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ABSTRACT
Perinatal hypoxic ischemic (HI) injury is a leading cause of long-term neurological complications in newborn babies. Matrix metalloproteinases (MMPs) are a family of endopeptidases that are capable of degrading the extracellular matrix (ECM) components. They are considered to be integral in many physiological processes. However, recently it has been demonstrated that the inappropriate activity of these proteases, particularly MMP-2 and 9, contribute to the pathogenesis of cerebral ischemia in the adult brain. Given that ECM disruption is frequently observed following injury to the developing brain, it is possible that MMPs play an important role in HI injury processes in the developing brain. Therefore, this thesis evaluated the hypothesis that MMP-2 and 9 participate in the pathophysiology of HI injury to the developing brain. Since ECM remodelling is a fundamental process during brain development it was important to first characterise the MMP-2 and 9 profiles in the normal developing forebrain. We demonstrated that MMP-2, which mainly was observed in cortical plate neurons, declined with age, thus indicating a potential role in the development and differentiation of the cortical plate. Conversely, MMP-9 was increased with age, particularly during active myelination, indicating that it may contribute in myelination. Secondly, we showed an upregulation of MMP-9 within the ischemic core during the early hours following HI injury, suggesting that MMP-9 may be involved in the development of delayed injury processes following hypoxic ischemia. On the contrary, MMP-2 was strongly upregulated during a later stage following injury surrounding the ischemic core possibly suggesting that it plays a role in wound repair processes. Thirdly, the profiles of tissue (tPA) and urokinase (uPA) plasminogen activators were characterised following HI injury since they are known to be major upstream activators of MMPs. uPA upregulation paralleled that of MMP-2 suggesting a function for uPA in wound repair processes following HI injury to the developing brain through activation of MMP-2. In contrast with uPA, tPA activity remained unaffected following injury at both ages. Finally, MMP-9 activity was inhibited using a very specific MMP-2/9 inhibitor, SB-3CT, to determine if the MMP-9 deficiency protects the developing brain from HI
injury. The elevated MMP-9 activity following HI injury was attenuated by the SB-3CT treatment. Although SB-3CT failed to confer any significant neuroprotection, we recommend that further investigations are needed before discounting the role of MMP-9 during HI injury to the developing brain. In conclusion, we suggest that MMP-9 is induced following an insult to the developing brain potentially contributing to the delayed neuronal death whilst MMP-2 is involved in essential developmental, differentiation and wound repair processes.
ACKNOWLEDGEMENTS

Completing this thesis has undoubtedly been an extraordinary journey. Throughout the past years I have learnt not only an abundance of scientific skills, but remarkable life skills too. It has not been an easy journey and it certainly would not have been possible without the invaluable support of many people.

I am greatly indebted to my current primary supervisor Dr Mhoyra Fraser, who went out of her way to ensure that I finish this thesis to a high standard. Thank you very much for lending a helping hand at the most difficult of times. I will always appreciate your guidance, support and interest in my research and all other aspects of my PhD. I am equally grateful to, Dr Arjan Scheepens, my former primary supervisor. I am deeply gratified to you for your invaluable contribution in designing this research project, bringing me up to speed in the lab and giving me constant encouragement throughout my PhD. I will fondly remember the great instances we shared as a group. I would also like to sincerely thank my former co-supervisor, A/Professor Chris Williams, for his scholarly advice in every aspect of this thesis from designing to writing.

I would also like to express my earnest gratitude to many people who were indirectly involved in making this thesis possible. To my advisor, Dr Hannah Gibbons, for being an extraordinary mentor as well as proof reading my thesis amongst her busy schedule. To Mr Ernest Sirimanne without whose magnanimous support in animal surgeries it would not have been possible to complete this research the way I envisaged. To Mr Eric Thorstensen for his generous help with the mass spectrometry experiments. To Dr Sam Mathai for his assistance with CSF collection. And, to Dr Mohan Kumar for teaching me numerous laboratory techniques amidst his own PhD research.

I would like to express my heartfelt gratitude to the fellow PhD students of the Perinatal Brain Injury Group. I am extremely grateful to Mrs Larissa Christophidis for providing me the cDNA that I needed to complete the studies, on top of innumerable other favours
she has done for me. Your input has greatly improved the quality of this PhD thesis. Above all, thank you for being an awesome friend, who I could always count on. Thank you, Ms Praneeti Pathipati for your assistance with countless tasks throughout the course of my PhD. Thank you for being there, as a great friend, to share some of the strenuous career experiences. Thank you, Dr Tanja Needham (nee Mödersheim) for giving us hope that there is light at the end of the tunnel. I will always cherish the wonderful moments we all had together. I would also like to acknowledge everyone in the Developmental Neuroendocrinology Research Group.

I would like to very warmly thank my good friend, Ms Vinthiya Paramanathasivam, although thank you would certainly be an understatement for her support during the past years from proof reading various reports to hearing my PhD horror stories almost all of the times we met. Also a gigantic “thank you” to, Dilini Hennayake, Kushil Ginige, Movin Kulathilaka, Nirosha Weerasinghe, Nishan Alahakoon and Ruchira Ginige for putting up with my PhD syndrome while helping in whichever way possible. And a very special “Winnie the Pooh Hug” to Baby Yeshika for being my biggest stress reliever through the last years without having the fainest idea of what I do. Above all, I love you all for making life outside PhD absolutely beautiful.

Last but certainly not the least, a very special mention to Mum, Dad and Bro. The warmth that you have given me throughout is beyond any measure that I know of. You truly are the inspiration of my strength. I love you.
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LIST OF ABBREVIATIONS

ADAM – A disintegrin and metalloproteinase family
AMPA - α-Amino-3-hydroxy-5-Methyl-4-Propionate
ANOVA – Analysis of variance
APS - Ammonium persulfate
BBB – Blood brain barrier
BCA – Bicinchoninic acid
BSA – Bovine serum albumin
BW – Body weight
CC – Corpus callosum
CNS – Central nervous system
CSF - Cerebrospinal fluid
DEPC – Diethylpyrocarbonate
DMSO - Dimethyl sulfoxide
cDNA – Complementary deoxy ribonucleic acid
DG – Dentate gyrus
DPX - Dibutyl-phthalate-xylene
ECM – Extracellular matrix
EDTA - Ethylene diamine tetra acetic acid
GAPDH - Glyceraldehyde-3-phosphate dehydrogenase
GAP-43 – Growth associated protein – 43
GFAP – Glial fibrillary acidic protein
HCl - Hydrochloric acid
HI - Hypoxic ischemic
ICV - Intracerebro ventricular
IP – Intraperitoneal
KPBS - Potassium phosphate buffered saline
M - Molar
MAP-2 – Microtubule associated protein -2
MCAO – Middle cerebral artery occlusion
MMP – Matrix metalloproteinase
mRNA - Messenger ribonucleic acid
NO - Nitric oxide
NOS - Nitric oxide synthase
NMDA - N-methyl-d-aspartate
NeuN - Neuronal specific nuclear protein
dNTP - Deoxyribonucleotide triphosphate
OD – Optical Density
PA – Plasminogen activator
tPA – Tissue plasminogen activator
uPA – Urokinase plasminogen activator
Postnatal Day 7 – P7
Postnatal Day 2 – P3
Postnatal Day 21 – P21
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PVL - Periventricular leukomalacia
ROS - Reactive oxygen species
RT - Room temperature
qRT-PCR – quantitative real time polymerase chain reaction
SDS – Sodium dodecyl sulphate
SEM – Standard error of the mean
SOD - Superoxide dismutase
SNN – Selective neuronal necrosis
TEMED - N,N,N,N-tetramethylethylenediamine
TIMP – Tissues inhibitor of matrix metalloproteinase
CHAPTER ONE: GENERAL INTRODUCTION

1.1 INTRODUCTION

Perinatal hypoxic ischemic (HI) injury is an important cause of long-term neurological complications (Volpe, 2001a, Low, 2004, Vannucci and Hagberg, 2004, Lawn et al., 2005, Rees et al., 2008). Statistically, HI brain injury occurs in two to three cases per 1000 full-term infants and nearly 60% of the pre-term infants in the developing world (Vannucci and Hagberg, 2004, Gunn and Bennet, 2008). Between 20-50% of the newborns that exhibit HI injury die within the immediate postnatal days. Approximately 25% of those who survive later develop neurodegenerative abnormalities that are recognised as they mature (Vannucci and Hagberg, 2004). These include cerebral palsy, mental retardation, learning disabilities, subtle motor abnormalities and vision or hearing impairments. Despite advances in perinatal care over the past three decades such neurological manifestations remain a major problem to date for which there is no specific treatment. (Takashima et al., 1995, Volpe, 1998, Inder and Volpe, 2000, Volpe, 2001a, Blumenthal, 2004, Ferriero, 2004, Vannucci and Hagberg, 2004, Rennie et al., 2007). A comprehensive knowledge of the pathophysiology of perinatal HI brain injury is essential in designing effective therapeutic interventions.

Following hypoxic ischemia neuronal death occurs in two distinct phases: acute and delayed phases. The acute neuronal death that occurs immediately following injury is associated with cellular hypoxia that leads to the cellular energy failure. Evidence suggests that cytotoxic edema, excitotoxicity, and oxidative stress are the major mechanisms of injury during the acute phase. The delayed phase, that commences from hours later and extends until days later, is triggered by a cascade of events that is initiated by the acute insult. Delayed neuronal death is associated with excitotoxicity, oxidative
stress and inflammation. It is now evident that the most significant amount of cell death occurs during the delayed phase rather than the acute phase (Inder and Volpe, 2000, Ferriero, 2004, Shalak and Perlman, 2004, Vannucci and Hagberg, 2004). Therefore, a better understanding of the cellular mechanisms associated with the delayed neuronal death is crucial in the development of novel therapeutic strategies for developing brain injury.

Disruption of the extracellular matrix (ECM) is a prominent characteristic of lesions that occur in the developing brain. Matrix metalloproteinases (MMPs) are a family of zinc dependant endopeptidases that are capable of degrading all components of the ECM. There are now more than 20 enzymes that are classified as matrix metalloproteinases (Nagase and Woessner, 1999, Stamenkovic, 2003). Recently, several lines of evidence have demonstrated that the inappropriate activity of MMPs, predominantly MMP-2 and MMP-9 contributes to the pathogenesis of cerebral ischemia in the adult brain. (Rosenberg et al., 1996, Romanic et al., 1998, Asahi et al., 2000, Asahi et al., 2001, Gasche et al., 2001, Planas et al., 2001, Rosenberg et al., 2001, Rivera et al., 2002, Lee et al., 2004). Given that cyst formation, disruption of developing white matter tracts and the presence of glial scars, where the ECM is disrupted, are prominent features of lesions to the developing brain (Meng et al., 1997, Volpe, 1998, Volpe, 2001b, Blumenthal, 2004, Sizonenko et al., 2005), it is possible that MMPs play an integral function in the delayed phase injury to the developing brain.

The following literature review aspires to provide a thorough understanding of the development of the mammalian central nervous system (CNS), HI injury in the
developing brain and importantly, MMP biology. The aim of our research project is to investigate the role of MMPs following hypoxic ischemic injury to the developing brain.

1.2 DEVELOPMENT OF CENTRAL NERVOUS SYSTEM

An understanding of the development of the nervous system is essential to our understanding of the pathophysiology of developing brain injury. Development of the mammalian brain can be divided into six major events; primary neurulation, prosencephalic development, neuronal proliferation, neuronal migration, organization, and myelination. Following is a brief review on the development of the human CNS.

1.2.1 Neurulation

The early embryo is a flat disk consisting of three distinct cell layers. The most inner cell layer is called the endoderm; the middle cell layer is called the mesoderm, while the most outer cell layer is the ectoderm. The endoderm gives rise to viscera that is the lining of all the internal organs. The mesoderm gives rise to the bones of the skeleton and the muscles. The ectoderm gives rise to the nervous system and skin (Bear et al., 2001). The nervous system is entirely derived from the dorsal ectoderm of the early embryo. Around the sixteenth day after gestation the neuroectoderm, termed as the neural plate, appears in the dorsal midline of the embryo (Figure 1.1). About two days later a groove, called the neural groove, appears in the neural plate running rostral to caudal. Alongside the neural groove are its walls, called the neural folds. By the end of the third week the neural folds subsequently move together and fuse dorsally, thus transforming the neural groove into the neural tube. Fusion of the neural folds proceeds rostrally and caudally, closing the openings at each end at about 24th and 26th days respectively. The entire CNS is derived from the walls of the neural tube. Neural crest cells that originate from the neuroectoderm
that is not incorporated into the neural tube produce the entire peripheral nervous system.

The process of forming the neural tube from the neural plate is called neurulation (Figure 1.1) (Garrod and Feldman, 1981, Smith and Schoenwolf, 1997, Bear et al., 2001, Volpe, 2001b).

Figure 1.1: Scanning electron micrographs of transverse sections through the chick embryo at the neuraxial levels of neurulation. Flat neural plat (a), progressive formation of the neural groove (b-d) and fusion of the neural folds (e) are shown. MHP = median hinge point DLHP = dorsolateral hinge point (Modified from Smith and Schoenwolf, 1997).

1.2.2 Prosencephalic Development

The rostral end of the neural tube differentiates greatly to develop into the brain while the caudal end develops into the spinal cord. Approximately a month after gestation, three primary brain vesicles appear at the rostral neural tube. The rostral-most vesicle is called the prosencephalon or the forebrain. Caudal to this is the mesencephalon or the midbrain.
The most caudally located vesicle is called the rhombencephalon or the hindbrain (Garrod and Feldman, 1981, Bear et al., 2001). During the fifth week the first and the third primary vesicles, especially the prosencephalon, further differentiate developing complex structures that later develop into the mature CNS. Development of the prosencephalon occurs in three sequential events; prosencephalic formation, prosencephalic cleavage, and midline prosencephalic development. Prosencephalic formation occurs at the end of first month and the beginning of second month of gestation. Prosencephalic cleavage, that included three basic cleavages, occurs during the fifth and sixth weeks of gestation. The prosencephalon is cleaved, a) horizontally to form the paired optical vesicles, olfactory bulbs, and tracts, b) transversely to separate the secondary vesicles telencephalon and diencephalons, and c) sagittally to form the paired cerebral hemispheres from the telencephalon. Commissural, chiasmatic and hypothalamic plates develop during the midline prosencephalic development that happens from late in the second month through the third month (Garrod and Feldman, 1981, Bear et al., 2001, Volpe, 2001b).

1.2.3 Neuronal Proliferation

The complex adult mammalian brain is entirely derived from a single neuroepithelial layer that spans the area between the inside ventricle and overlying pia. This neuroepithelium is called the ventricular zone because it lines the fluid filled ventricular space of the neural tube. It contains mitotically active radial glia cells extending processes across the width of the developing CNS that ultimately produce neurons, astrocytes and oligodentrocytes. These radial glia cells, first, extend their processes upwards towards the pia. Secondly, the nuclei of the cells follow their processes, thus migrating towards pia. When the nuclei touch the pial surface their DNA is copied.
Nuclei with two copies of DNA material migrate back to the ventricular surface. When the nuclei are in touch with the ventricular surface they retract their processes and divide producing two daughter cells (Garrod and Feldman, 1981, Bear et al., 2001, Volpe, 2001b, Brazel et al., 2003, Tramontin et al., 2003). Ventricular zone cells undergo two modes of divisions called vertical and horizontal division (Figure 1.2). Both of the vertically divided daughter cells remain in the ventricular zone to undergo more division (Figure 1.2b). This mode of division predominates early in development to expand the number of neural stem cells. Later in brain development horizontal division becomes the predominate mode. After horizontal division, only one daughter cell remains in the ventricular zone while the other migrates away to differentiate into mature cells (Figure 1.2c) (Bear et al., 2001). Evidence suggests that major proliferative events occur between second and fourth months of gestation, with the peak period of activity occurring in the third and fourth months (Garrod and Feldman, 1981, Bear et al., 2001, Volpe, 2001b, Brazel et al., 2003, Tramontin et al., 2003). Soon after neurogenesis begins in the ventricular zone, a second proliferate cell population appears adjacent to the ventricular zone between the ventricular zone and the differentiated cell population near the pia surface. During the later period of embryonic development this subventricular cell population rapidly expands with over 90% of cells proliferating. The subventricular zone is comprised of neuronal precursor cells that are capable of producing different types of cells that migrate away to differential areas of the developing brain. However, its cell population begins to decrease after the first postnatal week (Brazel et al., 2003).
1.2.4 Neuronal Migration

Neuronal migration refers to the process of newly formed neurons moving from their sites of origins at the ventricular zones to different loci where they reside permanently. The peak period of activity of neuronal migration occurs between third to fifth months of gestation. Initially, at approximately seven weeks of gestation a primitive group of cells migrates by translocation to form the preplate above the ventricular zone (Figure 1.3). These cells possess a long process that is attached to the pial surface and a short trailing process directed at the ventricular surface. They first extend their processes towards the pial surface. Then their bodies move along the extended processes to reach an appropriate
position in the preplate. Finally they gradually lose their pial and ventricular connections by withdrawing the processes. Later, approximately at ten weeks of gestation the preplate is divided into the superficial marginal zone nearest to the pial surface and the lower subplate nearest to the germinal zone by the sequential invasion of cortical plate neurons. This second wave of migratory cells predominately uses locomotion as their transport mechanism (Figure 1.3). They also possess a pial direct leading process but shorter and unattached compared to that of the earlier migratory cell. Experimental evidence suggests that these neurons migrate along the radial glial fibres that transverse the entire thickness from the ventricular zone to the pial surface. However, it is shown these cells use translocation at the end stage of their migration. It is also known that cortical plate neurons migrate in an inside–out sequence. The neurons that are developed early occupy the deepest layers next to the subplate layer while the most recently generated neurons migrate through them to the superficial layers below the marginal zone. By 20 to 24 weeks of gestation the cerebral cortex essentially has its full complement of neurons (Uylings, 2000, Nadarajah et al., 2001, Volpe, 2001b, Honda et al., 2003, Marin and Rubenstein, 2003, Kriegstein and Noctor, 2004).
Figure 1.3: Schematic illustration of the various modes of neuronal migration in the developing cerebral cortex. During early development the prevalent mode of radial migration is somal translocation (a). As development proceeds and the cortex thickens, the predominant mode of migration is glia-guided locomotion (b) (Adapted from Nadarajah et al, 2003).

1.2.5 Organization

Organizational events of the CNS are of particular importance because they establish the final circuitry of the mature brain that determines the various functions of the human body. The peak period of occurrence of such events is from approximately fifth month of gestation to several years of postnatal life. The major developmental features during this time include establishment and differentiation of the subplate neuronal layer, attainment of the proper alignment, orientation, and layering of cortical neurons, elaboration of dendritic and axonal ramification, establishment of synaptic contacts, selective elimination of cell, processors and synapses and glial proliferation and differentiation into astrocytes and oligodendrocytes (Volpe, 2001b).
1.2.6 Myelination

Myelination is the process where the plasma membrane of oligodendroglia is elaborated as the highly specific myelin membrane around the axons. Evidence suggests that in humans, the peak period of occurrence of myelination is from birth to several years postnatal. The oligodendrocyte progenitors that are originated in the ventricular zones migrate extensively similarly to neurons into the areas of future fibre tracts. During the second trimester of gestation these progenitor cells differentiate into preoligodendrocytes then into postmitotic immature oligodendrocytes. During the third trimester immature oligodendrocytes wrap around the axons in preparation for myelination, which in turn triggers them to differentiate into the mature oligodendrocytes. The myelination begins and continues until years after birth. The time point at which the mature myelin is reached varies within different brain structures (Back et al., 2001, Volpe, 2001b, Levitt, 2003).

1.2.7 Summary

In summary, the development of CNS comprises of six major events. The above table demonstrates the peak time of occurrence of each event in humans (Volpe, 2001b).

<table>
<thead>
<tr>
<th>Major Developmental Event</th>
<th>Peak time of Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurulation</td>
<td>3-4 weeks gestation</td>
</tr>
<tr>
<td>Prosencephalic development</td>
<td>2-3 months gestation</td>
</tr>
<tr>
<td>Neuronal Proliferation</td>
<td>3-4 months gestation</td>
</tr>
<tr>
<td>Neuronal Migration</td>
<td>3-5 months gestation</td>
</tr>
<tr>
<td>Organization</td>
<td>5 months gestation - years postnatal</td>
</tr>
<tr>
<td>Myelination</td>
<td>Birth – years postnatal</td>
</tr>
</tbody>
</table>

Table 1.1: Peak time of occurrence of major development events in the human CNS (Adapted from Volpe, 2001).
1.3 PERINATAL HYPOXIC ISCHEMIC BRAIN INJURY

Perinatal HI injury, which usually occurs as a consequence of asphyxia during the fetal, intrapartum and neonatal periods, is a foremost cause of neurological morbidity in human babies (Volpe, 2001a, Vannucci and Hagberg, 2004, Lawn et al., 2005, Rees et al., 2008). Asphyxia usually occurs as a result of interruption to placental blood flow by factors such as umbilical cord occlusion, maternal hypotension and placental abruption, which can be further superimposed by factors that include maternal-fetal infection (Volpe, 2001a, Low, 2004, Perlman, 2006, Rees et al., 2008). The following section discusses the consequent different neuropathologies of perinatal HI injury, mechanisms of cell death during hypoxic ischemia and current animal models of HI brain injury.

1.3.1 Neuropathology

The neuropathology of perinatal HI injury depends greatly on the duration and severity of asphyxia as well as the gestational age of the infant. Developmental variations in biochemical, cellular and anatomical constituents of the developing CNS determine the selective vulnerability at different gestational ages. The basic lesions of perinatal HI brain injury identified to date include periventricular leukomalacia, selective neuronal necrosis, parasagittal cerebral injury, and focal ischemic brain necrosis (Volpe, 2001a, Triulzi et al., 2006).

1.3.1.1 Periventricular Leukomalacia

Periventricular leukomalacia (PVL) loosely refers to the necrosis of white matter. The highest incidence of PVL is seen in premature infants of approximately 26 to 28 weeks of gestation (Figure 1.4a) PVL primarily consists of both a focal and a diffuse component (Figure 1.4b). The focal component is located deep in the cerebral white matter that is
located dorsally and laterally to the external angles of the lateral ventricle. The focal injury commonly occurs after 26 weeks of gestation. It involves necrosis of all cellular elements with subsequent cyst formation (Figure 1.4a). The cellular injury featuring round axonal swelling that represent axonal rupture occurs within 6 to 12 hours after a HI insult especially. The subsequent tissue dissociation that causes cyst formation occurs within one to three weeks. Until recently the focal necrotic lesions were believed to be the hallmark neuropathology of the periventricular leucomalacia. However, sophisticated techniques such as MRI have now demonstrated that noncystic diffuse injury is more common in surviving very low birth weigh infants. It is most apparent in very immature infants of below 28 weeks of gestation. It is largely a cell-specific lesion of which the major target is an early differentiating oligodendroglial precursor. Recent evidence suggests that subplate neuronal layer could be another possible target of PVL diffuse lesion (Meng et al., 1997, Volpe, 1997, Volpe, 1998, Inder and Volpe, 2000, Volpe, 2003, Blumenthal, 2004). Subplate neurons are a transient population of cortical cells that undergo programmed cell death postnatally. The activity of subplate layer peaks around gestation week 24. They play an extremely vital function in the establishment of cerebral circuitry (Volpe, 1996, Volpe, 2001b, McQuillen and Ferriero, 2004).
Figure 1.4: Coronal section of cerebrum from an infant who died at 8 weeks after birth, featuring PVL (a). Schematic representation of focal and diffuse PVL in relation to cerebral vascular supply (b) (Modified from Volpe, 1997).

The pathogenesis of periventricular leukomalacia in the premature brain consists of three interacting factors; cerebrovascular anatomic factors, cerebral vascular regulation and intrinsic vulnerability of oligodendroglial precursor. Localization of the white matter lesions in PVL suggests that it is closely correlated with the anatomical development of the blood supply to the cerebral white matter (Takashima and Tanaka, 1978, Inder and Volpe, 2000, Volpe, 2000). Blood vessels that are derived from the middle cerebral artery penetrate the cerebral wall from the pial surface with some ending in the subcortical areas (short penetrators) and some extending deep in the periventricular white matter (long penetrators) (Figure 1.4b). However, at 24-28 weeks of gestation they are not fully developed with long penetrators having few side branches and the short penetrators being infrequent as compared to the mature brain. Thus, border zones with poor blood supply within cerebral white matter may exist in the immature brain. These areas of already poor perfusion are likely to be particularly extremely vulnerable to ischemic insults. The deep focal necrotic lesions occur principally within the end zones of the long penetrating arteries whilst the diffuse lesions occur within the end zones of long penetrating arteries and short penetrating arteries (Figure 1.4b) (Takashima and Tanaka, 1978, Volpe, 1997,
Volpe, 1998, Inder and Volpe, 2000, Blumenthal, 2004). A portion of premature infants have been shown to exhibit impaired cerebrovascular autoregulation that is also referred to as pressure-passive circulation. Because of this impaired regulation, if the systemic blood pressure falls, as commonly occurs in such infants, the cerebral blood flow will fall too. Even if an intact autoregulation is present in these infants the range of mean arterial blood pressure over which it exists is narrow. Therefore, a marked reduction in blood pressure may lead to insufficient cerebral blood flow. Combined with the fact that cerebral white matter has a poor blood supply already, as discussed above, any further reduction would cause damage (Papile et al., 1985, Volpe, 1998, Inder and Volpe, 2000, Blumenthal, 2004). A maturation dependent vulnerability of the oligodendroglia is another important factor in the pathogenesis of periventricular leukomalacia. At 24 to 28 weeks of gestation oligodendroglial progenitors are the predominant oligodendroglia in human cerebral white matter. Evidence of lipid peroxidation was identified in these progenitors in autopsy brain tissues from infants with PVL suggesting that mechanism of death involves reactive oxygen species. In fact the oligodendroglial progenitors were shown to be extremely vulnerable to free radical attack while the mature oligodendrocytes are totally resistant. It may be due to elevated free radical formation because of enhanced iron uptake during differentiation or insufficient antioxidant defence at that particular developmental stage (Volpe, 1998, Inder and Volpe, 2000, Volpe, 2003, Blumenthal, 2004).

A significant number of infants with PVL demonstrate moderate weakness in the lower limbs during their neonatal lives. Premature infants that are affected by PVL incline to develop spastic motor deficits of lower limbs during the initial years of their lives. It is presumably due to the fact that focal necrotic lesion of PVL includes the region of
descending motor axons from the motor cortex that serve the lower extremities. When the injury is more severe, the necrosis may expand affecting the axons that serve the upper extremities thus developing motor deficits also in the upper limbs. The other important neurological outcome of PVL is the long-term development of cognitive deficits. Evidence suggests that incidence of cognitive impairment correlates with the incidence of diffuse noncystic lesion of PVL. Therefore, it can be suggested that a fraction of these cognitive deficits are due to the possible impairment of development of functional cerebral connections as a consequence of injury to subplate neurons (Volpe, 2001a, Ortibus, 2005)

### 1.3.1.2 Selective Neuronal Necrosis

Selective neuronal necrosis (SNN) is the most prevalently observed lesion following perinatal HI injury. It usually coexists with other varieties of lesions that are discussed here. As the name suggest it refers to necrosis of neurons in specific regions of the developing brain which is determined by the severity and duration of the asphyxia. Typically neuronal necrosis is initiated within 24 to 36 hours following injury continuing until several days. Four basic patterns of selective neuronal injury have been identified to date. Profound and prolonged asphyxia instigate a diffuse neuronal necrosis in which certain neurons at fundamentally all levels of the neuraxis are affected including cerebral cortex (neocortex and hippocampus), deep nuclear (striatum, globus pallidus and thalamus) brain stem, cerebellum and spinal cord. Neuronal necrosis in cerebral cortex to deep nuclear structures is perceived upon a moderately profound but prolonged asphyxia. An abrupt profound asphyxia causes neuronal degeneration particularly in deep nuclear structure to brain stem that is primarily observed in term infants. Pontosubicular neuronal death occurs predominately in premature infants but the nature of asphyxia which causes
it is yet to be defined (Inder and Volpe, 2000, Volpe, 2001a, Perlman, 2006, Triulzi et al., 2006, Nikas et al., 2008).

Differential regional vascular supply may contribute to the pathogenesis of SNN because the neuronal death in the vascular border zones such as parasagittal cortex is more prominent than that of the other areas in the developing brain. Also the development of the brain vascular supply would be different at different gestational ages thus presumably contributing to the age dependent regional selectivity. Another factor that seemingly plays a role in the pathogenesis is regional metabolic factors. The regional differences of energy requirements, mitochondrial function, free radical formation and scavenging ability may leave particular regions more vulnerable to hypoxic ischemia than others. For example, high metabolic requirements of deep nuclear neurons render them more vulnerable to abrupt profound HI injury. Glutamate induced exitotoxicity is considered to be the most prominent mechanism of neuronal death in SNN. Therefore, the maturation-dependant variations of the density of NMDA type glutamate receptors in differential brain regions greatly determines the regional vulnerability following perinatal asphyxia (Inder and Volpe, 2000, Volpe, 2001a, Perlman, 2006).

A variety of neurological abnormalities were detected in infants with SNN depending on which regions of the brain were affected. Derangement of the level of consciousness is the major manifestation detected in the neonatal period, which could be attributed to lesions in bilateral cerebral hemispheres or in the reticular activating system in the brain stem. Seizures, which transpire mainly due to cerebral cortical injury, are another important neonatal outcome of SNN. Furthermore, impairments of sucking, swallowing, tongue moments and oculomotor moments can be developed during the neonatal period.
following damage to brain stem cranial nerve nuclei. Mental retardation is the prevalent long-term consequence of SNN. It is principally a consequence of cerebral cortical injury. Impairment of cortical visual function presumably occurs largely as a consequence of cortical injury although striatal injury may also contribute to these visual deficits. Dystonia is another problematic long-term neurological outcome of SNN which is observed in infants who had bilateral basal ganglia injury without any injury to pyramidal tracts (Volpe, 2001a, Ortibus, 2005).

1.3.1.3 Parasagittal Cerebral Injury

Figure 1.5: Coronal view (T2-weighted MRI image) of an eight year old brain that suffered asphyxia at term showing bilateral parasagittal lesion (Arrows) (Adapted from Campistol et al., 1999).

Parasagittal cerebral injury, which typically occurs following a moderately severe but prolonged asphyxia, is the dominant HI neuropathology exclusively detected in full-term infants. It is characterised by bilateral necrosis of the cortex and subcortical white matter in the parasagittal and supermedial areas of the cerebral convexities (Figure 1.5) with the posterior aspect of the cerebral hemisphere being affected more than the anterior aspect.
The parasagittal cortex falls within the vascular border zones between the end zones of major cerebral arteries thus rendering it extremely vulnerable to reductions in cerebral perfusion. The higher vulnerability of the posterior parasagittal areas can be attributed to the fact that it is located between the end zones of all the three major cerebral arteries (anterior, posterior and middle) as compared to the anterior areas (Inder and Volpe, 2000, Volpe, 2001a, Perlman, 2006, Nikas et al., 2008). Another possibility of increased sensitivity is the relative high metabolic rate of the posterior regions of the newborn (Nikas et al., 2008). Also the parasagittal cerebral injury is found to be more severe in the depths of the sulci presumably due to another border zone of penetrating arteries that exists in the depth of the sulcus. The penetrating arteries that leave the meningeal vessels at right angles from each side of the sulcus tends to bend at the interface between gray and white matter thus leaving a relatively avascular region of white matter at the end of the sulci. They bend more acutely in the mature infants as compared to the premature infants because of the greater depth of their sulci therefore producing a more prominent vascular border zone in the mature infants. Another important determinant of parasagittal necrosis is the impaired vascular autoregulation in the asphyxiated infants. As discussed under section 1.3.1.1, reduction in arterial blood pressure markedly reduces the cerebral blood flow due the impaired regulation thus rendering particularly the vascular border zone regions more vulnerable to injury (Inder and Volpe, 2000, Volpe, 2001a, Nikas et al., 2008). Weakness of proximal limbs is the major neurological abnormality observed following parasagittal cerebral injury during the neonatal period. Generally, it continues into adulthood consistently demonstrating weakness in the upper limbs than the lower. Severe lesions may lead to spastic paraparesis of the arms. A considerable percentage of infants also develop specific intellectual deficits such as visuo-spatial abnormalities due to lesions in posterior parieto-occipito-temporal cortex (Volpe, 2001a, Ortibus, 2005).
1.3.1.4 Focal/Multifocal Ischemic Necrosis

Focal/multifocal ischemic necrosis is characterised by necrosis of all cellular elements (infarction) within a specific arterial distribution following interruption to a single or multiple cerebral arteries. The majority of focal necrosis lesions involve the unilateral occlusion of middle cerebral artery (Figure 1.6). A single infarction may later usually produce porencephaly while multiple infarctions may produce hydranencephaly or multicystic encephalomalacia. Generally, this particular variety of neuropathology is observed following asphyxia approximately between 28 weeks to 40 weeks after gestation. The propensity of cavitation appears to be higher in the developing brain than the adult brain. Paucity of myelinated fibres in the developing brain leaves it relatively less dense than the adult brain thus rendering it more prone to dissolution following necrosis. Further, the proliferation of astroglia is weak in the developing brain thus restraining the formation of the glia scar that also enhance the cavitation (Inder and Volpe, 2000, Volpe, 2001a, Perlman, 2006). Approximately 80 – 85% of the infants with unilateral cerebral infarction develop focal seizures within the initial postnatal days. Later, approximately 25% of the survivors of unilateral cerebral infarction demonstrate symptoms of hemiparesis with obvious motor disturbance after six months of life. Another 20 – 25% of the infants develop cognitive impairments following cerebral infarction. Seizure disorders such as epilepsy occur to approximately 10% of the infants (Volpe, 2001a, Ortibus, 2005).
1.3.2 Mechanisms of Cell Death following Hypoxic Ischemia

A clear understanding of the pathophysiology of the hypoxic-ischemia is essential to the innovation of therapeutic approaches to prevent perinatal neurological damage. An ample number of experiments provide evidence of three major types of mechanisms of cell death following hypoxic-ischemia; excitotoxicity, oxidative stress and, inflammation. These mechanisms mediate neuronal death by means of either apoptosis or necrosis mainly depending on the severity of hypoxic ischemia (Ferriero, 2004).

