Learning Deficits after Experimental Subarachnoid Hemorrhage (SAH)

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

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2011

Abstract

Survivors of subarachnoid hemorrhage (SAH) often have learning and memory deficits. This study tested the hypothesis that SAH in rats is associated with similar deficits and that they are due to neuronal injury in the hippocampus. SAH was induced in rats. Behaviour was investigated in the Morris water maze and brain injury by microscopy. Rats with SAH had deficits in spatial learning and working memory and had significantly more fluoro-Jade- and TUNEL-positive neurons in the hippocampus, cerebral cortex and cerebellum. Microthromboemboli in microvessels were more frequent in brains of rats with SAH and deficits there was vasospasm of the anterior and middle cerebral arteries. The amount of cell death in the hippocampus did not appear to be sufficient to cause the observed in the Morris water maze. This suggests that other factors such as dysfunction of neurotransmission or other pathology in hippocampal pathways might contribute to the impairment.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACA</td>
<td>Anterior cerebral arteries</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
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<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin &amp; eosin</td>
</tr>
<tr>
<td>ICP</td>
<td>Intracranial pressure</td>
</tr>
<tr>
<td>ITI</td>
<td>Inter-trial interval</td>
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<tr>
<td>LDF</td>
<td>Laser Doppler flowmetry</td>
</tr>
<tr>
<td>MCA</td>
<td>Middle cerebral artery</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>SAH</td>
<td>Subarachnoid hemorrhage</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
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Chapter I. Introduction

I.1. Overview
Subarachnoid hemorrhage (SAH) is bleeding into the cerebrospinal fluid (CSF)-filled space around the brain. The most common cause is trauma. Spontaneous cases, however are usually due to rupture of an intracranial aneurysm. Aneurysmal subarachnoid hemorrhage (SAH) represents a relatively small percentage of all stroke cases (5~7%) but the mortality and morbidity rate is among the highest. 1 Studies have shown that 15 to 20% of patients die before reaching hospital and another 20 to 25% die within the first 48 hours. 1, 2 The 30-day mortality is as high as 50%. 1, 3 There have been improvements in surgery, pharmacological treatment and intensive care. Lovelock, et al., analyzed the Oxford Vascular Study and conducted a metaanalysis of epidemiological studies of SAH. 4 They found that there was no evidence that the incidence of SAH or the 30-day case fatality was changing but that overall mortality was decreasing at 0.9% per year over the past 2 decades. In spite of this, the long-term outcome still is poor 4-7 with 30% of survivors remain dependent on others, mainly due to persistent cognitive impairment. Most patients do not have persistent focal neurological deficits. 8 The mechanisms of neurocognitive impairments after SAH have not been well studied, but they have been attributed to ischemic brain injury occurring either during the initial hemorrhage or as a consequence of macro- and microvascular dysfunction and delayed cerebral ischemia (DCI) (Fig. 1). 8
Most of the research on SAH has focused on cerebral vasospasm as a mechanism of DCI and poor outcome based on the finding that cerebral vasospasm can be associated with a substantial reduction in cerebral blood flow (CBF) and that reduced CBF can lead to cerebral ischemia and infarction. However, recent findings suggest that in addition to vasospasm, other processes may contribute to DCI and to poor outcome after SAH. This is based on the weak correlation between reduction in degree of the arterial luminal diameter, a measure of cerebral vasospasm, with the degree of CBF reduction, the development of infarction, and outcome. The clinical study by Dankbaar et al. found that in only 15 of 23 patients with moderate to severe vasospasm (65%), the territory of the vessel with the most severe vasospasm corresponded with the least perfused region. The difference in perfusion was more prominent between none and severe vasospasm than between none and moderate vasospasm. Four of seven (57%) patients with severe vasospasm and six of 16 (38%) with moderate vasospasm had DCI compared to three of 14 (21%) of patients with no vasospasm. The results also show that cerebral perfusion decreases with increasing degree of vasospasm and patients with severe vasospasm more often experience DCI than patients with moderate vasospasm. However, almost half of the patients with severe vasospasm do not experience DCI. The author concluded that although vasospasm causes a decrease in perfusion in the area behind the spasm, severe vasospasm alone is not sufficient to cause DCI. Most likely, other factors play role in decreasing cerebral perfusion to a level where DCI does occur.

Other clinical studies suggest that the vasospasm may not be the only factor that contributes to DCI after SAH. A clinical trial of clazosentan, an endothelin A receptor
antagonist, decreased angiographic vasospasm in a dose-dependent fashion in a phase 2b randomized, blinded clinical trial. Despite this, there was no improvement in clinical outcome. Thus there is a possibility that other processes, such as those mentioned above are associated with vasospasm and cause infarction and poor outcome even if vasospasm is eliminated. In the trial of clazosentan, it was suggested that this result might be due to insensitivity of the endpoint, the dichotomous Glasgow outcome scale (GOS), to finer aspects of clinical outcome. The GOS categories focus on physical changes that are not very common after SAH. These patients are more likely to have abnormalities in cognition and executive functioning. The sample size of the trial of the study was not planned to detect an effect on such a scale, a patient-centered outcome, as well.

Clazosentan increased the incidence of pulmonary complications, anemia and hypotension in this study. Drug side effects and off-target effects also can counterbalance any benefits of decreasing angiographic vasospasm.
Fig. 1. The pathophysiology of brain injury after SAH. Transient global ischemia (due to increased intracranial pressure and decreased cerebral perfusion pressure), subarachnoid blood clot and acute hypertension may lead to a variety of secondary effects including brain edema, delayed large artery vasospasm, breakdown of the BBB, microcirculatory changes, thromboemboli, cortical spreading depression and delayed neuronal death due to apoptosis or other mechanisms. The end result is focal and scattered brain injury. In the end, these processes have to cause neurological and neurobehavioural deficits to be important and these will depend on what areas of the brain or networks in the brain are disrupted. (extracted from Jeon HJ 2009)
Microvascular constriction was mainly investigated in animal models but the studies report conflicting results.\textsuperscript{13-15} In the double hemorrhage dog model that creates SAH generally without raised ICP, intraparenchymal brainstem arterioles were constricted 3 and 7 days after SAH,\textsuperscript{14,16} but these vessels were dilated after SAH in another study.\textsuperscript{14,16} Parenchymal arteries in humans with SAH did not exhibit autoregulatory vasodilation during vasospasm\textsuperscript{16,17} and peripheral cerebral circulation time was prolonged\textsuperscript{10} which indirectly indicated a potential narrowing of small arteries. Direct relationship of microvascular constriction to the pathogenesis of DCI after SAH was not able to be made.\textsuperscript{18} Another process hypothesized to cause DCI is cortical spreading ischemia. The hypothesis is based on the association between DCI and repeated spreading depolarizations with prolonged electrocorticographic depression periods after SAH.\textsuperscript{19} The presence of a delayed cluster of spreading depolarizations had positive predictive value of 86\% for development of DCI whereas the absence of spreading depolarization had a negative predictive value of 100\% for DCI.\textsuperscript{19} After SAH, the break down products of erythrocytes in the subarachnoid space have been suggested to cause spreading depolarizations.\textsuperscript{20} The local toxic effects of hemolysis products evoke spreading depolarizations, which induce transient but long-lasting microvascular spasm which may in turn cause cortical spreading ischemia.\textsuperscript{20} Cerebral blood flow or metabolism measurements were not performed in these studies so the mechanisms underlying the depolarizations could not be determined.\textsuperscript{16}
After a vessel injury, platelets are activated by several factors, such as collagen, von Willebrand factor (vWF) and thrombin. On activation of platelets, exocytosis of platelet granules results in the release of a wide array of procoagulant substances. This leads to initiation of the coagulation cascade, which ultimately results in the formation of microthrombi. There is emerging evidence to suggest that formation of microthromboemboli occur after SAH. Evidence suggesting that there is activation of the coagulation cascade after SAH is supported by increased serological levels of beta-thromboglobulin, thromboxane B2, soluble platelet selectin (sP-selectin), platelet-activating factor, and increased CSF level of tissue factor in humans with SAH. Patients with DCI after SAH also have significantly higher levels of plasminogen activator inhibitor-1 (PAI-1) antigen in the CSF compared with patients without DCI, suggesting fibrinolysis impairment is associated with DCI. Microthromboemboli were detected in autopsy studies in patients with cerebral infarction after SAH and transcranial Doppler ultrasound also detected embolic signals in patients with SAH. Lack of a marked beneficial effect of antiplatelet and anticoagulant drugs, such as aspirin and enoxaparin on outcome in patients with SAH was attributed to the hemorrhagic complication of these drugs which may counterbalance the beneficial effects.

Neurocognitive testing has been done for patients generally 3 ~ 6 months after SAH. Animal studies have usually relied on histological assessment of neuronal death which has several problems. Corbett et al. suggested that neurons may be alive and not detected by assays of apoptosis or necrosis, but they may not be functional. In fact, numerous
studies demonstrated that animals can have neurocognitive deficits after TBI or ischemia without having substantial neuronal death. 35 For instance, hippocampal neurons have been found to be dysfunctional but not dead by histological criteria after cerebral ischemia. 36, 37 Most studies of behaviour after experimental SAH have investigated neurological function rather than neurocognition. 6, 38-42 Only two animal studies investigated cognitive function after SAH. They used different models and there was some inconsistency in their results which may be in part due to the difference in the method of induction of SAH. 43, 44 Further investigation of neurocognitive function after experimental SAH is needed in order to determine if animals develop such deficits and understand their mechanisms.

I.2. Long-term Neurocognitive Impairment after SAH: Clinical Studies
The end result of SAH is focal and scattered brain injury, associated with neurological and neurobehavioural deficits that depend on the areas of brain or networks in the brain that are disrupted. The frontal lobes are responsible for motor functioning of all parts of body. They also contain Broca area, which is the main speech output center. 45 The prefrontal regions of the frontal lobes in particular play a key role in many types of higher-order intellectual behaviour such as judgment, planning, and decision-making which together are called executive functions. These areas are also responsible for verbal fluency, design fluency and emotional functioning. Another important function subserved by the prefrontal region is “working memory”, a brief window of mental processing (a minute or two) during which a limited amount of information is held. 45
The temporal lobes are comprised of the primary auditory cortex and auditory association cortices that are crucial for perception of auditory information. They also are involved in face, object and pattern recognition and retrieval of conceptual knowledge, names, and non-verbal information (Fig. 1). The medial temporal lobes, comprising the amygdala, hippocampus, entorhinal and perirhinal cortices and anterior portion of parahippocampal gyrus are known to play a crucial role in memory and learning of new information (anterograde memory). The hippocampal complex is extensively interconnected with higher-order association cortices located in the temporal lobe. In addition to their function in integrating various aspects of memory experiences, including visual, auditory and somatosensory information, their primary function is the acquisition of new factual (declarative) knowledge. Damage to hippocampus is associated with amnesia. The amygdala is important for the acquisition and expression of emotional memory. Other areas of the cerebral cortices are involved in vision (occipital lobe) and basic somatosensory perception, proprioception and visuospatial processing (parietal lobe). The cerebellum has key roles in motor coordination, gait balance, motor learning, eye movement and orientation of attentional resources.

The most frequently impaired cognitive functions after SAH are memory, executive function and language. This strongly implicates temporal (hippocampal) and frontal lobe dysfunction. Al-Khindi, et al., reviewed long-term effects of SAH on cognitive, memory, neuropsychological, and day-to-day functioning. Survivors of SAH also frequently reported to suffer from depression, anxiety, fatigue and sleep disturbances which further compound with the cognitive impairment and functional outcome. They
noted that impairments in activities of daily living (self-care such as feeding, grooming, dressing, bathing, personal hygiene, and toileting) tended to be associated with deficits in visual memory, visuospatial function and psychomotor functioning. In addition to activities of daily living, impairment in more complex measures such as instrumental activities of daily living also were associated with visual memory and psychomotor functioning. Cognitive difficulties including attention, planning and reasoning impairment after SAH also were found to affect the patient’s ability to work. A substantial proportion of survivors of SAH are unable to return to their previous occupation, working fewer hours or taking jobs with less responsibility.

Patients who are classified as having made favorable outcomes after SAH (GOS score 4 or 5) often have cognitive deficits on tests sensitive to temporal lobe dysfunction (verbal and visual memory, verbal fluency, pattern recognition and spatial working memory). Abnormalities in these domains, particularly verbal and visual memory, are the most commonly affected in patients with SAH. Deficits in these domains were associated with ruptured aneurysms in the anterior circulation along with older age, lower education level, poorer neurological grade on admission, and thick subarachnoid blood. Verbal and visual memory impairment have been correlated with hippocampal volumes measured with the volumetric MRI in patients after SAH, implicating brain injury and probably neuronal cell loss in the pathogenesis of these deficits.
Executive function as mentioned above, is predominantly mediated by the frontal lobes, and refers to higher-level cognitive functions such as planning complex behaviour, inhibiting inappropriate social activity, problem-solving, attention and decision-making. Al-Khindı et al. noted that the majority of studies of neurocognitive dysfunction after SAH treated executive function as a unitary construct rather than differentiating between different aspects of executive function. This resulted in a wide range of prevalence rates of executive dysfunction in survivors of SAH. Executive dysfunction after SAH is more pronounced in older patients, those with fewer years of education, and those with poorer neurological grade on admission. 62, 65

I.3. Animal Model of SAH
I.3.1. Overview
Simulating a disease in an animal model is an essential part of research in order to understand the pathogenesis of disease. Many species have been used to model SAH including mice, rats, rabbits, dogs and monkeys. 7 There is increasing use of rats in experimental SAH research. The advantages are they develop vasospasm and some delayed complications of SAH. They are also relatively inexpensive and easy to handle and there is considerable knowledge about rat biology and behaviour. 66 Disadvantages are there is conflicting and weak evidence that they develop prolonged vasospasm and the time course and severity of vasospasm is shorter and less than that occurring in dogs, monkeys and humans. These same issues apply to mice and rabbits. Additional advantages of mice are availability of transgenics and knockout animals whereas
disadvantages are very small size and difficulty doing invasive monitoring.\textsuperscript{67} Advantages of the dog model of SAH are acceptable cost, well characterized time course of vasospasm, and easier diagnostic procedures.\textsuperscript{67} Disadvantages are the low mortality rate which does not resemble clinical SAH. Advantages of the monkey model are the similarity of time course and severity of vasospasm to humans, pathological and pharmacological changes and ability to produce cerebral ischemia with a moderate mortality rate.\textsuperscript{67} Limitations of this model are need for complex equipment and technical expertise to create the model as well as cost.

