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Neuropilins: Their Transcriptional Regulation and Role in Ischemia-Induced Neuronal Cell Death

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NEUROPILINS: THEIR TRANSCRIPTIONAL REGULATION AND ROLE IN ISCHEMIA-INDUCED NEURONAL CELL DEATH

A Thesis Submitted to the Department of Graduate Studies within the Faculty of Medicine
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ABSTRACT

Elucidation of molecular mechanisms modulating the death and failure of neurons to regenerate after cerebral ischemia is important in developing therapeutics to stroke. Here, results demonstrate that the pro-death transcription factor E2F1 inhibits neuronal survival through up-regulation of the receptor for axon repulsive guidance molecule, NRP-1. First, NRP-1 is shown as a direct target of E2F1 based on: reactivation of NRP-1 expression in E2F1 -/- neurons after E2F1 replacement, EMSA and reporter assays confirming E2F1 binding and activation of the NRP-1 promoter, respectively. Second, pharmacological and genetic inhibition of NRP-1 conferred neuroprotection. Collectively, these findings support a model in which E2F1 targets NRP-1 to modulate axonal damage and neuronal death in response to cerebral ischemia. Future work should determine the differential contributions of NRP-1 and NRP-2 in neuronal survival. Studies *in vivo* will also confirm the therapeutic benefit of inhibition of NRPs in stroke recovery.

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LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AraC	Cytosine-β-D-arabinofuranoside
ATP	Adenosine triphosphate
BDNF	Bovine derived growth factor
bp	Base pair
BSA	Bovine serum albumin
C56/Bl6	C56/Black 6 mice
CDK	Cyclin dependent kinase
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
CGN	Cerebellar granular neuron
CHIP	Chromatin immunoprecipitation
c-myb	Transcription factor c-myb
CNS	Central nervous system
CT	Computed tomography
CTL	Control
CRMP	Collapse response mediator protein
Cy3	Cyanine 3
DAPI	4',6-diamidino-2-phenylindole
dCTP	2'-deoxycytidine 5'-triphosphate

ddH2O	Double distilled water
DIV	Days *in vitro*
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
dsDNA	Double stranded DNA
DTT	Dithiothreitol
ECL	Enhanced chemiluminscence
ED_{50}	Effective dose for 50% of treated cells
EDTA	Ethylene diamine tetracetic acid
E2F1	Transcription factor E2F1
EGTA	Ethylene glycol tetraacetic acid
EMSA	Electrophoretic mobility shift assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GPI	Glycosylphosphotidylinositol
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphate hydrolase
h	Hours
HEK 293	Human embryonic kidney cells

H_2O	Water
HRP	Horse radish peroxidase
KO	Knock-out
LTP	Long-term potentiation
MAP2	Microtubule associated protein 2
min	Minutes
MCAO	Middle cerebral artery occlusion
MEM	Minimal essential media
MGF	Mouse cross SV129 x C57Bl/6
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NF-kB	Nuclear factor kappa-B
NGF	Nerve growth factor
NIH	National Institutes of Health
NMDA	N-methyl-D-aspartic acid
NRP-1	Neuropilin-1
NRP-2	Neuropilin-2
Nt.	Nucleotide
OD	Optical density
OGD	Oxygen glucose deprivation
p53	Protein 53
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.05 %

	Tween
PCR	Polymerase chain reaction
PET	Positron emission tomography
PFA	Paraformaldehyde
PI	Propidium iodide
PLEX	Plexin
Poly-dIdC	Polydeoxyinosinic-deoxycytidylic acid
pRb	Phosphorylated retinoblastoma
Rb	Retinoblastoma
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
Sema	Semaphorin
TIA	Transient ischemic attack
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
VEGF	Vascular endothelial growth factor
WT	Wild type

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CHAPTER 1 LITERATURE REVIEW

1.1 Stroke Review

1.1.1 Stroke Statistics

According to the Heart and Stroke Foundation of Canada, stroke is the fourth leading cause of death in Canada, and while 300, 000 Canadians per year are living with the effects of stroke another 16, 000 have deceased as a result of it, in 2006 alone. (HSFO, 2006). About 80% of strokes are ischemic, meaning they are caused by the interruption of blood flow to the brain due to vessel blockage (HSFO, 2006). Fatty acid and cholesterol accumulation are common causes of atherosclerotic plaques, which narrow the arteries and may lead to transient or complete blockage of brain vessels (Andreoli et al., 2004). Given the high fat diets, sedentary lifestyles, and increasing stress levels of many Canadians today, stroke incidence is predicted to increase exponentially. Interestingly, women are more likely to incur a fatal stroke than males of equal age and after age 55 the risk of stroke doubles every 10 years (HSFO, 2006).

Aside from the severity and prevalence of this condition, the Canadian government spends more than 2.7 billion dollars per year treating it, further indicating its effect on our population (HSFO, 2006). Research teams and health care professionals world-wide have recognized the need to address this potent health issue. For example, the Heart and Stroke Foundation of Canada, a foundation from which this thesis is supported, had allocated more than 7.6 million dollars annually for stroke research. Although substantial progress has

been made over the years, many more questions still need to be addressed in order to prevent strokes, and improve treatment options and prognoses for stroke patients.

The primary focus of this thesis is *focal* cerebral ischemia, which is clearly differentiated from a *global* ischemic event such as general lack of oxygen delivery to the brain resulting from incidents including heart failure or drowning. In *focal* cerebral ischemia a specific brain vessel is blocked either partially or completely, leading to oxygen deprivation of tissue areas which are fed by the affected vessel. Although collateral vessel branching and hemodynamic autoregulation of brain vessels will allow partial perfusion of the ischemic area, this is often not sufficient to prevent tissue damage (Andreoli et al., 2004). To date, minimal clinical treatment options exist for cerebral ischemia (Gladstone et al., 2002); these treatments will be discussed in section 1.1.4. The lack of treatment options underlies the importance of studies such as this one designed to further elucidate underlying causes of neuronal loss and endogenous obstacles to neuroregeneration following focal ischemic insult.

1.1.2 Stroke History and Milestones

Some of the earliest descriptions of stroke are attributed to Hippocrates in 400 BC, when he described a sudden onset of paralysis he called *apoplexy* (Garrison, 1969). In his writings, he made the following observations: "Persons are most subject to apoplexy between the ages of forty and sixty" and "it is impossible to remove a strong attack of apoplexy, and not easy to remove a weak attack" (Garrison, 1969). With the development of more highly sophisticated research models, protocols, and methods of observing

physiological events, modern interventions have delayed onset of stroke and improved the prognosis of stroke patients albeit to limited extent (Garrison, 1969).

During the mid-1600s Johann Wepfer became the first person to investigate the pathology underlying this so called apoplexy. Among many accomplishments, he identified signs of post-mortem bleeding in patients who died of apoplexy and observed the carotid and vertebral arteries while performing autopsies (Thompson, 1996). An important conclusion drawn by Wepfer was that, besides being caused by bleeding of the brain (presently known as an arterial hemorrhage), apoplexy could be caused by blockage of one of the main arteries supplying blood to the brain (Gurdjian, 1979). Thus, stroke was first recognized as a cerebrovascular disease.

Further pathological observations of ischemic brains included the discovery of Pierre Marie in 1901 of "softenings" of brain tissue surrounding the occluded vessel. (Poirier and Derouesne 1985). C Miller Fisher made several important contributions to the understanding of stroke pathogenesis. He identified the relationship between obstruction of the internal carotid arteries in the neck and cerebrovascular disease and further suggested that thrombotic debris were responsible for the event (Fisher, 1951).

One of the biggest advances for studies of stroke was the development of neuroimaging techniques, the first of which being early cerebral angiography developed by Moniz in 1927 (Ferro, 1988). Ten years later, Moniz provided the first description of internal carotid artery occlusion through this method (Ferro, 1988). A significant step was made by Reid and Spencer in 1972 when the first ultrasonic image of the carotid arteries and the bifurcation were recorded (Reid and Spencer, 1972). Based on previous experiments using computed tomography, Hounsfield introduced computed tomography

(CT) for commercial use in 1972 (Webb et al., 1992) and the first neurological clinical application using CT of the brain was presented by Ambrose (Ambrose, 1973). The application of nuclear magnetic resonance to imaging, independently demonstrated by Bloch and Purcell, led to magnetic resonance imaging (MRI) (Andrew, 1992). Lauterbur and Damadian developed the first low quality medical images in the early 1970s (Seynaeve and Broos, 1995). Presently, MRI, CT as well as positron emission tomography (PET) technologies have enormous implications for earlier treatment, improved prognoses and determination of precise brain areas affected by a given vessel occlusion.

1.1.3 Clinical Synopsis of Acute Focal Ischemic Stroke

The circulation of the brain is divided into anterior and posterior circulating vessels, formed by the carotid and vertebral arteries, respectively. The common carotid artery bifurcates into the external and internal carotid arteries in the neck. The branches of the internal carotid artery, the anterior cerebral artery and middle cerebral artery, supply the medial and lateral surfaces of the cerebral cortex, respectively. Smaller ancillary branches of these arteries are commonly affected in focal cerebral ischemia and produce characteristic physical signs such as: ipsilateral blindness, contralateral loss of movement or sensation in the face, arm, and sometimes leg, as well as difficulty in swallowing (Andreoli et al., 2004). The posterior circulation may also be affected by focal cerebral ischemia. The posterior and anterior inferior cerebellar arteries are branches of the vertebral arteries and supply the cerebellum and brain stem regions. Vessel occlusion affecting the cerebellum is likely to produce symptoms such as loss of motor control, dizziness, and

memory loss (Andreoli et al., 2004). Partial physiological deficits may be recovered through physiotherapy, which makes use of the neuronal plasticity to reform lost network connections in the brain.

1.1.4 Stroke Treatments

Once causes and effects of stroke were classified, prevention was the next target of many researchers. For example, the first use of aspirin for vascular prevention is attributed to Craven in 1950 (Craven, 1950). McDevitt and colleagues described the effectiveness of anticoagulant therapy in patients with cerebral vesicular blockages in the 1950s (McDevitt et al., 1958). Three basic groups of drugs are currently in use for the treatment of stroke. These are the "clot-busters" such as tissue plasminogen activator (tPA), which lyse the blood clot within the vessel, anti-platelet drugs (such as aspirin), which prevent platelet aggregation and decrease chances of clot formation and anti-coagulants (such as heparin), which act to prevent further clotting from occurring by thinning the blood.

Currently, the only government approved treatment for acute ischemic stroke in North America is the administration of the thrombolytic agent tPA to lyse the clots (Wardlaw et al., 2000). Studies show that patients are 32% more likely to be protected from suffering disability but tPA must be delivered within a narrow window of 3 hours of stroke onset (National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). Further, there is some experimental evidence that this treatment, although beneficial at initial reduction of tissue damage, may have negative implication on post-ischemic recovery, including exacerbation of neuronal damage (Wang et al., 1998, Traylenis and

Lipton, 2001) and death (Nagai et al., 1999). Thus, physicians need to use strict inclusion and exclusion criteria in order to determine which patients may benefit from tPA treatment (Wardlaw et al., 2000). Because of strict prescription criteria, controversy over tPA use, and a narrow therapeutic window, on average in North America, only 5% of stroke patients actually benefit from tPA use (Weinberger, 2006). Current clinical and pre-clinical studies are aimed at strategies to extend the narrow therapeutic window through use of combinations of drugs, surgical interventions, and understanding of the ischemic cascade at the molecular level (Weinberger, 2006).

1.1.5 Animal Models of Stroke

An enormous driving force for stroke research has been the development of animal models for both global and focal cerebral ischemia. Although these models prove extremely useful in observation of a physical barrier to blood flow to the brain, it must be kept in mind that stroke in humans is a complex multi-factorial process, of which animal models can represent only certain aspects (Small and Buchan, 2000). Of interest to this thesis, is the focal ischemic model in mice and rats, known as middle cerebral artery occlusion (MCAO). This surgical procedure involves the insertion of a thin nylon filament from the carotid artery into the middle cerebral artery of the brain, (which is the most commonly affected vessel in human cerebral ischemia [Slater, et al., 2006]), where it stays to block blood flow for a given period of time. The filament may then be removed and the rodent brain allowed to reperfuse for a given period of time. (Koizumi et al., 1986; Longa et al., 1989). This tool has enabled a considerable amount of information to be obtained about the

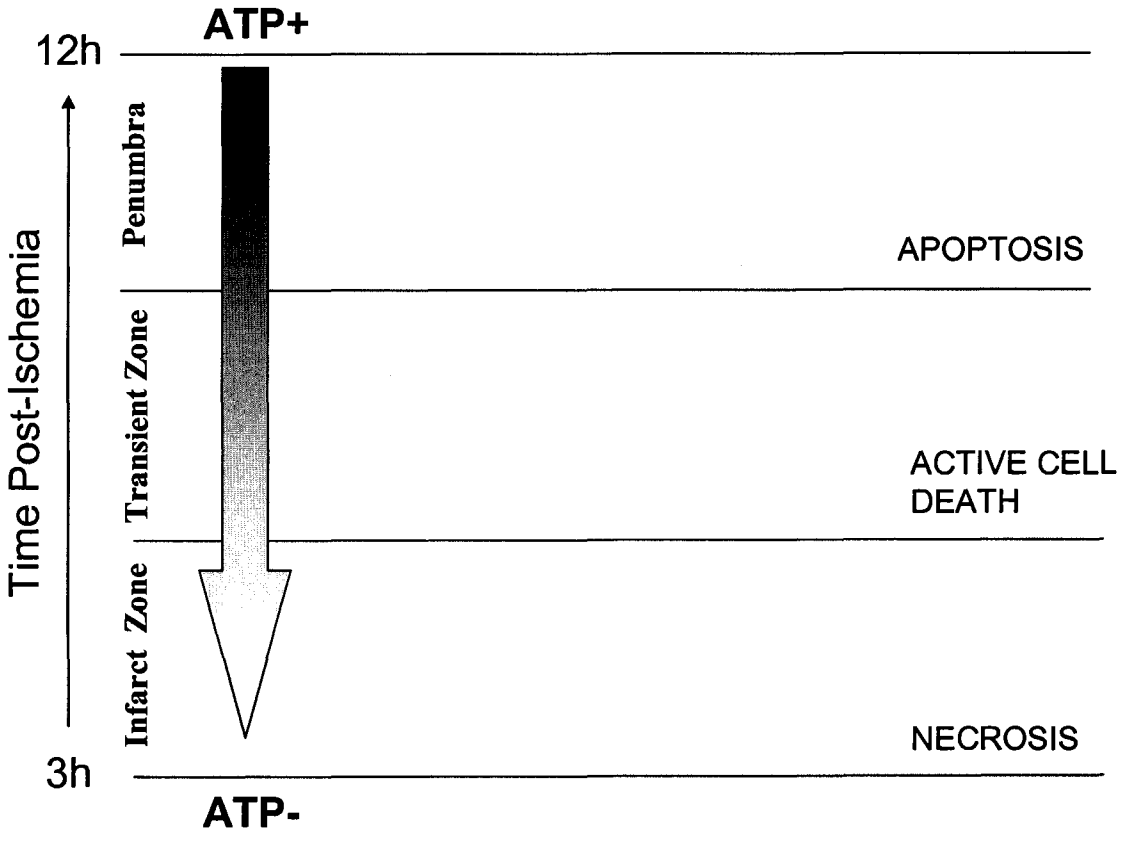
pathophysiological and biochemical effects of cerebral ischemia in mammals and from this, specific biochemical events have been characterized.

1.1.6 Specific Biochemical Stages in Stroke-Induced Damage and Cell Death

The first stage of tissue damage via cerebral ischemia is the sudden reduction of blood flow through a vessel, resulting from such causes as hemorrhages, induction of clotting cascades, and fatty plaque accumulation within the inner vessel wall (Weinberger, 2006). After acute, transient blood vessel occlusion in the brain, there is an induction of necrotic cell death in the region designated as the *infarct core* due to lack of oxygen and glucose; reviewed by Hou and MacManus (2002). Cell death occurs rapidly after vessel occlusion, creating a very narrow therapeutic window for the prevention of neuronal loss. The *infarct core* indicates the area of the brain tissue, which has been directly deprived of blood supply, and acute damage is typically observed within a 3 hour window (Hou and MacManus, 2002).

There exists however, more distal areas of the tissue which are affected by the ischemic event, and the resulting damage to these areas is often conspicuously delayed. A simplified diagram, modified from Hou and MacManus (2002), illustrates the importance of perfusion and ATP levels in determining the fate of neurons after cerebral ischemia (Fig. 1). In the infarct zone, where perfusion is limited and ATP levels are the lowest, neurons die rapidly by necrosis (Eguchi et al., 1997; Leist et al., 1997).

Fig.1 Post-ischemic zones of tissue damage. Energy supply to neurons as obtained from the circulation is critical not only to neuronal survival, but also to the type of cell death process that ensues. In the infarct core, where the blood and energy supply from the occluded vessel approaches zero, neurons die by a necrosis – a form of cell death, which is irreversible once initiated. Within 3 hours of vessel occlusion, energy supply drops rapidly in the surrounding brain tissue. Although the tissue is partially perfused by collateral vessels, energy supplies required for viability are not maintained. In the transient zone, the presence of a low level of residual ATP may initially activate apoptosis, which is intrinsically programmed and energy dependent. However, as energy levels continue to decrease with time, many neurons in this area abort the apoptotic pathway, and lyse via necrosis. The penumbra zone is of interest with respect to providing a longer window period for treatment. Here, neurons may survive up to 12 hours after stroke onset, and since they maintain adequate levels of ATP during this time, die by the slower, and more controlled process of apoptosis. Halting the delayed cell death in the penumbra is a target of current research. Adapted from Hou and MacManus, (2002).


ATP+
12h
Time Post-Ischemia
Penumbra
Transient Zone
Infarct Zone
APOPTOSIS
ACTIVE CELL DEATH
NECROSIS
3h
ATP-

The *transient* and *penumbra* ischemic zones designate areas surrounding the infarct core, with levels of ATP increasing in the direction of transient zone to penumbra (Asrup et al., 1981; Hossman, 1993). These zones display a more delayed and active cell death, where caspases become activated and the neurons die via a mechanism that is largely considered to be on the continuum between apoptosis and necrosis (Hou and MacManus, 2002; Benchoua et al., 2001; Martin, 2001). Post-ischemic neurons in the transient zone, where ATP levels are initially sufficient for induction of active death mechanisms typically exhibit hallmarks of apoptosis such as effector caspase activation and DNA fragmentation, yet cytologically the final step of the death process resembles necrosis (Benchoua et al., 2001). In the penumbra, where most of the energy is preserved, ATP levels are sufficient to complete an apoptotic pathway (Hu et al., 1999). The relative contribution of these delayed cell death mechanisms to the physiological outcome of human cerebral ischemia remains unclear (Heiss et al., 1999; Heiss, 2000), however, they currently remain the target of many researched therapeutic strategies.

Although the degree of initial neuronal cell loss induced by ischemia in the infarct zone is important to the outcome of ischemic insult, the failure of surrounding, more distal neurons to regenerate is also a hindrance to post-ischemic recovery prospects. Ramon y Cajal was the first neuroanatomist to theorize that learning and memory is not due to an increase in neuronal number but is due instead to strengthening, and remodeling of existing neuronal connections in the developed brain (Ramon y Cajal, 1894). This effect has since been studied both in terms of physiological plasticity, where learning and memory take place, and in terms of pathological plasticity which occurs after injury such as cerebral ischemia. Long term-potentiation (LTP), which is the stimulation-dependent increase in

chemical synaptic strength, is thought to play a role in both types of neuronal plasticity (physiological and pathological). An increase in glutamate release from neurons in the infarcted region, stimulate α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors and the consequent increase in calcium influx, induces a state of excitotoxicity. Interestingly, transient ischemic attacks (TIAs), wherein a brain vessel is blocked only partially and for a small amount of time, have been shown to induce protection from effect of a consequent acute focal cerebral event (Kitagawa et al., 1990; Stagliano et al., 1999). It is thought that transient excitotoxcity, leads to a form of LTP, which may stimulate delayed post-stroke recovery of neurological deficits (Calbresi et al., 2003). Yet, after a longer period of energy deprivation from focal cerebral ischemia, pathological plasticity of neurons is induced and is thought to play a role in the transition of the ischemic penumbra to the ischemic core, through induction of delayed cell death mechanisms as reviewed by Calbresi et al., (2003). The longer the period of energy deprivation, the more likely that physiological plasticity such as that which occurs in the context of learning and memory, may also be impaired (Calbresi et al., 2003). Thus the goal of current studies is to prevent physiological loss of motor, memory and learning skills post-ischemia through elucidation of molecular mechanisms that: 1) reduce the number of neurons lost to the ischemic event, and 2) encourage the formation of new synaptic routes which can replace those lost through cell death and pathological plasticity.

Neurons which are located in the ischemic penumbra, physically distal from the ischemic core, yet affected via diffusion-perfusion mismatch, show many initial adaptive responses after ischemia, as reviewed by Kier and Wardlaw (2000). These responses

include the axonal extension of growth cones towards the injured area (Stroemer et al., 1995), as well as induction of LTP (Calbresi et al., 2003) presumably attempting to restore synaptic connections that were lost by neuronal death in the infarct zone. The penumbra is thus a target of research for therapeutic interventions, which aim to overcome a central nervous system (CNS) environment which is generally inhibitory to neuronal regeneration (Sandvig et al., 2004).

Axonal regeneration in the injured CNS has long thought to be attenuated both physically and biochemically by the formation of a neural scar in the damaged area (Clemente, 1964; Windle, 1956). This scar is comprised of meningeal fibroblasts, astrocyte processes and macrophage/microglial cell infiltration (Sandvig et al., 2004). Although CNS neurons show initial evidence of axonal extension (Clemente, 1964; Windle, 1956) near the scar, this intrinsic capacity for regeneration is thought to be suppressed, both by myelin associated factors that are restricted to the CNS and the plethora of chemorepellant guidance cues secreted from the damaged infarct core (Sanvig et al., 2004). Semaphorin (Sema) 3A, for example, is known to inhibit axonal outgrowth through its ligation with its receptor, neuropilin-1 (NRP-1) (He and Tessier-Lavigne, 1997) and has been shown along with NRP-1 to be up-regulated in both neurons and meningeal fibroblasts that comprise a post-injury scar (Pasterkamp et al., 1999; Fujita et al., 2001; Zhang et al., 2001).

Post-ischemic stimuli for cell death include glutamate excitotoxicity, disruption of calcium homeostasis, oxidative stress leading to DNA damage, and pro-death signalling by cytokines, chemokines and adhesion molecules, as reviewed by Martin, (2001). Intracellular proteases and endonucleases become activated and excessive free radical generation during reperfusion of the tissue leads to the development of oxidative stress

(Martin, 2001). These stimuli have been shown to lead to an initial overall suppression of gene expression (Hossmann, 1993; Hu and Wielock, 1993; DeGracia et al., 1996; Althausen et al., 2001), wherein reduction of blood flow appears to have the greatest impact on gene expression (Jacewicz et al., 1986; Mies et al., 1991). The changes in gene expression in the brain that occur as a result of focal cerebral ischemia are of significant interest to this thesis, as certain pro-death molecular mediators may be targeted for therapeutic intervention. Despite overall suppression of translation, specific hypoxia-induced gene expression is observed in genes such as antioxidant molecules, heat shock protein 70, stress activated protein kinases, pro- and anti-apoptotic genes as reviewed by Hou and MacManus (2002). Interestingly, the pro-death transcription factor E2F1 has been found through cDNA microarray analysis to be significantly up-regulated post ischemia (Jin et al., 2001) and found to play a major role in post-ischemic neuronal death (MacManus et al., 1999; Hou et al., 2000; O'Hare et al., 2000). The elucidation of E2F1 target genes and their role post-ischemia are of significant interest to the Hou laboratory and the impetus behind this thesis.

