronal cultures, the whole midbrain, without meninges and blood vessels, was collected from embryos aged 13.5 days gestation and processed as above and as similarly described (Liu et al., 2000). Cultures were subject to 20 μM MPP^+ or rotenone (as indicated in text). In select experiments, neurons were also pretreated with the calpain inhibitor PD150606 (Calbiochem, Canada), the Cdk inhibitor,

roscovitine (Sigma, Canada), or lithium (BDH, Canada) for 3 hr and then cotreated with 20 μM MPP^+. For survival using $p35^{-/-}$ neurons, littermate controls, or siRNA knockdowns, the MTT assay was utilized as per manufacturer's instruction (Sigma, Canada). For transfection or infection cultures, the alternative strategy described below was utilized since only a small percentage of the neurons in culture were targeted.

ROS Imaging

Cortical neurons were incubated with 10 μM DCF for 20 min at 37°C and washed three times with NB medium. The fluorescence signal of oxidized DCF was observed by an inverted fluorescent microscope equipped with a 100 W xenon lamp and filter (for oxidized DCF, excitation = 488 nm and emission = 510 nm). At least four random fields were quantified for DCF-positive cells and/or average intensity by image analyses.

Infection and Calcium Phosphate Transfection of Cultured Neurons

Cortical neurons were mixed with adenovirus at MOI of 50 prior to plating and were then immediately seeded to 24-well plates and cultured for three days as previously described (Aleyasin et al., 2004; O'Hare et al., 2005; Zhang et al., 2006). The cultures were exposed to MPP^+ for 48 hr. Cultures were then fixed and stained with Hoechst 33258 (0.5 ng/ml) and neuronal survival was evaluated by assessing nuclear integrity of GFP-positive neurons as previously described (Aleyasin et al., 2004). For transfection, 3 days after plating cortical neurons were transiently transfected using a modified calcium phosphate precipitation protocol (Xia et al., 1996; Zhang et al., 2006). In brief, neurons were transfected with 1 μg of total plasmid DNA (0.75 μg of plasmid DNA and 0.25 μg of pEGFP as a reporter) purified using an EndoFree Plasmid Maxi kit (QIAGEN, Inc, Canada). Twenty-four hours post-transfection, neurons were treated with 20 μM MPP^+ (48 hr) and were fixed in 4% paraformaldehyde (containing 0.2% picric acid in 0.1M phosphate buffer [pH 6.9]) and evaluated as described above. Alternatively, neurons were transfected with double-stranded short-interfering RNA (siRNA) to Prx2 or CY3-labeled control duplex (60 pmol siRNA/24-well) as previously described (Zhang et al., 2006). We have observed that targeting of duplexes to neurons is much more efficient than that of plasmids and have used this procedure previously (Aleyasin et al., 2004; Zhang et al., 2006). The Prx2 duplexes (s1: GCUUUCG GACUACAGAGGG, s2: GGGAUUCUUUAAGAGCUCU, s3: CCAAAU AAUUACUAGGCCU) along with a Cy3-labeled control duplex were obtained from Ambion (Austin, TX, USA). Forty-eight hours post-transfection, neurons were treated with MPP^+ (20 μM). At appropriate times, the cells were assayed for survival by MTT method (48 hr) or ROS as described above. Alternatively, cultures were analyzed by Western blot analyses for Prx2 levels (24 hr).

Peroxidase Activity Assay

Peroxidase activity was carried out by measurement of the consumption of NADPH (Fisher) which was mediated by Trx (Sigma, Canada) and Trx reductase (Sigma, Canada) at 30°C for 10 min for bacterially expressed proteins and 1 hr for precipitated proteins from cultured neurons or the SNc. In brief, 0.5 μg of bacterially expressed proteins or the precipitated proteins was incubated with 5 μM Trx, 1 μM Trx reductase, and 100 μM NADPH in HEPES (pH 7.5). The reaction was initiated by the addition of H_2O_2 at a final concentration of 0.2 mM. The consumption of NADPH was measured at 340 nm by spectrophotometer.

MPTP Administration

Mice received one intraperitoneal (i.p.) injection of MPTP·HCl per day (25 mg of free base per kg of body weight per injection; Sigma) for 3 or 5 consecutive days (Crocker et al., 2001; Kalia et al., 2004; Kim et al., 2005; Smith et al., 2003); control mice received an equivalent volume of 0.9% saline. Brains were extracted at indicated times and either perfused for immunohistochemical analyses or quickly removed and dissected for biochemical analyses.

Intrastriatal Administration of Adenoviruses

The adenoviruses expressing Prx2, Prx2T89A, and Prx2T89E were engineered using pAdEasy system as previously described (Sedarous et al., 2003). We and others have previously shown that adenoviruses can target the SNc from the striatum by retrograde transport (Crocker et al., 2001, 2003; Kalia et al., 2004; Kim et al., 2005; Smith et al., 2003). Each adenovirus was injected directly into the striatum of animals 7 days before initiation of MPTP treatment (as described above). A GFP-containing construct was used as a control. A single unilateral injection of each virus (2 μl, 1×10^7 particles per μl) was delivered to the right striatum (0.5 mm rostral, 2.2 mm right of bregma, and 3.4 mm below the skull surface). Each adenovirus injection was given at a constant rate (0.5 μl/min) by using a syringe pump system. Brains were extracted for immunohistochemistry and western blot analysis 14 days after the first MPTP treatment. Double-labeling experiments with GFP (present in all viral vectors) and TH indicated that the majority of SNc TH-positive neurons at the level of the medial terminal nucleus were also GFP positive for all viruses injected (GFP control, Prx2, Prx2T89A, and Prx2T89E).

Immunocytochemistry

Mice were perfused transcardially and brains were fixed in paraformaldehyde and cryoprotected as previously described (Crocker et al., 2003). Serial coronal sections (14 μm thickness) of the ventral midbrain were collected as free-floating sections in 0.01 M PBS/0.02% sodium azide or collected on slides. Sections were then incubated in primary antibody (to TH, 1:10,000; p-T89, 1:100, in 0.3% Triton X-100/0.01 M PBS) for 24 hr at 4°C. For TH staining on floating sections, slices were then incubated with biotinylated secondary antibody and streptavidin horseradish peroxidase-conjugated tertiary antibody and visualized by using a 3,3′-diaminobenzidine/glucose oxidase reaction as previously described (Crocker et al., 2003). To examine the distribution of phosphorylated Prx2 in DAergic neurons, a double-labeling immunofluorescence approach was used. After incubation with the specific primary antibody at 4°C, immunolabeling was visualized by using either Alex-488-conjugated anti-rabbit IgG (1:2000) or Alex-594-conjugated anti-mouse IgG (1:2000).