There are three commonly-used methods to induce SAH in animals: injection of blood into the cisterna magna (single injection or double injection separated by one or two days) or prechiamatic cistern, perforation of an intracranial artery either endovascularly or transcranially, or placement of blood clot surgically into the subarachnoid space.

\textbf{1.3.2. Cisterna Magna Injection Model of SAH}
In this model, SAH is induced by injecting a preselected amount of autologous blood into the subarachnoid space via the cisterna magna. The rate of injection can be varied so as to vary how high the intracranial pressure rises and thus how much global cerebral ischemia occurs.\textsuperscript{66,68,69} Blood can be injected once (single hemorrhage) or twice (double hemorrhage) which are usually 48 hours apart.
This is the most commonly used method in rats, mice, rabbits and dogs. In rats, and rabbits and dogs, both single, and double injections are used, while a single injection is more preferred in mice. Animals in these models seldom or never develop cerebral infarctions due to SAH or vasospasm. This is in part because blood injected in the cisterna magna gets dispersed into the spinal canal, reducing the severity of SAH. The CBF reduction due to vasospasm does not usually reach the threshold (40~50%) to cause infarction. In an effort to try to produce cerebral infarction in an animal model, a single cisterna magna injection was combined with ligation of both carotid arteries in 21 rabbits. The authors were able to demonstrate cerebral ischemia from this combination of SAH, carotid ligation and vasospasm.

The severity of vasospasm induced by the cisternal injections in rodents is relative mild. Two injections in dogs are required to produce moderate to severe vasospasm. The reasons for this are unclear, but are probably due to the relatively small amount of blood that can be accommodated in the subarachnoid space of rodents. Whether there is a fundamental difference in the propensity to develop vasospasm lasting for days as it does in humans is unclear. Fisher et al. suggested that bleeding in the posterior fossa among patients might be better tolerated than supratentorial SAH, because of proximity of the spinal canal. In rats with a single cisternal injection was associated with no mortality and only a small fraction of animals exhibiting pathological lesions. The amount of subarachnoid blood was found to be variable in this model, and it was distributed around the brainstem and cerebellum with only a small amount around the circle of Willis.
This pattern does not resemble the blood distribution after SAH in humans. Changes in CBF also were only transient in the single cisternal injection model \(^{68}\) and returned to baseline levels within days whereas CBF remains decreased for weeks after SAH in humans.

I.3.3. Endovascular Perforation Model of SAH
The endovascular perforation model is a noncraniotomy model that mimics the mechanism of aneurysmal rupture in human SAH. \(^{68,72}\) This has been done in rats or mice. The carotid artery is exposed surgically and a suture is inserted into the external carotid artery and advanced up the internal carotid artery to the intracranial bifurcation of the internal carotid artery into the middle and anterior cerebral arteries. \(^{73,74}\) The suture has a sharp end and is pushed through the arterial wall to create a SAH. The advantages are that the SAH is associated with a substantial increase in intracranial pressure that reflects fairly accurately what happens in humans with SAH. The mortality also is reported to be up to 40% which is similar to that occurring clinically. Blood also was found to be deposited in the basal part of the brain, especially around the ipsilateral circle of Willis, as well as the brainstem and cerebellum. Disadvantages are that the subarachnoid blood volumes are highly variable, which was suggested to be caused by a difference in the perforation site. \(^{68}\)

Overall, this model has been suggested to have low reproducibility with variable severity of SAH. The low cost, relative ease of production and ability to simulate several aspects
of acute pathophysiology of SAH including rupture of an aneurysm makes this model valuable especially for study of the initial effects of SAH. This method is frequently used in rats \textsuperscript{75} and mice. \textsuperscript{76, 77}

I.3.4. Anterior Circulation Model of SAH (Prechiasmatic Injection)
The anterior circulation model of SAH was reported by Prunell and colleagues. In this model, a burr hole is drilled near the bregma and SAH is induced by advancing a needle through the burr hole and injecting autologous blood into the prechiasmatic cistern anterior to the optic chiasm. The amount of subarachnoid blood is obviously set at a consistent amount which increases reproducibility of the model. The blood is deposited mostly around the anterior circle of Willis with only small amounts over the hemispheres and cerebellum. This pattern resembles very well the pattern of blood distribution in patients with SAH. Other advantages are that there is a moderate mortality rate and a high incidence of pathological lesions such as apoptotic neuronal death. \textsuperscript{68, 69} There is a decrease in CBF for up to 90 minutes after SAH. \textsuperscript{68, 69, 78} This makes this model more valuable for study of long-term consequences of SAH. This method has so far been reported in rats and mice.

I.4. Behavioural Assessment of Animals with SAH
I.4.1. Overview
Behavioural consequences of SAH have been investigated in all animal models mentioned above, although the majority of studies involve rats and do not include
sophisticated neurobehavioural assessment. Most studies have assessed body weight and neurological deficits such as sensory, motor and general behavioural impairment. Some aspects of neurocognitive function such as learning and memory have been studied in rats. We previously reviewed neurological and neurobehavioural assessment after experimental SAH. 7

I.4.2. Body Weight
Body weight in animals can be used to assess appetite and motivation. SAH induced by cisterna magna injection in rats was associated with a significant decrease in body weight which persisted for 3 to 5 days. 6, 38-42 The amount of blood injected (300 or 400 µl) and the injection rate were associated with greater body weight reduction. Mice with SAH by endovascular perforation also exhibited significant weight loss 3 days after SAH compared to sham-operated animals. 77, 79

I.4.3. Neurological Function Scales and Qualitative Assessments
Several neurological function scales and qualitative assessments have been adapted to investigate general neurological function after SAH in various animal models. Many different scales for assessing neurological function of rats have been reported, some of which were developed for various diseases and then adapted for SAH, and a few developed specifically for SAH. Some scales include those developed for assessment of the effects of cardiac arrest 7, 80, 81, focal ischemia 7, 79, 82, and traumatic brain injury (TBI). 7, 83, 84 These scales use tests that evaluate general behaviour, reflexes, spontaneous
activity and motor and sensory functions such as balance and proprioception. Some of these scales were able to differentiate neurological function of rats with SAH induced by endovascular perforation compared to untreated rats \(^7, 82, 85\) when tested during acute phase of SAH. Germano and colleagues \(^41\) tested for simple nonpostural somatomotor functions (duration of suppression of the pinna reflex, corneal reflexes, startle response) and complex postural somatomotor functions (righting response, spontaneous locomotion, escape response) in rats subjected to a single cisternal injection of autologous blood and compared the responses to control animals that underwent injection of artificial CSF. This scale did not detect any differences between the groups after injection of blood on day 0.

Previously described scales were used to assess motor and sensory activity in mice with SAH created by endovascular perforation. The scale was comprised of spontaneous activity, symmetry of limb movements, climbing, balance and coordination (0-12 points) for motor and proprioception, vibrissae, visual, olfactory and tactile responses (5-15 points) for sensory assessment. \(^7, 77, 79, 86\) Neurological function was significantly impaired in SAH animals compared to sham-operated mice.

Rabbits with cerebral ischemia and SAH created by ligating both common carotid arteries and injection of blood into the cisterna magna were found to have neurological impairment. \(^70\) The severity of the neurological deficit was greater in these animals compared to animals with SAH alone or carotid ligation alone. In this study, neurological
deficits were categorized as normal, minimal or suspicious neurological deficit, or mild
deficit without abnormal movement. When rabbits with a double hemorrhage SAH were
assessed on a 6-point scale, first developed for dogs, the only difference compared to
rabbits with single hemorrhage SAH was in the appetite score which was significantly
higher (e.g. reduced appetite) 3 days after SAH in the double hemorrhage group. This
experiment did not include a saline-injected control group. Another neurological scale
developed to assess myelopathy in rabbits was applied to rabbits with SAH. The
neurological functions evaluated on this scale were posture, gait, and righting reflexes.

Neurological function in the dog model of SAH has only been assessed using broad
qualitative assessments. This has usually been done using the double-hemorrhage model
within hours of SAH. On a 6-point neurological function scale, dogs with SAH
induced by a single injection showed that sudoxicam, a nonsteroidal anti-inflammatory,
 improved function. Each function was graded as: (1) appetite: graded as finished meal =
2, left meal unfinished = 1, scarcely ate = 0. (2) activity: active, barking or standing = 2,
lying down, will stand and walk with some stimulation = 1, almost always lying down =
0 (3) neurological deficits: no deficit = 2, unable to walk because of ataxia or paresis = 1,
impossible to walk or stand because of ataxia or paresis = 0. The results are not consistent,
however, since a similar scale did not detect deficits after SAH in dogs compared to
untreated controls in other studies. The most widely used scale in dogs assessed
appetite, activity and neurologic deficits in the double injection model.
I.4.4. Rotarod, Horizontal Ladder, and Other Neurological Tests
The rotarod and horizontal ladder tests mainly measure motor function. Numerous studies have applied these tests to rats with SAH but the details of the methods have been inconsistent, which makes comparison between the studies difficult. Thal, et al., assessed rotarod performance in rats subjected to SAH using the endovascular perforation model. Rats were placed on the rotarod for 10 seconds. Rotation then started and accelerated to 40 revolutions per minute (rpm) within 90 seconds and then remained constant for 30 more seconds. The trial was repeated 5 minutes later and the test was stopped if the animal fell off or gripped the rungs and spun for 2 revolutions. No control groups were included in this experiment. In another study, rats with a double cisterna magna injection of blood were tested on a rotarod with the rotation rate increased to 4 rpm within 5 minutes. Animals performed 3 trials per day for 28 days. The performance of the SAH group was compared with that of sham and saline-injected control groups. The SAH group showed marked and persistent deficits compared to the other groups and these persisted for 28 days.

Silasi and colleagues found no differences between endovascular SAH and sham-operated rats in tapered beam walking or horizontal ladder tests when observed 3, 7, 14, or 21 days after SAH.

Few studies have assessed neurological functions in mice. Mice with endovascular SAH had significant deficits in their performance on the rotarod three days after SAH compared to sham-operated animals.
I.4.5. Beam Balance Test

In this test, rats are placed on a narrow wooden beam (diameter of 1-2.5 cm) for up to 60 seconds\(^6,39-42,98\) to test motor and vestibular function. The parameters are beam balance time (duration the animal steadily remains on the beam)\(^41\) and beam balance score (descriptive and examiner-dependent measure of the performance on the beam).\(^98\) Deficits usually have been detected only during the acute phase of SAH in rats (one to two days after SAH) after which time there is no difference between control and SAH animals.

Most studies using the beam balance test were reported by one laboratory using same method of induction of SAH and similar behavioural assessments. Despite this, their results have varied. The sensitivity of the test seems to be relatively low although it can be improved by decreasing the diameter of the wooden beam to some extent.\(^41\) For example, rats with a single hemorrhage SAH induced by cisterna magna injection exhibited a significantly increased beam balance score (decreased performance level) only one day after SAH compared to sham-operated and CSF-injected animals.\(^41\) Another limitation is that while the beam balance time may be objective, the beam balance score is subjective and descriptive.\(^41\) The beam balance test also is not well-standardized. The diameter, length, shape and composition of the beam can vary\(^98\) which may affect the results. The variable severity of SAH induced by cisterna magna injection might also contribute to inconsistency in the results.\(^68\)
I.4.6. Beam Walking Test
The beam walking test was used to assess memory, motivation, attention, somatomotor and locomotor function. The animal is encouraged to walk along a beam into a protected hole, so this test incorporates a learned avoidance component. This is a negative reinforcement paradigm in which termination of adverse stimuli (noise and light) serves as a reinforcement reward. The measure of the performance on the beam walking test is the time taken to traverse the beam and enter a darkened goal box which terminates the adverse stimuli. Most studies found that rats with SAH induced by a single cisterna magna injection took a significantly longer time to traverse the beam compared to before SAH and to sham-operated controls. Deficits generally persisted for up to four days and gradually improved from a maximal deficit one day after SAH.

