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Circadian Influences on Brain Damage, Regeneration, and Neurogenesis

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Circadian Influences on Brain Damage, Regeneration, and Neurogenesis

by

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Abstract

The number of people affected by brain damage each year ranges from millions to billions. Research into factors that affect brain damage and recovery from the disabilities incurred is pertinent to alleviating stress put on individuals, their families, healthcare systems, and society as a whole. Circadian rhythms are ubiquitous throughout the animal kingdom, throughout individual organisms, and even in each cell of an organism. Therefore, circadian influences on brain damage, recovery, and physical brain repair warrant further investigation. In this dissertation, the role of the circadian clock in stroke outcome, and in models of brain repair will be examined. I hypothesize that circadian rhythmicity is involved not only in outcomes following brain damage, but may also be a significant contributor to neuroregeneration. Here, I show the effects of the time of day that a stroke occurs on behavioural and anatomical outcome in rats. This is followed by an investigation into the rhythmic expression of clock genes in regenerated medial prefrontal cortex (MPFC), and the effects that circadian dysfunction has on regeneration in both the neonatal MPFC, and adult subgranular zone of the dentate gyrus in mice.

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To all my uncles

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List of Abbreviations

TIA – Transient ischemic attack

TBI – Traumatic brain injury

GCA – Glasgow Coma scale

MCA – Middle cerebral artery

LD – Light:Dark

DD – Dark:Dark (constant darkness)

SCN – Suprachiasmatic nucleus

CT – Circadian time

PER – Period protein

Per – Period gene

mPer – Mouse period mRNA

CRY – Cryptochrome protein

Cry – Cryptochrome gene

mCry – Mouse cryptochrome mRNA

ATCH – Adrenocorticotropin releasing hormone

HPA – Hypothalamic-pituitary axis

ILC – Infralimbic cortex

CIMT - Constraint induced movement therapy

BWSTT - Body weight-supported treadmill training

NIBS - Noninvasive brain stimulation

TMS – Transcranial magnetic stimulation

rTMS - Repetitive TMS

tDCS - Transcranial direct current stimulation

VR - Virtual reality

BCI - Brain-computer interface

PVT – Paraventricular thalamic nucleus

PRV - Pseudorabies virus

DG – Dentate gyrus

GCL – Granule cell layer

PH - Partial hepatectomy

MPFC – Medial prefrontal cortex

SVZ – Subventricular zone

SGZ – Subgranular zone

BrdU – Bromodeoxyuridine

WEE1 – Wee1 protein

Wee1 – Wee1 gene

Wee1 – Wee1 mRNA

CDK – cyclin-dependant kinase

Chapter 1: Introduction

1.1.1 Brain Damage

The brain is arguably the most valuable organ in the mammalian body. It controls nearly all functions of the body, from breathing, sleep and eating, to consciousness, emotion and cognition. Damage to such an important control center can have a myriad of effects on behavioural and cognitive processes. Depending on which region of the brain is damaged and to what extent, a person can be left unable to move, see, feel, talk, or remember. Severe damage to the brain can cause death, yet even minor damage can have devastating effects on the lives of those affected, their families, jobs, and society as a whole. Approximately 5.5 million Canadians live with some sort of brain dysfunction, whether due to a disorder, injury or disease (Duncan, 2012).

Accurate estimations of the total number of individuals directly affected by brain damage are difficult to obtain, due to many different factors. First, brain damage and dysfunction can easily go unreported, as variation in the severity of functional impairment following damage allows mild cases to be easily missed. In fact, it is common for the average person to experience "mini-strokes", or transient ischemic attacks (TIA), throughout their lifetime, most of which go unreported and even unnoticed. This fact alludes to a second reason why quantifying the number of people affected by brain damage is so difficult. Damage to the brain can be caused by numerous events, a lack of oxygen or blood flow to the brain, a bump to the head, or by developmental or hereditary disorders. Stroke, for example, affects 15 million people worldwide per year (Centers for Disease Control and Prevention, 2012; MacKay and Mensah, 2004). Traumatic brain injury (TBI), or concussion, is a very common

occurrence in many countries around the world. TBI has been called a silent epidemic, with estimates of nearly 1.7 million Americans suffering TBI or concussion each year (Centers for Disease Control and Prevention, 2012). These are just a few of the commonly known causes of physical brain damage, and already one can see the exceedingly large numbers of individuals directly affected.

Concurrently, the impact of brain damage can extend far beyond the individual. People with brain damage are often unable to work following the traumatic event. This often leaves families to care for these individuals, especially when healthcare cannot be afforded, or is simply unavailable. Jobs are lost or neglected due to the added strain of caring for family members, who are unable to care for themselves. Not only are entire families influenced, but the costs to healthcare systems are also extreme. For example, in 2000, both direct and indirect costs of TBI alone in America exceeded $76.5 billion (Centers for Disease Control and Prevention, 2012). Add on costs incurred from stroke, and estimates climb to over $115 billion annually. The stress that the cost of these two forms of brain injury alone can place on the healthcare system and society as a whole is only increasing. Research that focuses on prevention and repair following brain damage is necessary to try to help alleviate the burden being placed on our healthcare and government systems. If we can assist those affected by brain damage in returning to work, and to a more normal life, we can cut a significant portion of these costs.

1.1.2 Recovery from Brain Damage

Given that brain injury affects millions of people, and costs billions of dollars, prevention of brain injury is a high priority focus of current research, as is developing new treatments, and increasing the efficacy of available treatment methods. The fact

remains, however, that brain injury will never be completely avoidable, as accidents happen, and physiological events cannot yet be predicted. Therefore, research must focus on ways to enhance recovery in brain damaged patients.

Recovery from brain damage is highly variable due to many factors. Some of these include the extent of the initial damage, type and location of the injury, previous medical history, previous neurological problems, age, and blood pressure and oxygen levels after the injury. Recovery can also vary depending on current results from physical examinations, brain imaging examinations, radiological studies of the brain, and other common tests performed on hospitalized patients. Prognosis, therefore, is also very difficult, as each individual may have very different histories, and different current conditions.

A common tool used to assess brain damage, most commonly TBI, is the Glasgow Coma Scale (GCS) (Rehabilitation Institute of Chicago, 2012). This scale can be used to classify brain damage as mild, moderate, or severe, and these categories can assist healthcare professionals in generating more accurate prognoses for brain damaged patients. A prognosis of "mild" brain damage often means that the patient can return to normal life shortly after the damaging event. These patients do not require extended hospital stays, rarely need inpatient rehabilitation, and often experience full recovery of a lost function. Moderate and severe brain damaged patients, however, do require significant assistance in returning to daily life. Although recovery can also occur in these patients, it is often incomplete, and takes significantly more time to acquire. In many cases, these patients learn a new way of completing a task. True recovery, therefore, can be defined as a return of the initial behaviour, whereas compensation is that which occurs

when a patient reacquires the ability to complete a task without utilizing the exact initial movements and behaviours (Kolb and Whishaw, 1989). However, these terms are often used interchangeably, and here recovery will refer generally to a returned ability to complete a given task. Regardless of the definition and extent of recovery or compensation in these patients, however, outcomes following brain damage remain highly variable and difficult to predict. The GCS is a valuable tool for initial assessment and classification of the severity of brain damage, and can be utilized in various forms of brain damage, from coma, to TBI, to stroke.

1.2 Stroke

Stroke is a cardiovascular event characterized by a blockage or cessation of blood flow to or within the brain (Martin, 2006). Since the brain has very low energy storage capacity, it requires a constant supply of circulating blood to provide the nutrients necessary for cell survival, and to remove harmful waste products generated by the activity of neurons and glia. After only a few minutes of interrupted blood flow, brain tissue can be permanently damaged (Martin, 2006; Kandel et al., 2000).

There are two main types of stroke, ischemic and hemorrhagic. Approximately 80% of all strokes are ischemic, and occur when a blood clot blocks an artery supplying blood to a given region of the brain (Heart and Stroke Foundation, 2012). Ischemic strokes can be further divided into thrombotic strokes, where the clot forms in an artery within the brain, or embolic strokes in which a clot originating elsewhere in the body travels to the brain to cause a blockage there. Either form of ischemic attack can be permanent or temporary, the latter case being a TIA (Heart and Stroke Foundation, 2012). Hemorrhagic strokes comprise the other 20% of strokes, and are defined as a rupture of a

blood vessel in the brain. As with ischemic stroke, there are also two sub-classifications of hemorrhagic stroke; subarachnoid hemorrhage and intracerebral hemorrhage. A subarachnoid hemorrhage refers to bleeding on the surface of the brain, while intracerebral hemorrhage refers to bleeding deep within the brain (Heart and Stroke Foundation, 2012).

The most common sub-type of stroke is a unilateral middle cerebral artery (MCA) ischemic stroke (Heart and Stroke Foundation, 2012; Canadian Stroke Network, 2012). The MCA is directly linked to the incoming supply of blood from the heart to the brain through the internal carotid artery (Martin, 2006). The MCA supplies nearly 70% of the brain with blood, including regions of the cortex such as Broca's area, Wernicke's area, the pre- and post-central gyri, and the temporal and parietal lobes, as well as subcortical structures such as the basal ganglia and internal capsule (Kandel et al., 2000). Since the MCA supplies blood to motor areas of the brain, both cortical and subcortical, some of the most common deficits from this particular type ischemic attack occur within the realm of motor function, and more specifically, those related to limb movement.

Although stroke comprises only a small portion of all possible causes of brain damage, the results of population analyses specific to stroke alone are overwhelming. Any of the above mentioned sub-types of stroke can cause serious damage to brain tissue, which can result in severe behavioural and cognitive deficits, and even death. Current statistics from the Canadian population show that 10% of patients recover completely, and appear to be unimpaired by the stroke event (Heart and Stroke Foundation, 2012). Other patients recover only certain aspects of their original abilities (Bonita and Beaglehole, 1988; Sudlow and Warlow, 1997). Of the 50,000 stroke patients in Canada

each year, 25% are said to recover with minor impairments, and 40% are left with moderate to severe disabilities. Approximately 10% of stroke patients, however, do not show signs of recovery at all, and are so debilitated by the damage incurred that they require assistance with many aspects of daily care (Heart and Stroke Foundation, 2012; Rothwell et al., 2004). These statistics reveal the importance of stroke research and the development of treatment regimes, since the majority of patients do not have good prognoses and are unable to resume their normal lives.

It is not yet fully understood why stroke itself is so variable, or why patients with seemingly similar damage often have very different outcomes or prognoses. Physiological events throughout in the brain and body are under control of an endogenous daily time keeper. Therefore, numerous physiological parameters vary across the day, at both the whole body and cellular levels. One possibility, which has not been extensively investigated, is that daily rhythmic timing elements in stroke onset, manifestation, and outcome, as well as daily rhythms in related physiological parameters, may contribute to variability in brain damage, recovery, and potential brain repair.

1.3 Circadian Rhythms

The term "circadian" was first coined in 1959 by Franz Halberg. It stems from the Latin "circa", which means about, and "dies", a day. Circadian rhythms then, are cyclic variations that take approximately 24 hours to complete. The realization that organisms can maintain their own intrinsic circadian rhythm without exogenous input has been a topic of study for hundreds of years. The first noted observation of circadian rhythmicity took place as far back as 1729 when Jean Jacques d'Ortous de Marian observed a circadian rhythm in the opening and closing of the leaves of the *Mimosa* plant.

He found that once all external cues were removed, and the plant was maintained in constant conditions, it continued to behave as though it were in the presence of the sun. That is, the leaves of the *Mimosa* plant continued to open during the day, and close at night. Over time however, the rhythm of the opening and closing of the leaves drifted further from that of the natural light/dark (LD) solar cycle, yet the "behaviour" continued to exist. Since then research has vastly progressed, and it is now well understood that nearly all organisms from plants and bacteria to mammals, exhibit circadian rhythms in physiology and behaviour.

Circadian rhythms evolved as a means of optimizing survival. Within an organism's environmental niche there are also temporal niches that can be exploited. To efficiently occupy a temporal niche the organism must have some means of keeping time in absence of salient external cues such as the LD cycle, or some means of anticipating the LD cycle. Hence, the necessity of an intrinsic circadian time keeper emerged and was maintained. As an example, many rodent species have evolved to be nocturnal and conduct most of their active behaviours at night. This may promote their survival by making them more difficult for predators to locate. When rodents are foraging for food, looking for mates, or caring for their young, it is most beneficial to do so in the dark phase of the LD cycle to keep from being exposed and easy to detect in the light. Other benefits to occupying a temporal niche in one's environment relate to homeostasis and other metabolic processes.

In mammals the master circadian clock, aka the "master circadian pacemaker", is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. The SCN is a relatively small brain region comprised of approximately 20,000 neurons bilaterally

(Reppert and Weaver, 2001; Weaver 1998). These neurons, however, are not homogenous in nature and are comprised of various groups of cells with very different phenotypes (Weaver, 1998). Two of the most blatant demarcations within the SCN are termed the "core" and the "shell" regions. The retinorecipient cells of the ventrolateral region of the SCN, known as the "core", lie next to the rhythmic cells of the dorsomedial region, named the "shell". Within the core of the SCN, cells receive input from the retina via the retinohypothalamic tract regarding changes in external light cues. These cells then pass this information on to the shell cells, which oscillate intrinsically even without the presence of LD cycle information. It is the communication between these two areas that keep the circadian rhythm of the intrinsically oscillating cells in tune with the external environment. Without this information, the shell cells would maintain their own oscillatory rhythm, and like the *Mimosa* plant, would slowly drift away from the LD rhythm of the external world. When this drifting occurs, for example in experimentally induced constant conditions, the organism is said to be "free-running" on its internal clock's time only. Most often the internal clock maintains near, but not exactly, 24 hour rhythmicity which explains the gradual drift from the 24 hour solar cycle.

Many aspects of the master circadian pacemaker's mechanisms of action are well understood thanks to years of valuable research (Reppert and Weaver, 2001; Weaver 1998). Much of this information was initially retrieved from studies utilizing *Drosophila,* further exemplifying the comprehensive nature of circadian rhythms throughout the animal kingdom. Specific genes have been identified that are directly linked to the cycle of the circadian rhythm of an organism. One of the first of these "clock genes" was discovered in the 1970's by Konopka and colleagues. This was the Drosophila form of

the Period (Per) gene, and was later cloned and found to play a role in the circadian system of mammals (Reddy et al., 1984). After the identification of *per* in Drosophila, an understanding of the overall function of the Drosophila circadian system came to light, and this knowledge was expanded in studies of mammals.

1.4 Transcription-Translation Feedback Loop/Inner Workings of the Clock

The clocks of Drosophila and mammals share many similar molecular and genetic components, however, we will focus here on a description of the mouse circadian system and the role that clock genes play in creating and maintaining rhythmicity in these mammals. The circadian clock of the mouse is one of the best described, and the basics of the pacemaker function as well as many of the genes involved are similar in humans. Following the identification of *Per* other clock related genes, namely, *Clock*, *BMAL1*, and *Cryptochrome* (Cry) were noted as major components in the system. There are, of course, other genes involved, but these genes will outline the function of the clock sufficiently for the purposes of this description.

A basic principle that must be understood about the way in which circadian rhythms are formed in an organism is that of a transcription-translation feedback loop. Specifically in the mouse, the cycle begins in the nucleus of an oscillating cell within the SCN. CLOCK and BMAL1 (also known as MOP3 or ARNT1) heterodimerize within the nucleus and it is this pairing that begins the positive end of the feedback loop. As levels of the CLOCK:BMAL1 heterodimer increase, they bind to E-Box promoter regions of various genes, for our purposes *Per1* and *Per2* and probably *Cry1* and *Cry2*. This increases the transcription of the *mPer* and *mCry* genes. *Per* and *Cry* mRNA then move to the ribosomes of the cytoplasm, where they are translated into PER and CRY proteins.

These two proteins accumulate outside of the nucleus, where they dimerize and then translocate back into the nucleus. Once inside the nucleus, the PER:CRY dimer blocks the CLOCK:BMAL1 induced production of their own mRNA, thereby decreasing their own production (Dunlap et al., 2004). It is this feedback loop, which takes approximately 24 hours to complete, and makes the circadian clock "tick" in a daily fashion. Figure 1.1 shows a representation of this feedback loop.

The timing of these molecular events varies throughout the 24 hour day. The temporal story of the transcription-translation feedback loop begins in the mid-evening to late night, when CLOCK and BMAL begin to heterodimerize and initiate transcription of *mPer1*, *mPer2*, *mCry1* and *mCry2*. Levels of BMAL1 and CLOCK are highest in the late evening or approximately circadian time (CT) 18 (which is 6 hours after activity onset, defined by convention as CT12 in nocturnal animals). An increase in the formation of the CLOCK:BMAL1 heterodimer occurs by early morning or CT0, also resulting in a peak in the amount of *mPer* and *mCry* at around CT 6. Production of PER and CRY proteins also begins in the morning, and levels peak in the evening at around CT10-12, approximately 4-6 hours after the peak in their morning mRNA production. At this time, the increased levels of the PER:CRY complex take action in the nucleus, initiating the repression of the CLOCK:BMAL1 heterodimer activity. This results in a decrease in *mPer* and *mCry* near CT12. By CT18, the effect of the repression of PER:CRY on the CLOCK:BMAL1 complex is reduced enough by the degradation of the PER:CRY complex that the cycle can start again (Dunlap et al., 2004). Though this is a simplified view of all that occurs in an oscillating cell of the SCN, it is sufficient to give an

understanding of the inner workings of the clock and to highlight some of the major components necessary for appropriate clock functioning.

1.5 Peripheral Clocks

In mammals, endogenous rhythmicity has been noted in brain regions outside of the SCN, as well as in peripheral tissues. For example, rhythmic clock gene expression exists in cells of the hippocampus, stria terminalis, and more (Abe et al., 2002). Cells in the liver, alimentary tract, bone marrow, and oral and rectal mucosas (for review, see Balsalobre et al., 2002) also show circadian oscillation. These clocks function in a similar way to cells in the SCN, however, without some input from the SCN itself these peripheral rhythms fade and gradually disappear (Balsalobre et al., 1998). In animals that have had their SCN removed or disconnected from the surrounding brain tissue, rhythms in peripheral tissues slowly degrade over time (Balsalobre et al., 2002; Sakamoto et al. 1998). Studies have also shown that transplanting the SCN region of one animal into that of another, results in maintenance of the rhythm of the transplanted tissue, as opposed to the initial rhythm of the animal before the transplant (Balsalobre et al., 2002; Earnest et al., 1999; Silver et al., 1996). This is, therefore, significant evidence showing that the overarching influence of the master circadian clock is necessary to keep peripheral oscillators in time throughout the body.

The ways in which the master circadian oscillator communicates with other rhythmic tissues are many. Some brain regions are directly connected through synaptic projections from the SCN and are therefore influenced and kept in time via a direct flow of information from the SCN (Kalsbeek and Buijs, 2002). Other areas of the brain and body appear to have multi-synaptic connections to and from the SCN, which are likely

also important for maintaining those regions' rhythmicity (Kalsbeek and Buijs, 2002; Sylvester et al., 2002). Another major method of SCN influence over peripheral oscillators lies in diffusible humoral signals that leave the SCN and end in communication with other brain and body regions. In a study conducted in 1996, Silver and colleagues were able to encapsulate SCN grafts into animals with ablated SCNs. They showed that the encapsulated graft was able to restore rhythmicity without any direct connections to other parts of the body (Silver et al., 1996). This ability of the SCN to communicate through diffusible humoral signals is not surprising, given the vast amount of hormones produced in and released from the hypothalamus, and the extent to which this region of the brain uses hormones as a means of communication with other parts of the body. For example, another form of hypothalamic hormonal communication is the release of ATCH to communicate with the adrenal glands in the HPA axis and stress response (Kalsbeek and Buijs, 2002; Kalsbeek and Buijs 1992, Kandel et al., 2000).

1.6 The Clock and Disease

In knowing the ubiquitous nature of circadian rhythms throughout the animal kingdom, and their vast dispersion even throughout an organism, research in recent years has begun to focus on the role that these rhythms may have on disorders and disease. The most prominent example of this has taken place in human cancer treatment. This is one of the few areas in human disease research that has utilized circadian rhythms to optimize treatment regimes, and researchers and doctors have done so with much success (Lis et al., 2003; Focan, 1995). The idea of utilizing circadian timing for treatment of cancer came about shortly after the discovery that the formation of cancer itself is strongly

influenced by circadian rhythms (Granda and Levi, 2002). It is becoming more commonly known that individuals who are not entrained to their environment's light dark cycle, such as shift workers and those who travel frequently by air and are therefore subject to frequent jet lag, are at a much higher risk of developing cancer, as well as many other diseases and disorders such as cardiovascular disease (Caciari et al., 2013; Grundy et al., 2013a; Grundy et al., 2013b; Vyas et al., 2012; Lis et al., 2003). Even with the growing knowledge of circadian influence on disease, many other areas of human disease research have not investigated potential circadian influences. It is a growing field that will undoubtedly be able to help many patients with various aspects of their ailments in the future.

1.6.1 Circadian Rhythms and Stroke

To test the overarching hypothesis that circadian rhythms can affect various brain disorders and disease, the present dissertation will examine the relationship between circadian rhythmicity and stroke. Several factors involved in stroke are known to show daily rhythmic oscillations. It is well known that while a stroke or cardiac attack can occur at any time of day, there is a much higher frequency of stroke in the morning, referred to as the "waking hours", than at other times of day (Argentino et al., 1990; Toni et al., 1991). Interestingly, blood has many properties that fluctuate in a circadian manner and these could influence the occurrence of stroke, including clotting factors, red blood cell numbers, and hormone composition.

The activity of clotting factors in the blood change rhythmically throughout the day, and changes in the properties and composition of the blood appear to influence the occurrence of stroke (Kubota et al., 1987; Hartman and Ashkenazi, 1976; Scheving and

Pauly, 1967). Thrombogenesis is strongly influenced by the circadian clock, with some clock mutant animals exhibiting accelerated thrombogenesis after undergoing a model of stroke (Scheer et al., 2011; Westgate et al., 2008; Kubota et al., 1987), suggesting that this factor is highly regulated by the circadian system, and linked to stroke onset and outcome.

Another seemingly important variable and constituent of blood is hormones, as blood is often the carrier of hormones and humoral signals such as those mentioned earlier in the SCN's communication with peripheral clocks (Silver et al., 1996; Haapaniemi et al. 1992). There is, for example, a circadian rhythm in corticosteroid, insulin, and catecholamine concentration in the blood that peaks during the day and troughs at night (Tofler et al. 1987). Growth hormones, prolactin, thyroid stimulating hormone, arginine vasopressin, and melatonin are often higher during the night than during the day (Maywood et al., 2007). So not only do physiological components of the blood itself exhibit circadian rhythms, but many substances and molecules in blood show similar rhythms.

Other important rhythms that may relate to stroke affect the performance of the body, and may also influence blood flow. For example, the severity of stroke is known to be affected by body temperature, in that lower temperature can be neuroprotective (Linares and Mayer, 2009). Temperature can also influence blood flow and viscosity, and is one of the first physiological systems in which circadian rhythms were measured (Refinetti and Menaker, 1992). Heart rate, daily exercise and activity, and blood pressure also fluctuate on a daily cycle, and have been linked to stroke onset (Morris et al., 2013;

Scheer et al., 2010). The daily variation in these factors is likely to influence the temporal aspects of stroke as well.

Other evidence suggesting a circadian role in stroke and cardiovascular disease comes from the finding that cardiovascular disease itself can influence the cycle of circadian clocks in various tissues. A recent study utilizing a mouse model of atherosclerosis showed a change in the circadian rhythm of various clock genes which correlated with the severity of the disease (Xu et al., 2009). The circadian rhythm of some apoptosis genes is disrupted in this model as well, raising the possibility that cardiovascular disease can change the viability of cells themselves.

The fact that circadian rhythms are so prevalent throughout mammalian physiology has over the years lead to the prediction that diseases which affect these systems are also under some form of circadian control. Circadian rhythmicity has, in fact, been shown to influence disease (Litinski et al., 2011; Rajaratnam and Arendt, 2001). For example, cardiovascular accidents occur more frequently in the morning, asthma is often worse at night, and temporal lobe epileptic seizures commonly occur in the afternoon. Circadian rhythms and the systems they control may interact with the environment to influence the manifestation of many diseases, specifically cardiovascular disease (Reilly et al., 2007). Understanding which aspects of circadian rhythms in physiology are related directly to the severity of a stroke and subsequent recovery could help identify approaches which may aid recovery following an ischemic event.

1.7 Methods of Recovery

At present, options for aiding recovery from brain damage are quite limited. Though spontaneous functional recovery or compensation does occur in brain damaged

patients, it is usually only apparent in those with mild damage. Immediately after a stroke or brain damaging event, a number of factors contribute to this type of recovery. Complex cascades of cellular and molecular events are activated in both the immediate area of damage, as well as in surrounding areas (Schallert et al., 2000). These processes include inflammation, changes in transcription and translation, secretion of growth factors, changes in neurotransmitter receptors, formation of new synapses, and sprouting of axons (Cramer, 2008), all of which facilitate the "rewiring" of neuronal networks with the goal of compensating for the loss of damaged brain tissue.

Aside from spontaneous recovery, methods of inducing recovery or compensation are being developed. The most promising opportunity in practise today for inducing recovery in moderate to severe brain damaged patients lies in physical or occupational therapy. Persons suffering from stroke or other forms of brain damage show the greatest recovery when placed in therapeutic sessions which focus specifically on their acquired deficits. Some of these include constraint induced movement therapy (CIMT), body weight-supported treadmill training (BWSTT), robotic training, transcutaneous neuromuscular electrical stimulation, noninvasive brain stimulation (NIBS) such as repetitive TMS (rTMS) and transcranial direct current stimulation (tDCS), action observation, virtual reality (VR) training, and brain-computer interface (BCI) (Takeuchi and Izumi, 2013)

The time window for spontaneous or therapy induced recovery is not indefinite. Recovery is generally at its highest level in the first four weeks following the infarct, and is best accepted in the first 3-6 months following the damage (Grefkes and Ward, 2013; Cramer, 2008; Bonita and Beaglehole, 1988). Although, some practitioners also state

that changes and improvements in functional ability can be seen for as long as 2 years following brain damage (Rehabilitation Institute of Chicago, 2012).