1.2 E2F1

1.2.1 E2F nuclear transcription factors

First identified as a cellular factor that bound and activated the adenoviral E2 gene promoter, E2F proteins became of great interest in the oncology field of research when it was discovered that E2F could be associated with and regulated by the tumor suppressor

protein retinoblastoma (Rb) (Chellappan et al., 1991; Bandara et al., 1991). Exploiting the ability of E2F to bind Rb, three separate groups of researchers cloned E2F1, the first of the E2F gene family to be identified (Helin et al., 1992; Kaelin et al., 1992). The other family members were later cloned by searching for sequence homologies to E2F1, and by immunoprecipitation assays with Rb and Rb-associated proteins, as reviewed by DeGregori and Johnson (2006).

The E2F family of nuclear transcription factors consists of 8 family members to date, E2F1-E2F8, which act in a coordinated manner to regulate both cellular proliferation and cell death (DeGregori and Johnson, 2006). E2F family members are divided into two groups: transcriptional activators (E2F1, E2F2, and E2F3a) and transcriptional repressors (E2F3b-E2F8) (DeGregori and Johnson, 2006). The DNA binding domain shared by the E2F family members represents the area of greatest homology. In fact, each E2F transcription factor binds to a specific E2F consensus sequence to transcriptionally activate target genes, which are largely cell cycle regulating genes, especially at the G1/S phase checkpoint (DeGregori and Johnson, 2006). In addition, chromatin immunoprecipitation (CHIP) analyses have shown that E2F family members are also important for regulating the transcription of genes involved in DNA damage response, chromatin dynamics, and mitotic spindle checkpoint (Ren and Dynlacht, 2004).

The control of cell cycle transition through the G1/S phase check point is largely regulated by the physical interaction between Rb and E2F proteins as reviewed Crosby and Almasan (2004). When Rb is hypophosphorylated, it is able to bind to E2F family members and inhibit their activities. Through the action of cyclin dependent kinase (CDK) 4/6 and CDK 2 complexes, Rb becomes hyperphosphorylated and its physical inhibition on E2F is

released (Crosby and Almasan, 2004). Functional E2F molecules in complex with obligate binding partner DP protein, may act as either transcriptional repressors or derepressors for molecules that determine the fate of mitotic cells to live, divide, or die (Chellappan et al., 1991).

1.2.2 The Dual Functionality of E2F1

Of the eight E2F family members, E2F1 has been studied in the most depth, especially with respect to its dual ability, to function both as a promoter of cellular proliferation (Xu et al., 1995) and later as a trigger of apoptosis (Phillips et al., 1999). Some researchers suggest that these opposing roles of E2F1 are dependent on the degree to which E2F1 is expressed in the context of the cell cycle and/or following DNA damage and the activation of its target genes, (Crosby and Almasan, 2004).

Depending on the cell type and context of internal and external stimuli, E2F1 over-expression can lead to either tumorigenesis or apoptosis. For example, E2F1 has been shown to induce neoplasia in astrocytes *in vitro* (Miyajima et al., 1996) and E2F1 expression is significantly elevated in many forms of cancer, including colon cancer (Kashara et al., 2000). Yet in other situations, increased expression of E2F1 can lead to apoptosis through, for example, increasing the stability of the pro-death protein 53 (p53) (Heibert et al., 1995). Another way that increased E2F1 levels could lead to apoptosis is by causing a general deregulation of expression of many of the hundreds of E2F1 target genes (Stanelle et al., 2002). Evidence suggests in neuronal models that an increase in E2F1 plays a significant role in the biochemical induction of an active cell death process. Downstream

transcriptional targets of E2F1 in neurons are either repressed or activated as a result of this increase in E2F1 post-global ischemia (Jin et al., 2001). One such study has shown that E2F1 upregulation triggers neuronal death via activation of known pro-apoptotic molecules such as Bax and caspases (Giovanni et al., 2000). Elucidation of the downstream effects of this transcriptional regulation of E2F1 targets is critical to understanding effects of E2F1 in the context of ischemic insult. This type of investigation has fundamentally impacted upon cancer therapeutic strategies. Many pre-clinical trials have attempted to make use of E2F1's ability to induce cell death. Adenoviral delivery of E2F1 to certain human cancer cells has resulted in growth suppression and further has resulted in increased sensitivity to certain chemotherapeutic agents (Stanelle and Putzer, 2006). Salient to this thesis, although the role of E2F1 as a pro-death transcription factor may be useful in an oncological context, this function is likely extremely deleterious following ischemic attack and thus represents a possible therapeutic target.

1.2.3 E2F1 in Neuronal Cell Death

Although the transcriptionally regulated component of apoptotic neuronal death is considered a crucial stage of a developing nervous system (Oppenheim, 1991), evidence has accumulated to show that this mechanism is also likely responsible for the pathological cell death that occurs with insult, injury and disease of mature neurons (Greene et al., 2004). Mature neurons do not undergo mitosis, yet nonetheless maintain expression of cell cycle related molecules including E2F1. The level of E2F1 activity is decreased, however, compared to that found in proliferating neuronal precursors (Dagnino et al., 1997). In

differentiated rodent neurons, E2Fs are found to be in complex with phosphorylated retinoblastoma (pRb) and are transcriptionally inactive (Corbeil et al., 1995). Upon neuronal insult, cell cycle related proteins such as cyclins, CDKs and E2F1 become activated, and deregulation of these genes leads to induction of active cell death mechanisms (Park et al,. 1998; Park et al., 2000; Greene and Liu, 2004). Some researchers speculate that injured post-mitotic neurons may be initiating a failed and deregulated attempt at restoring cell number through mitosis, which leads instead to cell death (Husseman et al., 2000; Raina et al., 2000).

The exact role of E2F1 in ischemia-induced cell death is not known, however, previous studies have implicated E2F1 in the decision between neuronal survival and death. For example, Rb-deficient mice exhibit extensive apoptosis during neurogenesis as a result of the increase in free E2F1 molecules (Jacks et al., 1992, Lee et al., 1992, MacLeod et al., 1996). Transgenic mice over-expressing E2F1 were found to have increased cellular apoptosis, whereas mice genetically devoid of E2F1 had a developmental phenotype of increased apoptosis in the thymus (Field et al., 1996; Yamasaki et al., 1996). Over-expression of E2F1 using replication deficient recombinant adenoviral technology in primary cortical mouse neurons was also associated with caspase 3 activation, DNA fragmentation and punctate nuclei, characteristic of apoptotic loss (Hou et al., 2000). Further, E2F1 expression was found to be increased after UV-induced apoptosis in dividing mammalian cells (Huang et al., 1997) and later a temporal association of E2F1 up-regulation and neuronal apoptosis was shown (Hou et al., 2000; O'Hare et al., 2000). Cortical cultures and hippocampal slices from E2F1 -/- mice show attenuated neurotoxicity when challenged with known pro-apoptotic stimuli such as staurosporine and UV treatment

(Gendron et al., 2001). Further, when treated with oxygen-glucose deprivation (OGD), an *in vitro* ischemic insult, E2F1 -/- neuronal cultures and brain slices were less susceptible to neuronal death (Gendron et al., 2001). The same publication also demonstrated that messenger ribonucleic acid (mRNA) levels of E2F1 were increased following OGD, supporting a role for E2F1 in ischemia-induced neuronal cell death. Later experiments demonstrated that brains of E2F1 -/- mice showed significantly reduced infarct sizes after middle cerebral artery occlusion (MCAO) and improved post-ischemic behavior ratings (MacManus et al., 2003). Collectively, these studies implicate E2F1 in the control of ischemia-induced neuronal apoptosis.

The exact mechanism through which E2F1 is involved in active cell death is not clear, however, it may be a combinatorial result of induction or stabilization of pro-apoptotic genes and suppression of survival genes (Phillips and Vousden, 2001). In some cases, E2F1 may promote apoptosis via a mechanism that is independent of transcriptional activation, suggesting a possible inhibition of pro-survival genes such as nuclear factor kappa-B (NF-kB) (Hou et al., 2001). In other cases, E2F1 has been shown to act as a transcriptional activator of pro-apoptotic molecules, such as transcription factor B and transcription factor c-myb (Liu and Greene, 2001). Thus, identification of E2F1 target genes is a necessary step in elucidating a mechanism for its effects.

1.3 NRPs and Semas as Axonal Guidance Molecules

1.3.1 Neuronal Guidance

The neuronal network of the central nervous system during both the dynamic embryonic development and the relatively stable mature adult system depends highly on refined directional guidance of the axon by signals in its surroundings as reviewed by Fiore and Püschel, (2003). The motile structure at the tip of a growing axon, known as the growth cone, must integrate attractant, repellent, pro-death, pro-survival and neutral signals to form a functional response. In embryonic development, gradients of secreted ligands are formed in order to guide axons to their correct targets and allow them to form appropriate neuronal connections (Mueller, 1999). It is thought that a similar integration of external environmental cues must also occur in the adult brain in diseased or injured states, where damage repair may be attempted (Ming et al., 1997; Song et al., 1998; Song and Poo, 1999; Stein and Tessier-Lavigne, 2001).

1.3.2 Semas

Semas comprise a large family of proteins, including membrane bound and secreted forms which are grouped into 8 classes according to structural features (Semaphorin Nomenclature Committee, 1999). The vertebrate class 3 Semas are secreted and believed to form steep tissue gradients with cellular directional effects (Luo et al., 1993). The other vertebrate Sema classes are integral membrane proteins or glycosylphosphotidylinositol (GPI)-linked proteins, which seem to function in short-range intercellular signalling (Tamagnone and Comoglio, 2000). It has also been proposed that these membrane-bound Semas could act not only as receptors but also as ligands (Tamagnone et al., 1999).

The focus of this thesis lies on class 3 Semas, which are divided further into six family members, Sema3A-3F, according to structure and ligand specificity. These molecules have been known as strong chemorepellant factors for quite some time. In fact, Sema3A was the first chemorepellant cue to be discovered. It was found in 1993 as a secreted soluble protein isolated from chick brain to have the ability to repel and collapse of sensory dorsal root ganglion axons (DRG)s (Luo et al., 1993, Kolodkin et al., 1993) and was known at that time as collapsin-1. Presently, it is recognized that Class 3 Semas are secreted by both neurons and glial cells and are of particular interest since they are thought to mediate chemorepulsion of axons surrounding the post-ischemic infarct core in a paracrine-type manner (De Winter et al, 2002). According to research by Giger et al., (1998), Class 3 Semas are widely expressed within the central nervous system of both rats and humans. Using *in situ* hybridization it was determined that specific areas of the cerebral cortex, as well as the cerebellum express Class 3 Semas (Giger et al., 1998).

The role of class 3 Sema signalling in axonal repulsion is exemplified by a Sema3D knock-out (KO) mouse model, in which cranial and spinal sensory neurons are found to enter areas that are typically not innervated (Taniguchi et al., 1997). Further, in a mouse model of cerebral ischemia, Sema3A mRNA has been found to be increased in the ischemic parietal cortex (Fujita, 2001; Zhang et al., 2001), indicating that this pathway may play a role in post-ischemic cellular responses, such as neuronal cell death and axonal damage. Attenuation of Sema3A signalling has also been found to confer significant protection against cell death as induced, for example, by dopamine stimulation (Shirvan et al., 1999).

In is interesting to note, however, that there have been literature published which indicate a bifunctionality of Sema proteins as both chemorepellants and chemoattractants depending on the intracellular cyclic nucleotide content of the stimulated cell. For example, transient high levels of cyclic guanosine monophosphate (cGMP) in one end of a developing axon causes it to grow toward a Sema gradient, whereas low levels of cGMP cause the dendrites to form by repulsion towards the opposite direction (Song et al., 1998; Rohm et al., 2000). Thus, it is also possible that a neural response to extracellular ligands may be regulated by intracellular means.

1.3.3 Neuropilins as Sema Receptors

Working to elucidate a receptor for Class 3 Semas, researchers made use of Sema-alkaline phosphatase (AP) and Sema-Fc fusion proteins to detect binding partners via immunoprecipiation. In 1997 a membrane protein was identified as the neuronal receptor for Sema3 (He and Tessier-Levigne, 1997; Kolodkin et al., 1997) and the following year Plexins were found to be receptors for class 1,4,7 Semas (Comeau et al., 1998, Winberg et al., 1998) and requisite co-receptors for Class 3 Semas. Ironically, seven years earlier, a research group in Japan had already discovered Neuropilin-1 (NRP-1) and Plexin receptor proteins (Tagaki et al., 1991). They had been found as antigens to two monoclonal antibodies in a screen for epitopes with specific distributions in the nervous system (Tagaki et al., 1991), yet their functionalities had not yet been determined.

Since identification of NRP-1 as a Sema3 binding receptor, a second NRP family member, Neuropilin-2 (NRP-2), was found (Chen et al., 1997). Presently, it is known that

Semas exert very specific effects on neurons depending on their expression levels of NRP-1 and NRP-2. For example, studies using antibodies against NRPs suggested that NRP-1 has the highest affinity for Sema3A, NRP-2 for 3F, and heterodimers of NRP-1/NRP-2 have the highest affinity for Sema3C (Giger et al., 1998; Chen et al., 1997). Sema 3A has not been found to interact specifically with NRP-2 alone (He et al., 2002). Interestingly, there is a discrepancy in the literature with respect to the biochemical explanation of this preferential binding. Takahashi et al., (1999) report it is due to an increased specificity of the different receptor complexes, and another group reports it is due to increased expression and availability of receptors (Rohm et al., 2000).

The interaction of NRPs with their Sema ligands has since been well characterized. Bioactive Sema3A has been found to bind NRP receptors as a disulfide-linked dimer (Koppel et al., 1998). This pathway is important for mediating neuronal responses, such as axon fasciculation, branching, axonal repulsion and growth cone collapse in the developing CNS; reviewed in He et al., (2002). This signaling pathway is extremely important in guiding developing axons and leading to the establishment of correct synaptic targets at the proper developmental stage.

Although important during embryogenesis, studies over the past decade additionally reveal an important role of NRP-Sema signaling in the mature, adult CNS (de Wit and Verhaagen, 2003). Several Sema family members, including Class 3 Semas have found to be expressed in both rat and human brains past development and fairly constant expression levels are maintained throughout adulthood (Giger et al., 1998). One hypothesis to explain this expression pattern is the suggestion that NRP-Sema signaling is involved in the maintenance and stability of adult neuronal networks wherein uncontrolled axonal

sprouting and synaptogenesis could have devastating consequences (deWit and Verhaagen, 2003). Although generally beneficial, the attenuation of neuronal migration and axonal outgrowth poses a severe restriction on the possibility of regeneration after injury or neurodegenerative disease.

Presently, it is known that the NRP family of receptors are composed of two homologous members NRP-1 and NRP-2, both of which have recently been found to exist in various truncated forms as a result of alternative splicing processes (Rossignol et al., 2000). Both have an approximate molecular weight of 130 kDa and are known to interact with ligands of the Sema family of proteins; reviewed by (Shirvan et al., 1999). Antibody perturbation, genetic knockout experiments and gene transfer studies have confirmed that NRP-1 is essential for Sema3A function (He and Tessier-Lavigne, 1997; Kitsukawa et al., 1997; Kolodkin et al., 1997; Takahashi et al., 1998). Interestingly, the cytoplasmic portion of these integral membrane proteins do not have any enzymatic activity and are believed to be dispensable with respect to the activation of the NRP/Sema pathway (Nakamura et al., 1998) , implicating another protein as the signal transducing component for Sema action. The binding activity of the NRPs however, is not indispensable as indicated by the phenotypes of the corresponding genetic knock out animals. Targeted disruption of NRP-1 in mice leads to an embryonic lethal phenotype, as a result of defective neuronal connectivity and insufficient vascularization (Kitsukawa et al., 1995). The NRP-2 null mutant mice are viable, however they display abnormal axonal patterning without apparent vascular phenotype (Chen et al., 2000; Giger et al., 2000).

1.3.4 The Role of NRP-1 in Angiogenesis

NRP-1 may not only regulate ischemic injury through neuronal impact but has also been shown to participate in angiogenesis (Shima and Mailhos, 2000; Mukouyama et al., 2002). Analogous to axonal growth cone guidance, angiogenic sprouting of endothelial cells involves specialized endothelial tip cells that can sense and are attracted to gradients of vascular endothelial growth factor (VEGF) (Gerhardt et al., 2004). Curiously, NRP-1 is also a receptor for VEGF165 as reviewed by Pasterkamp and Kolodkin (2003) and has been found to promote chemotaxis of endothelial cells (Soker et al., 1998). It is interesting to note that this response is antagonized by Sema3A which has been found to share the same extracellular NRP-1 binding region as VEGF165 (Miao et al., 1999). Whether NRP-1/Sema3A signaling plays a role in angiogenesis is not yet clear, however some evidence exists that Sema3A may also have repulsive effects on endothelial cells as well as on axons (Bates et al., 2003). NRP-1/VEGF165 signaling, however, seems to promote angiogenesis since mice expressing a mutated form of NRP-1 was only capable of binding to the VEGF165 ligand and not to Sema3A, developed normal blood vessels, but displayed markedly aberrant axonal projections (Gu et al., 2003). Of pertinence to cerebral ischemia, one group has found that application of VEGF165 to post-ischemic mouse brains *in vivo* lead to significantly reduced brain infarct volume and neuronal injury (Hayashi et al., 1998). Sema3A, VEGF, and NRPs have all been found to be expressed at CNS injury sites (Fujita et al., 2001; Skold et al., 2000., Bartholdi et al., 1997). It is possible that VEGF165 and Sema3A are competitors for NRP-1, and that relative amounts of each ligand may

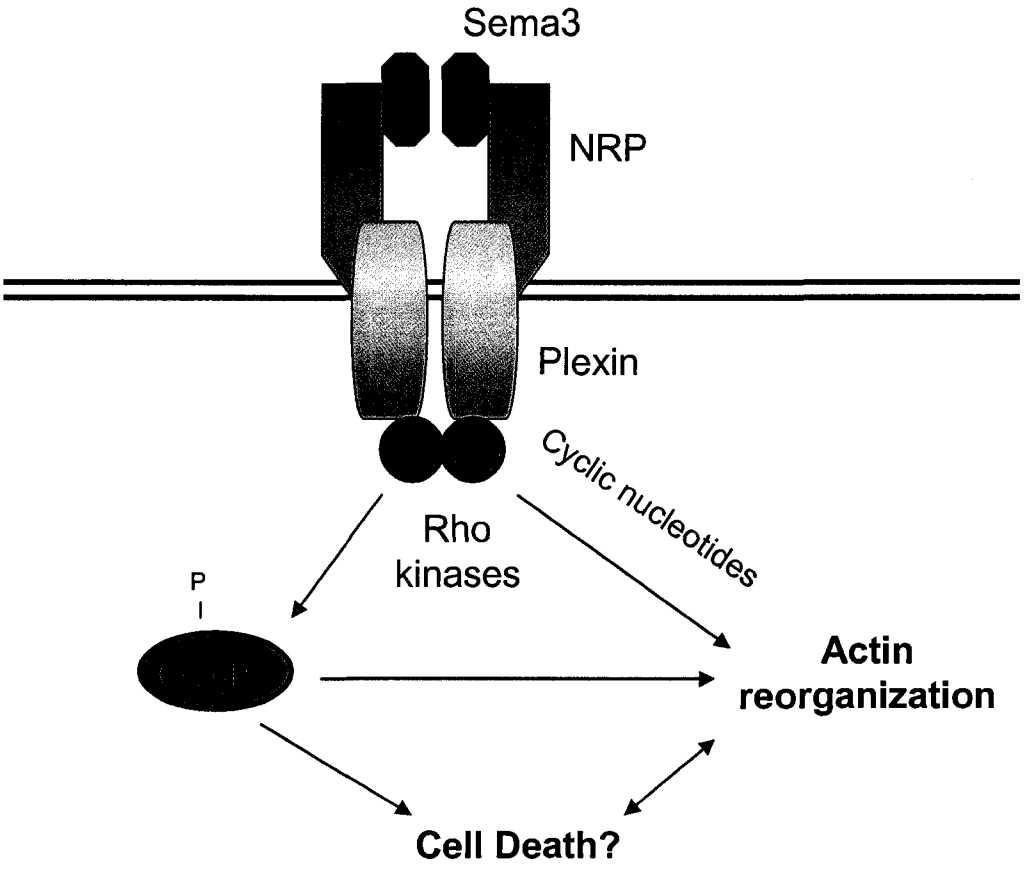
determine the signaling pathway transduced by the receptive cell. For this to be the case, different signal transducing co-receptors are likely required for this differential signaling.

1.3.5 Plexins as NRP Co-Receptors

Plexins (classes A-D) mediate NRP-1 signalling by acting as independent binding receptors for membrane bound Semas or in complex with NRPs for secreted class 3 Semas (Comeau et al., 1998; Windberg et al., 1998). Class A Plexins have been most thoroughly studied especially with respect to their role as obligate signal transducing component of the receptor complex activated by Sema3A binding to NRP-1 (Takahashi et al., 1999).

Presently, the known functional Sema receptor is a multi-protein complex composed of NRP as the ligand-binding component and Plexin as the signal-transducing component (Rohm et al., 2000). Upon Sema binding to NRP, the cytoplasmic tyrosine kinase domain of the plexin is then thought to induce, directly or indirectly, F-actin depolymerization along the axon, and growth cone collapse likely through the activation of small Rho family guanosine triphosphate hydrolases (GTPases), RhoA, Rac1 and Cdc42 (He and Tessier-Levigne, 1997; Rohm and Rahim, 2000; Gallo and Letourneau, 2004). Figure 2 illustrates potential binding partners in this dynamic multi-protein complex and gives a schematic of possible downstream signaling responses. The exact signal transduction cascade that includes these signaling proteins and links Semas to the cytoskeleton and to pro-death outcomes is complex and still incompletely understood (Fiore and Puschel, 2003), however, it is thought that collapse response mediator proteins (CRMP)s may be a central downstream target, and integrators of neuronal stimuli. For

Fig.2. Schematic representation of possible signal transduction pathways triggered by Sema3A. Class3 Sema dimers bind to NRPs directly. The full membrane receptor complex is yet to be elucidated, but is known to include clustered Plexin membrane co-receptor molecules. Upon Sema3 binding to NRP-1/-2 homo- or heterodimers, physical auto-inhibition of Plexins is thought to be removed through conformational changes (Takahashi and Strittmater, 2001). Unknown transducer kinases likely form a link between Plexins and induction of Rho kinases, which then act to induce actin reorganization (Goshima et al., 2002). Evidence indicates that concentrations of intracellular cyclic nucleotides can affect whether axons are repelled or attracted by the Sema3 gradient (Song et al., 1998; Rohm et al., 2000). Central to the downstream pathway, are CRMPs, which have been shown to be phosphorylated by Rho kinases, and may have effects both on axonal dynamics as well as neuronal death (Fiore and Puschel, 2003; Hou et al., 2006). It is not yet clear whether activation of this pathway alone may be responsible for induction of neuronal death *in vivo*. The temporal relationship of cytoskeletal changes and commitment of the cell body to death have yet to be determined.


Sema3
NRP
Plexin
Cyclic nucleotides
Rho
kinases
P
I
Actin
reorganization
Cell Death?

example, neutralization of CRMP-2 blocks the ability of Sema3A to induce growth cone collapse of DRGs growth cones (Goshima et al., 1995). Further, evidence shows that Rho kinases may phosphorylate and activate CRMPs since, for example, introduction of a dominant negative Rac mutant blocks Sema3A-induced growth cone collapse. Recently, our laboratory has suggested a novel role for CRMP-3 within the neuronal cell death pathway as induced by glutamate excitotoxicity (Hou et al., 2006). Thus evidence supports that the CRMP family may be future downstream targets of the NRP-1/Sema3 pathway, to facilitate neuroprotection and neuronal regeneration therapeutically (Hou et al., 2006).