Quantification of DAergic Neuron Loss

The number of DAergic (TH-positive) neurons was only counted from the sections in the region containing the medial terminal nucleus (MTN) because this region has been previously shown to be expressed at the highest level of virus-mediated gene expression after intrastriatal infection (Crocker et al., 2001). We also used the MTN as a landmark to evaluate consistent levels of the SNc. Neurons ipsilateral and contralateral to the viral injection were assessed as described above in at least three sections per animal. The number of neurons from ipsilateral or contralateral was then counted as previously described. Alternatively, cresyl violet staining was performed to validate determination of nigral counts as previously described (Crocker et al., 2003).

Western Blot Analysis for the SNc

The western blot assay was performed as previously described (Smith et al., 2003). In brief, 50 μg of protein was analyzed by SDS-PAGE using antibodies to phospho-Prx2T89, Prx2, and β-actin.

Human Brain Samples

Paraffin-embedded blocks of postmortem human midbrain were collected from the Ottawa Hospital Department of Pathology. Autopsies were performed according to the policies and procedures of The Ottawa Hospital with consent from the next-of-kin. The tissue was deparaffinized in xylene and subjected to citrate antigen retrieval (Martins et al., 1999) prior to DAB staining. Diagnoses of PD were made based on medical histories and postmortem confirmation (J.M.W.).

The mean average age for PD (n = 5; 4 males and 1 female) and control patients (n = 6; 6 males) was 72.6 ± 3.7 and 72.5 ± 3.6, respectively, and showed no significance ($p < 0.986$). Postmortem intervals for PD and control samples did not differ significantly ($p < 0.3$).

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/55/1/37/DC1/.

ACKNOWLEDGMENTS

This work was partially supported by the Parkinson's Disease Foundation and the Parkinson's Society of Canada (D.Q.) and the Heart and Stroke Foundation (J.R., H.A.) and by funds from the Canadian Institutes of Health Research, the Parkinson's Society Canada, the Parkinson's Disease Foundation, the Parkinson's Research Consortium, the US army, and the Heart and Stroke Foundation of Ontario (D.S.P.).

Received: September 27, 2006
Revised: February 8, 2007
Accepted: May 24, 2007
Published: July 5, 2007

REFERENCES

Abou-Sleiman, P.M., Muqit, M.M., and Wood, N.W. (2006). Expanding insights of mitochondrial dysfunction in Parkinson's disease. Nat. Rev. Neurosci. *7*, 207–219.

Adam-Vizi, V. (2005). Production of reactive oxygen species in brain mitochondria: contribution by electron transport chain and non-electron transport chain sources. Antioxid. Redox Signal. *7*, 1140–1149.

Aleyasin, H., Cregan, S.P., Iyirhiaro, G., O'Hare, M.J., Callaghan, S.M., Slack, R.S., and Park, D.S. (2004). Nuclear factor-(kappa)B modulates the p53 response in neurons exposed to DNA damage. J. Neurosci. *24*, 2963–2973.

Ara, J., Przedborski, S., Naini, A.B., Jackson-Lewis, V., Trifiletti, R.R., Horwitz, J., and Ischiropoulos, H. (1998). Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Proc. Natl. Acad. Sci. USA *95*, 7659–7663.

Bonifati, V., Rizzu, P., van Baren, M.J., Schaap, O., Breedveld, G.J., Krieger, E., Dekker, M.C., Squitieri, F., Ibanez, P., Joosse, M., et al. (2003). Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science *299*, 256–259.

Braak, H., Del Tredici, K., Rub, U., de Vos, R.A., Jansen Steur, E.N., and Braak, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol. Aging *24*, 197–211.

Bu, B., Li, J., Davies, P., and Vincent, I. (2002). Deregulation of cdk5, hyperphosphorylation, and cytoskeletal pathology in the Niemann-Pick type C murine model. J. Neurosci. *22*, 6515–6525.

Burns, R.S., Chiueh, C.C., Markey, S.P., Ebert, M.H., Jacobowitz, D.M., and Kopin, I.J. (1983). A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Proc. Natl. Acad. Sci. USA *80*, 4546–4550.

Burns, R.S., LeWitt, P.A., Ebert, M.H., Pakkenberg, H., and Kopin, I.J. (1985). The clinical syndrome of striatal dopamine deficiency. Parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). N. Engl. J. Med. *312*, 1418–1421.

Busser, J., Geldmacher, D.S., and Herrup, K. (1998). Ectopic cell cycle proteins predict the sites of neuronal cell death in Alzheimer's disease brain. J. Neurosci. *18*, 2801–2807.

Canet-Aviles, R.M., Wilson, M.A., Miller, D.W., Ahmad, R., McLendon, C., Bandyopadhyay, S., Baptista, M.J., Ringe, D., Petsko, G.A., and Cookson, M.R. (2004). The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. Proc. Natl. Acad. Sci. USA *101*, 9103–9108.

Crocker, S.J., Lamba, W.R., Smith, P.D., Callaghan, S.M., Slack, R.S., Anisman, H., and Park, D.S. (2001). c-Jun mediates axotomy-induced dopamine neuron death in vivo. Proc. Natl. Acad. Sci. USA *98*, 13385–13390.

Crocker, S.J., Smith, P.D., Jackson-Lewis, V., Lamba, W.R., Hayley, S.P., Grimm, E., Callaghan, S.M., Slack, R.S., Melloni, E., Przedborski, S., et al. (2003). Inhibition of calpains prevents neuronal and behavioral deficits in an MPTP mouse model of Parkinson's disease. J. Neurosci. *23*, 4081–4091.

Dawson, T.M., and Dawson, V.L. (2003). Molecular pathways of neurodegeneration in Parkinson's disease. Science *302*, 819–822.

