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Maheen Nadeem April 9, 2016

Assessment of Cognitive Impairment in Subarachnoid Hemorrhage Induced Brain Injury:

Neurobehavioral Outcomes in a Murine Model

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Abstract

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A large amount of money is spent on clinical trials for subarachnoid hemorrhage (SAH) treatments and many of them have been unsuccessful. Clinical trials should be pursued after improvements have been shown in clinically accurate animal models. Disability is highly prevalent in SAH survivors. This population has more individuals that fit diagnostic criteria for anxiety and depression than healthy reference populations and 25% of employed SAH survivors leave their jobs. Neurobehavioral deficit is thus a key outcome for animal models of SAH.

The two most commonly studied animal models of SAH are either arterial puncture models or blood injection models. Larger animals are generally easier to perform surgical methods, however small rodents are more cost-effective than larger animals. Mice provide opportunity for future transgenic study. Blood injection models are less variable between animals since hemorrhage volume is controlled via injection. In the present study, a blood injection SAH model of mice was evaluated for neurobehavioral deficits using three techniques: Modified Garcia Neurological Score, Elevated Plus Maze, and Morris Water Maze. Degeneration of hippocampal and cortical neurons was also measured with Flouro-Jade-C staining to compare SAH animals with sham controls.

Our cisterna magna injection model of SAH did not produce significant deficit in sensorimotor ability, anxiety-related behavior, or spatial learning. Fluoro-Jade-C staining also did not show enhanced neurodegeneration in SAH animals. Failure to show deficit in this model restricts the value of SAH studies in mice given that our blood injection model did not show significant functional deficit. Rat models provide a better opportunity for preclinical SAH research as they have repeatedly been shown to have deficits in our performed tests. Further investigation should be done to show that mice models of SAH that are used in justifying clinical trials do indeed present with functional neurobehavioral deficits.

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1. Introduction/Background

1.1 Subarachnoid Hemorrhage in the Clinical Population

Aneurysmal subarachnoid hemorrhage (SAH) is a form of hemorrhagic stroke that develops after an intracranial aneurysm rupture and results in pooling of blood in the subarachnoid space (Macdonald, 2014). Mortality from SAH can be as high as 50% on presentation with high rates of delayed mortality and severe disability for survivors. Reports have indicated that SAH affects 9.7-14.5 individuals per 100,000 people per year in the United States (Labovitz et al., 2006, Shea et al., 2007, Feigin et al., 2009). A recent study calculated an adjusted odds ratio to be 1.34 for SAH in women (95% CI 1.05-1.64) between 1985 and 2011 (Algra et al., 2012) and average age of incidence is around 50 for SAH (Ingall et al., 2000). Black individuals are 2.1 times (95% CI 1.3-3.6) more likely to develop SAH (Broderick et al., 1992).

1.2 Epidemiology

Explanations for the cause of aneurysmal rupture in the brain are varied and inconclusive. Risk factors for SAH generally contribute to increased vulnerability of blood vessels in the brain through loss of strength and/or stability of the arterial wall. Cigarette smoking, hypertension, heavy alcohol consumption and diabetes mellitus are associated with higher likelihoods of aneurysm development and rupture leading to SAH (Andreasen et al., 2013). Evidence that smokers are at significantly higher risk of SAH compared to non-smokers has been repeatedly shown in studies that suggest smokers are between 3.8-11.1 times more likely to develop SAH (Petitti and Wingerd, 1978, Bonita, 1986, Longstreth et al., 1992, Juvela et al., 1993). Cigarette smoking has been shown to inhibit α1-antitrypsin which is an important inhibitor of proteolytic enzymes (Schievink et al., 1996). Dysregulation between proteases and their inhibitors can promote degradation of arterial walls, which increases the likelihood of aneurysm formation.

Hypertension combined with smoking, especially in women, greatly increases risk of SAH (Knekt et al., 1991). Heavy binge drinking also increases the risk of SAH (Longstreth et al., 1992, Juvela et al., 1993). Consequently, patterns of behavior in patients can be helpful for clinicians to counsel patients with unruptured aneurysms.

1.3 Presentation

The most common symptom in SAH patients that brings them to hospitals is acute onset of severe headache (Schievink, 1997). Minor leaks that present with earlier, milder headaches can serve as preemptive warning signs of SAH (Leblanc, 1987). Computerized tomography (CT) scans missed 55% of minor leaks in patients while lumbar puncture was always positive for minor leaks in Leblanc's 1987 study. Intraocular hemorrhage can be found in one fourth of SAH patients so ophthalmologic examination, in addition to CT scans of the brain, may be useful in diagnosis (Garfinkle et al., 1992). Hydrocephalus was found in half of patients (N=3521) in an international clinical trial study evaluating timing of surgery in patient outcome (Kassell et al., 1990). Meningeal irritation can be found in SAH patients and results in significant back and neck pain caused by hemorrhagic cerebrospinal fluid (CSF) (Schievink, 1997). Complications mentioned in the following section can serve as additional poignant markers of SAH during diagnostic evaluation.

1.4 Complications leading to poor outcome

1.4.1 **Sudden Death:** While mortality rates of SAH, ischemic stroke, and intracerebral hemorrhage have decreased in high-come countries since the late 1980s (Ingall et al., 1989, Hop et al., 1997, Graeff et al., 1998, Feigin et al., 2009, Poisson et al., 2014), sudden death is not uncommon in SAH victims. In an epidemiological study that evaluated the SAH population of Rochester, Minnesota from 1960-1989, 13 out of 113 (12%) of victims died before hospital

admission and 92% of those patients showed intraventricular hemorrhage at autopsy (Schievink et al., 1995).

1.4.2 Vasospasm leading to Delayed Cerebral Ischemia: Cerebral vasospasm is a secondary event in SAH patients that accounts for major morbidity and mortality (Kassell et al., 1990). Cerebral vasospasm is confirmed with cerebral angiography, occurs 3 to 15 days after the initial hemorrhage, and is related to the extent of hemorrhage found in the CT scan (Weir and MacDonald, 1993). Delayed cerebral ischemia (DCI), a later pathophysiological outcome due to restricted blood flow caused by vasospasm, is also a key contributor to poor outcome in SAH survivors (Macdonald, 2014) and can result in brain infarcts in up to 40% of SAH patients (Rabinstein et al., 2004). Symptoms of ischemia develop in 50% of patients with vasospasm that is clearly visible in angiography (Connolly et al., 2012). Large artery spasm does not alone indicate DCI since extent of distal vessel autoregulation, or helpful vasodilation in response to increased cerebral pressure, varies from person to person (Yundt et al., 1998).

1.4.3 Inflammation: Inflammation has been proposed as a major contributor in the pathophysiology of cerebral vasospasm and DCI after SAH (Pradilla et al., 2010). Red blood cells that leave a ruptured aneurysm release free hemoglobin (Hgb) that is toxic to the cerebral microenvironment (Ascenzi et al., 2005). The immune system upregulates cell adhesion molecules (CAMs) to counteract Hgb toxicity and allow for macrophages and neutrophils to phagocytose RBCs and remove Hgb (Gallia and Tamargo, 2006, Pradilla et al., 2010). Reduced CSF flow due to vasospasm causes macrophages and neutrophils to collect in the subarachnoid space and eventually die and degranulate (Dietrich and Dacey, 2000). These immune cells release cytokines, chemokines, oxygen free radicals, and other toxic substances that can lead to chronic inflammation (Springer, 1994). Injured endothelium then recruits endothelin-1 (ET-1), a

potent vasoconstrictor that worsens cerebral vasospasm (Nishizawa and Laher, 2005). Another contributing phenomenon to post-SAH vasospasm relates to nitric oxide (NO) metabolism. Free Hgb preferentially binds NO and reduces its circulation that would otherwise combat vasospasm through vasodilation (Budohoski et al., 2014). NO has been shown to be significantly reduced in endovascular perforation rat models of SAH (Sehba et al., 2000).

Other circulating explanations for post-SAH mechanisms that lead to poor outcome implicate free radicals and oxidative stress (Gaetani and Lombardi, 1992, Gaetani et al., 1998, Kamezaki et al., 2002), diminished microcirculation (Yundt et al., 1998), microthrombosis (Ohkuma et al., 1991, Ikeda et al., 1997, Suzuki et al., 1999, Frijns et al., 2006), increased blood-brain barrier permeability (Germano et al., 2000), and hydrocephalus (Hasan et al., 1989, Nakazato et al., 2002, Budohoski et al., 2014).