I.4.7. Morris Water Maze
The Morris water maze (MWM) is a behavioural test that is frequently employed to test numerous aspects of learning and memory in rodents. It has been used for rats and mice. Animals are placed into a circular swimming pool and have to swim until they find a hidden platform submerged in the pool. There are a number of different paradigms that can be used, but a common one requires the animal to learn the spatial location of the platform in relation to extramaze visual cues in order to escape from the water. Learning and memory are measured by escape latency, swimming distance and time spent in the quadrant in which the platform is hidden. Detailed methods and discussion of the MWM are provided in chapters II and IV.
There are two studies investigating effects of SAH on learning and memory in rats in the MWM. \(^43, 44\) Takata, et al. studied short-term memory and long-term learning in rats with SAH created by two injections of blood into the cisterna magna. They used a modified version of the MWM in which the platform location was randomized each day. All animals performed 16, 60-second trials 29 to 35 days after SAH. Animals with SAH had significantly longer escape latency, swimming distance and faster swimming speed. Performance in the MWM was correlated with neuronal counts in the hippocampus and neocortex. Silasi and Colbourne used a similar procedure in rats but with four trials of 90 seconds each day from day 21 to 40 after SAH. \(^43\) The hidden platform location was randomized every second day. SAH was induced by endovascular perforation. Rats with SAH showed longer escape latency and swimming distance, compared to sham-operated animals, on the days the platform was moved to a new location. Four of five rats with SAH exhibited fluoro-Jade positive neurons although no other histopathological changes were detected three days after SAH.

**I.5. Behavioural Changes After Treatment of SAH**

**I.5.1. Overview**

Fig. 1 shows possible pathways that could cause brain injury after SAH and result in neurocognitive impairment. Many of these have been targeted for treatment in animal models. End points such as signs of brain injury (eg. infarction, neuronal death, blood brain barrier (BBB) permeability) were examined in animals undergoing SAH. Neurological function tests and assessments were used in some studies. Improvement in one measure was not always consistently associated with improvement in another.
measure. The results also seem to differ depending on the animal model, method of creating SAH and on the behavioural measures used. Again, most of these studies have employed rat and mouse models.

I.5.2. Mortality
In rats, mortality tends to be lowest with a single cisternal injection, higher with double injection and highest with the endovascular perforation method.\textsuperscript{41, 42, 73, 81, 82, 85, 101-105} Hyperbaric oxygen\textsuperscript{106} and pifithrin $\alpha$\textsuperscript{101,107} are the only treatments that have been shown to reduce mortality in rats with endovascular SAH. These two treatments also improved behavioural and neurological function in this model.\textsuperscript{101,106,107} Hyperbaric oxygen decreased expression of hypoxia-inducible factor 1 (HIF1$\alpha$) and its target genes BNIP3 and vascular endothelial growth factor (VEGF). There was less neuronal injury and improved CBF. Pifithrin $\alpha$, which reduced vasospasm, improved BBB integrity and neurological function, is known to reversibly suppress p53 and p53-dependent apoptosis.\textsuperscript{101, 107}

SAH in mice was associated with 4\% mortality\textsuperscript{76} after cisterna magna injection of 60 $\mu$1 of blood whereas it is 20 ~ 29\% after endovascular perforation.\textsuperscript{108} An apoE4 peptide mimetic reduced mortality, improved neurological score and reduced vasospasm in wild-type mice after SAH.\textsuperscript{103} ApoE mimetic peptide was neuroprotective in other brain injuries and was suggested to decrease vasospasm by anti-inflammatory mechanisms.\textsuperscript{103} Many studies in mice did not report mortality.
Mortality rates are not well-described in the rabbit model of SAH. One group reported 40% mortality after single cisternal injection SAH compared to 0% after saline injection. Another study reported 6% mortality with double injection SAH compared to 0% with single injection. Mortality was significantly reduced in rabbits with single hemorrhage SAH when erythropoietin was administered. Mortality in the dog model of SAH was seldom reported but is believed to be low in this model (R.L. Macdonald, personal observation).

I.5.3. Neurobehavioural Impairment after Treatments
The oxygen free radical scavenger +/− N,N′-propylenedicinicotinamide (AVS) decreased vasospasm and improved the balance beam score 1 and 2 days and beam walking time 1 to 4 days after SAH induced by a single cisterna magna injection in rats. This was taken as evidence that free radicals are important in the pathogenesis of vasospasm and brain injury after SAH. AVS did not affect weight loss. The calpain inhibitor, N-acetyl-leu-methioninal and the anticonvulsant, felbamate, on the other hand, prevented body weight reduction for up to 5 days after SAH in the same model. Both drugs also significantly improved beam balance scores for 1 to 3 days after single hemorrhage SAH. Calpains are calcium-activated neutral proteases that may be activated in cerebral vessels after SAH and lead to vasospasm and increased BBB permeability. Felbamate inhibits voltage-dependent sodium and calcium channels, potentiates amino-butyric acid (GABA)–mediated chloride currents and reduces excitatory glutaminergic neurotransmission via N-methyl-D-aspartate receptors thereby providing neuroprotection. In another study, rats with endovascular SAH were treated with
SP600125, a c-Jun N-terminal kinase inhibitor. They showed improvement on a 16-point scale assessing general neurological function, which was originally developed for TBI. This scale may be relatively more sensitive than others since it includes measures of motor and sensory function as well as beam walking and mobility that may assess higher neurological functions more likely to be affected after SAH.

Numerous studies demonstrated that general neurological scores may not be very sensitive to alteration by treatment. Treatments that are have beneficial effects on the brain such as neuronal preservation, less vasospasm, less BBB breakdown found to have only minimal effects on behavioural outcomes. A 100 point neurological evaluation scale detected a difference between rats with SAH treated with hypertonic saline and hypertonic saline plus dextran only on one day after endovascular SAH. A study comparing effects of NaCl, mannitol, dextran and hydroxyethylstarch also found no differences between groups for up to 7 days after SAH despite reduced neuronal loss in some treated groups. The scale of Bederson, et al., prehensile traction test and rotarod testing tend to assess focal motor deficits which all showed minimal deficits after endovascular SAH. They did not differentiate treatment effects.

In a rabbit model of SAH induced by cisternal injection with ligation of both carotid arteries, neurological deficits were categorized was normal, minimal or suspicious of neurological deficit, mild deficit without abnormal movement or severe neurological deficit with abnormal movements. Only subtle transient decrements in neurological function were detected in rabbits with SAH after treatment with intravenous...
anticardiolipin antibodies compared to SAH with no treatment. Whether nimodipine and ecdysterone improved the neurological function on the same neurological scale in the same rabbit model was difficult to discern from the paper. Intravenous injection of erythropoeitin improved mortality and open-field locomotor activity in rabbits with single SAH.

In the dog model of SAH, there are only a few reports of improvement in behaviour such as appetite and activity following treatment with mitogen associated protein kinase inhibitor and the caspase inhibitors Ac-DEVD-CHO and Z-VAD-FMK.

1.5.4. Cerebral Vasospasm and Behavioural Deficits after SAH
DCI is attributed to vasospasm and thought to contribute to poor outcome. Thus much work on SAH has focused on cerebral vasospasm. In fact, some studies have shown that improvement in neurological outcome occurs in association with decreased vasospasm, and decreases in other aspects of brain injury such as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and caspase 3 in endothelial cells, caspase 3 activation, apoptosis, BBB permeability and brain edema in rats with endovascular SAH treated with the pancaspase inhibitor z-VAD-FMK and pifithrin α. Mice with perforation induced-SAH treated with nimodipine alone or with an apoE mimetic peptide, acetyl-AS-Aib-LRKL-Aib-KRLL-amide, also had improved neurological scores and rotarod latency and decreased vasospasm. On the other hand, rats with double hemorrhage SAH showed deficits in rotarod, vertical screen, balance beam, MWM, as well as decreased CBF, neuronal loss in the hippocampus, and microvascular filling.
defects despite minimal large artery vasospasm. There was a lack of relationship between the severity of vasospasm and neurological function in rabbit and dog models of SAH. 91, 97, 112, 114 The lack of correlation between vasospasm and other endpoints has several possible explanations, one is that there may be multiple pathways to brain injury and poor outcome after SAH. Another is that the measurement of vasospasm does not accurately reflect its effects on CBF. It also is possible that the neurological functions measured are not those that are affected by vasospasm.

I.6. Summary
SAH is associated with neurocognitive impairment in humans. The most frequently impaired functions are memory, executive function and language, which implicate the temporal and frontal lobes as locations of brain injury and dysfunction. SAH is also associated with neuropsychological dysfunction such as depression, anxiety, fatigue and sleep disturbances which may further compound cognitive impairment and functional outcome.

Mice, rats, rabbits, dogs and monkeys have been used to model SAH. There are advantages and disadvantages of each model. The rat model is simple to perform, inexpensive and reflects the changes that occur in humans in that the animals develop vasospasm and exhibit some delayed complications of SAH. There also is considerable knowledge about rat biology and behaviour. The high mortality that is a characteristic of SAH in humans can be produced mainly be endovascular perforation models in rats and
mice. Several treatments have reduced mortality in rodent SAH models but effects of treatments on mortality are not well-described in rabbits and dogs.

SAH in rats and mice was associated with reduction in body weight after SAH but this endpoint has not been assessed in other animal models. Neurological scales testing motor, sensory and reflex functions have been used in rats, mice and dogs. The results are not consistent because these scales involve subjective and descriptive methods of assessment and have varied between species. Different animal models and methods of SAH induction also contribute to the inconsistency in the results. In general, neurological dysfunction after SAH in animals tends to be minimal and transient when assessed on these scales.

Rotarod, horizontal ladder, beam balance and beam walking tasks mainly measure motor and vestibular function. They have not been widely used. Animals with SAH tend to have small and transient deficits when tested on these tasks and compared to sham-operated animals. There were treatment effects on animals with SAH. The details of methods of rotarod and beam balance task are not well-standardized and showed inconsistent results.

Neurocognitive tests (MWM) have only recently been reported in rats and reported results are conflicting. Robust effects were shown in the double hemorrhage model and only minor differences were found in the endovascular model.\textsuperscript{43, 44} The degree of neuronal damage correlated with neurocognitive deficits in the MWM in the double hemorrhage model. There are no studies investigating learning and memory in animals
with anterior circulation SAH. Further investigation of neurocognitive function after experimental SAH is needed in order to determine if animals develop such deficits and to then be able to begin to investigate and understand their mechanisms.

Much research on SAH has focused on cerebral vasospasm as a mechanism of DCI and poor outcome. However, emerging evidence indicates that other factors might contribute to DCI and poor outcome after SAH. These include microvascular constriction, microthromboemboli and cortical spreading ischemia. The presence of these in many animal models and in humans with SAH, their causal association with outcome and their mechanisms are not well studied.
Chapter II. Hypothesis and Specific Aims

II.1. Summary
Review of the literature shows that SAH in humans is frequently associated with neurocognitive deficits as mentioned in Chapter I.2. The etiology of these deficits is unclear and could relate to multiple processes such as increased intracranial pressure and transient global ischemia occurring at the time of the SAH, as well as direct effects of the subarachnoid blood, delayed neuronal apoptosis, cerebral vasospasm, microthromboemboli and other processes. Previous data suggests that in humans there can be poor outcome even when drug treatment decreases angiographic vasospasm. The goal of this thesis was to determine in a rat model of SAH and vasospasm whether cognitive and memory impairment occur and to begin to elucidate the mechanisms by which it does.

II.2. Hypothesis and Specific Aims
We hypothesized that behavioural deficits after SAH are caused by dysfunction of neuronal pathways in the hippocampus and/or death of neurons in the brain, especially in the hippocampus, an area implicated in learning and execution of memories, and that these changes are independent of vasospasm and increased intracranial pressure and are due to direct effects of the SAH.

The specific aims to test this hypothesis are:

1. Aim 1: To determine if neurocognitive deficits occur in a rodent model of anterior circulation SAH that may mimic the pathophysiology of SAH seen in humans.
2. Aim 2: To determine if these deficits are associated with vasospasm, microthromboemboli and neuronal injury.

These studies would produce new data that would describe the associations, if any, between the neurocognitive deficits seen in the first aim, and the morphological changes seen in the second aim. We did not aim to test the causative nature of the associations at this preliminary stage. This is a key goal for subsequent investigations.
Chapter III. Materials and Methods

III.1. Animal Surgery

III.1.1. Rat Anterior Circulation Model of SAH

Procedures on animals were approved by the Institutional Animal Care Committee and complied with regulations of the Canadian Council on Animal Care. Thirty-three male Sprague Dawley rats, weighing 300 - 400 g, were randomly assigned to one of three groups: sham operated (n = 9), saline injection (n = 11), and blood injection (subarachnoid hemorrhage [SAH], n = 13). Animals that survived with no apparent motor deficits (n = 9 for each group) underwent testing in the Morris water maze (MWM).