Pharmacologic interventions are another possible option for assisting recovery. At this point, most pharmacologic interventions aimed at alleviating brain damage have not progressed into clinical use. Currently, there is only one approved and commonly utilized drug intervention for stroke patients, yet it has many downfalls. This substance is known as tissue plasminogen activator (tPA), and is now frequently administered to stroke and TBI patients. However, it must be administered within a small time window after the onset of the stroke event, in as little as 2 to 3 hours. Its use also requires that the type of stroke, ischemic or hemorrhagic, be known prior to administration, as tPA can exacerbate hemorrhagic stroke. Ultimately, it is a clot busting, blood thinning substance, and therefore one can imagine the effects of this substance on hemorrhagic stroke (Nadeau et al., 2005). Difficulties in the use and misuse of tPA have arisen as well, to the point of resulting in legal action against neurologists and hospitals, further indicating the need for a more precise pharmacologic intervention (Weintraub, 2006).

One other avenue that is currently being explored as a means of intervention in brain damage recovery is that of inducing neurogenesis (Xiong et al., 2010). In areas of damage, it is possible that the generation of new neurons and the ensuing growth of new brain tissue may increase functional recovery or compensation following damage. Manipulations that enhance neurogenesis have been shown to promote functional recovery following brain injury (Zhang and Chopp, 2009). This suggests that stimulation of endogenous neural precursors into a proliferative state may be a promising potential therapy for functional compensation and recovery following brain damage.

There are currently many PhaseII and III trials utilizing factors such as erythropoietin (EPO) and carbamylated erythropoietin CEPO (Ehrenreich et al, 2009; also available at clinicaltrials.gov). These studies aim to show a greater functional recovery that is reliant on the generation of new neurons. However, issues have arisen in getting these substances into our healthcare system, such as dosage, single vs. multiple doses, administration route, timing of administration, and potential combination therapies (Xiong et al., 2010).

Research is still near infancy with regard to inducing neurogenesis as a means of functional repair following brain damage. The induction of neurogenesis, such as that which occurs naturally in the subgranular zone (SGZ) or subventricular zone (SVZ) in the brain, aiding those new cells in transport to damaged areas, and aiding in their incorporation into neural networks holds much promise for inducing neural regeneration and aiding functional recovery from brain damage.

1.8 Cell division

The key to enhancing recovery through neuroregeneration is to identify and ultimately exploit endogenous regulators of the cell cycle. Numerous cells in the mammalian body are constantly undergoing regeneration, and this regeneration occurs in a cyclic manner (Smaaland, 1996; Alberts et al., 1994). Skin cells are constantly being replenished, liver cells undergo proliferation and maturation throughout life, and as has been mentioned above, even parts of the brain are constantly undergoing mitotic cell division. Studies have shown that the mechanisms behind cell division are complex, yet very similar across species, from yeast to humans. Therefore, studies in simple organisms, such as yeast, have allowed for a much greater understanding of the cell

division cycle (Alberts et al., 1994). Describing the entire cell cycle and its control systems is beyond the scope of this dissertation, however a brief outline of the major events that occur in a dividing cell is necessary before continuing on.

The cell division cycle (often referred to as "cell cycle", or "mitotic cell division") in its simplest explanation involves three major stages; chromosome replication, chromosome segregation, and cell division. These stages are also commonly referred to as interphase and metaphase (or M phase). In M phase (also referred to as mitosis) the nuclear envelope breaks down, its contents condense into chromosomes, and microtubules align in what is called the mitotic spindle, which will eventually separate duplicated chromosomes (Schafer, 1998; Alberts et al., 1994). While M phase can be subdivided into various other phases, an overall idea of what occurs in M phase as a whole is sufficient for our descriptions. In interphase, however, understanding of the various substages becomes more important, as later we will see that various techniques for visualizing dividing cells take advantage of the events that occur within interphase.

Interphase, in initial observations under a microscope, seemed like an uneventful time in the cell division cycle, where the cell grows in size. Yet further investigations using various techniques have shown that the seemingly extended interphase period comprises many of the necessities of cell division. The first portion of interphase is referred to as the G_1 phase. This stage comprises the time between the completion of the most recent mitosis, and the beginning of DNA synthesis. Following G_1 phase, S phase begins and replication of nuclear DNA occurs. The interval between the end of DNA replication and the beginning of actual cell division (or M phase) is called G_2 phase.

From the initiation of events that lead to cell division, cells progress through G_1, S, G_2, and on to M phase, where division occurs (Alberts et al., 1994).

When discovering these different stages of the cell cycle, researchers found that cells in the S phase can be visualized by supplying them with labelled molecules of thymidine. Cells use excessive amounts of thymidine in DNA replication, therefore cells in the S phase take up significant amounts of the labelled compound. The label can be radioactive, as in the case of ^{3}H-thymidine, or a more commonly used chemical label works in much the same way, namely bromodeoxyuridine, (BrdU), an artificial thymidine analog. BrdU labelling can then be visualized by staining the tissue with anti-BrdU antibody (Alberts et al., 1994). Not only is this one of the most common methods of identifying cells that have recently undergone S phase replication, but the ease with which BrdU labelling is accomplished makes it a valuable tool for investigating cells undergoing cell division. In later chapters, we chose to use BrdU labelling for its ease and accuracy in identifying dividing cells.

1.9 Regeneration

1.9.1 Peripheral tissues

Many tissues throughout the body are continuously regenerating throughout an organism's lifespan. These include, but are not limited to epithelium, tongue, blood cells, liver, skeletal muscle, and bone marrow (Khapre, 2010; Garcia et al., 2001; Bjarnason and Jordan, 2000; Buchi et al., 1991; Brown, 1991). The process of regeneration in the cells of these bodily tissues follow that of the cell cycle, described above, and have been shown to be influenced by circadian controlled factors (Matsuo et al., 2003; Schibler, 2003). Rates of regeneration can differ in various tissues, depending on factors such as

body temperature and food availability, but each area undergoes similar processes of progression from G_1 through M phase (Khapre, 2010; Alberts et al., 1994).

1.9.2 Neurogenesis

For decades it was thought that the adult brain is incapable of neural regeneration, that is, of growing new neural tissue. This is true in many cases and brain regions, for example if the cerebral cortex is damaged by TBI or stroke it will not sporadically regrow. In fact, there is little evidence for the existence of any cortical re-growth in an adult brain, be it in human, dog, rat, or mouse. However, the inability to produce new cells is not a ubiquitous feature of the adult brain. There are two well known regions of the brain that are continuously producing new cells throughout an organism's lifetime. These two areas are located in quite different regions of the brain, and it is known that the processes occurring in each region are not linked to one another. That is, the production of new cells in one area does not appear to instigate or influence the growth of cells in the other.

The first of these perpetually proliferating regions is the SVZ. Newly generated cells travel from the SVZ along the rostral migratory stream (RMS). The RMS begins at the SVZ, and extends to the olfactory bulb (OB), the most rostral portion of brain matter. In the ventricles, new cells are formed daily, and travel rostrally along the RMS to be incorporated into the functioning networks of OB. The olfactory system is one that requires constant regeneration, as cells in this area have a lifespan of only 30-60 days (Gould, 2007; Kandel et al., 2004).

1.9.3 The Hippocampus

Another area of the brain that is continually generating new cells lies in the dentate gyrus (DG) of the hippocampus, and is called the SGZ. The hippocampus is known to be involved in many important processes, such as learning and memory (Milner et al., 1998; Corkin et al., 1997; Maguire et al., 1996; Muller, 1996; Squire and Zola-Morgan, 1991). Specifically, spatial memory seems highly reliant on the hippocampus, as does the formation of new implicit memories in rodents (McHugh et al., 1996; Silva et al., 1992; O'Keefe and Dostrovsky, 1971). Since memory is constantly changing, new memories being formed and old memories being altered or forgotten, it makes some logical sense that newly generated neurons and glia, as well as apoptotic cells in this area, may be contributing to the functional role of this brain region.

Some of the first noted instances of DG neurogenesis were largely ignored. These studies showed that there were, in fact, new cells being generated, and shortly after it was shown that these cells hold properties of new neurons (Altman and Das, 1965, Kaplan and Hinds, 1977). This exciting new finding, though largely ignored at the time, set the stage for the years of research to follow beginning in the 1990's with the development of BrdU-technique (Kuhn et al., 1996). Other methods and technological advancements, such as retroviral methods, genetic marking, electron microscopy, electrophysiology, and transgenic mouse models have further added to the vast field of hippocampal neurogenesis research (Deng et al., 2010; Zhao et al., 2008; Ming and Song, 2005; Carleton et al., 2003; van Praag et al., 2002)

Numerous studies have evaluated the fate of SGZ progenitor cells. The time course of development of cells in this area has also been extensively investigated,

although some further clarification may be necessary with regard to species differences (see Zhao et al., 2008 for review). Overall however, it is thought that cells of the SGZ take at least a month to become functionally integrated into DG/hippocampal neural circuits, and their complete connectivity and incorporation into a functional network may be occurring for as many as 3 months after the birth of the new cell (Vivar and van Praag, 2013; Toni et al., 2007; Zhao et al., 2006; van Praag et al., 2002). The progression of growth leading up to functional incorporation is important to understand for the purposes of experimental design, as well as to understand cell fate after division.

One week after new cells are born in the SGZ, those destined to become neurons (Type II cells) begin to migrate from the SGZ into the granule cell layer (GCL) of the DG. These cells do not yet show spontaneous firing (Esposito et al., 2005), nor do they have any dendritic processes (Vivar and van Praag, 2013). Like cells in the developing brain, they are activated by GABA, and tonically applied GABA causes depolarization of these cells (Laplagne et al., 2007). As their growth continues into their second week of life, with the aid of various growth factors and the supporting DG environment, spineless dendrites begin to form. By 14 days after their birth, the new cells show projections that lead to pyramidal cells, as well as to both inner and middle layers of the DG (Zhao et al., 2006). GABA continues to have a depolarizing effect on these new cells, and their spontaneous firing rate begins to increase (Lo Turco and Kriegstein, 1991). Near the end of two weeks after cell birth, glutamatergic inputs become more important to survival (Tashiro et al., 2006). Since the major glutamatergic inputs to the DG do not form until approximately one month after these cells are born, up to 50% of the newborn cells undergo programmed cell death (apoptosis) at this 2 week time point (Cameron et

al.,1993; Dayer et al., 2003). Those that survive are thought to receive glutamatergic stimulation from mossy fibers and possibly from surrounding cells of the DG (Vivar et al., 2012). By four weeks of age, new cells have some normal projections to all layers of the DG, and to other areas of the hippocampus. Spines have formed on the dendritic outgrowths, and GABA is now hyperpolarizing (Ge et al., 2006; Ming and Song, 2011). Glutamate becomes the main excitatory influence on these cells and they exhibit normal spontaneous activity (Esposito et al., 2005, Vivar et al., 2012). So at four weeks after the initial mitotic activity resulting in the generation of new cells, those that have survived are showing characteristics of functional DG cells.

There are indications that these cells remain somewhat immature, in that they show higher input resistance and smaller membrane capacitance (Mongiat et al., 2009; Couillard-Despres et al., 2006; Esposito et al., 2005; Ambrogini et al., 2004; van Praag et al., 2002). Functionally, studies showing the unique connectivity of new DG cells suggest that they may be preferentially involved in novel object recognition, spatial learning, visual recognition, contextual learning, and memory as a whole, due to the function of the areas from which they receive direct and indirect projections. The exact details of the function of new DG cells are currently being discovered (for a brief review see Vivar and van Praag, 2013). For the purposes of the studies to follow, it is important to note that DG cells that survive to 4 or more weeks after they are generated become part of DG networks (Faulkner et al.,2008; Ide et al.,2008; Toni et al.,2008; Zhao et al.,2006). From this time on, they continue to grow, fire, and prune according to input and feedback from other areas within these networks, and from intra-DG communication.

Due to the time course of cellular maturation as explained briefly above, studies aiming to examine cell survival and the fate of new cells which are incorporated into DG networks should focus such examinations to time points beyond 4 weeks after cell birth. For example, to examine cell survival with BrdU, one should visualize BrdU labelling at least 6 weeks following the injection. In examination of immature cells, one can choose a time point that corresponds to the cellular activity and connectivity at various weeks and days following mitosis.

1.10 Medial Prefrontal Cortex

Another area of the brain has been shown to have regenerative properties. This less notorious model of brain regeneration has many more limitations than the continuous neurogenesis that occurs throughout adulthood in the SGZ and SVZ. However, it has been repeatedly shown in both rat and mouse models that the medial prefrontal cortex (MPFC) can regenerate after aspiration lesions induced on days P7-10 (Dallison and Kolb, 2003; Kolb et al., 1998a; Kolb et al., 1998b; Kolb et al., 1996; Phillips et al., 2009; unpublished data). The MPFC is involved in attention, cognition, emotion, mood, executive function, and temporal aspects of behaviour, to name just a few of its primary functions (Dalley et al., 2004; Eslinger et al., 2004; Fuster, 2002; Ragozzino et al., 2002; Delatour and Gisquet-Verrier, 2000; Dias and Aggleton, 2000; Kraemer et al., 2000; Owens et al., 2000; Cho et al., 2000; Mitchell et al., 1998; Drevets et al., 1997). The link to temporal aspects of behaviour is shown in studies where MPFC damaged maternal rodents show abnormalities in the timing and sequence of maternal behaviours such as nest building. These females have the motor ability to build a nest, yet the temporal aspects of the behaviour are out of sequence, and therefore building of the nest is not

cohesive as in intact maternal females (Afonso et al., 2007). This example, as well as others showing deficits in temporal aspects of behaviour (Adan and Sanchez-Turet, 2001; Kraemer et al., 2000; Owens et al., 2000; Cho et al., 2000; Mitchell et al., 1998; Drevets et al., 1997), suggest a link with the circadian timing system. Other studies have shown a circadian rhythm in attention, as tasks that require high levels of attention are performed better at certain times of day in individual subjects, in both rats and humans (Delatour and Gisquet-Verrier, 2000; Kraemer et al., 2000).

Further evidence of circadian influence on MPFC function comes from studies that have shown multi-synaptic connections between the MPFC and the SCN. Using a viral transneuronal labeling method, specifically, a pseudorabies virus (PRV) injection, Sylvester and colleagues (2002) investigated inputs to various regions of the MPFC. They showed that there is a multisynaptic pathway from the SCN to the infralimbic cortex (ILC) of the MPFC. They also determined that this may be the only region of the MPFC to receive such SCN innervation, as injections of PRV into other MPFC regions, such as the cingulate, prelimbic, and cortical areas showed very low numbers of trasneuronally labelled cells stemming from the SCN. The majority of the transneuronally labeled cells appeared in the dorsomedial shell of the SCN, the area responsible for maintaining intrinsic free-running rhythms. It may be the case that the information received by the ILC is sufficient to keep the entire MPFC in tune with SCN oscillation. It may also be the case that the ILC is the only MPFC region that requires rhythmic information, as it is involved in many of the roles associated with MPFC function (Maroun et al., 2012; Lehmann and Herkenham, 2011; Mueller et al., 2008). Another finding from Sylvester and colleagues' (2002) study was that the paraventricular

thalamic nucleus (PVT) is a critical relay station in the communication between the SCN and ILC. Once ablating the PVT region, tracing from the ILC to the SCN was eliminated. As mentioned earlier, however, there are many other ways in which circadian information can reach the MPFC, and these have not yet been fully investigated (i.e., hormonal communication).

Circadian rhythmicity in the MPFC itself has also been examined. The projections noted by Sylvester and colleagues (2002) may be involved in the rhythmic expression of clock genes in the MPFC, yet data regarding rhythmicity in this area have differed. In 2001 and in 2004, Abe and colleagues examined circadian rhythms in clock gene mRNA, namely *mPer1* and *mPer2*, in various regions of the brain. They found rhythmic expression of these in the cingulate area of the MPFC. Other studies have failed to replicate these findings however (unpublished data), and so rhythmicity of the MPFC region need be investigated further, possibly with greater control over the specific region within the MPFC being investigated. Since there are numerous temporal aspects to MPFC dependant behaviours, and it has been determined that clock genes are expressed in MPFC cells (Abe et al., 2001; Abe et al., 2004), it is likely that MPFC function is under circadian control and regeneration that occurs following damage to the MPFC region may be influenced by circadian oscillations (see section 1.12 below).

1.11 BMAL1 Deficiencies

As was described earlier, the function of the circadian clock revolves around four major cellular components, CLOCK, BMAL1, Per, and Cry. Knockout strains of animals lacking each of these core clock components have been developed. Interestingly, BMAL1 is the only of these core clock genes that does not have a functional paralog

(Bunger et al., 2000). This results in a BMAL1 deletion being the most detrimental to circadian rhythmicity, both at the behavioural and cellular levels. These animals often lack behavioural rhythmicity, as is shown in Figure 1.2 actograms of daily activity, as well as in Bunger and colleagues' (2000) study. Rhythmicity in associated clock genes, such as *Per* and *Cry* become aberrant in BMAL1 knockouts as well, since the transcription-translation feedback loop lacks the initiating component BMAL1 (Bunger et al., 2000). Therefore, the best model for showing effects of circadian rhythmicity and the overall functioning of the circadian clock is that of the BMAL1 knockout. Ablating other portions of the transcription translation feedback loop does not affect oscillatory behaviours or cellular functions in such an extreme and overarching manner. For example, while Clock mutants eventually become arrhythmic, bona fide clock knockouts exhibit robust circadian rhythms (Vitaterna et al., 1994).

1.12 Circadian Influence on Cell Division

1.12.1 Circadian Influences on Peripheral Tissue

Since the late 50's and early 60's, circadian rhythms in mitotic activity and cell proliferation have been noted in mice. In these studies, examination of such rhythms commonly took place in epithelial cells of various regions of the alimentary tract (Scheving et al. 1983 for list of old studies). More recently, such rhythms have also been described in tongue keratinocytes, bone marrow in both mice and in humans, human rectal epithelium, and skin (Garcia et al., 2001; Bjarnason and Jordan, 2000; Buchi et al., 1991; Brown, 1991). These studies suggest a circadian influence on the cell cycle and therefore in cell division and proliferation. There is a rhythm in the phases of cell division in mice, with a peak in M phase near activity onset. This also applies to humans,

where M phase activity is highest in the morning hours, and respective peaks in S phase correspond with this rhythm (Alberts et al., 1996).

Even more recently, however, studies have discovered a unique role of the circadian timing system in cellular regeneration in the liver, again exemplifying the overarching control that the circadian system has over physiology and cell division. Because the liver has the ability to regenerate following damage or partial hepatectomy (PH), and it is also known to exhibit clock gene oscillation intrinsically (under the influence of the master circadian pacemaker), Matsuo and colleagues (2003) examined circadian aspects of liver regeneration after PH. Though they looked at many different components of known circadian genetics, one of the major findings was a discrepancy in the rate of re-growth of the hepatic cells in animals lacking a fully functional circadian clock. By performing PH on *Cry* deficient knockout mice as well as controls, they discovered that liver weight was significantly lower 72 hours after PH in the *Cry* knockouts than in healthy wild-types. This difference was no longer apparent by 10 days post injury, yet does suggest impaired cellular proliferation in the Cry-deficient mice, as well as suggesting the necessity of normal circadian clock function in certain stages of cell division. Further analysis revealed that the transition from S to M phase and the differences in genes associated with the transition between phases was slowing proliferation in the Cry-deficient mice. Overall, however, this study shows that a fully functioning circadian clock may be necessary for normal cell division to occur in liver tissue.

1.12.2 Circadian Influences on Brain Tissue

The specific details of the circadian influence on brain tissue regeneration have not been extensively investigated. One study examining the role of Per2 in hippocampal neurogenesis found that although rhythmic expression of PER2 did not occur, its presence there played a role in regulating neurogenesis. PER2 was expressed in NPSCs as well as in new born/early post-mitotic cells within the DG. Using a Per2 knockout strain of animals, as well as *in vitro* analysis of Per2 knockout brain tissue, Borgs and colleagues (2009) found that cellular proliferation was significantly increased in these animals. However, the overall number of surviving DG cells was not increased, as an upregulation of cell death accompanied the increase in proliferation. This study suggests that Per2 plays a regulatory role in cell proliferation, and is linked to an increase in cell death (Borgs, et al, 2009). Yet as was mentioned earlier, Per2 plays a subtle role in the overall control of circadian rhythmicity, and therefore these studies do not show the role of circadian clock function on hippocampal neurogenesis.

Previous studies mentioned here may provide preliminary evidence that circadian clock genes play a role in cell division that is separate from their role in keeping time. Many genes have evolved with numerous purposes, depending on their location in the body, and this diversity in function may apply to clock genes as well. However, it is important to first determine if there is an overarching role of circadian rhythms on cell division: proliferation, differentiation, survival, and death.

1.13 Present studies: Objectives

In the following chapters, the circadian influence on stroke and brain regeneration models will be examined. The studies presented here are aimed at furthering our

knowledge of how the circadian clock might affect outcome after brain damage, as well as how clock genes influence cell division in various parts of the brain. It is commonly accepted that not only is there a circadian rhythm in the occurrence of cardiovascular events, which is not well understood, but that there is also a circadian influence on tissue regeneration, a natural occurrence that holds great promise in aiding recovery from various forms of brain damage, including cerebrovascular events. Therefore, the following studies aim to show effects of time of day on outcome following brain damage, as well as to characterize circadian clock gene expression in newly generated tissue, and to examine the effects of arrythmicity on neurogenesis.

1.14 Summary and Hypotheses

1.14.1 Experiment 1: Lesion size and behavioral deficits following endothelin-1-induced ischemia are not dependent on time-of-day

A circadian rhythm in stroke onset is known to occur (Elliott, 1998), with a peak in the morning hours after waking. Many factors are likely to contribute to this peak in stroke onset, as circadian rhythmicity occurs in many aspects of physiology that are related to stroke, such as blood pressure, body temperature, blood clotting agents, hormones, and overall activity, to name a few. Few studies have examined whether this time of day variation influences the overall outcome following the stroke. The study described here in Chapter 2 aims to determine if the time of day that a stroke occurs may be linked to lesion size, behavioural deficits, and recovery. I hypothesized that greater lesion size and behavioural deficits would be observed, along with a reduction the rate or severity of recovery from early morning stroke compared to stroke at other times of day. Results, however, do not suggest differences in any of these factors based on the time of

day that the stroke occurred. The time of day that a stroke occurs, therefore, may not significantly affect overall outcome.

1.14.2 Experiment 2: Clock gene Expression in Regenerated Medial Prefrontal Cortex

Understanding neonatal MPFC regeneration may be useful as a potential method of inducing regrowth and recovery following damage from stroke or TBI. Due to the variation in circadian behaviours following MPFC aspiration and regrowth (Phillips et al., 2009), and the multi-synaptic inputs from the SCN to the MPFC, circadian influences on MPFC regeneration will undoubtedly be important aspects of the cellular environment necessary for inducing this type of neurogenesis. Therefore, Chapter 3 will look at clock gene expression in MPFC of healthy adult mice and those with P7 aspiration to determine if circadian clock gene expression in this area differs between normal and regenerated tissue. The prediction was made that clock gene expression in regenerated tissue would significantly differ from that in healthy MPFC, and that these differences would suggest inefficient communication between the SCN and the newly generated tissue. Results, however, show a lack of rhythmicity in MPFC tissue of both aspirated animals and controls, yet significant differences in overall gene expression between groups were not apparent. This suggests that clock gene expression in newly generated tissue may not be significantly altered following MPFC aspiration.

1.14.3 Experiment 3: Medial Prefrontal Cortex Regeneration in Arrhythmic BMAL1 knockout mice

Previous studies have claimed that cell division is under circadian control (Matsuo et al., 2003) since hepatocyte proliferation following PH is delayed. Yet this

phenomenon has not been extensively investigated in brain tissue. Chapter 4 will utilize arrhythmic animals lacking a fully functioning circadian clock, BMAL1 knockouts, to investigate the role that the clock has on this type of mitotic activity. Predictions were that brain tissue in the MPFC area will regenerate at a significantly slower rate in animals lacking a fully functioning circadian clock than in healthy controls. However, in the first week following aspiration, there were no differences in MPFC tissue volume or weight.

1.14.4 Experiment 4: Hippocampal Neurogenesis in Arrhythmic BMAL1 Knockout mice

Lastly, Chapter 5 examines hippocampal neurogenesis as an adult model of central nervous system mitosis. Hippocampal neurogenesis in the SGZ will be examined in animals lacking a fully functioning circadian clock. It is hypothesized that neurogenesis will be negatively affected by a lack of circadian clock function, as occurs in liver tissue regeneration. Results of this study, however, show no effect of BMAL1 knockout on proliferation. Significant increases in cell survival and associated decreases in apoptosis were evident, without altering GCL volume.

Together, these findings aim to further our knowledge about the role of the circadian clock in brain damage and regeneration. We have found that the time of day in which damage occurs may not affect behavioural outcome or lesion size, and that neonatal brain regeneration may also lack significant circadian control. However, in adulthood is seems that the circadian influence on neurogenesis is strong.

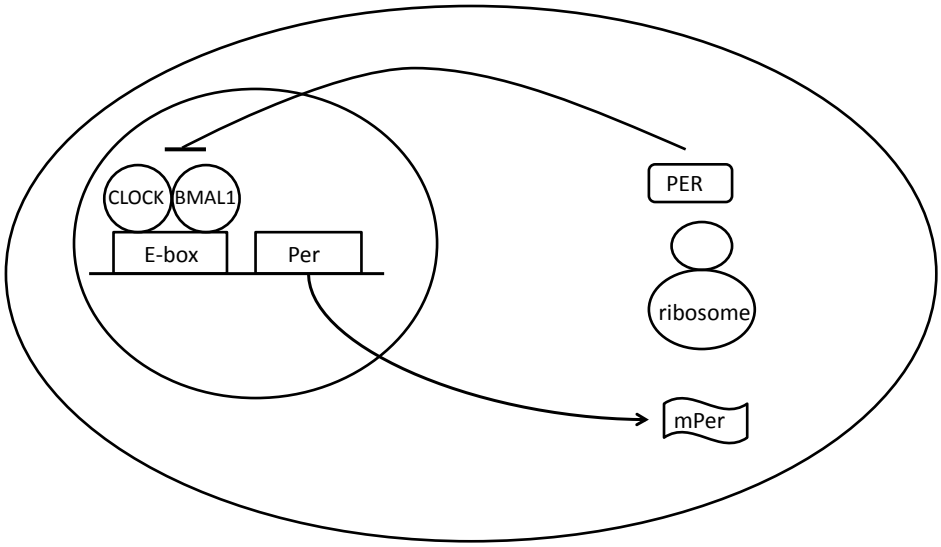

Figure 1.1 Representation of the Period driven transcription-translation feedback loop within a time keeping cell of the SCN. Identical parallel process describes Cry transcription and translation.

