The Effects of Cerebellar Hemorrhage on the Development of the Postnatal Cerebellum

by

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Abstract

With the improvement in neuroimaging technology and the increase in survival of preterm infants, the detection of abnormalities in the cerebellum has become increasingly common. Human and animal studies suggest that the preterm cerebellum is particularly vulnerable to damage because of its dramatic growth and complex developmental processes. Cerebellar hemorrhage (CBH) is the most frequently detected cerebellar lesion in premature infants and often leads to long-term neurological sequelae, such as motor, affective and cognitive dysfunction. However, how CBH affects the development and function of the cerebellum remains largely unknown. Therefore, our study focuses on developing a mouse model of CBH to determine the anatomical, behavioural, and molecular phenotypes resulting from the hemorrhage in the developing cerebellum. To induce hemorrhage in the fourth ventricle and germinal matrix of the cerebellum, we injected bacterial collagenase, which breaks down surrounding blood vessel walls, into the fourth ventricle of postnatal (P) day two mice. Controls were injected with saline. We then performed various anatomical, behavioural and molecular assessments to detect any changes in the morphology and function of the cerebellum and to unravel the mechanisms of injury and neuroprotection activated as a result of CBH. In our model, we found a delay in cerebellar development, reduction in granule cell and interneuron density, and persistent neurobehavioural abnormalities similar to the abnormalities found in premature infants with CBH. Furthermore, we found a significant upregulation of neurotrophic factor expression and of genes in the sonic hedgehog pathway, which indicate the activation of endogenous neuroprotective mechanisms. Thus, our study provides a novel preclinical model of CBH that can be used to understand the pathophysiology of the disease and for the development and evaluation of preventive therapies and post-hemorrhagic treatments.
Preface

The project described in this thesis was initiated by Dr. D. Goldowitz and Dr. G. Mak. Dr. G. Mak provided technical training for sample collection and the behavioural tests used for this thesis. Morris Water Maze testing was conducted by Dr. G. Mak. I performed all data analysis, created tables and figures, wrote the manuscript which is in preparation for submission.

Ethics approval was given by the Animal Care Committee of the UBC Research Ethics Board (Certificate A10-0279: The effects of intraventricular hemorrhage on cerebellar development).
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<tr>
<td>ADHD</td>
<td>attention deficit hyperactivity disorder</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>ASD</td>
<td>autism spectrum disorder</td>
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<tr>
<td>B6</td>
<td>C57BL/6J</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
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<tr>
<td>CBH</td>
<td>cerebellar hemorrhage</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
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<tr>
<td>EGL</td>
<td>external granule layer</td>
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<tr>
<td>EPO</td>
<td>erythropoietin</td>
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<tr>
<td>EPOR</td>
<td>erythropoietin receptor</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<tr>
<td>IAP</td>
<td>inhibitors of apoptosis</td>
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<td>ICH</td>
<td>intracerebral hemorrhage</td>
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<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>IGL</td>
<td>internal granule layer</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin-1 beta</td>
</tr>
<tr>
<td>IVH</td>
<td>intraventricular hemorrhage</td>
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<tr>
<td>Max</td>
<td>maximum</td>
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<tr>
<td>MBP</td>
<td>myelin basic protein</td>
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<tr>
<td>Min</td>
<td>minimum</td>
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<td>ML</td>
<td>molecular layer</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<td>MWM</td>
<td>Morris water maze</td>
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<tr>
<td>OCT</td>
<td>optimal cutting temperature</td>
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<tr>
<td>P</td>
<td>postnatal day</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PCL</td>
<td>Purkinje cell layer</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<tr>
<td>PVL</td>
<td>periventricular leukomalacia</td>
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<tr>
<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RL</td>
<td>rhombic lip</td>
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<tr>
<td>RM-ANOVA</td>
<td>repeated measures of analysis of variance</td>
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<tr>
<td>RQ</td>
<td>relative quantity</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SHH</td>
<td>sonic hedgehog</td>
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<tr>
<td>TNFα</td>
<td>tumor necrosis factor-alpha</td>
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<tr>
<td>VZ</td>
<td>ventricular zone</td>
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<tr>
<td>WM</td>
<td>white matter</td>
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<tr>
<td>XIAP</td>
<td>X-linked inhibitors of apoptosis</td>
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I would like to express my heartfelt gratitude to my supervisor, Dr. Daniel Goldowitz for all his support and guidance throughout my undergraduate and graduate years in his laboratory. To Dr. Diana Juriloff and Dr. Steven Miller, my advisory committee members, I am grateful for their expert guidance, ideas for improvement of the study, and thoughtful comments on my thesis.

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I thank my parents for their unconditional love, support, and prayers throughout this journey. Also, I would like to express my gratitude to my spiritual family for all the encouragement, guidance, and prayers that kept me going in the right direction.

I give thanks to the LORD for his unfailing love and his wonderful deeds for me (Psalms 107:31).
1. General Introduction

1.1 Preterm Infants and Brain Abnormalities

Preterm birth, defined as childbirth before a gestational age of 37 complete weeks, is the leading cause of neonatal mortality and morbidity (Beck et al 2010; Goldenberg et al 2008; McCormick 1985). In the developed regions of the world, the rate of preterm birth is about 8.6%, while the average rate in the developing countries is approximately 12% (Blencowe et al 2012). With the recent improvement in neonatal intensive care, especially in the developed countries, the survival rate of premature infants is rising, reaching the current rate of 90% (Deng 2010). In particular, the rate of survival for infants born with a very low birth weight (≤1500 g) and at very early gestational age has increased remarkably in the last decade (58% for 24 weeks, 85% for 26 weeks, and 95% for 28 weeks gestational age) (Saigal & Doyle 2008). However, these prematurely born infants are at an increased risk for various adverse outcomes such as neurodevelopmental disability and respiratory and gastrointestinal complications (Saigal & Doyle 2008). In recent studies, it has been estimated that the rate of short-term and long-term disability for these premature infants is more than 50%, resulting in enormous physical, psychological, and economic costs (Tyson et al 2008; Watts & Saigal 2006; Wood et al 2005).

Surviving preterm infants are especially susceptible to brain abnormalities that involve both grey and white matter. Preterm brain injuries are significantly associated with various neurodevelopmental disabilities, such as motor deficits (ie, cerebral palsy), cognitive, behavioural and socialization deficits, and sensory deficits (ie, visual and auditory impairments). The prevailing neuropathology in very preterm infants has been considered to be periventricular leukomalacia (PVL) and intraventricular hemorrhage (IVH) (Childs et al 2001; Counsell et al 2003; Volpe 2003). PVL, which is characterized by necrosis near the lateral ventricles, occurs in 3-5% of surviving preterm infants (Volpe 2003). IVH is also a very commonly detected brain hemorrhage which occurs from the periventricular germinal matrix and fills up the ventricular system (Volpe 2008). In addition, many preterm infants show damages in the cortical grey matter which often leads to the reduction in cerebral cortical grey matter volumes (Inder et al 2005; Isaacs et al 2001; Isaacs et al 2000; Peterson et al 2000). In recent years, many clinical studies have reported that abnormalities
of the cerebellum and posterior fossa are detected frequently in the preterm infants (Miall et al 2003).

Cerebellar abnormalities are an increasingly recognized complication of premature birth due to the recent improvement in imaging technology. In the past, cerebellar injury has been difficult to visualize using conventional anterior fontanelle views on ultrasonography. However, the increase in the use of magnetic resonance imaging (MRI) and the mastoid fontanelle view on ultrasonography has greatly improved diagnosis of cerebellar abnormalities (Limperopoulos et al 2005; Sehgal et al 2009; Steggerda et al 2009). Thus, the findings of recently published neuroimaging studies suggest that the incidence of cerebellar injury may be as high as 19% in preterm infants (Limperopoulos et al 2005; Steggerda et al 2009). In addition to the high incidence rate, a high prevalence of long-term neurological deficits, including motor, cognitive, learning, social and behavioural disturbances, significantly associated with premature cerebellar injury, contributes to the overall burden of the disease (Limperopoulos et al 2007). Despite the increased recognition, the precise pathophysiology of preterm cerebellar injury still remains unknown and further clinical data analysis and studies using animal models are necessary to enhance our understanding of the disease and to improve the preventive therapies and post-hemorrhagic treatments.

1.2 Cerebellar Development and the Period of Particular Vulnerability

Development of the cerebellum begins at four weeks after conception; the cerebellum achieves maturity two years postnatally. Cerebellar growth is most rapid during 24 weeks to 40 weeks gestation, the period of preterm birth; the increase in cerebellar volume is about five-fold and the increase in surface area is more than 30-fold (Chang et al 2000; Lemire 1975).

Unlike other structures of the brain, the cerebellum possesses two germinal matrices that give rise to cerebellar neurons (Figure 1.1(A)). The first germinal zone, the dorsomedial ventricular zone (VZ), is formed in the early embryonic stage and gives rise to γ-aminobutyric acid (GABA-ergic) neurons. The VZ first generates the cerebellar nuclear neurons (Week 9 in humans; E10-12 in mice). Purkinje cells are also generated from the VZ (Week 10 in humans; E11-13 in mice) and migrate radially to form the cerebellar plate (Figure 1.1(B)). From the cerebellar plate, Purkinje cells
Figure 1.1 Development of the cerebellum. (A) Early embryonic cerebellum possesses two proliferative zones, the ventricular zone (VZ) and the rhombic lip (rl). (B) Major histogenic events from 9 weeks gestation to 7 months after birth. Purkinje cell (P-cells) and dendate (De) cells born from the VZ migrate upward. Granule cell precursors from the rl migrate tangentially to form the external granule layer (EGL). Granule cells continue to proliferate under the positive control by Sonic hedgehog (shh) from P-cells. When granule cells mature, they migrate inward to form the internal granule layer (IGL). ML, molecular layer; IZ, intermediate zone, WM, white matter. (C) The developing cerebellum and surrounding brain structures. Black region shows CSF-filled cavities, the 4th ventricle and subarachnoid space and white arrows show the direction of CSF flow. Red line indicates the cerebellar surface covered with capillary beds. SOURCE: (A) modified from (Dastjerdi et al 2012) and used under Creative Commons Attribution License; (B) Volpe, J. J. Journal of Child Neurology (Volume 24, Issue 9) pp. 1085-1104, copyright © 2009 by SAGE Publications Inc. Journals
continue to migrate and establish a monolayer, called Purkinje cell layer (PCL). As Purkinje cells mature (gestational week 24 – postnatal week 40 in humans; P0-P15 in mice), synaptogenesis occurs to establish connections with granule neurons through parallel fibers and with inferior olivary nuclei with climbing fibers. Also, Purkinje cells make axonal projections to the cerebellar nuclear neurons. The VZ also gives rise to GABAergic interneurons, including Golgi, basket and stellate cells which colonize the molecular layer (ML). The second proliferative zone is the rhombic lip (RL), which gives rise to glutamatergic neurons (Figure 1.1(A)). Granule cell precursors are generated from the RL, starting as early as week 11 in humans and E13-19 in mice. These primitive cells migrate tangentially to the surface of the cerebellum to form the external granule layer (EGL) (Figure 1.1(B)). Within the EGL, the outer part is populated by rapidly proliferating granule cells and the inner EGL contains mature granule cells. When the maturation process is complete, granule cells begin to migrate radially along the fibers of Bergmann glia into the cerebellar cortex to form the internal granule layer (IGL). This inward migration continues to the seventh month after the birth in humans and to P15 in mice. The axons of granule cells, parallel fibers, project to the ML and establish synaptic connections to the dendrites of Purkinje cells and interneurons.

During the preterm period, the cerebellum undergoes the most drastic increase in volume and surface primarily due to the extensive proliferation of granule cells in the EGL. The EGL becomes six to nine cells thick during its peak proliferative period and in the mature cerebellum, the total number of granule cells reaches approximately $10^{11}$ and accounts for 95% of all neurons in the brain (Carletti & Rossi 2008). This period of rapid expansion in the number of neurons (ie, “growth spurt”) is known to be the period during which the brain structure is particularly vulnerable to disturbances in its growth program (Dobbing 1974). In the context of the cerebellum, undernutrition, glucocorticoid exposure, and x-irradiation, during the rapid growth, have shown to cause long-term structural and functional changes in experimental animal models (Chase et al 1969; Dobbing 1974; Dobbing et al 1970; Dobbing & Sands 1973; Howard 1968). Moreover, since the cerebellar germinal matrices, the EGL and VZ, possess rich capillary beds that are especially vulnerable to rupture during the preterm period and are in close proximity to the subarachnoid space and fourth ventricle, the most highly proliferative cells are directly exposed to the cerebrospinal fluid which may contain blood products in case of cerebellar, intraventricular, or subarachnoid hemorrhage (Figure 1.1(C))(Messerschmidt et al 2008).


1.3 Function of the Cerebellum

The major function of the cerebellum is the coordination of movement. The cerebellum is known to be utilized primarily in control of voluntary movement, equilibrium, muscle tone, and postural control. It receives sensory input from the spinal cord and other parts of the brain, and integrates the information to generate and fine-tune the motor activity. In addition, the cerebellum has been suggested to play an important role in the process of motor learning (Desmond & Fiez 1998; Doyon et al 1996). The acquisition of a new motor skill is thought to involve the cerebellum to act as a feedback processing center by detecting errors from already performed movement and making corrections to the same type of movement (Halsband & Lange 2006; Jueptner & Weiller 1998). Also, neuroimaging studies have shown a strong activation of cerebellar neurons during the initial phase of learning a new motor task and clinical studies have reported that patients with posterior cerebellar hemisphere damage exhibit problems in motor learning (Flament et al 1996; Jueptner & Weiller 1998).

Recently, there has been increasing recognition of the role of the cerebellum in non-motor functions. Functional neuroimaging and neurobehavioural studies provide evidence for the cerebellum’s participation in cognitive and affective functions, including attention, sensory discrimination, memory, and verbal learning (Andreasen et al 1995; Courchesne et al 1994; Gao et al 1996; Klingberg et al 1996). The role of the cerebellum in these functions seems to be facilitated through the cerebrocerebellar system, the interconnection between the cerebellum and cerebral cortical areas, including not only the motor cortex, but also other regions, such as limbic, prefrontal, posterior parietal, and paralimbic cortices (Schmahmann 2001). Clinical studies also corroborate the cerebellum’s involvement in cognition; there have been consistent reports on cognitive impairment following the isolated damage to the cerebellum. Schmahmann and Sherman coined the term “cerebellar cognitive affective syndrome” to describe the disturbances in executive, visual, spatial, and linguistic function in patients with cerebellar injury (Schmahmann & Sherman 1998).

1.4 Cerebellar Hemorrhagic Injury in Preterm Infants

Cerebellar abnormalities associated with preterm birth can occur in two ways – destructive lesions and impaired development (Volpe 2009). The major types of destructive lesions include hemorrhage and infarction. Cerebellar underdevelopment may occur as a result of intraventricular
blood, hypoxia-ischemia, infection-inflammation, glucocorticoid treatment, and undernutrition (Messerschmidt et al 2005; Parikh et al 2007; Tam et al 2011b; Volpe 2009). In many clinical cases, there may be overlap of destructive lesion and underdevelopment.