Dhavan, R., and Tsai, L.H. (2001). A decade of CDK5. Nat. Rev. Mol. Cell Biol. *2*, 749–759.

Fortin, A., Cregan, S.P., MacLaurin, J.G., Kushwaha, N., Hickman, E.S., Thompson, C.S., Hakim, A., Albert, P.R., Cecconi, F., Helin, K., et al. (2001). APAF1 is a key transcriptional target for p53 in the regulation of neuronal cell death. J. Cell Biol. *155*, 207–216.

Gong, X., Tang, X., Wiedmann, M., Wang, X., Peng, J., Zheng, D., Blair, L.A., Marshall, J., and Mao, Z. (2003). Cdk5-mediated inhibition of the protective effects of transcription factor MEF2 in neurotoxicity-induced apoptosis. Neuron *38*, 33–46.

Greenamyre, J.T., Betarbet, R., and Sherer, T.B. (2003). The rotenone model of Parkinson's disease: genes, environment and mitochondria. Parkinsonism Relat. Disord. *9 (Suppl 2)*, S59–S64.

Hoehn, M.M., and Yahr, M.D. (1967). Parkinsonism: onset, progression and mortality. Neurology *17*, 427–442.

Ito, M., Yasui, A., and Komamine, A. (1993). Precise mapping and molecular characterization of the MFT1 gene involved in import of a fusion protein into mitochondria in Saccharomyces cerevisiae. FEBS Lett. *320*, 125–129.

Jenner, P. (1998). Oxidative mechanisms in nigral cell death in Parkinson's disease. Mov. Disord. *13 (Suppl 1)*, 24–34.

Jin, M.H., Lee, Y.H., Kim, J.M., Sun, H.N., Moon, E.Y., Shong, M.H., Kim, S.U., Lee, S.H., Lee, T.H., Yu, D.Y., and Lee, D.S. (2005). Characterization of neural cell types expressing peroxiredoxins in mouse brain. Neurosci. Lett. *381*, 252–257.

Jonsson, G., Nwanze, E., Luthman, J., and Sundstrom, E. (1986). Effect of MPTP and its pyridinium metabolites on monoamine uptake and on central catecholamine neurons in mice. Acta Physiol. Scand. *128*, 187–194.

Kalia, S.K., Lee, S., Smith, P.D., Liu, L., Crocker, S.J., Thorarinsdottir, T.E., Glover, J.R., Fon, E.A., Park, D.S., and Lozano, A.M. (2004). BAG5 inhibits parkin and enhances dopaminergic neuron degeneration. Neuron *44*, 931–945.

Kim, R.H., Smith, P.D., Aleyasin, H., Hayley, S., Mount, M.P., Pownall, S., Wakeham, A., You-Ten, A.J., Kalia, S.K., Horne, P., et al. (2005). Hypersensitivity of DJ-1-deficient mice to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrindine (MPTP) and oxidative stress. Proc. Natl. Acad. Sci. USA *102*, 5215–5220.

Lang, A.E., and Lozano, A.M. (1998). Parkinson's disease. First of two parts. N. Engl. J. Med. *339*, 1044–1053.

Langston, J.W., Ballard, P., Tetrud, J.W., and Irwin, I. (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science *219*, 979–980.

Lee, K.Y., Rosales, J.L., Tang, D., and Wang, J.H. (1996). Interaction of cyclin-dependent kinase 5 (Cdk5) and neuronal Cdk5 activator in bovine brain. J. Biol. Chem. *271*, 1538–1543.

Lee, M.S., Kwon, Y.T., Li, M., Peng, J., Friedlander, R.M., and Tsai, L.H. (2000). Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature *405*, 360–364.

Li, B.S., Zhang, L., Takahashi, S., Ma, W., Jaffe, H., Kulkarni, A.B., and Pant, H.C. (2002). Cyclin-dependent kinase 5 prevents neuronal apoptosis by negative regulation of c-Jun N-terminal kinase 3. EMBO J. *21*, 324–333.

Liu, B., Du, L., and Hong, J.S. (2000). Naloxone protects rat dopaminergic neurons against inflammatory damage through inhibition of microglia activation and superoxide generation. J. Pharmacol. Exp. Ther. *293*, 607–617.

Mao, H., Zhao, Q., Daigle, M., Ghahremani, M.H., Chidiac, P., and Albert, P.R. (2004). RGS17/RGSZ2, a novel regulator of Gi/o, Gz, and Gq signaling. J. Biol. Chem. *279*, 26314–26322.

Martinat, C., Shendelman, S., Jonason, A., Leete, T., Beal, M.F., Yang, L., Floss, T., and Abeliovich, A. (2004). Sensitivity to oxidative stress in DJ-1-deficient dopamine neurons: an ES-derived cell model of primary Parkinsonism. PLoS Biol. *2*, e327. 10.1371/journal.pbio.0020327.

Martins, A.R., Dias, M.M., Vasconcelos, T.M., Caldo, H., Costa, M.C., Chimelli, L., and Larson, R.E. (1999). Microwave-stimulated recovery of myosin-V immunoreactivity from formalin-fixed, paraffin-embedded human CNS. J. Neurosci. Methods *92*, 25–29.

McShea, A., Harris, P.L., Webster, K.R., Wahl, A.F., and Smith, M.A. (1997). Abnormal expression of the cell cycle regulators P16 and CDK4 in Alzheimer's disease. Am. J. Pathol. *150*, 1933–1939.

Moon, J.C., Hah, Y.S., Kim, W.Y., Jung, B.G., Jang, H.H., Lee, J.R., Kim, S.Y., Lee, Y.M., Jeon, M.G., Kim, C.W., et al. (2005). Oxidative stress-dependent structural and functional switching of a human 2-Cys peroxiredoxin isotype II that enhances HeLa cell resistance to H2O2-induced cell death. J. Biol. Chem. *280*, 28775–28784.

Mouatt-Prigent, A., Karlsson, J.O., Agid, Y., and Hirsch, E.C. (1996). Increased M-calpain expression in the mesencephalon of patients with Parkinson's disease but not in other neurodegenerative disorders involving the mesencephalon: a role in nerve cell death? Neuroscience *73*, 979–987.