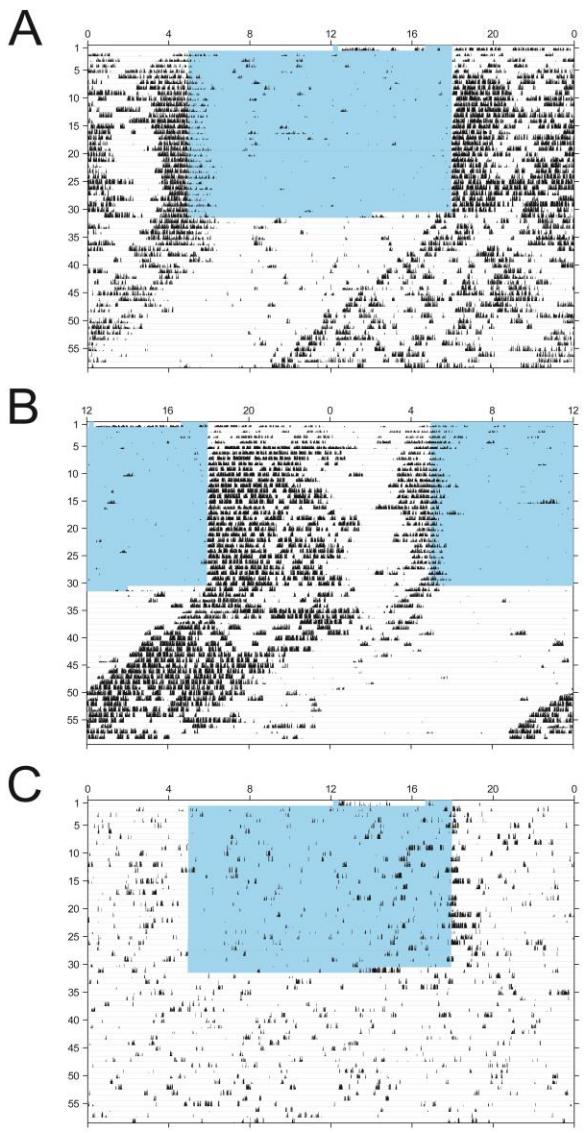

Figure 1.2 Images of actograms taken directly from Clocklab data collection software package (Coulbourn Instruments, Allentown, PA, USA) showing activity rhythms of BMAL1 A) (+/+), B) (+/-), and C) (-/-) mice. Days are plotted on the Y axis, the X axis shows the 24 hour day. Areas shaded blue represent the light phase of the 12:12 LD cycle. Unshaded areas show when lights are off, and constant darkness conditions. Black bars show wheel running activity. Arrhythmic behaviour is noted regardless of light phase in the BMAL1 knockout animals (C).

Chapter 2

Lesion size and behavioral deficits following endothelin-1-induced ischemia are not dependent on time-of-day

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2.1 Abstract

The occurrence of stroke exhibits a strong circadian pattern with a peak in the morning hours following waking. The factors that influence this pattern of stroke prevalence may confer varying degrees of neuroprotection and therefore influence stroke severity. This question is difficult to address in clinical cases, due variability in the location and duration of the ischemic event. The purpose of this study was to determine if time-of-day affected the severity of stroke targeting the motor cortex in rat. Strokes were produced using topical application of the vasoconstrictor endothelin-1 to motor cortex of unanesthetized animals at two time points; early-day and early-night. Behavioral deficits were measured using reaching, cylinder, and horizontal ladder tasks, and the volume of the lesion was quantified. Behavior on a reaching and horizontal ladder tasks were both severely impaired by endothelin-1 treatment compared to vehicle treated animals, but deficits did not differ according to time of treatment. Similarly, while endothelin-1 produced larger lesions of the motor cortex than did vehicle treatment, the size of the lesion did not differ according to time of treatment. These results suggest that while many factors under circadian control can influence the prevalence of stroke, the magnitude of lesion and behavioral deficit resulting from an ischemic event may not be influenced by time-of-day.

Key Words: cerebral infarction, rat, circadian rhythm, forelimb, single pellet reaching, horizontal ladder

2.2 Introduction

Stroke is more likely to occur in the morning hours following waking (Elliott, 1998). Ischemic strokes exhibit a prominent circadian rhythm in their onset, with a pronounced peak observed between 06:00 h and 12:00 h, often within a couple hours of waking (Tsementzis et al., 1985; Marler et al., 1989; Argentino et al., 1990; Marsh et al., 1990; Gallerani et al., 1993; Kelly-Hayes et al., 1995; Lago et al., 1998; Chaturvedi et al., 1999; Casetta et al., 2002b; Gupta and Shetty, 2005; Omama et al., 2006; Turin et al., 2009), although some studies have reported a nighttime peak during sleep (Marshall, 1977; Kocer et al., 2005; Naess et al., 2010). A link between waking and stroke is suggested by the observation that the morning peak in stroke is shifted later on days when individuals are not working and have later wake times (Haapaniemi et al., 1992). Hemorrhagic strokes also exhibit a morning peak (Tsementzis et al., 1985; Sloan et al., 1992; Gallerani et al., 1996; Passero et al., 2000; Casetta et al., 2002a; Omama et al., 2006; Turin et al., 2010) or nighttime trough (Feigin et al., 2001) in their onset, although due to lower frequency of hemorrhagic stroke, this pattern is not always observed (Marshall, 1977; Gallerani et al., 1993; Gupta and Shetty, 2005; Kocer et al., 2005). Both acute myocardial infarction and sudden cardiac death also show morning peaks (Muller et al., 1985; Muller et al., 1987) suggesting that circadian rhythms in cardiovascular and hemodynamic variables may underlie the morning peak in cardiovascular and cerebrovascular attacks. Indeed, many physiological parameters that facilitate ischemic events peak in the morning around waking, such as blood pressure (Mann et al., 1980), a rise in heart rate (Mann et al., 1980), blood viscosity (Kubota et al., 1987), platelet aggregability (Tofler et al., 1987) and catecholamine levels (Tofler et al., 1987), while

factors that decrease the likelihood of an ischemic event, such as fibrinolytic activity (Andreotti et al., 1988) are at their lowest levels around waking. Some of these variables also exhibit a bimodal circadian rhythm with secondary peaks in the late afternoon that correspond well with secondary peaks in stroke occurrence (Tsementzis et al., 1985; Muller et al., 1987; Argentino et al., 1990; Marsh et al., 1990; Sloan et al., 1992; Gallerani et al., 1996; Casetta et al., 2002b; Casetta et al., 2002a; Omama et al., 2006; Turin et al., 2010). While many of these variables are driven in part by behavior, their levels and reactivity to exercise display endogenously regulated circadian rhythms (Scheer et al., 2010), leaving open the possibility that these endogenous rhythms underlie the daily pattern observed in stroke occurrence.

Despite the well characterized diurnal pattern of stroke onset, strokes can occur at any time of the day. It has been hypothesized that the severity of stroke may also exhibit a circadian rhythm (Manev and Uz, 1998). This may be due to some of the same variables that influence the circadian rhythm in onset time. Additionally, melatonin (Manev and Uz, 1998) and GABAergic tone (Clarkson et al., 2010) have been suggested to be neuroprotective at the peak of their circadian rhythms.

Comparing the severity of ischemic strokes is complicated by a number of factors. The severity of a stroke is influenced by the location of the occlusion, and the amount of cell death can be influenced by the latency to treatment and reperfusion. Rats, which also exhibit circadian rhythms in heart rate, blood pressure, catecholamine levels, and hemodynamic factors (Miki and Sudo, 1996; Turner et al., 2001; Basset et al., 2004; Matsumoto et al., 2009; Ohkura et al., 2009), present a model in which location and duration of ischemia can be controlled. Vinall and colleagues (2000) found that the size

of the infarct caused by intraluminal occlusion of the rat middle cerebral artery was nearly three times as large when the occlusion occurred during the animal's active phase (i.e., night) than when occlusions occurred during the animal's rest phase. There is also a circadian rhythm in activation of proteins in the hippocampus associated with cell death (i.e., caspase-3, -8, and -9) following transient global ischemia that models sudden cardiac death (Tischkau et al., 2007). The greatest amount of mRNA and the highest number of immunoreactive cells for these caspases in the CA1 area of the hippocampus were observed following transient global ischemia induced in the early night, compared to in the late night or mid day. These studies suggest that the brain is most sensitive to ischemia during the active phase. The pattern is somewhat different for a model of closed head injury in rats. Mortality following a traumatic brain injury using this model is greatest during the day, and size of the resulting damage in the brain exhibited a trend towards being larger during the day (Martinez-Vargas et al., 2006). However, there was no effect of time-of-day on impairment following the traumatic brain injury on either motor performance tests or neurological assessments (Martinez-Vargas et al., 2006). While these studies suggest that severity of stroke may differ across the day, all of these models of brain injury were performed in anesthetized animals. Anesthesia may override subtle circadian influence. Anesthetics have been shown to confer a degree of neuroprotection to ischemic insult either directly through their action on cells or through preconditioning (Clarkson, 2007; Karmarkar et al., 2010). Given prominent circadian rhythms in response to anesthetics (Scheving et al., 1968), it is possible that the degree of neuroprotection produced by the anesthetics may have differed across the day, obscuring any circadian rhythm of stoke severity. This problem is nullified by avoiding the use of

anesthetics, which has the added benefit of better modeling the natural state in which the majority of strokes occur.

The goal of the present study was to generate a small focal ischemic event in conscious animals at different times of the day. These focal ischemic events were focused on the area of the motor cortex that controls the forelimb so that functional deficits and recovery from the ischemic event could be assessed with sensitive behavioral assays. I hypothesize that stroke at waking will result in greater behavioural deficits, reduced recovery, and increased lesion size when compared to animals with stroke during sleep.

2.3 Materials and Methods

2.3.1 Animals

Sixteen Long Evans rats were used in this study, obtained from the University of Calgary Breeding Colonies. All rats were housed individually in a colony room maintained on a 12:12 LD cycle, with lights on at 07:00 h and lights off at 19:00 h. A small window in the colony room allowed some light into the room during night which was sufficient for animal husbandry and injections during the dark phase. Animals were provided food and water *ad libitum* until the commencement of reach training, at which point food was restricted as described below. All procedures were performed in accordance with the Canadian Council on Animal Care guidelines, and were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary.

2.3.2 Experimental Timeline & Treatment groups

Animals were exposed to the reaching and horizontal ladder tasks until successfully trained. Once animals were fully trained, they received cannula implant surgery, were given 2 days to recover, and were then tested for one week to ensure that their success in the task was not affected by the surgery. Animals were trained on the reaching task for 5 consecutive days and then rested for 2 days each week. Training on the cylinder and horizontal ladder occurred once a week. Animals were given two weeks of baseline assessments. After this time, stroke induction was initiated. Six animals were subject to stroke within the first hour after lights out (19:00-20:00 h). One animal changed its preferred paw in testing sessions after stroke induction, and so was removed from analyses. Five animals were exposed to the same procedure during the rest/sleep phase, at lights on (07:00-08:00 h). Control animals (n=5) were given a sterile saline microinjection after lights-out (n=2) or lights-on (n=3). The day following the stroke induction, reaching assessments resumed for 5 consecutive days, followed by 2 rest days each week. The horizontal ladder and cylinder tasks occurred on the second day of each week. For analysis, the performance on the horizontal ladder and cylinder tasks was averaged over the first 2 weeks post stroke (designated Post Stroke period) and a averaged again over the 8^{th} and 9^{th} weeks post stroke (designated the Recovery period). The timeline for the various treatments and tests are depicted along the X-axis of Figure 2.1.

2.3.3 Surgery

A 3mm cannula was implanted contralateral to the preferred paw, as determined by reach training prior to surgery (see below for training protocol). Animals were

anesthetized using 90 mg/kg ketamine and 5 mg/kg xylazine, administered intramuscularly. Surgical procedures were modified from Sharkey and colleagues' (1993) methods, with cannula placement coordinates at 1 mm anterior and 3 mm lateral to bregma with the skull leveled between bregma and lambda. Dura was cut with a sterile needle, and the cannula was lowered 1.5 mm ventral to the surface of the skull through the burr hole and incision in dura, so that it was just touching the surface of the brain. Jewelers' screws were placed around the cannula to support a dental acrylic head cap. A dummy cannula extending 0.5mm beyond the end of the guide cannula was then inserted to maintain patency. All animals received 0.03mg/kg of buprenorphine and 1ml sterile saline, both delivered subcutaneously.

2.3.4 Stroke Induction

Endothelin-1 (Sigma Aldrich, St. Louis, MO) was dissolved in sterile saline at a concentration of 1µg/µl. After either lights-out (19:00h) or lights-on (7:00h), animals were removed from their home cages, the dummy cannula was removed, and a microinjector that extended 0.5mm beyond the end of the guide cannula and that contained either sterile saline or endothelin-1 solution was inserted into the cannula. The solution was injected at a rate of 0.2 µl/minute for a total of a 1µl injection. After the full amount of solution had been injected, the injector tip was left in place for 5 minutes to ensure that the solution had fully dispersed and would not be taken back up with the injector. Animals were observed for freezing or rotating behavior during the injection, as these behaviors have been shown to be an indicator of brain damage in the conscious animal (Callaway et al., 1999).

2.3.5 Single Pellet Reaching

Beginning two days prior to single pellet reach training, animals' were started on a food restriction regimen initially set at the average daily intake for Long Evans rats, 30g/day. If animals were not attempting to reach for pellets by day 4 of training, they were reduced to 25g/day, and this amount was reduced daily if a failure to reach continued. No animals were restricted to less than 15g of food/day. This amount of food kept their body weight at 95%-100% of free-feeding weight or greater for younger animals.

Single pellet reaching boxes were made of clear Plexiglas (40cm x 45cm x 13.1cm). Animals are trained to reach through a small vertical opening on one end of the box, 1.3cm wide x 15cm high. A 4cm wide platform sits 4cm above the floor on the outside of the opening to place the food pellets. Two small indentations, 5mm in diameter and 1.5mm deep are evenly spaced on the platform in line with the edges of the vertical opening. This placement encourages the animals to use only their preferred paw, as they need to extend their forelimb across their midline to obtain the pellet (Whishaw and Pellis, 1990).

For the first 2 days of training, animals were placed in the reaching apparatus with pellets scattered both inside the reaching box and on the reaching platform. On day 3, pellets were placed only on the platform and the preferred paw, that which the animal reached through the vertical opening with most often, was observed and recorded. By the end of one week of reaching, only a single pellet was placed on the platform, opposite the animal's preferred paw. Animals were trained to travel to the back of the box to "reset" after each reach attempt. By day 12 of training, animals were familiar with the task, and

scoring of the reach attempts began. Though light is the most powerful stimuli in entraining the circadian clock, it has been shown that rats will entrain to the presentation of food or a palatable treat (Mistlberger and Rusak, 1987; Antle and Silver, 2009; Mistlberger, 2009). To ensure that animals remained entrained to the light/dark cycle, and not to the palatable pellets used in training or to restricted food schedules, training and feeding occurred at random times each day, with no two consecutive days having the same training or feeding times.

Scoring consisted of recording the number of "hits" vs. "misses". A hit was defined as a reach attempt where the animal extended the paw through the vertical opening, grasped a single pellet, and immediately ate it, while misses consisted of attempts that did not result in grasping and immediately eating the pellet. Each animal was given 20 reach attempts per session, and percent success was determined. Once animals reached 50% success or greater they were considered fully trained in the task. Animals received one to two days rest without reaching per week.

2.3.6 Horizontal Ladder

The horizontal ladder apparatus was made of clear Plexiglas. The walls of the apparatus were 50 cm in height and 100 cm in length. The metal rungs, which spanned the 100 cm length of the apparatus, were 0.3 mm in diameter and separated by a distance of 1 cm. One end of the apparatus had an open starting box, while the animals' home cage was placed at the "finish" end as an incentive for the animal to cross the ladder.

Animals were trained to cross the ladder for one week, at which time they were comfortable with the apparatus and successfully crossed the runway by accurately placing fore- and hind-limbs on the bars. During rung walking sessions, skilled walking

was tested by removing 20 metal rungs in random sequence with no more than 2 rungs removed consecutively, thereby allowing a maximum gap length of only 3 cm (Henry et al., 2008). This pattern was changed for each session to ensure that the animals did not learn a specific rung pattern.

Testing sessions were video recorded and later analyzed. The number of steps and faults (errors or "slips") for each forelimb were counted. The scoring system used (Metz and Whishaw, 2002) rated a successful foot placement on the rung as 0, 1 point was allocated for a corrected fault, 2 points for correct placement followed by removing the paw and replacing it correctly, 3 points for a slip after correct placement, and 4 points were given for a paw that missed a rung completely.

2.3.7 Cylinder

The cylinder/limb-use asymmetry task (Schallert et al., 2000) consisted of a clear plastic cylinder 20 cm in diameter and 30 cm in height. The cylinder was placed on a clear Plexiglas base, with a mirror angled beneath it so that the session could be videotaped from below the animal.

Each testing session consisted of placing an individual animal in the cylinder for 5 minutes. The animal's behavior was video recorded during this time. While in the cylinder animals spontaneously reared, placing their paws on the walls of the cylinder. A ratio of paw use was determined by counting the number of times the animal placed the affected paw (contralateral to the lesion) or unaffected paw on the cylinder walls. The percentage of limb use was calculated as the number of touches with the affected paw, plus the number of touches with the unaffected paw, divided by the total number of wall touches (Schallert et al., 2000; Woodlee et al., 2005). Wall touches using both paws were

eliminated from the analysis here, due to the low percentage of overall wall touches that they comprised.

2.3.8 Infarct measurements

At the end of the study, animals were given an overdose of barbiturate and were perfused transcardially with saline followed by 4% paraformaldehyde. Brain tissue was collected and examined to determine the extent of the lesion. Thirty-five µm slices of brain tissue were collected from the region of damage (and comparable rostro-caudal levels in control animals) and stained with Cresyl Violet. Digital images of these sections were captured (Image-Pro Plus 5.1; Media Cybernetics, Silver Spring, MD), and volume analysis was completed, using ImageJ (NIH), by outlining the sections, and calculating the area of each hemisphere. The lesioned hemisphere was compared to the intact hemisphere as a control, as the lesions were unilateral. Lesion volume was calculated using the following equations: Volume of tissue lost = [remaining volume of contralateral hemisphere] - [remaining volume of ipsilateral hemisphere], and Volume of hemisphere = [(average (area of the complete coronal section of the hemisphere - area of damage)) x (interval between sections x number of sections)]. To examine overall extent of damage, images of the brains were resized to fit templates of coronal section in Paxinos and Watson (2008), and the edge of the tissue was traced onto the template.

2.3.9 Statistical Analyses

All behavioral data were analyzed using repeated measures ANOVA (SigmaStat, Version 2.03, Systat Software, Inc., Chicago, IL). Saline and endothelin-1 treatments were analyzed separately in the behavioral tests. Tukey's post hoc comparisons were used to examine pair-wise differences between groups. Independent-samples t-tests were

used to specifically compare the performance of the morning- and evening-treated endothelin-1 animals immediately after the stroke surgery. Infarct measurements were analyzed using unpaired t-tests, comparing the two lesion groups. A significance level of 0.05 was considered significant in all analyses. The number of subjects was selected to provide sufficient power to detect differences in performance of about 10%.

2.4 Results

2.4.1 Single Pellet Reaching

Performance on the reaching task was significantly impaired by the endothelin-1 treatment ($F_{(2,16)}$=45.641, p<0.001; Figs. 2.1&2.2). Endothelin-1 animals exhibited significantly less success in reaching in the ten days following the endothelin-1 treatment relative to baseline. After about 5 weeks, performance had significantly improved relative to performance immediately after the endothelin-1 treatment, but was still significantly poorer than during baseline. There was no effect of time of endothelin-1 treatment on reaching performance ($F_{(1,16)}$=0.03, p=0.86), and there was no interaction between the time of treatment (morning vs. evening) and testing session (baseline, post-stroke, recovery; $F_{(2,16)}$=0.029, p=0.97). Specific examination of the performance of the morning and evening-treated endothelin-1 animals revealed no significant difference in performance immediately after the stroke treatment ($t_{(8)}$=0.32, p=0.376). Saline treatment did not affect performance on the reaching task ($F_{(1,16)}$=0.316, p=0.74) as performance following treatment did not differ from baseline.

2.4.2 Horizontal Ladder

Performance on the horizontal ladder task was significantly impaired by the endothelin-1 treatment ($F_{(2,16)}$=64.858, p<0.001; Fig. 2.3). Endothelin-1 animals

exhibited significantly poorer performance in the first 2 weeks following the endothelin-1 treatment relative to baseline. After seven weeks, performance had significantly improved relative to performance immediately after the endothelin-1 treatment, but was still significantly poorer than during baseline. There was no effect of time of endothelin-1 treatment on horizontal ladder performance ($F_{(1,16)}$=0.545, p=0.481), and there was no interaction between the time of treatment (morning vs. evening) and testing session (baseline, post-stroke, recovery; $F_{(2,16)}$=2.042, p=0.162). Visual inspection of average performance following the stroke suggested that the group treated with endothelin-1 in the morning performed worse than the group treated in the evening, however this difference was not significant ($t_{(8)}$=1.26, p=0.12). Saline treatment did not affect performance on the horizontal ladder task ($F_{(1,16)}$=1.018, p=0.416) as performance following treatment did not differ from baseline.

2.4.3 Cylinder Task

Neither endothelin-1 nor saline treatment altered the pattern of forepaw use assessed in the cylinder task (endothelin-1, $F_{(2,16)}$=1.92, p=0.179; saline, $F_{(2,16)}$=0.907, p=0.453; Fig. 2.4). There was no effect of time of treatment ($F_{(1,16)}$=0.838, p=0.387) and no interaction between testing session and time of treatment ($F_{(2,16)}$=0.728, p=0.498) for the endothelin-1 treated rats.

2.4.4 Lesion Volume Analysis

Endothelin-1 treatment produced significantly larger lesions than did saline control treatment ($F_{(1,11)}$=8.476, p<0.05; Figs. 2.5&2.6). Time of treatment had no effect on the resulting lesion size ($F_{(1,11)}$=0.515, p=0.488), and there was no interaction between treatment and time of treatment. ($F_{(1,11)}$=0.643, p=0.439).

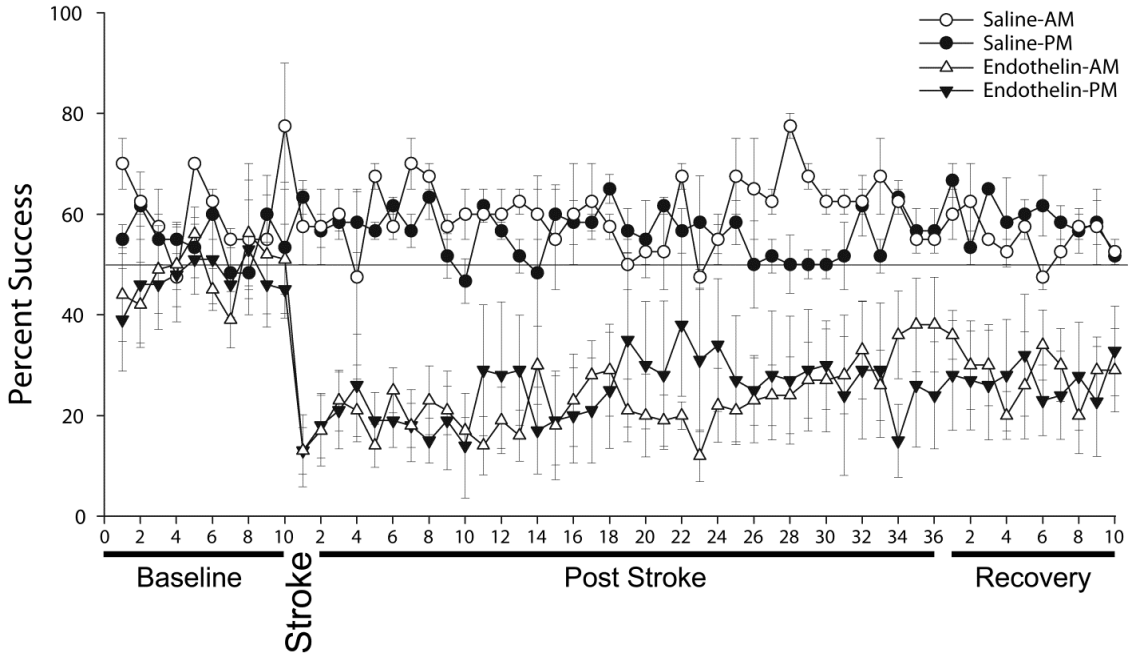

Figure 2.1. Overall reaching success of experimental and control groups prior to stroke and throughout the post-stroke and recovery periods. Both endothelin-1 groups displayed a significant decrease in reaching success following stroke, without significant recovery up to 40 days after the stroke. Testing was conducted Monday to Friday, with rest days over the weekend. Black bars along the bottom depict weeks, while numbers represent testing days within each block of the timeline. The four circles along the x-axis represent days when the horizontal ladder and cylinder tasks were performed.