Among the different types of preterm cerebellar injuries, cerebellar hemorrhagic lesion is the best studied type of injury. More frequent use of ultrasonographic imaging through mastoid fontanelle views and MRI scanning has revealed that cerebellar hemorrhage (CBH) is much more common than previously thought. A study that used MRI, which allows identification of hemorrhages to sizes of 1-3mm in diameter, has shown 10-20% incidences of CBH in preterm infants (Steggerda et al 2009). The incidence appears to be dependent on the degree of prematurity. The incidence of CBH in infants who weigh less than 750g at birth was 17% and in the infants in between 750g to 1499g was only 2% (Limperopoulos et al 2005). Also, CBH occurs most frequently in the neonates born before 28 weeks gestation (Limperopoulos et al 2005).

Major categories of CBH include primary cerebellar hemorrhage, venous infarction, and extension of intraventricular or subarachnoid hemorrhage into the cerebellum (Volpe 2008). The primary cerebellar hemorrhage usually occurs from the germinal matrices of the cerebellum, including the EGL and VZ, the structures that are richly vascularized with capillaries (Figure 1.1(C)). The bleeding that originates from the EGL leads to focal unilateral hemispheric lesion (Limperopoulos et al 2005; Volpe 2009). The lesion that arises from the VZ is less common and results in vermian hemorrhage (Volpe 2009). In addition, a large proportion of CBH cases occurs from secondary invasion of intraventricular or subarachnoid blood; Donat et al. (1979) have reported that half of CBH cases appear to be from blood accumulated in the fourth ventricle. In these cases, massive supratentorial IVH from the cerebral periventricular germinal matrix first fills up the lateral ventricle and then spreads into the fourth ventricle and subarachnoid space that surround the cerebellum. Indeed, 77% of CBH has been found to be associated with supratentorial IVH (Limperopoulos et al 2005; Miall et al 2003).

The high rate of co-occurrence of CBH and supratentorial IVH suggests that the etiology of CBH is likely to be similar to the mechanisms that lead to IVH. The major risk factors that predispose the development of preterm supratentorial IVH and CBH include traumatic delivery, respiratory distress syndrome, hypoxia, hypercapnia, patent ductus arteriosus, thrombocytopenia, and infection (Ballabh 2009; Limperopoulos et al 2005; Volpe 2008). In addition, the fragility of immature vasculature of cerebellar germinal matrices, disturbance in the cerebellar blood flow, and abnormalities in coagulation may contribute to pathogenesis of preterm CBH (Volpe 2008).
Previous clinical studies have investigated the short-term and long-term outcomes of preterm CBH. The mortality rate reported from 35 cases of preterm CBH was 14% (Limperopoulos et al 2005), but another clinical study conducted Zayak and colleagues (2011) has shown that CBH is not significantly associated with higher mortality rate. However, both studies others have consistently shown a significant association between CBH and various adverse neurodevelopmental outcomes. The surviving infants have high rate of severe morbidity with various complications, such as longer requirement for oxygen, ventilator support and duration in the neonatal intensive care unit (Zayek et al 2011). In addition, in 66% of cases of preterm CBH, long-term neurologic abnormalities have been observed at age of ~2.5 years (Limperopoulos et al 2007). Motor impairments, including hypotonia, gait abnormalities, and extraocular disturbances, were also noted in the affected infants (Limperopoulos et al 2007; Messerschmidt et al 2005). Furthermore, recent CBH studies have demonstrated significant deficits in cognitive, affective, language, and social behavioural functions, suggesting the effect of cerebellar injury on non-motor functions (Biran et al 2011b; Limperopoulos et al 2007).

The outcomes of CBH are dependent on the size and location of lesion. Compared to Limperopoulos and colleagues’ study (2007), which reported a significant association between relatively large CBH and impairments in motor, cognitive, and social behaviour, Tam et al.’s study (2011c), which focused on MRI-diagnosed smaller hemorrhages, showed the significant association only with abnormal neurologic outcomes, but not with cognitive disturbances. The severity and the type of morbidity are not only dependent upon the size of the lesion, but also on the location of hemorrhage; Zayek et al. (2011) found that the infants with vermian hemorrhage have a higher rate of cerebral palsy, motor, and mental abnormalities, compared to hemispheric hemorrhage which showed significant association only with mental impairment. In addition, multiple neuroimaging studies have described the lesions in the cerebellar vermis in children with autism (Courchesne 2004; Courchesne et al 2001; Hashimoto et al 1995).

The neurodevelopmental abnormalities that are found in the survivors of preterm cerebellar injury are probably due to the disturbances in cerebellar growth during the critical phase of development. As an effect of primary CBH, volume loss in the cerebellum has been reported (Limperopoulos et al 2010). Furthermore, cerebellar volume reduction has been noted from the preterm infants with supratentorial IVH, which may cause deposition of blood products in the fourth ventricle and cerebellar germinal matrices (Messerschmidt et al 2005; Tam et al 2011b). However, detailed topographical and morphological studies have not yet been done. Although it has been postulated that the granule cell precursors in the EGL may be the key target of preterm CBH,
no experimental evidence that elucidate the damages at the anatomical, cellular, and molecular levels has been provided from clinical studies. Therefore, further investigation on the effects of hemorrhagic injury on the developing cerebellum using a preclinical animal model would be necessary to enhance the current understanding of the disease.

1.5 Animal Studies on Cerebellar Hemorrhage

To investigate the effects of hemorrhagic injury on the developing cerebellum, a preclinical animal model is required. The animal model would be used as a tool to study the morphological, behavioural and molecular phenotypes that result from preterm CBH and to address the mechanisms that underlie the outcomes associated with CBH. Furthermore, an effective animal model for CBH would allow investigation on the effects of different preventive therapies and post-hemorrhagic treatments. Although several animal models have been developed and used for studies on supratentorial intracerebral hemorrhage (ICH) (Rosenberg et al 2008), animal studies on CBH have been very rare. In fact, a rat model for adult spontaneous CBH developed by Lekic and colleagues (2011) is the only currently available animal model. In their report, Lekic et al (2011) emphasized the importance of the CBH model, because the pathophysiology of cerebellar disease is distinct from cortical injuries. In vitro studies using cerebellar and cortical neuronal cultures have revealed the differences in the levels of vulnerability and injury mechanisms (Scorziello et al 2001). In addition, surgical and treatment approaches for CBH differ from the supratentorial hemorrhage (Mendelow et al 2005; Morgenstern et al 2010).

Although the adult CBH model proved the feasibility of experimental induction of hemorrhage in the adult cerebellum, neonatal CBH has never been modeled in animals. The etiology, pathophysiology, and neurobehavioural outcome of hemorrhagic injury to the developing immature cerebellum of preterm neonates are likely to be very different from the mechanisms and outcomes of adult cerebellar injury. Additionally, the particular vulnerability of rapidly developing cerebellum during the third trimester (discussed in Section 1.2) makes the preterm CBH a unique issue which cannot be sufficiently parsed by the adult CBH model. Therefore, the development of the preterm CBH model is especially important for translational purposes to enhance the understanding of the disease in the preterm population.

In order to employ an animal model in the study of preterm CBH, a complete assessment of the relevance to the human condition and understanding of a specific animal model's advantages
and limitations would be necessary. Many different species, ranging from larger animals such as primates, pigs and sheep to smaller species, including mice and rats, have been utilized for developmental cortical injuries (Hagberg et al 2002; Vannucci & Vannucci 2005). Among these, rodents, especially mice, are the most commonly used animal species for studying developmental brain injuries because of many practical and economical advantages. With rodent models, various environmental and genetic influences are easy to control and they are relatively inexpensive to maintain and reproduce. Also, there is vast amount of information on mouse neurobehavioural development and experimental approaches for anatomical, molecular, and functional assessments after the injury. Despite the differences in anatomy, the human and the mouse brain follow the same basic neurophysiological maturational processes. In particular, the cerebellum of humans and rodents possesses highly analogous processes of morphological and cytological development, as well as histological layers of the cerebellar cortex, which make mouse cerebellum an appropriate model system for studying preterm CBH (Biran et al 2011b). Therefore, careful replication of the disease and thorough evaluation of the phenotypes in the model animal would enable researchers to gain valuable information for understanding, treating, and preventing CBH.

1.5.1 Timing of Hemorrhage Induction

![Diagram of cerebellar development in rodent and human](image)

**Figure 1.2 Comparison of timing of cerebellar development in rodent and human.** The period of preterm birth is marked in red. Purkinje cell (PC), granule cell (GC), external granule cell (EGC), embryonic day (E), postnatal day (P), gestational week (gw), postnatal week (pnw), postnatal month (pnm). **SOURCE:** modified from (Biran et al 2011b) and used under Creative Commons Attribution License
Brain maturation at birth is one of the major differences between the two species (Figure 1.2). Therefore, many existing models of preterm brain injury, such as supratentorial IVH and PVL, utilize rats and mice during the early postnatal period, from P0 to P7, the age that is comparable to 24 to 32 weeks gestation in humans (Figure 1.2) (Balasubramaniam et al 2006; Lekic et al 2012; Xue et al 2003). To study the effects of preterm-associated forebrain injuries on the development of the cerebellum, Biran and colleagues (2011a) have used rats at P2, which is comparable to 28 weeks gestation in humans. Since CBH occurs most frequently in preterm infants who are born before 28 weeks gestation, preterm cerebellar hemorrhagic injury would be best modeled in mice or rats at the age between P0 and P2.

1.5.2 Methods of Hemorrhage Induction

Several preclinical animal models have been developed to understand the pathophysiology of ICH. Although many of these animal models were developed to replicate adult cortical hemorrhage, some studies have shown that similar experimental procedures can be applied to model hemorrhage in the neonatal brain (Alles et al 2010; Balasubramaniam et al 2006; Lekic et al 2012; Xue et al 2003). Two paradigms have been most commonly utilized to induce ICH in rodents: direct injection of autologous blood and administration of bacterial collagenase. Each of these methods has its inherent strengths and weaknesses and possesses the potential to enhance our understanding of the pathophysiology and therapies for hemorrhagic brain injury.

The first commonly used approach of hemorrhage induction is the injection of autologous blood, which mimics a single large bleeding in human ICH cases (Bullock et al 1984; Herbstein & Schaumburg 1974). This method uses the injection of a precise volume of whole blood into a specific area of the brain over a specified period. Therefore, the advantage of this method is the control in production of a consistent hemorrhage size. However, blood injection does not reproduce spontaneous bleeding from vessel rupture, which occurs in ICH patients. Also, other drawbacks include back flow of blood, which may lead to variable lesion size, and potential side-effects of anticoagulants (Yang et al 1994). This blood infusion model is therefore more useful for studying the pathophysiology and biochemical mechanisms that result from the presence of blood in the brain tissue.

In comparison to the autologous blood model, bacterial collagenase infusion model is considered as a better paradigm to replicate clinical ICH (James et al 2008). The injection of collagenase disrupts the basal lamina of blood vessels, leading to spontaneous blood leakage into the surrounding brain tissue (Rosenberg et al 1993; Rosenberg et al 1990a). Moreover, collagenase
injection may simulate the bleeding-bleeding phenomenon in ICH patients. The technical advantages of the collagenase model are the relative simplicity of the procedure and the roughly dose-dependent formation of hemorrhagic mass (Rosenberg et al 1990b). A potential disadvantage of bacterial collagenase model is that collagenase is thought to induce an exaggerated inflammatory response (Participants 2005). However, in vitro assays have reported that collagenase, at the concentration used for in vivo studies, does not have direct toxicity that leads to inflammation and neuronal apoptosis (Chu et al 2004; Matsushita et al 2000). Also, diffuse bleeding from rupture of small blood vessels in the collagenase model does not completely emulate clinical ICH where the hematoma formation is due to the bleeding from the arterial source.

Despite some drawbacks, the collagenase-induced hemorrhagic injury to developing cerebellum is most suitable for our study. Through previous adult ICH animal model studies, the bacterial collagenase injection approach has proven its ability to produce spontaneous bleeding and long-term histological and behavioural outcomes which we aimed to replicate in our preterm cerebellar hemorrhagic injury model (MacLellan et al 2008; Manaenko et al 2011). Additionally, unlike the blood infusion model, the collagenase injection method has been used in a rat model for CBH, showing that the injection paradigm can be adapted to the cerebellum (Lekic et al 2011). However, further modification to the existing collagenase injection protocol was necessary to induce hemorrhage in the neonatal mouse cerebellum.

1.5.3 Animal Behavioural Tests for Assessment of Neurobehavioural Phenotypes

Behavioural measurements are crucial parameters that help to identify the neurobehavioural phenotypes and functionality that result from brain lesion. Analysis of mouse behaviour using behavioural paradigms that are developed and validated for rodents allows the evaluation of cerebellar function and the comparison to the behavioural phenotypes seen in patients with the preterm cerebellum injury. In this study, a battery of behaviour tests consisting of open field, rotarod, horizontal ladder rung, goal-directed task, and Morris water maze (MWM) was used to assess the motor, affective, and cognitive function of the animals with the cerebellar injury.

1.5.3.1 Rotarod

The rotarod performance test is commonly used to assess coordination and motor learning in animal models of disease. In this test, a mouse is placed on a horizontally oriented, rotating rod and must move to avoid falling to the ground. The duration that a mouse stays on the rod is used as a measure of its balance, coordination, and motor-planning. The complexity of task can be increased
by accelerating the speed of rotation (Jones & Roberts 1968). Furthermore, by training the mice on the accelerating rotarod over several days and comparing the performance between training sessions, motor skill learning can be evaluated (Buitrago et al 2004). The rotarod test has been used in numerous studies to assess cerebellum-dependent coordination and motor learning (Cendelin et al 2008; Lekic et al 2011; Miyata et al 2001).

1.5.3.2 **Horizontal Ladder Walking Test**

The horizontal ladder walking test is used to assess skilled walking and forelimb and hindlimb placing. The test requires animals to spontaneously walk across a horizontal ladder. The spacing between the ladder rungs can be changed periodically to prevent compensating impairment through learning the spacing and location of the rungs (Metz & Whishaw 2002). Animals are first trained to cross the ladder to reach the refuge. Then, on test days, the performance is video-recorded for a foot placement accuracy analysis. The horizontal ladder walking test was originally developed to test the effects of cortical and subcortical lesions and is now widely used for testing animal models for stroke, spinal cord injury, and neurodegenerative diseases due to its high sensitivity to detect even a subtle impairment in movement capacity (Metz & Whishaw 2002; Rha et al 2011; Sgado et al 2006). However, relatively little is known about the effects of postnatal cerebellar injury on horizontal ladder walking performance.