1.3.2.1 Excitotoxicity

Excitotoxicity is considered as the major cause of injury during the initial phase after hypoxic ischemic insult in the brain. Unlike other organs, the brain is incapable of synthesising and storing energy reserves. Therefore any interruptions to the oxygen supply cause an impairment of the mitochondrial function leading to a dramatic reduction
of cellular energy source ATP. Energy failure impairs the function of sodium potassium pumps thus causing an accumulation of sodium ions inside the cells. On one hand, this causes membrane depolarization which renders the neurons to fire action potentials. On the other hand, disrupted osmotic balance between the extracellular and intracellular space attract water into the cells leading to osmotic cell lysis. The above events cause the uncontrolled release of neurotransmitters, in particular, glutamate into the extracellular space. Although, during normal conditions energy-dependant pumps that are located at the nerve terminals and on presynaptic glials uptake the excess glutamate from the synapses, energy failure following hypoxic ischemia impairs the activity of these glutamate pumps. Therefore, hypoxic ischemic insult causes a massive elevation of the extracellular concentration of glutamate in the brain (Choi, 1992, Johnston, 1995, Doble, 1999).

Glutamate is the major excitatory neurotransmitter in the mammalian brain. Glutamate exerts its effect through a variety of inotropic receptors that include NMDA, AMPA, kainite receptors and metabotropic glutamate receptors (Seeburg, 1996). NMDA channels are hetero-oligomers that are constituted of NR1 subunit and NR2 subunit that has four subtypes (NR2A-NR2B). The NR1 subunit is compulsory for the formation of a functional ligand gated ion channel while the pharmalogical and biophysical properties are determined by the component NR2 subunit. These receptors exhibit low gating kinetic, high Ca\(^{2+}\) permeability, Mg\(^{2+}\) block at resting potential and a requirement of glycine as coagonist (Seeburg, 1996, Cull-Candy et al., 2001, Vannucci and Hagberg, 2004). AMPA receptors are tetrameric complexes that are formed of subunits GluR1-4. They exhibit fast gating kinetics and low calcium permeability. It has been shown that the receptors containing GluR2 subunits are completely impermeable to calcium. Kainate
receptors consist of various combinations of GluR5/6/7 and KA1/2 subunits. They exhibit fast desensitization in the presence of an agonist. Metabotropic glutamate receptors are G-proteins linked proteins that are coupled to phospholipase C (Seeburg, 1996).

Although the molecular basis of glutamate toxicity is uncertain, there is general agreement that it is in large part Ca\(^{2+}\)-dependent (Figure 1.7). Prolonged activation of glutamate receptors leads to an overload of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)). Glutamate initially activates AMPA/Kainate receptors causing a Ca\(^{2+}\) influx through the GluR2 lacking receptors. Ca\(^{2+}\) influx through these channels can be limited because of their rapid desensitisation following prolonged exposure to an agonist. Currents through both Ca\(^{2+}\) permeable and impermeable AMPA/kainate receptors depolarize the membrane thus releasing the Mg\(^{2+}\) block from NMDA receptors. Prolonged activation of NMDA receptors results in further influx of Ca\(^{2+}\). It is considered that in excitotoxicity Ca\(^{2+}\) influx is predominantly mediated through NMDA channels. Further depolarization of membrane results in the activation of voltage-gated Ca\(^{2+}\) channels causing a further increase of Ca\(^{2+}\) influx. Another important mechanism in elevating [Ca\(^{2+}\)]\(_i\) is the release of Ca\(^{2+}\) from intracellular store, endoplasmic reticulum (ER). Ca\(^{2+}\) release from ER is mediated via IP\(_3\) receptors and Ca\(^{2+}\) sensitive ryanodine receptors. The activation of metabotropic glutamate receptors, which are coupled to phospholipase C via G-proteins, increases intracellular IP\(_3\) concentration thus inducing Ca\(^{2+}\) release from ER. Increased [Ca\(^{2+}\)]\(_i\) induces further Ca\(^{2+}\) release from stores by acting on Ca\(^{2+}\) sensitive channels (Mody and MacDonald, 1995, Durand et al., 1996, Conti and Weinberg, 1999, Mattson et al., 2000, Reynolds, 2002).
The high concentration of Ca\(^{2+}\) leads to activation of numerous catabolic enzymes that disintegrate cells, leading to cell death by necrosis. Activation of nuclease causes disruption of DNA in the nucleus. Cytosolic proteases such as calpain destroy the cytoskeleton and cellular organelles. Activation of Ca\(^{2+}\)-dependant kinases such as protein kinase C results in modification of phosphorylation state of other proteins disrupting their function. Lipases such as phospholipase A2 attack plasma and internal membranes. Ca\(^{2+}\) also induces the production of free radicals. Free radicals cause cell death by interrupting functions of cellular molecules. Ca\(^{2+}\) can also cause cell death by inducing apoptosis. Depletion of Ca\(^{2+}\) in ER and overload of Ca\(^{2+}\) in mitochondria that happens subsequent to increased [Ca\(^{3+}\)]\(_{i}\) signals the nucleus to induce the expression of apoptotic genes (Choi, 1992, Berridge et al., 1998, Doble, 1999).

![Figure 1.7: A systematic diagram demonstrating the cellular events occurring during excitotoxicity (Adapted from Doble, 1999).](image-url)
The developing brain demonstrates an increased vulnerability to excitotoxic damage compared to the adult brain. Apart from its role as a neurotransmitter, glutamate plays an important role in the developing brain in neuronal growth, differentiation, and survival, neuronal circuitry formation and synaptic plasticity. NMDA receptors expressed in the immature brain thus have subunit composition that allows them to mediate the effects of glutamate more easily. During development the predominance of the NR2 subunit expressed are of 2B subtype thus rendering the NMDA receptors to posses an enhanced calcium permeability (McDonald and Johnston, 1990, Zhong et al., 1995, Cull-Candy et al., 2001, Johnston, 2001, Vannucci and Hagberg, 2004). Also the developing brain demonstrates an increased tyrosine kinase activity that causes an increased tyrosine phosphorylation of the NMDA receptor, which in turn results in the increased excitability of the receptor (Gurd et al., 2002, Vannucci and Hagberg, 2004).

1.3.2.2 Oxidative Stress

Free radicals are molecular species that have unpaired electrons in the outer orbit of the atom. They contribute to cellular injury via membrane peroxidation, protein oxidation and nucleic acid oxidation (Delivoria-Papadoopoulos and Mishra, 2000). Production of an excess of free radicals, namely superoxide anion (O$_2^-$), nitric oxide (NO), hydrogen peroxide (H$_2$O$_2$) and hydroxyl ions (OH$^-$) upon reperfusion is considered another major mechanism of injury in the hypoxic ischemic brain.

Normally about 10-20% of the oxygen consumed by the cell undergoes reduction to form oxygen free radicals such as O$_2^-$, H$_2$O$_2$ and OH$. Under normal conditions they are scavenged by cellular antioxidants such as superoxide dismutase (SOD), glutathione peroxidase and catalase to prevent damage to the cell. But upon reperfusion after a HI
insult the production of oxygen free radicals is dramatically elevated over the scavenging capacity of the antioxidants. The major sources of reactive oxygen species (ROS) are xanthine oxidase, cyclooxygenases, and lipoxygenase (Delivoria-Papadopoulos and Mishra, 2000, Chan, 2001, Shalak and Perlman, 2004). Experimental evidence suggests that these free radical producing pathways are directly or indirectly activated due to the increased cytosolic free calcium concentration. Calcium is known to activate the protease that converts the xanthine dehydrogenase into xanthine oxidase. Xanthine oxidase reduces oxygen to the superoxide anion and hydrogen peroxide while oxidising both hypoxanthine and xanthine (Delivoria-Papadopoulos and Mishra, 2000, Dani et al., 2004, Calvert and Zhang, 2005). As mentioned above, phospholipase A2 is also a major lipase that is activated by intracellular free calcium. It causes the release of arachidonic acid from membrane phospholipids that is subsequently metabolised into prostaglandins and leukotrienes by cyclooxygenases and lipoxygenase producing more oxygen free radicals (Walton et al., 1997, Delivoria-Papadopoulos and Mishra, 2000, Dani et al., 2004, Shalak and Perlman, 2004).

NO is another important participant in the cellular injury processes following hypoxic ischemia in the brain. It is formed during the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS). Three isoforms of NOS exists in the CNS. Neuronal NOS (nNOS) is a constitutively produced enzyme that is localised in neurons. Inducible NOS (iNOS) is induced in microglia/macrophages, astrocytes and endothelial cells. Endothelial NOS (eNOS) is another constitutively expressed isoform that is localised to endothelium. Constitutively expressed, NOS isoforms are intracellular free calcium-dependent while the inducible isoform is calcium-independent. However, the role of NO in neuronal injury has been controversial because it has been shown that NO produced
from nNOS and iNOS are neurotoxic while NO produced from eNOS is shown to be neuroprotective (Delivoria-Papadopoulos and Mishra, 2000, Chan, 2001, Shalak and Perlman, 2004). The synthesis of NO during hypoxia is limited due to the lack of oxygen but is markedly elevated upon reperfusion (Calvert and Zhang, 2005). NO itself is a weak free radical but hypothesised to cause neuronal damage mainly by producing more potent secondary oxidants. It is capable of producing peroxynitrite by combining with superoxide radicals that are also over-produced during reperfusion. Then the peroxynitrite rapidly decomposes to produce nitrogen dioxide and hydroxyl radicals that are strong cytotoxic oxidants (Delivoria-Papadopoulos and Mishra, 2000, Shalak and Perlman, 2004, Calvert and Zhang, 2005).

Under physiological conditions iron is maintained in a non-toxic, ferric state by binding to proteins such as ferritin and transferrin. However, during a HI insult, iron is released into the cytoplasm as free ions. It can generate toxic hydroxyl radicals by reacting with peroxidases via a fenton reaction. Also free ferric iron can be reduced into a ferrous form that can also be neurotoxic (Shalak and Perlman, 2004, Calvert and Zhang, 2005).

The perinatal brain exhibits an increased vulnerability to the oxidative stress compared to that exhibited in the adult brain. It has been demonstrated that the developing brain has a reduced capacity of antioxidant enzymes, relative to the developed brain and, in particular, glutathione peroxidase. Therefore, the detoxification of H$_2$O$_2$ produced during the reperfusion is limited in the developing brain rendering it more vulnerable to oxidative stress. The higher level of free ferric iron in the developing brain is another factor that contributes to its higher sensitivity to oxidative stress. As mentioned above,
iron reacts with peroxidases to produce H$_2$O$_2$ that cause further damage (Vannucci and Hagberg, 2004).

1.3.2.3 Inflammation

Inflammatory mediators are known to play a critical role in the development of normal CNS (Saliba and Henrot, 2001). Recent data suggests that they also play a prominent role in the pathogenesis of HI injury in the brain (Plessis and Volpe, 2002, Shalak and Perlman, 2004, Calvert and Zhang, 2005). Recent experimental evidence suggests that proinflammatory cytokines such as Interleukin (IL)-1β, Tumour necrosis factor-α (TNF-α) and IL-6 are expressed by brain cell types that include microglia, astrocytes and neurons (Saliba and Henrot, 2001). Several studies have demonstrated an increased of expression and bioactivity of IL-1β, TNF-α and IL-18 following hypoxic ischemia in the brain (Szafarski et al., 1995, Hagberg et al., 1996, Hedtjarn et al., 2002). Furthermore, the inhibition of these cytokines were shown to be neuroprotective against HI insults (Martin et al., 1994, Yamasaki et al., 1995, Eun et al., 2000). Cytokines may cause neuronal damage directly by stimulating the production of free radicals via activation of prooxidants such as iNOS and cycloxgenase (Shalak and Perlman, 2004). Also they indirectly contribute to the injury by impairing the glutamate transporters in the astrocytic membranes, thus initiating excitotoxicity (Plessis and Volpe, 2002, Shalak and Perlman, 2004). It is further suggested that cytokines might enhance the HI injury by reducing the cerebral blood flow due to their potent vasomotor and vaso-occlusive effects (Plessis and Volpe, 2002).
1.3.3 Animal Models of Hypoxic Ischemia in Brain

Experimental animal models in a range of species including; juvenile and fetal rats, rabbits, guinea pigs, sheep and monkeys have provided important information of pathophysiology of perinatal cerebral hypoxic-ischemia over the years (Vannucci, 1993). However, of the several available animal models, the immature rat has been studied most extensively because of its similarity to humans in respect to physiology of reproduction and neuroanatomy (Vannucci, 1993, Yager, 2004). The method of producing HI damage in the juvenile rat brain is adopted from the method devised by Levine et al., in the adult rat. It consists of unilateral common carotid artery ligation followed by hypoxia for a particular time period (Levine and Marvin, 1960). The following section discusses three rat models of unilateral HI injury that have been used to elucidate the pathophysiology of perinatal HI brain injury at various stages of development.

1.3.3.1 Postnatal Day Seven Rat

The postnatal day seven (P7) rat was originally chosen for the study of hypoxic-ischemia in the immature brain because the development stage of P7 rat brain is similar to that of 30-34 weeks gestation human fetus with completed cortical plates, involuting germinal matrix and little myelination (Vannucci et al., 1999). The P7 HI brain injury model was first introduced by Rice et al., in 1981. They performed permanent unilateral carotid ligation followed by exposure to 3.5 hours at hypoxia with 8% oxygen in a warm environment on 7 day old rats (Rice et al., 1981). Thereafter P7 model has been used widely with some modifications for studying HI injury in the juvenile brain (Yager, 2004). In this model, damage is predominately seen in the ipsilateral cerebral cortex, striatum, hippocampus, and subcortical and periventricular white matter. Neocortical damage often occurs in columns of dead neurons at right angles to the pial surface.
Occasionally laminar neuronal death involving in particular cortical layers III, V and VI are also seen (Rice et al., 1981, Vannucci et al., 1999, Yager, 2004). However, the inconsistency of the degree of neuronal loss and high mortality rate of the model are considered disadvantageous for finer studies (Sirimanne et al., 1994).

1.3.3.2 Postnatal Day 21 Rat

The juvenile rat model was further refined by Sirimanne et al., for use in the 21 day old (P21) rats. The P21 rat was chosen because its brain shows a greater plasticity that may partly be due to its enhanced capacity to produce neurotrophic factors (Nieto-Sampedro et al., 1982). Sirimanne et al., performed two distinct neuronal insults, a moderate and a severe HI injury, resulting in two distinct patterns of neuronal damage (Figure 1.8). P21 rats were subjected to permanent unilateral right carotid artery ligation followed by 15 (moderate) or 60 (severe) minute hypoxia in 8% oxygen (Sirimanne et al., 1994). The moderate injury resulted in the neuronal loss predominately in the CA1-CA2 region of the hippocampus and cortical layers IV and V and moderately in dentate gyrus, striatum and thalamus. Neuronal degeneration started approximately at one day after the injury peaking at three days. Appearance of apoptotic morphology, DNA laddering and acidophilia suggested that apoptosis is the major form of cell death following the moderate HI injury. Severe injury resulted in a massive neuronal loss in the frontoparietal cortex in particular in the cortical layers III and IV and in the entire hippocampus. Also less prominent neuronal loss was seen in stratum, thalamus and amygdala. A slight neuronal degeneration that appeared to involve apoptotic mechanisms was observed at as early as 10 hours after the injury. However, the lack of DNA laddering and morphological changes indicated the involvement of necrosis rather than apoptosis during
the massive cell death at 24 hours after the injury (Sirimanne et al., 1994, Beilharz et al., 1995).

Figure 1.8: Fluoro-jade B staining at 3 days following severe (a and b) and moderate (c and d) unilateral HI injury at P21. Following severe injury, neuronal loss was mainly seen in layers III–V of the parietal cortex (a and inset) and pyramidal layer of the hippocampus (b) of the ipsilateral hemisphere. In the moderately injured brain, selective neuronal loss was seen in cortical layers III–V (c). A moderate amount of neuronal loss was also seen in the basal ganglia (inset c) and CA1/2 pyramidal layer (d) Scale bars = 50 μm (a, b and c inset); 500 μm (a inset); 100 μm (c and d) (Adapted from Möderscheima et al., 2007).

1.3.3.3 Postnatal Day Three Rat

Most animal models that are developed to mimic hypoxic ischemic injury in the developing brain correspond more to the injury seen in full-term or near-term infants rather than very premature infants. Therefore a newer model was needed to investigate
the pathophysiology of diffuse white matter injury that is followed by the cortical developmental alteration seen in very premature infants.

Cortical development of the postnatal day three (P3) rat brain closely resembles that of human fetus of 24-26 weeks of gestation. Human (Uylings, 2000, Volpe, 2001b, Honda et al., 2003) and rat (Clancy et al., 2001) cerebral corticies has accomplished its full complement of neurons by gestation week 24 and postnatal day 3 respectively. In both species, brains have recently started undergoing organizational events such as lamination, dendritic and axonal ramification, selective elimination of cell processes and synapses (Clancy et al., 2001, Volpe, 2001b). Myelination is also at a similar developmental stage in both gestation week 24-26 old human brain and day three old rat brain. At this development stage preoligodendrocytes is the predominate population of oligodendroglia with a minority of immature oligodendrocytes in both human (Gard and Pfeiffer, 1989, Back et al., 2001, Volpe, 2001b, Back et al., 2002) and rat (Craig et al., 2003) brains. Therefore in both species myelination has not yet begun. According to the above evidence it can be safely suggested that postnatal day three old rat brain parallels the developmental stage of the brain of a 24-26 weeks gestation human fetus.

In the P3 model of HI brain injury, significant neuronal degeneration begins in the ipsilateral cortex from 12 hours after the injury reaching a peak at 24 hours. Cellular degeneration is organised in a columnar pattern perpendicular to the surface of the cortex within the cortical layers IV, V and VI. Highest cell loss occurs in the deepest cortical layer, which may comprises the subplate neurons (Figure 1.9) (Stadlin et al., 2003, Sizonenko et al., 2005). Degenerating neurons express apoptotic morphology chromatin clumps and nuclear and cytoplasmic shrinkages suggesting that apoptosis is the major
form of cell death. However, necrotic cells featuring small irregular shaped chromatin clumps and condensed granular cytoplasm are occasionally found too (Stadlin et al., 2003). White matter damage is observed at approximately 24 hours after hypoxic ischemia with similar pattern to that of neuronal degeneration. Clear retraction and clubbing of the axons are observed within the damaged areas. Glial activation is another prominent feature seen after a HI injury in P3 rat brain. Expression of reactive astrocytes is increased within the damaged cortices and the corpus callosum at approximately 24 hours after injury and remained elevated until about 20 days (Stadlin et al., 2003, Sizonenko et al., 2005).

![Figure 1.9: Fluoro-jade B staining of P4 rat brain following HI injury at P3 showing the columnar pattern of severe (a) and moderate (b) neuronal death in the ipsilateral hemisphere. Scale bars = 500μm (a); 100μm (b) (Modified from Sizonenko et al., 2005).](image)

A study by Sizonenko and colleagues characterized the effects of a focal HI brain injury at P3 on subsequent cerebral development at P21. The volume of the cerebral cortex of the hypoxic ischemic rat brain was significantly lower than that of the controls. Thionin staining demonstrated a selective loss of ipsilateral deep cortical layers IV–VI in the injured cortex. Also a disorganisation of the neuroarchitectural pattern was observed in
the ipsilateral cortex compared to the controlateral cortex. Immunostaining for myelin basic protein revealed a clear loss of white matter in the injured animal. The reminder of the myelinated fibres demonstrated abnormal arrangements such as compacting or clumping within the ipsilateral cortex. GFAP immunoreactivity was markedly higher in the ipsilateral cortex compared with contralateral cortex suggesting that gliosis is persistent through the development following hypoxic ischemia in P3 rats. These developmental alterations such as loss of cortical volume, altered myelination, and persistent gliosis observed following HI injury at P3 are similar to alterations witnessed in the human premature infant brain with PVL. Therefore, HI brain damage in P3 rat may provide a comparable model of brain injury to that observed in human preterm infants (Sizonenko et al., 2003).

1.4 MATRIX METALLOPROTEINASES

1.4.1 Introduction

The ECM is a complex structure that influences the behaviour of its resident cells and migrating cells by providing specific contextual information. It consists of two main domains: the basement membrane that is adjacent to epithelial cells and the interstitial matrix that covers the cell sheets. The basic structure of both domains is defined by a collagen scaffold. Adhesive laminin, tenascin and proteoglycans adhere to the collagen scaffold to interact with the cells in, or adjacent to, the matrix. The interaction between the matrix proteins and cells are conducted through matrix receptors that are mainly constituted of integrins. The ECM is constantly being remodelled, a process that involves breakdown of existing and synthesis of new ECM proteins, thus altering the interaction between the matrix and the cells. One major class of proteolytic enzymes that plays a
dominant role in ECM degradation is that of MMP (Sethi et al., 2000, Bosman and Stamenkovic, 2003).

1.4.2 Matrix Metalloproteinase Family

There are now more than twenty enzymes that are classified as MMPs. These enzymes have both a descriptive name and a numerical name (Nagase and Woessner, 1999, Sethi et al., 2000, Stamenkovic, 2003). Several subclasses of MMP family have been identified, some of which are membrane bound while as others are secreted (Stamenkovic, 2003). The membrane bound MMP comprises five members that function while attached to the cell membrane (Sethi et al., 2000). The secreted MMPs are further allocated into five subclasses based on their substrate preference; collagenases, gelatinases, stromelysins, metrilysins and metalloelastase (Nagase, 1997). The collagenases predominately degrade fibrillar collagens type I, II and III. Gelatinases demonstrate a preference for triple helical type IV collagen. The stromelysins cleave a variety of proteins including aggrecans, fibronectin, laminin and collagen IV (Nagase, 1997, Sethi et al., 2000, Rosenberg, 2002). However, they have a high degree of overlap among the substrates thus rendering this nomenclature imprecise at best (Sethi et al., 2000, Stamenkovic, 2003). The following table provides a summary of the MMP family subclasses and their substrate specificities (Table 3) (Nagase, 1997, Wojtowicz-Praga et al., 1997, Rosenberg, 2002).
Table 1.2: Major class of MMP and their substrates (Nagase, 1997, Wojtowicz-Praga et al., 1997, Rosenberg, 2002).

<table>
<thead>
<tr>
<th>Descriptive Name</th>
<th>Numerical Name</th>
<th>Substrate</th>
</tr>
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<tbody>
<tr>
<td><strong>Collagenases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interstitial collagenase</td>
<td>MMP-1</td>
<td>collagen I, II, III, IV, and X, gelatin, entactin, aggrecan, link proteins</td>
</tr>
<tr>
<td>Neutrophil collagenase</td>
<td>MMP-8</td>
<td>collagen I, II, and III, aggrecan, link proteins</td>
</tr>
<tr>
<td>Collagenases 3</td>
<td>MMP-13</td>
<td>collagen I, II, and III</td>
</tr>
<tr>
<td><strong>Gelatinase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatinase A</td>
<td>MMP-2</td>
<td>collagen I, IV, V, VII, X and XI, gelatin, fibronectin, laminin, elastin, aggrecan, vitronectin, large tenascin C</td>
</tr>
<tr>
<td>Gelatinase B</td>
<td>MMP-9</td>
<td>collagen IV, V, and XIV, gelatin, elastin, aggrecan, vitronectin, entactin</td>
</tr>
<tr>
<td><strong>Stromelysins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromelysins 1</td>
<td>MMP-3</td>
<td>collagen III, IV, IX and X, aggrecan, gelatin, fibronectin, laminin, large tenascin-C, vitronectin</td>
</tr>
<tr>
<td>Stromelysins 2</td>
<td>MMP-10</td>
<td>collagen IV, aggrecan, fibronectin</td>
</tr>
<tr>
<td>Stromelysins 3</td>
<td>MMP-11</td>
<td>collagen IV, fibronectin, laminin, aggrecan, gelatin</td>
</tr>
<tr>
<td><strong>Matrilysin</strong></td>
<td>MMP-7</td>
<td>collagen IV, aggrecan, fibronectin, laminin, gelatin, elastin, entactin, small tenascin-C, vitronectin</td>
</tr>
<tr>
<td><strong>Metalloelastase</strong></td>
<td>MMP-12</td>
<td>elastin</td>
</tr>
<tr>
<td><strong>Membrane-type</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>MMP-14</td>
<td>collagen I, II, III, fibronectin, laminin-1, vitronectin</td>
</tr>
<tr>
<td>MT2-MMP</td>
<td>MMP-15</td>
<td>not known</td>
</tr>
<tr>
<td>MT3-MMP</td>
<td>MMP-16</td>
<td>not known</td>
</tr>
<tr>
<td>MT4-MMP</td>
<td>MMP-17</td>
<td>not known</td>
</tr>
</tbody>
</table>

1.4.3 Structure

Matrix metalloproteinases contain several highly conserved common structural domains (Figure 1.10). The signal sequence, which is usually removed during translation, directs the translated protein for secretion. The pro-domain at the amino terminal consists of 80-
90 amino acids that form a secondary structure of three $\alpha$ helix and connecting loops. It has a conserved PRCGXPDV sequence motif within which the cycteine-sulphydryl group binds the zinc atom in the active site of the protein. A bait region that is susceptible to proteinases is located between $\alpha$-helix 1 and 2 of the pro-domain.

![MMP structure](image)

**Figure 1.10:** Protein structure of MMPs. The principal structural subclasses of MMPs are shown and the different domains indicated. Individual MMPs that belong to each structural subclass are listed (Adapted from Stamenkovic, 2003).

A peptide of about 170 amino acids that is situated adjacent to the pro-domain makes the catalytic domain that is common to all MMP. It folds to compose a five stranded $\beta$-sheet, three $\alpha$-helices and bridging loops (Figure 1.11). The catalytic domain contains a zinc binding motif that has an amino acid sequence of HEBXHXBGBXH where X is a variable residue and B is a bulky hydrophobic residue. It, together with a conserved
methionine, forms a unique “Met-turn” structure that presumably provides a hydrophobic core for the functional zinc ion. In addition to the functional zinc ion at the zinc binding motif, the catalytic domain contains another structural zinc ion and up to three calcium ions to facilitate the stability of the structure. MMP-2 and MMP-9 have a unique domain consisting of three repeats of a 58 amino acid fibronectin II-like module inserted into their catalytic sites. It has been demonstrated that these repeats interact with collagens and gelatins (Bode et al., 1993, Murphy and Knauper, 1997, Nagase and Woessner, 1999, Stamenkovic, 2003, Nagase et al., 2006).

![Figure 1.11: Ribbon stereo plot of a representation of the catalytic domain of MMPs (Adapted from Bode et al, 2003).](image)

Apart from minimal domain MMPs, MMP-7 and MMP-26, which are composed only of the signal peptide, pro-peptide, and catalytic domain, most of the remaining MMPs contain a C-terminal haemopexin-like domain (Stamenkovic, 2003). The 210 amino acid chain is folded into a four bladed β-propeller structure, in which each blade is composed of four antiparallel β-strands and an α-helix (Figure 1.12). This confers it a tertiary figure of an ellipsoidal disk shape with the four β-propeller blades arranged around a funnel-like
tunnel. The haemopexin-like domain is an absolute requirement in determining the substrate specificity of collagenase in proteolysis of triple helical collagens. Another important function of this domain is that it facilitates the interaction of the enzymes with their tissue inhibitors (Gomis-Ruth et al., 1996, Murphy and Knauper, 1997, Nagase and Woessner, 1999, Sethi et al., 2000, Stamenkovic, 2003, Nagase et al., 2006).

Figure 1.12: Ribbon stereo plot of a representation of the haemopexin-like domain of MMPs (Adapted from Gomis-Ruth et al., 1996).

1.4.4 Regulation of Matrix Metalloproteinase Activity

A delicate balance of MMP activation and inhibition is essential to maintain a controlled ECM proteolysis. Among the many regulatory mechanisms that influence the ultimate effect of MMP, the most commonly observed three mechanisms are transcriptional regulation, proenzyme activation and the action of tissues inhibitors of metalloproteinases (TIMP).
1.4.4.1 Transcriptional Regulation

Accumulating evidence suggests that the key regulatory method of MMP expression is the transcription regulation because most MMPs are only expressed when physiological or pathological tissue remodelling occurs (Nagase and Woessner, 1999, Ye, 2000). It is evident that the MMP gene expression can be influenced by many effectors including growth factors, cytokines, chemical agents, physical stress, and cell-cell and cell-matrix interactions (Wang and Keiser, 1998, Ma et al., 2001, Allen et al., 2003, Wright and Friedland, 2004). The effect of such factors is mediated through regulatory elements in the MMP gene promoters. The promoters of inducible MMP genes encompass an AP-1 element, which binds members of the AP-1 transcription family, at position approximately -70. AP-1 transcriptional factors are leucine zipper proteins of Jun and Fos family that bind to a consensus DNA sequence as a dimeric complex (Westermarck and Kähäri, 1999, Ye, 2000). Accumulating evidence strongly suggests that the activation of AP-1 element by AP-1 transcriptional factors induce the MMP gene expression (Qin et al., 1999, Ishii et al., 2003, Woo et al., 2004). PEA-3 is another transcriptional control element that is found in the MMP gene promoters at positions between -140 and – 200. EST is the helix-turn-helix transcription factor that binds to the PEA-3 element at a purine rich sequence. Evidence showing colocalisation of EST with several MMP proteins, expression of EST during various cases of tissue remodelling and enhanced MMP expression following overexpression of EST suggest that EST influences the MMP expression (Westermarck and Kähäri, 1999, Ye, 2000).

1.4.4.2 Proenzyme Activation

MMPs are first expressed as proenzymes of which the enzymatic activities are suppressed by the action of their propeptide. They can be activated through removal of the
propeptide by various endogenous proteinases (Table 1.3). As mentioned in section 1.4.3, most MMP proteins have a cysteine-sulphydryl group within their propeptide that binds to the active site zinc atom thus preventing the activity of the protein. Therefore proenzyme activation includes disruption of Cys-Zn$^{2+}$ interaction allowing the functional zinc atom to interact with a water molecule that is required for catalysis (Nagase, 1997, Visse and Nagase, 2003, Nagase et al., 2006).

<table>
<thead>
<tr>
<th>Zymogen</th>
<th>Activation by</th>
<th>Final Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>proMMP-1</td>
<td>Trypsin, plasmin, plasma kallikrein</td>
<td>MMP-3, MMP-2, MMP-7, MMP-10, Chymase$^a$</td>
</tr>
<tr>
<td>proMMP-2</td>
<td>MT1-MMP, MT3-MMP, MMP-1</td>
<td>MMP-2, MMP-7, MMP-3</td>
</tr>
<tr>
<td>proMMP-3</td>
<td>Many proteinases but not by MMPs</td>
<td>MMP-3</td>
</tr>
<tr>
<td></td>
<td>(e.g., trypsin, chymotrypsin, plasmin, chymase, leukocyte elastase, pseudocollin, thermolysin, etc.)</td>
<td></td>
</tr>
<tr>
<td>proMMP-7</td>
<td>Trypsin, plasmin, leukocyte elastase</td>
<td>MMP-5, MMP-7</td>
</tr>
<tr>
<td>proMMP-6</td>
<td>Tissue kallikrein, leukocyte elastase, cathespin G, trypsin</td>
<td>MMP-5, MMP-10$^b$</td>
</tr>
<tr>
<td>proMMP-9</td>
<td>MMP-1, MMP-2, MMP-3, MMP-7</td>
<td>MMP-1, MMP-2, MMP-3, MMP-7</td>
</tr>
<tr>
<td>proMMP-10</td>
<td>Plasmin, trypsin, chymotrypsin</td>
<td>MMP-10</td>
</tr>
<tr>
<td>proMMP-11</td>
<td></td>
<td>furin$^b$</td>
</tr>
<tr>
<td>proMMP-13</td>
<td>MMP-3, MT1-MMP</td>
<td>MMP-3, MMP-10, MMP-2</td>
</tr>
<tr>
<td>proMT1-MMP</td>
<td></td>
<td>furin$^b$</td>
</tr>
</tbody>
</table>

**Table 1.3: Various activators of pro-MMPs (Adapted from Nagase, 1997).**

Proteolytic activation frequently happens in a stepwise manner (Figure 1.13). It has been identified to date that thirteen MMPs are secreted as proMMPs before getting activated extracellularly by various EMC proteinases. The proteinases initially attack the proteinase susceptible bait region in the middle of the propeptide resulting in an intermediate product. The conformation change of the intermediate product not only disturbs the Cys-Zn$^{2+}$ interaction but also renders the activation site at the end of the propeptide to be readily cleaved by a second proteolysis. This second proteolysis is usually catalyzed by other active MMPs but not by the initial proteinase. The proteolytic cleavage of the propeptide (pro-domain) leads to the irreversible loss of the cysteine-sulphhydryl group. Activation by exogenous chemical follows the similar step wise
manner, but with greater number of intermediate products (Nagase, 1997, Visse and Nagase, 2003, Nagase et al., 2006).