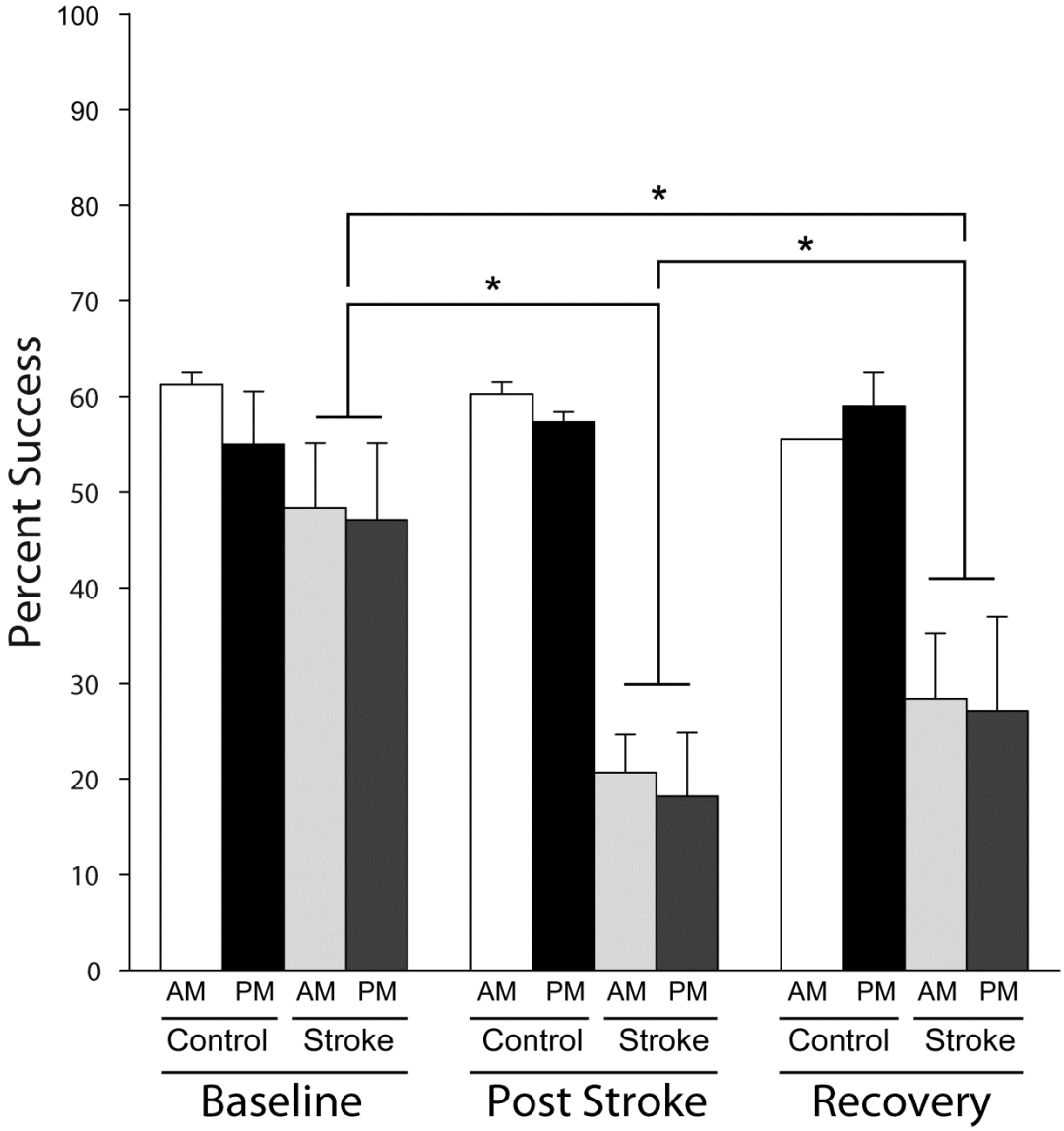

Figure 2.2. Group means in reaching success pre- and post-stroke, and after more than one month recovery time. Asterisks (*) represent $p < 0.05$.

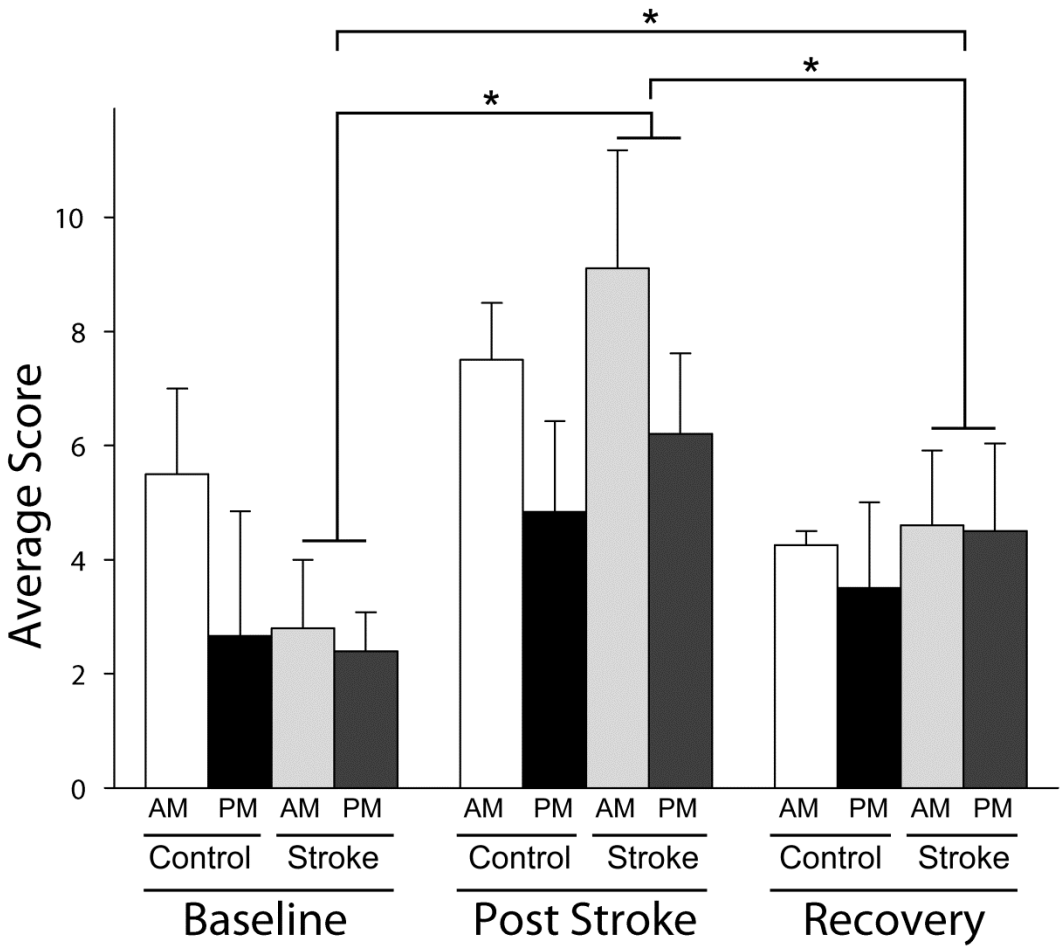

Figure 2.3. Group mean scores on contralateral to lesion forelimb placement in the ladder task. Higher score represents more errors in forelimb placement. Animals made significantly more errors in forelimb placement following stroke induction. Asterisks (*) represent $p < 0.05$.

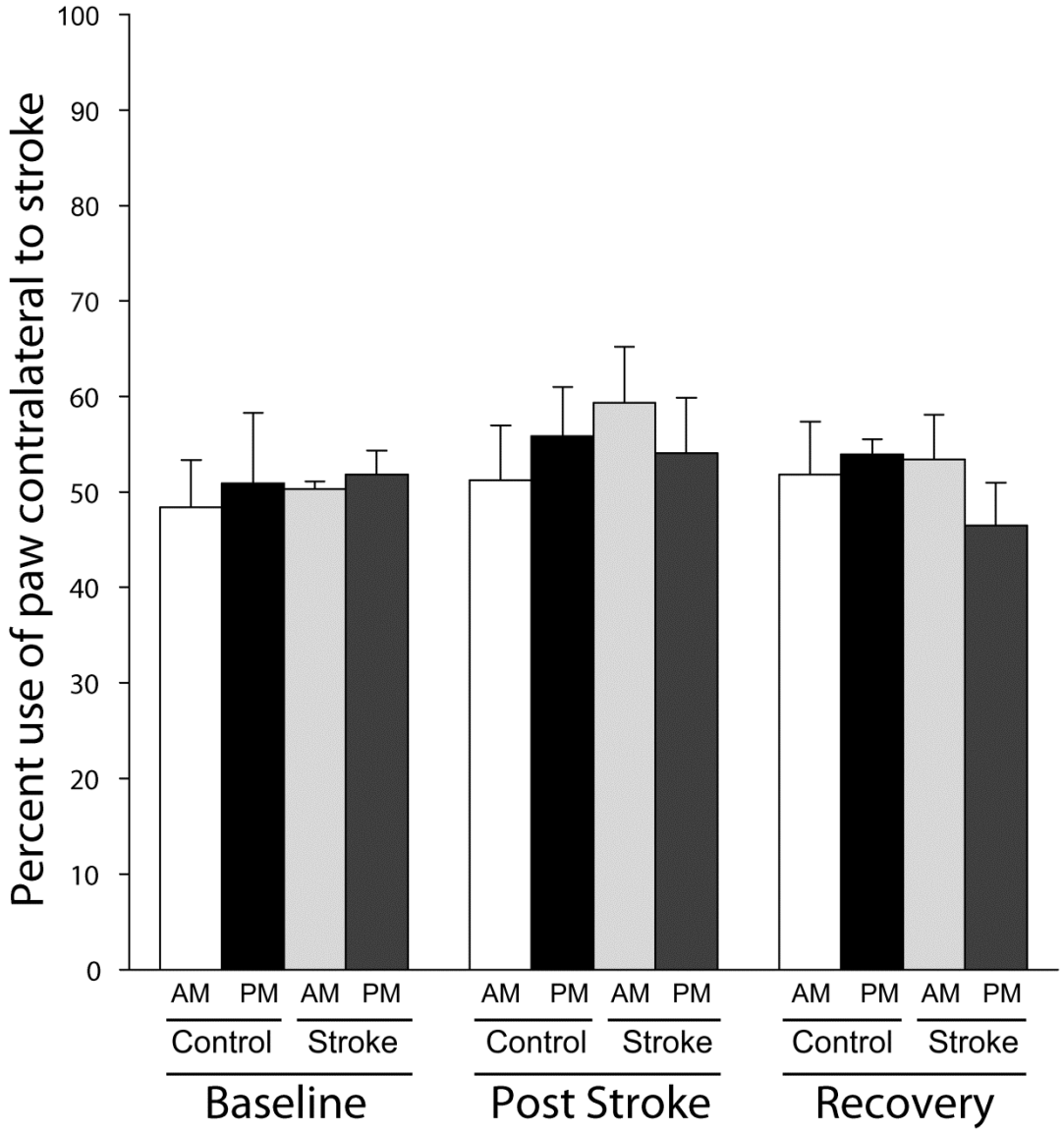

Figure 2.4. Percent use of preferred (contralateral) and non-preferred (ipsilateral) paw use in rearing exploration of the cylinder task for experimental and control groups. Animals used their preferred paw to explore the cylinder more often than the non-preferred paw at all three time points, pre- and post-stroke, and recovery.

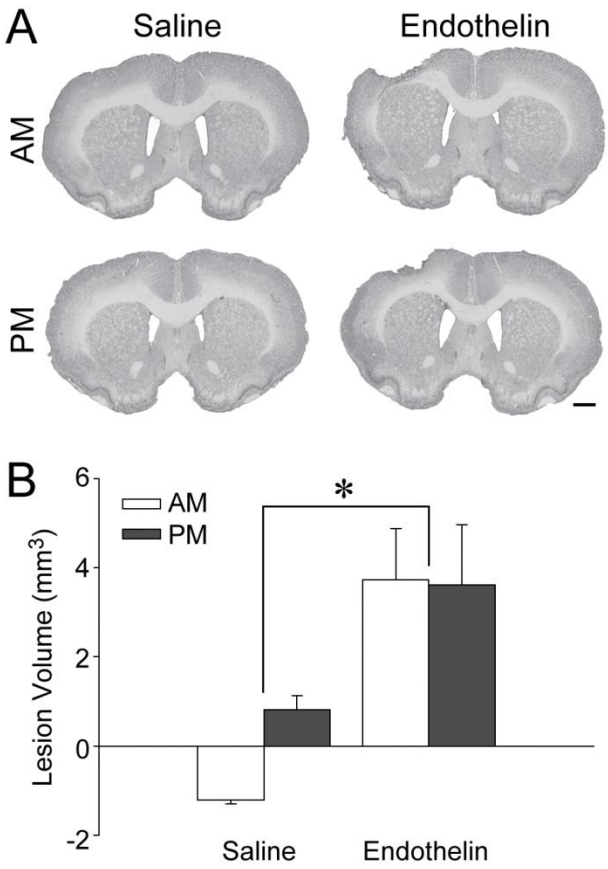

Figure 2.5. A) Sample photomicrographs from mid lesion levels for each of the treatment conditions. Scale bar = 1mm. B) Mean lesion volumes (mm^3) from morning (AM) and evening (PM) for the saline control and endothelin-1-induced stroke treatments groups. There were no significant differences between lesion volumes of the two endotheline-1 groups ($p>0.05$), nor did lesion volumes differ between the two saline groups. Endothelin-1 treatments produced larger lesion volumes than saline treatments ($p<0.05$).

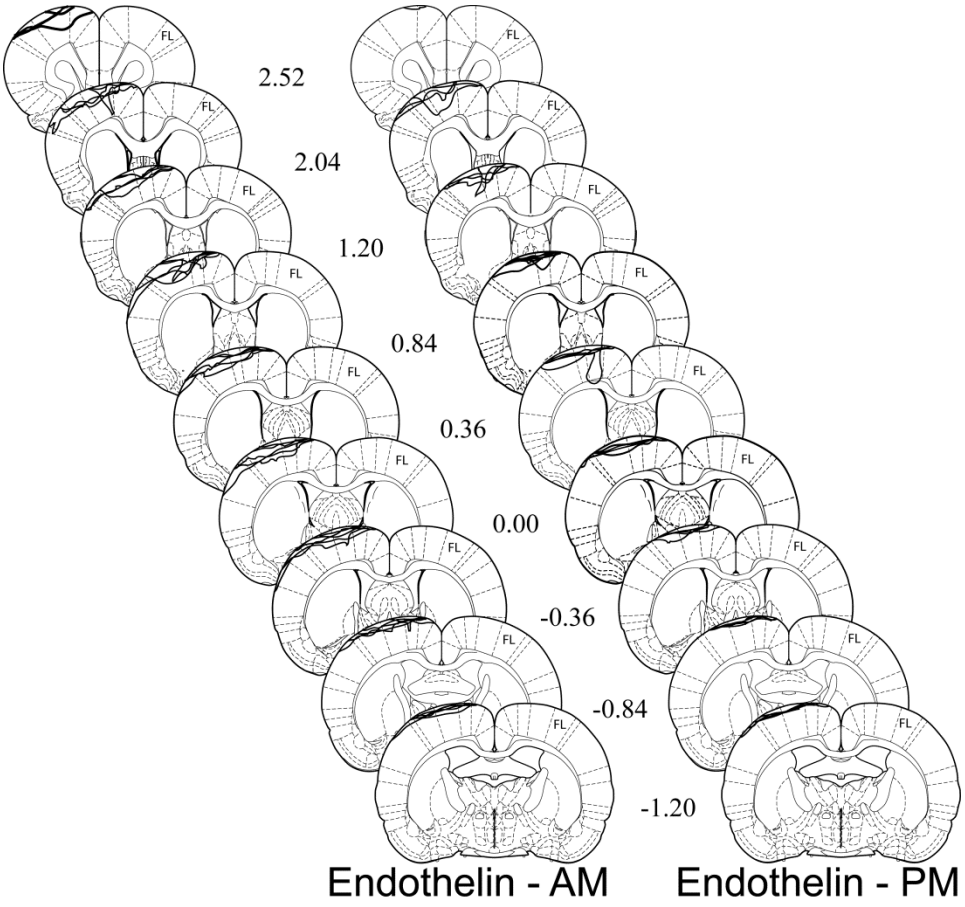

Figure 2.6. Nine representative schematics through the forelimb area of both endothelin-1 groups. Brain templates based on Paxinos and Watson (2008). Thick black lines denote actual lesion boundaries from morning and evening stroke groups. FL = forelimb region.

2.5 Discussion

Cortical ischemia induced by application of endothelin-1 to the forelimb motor cortex in the rat produced focal infarcts that lead to significant deficits in skilled reaching and horizontal ladder walking. Some recovery in the performance of these behaviors was observed over time. However, the size of the infarct, magnitude of behavioral deficits and recovery did not differ according to the time of day that the endothelin-1 was applied.

There is a prominent circadian rhythm in prevalence of ischemic stroke (Tsementzis et al., 1985; Marler et al., 1989; Argentino et al., 1990; Marsh et al., 1990; Gallerani et al., 1993; Kelly-Hayes et al., 1995; Lago et al., 1998; Chaturvedi et al., 1999; Casetta et al., 2002b; Gupta and Shetty, 2005; Omama et al., 2006; Turin et al., 2009) and it has been suggested that many of the factors under circadian control that influence the changing prevalence of stroke over the day may also modulate the severity of a stroke (Manev and Uz, 1998). The present results suggest that this is not the case for ischemia caused by vasoconstriction induced by application of endothelin-1 to the surface of the cortex. Three other studies have sought circadian rhythms in the response to various cerebral injuries. While Martinez-Vargas et al. (2006) found a circadian rhythm in survival following a moderate close head injury (more rats died when injured during the day), they too found no difference in the magnitude of motor and neurological deficits and size of the resulting lesion between the survivor rats injured in the day or night. These findings differ from those of Vinall et al. (2000) who found a prominent time of day effect on lesion size resulting from middle cerebral artery (MCA) occlusion, with the largest lesions occurring after a late-night occlusion and the smallest lesions occurring with late-day occlusions. Tischkau et al. (2007) found that activation of the cell death

markers caspases-3, 8, and 9 in the hippocampus following a global ischemia was greatest during the early-night. Daytime global ischemia activated these pathways to a lesser extent, and late-night global ischemia produced small or insignificant activation of these pathways.

The approach used here to elicit a stroke has a number of advantages over other approaches to study ischemic insult. Previous studies investigating time of day effects on brain injury all used anesthetized animals (Vinall et al., 2000; Martinez-Vargas et al., 2006; Tischkau et al., 2007). It has been reported that anesthesia can provide some protection during an ischemic event (Clarkson, 2007), and it is well established that there is a prominent circadian rhythm in response to anesthetics (Scheving et al., 1968), raising the possibility that any circadian rhythms to a brain injury observed under anesthetic could be due to a circadian rhythm in the protection afforded by the anesthetic. The approach used in this study avoids this problem by producing a focal ischemic event in an un-anesthetized animal, mimicking the physiological state in which most humans experience a stroke. Comparing severity of stroke across time of day in humans is complicated by the different locations and durations of the ischemic events. By administering a specific dose of endothelin-1 to a particular location, we are able create a reproducible stroke in terms of location and severity. The endothelin-1 model is reliable in terms of the infarct volume it produces (Sharkey and Butcher, 1995; Fuxe et al., 1997). The chief disadvantage of the endothelin-1 model is that the duration of the vasoconstriction that it produces endures for at least 4 hours (Fuxe et al., 1997) and possibly longer (Windle et al., 2006), whereas the MCA occlusion (Vinall et al., 2000) and global ischemia (Tischkau et al., 2007) models used elsewhere are able to produce

ischemic events of very discrete durations (i.e., 2 hours and about 4 minutes in these previous studies, respectively). If the duration of the vasoconstriction produced exceeds the temporal spacing of treatment groups, then that could be an explanation for the lack of observance of a circadian rhythm in severity of the stroke. The duration of decreased blood flow following topical application of endothelin-1 to the cortex does not appear to be dose dependent (Fuxe et al., 1997), thus lowering the dose would not decrease the duration of the ischemic event. Other approaches with shorter durations of action, possibly infusion of a low dose of an excitotoxic agent such as quinolinic acid (Gilmour et al., 2004), may be better able to mimic the effect of a brief and focal ischemic event. Additionally, the degree and area of ischemia following endothelin-1 application may not produce much of an ischemic penumbra. Tissue within the ischemic core will likely become necrotic regardless of time of day while survival of the tissue in the ischemic penumbra may be influenced by time of day. If endotelin-1 produced near-complete vasoconstriction wherever it reached with an infusion over the cortex, then most of the ischemic zone would be core, with little penumbra. Other approaches that produced more targeted ischemia may uncover a circadian rhythm in stroke severity, such as middle cerebral artery infusions of Endothelin-1, although such an approach would produce much larger ischemic areas, there may also be a larger penumbra.

The behavioral tests used here are quite sensitive to strokes of the forelimb motor cortex. The single-pellet reaching task and horizontal ladder tasks are both very sensitive to forelimb motor cortex damage (Whishaw et al., 1991; Metz and Whishaw, 2002; Riek-Burchardt et al., 2004; Whishaw et al., 2008). These tasks can be used to quantify the magnitude of the deficit as well as the rate and nature of recovery. The deficits observed

here match those reported previously in reaching (Gilmour et al., 2004) and horizontal ladder (Riek-Burchardt et al., 2004). While no deficit was detected in the forepaw use in the cylinder task, this also matches deficits observed following focal electrolytic lesions of the forelimb motor cortex and MCA occlusions (Schallert et al., 2000). Significant asymmetry in forelimb use following larger aspiration lesions in this cortical area have been found by Woodlee et al. (2005), suggesting that this task can be used to distinguish between large and small infarcts of the motor cortex.

The times selected in this study correspond to the start of the active period and start of inactive period. Cortical inhibition due to high GABAergic tone should be highest in the early day when sleep propensity is high, while cortical arousal due to high cholinergic tone should be maximal during the early night when wake propensity is highest (Jones, 2005). Additionally, many factors that influence the prevalence of stroke in humans peak after waking, thus we expected that this phase would be most sensitive to stroke. The species used in the present study exhibits similar circadian variation, although in antiphase to humans given that rats are nocturnal (Miki and Sudo, 1996; Turner et al., 2001; Basset et al., 2004; Matsumoto et al., 2009; Ohkura et al., 2009). The combined effect of high GABAergic tone during the early part of the sleep phase and the trough in many factors that influence stroke prevalence (e.g., blood pressure, heart rate, catecholamine levels) was predicted to decrease the severity of the stroke at this phase. While no difference was noted, it is possible that other phases may be more or less sensitive than those examined here.

While strokes exhibit a circadian rhythm in their prevalence, peaking in the morning hours following waking (Elliott, 1998), stroke can occur at any time of the day.

The present findings suggest that the severity of the stroke is not time-of-day dependent. Other factors, such as latency to treatment, location of thrombolic/embolic occlusion, and duration of ischemic event are more likely to influence stroke severity.

Chapter 3

mPer1, *mPer2*, PER1 and cFos Expression in Regenerated Medial Prefrontal Cortex

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3.1 Abstract

There is a strong temporal influence over medial prefrontal cortex (MPFC) dependent behaviours. The MPFC is not only connected to the master pacemaker through multisynaptic pathways, but has also been shown to experience rhythmic clock gene expression. Aspiration of the MPFC of neonatal rats on P10 and mice on P7, show not only a more favourable behavioural outcome but also apparent regrowth or filling in of the lesion cavity. However, these animals show abnormalities in circadian behaviours such as wheel running and phase shifting in response to light pulses. Therefore, we sought to examine circadian clock genes *Period1*, *Period2*, and the immediate early gene *cFos* in the MPFC of P7 aspirated mice. Our results show no significant differences in clock gene expression between neonatal decorticates and sham lesioned or healthy adult controls. However, rhythmicity in expression of clock genes in MPFC were also not significant in any groups.

Keywords: medial prefrontal cortex, clock genes, period genes, mRNA, cFos, regeneration

3.2 Introduction

The medial prefrontal cortex (MPFC) and its connections are thought to be necessary for normal execution of executive function. The term executive function encompasses behaviours and concepts such as cognition, social behaviour, mood, planning, attention, learning from past experiences, and more generally, the temporal organization of behaviour (Dalley et al., 2004; Eslinger et al., 2004; Fuster, 2002). Studies of MPFC damaged patients have described a frontal lobe syndrome, which outlines deficits in social and moral development, as well as emotional and personality changes (Eslinger et al., 2004).

Many of the complex behaviours controlled by the MPFC rely heavily on keeping time and fluctuate according to time of day (Adan and Sanchez-Turet, 2001; Kraemer et al., 2000; Owens et al., 2000; Cho et al., 2000). Recalling the order in which past events occurred and organizing behaviours in an appropriate sequence to obtain a specific outcome are examples of temporally related behaviours which rely on normal MPFC function. In rodents, MPFC damage has resulted in deficits in temporal order memory object recognition, attentional tasks, alternation learning, as well as decreases in the rate of reinforcement reacquisition, and changes in running wheel activity (Phillips et al., 2009; Hannesson et al., 2004; Ragozzino et al., 2002; Mitchell et al., 1998; Nonneman & Corwin, 1981; Kolb et al., 1974). Disturbances in recalling the order and recency of events or objects encountered previously have also been shown in rodent models (Mitchell et al., 1998). Other studies have shown deficits in the sequential ordering of maternal behaviours and nest building in MPFC decorticate females (Alfonso et al.,

2007). These studies suggest a strong temporal influence over MPFC dependent behaviours.

Studies examining behavioural and anatomical effects of adult MPFC lesioned animals, neonatal MPFC lesioned animals, and perinatal MPFC lesioned animals have shown very different results based on the age of the subject at the time of brain injury (Dallison & Kolb, 2003; Kolb et al., 1998a; Kolb et al., 1998b; Kolb et al., 1996; Nonneman & Corwin, 1981). In fact, it has been shown that neonatal MPFC decorticates of a particular age (i.e., aspiration of the MPFC specifically between 7 and 10 days of age in mice and rats, Phillips et al., 2009; Kolb et al., 1974, 1996, 1998a, 199b) consistently show less impairment on tasks thought to tap into MPFC function than animals subject to aspiration at other ages, either older or younger. (Nonneman & Corwin, 1981; Kolb et al., 1974; 1996; 1998). This finding has lead researchers to investigate this behavioural phenomenon, and it has been reliably shown that the MPFC of neonatal rats with MPFC decortication on P10, show not only a more favourable behavioural outcome but also apparent regrowth of the MPFC tissue (Kolb et al., 1998). In their study, Kolb and Gibb (1998) discovered that this filling of the lesion cavity following bilateral neonatal MPFC aspiration on day P10 in rats, was apparent as early as 3 postoperative days. This cavity continued to fill and was virtually indistinguishable to the naked eye from control tissue in adulthood. Laminar organization was evident, and some normal connectivity with other brain areas has been reported following these specific lesions (Kolb et al., 1998; Driscoll et al., 2007). Phillips and colleagues (2009), however, discovered abnormalities in circadian behaviours such as wheel running and phase shifting in response to light

pulses in neonatal MPFC decorticates using the same aspiration procedure as Kolb's studies (1974; 1996; 1998).

This most recent finding by Phillips and colleagues (2009) has raised interest, as most research until this point has supported near normal functioning of the filled in region, at least when tested behaviourally in rats. That circadian behaviours may be affected by MPFC lesions indicates that the temporal aspect of MPFC function may be more prominent and disturbed than MPFC dependant behaviours that rely less on keeping time.