1.5.3.3 **Open Field**

The open field test provides a comprehensive evaluation of the general locomotor activities and emotionality of the mouse. The open field was first introduced by Hall (1934) as a behaviour test for rats. Principally, the open field measures many qualities of the movement in an arena, generally square, rectangular or circular in shape, with surrounding walls that prevent escape (Gould et al 2009). The open field-dependent parameters, most commonly measured to assess the qualities of movement, include distance moved, velocity of movement, and time spent moving, rearing, freezing, and grooming (Walsh & Cummins 1976). In addition, the open field test is frequently employed to evaluate anxiety-like behaviour. In the open field, two factors trigger anxiety-like behaviour: individual testing (test subject is separated from its social group) and agoraphobia (the open field arena is very large compared to the subject’s breeding environment) (Prut & Belzung 2003). This stressful environment results in an animal spending a majority of their time in close proximity to the walls while avoiding the brightly lit center arena. Therefore, the duration in the center of the field and frequency of entering the center are used to interpret the
level of anxiety in the test subject. In general, cerebellar damage has been shown to increase open-field activity and decrease anxiety-related behaviour, providing evidence for involvement of the cerebellum in locomotion and fear-related behaviour (Le Marec et al 1997a; Supple Jr et al 1987).

1.5.3.4  Goal-directed Task

To measure the attention and motivation of mice, we developed a behavioural test paradigm, which we called “goal-directed task.” The animals are water-deprived for 16 hours prior to the test. Then, the animals are put into an open field box which contains a small Petri dish with a droplet of water. The dish is located at the corner of the square open field box and remained at the same location throughout three days of testing. We measured the latency for each mouse to drink from the water dish. The latency does not show significant decrease over the three days, suggesting that the test does not involve memory and learning the location of the dish. In this test, drinking from the water dish, which relieves thirst, serves as a “goal,” and the goal “directed” behaviour is mediated by knowledge of the relationship between the action and the outcome (Dickinson & Balleine 1994). The performance of goal-directed behaviour involves motivational, emotional, cognitive processes (Brown & Pluck 2000), making this behavioural paradigm an appropriate test for the attention and motivation for mice.

1.5.3.5  Morris Water Maze

The MWM is the most frequently used cognitive tool to assess spatial learning and memory for rodents (Morris 1984). Briefly, animals are required to find a hidden platform within a pool, filled with water, by using visual cues surrounding the pool. During a number of training trials, the time to locate the platform decreases as the animals learn to swim directly to the hidden platform. To assess spatial learning and memory, the time it takes to reach the platform (escape latency), distance travelled (swim distance), average swimming speed, and time spent in the target quadrant are measured. In addition, after the training sessions, probe trials are given: the escape platform is removed and the mice are allowed to swim for a fixed duration. The swimming patterns and time spent in the target quadrant are recorded. Many methodological modifications and variations have been used by numerous research groups to test different aspects of learning and memory (Vorhees & Williams 2006). Several studies have used the MWM to assess the spatial data processing, learning and memory after the cerebellar lesion and showed that that the animals with cerebellar damage are more likely to exhibit inferior performance compared to controls (Mandolesi et al 2001; Martin et al 2003; Petrosini et al 1996). These studies suggest the involvement of cerebellum in
cognitive function. However, this test has never been used to test the cognitive function of the cerebellum after neonatal cerebellar injury.

1.6 Molecular Mechanisms of Brain Injury and Neuroprotection

1.6.1 Potential Mechanisms of Injury

1.6.1.1 Apoptotic Pathway

Apoptosis is a process of programmed cell death that possesses distinct morphological and biochemical changes, such as cell shrinkage, DNA laddering, and caspase activation (Lipton 1999). In contrast to necrosis, a type of traumatic cell death that occurs after an exogenous injury, apoptosis is an ordered physiological process involved in forming a new structure and eliminating unwanted cells. Therefore, apoptosis is considered to be a normal developmental process. In the developing cerebellum, excess granule neurons are produced and subsequently eliminated by apoptotic cell death (Wood et al 1993). In addition, increasing evidence suggests that apoptotic cell death is also involved in brain injury. In adult ischemia animal models and human patients, there is a strong evidence for DNA laddering and activation of caspase, suggesting its involvement in mediating some of the neuronal death after the injury (Cahill et al 2006; Matsushita et al 2000; Raghupathi et al 2000). Previous studies have shown that in response to brain injury, the apoptotic pathway can be activated extrinsically by Fas ligand or tumor necrosis factor-alpha (TNFα) and intrinsically by mitochondrial outer membrane permeabilization (Hagberg et al 2006). Both intrinsic and extrinsic activation of pathway results in the activation of Caspase-3. Then, Caspase-3 leads to cleavage of caspase activated DNase which causes DNA fragmentation and cell death. Therefore, in this study, we hypothesized that the cerebellar injury will lead to increased expression of activated Caspase-3, resulting in the increase in the level of apoptosis. Currently, there is only a limited number of studies that examined the activation of apoptosis in neonatal or preterm cerebellar injury models. Also, the extent of its involvement and the timing of activation have not been clearly defined.

1.6.1.2 Inflammatory Pathway

Inflammation is systemic and local immune reaction to injury. While it is well known that infectious insults initiate inflammatory responses, non-infectious insults, such as brain hemorrhagic injury and hypoxia-ischemia have also been found to lead to inflammatory responses
(Szaflarski et al 1995). In perinatal brain, inflammation has also been shown to be involved in response to non-infectious injury (Hagberg et al 2006). Several pro-inflammatory cytokines (IL-1α, IL-1β, IL-18, TNFα, IL-6) and chemokines (MIP-1a, MIP-1b, Rantes, MCP-1, MIP-2, IP-10, MCP-3) are produced by microglia and astrocytes (Brunswick et al 2012; Silverstein et al 1997). These proteins serve to attract and activate inflammatory cells and coordinate the immune response to inhibit potential infectious process after brain lesion. However, the inflammatory process can lead to tissue destruction and the secondary injury to the brain (Nathan 2002). Therefore, previous studies have reported that inhibition of proinflammatory mediators, such as IL-18 and IL-1, reduce the extent of brain injury (Hedtjarn et al 2002; Martin et al 1994). Although there are only limited experimental studies on inflammation in the developing cerebellum, Dean and colleagues have recently reported that the immune responses that are triggered by *Escherichia coli* lipopolysaccharide exposure in preterm fetal sheep result in cerebellar white matter (WM) lesion (Dean et al 2009). However, it remains unknown whether non-infectious insults, such as cerebellar hemorrhagic injury can lead to the activation of inflammatory pathway in the preterm infants and therefore, our study explored the expression of transcripts for pro-inflammatory cytokines, *Tnfα* and *Il-1β*.

**1.6.2 Potential Mechanisms of Neuroprotection**

**1.6.3 Neurotrophic Factors**

Neurotrophic factors are a group of proteins that play important roles in cell survival, growth, and function of neurons (Reichardt 2006). In the developing brain, many neurotrophic factors are expressed and show specific expression patterns, affecting selected neuronal populations (Semkova & Krieglstein 1999). The expression of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), insulin like growth factor (IGF-1), ciliary neurotrophic factor (CNTF), and erythropoietin (EPO) and its receptor, EPOR, have also been described in the cerebellum, promoting cerebellar cell survival and exonal elongation (Dame et al 2000; Schwartz et al 1997; Wang & Zoghbi 2001). When brain injury occurs, these neurotrophic factors have been reported to be up-regulated and the increased levels of neurotrophic factors have been proposed to play a role in a protective response (Guan et al 2003; Lin et al 1998; Siren et al 2001). Studies have suggested that the levels of the neurotrophic factors may determine the susceptibility to neuronal death after brain injury and trigger regeneration neurons (Larsson et al 1999; Mattson & Scheff 1994). Therefore, the exogenous administration of these neurotrophic factors recently emerged as a potential neuroprotective therapy (Wu 2005). However, it remains unknown whether CBH leads
to increased production of these neurotrophic factors endogenously and whether the up-regulation would result in neuroprotection in the developing cerebellum.

1.6.4 Sonic Hedgehog Pathway

Sonic hedgehog pathway is an important signaling pathway that plays a role in patterning of many tissues, including nervous system, and regulates the development of many neurons in the mammalian brain (Hammerschmidt et al 1997). In addition to its role in development, in many studies, sonic hedgehog pathway has been reported to be involved in neuroprotection after brain injury (Amankulor et al 2009; Bambakidis et al 2003). In response to hypoxia-ischemia, Sonic hedgehog (SHH) expression was found to be increased in neuronal precursors and mature neurons in vitro and in vivo and suggested to account for the subsequent increase in proliferation (Sims et al 2009). However, the expression of SHH and other players of the sonic hedgehog pathway has never been studied in context of neonatal cerebellar injury despite the critical role of this molecular pathway on the development of the postnatal cerebellum. During cerebellar development, the sonic hedgehog pathway controls the proliferation of granule cell precursors in the EGL (Wechsler-Reya & Scott 1999). SHH, produced and secreted from Purkinje cells, binds to its receptor Patched (PTCH) on granule cell precursors and releases PTCH inhibition of a transducing protein, Smoothened (SMO), which in turn activates transcription factors, such as GLI1 and GLI2. These transcription factors then lead to the increase in Nmyc expression which regulates cell cycle progression in granule cell precursors (Kenney et al 2003).

1.7 Thesis Objectives

CBH is a severe problem in preterm infants because of its significant association with higher mortality rate and various adverse neurodevelopmental outcomes. Despite growing recognition of preterm CBH and its neurodevelopmental outcomes, the information on the precise pathophysiology of CBH is still very limited. The detailed investigation of the disease has not been possible partly due to the limitations in clinical studies and the lack of animal models. Therefore, our study focuses on developing an effective animal model of the preterm CBH and investigating the anatomical, behavioural, and molecular phenotypes resulting from the injury.
2. The Effects of Cerebellar Hemorrhage on the Development of the Postnatal Cerebellum

2.1 Introduction

Human and animal studies suggest that the cerebellum is particularly vulnerable during the preterm period. Hemorrhagic injury to preterm cerebellum may disturb its rapid and complex developmental processes and result in various short-term and long-term outcomes. However, despite the increased recognition of neurodevelopmental outcomes, the information on the precise pathophysiology is still very limited. Although the cerebellar volume reduction has been noted as an effect of CBH, exact morphological phenotypes and changes at the molecular level that lead to volume reduction remain to be elucidated. The detailed pathological investigations on preterm CBH have not been possible due to the limitations in the clinical studies and the lack of animal models. Therefore, it is clinically important to develop and characterize an animal model of preterm CBH to enhance our understanding of the mechanisms of injury and neurodevelopmental consequences. Although several groups have developed animal models for cerebellar abnormalities through different mechanisms, such as infection, hypoxia, ischemia, exposure to drugs, and undernutrition (Vorhees & Williams 2006), these existing models do not adequately resemble preterm CBH with respect to the etiology, pathology, and neurodevelopmental outcomes.

Therefore, in the present study, we developed a model of the preterm human CBH in neonatal mouse pups. We used the pups at postnatal day P2, which is developmentally comparable to 26-30 weeks gestation in human. Using this animal model, we aimed to investigate the effects of preterm CBH on the development and function of the cerebellum. Our general hypothesis was that CBH leads to abnormalities in the cerebellum development, deficits in cerebellar-specific behaviours, and the activation of molecular pathways for brain injury and neuroprotection. Therefore, we examined anatomical features of the cerebellum, behavioural phenotypes, and expression level changes of genes that are important for cerebellar injury and neuroprotection after the cerebellar hemorrhagic injury. Our hypothesis was addressed by three experimental questions: (1) Does CBH affect the development of the cerebellum?; (2) Does CBH result in altered motor function, anxiety-associated behaviour, or cognitive and affective processing?; (3) Does CBH activate the molecular pathways involved in damage mechanisms and endogenous neuroprotection?
2.2 Methods

2.2.1 Experimental Model for Preterm Cerebellar Hemorrhage

2.2.1.1 Animals and Breeding

Mice were bred at the Centre for Molecular Medicine and Therapeutics (CMMT) Mouse Core Facility, University of British Columbia (UBC). Mice were maintained on a 12:12 hr light/dark cycle (light onset at 7:00 AM), with controlled temperature at 21 – 22 °C. Mice were provided a standard laboratory chow, Prolab Isopro RMH 3000 5P76 (LabDiet), and had access to tap water. For mating, female mice aged from P60 to P150 were housed together with a male stud until a vaginal plug was detected. Pregnant females were housed together until two days before giving birth. The day that pups were born was considered P0. Pups were kept with their mother until weaning on P21. This study was conducted in accordance with the guidelines defined by the Canadian Council of Animal Care, and was approved by UBC Animal Care Committee (Animal Care Application ID: A10-0279).

In this study, we used ICR mice for all experiments for anatomical, behavioural, and molecular evaluation. However, for Morris water maze (MWM) behavioural test, B6 mice were used instead of ICR. Since the MWM is a task that involves visual-spatial learning, we could not use ICR mice which are known to have a high incidence of retinal degeneration. The visual impairment in ICR mice have previously been shown to result in poor performance not due to the deficits in learning and memory (Brown & Wong 2007). Therefore, in this study, B6 mice were used for MWM.

2.2.1.2 Induction of Cerebellar Hemorrhage

Hemorrhage induction was performed at P2. Pups were anesthetized with isoflurane (4% for induction, 1-2% for maintenance, mixed with air and oxygen from a precision vaporizer). To induce the hemorrhage in the cerebellum, bacterial collagenase was injected into the fourth ventricle, by inserting a 26 gauge needle attached to a 10µl Hamilton syringe into the cerebral aqueduct, through which collagenase flows into the fourth ventricle. The stereotactic coordinates for the cerebral aqueduct, measured from lambda are 1.5mm (caudal) and 1.5mm (deep). After delivering collagenase, the syringe remained in place for 2 minutes to prevent back-leakage before being withdrawn. 1µl of bacterial collagenase at a concentration of 0.057U/µl and 0.045U/µl was administered for ICR and B6, respectively. The specific collagenase doses for both ICR and B6 mice were determined by assessing the size of hemorrhage, as well as the survival rate after the injection. 0.6 U, the dose previously used for CBH induction in adult animals (Lekic et al 2011), was initial dose tested and the injection of 0.6 U collagenase resulted in death of all the injected pups.
Therefore, we gradually lowered the dose to determine the optimal dose for both ICR and B6. Controls were injected with 1 μl of saline (0.9% NaCl dissolved in dH₂O), using the same injection methods. After the injection, animals were placed on a warmed heating pad and then returned to their nest with the mother following recovery from anesthesia. Identification numbers were applied to the back of the pups with a marker and reapplied as needed until weaning when a permanent ear punch was made.

### 2.2.2 Procedures for Anatomical Evaluation

#### 2.2.2.1 Tissue Collection and Processing

After the collagenase/saline injection at P2, cerebellar tissue of the surviving animals at 6 hours, and at 1, 3, 5, and 13 days were collected (ie, tissue collection at P2, P3, P5, P7 and P15). The animals were randomly allocated to each survival group and a minimum of five brains per treatment group were collected at each time point. The number of subjects used in this part of study is summarized in Table 2.1. Adult brain tissues were obtained from the P75-P78 after the completion of behaviour testing. To harvest the brain tissue, animals were overdosed with the anesthetic avertitin (1.25% (w/v), 2,2,2-tribomoethanol in tert-amyl alcohol) through intraperitoneal injection. Subsequently, mice were transcardially perfused with cold phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA) in 0.1M PBS. The brains were carefully dissected from the skull and post-fixed in 4% PFA solution for overnight. After the postfix, the brains were cryo-protected in ascending sucrose concentrations in PBS (10, 20 and 30%), embedded in Optimal Cutting Temperature (OCT; Sakura) in cryomolds and stored at -80°C until use. Sagittal whole-brain sections were cut at 14μm thickness by using a cryostat and directly mounted on glass slides. Slides were stored at -20°C until stained.