1.3.6 NRP-1 as a Possible Neuronal Death Receptor

Previous literature has focused on the NRP-1/Sema3A pathway as a mediator of axonal dynamics, however, recent work has demonstrated that upregulation of this pathway may be involved in apoptosis (Shirvan et al., 1999). During dopamine-induced apoptosis of cultured DRG neurons, Sema3A expression was found to be increased before the time point of commitment to cell death (Shirvan et al., 1999). Further, the direct application of high concentration of Sema3A protein to neurons was found to induce apoptosis (Shirvan et al., 2000). These authors speculate that it is possible that varying concentrations of Sema3A may induce different neuronal responses including axonal repulsion to cell death (Shirvan et al., 2000). The role of NRPs in CNS neurons and contribution of this pathway to post-ischemic effects, however, have yet to be studied and represent the starting point of the studies described in this thesis.

CHAPTER 2 SPECIFIC AIMS

Therepeutic prospects for post-ischemic brain tissue should target one of two factors:

1. Neuronal cell death, as preceded by or coordinated with axonal degeneration (Hou and MacManus, 2002) and
2. Regenerative failure of surviving neurons to compensate for neuronal loss, due to lack of growth stimulatory guidance cues, abundance of inhibitory cues, or relative imbalance of both types of signalling (Dontchev and Letourneau, 2003; Hou and MacManus, 2002). It is of interest to this work that NRPs and their subsequent signaling pathway are believed to play a role in ***both*** of these processes, through binding of their Sema ligands (He and Tessier- Levigne, 2003).

Since previous studies demonstrate that E2F1 plays a significant role in the death cascade in post-ischemic neurons (Hou, et al., 2000; Heibert et al., 1995; Stanelle et al., 2002), the major aim of this project was to focus on NRPs as potential molecular targets of E2F1, and to determine whether E2F1-dependent expression and activation of NRP-1 is significant in post-ischemic axonal retraction and neuronal death. To address these issues, this thesis utilized a variety of methodologies focused on two major research aims:

AIM 1. To better demonstrate that transcription factor E2F1 specifically participates in the transcriptional regulation of NRP-1.

NRP-1 expression in E2F1-/- and E2F1 +/+ were compared at the mRNA and protein level. A gene replacement experiment was used to determine whether NRP-1 expression in E2F1 -/- neurons could be restored through adenoviral-mediated expression of E2F1 in these cells. Further examination of potential E2F1 binding and activation of the NRP-1 promoter were assessed by electrophoretic mobility shift assay (EMSA) and luciferase reporter assay respectively. EMSA was used to assess the dynamics of E2F1 occupation at the NRP-1 promoter *in vivo*, and to determine whether they would correlate to the temporal expression profile of NRP-1 after cerebral ischemia.

> **AIM 2.** To determine whether the NRP-1/Sema3A pathway can induce neuronal death, alone and in the context of ischemia and whether inhibition of this pathway may confer protection.

Experiments were designed to elucidate whether activation of the NRP-1/Sema3A pathway would increase post-ischemic neuronal loss and axonal retraction, and whether inhibition of this pathway would be protective. Expression levels of NRP-1 and NRP-2 at the protein level were determined post-ischemia both *in vivo* and *in vitro* in order to establish a temporal profile of expression. It was of further interest to observe whether the over-activation of the NRP-1/Sema pathway could be a cause of axonal retraction and/or cell death. Lastly, *in vitro* ischemia was performed on NRP-2 -/- cerebellar granular neuron (CGN) cell cultures to determine whether loss of NRP-2 expression would be protective against post-ischemic processes.

CHAPTER 3 E2F1 AS A TRANSCRIPTIONAL REGULATOR OF NRP-1

Contributions of Collaborators

The results in this Chapter may also be found in Jiang et al., (2007), a shared first author publication by Dr. S.X. Jiang and myself. Referral to this work does not imply (unless otherwise stated) that the figure or statement was contributed by Dr. Jiang but does signify that this information may be found also in the paper. Dr. Jiang has contributed all RT-PCR data, and statistical analyses. Angele Desbois performed primary cell cultures.

3.1 Introduction

Since E2F1 is believed to affect a multi-faceted group of downstream effectors, therapeutically intervening at this level may have both beneficial and damaging effects. The determination of specific downstream effectors of E2F1, will aid not only elucidating downstream signalling pathways, but may also allow research to focus on specific facets of this pathway. In this way specific molecular contributions relevant to eventual development of safe therapeutics may be determined.

With the availability of viable E2F1 -/- mice, recent findings from our laboratory have provided an indication that E2F1 may regulate the expression of a large number of genes in the ischemic mouse brain. By performing immunoprecipitation assays in post-ischemic brain tissue, it was found that levels of E2F1 were transiently increased following 2h focal ischemia and were maximally increased at 6 hours (h) post-ischemia (Hou et al., 2002; Hou and MacManus, 2002). In order to determine expression profiles of genes under the regulation of E2F1, 6 hours after focal ischemia, gene profiles of E2F1 -/- and E2F1

+/+ cortical tissues were compared by microarray analysis (Jiang et al., 2007). In this way hundreds of genes whose expression had either increased or decreased more than 5-fold post- ischemia were identified. Interestingly, among the many genes identified, a number of them were associated with axonal collapse and brain plasticity, including NRP-1, cofilin-1, and collapse response mediator proteins (Jiang et al., 2007 and unpublished data). As well as being a modulator of axonal retraction, collapse and possibly neuronal death, the NRP-1/Plexin complex is also known to be a receptor for VEGF165, a ligand which plays a significant role in angiogenesis (Neufeld et al., 2002). Many studies have shown that focal cerebral ischemia in mammalian brains is associated with the growth of new blood vessels and neuronal remodelling (Beck et al., 2002). Since NRP-1 may be a common molecule regulating these processes, it was selected for further analyses to determine whether there is a true biochemical interaction between E2F1 and NRP-1. In order to directly answer the question of whether or not E2F1 transcriptionally activates NRP-1, and whether the presence of E2F1 is requisite for basal NRP-1 expression before or after ischemia, methods including Western analysis, EMSA, gene replacement, and reporter assays were utilized.

Overall, this Chapter examines Specific Aim 1: to better demonstrate that transcription factor E2F1 specifically participates in the transcriptional regulation of NRP-1.

3.2 Material and Methods

3.2.1 Animal Breeding

C57Black/6 mice (C57/BL6) of 20-30 g were obtained from Charles River (St Foie, Quebec, Canada). The E2F1 -/- (stock 2785) and E2F1 +/+ (stock 101045) mice

representing a hybrid genetic background of C57BL/6 and SV129 strains were initially obtained from Jackson Laboratories (Bar Harbor, ME) but were bred and genotyped in house (Hou et al., 2000). Breeding and genotyping was performed by our laboratory technician, Angele Desbois. All procedures using animals were approved by the local Animal Care Committee.

3.2.2 Cell Culture and Treatment

Primary cultures of CGNs were prepared from 6 – 8 day postnatal mice (E2F1 +/+ and E2F1 -/-), taking account of the importance in ensuring congenicity (Smith, 2003). Mouse pups were anesthetized with 4% isofluorane for 3-5 min at a flow rate of 4 L/min. Pups were decapitated and the skullcap removed. The cerebella were removed and meninges dissected from tissue by hand with fine forceps. The remaining tissue was washed with phosphate buffered saline (PBS) and placed in a 35 mm dish. The tissue was diced with a scalpel and suspended in Solution A (124 mM NaCl, 5.4 mM KCl, 1 mM NaH_2PO_4-H_20, 14.5 mM glucose, 1 M Hepes buffer pH 7.2, 0.08% phenol red (Sigma P0209), 0.3% bovine serum albumin (BSA; Sigma A8412), 1.2 mM $MgSO_4$ lyzed enzymatically with 0.025% trypsin solution (Sigma T4665) for 25 min, with mechanical dissociation. Trypsin inhibitor (Roche #109878) was then added to stop the reaction and 0.05% DNase (Roche #1284932) was added to digest nucleic acid from dead cells and cellular fragments. Neurons were dispersed mechanically by trituration with a Pasteur pipette and centrifugation. Cell pellets were then resuspended in Eagle's minimum essential medium (MEM) with Earle's salts and $NaHCO_3$ without glutamine (Sigma M-2279) supplemented with 0.8 mM L-glutamine, 27 mM glucose, 0.01% gentamycin, 9%

(v/v) fetal bovine serum (FBS), 20 mM KCl, 27.6 mM D+ glucose. Cells were plated onto 10 cm dishes coated with poly-L-lysine coated coverslips at a density of 21 x 10^6 cells per dish. After approximately 18 h, cytosine-β-D-arabinofuranoside (AraC) was added to the media to a final concentration of 5 μM, to prevent glial cell proliferation.

Glial cells were also prepared for transfection in a luciferase reporter assay (see protocol 3.2.7). Cell culture was prepared as per CGN preparation, but without the addition of AraC. Cultures were grown for 7 days at which point 120 uM of glutamate was added overnight to kill neurons. On day 8, dead cells were removed by gentle trituration, media was removed and replaced with fresh MEM culture media. The majority of primary cell culture preparations were performed by our laboratory technician Angele Desbois.

3.2.3 Semi-quantitative RT-PCR

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed as a preliminary experiment by Dr. Susan X. Jiang. Total ribonucleic acid (RNA) was extracted from cultured neurons using the TRIzol reagent (Invitrogen Life Technologies # 15596-026) as per manufacturer's instructions. First strand complementary deoxyribonucleic acids (cDNA)s were reverse transcribed from 4 μg of total RNA using the First Strand cDNA Synthesis Kit (Amersham Biosciences) as per the manufacturer's instructions. The optimal polymerase chain reaction (PCR) amplification conditions and cycle number were determined experimentally to ensure specific and exponential signal generation. The primer sequences for β-actin were as follows: 5'-AAC ACC CCA GCC ATG TAC GTA G-3' and 5'-GTG TTG GCA TAG AGG TCT TTA CGG-3'. Primers (Qiagen) to β-actin span the intron D, which allows the detection of genomic contamination

in PCR products. The cycle conditions for β -actin were: 1) 94 °C for 45 s, 2) 58 °C for 45 s, 3) 72 °C 1 for min. Steps 1-3 were repeated 30 times. The primer sequences (Qiagen) for NRP-1 were as follows: 5'-GTC CGG CTC TCA TGT GGG TAC A-3' and 5'-GAG CACTCC AGG ATA TGA AGG A-3'. The cycle conditions for NRP-1 were: 1) 94 °C for 1 min, 2) 59 °C for 1 min, 3) 72 °C for 1 min. Steps 1-3 were repeated 32 times. Taq deoxyribonucleic acid (DNA) polymerase, buffers, $MgCl_2$, deoxyribonucleotide triphosphate (dNTP) mix were purchased from Invitrogen. Expression of β-actin mRNA was used as an internal standard to quantify the relative gene expression as described before (Pasterkamp et al., 1998). A negative control (without the addition of cDNA) was included in each PCR run.

3.2.4 Western Blot

The procedures for Western blotting were exactly as described previously (Hou et al., 2000; Smith et al., 2003). Total protein was extracted from the cortices of E2F1-/- and E2F1 +/+ mice using Western lysis buffer (1% NP40, 10% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris pH 7.5, fresh protease inhibitor cocktails), followed by 3 x 10 s of sonication, in order to dislodge NRP-1 from the cell membrane. Protein samples were quantified using the Bradford Assay, and 20 µg per sample were boiled in 5X loading buffer [62.5 mM Tris, 2.5 mM EDTA, 2.5 mM ethylene glycol tetraacetic acid (EGTA), 10% glycerol, 2% SDS, 0.001% bromophenol blue, 5% mercaptoethanol] prior to being loaded on an 8% polyacrylamide gel. Proteins were electro-blotted using the wet transfer technique onto a nitrocellulose membrane in ice-cold transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.5) for 1 hour.

Membranes were blocked with 5% skim milk in PBS containing 0.05% Tween-20 for at least 20 min at room temperature. Membranes were cut according to the size of expected protein marked by the ladder, and incubated overnight at 4 °C with either rabbit anti - NRP-1 antibody (Calbiochem PC 343) at a dilution of 1:100, rabbit anti - NRP-2a antibody (Upstate Biotechnology #22108) at a dilution of 1:100 or mouse anti -glyceraldehyde-3-phosphate (GAPDH) antibody (Sigma) at a dilution of 1:10,000. GAPDH was used as a loading control. The following day, membranes are rinsed with 0.05% Tween in PBS (PBST) and horse radish peroxidase (HRP)-conjugated secondary antibodies against mouse or rabbit were added in 5% milk solution for 30 min at room temperature. Enhanced chemiluminescence (ECL) detection of the target proteins was achieved with the ECL-plus detection system from Amersham Biosciences. The intensities of the bands were quantified using the Molecular Dynamics ImageQuant Software.

3.2.5 Adenoviral Gene Delivery of E2F1 to Mouse Neurons

The replication defective wild-type free adenoviruses encoding full length human E2F1 cDNA, or E2F1 mutant (with a point mutation at amino acid 132 to abolish DNA binding) cDNA in an adenoviral shuttle vector containing a green fluorescent protein (GFP) marker and under the cytomegalovirus promoter/enhancer, were used from stocks created previously in the lab ([A]Hou et al., 2001). Neurons at 80% confluence were infected at a multiplicity of infection of 10, in 10 cm dishes. Infection was detected at a maximum level 3 days post infection and equal infectivity between cell samples was ensured by manually counting neurons expressing GFP under a fluorescent microscope. Three days post-infection, cells were scraped from the dish and total protein was extracted as per section

3.2.3. Western blotting was performed using 7 μg per protein sample, and levels of NRP expression were compared between E2F1-/- and +/+ CGNs.

3.2.6 MCAO

All procedures using animals were approved by the local Animal Care Committee. C57BL/6 mice (20-23 g) were obtained from Charles River (St.Foie, PQ). Under temporary isofluorane anesthesia, mice were subjected to MCAO using an intraluminal filament as previously described (MacManus et al., 2003). After 1 h of MCAO, the filament was withdrawn, blood flow restored to normal and wounds were sutured. Sham operated mice, which were subjected to the same brain surgery but no MCAO were used as controls. Animals were sacrificed after 0 h – 24 hof reperfusion.

3.2.7 EMSA

EMSA was performed using nuclear extracts essentially as described (Cobrinik et al., 1993). Nuclear protein was extracted from mouse brain following the protocol described previously (Saunders et al., 1999). Double- stranded E2F binding oligonucleotide with the sequence of 5'-TGC TCT AAG AAA GTC TGC CTA TGC TTT ACG TGG CAG ACT GGG-3' in the NRP-1 promoter region was labeled with [α-^{32}P] 2'-deoxycytidine 5'-triphosphate (dCTP) (Amersham) using T4-polynucleotide kinase (Amersham). A completely random scrambled sequence at the same length was also made as a control. Eight μg of nuclear extract was incubated with an excess of [α-^{32}P] dCTP labeled double-stranded DNA (ds-DNA) probe (60,000 cpm/0.2 ng of DNA). The binding reaction (20 μl)

was carried out at room temperature for 20 min in binding buffer [20 mM Hepes, pH 7.6, 0.2 mM ethylene diamine tetracetic acid (EDTA), 100 mM KCl, 5% glycerol, and 2 mM dithiothreitol (DTT)] and 0.1 μg of nonspecific competitor polydeoxyinosinic-deoxycytidylic acid (poly-dIdC; Amersham). Each new preparation of poly-dIdC was titrated to determine the ideal concentration for EMSA. Unlabelled oligonucleotides (or competitors) were used for the cold competition binding assay. Monoclonal E2F1 antibody (2 μl / reaction, Santa Cruz Biotechnology # KH95 X) was used. To obtain the super-shift, no poly-dIdC was used for non-specific blocking, and 6 ul of anti-E2F1 were required to detect the super-shift. Binding reaction was performed at room temperature for 45 min. Complexes were resolved on a 5 % acrylamide gel and run for 2 h, dried with a gel dryer and visualized by autoradiography.

3.2.8 Luciferase Reporter Assay

A 150 base pair (bp) sequence of the predicted E2F1 binding region of the NRP-1 promoter (560 nt. - 709 nt.) was cloned into the PGL3 Basic vector using the Kpn1 and HindIII restriction sites to create the NRP-1 promoter/Luciferase reporter vector construct. See Appendix B for cloning strategy and primer design. See Figure 7A for PGL3 Basic Vector Map. PCR reaction conditions were as follows: 10X PCR buffer, 2 mM dNTPs, 2 μM of each primer, 0.5 μg of total genomic DNA isolated from mouse cross SV129 x C57Bl/6 (MGF) brains, 5 units taq polymerase, 50 μM $MgCl_2$, ddH_20 to a total reaction volume of 50 μl. PCR amplification conditions were: 1) 3 min at 95 °C, 2) 45 s at 94°C, 3) 45 s at 61 °C, 4) 1 min at 72 °C. Steps 2-4 were repeated 30 times. The construct was

amplified in Top 10 Bacteria and purified using a DNA midi-prep kit as per manufacturer's protocol (Qiagen).

Cultured primary glial cells from E2F1 +/+ and E2F1 -/- mouse brains were transfected with 10 μg PGL3-luciferase-NRP-1 promoter construct using Lipofectamine 2000 following the manufacturer's instructions (Invitrogen). After 3 days of transfection to allow for gene expression, cells were collected and lysed in luciferase buffer. Luciferase activity was determined with the luciferase reporter assay system following the manufacturer's instructions (Promega Corporation). Briefly. GLO buffer (which contains the luciferase substrate) was added to the protein samples in an opaque 96 well plate. Luminescence was detected using a luminometer, and was normalized against background luminescence from GLO buffer alone.

3.3 Results

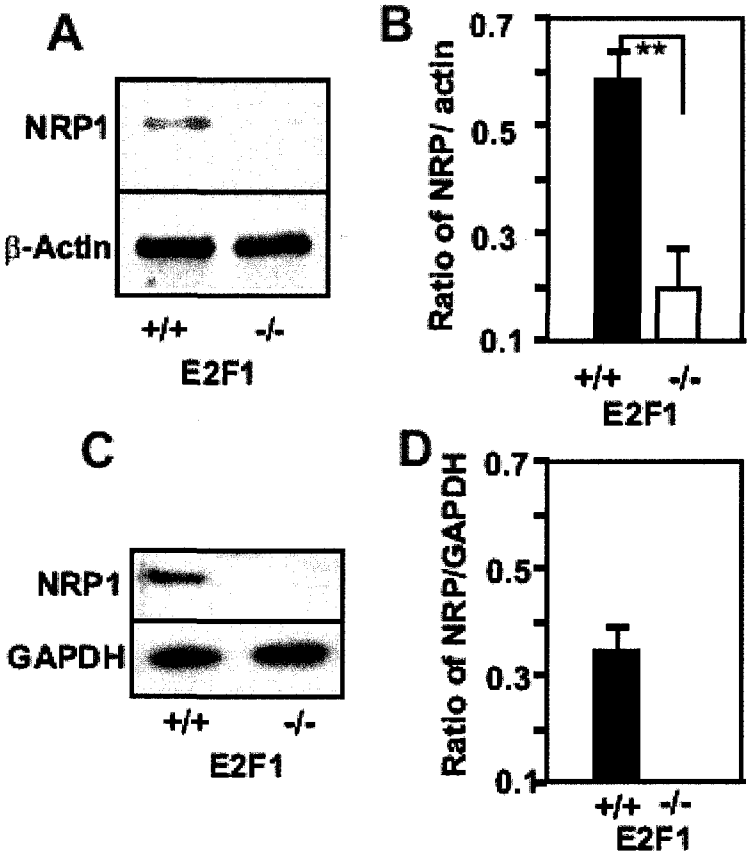
The results in this Chapter may also be found in Jiang et al., (2007), a shared first author publication by Dr. S.X. Jiang and myself. Referral to this work does not imply (with the exception of microarray and CHIP assays) that the figure or statement was contributes by Dr. Jiang but does signify that this information may be found also in the paper.

3.3.1 Expression of NRP-1 in E2F1 -/- and E2F1 +/+ brain

After identifying NRP-1 as having a differential expression in cultured E2F1 -/- and E2F1 +/+ mouse neurons (Jiang et al., 2007) and knowing that NRPs have been shown to play important roles in ischemia-induced angiogenesis and neuronal remodeling in the

brain (Beck et al., 2002), it was of interest to investigate the significance of the differential transcription of NRP-1 in neurons in ischemic brain tissue. To determine whether NRP-1 is expressed differentially in E2F1 -/- and E2F1 +/+ cortical tissue, levels of NRP-1 mRNA and protein were assessed by using semi-quantitative RT-PCR and Western blotting, respectively. RT-PCR data (contributed by Dr. S.X. Jiang) demonstrated that in E2F1-/- cortical brain tissue the amount of NRP-1 transcript compared to that of E2F1 +/+ littermates is significantly reduced (Fig. 3A and 3B). Data was normalized against the expression of β-actin (Fig. 3B). To determine whether this effect translates into the decreased production of NRP-1 protein in E2F1 -/- compared to E2F1 +/+ brain tissue, western blot was performed. A single band was observed at an approximate molecular weight of 130 kDa which corresponds to that of the predicted NRP-1 (Luo et al., 1993). Based on the band intensity from western blot analysis and subsequent quantification using GAPDH as the internal loading control, it was found that NRP-1 expression was almost absent in E2F1-/- mouse brain tissue compared to that of E2F1 +/+ littermates (Fig. 3C and 3D). These results suggest that the nuclear transcription factor E2F1 positively regulates NRP-1 gene expression in mouse brain tissue.

Fig. 3: E2F1-/- CGNs have significantly reduced NRP-1 expression. (A) mRNA was extracted from brain lysates of E2F1 +/+ and E2F1 -/- mice (littermates). Equal amount of total RNA was used for the 1st strand cDNA synthesis. PCRs were performed using the cDNAs to detect NRP-1 and β-actin levels. β-actin was used as an internal control for equal mRNA loading (data and figure contributed by Dr. S.X. Jiang). (B) The intensities of the bands were quantified using densitometry and the ratio of NRP-1 against β-actin was calculated and plotted (contributed by Dr. S.X. Jiang). (C) Western blotting was performed on total protein extracts from brain lysates to detect NRP-1 protein expression. GAPDH was used as control for equal protein loading. No detectable NRP-1 protein was found in E2F1-/- brain lysate (C and D). Statistical analysis was performed with ** to indicate $p < 0.001$ by Student t-test (Jiang et al., 2007).

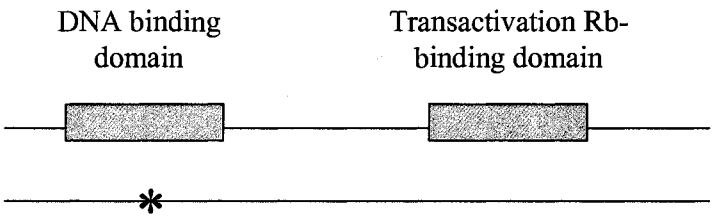

A
NRP1
β-Actin
+/+
-/-
E2F1
B
Ratio of NRP/ actin
0.7
0.5
0.3
0.1
**
+/+
-/-
E2F1
C
NRP1
GAPDH
+/+
-/-
E2F1
D
Ratio of NRP/GAPDH
0.7
0.5
0.3
0.1
+/+
-/-
E2F1

In order to demonstrate that E2F1 indeed transcriptionally activates NRP-1, a gene replacement protocol was used, wherein replication defective adenoviruses were used to reintroduce E2F1 into E2F1 -/- CGNs (Fig. 4). CGNs from E2F1-/- and E2F1+/+ mice were cultured in 24 well dishes (6 wells/treatment). Cells at 7 days in culture (DIC) were infected at a multiplicity of infection of 10 with adenoviral constructs expressing either GFP alone (Ad-GFP), E2F1 mutant/GFP (Ad-Emut: a point mutant of E2F1 at 130 amino acids which abolished promoter binding capability as shown in Fig. 4A.) or the full length E2F1/GFP (Ad-E2F1).