The importance of time keeping in MPFC dependent behaviours may be outlined by the link between the MPFC and circadian brain centers. The MPFC is connected with the master circadian pacemaker of the SCN through multi-synaptic pathways, shown in viral transneuronal tracing studies (Sylvester et al., 2002). The SCN itself is endogenously rhythmic, maintained by an approximately 24 hour transcription-translation feedback loop. It receives information from and entrains to the external cyclic environment (principally, the light/dark cycle) via the retinohypothalamic pathway, and projects rhythmic timing information to other areas of the brain (Antle and Silver, 2005; Reppert and Weaver, 2001). The SCN has an overarching entraining influence on circadian rhythmicity in the entire brain, as well as influence over peripheral oscillators throughout the body (Balsalobre, 2002). The endogenous rhythmicity of the SCN is apparent in expression of clock genes, such as *Period1* and *Period2* (Antle and Silver, 2005; Reppert and Weaver, 2001). Not surprisingly, these genes are also expressed in other brain regions which appear to keep time as well, and are thought to be under the influence of the master circadian clock.

The MPFC is not only connected to the master pacemaker, but also has been shown to experience rhythmic gene expression itself (Abe et al., 2001; Abe et al., 2004). *Period1* and *Period2* mRNA expression has been shown previously to oscillate in the cingulate area of the MPFC, an area also independently linked to attention and temporal organization of behaviour (Kolb et al., 1974).

The oscillation of clock gene expression may potentially play an important role in the generation of new tissue. Matsuo and colleagues (2003) found in the peripheral tissue of the liver, that a dysfunctional circadian clock negatively influenced liver regeneration. Their results suggest that normal influence from the master circadian clock as well as normal clock gene expression and oscillation is necessary for new tissue to develop. This has not been tested in brain/central nervous system regeneration models however.
The following study aims to determine the expression profiles of clock genes, namely *Period1* and *Period2* mRNA and protein synthesis, as well as the immediate early gene cFos, in the regenerated tissue of neonatal (P7) MPFC decorticates. I hypothesize that healthy MPFC will show rhythmicity in gene expression, and regenerated tissue will not exhibit coherent rhythmicity. Patterns of protein expression of mouse PER1 and the immediate early gene cFos, as well as mRNA expression of *Period1* and *Period2* will be examined over 24 hours in neonatal decorticates once they have reached adulthood and cortical regeneration is complete.

3.3 Materials and Methods

3.3.1 Animals

Animal care, handling, and surgical procedures were carried out under the guidelines of the Canadian Council on Animal Care, and were approved by the Life and

Environmental Sciences Animal Care Committee at the University of Calgary. Animals were provided food and water ad libitum throughout the study, in a colony room maintained at 31% humidity and on a 12:12 light:dark cycle. Experimental animals were bred in the Antle lab mouse breeding colony, and weaned at 21 days of age. A total of 73 C57 mice (46 P7 aspirations and 27 shams) were generated for the purposes of this study. One hundred thirty-six adult control C57 mice were obtained from the University of Calgary breeding colony.

3.3.2 Surgery

Bred mice were subject to MPFC aspiration lesions on P7. At this time, the dam was removed from the cage, and placed in a clean cage until 1 hour post-surgery, at which time she was returned to her pups. Anesthesia was induced with 4-5% isoflurane, and maintained at 2-3% throughout the surgery, with a constant flow rate of oxygen of 800-900mm^3 per min. A midsagittal incision was made at the rostral portion of the skull to expose the MPFC area. Craniotomy was performed bilaterally at 1 mm lateral to the midline, and from bregma forward to the anterior edge of the frontal bone. A 23 gauge cannula was attached to a vacuum, and used to gently aspirate the exposed tissue. Care was taken to not aspirate ventrally to the lateral ventricles, as extending the lesion this far ventrally has been known to prevent regrowth of MPFC tissue. Arteries were also avoided to ensure stable blood-flow to the area following surgery. Animals were sutured, and allowed to recover for 1 hour on a thermal blanket (37 degrees C), after which time the dam was returned to the cage, and all were placed back in the colony room. The surgery itself took no longer than 15 minutes including anesthesia induction. Sham surgeries consisted of the same procedure, however without craniotomy and aspiration.

Once the animals reached adulthood (60 days of age), they were sacrificed via anesthetic overdose and perfused with paraformaldehyde at 12 different circadian time points over a full 24 hour circadian period. The brains were extracted and slices were taken throughout the MPFC. Tissue was examined for lesion cavity filling, and in all P7 aspirated animals, apparent lesion filling had occurred. Therefore, tissue was then processed for *mPer1*, *mPer2*, PER1 and cFos expression using immunohistochemistry procedures (Smith et al., 2008).

3.3.3 Histology

Animals were deeply anesthetized with euthanyl, and perfused transcardially with 4% paraformaldehyde. Brains were left in this solution for 12 hours, after which time they were moved into a 20% sucrose solution. After 24 hours in the sucrose solution, brains were sliced on a cryostat at 35um thickness.

3.3.4 Immunocytochemistry (ICC)

At room temperature (unless otherwise specified) sections were rinsed for 30 minutes in 0.5% H_2O_2 in PBSx to inactivate endogenous peroxidase. Following this, three 10 minute PBSx rinses and a 90-minute incubation in 1% normal horse serum in PBSx were completed. Tissue was then incubated for 2 days 4°C in the primary antibody (1:10,000 goat anti-Per1 or goat anti-cFos, Santa Cruz). Subsequently, sections were exposed to three 10 minute PBSx rinses and a 1-hour incubation in the secondary antibody biotinylated horse anti-goat (1:200; Vector Laboratories, Burlingame, CA). This was followed by three 10 minute PBSx rinses, a 1-hour incubation in ABC (Vectastain Elite kit, Vector Laboratories, Burlingame, CA) and three 10 minute PBSx rinses. Slices were then developed for approximately 5 min in 0.05% DAB and 0.02%

NiCl in 25mL of 0.1M Tris buffer activated with 80µL of 30% H_2O_2. Finally, slices were mounted on gelatin-coated slides, air-dried, dehydrated through an alcohol series (75%, 90%, 100%, 95%, 75% EtOH, 3 minutes in each), cleared in xylenes for 3 minutes, and coverslipped with Permount.

3.3.5 In Situ Hybridization

For *mPer1* and *mPer2 in situ* hybridization, all instruments and trays were cleaned with RNase Zap (Ambion, Austin, TX) and all solutions were prepared with RNAase-free water. Free floating tissue sections were processed according to the digoxigenin (DIG) protocol of Yan and Silver (2002). First, tissue was treated with proteinase K (1 mg/ml, 0.1 M Tris buffer pH 8.0; 50 mM EDTA; 10 min) at 37°C. This reaction was then stopped by adding 4% paraformaldehyde. Following rinses in saline sodium citrate (300 mM NaCl, 30 mM sodium Citrate) the tissue was treated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. The sections were then incubated in hybridization buffer (50% formamide, 60 mM sodium citrate, 600 mM NaCl, 10% dextran sulphate, 1% N-laurylsarcosine, 25 mg/ml tRNA, 1X Denhardt's, 0.25 mg/ml salmon sperm DNA) containing the DIG-labelled *mPer1* or *mPer2* antisense cRNA probes (0.1 µg/ml; for details see Hamada et al., 2001) for 16 h at 60°C. After two 30 minute high-stringency posthybridization washes (50% formamide/saline sodium citrate at 60°C), sections were treated with RNase A. After 2 more high-stringency washes, the tissue was processed for immunodetection with a nucleic acid detection kit (Roche, Indianapolis, IN). The sections were incubated in 1% of blocking reagent in buffer 1 (100 mM Tris-HCl buffer, 150 mM NaCl, pH 7.5) for 1 h at room temperature. Sections were then incubated at 4°C in alkaline phosphatase-conjugated DIG antibodies diluted 1:5000

in buffer 1 for 3 days. On day 4, sections were washed in buffer 1 twice (15 min each), and then incubated in buffer 3 (100 mM Tris-HCl buffer, pH 9.5, containing 100 mM NaCl and 50 mM $MgCl_2$) for 5 min. They were then incubated in a solution containing nitroblue tetrazolium salt (0.34 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (0.18 mg/ml) (Roche) for 16 h. The colorimetric reaction was stopped by immersing the sections in buffer 4 (10 mM Tris-HCl containing 1 mM EDTA, pH 8.0). The tissue was then mounted on gelatine coated slides, dehydrated in an alcohol series (75%, 90%, 100%, 95%, 75% EtOH, 3 minutes in each), cleared in xylenes for 3 minutes, and coverslipped with Permount. Brains were numbered using a system of nicks, and each assay contained tissue from animals representing each timepoint for comparison (Smith et al., 2008).

3.3.6 Relative Optical Density (ROD)

Images were captured and analyzed on a Olympus BX51 microscope using Image-Pro Plus 5.1 software (Media Cybernetics, Silver Spring, MD). Relative optical densities (ROD) were collected bilaterally in layers 2 and 3 of the MPFC. ROD was also examined in the SCN (to validate that we can detect a circadian rhythm of gene expression in an area where rhythms are well known). The ROD calculations were performed using ImageJ (NIH) and were expressed as a ratio of mean grey level in area of interest/mean grey level in an adjacent patch of tissue (layer 1) on the same section with no signal.

3.3.7 Statistical Analysis

ROD data were analyzed using analysis of variance (ANOVA) comparing healthy adult controls and sham lesion controls to P7 aspirated adults at various circadian time

points (CT0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22). The Bonferroni t-test was used to follow-up significant ANOVA results. Significance was set at an alpha of 0.05.

3.4 Results

3.4.1 PER1

ANOVA did not reveal a significant interaction between groups (P7 aspiration X healthy adult controls) and circadian time points in PER1 expression ($F_{(22,114)} = 0.576$, $p = 0.928$). This is shown in Figure 3.1. The main effect of group ($F_{(2,114)} = 3.348$, $p = 0.040$), however, was statistically significant, yet main effect of time point ($F_{(11,114)} = 0.756$, $p = 0.682$) was not, suggesting that the overall amount of PER1 protein may have been different in the two groups of animals. Bonferroni follow up t-tests did not result in significance between the groups ($t = 2.170$, $p = 0.099$). Results for comparisons of P7 aspirated animals and P7 sham controls showed no significant differences in interactions or main effects of group ($F_{(2,65)} = 0.238$, $p = 0.628$) or time ($F_{(11,65)} = 0.742$, $p = 0.693$).

Neonatally aspirated animals' PER1 expression was also not significantly different between any time points ($F_{(11,45)} = 0.854$, $p = 0.590$) suggesting a lack of overall intrinsic rhythmicity. However, this lack of rhythmicity was also apparent in healthy adult control groups ($F_{(11,68)} = 0.902$, $p = 0.544$) and in animals with P7 sham lesions ($F_{(7,17)} = 2.057$, $p = 0.145$), contrary to previous studies (Abe et al., 2001; 2004).

3.4.2 cFos

Data for cFos expression showed similar results, as portrayed in Figure 3.2. Between P7 aspirated animals and healthy adult controls, no significant interaction was found ($F_{(22,113)} = 0.329$, $p = 0.998$), nor were significant main effects of group ($F =_{(2,113)} 0.0142$, $p = 0.986$) or circadian time ($F_{(11,113)} = 0.163$, $p = 0.999$). Significant differences

due to CT also failed to be produced when comparing P7 aspiration animals in adulthood with P7 shams in adulthood. Yet there was a significant main effect of group (P7 aspiration vs P7 shams, $F_{(2,67)} = 3.806$, $p = 0.028$) suggesting that the overall cFos expression may have differed between these groups. Bonferroni t-test results ($t = 2.754$, $p = 0.024$) and group means suggest that P7 aspiration ($M = 1.104$, $SEM = 0.00866$) animals expressed greater amount of cFos than P7 shams ($M = 1.087$, SEM = 0.0211).

Within each group, again there were no significant differences between time points and therefore no significant rhythmicity for neonates ($F_{(11,45)} = 0.875$, $p = 0.572$), adult controls ($F_{(11,67)} = 0.653$, $p = 0.775$), or neonatal shams ($F_{(7,17)} = 1.421$, $p = 0.296$). Representative photomicrographs of PER1 and cFos expression are provided in Figure 3.1.

3.4.3 *mPer1*

Expression profiles of mPer1 data can be seen in Figure 3.2. Expression of *mPer1* in P7 aspirated animals and healthy controls did not reveal a significant interaction ($F_{(22,113)} = 0.566$, $p = 0.933$), nor were there significant main effects of group ($F_{(2,113)} = 0.864$, $p = 0.425$) or time ($F_{(11,113)} = 0.381$, $p = 0.960$). Also, when P7 aspirated animals *mPer1* profiles were compared to sham lesion controls, there were also no apparent differences, no interaction ($F_{(11,70)} = 0.557$, $p = 0.853$), nor main effects (group: $F_{(1,70)} = 0.001$, $p = 0.975$; CT: $F_{(11,70)} = 0.583$, $p = 0.833$).

Overall rhythmicity in *mPer1* expression in each group was null, as significant differences between time points did not appear in MPFC of P7 aspirated animals ($F_{(11,44)} = 0.701$, $p = 0.728$), healthy adult controls ($F_{(11,68)} = 0.697$, $p = 0.736$)., or sham lesioned animals ($F_{(10,24)} = 0.407$, $p = 0.921$).

For comparison to Abe and colleagues (2001; 2004) studies, we also compared peak and trough *mPer1* expression times from their study (CT10 vs CT14) in our MPFC tissue. In healthy control animals, there was not a significant difference between these two time points (t = -0.869, p = 0.407). The same was true for regenerated MPFC tissue (t = -0.361, p = 0.730).

3.4.4 *mPer2*

Data for *mPer2* were similar, showing no significant interactions or main effects (interaction: $F_{(22,113)}$ = 0.152, p >0.999; group: $F_{(2,113)}$ = 0.298, p = 0.744; time: $F_{(11,113)}$ = 0.433, p = 0.936) when comparing P7 aspirated animals to healthy controls, nor when comparing them to sham lesioned animals (interaction: $F_{(11,72)}$ = 1.607, p = 0.126; group: $F_{(1,72)}$ = 0.113, p = 0.738; time: $F_{(11,72)}$ = 0.463, p = 0.917).

Analysis of the overall rhythmicity of *mPer2* in each group (P7 aspirated animals, healthy controls, and P7 sham animals) did not show significant differences between peaks and troughs in expression ($F_{(11,45)}$ = 1.928, p = 0.070; $F_{(11,67)}$ = 0.888, p = 0.557; $F_{(7,15)}$ = 0.373, p = 0.894), suggesting a lack of overall rhythmicity in this area, contrary to Abe and colleagues' (2001; 2004) findings in the cingulate cortex.

In accordance with Abe and colleagues findings, CT10 and CT14 alone were compared for *mPer2* expression in MPFC as well, and significant difference between the two time points were not found (t = -0.782, p = 0.464).

3.4.5 SCN

As an added control, protein, mRNA, and cFos expression were examined in the SCN of control animals, to ensure that labelling of cells was reliable.

In control tissue (sham lesioned animals and healthy controls), PER1 expression was significantly different at various time points in the SCN ($F_{(5,11)} = 4.419$, $p = 0.049$). This was also true of cFos expression within the SCN as well ($F_{(9,31)} = 2.349$, $p = 0.049$) suggesting that our immunocytochemistry protocol, as well as our method of quantification (ROD), was sensitive enough to detect rhythmic differences in both cFos and PER1 expression. Also, in the SCN of control animals labelled using our *in situ hybridization* methods, significant differences in *mPer1* arose between time points ($F_{(11,68)} = 2.215$, $p = 0.026$), which was also true for *mPer2* ($F_{(11,68)} = 2.317$, $p = 0.020$). These data suggest that our *in situ* methodology was also sensitive enough to detect significant differences in mRNA expression across a 24 hour period.

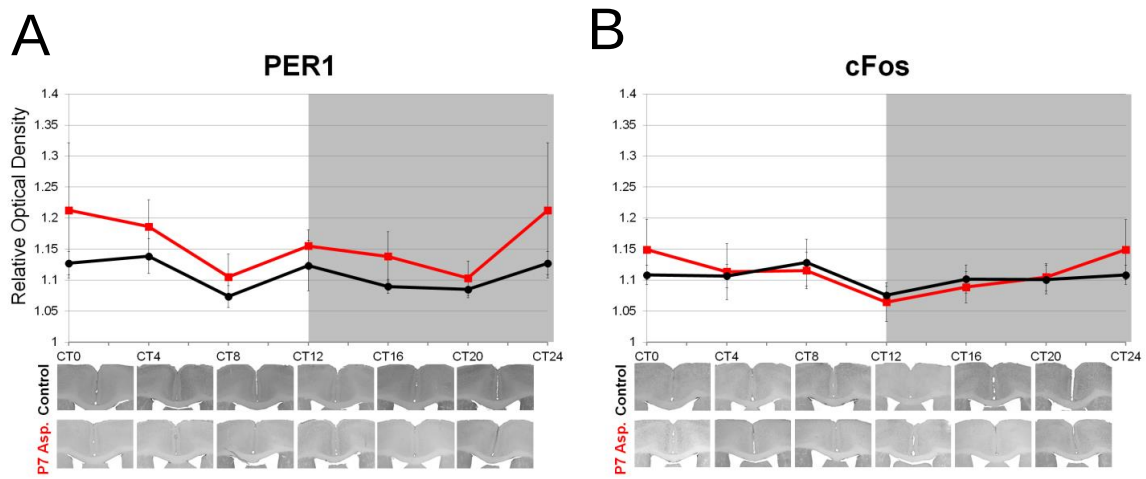

Figure 3.1. A) PERIOD1 protein expression, calculated via relative optical density throughout the extent of the MPFC, in adult regenerated MPFC following P7 aspiration and healthy adult control MPFC. B) cFos expression throughout the MPFC of adult P7 aspirated animals and healthy controls. The figures show every second time point from which samples were taken. There were no significant differences between time points or groups (p>0.05).

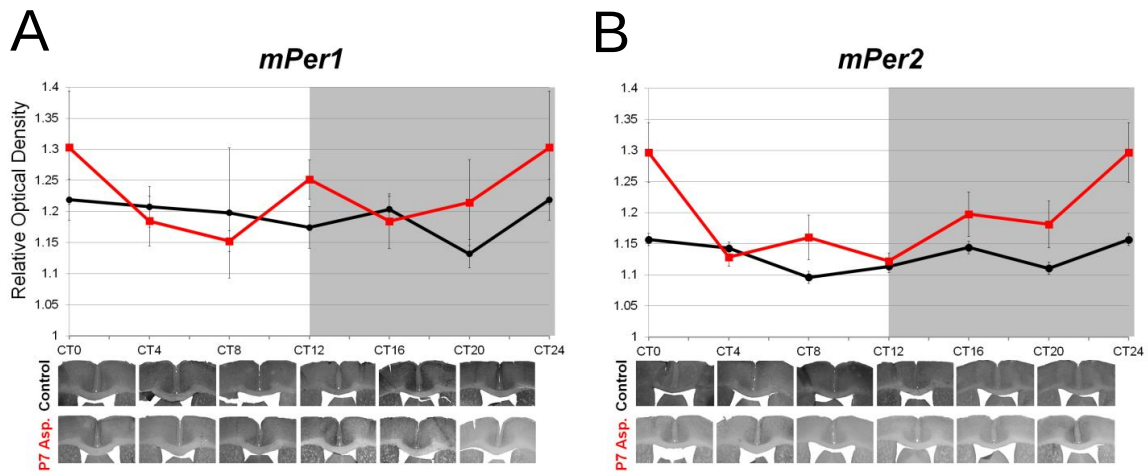

Figure 3.2 A) *mPer1* expression, calculated via relative optical density throughout the extent of the MPFC, in adult regenerated MPFC following P7 aspiration and healthy control MPFC. The lower half of A shows representative photomicrographs of mid-MPFC of control and regenerated MPFC tissue. B) mPer2 expression throughout the MPFC of adult P7 aspirated animals and healthy controls and associated representative photomicrographs of mid-MPFC of control and regenerated MPFC tissue. The figures show every second time point from which samples were taken. There were no significant differences between time points or groups ($p>0.05$).

3.5 Discussion

This study examined Period mRNA and protein, as well as cFos expression patterns over a 24 hour period in the MPFC of adult mice. One group was subject to P7 aspiration lesions and were allowed to mature to adulthood before their brain tissue was examined, while other groups consisted of healthy adult control animals or sham lesioned animals. It has been shown previously that the cingulate area of the MPFC shows rhythmic *mPer1* expression in mice, yet our study failed to replicate these results when examining rhythms in both *mPer1* and PER1 protein expression. In the present study, differences in PER1 expression in healthy control MPFC versus P7 aspirated MFPC in adulthood were not apparent. This was also true of cFos expression. Individually, each group did not show clear rhythmicity in *mPer1* or *mPer2* expression, PER1 expression, or in cFos expression, and this also contributed to the lack of significant differences between the expression profiles of the two groups when compared to one another.

Interpretation of the current results can take three main avenues. First, if taken at face value, these results suggest that there is a lack of rhythmicity in this brain region, at least in our animals. This would not coincide with previous research which examined mRNA levels in this area (Abe et al., 2004; Abe et al., 2001). Although the results of this study contradict previous knowledge on rhythmic expression of clock genes in the MPFC, previous studies in this lab have found results similar to Abe (2001, 2004). Abe and colleagues (2001; 2004) found the greatest difference in *Period1* mRNA expression between CT10 and CT14, and while preliminary results from our lab suggested that there may be a circadian rhythm, based on analysis of only CT10 and CT14 (Rakai and Antle, SRBR 2010), this result was not robust and did not persist once the full data-set was

processed and analyzed. In our overall analysis, inclusion of numerous time points may have reduced the power of the statistical tests, enough to eliminate differences seen between peaks and troughs of gene expression. There may also be differences in delineation of brain areas when comparing our results to those of Abe and colleagues (2004; 2001). Their studies examined expression patterns in the cingulate cortex, yet they do not describe how they delineated this area. Therefore, our method of examining the entire MPFC area, from the most rostral slice of tissue through to bregma, may not coincide with the area of cingulate that they examined. If it is the case that we were examining a much larger area of brain tissue, significance in our results may have been washed out.

A second interpretation of these results may suggest that the Per proteins are expressed differently than mRNA. That is, mRNA expression as shown in Abe's (2001; 2004) studies may not be translated into greater protein expression at corresponding circadian times. Therefore, our analysis of PER1 expression may be unrelated to actual mRNA expression. Should this be the case, a viable explanation for the presence of mRNA but not its end result, protein, will be necessary. However, this explanation is unlikely, considering that our lab has matched Abe's (2001; 2004) mRNA measures with protein production in the past, albeit at only two circadian time points.

A third, and more likely interpretation, may be that the results obtained here provide evidence of similarities in healthy and aspirated animals' clock protein expression in the MPFC. In examining the profiles of PER1 expression in Figure 3.1, it does appear that there may be some strong similarities between MPFC area PER1 and cFos expression in the aspirated and control animals, although with minor differences at

CT0 and CT18. Overall, protein expression is largely the same with only a few subtle differences between MPFC aspirated animals and controls. This trend suggests that the regenerated MPFC tissue may make some normal connections with the SCN, and therefore may be under its control. This would be contrary to predictions, which had assumed that the newly generated tissue would not connect sufficiently with the circadian pacemaker to induce rhythmicity. However, other reasons for such a similar labelling in PER1 expression between controls and aspirated animals would be that some other form of communication, possibly communication by diffusion, between the SCN and MPFC is sufficient without the normal multi-synaptic connections (Sylvester et al., 2002) to keep time in the new tissue.

Further evidence to suggest that this interpretation is appropriate comes from our analysis of control SCN tissue. We were able to identify significant differences in SCN mRNA and protein expression, which suggests that our staining protocols were sensitive to time of day variation. Rhythms in MPFC may be significantly muted compared to the robust rhythms of the SCN, and with such a blunted rhythm, significance was not achieved. Therefore, the fact that our data suggest strong similarities between aspirated and control MPFC is likely to be valid, as our control data showed sufficient sensitivity in our methods.

Other studies using rats may be relatable to our data. Many of these studies have shown that the newly generated tissue in the MPFC area may have the ability to behave similarly to normal tissue. Driscoll and colleagues (2007) found similarities in firing rates and patterns in the regenerated tissue when compared to healthy controls. However, there were differences in the peak frequencies of these EEG recordings, suggesting that

although the tissue is behaving similarly to healthy animals, subtle differences still arise in certain aspects of the overall output. If one takes the third interpretation of the above results to be accurate, that gene expression profiles may be similar with only minor differences at specific circadian times, then our findings may be in accordance with the Driscoll and colleagues (2007) results. The differences in the peaks and troughs in gene or protein expression may be muted in the regenerated MPFC just as EEG peaks and troughs are lower than that of controls. Further investigation is necessary to deduce the true significance of the minor differences seen in Figures 3.1 and 3.2, and their relation to the differences seen in EEG patterns.

In the most recent study conducted by Phillips and colleagues (2009), circadian patterns of activity were significantly different between aspirated animals and controls. Their results also showed a very similar overall activity pattern, but with significant differences in the day-time activity, and a general muting of night-time activity. These results appear to suggest some miscommunication between circadian centers in the brain and the newly generated tissue. If one accepts the interpretation that mRNA and protein expression are largely the same between controls and MPFC lesioned animals with slight differences at specific circadian times, then the apparent delay of the animals' phase in Phillips' (2009) study would lead to the prediction that the PER1 protein expression peaks and troughs have also shifted. This would also be in accordance with Abe's (2001; 2004) studies, which showed peaks and troughs not at CT0 and CT8 respectively, as it appears in Figure 3.1 for MPFC lesioned animals, but at CT14 and CT10, which correspond with CT's 20 and 14 for the production of the protein. If, as Abe's (2001, 2004) reports suggest, the trough of protein production (which is known to be about 6

hours after mRNA expression) is at CT16 and the peak at CT20, then the shift in our animals' trough to CT8 and peak at CT0 may be linked to the changes in activity patterns seen by Phillips and colleagues (2009) and may help to explain the increase in day-time activity. This is yet another reason to believe that the most reliable interpretation of the current findings is that overall expression is similar, with slight changes in peaks and troughs. At present, however, the lack of significance and the direction in the shift of the peaks and troughs compared to the shift in activity warrant further clarification.

Overall, the results of this study do not match previous studies nor do they offer overwhelming evidence for differences in MPFC circadian function in P7 aspirated animals compared to controls. The above results may be indicative of only minor differences in clock gene expression in the MPFC following neonatal aspiration lesions.

Other future directions for this research are abundant. First, the role of clock genes themselves in the regeneration process should also be examined. To do this, MPFC tissue in animals without a fully functioning circadian clock, and therefore without rhythmic clock gene oscillation, should be examined following P7 MPFC aspiration. As was mentioned earlier, it is thought that a fully functioning circadian clock is necessary for tissue regeneration in other areas of the body, and so cortical regeneration may rely on the circadian clock as well. Future studies may also examine clock gene expression at different ages following MPFC aspiration, as this may shed light on the processes that are occurring in the new tissue as it develops.