#### 2.2.2.2 BrdU Injection

Analysis of cell proliferation was performed by using BrdU labeling. Animals received 50mg/kg BrdU through a single intraperitoneal injection 24 hours after collagenase or saline treatment.
Table 2.1 Summary of the number of animals used for anatomical evaluation

<table>
<thead>
<tr>
<th>Age</th>
<th>Treatment</th>
<th># of Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>Saline</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Collagenase</td>
<td>11</td>
</tr>
<tr>
<td>P3</td>
<td>Saline</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Collagenase</td>
<td>9</td>
</tr>
<tr>
<td>P5</td>
<td>Saline</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Collagenase</td>
<td>11</td>
</tr>
<tr>
<td>P7</td>
<td>Saline</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Collagenase</td>
<td>20</td>
</tr>
<tr>
<td>P15</td>
<td>Saline</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Collagenase</td>
<td>21</td>
</tr>
<tr>
<td>Adult Male (~P75)</td>
<td>Saline</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Collagenase</td>
<td>7</td>
</tr>
<tr>
<td>Adult Female (~P75)</td>
<td>Saline</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Collagenase</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>Saline</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Collagenase</td>
<td>85</td>
</tr>
</tbody>
</table>

2.2.2.3 Cresyl Violet Staining

For cresyl violet staining, brain sections were rinsed in distilled water and immersed in 0.1% cresyl violet (dissolved in 1L dH2O with 3mL glacial acetic acid and 0.205g sodium acetate) for 10-15 minutes. After rinsing in water to remove excess stain, slides were dehydrated and decolorized in 50 and 70% ethanol solution (3 minutes each). When further decoloration was required, slides were immersed in Differentiation solution (2 drops glacial acetic acid in 95% ethanol). Dehydration was continued in 95% ethanol, and 3 changes of 100% ethanol (3 minutes each). Then, sections were cleared in three consecutive baths of xylene (3 minutes each) and coverslipped with Permount.
2.2.2.4 Immunohistochemistry

Immunohistochemistry was performed to label specific cell types in the cerebellum. Slides were rinsed in 0.1M PBS, and treated with 0.3% hydrogen peroxide to quench endogenous tissue peroxidases. Then, sections were blocked in blocking solution, which contains 30% bovine serum albumin (1:100, Sigma-Aldrich), normal goat serum (1:10, Bethyl Laboratories), and triton X-100 (1:100, Fisher Scientific) in PBS. After 30 minutes of blocking, sections were incubated in primary antibody at 4°C overnight. Sections were then rinsed and incubated for 1 hour with biotinylated goat anti-rabbit or goat anti-mouse IgG (ABC Elite Kit, Vector Laboratories). Following intermittent rinses in PBS, avidin and biotinylated horseradish peroxidase (Vector Laboratories) were applied for 30 minutes. Immunostaining was visualized with diaminobenzadine (DAB; Sigma-Aldrich). Slides were dehydrated in ethanol, cleared in xylene and coverslipped using Permount. Antibodies and final dilutions used for immunohistochemistry are listed on Table 2.2.

2.2.2.5 Immunofluorescence

Immunofluorescence was used to perform double-labeling with anti-BrdU and a cell-type specific marker. For detection of BrdU-labeled nuclei, DNA denaturation (30 minutes incubation in 1 M HCl at 37°C) preceded the incubation with primary antibodies. The list of primary antibodies and final concentrations used for immunofluorescence are listed on Table 2.2. After overnight incubation with anti-BrdU and a cell type-specific antibody, secondary antibodies, Alexa Fluor goat anti-rat and Alexa Fluor goat anti-mouse or rabbit, were applied for 2 hours to label BrdU-positive nuclei and a specific cerebellar cell type, respectively. After rinsing, sections were counterstained with DAPI (4',6'-diamidino-2-phenylindole) and then coverslipped with Fluorosafe mounting medium (Calbiochem).

2.2.2.6 Image Collection and Morphometric Analysis

For all morphometric analyses, we used 4 medial (vermian) sagittal cerebellar sections per animal. The immunostained tissues were examined with a Zeiss fluorescence microscope and photomicrographs were taken with Axio Vision Rel. 4.6 software. Morphometric analysis of cresyl violet or DAPI-stained medial cerebellar sections involved computer-assisted (ImageJ 1.46; National Institutes of Health) hand delineation of the cerebellum and cerebellar cell layers including EGL, IGL, ML, and WM.
2.2.2.7 Cell Quantification

Four medial sagittal cerebellar sections per animal (n=5 animals per CBH and control groups) were used for measurement of granule cell density in adult IGL. Using ImageJ software, a counting grid was superimposed on a 20X magnification image of CV stained IGL region. Each box in the counting grid has the area of 0.05mm$^2$. We counted the number of granule cells in 32 randomly chosen grid boxes per animal. All counting was done by the same person and image files were numbered to hide the treatment group information. All statistical analyses were performed after counting was completed. The average number of cells per 0.05mm$^2$ per animal was calculated.

The interneuron density in the ML of adult cerebellum was also measured from four medial sagittal sections per animal. We counted the number of CV stained interneurons in the four 20X magnification images taken from the ML of a cerebellar section. Then, we used ImageJ to measure the area the ML and calculate the density of cells.

Purkinje cell counting was done on four medial sagittal cerebellar sections per animal. To label the Purkinje cells, tissue sections were immunostained with anti-Calbindin prior to counting. Purkinje cell nuclei were identified with the aid of a brightfield microscope with 40X objective. Purkinje cells were counted from the whole sections and data obtained from four sections were averaged to estimate the number of Purkinje cells per mid-sagittal cerebellar section.

2.2.2.8 Statistical Analysis for Anatomical Evaluation

All statistical analyses were performed using IBM SPSS Statistics 20 software (IBM SPSS Statistics). Data were expressed as means ± 1 standard error of the mean (SEM). All data collected

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Antibody</th>
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<tr>
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<td>Anti-Caspase-3</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>1:1000</td>
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</tbody>
</table>
for anatomical evaluation were analyzed using unpaired 2-tailed $t$ tests to detect a significant difference between collagenase-injected CBH and saline-injected control animals.

### 2.2.3 Procedures for Behavioural Evaluation

#### 2.2.3.1 Behaviour Test Schedule

Tests began when animals became 60 days old. The animals were transferred to the behaviour testing suite, located in the CMMT Animal Core Facility, one week before the testing to acclimate to the room and altered light/dark cycle (light onset at 7 PM). All tests were performed during the dark phase, between 9 AM and 5 PM. Two behavioural tests, the rotarod and horizontal ladder rung, were used to assess the motor function. The cognitive and affective behaviour was tested by using open field, goal-directed task and the MWM.

For the ICR mice, 10 saline female, 12 collagenase female, 10 saline male, and 13 collagenase male mice were tested. A behavioural battery for the ICR mice consisted of 4 tests carried out over 10 days. The open field test was conducted on the first day and then 3 days of rotarod, 3 days of horizontal ladder test, and another 3 days of goal-directed task were performed. The same animals were used for the open field, rotarod, horizontal ladder test, and goal-directed task.

For B6 strain, 10 saline female, 11 collagenase female, 8 saline male, 9 collagenase male mice were tested. The B6 strain was used only in the MWM test. The MWM consisted of acquisition phase (day 1-3), short-term retention (day 4), and long-term retention (day 14).

#### 2.2.3.2 Open Field Activity

General locomotor activity and the level of anxiety were assessed by open field test performed at P60. Mice were placed in the center of a 50cm x 50cm x 20cm arena. The activity of each mouse was digitally recorded for 10 minutes and subsequently analyzed by Noldus Ethovision XT software (Noldus Information Technology). Total distance travelled, frequency of entering center, time spent in the center, mean velocity were measured. The center of the arena was defined as a 20cm x 20cm central square.

#### 2.2.3.3 Rotarod

After the completion of open field test, the mice were assessed for balance, motor coordination, and motor learning on an accelerating rotarod (Ugo-Basile). Revolutions per minute (rpm) were set at 4 as an initial value, with a progressive increase to a maximum of 40rpm across
the five minute test session. The duration on the rotating bar was recorded for each animal for three consecutive days. On day 1, mice were given four trials and on day 2 and 3, two trials were given. Mice clinging on to the rod and rotating for three consecutive rotations were scored as a fall. If a mouse fell off within five seconds from the beginning of the trial, that trial was not counted, and the mouse was given a new trial.

The performance improvement during day 1 and the improvement across three days were analyzed within the control and CBH groups separately. For performance improvement on day 1, all four trials were used in analysis of variance (ANOVA). For performance improvement across three days, the first two trials from each day were used in a repeated measure (RM) ANOVA. Then, the performance of the controls was compared with CBH mice by using RM-ANOVA.

### 2.2.3.4 Horizontal Ladder Rung Walking Test

The horizontal ladder rung walking test was conducted to assess skilled forelimb and hindlimb walking and to determine subtle loss of movement capacity. The test apparatus consisted of clear Plexiglas side walls (1m long and 20cm high) and variably spaced metal rungs (3mm diameter). The ladder was elevated 30cm above the ground with a home cage at the end of the ladder. Animals were habituated to the ladder and the height prior to the test through a training session consisted of five trials. All animals crossed the ladder in the same direction.

The level of difficulty was modified by varying the position of the rungs. We arranged the rungs in an irregular pattern; the distance between rungs varied from 1 to 3cm. Two different irregular patterns of the rungs were used over two days. On the first day, all animals were trained over 5 trials to cross the ladder. Then, on the following day, using the different rung pattern, mice were tested in 5 trials and their performance was video-recorded. The video-recordings were inspected using frame-by-frame analysis to score the number of steps and foot misplacements. Then, an error rate was calculated using the following formula:

\[
\text{Error rate} = \frac{\text{Number of slips}}{\text{Number of steps}}
\]

The error rate for each trial was calculated and the average across the five trials was used as an indication of the performance level for each animal.

### 2.2.3.5 Goal-directed Task

The goal-directed task was used to assess attention and motivation. Mice were water-deprived for 16 hours prior to the test. Then, on the test day, mice were placed in the open field box.
which contained a 35 x 10mm Petri dish at a dimly-lit corner of the box. The water dish remained at the same location throughout three days of testing. Mice were allowed to navigate in the open field box and maximum time allowed to drink from the water dish was 5 minutes. We measured the latency for each mouse to drink from the water dish and RM-ANOVA was performed to evaluate the task performance between the treatment groups. We repeated the test for three days and upon the completion of the test for each day, mice were allowed to have access to water for approximately 8 hours between each test over three days.

2.2.3.6 *Morris Water Maze*

The MWM task was based on the standard procedure for spatial learning in rodents. The water maze consisted of a circular swimming pool (155cm in diameter) and constructed of white fiberglass. The water maze was located in a room with numerous visual cues. The pool was filled with water, allowed to acclimate to room temperature overnight, and the water was then made opaque with non-toxic tempera paint (Demco). A platform which served as a refuge from the water was located in the center of an arbitrarily defined quadrant and submerged 2cm below the surface of the water. The behaviour of mice in the water maze was recorded and analyzed using an automated video tracking system (Noldus Ethovision) for the total distance taken to get to the hidden platform, latency to reach the platform, average swimming speed, and percent time spent in each quadrant. Mice were tested for their ability to find an escape platform on two components: hidden platform acquisition for 3 days to assess learning and two probe trials in the absence of the platform to test short-term and long-term memory.

For the learning test, each animal underwent two training sessions, which consisted of 4 trials, across 3 days. In each trial, a mouse was released from one of four equally spaced points along the perimeter of the pool, and then given 60 seconds to find the platform. Once the platform was found, the animal remained on the platform for 10 seconds. When the platform was not found, the mouse was placed on the platform for 10 seconds. At the end of each trial, mice were placed in a clean cage with paper towel, and then returned to the home cage for a 10 minute inter-trial period. The platform remained in the same location for 3 days of testing. The criterion for learning was an average latency to locate the platform.

Probe trials were introduced following the learning test on days 4 and 11 to test short-term and long-term memory, respectively. During probe trials, the escape platform was removed from the pool. Each mouse was allowed to swim for 60 seconds and then removed from the pool. The
The criterion used to assess memory was an average duration in the target quadrant in which the platform was located during the training trials.

2.2.3.7 Statistical Analysis for Behavioural Evaluation

All statistical analyses for behavioural evaluation were performed using IBM SPSS Statistics 20 software (IBM SPSS Statistics). Data were expressed as means ± 1 SEM. Open field and the horizontal ladder rung walking test were analyzed using unpaired 2-tailed t test. The goal-directed task and MWM behaviour data were evaluated by RM-ANOVA with treatment group as a between subject factor. Rotarod performance was similarly analyzed except that performance improvement was first analyzed within the control and CBH groups. When the assumption of sphericity was violated in RM-ANOVA, the Greenhouse-Geisser (GG) correction was applied to obtain the p-value. A value of p < 0.05 was considered statistically significant.

2.2.4 Procedures for Molecular Evaluation

2.2.4.1 Tissue Collection for RNA Extraction

A total of 16 ICR mice (P2, n=4/treatment group; P15, n=4/treatment group) were used to determine the gene expression changes after CBH. The cerebellum was dissected and snap-frozen in liquid nitrogen. Then, the tissues were stored at -80°C before RNA extraction was performed. Total RNA was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. The RNA quantity and quality were measured using spectrophotometry and the samples were stored at -80°C until use.

2.2.4.2 Reverse Transcriptase PCR and Quantitative Real-time PCR

1µg of total RNA was used as template to perform reverse transcription–polymerase chain reaction (RT-PCR) in a total reaction volume of 20µl. Reverse transcription reactions were primed by 1µl of oligo d(T)20 and incubated at 65°C for 5 minutes. The reaction tubes were cooled on ice for 2 minutes, followed by the addition of SuperScript III reverse transcriptase (SuperScript III First-Strand Synthesis Systems; Invitrogen) and incubated at 50°C for 50 minutes. After the termination of the reaction at 85°C for 5 minutes, RNase H was added.

Quantitative real-time PCR (qRT-PCR) was conducted to compare the expression levels of selected genes from saline and collagenase treated cerebellum samples. Fast SYBR Green Master Mix (Applied Biosystems) was used to set up qRT-PCR reactions (10 µl total volume) containing
cDNA as template. The reactions were carried out in an ABI 7500 Fast Real Time PCR Systems (Applied Biosystems). Amplicons were designed to amplify between 100 and 200 bp in length (see Table 2.3 List of primer sequences for the primer sequences). All samples were tested in triplicate with the reference gene 18S to normalize the data to correct for variations in cDNA quantity.

2.2.4.3 Tissue Collection and Processing for Immunofluorescence

Detailed procedures on tissue collection for immunofluorescence are described in Section 2.2.2.1. The protocol for immunofluorescence is also described in Section 2.2.2.5. We used mouse anti-Caspase3 (Abcam) at 1:1000 dilution to label apoptotic cells.