1.5 SAH survivors: Independently maintaining health, not day-to-day functioning

Based on 21 SAH patient population studies between 1960 and 1992, mortality, including pre-hospital deaths, varied between 32 and 67% with a weighted average of 51% (Hop et al., 1997, van Gijn and Rinkel, 2001). While case-fatality of SAH was shown to have gradually decreased in this study, dependence measured by the modified rankin scale (MRS) of SAH patients after they were discharged ranged between 10 and 20% (Hop et al., 1997). A randomized, double-blinded, placebo-controlled clinical trial showed an association between decreased infarction and better functional outcome as measured by the widely used MRS or extended Glasgow outcome scale (Table 1) (Bonita and Beaglehole, 1988, Vergouwen et al., 2011). Outcome evaluation through these scales only signify how likely an SAH survivor is to require medical assistance. These scales are not sensitive enough in elucidating the effect on quality of life in independent SAH survivors (Mayberg et al., 1994). One study asked SAH survivors about their quality of life

post-SAH, and only 19% of independent survivors claimed that they had no reduction in quality of life (Hop et al., 1998). Reevaluation 18 months later of that cohort found an increase to only 31% (Hop et al., 2001). Hop et al.'s 1998 study used the short form 36 (SF-36) health survey questionnaire (SF-36). This questionnaire asks patients about their health perception on eight dimensions (Table 2) (Brazier et al., 1992). Physical role limitations and social functioning were the lowest scoring categories in this study.

SAH survivors are also more likely to develop anxiety and/or depression-related disorders (Wermer et al., 2007). The hospital anxiety and depression scale is commonly used to evaluate mood states in SAH survivor populations. While ~5% of control populations are diagnosed with anxiety/depression with this test, SAH survivors have shown a 14 to 16% prevalence for these disorders (Powell et al., 2002). A prospective study of 105 patients found 37% of SAH survivors to fit diagnostic criteria for PTSD three months after SAH (Noble et al., 2008). Anxiety was either moderate or severe in 40% of patients evaluated as late as sixteen months after the initial hemorrhage (Morris et al., 2004). In Wermer et al.'s study, 59% of patients complained of alterations in personality with irritability and emotionality being the most commonly described changes (Wermer et al., 2007).

SAH survivors experience many other neurological impairments including deficits in executive functioning, memory, language, and attention (Al-Khindi et al., 2010). At three months after hospital admission, 29% of SAH patients were found to score below the tenth percentile in a prose recall test that asked them to recall as many ideas or facts that they had just heard from a spoken story (Powell et al., 2002). In another study, 42% of patients showed significantly impaired verbal memory (Mayer et al., 2002). Global cognitive impairment is present in many SAH survivors up to a year after hospitalization (Springer et al., 2009). Using

the Telephone Interview for Cognitive Status in 232 SAH survivors, Springer et al. found cognitive impairment in 27% of subjects three months after hospitalization and in 21% of subjects 12 months after hospitalization that was predicted by DCI (Springer et al., 2009). Cognitive impairment can influence an SAH survivor's working life. In a study that interviewed independent SAH survivors who received clipping, one quarter of employed survivors stopped working and about another quarter of those independent survivors took on fewer hours or a position with lesser responsibility (Wermer et al., 2007). Only half of those survivors' working lives remained unchanged after SAH.

Deficits impose difficulties for survivors' day-to-day functioning and demand that patients remain dependent on others in their lives even if they are technically independent from reliance on medical care. Those affected by SAH are on average younger than most ischemic stroke survivors (52 years versus 70-80 years) (Taylor et al., 1996). Thus, SAH survivors impose a larger cost to society, especially their loved ones, than ischemic stroke survivors. The low prevalence of favorable outcome after SAH demands that preclinical research continue to investigate cognitive decline in this disease.

Modifed Rankin Scale	Extended Glasgow Outcome Scale	Score
No symptoms at all	/	0
No significant disability despite symptoms; able to carry out all usual duties and activities	**Death (D)**	1
Slight disability; unable to carry out all previous activities, but able to look after own affairs without assistance	**Vegetative state (VS)**	2

Moderate disability; requiring some help, but able to walk without assistance	**Lower severe disability (SD-)**	3
Moderately severe disability; unable to walk without assistance and unable to attend to own bodily needs without assistance	**Upper severe disability (SD+)**	4
Severe disability; bedridden, incontinent and requiring constant nursing care and attention	**Lower moderate disability (MD-)**	5
Dead	**Upper moderate disability (MD+)**	6
/	**Lower good recovery (GR-)**	7
/	**Upper good recovery (GR+)**	8

Table 1: Modified Rankin Scale (mRS) and extended Glasgow Outcome Scale (GOS) Ascending mRS scores represent poorer functional outcome while the extended GOS scores represent positive recovery with ascending scores.

Area	Dimension	No of questions
Functional status	Physical functioning	10
	Social functioning	2
	Role limitations (physical problems)	4
	Role limitations (emotional problems)	3
Wellbeing	Mental health	5
	Vitality	4
	Pain	2
Overall evaluation of health	General health perception	5
	Health change*	1
Total		36

Table 2: SF-36 Questionnaire asks patients about their well-being in eight different categories using 36 various questions.

1.6 SAH Clinical Trials

Of the 50 randomized clinical trials for SAH reviewed in the process of creating a large repository of SAH patients, only two trials succeeded in finding a beneficial effect of treatment (Jaja et al., 2014). There is an extensive amount of money invested in clinical trials for SAH but the money does not currently seem well spent with such a low success rate. Failed translation of results in SAH animal studies to results in clinical trials can be explained by insufficient external validity of disease models (van der Worp et al., 2010). Using a clinically relevant animal model is of high importance in preclinical SAH research so that clinical trials are more likely to be successful. In the present study, we evaluate neurobehavioral and cognitive deficit in a murine SAH model so that its efficacy can be evidenced as translationally useful.

1.7 Animal Models

Induction of SAH in animal models occurs through one of three ways: arterial puncture, autologous blood injection into the subarachnoid space or around an intracranial artery of interest, or clot placement around an intracranial or extracranial artery (Megyesi et al., 2000). Rats and mice have been commonly used given that they are inexpensive and simple to house, but rats have been more thoroughly studied compared to mice (Titova et al., 2009). The first rat model of SAH was reported in 1979 and utilized a basilar artery puncture technique (Barry et al., 1979). Arterial puncture models, or endovascular perforation models, involve inserting a suture into the external carotid artery to perforate the middle cerebral artery or another vessel, however Barry et al.'s model also involved craniotomy (Park et al., 2004). Since Barry et al.'s first perforation model published in 1979, other groups have developed perforation models of internal carotid (Bederson et al., 1995) or anterior cerebral artery puncture without craniotomy (Sheffield model) (Veelken et al., 1995).

Blood injection models involve injection of arterial blood, commonly from the femoral artery, to cause blood collection in the subarachnoid space around the Circle of Willis (Titova et al., 2009). Most blood injection models introduce blood to the cisterna magna, the largest gap between arachnoid and pia mater layers of the brain and consequently a convenient space for injection. The most popular rabbit model of SAH is a cisterna magna injection model that places animals 30° head-down after surgery (Chan et al., 1984). Subsequent cisterna magna injection models were developed in rats (Delgado et al., 1985, Solomon et al., 1985). In 1995, a group developed a prechiasmatic cistern injection model in rats (Piepgras et al., 1995). Injection models diverged into either single-injection (Marzatico et al., 1988, Jackowski et al., 1990) or double-injection models (Meguro et al., 2001, Gules et al., 2002). Dogs, which were the first animals to receive subarachnoid blood injection, have been extensively used in SAH research primarily through injection methods (Megyesi et al., 2000). Primates are popular options for vasospasm research given their large size that facilities angiography (Megyesi et al., 2000) but their use is limited by availability and cost.

Even though larger animals allow for easier vasospasm measurements, mice models allow for convenient transgenic study to compare knock-out or knock-in effects on vasospasm (Sabri et al., 2011, Egashira et al., 2014, Pena Silva et al., 2014, Vergouwen et al., 2014, Chu et al., 2015, Shimada et al., 2015, Siler et al., 2015). The present study utilized a mice model primarily for the animal's potential in transgenic modeling. We used a single cisternal blood injection model that has been proven to produce a clinically relevant SAH (Lin et al., 2003, Kamp et al., 2014).

1.8 Neurobehavioral Testing in Rodents

1.8.1 Neurological Scoring Systems: Neurological scores are used in preclinical SAH studies to distinguish sensorimotor deficit in SAH animals in comparison to sham-operated animals. These scores are subject to experimental variability so a single blind observer is preferred for all data collection. While other observational behavior tests have been used in studying SAH animals (He et al., 2015a, Zhao et al., 2015, Zhou et al., 2015), the Modified Garcia Neurological Score has been most commonly used in testing severity of neurological injury in SAH animals. The Garcia score was originally developed for neurological evaluation after middle cerebral artery occlusion in a rat stroke model (Table 3) (Garcia et al., 1995). This scoring scale includes six different tests that lead to a maximum score of 18 (maximum three points per test) for each animal. The six tests include spontaneous activity, symmetry of movement of four limbs, forepaw outstretching, cage climbing, body proprioception, and vibrissae touch (Additional Details in Methods Section). A modified version of the Garcia score, which adds lateral turning to the original four sensory and two motor tests to make a 21-point scale, has also been used for evaluation of SAH rodents (Lee et al., 2008, Khatibi et al., 2011, Tso et al., 2015, Zuo et al., 2016). Studies that have used unique neurological scores have included observation of visual and tactile sensory response in addition to appetite (Milner et al., 2014, Zhou et al., 2015). In recent rat studies, neurological deficits 24 hours after hemorrhage were shown with Garcia scores decreasing 27.5-30.8% between sham and SAH animals (He et al., 2015b, Teng et al., 2016). However, relative paucity of the effectiveness of the Garcia score exists in mice SAH models. Our work aims to elucidate the utility of the Garcia score in an injection model of SAH in mice.