We used a prechiasmatic injection model of SAH. On day 0, rats were weighed and anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), intraperitoneally. They were positioned prone in a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA). A midline scalp incision was made and a one mm hole was drilled 7.5 mm anterior to the bregma in the midline, at an angle of 30° caudally (Fig. 2.A). A 27 gauge spinal needle was advanced 11 mm into the prechiasmatic cistern through this burr hole (this is equivalent to a stereotaxic coordinate of zero mm from the middle line, 2 mm from the bregma with depth of 9.5 mm from the skull for the prechiasmatic cistern). The skull was thinned on the left side posterior to the bregma until the blood vessels of the cortex could be seen. A laser Doppler probe (Transonics model BLF21, type n flow probe, Ithaca, NY, USA) was placed on this area to monitor local cerebral blood flow (CBF) as measured in tissue perfusion units (TPU). Blood pressure was monitored with a 24 gauge catheter inserted into a tail artery. The catheter was connected to a pressure
transducer (World Precision Instruments Inc., Sarasota, FL, USA) through a tubing system filled with 0.9% NaCl. Body temperature was maintained and monitored with a heating pad (homeothermic system, Harvard Apparatus, Holliston, MA, USA) and a probe placed into the rectum. Baseline CBF, blood pressure and body temperature were recorded for 15 minutes. 300 µl fresh blood was withdrawn from the tail artery catheter and injected into the subarachnoid space through the 27 gauge needle over 20 seconds using a syringe pump (Harvard Apparatus). The saline control group underwent injection of 300 µl 0.9% NaCl and the sham-operated group was subjected to the same surgical procedures without injection of anything into the subarachnoid space. Fig. 2.B shows the gross appearance of a rat brain after autologous blood injection into the prechiasmatic cistern. The blood was seen throughout the basal surface of the brain and around the brain stem and only sparse amounts of blood clots were found over the hemispheres. Blood pressure, CBF and body temperature were monitored for 45 minutes after which the needle, catheter and probes were removed. The incisions were closed, a subcutaneous injection of 20 ml 0.9% NaCl was given and the rats were placed in a 27°C incubator until they recovered.
**Fig. 2.** Rat anterior circulation model of SAH. (A) schematic diagram (sagittal view) showing trajectory of the spinal needle passing through an incision made at 7.5 mm anterior to the bregma in the midline, at an angle of 30° caudally (brain is in the saggital view) (B) a gross appearance of a rat brain showing distribution of blood immediately after injection into the chiasmatic cistern anterior circulation.
III.1.2. Intracardiac Pressurized Perfusion
Animals were euthanized eight days after SAH or sham surgery on day 8 after completion of MWM testing. The perfusion system was filled with 0.9% NaCl in one bottle and 4% paraformaldehyde in phosphate-buffered saline (PBS) in the other. Rats were put in a dorsal position in a tray and, the thorax were opened to expose the heart. An incision was made in the left ventricle of the heart and an 18 gauge blunted needle connected to of the perfusion system was inserted. The needle was fixed with a clamp and open the 3-way tap to 0.9% NaCl. The right atrium was cut and the system was pressure infusor pressurized to perfuse the rat for 20 minutes with 0.9% NaCl followed by 200 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS) at a constant pressure of 80 mmHg.

III.1.3. Brain Sectioning
Brains were post-fixed in the same solution for another 48 hours and then were cut into blocks in a rat brain matrix (Harvard apparatus). Three coronal cuts were made first; at the groove between the forebrain and the cerebellum, and then 3 mm posterior and 6 mm anterior to this groove for visualization of the cerebellum and hippocampus, respectively (Fig. 3A). The anterior portion of the forebrain was then rotated 90° and two sagittal cuts were made at the crossing of the olfactory tract and the middle cerebral artery (MCA) (Fig. 3B). These blocks were used for viewing the MCA. The middle piece of the block left from these cuts was cut coronally just above and parallel to the arc formed by the two anterior cerebral arteries (ACA) in order to obtain a cross-section of the ACA (Fig. 3B). The blocks were embedded in paraffin and 7 µm sections were cut.
Fig. 3. Brain sections. (A) sagittal section of a rat brain. A, B and C are the points where the three coronal cuts were made. (B) two sagittal cuts were made through the anterior portion of the forebrain (block 1) to expose the MCA and ACA.
III.2. Behavioural Testing

III.2.1. Overview
Spatial learning and memory were assessed using a modified version of the MWM. The MWM consisted of a circular pool 2 m in diameter and 0.75 m in height (Fig. 4A). It was filled with water to a depth of 0.4 m and kept at room temperature. Non-toxic blue paint was added to the water. Four equally-spaced points were arbitrarily designated as north (N), south (S), east (E) and west (W) around the circumference of the pool. This established four quadrants (NW, NE, SE, SW). The area of the pool within 20 cm of the outer wall was designated as the perimeter for the assessment of thigmotaxis. The annulus was defined as a circle surrounding the hidden platform that indicates the exact position of the platform. A clear plexiglass platform (10 x 10 cm) was submerged 2 cm below the water level in the middle of one of eight equally spaced arbitrary lines (N, S, E, W, NW, NE, SE, and SW) (Fig. 4B). Six large unique shapes were placed on three walls above the maze to function as distal cues around the pool. A camera mounted in the centre of the ceiling above the pool track the rat’s performance (Poly Track System, San Diego Instruments, San Diego, CA, USA) (Fig. 4A). Behavioural testing was performed between 10:00 and 18:00 hours. All animals were housed at a constant temperature of 22°C, under a 12-hour, light–dark cycle (light switched on at 6:00 a.m.), with free access to food and water.
**Fig. 4.** Morris water maze (A) schematic diagram of MWM: pool with submerged hidden platform is surrounded by unique shapes which provide distal cues. Camera mounted in the ceiling records motions of the animal in the pool. (B) camera view of the MWM arena with arbitrarily designated quadrants, perimeter, annulus and platform regions.
III.2.2. Body Weight
All animals were weighed from day 0 (the day of SAH or sham surgery) to day 8, before any surgical or experimental procedures were performed that day. Body weight was standardized to the initial body weight on day 0 and expressed as percent body weight. Body weight was used to assess feeding and drinking behaviour.

III.2.3. Cued Learning Procedure
Two days after surgery (day 2), cued learning was begun. The location of the platform was indicated by a flag mounted on the platform, which extended 12 cm above the surface of water. White curtains surrounded the MWM to occlude extra-maze cues. Both the location of the platform and the starting positions were pseudo-randomized among the eight locations (N, S, E, W, NW, NE, SE, and SW) for each trial (Table 1). We performed eight trials of 60 second duration for each rat with an inter-trial interval (ITI) of 5 minutes. Rats were allowed to spend 10 seconds on the platform after they climbed onto it. If they could not find the platform within the 60 seconds, they were guided to it and allowed to stay on it for 10 seconds. This was counted as a failed trial. Escape latency and number of failed trials were recorded. All rats were returned to the cage for rest and had full access to food and water.
Table 1. Morris water maze schedule for cued learning procedure. Both the starting and platform positions were pseudo-randomized for each trial among the eight locations (N, S, E, W, NW, NE, SE, and SW) around the circumference of the pool. Each rat performed eight, 60s cued learning trials which was separated by ITI of 5 minutes.

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III.2.4. Spatial Acquisition Procedure

Five hours after the last trial of the cued learning task on day 2 after surgery, rats underwent spatial acquisition procedure over four consecutive days. Each rat performed four, 60 second trials of a hidden platform task (days 2 to day 5 after surgery). The animals performed five trials on day 1 and the last four trials were included for data analysis because the animals find the hidden platform only by chance on the first trial. The platform was hidden in the middle of the NE quadrant (Fig. 3B). The starting position was pseudo-randomized among the seven locations (N, S, E, W, NW, SW, SE) to exclude the shortest path to the goal (platform) (Table 2). By using the maximum number of starting locations, we also tried to avoid allowing the animals to memorize specific routes, rather than using the distal cues. Rats were allowed to remain on the platform for 10 seconds after they either climbed on to it or were guided to it if they failed to find the hidden platform. The ITI was 10 seconds. Escape latency, swimming distance, number of failed trials, and swimming speed were recorded. The following sensorimotor disturbances were measured during spatial acquisition training (1) time
spent in the perimeter: measure of thigmotaxic swimming, the tendency to cling or follow the wall around the outer perimeter of the tank, as an index to reflect that the animal is not problem-solving. (2) swim-overs: when rats swim over the platform on contact; (3) jump-offs: when rats jump off from the platform with their forelimbs on contact. Rats that reach the platform but do not climb on it, or do not stay on it, are not acquiring the requisite association between the platform and escape. These sensorimotor disturbances correlated highly with conventional measures of maze acquisition, accounting for more than 98% of the variance in some cases. Our pilot study showed that rats might exhibit such sensorimotor deficits after SAH when they perform MWM tasks. One rat with hemiparesis had significantly increased time spent in the perimeter, swim-overs and jump-offs in this study was excluded from the data analysis.

Table 2. Morris water maze schedule for spatial acquisition procedure. The starting position was pseudo-randomized among the seven locations (N, S, E, W, NW, SW, SE) around the circumference of the pool while the platform was hidden in the middle of NE quadrant. Each rat performed four, 60s spatial acquisition trials with ITI of 10s on day 2 to day 5.

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<tr>
<th>Trial No.</th>
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<td>SE</td>
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III.2.5. Spatial Reference Memory Test
24 hours after the last trial of the spatial acquisition task on day 6, long-term reference memory of each animal was tested on a probe trial. The platform was removed from the pool and rats were permitted to swim freely for 60 seconds. The starting position was “SW”. The percentage of time spent in each quadrant and the number of annulus crossings as platform-site crossovers were recorded.

III.2.6. Spatial Working Memory Procedure
Rats underwent working memory testing on days 6 to 8. On each day, each animal performed two 60 second trials, the sample (first) trial and the test (second) trial. During this time, rats were allowed to swim freely to find and climb on to the hidden platform positioned in a new location, in one of the four quadrants (NE, NW, SW, SE). The starting position was pseudo-randomized among the eight equally spaced points (Table 3). The ITI was 10 seconds as in the previous spatial acquisition task. Rats were left on the platform for 10 seconds after they climbed on to it or they were guided to the platform after 60 seconds. The time saved in escape latency (seconds) for finding the platform on the test (second) trial from the sample (first) trial was calculated for each day.
Table 3. Morris water maze schedule for working memory procedure. The starting position was randomized among the eight locations (N, S, E, W, NW, SW, SE) as in cued learning tasks while the platform position was randomized among the four quadrants (NE, NW, SW, SE). Each rat performed two, 60s working memory trials with ITI of 10s on day 6 to day 8 after SAH.

<table>
<thead>
<tr>
<th>Day – Trial No.</th>
<th>Platform position</th>
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<td>7 - SW</td>
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<td>8 - SE</td>
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III.3. Morphology

III.3.1. Hematoxylin & Eosin Staining
Sections were obtained as described above and stained with hematoxylin and eosin and representative fields containing cross-sections of the MCA and ACA were scanned at 200 x (Carl Zeiss, MIRAX, Standort Gottingen Vertrieb Deutschland). The lumen area and wall thickness were quantified on using ImageJ (NIH, Bethesda, MD, USA) by an observer blinded to the animal group. The radius of the arteries was calculated from the circumference and artery wall thickness was measured at four perpendicular points around the circumference and averaged. The sectioning procedure was performed in order to ensure cross sections of arteries were obtained perpendicular to the long axis of the artery.
III.3.2. Fibrinogen staining
Fibrinogen was detected in sections using a specific affinity-purified chicken anti-rat antibody to fibrinogen (1:200, Immunology Consultants Laboratory, Newberg, OR, USA). After blocking with PBS containing 5% normal goat serum for 30 minutes at room temperature, sections were washed three times with PBS and incubated with primary antibody for 30 minutes at room temperature. After washing with PBS, sections were incubated with biotinylated goat anti-chicken antibody (1:1000, Millipore, Billerica, MA, USA) for 30 minutes at room temperature. Sections were incubated for 30 minutes with VECTASTAIN ELITE ABC ready-to-use reagent (Vector Laboratories, Burlingame, CA, USA) and fibrinogen immunoreactivity was detected with the VIP peroxidase substrate kit (Vector) and counterstained with methyl green. Negative controls were obtained by omitting the primary antibody.
Fig. 5. Photomicrographs of the ACA. Arrows indicating the wall thickness and lumen radius used to calculate the wall thickness to lumen ratio.
III.3.3. TUNEL and Fluoro-Jade Staining
Apoptosis was detected using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method (DeadEnd Flurometric kit, Promega, WI). Slides were counter-stained with 4',6-diamidino-2-phenylindole (DAPI), washed and cover slipped with a water-based mounting medium, and sealed with nail polish. Neurons in the dorsal hippocampal (CA1, CA3 and dentate gyrus) and cortical regions showing positive staining for both TUNEL and DAPI were counted as apoptotic.

Fluoro-Jade B (Histo-Chem Inc., Jefferson, AR, USA) was used as a marker of neurodegeneration. After incubation with deionized water for one minute, slides were incubated in 0.06% potassium permanganate for 15 minutes with gentle shaking. Slides were then rinsed in deionized water and then immersed in 0.001% fluoro-Jade working solution (0.1% acetic acid) for 30 minutes. Slides were then washed and heat dried at 50-60°C for 10 minutes. Fluoro-Jade positive neurons were counted in hippocampal (CA1 and CA3) and cerebellar regions. They were viewed under a confocal microscope with representative fields digitized. The same parameters (laser power, exposure time, pin hole size) were used for all sections.