2.2.4.4 Cell Quantification

To quantify apoptotic cells following the injury, anti-Caspase-3 positive cells were counted from four medial sagittal cerebellar sections per animal. Anti-Caspase-3 positive cells were identified with the aid of a Zeiss Fluorescence microscope with 20X objective. Apoptotic cells were counted from the whole section and data obtained from four sections were averaged to estimate the number of apoptotic cells per mid-sagittal cerebellar section per animal.
<table>
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2.3 Results

2.3.1 Experimental Model of Preterm Cerebellar Hemorrhage

The ICR mouse pups injected with 0.057U/μl collagenase had a 35% mortality rate within the first 24 hours after the injection and most of death by collagenase treatment occurred within the first 6 hours. The collagenase dose for ICR was determined by testing 0.6U/μl previously used by Lekic and colleagues (2011) to induce CBH in adult rats. Our experiment showed that the dose was not tolerated when injected into the fourth ventricle of P2 mouse pups. By gradually lowering the dose, we found that the dose higher than 0.057U results in very widespread destruction of vasculature and a large hematoma extended to both infratentorial and supratentorial regions of the brain, leading to extremely high mortality rate. Also, when lower collagenase doses were tested, relatively smaller hematoma size was observed and there were no significant changes in mortality rate and the size of the postnatal cerebellum between the treatment groups. Therefore, in our study, 0.057U was determined to be the most appropriate dose for inducing CBH in ICR. However, when the same collagenase dose, 0.057U, was used for the B6 strain, approximately 80% mortality was observed. Therefore, we chose to lower the dose for B6 strain to 0.045U/μl, which lowered the mortality rate to approximately 35%. All pups that survived the first 24 hours after collagenase injection survived to the end of the study. Among the collagenase-treated survivors, approximately 7.5% of the animals showed stunted growth. Gross examinations of the brain 30 minutes after collagenase injection revealed the presence of hematomas in the fourth ventricle and on the cerebellar surface (Figure 2.1). We confirmed that the hematoma size becomes larger in the fourth ventricle area 1.5 hours after the injection and smaller hematomas were consistently observed up to P7. From the saline injected control animals, we did not observe any death or stunted growth.

Figure 2.1 Photograph of a mouse brain taken after the induction of hemorrhage. (A) Dorsal and (B) ventral brain show blood accumulated in the fourth ventricle and the cerebellum (CB) 30 minutes after collagenase injection.
2.3.2 Anatomical Evaluation

2.3.2.1 Cerebellum Size

The area of medial (vermian) cerebellar sections of developing and adult cerebellum was measured to assess the changes in size of the cerebellum after cerebellar hemorrhagic injury. Unpaired 2-tailed t test was performed to examine the effect of treatment (saline and collagenase) on cerebellar size at each time point (Figure 2.2). In short-term, 6 hours (P2), 1 day (P3) and 3 days (P5) after the injection, the CBH group did not show any significant differences in the size of the cerebellum, compared to the saline-injected controls. However, at P7 and P15, the CBH group showed a significant decrease in the area of the cerebellum (p = 0.024 and 0.01, respectively). In the adult animals, the mean areas of the cerebellum were smaller in the CBH group, but the differences were not statistically significant.

![Bar chart showing the effects of CBH on cerebellar size](image.png)

**Figure 2.2 The effects of CBH on cerebellar size.** Area (mm²) measurements were taken at postnatal and adult time points and from four medial cerebellar sections collected from CBH and control groups. Error bars show the 95% confidence interval for SEM. Asterisks (*) indicates significance at the 0.05 level from 2-tailed t-test.
2.3.2.2  **Granule Cell Layers and Granule Cells**

We examined whether collagenase infusion in the posterior fossa promotes short-term and long-term morphologic alterations in the granule cell layers. We first measured the area of the EGL, the proliferative zone for granule cell precursors, at P2, P3, P5 and P7 (Figure 2.3(A)). The area of the EGL in P2 CBH group was slightly increased, compared to the EGL area of saline-infused controls ($p = 0.032$). This area increase disappeared at P3 and P5. At P7, we detected a significant reduction in the EGL area measured from CBH group ($p = 0.017$).

At P15, when the EGL starts to diminish and the IGL becomes more prominent, we measured the area of the IGL to estimate the IGL size change in CBH animals (Figure 2.3(B)). The IGL area of CBH animals showed a trend of decrease, but the difference was not statistically significant ($p = 0.2$ at P15; $p = 0.63$ for adult females and $p = 0.27$ for adult males).

The density of mature granule cells was measured for the IGL of adult male and female animals subjected to collagenase or saline injection (Figure 2.3(C)). Our data showed that granule cell density was decreased in the collagenase injected males ($p = 0.003$). In the adult female mice, we did not detect a significant change in granule cell density ($p = 0.85$).
Figure 2.3 The effects of CBH on granule cell layers. The area (mm²) of EGL (A) and IGL (B) and granule cell density (cells/0.05mm²) in the IGL (C) were measured from CBH and control animals. All measurements were taken from four medial cerebellar sections. Error bars show 95% confidence interval for SEM. Asterisks (*) indicates significance at the 0.05 level from 2-tailed t-test.
2.3.2.3 *Inhibitory Interneurons*

The density of inhibitory interneurons located in the ML of adult cerebellum was measured to investigate the effects of CBH on cerebellar interneurons (Figure 2.4). From the female mice, we did not observe any significant difference, but from the male mice, we detected a significant reduction in interneuron density in the collagenase group (p = 0.041).

![Bar chart showing interneuron density](image)

**Figure 2.4 The effects of CBH on interneuron density.** Interneuron density (cells/mm\(^2\)) measurements were taken at adult time point and from four medial cerebellar sections collected from CBH and control animals. Error bars show the 95% confidence interval for SEM. Asterisks (*) indicates significance at the 0.05 level from 2-tailed t-test.

2.3.2.4 *Purkinje Cells and Molecular Layer*

Purkinje cells were counted from medial cerebellar sections of adult mice and the average Purkinje cell number per section was compared between CBH and control groups (Figure 2.5(A)). Purkinje cell counts obtained from both females and males showed no significant difference between collagenase and saline injected animals (p = 0.38 for males and p = 0.35 for females).

The area of the ML, which possesses Purkinje cell dendrites, was also measured to compare the size difference of ML in CBH and control animals (Figure 2.5(B)). There was a significant effect of collagenase-induced hemorrhage on the size of the ML at P15: the area of ML in CBH group was
smaller than the ML area in controls ($p = 0.013$). In the adult females and males, we did not observe any significant difference in ML area, but the trend was the same as at P15.

![Bar chart A](image)

![Bar chart B](image)

**Figure 2.5 The effects of CBH on Purkinje cells and Purkinje cell dendrites.** Purkinje cell counts (cells/section) (A) and ML area measurement from medial cerebellar sections (B) were taken from four medial cerebellar sections. Error bars show the 95% confidence interval for SEM. Asterisks (*) indicates significance at the 0.05 level from 2-tailed $t$-test.
2.3.2.5 Oligodendrocytes and White Matter

To determine whether the hemorrhagic insult causes cerebellar WM injury, we measured the area of WM in medial cerebellum (Figure 2.6(A)). The area of P15 and adult cerebellar WM, immunostained with anti-MBP, showed no difference in area between collagenase and saline injected groups.

In addition, to investigate the effects of hemorrhage on the proliferation of oligodendrocytes, we performed immunofluorescence with anti-Olig2 and anti-BrdU (Figure 2.6(B)). Our double-labeled cell counting data shows that the number of proliferating oligodendrocyte significantly increases 1 day after the hemorrhage (p = 0.024), and the level of proliferation in CBH group becomes similar in the older animals.

Figure 2.6 The effects of CBH on white matter development. The area of white matter (mm²) (A) and Olig2 BrdU positive cell counts (cells/section) (B) were taken from the control and CBH animals. Bar represents mean area of WM from 3 most medial cerebellar sagittal sections ± SEM. Asterisks (*) indicates significance at the 0.05 level from 2-tailed t-test.
2.3.3 Behavioural Evaluation

2.3.3.1 Rotarod

The assessment of motor coordination, balance and, motor learning in mice affected with neonatal CBH was done by performing the rotarod test after P60. From the four trials given on day 1, there was significant difference in performance (motor coordination and balance) between male mice with CBH and the controls (Figure 2.7(A); \( p = 0.021 \)). Also, significant performance difference was also observed from female mice on day 1 (Figure 2.7(B); \( p = 0.015 \)). Statistically significant improvement in performance over the 4 trials on day 1 was detected from both control and CBH males, suggesting that both groups have normal motor learning ability (Figure 2.7(A); \( p = 0.012 \) for control and \( p < 0.0001 \) for CBH). From the female mice, significant learning was only observed from the CBH group (\( p = 0.008 \)), since the control group showed the ceiling effect at the maximum time allowed for each trial (Figure 2.7(B)).

From the data obtained from the rotarod test repeated over 3 days, both males and females showed the performance difference: the control group significantly outperformed the CBH group (Figure 2.7(C) and (D); \( p = 0.032 \) for males and \( p = 0.02 \) for females). Also, significant performance improvement was observed from all of the groups, except for the female control mice whose performance showed the ceiling effect (\( p = 0.028 \) for male control, \( p < 0.0001 \) for male CBH, \( p = 0.18 \) for female control, and \( p = 0.006 \) for female CBH).
Figure 2.7 The effects of CBH on Rotarod performance. (A) Male mice performance for 4 trials given on day 1. (B) Female mice performance for 4 trials given on day 1. (C) Male mice performance over 3 days. (D) Female mice performance over 3 days. Values are expressed as mean ± S.E.M.
2.3.3.2  **Horizontal Ladder Rung Walking Test**

The impairments in limb coordination and placing were investigated by assessing the mouse’s ability to cross the horizontal ladder with irregularly spaced rungs. Male adult mice in CBH group showed significantly higher error rate, compared to the control animals (Figure 2.8; Control $= 0.0148 \pm 0.00792$, CBH $= 0.0479 \pm 0.01231$, $p = 0.047$). Also, female CBH mice showed higher error rate (Control $= 0.0287 \pm 0.01132$, CBH $= 0.0644 \pm 0.01392$, $p = 0.033$).

![Figure 2.8 The effects of CBH on skilled rung walking performance. Error rates from each group are expressed as mean ± S.E.M. An asterisk (*) denotes the value for which CBH group differed significantly ($P < 0.05$) from the control group.](image-url)
2.3.3.3 Open Field

To assess the general movement, activity, and anxiety, we used the open field test. The level of anxiety was measured from the duration in the center of the open field. This test showed that the adult male mice from CBH group spend more time in the center of the open field (Figure 2.9; Control = 60.87 ± 8.05s, CBH = 83.48 ± 6.19s, p = 0.035). In contrast, the female mice from CBH group did not show significant difference in time spent in the center, compared to the controls (Control = 50.16 ± 5.41s, CBH = 54.52 ± 6.99s, p = 0.638). In addition, we observed that the male mice from CBH group make more frequent attempts to jump on the wall of the apparatus. Also, we detected abnormal stereotypic behaviour, constant circling, in two male CBH mice which remained in the study. We did not observe any significant effects of CBH on other measurements, such as total distance travelled, frequency of entering center, and mean velocity (data not shown).

![Figure 2.9 The effects of CBH on duration spent in the center of the Open Field.](image)

*Measurements were taken from the adult male and female mice injected postnatally with either saline (Control) or collagenase (CBH). Values are expressed as mean ± S.E.M. An asterisk (*) denotes the value for which CBH group differed significantly (P < 0.05) from the control group.*
2.3.3.4  **Goal-directed Task**

The goal-directed behaviour of the mice with neonatal CBH was assessed with the goal-directed task. The latency to drink from the water dish measured from the male controls and CBH mice showed that CBH males spend significantly longer time to attain the goal (Figure 2.10(A); \( p = 0.039 \)). The CBH females also showed the longer latency to drink from the water dish, compared to the control mice (Figure 2.10(B); \( p = 0.003 \)).

![Figure 2.10 The effects of CBH on goal-directed behaviour test performance. Average latency to drink from the water dish was measured from the water-deprived male (A) and female (B) control and CBH mice. Values from each group are expressed as mean ± S.E.M.](image)

2.3.3.5 Morris Water Maze

The MWM test was used to assess the memory and learning in mouse affected with CBH. From the learning test, no significant difference in the average latency to find the platform between the controls and CBH males and females was observed (Figure 2.11(A) and (B)). In addition, from the short-term memory test, the time spent in the target quadrant did not differ between the CBH and control groups (Figure 2.11(C)). Also, the long-term memory test showed that the duration in the target quadrant is not different (Figure 2.11(D)).

Figure 2.11 The effects of CBH on MWM performance. (A) Average latency to find the platform for males. (B) Average latency to find the platform for females. (C) Short-term memory test measured by duration in target zone. (D) Long-term memory test measured by duration in target zone. All values are expressed as mean ± S.E.M.
2.3.4 Molecular Evaluation

2.3.4.1 Apoptosis

Activation of the apoptotic pathway in the cerebellum after the hemorrhagic insult was assessed by quantifying anti-Caspase-3-positive cells and examining the Caspase-3 transcript levels. The anti-Caspase-3-positive cell counting data collected from different time points showed that there is no significant increase in the number of Caspase-3-expressing cells in the CBH animals, compared to the controls (Figure 2.12(A)). At P2 and P5, the collagenase-injected pups showed a trend of increase in cell death, although it did not reach a statistical significance (\( p = 0.065 \) for P2 and \( p = 0.151 \) for P5).

The levels of Caspase-3 transcripts were compared between the control and CBH groups at P2 and P15. Although we did not detect a significant change in Caspase-3 level at P2, we found approximately 2 fold increase in the transcript at P15 in CBH mice (Figure 2.12(B); Control: \( RQ = 1.00, RQ \text{ Min} = 0.714, RQ \text{ Max} = 1.4 \); CBH: \( RQ = 1.814, RQ \text{ Min} = 1.617, RQ \text{ Max} = 2.033 \)).

Figure 2.12 The effects of CBH on the levels of cell apoptosis. (A) The number of Caspase-3 positive cells on cerebellar cryosections, immunostained with anti-Caspase-3. (B) the relative quantity of Caspase-3 transcript from the cerebella of the control and CBH group. Error bars show the 95% confidence interval for SEM. Asterisks (*) indicates significance at the 0.05 level from 2-tailed t-test.
2.3.4.2 Inflammation

We also compared the levels of transcripts involved in the inflammatory pathway in the cerebella collected from P2 and P15 control and CBH animals. The RT-PCR results showed that the levels of Tnf-α and Il-1β are not detectably affected by the hemorrhage at both P2 and P15 time points (Figure 2.13(A) and (B)). However, there may be a trend for both Tnf-α and Il-1β to be elevated in the CBH animals.

![Bar chart showing the relative quantity of Tnf-α and Il-1β at P2 and P15 for control and CBH groups.](image)

Figure 2.13 The effects of CBH on the inflammatory pathway. (A) Tnf-α and (B) Il-1β. Error bars show the 95% confidence interval for SEM.