![Diagram of proMMP activation](image)

Figure 1.13: Stepwise activation of proMMPs (Adapted from Nagase, 1997).

Ten proMMPs including the MT-MMPs and MMP-11 contain a furin recognition motif adjacent to their propeptide at C-terminal end. Therefore, they are likely to be activated intracellularly by furin-like proteinases before being secreted into the ECM (Nagase, 1997, Nagase and Woessner, 1999, Sethi et al., 2000, Stamenkovic, 2003). In 1995 Pei and Weiss demonstrated that MMP-11 is activated by Golgi-associated proteinase furin and secreted as the low molecular weight active form (Pei and Weiss, 1995). Thereafter, other MMPs with the furin recognition site were also shown to be activated in a similar manner (Nagase, 1997, Nagase and Woessner, 1999, Stamenkovic, 2003, Visse and Nagase, 2003, Nagase et al., 2006).
Several proMMPs are not readily activated by general ECM proteinases. In 1994 Sato et al., first demonstrated that pro-MMP-2 is activated on the plasma membrane by MT1-MMP. Further experiments have shown that MMP-2 proenzyme interacts with MT1-MMP via TIMP-2. This evidence suggests that C-terminal domain of TIMP-2 binds to the homepepxin domain of pro-MMP-2 and localizes the proenzyme close to the active MT1-MMP, which then activates it by proteolytic cleavage. To date only MMP-2 and MMP-13 have been identified to be activated at the cell surface by MT1-MMP (Nagase, 1997, Nagase and Woessner, 1999, Visse and Nagase, 2003).

1.4.4.3 Tissue Inhibitors of Matrix Metalloproteinases

Activity of the cleaved MMPs is tightly controlled by their endogenous inhibitors called the tissue inhibitors of metalloproteinases (TIMPs). Four isoforms termed TIMP-1-4, of which the molecular weights range from 20-30kDa, have been identified to date. They reversibly inhibit the activated MMPs by forming a 1:1 non-covalent complex. They consist of 184-194 amino acids that are subdivided into N and C-terminal domains (Figure 1.14). Six conserved disulphide bonds maintain the protein in rigid conformation. The N-terminal domain forms an independent unit that is capable of inhibiting the MMPs by itself. The final figure of TIMPs appears similar to that of a wedge. It slots into the active-site cleft of MMPs similarly to their substrates. The N-terminal amino group of the conserved cysteine at residue 1 of the TIMPs chelates the catalytic zinc atom of the MMPs thus expelling the water molecule bound to it. TIMPs are capable of inhibiting all the MMPs that has been tested to date. However, TIMP-1 demonstrates lesser capacity in inhibiting MT1-MMP, MT3-MMP, MT5-MMP and MMP-19. Conversely, TIMP-3 shows affinity towards other MMP related proteins such as ADAMs and aggrecanases (Gomis-Ruth et al., 1996, Gomis-Rüth et al., 1997, Nagase and Woessner, 1999, Brew et

Figure 1.14: Ribbon stereo plot of a representation of TIMP-2 illustrating the N (NTD) and C (CTD) terminal domains (Adapted from Visse and Nagase, 2003).

1.4.5 Matrix Metalloproteinases in Brain Development

The members of MMP family are crucial regulators of mammalian development. They likely participate in the developmental processes through a) allowing cellular movement by degrading ECM macro molecules; b) affecting cellular behaviour by altering the ECM-cell interactions; and c) regulating the activity of biologically essential molecules by proteolytically cleaving the pro-forms producing the active-forms, releasing them from bound stores or by regulating the activity of their inhibitors. MMP activity is implicated in many developmental processes that include embryonic implantation, angiogenesis, and bone morphogenesis (Nagase and Woessner, 1999, Vu and Werb, 2000, Yong et al., 2001). As mentioned previously, during development of the CNS, neurons are generated in the ventricular zone, migrate to their final destinations along radial glia tracts, extend neurites to develop the connectivity necessary for information transductions. Since these processes potentially require many complex interactions with
brain ECM, it is plausible that MMPs has an important contribution during development of the brain (Letourneau et al., 1994, Conant and Gottschall, 2005).

### 1.4.5.1 Developmental Regulation of Matrix Metalloproteinases

Studies investigating the spatiotemporal expression of MMPs clearly indicate that MMP expression is developmentally regulated. Several MMPs showed elevated expression during developmental periods of the brain as compared to the mature periods. For example, MMP-2 (Frolichsthal-Schoeller et al., 1999), MMP-8 (Giambernardi et al., 2001) and MMP-9 (Soler et al., 1995) were demonstrated to be highly expressed in the embryonic mammalian brain as compared to the mature brain. Studies on the developing rat cerebellum illustrated that cerebellar MMP-2 (Ayoub et al., 2005) and MMP-9 (Vaillant et al., 1999, Ayoub et al., 2005) mRNA expression gradually decreased with age. In agreement with the mRNA expression, MMP-2 and 9 activities in the rat cerebellum also decreased with increasing age (Ayoub et al., 2005). Conversely, Sekine-Aizawa et al., found that MT5-MMP expression in the postnatal cerebellum increased progressively with increasing age (Sekine-Aizawa et al., 2001). Furthermore, a recent detailed reverse transcriptase polymerase chain reaction study demonstrated that MMP-2, -9, -11, -13, -14, -15 and -24 (Mt5-MMP) expression levels were significantly higher at one week after birth as compared to the later times in the mouse prosencephalon, rhombencephalon and spinal cord while MMP-12 was upregulated at later time points in the rhombencephalon and spinal cord (Ulrich et al., 2005). Above evidence strongly suggests that members of the MMP family are robustly regulated during mammalian brain development.
1.4.5.2 Mechanisms of Action

Accumulating evidence suggests that MMPs contribute to fundamental developmental processes of the CNS that include migration of neuronal precursors, neurite outgrowth and myelogenesis.

Neuronal Migration

Neuronal migration is an essential process during brain development that requires vigorous ECM modulation (Nadarajah et al., 2003) thus presumably requiring a high level of MMP activity. In fact, MMP-2 (Ayoub et al., 2005) MMP-3 (Vaillant et al., 1999) and MMP-9 (Vaillant et al., 1999) were shown to be expressed by external granular layer (EGL) cells of the developing rat cerebellum approximately at P9-10 when the migration of these cells is known to be maximal. Moreover, RT-PCR analysis of the mouse CNS suggested that the reduction of MMP-2, -9, -14 and -15 expression with increasing age correlates with the reducing amount of migratory cells, mainly from the cerebellar EGL (Ulrich et al., 2005). Importantly, Vaillant et al., demonstrated that a specific MMP-9 blocking antibody on cerebellar explant cultures inhibited the granular cell migration in a dose dependant manner. They also showed that granular precursor cell migration was significantly delayed in the MMP-9 deficient mice (Vaillant et al., 2003). Above evidence strong suggest that MMPs assist in granular cell migration during cerebellar development. Likewise, Bovetti et al., recently showed that an array of MMPs including MMP-2, MMP-9, MMP-11, MMP-19, MMP-20, MT1-MMP, MT2-MMP, MT3-MMP, MT4-MMP and MT5-MMP were expressed in the mouse rostral migratory stream (RMS). Furthermore, administration of an furin-inhibitor that inhibits the furin-convertase enzymes including MMP-11, MMP-23 and all the MT-MMPs significantly attenuated the individual neuroblast migration indicating that these MMPs may contribute
to the neuroblast migration in the developing mouse brain (Bovetti et al., 2007). Together, these evidences strongly suggest that MMPs contribute fundamentally to the migration of neuronal precursors during brain development.

**Neurite Outgrowth**

It was demonstrated that MMP-2 (Ayoub et al., 2005) MMP-3 (Vaillant et al., 1999) and MMP-9 (Vaillant et al., 1999) were strongly expressed in the Purkinje (PK) cell layer in the rat cerebellum approximately at P10 that correlates to the period of arborisation of the PK cells suggesting a role for these MMPs in neurite outgrowth. Further, a specific MMP-9 blocking antibody was shown to inhibit the neurite outgrowth in cerebellar explant culture (Vaillant et al., 2003) confirming that MMP-9 supports arborisation of cerebellar neurons. Moreover, Hayashita-Kinoh et al., demonstrated that MT5-MMP was expressed at the edge of the growth cone of the dorsal root ganglion neurons suggesting that MT5-MMP may participate in axonal growth. They then confirmed that MT5-MMP indeed facilitated neurite growth by showing that administration of recombinant MT5-MMP eliminated the inhibitory effect of the proteoglycans on neurite extension of these neurons (Hayashita-Kinoh et al., 2001).

**Myelination**

Myelination is another important brain developmental process that seemingly requires MMP activity. Indeed, it was demonstrated that period of MMP-9 (Uhm et al., 1998) and MMP-12 (Ulrich et al., 2005) expression in the postnatal mouse brain positively correlates with the period of myelination (Clancy et al., 2001). Crucially, Larsen et al., showed that MMP-9 and/or MMP-12 null mice had deficient myelination in the corpus colossum from P7 to 14 as compared with the wild type mice, further proving that MMP-
9 and MMP-12 are important during developmental myelination (Larsen et al., 2006). MMP-9 (Uhm et al., 1998) and MMP-12 (Larsen and Yong, 2004) were also shown to facilitate oligodendrocyte (OL) process outgrowth. Moreover, it was also shown that elimination of MMP-9 activity by pharmacological MMP-9 inhibitors (Uhm et al., 1998), function-perturbing anti-MMP-9 antibodies and MMP-9 gene knockout (Oh et al., 1999) retarded the process outgrowth of OLs in cultures. Therefore, it can be suggested that MMP-9 may contribute to myelination partly or fully by facilitating the OL process outgrowth.

1.4.6 Matrix Metalloproteinases in Cerebral Ischemia

1.4.6.1 MMP Response following Ischemia

Accumulating evidence indicates that MMPs, predominately MMP-2 and MMP-9 and occasionally MMP-3, are upregulated following cerebral ischemia in the adult brain. In one of the earliest studies of investigating MMPs in cerebral ischemia, Rosenberg et al., 1996 demonstrated increased activities of MMP-9 and 2 respectively at 12 hours and five days following injury in a rat model of focal cerebral ischemia (Rosenberg et al., 1996). In 1997 Clark et al., then produced the first evidence of MMP association in cerebral ischemia in humans. They showed that brain MMP-9 activity was markedly increased two days following infarction while MMP-2 activity was only significantly increased after four months or longer in stroke patients dying at different time points after focal cerebral ischemia (Clark et al., 1997). Thereafter, ample animal studies have revealed that MMP-9 was induced within the initial hours to days following transient focal cerebral ischemia in the adult brain (Rosenberg et al., 1998, Fujimura et al., 1999, Gasche et al., 1999, Planas et al., 2001, Rosenberg et al., 2001, Koh et al., 2005, Gao et al., 2006). Similar findings demonstrating MMP-9 upregulation immediately following injury was
also demonstrated subsequently to permanent focal cerebral ischemia (Romanic et al., 1998, Gasche et al., 1999, Asahi et al., 2000, Asahi et al., 2001). Furthermore, several studies on global cerebral ischemia has also demonstrated an early elevation of MMP-9 activity following injury (Rivera et al., 2002, Zalewska et al., 2002, Lee et al., 2004). Importantly, a recent study on human stroke patients showed that serum MMP-9 levels of ischemic patients were increased dramatically by day one and remained elevated until day 12 (Horstmann et al., 2003). Conversely, MMP-2 protein expression was illustrated to be predominately increased several days following injury in the animal models of transient focal cerebral ischemia, (Rosenberg et al., 1998, Fujimura et al., 1999, Planas et al., 2001, Rosenberg et al., 2001), permanent focal cerebral ischemia (Romanic et al., 1998, Gasche et al., 1999) and global cerebral ischemia (Zalewska et al., 2002, Lee et al., 2004). However, several studies indicated that following transient cerebral ischemia, a moderate elevation of MMP-2 protein expression was also observed within the early hours (Fujimura et al., 1999, Gasche et al., 1999, Planas et al., 2001, Magnoni et al., 2004, Yang et al., 2007). Likewise, MMP-3 was also shown to be elevated after transient cerebral ischemia in rat brains (Rosenberg et al., 2001, Sole et al., 2004). Horstmann et al., provided further evidence of a MMP-3 involvement during cerebral ischemia by showing that serum MMP-3 amount was increased at one day following injury in the human patients that suffered stroke. Above evidence strongly suggests that principally MMP-2 and MMP-9 are significantly upregulated following an ischemic injury to the adult brain. MMP-9 appears to be predominately induced within early periods following an ischemic injury, whereas MMP-2 is strongly upregulated during the later periods following ischemia. However, to date there is little evidence of MMP upregulation following an ischemic injury to the developing brain. Recently, Schulz and colleagues demonstrated that plasma levels of MMP-9 were significantly higher in infants with
intraventricular haemorrhage suggesting a possible role of MMP-9 in perinatal brain injury (Schulz et al., 2004)

1.4.6.2 Effect of MMP Deficiency

The strongest evidence that suggest MMPs contribute to the pathophysiology of cerebral ischemia arises from studies that investigate the effect of MMP deficiency following a HI injury. Romanic et al., showed that intravenous administration of a neutralizing monoclonal antibody directed against MMP-9 reduced the infarct volume developing following permanent middle cerebral artery occlusion (MCAO) (Romanic et al., 1998). Treatments with synthetic broad spectrum MMP inhibitor that include BB-94 (Asahi et al., 2000, Jiang et al., 2001, Pfefferkorn and Rosenberg, 2003, Lee and Lo, 2004, Lee et al., 2004), KB-R7785 (Jiang et al., 2001), and GM6001 (Amantea et al., 2007) significantly reduced infarct volumes following cerebral ischemia in various animal models. Several naturally occurring molecules including estrogen (Liu and Rosenberg, 2005), resvertrol (Gao et al., 2006), and quercetin (Gao et al., 2006) that were demonstrated to be neuroprotective following cerebral ischemia also reduced the ischemic-induced MMP-2 and 9 activity indicating that their beneficial effects were at least partly mediated through inhibition of MMPs. Also several studies illustrated that hypothermia induced reduction of ischemic infarction following cerebral ischemia was presumably mediated through inhibition of MMP-2 and MMP-9 (Hamann et al., 2004, Lee et al., 2005). Importantly, Gu et al., 2005 demonstrated that a highly specific inhibitor of MMP-9, SB-3CT, considerably attenuated the infarction following transient MCAO in mice, providing strong evidence for MMP-9 participation the pathogenesis of brain ischemia (Gu et al., 2005). Furthermore, MMP-9 knock-outs were demonstrated to develop significantly diminished infarcts following an ischemic injury as compared to the
wild-types (Asahi et al., 2000, Asahi et al., 2001, Lee and Lo, 2004, Lee et al., 2004, Copin et al., 2005). However, a contradictory argument is produced by few studies demonstrating that MMP deficiency was neuroprotective. A recent study demonstrated that treatments with MMP broadspectrum inhibitor, BB-1101, not only failed to attenuate the lesion volume but also exacerbated the neurological behaviour of rats following transient MCAO (Sood et al., 2008).

1.4.6.3 Localization of Matrix Metalloproteinases in Cerebral Ischemic Brain

MMP-2 mRNA, protein and enzymatic activity have been reported to be expressed in a variety of cell types after cerebral ischemia. Evidence suggest that MMP-2 was observed in ischemic neurons during early hours following an ischemic injury (Planas et al., 2001, Magnoni et al., 2004, Lee et al., 2005, Wang et al., 2007, Yang et al., 2007) possibly indicating that it participates in neuronal death. Furthermore, it may also contribute to blood brain barrier (BBB) disruption as indicated by its localization in endothelia cells immediately following ischemia (Planas et al., 2001, Magnoni et al., 2004, Yang et al., 2007). MMP-2 was also observed in astrocytes (Figure 1.15) during the early period following cerebral ischemia (Planas et al., 2001, Magnoni et al., 2004, Lee et al., 2005, Yang et al., 2007) mainly in the end feet adjoining the blood vessels. Conversely, MMP-2 was principally located in reactive astroglia (Rosenberg et al., 2001, Magnoni et al., 2004), microglia or macrophages (Romanic et al., 1998, Planas et al., 2001, Magnoni et al., 2004) during later periods following cerebral ischemia suggesting a possible function in recovery mechanisms. Above evidence suggest that MMP-2 is differentially localised during the different phases following ischemia presumably contributing differentially to the injury/recovery process.
Figure 1.15: MMP-2 immunoreactivity in an adult rat brain following 90 min MCAO and 3 hours of reperfusion showing astrocytic-like immunoreactivity (Adapted from Rosenberg et al, 2001).

Similarly MMP-9 immunoreactivity is also detected in a wide range of cell types following ischemic injuries. Immunohistochemical studies have illustrated that MMP-9 was predominately located in morphologically identified ischemic neurons (Figure 1.16a) following injury (Planas et al., 2001, Rosenberg et al., 2001, Magnoni et al., 2004, Lee et al., 2005, Wang et al., 2007). Double labelling experiments demonstrating colocalisation of MMP-9 with the neuronal marker, NeuN, in the ischemic core further proved that MMP-9 was expressed by neurons (Gu et al., 2002, Rivera et al., 2002, Gu et al., 2005). These studies suggest a strong participation of MMP-9 in neuronal degeneration. Furthermore, MMP-9 was frequently observed in endothelia cells (Figure 1.16b) within the ischemic lesion immediately after injury (Romanic et al., 1998, Rosenberg et al., 2001, Kim et al., 2003, Rosell et al., 2006) presumably indicating a role in BBB disruption. Moreover, MMP-9 was also demonstrated to be located in neutrophils (Romanic et al., 1998, Rosenberg et al., 2001). Several studies have demonstrated a positive correlation between MMP-9 increase and neutrophil infiltration after transient MCAO further suggesting that neutrophils may be an important source of MMP-9 during ischemia (Planas et al., 2001, Justicia et al., 2003, Maier et al., 2004). Additionally, MMP-9 was observed in microglia (Rivera et al., 2002, Lee et al., 2005) and
macrophages (Romanic et al., 1998, Magnoni et al., 2004) respectively during the initial and latter phases following ischemia in several animal models.

Figure 1.16: MMP-9 immunohistochemistry in an ischemic cortex at 48 h after 90 min MCAO and 3 hours of reperfusion showing diffuse extracellular staining with some neuronal immunoreactivity (a), endothelial immunoreactivity (b), and occasional glial immunoreactivity (c) (Adapted from Rosenberg et al, 2001).

In contrast to MMP-2 and MMP-9, MMP-3 is less frequently studied in the ischemic brain. Rosenberg et al., have shown MMP-3 immunoreactivity in neurons, activated microglia/macrophages and in extracellular space around blood vessels in the ischemic cortex (Rosenberg et al., 2001). Sole et al., also showed MMP-3 expression in neurons, oligodendrocytes, microvasculature and reactive microglia/macrophages in ischemic rat brains (Sole et al., 2004).

1.4.6.4 Mechanism of Activation

As mentioned earlier, MMP-2, MMP-3 and MMP-9 are mainly activated in a stepwise manner either by endogenous proteinases in the ECM (Nagase, 1997). Although the factors that are involved in the activation process of these proteinases during cerebral ischemia have not been clearly identified, evidence to date suggests few possibilities. It has been found that inhibition of nitric oxide synthase resulted in declined MMP-9 expression (Gursoy-Ozdemir et al., 2000). Also Gasche et al., demonstrated that MMP
activity, in mice deficient in superoxide dismutase (SOD), is much higher than that of wild type after transient focal cerebral ischemia. They also observed colocalization of MMP activity with ROS in capillary walls and astrocytic processes (Gasche et al., 2001). Further Cho et al., showed that administration of a strong antioxidant, quercetin, significantly reduced the ischemia-induced MMP-9 activity (Cho et al., 2006). According to the above results it can be suggested that oxidative stress plays an important role in MMP activation. It has also been demonstrated that oxidative stress induced MMP-9 expression in human brain endothelial cell cultures was accompanied by a significant elevation of NF-κB immunoreactivity localised to the nuclei suggesting that oxidative stress may increase MMP-9 gene transcription via the transcription factor NF-κB (Kolev et al., 2003). Koh et al., complemented above evidence by showing that MMP-9 reduction by an inhibitor of nuclear enzyme PARP also accompanied a reduction of NF-κB (Koh et al., 2005). Also inflammation possibly contributes to the upregulation of MMPs following an ischemic insult. A recent study showed that an anti-inflammatory agent, nimesulide, attenuated the ischemia-induced MMP-2 and 9 protein expression following transient cerebral ischemia in rats (Wang et al., 2007). Tissue plasmingen activator (tPA) is another well known activator of MMPs. Sumii et al., showed that treatment with tPA after focal ischemia significantly increased the levels of both pro and cleaved forms of MMP-9, which suggests that tPA may promote MMP-9 induction (Sumii and Lo, 2002). Administration of the broad spectrum MMP inhibitor BB-94 reduced the recombinant tPA (rtPA) induced mortality in delayed reperfusion after MCAO, which again suggests that rtPA may exert its effect through MMP (Pfefferkorn and Rosenberg, 2003). The role of plasminogen activators in activation of MMPs will be discussed more thoroughly in section 1.5.
1.4.6.5 Mechanism of Action

An abundance of evidence indicates that MMP deleteriously contributes to the pathogenesis of brain ischemia. A recent study on human ischemic stroke showed that plasma MMP-9 concentration seven days following stroke positively correlated with the worsening of clinical outcome at three months (Lucivero et al., 2007). Accumulating evidence indicates that BBB permeability increases with the increased MMP expression after cerebral ischemia (Rosenberg et al., 1996, Fujimura et al., 1999, Gasche et al., 1999). Also deficiency of MMPs, in particular MMP-9, was shown to attenuate the BBB permeability following an ischemic injury in ample animal studies (Asahi et al., 2001, Lee et al., 2005, Liu and Rosenberg, 2005, Shigemori et al., 2006, Yang et al., 2007). Together, this evidence suggests that MMP activity causes blood brain barrier disruption, thus presumably contributing to the injury mechanisms following cerebral ischemia. It is further proven by showing that MMP-9 knock-out mice have reduced BBB component zonae occludens-1 in a mouse model of transient focal cerebral ischemia (Asahi et al., 2001). Further, inhibition of MMP-2 and MMP-9 by hypothermia reduced degradation of basal lamina collagen type IV loss in rats after transient MCAO (Hamann et al., 2004). Recently, Yang et al., also showed that degradation of two major tight junction proteins of cerebral vascular endothelia, claudin-5 and occluding, following focal ischemia could be restrained by a MMP inhibitor (Yang et al., 2007). Apart from its effect on the blood brain barrier, MMPs are also known to affect the cell survival by modifying the cell-matrix interactions (Nagase, 1997, Sethi et al., 2000, Bosman and Stamenkovic, 2003). Localization of MMP-9 activity after global cerebral ischemia has been shown to correlate strongly with the location of maximal laminin degradation within the hippocampus (Zalewska et al., 2002). Recently Gu et al., suggested that laminin degradation that was induced by MMP-9 activity was positively associated with the
apoptotic neuronal death following transient cerebral ischemia in mice (Gu et al., 2005). Another study supplemented above evidence by showing that broad-spectrum MMP inhibitor was capable of attenuating neuronal apoptosis in a rat model of focal cerebral ischemia (Copin et al., 2005). According to above evidence, it is possible that MMPs are affecting the neuronal survival by modulating their interaction with the ECM by degrading prevalent brain ECM molecules such as laminin. In addition, Asahi et al., have previously demonstrated MMP-9 knock-out mice to have reduced white matter associated myelin basic protein after transient cerebral ischemia (Asahi et al., 2001) suggesting another possible role for MMPs in axonal degeneration. Conversely, given their wide range of substrates and differential expression profiles it can be suggested that MMPs beneficially contribute in the recovery mechanisms following an ischemic injury. In fact, a recent study showed that plasma concentration of MMP-2 negatively correlated with the severity of injury in human stroke patients suggesting an advantageous role for MMP-2 in brain ischemia (Lucivero et al., 2007). Furthermore Lee et al., demonstrated that migrating neuroblast cells from the SVZ also expressed MMP-9 during the two week recovery period after transient focal cerebral ischemia in mice. They further showed that broad spectrum MMP inhibitor, GM6001, considerably reduced the migration of these neuroblasts from SVZ into the striatum confirming that MMPs play an important role in their migration (Lee et al., 2006).

1.5 PLASMINOGEN ACTIVATORS

1.5.1 Introduction

Plasmin is a broad–spectrum serine protease of trypsic specificity that can cleave various ECM proteins, activate proteinases and deliver growth factors. Plasminogen is the precursor protein that is cleaved by plasminogen activators to produce active plasmin
(Figure 1.17). Tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) are the major types of plasminogen activators (PAs) identified in mammals to date. Similar to plasmin, they are serine proteases with tryptic specificity but with restricted substrate specificity. tPA and uPA are products of distinct genes that differ in the domain organisation and function of their noncatalytic regions (Saksela and Rifkin, 1988, Mohanam et al., 1994, Parry et al., 2000, Castellino and Ploplis, 2005).

1.5.2 Plasminogen to Plasmin

Plasminogen is secreted as a single-chain glycoprotein with a molecular mass of ~92kDa. It is comprised of a N-terminal pre-activation peptide, five kringle domains and a catalytic C-terminal serine proteinase domain (Wiman and Wallen, 1975, Forsgren et al., 1987). It is found abundantly in the vasculature and also in other body fluids. It circulates in a globular, “closed” confirmation which is not readily susceptible to proteolysis by the PAs. It adopts an extended, “open” confirmation that is more rapidly activated to plasmin when it is bound to a surface. The lysine-binding sites in the kringle domains of the protein mediate its localization to surfaces by binding to various receptors on cell membranes and ECM proteins. Circulating plasminogen has a glutamine acid at its NH₂-terminus end and, thus is known as the Glu-plasminogen. Active plasmin mediates the cleavage of the pre-activation peptide of Glu-plasminogen producing Lys-plasminogen, which is more readily activated by PAs. Then PAs catalyse the cleavage of the peptide bond Arg₅₆₁-Val₅₆₂ at the end of the kringle domains to produce a two-chain plasmin held together by two disulfide bonds (Saksela and Rifkin, 1988, Angles-Cano, 1994, Plow et al., 1995, Parry et al., 2000, Castellino and Ploplis, 2005). Plasmin displays a wide range of activities. Although fibrin is the classical substrate of the proteinase it is now evident that it can also cause the proteolysis of other extracellular molecules such as laminin and
fibronectin. Plasmin also acts as a major activator of several pro-MMPs including MMP-1, MMP-3, and MMP-9 which also degrade the ECM. Therefore, activation of plasminogen to plasmin may lead to excessive degradation of the ECM (Figure 1.17). However, plasmin may also be beneficial by enhancing the activity of growth factors that include TGF-β, bFGF and VEGF by either activating the latent forms or releasing them from the ECM (Saksela and Rifkin, 1988, Mayer, 1990, Parry et al., 2000, Castellino and Ploplis, 2005).

**Figure 1.17: Schematic diagram of protease cascade that leads to ECM modulation**

### 1.5.3 Tissue Plasminogen Activator

tPA is believed to be the major physiological vascular activator of plasminogen. It is secreted as a single chain glycoprotein with a molecular mass of 64kDa. It consists of a finger domain at the NH$_2$ terminus followed by a growth factor domain and two kringles and a serine protease domain at the COOH$_2$ terminus. The finger domain and the second kringle account for the high fibrin binding affinity of the enzyme. The growth factor domain is not known to be coupled with a specific function. Unlike any of the other proteinases the single chain form of tPA (sctPA) possesses high catalytic activity. In the presence of plasmin or other proteases including trypsin, sctPA can be cleaved at the end of the second kringle producing a two chain form (tctPA) held together by a single
disulfide bond. The catalytic activity of the tctPA appears to be 5-10 fold higher than that of the sctPA. tPA is produced predominately by endothelial cells but also expressed by keratinocytes, melanocytes and neurons (Saksela and Rifkin, 1988, Angles-Cano, 1994, Plow et al., 1995, Parry et al., 2000, Castellino and Ploplis, 2005).

1.5.4 Urokinase Plasminogen Activator

It has been demonstrated that uPA is the most important activator of plasminogen in the extravascular space. Similarly to plasminogen and tPA, pro-uPA is also secreted as a single chain glycoprotein with a 54kDa. scuPA, in contrast to the sctPA, has no or little catalytic activity. The enzyme is transformed into a two chain active form held together by a single disulfide bond following cleavage of a single peptide bond that can be mediated by proteases such as plasmin, trypsin and kallikrein. The N-terminal region (A chain) includes a growth factor domain followed by one kringle domain. The C-terminal region (B chain) consists of the serine protease domain. The growth factor domain of the protease contains the receptor binding site (Blasi et al., 1987, Saksela and Rifkin, 1988, Mayer, 1990, Angles-Cano, 1994, Plow et al., 1995, Spraggon et al., 1995, Parry et al., 2000, Castellino and Ploplis, 2005).

The uPA receptor (uPAR) is a carbohydrate-rich integral membrane protein that is present on the plasma membrane of many cell types. It can facilitate the recruitment of bound uPA to areas where proteolysis is needed. It binds to both the scuPA and tcuPA with similar kinetics. The receptor bound scuPA is shown to be converted to tcuPA with high affinity. uPA bound to the receptor maintain its catalytic activity thus attributing the cell a pericellular proteolysis activity (Saksela and Rifkin, 1988, Mohanam et al., 1994, Parry et al., 2000).
Plasmin can further process the tcuPA to a 33kDa molecule that lacks the growth factor domain and the kringle domain. Although this lower molecular weight enzyme lacks the ability to bind to its receptor, it possesses full catalytic activity (Blasi et al., 1987, Sakse and Rifkin, 1988).

1.5.5 Plasminogen Activators in Cerebral Ischemia

1.5.5.1 Evidence

Tissue plasminogen activator is the only FDA approved treatment for ischemic stroke. However, emerging data suggest that both plasminogen activators (tPA and uPA) can be potentially neurotoxic.

A considerable body of literature derived from animal experiments proposes that both tPA and uPA are upregulated after cerebral ischemia. uPA activity in the ischemic hemisphere was increased from four hours to one day after embolic focal cerebral ischemia in mice (Ahn et al., 1999). Yepes et al., 200 showed that both tPA and uPA activities were significantly increased within the region of the infarct within six hours in their rat model of stroke. Further they showed that injection of neuroserpin, a PA inhibitor, reduced the tPA and uPA activities and the infarct volume (Yepes et al., 2000). Increased levels of tPA and uPA immunoreactivity were seen in reactive astrocytes, microglia and endothelial cells surrounding the lesion zone in ischemic human brain (Dietzmann et al., 2000). Hosomi et al., showed that both tPA and uPA mRNA levels were significantly increased in the ischemic cortex following MCAO in rats. uPA activity and antigen was shown to be significantly increased within the ischemic basal ganglia one hour after MCAO in primates (Hosomi et al., 2001).
Most compelling evidence of a neurotoxic potential of PAs comes from tPA knockout studies. In 1998 a study by Wang and colleagues showed that tPA knockout mice had decreased microglia activation, neuronal loss and smaller infarct volume than wild-type mice following MCAO. Further, they showed that intravenous administration of exogenous rtPA exacerbated the injury in both wild-type and knockout mice (Wang et al., 1998). In a mouse model of transient focal cerebral ischemia, tPA gene inactivation caused a reduction in infarct volume while plasminogen activator inhibitor (PAI) gene inactivation caused an increase in infarct volume (Nagai et al., 1999). Tsuji et al., demonstrated that infarct size and edema were significantly lower in tPA knockouts than in wild types after focal cerebral ischemia (Tsuji et al., 2005). tPA deficiency was also demonstrated to reduce the hippocampal cell death in a global cerebral ischemia (Lee et al., 2007).

The above evidence strongly suggests that plasminogen activators may have a deleterious effect during cerebral ischemia. However, several studies have provided contradictory evidence suggesting that PAs play beneficial role during ischemia. In a mouse model of transient cerebral ischemia tPA knockout mice were found to have larger infarct volumes than wild type mice after MCAO (Tabrizi et al., 1999). However, Sheehan and Tsirka suggested that it could be due to the fact that in this study the nylon filament used to induce MCAO was not coated with silicon to prevent thrombosis thus coat formation occurred during the procedure (Sheehan and Tsirka, 2005). Atochin et al., showed that tPA knockouts aggravates ischemic thrombotic infarction after microembolic infusion compared to wild types (Atochin et al., 2004). Several other studies have demonstrated that exogenous administration did not have any detectable detrimental effect in rat...
models of global, focal (Klein et al., 1999), embolic and mechanical (Meng et al., 1999) cerebral ischemia. These studies suggest that plasminogen activators are likely to have an advantageous effect on forms of cerebral ischemia that require clot lysis for resolution (Sheehan and Tsirka, 2005).