1.8.2 Anxiety: Several testing methods exist to evaluate anxiety in rodents (Bailey and Crawley, 2009). The open field test places animals in a square chamber and observes their exploratory

behavior. If animals spend more time away from the periphery of the chamber, their behavior is considered to be anxiolytic in nature (Prut and Belzung, 2003). Elevated plus maze (EPM) is a well-established experimental paradigm used in measuring anxiety-like behavior in rodents (Rodgers and Dalvi, 1997). Animals have the option to explore open or closed lanes on an apparatus that is usually elevated about one meter off the ground (Bailey and Crawley, 2009). Anxiolytic behavior is concluded whenever animals spend more time in closed lanes because they prefer darker and more enclosed spaces. The light and dark exploration test was first developed by Crawly and Goodwin to test the anxiolytic effect of benzodiazepines (Crawley and Goodwin, 1980). This test places animals in an apparatus that has two sides: one brightly lit with transparent sides and the other painted black on all sides. A small aperture between the two sides allows for animals to go back and forth between sides. An increase in exploratory behavior by entering the lit side is interpreted as a release of exploratory inhibition (Bailey and Crawley, 2009). EPM's sturdy place in the history of anxiety-related behavior evaluation in rodents convinced our group to use this method to evaluate the anxiety in induced-SAH animals.

1.8.3 Spatial Learning and Memory: Navigational learning is a complex process highly relevant to survival. It requires remembering locations and routes and combining information to ultimately reach a destination. Allocentric wayfinding, or spatial navigation, involves consolidating distal visual cues that are far away from the subject to navigate (Vorhees and Williams, 2014). The hippocampus was identified decades ago for its role in forming cognitive maps (O'Keefe and Conway, 1978). Morris water maze (MWM) is the most commonly used test to investigate allocentric navigation in rodents and was developed in the early 80s (Morris, 1984). MWM uses a large tub of opaque water and an escape platform to observe navigational learning patterns in rodents.

Morris confirmed that animals do indeed use distal cues for navigation. He tested animals with platforms painted black or white to either distinguish or hide the platform, and rats tested with a black platform learned much faster than animals tested with a white platform. Those tested with a white platform whose position moved for each trial showed the poorest performance; Morris interpreted this to mean that animals need initial learning with a distal cue to learn the test. It has been shown that after rats learn platform location, they tend to start their first testing trials with thygomotaxis or swimming around the perimeter of the pool (Dalm et al., 2000). Smaller circling patterns follow thygomotaxis in order to find the platform (Hodges, 1996). Morris' following work focused on showing the role of the hippocampus in MWM. Animals with hippocampal lesions or treated with an N-methyl-D-aspartate (NMDA) receptor antagonist, AP5, showed severely impaired performance on MWM (Morris et al., 1982, Morris et al., 1986). Morris et al. (1986) showed how crucial hippocampal long-term-potentiation was in place learning. Many testing variations have now been crafted for MWM and testing is common in both mice and rats.

There are some problems that may be associated with using MWM in mice (Sharma et al., 2010). Stress associated with swimming is enough to alter cognitive performance on MWM; foot shock treatment in rats and the glucocorticoids that are released in response to foot shock significantly affected memory retention (de Quervain et al., 1998). Species differences do exist in mice, however the strain of mice used in the present study (C57BL/6J) has been shown to perform better on MWM than other strains (Patil et al., 2009).

1.9 Histological Studies for Neuronal Injury and Degeneration

Fields of research on neurodegenerative diseases like Alzheimer's Disease and Parkinson's Disease provide other neuroscientific investigations with useful information about histopathological methods for neurodegeneration in animals (Yamaguchi and Shen, 2013). These fields of research are often interested in differentiating between different modes of cell death including necrosis, apoptosis, and autophagy which can be done with morphological analysis (Schweichel and Merker, 1973). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) marks apoptotic cells while Fluoro-Jade-B reliably marks any degenerating neurons regardless of mode of death (Negoescu et al., 1996, Schmued and Hopkins, 2000).

2. Specific Aims of the Study

While our investigation utilizes a previously studied murine model of SAH (Lin et al., 2005), our study profiles behavior that has not yet been investigated in this model.

Aim 1: To test the neurocognitive deficits in a murine model of subarachnoid hemorrhage. The purpose of the proposed study is to investigate the effect of SAH induced brain injury on neurological dysfunction, anxiety, and cognitive impairment using three behavioral testing methods frequently tested in rats, but not validated in mice. The three techniques we used to assess neurological and cognitive dysfunction in SAH mice were Garcia Neurological Score, Elevated Plus Maze, and Morris Water Maze.

Aim 2: To quantify the neuronal degeneration in the hippocampus and cortex following SAH. Histopathological outcome was evaluated by quantifying the number of degenerating neurons stained by Fluoro-Jade-C on tissue sections in the hippocampus and cortex induced by inflammation and delayed cerebral ischemia. We quantitatively measured the number of Fluoro-

Jade-C positive cells in 3 separate areas in both right and left hemispheres separately and compared the mean for each area between the sham operated and SAH induced mice.

Significance. Our results address whether or not a cisternal autologous blood injection model in mice is suited to study the neurobehavioral deficits seen in SAH. If we find deficits, this model can be used to test potential therapeutic agents to evaluate their efficacy in preclinical studies.

3. Hypothesis

Induced-SAH animals will show significant deficits in MWM and EPM performance, and worsening in Garcia Neurological Scoring as well as increased number of degenerating neurons secondary to SAH induced injury when compared to sham operated mice.

4. Methods and Materials

4.1 Animals

Six week-old C57BL/6 male mice underwent SAH induction by single cisternal blood injection or sham operation. Animals were housed in colonies of 5 animals with free access to pellet chow and water, quarantined for 1 week, and handled at least five times for 1 week to reduce experimenter induced stress before starting the experiments. Room temperature was maintained at 21–25°C and humidity was controlled at 45–50%. Mice were housed under a 12:12-h light–dark cycle that switched at 7 AM and 7 PM. All behavioural testing occured in the light phase between 9 AM and 3 PM excluding the first day of MWM which included two testing phases. All experimental animal procedures were approved by the Emory University Animal Care and Use Committee (IACUC), Protocol # 2001517.

4.2 Groups

30 Animals were randomized to two groups: sham (n=15) and SAH (n=15). A total of three sets of experiments were performed over several months. Each period planned for ten mice (5 sham,

5 SAH) however some were lost during surgery (n=4), most likely through SAH induced injury and anesthetic complications, and one was lost to entrapment. Most results are based on a sample size of 25, however some only include 24 due to the timing of the entrapment incident.

4.3 Surgical Technique and Post-operative Care

A modified murine SAH model was used for this study. Surgical anesthesia was induced and maintained with ketamine/xylazine with an intraperitoneal (IP) injection, dosed at 85 mg/kg and 4 mg/kg respectively. Surgical sites were shaved and skin was prepared with povidone-iodine solution and alcohol. Eyes were applied with antibiotic ointment before surgery. Body temperature was monitored and maintained at 36.5±0.5 ^{o}C with an infrared heating lamp, controlled by rectal thermistors. Animals were positioned prone and the atlanto-occipital membrane was exposed under microscopic visualization. Animals were then placed supine for exposure of the femoral artery. Sixty μL of autologous blood were withdrawn from each animal's right femoral artery. An IP injection of normal saline solution (60 μL) was given after blood removal. After reorienting the animal prone, the atlanto-occipital membrane was subsequently punctured and 60 μL of autologous blood were slowly injected into the cisterna magna. Sham animals received an identical procedure up until puncture to the atlanto-occipital membrane with blood injection excluded. Animals were positioned head-down at 30 degrees for 30 minutes to facilitate pooling of the blood and ensure clot formation. The neck tissue was approximated with auto-clips, and leg incisions were sutured with 4-0 nylon monofilament.

All mice undergoing SAH surgery received moistened chow on the cage floor for the first 2 days. All mice were weighed daily both pre- and post-surgery. During this handling time, they were inspected for signs of stress or pain as described by the IACUC Endpoint guidelines, such as (or in addition to) >25% loss of weight, hunched appearance, excessive eye secretions,

excessive vocalizations upon handling, bleeding from the incision site, lack of grooming, etc. Buprenorphine (0.05-0.1 mg/kg) was administered subcutaneously for postoperative pain alleviation.

4.4 Assessment of neurobehavioral outcome

Behavioral response was evaluating using the Garcia Neurological Score, Elevated Plus Maze, and Morris Water Maze. Timing of measurements is shown in Table 3.

BASELINE Garcia Scores EPM	SURGERY	POD1
POD2 Garcia Scores	POD3	POD4
POD5 EPM	POD6 MWM	POD7 MWM
POD8 MWM	POD9 MWM	POD10 MWM
POD11 MWM	POD12 MWM	POD13 MWM
POD14 MWM	POD15 MWM	POD16 MWM EPM (Sacrifice)

Table 3: Neurobehavioral Testing Timeline The timeline for neurobehavioral experiments was spread across 16 days and all experiments were performed on three sets of animals (30 animals ordered).