Nakamura, S., Kawamoto, Y., Nakano, S., Akiguchi, I., and Kimura, J. (1997). p35nck5a and cyclin-dependent kinase 5 colocalize in Lewy bodies of brains with Parkinson's disease. Acta Neuropathol. (Berl.) *94*, 153–157.

Nguyen, M.D., Boudreau, M., Kriz, J., Couillard-Despres, S., Kaplan, D.R., and Julien, J.P. (2003). Cell cycle regulators in the neuronal death pathway of amyotrophic lateral sclerosis caused by mutant superoxide dismutase 1. J. Neurosci. *23*, 2131–2140.

O'Hare, M.J., Kushwaha, N., Zhang, Y., Aleyasin, H., Callaghan, S.M., Slack, R.S., Albert, P.R., Vincent, I., and Park, D.S. (2005). Differential roles of nuclear and cytoplasmic cyclin-dependent kinase 5 in apoptotic and excitotoxic neuronal death. J. Neurosci. *25*, 8954–8966.

Osuga, H., Osuga, S., Wang, F., Fetni, R., Hogan, M.J., Slack, R.S., Hakim, A.M., Ikeda, J.E., and Park, D.S. (2000). Cyclin-dependent kinases as a therapeutic target for stroke. Proc. Natl. Acad. Sci. USA *97*, 10254–10259.

Patrick, G.N., Zukerberg, L., Nikolic, M., de la Monte, S., Dikkes, P., and Tsai, L.H. (1999). Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature *402*, 615–622.

Przedborski, S. (2005). Pathogenesis of nigral cell death in Parkinson's disease. Parkinsonism Relat. Disord. *11 (Suppl 1)*, S3–S7.

Qu, D., Li, Q., Lim, H.Y., Cheung, N.S., Li, R., Wang, J.H., and Qi, R.Z. (2002). The protein SET binds the neuronal Cdk5 activator p35nck5a and modulates Cdk5/p35nck5a activity. J. Biol. Chem. *277*, 7324–7332.

Rashidian, J., Iyirhiaro, G., Aleyasin, H., Rios, M., Vincent, I., Callaghan, S., Bland, R.J., Slack, R.S., During, M.J., and Park, D.S. (2005). Multiple cyclin-dependent kinases signals are critical mediators of ischemia/hypoxic neuronal death in vitro and in vivo. Proc. Natl. Acad. Sci. USA *102*, 14080–14085.

Rhee, S.G., Yang, K.S., Kang, S.W., Woo, H.A., and Chang, T.S. (2005). Controlled elimination of intracellular H(2)O(2): regulation of peroxiredoxin, catalase, and glutathione peroxidase via post-translational modification. Antioxid. Redox Signal. *7*, 619–626.

Rideout, H.J., Wang, Q., Park, D.S., and Stefanis, L. (2003). Cyclin-dependent kinase activity is required for apoptotic death but not inclusion formation in cortical neurons after proteasomal inhibition. J. Neurosci. *23*, 1237–1245.

Sarafian, T.A., Huang, C., Kim, A., de Vellis, J., and Shau, H. (1998). Expression of the antioxidant gene NKEF in the central nervous system. Mol. Chem. Neuropathol. *34*, 39–51.

Schapira, A.H. (2001). Causes of neuronal death in Parkinson's disease. Adv. Neurol. *86*, 155–162.

Sedarous, M., Keramaris, E., O'Hare, M., Melloni, E., Slack, R.S., Elce, J.S., Greer, P.A., and Park, D.S. (2003). Calpains mediate p53 activation and neuronal death evoked by DNA damage. J. Biol. Chem. *278*, 26031–26038.

Shendelman, S., Jonason, A., Martinat, C., Leete, T., and Abeliovich, A. (2004). DJ-1 is a redox-dependent molecular chaperone that inhibits alpha-synuclein aggregate formation. PLoS Biol. *2*, e362. 10.1371/journal.pbio.0020362.

Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. *68*, 850–858.

Smith, P.D., Crocker, S.J., Jackson-Lewis, V., Jordan-Sciutto, K.L., Hayley, S., Mount, M.P., O'Hare, M.J., Callaghan, S., Slack, R.S., Przedborski, S., et al. (2003). Cyclin-dependent kinase 5 is a mediator of dopaminergic neuron loss in a mouse model of Parkinson's disease. Proc. Natl. Acad. Sci. USA *100*, 13650–13655.

Smith, P.D., Mount, M.P., Shree, R., Callaghan, S., Slack, R.S., Anisman, H., Vincent, I., Wang, X., Mao, Z., and Park, D.S. (2006). Calpain-regulated p35/cdk5 plays a central role in dopaminergic neuron death through modulation of the transcription factor myocyte enhancer factor 2. J. Neurosci. *26*, 440–447.

Smythies, J., and Galzigna, L. (1998). The oxidative metabolism of catecholamines in the brain: a review. Biochim. Biophys. Acta *1380*, 159–162.

Songyang, Z., Lu, K.P., Kwon, Y.T., Tsai, L.H., Filhol, O., Cochet, C., Brickey, D.A., Soderling, T.R., Bartleson, C., Graves, D.J., et al. (1996). A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, phosphorylase kinase, calmodulin-dependent kinase II, CDK5, and Erk1. Mol. Cell. Biol. *16*, 6486–6493.

Sorimachi, H., Ishiura, S., and Suzuki, K. (1997). Structure and physiological function of calpains. Biochem. J. *328*, 721–732.

Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G., et al. (2004). Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science *304*, 1158–1160.

Wang, F., Corbett, D., Osuga, H., Osuga, S., Ikeda, J.E., Slack, R.S., Hogan, M.J., Hakim, A.M., and Park, D.S. (2002). Inhibition of cyclin-dependent kinases improves CA1 neuronal survival and behavioral performance after global ischemia in the rat. J. Cereb. Blood Flow Metab. *22*, 171–182.

Wang, K.K., and Yuen, P.W. (1994). Calpain inhibition: an overview of its therapeutic potential. Trends Pharmacol. Sci. *15*, 412–419.