1.5.5.2 Mechanism of Action

A large body of work derived from animal experiments proposes that plasminogen activators can be harmful when present in high concentration in brain parenchyma. Plasminogen activators could be exerting their deleterious effect principally through their interaction with plasminogen thus promoting ECM degradation.

Both tPA and uPA cleaves plasminogen, the inactive precursor protein, to produce active plasmin. Mice lacking plasminogen were shown to be resistant to excitotoxin-mediated hippocampal neuronal degeneration (Tsirka et al., 1997). Increased plasminogen activity was observed at three hours in the areas of structural injury following MCAO in rats (Pfefferkorn et al., 2000a). Increased plasminogen activation was seen in the basal ganglia and cortex of ischemic hemisphere at two, three and seven days following unilateral permanent focal cerebral ischemia. Further, areas of these plasminogen activation colocalised with the areas of ischemia-induced MAP-2 degradation (Pfefferkorn et al., 2000b). Administration of plasmin or plasminogen to the rat brain significantly increased the number of apoptotic neurons in the striatum (Xue and Bigio, 2001). The above evidence proposes that plasmin have a detrimental effect on neuronal degeneration. Therefore it could be suggested that tPA exerts its deleterious effects by activating the plasminogen/plasmin system. Plasmin might exert a detrimental effect on brain parenchyma by directing cleaving certain ECM molecules such as laminin or
indirectly by activating ECM degrading MMPs such as MMP-2 and 9 (Tsirka et al., 1997, Kaur et al., 2004, Sheehan and Tsirka, 2005).

Hosomi et al., showed that increase of uPA activity following MCAO in primates coincided with the increase of MMP-2 and ECM damage (Hosomi et al., 2001). A study that investigated temporal profiles of MMPs in human stroke under different treatment paradigms showed that MMP-9 levels were significantly higher in patients treated with tPA as compared with patients treated with hypothermia (Horstmann et al., 2003). MMP-9 activity was reduced in tPA knockouts as compared to wild-types after focal cerebral ischemia (Wang et al., 1998, Tsuji et al., 2005). Furthermore, administration of exogenous tPA into tPA knockout animals reinstated their MMP-9 activity levels to levels comparable to that of wild type cerebral ischemic animals (Wang et al., 1998, Tsuji et al., 2005). Similar outcomes were obtained in a mouse model of global ischemia where they demonstrated that tPA deficiency amolirated the MMP-9 activity upregulation after injury (Lee et al., 2007). Kelly et al., showed that treatment with tPA was associated with the activation of MMP-9 at six, 12 and 24 hours following ischemia in rats (Kelly et al., 2006). A recent study showed that doxycyclin treatment reduces the cerebral damage after focal cerebral ischemia by reducing MMP-2, MMP-9 and uPA suggesting an involvement of plasmingen activator-plasmin-MMPs axis in the pathophysiology of injury (Burggraf et al., 2007).

Another body of evidence proposes a plasminogen activation independent neuronal effect for tPA (Pawlak and Strickland, 2002). In a mouse model of transient cerebral ischemia, tPA -/- mice were found to be resistant to neuronal death, whereas Plasminogen -/- mice has increased injury (Nagai et al., 1999). In 2001 Nicole et al., demonstrated that tPA
treatment potentiates NMDA-induced calcium influx and neuronal death in cortical neuron cultures. They also showed that tPA forms a direct complex with NR1 subunit of the NMDA receptor then cleaving a fragment of approximately 15–20 kD from its amino terminus (Nicole et al., 2001). These findings suggested that the direct action of tPA on NMDA receptors promotes excitotoxic neuronal death. Several other evidence from animal models of extotoxin induced injury further supports this argument. Injection of kainite into the amygdala and hippocampus resulted in simultaneous upregulation of tPA and seizures causing neuronal death. The propagation of seizures were attenuated in tPA knockout mice but not in plasminogen knockouts (Yepes et al., 2002). Treatment with a synthetic tPA inhibitor reduced the NMDA-mediated neuronal death in mouse cortical cell cultures and NMDA-induced lesions in rats (Liot et al., 2004). A recent study showed that exogenous neuroserpin protects the cortex and the striatum against NMDA-induced injury. It further showed that neuroserpin prevents the NMDA induced cell death presumably by decreasing the NMDA receptor-mediated intracellular calcium influx (Lebeurrier et al., 2005).

1.6 AIMS
Perinatal hypoxic-ischemic (HI) brain injury following birth asphyxia is considered one of the uppermost causes of enduring neurological deficits worldwide. Infants that survive perinatal brain injury are at risk of developing neurodevelopmental abnormalities that include mental retardation, learning disabilities, subtle motor abnormalities and vision or hearing impairments later in life. Such neurological handicaps remain a major problem to date for which there is no specific treatment. It is understood that neuronal death occurs in two distinct phases; acute and delayed phases following an HI injury to the brain. Although the mechanisms of neuronal death during the acute phase are fairly well
established, the mechanisms involved during the delayed phase are yet to be fully elucidated. It is now evident that the most significant amount of cell death occurs during the delayed phase rather than the acute phase (Inder and Volpe, 2000, Volpe, 2001a, Ferriero, 2004, Shalak and Perlman, 2004, Rennie et al., 2007). Therefore a better understanding of the cellular mechanism associated with the delayed neuronal death is vital in developing novel therapeutic strategies for developing brain injury.

MMPs are a family of zinc dependant endopeptidases that are capable of degrading all components of the ECM. They are considered to be integrally involved in CNS development (Nagase and Woessner, 1999, Stamenkovic, 2003). However, recent evidence strongly suggests that the inappropriate activity of these proteases contribute to the pathogenesis of cerebral ischemia in the adult brain (Planas et al., 2001, Pfefferkorn and Rosenberg, 2003, Gu et al., 2005). Given that ECM disruption is a prominent feature of lesions in the developing brain (Meng et al., 1997, Volpe, 1998, Volpe, 2001b, Blumenthal, 2004, Sizonenko et al., 2005), it is possible that MMPs play an important role in injury to the developing brain.

The overall objective of this thesis was to evaluate the hypothesis that MMP-2 and 9 participate in the pathophysiology of hypoxic ischemic injury in the developing brain using a well characterised rat model of unilateral HI injury at two developmental stages; P3 and P21. Firstly, we characterised the MMP-2/9 expression in the normal developing forebrain. Given that ECM remodelling is an essential process during CNS development, it was fundamental to firstly define the developmental changes of MMPs in the normal brain. Secondly, we determined the MMP-2 and 9 responses following HI injury at the two developmental stages; P3 and P21. Thirdly, we characterised changes in plasminogen
activators, tPA and uPA, which are upstream activators of MMPs, to determine the mechanisms of MMP-2 and 9 activity induction after HI injury. Finally, we inhibited MMP-9 activity using a very specific MMP-2/9 inhibitor, to determine if the absence of MMP-9 activity is neuroprotective to the developing brain.
CHAPTER TWO: MATERIAL AND METHODS

2.1 HYPOXIC ISCHEMIC INJURY

All experiments were approved by the University of Auckland Animal Ethics Committee, New Zealand. Wistar rats were maintained under standard light (8am-8pm), temperature (22±2°C) and humidity (55±5%) conditions and fed *ad libitum*.

2.1.1 Postnatal Day 21

Postnatal day 21 (P21) old Wistar rats were obtained from litters bred at the Animal Resource Unit of the School of Medicine, University of Auckland. Breeding females were kept at 22°C with a 12-hour cycle of light and dark. Rats of either sex weighing 45±5g at day 21 of age were selected for use in the hypoxic ischemic (HI) preparation. Rats were initially anaesthetized with 5% halothane/oxygen (O₂) and then maintained on a 2% halothane/O₂ mixture. The right common carotid artery was exposed through a mid-ventral neck incision. The artery was then separated from the vagus nerve and doubly ligated with electrosurgical forceps (0.7mm; Bipolar Blunt Tip Forceps; Erbe ICC, Germany) or silk sutures (Fine Science Tools, Canada). The neck incision was stitched closed and rats were allowed to recover at room temperature (RT). Once recovered, the ligated rats were held for one hour in an infant incubator, kept at a stable thermoneutral environment of 34°C with a relative humidity of 85±5% and with circulating air, in order to equilibrate the animals for the conditions during hypoxia. While still in the incubator the ligated rats were exposed to hypoxia of 8% O₂ in nitrogen (N₂) for 60 minutes. The O₂ percentage was continuously monitored on an industrial gas analyser (Rapidx 2001; Cambridge Sensotec Ltd, UK). The O₂ percentage reading was also checked against a 6% O₂ in N₂ standard (BOC, New Zealand) to adjust for daily changes in barometric pressure. The desired O₂ percentage was achieved by flooding the incubator with N₂ (O₂
free) at 30L/min until the O₂ was around 9.8% after which the N₂ flow was dropped to approximately 12L/min and let in with normal air at 4-6L/min. The timing for the hypoxia was started once a stable O₂ percentage of between 7.9% and 8.2% was reached. At the end of 60 minutes, the animals were removed from the incubator and allowed to recover at RT. They were held at RT (22°C, 55%±5% relative humidity) until killed at an appropriate time by an overdose of sodium pentobarbitone (Pentobarb 300; Chemstock International, New Zealand) via intraperitoneal (IP) administration.

2.1.2 Postnatal Day Three

Postnatal day three (P3) old Wistar rats were obtained with the dam from the Animal Resource Unit of the School of Medicine, University of Auckland. Breeding females were kept at 22°C with a 12-hour cycle of light and dark. Rats of either sex weighing 8-10g at day three of age were selected for use in the HI preparation. Rats were initially anaesthetized with 5% halothane/O₂ and then maintained on a 2% halothane/O₂ mixture. They were placed on their back in a specially designed rat pad which included a thermal barrier. Under a surgical microscope (Zeiss, Germany) the right common carotid artery was exposed through a mid-ventral neck incision, separated from the vagus nerve and doubly ligated with electrosurgical forceps. They were allowed to recover from the anaesthetic under a heat lamp after closing the incision with Steri-Strip™ (3M, USA). Following recovery, they were kept in an infant incubator at 37°C with 86-90% relative humidity for 30 minutes. While still in the incubator the animals were subjected to hypoxia at 6% O₂ for 30 minutes. The O₂ percentage was continuously monitored on the industrial gas analyser. The O₂ percentage reading was also checked against a 6% O₂ in N₂ standard to adjust for daily changes in barometric pressure. The desired O₂ percentage was achieved by flooding the incubator with N₂ (O₂ free) at 30L/min until O₂ is around 7-
7.5% after which it was dropped to 13L/min and let in with normal air at 4.2L/min. Once a stable O₂ percentage of between 5.9% and 6.1% was reached it was maintained for 30 minutes with regular monitoring on the gas analysis. Following hypoxia the animals were allowed to recover in the incubator for about 30 minutes before returning to the dam. At the appropriate time point after the injury the animals were killed by administration of an IP overdose of sodium pentobarbitone (150mg/kg).

2.2 PARAFFIN EMBEDDING

The animals were killed by an overdose of sodium pentobarbitone. The ribcages were cut open to allow access to the heart. Blood was perfused out of the animals using sterile 0.9% saline solution by transcardial perfusion until no further evacuation of blood was obvious. The saline was then replaced by freshly prepared modified Bouin’s solution (0.1M phosphate buffered saline (PBS), 4% paraformaldehyde (w/v), 0.08% glutaraldehyde (v/v), 15% picric acid (v/v) (pH 7.4)). Approximately 50mL (P21) or 20mL (P3) of the fixative was perfused into the rats before the skulls were cut open and the brains were removed. The dissected brains were placed in the same solution for at least 24 hours to ensure complete fixation. They were then washed with 50% (P21) or 70% alcohol (P3) for a further three days replacing the solution daily. The brains were then cut into 2mm sections using a rat brain matrix (Activational Systems, Michigan, USA) and processed in an automated processing machine as outlined in Table 2.1 (P21) and Table 2.2 (P3). They were then blocked in wax and allowed to set on a cold plate.
### Table 2.1: Processing cycle used to process P21 rat brains in the processing machine

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Duration</th>
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<tbody>
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<tr>
<td>95% Alcohol</td>
<td>20 minutes</td>
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<td>95% Alcohol</td>
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<td>100% Alcohol</td>
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<td>100% Alcohol</td>
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<td>Chloroform</td>
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<td>Chloroform</td>
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<td>Wax</td>
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<td>Wax</td>
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<tr>
<td>Wax</td>
<td>10 minutes</td>
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### Table 2.2: Processing cycle used to process P3 rat brains in the processing machine

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Duration</th>
</tr>
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<tbody>
<tr>
<td>70% Alcohol</td>
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<tr>
<td>80% Alcohol</td>
<td>90 minutes</td>
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<tr>
<td>95% Alcohol</td>
<td>60 minutes</td>
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<td>90 minutes</td>
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<td>100% Alcohol</td>
<td>45 minutes</td>
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<tr>
<td>100% Alcohol</td>
<td>45 minutes</td>
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<tr>
<td>Chloroform</td>
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<td>Chloroform</td>
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<td>Wax</td>
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</table>

### 2.3 PROTEIN EXTRACTION

Animals were killed by an IP injection of sodium pentobarbitone at the appropriate time. They were then transcardially perfused with cold 0.01M potassium phosphate buffered saline (KPBS) (pH 7.4) until no evacuation of blood was observed. Brains were collected,
the olfactory bulbs and cerebellum were removed, and the two hemispheres were separated. Dissected hemispheres were frozen immediately in liquid N\textsubscript{2} and then stored at -80°C. The samples were later homogenised in lysis buffer (50mM tris-HCl (pH 7.4), 150mM NaCl, 1% Nonidet\textsuperscript{®} P-40, 0.1% sodium dodecyl sulfate (SDS), 0.1% deoxycholic acid, and 10% ethylene diamine tetra acetic acid (EDTA)-free mini tablets) using a mini bead beater. The resulting homogenate was then centrifuged at 14000xg for 10 minutes at 4°C and the supernatant was stored at -20°C. Protein concentrations of the tissue extracts were measured against a bovine serum albumin (BSA) (Sigma-Aldrich, New Zealand) standard curve using the bicinechonic acid (BCA) protein assay (Sigma-Aldrich, New Zealand).

2.4 RNA EXTRACTION

After the animals were killed, the brains were dissected immediately, separated into left and right hemispheres and further microdissected into specific regions, including the olfactory bulb, cortex, hippocampus, and cerebellum using a dissecting microscope. Tissues were frozen immediately in liquid N\textsubscript{2} and stored at -80°C. Total RNA from brain tissue was isolated using either TRIZOL\textsuperscript{®} (Invitrogen, New Zealand) or PureLink\textsuperscript{™} Microto-Midi kit (Invitrogen, New Zealand). The majority of the samples were homogenised in TRIZOL\textsuperscript{®} using a mini bead beater. The resulting homogenate was then centrifuged at 12000xg for 10 minutes at 4°C. Chloroform was added to the supernatant, mixed vigorously and incubated for two to three minutes at RT before centrifuging for 15 minutes at 12000xg at 4°C. The top layer aqueous phase was carefully transferred to a fresh tube, mixed with isopropl alcohol, incubated at RT for 10 minutes and centrifuged again at 12000xg for 10 minutes at 4°C. The supernatant was discarded and the remaining RNA pellet was washed in 75% alcohol. All the alcohol was carefully removed; the
pellet was air dried for about five minutes before dissolving in RNase-free water. Invitrogen’s PureLink™ Micro-to-Midi kit was used, according to the manufacturer’s instructions, for the total RNA isolation, which was performed by Mrs Larissa Christophidis. Finally, the RNA samples were treated with RNase-free recombinant DNase 1 (Ambion, USA; Invitrogen, New Zealand), according to the manufacturer’s instructions in order to remove any genomic DNA, before freezing them at -80°C. The quantity and quality of the extracted RNA were determined using the NanoDrop® ND-1000 (Biosciences, New Zealand) (Table 2.3). NanoDrop is a spectrophotometer that quantifies the nuclear acids (260nm), proteins (280nm) and other contaminants (230nm). A 260/280 and 260/230 ratio greater than 1.8 is considered to be an indicator of good quality RNA (Fleigea and Pfaffl, 2005).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day Injured Cortex (P21)</td>
<td>1099.75</td>
<td>27.494</td>
<td>2.08</td>
<td>1.95</td>
</tr>
<tr>
<td>1 day Injured Hippocampus (P21)</td>
<td>151.74</td>
<td>3.793</td>
<td>2.08</td>
<td>1.29</td>
</tr>
<tr>
<td>6 hours Injured cortex (P3)</td>
<td>224.3</td>
<td>5.608</td>
<td>2.08</td>
<td>1.48</td>
</tr>
<tr>
<td>6 hours Injured Hippocampus (P3)</td>
<td>216.96</td>
<td>5.424</td>
<td>2.07</td>
<td>1.47</td>
</tr>
</tbody>
</table>

Table 2.3: Nanodrop results of several representative samples

The integrity of the RNA extracted was further confirmed by electrophoresis of a representative sample on a denaturing agarose gel (Figure 2.1). Intact total RNA run on a denaturing gel is shown to produce sharp 28S and 18S rRNA bands. Approximately a 2:1 ratio of 28S:18S indicates that the RNA is of high integrity. Degraded RNA is shown to appear as a low molecular weight smear (Fleigea and Pfaffl, 2005).
2.5 cDNA SYNTHESIS

First-strand cDNA synthesis with oligo(dT) primers was carried out using a Superscript III First Strand Synthesis kit (Invitrogen, New Zealand) according to the manufacturer’s instructions.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>01 Total RNA</td>
<td>n</td>
</tr>
<tr>
<td>02. Oligo(dT) primers</td>
<td>1</td>
</tr>
<tr>
<td>03. 10mM dNTP mix</td>
<td>1</td>
</tr>
<tr>
<td>04. DEPC-treated water</td>
<td>10-n</td>
</tr>
<tr>
<td>05. 10x RT buffer</td>
<td>2</td>
</tr>
<tr>
<td>06. 25mM MgCl₂</td>
<td>4</td>
</tr>
<tr>
<td>07. 0.1M DTT</td>
<td>2</td>
</tr>
<tr>
<td>08. RNaseOUT™</td>
<td>1</td>
</tr>
<tr>
<td>09. SuperScript™ III RT</td>
<td>1</td>
</tr>
<tr>
<td>10. RNase H</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>21</strong></td>
</tr>
</tbody>
</table>

Table 2.4: Component composition for one reaction of cDNA synthesis
The protocol was as follows: Total RNA (500ng - 1μg) was first mixed with oligo(dT) primers, 10mM dNTP and DEPC-treated water (01-04: Table 2.4). The mix was incubated at 65ºC (Table 2.5) for 5 minutes and chilled on ice for at least one minute. cDNA synthesis mix (05-09: Table 2.4) was then added to the sample and incubated at 50ºC for 50 minutes, followed by 85ºC for 5 minutes (Table 2.5). Finally RNAse H (10: Table 2.4) was added and the mix was incubated at 37ºC for 20 minutes (Table 2.5). Samples were aliquoted and stored at -80ºC.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Temperature ºC</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denature</td>
<td>65 ºC</td>
<td>5</td>
</tr>
<tr>
<td>Annel</td>
<td>25 ºC</td>
<td>10</td>
</tr>
<tr>
<td>cDNA Synthesis</td>
<td>50 ºC</td>
<td>50</td>
</tr>
<tr>
<td>Termination</td>
<td>85 ºC</td>
<td>5</td>
</tr>
<tr>
<td>RNA Removal</td>
<td>37 ºC</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 2.5: Summary of cDNA synthesis procedure

2.6 ACID FUCHSIN AND THIONIN STAINING

Sections, which were of 8µm thickness, were cut onto 2,3-aminopropylsilane (Sigma-Aldrich, New Zealand) coated slides, deparaffinized in xylene, rehydrated in a descending alcohol series (Table 2.6). They were then stained in Thionin stain for 10 minutes. Following a quick wash in water, the sections were incubated in Acid Fuchsin stain for 30 seconds. They were briefly washed in water again, followed by six brief washes in 95% alcohol. Sections were then dehydrated by incubating for five minutes each in: 100% alcohol, 50/50 mix of 100% alcohol and xylene, and 100% xylene respectively. They were then mounted in dibutyl-phthalate-xylene (DPX) medium (BDH Laboratory Supplies, England) and left to air dry before examination under a light
microscope. Acid Fuchsin stained damaged cells pink while Thionin staining gave a blue nissl colour to the intact cells.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dewax Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Dewax Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Dewax Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Absolute Ethanol</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Absolute Ethanol</td>
<td>3 minutes</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>3 minutes</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>3 minutes</td>
</tr>
<tr>
<td>70% Ethanol</td>
<td>3 minutes</td>
</tr>
<tr>
<td>50% Ethanol</td>
<td>3 minutes</td>
</tr>
<tr>
<td>MQ Water</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>

Table 2.6: Descending alcohol series for hydrating paraffin embedded sections

2.7 IMMUNOHISTOCHEMISTRY

2.7.1 Single Immunohistochemistry

Sections of 8μm thickness were rehydrated as described in the above section (Table 2.6). These sections were subjected to an antigen retrieval procedures using 0.01M citrate buffer (pH 6) if necessary. They were then washed three times for five minutes in 0.01M KPBS. Non-specific binding of the secondary antibody was blocked with normal serum (from the animal in which the secondary antibody was raised) in 5mg/mL BSA/KPBS for two hours at RT. After a brief wash, they were further incubated with avidin and biotin (Biotin Blocking system; DAKO, Denmark) respectively for 10 minutes each. The sections were then incubated with the appropriate primary antibody in 5mg/mL BSA/KPBS for a specified time period (Table 2.7) at 4°C in a humidified chamber. At the end of the
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Dilution</th>
<th>Antigen-antireceptor</th>
<th>Wash</th>
<th>Incubation</th>
<th>Black</th>
<th>Block</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td>Enzo LifeSciences</td>
<td>1:200</td>
<td>Yes</td>
<td>0.01 M HEPES, pH 7.4</td>
<td>12 hours @ 4°C</td>
<td>5% NHS in 50 mM Tris, pH 8.0</td>
<td>Alkaline Phosphatase anti-alkaline phosphatase (APAAP)</td>
<td>Biotinylated HRP anti-mouse 1:1000</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Enzo LifeSciences</td>
<td>1:200</td>
<td>Yes</td>
<td>0.01 M HEPES, pH 7.4</td>
<td>12 hours @ 4°C</td>
<td>5% NHS in 50 mM Tris, pH 8.0</td>
<td>Alkaline Phosphatase anti-alkaline phosphatase (APAAP)</td>
<td>Biotinylated HRP anti-mouse 1:1000</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Enzo LifeSciences</td>
<td>1:200</td>
<td>Yes</td>
<td>0.01 M HEPES, pH 7.4</td>
<td>12 hours @ 4°C</td>
<td>5% NHS in 50 mM Tris, pH 8.0</td>
<td>Alkaline Phosphatase anti-alkaline phosphatase (APAAP)</td>
<td>Biotinylated HRP anti-mouse 1:1000</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Enzo LifeSciences</td>
<td>1:200</td>
<td>Yes</td>
<td>0.01 M HEPES, pH 7.4</td>
<td>12 hours @ 4°C</td>
<td>5% NHS in 50 mM Tris, pH 8.0</td>
<td>Alkaline Phosphatase anti-alkaline phosphatase (APAAP)</td>
<td>Biotinylated HRP anti-mouse 1:1000</td>
</tr>
<tr>
<td>GAP-43</td>
<td>Sigma-Aldrich</td>
<td>1:200</td>
<td>Yes</td>
<td>0.01 M HEPES, pH 7.4</td>
<td>12 hours @ 4°C</td>
<td>5% NHS in 50 mM Tris, pH 8.0</td>
<td>Alkaline Phosphatase anti-alkaline phosphatase (APAAP)</td>
<td>Biotinylated HRP anti-mouse 1:1000</td>
</tr>
<tr>
<td>GAP-43</td>
<td>Sigma-Aldrich</td>
<td>1:200</td>
<td>Yes</td>
<td>0.01 M HEPES, pH 7.4</td>
<td>12 hours @ 4°C</td>
<td>5% NHS in 50 mM Tris, pH 8.0</td>
<td>Alkaline Phosphatase anti-alkaline phosphatase (APAAP)</td>
<td>Biotinylated HRP anti-mouse 1:1000</td>
</tr>
</tbody>
</table>

Table 2.7: Primary antibodies used in the study and their optimal conditions
incubation period any excess primary antibody was removed by washing in 0.01M KPBS three times for five minutes at RT. The sections were then incubated in the biotinylated secondary antibody (Vector Laboratories, UK) at 1/200 in 5mg/mL BSA/KPBS for one hour at RT. They were then washed in KPBS three times at five minute intervals before incubating with Alexa 568 (1:200; Vector Laboratories, UK) for one hour at 37°C. They were mounted in Vectashield (Vector Laboratories, UK) after washing in 0.01M KPBS and stored at 4°C.

2.7.2 Double Immunohistochemistry

Procedures for the first antibody were carried out as described above, followed by extensive washing and reblocking with 5% or 10% normal serum from the animal in which the second secondary antibody was raised in. Sections were incubated with the second primary antibody in 5mg/mL BSA for the appropriate time period (Table 2.7) at 4°C. At the end of the incubation the antibody was washed off with 0.01M KPBS, the sections were incubated with the second biotinylated secondary antibody (1:200; Vector Laboratories, UK) for one hour at RT followed by Alexa 488 (1:200; Vector Laboratories, UK) for one hour at 37°C. Alternatively, sections were incubated with an Alexa 468 conjugated secondary antibodies. These sections were then mounted in vectashield and stored at 4°C.

2.8 GELATIN ZYMOGRAM

Gelatin zymography was used to determine the activity levels of matrix metalloproteinase (MMP) 2 and 9. 10% gelatin zymograms were either bought from Invitrogen (New Zealand) or prepared in-house using Bio-Rad Laboratories (USA) gel casting apparatus. For in-house gels separating and stacking gel mixers were prepared as outlined below
(Table 2.8). The separating gel mixer was loaded between the glass plates of the gel casting apparatus immediately after adding ammonium persulfate (APS) and N,N,N,N-tetramethylethylenediamine (TEMED) that initiate polymerisation. It was overlaid with MQ water to exclude oxygen from the surface of the polymerizing gel and decanted after the gel was allowed to polymerise for approximately 45 minutes. A stacking gel mixer was loaded on top of the separating gel and allowed to polymerise for approximately 15 minutes.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Separating mL</th>
<th>Stacking mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>4.0</td>
<td>2.1</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>3.3</td>
<td>0.5</td>
</tr>
<tr>
<td>0.5M tris-HCl (pH 6.8)</td>
<td></td>
<td>0.38</td>
</tr>
<tr>
<td>1.5M tris-HCl (pH 8.8)</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>10% Gelatin</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1</td>
<td>0.03</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.1</td>
<td>0.03</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.004</td>
<td>0.003</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 2.8: Reagent composition for casting 10% gelatin zymograms

36μg of protein in non-reducing sample buffer (0.4M tris (pH 6.8), 5% SDS, 20% glycerol, 0.05% bromophenol blue) was loaded onto 10% gelatin zymograms. Electrophoresis was performed in tris-glycine running buffer (25mM tris, 192mM glycine and 0.1% SDS), until the sample buffer reached the bottom of the gel, for 90 minutes at 150V for in-house cast zymograms and three hours at 100V for Invitrogen pre-cast zymograms. They were then washed in renaturing buffer (2.7% Triton® X-100) twice for 15 minutes at RT with gentle agitation. Gels were then equilibrated in the Invitrogen (New Zealand) developing buffer (50mM tris, 40mM 6N HCl, 200mM NaCal, 5mM...
CaCl$_2$-2H$_2$O, 0.02% Brij-35) for 30 minutes at RT with gentle agitation before replacing with fresh developing buffer and incubating at 37$^\circ$C for 40 hours. In order to visualise the areas of protease activity, gels were first fixed in destaining/fixing solution (45% methanol, 10% acetic acid, 45% H$_2$O) for 15 minutes, stained with 0.5% Coomassie Blue R-250 (in destaining/fixing solution) for 30 minutes and then destained for 20 minutes. Gelatinolytic activities were evidenced as clear bands against the blue background of stained gelatin. Gels were scanned with a GS-800 Calibrated Densitometer (Bio-Rad) and analysed with the appropriate Quantity One 1-D Analysis Software (Bio-Rad). Optical density (OD) of each gelatinolytic band was measured and adjusted by subtracting the background OD of the corresponding lane. In order to make comparison between different gels, the gelatinolytic band OD values of the samples were calculated as a percentage of the band OD of a MMP-2/9 standard (BIOMOL, USA) in each gel. The final OD values were presented as a measure of relative MMP activity.

The quantitative nature of this protocol was validated by densitometric analysis of serial dilutions of a sample that demonstrates the strongest MMP activity. The gelatin zymography protocol described above was performed on a 1.25-fold serial dilution (48, 38, 31, 25, 20, 16, 13, 10, 6, and 5ug) of an embryonic day 18 brain protein sample. The relative activity of MMP-2 (the most abundant gelatinase) for each amount of protein loaded was measured (Figure 2.2) as detailed previously. As shown in Figure 2.2a the coefficient of regression ($r^2$) of the relationship between the amount of protein loaded and the measured MMP activity was calculated to be 0.9522 confirming that it is indeed a linear relationship.
Figure 2.2: Validation of the gelatin zymography analysis of the gelatinase activity. The graph demonstrates the linear relationship ($r^2 = 0.9522$) between the amount of protein loaded and the measured MMP-2 activity (a). The gelatin zymogram illustrates the reduction of MMP-2 activity detected with the decreasing amount of protein loaded (b).

Furthermore, a negative control experiment was performed by adding 20 mM EDTA, a known inhibitor of MMPs, into the developing buffer. It was found that gelatinolytic activity was completely abolished by EDTA (Figure 2.3).
2.9 PLASMINOGEN ZYMOGRAPHY

Plasminogen zymography was used to determine the activity levels of tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). 10% plasminogen zymograms were prepared using the Bio-Rad Laboratories (USA) gel casting apparatus as described in section 2.8 using the mixers outlined in the Table 2.9.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Separating mL</th>
<th>Stacking mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>4.0</td>
<td>2.1</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>3.3</td>
<td>0.5</td>
</tr>
<tr>
<td>0.5M tris-HCl (pH 6.8)</td>
<td></td>
<td>0.38</td>
</tr>
<tr>
<td>1.5M tris-HCl (pH 8.8)</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>10% Casein</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>4mg/mL Plasminogen</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1</td>
<td>0.03</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.1</td>
<td>0.03</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.004</td>
<td>0.003</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 2.9: Reagent composition for casting 10% plasminogen zymograms
36μg of protein in non-reducing sample buffer (0.4M tris (pH 6.8), 5% SDS, 20% glycerol, 0.05% bromophenol blue) was loaded onto 10% plasminogen zymograms. Electrophoresis was performed in tris-glycine running buffer (25mM tris, 192mM glycine and 0.1% SDS) until the loading dye reached the bottom of the gel for 90 minutes at 150V. They were then washed twice in renaturing buffer (2.7% Triton® X-100) for 15 minutes at RT with gentle agitation. Gels were then equilibrated in the developing buffer (100mM tris-HCl (pH 8.2)) for 20 minutes at RT with gentle agitation before replacing with fresh developing buffer and incubating at 37°C for three hours. Proteolytic activity was visualised and quantified as described in section 2.8. A tPA recombinant protein was run in each gel to allow comparison between gels. A negative control experiment was performed by eliminating plasminogen from the gels. Proteolytic activity at approximately 60 and 36kDa was completely eliminated confirming they corresponded to the cleavage of plasminogen (Figure 2.4).

![Figure 2.4: Representative plasminogen zymogram illustrating the elimination of proteolytic activity in the absence of plasminogen](image)
2.10 QUANTITATIVE REAL-TIME PCR

Taqman® quantitative real-time PCR (qRT-PCR) was used to quantify MMP-2 and 9 (Table 2.10) gene expression levels in the rat brain. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 2.10) was used as an endogenous control to normalize gene expression within each sample.