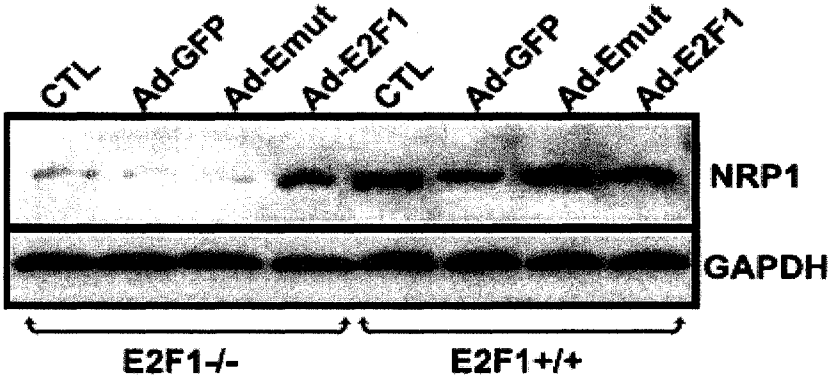
These constructs were previously generated and purified and have been shown to be effective in expressing the encoded genes in neurons ([A]Hou et al., 2001; O'Hare et al., 2000). Non-infected controls were also used to detect basal levels of NRP-1 expression and to ensure the infection process itself did not alter NRP-1 expression. Three days post-infection, cells were observed under a fluorescent microscope to allow for equal numbers of GFP expressing cells (visualized as bright green cells) per sample well. The cells were collected, pelleted and total protein was extracted. Western blot analysis was performed using anti-NRP-1 monoclonal antibody. As shown in Figure 4B, results from this experiment have determined firstly that NRP-1 expression in E2F1 -/- CGNs is significantly lower as compared to E2F1 +/+. These data corroborate the results of experiment 3.3.1(Fig. 3) wherein expression analyses between E2F1 -/- and E2F1 +/+ neurons was performed. Of specific interest with respect to the data in Figure 4 is that when the E2F1 gene was replaced in the E2F1 -/- cells, the wild-type level of NRP-1 expression was restored. Controls where cells were infected with GFP alone or mutant E2F1, did not

Fig 4. E2F1 gene replacement in E2F1-/- neurons leads to up-regulation of NRP-1 expression. Replication defective adenoviral constructs expressing GFP only (Ad-GFP), and mutant E2F1 (Ad-Emut) and the full-length E2F1 (Ad-E2F1) made and used to infect CGNs derived from the brains of E2F1 -/- and E2F1 +/+ mice. (A) Emut contains a point mutation at amino acid 132 in the DNA binding domain (at amino acid 132), preventing it from activating target genes, as indicated by the *(B) Non-infected cells were used as the baseline control for NRP-1 expression (CTL). After 3 days of infection, cells were collected for Western blotting to detect NRP-1 expression. GAPDH was used as a loading control. (Jiang et al., 2007).

A


DNA binding domain
Transactivation Rb-binding domain
E2F1
Emut (E132)

B


CTL
Ad-GFP
Ad-Emut
Ad-E2F1
CTL
Ad-GFP
Ad-Emut
Ad-E2F1
NRP1
GAPDH
E2F1-/-
E2F1+/+

show induction of NRP-1 expression. GAPDH was used as a loading control to demonstrate equal protein loading (Fig. 4B). The infection of E2F1 +/+ neurons with the full length E2F1 adenoviral vector did not result in a detectable increase in NRP-1 compared to uninfected, Ad-GFP and Ad-EMut controls used in E2F1 +/+ neurons. NRP-1 levels were increased in all E2F1 +/+ samples compared to E2F1 -/- neurons expressing adenovirally delivered full length E2F1 (Fig. 4B). These results further establish that E2F1 is a specific transcriptional activator of NRP-1, since the presence of E2F1 with transcriptional activity is requisite for NRP-1 expression.

3.3.3 E2F1 binding of the NRP-1 promoter

The next step was to determine whether transcription factor E2F1 binds to the NRP-1 promoter. Firstly, a bioinformatics approach was taken to search for putative E2F1 binding sequences in the promoter region of NRP-1 gene. A known mouse E2F1 DNA binding sequence (Slansky and Farnham, 1996) was used to compare with the NRP-1 promoter sequence using CLUSTAL W software by pair-wise alignment. Interestingly, a small sequence of the NRP-1 promoter showed a region of high similarity to the pre-determined consensus sequence (Fig. 5A). Appendix B shows the full CLUSTAL W alignment between the putative E2F1 binding site and the full-length NRP-1 promoter sequence.

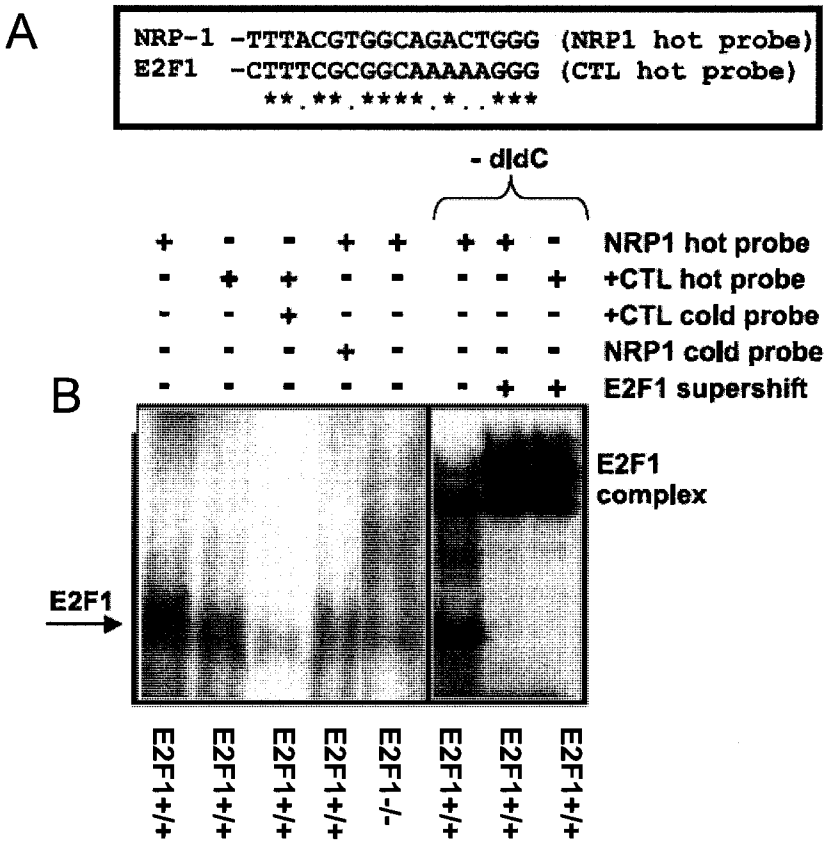
In order to demonstrate that E2F1 is physically capable of binding to the NRP-1 promoter, EMSA was selected as the most definite experimental method. The double stranded oligonucleotide sequence corresponding to the E2F1 binding site on NRP-1 promter (NRP-1) was synthesized commercially and used for EMSA to demonstrate a

theoretical sequence specific binding by E2F1 (Fig. 5A). As a positive control, the known E2F1 binding sequence (CTL) was also synthesized and used in EMSA. A negative control oligonucleotide probe was also made, and consisted of a randomly scrambled NRP-1 promoter sequence. Total E2F1 -/- and E2F1 +/+ mouse brain nuclear protein extracts were used in this experiment since nuclear extracts reduce the chance of non-specific binding by cytosolic molecules and enrich the samples for E2F1.

Referring to Figure 5B, when E2F1 +/+ brain nuclear extracts were incubated with the ^{32}P –radiolabelled oligonucleotide probes from either the NRP-1 promoter (NRP-1), or the known consensus positive control sequence (CTL), a single strong band was observed in both samples (Fig. 5B). To further demonstrate that the band observed is a specific E2F1:probe complex, cold probe competition was performed wherein 100 times excess unlabelled (cold) CTL or NRP-1 probe was incubated with respective radiolabelled (hot) probe samples, and resulted in a decrease in band intensities. This control further aids in the confirmation of a specific E2F1: probe complex. A further control used was E2F1 -/- nuclear brain extract incubated with the hot NRP-1 probe, wherein decrease in band intensity was seen, as compared to E2F1 +/+ nuclear extract incubated with same probe.

The key element in this experiment was the E2F1 super-shift. The addition of anti-E2F1 into the reaction mixture formed a higher molecular weight E2F1:probe:antibody complex which appeared at the top of the EMSA gel. This band is known to be specific since the samples without antibody added under otherwise identical reaction conditions, showed a lower molecular weight band, which disappeared and shifted upwards with the addition of the antibody. Hence the supershift was successful and a specific E2F1 binding site within the NRP-1 promoter was identified. Three modifications to protocol were required to demonstrate the supershift: 1) the elimination of the usual addition of poly dIdC

Fig 5. Identification of E2F1 binding sequence in the NRP-1 promoter. (A) Mouse E2F1consensus binding sequence (CTL) was used to identify homologous sequences in the NRP-1 promoter region (NRP-1) by pair-wise alignment using Clustal W software. The sequence identified on the NRP-1 promoter was chemically synthesized to make a ds-DNA which was labeled with radio-active [α- ^{32}P] dCTP for EMSA. (B) Nuclear fractions were isolated from E2F1+/+ and -/- brain extracts and mixed with radio-labelled ds-DNA probes. Several controls were also used in the study including 100 times excess cold probe, random sequence control, and E2F1 antibody super-shift. A specific E2F1 binding only occurred to the NRP-1 promoter sequence and positive control sequence as indicated by the arrow. The super-shift pattern is characteristic of E2F1, which when mixed with the antibody completely abolished the binding of E2F1 to the ds-DNA probe. (Jiang et al., 2007).


A
NRP-1 -TTTACGTGGCAGACTGGG (NRP1 hot probe)
E2F1 -CTTTCGCGGCAAAAAGGG (CTL hot probe)
..****.*..***
- dIdC
NRP1 hot probe
+CTL hot probe
+CTL cold probe
NRP1 cold probe
E2F1 supershift
B
E2F1 complex
E2F1
E2F1+/+
E2F1+/+
E2F1+/+
E2F1+/+
E2F1-/-
E2F1+/+
E2F1+/+
E2F1+/+

(which is the blocking agent to prevent non-specific binding to the probe), 2) a larger amount of E2F1 monoclonal antibody (6 µl compared to 2 µl used in all other samples) was required and 3) the incubation time was increased to 45 min at room temperature. As seen in Figure 5B, it is preferable to use poly-dIdC in the non-supershift samples, however, since this agent eliminates the appearance of non-specific bands that would otherwise complicate the identification of an E2F1 specific band.

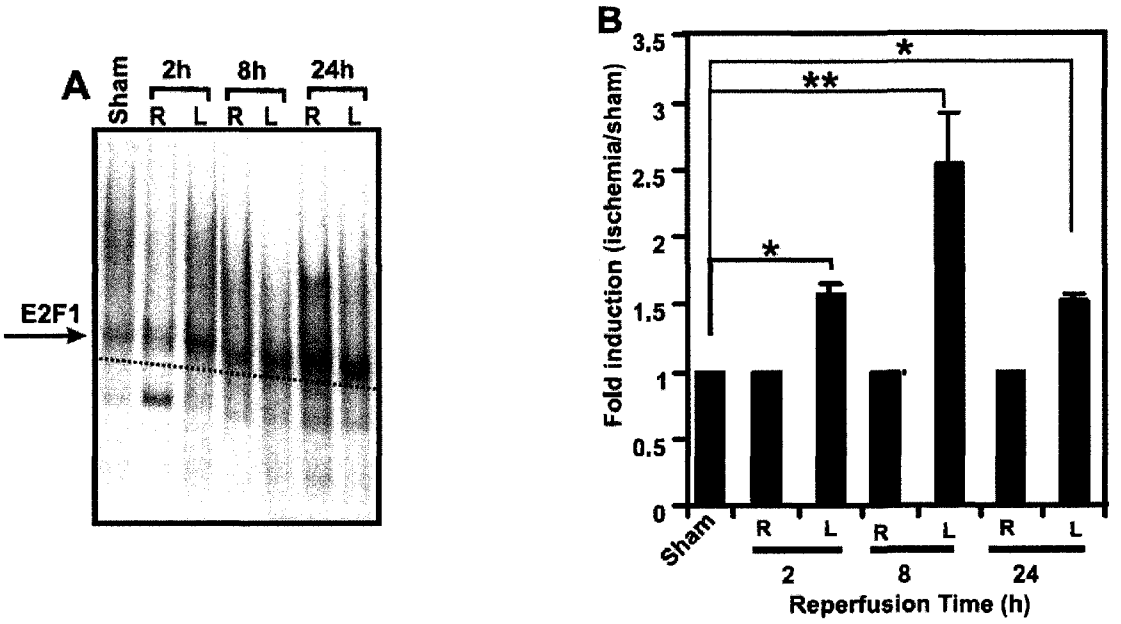
In summary, an E2F1 *specific* binding site was detected in the mouse brain nuclear extract. This band was demonstrated to be specific since: 1) super-shift with an E2F1 monoclonal antibody was observed, 2) competition with 100X excess cold probe reduced the binding reaction and 3) a scrambled oligonucleotide failed to induce E2F1 binding to the NRP-1 sequence. The results of the EMSA thus show that transcription factor E2F1 is able to physically bind to the putative E2F1 binding sequence within the NRP-1 promoter.

3.3.4 E2F1 occupation of NRP-1 promoter in the post-ischemic brain

To determine whether E2F1 targets NRP-1 specifically after focal cerebral ischemia in the brain, EMSA was performed to compare the level of E2F1:NRP-1 promoter association with or without an ischemic insult. Making use of the fact that mice treated with MCAO typically show effects limited to the infarcted brain hemisphere (left), the contralateral (right) hemisphere was unaffected and served as an internal control.

Nuclear extracts from MCAO-treated brains were subjected to EMSA using the NRP-1 promoter sequence which is specific to E2F1 binding. As shown in Figure 6, E2F1 binding to the NRP-1 promoter sequence significantly increases after 2 h, 6 h and 8 h of reperfusion in the ischemic side of the cortex compared to the corresponding contralateral

Fig. 6. E2F1 positively regulates NRP-1 expression during cerebral ischemia. (A) Nuclear fractions were isolated from both the ischemic (L) and contralateral (R) sides of MCAO mouse brains. EMSA was performed using these nuclear extracts. Sham-operated brains were used as a control to determine the baseline level of E2F1 binding to the promoter. (B) The E2F1 band was quantified by densitometry measurement and plotted. Temporal changes in E2F1 binding on the ischemic side of the brain were normalized against those on the contralateral side of the brain. * and ** indicate statistical significance at $p < 0.05$ and $p < 0.01$, respectively. (Jiang et al., 2007).


A
Sham
2h
8h
24h
R L R L R L
E2F1
B
Fold induction (ischemia/sham)
3.5
3
2.5
2
1.5
1
0.5
0
*
**
*
Sham
R L R L R L
2 8 24
Reperfusion Time (h)

(non-ischemic) side of the brain. The increase in E2F1 binding to the NRP-1 promoter in ischemic versus contralateral sides of the brain was statistically significant as measured by densitometry. No significant changes in E2F1 binding occurred between the various time points of reperfusion on the contralateral side of the brain (the band intensity remained constant), whereas on the ischemic side band intensity increased with increased reperfusion time. These binding profiles correlate temporally with the induction in NRP-1 mRNA levels in ischemic brain hemispheres observed by RT-PCR (Dr. S.X. Jiang, 2005, unpublished). Collectively, these results demonstrated that E2F1 occupied the promoter site of NRP-1 in response to ischemia and lead to an increase in NRP-1 expression.

3.3.5 Activation of NRP-1 promoter by E2F1

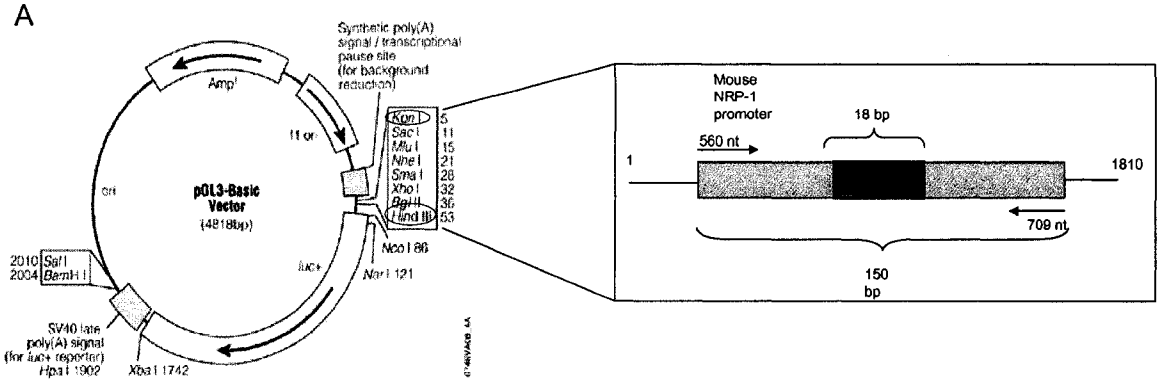
To further demonstrate that NRP-1 is a bona fide target of E2F1, a luciferase reporter assay was designed and performed to show the *activation* of the NRP-1 promoter by E2F1. A 150 bp region of the NRP-1 promoter sequence from nucleotides (nt.) 560 - 709, containing the putative E2F1 binding site (642 nt. – 658 nt.) was amplified using PCR and the fragment was subcloned into a promoterless PGL3 expression vector (Promega). The PGL3 vector contains a modified coding region for firefly (*Photinus pyralis*) luciferase that has been optimized for monitoring transcriptional activity in transfected eukaryotic cells. Since this vector does not contain a promoter, its transfection into cells will not lead to Luciferase expression. The PGL3 vector map is shown in Figure 7A.

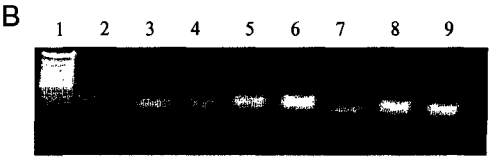
Restriction enzymes HindIII and Kpn1 were utilized to create a sticky-end insert:vector ligation (Appendix B). The construct was transformed into *E.coli* Top 10 bacterial stocks and multiple clones were picked from Ampicillin+ agar plates and

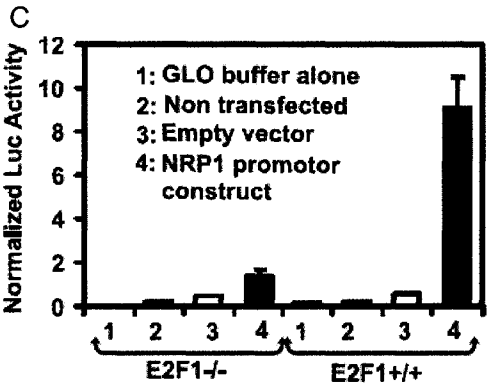
amplified by midi-preparation (Qiagen). A diagnostic PCR was performed to determine whether the 150 bp inserts were present (Fig. 7B). After midi-preparation, Gene Clean purification (Qiagen) and sequencing, the clones were transfected into actively proliferating E2F1 +/+ and E2F1 -/- glial cells. Glial cells are used in this case because they have been found to show significantly higher transfection efficiency (~40% transfection efficiency) than do the non-mitogenic CGNs. Two days post-transfection, cells were collected and total protein extracted. Controls include non-transfected glia and glia transfected with empty PGL3 Basic Vector.

The results of this experiment are shown in Figure 7C. When E2F1 -/- glial cells were transfected with the empty promoterless PGL3 Basic Vector, no appreciable luciferase activity was detected. This was comparable to control cells that were untreated or incubated with lucifase reaction (GLO) buffer alone. The GLO buffer contains the substrate for the luciferase enzyme. The same set of negative controls were used with E2F1 +/+ cells and yielded comparable lack of luciferase activity. When E2F1 +/+ glial cells were transfected with the PGL3 Basic Vector containing the NRP-1 promoter construct, an approximately 5 fold increase in luciferase activity was observed as compared to E2F1 -/- cells transfected with the same vector. A greater than 10 fold increase in luciferase activity was seen in the PGL3/NRP-1 transfected cells compared to those transfected with empty vector. These results confirmed that E2F1 is able to not only bind, but also activate transcription at the NRP-1 promoter in the theoretical E2F1 binding sequence.

Fig. 7. Activation of NRP-1 promoter by E2F1 was demonstrated by the use of a luciferase reporter assay. (A)The E2F1 binding site sequence from the NRP-1 promoter was subcloned into the PGL3 promoterless expression vector. This vector was used to carry the 150 bp of the promoter region of the NRP-1 promoter (560 nt. - 709 nt.), which encompassed the 18 bp core E2F1 binding region (642 nt. – 658 nt.) as shown in dark blue. Kpn 1 and Hind III were the restriction sites used to insert the promoter clones. Luciferase gene expression was detected when the promoter is activated. Amp+ resistance was used for clone selection on Amp+ agar plates. (B) A diagnostic PCR was run using the same primers used to amplify the 150 bp insert. After the insert was ligated into the vector and transformed into E.Coli Top 10 bacteria, various colonies were selected for the diagnostic PCR, to check for the inclusion of the correct 150 bp insert. Lane 1 = 100 bp ladder, lane 2 = bacteria transformed with empty PGL3 Basic vector, lanes 3-9 = bacteria transformed with vector:insert ligation reaction. Construct DNA from colonies 5,6,8 and 9 were used for the luciferase assay (C) Primary glial cells derived from E2F1 +/+ and E2F1 -/- mouse brains were transfected with the constructs for 3 days. Cells were then collected and their lysates were quantified. Equal amounts of proteins were subjected to luciferase assay using a Promega kit following the manufacturer's instructions. A 10-fold increase in luciferase activity was detected in the NRP-1 promoter construct transfected E2F1/ cells. Several controls were also used including GLO buffer alone (contains the luciferase substrate), non-transfected cells, and cells transfected with the empty PGL3 Basic vector. Luciferase activity was normalized against background activity by GLO buffer alone.(Jiang et al., 2007).


A
Synthetic poly(A) signal / transcriptional pause site (for background reduction)
Amp
f1 ori
ori
pGL3-Basic Vector (4818bp)
Kpn I 5
Sac I 11
Mlu I 15
Nhe I 21
Sma I 28
Xho I 32
Bgl II 36
Hind III 53
Nco I 86
Nar I 121
luc+
2010 Sal I
2004 BamH I
SV40 late poly(A) signal (for luc+ reporter)
Hpa I 1902
Xba I 1742
Mouse NRP-1 promoter
18 bp
560 nt
1
1810
709 nt
150 bp


B
1 2 3 4 5 6 7 8 9


C
Normalized Luc Activity
12
10
8
6
4
2
0
1: GLO buffer alone
2: Non transfected
3: Empty vector
4: NRP1 promotor construct
1 2 3 4 1 2 3 4
E2F1-/-
E2F1+/+

3.4 DISCUSSION

3.4.1 Summary of Results

In addition to *in vivo* CHIP-generated evidence from Dr. Hou's lab that nuclear transcription factor E2F1 binds the NRP-1 promoter (Jiang et al., 2007), the results of this section provide further evidence to demonstrate that nuclear transcription factor E2F1 is a positive transcriptional regulator of NRP-1 (Jiang et al., 2007). The following evidence was found in support of this conclusion: (i) NRP-1 expression at both the mRNA and protein level was significantly reduced in E2F1 -/- neurons and brain tissue as compared with E2F1 +/+ littermates, (ii) replacement of E2F1 using a replication defective adenoviral vector in E2F1 -/- neurons restores the expression of NRP-1 protein, (iii) a bioinformatics analysis using the CLUSTAL W pair-wise alignment demonstrated the presence of a region in the NRP-1 promoter sequence with a high degree of similarity to a known core E2F1 binding sequence, (iv) experiments using EMSA demonstrated that E2F1 protein is able to physically bind to a cloned region of the NRP-1 promoter encompassing the putative E2F1-binding sequence, (v) levels of E2F1 occupation at the NRP-1 promoter found by EMSA were consistent with increases in NRP-1 expression in post-ischemic brain tissues and (vi) a luciferase reporter assay shows that the presence of E2F1 is requisite for the activation of the NRP-1 promoter. From these collective findings it is reasonable to conclude that NRP-1 is transcriptionally regulated by nuclear transcription factor E2F1 (Jiang et al., 2007). This knowledge may aid in the elucidation of mechanisms of ischemia-induced neuronal cell death.