2.3.4.3 Neurotrophic Factors

The changes in expression of neurotrophic factors were examined in the cerebella of collagenase-injected CBH animals and saline-injected controls. Immediately after the hemorrhage, at P2, a slight but significant increase in Igf-1 expression was detected from CBH group (Figure 2.14(A); Control: RQ = 1.00, RQ Min = 0.909, RQ Max = 1.1; CBH: RQ = 1.231, RQ Min = 1.159, RQ Max = 1.307). In contrast, other neurotrophic factors, Bdnf, Epor, and Cntf did not show any significant increase in expression at P2 (Figure 2.14(B), (C), and (D)). At P15, Igf-1 expression increase was no longer statistically significant, but Cntf showed a significant increase in expression (Figure 2.14(D); Control: RQ = 1.00, RQ Min = 0.837, RQ Max = 1.194; CBH: RQ = 1.426, RQ Min = 1.229, RQ Max = 1.656). The expression of other neurotrophic factors did not differ significantly from the control group at P15 (Figure 2.14(A), (B), and (C)).
Figure 2.14 The effects of CBH on neurotrophic factor expression. (A) Igf-1, (B) Bdnf, (C) EpoR, and (D) Cntf. Error bars show the 95% confidence interval for SEM. Asterisks (*) indicates significance at the 0.05 level from 2-tailed t-test.
2.3.4.4 Sonic Hedgehog Pathway

The transcript expression levels of the members of the sonic hedgehog signaling pathway were investigated. *Shh* expression showed approximately 1.5 fold increase immediately after the induction of hemorrhage (Figure 2.15(A); Control: RQ = 1.00, RQ Min = 0.896, RQ Max = 1.116; CBH: RQ = 1.524, RQ Min = 1.382, RQ Max = 1.68). This increase in *Shh* was not persistently significant in the P15 CBH animals (Figure 2.15(A)). The mediators of the sonic hedgehog pathway, *Gli1* and *Gli2*, were found to be increased in their expression at P2 (Figure 2.15(B) and (C); *Gli1* Control: RQ = 1.00, RQ Min = 0.926, RQ Max = 1.08; *Gli1* CBH: RQ = 1.218, RQ Min = 1.144, RQ Max = 1.297; *Gli2* Control: RQ = 1.00, RQ Min = 0.915, RQ Max = 1.092; *Gli2* CBH: RQ = 1.221, RQ Min = 1.101, RQ Max = 1.354). The magnitude of expression increase for *Gli1* and *Gli2* at P15, compared to the control, was greater than that of P2 (Figure 2.15(B) and (C); *Gli1* Control: RQ = 1.00, RQ Min = 0.861, RQ Max = 1.161; *Gli1* CBH: RQ = 1.449, RQ Min = 1.303, RQ Max = 1.611; *Gli2* Control: RQ = 1.00, RQ Min = 0.856, RQ Max = 1.169; *Gli2* CBH: RQ = 1.658, RQ Min = 1.461, RQ Max = 1.882). The *Nmyc*, the downstream effector of the sonic hedgehog pathway, showed a slight increase in the expression at P2 (Figure 2.15(D); Control: RQ = 1.00, RQ Min = 0.913, RQ Max = 1.095; CBH: RQ = 1.289, RQ Min = 1.203, RQ Max = 1.381). The expression of *Nmyc* in P15 CBH cerebella was approximately 3.5 fold higher than the expression in the control group (Figure 2.15(D); Control: RQ = 1.00, RQ Min = 0.587, RQ Max = 1.703; CBH: RQ = 3.419, RQ Min = 3.029, RQ Max = 3.86).
Figure 2.15 The effects of CBH on the sonic hedgehog pathway. (A) Shh, (B) Gli1, (C) Gli2, and (D) Nmyc. Error bars show the 95% confidence interval for SEM. Asterisks (*) indicates significance at the 0.05 level from 2-tailed t-test.
2.4 Discussion

2.4.1 Overview

CBH is a serious neurological problem in the preterm infant population. The hemorrhagic injury to the rapidly developing preterm cerebellum may lead to devastating short-term and long-term sequela, such as infant mortality and adverse neurodevelopmental outcomes (Volpe 2008). Despite the increased recognition as a severe preterm brain injury that shows strong association with various adverse outcomes, pathophysiology of CBH has never been adequately explored. This is probably due to the limitations in clinical studies, as well as a lack of preclinical animal models. Therefore, in the present study, we developed a mouse model for preterm CBH and investigated the effects of hemorrhage on the development of the cerebellum, motor, cognitive and affective behaviour, and molecular pathways for injury and protection.

The mouse pups injected with collagenase developed CBH, as shown by hematoma in the fourth ventricle and the cerebellum. The anatomical evaluation of the cerebellum collected at various postnatal ages and the adult stage revealed that the hemorrhage leads to a decrease in size of the cerebellum during postnatal growth, as well as a reduction in granule cell and interneuron density in adults. These anatomical abnormalities seem to correlate well with various behavioural deficits detected in our behavioural evaluation performed on the adult animals. The collagenase-induced CBH in the neonatal mouse pups resulted in long-term behavioural abnormalities, including motor deficit and abnormal affective behaviour, such as reduction in anxiety and attention. From molecular evaluation, we found a significant increase in Caspase-3 expression, which suggests an increase in apoptosis. Here, we also report that CBH results in the up-regulation of the expression of neurotrophic factors and the members of the sonic hedgehog pathway, which may act as endogenous protective mechanisms by promoting cell proliferation after the hemorrhage. Overall, the present study enhances our understanding on the pathophysiology of CBH in the preterm infants, and also this novel animal model can potentially be employed to evaluate post-hemorrhagic therapies and neuroprotective interventions.

2.4.2 Evaluation of the Cerebellar Hemorrhage Mouse Model

To model CBH in mouse pups, we injected bacterial collagenase (0.057U/μl for ICR strain and 0.045U/μl for B6) into the cerebral aqueduct, through which CSF flows into the fourth ventricle. Within 30 minutes after collagenase infusion, animals began to develop hematoma in the fourth ventricle and on the surface the cerebellum. The location of hematoma observed in our
model closely resembles CBH in preterm infants. The bleeding detected from the cerebellar germinal matrices (cerebellar surface) mimics primary CBH (Volpe 2008). Also, the fourth ventricular blood, accumulated from the rupture of blood vessels in the posterior fossa, including the brainstem and cerebellum, may replicate the type of CBH which result from the extension of ventricular hemorrhage into the cerebellum (Volpe 2008). Therefore, CBH induced by collagenase injection in our experimental model shows similarity to CBH in premature infants.

Previous studies have demonstrated that the bacterial collagenase administration is an effective method of hemorrhage induction in animal models of hemorrhagic brain injury (discussed in more detail in Section 1.5.2). Collagenase disrupts the basal lamina of blood vessels at the site of injection, leading to spontaneous blood leakage into the surrounding brain tissue (Rosenberg et al 1993; Rosenberg et al 1990a). The collagenase administration also reproduces gradual formation of hematoma and bleeding-rebleeding phenomenon, which are frequently observed in human patients (Brott et al 1997). Therefore, in this study, the collagenase-injection paradigm was selected as an experimental method to induce hemorrhage in the neonatal mouse cerebellum.

2.4.3 Anatomical Evaluation

We investigated the effects of CBH on the development of mouse postnatal cerebellum, by examining changes in gross and microscopic anatomy of the cerebellum after the injury. Since mouse cerebellum undergoes very rapid development during early postnatal period, which is comparable to human preterm period, hemorrhagic injury to the cerebellum may disturb crucial developmental processes. In our study, by measuring the size of the developing cerebellum, we found significant early postnatal cerebellar growth deficiency in the animals with the hemorrhage. At P7 and P15, the size of the collagenase-treated mouse cerebella was found to be significantly reduced, compared to the saline-treated control mice cerebella. The adult females and males also showed a trend of reduction in the cerebellar size. Although the data collected from adult animals did not reach a statistical significance, the trend of cerebellar area reduction is shown in both males and females suggests that increasing the sample size to the number of animals used for P7 and P15 analyses may allow us to detect significant difference in adults. Our experimental data from the animal model seems to be in accordance with previous clinical studies; cerebellar atrophy has been reported to be detectable from 37% of infants 2 months after CBH (Volpe 2009). In addition to direct hemorrhage, blood deposition in the fourth ventricle and on cerebellar surface has also been shown to cause cerebellar underdevelopment (Messerschmidt et al 2008).
The significant association between the blood breakdown products and hemosiderin deposition and cerebellar atrophy has been noted in multiple studies (Janss et al 1993; Koeppen et al 2008; Levy et al 2007; Messerschmidt et al 2005). In particular, developing cerebellum appears to be especially vulnerable to toxic effects of blood products and hemosiderin (Steggerda et al 2009). Previous studies have shown growth impairment from immature cerebellum exposed to blood products and hemosiderin deposition, which may have been produced from direct CBH or supratentorial brain hemorrhage. A brain imaging study conducted by Tam et al (2011b) revealed that preterm infants with supratentorial IVH show reduced cerebellar volume, significantly associated with the severity of IVH. Moreover, postmortem examination of late preterm infants' brain with IVH and post-hemorrhagic hydrocephalus also revealed that the presence of blood products on the cerebellum surface is linked to anatomical abnormalities, affecting the overall growth of the cerebellum (Fukumizu et al 1995). In addition to clinical findings, multiple studies with rodent models provide further evidence on toxic effects of blood products (Hua et al 2006; Yan et al 2008; Zhang et al 2012).

Among various cell types in the developing cerebellum, the granule cell precursors may be the key target for the adverse effects of blood from CBH. The EGL, the most prominent germinal matrix in the developing cerebellum is richly vascularized and it capillaries are very vulnerable to rupture during the preterm period (Volpe 2008). Furthermore, in CBH cases that originate from the extension of intraventricular or subarachnoid hemorrhage, blood deposition is likely to occur on the superficial layer of the cerebellum, the EGL. Therefore, the highly proliferative granule cell precursors in the EGL are the primary target for toxic effects of blood products. In our study, by measuring the area of the EGL at different time points, we found that hemorrhage affects the normal development of the EGL. Transient swelling can account for the observed increase in the area of EGL at P2. Then, at P7, we detected a subsequent size reduction of EGL as a result of CBH. Although this size reduction detected from the EGL was not persistently found in the IGL of the CBH group, we detected a significant decrease in granule cell density in the male CBH animals, compared to the controls. Therefore, our data suggests that the blood deposition resulting from the hemorrhage may have a significant impact on proliferating granule cells located in the EGL, leading to a subsequent reduction of granule cells in the IGL.

In many CBH cases, blood deposition also occurs in the walls of the fourth ventricle, where ventricular neuroepithelium is actively generating GABAergic interneurons (Donkelaar et al 2006). The proliferation of inhibitory interneurons, such as basket and stellate cells, reaches its peak during the first postnatal week in mice and 19 weeks gestation until birth in human (Donkelaar et
al 2006; Weisheit et al 2006). The permanent reduction in the interneuron density observed from our hemorrhage model suggests that the blood deposition disrupts the normal development of not only granule cells, but also cerebellar interneurons that arise from the ventricular neuroepithelium. In addition to the direct effects of blood products, granule cell loss may have an indirect influence on the density of interneurons. Transneuronal degeneration, the death of neurons due to the disruption of input or output of other neurons, is one of the likely mechanisms by which granule cell reduction causes adverse effects on the development of other cell types in the cerebellar circuitry (Fukumizu et al 1995; Uchino et al 1993; Uchino et al 2006). Cerebellar inhibitory interneurons receive excitatory inputs from granule cells via parallel fibers and it has been suggested that the loss of parallel fibers results in anterograde transneuronal degeneration (Anderson & Flumerfelt 1986).

Purkinje cells are another major cell types that receive excitatory input from granule cells through parallel fibers. The normal development of Purkinje cell is known to be dependent on the input from granule cells and failure to establish a proper connection between granule cells and Purkinje cells may result in death of Purkinje cells. Previous studies with weaver mutant mouse, which possesses autosomal mutation leading to the death of granule neurons during the first 2 postnatal weeks, have revealed that this granule cell loss results in abnormal Purkinje cell dendrite arborization and orientation, as well as deficits in Purkinje cell number (Maricich et al 1997; Rakic & Sidman 1973a; b; Smeyne & Goldowitz 1989). In the current study, we did not observe any significant difference in the number of Purkinje cells in CBH animals, compared to the controls, despite the reduction in the size of EGL and the density of granule cells. Since the degree of granule cell loss is much smaller in the CBH model, compared to the mutant, it is likely that the degree of granule cell reduction in CBH animals was not sufficient to affect Purkinje cells number. However, we cannot exclude the possibility that granule cell loss may have impacted proper development and establishment of axonal and dendritic connections. The reduction in the size of the ML, which is primarily comprised of Purkinje cell dendrites, parallel fibers, and inhibitory interneurons, has been considered to be a reasonable indication for the decrease in the number of synapses per neuron (Anderson 1994; Greenough & Anderson 1991). Although further investigation is necessary, the decrease in the ML area at P15, observed in the CBH group, may indicate aberrant development of synaptic connections between Purkinje cells and parallel fibers from granule neurons.

In the current study, we did not detect any significant changes in the WM in the adult animals in the CBH group. This finding contrasts with the MRI data collected from preterm CBH
patients, which showed a significant reduction in WM volume (Limperopoulos et al. 2005). Furthermore, cerebellar WM volume reduction at term gestational age equivalent was also reported in the infants with supratentorial diffuse PVL and hemorrhagic infarction, even in the absence of direct cerebellar hemorrhagic injury (Limperopoulos et al. 2005). The discrepancy between our animal data and the human reports may be explained by the difference in age at which WM size was measured. Also, as our anti-BrdU and anti-Olig2 double labeling experiment suggests, CBH leads to an immediate increase in oligodendrocyte proliferation, which may act as a compensatory mechanism for the damage on the WM after the hemorrhage. Currently, the long-term effects of direct CBH or supratentorial brain lesion on the WM remain unclear in preterm infants. Therefore, further clinical and animal studies need to be conducted to determine the effects of CBH on the development of the WM.

2.4.4 Behavioural Evaluation

The clinical follow-up of premature infants with CBH has shown a significant association of the injury with various neurobehavioural abnormalities, including motor, social, behavioural, and cognitive deficits (Limperopoulos et al. 2007; Limperopoulos et al. 2005). Therefore, in the present study, we used a series of behavioural tests to assess the long-term neurobehavioural consequences in the mice affected by neonatal CBH. Specifically, we examined motor performance and cognitive and affective behaviour to investigate the effects of CBH on the function of the cerebellum and other brain areas that are connected with the cerebellum.