Once the environment necessary for cortical regrowth is fully understood, models may be able to be developed to initiate this process in animals of a different age. The

possibility of inducing brain regeneration is exciting and promising for the development of brain damage recovery models.

Chapter 4

Medial prefrontal cortex regeneration in BMAL1 knockout mice

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4.1 Abstract

For years, it was widely believed that the brain cannot regenerate following injury. After discovery that the subgranular and subventriclar zones regenerate throughout adulthood, more dramatic regeneration was also observed following aspiration of the medial prefrontal cortex (MPFC) in neonatal rodents aged 7-10 days. Once these animals reach adulthood, those subject to the aspiration procedure are nearly indistinguishable from healthy adult animals, except for altered circadian activity patterns and responses to light pulses. The MPFC is thought to exhibit clock gene expression, and therefore may be under circadian control. The circadian clock also plays a role in the cell division cycle in the liver, but examination of its role in brain regeneration has been less extensively studied. Using arrhythmic BMAL1 knockout mice and littermate controls, we examined MPFC lesion volumes and brain weights 3 and 7 days after P7 aspiration. Regeneration was already apparent in these animals, and we found no significant differences in either of these measures at either time point. These results suggest that MPFC regeneration may not be affected by BMAL1 deletion in the week following aspiration. We conclude that brain regeneration is affected differently by circadian arrhythmicity and a dysfunctional circadian clock than in the liver.

Keywords: medial prefrontal cortex, regeneration, BMAL1, arrhythmic, circadian

4.2 Introduction

For years, it was widely believed that the brain cannot regenerate. This dogma has been challenged with the observation of adult neurogenesis in the subgranular zone (SGZ) of the dentate gyrus (DG) and the subventricular zone (SVZ) leading to the olfactory bulb (OB). However, even more dramatic regeneration has been observed following damage to the medial prefrontal cortex (MPFC). Specifically, in neonatal rodents, aspiration of the MPFC results in a filling in of the lesion cavity by adulthood (Dallison et al., 2003; Kolb et al., 1998; Kolb et al., 1996; Kolb, 1987). There are caveats to this regeneration, however. First, the MPFC must be aspirated via suction for the regrowth to occur. Secondly, this aspiration must occur between 7 and 10 days of age (Phillips et al., 2009; Dallison et al., 2003; Kolb et al., 1998; Kolb et al., 1996; Kolb, 1987). To the naked eye, the regenerated area is almost unnoticeable when examined in adulthood. Kolb and colleagues (Dallison et al., 2003; Kolb et al., 1998; Kolb et al., 1996; Kolb, 1987) have also shown that behaviourally, animals subject to the aspiration procedure are nearly indistinguishable from healthy adult animals (this has also been shown in unpublished data in mice). Furthermore, Driscoll and authors (2007) determined that the electroencephalographic (EEG) profile of new cell activity in regenerated MPFC is similar to healthy controls, although peaks in EEG output are slightly reduced in most cases. However, Phillips and colleagues (2009) discovered that circadian behaviours are disturbed in these animals. Others have noted circadian rhythms in clock gene expression in the cingulate area of the MFPC (Abe et al., 2001; 2004), and multi-synaptic innervation from the SCN to the ILC of the MPFC, which may further suggest circadian control over the MPFC region.

The circadian clock plays a role in the cell division cycle (Matsuo et al., 2003). Yet, the details of this role remain rather elusive. In peripheral tissues a fully functioning circadian clock may be necessary to control the rate and timing of tissue regeneration. Matsuo and colleagues (2003) examined liver regeneration following partial hepatectomy (PH) in *Cry* deficient mice. They show that liver regeneration is delayed in animals without stable intrinsic rhythmicity. In tissue culture, Grechez-Cassiau and authors (2007) found similar results in BMAL1 knockout hepatocytes. Their study showed a reduced rate of proliferation, suggesting that the circadian clock regulates this aspect of cell division. Another study examined regeneration of blood cells in BMAL1 knockout mice (Sun et al., 2006). Results of this study found altered B-cell development, while other cell types were unaffected. These data suggest that the circadian clock bares some control over cellular proliferation in regenerating cells.

These studies are promising for unveiling the importance of circadian timing and rhythmicity on tissue regeneration. However, examination of regeneration in central nervous system or brain tissue has not been extensively undertaken. The following study aims to investigate whether a fully functioning circadian clock is necessary for MPFC tissue regeneration in neonates. Using arrhythmic BMAL1 knockout strains of mice, cortical regrowth as seen in P7 aspiration models of MPFC regeneration will be examined and compared between arrhythmic mice and healthy littermate controls. MPFC volume and wet brain weights of BMAL1 knockout animals and littermate controls will be calculated 3 and 7 days following aspiration. I hypothesize that BMAL1 knockout mice, who lack any form of intrinsic circadian rhythmicity (Bunger et al.,

2000), will show a delayed or otherwise impaired rate of cortical regeneration compared to littermate controls.

4.3 Materials and Methods

4.3.1 Animals

Animal care, handling, and surgical procedures were carried out under the guidelines of the Canadian Council on Animal Care, and were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary. Animals were provided food and water ad libitum throughout the study, in a colony room maintained at 31% humidity and on a 12:12 light:dark cycle. Experimental animals were bred in the Antle lab mouse breeding colony, and weaned at 21 days of age. A total of 18 mice (6 +/+, 6 +/-, and 6 -/-) were generated for P14 lesion volume analysis. The number of animals utilized for P10 analysis totaled 15 (5 +/+, 5 +/-, and 5 -/-).

4.3.2 Surgery

Bred mice were subject to MFC aspiration lesions on P7. At this time, the dam was removed from the cage, and placed in a clean cage until 1 hour post-surgery, at which time she was returned to her pups. Anesthesia was induced with 4-5% isoflurane, and maintained at 2-3% throughout the surgery, with a constant flow rate of oxygen of 800-900mm^3 per min. A midsagittal incision was made at the rostral portion of the skull to expose the MFC area. Craniotomy was performed bilaterally at 1 mm lateral to the midline, and from bregma forward to the anterior edge of the frontal bone. A 23 gauge cannula was attached to a vacuum, and used to gently aspirate the exposed tissue. Care was taken to not aspirate ventrally to the lateral ventricles, as extending the lesion this far ventrally has been known to prevent regrowth of MFC tissue. Arteries were also avoided

to ensure stable blood flow to the area following surgery. Animals were sutured, and allowed to recover for 1 hour on a thermal blanket (37 degrees C), after which time the dam was returned to the cage, and all were placed back in the colony room. The surgery itself took no longer than 15 minutes including anesthesia induction.

4.3.3 Histology

Animals were deeply anesthetized with euthanyl, and perfused transcardially with 4% paraformaldehyde on P14 or P10. Brains were left in paraformaldehyde solution for 12 hours, after which time they were moved into a 20% sucrose solution. After 24 hours in the sucrose solution, brains were sliced on a cryostat at 35um thickness.

4.3.4 Lesion Volume Analysis

Thirty-five µm slices of frozen brain tissue were collected from the MFC region and stained with Cresyl Violet. Digital images of these sections were captured (Image-Pro Plus 5.1; Media Cybernetics, Silver Spring, MD), and volume analysis was completed using ImageJ (NIH), by outlining the sections and calculating the area of each slice. The total volume of the MFC region was then calculated by multiplying the volume of each 35 µm slice by the total number of slices in the region. Here, every fourth slice was kept, so the resulting formula for calculating MFC volume was as follows: Sum of [(area of slice x 35 µm) x 4] for each slice kept, starting at the olfactory bulb through to bregma.

4.3.5 Brain Weights

Immediately following perfusion, brains were extracted and wet brain weight was measured. The brain was separated from the brainstem just posterior to the cerebellum.

olfactory bulbs removed, and the brains were placed on a standard laboratory scale for measurement.

4.3.6 Statistical Analyses

All data were analyzed using analysis of variance (ANOVA), with significant results being followed up by Bonferroni correction t-tests (SigmaPlot V11.0)

4.4 Results

Initially, comparison of the three genotypes (+/+, +/-, -/-) were completed on P14, 7 days following P7 aspiration. As shown in Figure 4.1, results from this one way ANOVA revealed no significant differences in lesion volumes between the three genotypes ($F_{(2,17)} = 0.275$, $p = 0.763$). These data were further supported by comparing wet brain weights immediately after extraction, where significant differences between groups were also null ($F_{(2,17)} = 0.862$, $p = 0.442$).

The same comparison 3 days after P7 aspiration (P10 perfused animals) showed similar results ($F_{(2,14)} = 0.0863$, $p = 0.918$ for MPFC volume; $F_{(2,14)} = 0.946$, $p = 0.416$ for wet brain weights), suggesting that the amount of cortical regrowth that occurred did not vary by genotype. These data are shown in Figure 4.2.

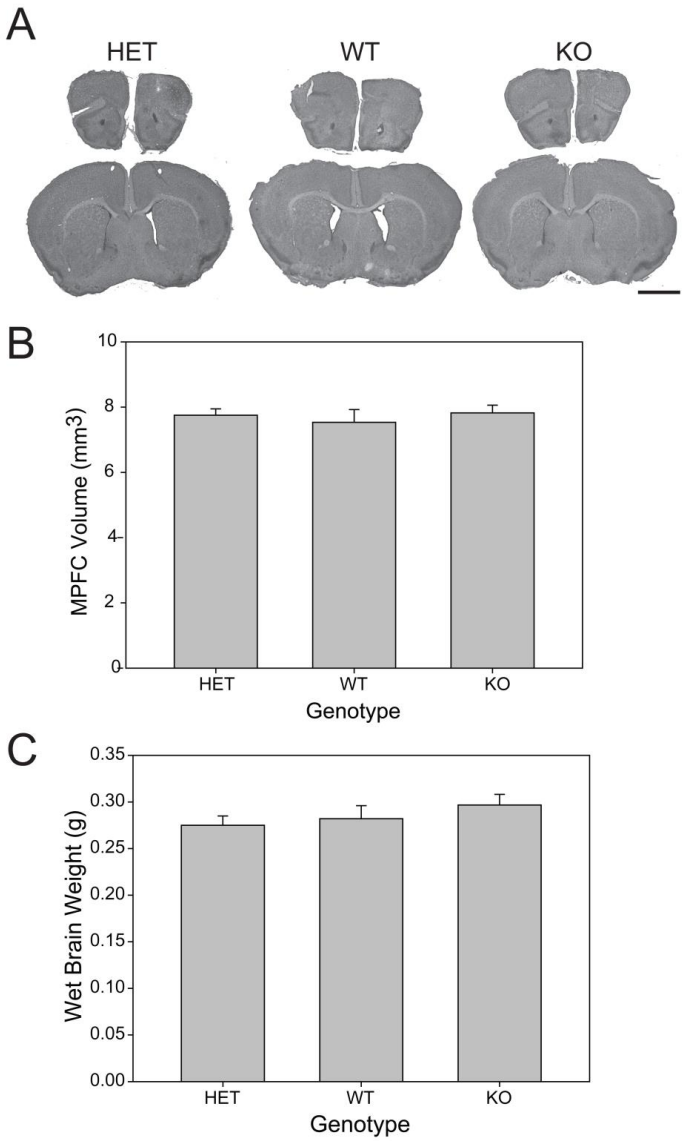

Figure 4.1 Data from MPFC on P14 in BMAL1 heterozygotes, wildtype, and knockout animals, one week after the P7 aspiration lesion. A) Representative photomicrographs of rostral (top) and mid (bottom) sections of MPFC 7 days after P7 aspiration. Scale bar represents 1mm. There were no significant differences in MPFC volume on P14 following P7 aspiration, shown in graphical representation B). Wet brain weights C) also did not differ between knockout animals and littermate controls.

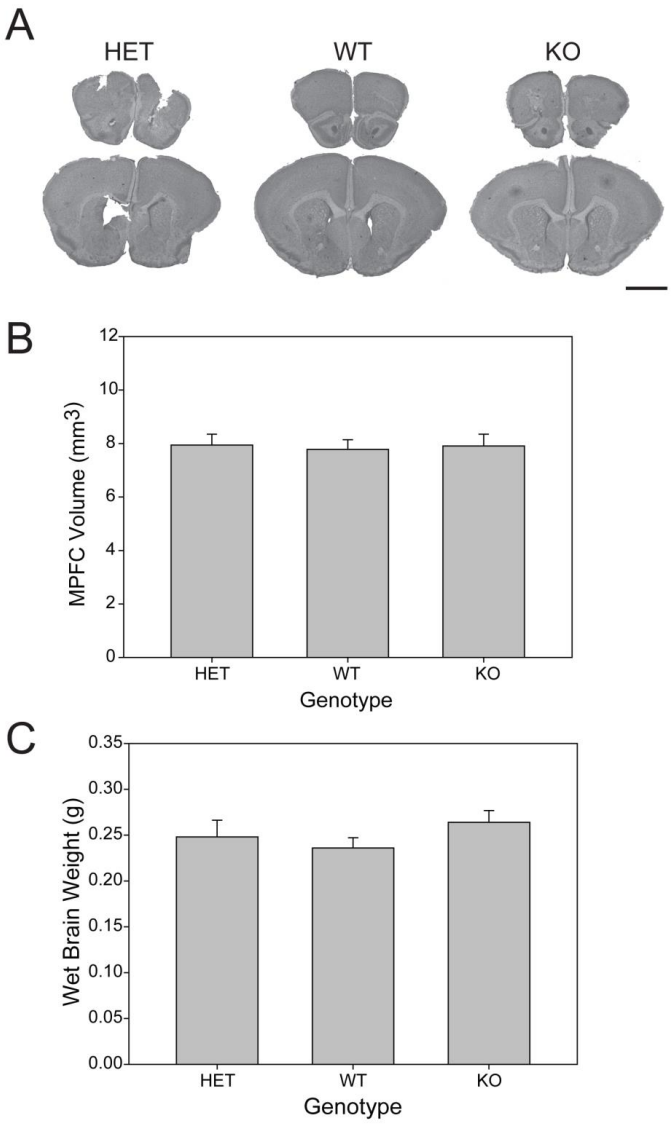

Figure 4.2 Data from MPFC on P10 in BMAL1 heterozygotes, wildtype, and knockout animals, three days after the P7 aspiration lesion. A) Representative photomicrographs of rostral (top) and mid (bottom) sections of MPFC 3 days after P7 aspiration. Scale bar represents 1mm. There were no significant differences in MPFC volume on P10 following P7 aspiration, means shown in graphical representation B). Wet brain weights C) also did not differ between knockout animals and littermate controls.

4.5 Discussion

Animals with dysfunctional circadian clocks did not differ in the rate of regeneration of aspirated MPFC tissue when compared to littermate controls. The volume of the MPFC region was calculated, and values were not significantly different across genotypes (Figures 4.1 and 4.2). Wet brain weight also did not differ, suggesting a normal rate of regrowth over the first week following aspiration, from P7 to P14. Since studies have shown a delayed proliferative stage in the cell cycle of animals with circadian disturbances (Matsuo et al., 2003), we hypothesized that regeneration in the MPFC region would therefore also initially be slightly delayed. However, our results did not coincide with our predictions, and there may be numerous reasons for the discrepancy.

First, in comparing our results to those of Sun and colleagues (2006), one explanation stands out. As was mentioned previously, Sun and colleagues (2006) examined the effects of knocking out BMAL1 on multi-potential progenitor cells. What they found was that a lack of BMAL1 resulted in a disturbance in B-cell proliferation, yet other types of cells were not affected. In our study, we were examining the regeneration of MPFC cells, which hold many differences from the characteristics of B-cells in bone marrow. The differences between these two types of cells in morphology, intracellular components, and even downstream communications may be cause for the differences in results. Direct comparison of B-cell proliferation and that of new neurons in the brain may not be valid due to the significant number of differences in the cellular structure, function, and molecular characteristics of these two populations of cells.

Secondly, many studies of the influence of the circadian clock on tissue regeneration have come from studies of hepatocytes in liver. Though this model is a good example of regeneration within the body, again there are numerous differences between cellular characteristics of hepatocytes and MPFC neurons, or neurons in general.

Matsuo's (2003) study examined hepatocyte proliferation in animals with *Cry* deficiencies. Although *Cry* is a key component of the healthy circadian clock, *Cry* deletions do not have an astounding effect on circadian rhythmicity. Although these animals do show a lack of free running rhythmicity (van der Horst et, al., 1999), there are functional homologs for *Cry*, and therefore the circadian clock is still able to function at some level. BMAL1 has no known functional replacements, therefore its deletion is thought to be much more detrimental to circadian clock function (Bunger et al., 2000). This may be a reason for the discrepancy between our results and those of Matsuo's (2003) study, as some downstream component of the circadian clock, or the presence of a functional replacement, may have very different effects on the cell cycle in parts of the body far removed from the SCN than in central nervous system tissues.

However, Grechez-Cassiau and her group (2007) conducted a similar study to that of Matsuo and colleagues (2003), using BMAL1 deficient animals as opposed to *Cry* knockouts. Both these studies' results were in accordance with one another, as a delayed proliferative stage was apparent in hepatocyte regeneration. One difference, however, was that the Grechez-Cassiau and colleagues utilized *in vitro* as opposed to *in vivo* methodology. Though this method is commonly used, one must take caution in extrapolating *in vitro* results to the living animal, where numerous variables can affect outcomes. Therefore, one cannot fully conclude that BMAL1 knockout liver tissue will

perform in the same manner in a living animal, and the effects of BMAL1 deletion on *in vivo* liver regeneration need still be investigated. Overall, however, these two studies combined show a strong role for the circadian clock in liver regeneration.

Although both Mastuo (2003) and Grechez-Cassiau (2009) were able to show very similar deficits in cellular proliferation using two very different models of circadian disruption (*Cry* deficiencies and *BMAL1* deficiencies), the fact that these studies used liver tissue as opposed to brain tissue may have been an important factor leading to the discrepancies between their results and ours. These two tissues are known to behave in very different ways. Of particular importance is that mature neurons in the brain are postmitotic, and only certain subsections of neural precursor cells have the ability to divide, while liver cells consistently regenerate throughout life. Therefore it is not surprising that circadian influences on central nervous system tissue regeneration may yield quite different results than in peripheral tissues, which we were able to show with our study.

Previous studies have noted differences in cortical thickness and lamination of the regenerated tissue, and in overall brain weight when comparing regenerated MPFC to healthy controls (Dallison et al., 2003; Kolb et al., 1998; de Brabander and Kolb, 1997; Kolb et al., 1996; Kolb, 1987; Kolb and Whishaw, 1981). Although we did not quantify cortical thickness or laminar organization of the new tissue, we did not note the same decrease in MPFC volume, nor did we witness the same 10% decrease in overall brain weight. This suggests that there may be differences in MPFC regeneration between rats, used in Kolb's studies (Dallison et al., 2003; Kolb et al., 1998; de Brabander and Kolb, 1997; Kolb and Whishaw, 1981), and mice, utilized in our lab. Upon gross examination

of MPFC areas in our study (Figures 4.1 and 4.2), clear delineation of the lesion boundaries are less prominent than those seen in the previous studies. Abnormalities in the shape of the corpus callosum are apparent, with a strong resemblance to the lesion boundaries seen in Kolb's (1998) rat studies. Yet, due to differences in procedure, induced simply by different surgeons, initial lesion size may have varied between the studies. However, in both species, regeneration will not occur should the lesion be deep enough to involve the ventricles, located just under the area of aspiration. Therefore, it seems unlikely, since cortical regeneration did occur in all three groups that the induced lesions differed in size across studies. One other possibility is that the lesions produced in our study were slightly smaller than those of Phillips and colleagues (2009) or of those produced by Kolb's group (1998). Yet, regardless of this possibility, regeneration occurred, and the foundation of the phenomenon was the same.

Kolb's studies found various rates of cavity filling, but stated that it is complete by adulthood (Dallison et al., 2003; Kolb et al., 1998; de Brabander and Kolb, 1997; Kolb and Whishaw, 1981). Previous unpublished data in mice has suggested that the cavity filling is well underway by 3 postoperative days. Therefore, our postoperative times of brain examination, 3 and 7 days, may be too long after the lesion is induced to note significant differences in brain regeneration. We chose these time points also based on liver regeneration studies, which show that although proliferation is initially delayed in animals with circadian deficiencies, the regeneration process becomes equal to control animals by 10 days following partial hepatectomy. It may be the case that the process occurs faster in mouse MPFC tissue, and we were then simply too late to find the discrepancies. Since regeneration of mouse MPFC has not been fully quantified, the

possibility that proliferation and regeneration differ only in the first day or two after aspiration is somewhat valid. Yet a finding such as this would make it difficult to state that regeneration differs at all, since the exact time of the lesion induction cannot be highly controlled (ie, it is impossible for the same surgeon to conduct surgery on two animals at the very same time). Future studies may also investigate if there are variations in MPFC regeneration depending on time of day, as Matsuo and colleagues (2003) found differences in liver regeneration based on the time of day that partial hepatectomy was performed.

Age may be an important factor for future studies to address as well. Adult liver regeneration (Matuso et al., 2003) as well as adult neurogenesis (described in the next chapter) are influenced by the circadian clock, whereas we examined regeneration following neonatal injury. It is possible that the circadian clock regulates the cell cycle in adult animals, and that the infancy or incomplete development of the circadian clock in neonates reduced circadian influence on tissue regeneration. However, the role of the circadian clock in liver regeneration in neonates has not been investigated. Since it is likely that development occurs at a faster rate in neonates, and we were unable to notice differences in MPFC regeneration by post-surgical day 3, investigation into the rate of liver regeneration may shed light on our current findings. That is, if tissue regeneration in the liver is accelerated in neonates compared to adults, then it is likely that MPFC tissue would show a similar increased rate of regeneration, and our study may have, in fact, examined MPFC regrowth too long after the aspiration procedure. Therefore, future studies may need to investigate liver, as well as MPFC regeneration sooner after damage.

Overall, interpretation of our findings may take many avenues. Differences in procedure, time of tissue examination, and species differences may all be valid reasons for the end results and the differences seen when comparing to previous literature. However, the most plausible explanation is that in brain tissue, in the MPFC specifically, the influence of the circadian clock's role on the cell cycle is different than that which occurs in peripheral tissues such as the liver. MPFC regeneration is a unique process, and future research should focus on the circadian clock's role in more common forms of neurogenesis. Those which occur naturally in adulthood may hold more promise for adult brain damage repair than this elusive neurogenesis induction model. Yet our results still hold significant promise in adding to the literature regarding neonatal stroke and brain repair, and in furthering our knowledge of the importance of circadian clock function in brain damage and recovery.

Chapter 5

Hippocampal neurogenesis in arrhythmic mice lacking the BMAL1 gene

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5.1 Abstract

The subgranular zone gives rise to new neurons that populate the dentate gyrus of the hippocampal formation throughout life. Cells in the hippocampus exhibit rhythmic clock gene expression. The circadian clock is known to regulate the cycle of cell division in a number of areas of the body. These facts suggest that the circadian clock may regulate adult neurogenesis in the hippocampus as well. In the present study, neurogenesis in the hippocampal subgranular zone was examined in arrhythmic BMAL1 knockout mice and their rhythmic heterozygous and wildtype littermates. Proliferation and survival of newly generated subgranular zone cells were examined using BrdU labelling, while apoptotic cell death and hippocampal volume was examined in cresyl violet stained sections. There was no significant difference in cellular proliferation between any of the groups, yet survival of proliferating cells was significantly greater in the BMAL1 knockout animals. Cell death was significantly decreased in BMAL1 knockout animals as well, yet hippocampal volume remained the same across genotypes. These findings suggest that, while a functional circadian clock is not necessary for normal proliferation of neuronal precursor cells, the normal apoptotic pruning of newly generated neurons in the hippocampus does require a functional circadian clock.

5.2 Introduction

Mitotic cell division is under circadian control (Matsuo et al., 2003). In tissues such as the liver, deficiencies in clock genes interrupt normal cell division after injury. Following partial hepatectomy (PH), liver tissue in *Cry* deficient mice shows delayed regeneration (Matsuo et al., 2003). A key component of the positive arm of the circadian transcription-translation feedback loop, BMAL1, has also been shown to be involved in mitotic cell division in the liver. The progression of hepatocytes from quiescence into G1 of the cell cycle is disturbed when BMAL1 is knocked out. Proliferation in liver tissue is delayed initially, yet regeneration of liver tissue does reach normal levels 10 days post PH (Matsuo et al., 2003; Gréchez-Cassiau et al., 2007).

In central nervous system (CNS) tissues, and in brain tissue specifically, circadian influences on cell division have not been extensively examined. Since the first discovery of adult hippocampal neurogenesis in the 1960's, it has become commonly known that the subgranular zone (SGZ) of the dentate gyrus (DG) undergoes mitotic cell division throughout an organism's lifespan (Altman and Das, 1965). Throughout the hippocampus, extensive expression of clock genes has been reported, many of which exhibit circadian oscillation (Golini et al., 2012; Borgs et al., 2009; Fonzo et al., 2009; Wang et al., 2009; Abe et al., 2004). Time of day variation in hippocampal dependent behaviours persist when the SCN is ablated (Cain and Ralph, 2009) suggesting that there may be a role of circadian clock genes in the normal function of the hippocampus. It is possible that the circadian clock plays a role in hippocampal neurogenesis. Borgs and colleagues (2009) found that PERIOD2 protein, part of the negative limb of the circadian transcription-translation feedback loop, plays a regulatory role in neural stem/progenitor

cell (NSPC) division in the hippocampus. Per2 knockout animals showed an increase in the number of dividing NSPCs, and a higher rate of proliferation. However, later in life these increases were no longer apparent when knockouts were compared to wildtype animals, as the increase in proliferation was counteracted by an increase in cell death (Borgs et al., 2009). The results of these previous studies in both the liver and hippocampus suggest that the role of circadian clock genes in cell division may differ between peripheral and CNS tissues.

In the following study, I examine the role of the circadian clock on CNS cell division in the SGZ. Using BMAL1 knockout mice and littermate controls, I examined SGZ cell proliferation, survival, apoptotic cell death, and granule cell layer (GCL) volume to see if a functional circadian clock is necessary for normal cell division and survival of new hippocampal cells generated during adulthood. I hypothesize that cellular proliferation and survival will be impaired in the SGZ of BMAL1 knockout mice.