3.4.2 Experimental Review and Critique

Examining in more detail the results and methods of each of the experiments performed, some critique becomes relevant to each one. In experiment 3.3.1 (Fig. 3), mouse CGNs were used *in vitro* to determine that the expression of NRP-1 is reduced in E2F1 -/- neurons compared to those from E2F1 +/+ littermates. Given that the overall goal of these studies is to determine pathways that may be of importance in the post-ischemic brain, selecting cerebellar-derived neurons is less relevant than would be selecting cortical neurons for this study, since focal cerebral ischemia in the MCAO mouse model targets cortical tissue (Koizumi et al., 1986). However, clinical acute ischemic stroke in human patients may affect vessels of the cerebellum, brain stem, as well as the cerebral cortex (Andreoli et al., 2004). CGNs were selected in this and other experiments for a number of reasons including: shorter culture time, ease of obtaining purified neuronal cultures, increased number of neurons per dish/well, and to be consistent with previous experiments performed in Dr. Hou's laboratory. To further credit this choice, however, it should be noted that the majority of studies in the literature involving neuronal death make use of DRGs from the peripheral nervous system (PNS), instead of neurons derived from the CNS, which are less relevant to stroke pathology than CGNs. In any case, data at both the transcriptional and translational level show that the presence of E2F1 in the cells used is requisite for wild-type (WT) levels of NRP-1 expression (Jiang et al., 2007). This is an important piece of information since it corroborates the correlative information obtained from the DNA microanalyses showing a difference in NRP-1 gene expression in E2F1 -/- and E2F1 +/+ brain tissue post-ischemia (Jiang et al., 2007). It does not, however, demonstrate that there is a *concrete* association between expression of NRP-1 and presence

of E2F1 since DNA microarray analyses has shown that the genetic absence of E2F1 affects hundreds to thousands of genes (Jiang et al., 2007; Müller et al., 2001), and it is possible that the dysregulation of any of these genes may secondarily affect NRP-1 expression.

Experiment 3.3.2 (Fig. 4) made use of previously generated adenoviral vectors in Dr. Hou's lab. Adenoviral constructs encoding full-length E2F1-GFP construct, an E2F1 mutant-GFP construct (containing a point mutation in the DNA binding region), or GFP alone, were used to determine whether replacement of the E2F1 gene in E2F1 null mice neurons would restore NRP-1 expression. The results show that E2F1 gene delivery to E2F1 null neurons did increase NRP-1 expression. It is interesting to note that in this experiment and in experiment 3.3.1 (Fig. 3), that even in E2F1-/- neurons or cortical tissue, there is still some, albeit extremely low, expression of NRP-1. This likely indicates that E2F1 is not the sole regulator of NRP-1 expression. It is possible that one or more of the other E2F family members may participate in the regulation of NRP-1 expression, since highest degree of homology between the E2F family members is within their DNA binding domains (DeGregori and Johnson, 1997). Thus other E2F family members such as E2F2 and E2F3 may also be capable of inducing cell death (Dirk et al., 1998; Vigo et al., 1999). Also previous experiments using adenoviral gene delivery of E2F1 to E2F1 +/+ neurons as a sole insult lead to an increase in cell death after 3 days (Hou et al., 2000). It was noted in during cell collection in this experiment (3.3.2), that in all samples where viruses were used, some cell death occurred, secondary to the infection process itself. In E2F1 +/+ cells infected with E2F-carrying adenovirus, more death was noted such that more cell plates were needed to obtain the same amount of protein for the western blot as the E2F1 -/- sample, corroborating previous work (Hou et al., 2000). Interestingly, the E2F1 +/+ cells

did not show an appreciable increase in NRP-1 expression compared to negative controls. Expression of NRP-1 was, however, increased compared to E2F1 -/- cells transfected with E2F1. This is somewhat unexpected since an increase in E2F1 expression post-ischemia is hypothesized to increase expression of NRP-1. However in this case, no insult was given, and it is possible that Rb, the negative regulator of E2F1 is acting to prevent over-activity of E2F1 in cells which already express E2F1. In differentiated rodent neurons, E2Fs are found to be in complex with pRb and thereby are not promoting expression of their transcriptional targets (Corbeil et al., 1995). To determine if this is the case, further experiments would be required to quantify Rb expression after gene delivery of E2F1 to these neurons. Another explanation is that the E2F1 +/+ neurons were being committed to apoptosis by the overexpression of E2F1 (Hou et al., 2000), and gene expression of NRP-1 at the protein levels was not upregulated to an extent that was detectable.

In experiment 3.3.3 (Fig. 5A), a putative E2F1 binding region was identified in the NRP-1 promoter. It is interesting to note that no such binding region was found in the NRP-2 promoter, nor did microarray analysis (performed by Dr. S.X. Jiang in Jiang et al., 2007) show a significant difference in NRP-2 expression in E2F1-/- and E2F1 +/+ cortical tissue (Jiang et al., 2007). Although both NRP-1 and NRP-2 mRNA have been shown to be up-regulated post-ischemia (Fujita et al., 2001; Zhang et al., 2001) and it is likely that NRP-2 is biochemically regulated in a different manner than NRP-1 and may have a different role during neuronal cell death. Further, no putative binding sequences were found in promoters of proteins that complex with NRP- 1 such as Plexins and L1 adhesion molecule (Jiang et al., 2007).

Also in experiment 3.3.3 (Fig. 5B), EMSA was used to show that E2F1 is capable of physically binding to a putative E2F1 binding region within the NRP-1 promoter

sequence. This is an important finding since it demonstrates that NRP-1 is a direct target of E2F1. In Figure 5B the band intensity with use of the NRP-1 hot probe compared to that of the positive CTL probe was significantly higher. This is likely an artifact of the probe labelling given that each probe was prepared separately. As such band intensities between different probes are not comparable. Thus this increased band intensity does not necessarily represent an increase in E2F1 binding affinity to the NRP-1 binding site than to the known core E2F1 binding site. As shown in Figure 6, quantification was only performed on samples that were incubated with the same (NRP-1) probe.

It is interesting to note that in Figure 5B, a light band was present even in the E2F1 -/- nuclear protein sample that was incubated with the NRP-1 probe. As previously mentioned, it is possible that other members of the E2F family such as E2F2 and E2F3, may be also able to bind to the putative NRP-1 promoter region (DeGregori and Johnson, 1997), albeit with likely less affinity and could also be responsible for cellular death processes (Dirk et al., 1998; Vigo et al., 1999). Note that in Figure 5B that the addition of poly-dIdC removed the presence of background and non-specific bands in the EMSA. In order to achieve the super-shift (ie: obtain a complex of anti-E2F1 antibody, E2F1 protein and NRP-1 promoter hot probe), poly-dIdC had to be omitted from the protocol, indicating the sensitivity of this complex formation. Thus EMSA was performed without this blocking agent, and a super-shift was confirmed using both the NRP-1 experimental probe, and a probe that contained the exact sequence of the known E2F1 core binding site. A supershift was not necessary in the E2F1 -/- samples, since only one band was present, which was used as a negative control against E2F1 +/+ samples.

Having established that E2F1 can bind the NRP-1 promoter, performing EMSA on post-ischemic brain samples demonstrated an increase in E2F1 occupation at the NRP-1

promoter on ischemic sides of the brain compared to contralateral sides of the same animal for controls. Although focal cerebral ischemia induced by MCAO appears to produce a localized focal lesion on the ipsilateral side of the brain based on gross pathological appearance, increasing genomic evidence indicates changes in gene expression profiles also on the contralateral side perhaps due to subtle molecular damages (Hou et al., 2003; Keyvani et al., 2002). This may explain why, in Figure 6, there exists a temporal increase in the occupation of the NRP-1 promoter by E2F1 on the contralateral (right) brain hemispheres. This expression change however, did not mask the significance of the increase of E2F1 complex formation on the ipsilateral side seen in Figure 6 and so use of the contralateral side of the brain was justified as an internal negative control.

Overall the EMSA has lead to an interesting observation: post-ischemia, there is an increased occupation of E2F1 at the NRP-1 promoter. This shows that E2F1 is indeed capable of binding to the NRP-1 promoter, and hence this gives us further evidence to show that NRP-1 expression is regulated by E2F1. Further, the increased accumulation of E2F1 at the NRP-1 promoter after ischemia is consistent with the knowledge that E2F1 is capable of activating its own expression, and that during cell death Rb becomes phosphorylated by cyclin/CdK complexes leading to more free and active E2F1 (Johnson et al., 1994; Crosby and Almasan, 2004). This leads us to hypothesize that the expression of NRP-1 may be increased after ischemia. Previous evidence indicates that NRP-1 mRNA levels increase post-ischemia (Fujita et al., 2001) and whether functional NRP-1 protein is increased as well in CGNs and brain tissue after MCAO is explored in Chapter 4.

In experiment 3.3.5 (Fig. 7), a luciferase reporter assay demonstrated that the presence of E2F1 in mouse glia was requisite for the activation of the NRP-1 promoter and the expression of luciferase, as the reporter protein (Jiang et al., 2007). One critique of this experiment is that due to lack of time, no internal control, such as β-galactosidase was utilized to demonstrate equal transfection efficiency between E2F1-/- and E2F1 +/+ cells. Further it the results would have been better demonstrated with the inclusion of a mutated E2F1 binding site sequence, showing abolishment of NRP-1 promoter activation. However, in context of the cumulative evidence in this chapter and in the published work (Jiang et al., 2007), we are sufficiently confident to conclude that E2F1 does regulate the transcription of NRP-1.

Another critique of this experiment (Fig. 7) is the use of glial cells as opposed to CGNs. It may then be argued that the results of this experiment may not be comparable with the results of the EMSA (Fig.5 and Fig. 6) and expression analyses (Fig 3. and Fig. 4), which utilized CGNs. However, regardless if E2F1 expression or regulation differs between the cell types, a positive activation of the NRP-1 promoter by the E2F1 transcription factor was shown. Interestingly, the results of the luciferase assay (Fig. 7) also corroborate a trend which has been shown in all experiments in this chapter: that there is a low level of activation of the NRP-1 promoter in the absence of E2F1.This is shown by a slight increase in luciferase activity in the E2F1 -/- glia transfected with the PGL3 Basic/NRP-1 promoter construct. This indicates, once again, a role for other transcription factors such as E2F2 and E2F3 in the activation of NRP-1 expression.

Collectively, the results in this chapter contribute to the understanding of the molecular pathways involved in the process of cell death by focal cerebral ischemia. NRP-1 is thus implicated as a possible mediator of E2F1-regulated neuronal death post-ischemia.

Previous studies have shown E2F1 is certainly a key mediator in inducing apoptotic cell death in post-mitotic neurons, yet just how this occurs is not yet clear. What is known is that the cells which are genetically devoid of the nuclear transcription factor E2F1 are largely more resistant to ischemia induced neuronal death both *in vitro* via OGD (Gendron et al., 2001), and *in vivo* via MCAO (McManus et al., 1999). Experimental evidence has shown that E2F1 can induce cell death responses in a number of ways, including the induction of expression of *pro-death genes* such as effector caspases and the repression of expression of pro-survival genes (Hou et al., 2000), as well as p53-dependent apoptosis. It is of interest that along with known pro-death genes, NRP-1 was also found to be a molecular target of E2F1 (Jiang et al., 2007). Up until recently, NRP-1 had not been implicated as a pro-death gene. Researchers have shown that direct application of Sema3A (a known NRP-1 ligand) to DRG neurons will induce what appears to be apoptotic cell death. (Shirvan et al., 1999; Shirvan et al., 2000). However, high concentrations of purified Sema3A ligand were used, and may or may not be of physiological relevance, even though Sema3A transcript is shown to be increased in the post-ischemic brain (Fujita et al., 2001; Zhang et al., 2001).

Collectively, the results in this chapter lead to the possibility that NRP-1 up-regulation may contribute to pro-death stimuli within a post-ischemic neuron. Whether or not the NRP-1 pathway is able to act as a pro-death mediator is explored in Chapter 4 of this thesis.

CHAPTER 4 NRP/SEMA PATHWAYS AS CONTRIBUTORS TO POST-ISCHEMIC DAMAGE

Contribution of Collaborators: Primary cell culture was performed by Angele Desbois and Dr. Susan Jiang performed initial statistical analyses.

4.1 Introduction

Evidence was provided in Chapter 3 demonstrating that the nuclear transcription factor E2F1 is an important mediator of post-ischemic neuronal cell death (Jiang et al., 2007). E2F1 transcriptional targets include known pro-death genes, such as certain caspase family members, as well as the receptor for Sema3A and VEGF165, NRP-1 (Giovanni et al., 2000; Jiang et al., 2007). EMSA analysis in Chapter 3 showed that there was an increased accumulation of E2F1 at the NRP-1 promoter post-ischemia (Jiang et al., 2007), and as such it was of interest to determine whether this increased promoter binding by E2F1 translated to an increase in NRP-1 protein expression.

Only a few studies in DRGs have shown that direct activation of the NRP-1/Sema3A pathway is capable of inducing neuronal death (Shirvan et al., 1999; Shirvan et al., 2000). Since E2F1 has been implicated in post-ischemic neuronal cell death, it is essential to determine whether NRP-1 as its downstream target is a contributor to the cell death process and if so, whether inhibition of this pathway could confer neuronal protection from ischemic insult. Since NRP-1 and NRP-2, as well as Sema3A mRNA have been shown to be transiently up-regulated post-ischemia in rats (Fujita et al., 2001), NRP-2 was

also included in the study. The long term goal with respect to NRP-2 experimentation is to elucidate the differential function of the NRP family of proteins in signaling neuronal death.

Overall, this Chapter examines Specific Aim 2: to better understand the role of the NRP-1/Sema3A pathway in neurons in the context of ischemic insults

4.2 Methods

4.2.1 Cell Culture

Primary CGN cell cultures were obtained exactly as per section 3.2.2. CGN cultures are approximately 95% neurons. NRP-2 -/- mice were obtained from Dr. David Ginty and Dr. Alex Kolodkin (John Hopkins Medical University, Baltimore, MD).

4.2.2 Western Blot

The procedures for Western blotting were exactly as described previously in section 3.2.3. The intensities of the bands were quantified using the Molecular Dynamics ImageQuant Software (Sunnyvale, CA). Anti-Neuropilin-1 monoclonal antibody (Calbiochem) was used at a dilution of 1:100.

4.2.3 Sema3A Treatment of CGNs

Recombinant Sema3A/Fc chimera (R & D Systems # 1250-S3) was added at a concentration of 0.1 μg/ml or 5 μg/ml to the E2F1 -/- and E2F1 +/+ CGN cultures at either 0 or 3 DIC for 18 h. The Sema3A/Fc chimera was added to the cultures with 10 ng/mL NGF (nerve growth factor). The cells were then either fixed with 4 % paraformaldehyde (PFA) and phase imaged or added with 10 μg/ml propidium iodide (PI) for cell viability assays.

4.2.4 Measurement of Neurite Outgrowth and Cell Death

CGNs were fixed with 4 % PFA for 20 min and subjected to immunostaining with primary antibody against myelin associated protein 2 (MAP-2) or β-III-tubulin (Chemicom International). Under a fluorescent microscope, digital images were taken of 10 randomly selected fields that contained more than 300 cells. Axonal lengths on the digitized images were measured using Image J, a National Institutes of Health (NIH) image analysis system (http://rsb.info.nih.gov/ij/). Cells were also counted using Image J. These analyses were performed in a double-blinded fashion, and the data were averaged and plotted for statistical analysis. Neuronal death was also measured by adding PI at 10 μg/ml concentration to the live cultures. Cellular fluorescence was measured at an excitation wavelength of 530 nm and an emission wavelength of 645 nm in a Cytofluor 2350 fluorescence measurement system (Millipore). Viability was normalized to the control cell readings.

4.2.5 OGD

OGD was performed as described by Gendron et al., (2001) in a 37 °C incubator housed in an anaerobic glovebox (Forma Scientific, Marjetta, OH, USA). Cultures were washed twice with OGD media: 140 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 10 mM HEPES, pH 7.4. Cultures were then incubated with OGD media and subjected to an anaerobic environment of 95% N_2 / 5%CO_2 for 1.5 h. OGD insult was terminated by removing the plates from the anaerobic chamber, returning the original media to the cells, and placing the plates back in a standard incubator maintained at normal atmospheric O_2 with 5% CO_2 at 37 °C. Control cultures were not treated with either the glucose deprived OGD media or anaerobic conditions. At the pre-determined end points, the cells were collected, pelleted and total protein extracts collected in Western Lysis Buffer. CGNs, were selected as the primary neuronal cell culture of choice for this and subsequent experiments due to their simple cell culture protocol, ease of maintenance, reliable maturation rate, and to be consistent with past experiments in our laboratory.

4.2.6 Sema3A Antagonist Treatment of CGNs

CGNs that were 7 DIC from either NRP-2 -/- or NRP-2 +/+ mice were pre-incubated for 30 min with or without 0.5 μg/ml of a 17 amino acid synthetic peptide inhibitor (GenScript) of sequence: HAVEHGFMQTLLKVTLE. This inhibitor is

antagonistic to the interaction between NRP-1 and its ligand Sema3A (Williams et al., 2005). A scrambled peptide of the same length and molecular weight was also synthesized (GenScript): LKHEVMFLQETVTHLAG and was used as a negative control. Cells were subsequently subjected to 1.5 h OGD and reperfused for 24 h at which point the cells were fixed and double immunostained for 4',6-diamidino-2-phenylindole (DAPI) and microtubule associated protein 2 (MAP2). Cell death was quantified by counting of condensed DAPI stained nuclei. Neuritic morphology and length was established following MAP2 staining.

4.2.7 Double Immunofluorescent Staining

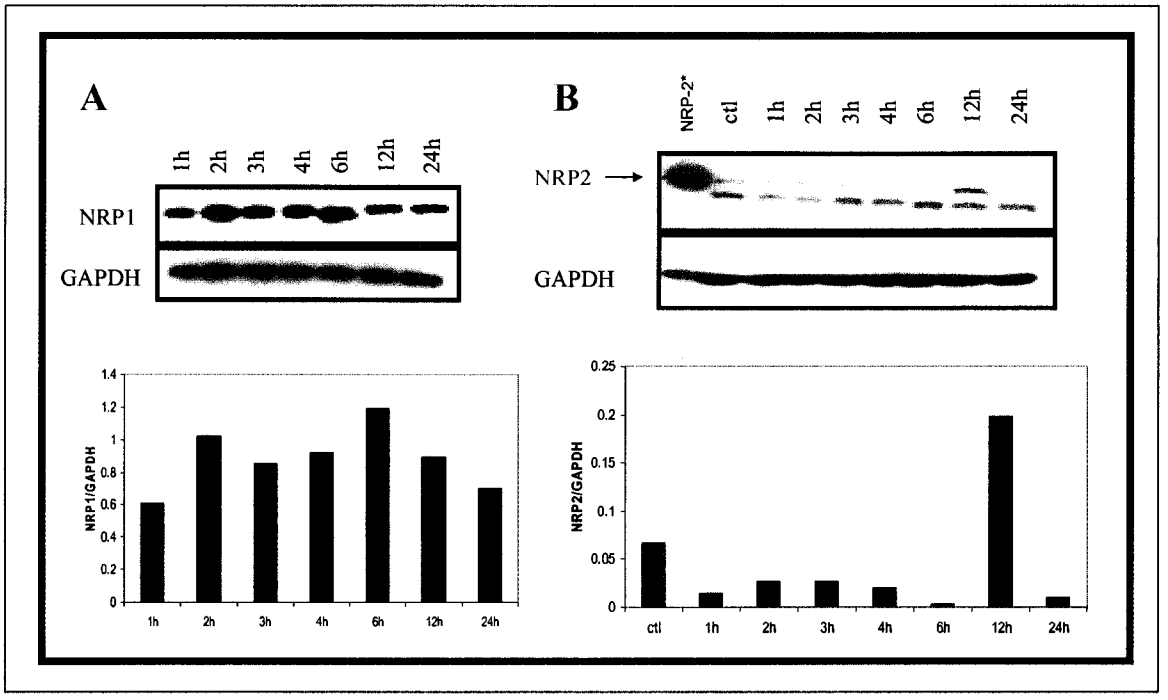
The procedures for fluorescent immunocytochemistry were exactly as previously described (Hou et al., 2002). Briefly, cells were fixed with 4% PFA for 20 min at room temperature and then washed gently, 3 times with PBS. Cells were blocked with 1.5 % BSA in PBS with 0.3% Triton-X for 30 min. Incubation with primary antibody, diluted in blocking solution, was performed overnight at 4 °C. Cells were then washed 3 times with PBS and incubated with the secondary antibody at room temperature for 1 h. Cells were washed 3 times again, with PBS and mounted on glass slides using mounting media containing DAPI for nuclear staining. The primary antibody raised in rabbit against MAP2 was purchased from Sigma and used at a 1:200 dilution. Goat anti-rabbit secondary antibody was used at a dilution of 1:200, either conjugated to fluorescein isothiocyanate (FITC) or cyanine 3 (Cy3) for detection under a fluorescent microscope.

4.3 Results

4.3.1 Expression of NRP-1 and NRP-2 in CGNs after OGD

In order to determine if NRP expression changes after *in vitro* ischemic insult, 7 DIV CGNs were plated in 10 cm dishes and subjected to OGD treatment for 1.5 h. Individual plates were reperfused for either 1 h, 2 h, 3 h, 4 h, 6 h, 12 h, and 24 h with regular culture media. At these time points, the cells were scraped from the plate with a rubber policeman, pelleted and total protein extracts collected in western Lysis Buffer followed by 30 seconds of sonication. Figure 8 illustrates the subsequent Western blotting. The results show that NRP-1 expression transiently increases at 2 -6 h of reperfusion and then returns to normal levels at 12 h-24 h (Fig. 8A). NRP-2 expression increased at 12 h and then decreased at 24 h (Fig. 8B). GAPDH is used as an internal loading control for both westerns and densitometric quantification is shown graphically. The results indicate that both NRP-1 and NRP-2 expression in CGNs is induced post-ischemia *in vitro*. Since two bands were shown in the western for NRP-2 in Figure 8B, a positive control was used to identify the correct band for NRP-2. This control consisted of total protein from human embryonic kidney (HEK-293) cells that had been transfected with a plasmid encoding the full length mouse NRP-2 protein (plasmid was a gift from Dr. Stephen Strittmatter, Yale University School of Medicine, USA)

Fig 8. NRP-1 and NRP-2 expression in CGNs is induced post-ischemia *in vitro*. 7 day CGNs were subjected to OGD treatment for 1.5 h and reperfused for 1, 2, 3, 4, 5, 6, 12, 24 h respectively. At these time points, the cells were pelleted and total protein extracts collected in Western Lysis Buffer with 30 seconds sonication. (A) Western blotting is performed for NRP-1showing that NRP-1 expression transiently increases at 3 h-6 h and then return to normal levels at 12 h-24 h. (B) NRP-2 expression increases at 12 h post- ischemia and then decreases at 24 h. NRP-2* is a positive control and consisted of a total protein sample from HEK-293 cells which had been transfected with a plasmid encoding NRP-2. This control was purified by Angele Debois. GAPDH is used as an internal loading control for both westerns and quantification by densitometry is shown graphically.