Wang, K.K., Nath, R., Posner, A., Raser, K.J., Buroker-Kilgore, M., Hajimohammadreza, I., Probert, A.W., Jr., Marcoux, F.W., Ye, Q., Takano, E., et al. (1996). An alpha-mercaptoacrylic acid derivative is a selective nonpeptide cell-permeable calpain inhibitor and is neuroprotective. Proc. Natl. Acad. Sci. USA *93*, 6687–6692.

Watabe, S., Kohno, H., Kouyama, H., Hiroi, T., Yago, N., and Nakazawa, T. (1994). Purification and characterization of a substrate protein

for mitochondrial ATP-dependent protease in bovine adrenal cortex. J. Biochem. (Tokyo) *115*, 648–654.

Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T., and Mann, M. (1996). Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. Nature *379*, 466–469.

Xia, Z., Dudek, H., Miranti, C.K., and Greenberg, M.E. (1996). Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. J. Neurosci. *16*, 5425–5436.

Xiang, H., Hochman, D.W., Saya, H., Fujiwara, T., Schwartzkroin, P.A., and Morrison, R.S. (1996). Evidence for p53-mediated modulation of neuronal viability. J. Neurosci. *16*, 6753–6765.

Yang, K.S., Kang, S.W., Woo, H.A., Hwang, S.C., Chae, H.Z., Kim, K., and Rhee, S.G. (2002). Inactivation of human peroxiredoxin I during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfinic acid. J. Biol. Chem. *277*, 38029–38036.

Zhang, M., Li, J., Chakrabarty, P., Bu, B., and Vincent, I. (2004). Cyclin-dependent kinase inhibitors attenuate protein hyperphosphorylation, cytoskeletal lesion formation, and motor defects in Niemann-Pick Type C mice. Am. J. Pathol. *165*, 843–853.

Zhang, Y., Qu, D., Morris, E.J., O'Hare, M.J., Callaghan, S.M., Slack, R.S., Geller, H.M., and Park, D.S. (2006). The Chk1/Cdc25A pathway as activators of the cell cycle in neuronal death induced by camptothecin. J. Neurosci. *26*, 8819–8828.

JOURNAL OF NEUROCHEMISTRY | 2008 | 105 | 703–713 doi: 10.1111/j.1471-4159.2007.05166.x

Delayed combinatorial treatment with flavopiridol and minocycline provides longer term protection for neuronal soma but not dendrites following global ischemia

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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.

Keywords: cerebral ischemia, cyclin-dependent kinases, flavopiridol, minocycline.
J. Neurochem. (2008) **105**, 703–713.

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.* 2003; 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, is observed following both focal and global ischemia. DNCdk5

Received October 27, 2007; revised manuscript received November 26, 2007; accepted November 26, 2007.

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Abbreviations used: 4VO, four vessels occlusion; ACSF, artificial CSF; CDK, cyclin-dependent kinase; DMSO, dimethyl sulfoxide; DN, dominant negative; fEPSP, field excitatory post-synaptic potentials; GFAP, glial fibrillary acidic protein; PBS, phosphate-buffered saline.

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* models 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 4 h following global ischemia protected CA1 neurons and resulted in improved behavioral 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 (Osuga *et al.* 2000; 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, 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 regiment 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

Global ischemia

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 was performed on male Wistar rats (180–220 g; Charles River, Saint-Constant, Quebec, Canada) using the four vessels 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 (assess 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 10–15 s 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 10 min 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 intraperitoneal 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 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, 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 (7 μm) 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 sample were collected in the anesthetized animals by heart puncture in 1 mL 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 1).

Table 1 Summary of selected physiological parameters 1 h after reperfusion or flavopiridol infusion

Variables	Sham operated	4VO	4VO + flavopiridol (500 μmol/L)
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. 4VO, four vessels occlusion.

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 step 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-color where appropriate using Northern eclipse. Cells positive for each appropriate staining was 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 neuron was 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 (Fig. 1b) than rats receiving either minocycline (Fig. 1d) or flavopiridol alone (Fig. 1c) or vehicles + saline treatment (Fig. 1e). Eighty-eight percentage 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 (Fig. 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 protected CA1 neurons 2 weeks following ischemia (Fig. 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.

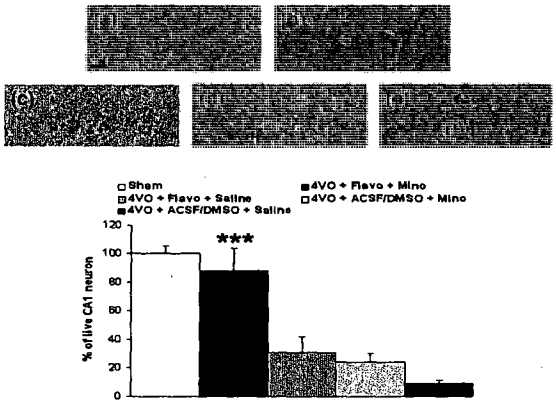

Fig. 1 Combined treatment of flavopiridol and minocycline provides synergistic protection neurons 2 weeks following 10-mins 4VO. (a–e) Hematoxylin and eosin stained representative sections of CA1 of (a) sham control, $n = 5$, (b) 4VO + flavopiridol + minocycline, $n = 5$, (c) 4VO + flavopiridol + saline, $n = 4$, (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 (Fig. 2a) which is in sharp contrast to a robust microgliosis seen in the untreated ischemic rats both at 2 and 5 days (Fig. 2b and f) following ischemia. The number of CD11b expressing microglia was increased significantly at 5 days, 95.7 ± 15.5 (Fig. 2f) following global ischemia compared with 41.3 ± 5.3 at 2 days ($p < 0.02$). Treatment of ischemia-induced rats with minocycline alone (Fig. 2d) or minocycline + flavopiridol (Fig. 2e) blocked increases in microglia. Surprisingly, flavopiridol also inhibited microglia CD11b expression (Fig. 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 (Fig. 2h–l) but not in the sham operated (Fig. 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 (Fig. 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 (Fig. 2h–l) compared with untreated ischemic (4VO + ACSF/DMSO + saline) rats. 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 (Fig. 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 (Fig. 3c and e vs. b, and f). Minocycline alone also appears to reduce astrogliosis (Fig. 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.