III.4. Quantification & Statistical Analysis
Data are expressed as means ± standard error of the mean (SEM). Raw CBF values in tissue perfusion units (TPU) were standardized to the baseline and converted into percent CBF. Body weight was also calculated as a percent of baseline. Vasospasm was quantified by calculating the lumen radius to the wall thickness ratio of the ACA. For
counting positively stained cells or microthromboemboli, we used modified stereologic counting rules\textsuperscript{121}. We chose three regions, namely cerebral cortex, hippocampus and cerebellum for all the quantifications performed on the three histologic endpoints. Cerebral cortex and hippocampus are the regions known for their direct involvement in spatial learning. The histological quantification in these regions would allow us to most directly correlate histological changes to the behavioural changes, although we recognize that multiple brain regions are involved and that this study only correlates the changes observed. At the same time, we also included histological quantification of changes in the cerebellum, to determine see whether histological damages in the cerebellum would also contribute to behavioural deficits. For cerebral cortex, we pre-determined six fixed symmetrical areas on a proper coronal section\textsuperscript{122} for each half of the coronal rat brain slides. We took a total of 12 images for each cerebral cortex section (Fig. 6A). Similarly, for cerebellum, we took a total of six images for each cerebellar cortex (three from each hemisphere both side of the section). These images were used for counting cells in all three stained positively with each staining (TUNEL, fluoro-Jade and fibrinogen). For hippocampus, we counted all the positive cells for TUNEL and fluoro-Jade staining along the pyramidal cell layer (stratum pyramidale) from dentate gyrus to CA1. For fibrinogen staining in the hippocampus, one image was taken from each of the three areas (dentate gyrus, CA3 and CA1). The images were taken in such a way that the image covers most of the tip area of the dentate gyrus (mainly stratum moleculae). For CA1 and CA3, the image centered on the cell layer and covered both the stratum oriens and stratum radiatum. In the cerebellum, the images were taken in the cerebellar cortex area including the granule cell layer, Purkinje cell layer and molecular layer. Purkinje cells were
counted for the TUNEL and fluoro-Jade staining, and fibrinogen positive stained microthromboemboli were counted from all three layers. All counting were done by blinded researchers. All images were of 200 x magnification. The total area of each image covered about 1.1 x 1.5 mm. Cell counts are for the actual images taken, not the estimated total for the whole region or slice.

Behavioural data were analyzed using repeated measures of analysis of variance (ANOVA). CBF, blood pressure, body weight and all histological data, including vasospasm, quantification of TUNEL, fluoro-Jade positive cells and fibrinogen positive microthromboemboli were analyzed using one-way ANOVA with secondary pairwise multiple comparison procedures (Holm-Sidak method). The $\chi^2$ test was used to compare the percentage data such as mortality rate. Correlations were assessed by Pearson coefficients. A p value < 0.05 was considered significant for all statistical analysis.
Fig. 6. Brain regions in the cerebral cortex and the hippocampus taken for histological quantification.
Chapter IV. Physiological Changes after SAH in Rats

IV.1. Introduction
One of the main causes of poor outcome in patients sustaining an aneurysmal subarachnoid hemorrhage (SAH) is the initial effect of the hemorrhage. In fact, the mortality is the highest in the first seven days after the ictus and 15% of the patients die even before reaching the hospital. Investigation of the events occurring immediately after the aneurysmal rupture is not usually possible in humans so most of the data has come from animal models. This has some limitations for the study of SAH, however. Available clinical data and experimental studies do show that several physiological changes occur acutely after SAH.

Decreased cerebral blood flow (CBF)\textsuperscript{73, 123, 124, 125, 126} and the associated transient global ischemia is one of the major cause of morbidity and mortality after SAH.\textsuperscript{32, 127} When an aneurysm ruptures, blood extravasates into the subarachnoid space at close to arterial pressure. The acute increase in intracranial volume elevates intracranial pressure (ICP) dramatically, leading to a sharp reduction in CBF. This perfusion deficit causes transient global ischemia.\textsuperscript{123, 128, 129, 130} Even after the initial increase in intracranial pressure has resolved and the perfusion deficit should have recovered, reduced CBF may persist for hours to days.\textsuperscript{131, 132} These changes are more marked in patients who are admitted in poor neurological grade after SAH and the increase in intracranial pressure (ICP) along with global ischemia probably never resolves and is the cause of death in patients who die immediately after SAH. On the other hand, in cases of milder SAH, the patient may not loose consciousness so it is unlikely that there is any substantial early global ischemia.
in these cases. Thus in many animal models, the changes in physiological parameters especially the increase in ICP followed by sustained reduction in CBF have been used to characterize the acute phase of SAH. More chronically, body weight has been an important physiological as well as behavioural parameter.\textsuperscript{38-40} In this work, we endeavored to separate the effects of SAH from those of transient global ischemia by inclusion of appropriate control groups. This was done to allow testing of the hypothesis that SAH itself is an independent factor leading to brain injury and neurocognitive deficits, in addition to any global ischemic insult.

In this experiment, we recorded acute CBF and arterial blood pressure (BP) changes before, during and acutely after injection of blood or physiological saline into the chiasmatic cistern. CBF was measured by laser Doppler flowmetry (LDF), which gives a measure of relative changes in CBF. Body weight was measured before surgery on day 0 and up to day 8. The main purpose of obtaining these physiological parameters were first, to ensure that the model mimics the early pathophysiology of SAH consistently across the animals and second, to study the initial effect of SAH on these parameters in relation to mortality and other behavioural consequences.

**IV.2. Results**

**IV.2.1. Mortality**
Four of 13 (31\%) and two of 11 (18\%) animals injected with blood and saline died respectively ($p = 0.48, \chi^2$ test). All mortality occurred within 24 hours of surgery. One rat with SAH was excluded from the MWM experiment due to hemiparesis.
Table 4. Mortality and morbidity rates (percent number of animals with any signs of neurological deficit) for the experimental animals after injection of blood, saline or sham surgery. All mortality occurred within 24 hours of surgery. Animals with neurological deficits including general behavioural deficits, paralysis, paresis, impairments in reflexes and any signs of sensorimotor disturbances exhibited on MWM experiments such as jump-offs and swim-overs were excluded from the behavioural experiments.

<table>
<thead>
<tr>
<th>Rates</th>
<th>Group</th>
<th>SAH (n = 13)</th>
<th>Saline (n = 11)</th>
<th>Sham (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality rate</td>
<td>31 %</td>
<td>11 %</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Neurological deficits</td>
<td>7 %</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

IV.2.2. Cerebral Blood Flow (CBF)
There was no significant change in CBF after sham surgery. There was a decrease in CBF immediately after injection of saline or blood. The minimum percent CBF was 6.4 ± 2.5% and 17.7 ± 5.3% of baseline in blood and saline-injected groups, respectively. CBF returned to near-baseline values within 2.5 minutes of the injection (Fig 7). Mild reduction in CBF persisted up to 30 minutes after injection of blood. Animals that died immediately after the injection or within 24 hours tended to have lower minimum CBF (< 10 % of baseline CBF) and a slower return to baseline CBF.

IV.2.3. Arterial Blood Pressure
The only significant change in blood pressure was an increase up to a maximum of 121 ± 10 mm Hg from 95 ± 6 mm Hg at five minutes after SAH compared to an increase from 89 ± 5 to 101 ± 10 mm Hg after injection of saline (p < 0.05, Fig. 8). There were no significant differences in arterial blood pressure between the groups after 5 minutes.
Fig. 7. Percent CBF of the baseline CBF values (average CBF value for 15 min prior to the injection of blood). There was an immediate decrease in CBF after injection of blood or saline ($p < 0.05$ to $0.01$ at different time points). The minimum percent CBF value was lower in SAH group as compared to both of the control groups and there was no difference in the nadir CBF values between the SAH and saline control groups. Animals with SAH showed relatively lower CBF values compared to both of the control groups throughout the observational period.
Fig. 8. Blood pressure measurement. Blood pressure increased to the maximum value 5 minutes after blood or saline injection although only blood injection caused a significantly higher blood pressure than the sham-operation group at this time (p < 0.05).
IV.2.4. Percent Body Weight
Feeding and drinking behaviours were disturbed after SAH as reflected in a significant decrease in percent body weight that persisted for up to four days after SAH (Fig. 9).

Reduction in body weight was $2 \pm 1 \%$ in the control groups and $7 \pm 1 \%$ in the SAH group two days after surgery ($p < 0.05$).
Fig. 9. Body weight expressed as a percent of baseline body weight was lowest 2 days after SAH, saline-injection or sham-operation. Animals with SAH showed the greatest reduction in percent body weight and this persisted up to day 4 (p < 0.05, values are means ± SEM, n = 9 per group).
**IV.3. Discussion**

In this anterior circulation model of SAH in rats, there were disturbances in CBF, arterial blood pressure and percent body weight demonstrating that the model mimics the pathophysiology of SAH that is believed to occur during the early phase of the disease in humans. We did not investigate the effects of ICP and cerebral perfusion pressure directly but it is reasonably safe to assume that there was an increase in ICP following blood or saline injection because an immediate drop in CBF was consistently seen across the animals. This also is consistent with as in previous experimental studies that did measure ICP.\(^1\),\(^2\),\(^26\) Comparison of the SAH group with the saline-injected control group allowed us to separate the effect of ICP from the effect of blood (SAH itself) on CBF and other consequences after SAH. We attempted to standardize the additional injury from increased ICP\(^27\) by maintaining the injection rate at ~ 15 µl/s and by including the control, saline-injected group.

At the time of injection of 300 µl of blood or saline into the prechiasmatic cistern, there was a sharp decrease in percent CBF. The SAH group reached a lower minimum percent CBF and took much longer to return to the baseline CBF as compared to the saline-injected group. Thus, a sustained CBF reduction seems to be mainly associated with blood in the subarachnoid space as opposed to simply an increase in ICP. Formal measurement of ICP would be necessary to prove this but several other findings suggest that blood itself but not the increase in ICP and/or reduction in cerebral perfusion pressure (CPP) is related to sustained reduction in CBF in this model. Sustained reductions in CBF may also contribute to poor outcome of patients. First, SAH and
saline-injected animals had a similar ICP peak but the CBF of SAH animals decreased for at least one hour even though CPP had normalized within minutes. In animals injected with saline, CPP and CBF normalized within minutes. Second, the ICP increase after SAH caused a reduction in CBF only in cases of rebleeding in human patients. Third, the CPP usually does not decrease to the point of perfusion arrest and does not correlate with neurological outcome of patients. Significant elevation in arterial blood pressure after SAH seen in this experiment might also support that the notion that the effects of increased ICP are relatively minor since this would tend to maintain CPP. The hypertensive response has been attributed to ischemia in brainstem centers that control blood pressure.

Causes of reduction in CBF during the acute phase of SAH have been suggested to be acute vasospasm and primary metabolic depression. However acute vasospasm has seldom been directly demonstrated. Indirect evidence includes data showing a decrease in CBF out of proportion to reduced CPP after SAH in a rat model. In humans elevation in blood flow velocity by transcranial Doppler ultrasound was demonstrated. Some studies in humans document reduced cerebral metabolism sometimes out of proportion to the reduction in CBF. Microdialysis studies that measure only very focal areas of the brain also suggest decreased brain metabolism after SAH. Animal studies show that cerebral metabolism may be reduced after SAH although this is not necessarily in direct proportion to the ischemic insult.

Mortality occurred in this model within 24 hours of injection (Table 4) and was
associated with the minimum percent CBF at the time of the injection. Animals tended to
die prematurely when the minimum percent CBF was close to the near arrest value (< 5% of baseline CBF) at the time of injection. This was true for both saline-injected and SAH groups. The higher mortality rate in the SAH group as compared to the saline-injected group (Table 4) suggests additional deleterious effects of blood, which also were associated with lower minimum and sustained reductions in CBF during the acute phase of SAH.