A
1h
2h
3h
4h
6h
12h
24h
NRP1
GAPDH
B
NRP-2*
ctl
1h
2h
3h
4h
6h
12h
24h
NRP2
GAPDH
NRP1/GAPDH
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1h
2h
3h
4h
6h
12h
24h
NRP2/GAPDH
0.25
0.2
0.15
0.1
0.05
0
ctl
1h
2h
3h
4h
6h
12h
24h

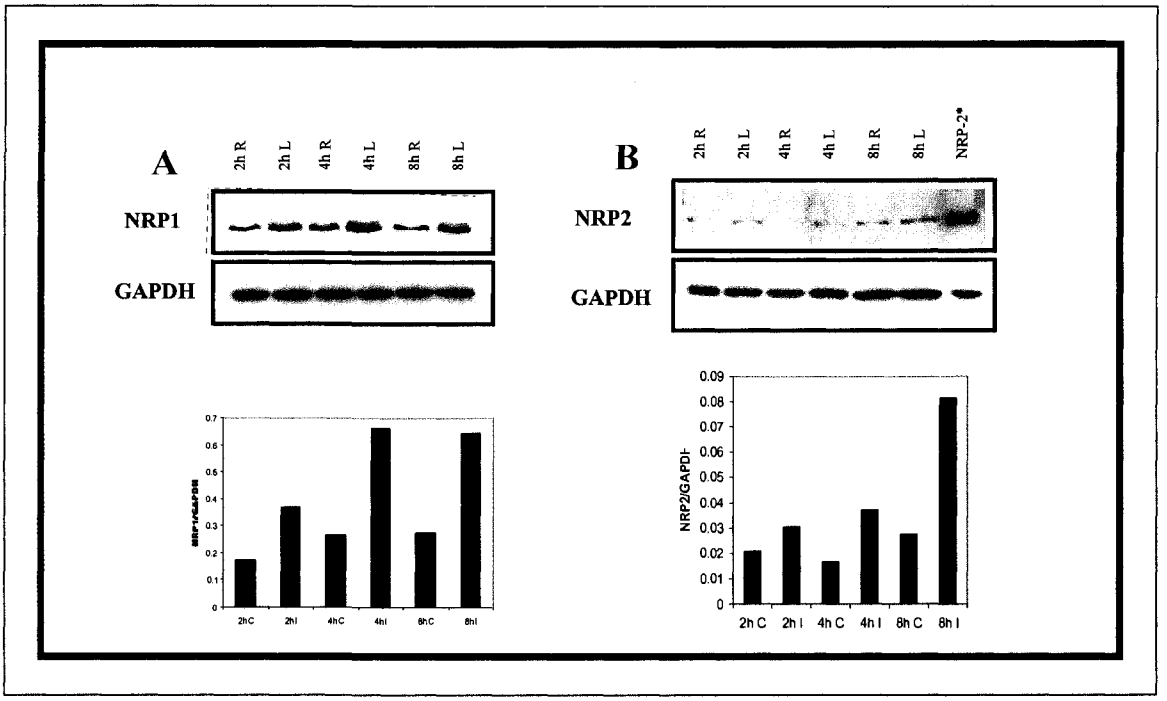
4.3.2 Expression of NRP-1 and NRP-2 in CGNs after MCAO

To determine whether NRPs are indcued post-ischemia *in vivo*, western blots were performed from total protein extraction from MCAO treated mouse brains. After treatment of mice with MCAO, followed by 2 h, 4 h, and 8 h reperfusion the mice were sacrificed and brains were sectioned into right (R) and left (L) hemispheres. The left hemisphere is the ischemic side and the right is the contralateral, non-ischemic side. Total protein was run on western blot and probed for expression of NRP-1 and NRP-2 (Fig. 9). GAPDH was used as the internal loading control. Densitometric quantification illustrates consistent increase in both NRP-1 and NRP-2 protein expression in the ischemic versus contralateral sides of the brains treated with MCAO (Fig. 9). The other evident trend is that with increasing reperfusion time up to 8 h, expression of both NRPs tends to increase. This correlates with previous RT-PCR data showing peak expression of NRP-1 at 2 h and 8 h of reperfusion (Jiang 2005, unpublished).

4.3.3 Differential responses of E2F1 -/- and E2F1 +/+ CGNs to Sema3A

To determine the role of NRP-1 in mature primary neurons, and to demonstrate that E2F1 -/- neurons do not express a functional level of NRP-1, CGNs on the first day of culture (day 0) were treated with 0.1 μg/ml or 5 μg/ml of commercially produced recombinant Sema3A/Fc fusion protein (R&D Systems). This protein is recombinant Sema3A disulfide-linked dimer. Its activity is measured by its ability to collapse DRG

Fig 9. Ischemia induces increase in NRP-1 and NRP-2 expression *in vivo*. After treatment of C57/Bl6 mice with MCAO, followed by 2 h, 4 h, and 8 h reperfusion, the mice were sacrificed and brains were sectioned into right (R) and left (L) hemispheres. Total protein was run on western blot and probed for expression of (A) NRP-1 and (B) NRP-2. Left hemispheres are stroked and right are contralateral. GAPDH is used as the internal loading control.


A
2h R
2h L
4h R
4h L
8h R
8h L
NRP1
GAPDH
B
2h R
2h L
4h R
4h L
8h R
8h L
NRP-2*
NRP2
GAPDH
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2hC
2hI
4hC
4hI
8hC
8hI
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
NRP2/GAPDH
2h C
2h I
4h C
4h I
8h C
8h I

growth cones (R&D Systems). Sema3A has been shown to inhibit axonal outgrowth through NRP-1-mediated signal transduction (Kawasaki et al., 2002). As shown in Figure 10, the addition of 0.1 μg/ml or 5 μg/ml Sema3A/Fc fusion protein to 0 DIV E2F1 +/+ CGNs significantly inhibited axonal outgrowth after 3 days in culture, but this effect was not observed in E2F1 -/- CGNs. No difference in axonal length was found between cultures treated with 0.1 μg/ml or 5 μg/ml Sema3A/Fc fusion protein. This shows that the absence of E2F1 expression ameliorated Sema3A mediated inhibition of axonal outgrowth.

Although axonal effects were observed with 0.1 μg/ml of Sema3A/Fc fusion protein, this concentration did not induce cell death in the neurons, as did higher concentrations (5 μg/ml). As shown in Figure 11, the addition of 5 μg/ml Sema3A to 3 DIC CGNs for 18 h not only led to contraction of the axons of E2F1 +/+ CGNs, but also caused a significant induction in neuronal death as quantified by PI. E2F1 -/- CGNs were protected from these effects. These studies provide initial evidence that NRP-1 plays a role in modulating axonal retraction *and* neuronal death in post-mitotic CNS neurons further substantiating that NRP-1 is a part of the death signal transduction pathway of E2F1.

Fig. 10. E2F1 -/- CGNs are less susceptible to Sema3A-mediated inhibition of axonal outgrowth. Sema3A/Fc fusion protein was added to CGNs at the indicated concentrations at the time of plating. After 3 days in culture, cells were fixed in 4% PFA and immunostained with β-III-tubulin to detect axons. As shown in panels A and C, untreated E2F1|+/+ and E2F1 -/- neurons had developed long axons and neurites and started to form networks of connections at 3 DIV. However, Sema3A treatment caused a significant inhibition of axonal outgrowth in E2F1 +/+ CGNs (B and E), but not in E2F1 -/- CGNs (D and E). Digital images of axons were taken, and axon lengths were measured using Image J software. **, $P < 0.01$ by one-way ANOVA and post-hoc analysis (Tukey's test). The error bars indicate standard deviations (Jiang et al, 2007). Figure generated by Dr. S.X. Jiang.

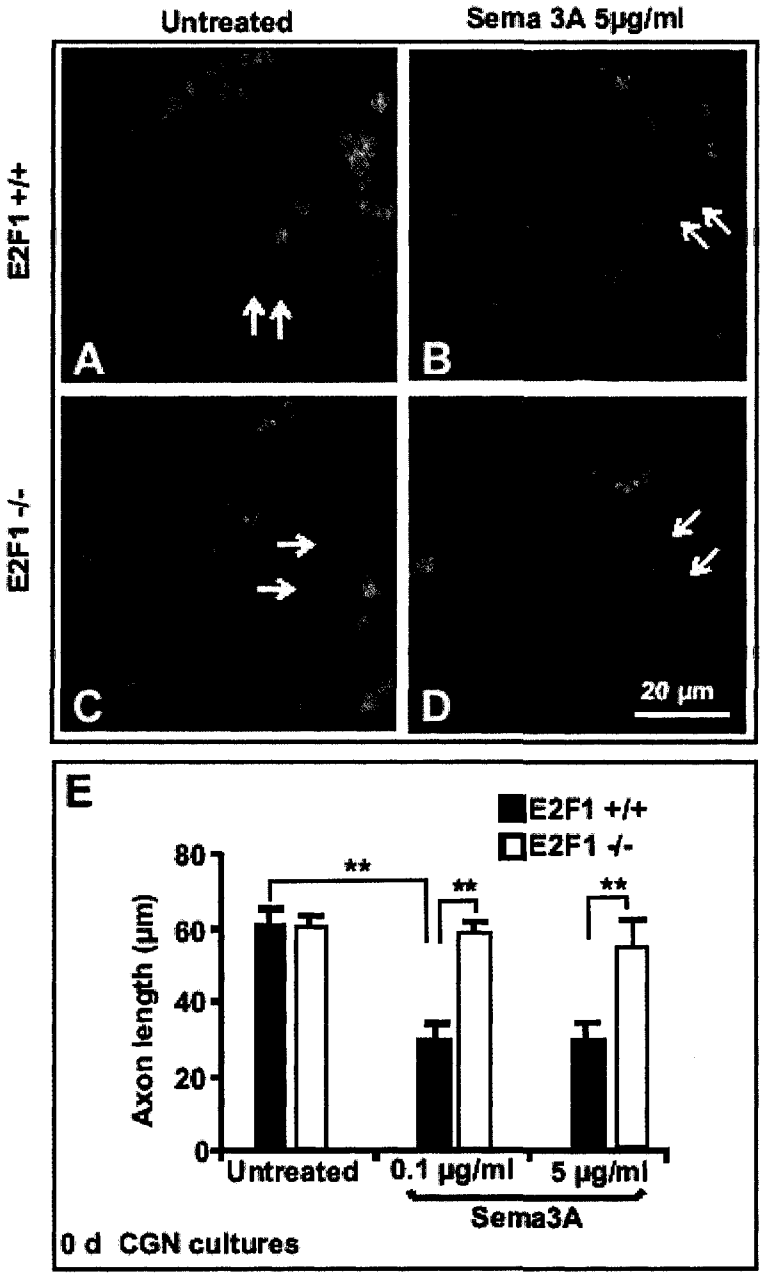
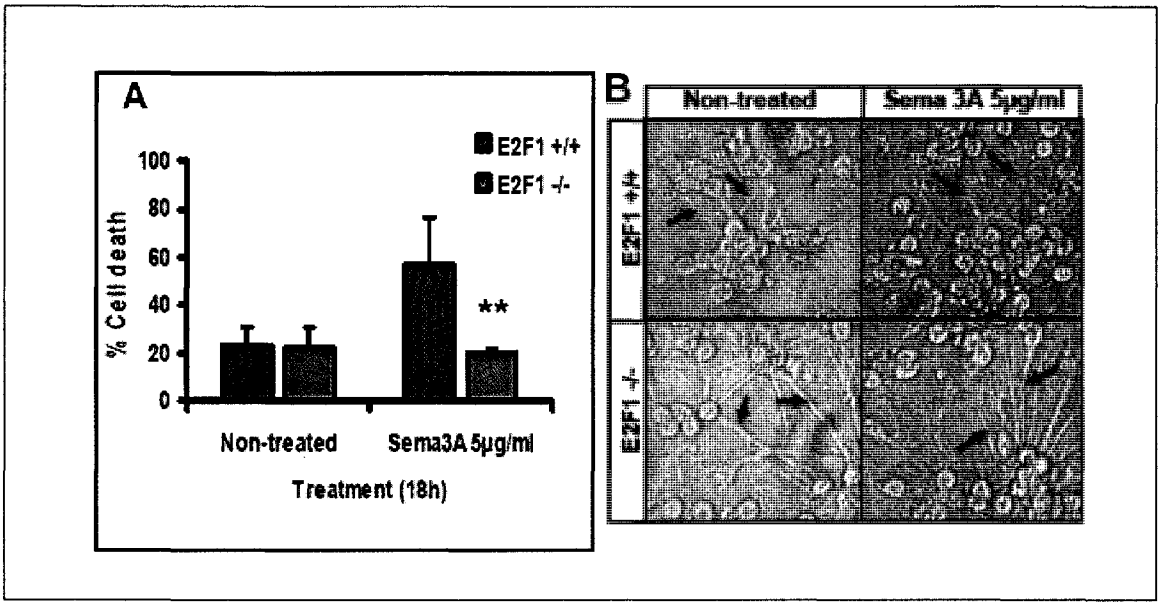

Untreated
Sema 3A 5µg/ml
E2F1 +/+
E2F1 -/-
A
B
C
D
20 µm
E
E2F1 +/+
E2F1 -/-
Axon length (µm)
80
60
40
20
0
**
**
**
Untreated
0.1 µg/ml
5 µg/ml
Sema3A
0 d CGN cultures

Fig 11. Semaphorin 3A causes axonal damage and neuronal death. 3day CGNs from either E2F1-/- or E2F1 +/+ brains were treated with 5ug/ml Sema3A for 18 h. CGNs were either fixed with 4% PFA and phase imaged (B) or added with 10 μg/ml PI for cell viability assays (A). E2F1-/- CGNs axons were not affected by Sema3A treatment, while E2F1+/+ neurons had significantly shortened axons following 5 μg/ml Sema3A treatment. Cell death assay using PI staining detected significant amount of neuronal death after 18 h Sema3A treatment (B), whereas E2F1-/- cells show protection from cell death. ** is $p < 0.001$ by T-test.


A
E2F1 +/+
E2F1 -/-
100
80
60
40
20
0
% Cell death
**
Non-treated
Sema3A 5µg/ml
Treatment (18h)
B
Non-treated
Sema 3A 5µg/ml
E2F1 +/+
E2F1 -/-

4.3.4 Inhibition of NRP-1 and genetic ablation of NRP-2 confer protection from OGD

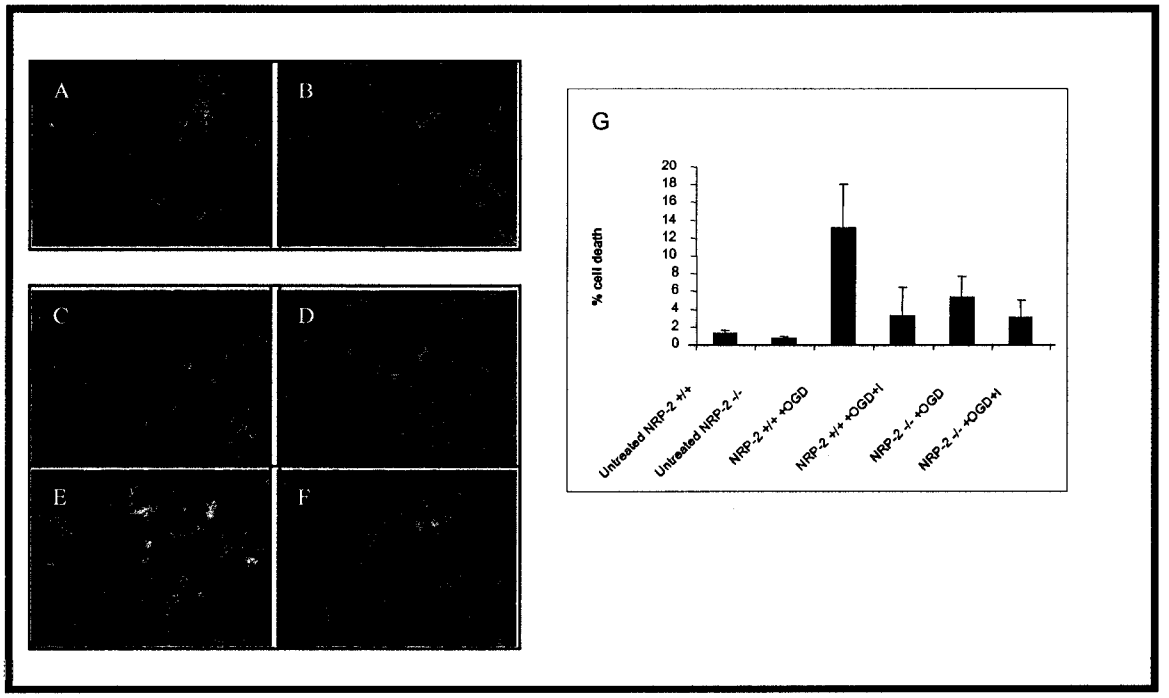
We next sought to determine whether or not the resistance of E2F1-/- neurons to OGD (Gendron et al., 2001) is due solely to the lack of the death transcription factor itself, or to the significantly lower expression levels of its target genes, especially NRP-1. Unfortunately a NRP-1 -/- model is unavailable due to the embryonic lethality of this mutation (Kitsukawa et al., 1995), and thus genetic tests to identify the contribution of the NRP-1 pathway in post-ischemic processes becomes more difficult. The synthesis and use of a peptide inhibitor which binds to Sema3A and inhibits its interaction with receptor NRP-1 is an alterative method for observing the effect of inhibiting this pathway (Williams et al., 2005). Although NRP-2 expression has not been shown to be affected by E2F1, it was of interest nonetheless to observe whether this receptor could affect post-ischemic neuronal outcomes after OGD. Fortunately, a viable NRP-2-/- mouse model was available (provided by Dr. David Ginty and Dr. Alex Kolodkin from John Hopkins Medical University, USA) and CGNs from this model were used in the following experiment.

CGNs at 7 DIC from either NRP-2 -/-, or NRP-2 +/+ mice were pre-incubated for 30 min with or without 0.5 μg/ml of synthetic NRP-1/Sema3a peptide inhibitor (commercially synthesized by GenScript). This inhibitor is antagonistic to the interaction between NRP-1 and its ligand Sema3A (Williams et al., 2002). Cells were subsequently subjected to 1.5 h OGD and reperfused for 24 h at which point the cells were fixed with 4% PFA and double immunostained as per protocol for DAPI (identifies nuclei) and MAP2

(identifies neurites) (Fig. 12). Cell death was quantified by manual counting of condensed DAPI stained nuclei as observed under fluorescence microscopy. Neuritic morphology and length was observed by MAP2 staining.

As shown in Figure 12 axonal appearance differs in untreated NRP-2 -/- and NRP-2 +/+ cultures. NRP-2 -/- CGNs (Fig. 12B) tend to have longer axons in culture than CGNs from NRP-2 -/- mice (Fig. 12A). When NRP-2 +/+ CGNs are treated with OGD, axonal networks collapse and condensed DAPI nuclei are visible, indicating apoptotic cell death is taking place (Fig. 12C). NRP-2 -/- CGNs seem to show some protection from axonal shortening and cell death after OGD in comparison (Fig. 12E). NRP-2-/- CGNs and wild-type CGNs pre-treated with the NRP-1/Sema3A inhibitor were found to be protected from OGD induced cell death as detected by DAPI, and axonal retraction, and appearance of varicosities within the neurites. NRP-2-/- cells show typically longer axons both in untreated and post-ischemic states (Fig. 12B, 12E, 12F). Quantification of cell death from the above results is shown in Figure 12G and was obtained by microscopic counting of condensed DAPI stained nuclei. The combination of NRP-2 gene knockout culture plus treatment with NRP-1/Sema3A pathway inhibitor did not show more protection that treatment with NRP-1/Sema3A pathways inhibitor on NRP-2 +/+ cultures (Fig. 12G). Together, these data suggest that antagonism of the NRP-1/Sema pathway effectively reduces OGD induced neuronal damage. These results suggest that the NRP-1/Sema3A pathway may play a more significant role in ischemia induced cell death than the NRP-2 pathway. Further experimentation is needed to conclusively demonstrate this.

Fig 12. Genetic reduction or ablation of NRP-2 expression and biochemical inhibition of the NRP-1/Sema3A interaction confer protection to CGNs from *in vitro* ischemic insult. After 7 DIC CGNs from either NRP-2-/-, NRP-2 +/-, or wild-type CGNs were pre-incubated for 30 minutes with or without 0.5 ug/ml of synthetic peptide inhibitor (I). This inhibitor is antagonistic to the interaction between NRP1 and its ligand Sema3a (Williams et al., 2002). Cells were subsequently subjected to 1.5 h OGD and reperfused for 24 h at which point the cells were fixed and double immunostained for DAPI and MAP2. Cell death was quantified by counting of condensed DAPI stained nuclei and neuritic morphology and length was observed by MAP2 staining. NRP2-/- CGNs and wild-type CGNs pre-treated with the NRP1/Sema3a inhibitor were found to be protected from OGD induced cell death as detected by DAPI, and axonal retraction, and appearance of varicosities within the neurites. NRP2-/- cells show typically longer axons both in untreated and post-ischemic states.I = NRP1/Sema3a inhibitor; wt = wild-type. (A) Untreated NRP-2 +/+ CGNs stained with DAPI and MAP2. (B) Untreated NRP-2 CGNs stained with DAPI and MAP2. (C) NRP-2 +/+ CGNs treated with OGD (D) Protection conferred to NRP-2 +/+ CGNs from OGD by pre-incubation with (I). (E) NRP-2 -/- CGNs are protected from OGD (F) NRP-2 -/- CGNs pretreated with (I) are slightly more protected from OGD than NRP-2 -/- cells without (I) added. (G) Quantification of cell death.


A
B
C
D
E
F
G
% cell death
20
18
16
14
12
10
8
6
4
2
0
Untreated NRP-2 +/+
Untreated NRP-2 -/-
NRP-2 +/+ +OGD
NRP-2 +/+ +OGD+I
NRP-2 -/- +OGD
NRP-2 -/- +OGD+I

4.4 DISCUSSION

4.4.1 Summary of Results

Collectively, the results from this chapter provide evidence to indicate that NRPs may play a significant role in ischemia-induced neuronal cell death. To summarize, the evidence is as follows: 1) expression of NRP-1 and NRP-2 increased after *in vitro* ischemic insult, 2) expression of NRP-1 and NRP-2 were increased on ischemic sides of the brain in a mouse model of in vivo focal cerebral ischemia, correlating with increased occupation of E2F1 at the NRP-1 promoter, 3) axonal repulsion mediated by Sema3A was attenuated in E2F1 -/- neurons, further supporting the role of E2F1 in regulating NRP-1 expression, 4) Sema3A added to neuronal cultures as a sole insult was able to induce significant cell death in E2F1 +/+ neurons but E2F1 -/- neurons were protected, 5) an inhibitor of the NRP-1/Sema3A pathway conferred protection from *in vitro* ischemic insult and 6) NRP-2 -/- neurons cultures appeared to be slightly protected from *in vitro* ischemic insult. The function of NRP-2 within the context of ischemia is much less clear than that of NRP-1 and further experimentation is needed to determine whether it does or does not contribute to ischemia-induced neuronal damage.

4.4.2 Experimental Review and Critique

4.4.2.1 Differential Regulation of NRP-1 and NRP-2 Expression

In experiments 4.3.1 (Fig. 8) and 4.3.2 (Fig.9), it is interesting to observe that both NRP-1 and NRP-2 are up-regulated post-ischemia *in vitro* and *in vivo.* According to the results in Chapter 3, NRP-2 does not appear to be regulated by transcription factor E2F1, as is NRP-1 (Jiang et al., 2007). No matching E2F1 binding site was found within an NRP-2 promoter, nor was NRP-2 found to be differentially regulated on microarray analyses of E2F1 -/- and E2F1 +/+ mouse cortical tissue (Jiang et al,. 2006). The role of NRP-2 has not been studied in the literature nearly as extensively as that of NRP-1, and as such it is difficult to speculate whether this increase in NRP-2 expression plays a significant role in post-ischemic effects. However, previous studies indicate that NRP-1 and NRP-2 are able to function as hetero-dimers within the multi-protein Sema3 receptor complex (Chen et al., 1997). Whether this association alters downstream signaling, or changes ligand affinity is still to be determined. Further, the expression of NRP-1 and NRP-2 as shown in Figures 8A and 8B seems to oscillate in a time dependent manner, with expression levels of NRP-1 being highest at 2 h and 6 h reperfusion and expression of NRP-2 being highest at 12 h reperfusion. Because of this difference in expression profiles it is likely that NRP-1 and NRP-2 play different roles in the neuronal cell death process. NRP-1 may act as an earlier

mediator and NRP-2 expression perhaps is increased after the commitment to cell death has already occurred.