5.3 Materials and Methods

5.3.1 Animals

Thirty two mice were generated for this study (11 BMAL1+/+, 10 BMAL1 +/-, and 11 BMAL1-/-). Original heterozygous breeders were obtained from Jackson Laboratories, MA, and bred to produce heterozygous (+/+), wildtype (+/-), and knockout (-/-) animals. Mice were weaned at 21-22 days of age, and were housed with same sex littermates in a colony room maintained on a 12:12 light/dark cycle, with lights on at 7 AM and lights off at 7 PM. Animals were provided food and water ad libitum and all procedures were performed in accordance with the Canadian Council on Animal Care guidelines, and

were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary.

5.3.2 Genotyping

Animals were genotyped according to the protocol of Bunger et al., (2000). Specifically, genotyping was performed using multiplex PCR with one forward primer (CCACCAAGCCCAGCAACTCA) and two reverse primers, one specific to the portion of the BMAL1 gene that is deleted in the knockout animals (ATTCGGCCCCCTATCTTCTG) and the other specific to the Neomycin gene that replaces the deleted portion of the BMAL1 gene in the engineered gene (TCGCCTTCTATCGCCTTCTTGACG). The first reverse primer amplifies a 400 bp band corresponding to the wild-type allele, while the second reverse primer amplifies a 600 bp band. PCR was performed on genomic DNA from tail biopsies for 40 cycles of 95°C, 15 s: 60°C, 15 s: 72°C, 1 min in 1× PCR buffer (Sigma-Aldrich, ON) containing 3.5 mM $MgCl_2$.

5.4 Experiment 1 – Assessment of Cell Proliferation

5.4.1 BrdU

Animals in experiment 1 were injected intraperitoneally (i.p.) with 50mg/kg bromodeoxyuridine (BrdU) (Sigma-Aldrich, ON) on P60 at midday, approximately CT6. There is contradicting evidence for variation in hippocampal neurogenesis across the day (Kochman et al., 2006; Van der Borght et al., 2006; Holmes et al., 2004; Ambrogini et al., 2002) with a majority of studies suggesting no rhythm. BMAL1 knockout animals lack rhythmicity, and are likely to also lack rhythms in neurogenesis. Therefore, we chose to use one injection of BrdU, to test a direct difference in daytime proliferation,

and so as to not complicate the analysis with potential day/night variation in proliferation. Killing animals 24 hours after BrdU injection allowed us to examine the number of new cells born near midday. Animals were killed using an overdose of barbiturate, and were perfused transcardially with phosphate buffered saline followed by 4% paraformaldehyde. After twenty four hours in paraformaldehyde, the brains were moved to a 20% sucrose solution until sectioned At slicing, four series of tissue were collected throughout the extent of the hippocampus at 35 micrometer thickness and immunohistochemistry was performed on one series of the tissue.

5.4.2 Immunocytochemistry

Tissue staining protocols were altered from Antle and colleagues (2005). Briefly, following slicing tissue was first rinsed in Tris-buffered saline (TBS). Brain sections were then transferred into a 0.5% H_2O_2 in 0.1%TBS solution for 10 minutes, followed by transfer into 50% formamide/2X saline-sodium citrate (SSC) solution where they were incubated for two hours at 65 degrees Celsius. The sections were then rinsed for 15 minutes at room temperature in 2XSSC without formamide, followed by a 30 minute incubation in 2N HCl, at 37 degrees. The sections were then rinsed in 0.1M Borate buffer (ph 8.5) for 10 minutes. Six rinses in TBS preceded a 60 minute incubation in TBS-Plus (3% Normal rabbit Serum [NRS] solution of TBS and Triton X-100). Following incubation in 3% normal serum, sections were incubated for 48 hours in sheep anti-BrdU (1:1000, in TBS-Plus, AB1893, Abcam, ON) at 4ºC.

After the 48 hour incubation in primary antibody, the sections were washed 9X in TBS, followed by a 1 hour incubation in secondary antibody (Biotinylated rabbit anti-sheep, 1:200, Vector Labs, ON). Three TBS rinses were then performed before allowing

the tissue to incubate in Avidin-biotin Complex (Vector Labs, CA) for 1 hour. Three additional TBS rinses followed, and the sections were then stained in 25ml TBS containing 0.05% diaminobenzidine (DAB), 0.02% NiCl, and 80 µl of 30% H_2O_2 for 3 minutes, followed immediately by nine TBS rinses. The sections were mounted on gel coated slides, dehydrated through an alcohol series (75%, 90%, 100%, 95%, 75% EtOH, 3 minutes in each), cleared in xylenes for 3 minutes, and coverslipped using Permount. Digital images of hippocampal sections were captured using Image-Pro Plus 5.1 software (Media Cybernetics, MD), and the number of nuclei stained for BrdU was counted on thirteen sections, identified by labelling of at least 30-50% of the nucleus when visualized using Image-Pro Plus 5.1 software (Media Cybernetics, MD). As this represented only every 4^{th} section, this final count was multiplied by 4 to provide an estimate of the total number of BrdU-labeled cells,

5.5 Experiment 2 - Assessment of Surviving Cells

5.5.1 BrdU

In experiment 2, mice were administered a 100mg/kg i.p. injection of BrdU at midday and killed 6 weeks post injection and the brains prepared for immunohistochemistry using the same methods as described for experiment 1.

5.5.2 Immunocytochemistry

In experiment 2, we used fluorescent labelling in hopes of identifying the phenotype (neuron or glial cell) of the surviving cells (stereology to be completed, data not shown). The tissue was treated much the same in experiment 2 as in experiment 1, and again, modified from Antle and colleagues (2005). However, On the first day of staining there was no rinse in 0.5% H_2O_2, a 10% Normal Donkey Serum was used instead

of 3% NRS, and tissue was incubated for 60-72 hours in a TBS-Plus solution containing the following antibodies: sheep anti-BrdU (1:100, Abcam), mouse anti-NeuN (1:2000, Millipore), and rabbit anti-GFAP (1:1000, Dako). After 60-72 hours incubation in primary antibody at 4 degrees, tissue was rinsed twice in TBS and once in TBS-Plus for 15 minutes each. It was then transferred into secondary antibodies (Donkey anti-sheep Alexafluor 488, donkey anti-mouse CY5, and donkey anti-rabbit CY3; Jackson ImmunoResearch, PA) for 4 hours at room temperature. Six TBS rinses, 10 minutes each, were then undertaken to decrease background staining and tissue was mounted from TBS onto gel coated slides, left to air dry briefly, coverslipped with Krystalon and stored until dry. Digital images of Alexafluor 488 channel were captured from these sections (Image-Pro Plus 5.1; Media Cybernetics, MD), and the number of cells labelled with BrdU were counted as in experiment 1, and multiplied by 4 to obtain an estimate of total numbers expected in the entire dentate gyrus.

5.5.3 Pyknotic Cells

One series of sections from experiment 2 was stained with cresyl violet to examine differences in numbers of pyknotic/apoptoic cells in the dentate gyrus GCL. A pyknotic cell was defined as one in which the nucleus exhibited the following features: strong and homogeneous staining, condensed chromatin and nuclear fragmentation (Ben Abdallah et al., 2010). This series of tissue was utilized for Cavalieri hippocampal volume analysis (Gundersen and Jensen, 1987)

5.5.4 Statistical Analysis

All cell counting was performed blind to genotype. All data were analyzed using one-way analysis of variance (SigmaStat, v 2.03; Systat Software, Inc, IL) with Tukey test follow up analyses where significant main effects were found.

5.6 Results

5.6.1 Experiment 1 – Proliferation

Analysis of variance did not reveal significant differences between genotypes in the overall number of BrdU-positive cells 24 hours after BrdU injection ($F_{(2,13)}$=1.289, p=0.314, Figure 5.1). A single BMAL1 knockout animal had exceptionally high number of BrdU labelled cells, leading to a high amount of variance, and a slight increase in the mean number of newly generated cells in the knockout group. All other knockout animals had virtually the same number of BrdU labeled cells as did the wildtype and heterozygous controls. These data suggest that cellular proliferation in the SGZ is not affected by a BMAL1 deletion.

5.6.2 Experiment 2 – Cell Survival, Cell Death, and GCL Volume

BMAL1 knockout mice had significantly more BrdU labelled cells 6 weeks after BrdU injection ($F_{(2,17)}$=7.628, p=0.005, figure 5.2). Follow up Tukey tests showed that this difference was significant when compared to heterozygous (p=0.016) or wildtype controls (p=0.008). Differences between the number of surviving new cells in wildtype animals and heterozygotes were not significant (p = 0.922).

BMAL1 knockout animals had significantly fewer pyknotic cells in the SGZ than littermate controls ($F_{(2,17)}$=24.115, p<0.001, figure 5.3). Follow up Tukey tests showed that BMAL1 knockout animals had significantly fewer pyknotic cells than either the

heterozygous ($p<0.001$) or the wildtype controls ($p<0.001$). There was no difference between wildtype and heterozygous groups ($p=0.349$). These data suggest that apoptotic cell death in the dentate gyrus is diminished in BMAL1 knockout animals.

Cavalieri volume estimates did not show significant differences in hippocampal volume across genotypes ($F_{(2,17)}=0.534$, $p=0.597$, figure 5.4).

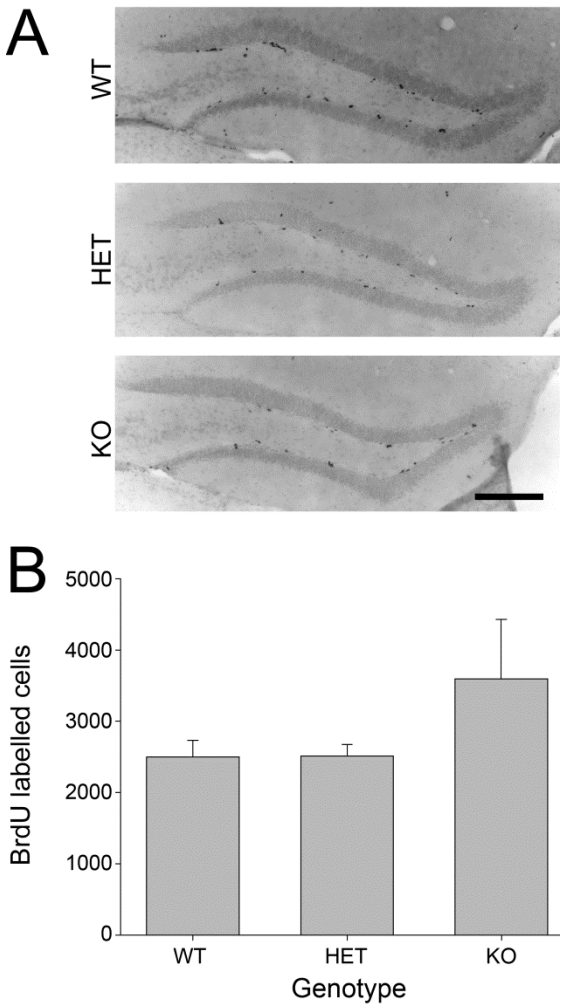

Figure 5.1. Photomicrographs of DAB labelled BrdU positive cells in BMAL1 (+/+), (+/-) and (-/-) SGZ of the DG, 24 hours after BrdU i.p. injection (A). Graph representing an estimate of the number of cells labelled with BrdU throughout the extent of the hippocampus (B), calculated by counting cells in every fourth slice and multiplying by 4. Differences between genotypes were not significant ($p > .05$).

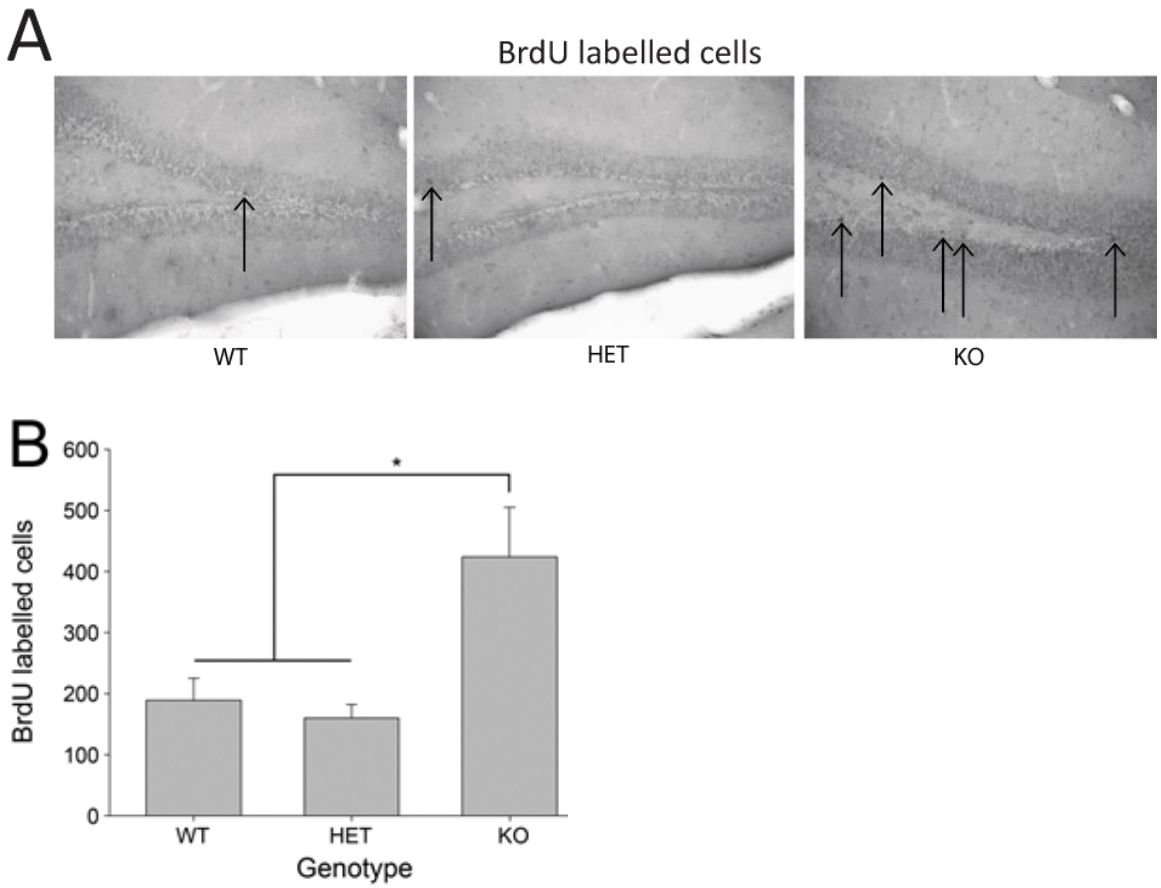

Figure 5.2. Representative photomicrographs of BrdU labelled cells in the SGZ of BMAL1 (+/+), (+/-), and (-/-) mice 6 weeks after BrdU i.p. injection (A). Graph representing estimates of the total number of BrdU labelled cells throughout the hippocampus (B), calculated by counting cells in every fourth slice and multiplying by 4. *$p < .05$.

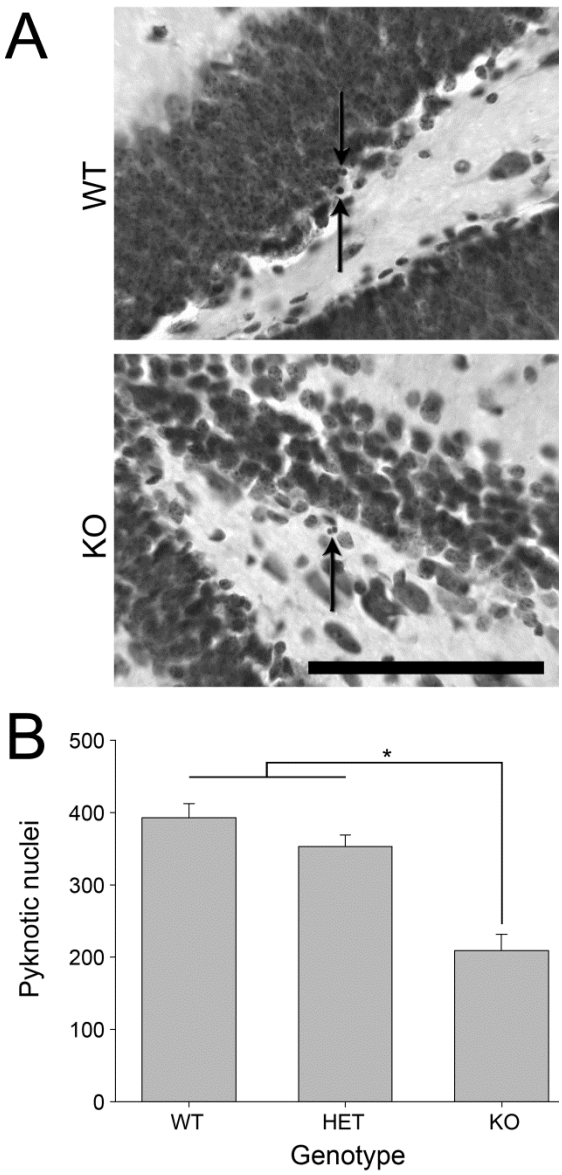

Figure 5.3. Photomicrograph of cresyl violet stained pyknotic cell, shown by the white arrow (A). Estimates of the total number of GCL pyknotic cells throughout the extent of the hippocampi of BMAL1 (+/+), (+/-), and (-/-) mice (B), calculated by counting pyknotic cells in every fourth slice and mulitplying by 4. *p < .05.

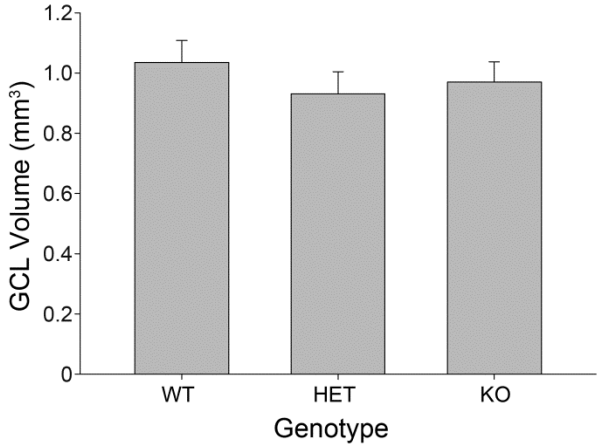

Figure 5.4 Cavalieri estimates of total hippocampal volume in BMAL1 (+/+), (+/-), and (-/-) mice at approximately 100 days of age. Estimates were calculated from the cresyl violet stained tissue series utilized in Figure 3 for pyknotic cell counts. Hippocampal volume estimates were not significantly different across genotypes.

5.7 Discussion

Circadian clock genes are expressed throughout the hippocampal formation, many of which exhibit rhythmic expression there (Golini et al., 2012; Borgs et al., 2009; Fonzo et al., 2009; Wang et al., 2009; Abe et al., 2002). It has been suggested that there is some circadian regulation of SGZ neurogenesis, though it is not well understood (Borgs et al., 2009). We show here that while a functioning circadian clock may not be necessary for normal mitotic proliferation in the hippocampal dentate gyrus SGZ, a functioning circadian clock is necessary for normal levels of apoptotic pruning of these newly generated cells. BMAL1 knockout mice had significantly more BrdU-labelled cells 6 weeks after the BrdU injection, and a significant reduction in the number of pyknotic cells in the dentate gyrus, indicative of reduced apoptosis. These data are accompanied by a stable hippocampal volume across genotypes, suggesting that the increase in cell survival and cell death is not affecting the volume of the structure. Overall, our study suggests that BMAL1, or the lack of rhythmicity that occurs in BMAL1 knock-out animals (Bunger et al., 2000), may diminish apoptosis, thereby allowing for more new cells to survive and potentially be incorporated into the hippocampal neural network than would occur normally.

There are three possible mechanisms by which apoptosis could be impaired in BMAL1 knockout mice. First, it is possible that a functioning circadian clock is necessary within individual cells in order to initiate programmed cell death. Deletion of the BMAL1 gene is the only single-gene manipulation which abolishes circadian rhythmicity in behaviour and gene expression derived from the transcription translation feedback loop. Alternatively, a functional circadian clock may be necessary at the

organism level. BMAL1 knockout mice are completely arrhythmic, on both the behavioural and cellular level, and it may be a rhythm exogenous to the individual cell that is important for supporting normal programmed cell death. Finally, it is possible that it is the loss of the BMAL1 gene itself, and not the concomitant loss of rhythmicity at the cellular and organism level, that is responsible for altered apoptosis. BMAL1 participates in e-box-mediated transcription of other genes, only some of which are core components of the circadian clock. Loss of BMAL1 may prevent normal expression of non-circadian genes activated by BMAL1 heterodimers that may be critical for normal programmed cell death.

A number of studies have suggested that the circadian clock may play a role in neurogenesis. The period of the cell cycle in neural stem/precursor cells (NSPC) cells is approximately a day (Cameron and McKay, 2001). Exercise enhances neurogenesis and is gated by the circadian clock (Holmes et al., 2004). Disruption of normal sleep wake patterns disrupts neurogenesis (Mueller et al, 2008), although this is independent of disruption of the circadian clock (Mueller et al. 2011). A daily rhythm in proliferation has been noted (Kochman et al., 2006) although this phenomenon is restricted to more proliferation during the day in the hilus that gives rise to glial cells. The hippocampus and the dentate gyrus express a number of clock genes, including *mPer2* (Borgs et al., 2009). This expression starts when cells are immature proliferating cells, and persists into adulthood. Interestingly, Borgs and colleagues (2009) found no circadian variation in the levels of PER2 protein, suggesting that the circadian clock may not be operating in these cells (although others have found rhythms in PER2 protein and mRNA; Wang et al., 2009). Disrupting mPer2 affects neurogenesis in a number of ways (Borgs et al.,

2009). Proliferation is enhanced in mice in which the PAS domain of the mPer2 gene has been deleted (*Per2*^{brdm1}, Borgs et al., 2009). Mice with this mutation are capable of sustained circadian activity in constant conditions; albeit with a shortened freerunning period before becoming arrhythmic after a number of weeks (Zheng et al, 1999). After 3 weeks, the number of surviving neurons in *Per2*^{brdm1} knockout mice is not different from wild type controls, owing to enhanced apoptosis (Borgs et al., 2009).

The differences between the present study and those with the *Per2*^{brdm1} mice may be due to a number of reasons. First, *Per2*^{brdm1} are rhythmic in that they show cyclic variation in activity. BMAL1 mice are arrhythmic, with no cyclic variation in activity or gene expression, yet overall activity levels are similar in BMAL1 knockouts and controls (data not shown). Second, PER2 is constitutively present in the DG, suggesting that it does not contribute to circadian oscillations there. Finally, PER2 is on the negative limb of the e-box mediated transcription-translation feedback loop, while BMAL1 constitutes the positive limb of these loops. If neurogenesis or apoptosis factors were expressed in part due to activation/inactivation of the e-box, one might expect the opposite results when disrupting the positive versus the negative limbs of the loop.

One study has examined the role of BMAL in neurogenesis. BMAL1 mRNA is rhythmically expressed in differentiating NSPCs (Kimiwada et al., 2009). When BMAL1 expression is disrupted in vitro using siRNA, markers for neuronal differentiation were also decreased. While this finding appears different than the present results where BMAL1 knockouts had normal proliferation, a number of differences in approach might account for differences in findings. First we examined neurogenesis in the SGZ of the dentate gyrus, whereas Kimiwada and colleagues (2009) looked at the subventricular

zone. Furthermore, we assessed proliferation and survival in vivo, while they examined the behavior of NSPCs in vitro. We were also examining different phenomena; proliferation and survival in the present study as opposed to differentiation in vitro (Kimiwada et al., 2009). Furthermore, Kimiwada and colleagues (2009) did not find effects of BMAL1 or Clock knockdown on proliferation, findings which are consistent with our results.

In peripheral tissues, such as the liver, the circadian clock seems to be necessary to maintain a normal rate of cellular proliferation (Matsuo et al., 2003). In mice lacking both *Cry1* and *Cry2*, thereby rendering them arrhythmic, regeneration of the liver following partial hepatectomy was impaired (Matsuo et al, 2003). However, cell survival appears to be normal, and regeneration of hepatocytes return to near normal levels within days after hepatectomy, demonstrating that regeneration was not prevented in arrhythmic animals, just delayed.

Differences in tissue (liver vs. brain) and genetic manipulations (double Cry knockout vs. BMAL1-knockout) may explain the differences between the present study and that of Matsuo and colleagues (2003). Like PER2 discussed above, the cryptochrome proteins are on the negative side of the transcription translation feedback loop, so opposite results might be expected if it is not loss of the circadian clock per se, but rather altered transcriptional regulation at e-box elements. Studies have suggested an indirect repressing role of BMAL1 on *p21*, *p53*, and *Wee1*, each of which inhibit or promote various aspects of the cell cycle, as possible mechanisms in proliferative stages in the liver (Khapre et al., 2010 for review; Grechez-Cassiau et al., 2008; Matsuo et al., 2003). The functional consequences of increased cell survival and the associated decrease in cell

death in the DG as seen in our study have yet to be determined. Since BMAL1 knock-out animals have increased mortality in adulthood at about 8 months, as well as numerous health problems (Bunger et al., 2000; 2005), the lack of modulation of SGZ cell survival may in fact be detrimental. These animals are known to experience an accelerated rate of aging after about 16 weeks of age, therefore the effects that we have seen in the hippocampus may be due to senescence, and may be indicative of an aged hippocampus. That is, our results may not necessarily be related to their circadian rhythmicity, but to some other function of BMAL1 (Bunger et al., 2005). Neurogenesis in the hippocampus has been related to learning and memory (Deng et al, 2010). With enhanced cell survival, there may be alterations in various hippocampal dependant tasks between BMAL1-knockouts and wildtype animals. However, the circadian clock also participates in learning hippocampal dependent tasks (Valentinuzzi et al., 2004), thus any enhancement due to increased cell survival in the DG in BMAL1-knockouts may be mitigated by diminished performance due to being arrhythmic.

The fact that there has been little investigation into the circadian role in adult neurogenesis points to the broad direction in which future studies may follow. Our study did not examine various times of day, with a single injection of BrdU being given in the light phase, the time at which cellular proliferation may be at its peak, at least in hippocampal areas outside of the DG, ie) the hilus (Kochman, et al., 2006). A full characterization of SGZ cell division, in all its stages and across the entire day/night cycle, in BMAL1 knockouts and controls may shed light on finer details of the circadian clock's role in neurogenesis in this brain region. Further investigation into subventricular zone cell division may also reveal differences that will expand our knowledge of exactly

how clock genes are involved in neurogenesis. Beyond characterization of what occurs in these brain regions throughout the 24 hr cycle, it will become important to determine the functionality of these new cells. Behavioural analysis of these animals is necessary to identify if there are any benefits to the increase in surviving cell in the SGZ. Studies investigating the connectivity of these new cells may also help to determine their functionality.