4.4.1 Garcia Neurological Score: Garcia score was used to rate animals on their basic abilities in four motor and two sensory tests (Garcia et al., 1995). This scoring scale includes six different

tests that lead to a maximum score of 18 (maximum three points per test) for each animal. The six tests include spontaneous activity (0-3), symmetry of movement of four limbs (0-3), forepaw outstretching (0-3), cage climbing (1-3), body proprioception (1-3), and vibrissae touch (1-3). Spontaneous activity expects animals to approach all four walls of a test cage in five minutes. Symmetry of the four limbs is confirmed if holding mice by the tail produces equal power of movement across all four limbs. For forepaw outstretching, the mouse is held up by its tail and observed to walk across a surface symmetrically with just forepaws. Observing the animal's ability to climb a wire cage placed at 45 degrees to a wall assessed climbing. To assess body proprioception and vibrissae touch, a blunt object was used to prod the side body or to graze the whiskers outside of the animal's field of vision. Symmetrical and equally energetic responses are given three points. Garcia et al. have defined the circumstances for two points and one point in each test (Table 4). Testing took place one day before injury for baseline and 48 hours after injury. We chose to test quickly after hemorrhage as neurological deficits in SAH have been shown to disappear after one week (Germano et al., 2002, Jeon et al., 2009).

Table 1.

Neurological Evaluation After Middle Cerebral Artery Occlusion in Wistar Rats

Test	Score			
	0	1	2	3
Spontaneous activity (in cage for 5 min)	No movement	Barely moves	Moves but does not approach at least three sides of cage	Moves and approaches at least three sides of cage
Symmetry of movements (four limbs)	Left side: no movement	Left side: slight movement	Left side: moves slowly	Both sides: move symmetrically
Symmetry of forelimbs (outstretching while held by tail)	Left side: no movement, no outreaching	Left side: slight movement to outreach	Left side: moves and outreaches less than right side	Symmetrical outreach
Climbing wall of wire cage	...	Fails to climb	Left side is weak	Normal climbing
Reaction to touch on either side of trunk	...	No response on left side	Weak response on left side	Symmetrical response
Response to vibrissae touch	...	No response on left side	Weak response on left side	Symmetrical response

Table 4: Garcia Neurological Score 4 motor and 2 sensory tests are done in animals to make a total maximum score of 18. Lower scores are indicative of deficit (Suzuki and Zhang 2012).

4.4.2 <u>Elevated Plus Maze (EPM):</u> EPM evaluated anxiety in animals. A plus-shaped apparatus stood above the ground (above the MWM tub) with two opposite lanes enclosed by high walls. After animals were acclimated to the study room for about 30 minutes, each animal was allowed to explore the maze for five minutes after being dropped at the center cross. Video recordings of each animal's performance were evaluated by an observer to measure time spent in uncovered lanes. Lane entries were also counted so that a proportion of closed/open lane entries could be quantified (Komada et al., 2008). Testing was performed one day before injury for recording baseline and 5 days and 16 days after injury.

4.4.3 Morris Water Maze: Spatial learning was evaluated in SAH rodents using the Morris Water Maze (MWM). This technique used a circular pool of opaque water with a hidden platform available for escape. The platform is hidden because its top surface lies just below the surface of the water. Opacity of the water is controlled with pure or powdered milk (Morris, 1984, Rapp et al., 1987). Animals were tested over repeated trials to track spatial learning over time. For every MWM trial, animals had 60 seconds to successfully find the platform and had to remain on the platform for 10 seconds. Travelling patterns were recorded by labeling the pool with North, South, East, and West markers and using a video camera placed above the pool. Topscan Lite Version 2.00 tracking software (Clever Sys Inc., Reston, VA, 2000-2012) was used to compute travelling distances and velocities. A curtain was used to remove any cues from around the testing area and the same lighting environment was applied for all testing days. Animals were given 15 minutes of acclimation time to the room before any testing occurred. To quickly dry animals and avoid hypothermia, mice were placed in front of a heater in between or after trials.

The present study's MWM testing included four different phases that were chosen based on Vorhees' and Williams' protocols (Vorhees and Williams, 2006). Five hours after cued learning was performed with the assistance of a cue flag mounted to the platform, spatial acquisition phase began. During spatial acquisition, jump-offs, swim overs, and thygmotaxis were recorded in addition to escape latency. Swim overs were noted if mice did not remain on the platform at all but still swam over it and jump-offs were instances when the mice left the platform before 10 seconds was reached. Thygmotaxis was noted whenever mice traced more than a quarter of the tank's perimeter. The platform was not moved from the northeast spot during the spatial acquisition phase, however the platform was removed for a probe test to track

free-swim patterns on post-operative day 11 (POD11). Video footage was used after testing to measure proportion of time spent in each quadrant. Animals who performed well on POD11 of the Morris Water Maze spent most of their time in the northeast quadrant (Morris, 1984). An annulus, or circle around the platform, was made using tracking software to also count crossings into this area surrounding the platform. Working memory was more specifically evaluated on the last five days of MWM testing as difference in latency escape time between two consecutive, identical drops served as the primary measurement. For failed trials, 60 seconds was used as an escape time to ensure appropriate statistical analysis.

MWM Phase	Days	Description	Parameters
Cued Learning	POD 6	- 8 trials (N, S, W, E, NE, NW, SE, SW) - flag marker mounted on platform	Latency Failed Trials
Spatial Acquisition	POD 6-10	- 4 trials, randomized drops - platform in NE	Latency Failed Trails Distance Velocity Thygmotaxis Swim-overs Jump-Offs
Reference Memory	POD 11	- 1 trial - Platform removed	Quadrant time Annulus Crossings
Working Memory	POD 12-16	- 2 trials, identical drops - platform randomized each day	Latency difference

Table 5: Morris Water Maze Testing Phases After cued learning on the first testing day, three measurement phases followed.

4.5 Histopathological Study

4.5.1 Transcardiac Perfusion and Harvesting of Tissue: Deep anesthesia was induced with ketamine/xylazine with an IP injection of 0.1cc/10 grams body weight, or 0.2 ml of stock solution containing ketamine and xylazine, dosed at 85 mg/kg and 5 mg/kg respectively, using an insulin needle/syringe. The anesthesia level was monitored by response to toe pinch following

anesthesia. Incision was made through the sternum and the rib cage was lifted to expose the heart. The heart was released from the surrounding tissue and the rib cage was held back with hemostats. Animals were perfused with a 0.1M Phosphate Buffered Saline (PBS) using a 23 Gauge needle and infusion set pierced through the left lateral ventricle diagonally into the ascending aorta. An incision was made in the right atrium to allow the fluid to flow through. 20mL of 0.1M PBS (Phosphate buffered saline) to clear the blood from the animal followed by 50mL of 4% Paraformaldehyde (PFA) in 0.1 mol/L phosphate-buffered saline were perfused. Upon completion of the perfusion the animal was decapitated and the brain tissue was removed.

4.5.2 Fluoro-Jade-C staining for quantification of degenerating neurons: Harvested brains were placed in 4% paraformaldehyde overnight before being transferred to 10%, 20%, and then 30% sucrose solutions successively until all the tissues equilibrated. Brains were frozen by submerging them in methylbutane surrounded by dry ice. The tissues were then stored at -80 °C freezer until processing. Tissue samples were then embedded in OCT Tissue Tek compound, and coronal slices (10 μm) of the hippocampus were then cryosectioned using a cryostat. Slices 1.58-2.54 mm posterior to the bregma were used for staining of the hippocampus and cortex (Franklin and Paxinos). Franklin and Paxinos' stereotaxic atlas was used to confirm presence of both regions.

Fluoro-Jade C staining was done to identify degenerating neurons in the hippocampus and cortex of each section. For Fluoro-Jade-C staining, a modified version of Zhang et al.'s protocol was used (Zhang et al., 2012). Briefly, slides were immersed in a basic alcohol solution for 5 minutes before being transferred to 70% ethanol and distilled water for two minutes each. Incubation in potassium permanganate solution followed for 15 minutes and then slides were rinsed in distilled water for three one minute washes. Slides were then incubated in acidic Fluoro-Jade-C solution

for thirty minutes. Slides were then dried for five minutes before being cleaned in xylene for up to five minutes. Slides were coverslipped using DPX mounting medium and clear nail polish. An Axioskop 2 epifluorescence microscope was used with Axiovision 4.8.1 software to capture images of stained neurons. Images were taken at 20X magnification and three pictures of each side of the hippocampus and cortex were used to take the average for each side of both regions. ImageJ software was used to count positively stained degenerated neurons. Slides' animal IDs were taped over and coded for blinded microscopy and counting.

4.6 Statistics

All data were analyzed with IBM SPSS 23.0 software. All results were expressed as mean±SEM. All behavioral data was analyzed using repeated measures ANOVA for within-subjects and between-subjects comparisons, and Independent Samples T-Test was used for individual comparisons for each testing date. The criterion for statistical significance was set at $p<0.05$.

5. Results

5.1 Sensory and motor ability unaffected by single injection SAH injury

When animals were scored two days after surgery, Garcia scores were not significantly different between sham animals and SAH animals (Table 6). All animals received maximum scores of three points for all testing categories except vibrissae touch and side-body reaction.