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Assay ID</th>
<th>Reference Sequence</th>
<th>Amplicon Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td>Rn00579162_m1</td>
<td>NM_031055.1</td>
<td>72</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Rn01538176_m1</td>
<td>NM_031054.2</td>
<td>89</td>
</tr>
<tr>
<td>uPA</td>
<td>Rn00565261_m1</td>
<td>NM_013085.3</td>
<td>72</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Rn99999916_s1</td>
<td>NM_017008.3</td>
<td>87</td>
</tr>
</tbody>
</table>

Table 2.10: Applied Biosystems (New Zealand) TaqMan® gene expression assays

Real-time PCR for genes of interest was performed in triplicate on 4.8 – 43 ng of cDNA using TaqMan® PCR Master Mix in a reaction volume of 12 µL (Table 2.11). Firstly, a master mix that includes PCR reaction mix, primer probe mix and nuclease-free water was made for the total number of wells required and then 7.2 µL of this mixture was then aliquoted into each well. Thereafter, 4.8 µL of cDNA was added, resulting in a total volume of 12 µL.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume per Well µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Reaction Mix</td>
<td>6.24</td>
</tr>
<tr>
<td>20x Primer/Probe Mix</td>
<td>0.6</td>
</tr>
<tr>
<td>Water</td>
<td>0.36</td>
</tr>
<tr>
<td>cDNA</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Table 2.11: Reaction mix for qRT-PCR
Taqman® qRT-PCR reactions (Table 2.12) were performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The comparative threshold cycle (Ct) method for relative quantification ($\Delta \Delta \text{Ct}$) ($2^{-\Delta \Delta \text{Ct}}$) was used to quantitate gene expression according to Applied Biosystem recommendations.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holding</td>
<td>50°C</td>
<td>2 Minutes</td>
</tr>
<tr>
<td>Holding - Denaturing</td>
<td>95°C</td>
<td>10 Minutes</td>
</tr>
<tr>
<td>Amplication - Denaturing</td>
<td>95°C</td>
<td>15 Seconds</td>
</tr>
<tr>
<td>Amplication - Annealing</td>
<td>60°C</td>
<td>1 Minutes</td>
</tr>
</tbody>
</table>

Table 2.12: qRT-PCR cycle steps

The validity of GAPDH as a housekeeping gene that can be used for effective normalisation of the target gene expression was tested by comparing the mean Ct values of injured ipsilateral and uninjured contralateral cortices at one day following injury at the two developmental ages. It was determined that the Ct values of GAPDH does not change significantly following injury (Figure 2.5), thus confirming that it is a valid normalization control.
Figure 2.5: The rat brain GAPDH gene expression 1 day following HI injury at P21 and P3. No statistical significant difference was observed between the injured and uninjured hemisphere at either of the ages. n = 4

For a valid $\Delta\Delta C_t$ calculation the relative application efficiencies of the target(s) and endogenous control(s) must be relatively equivalent. Comparability of the PCR reaction efficiencies of MMP-2, MMP-9 and uPA gene expression assays and GAPDH gene expression assay were determined by investigating the variation of $\Delta C_t$ ($C_t$ target - $C_t$ reference) with the template dilution. qRT-PCR was performed for MMP-2 and 9 and GAPDH across five dilutions of the same cDNA template. The resulting $\Delta C_t$ ($C_t$ target - $C_t$ reference) values were plotted against the log of RNA input amount to create a semi-log regression line. According to the Applied Biosystems recommendations, if the slope of the semi-log regression line is less than 0.1, the PCR amplification efficiencies of the two genes are considered relatively equal, and thus can be used for $\Delta\Delta C_t$ calculations. As shown in the Figure 2.4, the slopes of the MMP-9/GAPDH (Figure 2.6a), MMP-2/GAPDH (Figure 2.6b) and uPA/GAPDH (Figure 2.6c) were less than 0.1.
Figure 2.6: Validation plots of $\Delta C_t$ against the long input amount of RNA for MMP-9 (a) MMP-2 (b) and uPA (c) with GAPDH.
The fold differences in gene expression were calculated utilizing the mean \( C_t \) value for each triplicate and the standard deviations of the mean as outlined in the following table (Table 2.13).

<table>
<thead>
<tr>
<th></th>
<th>Injured (I)</th>
<th>Uninjured (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ( C_t ) MMP-9</td>
<td>( M_1 \pm S_1 )</td>
<td>( M_1 \pm S_1 )</td>
</tr>
<tr>
<td>Mean ( C_t ) GAPDH</td>
<td>( M_2 \pm S_2 )</td>
<td>( M_2 \pm S_2 )</td>
</tr>
<tr>
<td>( \Delta C_t ) (Mean ( C_t ) MMP-9 – GAPDH)</td>
<td>( \Delta C_{tI} \pm S_{tI} = (M_1 - M_2) \pm (S_1^2 + S_2^2)^{1/2} )</td>
<td>( \Delta C_{tU} \pm S_{tU} = (M_1 - M_2) \pm (S_1^2 + S_2^2)^{1/2} )</td>
</tr>
<tr>
<td>( \Delta \Delta C_t ) (( \Delta C_t ) Injured - ( \Delta C_t ) Uninjured)</td>
<td>( \Delta \Delta C_{tI} \pm S_{tI} = \Delta C_{tI} - \Delta C_{tU} \pm S_{tI} )</td>
<td>( \Delta \Delta C_{tU} \pm S_{tU} = \Delta C_{tU} - \Delta C_{tU} \pm S_{tU} )</td>
</tr>
<tr>
<td>Fold Difference</td>
<td>( 2^{-\Delta \Delta C_t} \pm S )</td>
<td>( 2^{-\Delta \Delta C_t} \pm S )</td>
</tr>
</tbody>
</table>

Table 2.13: An example of \( \Delta \Delta C_t \) method illustrating the stepwise calculations involved in determining the fold difference in MMP-9 gene expression in an injured (I) sample compared to the uninjured (U) sample. \( M = \) Mean \( C_t \), \( S = \) Standard deviation.

### 2.11 STATISTICAL ANALYSIS

For time course studies block design was applied, in which a particular batch of animals was allocated equally into all the time points. Multiple litters were used to correct for litter variability. Parameters were compared using Two Way Analysis of Variance (ANOVA). When the overall difference was found, sub-groups were subjected to Holm-Sidak post hoc test. A paired block design was applied when two groups were evaluated. The paired animals/samples were treated exactly the same at all times. Parameters were compared using the t-test (normal distributions) or the Mann-Whitney U-test (non-normal distributions). Statistical analysis was performed using the Sigma Stat for Windows.
version 3.11. Graphs were plotted, and data were transformed using Graph Pad Prism 3.02. Data were considered to be significant at $P \leq 0.05$ and presented as mean ± standard error of the mean (SEM).
CHAPTER THREE: MMP-2 AND MMP-9 EXPRESSION IN THE NORMAL DEVELOPING RAT FOREBRAIN

3.1 INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are involved in degradation of the proteinaceous components of the extracellular matrix (ECM). There are now more than twenty enzymes that are classified as MMPs (Nagase and Woessner, 1999, Sethi et al., 2000, Stamenkovic, 2003). Their substrates include collagen, fibronectin, laminin, elastin and gelatin (Nagase and Woessner, 1999, Rosenberg, 2002). The family of MMPs are crucial regulators of mammalian development. Evidence suggests that they may participate in developmental processes through a) allowing cellular movement by degrading ECM macro molecules; b) affecting cellular behaviour by altering ECM-cell interactions; and c) regulating the activity of biologically active molecules by direct cleavage, release from bound stores or regulating the activity of their inhibitors (Nagase and Woessner, 1999, Yong et al., 2001, Conant and Gottschall, 2005).

As mentioned previously in Chapter One, during development of the central nervous system (CNS), neurons migrate from the subventricular zone to their final destinations and send out axons and dendrites to make connections with target cells. Since these processes potentially require many complex interactions with their ECM, it is probable that MMPs play an important role in the development of the CNS (Letourneau et al., 1994, Conant and Gottschall, 2005). Indeed, recent evidence indicates that several MMPs are crucial to many CNS developmental processes, which include neuronal survival, neuronal migration, neurite outgrowth and myelination (Soler et al., 1995, Oh et al., 1999, Yong et al., 2001, Ayoub et al., 2005, Hehr et al., 2005). In particular, MMP-2 and
9 appear to be involved in several developmental processes within the developing cerebellum (hindbrain) (Vaillant et al., 2003, Ayoub et al., 2005, Ulrich et al., 2005). However, the developmental expression of MMP-2 and 9 in the normal developing forebrain has not been fully characterised as yet.

The overall aim of this thesis was to determine the role of MMP-2 and 9 in the pathophysiology of hypoxic ischemic injury in the developing brain. Given that ECM remodelling is a fundamental requirement during CNS development, it was fundamental to first define the developmental changes of MMPs in the normal brain. Therefore, in the current study we defined the pattern of MMP-2 and 9 activity, mRNA expression, and protein localisation in the normal developing rat forebrain at different time points, ranging from gestation day 18 (E18) to postnatal day 120 (P120).

3.2 MATERIALS AND METHODS

Gelatin Zymography

Samples of normal rat brains were collected at E18, P3, P4, P6, P8, P10, P13, P21 and P120. In order to collect the E18 brain samples, one pregnant rat of 18 days gestation was anaesthetized with carbon dioxide and then killed by cervical dislocation. Embryos were removed from the mother, the brains were dissected out using a dissection microscope, separated into left and right hemispheres and washed in cold 0.01M KPBS (pH 7.4) before freezing in liquid nitrogen. From P3 onwards the brains were collected after killing the animals with an overdose of sodium pentobarbitone. They were then transcardially perfused with cold 0.01M KPBS (pH 7.4). The brains were then processed to extract the proteins according to the instructions outlined in Chapter Two; Section 2.3. Gelatin Zymography was then performed using pre-cast gelatin zymograms (Invitrogen,
New Zealand) as detailed in Chapter Two; Section 2.8 to determine the MMP-2 and 9 activity levels.

**Quantitative Real-Time PCR**

Samples of rat forebrain (excluding the olfactory bulbs) were collected at E18, P1, P4, P7, P14 and P21 as described above. The samples were washed in RNALater® overnight at 4°C before storing at -80°C. The cortex and hippocampus were dissected out from either hemisphere using a dissection microscope. Total RNA was extracted using Invitrogen’s PureLink™ Micro-to-Midi™ Total RNA purification system (Chapter Two; Section 2.4), treated with DNases using Ambion’s DNA-free™ kit, and first-strand cDNA was synthesised using Invitrogen’s SuperScript™ III Reverse Transcriptase kit (Chapter Two; Section 2.5) according to the manufacturer’s instructions. Sample collection, RNA extraction and cDNA synthesis, as described above, were performed by Mrs Larissa Christophidis. Thereafter, quantitative real-time PCR (qRT-PCR) was carried out on 30ng of cDNA as described in Materials and Methods (Chapter Two; Section 2.10) to determine the pattern of MMP-2 and 9 gene expression. Expression of target genes was normalized to the level of GAPDH and was expressed relative to the sample with the lowest expression.

**Immunohistochemistry**

Animals were killed; the brains were processed and embedded in paraffin as described in Chapter Two; Section 2.2. Single and double immunohistochemical procedures were carried out as described in Chapter Two; Section 2.7 according to the conditions outlined in Chapter Two; Table 2.7.
After rehydration, the sections were blocked for two hours for endogenous IgG (5% normal horse serum (NHS) or normal goat serum (NGS) in 5mg/mL BSA/KPBS (pH 7.4)) and endogenous biotin (DAKO: Denmark). They were then incubated with primary antibody against MMP-2 (1:25; IM33L; Calbiochem, USA) in 5mg/mL BSA/KPBS for 48 hours at 4°C. They were further incubated with horse anti-mouse biotinylated secondary antibodies (1:200; Vector Laboratories, UK) in 5mg/mL BSA/KPBS for one hour at room temperature followed by Alexa 568 (1:200) for one hour at 37°C. Sections were mounted in vectashield and stored at 4°C. Negative controls included using antibody diluting buffer or the anti-MMP-2 antibody pre-absorbed with affinity-purified MMP-2 (Calbiochem, USA) or protein diluting buffer at a ratio of 1:10 (antibody: protein) instead of the primary antibody. It was observed that any specific immunoreactivity was absent on these sections (Figure 3.3a).

Double immunohistochemistry was performed to determine the specific cell types that expressed MMP-2. Procedures for MMP-2 were carried out as above, followed by extensive washing and reblocking with 5% NHS in 5mg/mL BSA/KPBS. Sections were incubated with primary antibodies for neuronal nuclei maker (NeuN; 1:400; Chemicon, USA), microtubule-associated protein-2 (MAP-2; 1:200; Chemicon, USA) in 5mg/mL BSA for 12 hours at 4°C. This was followed by incubation with horse anti-mouse biotinylated secondary antibody (1:200; Vector Laboratories, UK) for one hour at room temperature followed by Alexa 488 (1:200) for 1 hour at 37°C. The sections were then mounted in vectashield and stored at 4°C.
a) Image of a gel with bands at 98kDa, 64kDa, and 50kDa markers.

b) Bar graph showing relative activity of Pro MMP-2 from E18 to P120. The bars are divided into two groups, RH and LH, with RH showing higher activity at younger ages.

c) Bar graph showing relative activity of Cleaved MMP-2 from E18 to P120. The bars are divided into two groups, RH and LH, with RH showing higher activity at younger ages.
Figure 3.1: MMP-2 and 9 activities in the normal developing forebrain of the rat. Representative gelatin zymography demonstrating the high levels of MMP-2 activity in the normal developing rat brain and its gradual down regulation from E18 to P120 (a). Intensity measurements of the pro (P ≤ 0.001) (b) and cleaved (P ≤ 0.001) (c) forms of MMP-2 confirm a gradual down regulation of MMP-2 levels with age. Low levels of pro (d) and cleaved (e) MMP-9 activity was detected in the developed brain (arrows). Results are presented as mean ± SEM. n = 6. RH = Right Hemisphere, LH = Left Hemisphere
3.3 RESULTS

MMP-2 activity in the normal rat forebrain decreased with age while MMP-9 activity increased

Gelatin zymography was performed to characterise the developmental changes in MMP-2 and MMP-9 activity of normal rat forebrain aged from embryonic day 18 to postnatal day 120. MMP-2 activity was observed at two molecular weights corresponding to the pro and cleaved forms of the enzyme (Asahi et al., 2000, Planas et al., 2000) (Figure 3.1a). However, activity was predominantly observed in the higher molecular weight latent form. MMP-2 activity at both molecular weights was very high from E18 to P3 brains and gradually decreased with age ($P \leq 0.001$) (Figure 3.1b and c). Both pro (Figure 3.1d) and cleaved (Figure 3.1e) forms of MMP-9 activity were scarcely present in detectable amounts in the immature brains from E18 to P13. However, at P21 and P120 low levels of pro (92kDa) and cleaved (87kDa) MMP-9 activities were visible on the gelatin zymograms (Figure 3.1a; arrows). Densitometric analysis proved that pro MMP-9 activity did not vary significantly over time (Figure 3.1d). Conversely, cleaved MMP-9 activity significantly increased ($P \leq 0.05$) with maturity (Figure 3.1e).

MMP-2 gene expression decreased with age while MMP-9 gene expression increased

qRT-PCR was performed to determine the changes in MMP-2 and 9 gene expression in the rat brain during development (Figure 3.2). Cortical MMP-2 gene expression significantly decreased ($P \leq 0.001$) with age reflecting the previously observed pattern of MMP-2 activity. MMP-2 mRNA expression was shown to be about 10 and six fold higher in the E18 and P1 cortices respectively compared to the P21 rat cortex (Figure 3.2a). Thereafter, it gradually decreased reaching a plateau by P14. However, MMP-2
Figure 3.2: MMP-2 and 9 gene expression in the normal developing rat brain E18 to P21. MMP-2 mRNA expression in the cortex (a) gradually declined with age ($P \leq 0.001$) while it did not vary significantly in the hippocampus (b). Conversely, MMP-9 mRNA expression increased ($P \leq 0.001$) with age in both the cortex (c) and hippocampus (d). Results are presented as mean ± SEM. $n = 3-5$
gene expression in the hippocampus was shown not to vary significantly with age (Figure 3.2b). It was shown that MMP-2 expression decreased about 2 fold from P1 to P14 but returned back to original levels by P21. In contrast, MMP-9 gene expression in both the cortex (Figure 3.2c) and hippocampus (Figure 3.2d) significantly increased (P ≤ 0.001) with age in agreement with the gelatinolytic activity. MMP-9 expression appears to be constant in low levels from E18 to P7 both in the cortex and hippocampus. It increased by approximately five fold and 10 fold at P14 and P21 respectively in both the structures. However, it is noteworthy that the raw C_t (cycle threshold) values of MMP-9 gene expression ranged from 28 to 32 while that of MMP-2 gene expression ranged from 22 to 26 using similar cDNA amounts. In qRT-PCR, the C_t value reflects the number of PCR cycles required to amplify the cDNA of gene of interest above the background levels (Applied Biosystems qRT-PCR Guide). Therefore, this suggests that MMP-9 mRNA expression was low compared to that of MMP-2.

**MMP-2 Immunoreactivity in the developing cortex also decreased with age**

MMP-2 immunoreactivity was determined at mid-striatal and mid-hippocampal levels of the normal rat brain from P3 to P21. P3 cortices were highly immunoreactive for MMP-2. Immunoreactive cells were primarily found in the cortical plate and subplate from layers II to layer VII (Figure 3.3a) of sagittal and frontoparietal cortices. MMP-2 positive cells were absent from the marginal zone. Most of the cells in the CA1-4 areas and some in dentate gyrus of the hippocampus were also found to be MMP-2 positive. Similar to the trend seen with gelatin zymography, the intensity and the number of MMP-2 immunoreactive cells decreased with age. At P6 the number of immunoreactive cells was decreased in layers II and III and the intensity of staining was reduced throughout
Figure 3.3: MMP-2 Immunoreactivity in the normal rat forebrain. P3 (a) and P21 (b) cortex had a punctuate pattern that was distributed homogeneously across the cell (from a P3 brain) (c). Negative control showed that incubation of MMP-2 primary antibody with its specific antigen abolishes its immunoreactivity at P3 (a inset). Double labelling showed that MMP-2 (d) immunoreactivity colocalised with NeuN (e) positive neurons (f). Double labelling for MMP-2 (g) and MAP-2 (h) showed that MMP-2 appears to be colocalising with microtubules in neuronal processes (i). Scale bars = 50μm (a-b) and 10μm (c-i)
the cortex. At P13, immunoreactivity was mainly seen in the deeper pyramidal layers V and VI. At P21, very few immunoreactive cells were seen in the mid to deep layers (Figure 3.3b). MMP-2 immunoreactivity had a punctuate pattern that was distributed homogeneously across the cytoplasm and nucleus of the neurons (Figure 3.3c). Double labelling studies showed that the neuronal marker NeuN colocalised with almost all of these MMP-2 positive cells (Figure 3.3d-f). MMP-2 also colocalised with the microtubule associated protein (MAP-2), which is expressed by the cytoskeleton of the cytoplasm and processes emerging from the cell body (Figure 3.3g-i). MMP-2 and MAP-2 co-labelled cells were predominantly evident on cortical layers V and VI. MMP-9 immunoreactivity was not observed in significant levels in the rat brain during early postnatal days (Refer to Chapter Four; Figure 4.5i). At P21, moderate MMP-9 immunoreactivity, which had the morphology of neuronal processes, was occasionally observed in the cortex (Refer to Chapter Four; Figure 4.5g).

### 3.4 DISCUSSION

MMP-2 and 9 activities have been implicated in the development of the CNS. However, the majority of *in vivo* evidence originates from studies on the development of the cerebellum. The developmental regulation of MMP-2 and 9 in the normal developing forebrain has not yet been fully characterised. In this study we showed that MMP-2 mRNA, protein and activity levels were high in the normal rat forebrain during early postnatal ages and declined with age. This was in accordance with the MMP-2 expression patterns found in the rodent cerebellum (Vaillant et al., 1999, Ayoub et al., 2005) and mouse brain (Ulrich et al., 2005). Conversely, MMP-9 mRNA, protein and activity were present at low levels early in postnatal life and increased with maturity. Interestingly, these findings conflict with those of Vaillant *et al.*, 1999 and Ayoub *et al.*, 2005 in the
rodent cerebellum and Ulrich et al., 2005 in the mouse brain but concur with that of Uhm et al., 2005 in the rodent corpus callosum.

Gelatin zymography revealed that MMP-2 activity in the normal rat forebrain gradually declined with age. It was observed in high levels from E18 to P3. MMP-2 gene expression in the normal rat cortex reflects the same pattern showing a peak at E18 which declined with age. However, MMP-2 gene expression in the hippocampus did not show a significant trend for reduction with age within P1 to P21. Immunohistochemical staining demonstrated that MMP-2 protein was localised to the cortical plate of the developing brain. Double labelling with the neuronal marker NeuN showed that its localisation was predominantly neuronal. Current literature suggests that the cortical plate development occurs within E15 to P4-6 in the rodent brain (Uylings, 2000, Clancy et al., 2001). Therefore, the radial migration of neurons involved in the formation of the cortical plate lasts until approximately P5-6 (Vanier et al., 1971, Uylings, 2000). The period of these neuronal migrations correlates with the time period where MMP-2 is highly expressed in the cortical plate. It has also been suggested previously that MMP-2 assists granular cell migration in the cerebellum (Ayoub et al., 2005, Ulrich et al., 2005). Therefore, it could be suggested that MMP-2 may play a role in neuronal migration during the development of the rat cortex. However, the fact that MMP-2 was present in NeuN immunoreactive cell, which have presumably withdrawn from the cell cycle and initiated neuronal differentiation (Mullen et al., 1992), may discount the possibility of it primarily assisting the migration process in the early postnatal rat cortex. Alternatively, a role in neurite outgrowth has also been suggested for MMP-2 by Zuo et al., 1998 who demonstrated that MMP-2 degrades and inactivates a neurite-inhibiting protein. According to the literature, neuronal differentiation, which mainly involves neurite outgrowth, occurs approximately
within P0-P10 during rodent brain development (Vanier et al., 1971, Uylings, 2000, Clancy et al., 2001). Our results showed that both MMP-2 activity and gene expression were present in significantly high levels during this time period. Furthermore, our findings demonstrating that MMP-2 colocalises with MAP-2 further supports this argument. Therefore, a reasonable interpretation of the findings is that MMP-2 may have a prominent role in neurite outgrowth in the developing rat cortex. However, MMP-2 gene expression in the normal rat hippocampus did not show a significant trend towards reduction with age within the time period studied in this study. This could be due to the fact that formation of hippocampal structures starts later during development of the rat brain as compared to the cortical plate (Clancy et al., 2001). In addition, we cannot discount the possibility that the lack of a significant trend may be the result of inaccurate microdissection of the hippocampus: for example, contamination with choroid plexus could have been a possibility.

Both MMP-9 proteolytic activity and gene expression were found to be extremely low or non-detectable in the normal rat forebrain until 13-14 days after birth. MMP-9 gene expression was significantly upregulated at P14 and P21. Cleaved MMP-9 activity was also elevated at P21. Interestingly, the upregulation of MMP-9 expression closely correlated with the period of myelination in the normal rodent brain, which is initiated approximately between P10 to P15 (Davison and Dobbing, 1966, Vanier et al., 1971, Uhm et al., 1998). In 1998, Uhm et al demonstrated that MMP-9 protein expression in the mouse corpus callosum steadily increased from P14 to P28. They further showed that oligodendrocytes utilise MMP-9 to extend their processes by demonstrating that in response to pharmacological activators, cultured human oligodendrocytes (OLs) augment their process extension with a proportional increase of MMP-9 activity (Uhm et al.,
1998). It was also shown that elimination of MMP-9 activity by pharmacological MMP-9 inhibitors (Uhm et al., 1998), function-perturbing anti-MMP-9 antibodies and MMP-9 gene knockout (Oh et al., 1999) retarded the process outgrowth of OLs in cultures suggesting that MMP-9 may contribute to the myelination process by facilitating the OL process outgrowth. Therefore, we can suggest a role for MMP-9 in myelination in the rat forebrain during development. Our results showing that MMP-9 immunoreactivity was observed in a process-like pattern, which may correspond to myelinating neuronal processes, further strengthen the above argument. However, as mentioned above, MMP-9 gene expression was found to be at low levels compared to that of MMP-2. Furthermore, the MMP-9 activity was also extremely low and was barely detectable by gelatin zymography. For this reason, it is debatable whether one can assume that MMP-9 plays a major role in the normal brain. It may be plausible that MMP-9 gene expression was upregulated with maturity as a defence mechanism to be used in pathological situations such as an immunological situation. Indeed, there is substantial evidence to suggest that MMP-9 has a role in inflammation (Gong et al., 2008).

In conclusion, the aim of our study was to define the pattern of MMP-2 and 9 mRNA expression, protein expression and activity in the normal developing forebrain. It was fundamental to firstly understand the degree of participation of MMP-2 and 9 during development of the rodent brain before investigating their possible participation during injury in the developing brain. Our study was the first study to fully characterise developmental changes of MMP-2 and 9 in the normal developing rodent forebrain. We showed that MMP-2 mRNA expression and activity progressively declined with maturity before reaching a plateau, whilst MMP-9 mRNA expression and activity increased with maturity. We suggest that these results support an important role for MMP-2 in the
development and differentiation of the cortical plate, whereas MMP-9 could potentially play a role in myelination.
CHAPTER FOUR: MMP-2 AND MMP-9 RESPONSE AFTER HYPOXIC ISCHEMIA IN THE DEVELOPING BRAIN

4.1 INTRODUCTION

Disruption of the extracellular matrix (ECM) is a prominent characteristic of lesions that occur in the brains of very preterm infants. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are involved in degradation of the proteinaceous components of the ECM and are integrally involved in central nervous system (CNS) development. Recently, several lines of evidence have demonstrated that the inappropriate activity of these proteases contribute to the pathogenesis of cerebral ischemia in the adult brain.

Accumulating evidence suggests that MMPs, predominantly MMP-2 and MMP-9 and occasionally MMP-3, are upregulated after cerebral ischemia in the adult brain (Rosenberg et al., 1996, Romanic et al., 1998, Asahi et al., 2000, Asahi et al., 2001, Gasche et al., 2001, Planas et al., 2001, Rosenberg et al., 2001, Rivera et al., 2002, Lee et al., 2004). A role of these MMPs in the exacerbation of ischemic damage is further supported by studies demonstrating that inhibition of MMP-9 activity reduces the severity of ischemic injury in the adult CNS (Romanic et al., 1998, Asahi et al., 2000, Jiang et al., 2001, Pfefferkorn and Rosenberg, 2003, Lee et al., 2004). Given that cyst formation, disruption of developing white matter tracts and the presence of glial scars, where the ECM is disrupted, are prominent features of lesions to the developing brain (Meng et al., 1997, Volpe, 1998, Volpe, 2001, Blumenthal, 2004, Sizonenko et al., 2005), it is possible that MMPs play an important role in injury in the developing brain. Indeed, MMP activity has also been implicated in the aetiology of injuries to the immature brain. Schulz and colleagues have demonstrated that plasma levels of MMP-9 are significantly
higher in infants with intraventricular haemorrhage suggesting a possible role of MMP-9 in neonatal brain injury (Schulz et al., 2004). More recently, Svedin et al., have shown that knock-out of the MMP-9 gene protects the immature mouse brain from hypoxic ischemic (HI) injury (Svedin et al., 2007). In spite of increasing evidence for their involvement in the health and injury of the developing brain, the spatiotemporal distribution of the MMPs in the injured developing forebrain has not been fully characterised.

In the current study, we determined the MMP-2 and 9 responses following HI injury in the developing brain. We characterised the pattern of MMP-2 and 9 activity, mRNA expression, and protein localisation following HI injury in the developing brain using a well characterised rat model of unilateral HI injury at two developmental stages; postnatal day 21 (P21) (Sirimanne et al., 1994) and postnatal day 3 (P3)(Sizonenko et al., 2005). Developmentally, the cortex of the P21 rat is comparable to that of a full-term to one year old infant while the P3 rat cortex approximately resembles that of a human pre-term fetus of 24-26 weeks of gestation (Clancy et al., 2001).

4.2 MATERIALS AND METHODS

Hypoxic Ischemic Injury

HI injury was induced in P21 and P3 rats as described in detail in Chapter Two; Section 2.1.

Gelatin Zymography

Animals were killed at three hours, six hours, 12 hours, one day, two days, three days, five days and seven days with an overdose of sodium pentobarbitone. They were then
transcardially perfused with cold 0.01M PBS (pH 7.4). The brains were then processed to extract the proteins according to the instructions outlined in Chapter Two; Section 2.3. Gelatin zymography was then performed as detailed in Chapter Two; Section 2.8. Invitrogen (New Zealand) pre-cast gelatin zymograms, which were of 1mm thickness, were used to analyse MMP-2 and 9 activity following HI injury at P21, whilst in-house-cast gelatine zymograms, which were of 0.75mm thickness, were used to analyse activities following injury at P3.

**Quantitative Real-Time PCR**

Animals were killed at one and five days and six hours following HI injury at P21 and P3 respectively. Total RNA from brain tissue was isolated (Chapter Two; Section 2.4), first-strand cDNA was synthesised (Chapter Two; Section 2.5) and quantitative real-time PCR (qRT-PCR) (Chapter Two; Section 2.10) was performed on either 4.8ng (P21) or 43.2ng (P3) of cDNA. Expression of target genes (MMP-2 and 9) in the ipsilateral injured hemisphere was normalized to the level of GAPDH and was expressed relative to the corresponding contralateral uninjured hemisphere.

**Immunohistochemistry**

Animals were killed; brains were processed and embedded in paraffin as described in Chapter Two; Section 2.2. Single and double immunohistochemical procedures were carried out as described in Chapter Two; Section 2.7 according to the conditions outlined in Chapter Two; Table 2.7.

Briefly, the sections were deparaffinized and rehydrated, and subjected to antigen retrieval procedures using 0.01M Citrate Buffer at pH 6. Sections were then blocked for two hours
for endogenous IgG (5% normal horse serum (NHS) or normal goat serum (NGS) in 5mg/mL BSA/PBS (pH 7.4)) and endogenous biotin (DAKO Biotin Blocking system) before incubating with primary antibodies for MMP-9 (1:100; IM37L; Calbiochem, USA/ 1:500; TP221; Torrey Pines Biolabs, USA) and MMP-2 (1:25; IM33L; Calbiochem, USA) in 5mg/mL BSA/PBS for 48 hours at 4°C. They were further incubated with horse anti-mouse or goat anti-rabbit biotinylated secondary antibodies (1:200; Vector Laboratories, UK) in 5mg/mL BSA/PBS for one hour at room temperature, followed by Alexa 568 (1:200; Molecular Probes, USA) for one hour at 37°C. Sections were mounted in Vectashield (Vector Laboratories, UK) and stored at 4°C. Negative controls included using antibody diluting buffer or the MMP-2/9 antibodies pre-absorbed with affinity-purified MMP-2/9 (Calbiochem, USA) or protein diluting buffer at a ratio of 1:10 (antibody: protein) instead of the primary antibody. It was observed that any specific immunoreactivity was absent on these sections (Figure 4.5j-n).

Double immunohistochemistry was performed to determine the specific cell types that expressed MMP-2 and 9. Procedures for MMP-2 and 9 were carried out as above, followed by extensive washing and reblocking with 5% NHS in 5 mg/mL BSA/PBS. Sections were incubated with primary antibodies for neuronal nuclei maker (NeuN; 1:400; Chemicon, USA), glial fibrillary acidic protein (GFAP; 1:200; Chemicon, USA) and growth associated protein-43 (GAP-43; 1:1000; Chemicon, USA) in 5mg/mL BSA for 12 hours at 4°C. This was followed by incubation with horse anti-mouse biotinylated secondary antibody (1:200) for one hour at room temperature followed by Alexa 488 (1:200) for one hour at 37°C. The sections were then mounted in Vectashield and stored at 4°C.
a) MMP-9 Complex 150kDa

b) MMP-9 Complex 150kDa

**IPSILATERAL (HI)**

**CONTRALATERAL (HI)**

**IPSILATERAL (C)**

**CONTRALATERAL (C)**

Pro MMP-9

**IPSILATERAL (HI)**

**CONTRALATERAL (HI)**

**IPSILATERAL (C)**

**CONTRALATERAL (C)**

Pro MMP-2
Figure 4.1: MMP-2 and 9 activities after HI injury in P21 rat brain. Representative zymogram shows the gelatinolytic activity at different time points after injury (a). MMP-9 activity (at 92kDa and 150kDa) was upregulated at 6 hours to 1 day after injury while pro MMP-2 (65kDa) was gradually upregulated from 1 day reaching a peak by 5 days. Histograms show the quantification of relative activities of MMP-9 - 150kDa (b), Pro MMP-9 (c), Cleaved MMP-9 (d) and Pro MMP-2 (e) by densitometry of gelatin zymography. Results are presented as mean ± SEM. Asterisks ** and * represent the statistical significance (P ≤ 0.005) between ipsilateral hemisphere (HI) and both the contralateral hemisphere (HI) and control brains and ipsilateral (HI) and either contralateral (HI) or control brains respectively. n = 3-9.
a) 

Pro MMP-9
Cleaved MMP-9
Pro MMP-2
Cleaved MMP-2

Time Point (Hours)

b) 

Pro MMP-9

<table>
<thead>
<tr>
<th>Time Point (Hours)</th>
<th>Relative Activity</th>
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<tbody>
<tr>
<td>3.0</td>
<td>0.25</td>
</tr>
<tr>
<td>6.0</td>
<td>0.50</td>
</tr>
<tr>
<td>24.0</td>
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Ipsilateral (HI) | Contralateral (HI) | Control

**

Cleaved MMP-9

<table>
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Ipsilateral (HI) | Contralateral (HI) | Control

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Figure 4.2: MMP-2 and 9 activities after HI injury in P3 rat brain. Representative zymogram shows the gelatinolytic activity at different time points after injury (a). Pro MMP-9 activity was upregulated at 6 hours while at 1 day both the pro and cleaved forms were upregulated. Cleaved MMP-2 was upregulated from 3 hours to 1 day after injury, whereas pro MMP-2 was unaffected (data not shown). Relative activities of pro MMP-9 (b), cleaved MMP-9 (c) pro MMP-2 (d) and cleaved MMP-2 (e) were quantified by densitometry of gelatin zymography. Results are presented as mean ± SEM. Asterisks ** and * represent the statistical significance (P ≤ 0.01) between ipsilateral hemisphere (HI) and both the contralateral hemisphere (HI) and control brains and ipsilateral (HI) and either contralateral (HI) or control brains respectively. n = 3-6.
4.3 RESULTS

MMP-9 and MMP-2 activity was increased following hypoxic ischemia

In normal 21 day old rat brains, a low level of pro-MMP-2 activity was detected, while MMP-9 activity was either very low or undetectable by gelatin zymography. MMP-9 activity was significantly induced in the ipsilateral hemisphere from six hours to one day following HI injury at P21 (Figure 4.1). By day two, levels were reduced but remained detectable and thereafter were below the level of detection (Figure 4.1a). Gelatinolytic activity of MMP-9 was strongly observed at three molecular weights; 150kDa, 92kDa and 87kDa. Evidence suggests that the 92kDa and 87kDa bands correspond to the pro and cleaved forms of MMP-9 respectively, while the 150kDa band is thought to represent a complex of MMP-9 with itself, other MMPs, or other molecules such as TIMPs or inflammatory molecules (Asahi et al., 2000, Gutiérrez-Fernández et al., 2007). Quantification of the relative activities as described in Chapter Two; Section 2.8 revealed that activities of both the 150kDa (P ≤ 0.005), (Figure 4.1b) and 92kDa (P ≤ 0.002), (Figure 4.1c) isoforms were upregulated while the 87kDa isoform (Figure 4.1d) remained unaffected. Pro MMP-2 activity gradually increased after injury, peaking at five days post injury (P ≤ 0.001) (Figure 4.1e), whereas cleaved MMP-2 activity was not detectable. At seven days after injury MMP-2 activity levels began to decline, but were still significantly higher than in control tissues.