2.4.4.1 Motor Control and Motor Learning

The effects of CBH on motor functions were examined in the current study from rotarod (duration on the accelerating bar) and the horizontal ladder rung walking test (error rate during walking performance). From rotarod test, which assesses gross motor coordination and balance, we found a significant reduction in performance in animals affected with CBH. In addition, the horizontal ladder rung test, which was used to detect fine motor deficits in forelimb and hindlimb walking, showed that CBH-affected animals exhibit a significantly higher error rate, compared to the control mice. Taken together, these results indicate that the neonatal cerebellar injury leads to gross and fine motor impairments. Since the cerebellum has long been known to play a major role in motor control, many previous studies of cerebellar injury also focused on motor outcomes. A clinical study conducted by Limperopoulos and colleagues (2007) has noted that severe motor deficits, affecting both gross and fine motor skills, were present in approximately 50% of infants.
with isolated CBH. Also, cerebellar injury in premature infants has been shown to be significantly associated with higher rates of cerebral palsy (Bodensteiner & Johnsen 2005; Johnsen et al 2005). More recently, Zayak and colleagues (2011) have reported that the infants with CBH in the medial (vermian) part of the preterm cerebellum are at a greater risk for motor disabilities and cerebral palsy, while lateral hemispheric CBH does not show a significant association with motor impairments. Therefore, the reduction in the size of medial cerebellum and motor disabilities that we observed from our CBH model closely parallel the effects of CBH on the development of the cerebellum and functional outcomes in humans. Furthermore, studies with animal models of various types of cerebellar lesions, whether of genetic origin or not, have shown that the cerebellar abnormalities induce motor deficiencies. For example, mutant mouse strains, such as *lurcher* and *weaver*, which have Purkinje cell loss and granule cell depletion, respectively, display deficits in motor coordination, shown by various motor tests, including the rotarod test (Lalonde & Strazielle 2007). Also, x-irradiation at the age between P10-14, that causes a partial loss of cerebellar granule cells, leads to motor disabilities in rats (Le Marec et al 1997b). Thus, CBH-induced cerebellar anatomical abnormalities, such as granule cell density reduction and potential aberrant Purkinje cell synaptic connections, suggested from decreased ML area, may contribute to the long-term deficits in motor control.

Despite the motor impairments seen in the CBH group of animals, we did not detect motor learning deficits in the mice with the neonatal cerebellar lesion. From the rotarod test, we found that there was significant improvement in performance by both the CBH and control mice on the first day of training and the subsequent three days of assessment. The past clinical and animal cerebellar lesion studies have shown contrasting reports on the effects of cerebellar damage on motor learning capabilities. Studies have generally indicated that the cerebellum plays a crucial role in motor learning and that cerebellar abnormalities result in motor learning impairments (Bracha & Shigemi Mori 2004; Himamizu 2000; Ito 2000; Maschke et al 2004). Yet, others argued that the observed deficits in motor learning are likely to be a consequence of motor performance deficit rather than the impairment in motor learning (Dennis et al 2006; Seidler et al 2002). Some of the possible explanations for the discrepancy between studies could be the differences in the size of cerebellar damage and the presence of additional extracerebellar lesions. Indeed, the x-irradiation study revealed that the motor learning functionality may depend on the degree of granule cell loss, by showing the deficits in motor learning with more severe granule cell depletion (Le Marec et al 1997b). Furthermore, cerebellar lesions, such as hemorrhage and tumor, frequently affect other parts of the brain either directly through co-occurrence of cerebral hemorrhage and tumor invasion.
or indirectly through hydrocephalus and increased intracranial pressure. These extracerebellar lesions, which are often overlooked in many studies, may interfere with motor learning. Thus, it is possible that the preserved motor learning skill in our CBH mice may be due to relatively smaller lesion size in the cerebellum or a lack of extracerebellar lesions.

2.4.4.2 Cognitive and Affective Behaviour

Recently, there has been increasing recognition of the contribution of the cerebellum in non-motor functions, such as anxiety, attention, memory, and verbal learning. In this study, to investigate the long-term effects of neonatal CBH on non-motor functions, we examined the behaviour of CBH-affected mice by using behavioural paradigms that assess affective and cognitive performance. Specifically, affective problems, such as anxiety-like behaviour and attention deficit, were evaluated by the open field test (duration in the center) and the goal-directed task (latency to drink from water dish), respectively. For the assessment of cognitive function, we used the MWM for testing short-term and long-term spatial learning and memory. We report here that the male mice with neonatal CBH engage in riskier behaviour, with more time exploring in the center area of the open field, compared to the control group. In addition, from the open field test, other abnormal behavioural phenotypes, such as more frequent attempts to jump on the wall of the open field apparatus and stereotypic circling behaviour, were detected from a subset of male CBH mice. The goal-directed task revealed that CBH group exhibits significantly higher latency to locate and drink from the water dish to relieve thirst, suggesting attention deficit and motivational impairment. However, from the MWM task, we did not detect any difference between CBH and control group and this result suggests that spatial learning and memory are preserved in CBH mice. Therefore, our data indicates that the CBH model exhibits affective processing deficit, while cognitive dysfunction is not found.

The abnormal behavioural phenotypes detected from CBH mice, such as risk-taking behaviour, attention deficit, and stereotypy are in accordance with clinical research findings, which showed that children with isolated CBH are much more likely to demonstrate such affective problems (Limperopoulos et al 2007). These overlapping behavioural features found in both human patients and our CBH model are very similar to some of the major behavioural symptoms of attention deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD) patients. Interestingly, both of neurodevelopmental disorders, ADHD and ASD, have been associated with abnormalities in developing cerebellum (Allen & Courchesne 2003; Berquin et al 1998; Fatemi et al 2012). The incidence rates of these disorders have been shown to be increased in infants with
cerebellar lesions, as well as in preterm infants who have higher susceptibility to various cerebellar developmental abnormalities (Hart et al 2008; Limperopoulos et al 2007; Limperopoulos et al 2008; Parker et al 2008). Furthermore, studies with adult patients with cerebellar damage corroborate the cerebellum’s contribution in affective behaviour. Attentional deficits, reduced motivation, personality changes, and disinhibited and inappropriate behaviour have been found to be present in adult patients following a traumatic injury in the cerebellum (Akshoomoff & Courchesne 1994; Schmahmann & Sherman 1998).

Atypical histological features and gross morphology of the cerebellum have been suggested to be associated with abnormal affective behaviour. For example, Purkinje cell abnormalities, such as reduction in number, have been consistently found in postmortem ASD studies (Bailey et al 1998; Lee et al 2002). Animal studies have revealed that Purkinje cell number is inversely related to the levels of stereotyped repetitive behaviour and hyperactivity, the behavioural features commonly seen in ASD patients (Martin et al 2010). Additionally, granule cell reduction has also been reported from postmortem brain of ASD patients (Bauman & Kemper 1994; Vargas et al 2005). Cerebellar histological abnormality has not yet been reported for ADHD; however, the partial loss of granule cells by x-irradiating mouse cerebellum has been shown to result in similar behavioural symptoms, such as a reduction in anxiety and attention and an increase in risk-taking behaviour (Le Marec et al 1997b). In addition to histological abnormalities, cerebellar structural abnormalities, found in neuroimaging studies, provided evidence for its association with abnormal affective behavioural phenotypes. In particular, an abnormality in the vermis has been consistently found in patients with affective problems. The reduction in the volume of cerebellar posterior vermis, particularly in the lobules VIII-X, has been reported in multiple imaging studies for ASD and ADHD (Berquin et al 1998; Ciesielski et al 1997; Courchesne et al 1988; Mackie et al 2007; Scott et al 2009). Also, a vermian lesion in adult cerebellar damage patients was found to be particularly important in the generation of affective problems (Schmahmann & Sherman 1998). Our anatomical evaluation which focused vermian cerebellum also showed a reduction in size in the CBH group. Therefore, our results provide further evidence for the link between vermian abnormality and affective behavioural problems.

It is generally accepted that the involvement of the cerebellum in non-motor functions is primarily through its connections with other parts of the brain, which play major role in affective and cognitive processing (Ito 2008; Ramnani 2006; Schmahmann & Sherman 1998). A recent clinical study, conducted by Limperopoulos and colleagues (2005), has demonstrated that the failure to establish proper neural connection in the injured preterm cerebellum leads to impaired
growth in specific regions of the uninjured contralateral cerebral cortex, influencing its function in higher order affective and cognitive processing. This remote trans-synaptic effect between the cerebellum and cerebrum is a form of diaschisis, a term used to describe a loss of function in a brain region connected to a distant damaged brain area (Von Monakow 1969). Therefore, the deficits in affective behaviour in our animal model and the patients with cerebellar damage are likely to arise from the dysfunctional neural connections between the cerebellum and cerebral cortex. In support for this hypothesis, the medial cerebellum which displayed anatomical abnormalities in our CBH animal model and patients with neurodevelopmental disorders, is known to be connected to limbic cortex, which modulates affective processing (Stoodley & Schmahmann 2010). The cerebellar vermin regions, particularly posterior vermis, is also known as “limbic cerebellum” (Konczak & Timmann 2007). Thus, the abnormalities in vermis may cause the interruption of the neural communication between the vermis and the limbic system, resulting in affective disturbances in the individuals with the CBH (Stoodley & Schmahmann 2010).

In our study, while the CBH mice presented deficits in affective behaviour, cognitive function in these mice seemed to be normal. Previous clinical studies have shown that adverse cognitive outcomes after the CBH may depend on the size of the lesion, as well as the topography of cerebellar damage (Limperopoulos et al 2009). Tam and colleagues (2011c) have shown that small cerebellar hemorrhagic lesions in preterm infants are associated with neurologic abnormalities, but not with cognitive deficits. Furthermore, cognitive deficits in cerebellar injury have been frequently associated with the lesions in the lateral cerebellar hemispheres, particularly hemispheric lobule VI and VII, the region also known as “cognitive cerebellum” (Stoodley & Schmahmann 2010). In preterm infants, lateral hemispheric lesion has been shown to be significantly associated with mental dysfunction (Zayek et al 2011). Therefore, it is possible that the negative findings for cognitive deficits in CBH mice may be accounted for by the size of the lesion and the location of the injury that occurs in our animal model. Further anatomical study would help understand sparing of cognitive function in our model.

2.4.5 **Sex Differences in Anatomical and Behavioural Outcomes**

The results obtained from our experiments suggest that males are more susceptible to the adverse effects of CBH. More specifically, from our anatomical evaluation, only males exhibited a significant reduction in granule cell and interneuron density. Also, from behavioural evaluation, the reduction in anxiety was only observed from the male animals affected with CBH. In general, it has been known that males have a higher incidence and poorer outcomes than females of many
neurodevelopmental deficits, including cerebral palsy, mental disabilities, ASD and ADHD. Likewise, in preterm infant population, several previous studies have indicated that the incidence and the outcome of preterm-associated brain injury, including CBH, are largely influenced by male gender (Kent et al 2011; Limperopoulos et al 2005; Limperopoulos et al 2012; Peacock et al 2012; van Kooij et al 2011). Animal studies have also documented that the equivalent lesion in both sexes results in greater damage and more adverse functional outcomes in male animals (Lauterbach et al 2001).

There are several potential mechanisms that may explain sex differences in response to preterm CBH. First, the sex discrepancy may be modulated by the effects of sex-specific hormones, such as testosterone and estrogen. In many brain injury studies with adult animal models, it has been shown that the presence of testosterone exacerbate the damage (Cheng et al 2008; Yang et al 2002). Therefore, the elevated levels of testosterone during gestation through the first year in human and perinatal period in rodents appear to be associated with enhanced neuronal excitotoxicity and increased long-term adverse outcomes in males (Hines 2008). In contrast, estrogen has been suggested to have protective effects on adult stroke models (McCullough & Hurn 2003). Although only minimal estrogen is circulating during perinatal period, it may have beneficial effects on recovery during the late brain development in females. Second, sex differences in preferred mechanisms of apoptosis may influence the outcomes following the injury. Previous studies have shown that females favor caspase-dependent apoptotic pathway, while males rely more on caspase-independent pathway after the brain injury (Lang & McCullough 2008). Third, previous data has shown that females may have a gene-linked advantage through the endogenous expression of inhibitors of apoptosis (IAP). Due to incomplete X inactivation, females express higher levels of X-linked IAP (XIAP), which halts the caspase-dependent apoptotic pathway. Since the caspase-dependent cell death pathway is a favored mechanism in females, the higher expression of XIAP may give selective protection for females following the injury (Askalan et al 2009; Hill & Fitch 2012).

2.4.6 Molecular Evaluation

2.4.6.1 Potential Mechanisms of Injury

In the current study, we aimed to understand the mechanisms underlying CBH-induced cerebellar abnormalities we found from our anatomical and behavioural evaluation. We first investigated whether apoptosis contributes to the cerebellar injury following the hemorrhage. The expression levels of Caspase-3 protein, as well as Caspase-3 transcript, were compared between the
control and the CBH animals at various developmental time points. Our Caspase-3-expressing cell counting data revealed that CBH does not lead to a detectable increase in apoptosis. Also, the transcript level of Caspase-3 measured 6 hour after the hemorrhage was not significantly different from saline-injected controls. However, at P15, we detected approximately two-fold increase in Caspase-3 transcript level in CBH group. In adult stroke models, apoptosis has been extensively studied and has been speculated to mediate some of the injury in brain (Cahill et al 2006; Matsushita et al 2000; Xue & Del Bigio 2000). In contrary to adult models, there is only a limited number of past studies which investigated the activation of apoptotic pathway in neonatal or preterm stroke models. Therefore, this study reveals a potential mechanism of injury and neuronal loss specific to the hemorrhage in the developing cerebellum. The lack of significant increase in Caspase-3-positive cells from 6 hours after hemorrhage to P15 suggests apoptotic pathway may not be activated as a direct response to the hemorrhage. Instead, as suggested by the significant increase in Caspase-3 transcript level at P15, the activation of apoptotic pathway may occur after P15 as a response to abnormal development of cerebellar synaptic connections. As evidenced from mutant mouse strains, such as weaver and lurcher, transneuronal degeneration in CBH animals may have been mediated by apoptotic cell death (Wullner et al 1995). Thus, our data suggests that the apoptosis may have a role in neonatal CBH-induced injury as a secondary mechanism dependent upon the initial cell loss.

Increasing evidence suggests that inflammatory responses are involved in the progression of hemorrhage-induced brain injury (Aronowski & Hall 2005; Xi et al 2006; Xue & Del Bigio 2000). High levels of various mediators of inflammation, such as TNFα, IL-1β, nitric oxide synthase, and intracellular adhesion molecule-1, have been detected in clinical and preclinical animal studies for ICH (Brunswick et al 2012; Castillo et al 2002; Lu et al 2005). However, in contrast to these previous studies, our animal model for preterm CBH did not show a significant increase in the expression of Tnfα and Il-1β transcripts at P2 and P15 time points. If real, this discrepancy can be explained by the difference between adult cortical hemorrhagic injury and neonatal CBH; it is also likely that the extent of cerebellar damage in our animal model may be insufficient to induce inflammatory response. However, relatively large SEMs and tendency for the observed means to be higher in the CBH samples suggest that our study may have been underpowered to detect the difference. Therefore, increasing the sample size or the use of alternative experimental approaches, such as quantitative analysis of leukocyte infiltration and microglia activation with tissues from denser time series, may help to determine the involvement of inflammation in CBH model.
Potential Mechanisms of Neuroprotection

When ICH occurs, not only cascades of brain damage are induced, but also endogenous protective mechanisms are activated. The expression levels of transcripts for many different proteins are known to be altered in response to experimental hemorrhagic induction and some of these changes may be important for protective pathways (Lu et al 2005). In our study, we examined the transcript expression level changes of four neurotrophic factors: Bdnf, Igf-1, EpoR and Cntf. These neurotrophic factors are known to play important roles in cell survival, growth and differentiation and have been shown in both human and animal studies to be up-regulated to mediate a protective response following various forms of brain injury (Guan et al 2003; Hicks et al 1999; Lin et al 1998; Siren et al 2001; Varela-Nieto et al 2005). We report here that neonatal CBH leads to the increase in expression of some of these neurotrophic factors. Our data detected that collagenase-treated animals show 1.2-fold increase in Igf-1 expression at P2 and 1.5-fold increase in Cntf at P15.