Total Garcia Neurological Scores POD2		***P* value**
Sham (n = 13)	**SAH (n = 13)**	0.54, NS
16.69±0.26	16.92±0.26	

Table 6: Garcia Neurological Score Results A deficit in SAH animals was not found based on Garcia Scoring

5.2 Lack of anxiety-associated behavior in SAH animals

EPM data showed no difference in anxiety between sham and SAH animals. Proportion of covered entries and time spent in uncovered lanes were not significantly increased, as hypothesized, in SAH animals. Proportion of covered entries in EPM was slightly higher in sham animals on both POD5 and POD16. This difference was more dramatic for sham animals 16 days after surgery (POD5 p=0.555, POD16 p=0.074). For the time spent in uncovered arms measurement, the difference between sham and SAH animals was, on the other hand, contrasting 5 days post-surgery (n=25) and 16 days post-surgery (n=24)(Figure 2)(p=0.742 and p=0.838, respectively). SAH animals spent less time than sham animals in uncovered arms on POD5, but this relationship reversed on POD16. SAH animals spent more time in uncovered regions on POD16 though without statistically significant difference from sham animals.

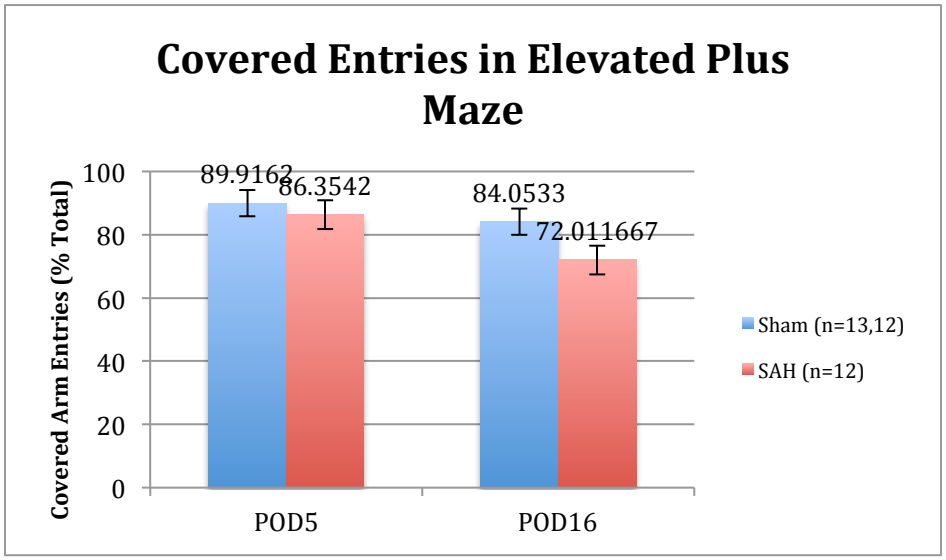

Figure 1: Covered Arm Entries in EPM Animal groups did not significantly differ in percent of covered lane entries in EPM (p>0.05, sham vs. SAH t-test for each day). Trend for significance was found between sham and SAH animals on POD16 (p=0.074).

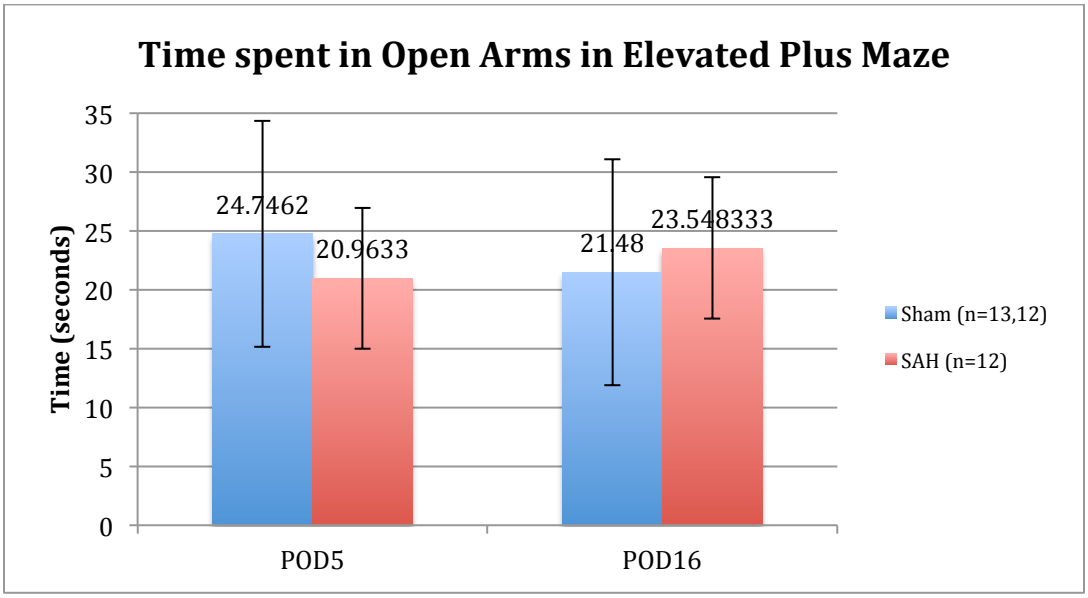

Figure 2: Time spent in Open Arms EPM data showed no significant difference between sham and SAH animals based on time spent in open lanes (p>0.05, sham vs. SAH t-test for each day).

5.3 Spatial and Working Memory in MWM

Both the cued learning and the spatial acquisition testing periods found no difference in mean escape times between both animal groups. However, SAH animals did show increased escape times compared to sham animals on four of the five spatial acquisition testing days (Figure 3). The starkest differences can be seen on POD6 and POD10 but no significance was found based on t-tests performed for each day. Velocity was significantly decreased in SAH animals on POD6 (p=0.029)(Figure 4).

Probe-test performed on POD11 found that SAH animals did not preferentially spend more time in any of the four quadrants when compared to sham animals. Annulus crossings also did not significantly differ between the two animal groups (p=0.88)(Figure 5 & Table 7).

Working memory testing from POD12-POD16 found significant difference between animal groups in saved escape latency only on POD12 (p=0.049). SAH animals on average saved more time between their first and second escape latencies than their sham counterparts. No

clear trend between the two animal groups was shown based on saved escape latencies (Figure 6).

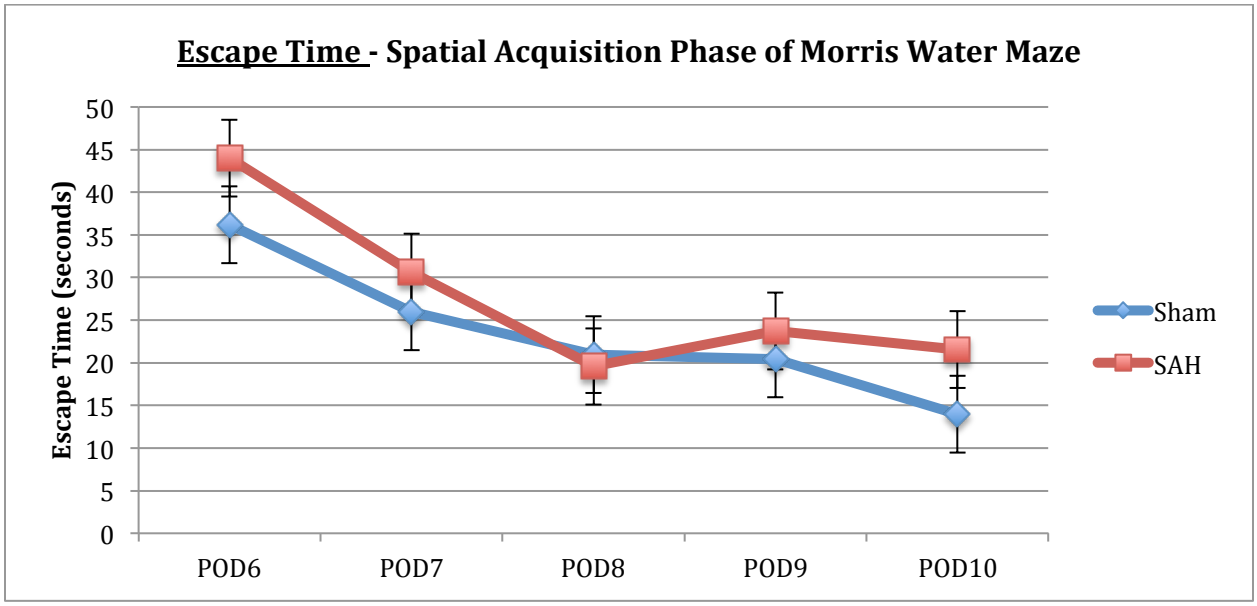

Figure 3: Spatial Acquisition Phase Escape Time With the platform kept in the northeast quadrant for five testing days, mice in both animal groups did not show contrasting abilities to find the hidden platform (p>0.05, Sham vs. SAH t-test for each day)(*[POD6]* n_{sham} = 13, n_{SAH} = 12, *[POD7-10]* n_{sham} = 12, n_{SAH} = 12). SAH animals did however show an increased escape time average on all testing days except for POD8.