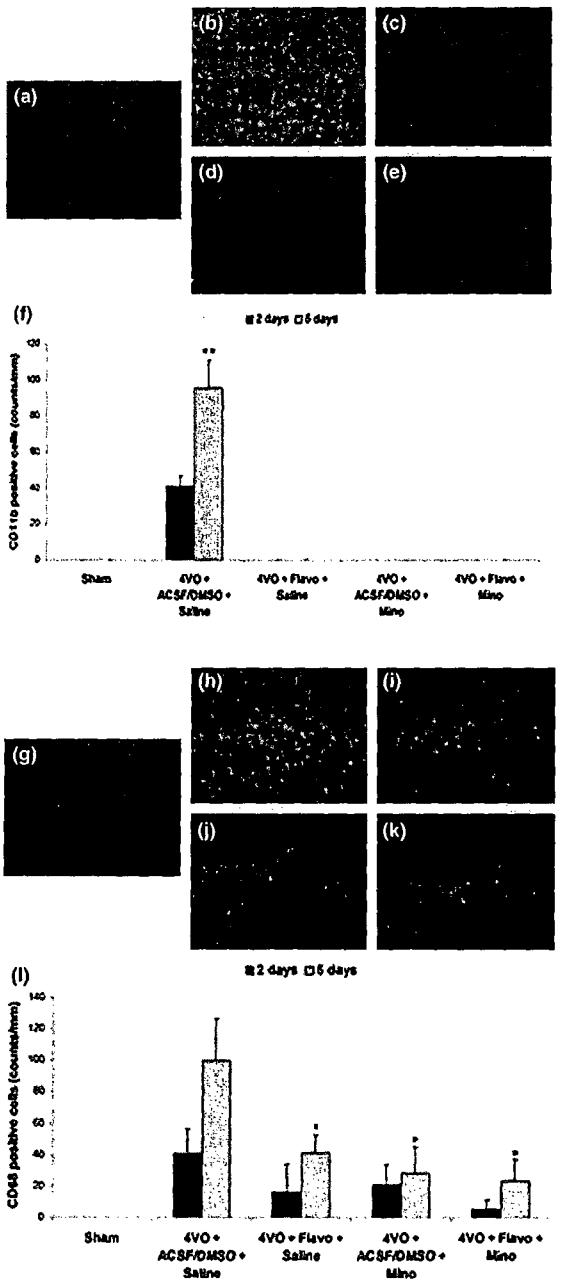

Fig. 2 Induction of CD11b and CD68 in the rat hippocampus 5 days following global ischemia. Photomicrographs of microglial CD11b expression using OX-42 antibody (a–e) and CD68 expression using ED1 antibody (g–k). (a and g) sham control, $n = 3$, (b and h) 4VO + ACSF/DMSO vehicle + saline, $n = 3$, (c 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.

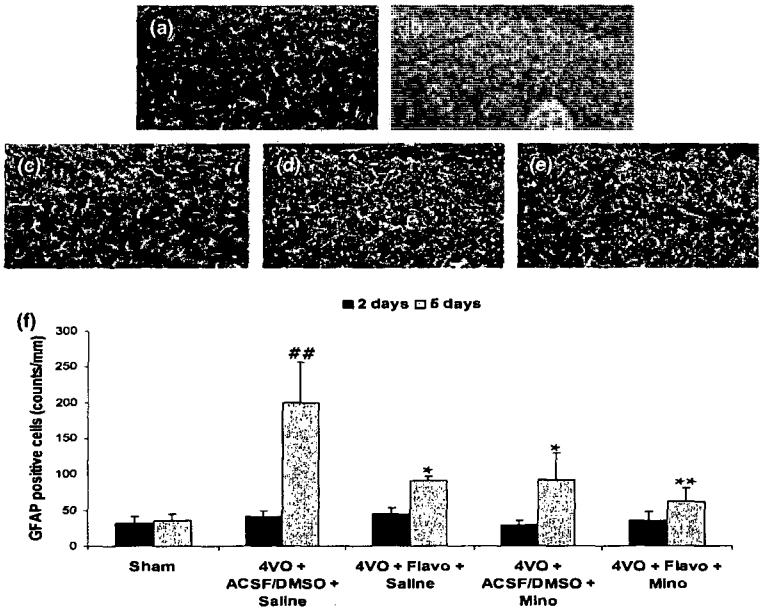

Fig. 3 Photomicrographs of GFAP stained rat brain sections 5 days following global ischemia. (a) Sham control, $n = 4$, (b) 4VO + ACSF/DMSO vehicle + saline, $n = 3$, (c) 4VO + flavopiridol + saline, $n = 5$, (d) 4VO + ACSF/DMSO vehicle + minocycline, $n = 3$, and (e) 4VO + flavopiridol + minocycline 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 describe 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%) (Fig. 4b vs. c–e). However, there was a dramatic reduction in the level of neuroprotection observed in the

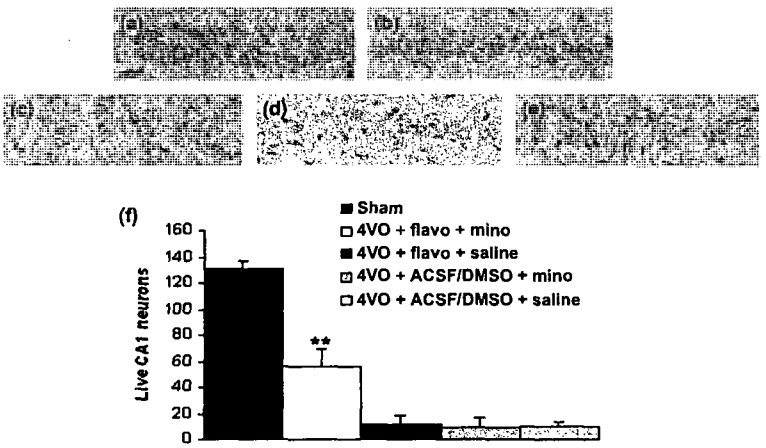

Fig. 4 Combinatorial treatment of flavopiridol and minocycline protects CA1 neurons 10 weeks following global ischemia. (a–e) Hematoxylin and eosin stained representative sections of CA1 of (a) sham control, $n = 9$, (b) 4VO + flavopiridol + minocycline, $n = 4$, (c) 4VO + flavopiridol + saline, $n = 5$, (d) 4VO + ACSF/DMSO vehicle + minocycline, $n = 4$, and (e) 4VO + ACSF/DMSO vehicle + saline treated rats, $n = 4$. Images were captured under 20x 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 + 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).