Following hypoxic ischemia at P3, increased MMP-9 levels were detected in the ipsilateral hemisphere at six hours after injury and remained significantly elevated until one day (Figure 4.2a). At six hours, MMP-9 activity was predominantly attributed to the 92kDa pro-form (P ≤ 0.001) (Figure 4.2b). At one day, activity at 92kDa was slightly reduced, while activity of the 87kDa molecular weight form, corresponding to the active
form of MMP-9, was significantly elevated ($P \leq 0.001$) (Figure 4.2c). Cleaved MMP-2 was up-regulated at six hours ($P \leq 0.01$) after injury (Figure 4.2e). However, the constitutively high levels of pro MMP-2 were unaffected by injury at any time point studied (Figure 4.2d).

**MMP-9 and MMP-2 gene expression was also increased following hypoxic ischemia**

qRT-PCR was performed to determine the changes in MMP-2 and 9 gene expression after HI injury, in order to further confirm the upregulation of MMP-2 and 9 activities as shown by zymography. Results showed that at one and five days following HI brain injury in the P21 rat, both MMP-2 and 9 exhibited low expression levels in the contralateral uninjured hemisphere (Figure 4.3). At one day following HI injury, MMP-9 expression was elevated two and four fold in the ipsilateral injured hippocampus ($P \leq 0.05$) and cortex ($P \leq 0.05$) respectively, as compared to the corresponding contralateral uninjured regions (Figure 4.3a). At five days following injury, MMP-9 expression in the injured and uninjured cortices did not differ significantly, similar to the activity levels. Interestingly, however, expression was significantly lower in the injured hippocampus ($P \leq 0.05$) as compared to the uninjured region (Figure 4.3b). This downregulation may correspond to the disruption of tissues as a consequence of injury. Conversely, MMP-2 gene expression was elevated in the injured cortex and hippocampus at both time points. At one day after HI injury at P21, MMP-2 mRNA levels were elevated two to three fold in both the injured cortex ($P \leq 0.05$) and hippocampus ($P \leq 0.05$), (Figure 4.3c). At five days after HI injury, it was very strongly upregulated approximately 25 fold in the injured cortex ($P \leq 0.05$) and seven fold in the injured hippocampus ($P \leq 0.05$) as compared to the uninjured regions reflecting the previously observed upregulation of MMP-2 activity (Figure 4.3d).
Figure 4.3: MMP-2 and 9 gene expression after HI injury in P21 rat brain. At 1 day post-injury MMP-9 gene expression was elevated 4 fold in the injured cortex and 2 fold in the ipsilateral injured hippocampus when compared to the contralateral uninjured hemisphere (a). At 5 days after injury, it was not significantly different in the uninjured and injured cortices, whereas it was significantly reduced in the injured hippocampus when compared to the uninjured (b). MMP-2 gene expression was slightly increased (2-3 fold) in both the injured cortex and hippocampus as compared to the contralateral hemisphere at 1 day after injury (c). At 5 days after injury it was highly increased approximately 27 fold in the injured cortex and 7 fold in the injured hippocampus when compared to corresponding regions of the uninjured hemisphere (d). * represents the statistical significance (P ≤ 0.05) between ipsilateral injured and contralateral uninjured hemispheres. n = 4
Figure 4.4: MMP-2 and 9 gene expression after HI injury in P3 rat brain at 6 hours after injury. MMP-9 gene expression was upregulated approximately 2 fold in the injured hippocampus and 3 fold in the injured cortex when compared to the uninjured contralateral hemisphere (a). However, MMP-2 gene expression was not significantly elevated in both the injured hippocampus and cortex when compared to the contralateral regions (b). * represents the statistical significance (P ≤ 0.05) between ipsilateral injured and contralateral uninjured hemispheres. n = 4
Levels of MMP-2 and 9 gene expression were determined at six hours following HI injury at P3. Both genes were constitutively expressed in the P3 brain. Following injury, MMP-9 expression was elevated three and two fold in the injured cortex and hippocampus respectively when compared to the contralateral uninjured hemisphere (Figure 4.4a) in agreement with gelatinase activity. However, MMP-2 gene expression was not significantly altered in either the injured cortex or hippocampus (Figure 4.4b), presumably as a result of already existing high constitutive levels of MMP-2 activity (Chapter Three: Figure 3.1).

MMP-9 and MMP-2 were located in the injured hemisphere following injury

At one day following hypoxic ischemia at P21, MMP-9 immunoreactivity was seen in the penumbra of the injured frontoparietal cortex (Figure 4.5a) and CA1-3 regions of the injured hippocampus (Figure 4.5b). Additionally, some immunoreactivity, which had the morphology of neuronal processes, was observed occasionally in the contralateral uninjured cortex (Figure 4.5g). Double labelling with NeuN confirmed that MMP-9 was mainly associated with neurons in the penumbra of the ipsilateral injured cortex (Figure 4.6a-c). Intense immunoreactivity was observed in the neuronal cytoplasm, neuronal processes and in extracellular space lining the cell membrane. Another subset of MMP-9 immunoreactive cells was observed along the hippocampal fissure and dentate gyrus (Figure 4.5c) in the injured hippocampus and occasionally in the penumbra of the injured cortex. These were found to be GFAP positive reactive astrocytes with their end feet surrounding the blood vessels (Figure 4.6d-f). By five days following injury at P21 a high level of MMP-2 immunoreactivity was present in the ipsilateral injured hemisphere. One group of immunoreactive cells was observed in the injured cortex, which appeared to form a cellular barrier around the infarct area (Figure 4.5d).
Figure 4.5: MMP-2 and 9 Immunoreactivity after HI injury to the developing rat brain. At 1 day after HI at P21, MMP-9 immunoreactive cells were seen in the penumbra of the injured cortex (a) and CA2-3 areas (b) and dentate gyrus (DG) (c) of the injured hippocampus. In the corresponding contralateral cortex occasional immunoreactivity was observed (g). At 5 days after HI at P21 MMP-2 immunoreactivity was seen in the injured cortex surrounding the infarct area (d) and along the corpus callosum (CC) (e). Low levels of immunoreactivity were observed in the contralateral cortex (h). At 1 day after HI at P3, MMP-9 immunoreactivity was observed in the deep layers of the injured cortex (f). No significant immunoreactivity was seen in the contralateral cortex (i). Omission of the primary antibody (j) and pre-absorption of the MMP-9 and 2 primary antibodies with MMP-9 (l) and MMP-2 (n) respectively abolished any specific immunoreactivity in areas of interest. Scale bars = 200μm (a-j) and 100μm (k-n).
Figure 4.6: MMP-2 and MMP-9 immunoreactivity after HI in P21 brain. Double immunostaining for MMP-9 (a) and NeuN (b) at 1 day after HI injury to P21 rat brain showed that MMP-9 is present in the neuronal cytoplasm, processes and some ECM (c). Double immunostaining for MMP-9 (d) and GFAP (e) confirmed that MMP-9 is also associated in reactive astrocytic cell bodies and end feet lining the blood vessels (f). Colocalisation of MMP-2 (g) and GFAP (h) immunoreactivity at 5 days after HI injury suggests that MMP-2 was located in the reactive astrocytes in the ipsilateral hemisphere (i). MMP-2 immunoreactivity (j) also colocalised (l) with GAP-43 immunoreactivity (k). Scale bars = 20μm
Another group of MMP-2 positive cells was observed along the white matter tracts of the corpus callosum (Figure 4.5e). Less intense MMP-2 immunoreactive cells were also seen in the hippocampus. Double staining with GFAP confirmed that MMP-2 was localised on GFAP positive reactive hypertropic astrocytes (Figure 4.6g-i). Some of these immunoreactive cells formed a fibrous network around blood vessels. In general, MMP-2 immunoreactivity was mainly concentrated in the cell body but also noted in the end feet of reactive astrocytes, where they formed a network around the blood vessels. Further, double staining of MMP-2 and GAP-43 showed that MMP-2 immunoreactive cells were co-localised with GAP-43 immunoreactivity (Figure 4.6j-l). GAP-43 is a brain specific cell membrane glycoprotein that is closely associated with neurite formation, regeneration, and plasticity (Skene, 1989, Meiri et al., 1998). GAP-43 immunoreactivity was observed in a punctuate pattern along the white matter tracts and in the penumbra of the injured cortex similar to the pattern of MMP-2 immunoreactivity.

At six hours following hypoxic ischemia at P3, intense MMP-9 immunoreactivity was seen in the deep layers (V-VII) of the injured cortex (Figure 4.5f). Staining of lesser intensity was also observed within the contralateral cortex (Figure 4.5i). The majority of the MMP-9 immunoreactive cells in the penumbra of the ipsilateral cortex had a neuronal morphology.

4.4 DISCUSSION
Several recent studies have demonstrated an upregulation of MMPs after cerebral ischemia in the adult brain (Rosenberg et al., 1996, Romanic et al., 1998, Asahi et al., 2000, Asahi et al., 2001, Gasche et al., 2001, Planas et al., 2001, Rosenberg et al., 2001, Zalewska et al., 2002, Lee et al., 2004). Svedin et al., showed that an MMP-9 knock-out
mouse was more resistant to HI injury during development (Svedin et al., 2007). However, the perinatal expression pattern of MMP-2 and MMP-9 in the injured cortex has not been described previously. First, we have shown that MMP-9 is markedly upregulated from six hours to one day following HI injury at both P3 and P21. Secondly, we have shown that MMP-2 is gradually upregulated after HI injury, peaking by five days post-injury.

We have demonstrated that MMP-9 activity was significantly upregulated following HI injury at both P21 and P3. HI injury to the P21 brain induced activity of two MMP-9 forms of 150kDa and 92kDa, from six hours to one day post-injury. The 92kDa form corresponds to the MMP-9 proform, whilst the 150kDa form likely represents a complex of MMP-9 with itself or another molecule (Asahi et al., 2000, Gutiérrez-Fernández et al., 2007). Following injury at P3, pro MMP-9 activity was significantly upregulated in the ipsilateral hemisphere within six hours, while at one day both the pro and cleaved forms were significantly elevated. The ratio of pro:cleaved forms decreased from 30:1 to 6:1 from six hours to one day after injury indicating that increased levels of pro forms are cleaved to produce active forms at one day. qRT-PCR studies showed that MMP-9 gene expression was also significantly upregulated in the injured hippocampus and cortex when compared to the contralateral uninjured regions at one day and six hours after injury at P21 and P3 respectively. Immunohistochemical studies showed that MMP-9 protein is highly expressed in the neuronal cytoplasm, neural processes and in the extracellular space adjacent to the neuronal membranes in the penumbra of the injured cortex and hippocampus. The spatiotemporal pattern of MMP-9 upregulation in both models strongly correlates with the period of highest delayed neuronal death (Williams et al., 1992, Sirimanne et al., 1994, Beilharz et al., 1995, Sizonenko et al., 2005). The neural
damage following severe hypoxic ischemia at P21 is most pronounced in the frontoparietal cortex and the entire hippocampal structure of the ipsilateral cortex, with the majority of cell death occurring within the first two days after injury (Sirimanne et al., 1994, Beilharz et al., 1995). It was also shown that neuronal and axonal degeneration peak at one day after inducing HI at P3 (Sizonenko et al., 2005). Therefore, our results together with results from previous studies on adult cerebral ischemia (Rosenberg et al., 1996, Romanic et al., 1998, Asahi et al., 2000, Asahi et al., 2001, Gasche et al., 2001, Planas et al., 2001, Rosenberg et al., 2001, Zalewska et al., 2002, Lee et al., 2004), and a recent study on HI injured neonatal brain (Svedin et al., 2007), strongly support a role for MMP-9 in the delayed injury processes following hypoxic ischemia at both P3 and P21. However, the relative change of MMP-9 activity in the injured ipsilateral hemisphere as compared to the control contralateral hemisphere after HI injury at P3 (two fold; estimated from Figure 4.2b) was considerably lower than that after HI injury at P21 (six fold; estimated from Figure 4.1c). Presumably, this could be due to the fact that constitutive protein and mRNA expression of MMP-9 was lower in the P3 compared to the P21 brain (Refer to Chapter Three). Therefore, the degree of MMP-9 participation in the injury processes after an HI insult at the two developmental ages could vary considerably.

Accumulating evidence suggests that MMP-9 plays a role in blood brain barrier (BBB) disruption during adult cerebral ischemia. Several studies have shown that the permeability of the BBB increases with increased MMP-9 activity after cerebral ischemia (Rosenberg et al., 1996, Fujimura et al., 1999, Gasche et al., 2001) and that deficiency of MMP-9 activity reduces BBB permeability (Asahi et al., 2001, Lee et al., 2005, Shigemori et al., 2006, Yang et al., 2007). Furthermore, MMP-9 inhibition reduced
degradation of the BBB components zonae occludens-1 (Asahi et al., 2001), basal lamina collagen type IV (Hamann et al., 2004), claudin-5 and occluding (Yang et al., 2007) following an ischemic attack to the adult brain. In fact, in the present study we demonstrated an increase in MMP-9 immunoreactivity in astrocytes adjoining cerebral blood vessels, suggesting a possible role for MMP-9 in BBB disruption in the developing brain. In addition to its role in mediating BBB permeability, MMP-9 activity has been shown to directly influence neuronal survival following cerebral ischemia, potentially by modulating cell-matrix interactions (Nagase and Woesnner, 1999, Sethi et al., 2000, Bosman and Stamenkovic, 2003). Pharmacological inhibition and gene deletion of MMP-9 has a neuroprotective effect during global ischemia (Jourquin et al., 2003) and the addition of exogenous MMP-9 into hippocampal slices and primary cortical neuronal cultures was shown to increase neuronal death (Gu et al., 2002). We observed that one day after injury, neurons in the penumbra of the injured cortex contained MMP-9 proteins in their cytoplasm and in the extracellular space lining their membranes. It is possible that MMP-9 is affecting their survival by modulating their interaction with the ECM. It has been demonstrated previously that the neuronal survival is strongly linked to extrinsic signals from the neighbouring environment (Jacobson et al., 1997, Goldberg and Barres, 2000). In addition to this, we found that neuronal processes in the injured cortex were intensely stained with MMP-9, suggesting a possible role for MMP-9 in axonal degeneration. Indeed, Asahi et al., have previously demonstrated MMP-9 knock-out mice to have reduced white matter associated myelin basic protein after transient cerebral ischemia (Asahi et al., 2001).

In contrast to MMP-9 activity, MMP-2 activity gradually increased after hypoxic ischemia at P21 reaching a peak at five days. In agreement with gelatinase activity levels,
MMP-2 expression was significantly upregulated in the injured cortex and hippocampus 25 and seven fold, respectively, when compared to the contralateral uninjured regions. Immunohistochemical studies revealed MMP-2 to be primarily associated with reactive hypertrophic astrocytes in the penumbra and along the corpus callosum of the injured cortex. Given that this occurs after the main period of delayed cell death (Beilharz et al., 1995), it is possible that this is not a pathogenic response but rather points to the involvement of MMP-2 in recovery processes. The pattern of MMP-2 elevation after hypoxic ischemia at P21 temporally and spatially parallels the formation of the glial scar around the ischemic core (Silver and Miller, 2004). A recent study has shown that astrocyte motility in vitro depends principally on MMP-2 activity (Ogier et al., 2006) suggesting that MMP-2 may potentially assist the migration and process outgrowth of reactive astrocytes during the formation of the glial scar. Conversely, it could also be participating in neuroregenerative processes. Several studies have demonstrated that MMP-2 promotes neurite outgrowth of peripheral nerves possibly by inactivating neurite inhibiting chondroitin sulfate proteoglycan (CSPG) (Zuo et al., 1998, Ferguson and Muir, 2000, Krekoski et al., 2002). Hsu et al., showed that elimination of MMP-2 activity, which was earlier shown to be increased between seven to 14 days after spinal cord injury by reactive astrocytes, resulted in increased levels of CSPG and reduced white matter sparing (Hsu et al., 2006). Further, Zhang et al., demonstrated that transplantation of retinal progenitor cells induced MMP-2 secretion by reactive glial cells resulting in proteolysis of neurite inhibiting CD44 and neurocan and increased neurite outgrowth (Zhang et al., 2007). Together, these studies suggest that increased MMP-2 expression evident on reactive astrocytes may have a role in the restoration of neuronal connectivity in the P21 rat brain. Indeed, we have shown that MMP-2 immunoreactive cells colocalised with GAP-43 immunoreactivity at five days after hypoxic ischemia at P21.
As mentioned above, GAP-43 is known to be very important role in neurite formation, regeneration, and plasticity in the brain (Skene, 1989, Meiri et al., 1998). In contrast to the response after hypoxic ischemia at P21, MMP-2 levels did not change significantly after hypoxic ischemia at P3, presumably as a result of already high constitutive levels during early postnatal brain development.

In summary, the aim of the present study was to determine the MMP-2 and 9 responses following HI injury to the developing brain. We demonstrated that MMP-9 activity was upregulated between six hours and one day following a HI injury at both P21 and P3. Similarly, qRT-PCR indicated MMP-9 gene expression was also significantly upregulated in the injured hemisphere following hypoxic ischemia at both ages. MMP-9 immunoreactivity was observed on neurons, peri-vascular astrocytes and neuronal processes in the ischemic regions. Conversely, MMP-2 progressively increased from one day following injury, reaching a peak by five days following HI injury at P21 whereas it was only moderately increased at six hours following HI injury at P3. MMP-2 mRNA expression was also increased in the injured hemisphere following injury at P21 similarly to its activity while it did not vary significantly following HI injury at P3. MMP-2 protein expression was localised predominantly on reactive astrocytes surrounding the infarct following injury at P21. In conclusion, we suggest that the above findings support an important role for proteolytic processes during injury in the preterm and early postnatal brain. MMP-9 potentially contributes in the development of delayed injury processes following HI injury to the developing brain whereas MMP-2 appears to be principally associated with wound repair processes following an injury.
CHAPTER FIVE: tPA AND uPA RESPONSE AFTER HYPOXIC ISCHEMIA IN
THE DEVELOPING BRAIN

5.1 INTRODUCTION

Plasmin is a broad–spectrum serine protease that can cleave various extracellular matrix (ECM) proteins including fibrin, laminin and fibronectin. Plasmin also acts as a major activator of several pro-matrix metalloproteinases (MMPs) including MMP-1, 2, 3, and 9. Plasminogen is the precursor protein that is cleaved by plasminogen activators to produce active plasmin. Tissue (tPA) and urokinase (uPA) plasminogen activators are the major types of plasminogen activators identified in mammals to date. They are also serine proteases similar to plasmin but have substrate specificity primarily on plasminogen (Saksela and Rifkin, 1988, Mohanam et al., 1994, Parry et al., 2000, Castellino and Ploplis, 2005). tPA is considered to be the major physiological vascular activator of plasminogen. It is produced predominately by endothelial cells although keratinocytes, melanocytes and neurons are also capable of producing it (Saksela and Rifkin, 1988, Angles-Cano, 1994, Plow et al., 1995, Parry et al., 2000, Castellino and Ploplis, 2005). However, it has been demonstrated that uPA is the principal activator of plasminogen in the extravascular space (Blasi et al., 1987, Saksela and Rifkin, 1988, Mayer, 1990, Angles-Cano, 1994, Plow et al., 1995, Spraggon et al., 1995, Parry et al., 2000, Castellino and Ploplis, 2005).

tPA is the only Food and Drug Administration (FDA) approved treatment for ischemic stroke. Nevertheless, emerging data suggest that both plasminogen activators (tPA and uPA) can be potentially neurotoxic. Recently, several studies have demonstrated that tPA and uPA are upregulated after cerebral ischemia in the adult brain (Ahn et al., 1999, Dietzmann et al., 2000, Yepes et al., 2000, Hosomi et al., 2001). Further studies have
illustrated that tPA deficiency is neuroprotective after cerebral ischemia (Wang et al., 1998, Nagai et al., 1999, Yepes et al., 2000, Tsuji et al., 2005), thus providing compelling evidence of a neurotoxic potential of plasminogen activators. A potential mechanism by which plasminogen activators are thought to be neurotoxic is through their activation of plasminogen to plasmin which then exerts a detrimental effect on brain parenchyma directly by cleaving certain ECM molecules such as laminin, or indirectly by activating ECM degrading MMPs such as MMP-2 and 9 (Tsirka et al., 1997, Kaur et al., 2004, Sheehan and Tsirka, 2005). Indeed, several studies have established a positive correlation between the tPA/uPA and MMP-2/9 expressions in cerebral ischemia (Hosomi et al., 2001, Horstmann et al., 2003, Wang et al., 2003, Kelly et al., 2006, Burggraf et al., 2007) proposing that plasminogen activators may affect pathophysiology of cerebral ischemia by activating MMP-2 and 9.

Our previous study (Chapter Four) showed that MMP-2 and 9 mRNA expression, protein expression and activity were strongly upregulated after a hypoxic ischemic (HI) injury to the developing brain. Given the evidence described above, we speculated that tPA and uPA activities would correlate with MMP-2 and 9 activity. Therefore, in the current study, we determined the tPA and uPA responses following HI injury in the developing brain using a well characterised rat model of unilateral HI injury at two developmental stages: postnatal day 21 (P21) (Sirimanne et al., 1994) and 3 (P3) (Sizomenko et al., 2005).
5.2 MATERIALS AND METHODS

Hypoxic Ischemic Injury

HI injury was induced in P21 and P3 rats as described in detail in Chapter Two; Section 2.1.

Plasminogen Zymography

Animals were killed at three hours, six hours, 12 hours, one day, two days, three days, five days and seven days post HI injury, with an overdose of sodium pentobarbitone. They were then transcardially perfused with cold 0.01M PBS (pH 7.4). The brains were then processed to extract the proteins according to the instructions outlined in Chapter Two; Section 2.3. Plasminogen zymography was then performed as detailed in Chapter Two; Section 2.9 to determine the tPA and uPA activity levels.

Quantitative Real-Time PCR

Animals were killed at five days and six hours following HI injury at P21 and P3 respectively. Total RNA from brain tissue was isolated (Chapter Two; Section 2.4), first-strand cDNA was synthesised (Chapter Two; Section 2.5) and quantitative real-time PCR (qRT-PCR) was performed (Chapter Two; Section 2.10) on 9.6ng of cDNA as described in Materials and Methods. Expression of target gene (uPA) in the ipsilateral injured hemisphere was expressed relative to the corresponding contralateral uninjured hemisphere after normalising to the level of the housekeeping gene (GAPDH).

Immunohistochemistry

Animals were killed; brains were processed and embedded in paraffin as described in Chapter Two; Section 2.3. Single and double immunohistochemical procedures were
carried out as described in Chapter Two; Section 2.8 according to the conditions outlined in Chapter Two; Table 2.7.

Briefly, the sections were deparaffinized and rehydrated, then blocked for two hours for endogenous IgG (5% normal horse serum (NHS) in 5mg/mL BSA/PBS (pH 7.4)) and endogenous biotin (DAKO Biotin Blocking system) before incubating with primary antibodies for uPA (1:25; 3689; American Diagnostica, USA) in 5mg/mL BSA/PBS for 24 hours at 4°C. They were further incubated with horse anti-mouse biotinylated secondary antibody (1:200; Vector Laboratories, UK) in 5mg/mL BSA/PBS for one hour at room temperature followed by Alexa 568 (1:200; Molecular Probes, USA) for one hour at 37°C. Sections were mounted in Vectashield (Vector Laboratories, UK) and stored at 4°C. Negative controls included using antibody diluting buffer instead of the primary antibody. Results showed that specific immunoreactivity was absent on these sections.

Double immunohistochemistry was performed to determine the specific cell types that expressed uPA. The immunohistochemical procedure for uPA was carried out as above, followed by extensive washing and reblocking with 5% NHS in 5mg/mL BSA/PBS. Sections were incubated with primary antibodies for glial fibrillary acidic protein (GFAP; 1:200; Chemicon, USA) and MMP-2 (IM33L; Calbiochem, USA) in 5mg/mL BSA respectively for 12 hours and 48 hours at 4°C. This was followed by incubation with horse anti-mouse biotinylated secondary antibody (1:200) for one hour at room temperature followed by Alexa 488 (1:200) for one hour at 37°C. The sections were then mounted in Vectashield and stored at 4°C.
Figure 5.1: tPA and uPA activities after hypoxic ischemia in P21 rat brain. Representative zymogram shows the proteolytic activity at different time points after injury (a). tPA activity remained unaffected while uPA activity was gradually upregulated from 12 hours reaching a peak at 3-5 days. Histograms show the quantification of relative activities of tPA (b), and uPA (c) by densitometry of plasminogen zymography. Results are presented as mean ± SEM. Asterisks ** and * represent the statistical significance (P ≤ 0.005) between ipsilateral hemisphere (HI) and both the contralateral hemisphere (HI) and control brains and ipsilateral (HI) and either contralateral (HI) or control brains respectively. n = 3-9.
Figure 5.2: tPA and uPA activities after hypoxic ischemia in P3 rat brain. Representative zymogram shows the proteolytic activity at different time points after injury (a). tPA activity increased with age but remained unaffected following HI injury while uPA activity was moderately increased between 12 to 24 hours following HI. Histograms show the quantification of relative activities of tPA (b), and uPA (c) by densitometry of plasminogen zymography. Results are presented as mean ± SEM. Asterisks ** and * represent the statistical significance (P ≤ 0.01) between ipsilateral hemisphere (HI) and both the contralateral hemisphere (HI) and control brains and ipsilateral (HI) and either contralateral (HI) or control brains respectively. n = 3-9.
5.3 RESULTS

uPA activity was increased following hypoxic ischemia while tPA activity remained unaffected

Proteolytic activity was detected at approximately 60 and 36kDa, presumably corresponding to tPA and the low molecular weight isoform of uPA respectively (Blasi et al., 1987, Saksela and Rifkin, 1988). In normal 21 day old rat brains, a high amount of tPA activity was detected, while uPA activity was detected in moderate amounts (Figure 5.1a). Quantification of the relative activities as described in Chapter Two; Section 2.9 revealed that uPA activity gradually increased after HI injury; it was significantly higher in the injured ipsilateral hemisphere at 12 hours ($P \leq 0.01$) after injury as compared to the uninjured contralateral hemisphere and control brains, and continued to increase reaching a peak at three to five days ($P \leq 0.001$) (Figure 5.1c). At seven days after injury uPA activity in the injured ipsilateral hemisphere began to decline, but was still significantly higher ($P \leq 0.01$) than in control tissues. Conversely, tPA activity remained unaffected after HI injury to the P21 brain (Figure 5.1b).

In normal three day old rat brains, both tPA and uPA activity levels were low (Figure 5.2a). Densitometric analysis showed that uPA activity was significantly upregulated from six hours to one day after injury in the injured ipsilateral hemisphere as compared to the uninjured contralateral hemisphere and control brains ($P \leq 0.05$) (Figure 5.2c). Thereafter, uPA activity did not vary significantly within the injured and control hemispheres. Similar to the pattern observed above, tPA activity remained unaffected following a HI injury to the P3 rat brain (Figure 5.2b). However, it was observed that tPA activity in the normal rat brains increased ($P \leq 0.001$) with age. tPA activity was detected in low levels in the rat brain from three hours (P3) to three days (P6). However,
it was significantly increased ($P \leq 0.005$) at five days (P8) as compared to the younger brains (Figure 5.2b). At seven days (P10) levels remained elevated ($P \leq 0.005$).

**uPA gene expression was also increased following hypoxic ischemia**

qRT-PCRs was performed to determine the changes in uPA gene expression after HI injury, in order to further confirm the upregulation of uPA activity that was demonstrated by zymography. uPA expression was shown to be minimal in the contralateral hemisphere both at five days and six hours following injury at P21 and P3 respectively, as indicated by the low $C_t$ (~30) values. At five days following HI injury at P21, uPA expression was significantly elevated; approximately 350 and 13 fold respectively in the ipsilateral injured cortex ($P \leq 0.05$) and hippocampus when compared to the corresponding contralateral uninjured regions (Figure 5.3a). At six hours following injury at P3, uPA expression was elevated approximately four and 11 fold in the injured cortex and hippocampus ($P \leq 0.05$) respectively, when compared to the contralateral uninjured hemisphere (Figure 5.3b) in agreement with uPA plasminogenic activity.

**uPA was located in the injured hemisphere following hypoxic ischemia**

By three days following HI injury at P21, a high level of uPA immunoreactivity was present in the ipsilateral injured hemisphere. The pattern of uPA immunoreactivity was observed in an identical pattern to that of MMP-2 immunoreactivity (*Chapter 4: Figure 4.5*). uPA immunoreactive cells were observed in the injured cortex, forming a cellular barrier around the infarct area (Figure 5.4a), along the white matter tracts of the corpus callosum (Figure 5.4b) and in the injured hippocampus (Figure 5.4c). Double staining with GFAP confirmed that uPA was localised on GFAP positive reactive hypertropic
Figure 5.3: uPA gene expression after hypoxic ischemia in P21 and P3 rat brain respectively at 5 days and 6 hours after injury. Following injury at P21 the uPA gene expression was upregulated approximately 350 fold in the injured cortex and 13 fold in the injured hippocampus when compared to the uninjured contralateral hemisphere (a). Following injury at P3, it was elevated 4 and 11 fold in the injured cortex and hippocampus respectively when compared to the contralateral regions (b). * represents the statistical significance ($P \leq 0.05$) between ipsilateral injured and contralateral uninjured hemispheres. $n = 3-4$
Figure 5.4: uPA Immunoreactivity after HI injury to the P21 rat brain. At 3 days uPA immunoreactivity was seen in the cortex surrounding the infarct area (a), along the corpus callosum (CC) (b) and hippocampus of the injured hemisphere. A higher magnification image (a inset) showed that uPA immunoreactivity has an astrocytic morphology. Double immunostaining (f) for uPA (d) and GFAP (e) confirmed that uPA was located in the reactive astrocytes in the ipsilateral hemisphere at 3 days after HI injury. uPA immunoreactivity (g) also colocalised (i) with MMP-2 immunoreactivity (h). Scale bars = 100μm (a-c), 20μm (a inset) and 10μm (d-i).
astrocytes (Figure 5.4d-f). Similar to MMP-2 immunoreactivity, it was mainly concentrated in the cell body of the GFAP immunoreactive cells. Furthermore, double staining of uPA and MMP-2 showed that uPA and MMP-2 were expressed by the same group of astrocytes (Figure 5.4g-i). Moreover, uPA and MMP-2 showed an identical localisation within their cellular source.