In the current study, we found that collagenase-treated CBH animals show slight increase in Igf-1 expression 6 hours after the induction of hemorrhage. Upregulation of IGF-1 has been noted in a number of studies, including both neonatal and adult models of brain injury research (Beilharz et al 1998; Lee et al 1992; Yamaguchi et al 1991). In the neonatal rats, the timing of IGF-1 induction is consistent with our finding which shows the significant increase 6 hours after the injury (Bergstedt & Wieloch 1993). Also, previous studies have shown that the expression peaks 5 days after the injury and gradually decreases by 10 days in infant rats (Guan et al 2003). This upregulation of IGF-1, a neurotrophic factor, has been suggested to play an essential role in promoting neuronal and glial proliferation, differentiation and maturation, neurite outgrowth, and myelination of oligodendrocytes after the injury (Guan et al 2003). Furthermore, IGF-1 possesses anti-apoptotic properties, which may prevent neuronal and glial cell death and improve the outcome of brain injury (Yin et al 1994; Zheng et al 2002). In mouse postnatal cerebellum, transgenically induced IGF-1 overexpression has been shown to stimulate granule cell precursor proliferation and anti-apoptotic activity (Chrysis et al 2001; Ye et al 1996). Therefore, in our animal model, upregulation of Igf-1 may have exerted anti-apoptotic activities and enhanced the survival and proliferation of granule neurons after the cerebellar injury to compensate for the initial cell loss and potential reduction of cell proliferation.

In addition to upregulation of Igf-1, we also observed a significant increase in the expression of Cntf, another neurotrophic factor that has been shown in previous studies to be upregulated after brain injury (Adler 1993; Ip et al 1993; Lee et al 1997; Oyesiku et al 1999).
increase in expression of Cntf mRNA has been noted to occur in a gradual and progressive manner. The level of Cntf expression peaks by 3 days after injury and is sustained for up to 20 days in adult rats with cortical contusion (Oyesiku et al 1999). This is consistent with our finding which indicated the delayed upregulation of Cntf expression after the CBH. Although the functional role and mechanisms of action for CNTF have not yet been clearly defined, many studies have proposed that CNTF plays an important role as a neuroprotective agent in various models of acute neuronal death (Sendtner et al 1994). Recent reports suggested that the neuroprotective effects of CNTF are mediated by astrocytes by reducing glutamate excitotoxicity (Beurrier et al 2009; Escartin et al 2006). In addition, CNTF upregulation has been shown to promote astrogliosis, which is known to play a protective role in ischemic stroke (Escartin et al 2007; Li et al 2007). As suggested from a rat lens injury model, CNTF expression from astrocytes may activate the axon regeneration upon brain injury (Muler et al 2007). Thus, CNTF upregulation, detected from the CBH animals, may result in protection or regeneration of cerebellar neurons and injured axons.

In this study, we also investigated the expression level changes of genes involved in the sonic hedgehog signaling pathway. The neuroprotective role of the sonic hedgehog pathway activation has been described in previous research with models of various forms of CNS injury (Amankulor et al 2009; Bambakidis et al 2003). In our study, we found significant upregulation of the sonic hedgehog pathway components, including Shh, Gli1, Gli2, and Nmyc, 6 hours after the induction of CBH. At P15, Shh expression returned to the similar level to the control group, while the expression levels of downstream targets, Gli1, Gli2, and Nmyc, seemed to be amplified. The upregulation of the sonic hedgehog pathway components likely represents a compensatory mechanism that may attenuate the adverse effects of hemorrhage in the rapidly developing cerebellum.

Several mechanisms may underlie the increased levels of the sonic hedgehog signaling in the animals with CBH. First, the sonic hedgehog signaling may have been increased in response to the loss of granule neurons or reduction of granule cell proliferation caused by the hemorrhagic injury. In this case, Purkinje cells are the likely source of SHH as in normal cerebellum and the cell type that increases expression of SHH targets would be proliferating granule neurons in the EGL. The upregulated Nmyc expression supports that the increase in the sonic hedgehog signaling is induced to promote proliferation of granule cells in our CBH model, since granule cell neuroblasts require Nmyc for the cell cycle progression (Kenney et al 2003; Knoepfler et al 2002). Second, the sonic hedgehog signaling may have been up-regulated to promote the proliferation of oligodendroglial cells. Previous studies have shown that SHH stimulates oligodendrocyte precursor
cell proliferation in the cerebellum (Bouslama-Oueghlani et al 2012; Loulier et al 2006). As shown from our double-labeling experiment with anti-BrdU and anti-Olig2, the number of proliferating oligodendrocytes showed a significant increase 1 day after the hemorrhage in the CBH group. This up-regulation of oligodendrocyte proliferation may act as an endogenous protective mechanism for WM injury in the animals with the hemorrhage.
3. General Discussion

3.1 Research Conclusions

The work presented in this thesis examines the effects of CBH on the development of the cerebellum. The mouse model for preterm CBH, developed in our study is the first animal model for preterm-associated cerebellar hemorrhagic injury. Using this animal model, we specifically investigated (1) the anatomical changes in the developing cerebellum, (2) the behavioural outcomes, and (3) the activation of cellular injury and protective mechanisms, following the hemorrhage induced during the neonatal period. Our findings indicate that CBH induces anatomical abnormalities of the cerebellum, motor and affective dysfunctions, and the activation of various molecular pathways leading to or preventing further damage.

In the first part of our study, we evaluated gross and microscopic anatomy of the developing and adult cerebellum. In the postnatal cerebellum, we found a size reduction, which may suggest developmental delay or an ongoing size reduction. In the adult cerebellum, a significant reduction in the density of granule cells and interneurons was found. Our findings support clinical studies which showed cerebellar atrophy in preterm CBH patients (Volpe 2009) and suggest that the cell types that are most severely affected in humans may be the granule cells and the interneurons.

In the second part of the study, we hypothesized that these anatomical abnormalities may lead to deficits in motor, cognitive, and affective functions of the cerebellum. Indeed, the behavioural paradigms that we used revealed that the animals with perinatal CBH exhibit abnormalities in motor, anxiety-like behaviour, and attention. These data collected from our animal model closely resemble the long-term adverse neurodevelopmental outcomes displayed in preterm CBH patients. Furthermore, our data that suggest sex difference in behavioural outcomes supports previous clinical studies which showed greater prevalence and more severe deficits in male gender (Limperopoulos et al 2012). Unlike motor and affective functions, cognitive processing seemed to be preserved in our CBH model. This may be due to the preservation of connectivity between the cerebellar lateral hemispheres and cortical cognitive processing centers from the damages caused by CBH. However, more extensive behavioural testing would be necessary to reveal the effects of CBH on cognitive function.

In our third study, our aim was to investigate the molecular mechanisms for injury or neuroprotection. We hypothesized that the genes involved in caspase-dependent apoptosis and
inflammatory pathway would increase in transcription. However, our data did not detect a significant increase in the expression of genes for the inflammatory pathway, suggesting that inflammation may not play a significant role as an injury mechanism. The upregulation of Caspase-3 detected only at P15 time point may indicate that apoptosis may act as a secondary injury mechanism following the immediate injury from the exposure to blood products. Furthermore, we report here that neurotrophic factors and genes involved in the sonic hedgehog pathway are significantly upregulated. The neurotrophic factors and sonic hedgehog genes may play a crucial role in compensating for the loss of cells after the damage.

Overall, the results indicate that CBH in mouse pups at the age comparable to the preterm in human leads to macroscopic and microscopic structural changes to the developing cerebellum, motor and affective dysfunctions, and the activation of injury and protective mechanisms. In this regard, the anatomical and behavioural abnormalities characterized in our preclinical mouse model for CBH closely mimic the anatomical features and behavioural outcomes that have been reported in clinical studies. Therefore, this new mouse model can serve as an effective animal model system to address many questions regarding the prevention and treatment of preterm CBH.

3.2 Strengths and Weaknesses of the Study

3.2.1 Strengths of the Study and Significance to the Research Field

This is the first study to investigate the effects of preterm associated CBH on the development of the cerebellum in an animal model. Despite the growing recognition of preterm CBH, detailed pathophysiological investigations have not been possible due to various limitations in clinical studies. Furthermore, unlike other major preterm-associated brain abnormalities (e.g. IVH and PVL), CBH has never been modeled in an animal. In this regard, the current study presents the first animal model for CBH with an extensive assessment of anatomical, behavioural, and molecular phenotypes. The data collected in our study does not only prove the animal model’s relevance to the disease in human patients but also bridges the gaps in the current understanding of the disease.

The mouse model for CBH used in our study closely reiterates the human condition and provides insights into the molecular and cellular mechanisms underlying the disease. In present study, the use of collagenase-injection method successfully reproduced spontaneous bleeding in the fourth ventricle and the cerebellar surface which is composed of rapidly proliferating granule cells. Also, the time point at which we induced the hemorrhage was comparable to the gestational age in
humans that CBH occurs most frequently in premature infants. The anatomical and behavioural phenotypes of the mice resembled the cerebellar morphological abnormalities and long-term neurodevelopmental outcomes previously reported in the CBH patients. Moreover, our data shows that males and females respond differently to the preterm CBH and supports previously established sex differences in neurodevelopmental outcomes of brain damage in preterm infants. While brain imaging or human tissue sections at one single time point cannot specify the mechanisms, our study, which examined various time points after the hemorrhage, allows us to more precisely determine the endogenous injury or protective mechanisms. Thus, our study provides a well-characterized preclinical animal model which can be utilized in enhancing our understanding of the disease and identifying effective preventive and therapeutic methods.

3.2.2 Limitations and Future Directions

Although the research has reached its aims in developing and characterizing the animal model for CBH, there were several potential limitations. First, while bleeding in the cerebellar germinal matrix was successfully replicated in our model, the collagenase injection using a Hamilton syringe produced variability in size of the hematoma. This inconsistency in the size of hemorrhage may have resulted from the backflow of collagenase along the needle track. Also, the collagenase injection into the fourth ventricle leads to rather diffuse bleeding in the cerebellum and often extends to the other regions of the brain, including pons. This variability in hematoma volume and location may have resulted in phenotypic variability within the treatment group. The large SEMs in some of our datasets suggest the presence of phenotypic variability, and increasing the number of animals per treatment group may allow us to detect a statistically significance from the moderate sized changes in phenotype. In particular, increasing the sample size for adult groups in our anatomical evaluation may lead to the detection of significant differences in cerebellar size between the treatment and control groups. Also, the sample size increase in molecular evaluation may lead to the stronger statistical power. Nevertheless, in terms of hemorrhage induction procedure, our collagenase model, compared to other currently available hemorrhage induction methods, is considered to more closely reproduce the hematoma progression and the bleeding pattern seen in the clinical presentation (Leonardo et al 2012).

Although we performed extensive assessments on the anatomical, behavioural, and molecular phenotypes resulting from CBH, further characterization of the animal model would not only strengthen the validity of the animal model but also provide more complete understanding of the disease. To obtain more thorough assessment of anatomical features, it would be necessary to
examine the gross and microscopic morphology of the lateral hemispheric cerebellar region in addition to the vermician cerebellum, the area where we primarily focused in our study. Additionally, histological and morphometric analyses on the cerebral cortical regions would provide information on the association between the cerebellar injury and cortical development. Also, the evaluation of social behaviour in CBH group would help us to elucidate the effects of CBH on the manifestation of autistic behavioural symptoms which have been noted in previous clinical studies (Limperopoulos et al 2007; Limperopoulos et al 2012). For the molecular evaluation, the use of denser time series would be necessary to more efficiently detect any transient changes in the levels of transcripts involved in various protective and damage pathways. Also, large-scale analysis of gene regulation through the use of microarray technology would enable us to investigate important endogenous molecular mechanisms that are activated or inactivated in CBH.

3.3 Potential Applications of Research Findings

In the present study, we describe a novel mouse model of neurological consequences following CBH that closely replicates anatomical and behavioural abnormalities seen in human preterm infants. Therefore, this model has a potential to be utilized as an invaluable tool for the evaluation of therapeutic targets in the prevention and treatment of the preterm-associated cerebellar injury. In the past decade, many neuroprotective strategies have shown their potential for improving the functional outcomes and minimizing damage (Robertson et al 2011). Some of the promising neuroprotective agents include melatonin (Luchetti et al 2010), xenon (Hobbs et al 2008), allopurinol (Peeters-Scholte et al 2003), erythropoietin (Kumral et al 2011), caffeine (Doyle et al 2010), and magnesium sulfate (Hunter & Gibbins 2011). For all of these agents, the neuroprotective properties, safety and efficacy have never been tested specifically as a treatment for preterm cerebellar hemorrhagic injury, and animal studies have not been possible due to the lack of CBH model. Therefore, using this CBH animal model system, the neonatal cerebellar specific responses to various neuroprotective therapies can be explored.

The CBH animal model presented in our study provides opportunities for integrating various co-morbid conditions to more accurately model the neonatal hospital course. The majority of preterm babies with CBH are likely to suffer from various other prematurity-associated complications. For instance, 77% of CBH cases are associated with supratentorial brain lesions, such as IVH (Volpe 2009). While the damages in the supratentorial brain regions are known to have
a significant impact on cerebellar development (Tam et al 2011b), the detailed examination on the
effects of co-occurrence of supratentorial and cerebellar hemorrhage have not yet been done in
clinical and preclinical studies. Other co-morbidities of preterm CBH that can be incorporated in
our model include infections, lung disease, and periventricular WM injury, which are the most
commonly observed complications among the preterm infants. Furthermore, the integration of
standard treatment practices that may have adverse effects on brain development to the CBH
model may provide important insights into potentially additive or synergistic responses. In
particular, glucocorticoid treatment, which is routinely used for prevention of bronchopulmonary
dysplasia in preterm infants, has been shown to be associated with cerebellar underdevelopment,
by increasing granule cell apoptosis (Tam et al 2011a). Since the prevalence of glucocorticoid
administration reaches 85% to 100% in premature babies, it is very difficult to study the additive
or synergistic effects of glucocorticoid exposure on CBH affected cerebellar development by
comparing to a control group without the glucocorticoid (Volpe 2009). Therefore, animal studies
would be crucial in order to precisely determine the effects of glucocorticoid in CBH patients on
cellular and gross morphology of the cerebellum and long-term neurodevelopmental outcomes.
Taken together, our animal model, which reproduces preterm CBH, provides a foundation for more
rapid progress towards a more thorough and clear understanding of the disease and may lead to
the development of more effective preventive and regenerative therapies.
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