A significant reduction in percent body weight persisted for 4 days after SAH. This suggests that appetite and motivation might be affected by SAH. A small reduction in percent body weight was also noted in saline-injected and sham-operated control groups. Since the rate of increase in percent body weight after day 2 in animals subjected to SAH was similar to that in the control groups, body weight also might be affected by the combined effects of the initial hemorrhage as well as increased ICP and the anesthesia itself. We did not measure the amount of food and water consumption for each animal. Thus this may have been caused either by decreased in food consumption or increased metabolic expenditure. Similar trends in body weight changes after SAH were seen in previous studies. 6, 38-41, 47

**IV. 4. Summary**
Injection of blood into the prechiasmatic cistern caused an acute decrease in percent CBF and an increase in arterial blood pressure. The initial decrease in CBF returned to near the baseline level within a few minutes but a mild reduction as compared to the control
groups and compared to the value before SAH in the SAH group continued acutely after SAH. The direct effect of blood seems to play a role in sustaining the CBF reduction while marked increases in ICP also contribute to the overall acute mortality. Mortality was frequent when the initial CBF reduction was almost to zero percent of the baseline value. Elevation of the arterial blood pressure may play role in compensating for the reduction in CPP. Decreased body weight, which reflects in part appetite and motivation, seems to be affected by the combined effects of SAH, increased ICP and anesthesia, with the most marked effect being due to SAH.
Chapter V. Behavioural Changes after SAH Assessed in the Morris Water Maze

V.1. Introduction

V.1.1. Overview
Patients with subarachnoid hemorrhage frequently have long-term neurocognitive and neuropsychological deficits. Al-khindi, et al., reviewed 61 empirical studies examining cognitive and functional outcome after aneurysmal SAH. The most common neurocognitive deficits were in memory, executive function and language. Review of this literature suggested that the cognitive impairments interact to affect day-to-day functioning, including activities of daily living, instrumental activities of daily living, return to work and quality of life of survivors of SAH. Deficits in cognition and day-to-day functioning may be further compounded by depression, anxiety, fatigue and sleep disturbances. Cognitive impairment tends to improve over the first 12 months after the ictus but patients who are classified as having a favorable outcome on standard outcome scales such as the Glasgow outcome scale still have cognitive deficits that may be related to temporal and frontal lobe dysfunction, such as verbal, spatial memory, spatial working memory, verbal fluency, pattern recognition, executive functioning, and attention which persist indefinitely. Numerous studies showed that visuospatial memory is particularly impaired and has an important effect on functional status, emotional health, and quality of life after SAH. Executive function, attention, concentration and short-term memory are also affected 1 to 5 years after SAH. Return to work is significantly decreased, demonstrating the clinical importance of long-term morbidity. Functional improvement of patients with SAH and chronic cognitive
impairment was associated with improved memory and executive function.\textsuperscript{152} Since spatial learning and memory are among the most frequently affected neurocognitive functions in patients with SAH, we investigated these two factors as well as spatial working memory in a rat model of SAH. We used the Morris water maze (MWM), which is widely used to test these functions, the anatomical basis of which is believed to include both hippocampal and cortical neuronal circuits.\textsuperscript{100} Unlike other maze tests, such as the T or Y mazes where the animal has only a small number of choices (eg. binary for T, tertiary for Y maze), spatial navigation in the MWM requires the animals to learn a specific path to the hidden platform, fixed in a location, by using the spatial relationship of the location of the hidden platform to extramaze visual cues. Numerous other test paradigms have been developed to test other cognitive functions.\textsuperscript{152, 153}

V.1.2. Cued Learning Task
The cued learning task is a control procedure which is sometimes omitted from MWM experiments. This task tests ability to learn to find the hidden platform guided by a proximal visual cue. Curtains are closed around the maze to remove distal cues and a visual cue such as a flag is mounted on the hidden platform.\textsuperscript{116} In order to ensure that the animals do not use any other cues to solve the problem, the goal (platform) and start locations for each trial are randomized among eight relatively equally spaced points around the pool. In the current experiments, a cued learning task was performed to eliminate the possibility of visual impairment as a cause of learning deficits. The model of SAH is by injection of blood into the prechiasmatic cistern in the vicinity of the optic chiasm so injury to the chiasm, visual pathways or possibly the visual cortex has to be
excluded. Thus, any deficits could be attributed to hippocampus-dependent spatial learning deficits as suggested by others. Also, the cued learning procedure is believed to require other basic neurological functions such as motor ability and motivation and thus is also useful to rule out other potential impairments that might affect performance in the spatial learning procedures.

V.1.3. Spatial Learning and Memory
It is accepted that the integrity of the hippocampal formation is essential for spatial learning and memory although there remain multiple theories about the actual neurologic mechanisms and pathways. For example, the hippocampus is necessary for acquisition and retrieval of spatial information as well as for consolidation and storage of this information. Spatial learning and memory tasks in the MWM are particularly sensitive to the effects of hippocampal damage in rats subjected to global cerebral ischemia and traumatic brain injury. Normal rats learn the location of the platform very rapidly.

In spatial learning procedures, animals must learn to navigate a direct path to the hidden platform. In the absence of proximal cues (no cue on the hidden platform), animals are forced to use distal extramaze cues to locate the hidden platform. Standard performance measures for spatial learning in the MWM include escape latency and length of the swimming path (swimming distance), which has been suggested to be the most appropriate index of cognitive performance in the MWM. Path directionality and
cumulative distance swum to reach the platform were also recommended in other studies.

Reference memory is memory that would typically be acquired with repeated training, and that persists from days to months. On the spatial memory task, the probe trial, time spent in different areas of the pool and number of target area crossings (annulus crossing numbers) are sensitive measures for reference memory as they require the animals to remember the precise location of the hidden platform.

There are few previous studies investigating spatial learning impairment after experimental SAH. One study used an intracisternal double injection model of SAH in rats and a second study used the endovascular perforation model. The deficits noted were variable with Takata and colleagues reporting quite substantial spatial learning deficits in a modified version of the MWM in animals weeks after SAH. Silasi and Colbourne, however, detected only very minimal changes. Investigation of neurocognitive impairment after SAH induced by anterior circulation injection (injection into the prechiasmatic cistern) has not been done previously.

V.1.4. Working Spatial Memory
Working memory comprises executive and attentional aspects of short-term memory which require the animal to acquire skills and rules to perform a task. Frontal/prefrontal function is probably necessary. Eichenbaum and Cohen defined working or trial-dependent memory as a type of short-term memory that involves active
manipulation by an individual of an object, stimulus, or location that is used within a testing session, but not typically between sessions. \cite{117,166,167}

Spatial working memory tests are often done by means of ‘matching-to-sample’ or ‘non-matching-to-sample’ method usually in T, Y or radial arm mazes. The MWM also has been used. In a working memory task in the MWM, animals are required to learn a new location of the hidden platform everyday. This occurs by trial-and-error during the first, sample trial. On the second, test (or matching) trial, savings in recall between the first and second trial are measured.\cite{116} Thus, this procedure is also called “matching-to-place” task. The two trials are usually separated by a short inter-trial interval for one specific location of the hidden platform. When the rat is introduced into the pool with a new hidden platform location (sample trial), they normally acquire the location of the platform very quickly and then find the platform on the second trial (test trial) with significantly reduced escape latency or swimming distance. Time saved in escape latency on the second trial is significantly less in animals with impaired working memory.\cite{169} Despite a high proportion of survivors of SAH having impairment in these neurocognitive functions, these sorts of deficits have never been studied in animal models of SAH.

\textbf{V.2. Results}

\textbf{V.2.1. Control Procedures}

For all behaviour procedures, swimming speed and thigmotaxis (percent time spent in the perimeter of the pool) were recorded and found not to differ significantly between the three groups except for one rat with hemiparesis that had significantly increased time
spent in the perimeter and that showed swim-overs and jump-off's. This animal was excluded from the study.

Rats rarely failed to find the visible platform and if they did, it was usually on the first trial, which happened equally across the groups. There was no difference in the escape latency in the cued learning procedure between the three groups (p = 0.44, degree of freedom = 2, Fig. 10).
Fig. 10. Bar graph of performance on the cued learning task. The escape latency (seconds [s]) to find the platform guided by a proximal visual cue (a flag) on the platform on day two was not significantly different between groups (p > 0.05, values are means ± standard error of the mean [SEM], n = 9 per group).
V.2.2. Spatial Learning and Memory
Spatial learning also was the same for all three groups during the 2\textsuperscript{nd}, 3\textsuperscript{rd} and 4\textsuperscript{th} days after SAH or sham-surgery. Spatial learning deficits, however, appeared 5 days after SAH (Fig. 11A and B). Animals from all three groups learned to find the location of platform each testing day (Fig. 11A-D), but animals with SAH were significantly impaired on this task on the last day (day five). Repeated measures analysis of variance (ANOVA) indicated a significant difference in escape latency (Fig. 11A, p < 0.001) and in swimming distance (Fig. 11C, p < 0.01) between the groups. When the escape latency and swimming distance from all of the testing days was separated into the four daily trials, an ANOVA demonstrated a significant difference between groups in escape latency (Fig. 11B, p < 0.001) and in swimming distance (Fig. 11D, p < 0.05). The percent improvement in animals subjected to SAH also decreased significantly by day five (Fig. 11E, p < 0.05) while animals in the control groups had gradual, non-significant decreases in percent improvements.

There was no significant difference between the groups in reference memory as measured by the percent time spent in the target quadrant (NE) in the probe trial, although animals with SAH exhibited a trend towards fewer annulus crossings (Fig. 12A-B).
Fig. 11. Graph of spatial learning and memory in the MWM. Escape latency and swimming distance presented over 16 trials (A, C) and averaged for each day (B, D) over days 2 to 5. The SAH group exhibited significantly longer escape latency (p < 0.001) and swimming distance (p < 0.01) on day 5 as compared to control groups. The percent improvement in escape latency from the previous training day was significantly lower on day 5 in the SAH group (E, p < 0.05, values are means ± standard error of the mean [SEM], n = 9 per group).
Fig. 12. Bar graph of spatial reference memory. There was no significant difference in percent time spent in each quadrant of the MWM (A) and number of annulus crossings (B) of the platform on the probe trial (reference memory test, p > 0.05, values are means ± standard error of the mean [SEM], n = 9 per group).
V.2.3. Spatial Working Memory
Animals with SAH showed no significant deficits on the working memory task (matching-to-place task) six to eight days after SAH as compared to both of the control groups, as shown by the time saved in latency for finding the platform on the second (test) trial compared to the first (sample) trial. There was a trend for animals in the SAH group to have showed a trend to longer times (Fig. 13).
Fig. 13. Graph of working memory. Working memory was measured on days six to eight after SAH or sham surgery as time saved on the second trial compared to the first trial. There was a tendency for reduction in time saved (seconds) in the SAH group on day eight (values are means ± SEM, n = 9 per group).
**V.3. Discussion**

This study found that rats with anterior circulation SAH develop deficits in spatial learning that appear days after the ictus. Behavioural changes in animals with SAH were detected starting five days after SAH. This was after 16 trials of hidden platform training. Significantly increased escape latency and swimming distance on this day coincided with decreased percent improvement in performance (ie. escape latency). Animals from the control groups had the most improvement on the first two days of training after which they plateaued. Animals with SAH, however, did not plateau in their performance but rather they decreased sharply. Similar patterns in escape latency and/or swimming distance were reported previously in animals with hippocampal damage caused by SAH or cerebral ischemia.\(^{35, 66, 73, 74}\) Significant loss of body weight in the SAH group was unlikely to account for the poor performance since the swimming speed was not significantly different among the groups. We did not examine animals for other signs of DCI, such as fine motor or sensory deficits that theoretically could result from SAH and DCI.

Interestingly, there were no significant differences in reference memory among the groups. The spatial learning task in the MWM requires the animal to effectively use distal cues around the pool to navigate to the hidden platform.\(^{116}\) Animals with SAH seem to be less able to process the spatial information regarding the platform location. The long-term memory regarding the general location of the hidden platform would have been acquired through training. This result is also consistent with clinical findings in that humans with SAH usually have relatively intact long-term memory.
Working memory has not been previously studied in an animal model of SAH. The anatomical basis of this form of memory may be different from other types of memory. The hippocampus is critically important for episodic memory function (memory for specific experiences that occur within a defined spatial/temporal context) whereas the prefrontal areas function in executive and/or organizational functions. We tested spatial working memory six to eight days after SAH or sham surgery by using a randomized location of the platform each day. Time saved in latency to swim to the hidden platform between the first and second trials on a given day was used as a measure of the rats’ working memory for the platform location. Working memory was not impaired by experimental SAH. The experimental results are consistent with clinical studies suggesting defects in executive function are less common after human SAH than are deficits in functions mediated by the temporal lobe. On the other hand, there may be clinically important deficits in executive function after SAH in humans. Hillis and colleagues suggested that deficits in frontal and prefrontal lobe function after SAH may be more related to effects of surgery, anesthesia, antiepileptic medications and general effects of bed rest and debility rather than SAH alone, which causes more hippocampal dysfunction. Knowledge is deficient in this area in both the experimental and clinical arenas. Further experiments are necessary to clarify deficits that are present after experimental SAH, as well as more refined testing of patients.

Few of the tested animals exhibited visible deficits in motor or sensory function, at least as assessed by measures such as thigmotaxis, swim-overs and jump-offs.
Detecting these deficits is important since they may impair performance in the MWM. One animal was excluded from the study due to hemiparesis. There was no difference in the performance on the cued learning task among the groups, which supports the contention that visual impairment did not contribute to the spatial learning deficits. Sensory and motor function have been investigated in many studies of experimental SAH in different species and the findings are consistent with ours and with those seen clinically. Most animals and patients that survive SAH do not have gross neurological deficits but rather suffer from cognitive deficits.  

Many cognitive deficits may occur after SAH, although the most common involve memory, executive function and language. 62, 176, 177 Few studies have investigated these functions experimentally. Earlier studies of experimental SAH assessed behaviour in rats using mainly tests of sensory and motor function. 6, 39-42, 47, 84 These include the beam walking test, which assesses locomotor activity but also some aspects of cognition in that it includes a learned avoidance test. 41 Animals with SAH usually had more enduring deficits on these tests as compared to other sensory and motor tests on which they tended to show only minor, transient deficits.

Two studies used the MWM to investigate spatial leaning deficits after SAH in rats. Findings were similar to the current study in that SAH caused minor cognitive impairment in the MWM but no motor deficits. 43, 44 There are some differences, however. Silasi and Colbourne used an endovascular perforation model of SAH in rats, which induces SAH as well as a substantial increase in intracranial pressure and
associated transient global ischemia. Thus, any abnormalities detected would be due to some combination of these processes. They concluded that this model would be problematic to use for studies testing treatments for SAH because there was great variability, mortality was high and surviving animals tended to have mild cognitive deficits. On the other hand, this is very much like human SAH. Takata, et al., noted marked abnormalities in rats that underwent SAH by two injections of blood into the cisterna magna. Animals showed significant, prolonged reductions in regional cerebral blood flow (CBF) that were associated with microvascular perfusion deficits, neuronal injury, impaired motor function and decreased spatial learning. Impaired motor function and indeed, the marked reduction in cerebral blood flow and many of the other findings of this study, were much greater than observed in prior studies although the spatial learning deficits are at least consistent with those observed here and by Silasi and Colbourne. On the other hand, the model used, which was the cisterna magna injection model of SAH is simple, standardizable, reproducible and widely used but is relatively less severe, in part due to most of the blood injected being cleared rapidly away into the spinal canal. For this reason, many studies use a “double” hemorrhage model in dogs, rats, and rabbits and dogs. Two injections are needed to increase the severity but this is unlike human SAH where there usually is only a single hemorrhage.