5.4 DISCUSSION

Recently, numerous studies have demonstrated that tPA and uPA upregulation is implicated in cerebral ischemia in the adult mammalian brain (Wang et al., 1998, Ahn et al., 1999, Nagai et al., 1999, Dietzmann et al., 2000, Yepes et al., 2000, Hosomi et al., 2001, Tsuji et al., 2005). Furthermore, several studies have illustrated a positive correlation between the tPA/uPA and MMP-2/9 activity levels in cerebral ischemia (Hosomi et al., 2001, Horstmann et al., 2003, Wang et al., 2003, Kelly et al., 2006, Burggraf et al., 2007) suggesting that plasminogen activators may affect the pathophysiology of cerebral ischemia by activating MMP-2 and 9. Our previous study (Chapter Four) showed that MMP-2 and 9 mRNA, protein and activity levels were strongly upregulated after HI injury in the immature brain. Therefore, in this study we investigated the possibility that tPA and uPA activities were upregulated in a similar manner. Our results showed that uPA activity and mRNA expression were upregulated after hypoxic ischemia at both P21 and P3 in a similar trend to that of MMP-2, whereas tPA activity remained unaffected.

uPA activity progressively increased following HI injury at P21 from 12 hours onwards, reaching a peak at three to five days. This pattern of uPA upregulation closely parallels that of MMP-2 after HI injury at P21 which also gradually increases reaching a peak at five days after injury (Chapter 4; Figure 4.1). Following injury at P3, uPA activity was
briefly upregulated within six hours to one day, again similar to the upregulation of MMP-2 in the same model (Chapter 4; Figure 4.2). Also the uPA and MMP-2 (Chapter 4; Figure 4.3) gene expression patterns following HI injury at P21 are closely correlated. Furthermore, immunohistochemistry showed that uPA and MMP-2 were co-expressed by GFAP positive reactive astrocytes in the HI injured rat brain in an identical manner. The above observations support the view that uPA may contribute to the pathophysiology of HI injury by activating MMP-2. In support, Hosomi et al., demonstrated that the increase of uPA activity following MCAO in primates coincided with the increase of MMP-2 activity (Hosomi et al., 2001). Furthermore, a recent study showed that doxycyclin treatment reduces the cerebral damage after focal cerebral ischemia by reducing MMP-2, MMP-9 and uPA suggesting an involvement of plasminogen activator-plasmin-MMP axis in the pathophysiology of injury (Burggraf et al., 2007). Several in vitro studies have demonstrated that uPA activates MMP-2 via activation of plasmin. Addition of plasminogen to human fibrosarcoma cells increased the conversion of pro MMP-2 to cleaved MMP-2, which was inhibited by an anti-uPA antibody, suggesting the involvement of a uPA/plasmin system in MMP-2 activation (Baramovaa et al., 1997). Pharmaceutical inhibition of uPA activity reduced the conversion of plasminogen to plasmin, which in turn reduced the cleaved MMP-2 activity in a cell line of pancreatic cancer (He et al., 2007). Furthermore, uPA is capable of directly activating MMP-2 in a plasminogen independent mechanism. Keski-Oja et al., 1992 showed that MMP-2 activation by uPA was inhibited by an anti-uPA antibody but was not affected by the plasmin inhibitors (Keski-Oja et al., 1992). These evidences suggest that uPA is proficient in activating MMP-2 via either a plasmin-dependant or a plasmin-independent mechanism. Previously, we suggested the MMP-2 is potentially involved in recovery processes following HI injury to the developing brain (Chapter 4). Therefore, given the
above evidence we propose that uPA may also contribute to the recovery processes following a HI injury by activating MMP-2. Indeed, several recent studies have demonstrated that uPA deficiency is not neuroprotective following cerebral ischemia in the adult brain (Nagai et al., 1999, Yepes et al., 2003, Nagai et al., 2008), suggesting that uPA may not contribute to the injury progression, thus indirectly supporting our suggestions.

tPA activity was not affected by HI injury at either P21 or P3, suggesting that tPA may not play an important role in the pathophysiology of HI injury in the developing brain. These results are supported by evidence that illustrate either a neutral or beneficial effect of tPA in the pathogenesis of injury in the adult brain. Several studies have demonstrated that exogenous administration of tPA does not confer any detectable negative effect in rat models of global and focal ischemia (Klein et al., 1999), embolic and mechanical cerebral ischemia (Meng et al., 1999). Also tPA deficiency has been shown to be detrimental in ischemic (Tabrizi et al., 1999) and thrombotic (Atochin et al., 2004) brain injuries. However, our finding contradicts those of Adhami et al., 2008, which demonstrated deleteriously increased tPA activity following HI injury in P7 mouse brain (Adhami et al., 2008). Numerous studies on the adult mammalian brain have also illustrated similar effects where tPA had detrimental processes following cerebral ischemia (Wang et al., 1998, Nagai et al., 1999, Tsuji et al., 2005).

In summary, the aim of the present study was to determine tPA and uPA responses following hypoxic ischemia in the developing brain. We showed that in the P21 rat brain, uPA activity gradually increased in the ipsilateral injured hemisphere reaching a peak at three to five days following injury, whereas tPA activity remained unaffected. In the P3
rat brain, zymography revealed uPA activity was moderately upregulated in the ipsilateral injured hemisphere from six hours to one day after injury, whilst tPA activity was unaffected. qRT-PCR showed that uPA expression was also increased in the injured hemisphere following HI injury at both P21 and P3 in agreement with the activity. Immunohistochemistry indicated that uPA co-localised with MMP-2 on reactive astrocytes surrounding the infarct. Consequently, we suggest a function for uPA in wound repair processes following hypoxic ischemia to the developing brain through activation of MMP-2, whereas tPA potentially may not play an significant role in the pathophysiology of HI injury to the developing brain.
CHAPTER SIX: EFFECT OF INHIBITION OF MMP-9 ACTIVITY AFTER HYPOXIC ISCHEMIA IN THE DEVELOPING BRAIN

6.1 INTRODUCTION

As mentioned in the introduction (Chapter One), perinatal hypoxic ischemic (HI) brain injury is a worldwide problem of enormous importance. Statistically, birth asphyxia occurs in 2-4 per 1000 full-term infants and nearly 60% of the pre-term infants worldwide. Approximately 20-50% of newborns that exhibit HI encephalopathy die within the immediate postnatal days. Approximately 25% of those who survive later develop neurodegenerative abnormalities that are recognised as they mature. These include mental retardation, learning disabilities, subtle motor abnormalities and vision or hearing impairments. (Takashima et al., 1995, Volpe, 1998, Inder and Volpe, 2000, Blumenthal, 2004, Ferriero, 2004, Vannucci and Hagberg, 2004). Such neurological handicap in infants surviving after perinatal HI remains a major problem for which there is no specific treatment.

Matrix metalloproteinases (MMPs) are believed to play an important role in the pathophysiology of cerebral ischemia. Indeed, accumulating evidence suggests that MMP-2 and MMP-9 activities are upregulated after cerebral ischemia in the adult brain (Rosenberg et al., 1996, Romanic et al., 1998, Asahi et al., 2000, Lee et al., 2004). Importantly, MMP-9 activity has recently been implicated in the aetiology of injuries to the immature brain. Schulz and colleagues have demonstrated that plasma levels of MMP-9 were significantly higher in infants with intraventricular haemorrhage suggesting a possible role of peripheral MMP-9 in neonatal brain injury (Schulz et al., 2004). Furthermore, Svedin et al., have shown that MMP-9 deficiency protected the immature mouse brain from HI injury (Svedin et al., 2007). Our previous study (Chapter Four)
showed that MMP-9 activity was strongly upregulated after HI injury to the developing brain within six hours. By 24 hours immunohistochemistry showed that MMP-9 was intensely present in the neuronal cytoplasm, neural processes and in the extracellular space adjacent to the neuronal membranes in the penumbra of the injured cortex and hippocampus. This spatiotemporal pattern of MMP-9 upregulation strongly correlated with the period of highest delayed neuronal death in the corresponding animal model (Williams et al., 1992, Sirimanne et al., 1994, Beilharz et al., 1995, Sizonenko et al., 2005). Given this, we therefore conclude that MMP-9 may be a useful target for rescue therapies in the injured developing brain.

Previous studies using synthetic inhibitors have provided evidence that inhibition of MMP-9 activity reduces the severity of ischemic injury in the adult brain (Romanic et al., 1998, Asahi et al., 2000, Jiang et al., 2001, Pfefferkorn and Rosenberg, 2003, Lee et al., 2004). The majority of these inhibitors are hydroxamates that bind to the zinc atom at the active site of MMPs, thus inactivating the enzymes (Woessner, 1999, Jacobsen et al., 2007, Nuti et al., 2007). Although these hydroxamates were highly successful in preclinical studies, they failed in the human clinical trials of cancer treatments due to adverse side effects such as inflammation (Coussens et al., 2002, Nuti et al., 2007, Fingleton, 2008). These undesirable effects were mainly a consequence of their inability to discriminate for a specific MMP active site and a specific ion, thus rendering them effective against a broad range of MMPs, other metalloenzymes such as ADAMs and possibly other physiological important enzymes (Woessner, 1999, Jacobsen et al., 2007, Nuti et al., 2007, Yong et al., 2007, Fingleton, 2008). Given these confounding problems as well as the fact that various other MMPs and related enzymes are known to have vital physiological roles during development of the brain (Yong et al., 2001, Ulrich et al.,
we chose to use SB-3CT, a highly selective inhibitor, that is known to target only MMP-2 and MMP-9 (Brown et al., 2000). SB-3CT inhibits the enzymes by acting as a suicide substrate that selectively binds within the active site cleft of MMP-2 and 9, therefore promising high in vivo selectivity. Firstly, the sulphur atom of the thiirane group of the inhibitor directly coordinates with the zinc atom in the active site of the MMP-2 and 9. This coordination then predisposes the thiirane to nucleophilic attack by the active site glutamate of these enzymes thus forming a covalent bond with the enzyme. Furthermore, the inhibitor prompts the enzyme to reinstate to the conformation of its latent form (Brown et al., 2000, Kleifeld et al., 2001, Ikejiri et al., 2005a, Ikejiri et al., 2005b). Recently Gu et al., showed that administration of SB-3CT prevents neuronal death in a mouse model of stroke (Gu et al., 2005).

In this study our primary objective was to determine the efficacy of SB-3CT in reducing the severity of HI brain injury to the developing brain using a postnatal day 21 (P21) old rat model. Firstly, we investigated the effect of SB-3CT on the activity of brain MMP-9 following an HI injury. Secondly, we determined that effect of SB-3CT administration on the total infarct area and neurological deficits of HI injured animals.

6.2 MATERIALS AND METHODS

Hypoxic ischemic Injury

HI injury was induced in P21 rats as described in detail in Chapter Two; Section 2.1.1.

Treatment

SB-3CT (BIOMOL, USA) was injected intraperitoneally (IP) as a suspension of 25, or 50mg/kg body weight (BW) with 10% dimethyl sulfoxide (DMSO) (density =
1.101 g/mL/ 90% saline, or 25% DMSO/ 75% polyethylene glycol (PEG) starting two hours after HI, followed by second and third injections at five and 14 hours. Weight matched control animals received injections of vehicle or saline only. A final volume of not more than 0.4% of BW was injected with an ultrafine 30 gauge insulin needle. An IP route of delivery was chosen since effective delivery of this inhibitor to the brain had previously been reported in adult rats (Gu et al., 2005). Furthermore, similar dosages have been shown to be effective in inhibiting of MMP activity in adult mouse models of ischemia (Gu et al., 2005) and liver (Kruger et al., 2005) and prostate (Bonfil et al., 2006) metastasis without any confounding toxic effects. Following treatment in the current study, the animals were returned to the normal rat housing rooms (22°C, 55% ± 5% relative humidity).

**Collection of Blood and Cerebral Spinal Fluid**

Blood and cerebral spinal fluid (CSF) samples were collected at six hours following the first two injections of SB-3CT to determine the presence of the drug in the system. The rats were placed into a stereotaxic head frame with the head flexed downward while under sodium pentobarbitone (50mg/kg BW) anaesthesia. The skin over the depression caudal to the occipital prominence was incised and a blunt dissection was made over the cisterna magna until the dura was exposed. CSF was then collected, using a fine 30 gauge insulin needle, into eppendorf tubes, chilled on ice and ultimately stored at -80°C. CSF samples that were contaminated with blood were excluded from the study. The rats were then killed by an i.p. administration of an overdose of sodium pentobarbitone. Blood samples were then taken transcardially into heparinised tubes, and centrifuged at 3000g for 15 minutes to isolate plasma, which was then stored at -80°C.
Liquid Chromatography and Tandem Mass Spectrometry

SB-3CT was extracted from the CSF and blood samples using ethyl acetate. 1mL of ethyl acetate (Merck KGaA Darmstadt, Germany) was added to 50μL of sample and 100uL of internal standard (corticosterone-d8 at 14ng/mL), vortex mixed and centrifuged to separate the organic and aqueous layers. The organic supernatant was dried using the freezer dryer, reconstituted in 80μL of 45% Methanol (Merck KGaA Darmstadt, Germany)/ 55% H2O, vortex mixed and transferred to high-performance liquid chromatography (HPLC) injector vials. The HPLC system consisted of a Waters 2690 Alliance separation module (Waters Corporation, Milford, MA, USA), a 50 × 3 mm C18 Phenomenex Luna column (Phenomenex, New Zealand) at 30°C, and a mobile phase of 55% Methanol/ 45% H2O at rate of 500μL/min. 25μL of each sample was injected into the HPLC column. The resolved samples were ionized using atmospheric pressure chemical ionization (APCI) (discharge current - 20.0V; vapouriser temperature - 244°C; sheath gas – 30; ion sweep gas – 8; capillary temperature – 350°C) in a Finnegan TSQ Quantum Ultra AM Mass Spectrometer (Thermo Electron Corporation, San Jose, CA, USA). Then the selected ion, SB-3CT (parent ion; mass/charge (m/z) - 307.00; retention time – 2.85 minutes; Q1 - 0.25 mass units) was fragmented using Argon (collision pressure – 1.2mTorr; collision energy - 15V) to produce a product ion (daughter ion; m/z 232.9; Q3 - 0.60 mass units). Corticosterone-d8 was analysed simultaneously to be used as an internal control (parent – m/z 355.30; daughter – m/z 125.2; collision pressure – 1.2mTorr; collision energy – 24V; Q1 - 0.30 mass units; Q3 - 0.70 mass units; retention time - 1.7 minutes).
**Gelatin Zymography**

Animals were killed at six hours after injury following two injections with an overdose of sodium pentobarbitone. They were then transcardially perfused with cold 0.01M PBS (pH 7.4). The brains were then processed to extract the proteins according to the instructions outlined in *Chapter Two; Section 2.3*. Gelatin zymography was then performed as detailed in *Chapter Two: Section 2.8* to determine the MMP-2 and 9 activity levels. The effect of SB-3CT on the MMP-2 and 9 activities *ex vivo* was determined by adding a range of concentrations (0, 0.001, 0.01, 0.1, 1, 10μM) of SB-3CT into the developing buffer.

**Behavioural Testing**

Neurological deficits following treatments were determined using a well characterised motor test (postural reflex) that was specifically designed to sensitively measure deficits produced by a unilateral model of brain injury (Bederson et al., 1986). The degree of abnormal posture can be estimated by suspending the rats by their tail 20cm above a tabletop and slowly lowering them towards the table top. They were scored as follows: Rats that extended both forelimbs towards the table surface were considered intact (score =0). Rats that only reached with the forelimb contralateral to the side of the injury were considered moderately injured (score =2). Animals that revolved their contralateral shoulder towards the tail were considered severely injured (score =4).

**Histology**

Areas of infarction and cell death were determined using acid fuschin and thionin staining on Stereo Investigator 6 (MicroBrightField, USA). Animals were killed at three
days following injury; brains were processed and embedded in paraffin as described in Chapter Two; Section 2.2. Sections were processed to determine the basic histology as described in Chapter Two; Section 2.6 according to the conditions outlined in Chapter Two; Table 2.6. Areas of infarction and cell death in the injured ipsilateral hemisphere were expressed as a percentage of the uninjured contralateral hemisphere in order to correct for possible brain edema.

6.3 RESULTS

*Ex vivo* inhibition of MMP-2 and 9 using SB-3CT

The effect of SB-3CT on the MMP-2 and 9 activity on gelatin zymograms was determined over a range of SB-3CT concentrations (Figure 6.1a). SB-3CT was dissolved in DMSO and diluted in the developing buffer to obtain the desired concentration. Both MMP-2 ($P \leq 0.001$) and 9 ($P \leq 0.05$) activities demonstrate a trend towards reduction with increasing SB-3CT concentration (Figure 6.1b). In the presence of approximately 3mg/L of SB-3CT in the developing buffer, the activity of MMP-2 and 9 were reduced approximately by 80%. As described in the Materials and Methods the gels were incubated for 40 hours in the developing buffer before they were analysed. Therefore, it is possible that the inhibitor might not have been active for the entire time period given its rapid metabolism.

Effect of SB-3CT on the MMP-9 activity after an intraperitoneal injection following hypoxic ischemia

It was essential to first determine if SB-3CT was delivered into the brain via the IP route, whether it would inhibit brain MMP-9 activity and if so which dose of SB-3CT had the maximal effect. Firstly, SB-3CT was intraperitoneally injected as a suspension
Figure 6.1: Effect of MMP-2/9 specific inhibitor, SB-3CT, on the MMP-2 and 9 activities on gelatin zymography. Representative gelatin zymography shows that both MMP-2 (P ≤ 0.001) and 9 (P ≤ 0.05) activities significantly decreased with increasing concentration of SB-3CT (a). Analysis of the gelatin zymography confirmed that MMP-2 and 9 activities shows significant negatively correlation with the concentration of SB-3CT (b)
of 10% DMSO in saline at a dose of 25mg/kg BW of the rats. The brain MMP-9 activity was determined at six hours following HI following two injections at two hours and five hours. As shown in Figure 6.2, SB-3CT treated animals did not show a significant reduction of pro and cleaved MMP-9 activity. It was also observed that SB-3CT precipitated immediately upon dilution in saline therefore alternative vehicle solvent options were considered in order to improve solubility. After several trials, 25% DMSO/75% PEG was selected as it suspended SB-3CT in solution most effectively for the longest period. Thus, animals were then treated with 25mg/kg of BW SB-3CT suspended in 25% DMSO/75% PEG or vehicle or saline only. Again, SB-3CT treatment failed to significantly reduce the MMP-9 activity in the brain as compared to the vehicle or saline treated groups (Figure 6.3). Therefore, the effect of a higher dose of 50mg/kg of BW of SB-3CT in 25% DMSO/75% PEG was then assessed (Figure 6.4a). Gelatine zymography showed that at this higher dose, animals had significantly reduced levels of pro MMP-9 as compared to the control groups (P ≤ 0.05) (Figure 6.4b). However, there was no statistically significant reduction in levels of cleaved MMP-9 activity with the SB-3CT treatment (Figure 6.4c). It was also noted that the vehicle and saline treated groups did not have statistically significant variations in either pro or cleaved MMP-9 activity at any dosage regime suggesting that vehicle alone did not have an effect on MMP-9 activity (Figure 6.3 and 6.4). Following the above experiments, it was decided that the dose of 50mg/kg BW of SB-3CT in 25% DMSO/75% PEG would be used in assessing the effect of SB-3CT on neuronal damage and neurobehavioural deficits since this dose regime was successful in reducing brain pro MMP-9 levels.
Figure 6.2: MMP-9 activity after IP administration of MMP-2/9 inhibitor SB-3CT at 25mg/kg BW in 10% DMSO, 90% Saline. Gelatin zymography analysis showed that both pro (a) and cleaved (b) forms of MMP-9 activity were not significantly different between the SB-3CT treated group and the vehicle treated group. n = 4.
Figure 6.3: MMP-9 activity after IP administration of MMP-2/9 inhibitor SB-3CT at 25mg/kg BW in 25% DMSO, 75% PEG. Gelatin zymography analysis showed that both pro (a) and cleaved (b) forms of MMP-9 activity were not significantly different between SB-3CT treated, vehicle treated and saline treated groups. n = 4.
Figure 6.4: MMP-9 activity after IP administration of MMP-2/9 inhibitor SB-3CT at 50mg/kg of BW of SB-3CT in 25% DMSO/ 75% PEG (a). Gelatin zymography analysis showed that the SB-3CT treated group has reduced pro MMP-9 activity compared to the vehicle treated group (b). However, cleaved MMP-9 activity was not significantly different between the groups (c). * represents the statistical significance ($P \leq 0.05$) between SB-3CT treated (n = 8) and vehicle treated (n = 8) groups.
a) 

b) Pro MMP-9

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* indicates significant difference.

c) Cleaved MMP-9

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Figure 6.5: Detection of SB-3CT in blood and CSF after the IP treatment of SB-3CT by mass spectrometry. Approximately 100ng/mL and 150ng/mL SB-3CT was detected in the blood samples of the rats that were injected with 25mg/kg BW and 50mg/kg BW respectively (a) but was not detected in the CSF sample. SB-3CT was eluted at 2.85 minutes (arrow heads; b) from the column, however another peak at 2.25 minutes (arrows; b) appeared in the SB-3CT standard, blood and CSF samples of SB-3CT treated (50mg/kg of BW of SB-3CT in 25% DMSO/ 75% PEG) rats but not in the vehicle treated rats.
SB-3CT Standard Blank
Blood SB-3CT Treated Blank
Blood Vehicle Treated CSF SB-3CT Treated CSF Vehicle Treated
Detection of SB-3CT in blood and CSF samples

A mass spectrometry method was developed for SB-3CT in order to determine if SB-3CT was delivered into the blood circulation and passed through the blood brain barrier into CSF. A standard solution of SB-3CT eluted from the HPLC column gave a peak at 2.85 minutes on the mass spectrometry (Figure 6.5b; row one; arrow head). Similarly, in the blood of animals treated with SB-3CT a peak was detected at 2.85 minutes (Figure 6.5b; row two; arrow head) but not in the vehicle treated animals. It was calculated using a standard curve that approximately 100ng/mL and 150ng/mL of SB-3CT was detected in the blood samples of the rats that were injected with 25mg/kg BW and 50mg/kg BW of SB-3CT respectively (Figure 6.5a). Interestingly, however, another peak was detected at 2.25 minutes (Figure 6.5b; arrows) in the SB-3CT standard, and blood and CSF samples of SB-3CT treated rats but not in the vehicle treated rats. This additional peak could possibly correspond to one of the metabolites of SB-3CT.

Effect of SB-3CT on body weight

One litter of rats was equally divided into all the three treatment groups taking care to equalize the weights. Their weights were measured before the HI injury was induced and every subsequent day until they were killed. All animals lost weight one day following injury but continued to gain weight from thereafter. There was no significant statistical variation of weights between the three treatment groups for each day (Figure 6.6). However, it was noted that the SB-3CT treated group was at the lower end of the weight range.
Effect of SB-3CT on neuronal damage

It has been described previously that a HI insult to a P21 rat brain induces extensive damage mainly in the cortex, hippocampus and striatum and to a lesser extent in the thalamus. The total area of neuronal damage of the total hemisphere measured at three coronal levels was not significantly different between the three treatment groups (Figure 6.7a). However, a tendency towards a reduction was observed in the vehicle treated and SB-3CT treated group respectively as compared to the saline treated group. The mean area of infarction and cell death of the SB-3CT treated group was about 20% and 7% less than that of the saline and vehicle treated groups respectively (Figure 6.7a). A similar trend was observed when the areas of damage were analyzed separately at the three coronal levels (Figure 6.7b). The greatest difference was shown at the mid-hippocampal level. Mean area of infarction at the mid-hippocampal level of the SB-3CT treated rats was approximately 25% and 6% less than that of saline and vehicle treated groups respectively. A significant difference was not observed even when the damage to different structures of the brain was analyzed separately (Figure 6.7c). Furthermore the majority of the neuronal damage was seen in the cortex. Although SB-3CT did not confer significant neuroprotection in the cortex over vehicle or saline, the cortical damage of the SB-3CT and vehicle treated brains was lower than that of the saline treated animals. Similarly, the hippocampal and thalamic damage in the SB-3CT animals appeared to be lower than that of the vehicle or saline treated groups although it did not reach statistical significance.

Effect of SB-3CT on neurobehavioural deficits

An analysis of the severity of the neurobehavioural deficits of these rats was also performed. Although animals treated with SB-3CT showed a trend towards a reduction
Figure 6.6: BWs from the day of the injury until the sacrifice of the animals. BWs were not significantly different in the SB-3CT (50mg/kg of BW of SB-3CT in 25% DMSO/ 75% PEG) and vehicle treated rats as compared to the saline treated. n = 12.
Figure 6.7: Effect of IP administration of MMP-2/9 inhibitor SB-3CT (50mg/kg of BW of SB-3CT in 25% DMSO/ 75% PEG) on the severity of the HI injury to the P21 brain. Infarct area measurements overall (a), at different coronal levels (b) and on different brain structures (c) showed that SB-3CT was ineffective in reducing infarct area. Results are presented as mean ± SEM. n =12.
Figure 6.8: Effect of IP administration of MMP-2/9 inhibitor SB-3CT (50mg/kg of BW of SB-3CT in 25% DMSO/75% PEG) on the severity of the HI injury to the P21 brain. Neurological behaviour scores (postural reflex test) (a) confirms that SB-3CT was ineffective in improving neurobehavioural outcome. However, the area of infarct directly correlated (P \leq 0.0001) with the neurological behaviour scores confirming that it is postural reflex test is reliable measure of injury. Results are presented as mean ± SEM. n = 12.
in neurobehavioural deficits as compared to the vehicle or saline treated animals, the
difference was not statistically significant (Figure 6.8a). In brief, our results showed that
the mean behavioural score of the SB-3CT treated rats was approximately 1.5 points, of
the 4 point scale that was used to gauge the injury, whilst that of vehicle and saline
treated groups were approximately 2 and 2.5 respectively. We evaluated the relationship
between the infarction and neurobehavioural measured by the postural reflex test to
determine if the neurological behaviour correlated with neuronal damage. Results showed
that the areas of infarct and cell death directly correlated \( (P \leq 0.0001) \) with the
neurological behaviour scores confirming that postural reflex test is a reliable measure of
injury.

6.4 DISCUSSION

MMP-9 has been highly implicated in the pathogenesis of injuries in the adult and
juvenile brain. Our previous study (Chapter 4) showed that MMP-9 potentially may play
a role in the development of delayed injury processes following HI injury in the
developing brain. Therefore, the aim of this study was to investigate the effect of
inhibition of MMP-9 following a HI injury to the P21 rat brain. Previously, numerous
studies have shown significant neuroprotection using broad-spectrum MMP inhibitors
following cerebral ischemia in the adult brain. This study for the first time investigated
the inhibition of MMP-9 following HI in the developing brain using a highly selective
MMP-2/9 inhibitor, SB-3CT. However, our results showed that despite significantly
inhibiting brain MMP-9 activity after HI, SB-3CT failed to confer significant
neuroprotection in P21 rats after an HI insult.
We first confirmed that SB-3CT indeed inhibited MMP-2 and 9 activities by performing an *ex vivo* study by incubating the gelatin zymograms with various concentrations of SB-3CT. It was found that 10μM of SB-3CT was required to inhibit activity by half. The requirement of the high concentration could be attributed to possible precipitation of the inhibitor given its poor solubility. Another possibility was that the inhibitor could have been metabolised completely by the end of the study given that they were incubated for 40 hours. Furthermore, penetration into the gelatin zymogram may have been limited. It was then essential to determine if SB-3CT was delivered into the brain after an IP administration before determining its effects of neuroprotection. The IP route has led to effective delivery of this inhibitor to the brain in a previous study in adult mice (Gu et al., 2005). In this study, we showed that SB-3CT treatment significantly reduced the brain pro MMP-9 activity confirming that it was indeed effectively transported into the brain. However, a significant reduction was not observed with cleaved MMP-9 activity. This could be due to the fact that cleaved forms of MMPs have a short half-life rendering it difficult to determine their actual activity levels (Birkedal-Hansen et al., 1993, Demestrea et al., 2005). It is also noteworthy that a significant change of levels of cleaved MMP-9 was not observed after HI injury to the P21 rat brain (*Chapter 4*). The presence of SB-3CT in blood and CSF was also determined using liquid chromatography and tandem mass spectrometry. SB-3CT was detected in the blood of treated animals in low concentrations while it was not detected at all in the CSF in the expected peak. It was recently reported that SB-3CT is rapidly metabolised *in vivo* in rats in particular (Lee et al., 2007). Interestingly, an unknown molecule was detected in higher levels than the expected SB-3CT peak in the CSF and blood of SB-3CT treated animals and also in SB-3CT standards but not in the vehicle or saline treated animals. The fact that this unknown compound elutes earlier than SB-3CT indicates that it is chemically different from SB-
3CT but it is able to later produce the same parent ion and then the daughter ion. It could be argued that this unknown molecule is a metabolite of the inhibitor. Lee et al, 2007 showed that SB-3CT is mainly metabolised via hydroxylation of the terminal phenyl ring producing a metabolite (M4) that is also potent in inhibiting MMP-2 and 9 (Figure 6.9). The unknown product we detected was eluted from the liquid chromatography column earlier than SB-3CT suggesting that it was more polar than SB-3CT. This may be a consequence of the unknown product possibly having more hydroxyl groups than SB-3CT proposing that it might be the hydroxylated metabolite (M4) of SB-3CT. However, in order to be detected via tandem mass spectrometry this unknown molecule should give the same molecular weight ion as SB-3CT in Q1. It is well established that losing water molecules upon ionisation is a common phenomenon in mass spectrometry (Chen et al., 2001, Glish and Vachet, 2003, Murphy et al., 2005). Consequently, it is a possibility that M4 could become dehydroxylated upon ionisation in the APCI source thus reverting back to the parent molecule at Q1. Therefore, there is a high possibility that the unknown molecule we detected in the CSF and blood samples of SB-3CT treated animals is actually the potent metabolite of SB-3CT. As mentioned earlier only the peak that may correspond to the hydroxylated metabolite of SB-3CT was detected in the CSF sample but not the peak corresponding to SB-3CT. Hence, it can be suggested that SB-3CT may have to be hydroxylated to be able to cross the blood brain barrier.

![Figure 6.9: SB-3CT (Parent) (a) mainly get metabolised via hydroxylation of the terminal phenyl ring producing a metabolite (M4) (b) (Lee et al., 2007).](image-url)
Our results showed that treatment with SB-3CT did not confer significant neuroprotection following HI injury in the developing brain despite significantly inhibiting the brain MMP-9 activity. However, there was a trend towards reduction of infarct area and neurological deficits with the SB-3CT treatment as compared to vehicle and saline treated groups. The lack of an effect of SB-3CT can be attributed to various reasons. Firstly, the inhibition of brain MMP-9 activity may not have been enough; only a two fold reduction was observed. However, practical reasons such as having to use a higher volume of DMSO and PEG prevented the usage a higher dosage. Secondly, it might be due to an adaptation mechanism, in which other MMPs, such as MMP-3, become upregulated to compensate for the reduced MMP-9 activity. It is a phenomenon specially characterised in knock-out animal models of various MMPs (Rudolph-Owen et al., 1997, Ducharme et al., 2000, Fingleton, 2008). As mentioned earlier, the rate of metabolism of SB-3CT was found to be the most rapid in the rats as compared to other species (Lee et al., 2007). Also recently, a comparative study of different MMP inhibitors showed that effectiveness of a inhibitor varies between different species, and also between different strains of the same species (Rosenberg et al., 2007). Therefore, we suggest that use of rats is another reason for the lack of effect of SB-3CT in this study as opposed to the significant neuroprotection observed with the same inhibitor in the mouse model of stroke (Gu et al., 2002). However, the limited effectiveness of SB-3CT is most probably a consequence of its poor solubility. It is likely that some of the inhibitor was precipitated inside the peritoneal cavity thus variably reducing the amount delivered into the brain. The amount of precipitation could be different from animal to animal depending on the fluid composition inside their peritoneal cavities. Another possibility is that the vehicle had an effect of its own on neuroprotection thus masking any effects of the inhibitor itself. Indeed, we found that vehicle also had reduced levels of infarction and
neurological behaviour as compared to saline treated group although it was not statistically significant. Importantly, DMSO has been demonstrated to reduce brain injury by relieving oxidative stress (Bardutzky et al., 2005, Rosenberg et al., 2007). However, it should be noted that these studies used higher doses of DMSO such as 1.5g/kg of BW. In our study, we used 25% DMSO in a volume 0.4% of the BW, thus only injecting approximately 1g/kg of BW. Finally, it can be suggested that MMP-9 may not play an important role in the pathogenesis of HI injury in the developing brain. However, it is yet very premature at this stage to formulate such a conclusion since evidence suggest otherwise. Indeed, the demonstration that knock-out of the MMP-9 gene protects the immature mouse brain from HI injury (Svedin et al., 2007) together with results of our previous chapter (Chapter 4) strongly suggest that MMP-9 may participate in the pathophysiology of injury in the developing brain.

Before disregarding the possibility that inhibition of MMP-9 could be neuroprotective in the developing brain as well as the adult brain it is important to investigate other experimental approaches. IP administration of SB-3CT was chosen for this study because it was the most practical therapeutic approach. It has been successful in many cancer treatment studies (Kruger et al., 2005, Bonfil et al., 2006) and in an adult stroke model in mice (Gu et al., 2005). However, it would be of interest to determine the effects of an intracerebral ventricular (ICV) injection of SB-3CT on the activity of MMP-9 within the brain, the infarction area and neurological behaviour. ICV route has been demonstrated to be an effective route of delivery of various treatments in the rat model of HI injury (Scheepens et al., 2001). Another possible approach would be pre-treatment with SB-3CT. It has been shown that pre-treatment of SB-3CT is more effective in conferring neuroprotection (Gu et al., 2005). However, these approaches are not favourable because
they are unachievable in a clinical situation. Therefore, immediate future experiments should mainly explore the possibility of using a more soluble version of SB-3CT (Ikejiri et al., 2005a) or another MMP-9 selective inhibitor (Lauer-Fields et al., 2008) that are currently in the development. It would be interesting to investigate the possibility of directly using the potent metabolite (M4) of SB-3CT described by Lee et al, 2005. Its extra hydroxyl group may provide it better solubility thus allowing the usage of a more neutral vehicle and more effective delivery into the brain.