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 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 (Fig. 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.

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 (Fig. 6c and d) as is evident from the loss of synaptophysin staining when compared with sham control (Fig. 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 (Fig. 6e and f, respectively). Generally, more synaptophysin stained processes were observed in the combined treatment group at 2 weeks than at 10 weeks (Fig. 6c vs. 6d). However, the difference between these two time points was not significant (Fig. 6e and f). Similarly, a decline in MAP-2 immunoreactivity was observed in the

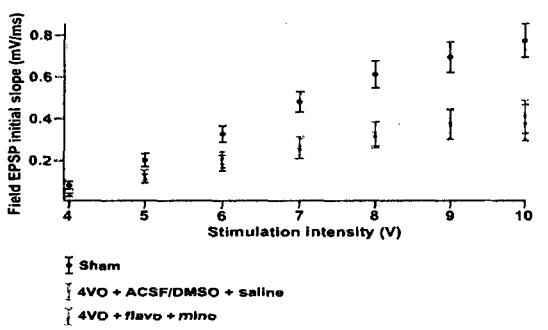

Fig. 5 CA1 neurons protected by combined treatment of flavopiridol and minocycline are synaptically impaired. Electrophysiology recording of CA1 neurons 10 weeks following global ischemia. The Shaffer-collateral pathway was stimulated at intensities ranging from 4 to 10 V. Field excitatory post-synaptic 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.

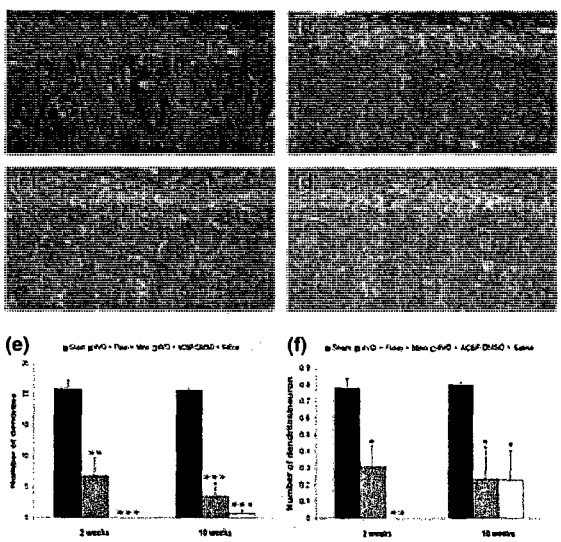

Fig. 6 Synaptophysin marker staining showed degenerating processes in animals treated with combination flavopiridol and minocycline. Representative photomicrographs of synaptophysin stained sections (a) sham control, $n = 3$, (b) 4VO + ACSF/DMSO vehicle + saline control, $n = 3$, (c) 4VO + flavopiridol + 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).

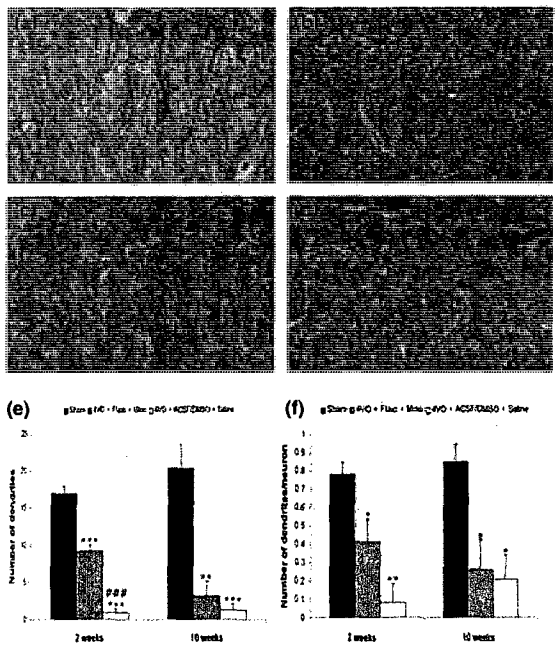

Fig. 7 MAP-2 marker staining showed degenerating processes in animals treated with combination flavopiridol and minocycline. Representative photomicrographs of MAP-2 stained sections. (a) Sham control, $n = 3$, (b) 4VO + ACSF/DMSO vehicle + saline control, $n = 3$, (c) 4VO + flavopiridol + minocycline 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).

combined treatment group both at 2 weeks ($p < 0.001$) and at 10 weeks ($p < 0.01$) when compared with sham control (Fig. 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 (Fig. 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.

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, 1999; 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 has been reported to contribute to inconsistent absorption when administered intraperitoneally (Fagan *et al.* 2004), the lack of CD11b staining at these time points suggest 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 them self 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 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 model of cerebral ischemia, including human stroke (Liu *et al.* 1998; Jin *et al.* 2001, 2006; Zhang *et al.* 2001). 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.* 2004; 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 death 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 agent (Wang *et al.* 2007). Accordingly, studies, including our own, targeting components of inflammatory reaction have demonstrated protection in models of cerebral ischemia (Yrjanheikki *et al.* 1998, 1999; Lucas *et al.* 2006; Wang *et al.* 2007).

In summary, our result using standard histological method show that we can provide enduring protection for neuronal soma using both CDKs and 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.

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).

References

Blum D., Chtarto A., Tenenbaum L., Brotchi J. and Levivier M. (2004) Clinical potential of minocycline for neurodegenerative disorders. *Neurobiol. Dis.* **17**, 359–366.

Chen M., Ona V. O., Li M. *et al.* (2000) Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. *Nat. Med.* **6**, 797–801.

Di Giovanni S., Movsesyan V., Ahmed F., Cernak I., Schinelli S., Stoica B. and Faden A. I. (2005) Cell cycle inhibition provides neuroprotection and reduces glial proliferation and scar formation after traumatic brain injury. *Proc. Natl Acad. Sci. USA* **102**, 8333–8338.