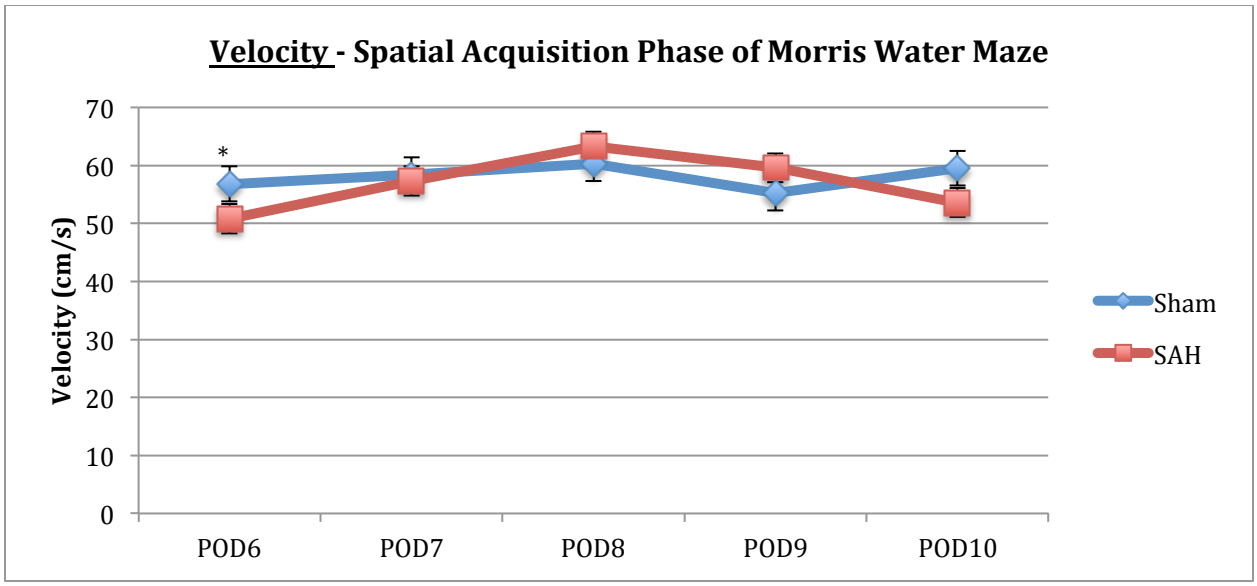

Figure 4: Spatial Acquisition Velocity SAH animals were significantly slower in their swimming patterns on POD6, the first day of testing, while showing no strong difference on

any other testing days (*[POD6]* $p<0.05$, *[POD7-10]* $p>0.05$, Sham vs. SAH t-test for each day)(*[POD6]* $n_{sham} = 13$, $n_{SAH} = 12$, *[POD7-10]* $n_{sham} = 12$, $n_{SAH} = 12$)..

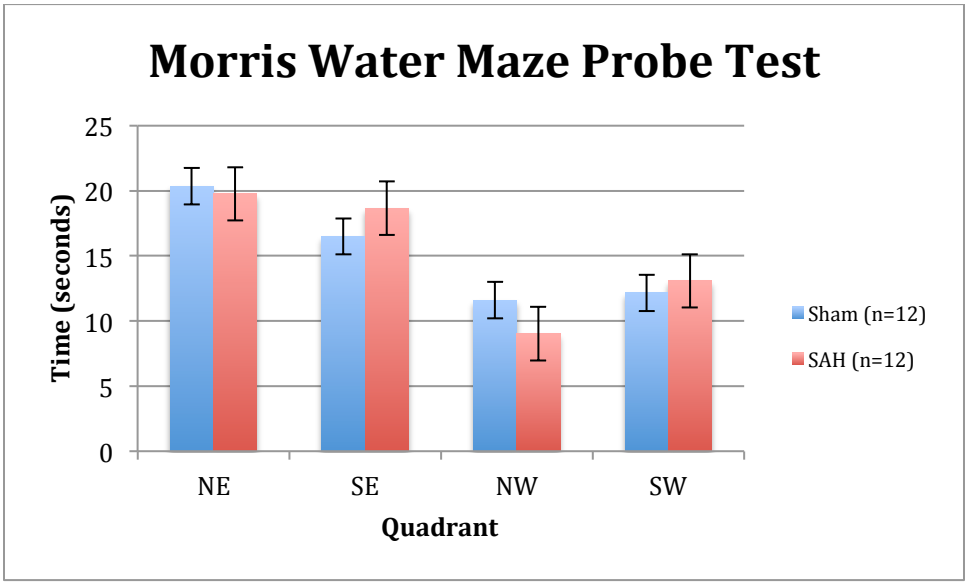

Figure 5: MWM Probe Test Removing the NE platform and observing swimming patterns did not reveal a deficit in SAH animals ($p>0.05$, Sham vs. SAH t-test for each region). They spent nearly equal amounts of time swimming in the NE quadrant (about a third of the testing time period).

Annulus Crossings in MWM Probe Test		
Sham (n = 12)	**SAH (n = 12)**	***P* value**
5.25±0.71	5.42±0.79	0.88, NS

Table 7: MWM Probe Test Annulus Crossings Removing the NE platform and observing swimming patterns did not reveal a deficit in SAH animals. When a circular zone was created and placed around the platform using tracking software, entries into this annulus could be counted. Hemorrhage animals did not enter the annulus on significantly fewer instances than sham animals.

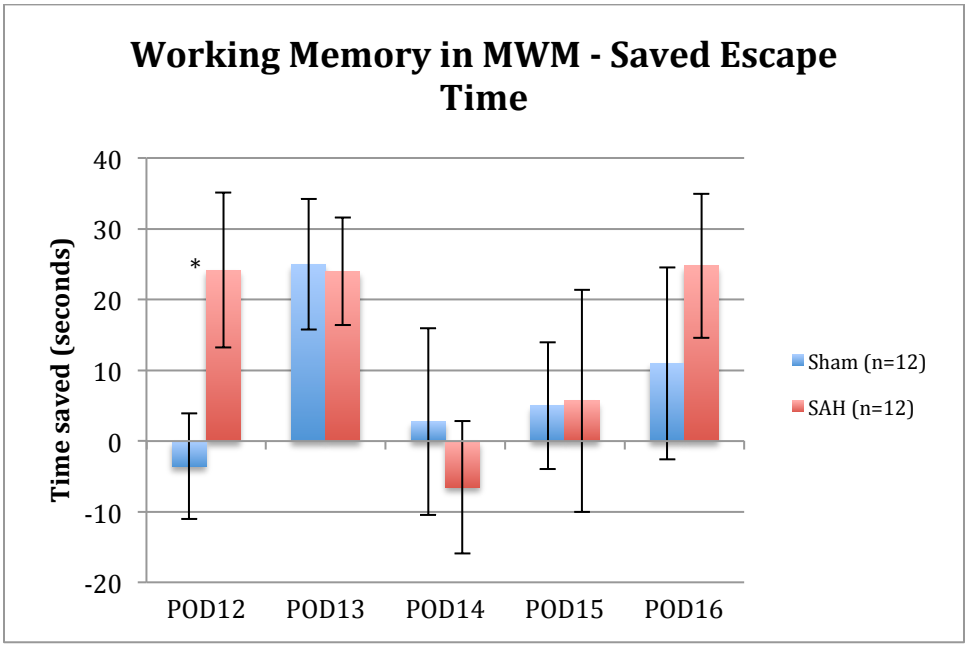

Figure 6: Saved Escape Time MWM Working Memory Testing SAH animals showed relatively enhanced working memory on POD12 ($p<0.05$, *[POD13-16]* $p>0.05$, Sham vs. SAH t-test for each day). They only showed a deficit on POD14 that was not significantly different from sham animals.

5.4 Neurodegeneration – Fluoro-Jade-C Staining

Histological study with Fluoro-Jade-C staining for degenerating neurons did not elucidate differences between SAH and sham animals. Hippocampal and cortical tissue were analyzed for both hemispheres separately (Figure 7). While a difference between animal groups were not found in any of the regions, significantly more cortical damage was found in animals overall. Figure 8 shows four representative images from the staining procedure.