The strength of the MWM is that it involves spatial components, the task depends primarily on analysis of latency or search patterns and the motivational level can be easily controlled via manipulations of the water temperature. This is useful
since rats are excellent swimmers. The MWM is a useful alternative to tasks where appetite is the motivating factor since these may pose difficulties for manipulation of experimental variables which involve deprivation such as in the force-choice alternation task. It also is not easy to test spatial discrimination and working memory on the basis of error scores. This is simpler in the MWM. Another use of the MWM would be for testing a conditional discrimination component, which is known to be very sensitive in detecting pre/frontal lobe dysfunction. Since our MWM working memory task did not have a conditional discrimination component, we may not have detected missed changes in pre/frontal lobe dysfunction. This is an important area for future investigation.

**V.4. Summary**

SAH induced by prechiasmatic injection of autologous blood in rats was associated with the delayed appearance of a spatial learning deficit. This coincided with a significantly decreased percent improvement in the performance. There was no significant memory (reference and working) impairment although there was a tendency for poorer performance in these tasks in animals with SAH. No major sensory or motor deficits seem to occur after SAH in this model in rats and they probably do not affect performance of spatial learning and memory in the MWM.
Chapter VI. Mechanisms of Learning Deficits after SAH

VI.1. Introduction

VI.1.1. Overview
The cause of poor outcome after SAH is certainly multifactorial, with contributions from transient global ischemia, effects of subarachnoid blood itself, cerebral infarction from treatment of the ruptured aneurysm, medical complications and delayed complications such as angiographic vasospasm and delayed cerebral ischemia (DCI). Long-term neurocognitive impairment after SAH has been attributed to all of these processes, meaning that the mechanisms involved are several. Delayed complications, however, generally manifest as DCI, have been postulated to be due to angiographic cerebral vasospasm, microthromboembolism, cortical spreading ischemia and microcirculatory dysfunction.16, 28, 186-188

Cerebral vasospasm is vasoconstriction of the intradural, conducting arteries of the circle of Willis that occurs 4 to 12 days after SAH. It has been associated with decreased cerebral blood flow (CBF), which can lead to death and stroke even after the patient survives the initial SAH.68, 72, 189-194 Treatments that decrease angiographic vasospasm, such as nimodipine195 and possibly magnesium sulfate196, also tend to reduce DCI and poor outcome. Thus it has been generally accepted that angiographic vasospasm is an important cause of DCI.78, 197, 198 However, there is emerging evidence that other processes contribute to DCI. This includes the observation that the degree of major artery luminal narrowing does not always correspond to development of DCI123, 199-201 or to CBF reduction.123 This suggests that there are other causes of
reduced cerebral perfusion and subsequent development of DCI after SAH in addition to vasospasm. These could be microcirculatory vasoconstriction \(^{10, 187, 202-205}\) cortical spreading ischemia and microthromboembolism. \(^{169}\)

**VI.1.2. Association of Prolonged Cerebral Perfusion Deficit and Poor Outcome after Subarachnoid Hemorrhage**

Decreased CBF occurs in the acute phase of SAH (Chapter III). There also, however, may be a prolonged reduction in CBF days after SAH in humans. \(^{32, 127}\) Numerous experimental studies also found an association between reduction in CBF and neurological and behavioural outcome. For example, decrease in laser Doppler flowmetry (LDF) in both hemispheres of rats with SAH was accompanied by a significant deficit in general behaviour on a gross neurological assessment scale. \(^{110}\) Magnetic resonance perfusion weighted imaging was used to measure regional CBF after SAH in rats. \(^{206}\) An association between reduced regional CBF and neurological deficits was documented. In a double hemorrhage model of SAH in rats, regional CBF reduction lasted as long as 14 days after SAH and correlated with spatial learning performance in the Morris Water Maze (MWM). \(^{44}\)

**VI.1.3. Ischemia-Induced Hippocampal Dysfunction as a Mechanism of Learning and Memory Deficits after Subarachnoid Hemorrhage**

The hippocampus is believed to be important for spatial learning and memory (Chapter IV). Hippocampal cells, especially CA1 pyramidal cells, are known to be highly vulnerable to effects of reduced blood flow which has been suggested to be the mechanism of neurocognitive impairment associated with other brain disorders such
as Alzheimer disease, vascular dementia, brain ischemia and traumatic brain injury. Focal and transient global cerebral ischemia also can cause learning and memory impairments associated with ischemic neuronal injury in the hippocampus. However, the correlation between cell loss in CA1 and the degree of impairment in learning and memory has been variable. For instance, Jaspers et al found water maze acquisition deficits after both two and four vessel occlusion models of transient global ischemia in rats even though there was no CA1 cell loss in animals with two vessel occlusion. Thus, necrotic or apoptotic neuronal cell loss may not be necessary for development of learning and memory deficits. Instead several neurochemical changes such as reduced cytosolic inositol phosphate levels have been suggested to cause these deficits. These authors argued that functional changes and reorganization within the hippocampus may be sufficient to produce behavioural deficits which would not necessarily be related to the extent of CA1 cell loss. Other studies showed that mild to moderate reduction in CBF (25~50% of control) can cause loss of long-term potentiation (LTP) in the hippocampus and that this can occur in the absence of cerebral infarction, neuronal loss and altered structure.

VI.1.4. Cerebral Vasospasm
DCI and cerebral infarction after SAH have been associated with angiographic vasospasm as mentioned above. Some evidence, however, suggests that other factors contribute to cerebral perfusion deficits and development of DCI after SAH. For instance, the incidence of angiographic vasospasm is up to 70% after SAH while
the incidence of DCI is only about 30%.\textsuperscript{213,214} It may be that the angiographic vasospasm in some cases is not severe enough to reduce CBF. Alternatively, other processes may reduce CBF. Rarely, patients with SAH may develop DCI and cortical band-like infarctions without any evidence of angiographic vasospasm.\textsuperscript{78,215,216} Observations that global reductions in CBF were not restricted to the area of the spastic artery also supports that concept that angiographic vasospasm is not the only cause of CBF reduction and DCI after SAH.\textsuperscript{123} When the correlation between the CBF in the territory of the most spastic artery and the least perfused brain region was evaluated in six patients with SAH, angiographic vasospasm was found to be present in most cases and to decrease CBF but the vasospasm corresponded with the least perfused region in only two thirds of the patients. Also, nearly half of patients with severe vasospasm did not have DCI.\textsuperscript{217}

VI.1.5. Evidence of Microthromboemboli after Subarachnoid Hemorrhage and Their Role in Delayed Cerebral Ischemia

Microthromboemboli may consist of a combination of fibrin thrombi and aggregated platelets. White and red blood cells eventually become incorporated. A role for cerebral microthromboembolism has been suggested to reduce global CBF and contribute to development of DCI after SAH. An autopsy study by Suzuki and colleagues\textsuperscript{217} showed significantly more microthromboemboli in clinically ischemic brain regions and in areas showing cerebral infarction on computed tomographic scans in patients who died from DCI after SAH. Alteration in several hematologic factors also was taken as evidence for formation of microthromboemboli after SAH.\textsuperscript{191} For
instance, in the acute phase after SAH, concentrations of tissue factor in the CSF were elevated\textsuperscript{218} and patients with DCI exhibit significantly higher levels of plasminogen activator inhibitor-1 antigen compared to those without DCI. Patients with DCI after SAH also showed increased concentrations of P-selectin, an adhesion molecule involved in leukocyte adherence to the endothelium. Finally, transcranial Doppler ultrasound monitoring of patients with SAH detected signals in the large basal arteries consistent with emboli.\textsuperscript{311} Experimental evidence for microthromboemboli seldom has been obtained, however, and whether they form after experimental SAH, whether they are thrombi or emboli, and whether they are primarily due to SAH or secondary to angiographic vasospasm is unknown.

In the current study, we examined markers of neuronal injury and ascertained their relationship to proximal large-artery cerebral vasospasm and peripheral microthromboemboli in order to begin to elucidate why spatial learning deficits may occur after SAH.

\textit{VI.2. Results}

\textbf{VI.2.1. Quantification of Vasospasm}
SAH was associated with a significant reduction in lumen radius to wall thickness ratio in the anterior cerebral artery (ACA, \(p < 0.01\), Fig. 14A - D).
Fig. 14.: Vasospasm in the ACA was determined by lumen perimeter to wall thickness ratio (A). The SAH group (D) exhibited significantly smaller lumen perimeter to wall thickness ratio compared to sham-operated (B) and saline-injected controls (C, values are means ± SEM, n = 9 per group, p < 0.01, scale bar is 200 µm).
VI.2.2. Microthromboemboli
SAH was associated with significantly more microthromboemboli in the cortex (p < 0.001, Fig. 15) and cerebellum (p < 0.005, Fig. 15). The number of microthromboemboli in the SAH animals also showed a trend to be increased in the hippocampus. The total number of microthromboemboli was significantly increased in SAH (p = 0.001) as compared to both of the control groups.
Fig. 15. Fibrinogen staining in cortical, cerebellar and hippocampal regions. SAH was associated with significantly higher numbers of microthromboemboli in cortical (p < 0.001) and cerebellar sections (p < 0.01) compared to both of the control groups (A, values are means ± SEM, n = 9 per group). There was a trend towards an increased number of microthromboemboli in the hippocampal region of the SAH group. Photomicrographs are sections of cortex, cerebellum and hippocampus from sham, saline and SAH groups (B, scale bar is 200 µm, arrows point to fibrinogen-positive microthromboemboli).
VI.2.3. TUNEL and Fluoro-Jade B Staining
Eighty nine percent 89% (8 of 9) of animals in the SAH group exhibited terminal
deoxyribonucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in the
cortex and hippocampal regions. TUNEL-positive cells were seen in the CA1 and
CA3 pyramidal cell layer. Eleven percent (1 of 9) of animals from each of the control
groups showed TUNEL-positive cells that were seen only in the cortex. The number
of TUNEL-positive cells in the SAH group was significantly higher than in either
control group in cortex, (p < 0.001) and hippocampal region (p < 0.01, Fig. 16) but not
in the cerebellum (p > 0.05). When we summed positive cells from all regions,
animals with SAH exhibited a significantly higher number of TUNEL-positive cells (p
< 0.001). Co-localization with 4',6-diamidino-2-phenylindole (DAPI) confirmed that
TUNEL-positive staining was generally in the nucleus (Fig. 16B).

Rats with SAH showed significantly more fluoro-Jade B positive cells in cortex (Fig.
17A, p < 0.001), hippocampus (p < 0.01) and cerebellum (p < 0.001, Fig. 17A). There
were no fluoro-Jade positive cells in either control group in cortex or hippocampus.
67% (6 of 9) of SAH compared to 11% (1 of 9) of saline-injected rats had fluoro-Jade
positive cells in the cerebellum while none of the sham-operated animals did (Fig. 17).
Fig. 16: TUNEL staining in cortex, cerebellum and hippocampus. SAH was associated with significantly more TUNEL positive cells in cortex ($p < 0.001$), hippocampus (A, $p < 0.01$, values are means ± SEM, $n = 9$ per group). There was a trend towards an increased number of TUNEL positive cells in the cerebellar region of the SAH group. Representative images are from cortex of sham, saline and SAH groups (B, scale bar is 200 µm). In animals with SAH, cells staining for TUNEL co-localized with DAPI nuclear staining (arrows).
Fig. 17. Fluoro-Jade staining in cortex, cerebellum and hippocampus. The SAH animals exhibited significantly more fluoro-Jade positive cells in cortical (p < 0.001), cerebellum (p < 0.001) and hippocampus (p < 0.01) (A, values are means ± SEM, n = 9 per group). Photomicrographs are from cortex, cerebellum and hippocampus (B, scale bar is 200 µm, arrows pointing to fluoro-Jade positive cells).
**VI.3. Discussion**

The deficit in spatial learning that we observed in rats with SAH in the MWM would classically localize to and correlate with neuronal injury in the hippocampus and particularly in the CA1 region. We did find some evidence of neuronal injury in hippocampal areas including CA1 and CA3 although the number of cells affected was relatively small. Evidence of cerebral vasospasm and microthromboemboli in animals with SAH suggests that these mechanisms might contribute to the neuronal injury and development of spatial learning deficits in this model. The delayed onset of spatial learning impairment is reminiscent of DCI occurring after SAH in humans. However, we cannot exclude the possibility that the changes in ICP were different between saline and SAH groups and that this could account for some of the differences in behaviour that were observed in current study.