Figures 9A and 9B show that both NRP-1 and NRP-2 are up-regulated in the ischemic side of the brain. This result coincides with the results of the EMSA in Chapter 3 (experiment 3.3.4) that demonstrate that levels of E2F1 occupation at the NRP-1 promoter are increased after ischemic insult. This validates the hypothesis that increased occupation by E2F1 at the NRP-1 promoter translates into NRP-1 up-regulation. The expression of NRP-2, however, is very barely detectable by western analyses, yet does show a temporal increase in expression at a later time point than NRP-1 (Fig. 9A and 9B). This coincides with the delayed up-regulation of NRP-2 shown *in vitro* (Fig 8B). This evidence suggests that NRP-2 signaling is likely not a significant determinant of early, salvageable cell death processes. More research is needed to determine the transcriptional regulation of NRP-2 and its function, if any with respect to post-ischemic prospects for cortical tissue in the penumbra.

With respect to experiment critique, statistical analyses are required to demonstrate a definite temporal expression profile of NRPs post-ischemia. In experiment 4.3.1 (Fig.8A and 8B), the expression of NRP-1 and NRP-2 was determined using western blot analyses of protein extracts from CGNs treated with OGD. Obtaining a clean western blot for both NRP-1 and NRP-2 was extremely difficult, since 1) NRPs are high molecular weight proteins and 2) they are GPI anchored and largely difficult to remove from the cell membrane. For this reason, statistical analyses could not be performed on this experiment due to the low quality of previous westerns (band concentration problems). However, all of the westerns consistently demonstrated the same temporal increases in NRP-1 and NRP-2

expression levels. As such, the last western blots performed, which were of highest quality were selected to represent the trend of NRP expression post-OGD. Further repeats will be necessary in order to establish statistics for publication. The same explanation is true for Figure 9, which illustrates representative western blots for NRP-1 and NRP-2 expression after MCAO.

4.4.2.2 The Contribution of the NRP-1/Sema3A Pathway to Neuronal Death

In further support of the concept that the pro-death nuclear transcription factor E2F1 is requisite for functional NRP-1 expression, Experiment 4.3.3 demonstrated that E2F1 -/- were significantly less susceptible to Sema3A-induced axonal retraction and cell death as shown in Figures 10 and 11. In fact, Sema3A incubation was not shown to have any statistical effect in this regard on E2F1 -/- cells. The fact that Sema3A was able to induce death in post-mitotic CNS neurons was a novel finding as well, since previous studies by Shirvan et al., (1999 and 2000) have shown this effect only on DRGs from the peripheral nervous system.

A criticism of the above experiments is that the physiological concentrations of Sema3A are likely significantly lower than the concentrations used in these experiments and other similar experiments performed by other researchers (Shirvan et al., 2000). The ED_{50} for the collapse of DRG growth cones with this Sema3A/Fc chimera as determined by the commercial produced was 20 ng/mL-60 ng/mL (R&D Bioscience). The concentration used to induce cell death in experiment 4.3.3 was 5000 ng/mL. Thus, whether this

concentration of Sema3A is relevant *in vivo* is still to be determined, as well as whether the Fc addition to this protein alters its effects.

Further it is possible that the cell death observed in these experiments is an artifact of cell culture, in the sense that a high concentration of Sema3A is maintained at an equal, high and unavoidable concentration in the dishes. *In vivo*, under normal physiological conditions, Sema3A is secreted as a gradient, and is able to diffuse. However, one argument in support of the *in vitro* experiments is that after cerebral ischemia, Sema3A mRNA has been shown to be increased over 1000 times that of normal conditions (Fujita et al., 2001). So it is possible, that post-ischemia, physiological gradients are abolished by a large wave of Sema3A expression and secretion. Sema3A, along with all other class 3 Semas have also shown to be very highly expressed and secreted by meningeal fibroblasts after injury to rat spinal cord (de Winter et al., 2002). These cells form a fibrotic scar that is non-permissive to axonal extension and regrowth and an analogous structure is formed in the post-ischemic brain; reviewed by de Winter et al., (2002). Future experiments need to be performed to determine whether the up-regulated Sema3A mRNA expression found in the CNS post-ischemia translates into increased translation and secretion of the Sema3A protein into the extracellular environment.

The assumption that makes the above experiments relevant is that ischemic insult induces the secretion of Sema3A by the neurons in culture and activation of the NRP-1/Sema3A pathway in an autocrine fashion. Further, since the neuronal cultures are never 100% pure neurons, it is possible that remaining glia may also play a role in the secretion of the Sema3 ligands. Future experiments will need to delineate Sema3A expression at the

protein level post-ischemia within neurons, glia, and the concentration found secreted in the media.

The fact that induction of the NRP-1/Sema3A pathway is sufficient to induce neuronal cell death is of interest in context of the results in Chapter 3, which suggest that NRP-1 may possibly be a death receptor induced by the pro-death transcription factor E2F1 (Jiang et al., 2007). Experiments performed in section 4.3.4 (Fig. 12) add to this conclusion by suggesting that inhibition of NRP-1 (and possibly NRP-2) signaling may confer protection from cell death and axonal damage from *in vitro* ischemic insult. Figure 12 shows firstly, that NRP-2 -/- cultures are morphologically similar to WT cultures with the exception that NRP-2 -/- CGNs tended to have axons with longer processes in general. This lends support to the role of NRP-2 as well as NRP-1 in axonal repulsion and inhibition of outgrowth. NRP-2 -/- CGNs were then subjected to OGD and cell death compared to that from NRP +/+ CGNs. In absolute numbers, cell death was reduced in NRP-2 -/- cultures, but only a minimal ~7%. Once again, in order for blocking NRP-2 pathway to be relevant, one would have to show that NRP2 ligands are activating theses receptors post-ischemia. NRP-2 receptors have been shown to be most responsive to Sema3F (He et al., 2002) and it is likely that Sema3F is also secreted post-ischemia, although no studies to date have examined this Sema3 subclass with respect to ischemia. Evidence does exist that all the Sema3 subclasses are secreted post-injury by spinal cord tissue in an autocrine fashion (deWinter et al., 2002).

However, it must be stated the results of this experiment should be questioned. Firstly, the OGD chamber was not producing a high enough cell death at the time of this experiment as inconsistent oxygen levels were produced due to a leak. Thus, this

experiment is preliminary. Secondly, repeats were preformed from cells from the same batches of mice, such that standard error bars cannot be interpreted as being statistically significant. Interestingly, this experiment was preformed again by Dr. M. O'Hare in Dr. Hou's lab, this time using cortical cultures (95% neurons), a functional OGD incubator and longer time of hypoxic incubation. His results clearly showed that there was no statistical protection using NRP-2 -/- cultures under better defined conditions. Thus, in the context of the collective results, NRP-2 does not seem to play a significant role in post-ischemic effects, and its regulation and function in this context remain to be resolved.

Within this same experiment (Fig. 12), the effect of blocking the NRP-1/Sema3A pathway specifically was pursued. Since the NRP-1 -/- mouse model is not viable, a synthetic peptide inhibitor was used to block the interaction of Sema3A with NRP-1 (Fig. 12D). This inhibition is known to be effective and specific (Williams et al., 2002). The use of the Sema3A inhibitor demonstrated protection from cell death in both the NRP-2 +/+ and NRP-2 -/- cultures. This aids in the confirmation the Sema3A pathway is activated during ischemic insult, and blocking this activation provides direct neuronal protection.

Statistical differences could not be obtained due to the aforementioned OGD chamber difficulties. However, the peptide inhibitor was able to protect the neurons both from cell death and axonal repulsion that is typically noted after OGD under normal conditions. This shows that there must be Sema3A being secreted in the cultures, and that the interaction of Sema3A with the NRP-1 receptor directly contributes to the post-ischemic cell death and axonal retraction.

Taken together, the results of Chapter 4 suggest that the NRP-1/Sema3 pathway can contribute to the death of neurons and inhibition of this pathway may have therapeutic effects in delaying or preventing post-ischemic neuronal damage and/or loss.

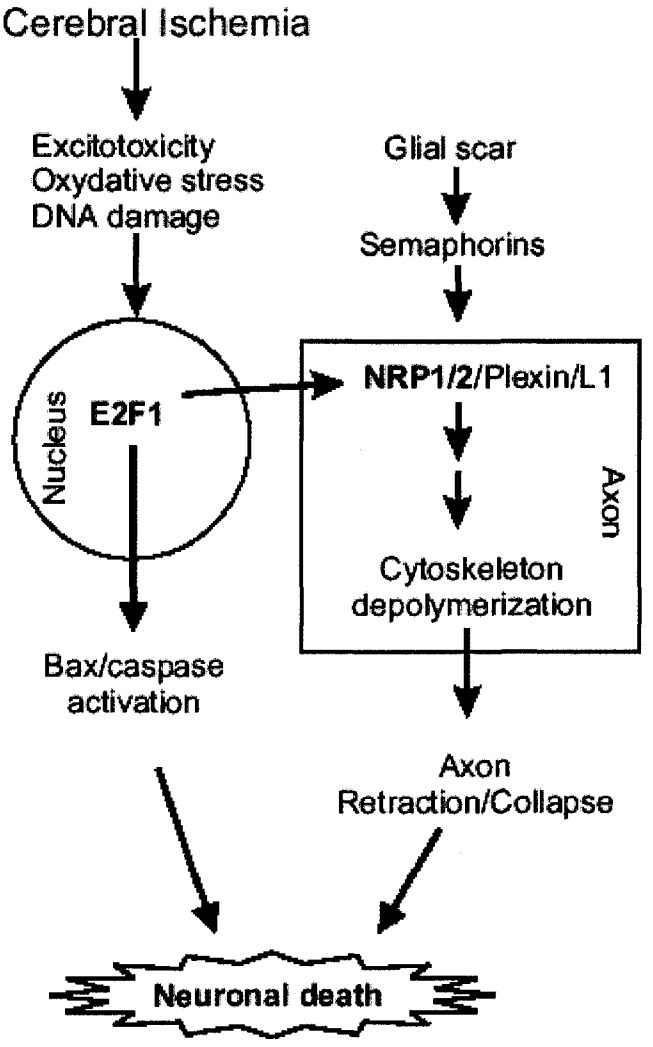
CHAPTER 5 OVERALL DISCUSSION AND CONCLUSIONS

5.1 Collective Summary of Results and Proposed Model

This thesis sought to expand the current understanding of the molecular mechanisms of ischemia induced neuronal death and axonal damage. The first half of this work (Chapter 3) examined the transcriptional regulation of NRP-1 by E2F1. Through NRP-1 expression analysis of E2F1 -/- and E2F1 +/+ tissue, EMSA for protein:DNA interaction between E2F1 and the NRP-1 promoter, and Luciferase reporter assays to show the activation of NRP-1 promoter by E2F1, we proposed that NRP-1 is transcriptionally up-regulated by E2F1 and that this E2F1-dependent expression of NRP-1 is increased post-ischemia. The second half of this research (Chapter 4) validates the induction of expression of NRP-1 and NRP-2 post-ischemia but implicates the NRP-1/Sema3A pathway activation alone in induction of cell death and axonal morphological changes associated with ischemic insult. Biochemical inhibition of the NRP-1 pathway conferred protection from ischemia induced axonal damage and cell death *in vitro*.

In summary we propose a model (Fig. 13), wherein effects of cerebral ischemia on the CNS neurons include excitotoxicity, oxidative stress, and DNA damage (Hou and MacManus, 2002). E2F1 nuclear transcription factor becomes activated (Greene and Liu, 2004), and acts to modulate neuronal cell death through different molecular pathways: 1) by induction of pro-apoptotic genes such as Bax and caspases (Giavanni et al., 2000) and 2) by induction of expression of NRP-1 and NRP-2 receptors, which upon exposure to

Fig. 13. Schematic diagram depicting pathways of axonal damaged and neuronal death as mediated by E2F1 activation of NRP-1 during cerebral ischemia (Jiang et al., 2006). Figure generated by Dr. S.T. Hou.


Cerebral Ischemia
Excitotoxicity
Oxydative stress
DNA damage
Glial scar
Semaphorins
Nucleus
E2F1
NRP1/2/Plexin/L1
Axon
Cytoskeleton
depolymerization
Bax/caspase
activation
Axon
Retraction/Collapse
Neuronal death

increased concentrations of Sema secreted from the neural scar, lead to axonal retraction and cell death (Fujita et al., 2001; Jiang et al., 2007).

5.2 Discussion of Model in Context of Relevant Literature

5.2.1 E2F1 Transcriptionally Regulates NRP-1

Nuclear transcription factor E2F1 has been shown in the literature to be an important mediator of cell death in post-mitotic neurons (Hou et al., 2000; Park et al., 2000; O'Hare et al., 2000). However, inhibiting E2F1 as a therapeutic target post-ischemia would be a very complex endeavor due to the enormous array of transcriptional targets under its regulation and the fact that E2F1 has been shown to have both pro- and anti-apoptotic effects. For example, E2F1 has been shown to induce neoplasia in astrocytes *in vitro* (Miyajima et al., 1996). Thus, this thesis aimed at determining the contribution of NRPs as potential specific downstream targets of E2F1 to ischemia induced axonal retraction and neuronal death. The knowledge that NRP-1 is regulated by E2F1 is a novel finding (Jiang et al., 2007) and is important in the context of cerebral ischemia since E2F1 has been shown to contribute significantly to neuronal death post-ischemia (Hou et al., 2000, Jiang et al., 2007; MacManus et al., 1999; MacManus et al., 2003). Therefore, elucidation of the downstream mediators of E2F1 is crucial for therapeutic development. Further exploration of other E2F1 target genes, especially those expressed at a functional level in both axons and cell bodies will be examined in the future.

5.2.2 The Complexity of NRP Regulation and Function

With respect to NRPs, research over the past decade has focused on the elucidation of how the NRP-1/Sema and NRP-1/VEGF pathways function and on the revelation of the molecular components of the NRP receptor complex for Sema and VEGF ligands. The NRP-induced signaling cascades have yet to be determined in their entirety, and their elucidation is complexed by the knowledge that intracellular mediators such as cyclic nucleotides can modulate axonal dynamics in response to the same Sema3 stimulus (Song et al., 1998; Rohm et al., 2000). Further, according to the results presented herein, NRP-1 and NRP-2 genes seem to be regulated in different manners (Jiang et al., 2007) and play diverse roles in the context of neuronal injury and regenerative potential. Suffice to say that the NRP pathway, its regulation and the outcomes of its stimulation are complex and incompletely understood.

The NRP receptor complex has been studied by numerous researchers over the past decade. Both NRP-1 and NRP-2 have been found to be critical components of the Sema receptor complex, and their dimeric combinations are believed to regulate the specificity of Sema3 binding and downstream signaling effects (Giger et al.1998; Chen et al., 1997). These Sema3 stimulated effects have been shown to be 1) beneficial in the developing nervous system with respect to regulation of immature neuronal populations and axonal guidance, 2) beneficial with respect to axonal fasciculation and guidance in the PNS post injury, 3) beneficial in the mature CNS to prevent spontaneous and unregulated neuronal plasticity, and 4) deleterious to the prospects of neuronal regeneration in the mature CNS after injury.

Only NRP-1, however, has been found as a receptor for angiogenic VEGF molecules, of which VEGF165 expression has been shown to enhance the potential for brain tissue repair post insult (Miao et al. 1999; Hayashi et al., 1998). This apparent paradox of beneficial and detrimental outcomes of the stimulated NRP receptor complex has made this area appealing to current research ventures.

5.2.3 Sema3A Contributes to Failure of Neuronal Regeneration in the CNS

The results presented in this thesis suggest a significant role for NRP-1/Sema3A signaling as inhibitory to CNS neuronal regeneration. However, results from research using PNS injury models describe a different role for NRP-1/Sema3A signaling. In the CNS, after ischemia or injury, Sema3A is up-regulated and along with other chemorepulsive ligands such as myelin associated inhibitors and proteoglycans, contribute significantly to the regenerative failure of neurons in the CNS (deWinter et al., 2002). Further, evidence provided in this thesis, as well as that from others show that activation of the NRP-1/Sema3A pathway by exposure to high concentrations of Sema3A can induce cell death in PNS and CNS neurons (Shirvan et al., 2000; Jiang et al., 2007). What is interesting to note, is the correlation between these observations and those observed in the PNS after injury. The PNS has long been known to have regenerative potential, and many studies have been conducted to determine the differences in expression profiles of various molecules within each system. In contrast to the CNS, NRP-1/Plexin positive neurons are able to regenerate after injury. One difference between CNS and PNS post-injury environments is that in the

PNS, the injured NRP-1 positive neurons do not encounter a Sema3A positive neural scar. Instead, it has been found that Sema3A mRNA is actually down-regulated post-injury in the PNS (Pasterkamp et al., 1998), and this could be one reason for the regenerative potential of neurons in a PNS environment.

5.2.4 VEGF Competes with Sema3A for the NRP-1 Receptor

NRP levels were shown in this thesis to be up-regulated in CNS neurons post-ischemia *in vitro* and *in vivo*. The purpose of this increase in receptor availability is not clear and confounded by the fact the NRP-1 has two ligands which appear to compete for its binding. Although a major focus of Class 3 Semas within the neural scar is their up-regulation and contribution to growth inhibition and cell death, VEGF, which is also a ligand of NRPs is also up-regulated after an injury or insult in the CNS (Skold et al., 2000; Bartholdi et al., 1997). Several conflicting results have emerged describing the role of VEGF and Sema3A as ligands for a shared receptor. One group has described results of their studies with post-ischemic rat brains that had been pre-treated *in vivo*, with VEGF (Hayashi et al., 1998). Their results demonstrated that increasing VEGF levels in the brain resulted in decreased infarct volumes post-ischemia (Hayashi et al., 1998). Angiogenesis is known to be induced soon after ischemia in the CNS, and it is conceivable then that the growth of new vessels is dependent upon the balance of VEGF and Class 3 Semas around the site of injury (deWinter et al., 2002). Other groups, however, have shown that VEGF positively influences survival, migration and proliferation of meningeal fibroblasts and epithelial cells, which are known to be both physical and biochemical barriers to CNS

neural regeneration (Miao et al., 1999; Bagnard et al., 2001). Thus, the increase in NRP-1 expression and activation of the Sema3A observed *in vivo* could also be influenced by levels of VEGF. Determining whether a benefit exists post-ischemia in tipping the balance towards VEGF signaling at the NRP-1 receptor instead of Sema3A will likely be investigated in the future.

5.2.5 The Relationship of NRP-1 Expression to Sema3A Binding

Another problem in context of our proposed model (Fig. 13) now becomes to untangle the concept of *activation* of the NRP-1/Sema3A pathway from *over-expression* of the NRP-1 receptor post-ischemia. Conceivably, up-regulation of E2F1 post -ischemia leads to the induction of NRP-1 expression, which coincides with increased expression of Sema3A mRNA (Fujita et al., 2001) as stimulated by the insult. It is interesting that the addition of Sema3A, an NRP-1 ligand (as sole insult) is sufficient to induce cell death. This suggests that the basal levels of NRP-1 expressed in the uninjured neuron are sufficient to engage enough Sema3A ligand to program cell death response. Thus, the increase in NRP receptor expression by E2F1 post ischemia, is not necessary to induce cell death, and leads us to question why this occurs at all. Interestingly, there is a discrepancy in the literature with respect to whether increased binding to NRP receptors by different subclasses of Sema3 is due to an increased affinity or due to increased expression and availability of receptors (Takahashi et al., 1999; Rohm et al., 2000). Using COS7 cells (immortalized monkey kidney fibroblast cells) to express NRP-1 and/or Plexin-A1, Takahasi et al., (2000) found an increase in the number of Sema3A binding sites when NRP-1 and Plexin A1 were

expressed together compared to NRP-1 expressed alone. Rohm et al., (2000) found instead that there is not an increase in binding sites but instead an increased affinity of Sema3A to an NRP-1/Plexin-A1 complex. Taking this into consideration, it is then possible that the same debate could exist with respect to the induction of the NRP-1/Sema3A pathway post-ischemia. Two possibilities are conceivable: 1) that there is an E2F1 regulated increase in NRP-1 expression, which increases receptor availability to accommodate the increase in Sema3A production and/or 2) that the increase in Sema3A secretion due to insult clusters more receptors and increases the affinity of the receptor complex for its ligand. These concepts may not be mutually exclusive, and could conceivably occur simultaneously in the context of cerebral ischemia. Further, the role of Plexins, and how they are regulated to form the functional NRP receptors in the context of cerebral ischemia should be examined. Given that the NRP pathways are known to be necessary guidance cues during development and during repair processes in the adult PNS (de Winter et al., 2002; de Wit et al., 2003), and developing CNS (de Winter et al., 2002; de Wit et al., 2003), a completely different model is also conceivable: that NRPs are up-regulated in anticipation of repair, re-growth or possibly mitosis - which of course fails in mature neurons. As seen in the OGD studies, at 24 h post-OGD, expression of NRPs significantly decreases, perhaps as the cells are committed to death processes. Thus, activation of the NRP pathway may be an *unintentional* side-effect of a failed attempt at cellular repair, and anticipation of a guidance role preserved from development.

5.2.6 Signal Transduction Between Axons and Cell Bodies

Our working model (Fig. 13) describes two death stimuli which occur in the neuron after ischemic insult. Firstly, increased free E2F1 leads to an increase in the expression of known pro-apoptotic molecules, such as caspases. Secondly, E2F1 induces the increased expression of NRP-1, which in turn, makes more binding sites available for Sema3A. NRP-1 expression on the neuronal membrane is mainly confined to the processes yet some researchers show low levels of NRP-1 expression on cell bodies as well (Kolodkin et al., 1997; He and Tessier-Levigne, 1997; Agudo et al., 2005). Basal levels of NRP-1 expression are sufficient to respond by axonal retraction and cell death as shown in Chapter 4 of this thesis. Thus, the NRP-1/Sema3A pathway could theoretically contribute to post-ischemic neuronal death.

The contribution of axonal stimulation as mediating effects in the cell body is incompletely understood. One strategy that neurons have to modulate their responses to stimuli is to modulate the expression of guidance receptor along the axon shaft and growth cone tip (Campbell and Holt, 2001). Research using ^{35}S-methionine to trace protein synthesis in whole neurons and presynaptic terminals (synaptosomes) reports that various mRNAs are stored in axons and may be translated locally upon stimulation to change the array of available axonal receptors (Crispino et al., 1993; Davis et al., 1992). It is not known whether NRP-1 is regulated in this way, yet Campbell and Holt (2001) have shown that Sema3A is able to induce rapid rises in the phosphorylation of translation initiation factor and protein synthesis in isolated retinal growth cones. Continuous Sema3A-induced axonal retraction was inhibited by protein synthesis inhibitors (Campbell and Holt, 2001),

indicating basal levels of certain protein components in the axon are not enough to maintain functional responses.

The results presented in Chapter 4 of this thesis demonstrate that the application of Sema3A as a sole insult to CNS neurons is able to induce cell death. This is a novel finding relevant to cerebral ischemia since previous experiments demonstrated Sema3A-induced cell death in DRGs of the PNS (Shirvan et al., 2000). In the context of axonal receptors, it would be interesting to determine whether or not application of Sema3A to axons alone is sufficient to induce cell death processes in the cell body. This could be accomplished by making use of a culture system known as Campenot chambers wherein axons are induced to extend beneath a barrier impermeable to organic solutes, ligands can be applied solely to axons (Campenot, 1982). If Sema3A application to axons were to induce cell death commitment, it would argue for molecular therapeutic targets which protect not only cell bodies, but also axons. Since NRP-1 is expressed in axons and has been shown in this thesis to be able to induce CNS neuronal death, it would make an excellent candidate for future research in this regard.