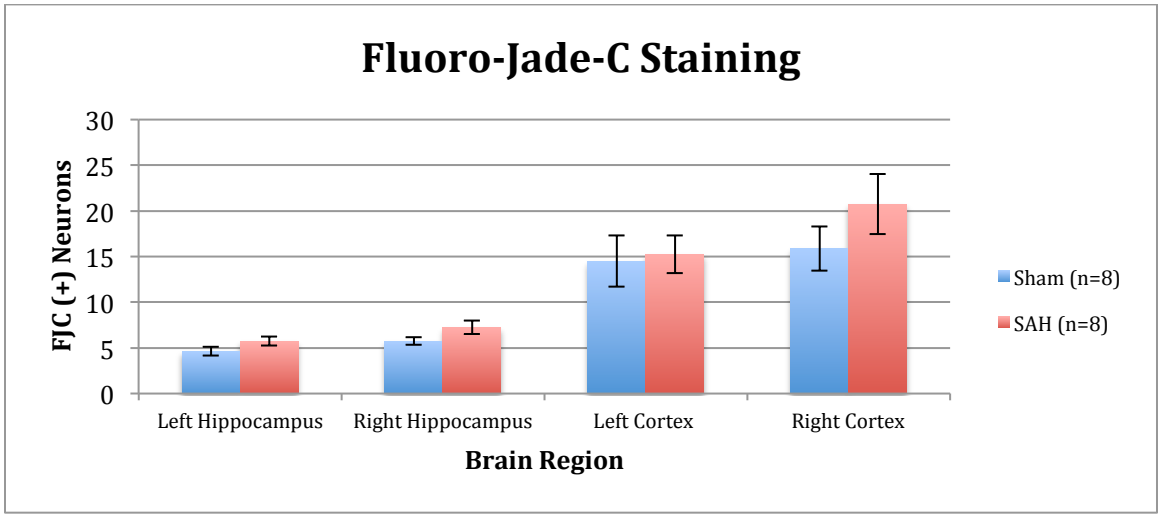

Figure 7: Neurodegeneration in SAH While a difference in degenerating neurons between sham and SAH animals was not found with Fluoro-Jade-C staining, enhanced cortical damage was found in all animals compared to hippocampal neurons (p>0.05, Sham vs. SAH separately for each side of each region).

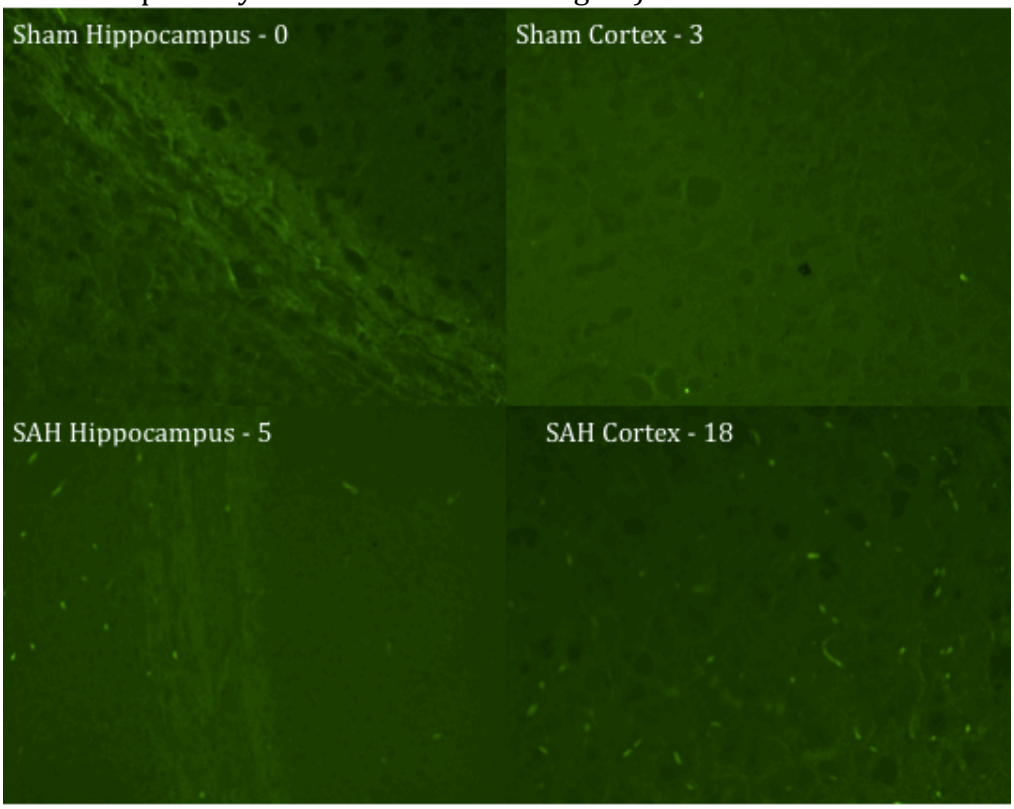

Figure 8: FJC Staining Images (20X magnification) Fluoro-Jade-C stained cortex and hippocampal tissues. While more Fluoro-Jade-C (+) cells were found in SAH animals, the difference was not significant (p>0.05, Sham vs. SAH for left and right sides of each region). Cortical damage was enhanced compared to hippocampal tissue in all animals.

6. Discussion

6.1 Summary

The current study investigated cognitive, functional and histopathological outcome in a cisterna magna single blood injection model of SAH in mice. Three sets of experiments were performed and results were collected for 25 animals in total (Sham=13, SAH=12). Sensory and motor function was measured with the Garcia Neurological Scoring system. Anxiety was evaluated with Elevated Plus Maze test and spatial/working memory was evaluated with Morris Water Maze. Fluorescence staining with Fluoro-Jade-C followed all the experimentation to conclude whether neurodegeneration differed between animal groups (sham=8, SAH=8). SAH induced injury in our animal model of SAH did not induce significant deficits in Garcia Neurological Score evaluated at 48 hours post-injury or Elevated Plus Maze evaluated 5 and 16 days post-injury. An anxiolytic effect was however close to being significant on POD16 closed-arm entry data ($p=0.074$). A relatively anxiolytic relationship can be seen on POD16 in both EPM measurements since SAH animals had a smaller proportion of covered arm entries and spent more time in open arms compared to sham animals. However, this relationship is not supported by significant statistical analysis. Significant findings from Morris Water Maze data were also sparse. SAH animals swam significantly slower than sham animals on the first day of spatial acquisition testing but the four other testing days for this period did not show any significant differences. SAH animals showed enhanced working memory compared to sham animals on POD12 in the last phase of Morris Water Maze testing; this significance was similarly only shown on the first day of the testing phase. Degeneration of neurons was quantified in hippocampal and cortical tissue of the tested animals; significant differences between animal

groups were not found however cortical damage was enhanced compared to hippocampal damage in all animals.

6.2 In the Field: SAH Rodent Research

6.2.1 **Neurological Score:** Sprague-Dawley rats subjected to both endovascular perforation and prechiasmatic cistern injection have recently been shown to have reduced Garcia neurological scores compared to sham animals (He et al., 2015b, Zuo et al., 2015, Teng et al., 2016). Mice subjected to perforation of the right anterior cerebral artery showed motor and sensory deficit with another scoring method (McGirt et al., 2002). The only study on mice using Garcia neurological score found deficits in SAH animals from prechiasmatic injection of donor blood at a larger volume (80 μL)(Tso et al., 2015). In our study, the most variability found in neurological deficit came from vibrissae and side-body tests, however significant deficit in SAH animals was not found in either of these tests nor with any of the other subcategories.

In general, the Garcia Neurological Score has been much more thoroughly used in ischemic stroke animal research (Shmonin et al., 2014). Within ischemic stroke animal research, it has also been shown to not be as sensitive as other measures for neurological deficit. Automated open field analysis was more sensitive to deficit than Garcia Neurological Score in one study (Desland et al., 2014). Ischemic stroke animals produce more sensory and motor deficits than SAH animals that Garcia neurological score is sensitive to measuring. Focal ischemic stroke models in particular produce significant motor deficits in limbs contralateral to infarcts (Liu et al., 2014, Zhang et al., 2015).

The difference in disease severity between ischemic and hemorrhagic stroke could serve as an explanation for the lack of significance found in our study. SAH mice may simply not produce significant enough deficit that can be found with our study's neurological assessment in

the same way that rats do. Milner et al. showed that an endovascular perforation SAH model of mice showed mild but significant neurological deficit with a neuroscore including balance, visual response, and tactile response in addition to the categories of Garcia neurological score. Our study's model may also not be severe enough compared to endovascular perforation. In a Huntington's Disease preclinical study, mice showed enhanced resilience to striatal degeneration with 3-nitropropionic treatment compared to more vulnerable rats (Alexi et al., 1998). Tso et al.'s prechiasmatic injection mice model involved a different surgical procedure, using burr holes and cerebral blood flow monitoring, and also used donor instead of autologous blood. Our study's surgical method may have also overwhelmed animals overall, hiding any differences between animal groups. High neurodegeneration found in sham animals supports this explanation.

Given that motor deficits are not found as commonly as cognitive deficits in SAH patients, Garcia Neurological Score may not be the best measurement of neurological deficit in hemorrhagic stroke research. An endovascular perforation rat model showed no significant motor deficit up to three weeks after surgery (Silasi and Colbourne, 2009). The test was reported to be effective up to 72 hours after hemorrhage in SAH rats, however, it hasn't been as widely studied in mice (Suzuki and Zhang, 2012). As mice have been shown to recover faster than rats, we chose our testing point to be 48 hours but without finding any deficits. Further studies at earlier time points such as 24 hours can clarify Garcia neurological score's role in hemorrhagic stroke research in cisternal blood injection models of SAH in mice. SAH's common symptoms of nausea, headache, and dizziness also point to behavioral tests looking at balance (CatWalk) as more relevant for measuring neurological deficit (Ahn and Balaban, 2010).