Du Y., Ma Z., Lin S. *et al.* (2001) Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. *Proc. Natl Acad. Sci. USA* **98**, 14669–14674.

Fagan S. C., Edwards D. J., Borlongan C. V., Xu L., Arora A., Feuerstein G. and Hess D. C. (2004) Optimal delivery of minocycline to the brain: implication for human studies of acute neuroprotection. *Exp. Neurol.* **186**, 248–251.

Giuliani F., Fu S. A., Metz L. M. and Yong V. W. (2005) Effective combination of minocycline and interferon-beta in a model of multiple sclerosis. *J. Neuroimmunol.* **165**, 83–91.

Hewlett K. A. and Corbett D. (2006) Delayed minocycline treatment reduces long-term functional deficits and histological injury in a rodent model of focal ischemia. *Neuroscience* **141**, 27–33.

Jin K., Minami M., Lan J. Q., Mao X. O., Batteur S., Simon R. P. and Greenberg D. A. (2001) Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. *Proc. Natl Acad. Sci. USA* **98**, 4710–4715.

Jin K., Wang X., Xie L., Mao X. O., Zhu W., Wang Y., Shen J., Mao Y., Banwait S. and Greenberg D. A. (2006) Evidence for stroke-induced neurogenesis in the human brain. *Proc. Natl Acad. Sci. USA* **103**, 13198–13202.

Katchanov J., Harms C., Gertz K. *et al.* (2001) Mild cerebral ischemia induces loss of cyclin-dependent kinase inhibitors and activation of cell cycle machinery before delayed neuronal cell death. *J. Neurosci.* **21**, 5045–5053.

Kim S. U. and de Vellis J. (2005) Microglia in health and disease. *J. Neurosci. Res.* **81**, 302–313.

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

Lucas S. M., Rothwell N. J. and Gibson R. M. (2006) The role of inflammation in CNS injury and disease. *Br. J. Pharmacol.* **147**(Suppl. 1), S232–S240.

Osuga H., Osuga S., Wang F., Fetni R., Hogan M. J., Slack R. S., Hakim A. M., Ikeda J. E. and Park D. S. (2000) Cyclin-dependent kinases as a therapeutic target for stroke. *Proc. Natl Acad. Sci. USA* **97**, 10254–10259.

Peeling J., Yan H., Buist R., Sitar D. S. and Corbett D. (2006) Protective effect of minocycline treatment on striatal ischemia. *J. Stroke Cerebrovasc. Dis.* **15**, 101–105.

Rashidian J., Iyirhiaro G., Aleyasin H., Rios M., Vincent I., Callaghan S., Bland R. J., Slack R. S., During M. J. and Park D. S. (2005) Multiple cyclin-dependent kinases signals are critical mediators of ischemia/hypoxic neuronal death in vitro and in vivo. *Proc. Natl Acad. Sci. USA* **102**, 14080–14085.

Shelton S. B. and Johnson G. V. (2004) Cyclin-dependent kinase-5 in neurodegeneration. *J. Neurochem.* **88**, 1313–1326.

Smith P. D., Crocker S. J., Jackson-Lewis V. *et al.* (2003) Cyclin-dependent kinase 5 is a mediator of dopaminergic neuron loss in a mouse model of Parkinson's disease. *Proc. Natl Acad. Sci. USA* **100**, 13650–13655.

Stefanis L., Park D. S., Friedman W. J. and Greene L. A. (1999) Caspase-dependent and -independent death of camptothecin-treated embryonic cortical neurons. *J. Neurosci.* **19**, 6235–6247.

Stoll G., Jander S. and Schroeter M. (1998) Inflammation and glial responses in ischemic brain lesions. *Prog. Neurobiol.* **56**, 149–171.

Sun Y., Jin K., Xie L., Childs J., Mao X. O., Logvinova A. and Greenberg D. A. (2003) VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. *J. Clin. Invest.* **111**, 1843–1851.

Thored P., Arvidsson A., Cacci E., Ahlenius H., Kallur T., Darsalia V., Ekdahl C. T., Kokaia Z. and Lindvall O. (2006) Persistent production of neurons from adult brain stem cells during recovery after stroke. *Stem Cells* **24**, 739–747.

Tikka T., Fiebich B. L., Goldsteins G., Keinanen R. and Koistinaho J. (2001) Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. *J. Neurosci.* **21**, 2580–2588.

Wang F., Corbett D., Osuga H., Osuga S., Ikeda J. E., Slack R. S., Hogan M. J., Hakim A. M. and Park D. S. (2002) Inhibition of cyclin-dependent kinases improves CA1 neuronal survival and behavioral performance after global ischemia in the rat. *J. Cereb. Blood Flow Metab.* **22**, 171–182.

Wang L., Zhang Z., Wang Y., Zhang R. and Chopp M. (2004) Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. *Stroke* **35**, 1732–1737.

Wang Q., Tang X. N. and Yenari M. A. (2007) The inflammatory response in stroke. *J. Neuroimmunol.* **184**, 53–68.

Weishaupt J. H., Kussmaul L., Grotsch P., Heckel A., Rohde G., Romig H., Bahr M. and Gillardon F. (2003) Inhibition of CDK5 is protective in necrotic and apoptotic paradigms of neuronal cell death

and prevents mitochondrial dysfunction. *Mol. Cell. Neurosci.* **24**, 489–502.

Yrjanheikki J., Keinanen R., Pellikka M., Hokfelt T. and Koistinaho J. (1998) Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. *Proc. Natl Acad. Sci. USA* **95**, 15769–15774.

Yrjanheikki J., Tikka T., Keinanen R., Goldsteins G., Chan P. H. and Koistinaho J. (1999) A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. *Proc. Natl Acad. Sci. USA* **96**, 13496–13500.

Zhang R. L., Zhang Z. G., Zhang L. and Chopp M. (2001) Proliferation and differentiation of progenitor cells in the cortex and the subventricular zone in the adult rat after focal cerebral ischemia. *Neuroscience* **105**, 33–41.

Zhu S., Stavrovskaya I. G., Drozda M. *et al.* (2002) Minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in mice. *Nature* **417**, 74–78.