Prolonged reduction in CBF has been associated with behavioural deficits and neuronal injury after SAH in clinical and experimental studies. Takata, et al., noted marked reductions in regional CBF associated with microvascular perfusion deficits, neuronal injury and impaired spatial learning after SAH induced by two injections of autologous blood into the cisterna magna of rats. The reduction in regional CBF occurred in various cortical and subcortical brain regions including cerebral cortex, cerebellum, hypothalamus, hippocampus and striatum. The authors also detected large artery vasospasm but whether this was severe enough to reduce CBF is uncertain. They did not examine brains for microthromboemboli.
Most of the data suggesting that microthromboemboli contribute to brain injury after SAH are associative or circumstantial. These include detection of embolic signals by transcranial Doppler ultrasound in patients with SAH. \(^{140}\) Alteration in hematological factors such as increased beta-thromboglobulin, thromboxane B2, soluble platelet selectin (sP-selectin) and platelet-activating factor \(^ {23, 224}\) also were taken to suggest that microthromboemoli form after SAH. In patients dying of SAH, multiple small patchy areas of infarction with associated thrombin-antithrombin III-positive intravascular thromboemboli were observed. \(^ {28}\) However, patients dying after SAH have numerous reasons to have infarcts and are a select subpopulation of those with SAH. The present experiments are the first to observe microthromboemboli in small vessels after SAH. Whether these are emboli or thrombi has not been determined, nor has their role in causing brain injury after SAH. Cerebral blood flow and metabolism studies as well as some other method to either modulate their presence or detect them in real-time after experimental SAH, are needed to clarify this.

Other possible explanations for reduction in CBF after SAH could be the “no-reflow” phenomenon \(^ {225}\) due to the obstruction of the microcirculation at the capillary level. This was observed in dogs with SAH that was induced by puncturing an intracranial artery under conditions of a closed skull. The intracranial pressure usually rose markedly, causing transient global ischemia. Perfusion of the brain revealed multiple perfusion deficits. These are likely due in part to reduced CBF rather than a cause of it. \(^ {226}\) What occurs after SAH and when has not been further investigated.

Microthromboemboli could contribute to a no-reflow phenomenon after SAH in rats.
Study of the time course of their appearance in relation to CBF may be helpful to clarify their role. Other possible factors are microvascular constriction and cerebral metabolic depression. Although some studies suggest that small penetrating arterioles are constricted after SAH, the reports concerning microvascular vasospasm have not always been consistent across animal models or in humans and animals. Vasoconstriction in the microcirculation was detected in some studies and suggested to be due to local toxic effects of blood which in turn likely result in focal as opposed to global ischemia. Other studies showed microcirculatory spasm after SAH in patients but were not able to show a direct relation to the pathogenesis of DCI. Finally, positron emission tomography studies reported increased cerebral blood volume after SAH, suggesting that the microcirculation is dilated.

Finally, depression in cerebral metabolic rate of oxygen (CMRO₂) also is frequently seen in patients with SAH who do not have vasospasm. The depression in CMRO₂ correlated with clinical outcome of patients. The relation of reduced CMRO₂ and CBF after SAH is controversial and whether cerebral metabolic disturbances are primarily due to the reduction in CBF after SAH or the CBF reduction is secondary to reduced metabolism is still a controversial topic. A decrease in the cerebral metabolic rate of glucose (CMRglu) was noted after SAH in various animal models. Other studies of CMRglu studied the acute stage after SAH and are conflicting. Further investigation is necessary.
The degree of neuronal injury was assessed semiquantitatively using TUNEL and DAPI double staining and fluoro-Jade staining. TUNEL is a widely used stain for measuring apoptosis. When double stained with DAPI, a nucleic acid stain which strongly binds to the minor groove of double-stranded DNA (dsDNA) and based on the morphology of stained cells, it is possible to state that the apoptotic cells were neurons. Fluoro-Jade staining also is widely used to identify neurons that are undergoing degeneration, although the method by which it does so is not entirely understood. The degree of neuronal injury observed in the hippocampus was mild and similar to that in other brain regions after anterior circulation SAH. Neuronal injury and loss did not appear to be severe enough to account for the deficits observed in the MWM. Prior studies have shown that severe reductions in CBF (> 40–50% of control) after SAH are correlated with extensive global ischemic damage and early mortality while mild to moderate (~ 25%) lasting global reductions in CBF after experimental SAH did not result in infarction or selective neuronal death. Based on this, we speculate that anterior circulation SAH in rats results in a prolonged mild to moderate reduction in CBF. This suggests some mechanism other than neuronal death is responsible for the spatial learning deficit. In fact, hippocampal neurons are highly vulnerable to hypoxic/ischemic insults and decreased cerebral perfusion and there is evidence that chronic but mild disturbances in the cerebral circulation can cause cognitive impairment in the absence of cerebral infarction, neuronal loss and altered structure. Measurements of CBF and cerebral metabolism are needed to confirm or refute this hypothesis.
Impairment of long-term potentiation (LTP) in hippocampal neurons was postulated to be an electrophysiologic correlate of the cognitive deficits in these studies. Loss of LTP has been reported in the hippocampus of rats with anterior circulation SAH as induced in this study.  

**VI.4. Summary**

There was evidence of proximal and distal cerebral circulatory abnormalities as shown by cerebral arterial vasospasm and microthromboemboli in rats with anterior circulation SAH. Neuronal injury was mild, suggesting that other functional abnormalities account for learning deficits observed in the MWM. Possible mechanism of this learning deficit is hippocampal dysfunction due to loss of LTP in the hippocampus. Cerebral blood flow and metabolism studies are necessary to investigate the relationship of microthromboemboli formation and CBF reduction after SAH.
Chapter VII. Conclusion

VII.1. Summary of Results

VII.1.1. Overview
The primary aim of this thesis was to investigate neurocognitive deficits in rats with anterior circulation subarachnoid hemorrhage (SAH) and begin to elucidate why they occur. Clinical evidence has suggested that cognitive, memory and neuropsychological impairments after SAH have considerable impact on the quality of life of patients who survive from the initial SAH. Experimental studies of cognitive and memory impairment after SAH have seldom been performed and there is a need for further studies to determine the mechanisms of these impairments. This was one of only a few studies to investigate these impairments after SAH in rats. The hypothesis that behavioural deficits after SAH are caused by dysfunction of neuronal pathways in the hippocampus and/or death of neurons in the brain was supported by spatial learning deficits found in animals with SAH when tested in the Morris water maze (MWM) using paradigms that rely on hippocampal function. Since only minimal hippocampal neuronal death was observed, we hypothesize that the cause of the spatial learning deficits involves mechanisms other than neuronal death. Inclusion of a control group that separates the effect of transient global ischemia from SAH itself shows that SAH was in fact an independent factor leading to brain injury and neurocognitive deficits, in addition to any global ischemic insult.
VII.1.2. Physiological Changes After Subarachnoid Hemorrhage in Rats
The initial event of the hemorrhage is known to be one of the main causes of poor outcome after SAH. Investigation of changes in several physiological parameters allowed us to study the initial effect of SAH in relation with mortality and other behavioural consequences. Anterior circulation SAH in rats caused an acute decrease in percent cerebral blood flow (CBF) and an increase in arterial blood pressure during the acute phase of SAH. The initial CBF decrease was probably due to the sudden increase in intracranial pressure (ICP). CBF tended to return to near baseline level within minutes but a mild reduction persisted in the SAH group. When the initial CBF reduction approached 100 percent of the baseline value, mortality was frequent. We concluded that the direct effect of SAH plays a role in sustaining the CBF reduction while a marked increase in ICP also contributes to the overall acute mortality. Body weight seems to be mostly affected by SAH itself, although the combined effects of SAH, increased ICP and anesthesia also contribute to effects on appetite and motivational function, which in part are reflected by body weight changes.

VII.1.3. Behavioural Changes after SAH Assessed in the Morris Water Maze
Neurocognitive impairment after SAH impacts on quality of life in patients with SAH by interacting with day-to-day functioning. Spatial learning and memory are among the most frequently affected neurocognitive functions in patients with SAH. Investigation of such impairments in an animal model of SAH was thus performed and the MWM was used to test these functions in this experiment. Anterior circulation SAH in rats was associated with delayed appearance of a spatial learning deficit,
which coincided with a significantly decreased percent improvement in the performance. There was no significant memory (reference and working) impairment and no major sensory or motor deficits seem to occur after SAH in this model. Thus motor and sensory deficits probably do not affect performance in spatial learning and memory tasks in the MWM.

VII.1.4. Mechanisms of Learning Deficit after Subarachnoid Hemorrhage

Neurocognitive impairments after SAH have been attributed to the immediate and delayed cerebral ischemia (DCI). The mechanism of development of DCI and how it causes the neurocognitive impairments are unknown. To test the hypothesis that the behavioural deficits after SAH are caused by hippocampal dysfunction and/or death of neurons in the brain, especially in the hippocampus, and that these changes are independent of vasospasm and are due to direct effects of the SAH, we examined markers of neuronal death and investigated if these occur with evidence of cerebral vasospasm and microthromboemboli. We found evidence of cerebral vasospasm and microthromboemboli that were associated with a minor degree of neuronal death. We concluded that functional abnormalities and processes other than direct neuronal death account for the learning deficit. Supporting evidence that learning was impaired was that there was loss of long-term potentiation (LTP) in the hippocampus of rats with SAH. Cerebral blood flow and metabolism studies are necessary to investigate whether vasospasm and microthromboemboli formation are sufficient to cause reduced CBF reduction after SAH and account for some of these deficits.
VII.1.5. Conclusion
Anterior circulation SAH in rats was associated with spatial learning deficits in the MWM, which is a well-established behavioural device for testing hippocampal-dependent learning and memory in rodents. The anterior model resulted in mild cognitive impairment and a minor amount of neuronal loss. Whether the neuronal loss was enough to cause the cognitive impairment requires further study. There was evidence of large-artery and peripheral cerebral circulatory disturbance shown by arterial vasospasm and microthromboemboli associated with anterior circulation SAH. Control animals injected with physiological saline did not display significant spatial learning deficits, neuronal death, cerebral vasospasm or microthromboemboli which supports our hypothesis that these changes are independent of increased intracranial pressure and are due to direct effect of the SAH.

VII.2. Future Directions
VII.2.1. Cerebral Blood Flow and Metabolism
Numerous prior studies investigated CBF and metabolism in various animal models and in humans. An initial step required in subsequent experiments is to determine in the rat anterior circulation model of SAH that the changes we observed are associated with reduced CBF and metabolism. If a reduction is not seen, or there is reduced metabolism with normal CBF, then this suggests other mechanisms for memory and learning deficits. If CBF and metabolism are reduced, with CBF being reduced primarily, then large-artery vasospasm and/or microthromboemboli may be responsible. We could use a drug such as the endothelin-receptor antagonist,
VII.2.2. Microthromboemboli
Chapter V discussed possible mechanisms of CBF reduction after SAH. Clinical and experimental studies including the current study point to microthromboemboli formation after SAH as a potential mechanism. It is assumed that these are a cause of brain injury after SAH, yet focal infarcts were not seen in brains of animals with microthromboemboli. Further experiments are needed as mentioned above to determine if these lesions reduce CBF after SAH. Next, it needs to be determined if they are thrombi, emboli or some combination of both. Proteins such as p-selectin and tissue factor (TF) are good candidates to be assessed, as well as electron microscopy to differentiate the two processes. For example, during cerebral ischemia, TF interacts with circulating factor VII to generate the TF and resulting VIIa complex initiates coagulation system activation and thrombosis. Thus it is the primary initiator of coagulation that activates thrombin, which in turn converts fibrinogen to fibrin. P-selectin is not normally detectable in the blood vessels of cerebral cortex and it is only up-regulated under pathogenic stimuli. It plays an essential role in the earliest step of the recruitment of leukocytes, capture and rolling along the endothelium thus
slow the circulating leukocytes under pathogenic conditions such as ischemia. This leads to break down of endothelium and leukocyte infiltration into the brain parenchyma eventually.

VII.2.3. Mechanism of Hippocampal Dysfunction
Long-term potentiation (LTP), a form of synaptic plasticity at hippocampal synapses, is a cellular mechanism that can be studied as a mechanism of hippocampal dysfunction and learning deficits after SAH. A previously study investigated LTP after SAH in rats and showed that SAH in rats was associated with loss of LTP in the CA3-CA1 pathway in the hippocampus while there was little neuronal or other structural damage that could account for this. Based on the findings of the current study, which included a minor but significant impairment in spatial learning and a minor amount of neuronal death, we postulate that the learning impairment in rats with SAH may be due to the loss of LTP in the hippocampus. There is evidence in other models that LTP is substantially attenuated in the hippocampus, without substantial neuronal death, after transient global ischemia.

A good candidate for the mechanism of loss of LTP is the 1-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptor and downstream processes mediated by it. Tariq et al. showed that SAH was associated with significant decrease in postsynaptic responses such as the excitatory post-synaptic potential and population spike but not in basic neurotransmission in CA3-CA1 pathway. This study also showed that the response level of LTP during the induction phase (quantified
immediately after the high frequency stimulation [HFS]) and the maintenance phase (in the last 10 minutes of the 1 hour recording after HFS) were similar in SAH, saline-injected and sham-operated rats. Thus, it was suggested that the molecular machinery for induction of LTP such as N-methyl-D-aspartate (NMDA) receptors were intact but the molecular components for maintenance of LTP such as AMPA receptors and their associated trafficking, calmodulin kinase II activation and other downstream processes may be impaired.  

This study was a critical but only a first step in long-term behavioural research in an animal model of SAH. Investigation on the pathological mechanism of neurocognitive impairment after SAH will require ongoing efforts using behavioural, electrophysiological, imaging, and molecular studies of SAH before the mechanisms can be unraveled.
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