In conclusion the aim of this study was to investigate the effect of MMP-9 inhibition on conferring neuroprotection following HI injury to the developing brain. We first showed that an IP administration of MMP-2/9 inhibitor, SB-3CT, reduced brain MMP-9 activity at six hours following HI. However, SB-3CT did not confer significant neuroprotection in the developing brain following HI. The lack of effect of SB-3CT may have been a consequence mostly of its poor solubility. Future experiments involving a more soluble version of SB-3CT or another MMP-9 selective inhibitor are required to resolve the role of MMP-9 in the aetiology of HI injury in the developing brain.
CHAPTER SEVEN: GENERAL DISCUSSION

7.1 OVERVIEW

Perinatal hypoxic ischemic (HI) brain injury is a major cause of long-term neurological deficits worldwide. Infants who suffer hypoxic ischemia within utero or during birth, develop brain damage leading to lifelong motor, cognitive and behavioural deficits. Despite major improvements in obstetric and neonatal care in the last decade, there are currently no effective neuroprotective therapeutic options consequently such neurological deficits remain a major problem (Volpe, 2001a, Ferriero, 2004, Vannucci and Hagberg, 2004, Barrett et al., 2007, Rennie et al., 2007, Rees et al., 2008). Following HI injury neuronal death occurs in two distinct phases; acute and delayed phases. The mechanisms that mediate cell death during the acute phase are extensively studied while understanding of those during the delayed phase is sparse. Nevertheless, it is now established that a very significant amount of neuronal death occurs during the delayed phase. Furthermore, the delayed phase commences from hours following injury and extends until days later, therefore providing a more achievable therapeutic window for treatments as compared to the acute phase (Takashima et al., 1995, Volpe, 1998, Inder and Volpe, 2000, Volpe, 2001a, Blumenthal, 2004, Ferriero, 2004, Shalak and Perlman, 2004, Rennie et al., 2007). Given this, further research in order to provide a comprehensive understanding of the mechanisms associated with the delayed neuronal death, is critical for the future development of suitable therapeutic strategies to reduce or prevent the debilitating consequences of perinatal HI injury.

The overall objective of this thesis was to evaluate the hypothesis that matrix metalloproteinase (MMP) 2 and 9 participate in the pathophysiology of HI injury in the developing brain using a well characterised rat model of unilateral HI injury at two
developmental stages; P3 and P21. MMPs are a family of zinc dependant endopeptidases that are capable of degrading all components of the extracellular matrix (ECM). Disruption of the ECM, that include cyst formation, disruption of developing white matter tracts and the presence of glial scars, is known to be a prominent characteristic of lesions in the developing brain (Meng et al., 1997, Volpe, 1998, Volpe, 2001b, Blumenthal, 2004, Sizonenko et al., 2005). Furthermore, inappropriate activity of several MMPs, in particular MMP-2 and 9 has been implicated in the pathophysiology of cerebral ischemia in the adult brain. Therefore, we hypothesised that MMP-2 and 9 participate in the pathophysiology of HI injury to the developing brain.

To address this hypothesis we undertook a series of studies. Since ECM remodelling is a fundamental process during brain development it was important firstly to characterise the MMP-2 and 9 expressions in the normal developing forebrain. Secondly, we determined the MMP-2 and 9 responses following HI injury at the two developmental stages; postnatal day 21 (P21) and 3 (P3). Thirdly, we characterised changes in plasminogen activators, tissue (tPA) and urokinase (uPA) plasminogen activator, since they were demonstrated to be major upstream activators of MMPs. Finally, we inhibited MMP-9 activity using a very specific MMP-2/9 inhibitor, to determine if the MMP-9 deficiency protects the developing brain from HI injury.

The findings obtained from this thesis have been discussed extensively in each respective results chapter. Therefore, the focus of the following chapter is to provide an overall narrative of the thesis as well as scientifically critiquing the findings obtained. Furthermore, possible limitations of this project are also discussed. Moreover, potential
future studies that would enhance the understanding of the field of this thesis are also suggested.

7.2 MAJOR FINDINGS AND THEIR IMPLICATIONS

Neuronal migration, arborisation and myelination are fundamental processes during development of the mammalian central nervous system (CNS) (Volpe, 2001a, Levitt, 2003, Nadarajah et al., 2003) that are believed to require vigorous ECM remodelling. One major class of proteolytic enzymes that plays a dominant role in ECM degradation is that of MMP (Sethi et al., 2000, Bosman and Stamenkovic, 2003). Therefore, it was fundamental to firstly determine the expression of MMP-2 and 9 during development of the normal rodent brain before investigating their possible participation during injury to the developing brain. Recent evidence indicates that MMP-2 and 9 are implicated during development of the mammalian cerebellum (hindbrain) (Vaillant et al., 2003, Ayoub et al., 2005, Ulrich et al., 2005). However, the developmental expression of MMP-2 and 9 in the normal developing forebrain has not been fully characterised previously. In Chapter Three, we characterised the developmental changes of MMP-2 and 9 in the normal developing rodent forebrain for the first time.

We demonstrated that MMP-2 protein activity as well as its mRNA expression in the normal rat forebrain gradually declined with age. MMP-2 protein was located in the cortical plate neurons of the developing rat forebrain. Literature suggests that the observed decline in MMP-2 mRNA and activity levels possibly parallels the degree of neuronal migration to the cortical plate (Vanier et al., 1971, Uylings, 2000, Clancy et al., 2001). Therefore, we proposed that MMP-2 might contribute to the migration of neurons from the ventricular zone to the cortical layer. However, we demonstrated that MMP-2
was localised in neurons that also expressed neuronal specific nuclear protein (NeuN) that is usually detected in neurons, which have withdrawn from the cell cycle and/or initiated the terminal differentiation (Mullen et al., 1992). Therefore, it is debatable that MMP-2 primarily assists the migration process in the early postnatal rat cortex. Alternatively, we suggested that MMP-2 might assist in neurite outgrowth that occurs approximately within P0-P10 during rodent brain development (Vanier et al., 1971, Uylings, 2000, Clancy et al., 2001). Moreover, our findings demonstrating that MMP-2 colocalises with MAP-2 further supported this argument. Indeed, it has been shown previously that MMP-2 assists neurite outgrowth by degrading the neurite-inhibiting protein (Zuo et al., 1998). Therefore, in conclusion, we suggest that MMP-2 may contribute in the development and differentiation of the rodent cortical plate.

In contrast to MMP-2, MMP-9 proteolytic activity and gene expression were extremely low in the early postnatal rat forebrain. They were only significantly upregulated between P14 and P21. Interestingly, the upregulation of MMP-9 expression closely correlated with the period of myelination in the normal rodent brain which is initiated approximately between P10 to P15 (Davison and Dobbing, 1966, Vanier et al., 1971, Uhm et al., 1998). Furthermore, we have demonstrated that MMP-9 immunoreactivity was observed in a process-like pattern in the P21 rat cortex that may correspond to myelinating neuronal processes. Given the aforementioned, we proposed a potential role for MMP-9 in myelination of the developing rat forebrain.

Recently, several lines of evidence suggested that inappropriate activity of MMP-2 and 9 may contribute to the pathogenesis of cerebral ischemia in the adult brain
(Rosenberg et al., 1996, Romanic et al., 1998, Zuo et al., 1998, Asahi et al., 2000, Asahi et al., 2001, Gasche et al., 2001, Planas et al., 2001, Rosenberg et al., 2001, Rivera et al., 2002, Lee et al., 2004, Gu et al., 2005, Amantea et al., 2007). Moreover, as mentioned in the overview, disruption of the ECM is a frequently observed feature of lesions in the developing brain (Meng et al., 1997, Volpe, 1998, Volpe, 2001b, Blumenthal, 2004, Sizonenko et al., 2005). Therefore, we speculated that MMP-2 and 9, which are believed to be prominent ECM degrading proteases, contribute to HI injury of the developing brain. Hence, in Chapter Four, we characterised the MMP-2 and 9 responses following HI injury to the developing brain using a well characterised rat model of unilateral HI injury at two developmental stages; P3 and P21.

We demonstrated that MMP-9 activity was upregulated between six hours and one day following HI injury at both P21 and P3. Similarly, qRT-PCR indicated MMP-9 gene expression was also significantly upregulated in the injured hemisphere following hypoxic ischemia at both ages. The temporal profile of MMP-9 upregulation in both ages strongly correlates with the period of highest delayed neuronal death in the this HI model (Sirimanne et al., 1994, Beilharz et al., 1995, Sizonenko et al., 2005). For this reason, we proposed that our results support a role for MMP-9 in the delayed injury processes following HI injury at both P3 and P21. Recently, Svedin et al., further supported our argument by demonstrating that MMP-9 deficiency protects the immature brain from an HI insult (Svedin et al., 2007). Our immunohistochemical studies indicated that MMP-9 protein expression was localised predominantly in neurons in the injured hemisphere, suggesting that MMP-9 presumably mediates neuronal death following injury. In addition we were also able to demonstrate an increase in MMP-9 immunoreactivity in astrocytes adjoining cerebral blood vessels, suggesting a possible role for MMP-9 in blood brain
barrier (BBB) disruption. Moreover, we found that neuronal processes in the injured cortex were intensely stained with MMP-9, suggesting a potential role for MMP-9 in axonal degeneration. Although MMP-9 was upregulated following injury at both ages, we speculate that the degree to which MMP-9 may contribute in the injury process following hypoxic ischemia, may vary according to the developmental age. We established that the relative change of MMP-9 activity in the injured ipsilateral hemisphere as compared to the uninjured contralateral hemisphere after HI injury at P3 was considerably lower than that after injury at P21. However, unfortunately, a direct comparison of the responses between the two developmental ages was not possible because of the variations of the quantification techniques used as discussed below under limitations (Section 7.3). Nevertheless, in summary, we advocate that MMP-9 potentially contributes in the development of delayed injury processes following HI injury to the developing brain.

Contrary to MMP-9, MMP-2 activity progressively increased from one day following HI injury at P21, reaching a peak at five days. MMP-2 mRNA expression was also increased in the injured hemisphere following injury similarly to its activity. MMP-2 protein was primarily associated with reactive hypertrophic astrocytes in the penumbra, surrounding the ischemic core, of the injured cortex following injury at P21. Given that MMP-2 upregulation occurs after the main period of neuronal death in this model (Beilharz et al., 1995) we propose that it is not a injury mechanism but rather a recovery mechanism from hypoxia ischemia. According to the literature (Ogier et al., 2006) it is possible that MMP-2 may play a role in the migration of astrocytes thus assisting the formation of the glial scar. Furthermore, we have shown that MMP-2 immunoreactive cells colocalised with GAP-43 immunoreactivity, a marker of new axonal growth, at five days after HI injury at
P21. Therefore, it is also plausible that MMP-2 may contribute to neurite outgrowth necessary for reformation of functional neuronal connectivity following injury by presumably inactivating neurite inhibiting chondroitin sulfate proteoglycan (CSPG) as reported previously (Zuo et al., 1998, Ferguson and Muir, 2000, Krekoski et al., 2002). MMP-2 levels did not change significantly after HI at P3, presumably as a result of already high constitutive levels during early postnatal brain development. In conclusion, our results indicate that MMP-2 is potentially more important during wound repair processes rather than injury developmental processes.

Plasminogen activators, tPA and uPA, are known to be prominent activators of MMPs (Tsirka et al., 1997, Sumii and Lo, 2002). Emerging data suggest that both plasminogen activators (tPA and uPA) can be potentially neurotoxic (Wang et al., 1998, Ahn et al., 1999, Nagai et al., 1999, Dietzmann et al., 2000, Yepes et al., 2000, Hosomi et al., 2001). Plasminogen activators possibly exert their deleterious effects by activating the plasminogen into plasmin which in turn, activates ECM degrading proteases such as MMP-2 and 9 (Tsirka et al., 1997, Kaur et al., 2004, Sheehan and Tsirka, 2005). In fact, there are several lines of evidence demonstrating a positive correlation between the tPA/uPA and MMP-2/9 expressions in cerebral ischemia (Hosomi et al., 2001, Horstmann et al., 2003, Wang et al., 2003, Kelly et al., 2006, Burggraf et al., 2007) suggesting that plasminogen activators may activate MMP-2 and 9 following an ischemic injury. Therefore, in Chapter Five, we established the tPA and uPA responses following HI injury to the developing brain to determine if they demonstrate a similar profile to that of MMP-2 and 9.
We showed that uPA activity progressively increased in the ipsilateral injured hemisphere reaching a peak at three to five days following injury at P21, whilst it was moderately upregulated in the ipsilateral injured hemisphere from six hours to one day following injury at P3. The pattern of uPA upregulation following HI injury at both developmental stages strongly correlated with that of MMP-2. qRT-PCR showed that uPA expression was also increased in the injured hemisphere following injury at both P21 and P3 in agreement with the activity. Immunohistochemistry indicated that uPA was co-localised with MMP-2 on reactive astrocytes surrounding the infarct. Hence, we proposed that it is likely that uPA functions in conjunction with MMP-2 following HI injury to the developing brain. Furthermore, several *in vivo* (Burggraf et al., 2007) and *in vitro* (Keski-Oja et al., 1992, Baramovaa et al., 1997, He et al., 2007) studies demonstrating that uPA inhibition negatively affects MMP-2 activity support our arguments. Taken together, our results suggest a function for uPA in wound repair processes following HI to the developing brain through activation of MMP-2.

In contrast with uPA, tPA activity remained unaffected following injury at both ages. Therefore, it is unlikely that tPA has a significant function in the injury or recovery processes following HI injury to the developing brain. Indeed, several studies of the adult brain have shown that tPA does not have any neurotoxic effects following ischemic injury (Klein et al., 1999, Meng et al., 1999, Tabrizi et al., 1999, Atochin et al., 2004). However, plasminogen zymography showed that tPA activity increased with maturity similar to the MMP-9 activity during development. Hence, it is likely that tPA participates during the development of the CNS. However, it was beyond the scope of our study to investigate the developmental involvement of the plasminogen activators, therefore further studies are required to draw finer conclusions.
As mentioned in the overview, neurological handicap following perinatal brain injury remains a major problem to date due to the lack of specific therapeutic treatments. Earlier we suggested that MMP-9 possibly participates in the delayed injury processes following an HI injury to the developing brain. In Chapter Six we investigated the effect of inhibition of MMP-9 within the period, in which it was upregulated following HI injury to the developing brain. Previous studies using synthetic inhibitors have provided evidence that inhibition of MMP-9 activity reduces the severity of ischemic injury in the adult brain (Romanic et al., 1998, Asahi et al., 2000, Jiang et al., 2001, Pfefferkorn and Rosenberg, 2003, Lee et al., 2004). However, the majority of these inhibitors were broad-spectrum MMP inhibitors, which were capable of inhibiting all MMPs, ADAMs and other MMP related enzymes. They have failed in the human clinical trials of cancer treatments because of their adverse side effects such as inflammation (Nuti et al., 2007, Fingleton, 2008). For this reason, we chose to use a highly selective inhibitor, SB-3CT, which targets only MMP-2 and MMP-9 (Brown et al., 2000). We investigated the effect of MMP-9 inhibition on conferring neuroprotection following HI injury at P21. This developmental age was chosen because the strongest MMP-9 upregulation was observed following HI injury at P21 as compared to P3. Furthermore, the variability of the injury that is produced following HI at P21 was less than that at P3 thus limiting the possibility of false positive results. We first showed that an intraperitoneal administration of MMP-2/9 inhibitor, SB-3CT, reduced brain MMP-9 activity following hypoxic ischemia. However, SB-3CT did not confer significant neuroprotection in the developing brain following HI injury despite the reduced MMP-9 activity. The lack of effect of SB-3CT most probably may have been a consequence of its poor solubility (Ikejiri et al., 2005). Although it can be suggested that MMP-9 may not play an important role in the
pathogenesis of HI injury in the developing brain, it is yet very premature at this stage to come to such a conclusion. The demonstration that the deficiency of MMP-9 gene expression protects the immature mouse brain from HI injury (Svedin et al., 2007) along with our findings from Chapter Four strongly suggests that MMP-9 may participate in the pathophysiology of injury in the developing brain. Therefore, future studies involving new approaches such as usage of a more soluble version of SB-3CT should be undertaken to clarify the role of MMP-9 before drawing further conclusions.

7.3 LIMITATIONS

One of the limitations of this thesis is the inability to directly compare the MMP-2 and 9 responses between the two developmental ages in Chapter Four. As discussed in Chapter Two MMP-9 expression was extremely low in the rat forebrain during early postnatal days. Therefore, it was necessary that the gelatin zymography and qRT-PCR techniques were modified to increase the sensitivity. Particularly, in-house gelatin zymography gels, which were of lesser thickness as compared to the Invitrogen pre-cast gels, were used to measure MMP-9 activity following HI injury at P3. Similarly, higher amounts of cDNA were used in qRT-PCR reactions to measure the MMP-9 gene expression following HI injury at P3. Inaccuracy in microdissection of the hippocampus is another possible limitation of this thesis. We speculated that the hippocampus might have been contaminated with choroid-plexus during dissection particularly at the early postnatal ages. It is plausible that such a contamination might have produced false positive results in Chapter Two. Furthermore, we were not able to investigate the profiles of tissue inhibitors of matrix metalloproteinases (TIMPs) following HI injury to the developing brain due to time and financial constrains. It would have provided information about the
extent of the endogenous MMP inhibition present following an injury thus allowing further validations of the roles of MMP-2 and 9 following an injury.

7.4 FUTURE DIRECTIONS

Several possible future studies that would enhance the understanding of MMP-2 and 9 contributions during health and disease in the developing brain can be suggested based on the findings of the present thesis.

We showed that MMP-2 and 9 were developmentally regulated in the rodent forebrain. However, the precise functions of MMP-2 and 9 during the rodent forebrain development require further clarification. The majority of information on the roles of MMPs during CNS development is based on the effects of broad-spectrum MMP inhibitors, such as GM6001 (Conant and Gottschall, 2005). However, they have not been enormously useful in determining the participation of single MMPs because they are capable of inhibiting a wide variety of enzymes including all the members of the MMP family as well as other related proteases (Coussens et al., 2002, Nuti et al., 2007). The other commonly used approach is the use of MMP knock-out animals. This approach also has not been very successful (Conant and Gottschall, 2005) presumably because of a possible adaptation mechanism, where other MMPs become upregulated to compensate for the deleted MMP (Rudolph-Owen et al., 1997, Ducharme et al., 2000, Fingleton, 2008), given the extensive overlap among their substrates. Therefore, we propose that a specific inhibitor such as SB-3CT should be used during the period of highest expression of the MMP of interest. An initial approach would be to inhibit MMP-2 or 9 activities using a selective inhibitor in vitro in a primary cell culture of the developing brain. Boyden chamber migration assays using an artificial ECM as the
membrane could be performed to determine the effect of MMP-2 inhibition on neuronal migration. The degree of neurite outgrowth could also be assessed by measuring the length of processes using MAP-2 immunoreactivity to identify the outgrowing processes. Determination of GAP-43 levels would provide further information of the degree of neurite outgrowth. GAP-43 is a brain specific cell membrane glycoprotein that is associated with neurite outgrowth (Skene, 1989, Meiri et al., 1998). Double labelling with GAP-43 and MMP-2 would distinguish whether growing neurites expressed MMP-2. MMP-2 has been reported to inactivate chondroitin sulfate proteoglycans (CSPGs) which are known to inhibit neurite outgrowth (Zuo et al., 1998, Ferguson and Muir, 2000, Krekoski et al., 2002). Therefore, analysis of the levels of neurite inhibiting proteins CSPGs by immunohistochemistry and western blotting would determine the extent of neurite outgrowth following MMP-2 inhibition. Double labelling of MMP-2 and CSPG would also provide important information about their association. Colocalisation studies with mature oligodendrocytes marker O1 (Oh et al., 1999) and MMP-9 could also be employed in order to determine the role of MMP-9 in the process of myelination. Another possibility would be using organotypic cultures at different ages of the cerebral cortex instead of primary cultures. Colocalization studies of MMP-2/CSPG, MMP-2/GAP-43, and MMP-9/O1 could be performed following MMP-2 or 9 inhibition and neurite outgrowth measured as described above. In addition, assessment of the effect of inhibition of MMP-9 on myelination could also be undertaken through measurement of the levels of myelin basic protein (MBP) either by immunohistochemistry or western blotting. Counting cells with fusiform somas after staining the cultures with a cell stain would provide an assessment of the migratory capacity of cells. Furthermore, the degree of angiogenesis could be determined by accessing the levels of related proteins such as vascular endothelial growth factor
(VEGF) (Ogunshola et al., 2000). Finally, a long-term histological analysis of the effects MMP-2 or 9 inhibition on the cortical plate development, neurite outgrowth, synapse formation and myelination would be essential to fully elucidate their role in the mammalian brain development. Furthermore, a long-term functional study that investigates cognitive behaviour as well as the motor behaviour would possibly provide evidence of the functional importance of MMPs during development.

A comprehensive understanding of the mechanisms of action of MMP-9 following HI injury to the developing brain would be beneficial in the design of possible therapeutic approaches targeting the MMPs. It has been demonstrated that MMP-9 contributes to injury by increasing the BBB permeability during cerebral ischemia in the adult brain (Asahi et al., 2001, Lee et al., 2005, Liu and Rosenberg, 2005, Shigemori et al., 2006, Yang et al., 2007). It is possible that MMP-9 has similar effects on the BBB following HI injury to the developing brain. Indeed, we have demonstrated that MMP-9 is located in reactive astrocytes adjoining the blood vessels within the HI injured ipsilateral hemisphere of the juvenile brain. Analysis of the BBB permeability using Evans blue extravasation assay (Asahi et al., 2001) following MMP-9 inhibition would further elucidate the function of MMP-9 in BBB breakdown. Also the degradation of BBB component including zonae occludens-1 (Asahi et al., 2001), basal lamina collagen type IV (Hamann et al., 2004), claudin-5 and occluding (Yang et al., 2007), which were shown to be targets of MMP-9 during cerebral ischemia in the adult brain, could be investigated following MMP-9 inhibition in the juvenile HI brain. Apart from its effect on the blood brain barrier, MMPs are also know to affect neuronal survival by either modifying the cell-matrix interactions or intracellularly activating apoptotic mediators (Nagase, 1997, Sethi et al., 2000, Bosman and Stamenkovic, 2003). Our results show that MMP-9 is located on
neurons in the penumbra of the injured cortex and hippocampus. Colocalisation of MMP-9 and apoptosis markers such as caspases (Teschendorf et al., 2008) cathepsin and calpain (Tsubokawa et al., 2006) may further clarify the role of MMP-9 in neurodegeneration. Furthermore, effect of MMP-9 inhibition in neurodegeneration during the period of maximal cell death could be investigated using Fluoro-Jade staining (Schmued et al., 1997, Sizonenko et al., 2005). Moreover, we found that neuronal processes in the injured cortex were intensely stained with MMP-9, suggesting a possible role for MMP-9 in axonal degeneration. To further clarify our suggestion, the degree of myelin loss should be investigated following MMP-9 inhibition using myelin basic protein immunohistochemistry.

We observed that MMP-2 and uPA were upregulated several days following HI injury to the developing brain outside the main period of neuronal death. Both MMP-2 and uPA were colocalised on reactive hypertrophic astrocytes in the penumbra of the injured hemisphere. Therefore, we proposed that uPA-MMP-2 system may participate in the recovery processes following hypoxic ischemia. We further showed that MMP-2 closely localised with GAP-43, a brain specific cell membrane glycoprotein that is closely associated with neurite formation. Hence, it would be of interest to further investigate the role of uPA-MMP-2 axis in restoration of neuronal connectivity. Firstly, the effect of MMP-2 and/or uPA on neurite outgrowth should be investigated in an in vitro study. Colocalisation of uPA/MMP-2 with CSPG and GAP-43 would provide primary evidence of their association. Determination of the levels of these proteins following pharmacological inhibition or exogenous administration of uPA and/or MMP-2 would provide further evidence of interaction. Finally the effect on neurite outgrowth can be estimated using MAP-2 immunoreactivity following manipulations. Then the effect of
uPA-MMP-2 axis could be similarly assessed in vivo following either exogenous administration or inhibition of MMP-2 and/or uPA during the recovery period following a HI injury. The effect of MMP-2 and/or uPA treatments on the functional recovery could be evaluated using various behavioural tests (Bederson et al., 1986, Gonzalez and Kolb, 2003). If MMP-2 essentially promotes neurite growth, it may presumably be applied in conjunction with growth hormone since this has been demonstrated to promote neurogenesis following an ischemic insult by members of our laboratory (Scheepens et al., 1999, Scheepens et al., 2001, Pathipati et al., In Press). Therefore, the proposed study would greatly facilitate designing of neuronal restoration strategies for HI injured brain.

As discussed in Chapter One, the activity of the MMPs is tightly controlled by their endogenous inhibitors (TIMPs). Therefore, characterisation of the TIMPs profiles following an HI injury to the developing brain would provide essential information about the extent of endogenous regulation already available following injury. qRT-PCR, western blotting and reverse zymography could be used to determine the levels of mRNA, protein and activity of the TIMPs. Subsequently, the existing regulation could be further enhanced by exogenously administrating relevant TIMPs or enhancing their gene expression at the relevant periods depending on their endogenous expression. Indeed, it has been illustrated that TIMPs are neuroprotective following cerebral ischemia in the adult brain (Fujimoto et al., 2008).

Most importantly, the effect of MMP-9 inhibition, using a specific MMP-9 inhibitor, following a HI injury to the developing brain should be re-investigated. As we have extensively discussed in Chapter Six the possible approaches for future studies include
use of an intracerebral ventricular injection of SB-3CT, pre-treatment with SB-3CT and, use of a more soluble version of SB-3CT or another MMP-9 selective inhibitor.

7.5 SUMMARY

In summery, the findings of the present thesis provide valuable information on MMP-2 and 9 involvements during health and disease of the developing brain. Firstly, we showed that MMP-2 mRNA expression and activity declined with age whilst, MMP-9 mRNA expression and activity increased with age. We suggest that these results support an important role for MMP-2 in the development and differentiation of the cortical plate, whereas MMP-9 potentially contributes in myelination. Secondly, we demonstrated that MMP-9 mRNA expression and activity was increased during the early hours following HI injury to the developing brain and that it was located predominately on neurons in the injured cortex suggesting that MMP-9 is presumably involved in the development of delayed injury processes following hypoxic ischemia. Conversely, MMP-2 mRNA and activity levels was progressively increased during later periods following HI injury to the developing brain and it was located on reactive astrocytes surrounding the ischemic core suggesting that MMP-2 may play a role in wound repair processes. Thirdly, we demonstrated that uPA mRNA expression and activity paralleled that of MMP-2 following HI injury suggesting a function for uPA in wound repair processes following HI injury to the developing brain through activation of MMP-2. Finally, we attenuated the elevated MMP-9 activity following HI injury in the juvenile brain using a highly specific MMP-2/9 inhibitor, SB-3CT. Although SB-3CT failed to confer any significant neuroprotection, we recommend that further investigations are needed before discounting the role of MMP-9 during HI injury to the developing brain.
7.6 CONCLUSIONS

In conclusion, we suggest that MMP-9 is induced in the developing brain following an insult potentially contributing to the delayed neuronal death whilst MMP-2 is constitutively expressed in the developing brain presumably participating in essential developmental, differentiational and wound repair processes following an insult.
APPENDIX

Acid Fuchsin

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid fuchsin</td>
<td>0.6g</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>15 drops</td>
</tr>
<tr>
<td>MQ water</td>
<td>Make up to 500mL</td>
</tr>
</tbody>
</table>

Bouin’s Solution

<table>
<thead>
<tr>
<th>Volume</th>
<th>1L</th>
<th>500mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraformaldehyde</td>
<td>40g</td>
<td>20g</td>
</tr>
<tr>
<td>Picric acid</td>
<td>150mL</td>
<td>75mL</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>800μL</td>
<td>400μL</td>
</tr>
<tr>
<td>0.1M PBS</td>
<td>Make up to 1L</td>
<td>Make up to 500mL</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
<td>7.4</td>
</tr>
</tbody>
</table>

0.5% Coomassie Blue

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie Blue R-250</td>
<td>2.5g</td>
</tr>
<tr>
<td>Fixing/Destaining solution</td>
<td>500mL</td>
</tr>
</tbody>
</table>

0.1 M Citric Acid

<table>
<thead>
<tr>
<th>Volume</th>
<th>1L</th>
<th>100mL</th>
<th>200mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount</td>
<td>19.24g</td>
<td>1.924g</td>
<td>3.848</td>
</tr>
</tbody>
</table>

0.01M Citrate Buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>500mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M Citric acid</td>
<td>8mL</td>
</tr>
<tr>
<td>0.1M Sodium citrate</td>
<td>42mL</td>
</tr>
<tr>
<td>MQ water</td>
<td>450mL</td>
</tr>
</tbody>
</table>

DEPC Water

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPC concentrate</td>
<td>1mL</td>
</tr>
<tr>
<td>MQ water</td>
<td>Make 1L</td>
</tr>
</tbody>
</table>
Fixing/Destaining Solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>1L</th>
<th>2L</th>
<th>3L</th>
</tr>
</thead>
<tbody>
<tr>
<td>MQH₂O</td>
<td>450mL</td>
<td>900mL</td>
<td>1350mL</td>
</tr>
<tr>
<td>Methanol</td>
<td>450mL</td>
<td>900mL</td>
<td>1350mL</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>100mL</td>
<td>200mL</td>
<td>300mL</td>
</tr>
</tbody>
</table>

0.01M Potassium Phosphate Buffered Saline (KPBS)

<table>
<thead>
<tr>
<th>Volume</th>
<th>1L</th>
<th>8L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8g</td>
<td>64g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.15g</td>
<td>9.2g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.2g</td>
<td>1.6g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2g</td>
<td>1.6g</td>
</tr>
<tr>
<td>H₂O</td>
<td>1L</td>
<td>8L</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
<td>7.4</td>
</tr>
</tbody>
</table>

0.1M Phosphate Buffered Saline (PBS)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M NaH₂PO₄·2H₂O</td>
<td>100mL</td>
</tr>
<tr>
<td>0.2 M Na₂HPO₄</td>
<td>400mL</td>
</tr>
<tr>
<td>NaCl</td>
<td>9g</td>
</tr>
<tr>
<td>MQ water</td>
<td>Make up to 1L</td>
</tr>
</tbody>
</table>

Sample Buffer 2X

<table>
<thead>
<tr>
<th>Reagent</th>
<th>10mL</th>
<th>50mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>0.015g</td>
<td>0.075g</td>
</tr>
<tr>
<td>HCl</td>
<td>To make it pH 6.8</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>0.4g</td>
<td>2g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2mL</td>
<td>10mL</td>
</tr>
<tr>
<td>Bromophenol</td>
<td>0.004g</td>
<td>0.02g</td>
</tr>
</tbody>
</table>

0.1M Sodium Citrate

<table>
<thead>
<tr>
<th>Volume</th>
<th>1L</th>
<th>100mL</th>
<th>200mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount</td>
<td>29.41g</td>
<td>2.941</td>
<td>5.882</td>
</tr>
</tbody>
</table>
### 0.2 M Sodium Phosphate Monohydrate (NaH$_2$PO$_4$·2H$_2$O)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaH$_2$PO$_4$·2H$_2$O</td>
<td>29.1g</td>
</tr>
<tr>
<td>MQ water</td>
<td>Make up to 1L</td>
</tr>
</tbody>
</table>

### 0.2 M Sodium Phosphate, Dibasic, Anhydrous (Na$_2$HPO$_4$)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>28.6g</td>
</tr>
<tr>
<td>MQ water</td>
<td>Make up to 1L</td>
</tr>
</tbody>
</table>

### Thionin

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Thionin</td>
<td>12.5mL</td>
</tr>
<tr>
<td>0.6% Glacial acetic acid</td>
<td>450mL</td>
</tr>
<tr>
<td>0.1M CH$_3$COONa$_3$H$_2$O</td>
<td>50mL</td>
</tr>
</tbody>
</table>

### 1.5M Tris-HCl pH 8.8

<table>
<thead>
<tr>
<th>Reagent</th>
<th>100mL</th>
<th>200mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>18.15g</td>
<td>36.3g</td>
</tr>
<tr>
<td>MQ water</td>
<td>100mL</td>
<td>200mL</td>
</tr>
<tr>
<td>HCl</td>
<td></td>
<td>To make it pH 8.8</td>
</tr>
</tbody>
</table>

### 0.5M Tris-HCl pH 6.8

<table>
<thead>
<tr>
<th>Reagent</th>
<th>100mL</th>
<th>200mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>6g</td>
<td>12g</td>
</tr>
<tr>
<td>MQ water</td>
<td>100mL</td>
<td>200mL</td>
</tr>
<tr>
<td>HCl</td>
<td></td>
<td>To make it pH 6.8</td>
</tr>
</tbody>
</table>

### 50mM Tris-HCl pH 8.2 (Plasminogen Developing Buffer)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>100mL</th>
<th>1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>0.6g</td>
<td>6g</td>
</tr>
<tr>
<td>MQ water</td>
<td>100mL</td>
<td>1L</td>
</tr>
<tr>
<td>HCl</td>
<td></td>
<td>To make it pH 8.2</td>
</tr>
</tbody>
</table>
### 2.7% Triton X-100

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton X-100</td>
<td>27mL</td>
</tr>
<tr>
<td>MQ water</td>
<td>To make 1L</td>
</tr>
</tbody>
</table>

### Zymography Running Buffer 10X

<table>
<thead>
<tr>
<th>Reagent</th>
<th>1L</th>
<th>2L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>29</td>
<td>58</td>
</tr>
<tr>
<td>Glycine</td>
<td>144</td>
<td>288</td>
</tr>
<tr>
<td>SDS</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>MQ water</td>
<td>to 1.0 L</td>
<td>to 2.0 L</td>
</tr>
</tbody>
</table>
BIBLIOGRAPHY


Bode, W., Gomis-Ruth, F. X. and Stockler, W., 1993. Astacins, serralysin, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. FEBS Letters. 331, 134-140.