6.2.2 <u>Anxiety:</u> Anxiety has not been studied thoroughly in preclinical hemorrhagic stroke research. Boyko et al.'s study evaluated anxiety using open field test and elevated plus maze in

single and double hemorrhagic rats three weeks after SAH (Boyko et al., 2013). Open field results consistently suggested that double hemorrhagic animals were significantly more anxious than control and single hemorrhagic animals. However, EPM results showed that single hemorrhagic animals were most anxious out of three animal groups based on time spent in open arms and closed arm entries. Double hemorrhagic animals were similar to control animals in EPM results of this study. Sasaki et al.'s study also used EPM to compare prechiasmatic blood model rats to double hemorrhagic cisterna magna injection model rats (Sasaki et al., 2016). Testing was done 20 days after hemorrhage, closely lining up with the Boyko et al.'s method. In this study, prechiasmatic blood injection animals exhibited decreased anxiety in EPM while double injection animals exhibited increased anxiety-related behavior. EPM results between Boyko et al.'s and Sasaki et al.'s studies are thus opposite in nature. The mentioned studies have both been done in rats. Mice, on the other hand, have rarely been investigated for anxiety in SAH. Our study most closely aligns with Sasaki et al.'s work showing an anxiolytic effect of single hemorrhagic stroke developing more than two weeks after hemorrhage. Significance may have been found in our study if measurements were taken past sixteen days after surgery or we had a bigger cohort of animals because an anxiolytic effect of SAH was close to being significant ($p=0.074$) on POD16 closed arm entries. Enhanced anxiety has been shown to persist in humans up to 12 months after stroke so elongated study is warranted in preclinical hemorrhagic stroke studies evaluating anxiety-related behavior (Latimer et al., 2013). Our study is the first one to assess anxiety using EPM in a mouse model of SAH.

6.2.3 <u>Spatial Memory and Learning:</u> Memory evaluation in SAH using Morris Water Maze (MWM) has been done almost solely in rats. MWM has been studied in both endovascular perforation (EP) and cisterna magna injection models of rats. Most of these studies involve

testing a treatment group for ameliorative, anti-inflammatory effects, however some involve identifying exacerbative contributors to disease severity. Even though the mentioned studies involve various treatments, they also involve sham and SAH without treatment animal groups that allow for appropriate comparison to the present study.

EP rat studies of SAH show reproducible dysfunction in SAH animals when compared to their sham counterparts. An EP rat model showed disrupted performance based on increased latency and swimming distance in SAH animals whenever the platform was moved to a new location after remaining in one spot for two days of testing (Silasi and Colbourne, 2009). In Silasi and Colbourne's study, increased cognitive load was necessary for a difference between animal groups to be shown. Initial MWM testing with the platform's first location did not show disrupted spatial learning. In Xie et al.'s study, EP SAH rats also showed impaired spatial reference memory functioning; they showed fewer appropriate platform crossings after the platform was removed in addition to increased latency and swim distance in SAH animals compared to control sham animals (Xie et al., 2015). Similar findings including increased latency and swimming distance in SAH animals compared to controls were also found in Hu et al.'s study that tested for improvement with hyperbaric oxygen (Hu et al., 2014). Milner et al.'s study, the only one that has tested MWM in SAH mice to date, found no significant difference in performance between SAH induced mice and controls in an EP model of SAH (Milner et al., 2014).

Disrupted spatial learning in SAH has been shown through MWM testing in many studies that used a prechiasmatic cistern injection model in rats (Takata et al., 2008, Jeon et al., 2010, Chen et al., 2013, Dong et al., 2013, Sun et al., 2013, Wang et al., 2013, Wang et al., 2014, Liu et al., 2015, Shen et al., 2015). All of the nine mentioned studies showed significantly increased

escape latencies and swimming distances in their SAH animals in comparison to sham controls. Four of the studies were able to show specific impairment in spatial working memory in SAH animals based on saved time between two trials (Jeon et al., 2010, Chen et al., 2013, Wang et al., 2013, Wang et al., 2014). The four studies showed that SAH rats with prechiasmatic blood injection did not find the platform as quickly on the second trial as sham controls did. A few of these studies used swimming speed as a measurement for MWM performance and their results are not in agreement. Takata et al.'s found increased speed in SAH animals while Shen at al.'s study and Jeon et al.'s study found no difference between animal groups (Takata et al., 2008, Jeon et al., 2010, Shen et al., 2015)

The present study is the first to evaluate outcomes of MWM in a cisternal blood injection mice model of SAH and the second to do any MWM testing in SAH mice. We expected to find both increased escape latency and swimming distance in SAH animals when compared to sham controls as these two observations have been so consistently shown in SAH blood injection models of rats. Instead, Milner et al.'s study that failed to show a deficit in EP SAH mice performing MWM most closely aligns with the results from our study. Significant findings from our MWM data only include decreased velocity on one of five testing days during the spatial acquisition period and unusually enhanced working memory based on a quicker average escape latency in SAH animals on the first day of the working memory testing phase. These significant results do not suggest that our mouse model of SAH produced relevant deficit in spatial learning and memory and thus disprove our study's hypothesis.

Velocity in MWM is commonly interpreted as a motor ability measurement whereas escape latency and swimming distance are interpreted as reliable measurements for spatial learning and memory (Shen et al., 2015). While significantly reduced velocity in SAH mice on

our first day of spatial acquisition testing could suggest enhanced motor deficit in SAH mice, this significant finding is not supported by deficits in the motor tests of the Garcia neurological score. POD6, the day on which this significant finding was found, also included cued learning testing five hours before spatial acquisition testing, so sheer exhaustion can explain decreased velocities in SAH animals on POD6 that were not found POD7-POD10. The highly unexpected enhanced working memory in SAH animals on the first day of the working memory testing phase is difficult to explain but our experimental paradigm could rationalize the significant findings. POD12 involved the first testing day with the platform moved from its northeastern position since POD6, so novelty on this testing day may have skewed results. As SAH animals showed somewhat anxiolytic behavior on POD16, perhaps less anxious states during a new MWM testing paradigm contributed to better performance compared to sham animals that simply disappeared POD13-POD16. However, the extensive variability in performance during the working memory testing phase suggests that significant findings are not particularly strong.

Failure to show deficit in MWM in our study decreases the cisterna magna blood injection mice model's relevance in SAH preclinical research. Combined with Milner et al.'s results, our study generally suggests that rats are better than mice at displaying significant neurocognitive deficit in SAH.

6.2.4 <u>Neuronal Degeneration in SAH mice:</u> In SAH preclinical research, disruptions to neuronal viability are often measured and correlated with impaired performance on behavior tests. Takata et al.'s 2008 study was able to correlate CA1 hippocampal neuron loss with disrupted performance on MWM (Takata et al., 2008). Fluoro-Jade-B (FJB) is an older version of the staining compound used in the present study that has been used on several occasions to measure cell death in SAH rodents. Silasi and Colbourne found FJB positive cells in the orbital

as well as cingulate prefrontal regions of SAH rats but they did not compare these findings with sham animals. Other groups were able to show significantly enhanced cell death using FJB in either the cortex, hippocampus, or cerebellum in cisterna magna blood injection SAH rats (Jeon et al., 2010, Wang et al., 2013, Wang et al., 2014, Liu et al., 2015). Other staining methods have been used in SAH preclinical research to measure cell death. Dong et al. stained for caspase-3 expression indicating apoptosis and found significantly increased caspase-3 expression in the hippocampus of SAH rats compared to vehicle controls (Dong et al., 2013).

Our study ultimately aimed to find cell death in the hippocampus that would correlate with disrupted performance in MWM. Performance on MWM was not disrupted in SAH animals and significantly different neurodegeneration was not found between the present study's two animal groups. FJC staining only showed overall cortical neuronal degeneration to be enhanced compared to hippocampal cell death in both animal groups. We theorize that enhanced cell death occurred in cortical injuries simply because cortical cells are anatomically closer to the injury site for surgery as well as the cortical inflammation that has been shown to be induced by the presence of blood in the subarachnoid space (Kooijman et al., 2014). Our findings similarly line up with Milner et al.'s work. Milner et al. used cresyl violet staining to visualize CA1 hippocampal neurons. Counting showed that intact neurons did not differ between SAH mice and controls (Milner et al., 2014). Jeon et al.'s work with cisterna magna injection rats also showed similar results to our study in that the cortex was shown to have the most neurodegeneration in comparison to the hippocampus and cerebellum, however this trend was shown in SAH animals only and not sham controls (Jeon et al., 2010). While cell death has been shown in SAH preclinical studies with rats, mice studies have failed to show cell death in SAH. This may represent an enhanced resilience in mice compared to rats or a need for more severe SAH models

in mice. Since reactive astrocytes have been previously found to be increased in SAH animals, our lab plans to investigate the astrocyte profile of SAH mice (Adelson et al., 2001).

7. Conclusion

In the present study, significant deficit was not found in a murine blood injection model of subarachnoid hemorrhage. Comparisons between sham operated and SAH animals using the Garcia Neurological Score, Elevated Plus Maze, and Morris Water Maze did not show impairment in SAH animals. Neuronal degeneration marked by Fluoro-Jade-C staining also did not elucidate a significant difference between animal groups. Future preclinical study in SAH animals should focus on testing in rats or arterial puncture models of mice since these models show clinically relevant disruption to neurological and behavioral functioning.

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