Chapter 6

6. Discussion

6.1 Summary of Experiments

The studies described here increase our understanding of the link between circadian rhythms and phenomena related to brain injury, recovery, regeneration and neurogenesis. In the first experiment, using a rat model of ischemic forelimb motor cortex stroke, I found that the time of day does not affect lesion size, behavioural deficits, or recovery. By applying endothelin-1, a potent vasoconstrictor, directly to the forelimb area of the cortex, I was able to show that animals with stroke in the waking hours had the same outcomes as rats with a stroke at the end of the active phase for months following the initial infarct. These results suggest that the time of day that a stroke occurs does not affect outcome, and in opposition to our hypotheses, when everything else is equal, morning stroke victims may not be left with greater deficits than those who experience stroke at other times of the day.

The second series of experiments focused on brain regeneration, models that have the potential to alleviate physical damage to the brain such as that which occurs in a stroke. I chose to investigate the MPFC region because it is known to regenerate following aspiration, it may be under circadian control as it receives indirect input from the SCN, many behaviours dependent on MPFC show circadian variation, and it may also exhibit rhythmic circadian clock gene expression. In the first of these experiments, I aimed to quantify circadian clock gene expression in the MPFC. Clock genes are present in this brain region, and therefore may be involved in temporal aspects of MPFC dependant behaviours. Furthermore, using a neonatal model of brain regeneration, where

the MPFC of mice is aspirated on P7 and the lesion cavity is naturally filled by adulthood, clock gene expression in the MPFC was compared to healthy controls and sham lesioned animals. Contrary to predictions, no rhythm in *mPer1* and *mPer2* were found, nor were rhythms apparent in the expression of PER1 or cFos. However, clock gene expression was not significantly different between MPFC aspirated animals, which suggests that the role of the master circadian clock of the SCN may be minimal in MPFC tissue.

This study was followed up with one which looked at regrowth of MPFC tissue in animals lacking a fully functioning circadian clock. It was hypothesized that, if the circadian clock as a whole is in control of the cell division cycle (as has been proposed elsewhere, Matsuo et al., 2003) regeneration in the MPFC area following aspiration would be impaired, as it is in the liver. However, our results showed no differences between brain weights and lesion volumes in aspirated animals and controls in the week following the lesion. These findings suggest that, in the neonatal MPFC, a dysfunctional circadian clock does not affect brain tissue regeneration.

Lastly, utilizing a model of adult hippocampal SGZ neurogenesis, I examined the effects of the circadian clock on the cell division cycle in CNS tissues. I was able to show that a dysfunctional circadian clock induces a deficit in apoptotic pruning of newly generated cells in the GCL of the hippocampus. Again, hypotheses assumed that the cell cycle would be disturbed in the knockout model, and therefore a reduction in proliferation and survival would occur. Yet we found no difference in cellular proliferation in BMAL1 knockout animals compared to controls, but a significant increase in the number of BrdU labelled surviving cells 6 weeks after BrdU

administration, and a significant decrease in apoptotic cell death. These results suggest a circadian role in SGZ cell cycle that differs from that which occurs in peripheral tissues.

Taken together, these data suggest that time of day that a stroke occurs may not be directly involved in stroke outcome after the insult. Circadian clock genes may also not be directly involved in brain regeneration during development. These genes do, however, show strong influences on adult neurogenesis, and therefore potential models of brain repair in the adult animal.

6.2 Summary of Conclusions

6.2.1 Stroke

With regard to Chapter 2, first, it is well known that the time of day that a stroke occurs shows a significant circadian rhythm (Elliot, 1998). This observation is linked to the numerous factors involved in stroke onset that are also rhythmically expressed. As mentioned in the introduction, many factors associated with blood and clotting abilities of blood vary depending on the time of day, as do measures such as heart rate, body temperature, hormonal levels, and activity (Mann et al., 1980; Kubota et al., 1987; Tofler et al., 1987). Results presented here, which suggest a lack of circadian influence on stroke outcome, do not contradict these previous findings. The fact that stroke manifestation and onset are influenced by circadian rhythmicity in various aspects of physiology does not necessitate that the overall outcome following the stroke event be dependent on the time of day that it occurs. Clock genes themselves may not be directly related to stroke outcome, yet other aspects of physiology that they control can affect timing of stroke onset. It may be a relief to think that, although the risk of stroke is higher at certain times of day, once the stroke has occurred circadian influences may be

minimal. That is not to say that treatment efficacy may not vary in a circadian manner, but the timing of a stroke may not lead to greater or lesser opportunity for recovery. This finding, though small in the grand scheme of stroke research and circadian influence on stroke, is novel and can have a significant impact on patient prognosis.

Other studies on rodent models of stroke which have examined time of day effects have had varying results. In comparison, our results did not contradict those of Martinez-Vargas and colleagues (2006). Although they found a circadian rhythm in survival following a moderate closed head injury, they too found no difference in the magnitude of behavioural deficits, neurological deficits, nor size of the lesion based on the time of day in their TBI model.

Compared to studies conducted by Vinall and colleagues (2000), and Tischkau et al. (2007), differences in results arise. Both of these studies found greater lesion size or greater activation of cell death pathways, respectively, when stroke occurred in the night. However, as was explained in Chapter 2, there are numerous explanations for the differences. The first relates to the method of stroke induction. We avoided the issue of anesthetic effects by inducing stroke in conscious animals, and therefore used very different stroke induction techniques than these previous studies, which used an intraluminal suture method and a transient global ischemia model, respectively. Secondly, the time points we chose to examine, and the endothelin-1 method of stroke induction specifically, may not have allowed for precise timing of the stroke, as the duration of vasoconstriction in our study may have exceeded the temporal spacing of treatment groups, or at least extended it past "morning" or "evening" hours. Overall, differences in our findings compared to others suggest that the time of day that a stroke

occurs in conscious animals, with all else being equal, may not greatly influence overall outcome and is unlikely to affect recovery.

6.2.2 Neonatal MPFC Regeneration

When looking specifically at neonatal models of brain regeneration, the studies described here suggest that the circadian clock and clock genes have little, if any, effect on outcome. That is, not only do clock genes appear to have little involvement on regeneration, but in neonatal regenerative MPFC clock gene expression is largely unaffected by the damage.

We initially proposed that newly generated MPFC tissue would not have sufficient input from the SCN to maintain normal rhythmicity, however, we were unable to replicate the finding of significant rhythms (Abe et al., 2004; 2001). What we did find was similar clock gene expression profiles in regenerated tissue to that seen in our healthy controls and sham lesions. This suggests that input from the SCN may have, in fact, been similar in healthy and regenerated tissue. If the pattern of clock gene expression in the MPFC is reliant on input from the SCN, whether that pattern is significantly rhythmic or not, the pathways from SCN to the new MPFC tissue were either re-established after the damage, or other possibly diffusible signals from the SCN or elsewhere were sufficient to keep clock genes in this area at similar expression levels as healthy controls (Silver et al., 1996). Overall, according to our results and in comparison to previous work, it is difficult to say with certainty whether the SCN input is necessary at all in neonatal MPFC regeneration.

There are numerous factors that may influence interpretation of our brain regeneration studies compared to others. First, in our examination of circadian clock

genes in the MPFC, we were unable to replicate previous findings of significant rhythms in this region (Abe et al., 2004; 2001). This is a cause for concern, in that we cannot directly relate our findings to those of the previous studies. One plausible reason for the discrepancy in our results and that of Abe and colleagues (2004; 2001) lies in the exact region being investigated. In their studies, Abe and colleagues (2004; 2001) investigated clock gene mRNA in the cingulate cortex. Though we limited our analyses to the cingulate area of the MPFC as well, the exact demarcations of what Abe's group consider cingulate were not described in their studies. Therefore, there was opportunity for differences in the exact areas being examined. We focused our ROD analysis on layers 2 and 3 of the midline of the MPFC cingulate area, from the most rostral portion of tissue that could be captured on slide extending caudally to bregma. It is unknown if Abe's group examined the entirety of the cingulate area, if they examined only midline mRNA expression, or if they examined only those regions shown pictorially in their publications. Because we examined only midline, and the full rostrocaudal extent of the MPFC, we may have included enough tissue in our analyses to wash out any significant differences in smaller more specific regions of the MPFC. However, we did address this (data not shown) by limiting analysis to rostral, or caudal portions of MPFC only, and still found no change in our results. So unless Abe and colleagues (2004; 2001) looked specifically at a mid-portion of cingulate, we have considered the other ways in which our demarcation of cingulate may have differed. The last possibility with regard to structure demarcation is that Abe's group may have examined the entire cingulate region, whereas we found gene expression only in layer 2 and 3 of medial cingulate area. Had they examined the full cingulate region, not just midline portions, that may have allowed for

their finding of rhythmicity versus non-rhythmicity found in our study. This may be addressed in future studies, with examination of smaller regions within the MPFC, or with more specificity in descriptions of regions examined.

However, the fact remains that our comparison of healthy animals and P7 aspirated animals were not significantly different from one another. Although we did not find different levels of expression (as calculated by ROD) of circadian clock gene mRNA or protein between our groups, a second explanation for the fact that we find rhythmicity in MPFC as reported by Abe and colleagues (2004; 2001) may be due to the number of time points that we chose to examine. As the number of variables increase in statistical analyses, the power of the test is reduced. Since we chose significantly more time points than previous studies, the statistical significance of our results may have been washed out due to having 12 levels of the independent variable. We did re-analyze the data using every second time point to address this issue (Figure 3.1 and 3.2), since many studies use 6 or less time points in their analysis (Abe et al., 2001; 2004), however results stayed the same.

Another reason that our results differed from those of Abe and colleagues (2004; 2001) may relate to the examination of mRNA vs. protein. Here, we examined both, yet Abe and colleagues examined only mRNA. It is well known that the presence of mRNA does not necessitate similar expression of constituent proteins. It is true that in most cases, the two are tightly related, yet there remains the possibility that all available mRNA will not be translated into protein. Therefore, it may not be surprising that a lack of rhythmicity in protein expression was found in our study. Regardless of this fact,

however, mRNA expression profiles still did not match up between studies, and reasons for this are likely related to the explanations mentioned above.

When examining the rate MPFC regeneration in the neonatal aspiration model, we found no significant effect of a BMAL1 deletion. This was also in opposition to our hypotheses, as it is well documented that in other tissue (i.e., the liver) the circadian clock has significant control over the cell cycle, and that circadian disruption leads to a delay in liver regeneration following damage (Matsuo et al., 2003; Grechez-Cassiau et al., 2007). Therefore, the process of cell division and tissue regeneration may be quite different in the periphery than in the brain. In the liver, circadian arrhythmicity has significant delaying effects on cellular proliferation, yet in neonatal regenerative MPFC there is no delay 3 days to 1 week following aspiration. The strongest conclusion that can be drawn from this and previous studies is that the process of cell division is differentially affected by the circadian system in these two areas. All in all, it is not surprising given the differences in tissue and cellular components between the liver and neonatal MPFC, that results are so variable between the two.

Due to our finding that the circadian clock does not influence neonatal MPFC regeneration, yet it does appear to do so in adult neurogenesis (described later in this chapter), it is important to examine circadian differences between the two ages. In healthy adults, it is safe to say that the circadian system is working properly, all aspects are fully developed, and in a consistent LD environment our healthy animals are exhibiting normal circadian function. Numerous studies have evaluated the ontogeny of the circadian system (for review, see Weinert, 2005). General conclusions about the maturity of circadian clock function suggest that all the components of the system are in

place by P10 in rats, and possibly earlier in mice. The molecular clockwork also becomes dependent on light input at or around P10 in rats as well (Sumova et al., 2006). Yet in peripheral tissues, clock gene expression may not be fully developed until P14-20 (Sakamoto et al., 2002) and prior to P10, circadian rhythmicity in the neonate is still largely dependent on maternal rhythms (see for review Weinert, 2005). Though all the details have not been as extensively investigated in mice, it is possible that components of the SCN clock are, at the very least, in place by P7. Shimomura and colleagues (2001) showed that the daily rhythm of *mPer1* and *mPer2* expression in the cerebral cortex is not apparent until P14 to P50 in rats. Therefore, our area of interest, the MPFC, is unlikely to be exhibiting circadian rhythmicity by the time we aspirate the area on P7. This may mean that there is enough time between aspiration and regeneration to allow for the new cells to be spared from abhorrent clock gene expression in the MPFC, and to either make normal connections with the SCN, or to be sufficiently influenced by other forms of SCN communication (ie, possibly diffusible signals). This may be one reason for the lack of circadian effects on neural regeneration in our P7 aspiration model. Influence of the circadian clock is likely to have a stronger effect in adulthood, when all clocks throughout the brain and body are in place and functioning properly.

6.2.3 Neurogenesis

Recently, numerous studies have stated that the circadian clock controls the cell cycle. This conclusion comes from the study of liver regeneration conducted by Matsuo and colleagues (2003). Not only did we find that circadian control of the cell cycle does not apply in neonatal MPFC regeneration, but the way in which the circadian clock "controls" cell division appears to be variable. In the liver, as mentioned throughout this

thesis, the clock seems to control and limit proliferation. Whereas our study has shown that in the brain, the clock seems to play a significant role in cell death and pruning. Also, when comparing the two studies, it becomes apparent that it may not be the overarching influence of the clock that is having an effect on proliferation, survival, or death of these newly generated cells. Because Matsuo and colleagues (2003) utilized *Cry* deficient mice and we utilized BMAL1 deficient mice, it may be that these components of the clock are playing slightly different roles in cell division the healthy animal. It could also be that disruption of the positive end of the circadian transcription-translation feedback loop affects cell division differently than disruption of the negative end, since BMAL1 and *Cry* are on opposite ends of this loop. Therefore, the broad statement that "the circadian clock controls the cell division cycle" may be better altered to say that "components of the circadian clock differentially affect the developmental time course and fate of new cells".

6.2.4 Overall Conclusions

In our investigation of circadian influences on brain damage, regeneration, and neurogenesis, we have found various roles for the circadian clock. First, though many aspects of circadian physiology are likely to be involved in stroke onset, we did not find significant circadian effects on outcome. That is, the time of day that a stroke occurs does not influence the behavioural, functional, and recovery outcome of the animal, at least in focal forelimb area stroke induced by endothelin-1. Secondly, we found no significant rhythms in circadian clock genes in the MPFC of healthy animals, nor in regenerated MPFC tissue. We also found that the rate of MPFC regeneration is not affected by a dysfunctional circadian clock and circadian arrhythmicity. These studies

suggest that the circadian system is not in control of tissue regeneration such as that which occurs in the MPFC neonatal aspiration model. Lastly, we were able to show that circadian arrhythmicity, induced by a BMAL1 deletion, strongly reduces apoptotic cell death and pruning in the adult hippocampal SGZ, resulting in a greater number of new cells surviving there.

Overall, these data show that the circadian role in the cell cycle is dependent on the specific area being investigated, and that the circadian role in cell damage and cell division differs, depending on the location and means of cellular proliferation, survival, and death.

6.3 Limitations and Future Directions

6.3.1 Stroke Study

In our stroke study, which examined anatomical and behavioural outcomes following a stroke event, there are numerous limitations that future research may address. First, our model of stroke, though it has many benefits, still differs from that which occurs in humans. Our rationale for choosing the endothelin-1 application method of stroke induction were numerous, in that we could control the exact time of stroke onset, the animals were in a conscious state during the stroke event, we were able to ensure that damage was occurring since these animals showed circling behaviours in the direction opposite the infarct as the endothlin-1 was being applied, and our animals were not under anesthetic conditions during the stroke. However, although our stroke induction methods had numerous advantages, some disadvantages remain. First, endothelin-1 itself is not the same as human stroke, in that a clot is not produced. The arteries and vessels constrict, reducing blood flow similar to that which occurs in stroke. Yet the presence of

a blood clot, as which occurs in human ischemic stroke, is not mimicked. Therefore, though results are similar to that which occurs when a clot is present, there remains the argument that our methodology is different from that which occurs in human stroke. Another limitation to the stroke induction method chosen here involves the areas surrounding the initial infarct. The extent of the penumbral area following an endolthelin-1 induced stroke is not known. As the penumbral area is often the main source of available "tissue recovery", if endothelin-1 creates no penumbral area, that may explain why our animals did not exhibit differences in recovery. If it is in fact the penumbral area that not only allows for recovery, but that also differs in size based on the time of day that a stroke occurs, our methods would be unable to detect these differences. Overall, as stated earlier, our methodology had numerous advantages, yet there remain some factors of stroke that we were unable to examine and control for, and therefore our study leaves room for further investigation of circadian effects on stroke outcome.

Future studies examining circadian effects on stroke outcome may more closely examine the penumbral area surrounding an infarct. They may also aim to look at larger, or smaller, infarct sizes. Due to the relatively small infarct that our stroke method induced, significant differences in outcome may have been undetectable. In conditions, such as MCA occlusion, that result in larger infarcts and potential differences in penumbral area, examination of lesion size, behavioural deficits, and recovery may be warranted. Time of day differences in these factors following MCA occlusion also warrants future investigation due to the fact that MCA occlusion is far more common than a forelimb area motor cortex stroke in humans. Using electrocoagulation or

injection of coagulated blood, a clot can be produced in the MCA, more closely mimicking the human stroke condition.

6.3.2 Clock Gene Expression in MPFC Study

Future studies can address the issues proposed in our study of MPFC rhythmicity in numerous ways. By changing the method of quantification of gene expression, more general, or more specific delineations of MPFC regions are possible. For example, instead of utilizing ROD methods, cell counting may result in greater detail in results. Our staining protocols for ICC could have allowed for cell counting, however determining the exact boundaries of a cell in our *in situ* hybridization tissue would have been more difficult. So as to keep methodology the same throughout our studies, we chose ROD quantification. However, in the future, cell counting methods would add to and clarify the results of our study.

As was mentioned earlier, our initial hypothesis in this study was that the multi-synaptic lines of communication between the SCN and MPFC would be severed in the P7 aspiration, and that the new tissue would not make these same connections after regrowth, or that any connections made would not be sufficient to maintain normal rhythmicity. Though we did not find rhythmicity in the MPFC, clock gene expression profiles in our aspirated animals did not differ significantly from healthy controls and shams. One possibility for future research in this area is to use transneuronal tracing methods in the MPFC regenerated animals, such as those used by Sylvester and colleagues (2002). Using their methods in animals with regenerated MPFC tissue, we could be certain as to whether the new tissue is, in fact, receiving normal indirect inputs from the SCN.

We also did not alter the LD cycle in our study. Animals were housed in a 12:12 LD cycle throughout the extent of the study. By examining free running rhythms in gene expression in MPFC, we may have had the ability to note much greater differences between our P7 aspirated animals and healthy controls. The fact that our animals were all consistently subject to the same lighting conditions may also have masked underlying differences in the regenerated tissue's ability to maintain its own circadian rhythm. Further investigation of free running rhythmicity in MPFC clock gene expression would surely add greater value to findings.

Another possibility for future research is to examine clock gene expression *in vitro*. Using Per1-lucferase or Per2-luciferase knock in mice, or Per1-GFP animals, clock gene expression can be visualized in healthy and regenerated MPFC tissue. These in vitro methods make visible the expression patterns of PER1 and PER2, and are commonly used in examinations of peripheral oscillators (Yoo et al., 2004; Yamazaki et al., 2000). Their ability to track the reporter in real time may be more sensitive for detecting a rhythm in the MPFC. If a rhythm was detected, then this procedure could be repeated with regenerated MPFC tissue to determine if it too is cable of rhythmic gene expression.

6.3.3 MPFC Regeneration in BMAL1 Knockout Study

In Chapter 4, we aimed to examine the MPFC of aspirated animals in adulthood as well as in neonates, however, survival rates of our aspirated animals were low beyond the juvenile period. Out of 15 P7 aspirated animals, only one knockout survived to P90. Most of these animals were subject to infanticide by the mother prior to weaning, while others appeared to die of natural causes, likely associated with an increased rate of aging

(Bunger et al, 2000). This may actually be preliminary data to suggest that differences in rhythmic animals survive this procedure better than arrhythmic animals. Differences in the development of the circadian system between BMAL1 knockouts and healthy controls may have resulted in the increase in mortality (data not shown). Since BMAL1 deletion increases the rate of aging, we may have uncovered a role for normal MPFC development with regard to mortality. That is, since we had greater rates of mortality in our P7 aspirated BMAL1 knockout animals than we did in un-operated BMAL1 knockouts, normal MPFC development may be a requirement in keeping mortality low in BMAL1 knockouts. With regard to our findings in adult neurogenesis, the fact that mortality was higher in MPFC lesioned animals than in littermate controls may warrant further investigation. However at this point, it is not known whether this mortality rate was higher than that seen in the previously reported accelerated senescence that accompanies the BMAL1 deletion (Khapre et al., 2011; Bunger et al., 2000).

At present, our results do not pin point why a BMAL1 deletion affects cell survival and apoptosis in the SGZ. The effects that we have shown in our study may be due to the overall loss of rhythmicity in BMAL1 knockout animals, or to the loss of BMAL1 specifically in SGZ cells. Therefore, to further examine the details of role that the master circadian clock has on neurogenesis, future studies should examine adult neurogenesis in SCN lesioned animals. If, in fact, our results are due to the loss of overall rhythmicity apparent in BMAL1 knockout animals, then lesioning the SCN in healthy animals should have the same effect on apoptosis in the SGZ as seen in our study. If, however, our findings were not linked directly to master circadian pacemaker function, but instead were the result of the loss of BMAL1 in SGZ progenitor cells, then

future studies may utilize RNAi techniques to temporarily knock down BMAL1 in the DG. These studies combined can truly answer the question of whether overall rhythmicity, or BMAL1 specifically, is involved in SGZ apoptosis.

Another possibility is to investigate recovery of normal apoptosis in BMAL1 knockout animals by injecting a viral vector containing the BMAL1 gene directly into the DG. This would temporarily activate BMAL1 in knockout animals, in a region specific manner. A study such as this would allow us also to determine if it is cellular BMAL1 in the SGZ that is linked to the reduction in apoptosis seen in our study, or if our results are linked to the broader influence of overall circadian rhythmicity.

A BMAL1 deletion, results in a dysfunctional circadian clock, but also has numerous effects throughout the body (Khapre et al., 2011, Bunger et al., 2000; 2005). Therefore our use of BMAL1 knockout animals may have influenced our results due to arrhythmicity in areas outside of the SCN. That is, circadian influences from the periphery or other parts of the body may be regulating apoptosis in the SGZ. Should the studies proposed above be unable to determine a role for the SCN, or for BMAl1 specifically in the DG, then investigation into the influence of other oscillators on the SGZ is also warranted. Some potential influences may be feedback from peripheral tissue oscillations, such as the adrenal glands which are known to communicate with the hippocampus, or from direct or indirect inputs from other rhythmic areas of the brain (Kandel et al., 2000).

Examination of naturally occurring apoptosis in other areas of the brain body in BMAL1 knockouts may further elucidate the role of BMAL1 in apoptosis. Using the methods described in our study, as well as those proposed above, areas outside of the

SGZ which show naturally occurring apoptosis could also be investigated. This would further our understanding of the role that the circadian clock and BMAL1 itself play in the CNS as a whole.

Future studies need also determine with greater specificity, if it is indeed BMAL1 that is influencing apoptosis in our study, or if it was due to regulatory roles that BMAL1 may have downstream of its dimerization with CLOCK. Since BMAL1 can dimerize with numerous factors other than CLOCK, and in its heterodimerized state it is known to act on E-box elements of target genes (Matsuo et al., 2003), it could be that BMAL1 deletion is affecting some other downstream regulator of the cell cycle. For example, BMAL1 is known to regulate p21 in the liver, a known cell cycle inhibitor (Grechez-Cassiau et al., 2007, 2004). In absence of BMAL1, p21, and other cell cycle inhibitors such as wee1 and p57, may not be activated. This would result in an increase in the rate of neurogenesis, and may have caused our increase in surviving cells. It is also possible that BMAL1 may be affecting cell cycle checkpoints such as cyclin-dependant kinases (CDK) or CDK inhibitors . One such CDK inhibitor is p57, which has been shown to be involved in SGZ neurogenesis, (Furturachi et al., 2013), and others exist which may be involved in progression through the stages of the cell cycle, and whose activity may be altered by a BMAL1 deletion.

Overall, future directions for this line of research can take many avenues, due to the infancy of this type of neurogenesis and regeneration research.

6.4 Implications

Brain repair following injury remains a major goal of neuroscience research. Following brain damage, repair of the tissue itself is nearly impossible, but we are

discovering new ways to induce some tissue regeneration in hopes of alleviating functional deficits afterwards.

Can these studies be used in developing interventions for brain regrowth in humans? Research investigating the possibility of inducing brain regeneration in humans is far from complete. The studies discussed here certainly have not unveiled all the aspects necessary for regeneration to occur. Our aim was to begin to determine the circadian influences and their significance on brain regeneration. We found that the circadian influence on neonatal brain regeneration is miniscule, at best. We also aimed to find and quantify a role of clock genes in MPFC regeneration, and found that disruption to the circadian clock's function did not alter regeneration. This may hold promise in future research which aims to expand this model. When trying to recreate the neonatal cellular environment that exists in the MPFC during the regeneration process, control over circadian clock genes may not be necessary. However, since we were able to show significant effects of circadian dysfunction in adult SGZ cell survival and apoptosis, this may imply that the cellular environment within the adult brain has changed significantly from the neonatal period, and that induction of P7-like regeneration may be difficult. Further research is necessary to pin down the details of the aspects of the regenerative cellular environment which are necessary, and how we may be able to recreate that environment in adult brain tissue.

There is still hope, however, since the results of our study in hippocampus show a decrease in apoptotic cell death, resulting in an increase in the number of surviving cells in the GCL. What we may have uncovered here is a factor which can potentially be temporarily altered to allow for more new cells to survive. Researchers are currently

working on ways to get SGZ proliferating cells to relocate to an area of damage. When knocking out BMAL1, we get more new cells surviving and fewer being pruned through apoptosis. Therefore, the potential for a BMAL1 knock down procedure to allow more new cells to survive, and hopefully migrate to an area of damage, without causing a great impediment to normal SGZ incorporation of new cells is high. That is, if the normal number of new cells that are consistently generated in the SGZ are necessary there, and we can increase the number of cells that survive, we may be able to have the excess surviving cells migrate to damage sites, while still having normal levels of new cells survive and stay within the hippocampus.

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