Further, the order of events that trigger axonal retraction and cell death has yet to be determined. We know that Sema3A as a sole insult can cause cell death and prevent axonal extension, as per results of Chapter 4. Yet in the context of the ischemic penumbra *in vivo*, it is not known whether E2F1 first relays death signals to the axon, which then increases expression of NRP-1 or whether axonal stimuli alone can mediate local up-regulation of the NRP-1 receptor and signal a commitment of neuronal death to the cell body. The fact that NRP-1 is specifically regulated by E2F1 post-ischemia (Jiang et al., 2007) suggests that the death signal occurs first in the cell body, which then leads to axonal retraction. However,

membrane proteins synthesized in the cell body are transported to the axons by slow axonal transport at a rate of 50-200nm per day (Campenot and Eng, 2000). Thus, temporally speaking, E2F1-induced increase in the expression of NRP-1 at the axons post-ischemia would likely not contribute to the cell death commitment. Yet it is also conceivable that stimuli to axons could induce retrograde death signals to the cell body and may mediate local axonal translation of stored mRNA to up-regulate NRP-1 expression in axons.

5.2.7 The State of Current Molecular Targets for Stroke Therapeutics

The biochemistry underlying the physiological deficits observed in acute stroke patients is fairly well understood and agreed upon. A review by Hou and MacManus (2002) describes that the core infarct area is rapidly depleted of viable neurons within the first 3 h of vessel occlusion through energy-independent necrotic processes. Areas more distal to the core, which have retained minimal collateral perfusion and ATP levels, increase commitment to the apoptotic pathway with time. Although the molecular pathways comprising the stage of post-commitment to neuronal death has been studied, it has been determined extremely difficult to target therapeutically because of the lack of therapeutic window. The peri-infarct tissue in the penumbra is known to have the longest delay before neuronal loss, and is thus an excellent opportunity to generate therapeutic targets (Hou and MacManus, 2002).

From 1996 onwards, a large number of drugs were developed that targeted neurotransmitter receptors, among other molecular pathways contributing to post-ischemic damage to the neuronal cell bodies (grey matter) (Dewar et al., 1999). Although a vast array

of mediators of the core apoptotic pathways, reactive oxygen pathways and neurotransmitter excitotoxicity are well elucidated within the penumbra and remain potential therapeutic targets, none have been successful past phase 3 clinical trials (Dewar et al., 1999).

The reasons for the lack of success of neuroprotective drugs include dose limiting side effects, difficulty with drug delivery across the blood brain barrier, different responses to drugs between different animal models, use of single drug therapy, and lack of focus on axonal integrity and preservation. Notably, rodents have been used largely for a convenient MCAO model of stroke, but it is argued by Dewar et al., (1999) that the use of larger animals with gyrencephalic brains more comparable to human brains may better indicate the potential benefit of the drug in humans. Further, Dewar et al., (1999) make the interesting suggestion that the failure of these neuroprotective agents in cerebral ischemia may also be due to the lack of focus on molecular events at axon. Experimental animal models such as rodents have less white matter (axonal) involvement than do humans post ischemia. NMDA receptor antagonists, for example, are targeted to receptors which are not present on axons or on the oligodendrocytes that myelinate them. In stroke patients, the goal is to enhance functional recovery, which has largely been shown to occur through axonal plasticity. Thus, preservation of neuronal cell bodies, with a failure to protect their axons from the effects of ischemia would not be a worthwhile endeavor. For example, the NMDA inhibitor, MK-801, failed to protect oligodendrocytes and myelinated axon tracts from ischemic damage and failed efficacy trials in humans (Irving et al., 1997; Yam et al., 1999). This is of particular interest to this thesis, since NRP receptors are expressed at the highest levels in axons and to a lesser extent on the membrane of the soma (Kolodkin et al.,

1997; He and Tessier-Levigne, 1997; Agudo et al., 2005). Functional NRP-1 expression has also been found on oligodendrocytes, which in the presence of Sema3A, showed dramatic decrease in process extension (Ricard et al., 2001). The results of this work show that inhibiting the NRP-1/Sema3A signaling pathway may prevent both neuronal death and inhibition of axonal outgrowth. Cell death and axonal length assays were performed in 95% pure neuronal cultures, and inhibition of this pathway in the brain would be of interest to future studies. These results contribute NRP-1/Sema3A pathways not only to the pool of possible molecular targets for cerebral ischemia, but also provide a common mechanism for the protection of both neuronal soma and axons.

5.2.8 Future Work

An important experiment which needs to be performed in the future is the determination of Sema3A expression after ischemic insult both *in vivo* and *in vitro*. For example, all of the experiments in this section that involve OGD are based on the assumption that this ischemic insult, among other effects, has the effect of inducing Sema3A expression and secretion in neurons. This assumption of autocrine signaling is the basis for which we conclude that the mechanism of protection from ischemic insults is via NRP-1 inhibition. The lack of adequate antibodies to detect the secreted Sema3A protein has hindered this progress so far.

Future work in general should also be aimed at delineating the importance of the up-regulation of the NRP receptors and whether modulation of this pathway at this level, or at the level of downstream effectors which are common to other death pathways as well,

could lead to protection, and encouragement of post-ischemic regeneration/repair in the brain. Awaited are the conclusive results from experiments in which MCAO was performed with NRP-2 -/- mice compared to wild type mice, to determine if there is protection from *in vivo* ischemic insult. Preliminary data (unpublished) from Dr. Laurie Karchewski in our laboratory have failed to show a significant difference, further demonstrating that NRP-2 may not be a significant determinant of post-ischemic outcomes.

What remains uncertain at this point is whether the up-regulation of NRPs is a consequence of an already progressing death pathway within the neuronal cell body as mediated by E2F1, or whether the shortening of axons as induced by NRPs is a preliminary response such that cell death may be arrested at this point, if intervention were to occur. Therefore, the temporal relationship of axonal shortening as induced by Sema3A, and the commitment to cell death still need to be delineated and future work should examine this question. Although much work remains to be done, it is clear that a detailed understanding of the molecular mechanisms underlying the damage induced by cerebral ischemia is essential for targeting this disease therapeutically. Based on the potential success of the NRP-1/Sema3A pathway inhibitor *in vitro*, it would be of interest to use this *in vivo*, and determine whether cortical infarct volumes could be reduced post-MCAO.

A popular trend in medicine today is the use of biological molecules, such as peptide inhibitors and humanized chimeric antibodies to inactivate ligands or enzymes whose activity within the context of illness is detrimental. An example is the use of anti-tumor necrosis factor (TNF) antibodies in the treatment of autoimmune inflammatory conditions such as rheumatoid arthritis and psoriasis. Conceivably, a similar mechanism of

Sema3A inhibition could be utilized, although future experiment will need to address the mechanism of delivery to the brain in human patients.

We suggest that further elucidation of biological mechanisms that will lead to strategies capable of blocking this detrimental signal transduction pathway may have potential therapeutic use. Research in this field is ongoing and with the results presented in this thesis which prove the regulation of NRP-1 by E2F1 and suggest the involvement of the NRP-1/Sema3 pathway in the detrimental effects of cerebral ischemia, we hope to spur further studies in the direction of stroke therapeutics.

CONTRIBUTIONS OF COLLABORATORS

The following people have contributed significantly to this thesis:

Jaqueline Slinn performed all mouse surgery for MCAO.

Angele Desbois performed the primary cell culture of CGNs and glia, animal breeding, genotyping and maintenance, expression of NRP-2 protein in HEK-293 cells and protein purification for positive control for westerns (Fig. 7B).

Dr. Susan X. Jiang performed all RT-PCR of NRP-1 (Fig. 3A, 3B), collaborated with the EMSA to establish a reliable protocol, quantification of EMSA in Figure 6B. All p-values were determined by Dr. Jiang's calculations. Figure 9 was a contribution from Dr. Jiang.

Dr. Sheng T. Hou generated the diagram used Figure 13.

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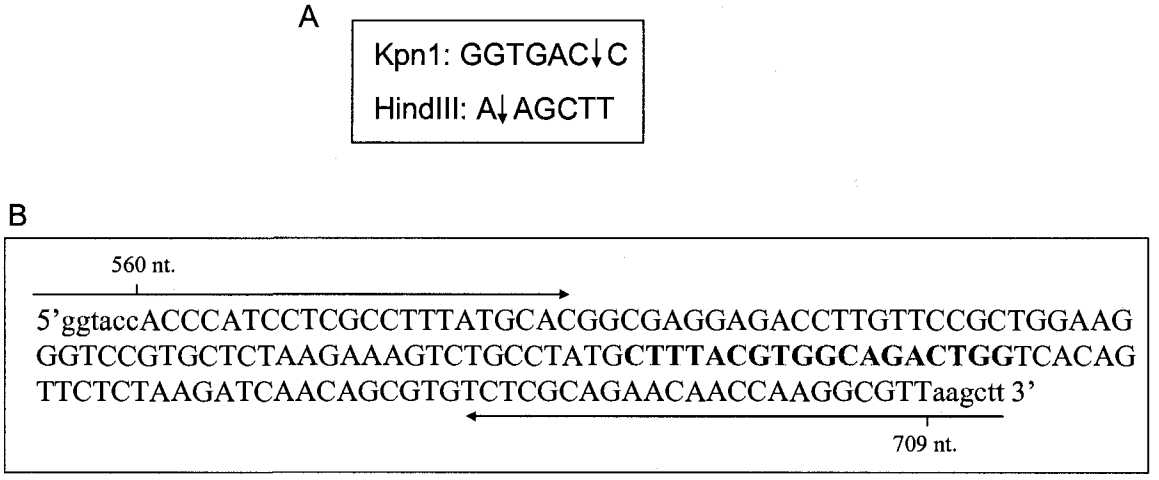
APPENDIX A

```
CLUSTAL W (1.83) multiple sequence alignment
Neuropilin-1 promoter sequence (AF482432) + E2F1 binding sequence (J.
Farnham)

neuropilin      ATATGTACTTGATCAGAAGCCTCAACCAGTTTATGTCCCTGGGTGTCCTTGCTATTCATA
60
E2F1            ------------------------------------------------------------

neuropilin      AACCAAAGCCACCTGCTGCAGTTCTCGGAAAGCCCTCGAGGATGGAACCGGACTACATGG
120
E2F1            ------------------------------------------------------------

neuropilin      GGGGAGGGGAGGCACTGGGCAGACACCAGGGACGCACCTCGTAAGTGGGTGGCGTATGAG
180
E2F1            ------------------------------------------------------------

neuropilin      GCTGGAGGATCAGTAGACACAATTGCCTGTAATCTAAACCTGGCACTAGGAGACCCTTGC
240
E2F1            ------------------------------------------------------------

neuropilin      AAAGATCATCCTGTCACCCTCACAAGGGGTCGTTGCCAGTCTTATCCTTCCCTCGGTGTG
300
E2F1            ------------------------------------------------------------

neuropilin      CACAGAAATGGGTCCCTCGACCCCTGATTCAGATTCTTGGAGTTCTACGTGGCTGGTCTG
360
E2F1            ------------------------------------------------------------

neuropilin      CGACTGTTGGCGGCTGCCCCCTAGCTGAAAGGCGCGCCACCATCCATCCTCCGAGCTCAT
420
E2F1            ------------------------------------------------------------

neuropilin      GTAACCCCAAAGAAGAGGGGACCAAATCTCGGGGAACAATGCAGCCTGAAGCGTCAGGGG
480
E2F1            ------------------------------------------------------------

neuropilin      TGGGGATGGGGGTATCAGGTACCCATCCTCGCCTTTATGCACGGCGAGGAGACCTTGTTC
540
E2F1            ------------------------------------------------------------

neuropilin      CGCTGCAGCCAGGGTCCGTGCTCTAAGAAAGTCTGCCTATGCTTTACGTGGCAGACTGGG
600
E2F1            -------------------------------------------CTTTCGCGGCAAAAAGGG
18
                                                           ** ** **** *  ***

neuropilin      TCACAGTTCTCTAAGATCAACAGCGTGTCTCGCAGAACAACCAAGGCGTTCTTCTTTTAG
660
```

```
E2F1            ------------------------------------------------------------

neuropilin      TCTAAGTCCTACTCGGAGCGTCCTGGAGCGCCCGACTGCTTCCTGCCCCAAACCTCAGGC
720
E2F1            ------------------------------------------------------------

neuropilin      TGACACCCAGGAAGTGAAAGCGGGGACTGACAGCGCGATCCACGCCTCCGGGGGCGCCGG
780
E2F1            ------------------------------------------------------------

neuropilin      TACCTAGGGATTAGGAGTTCCGTGAAGTAGCAGCTAACCCCGGTCTCTCGGAACTCCTTG
840
E2F1            ------------------------------------------------------------

neuropilin      ACTCCAGGATCCTCGCCTACACGAGCAGCCTAGTTCAGTTCTGGGGTGCAGAGATCTCGT
900
E2F1            ------------------------------------------------------------

neuropilin      CAAGGTAGACTAGCCTGCCACTGGGGCTGGTACCACTTGTACTGCCCCTTCTGACACACA
960
E2F1            ------------------------------------------------------------

neuropilin      TACGTGACCTTGCGCTCTGGGCGCAGACAACCTGCTCCCTAGTGACCTTCTTCCCGCGGA
1020
E2F1            ------------------------------------------------------------

neuropilin      GAGCACAGCAGGGTACCCGGAGGTGGGGTGGAGGGGGGAGCCCAGGGGACCAACCGGGAA
1080
E2F1            ------------------------------------------------------------

neuropilin      AGCACGCGGAGAGAAACTTGTGTCTCTGAATCTCCAGCCAGGCAATCACTGGCAGAGCTG
1140
E2F1            ------------------------------------------------------------

neuropilin      TGCGGGGAGGGGGCGGGAGGAAGGGAGGGAAGGGCTCTGGAAGGGAGGGGGAGGTCGGAG
1200
E2F1            ------------------------------------------------------------

neuropilin      AGAGAGGCGGGTGCCAGTGGAGAAAGTCCTTGCTCTCCCGCTCATCTTTTCATTGCTCTC
1260
E2F1            ------------------------------------------------------------

neuropilin      TCTCCTTCCCGCAGACAACCCGGACCTCCTCTGGGCGCCAGCTCCTCGGCTCCAACCCGT
1320
E2F1            ------------------------------------------------------------

neuropilin      CCAGAATCAAGCGGGATTTTTTTTTTTCTTTCCCTCTAGAAATTGGCTTTGGTGTTGTCG
1380
E2F1            ------------------------------------------------------------
```

```
neuropilin      CCCGCCCTCTCCCCTCCTCCTCGCAACTCCTCCCCTCTTTTTTTTCCTCCTTCTTCTTCT
1440
E2F1            ------------------------------------------------------------

neuropilin      TCCTGAGACATGGCCCGGGCAGTGGCTCCTGGAAGAGGAACAAGTGTGGGAAAAGGGAGA
1500
E2F1            ------------------------------------------------------------

neuropilin      GGAAATCGGAGCTAAATGACAGGATGCAGGCGACTTGAGACACAAAAAGAGAAGCGCTTC
1560
E2F1            ------------------------------------------------------------

neuropilin      TCGCGAATTCAGGCATTGCCTCGCCGCTAGCCTTCCCCGCCAAGACCCGCTGAGGATTTT
1620
E2F1            ------------------------------------------------------------

neuropilin      ATGGTTCTTAGGCGGACTTAAGAGCGTTTCGGATTGTTAAGATTATCGTTTGCTGGTTTT
1680
E2F1            ------------------------------------------------------------

neuropilin      TCGTCCGCGCAATCGTGTTCTCCTGCGGCTGCCTGGGGACTGGCTTGGCGAAGGAGGATG
1740
E2F1            ------------------------------------------------------------

neuropilin      GAGAGGGGGCTGCCGTTGCTGTGCGCCACGCTCGCCCTTGCCCTCGCCCTGGCGGGCGCT
1800
E2F1            ------------------------------------------------------------

neuropilin      TTCCGCAGCG 1810
E2F1            ----------
```

APPENDIX B



Appendix B. Fig 1.Cloning strategies for Luciferase Assay. (A) Restriction sites Kpn1 and HindIII were used to cut the PGL3 Basic Vector and insert cloned sequences. These restriction sites were added onto the 5' and 3' ends of the primer sequences respectively. (B) Cloning of a 150 bp region of the NRP-1 promoter encompassing an 18 bp region (blue letters) of similarity to the known core E2F1 binding region (642 nt. – 658 nt.), as found by CLUSTAL W alignment. Arrows indicate primer sequences used, with red letters designating the nucleotide additions designed to encode for Kpn I and Hind III restriction site for sticky end ligation into the PGL3 Basic Vector.

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EDUCATION:

2006-present	Current medical doctorate (M.D) student – Queen's University (Kingston)
2004-present	Current 3rd year M.Sc. student in Biochemistry –University of Ottawa
2000-2004	B.Sc. Honours (Magna Cum Laude) Biochemistry -University of Ottawa
1995-2000	High School Diploma (Honours -John McCrae Secondary School, Ottawa

INTERPERSONAL SKILLS

- Resourceful, motivated, dedicated, responsible and autonomous
- Genuine care and empathy for people; strong desire to work in medical field
- Strong oral and written communication skills
- Able to adapt to new environments, cultures, expectations, and to learn quickly
- Team player, able to develop trust and closeness in a team environment

RESEARCH

September 2004-2006: Masters Research Project (Biochemistry)
Co-supervised by Dr. Sheng Hou, NRC and Dr. Steffany Bennett, BMI

- ◊ Focused on learning the importance of Neuropilin/Semaphorin signalling in post-ischemic neuronal death and axonal damage *in vitro* and *in vivo*
- ◊ Used various methods (such as western blotting, immunostaining, primary neuronal culture, middle cerebral artery occlusion in mouse, oxygen-glucose deprivation in cerebral neurons, adenoviral gene replacement *in vitro*, immunoprecipitation, electrophoretic mobility shift analysis, axonal length measurements, behavioural studies, cloning, reporter assays) to determine that Semaphorin 3A plays a role in post ischemic neuronal death and damage, that neuropilins are upregulated after ischemic insult *in vivo* and *in vitro*, that the death-mediating transcription factor E2F1 can upregulate neuropilin transcription, and that cells from mice deficient in expression of neuropilin or E2F1 show protection from post-ischemic damage.
- ◊ Independent research proposal design, committee meeting presentations, conference presentations, problem solving, technical practice, critical understanding of current literature and relevance to project, courses and seminars, meetings, completed to date for degree.

September 2003-May 2004: Honours Research Project (virology)
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- ◊ Demonstrated the importance of presence of sialic acid on Enterovirus 70 receptor for effective cellular binding using techniques such as cell culture/sterile techniques, Enterovirus 70/ infections of clonal cell lines, virus binding assays, flow cytometry, intracellular FACS, viral entry and protein synthesis studies
- ◊ Critical data analysis and results summary, thesis writing, and seminar and poster presentations.

ABSTRACTS and PUBLICATIONS

1. March 2004→ Abstract and Poster Presentation, Keystone Neuroscience Symposium, Colorado USA:

"Neuropilin-1 is a target for up-regulation by transcription factor E2F1"

2. March 2007 →publication shared 1st author:

Neuropilin-1 Is a Direct Target of the Transcription Factor E2F1 during Cerebral Ischemia-Induced Neuronal Death *In Vivo*

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Dr. Steffany Bennett

Summer 2004: National Research Council – Institute of Biological Sciences
Dr. Sheng Hou (Neuroscience)

- ◊ Pre-training and planning for M.Sc. thesis

Summer 2003: Ottawa Health Research Institute
Dr. Johnny Ngsee (Neuroscience)

- ◊ Techniques include: sample preparation.SDS-PAGE, western blotting, co-IP, agarose gel electrophoresis, cloning, intro to site-directed mutagenesis, tissue culture, confocal microscopy.

Summer 2002: Lab Assistant
University of Ottawa -Dr. Harvey Kaplan (protein chemistry)

- ◊ Aid to two PhD students
- ◊ Sample preparation, SDS-PAGE + troubleshooting, agarose gel electrophoresis, novel i*n vacuo* methylation of proteins, vacuum sample preparation.

Summer 2001:Administrative Assistant
Coca-Cola Bottling Ltd (Ottawa)

- ◊ Use of strong administrative, computer skills and creativity to complete projects relating to sales and marketing of Coca-Cola products
- ◊ Using Microsoft Office Applications (Word, Excel and PowerPoint) as well as performing customer service, and basic accounting procedures.

1997 – 2001: Part-Time Medical Receptionist for Family Practice Walk-In Clinic
Dr. Nigel Spencer and Dr. Sandeep Nagpal (Ottawa)

- ◊ Greeted and scheduled patients appointments
- ◊ Entered medical information (electronic database - MD-5000)
- ◊ Created new patient files (recorded confidential information)
- ◊ Filed patient charts, filled out requisition forms
- ◊ Called-in prescriptions and answered telephone
- ◊ Learned basic medical techniques

Summers 1999 and 2000: various positions within restaurant industry (Ottawa)

- ◊ Nickels Restaurant (Waitress), Broadway Bar and Grill (Hostess), Outback Steakhouse (Bus Person)

VOLUNTEER EXPERIENCE:

April 2004-July 2004 → Health and Medical Volunteer in Guatemala

- ◊ Physiotherapy sssistant for young children with developmental disabilities in Hospital Hermano Pedro

May-June 2004 → Home-building within Villages in Guatemala

- ◊ Physical labour

June 2004 → Shadowed Physician in Rural Guatemalan Town

- ◊ Observed and participated in patient examinations, Spanish/English oral translation, dispensed medications and explained indications

2004 – present→ Recreational Therapy for Patients at the Ottawa Hospital

- ◊ Help patients participate in activities such as arts and crafts, music, story telling, baking and serving meals.

2003-2004 → Bio-X Club

- ◊ Oral presentations to students in the life sciences at the University of Ottawa to inform them of job availability, career and education options and to organize information and social evenings, as well as tours of labs.

2002-2006 → Pathmakers Science and Engineering Role Model Program

- ◊ Oral presentations to elementary school children with the purpose of educating particularly studentes about the benefits of pursuing careers in science and engineering.

2002→ Joints in Motion - The Arthritis Society

- ◊ Raised over $600 for the Arthritis Society through personally designed web-site, advertising and community events such as pub nights and BBQ's and media awareness.

AWARDS AND ACHIEVEMENT:

2006	Acceptance to Queen's Medical School M.D. Program
2006	1st place award at MSc. Student Poster Day (University of Ottawa)
2004-2006	Heart and Stroke Masters Studentship Award ($18,000 x 2yrs)
2004-2006	Post-graduate Award of Excellence (tuition), University of Ottawa
2004	Post-graduate Travel Award ($1,500)
2004	Certified formal Spanish advanced training- Zamora Academia,Guatemala
2004	Golden Key Honours Society (top 15% of class)
2002-2004	Award for Academic Achievement ($500/yr)
2001-2004	University of Ottawa Dean's List
2000	University of Ottawa Admission Scholarship ($2,000)
2000	Ontario "Aiming for the Top Tuition Scholarship" ($100)
2000	Black Belt in Karate (Greco Martial Arts Academy)
1995-2000	Honours Achievement
1995-1998	Member of award-winning Confederation High School (Ottawa) "*Jazz Connection Stage Band*" (saxophone player)
1995-1998	Gold Standard at National and Regional Music Festival Competitions
1995-1996	High School "Best of Class" Musical Performance Award

PERSONAL INTERESTS:

- Latin dancing (advanced training, teaching beginners classes)
- Spanish language training (ongoing with private teacher)
- Drumset, tabla, saxophone, guitar, piano
- Martial arts

** References will be provided upon request**