EFFECTS OF CHRONIC SLEEP RESTRICTION ON BLOOD VESSEL DENSITY IN THE PREFRONTAL CORTEX AND HIPPOCAMPUS IN RATS

By

Andrew Baldin

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ABSTRACT

Increased blood vessel density could increase the efficiency of the glymphatic system which removes waste from the brain. This study tested the hypothesis that chronic sleep restriction (CSR) increases blood vessel density in brain regions, such as the prefrontal cortex, where wake and sleep deprivation are associated with higher levels of neuronal activity compared to sleep, but not in those regions where neuronal activity does not increase during wake, such as the hippocampus. In the prefrontal cortex, a small increase in newly grown blood vessels was observed after four days of CSR while total blood vessel density did not increase. No increase in newly grown vessels occurred after 4 four-day cycles of CSR. In the hippocampus, there was no effect of CSR on blood vessel density. Based on these results, it is unlikely that CSR under the current condition has a major effect on the efficiency of the glymphatic system.

LIST OF ABBREVIATIONS USED

20-HETE	20-hydroxyeicosatreaenoic acid
ACTH	adrenocorticotropic hormone
Ang-1	angiopoietin-1
Ang-2	angiopoietin-2
ATP	adenosine triphosphate
BrdU	5-bromo-2'-deoxyuridine
CSF	cerebrospinal fluid
CSR	chronic sleep restriction
CYP	cytochrome P450
Dll4	delta-like 4
EC	exercise control
EET	epoxyeicosatrienoic acid
FITC	fluorescein isothiocyanate
FOXO1	forkhead box protein O1
GFAP	glial fibrillary acidic protein
GLUT1	glucose transporter 1
GLUT3	glucose transporter 3
HIF1α	hypoxia inducible factor 1α
Iba1	ionized calcium-binding adaptor molecule 1
iNOS	inducible nitric oxide synthase
LW	locked wheel
MBP	myelin basic protein
NeuN	neuronal nuclear antigen
NG2	neural/glial antigen 2
NGFI-A	nerve growth factor inducible protein A
nNOS	neuronal nitric oxide synthase

NO	nitric oxide
NREM	non-rapid eye movement
PFC	prefrontal cortex
PSA-NCAM	polysialylated neuronal cell adhesion molecule
pVHL	von Hippel Lindau protein
PVS	perivascular space
REM	rapid eye movement
SR	sleep restricted
TBS	tris-buffered saline
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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1: INTRODUCTION

1.1 Chronic sleep restriction

The function of sleep is not well understood, though the importance of sleep to one's health is readily apparent after a period of sleep deprivation. Impairments of perception, concentration, reaction time and decision making are accompanied by cardiovascular, hormonal and emotional disturbances (Banks and Dinges, 2007; Orzeł-Gryglewska, 2010). Chronic sleep restriction (CSR) is very common in our society. Due to work and family responsibilities and lifestyle choices, many individuals have insufficient sleep for weeks, months or years at a time. Additionally, for many individuals CSR is the consequence of a chronic medical condition. For example, it is estimated that 13% of Canadian adults meet criteria for insomnia (Morin et al., 2011) and 3% of Canadian adults suffer from obstructive sleep apnea (Public Health Agency of Canada, 2009), an ailment which occurs in all ages including young people (Raynes-Greenow et al., 2012). In addition to these conditions which affect sleep directly, many of the most common health problems such as pain and depression limit one's ability to obtain adequate sleep.

When Canadians are surveyed about their sleep habits, it is clear that CSR has become ubiquitous in our society. 74% of Canadians report that they sleep less than 7 hours each night, with 36% sleeping less than 6 hours (Morehouse et al., 2016). According to the national sleep foundation, most adults require 7-9 hours of sleep (Hirshkowitz et al., 2015). Individual sleep needs vary greatly. While sleeping less than 7 hours each night may be adequate for some

people, a large segment of the population is not getting enough sleep (Orzeł-Gryglewska, 2010).

Relatively mild forms of sleep restriction have been found to have significant impacts on cognitive abilities. For example, when people were restricted to 6 hours of sleep for fourteen days, their performance on a vigilance task became impaired after the first night and progressively declined every day that this restriction continued. Despite the ongoing decline in cognitive function, participants' subjective ratings of sleepiness increased very slowly after the first day of sleep restriction (Van Dongen et al., 2003). It is likely that many people are underestimating the degree to which they are affected by CSR. Understanding the changes which occur in the brain due to CSR is of great interest as the size of the population affected by CSR is immense. Knowing the effects of CSR also furthers our understanding of the function of sleep.

1.2 The glymphatic system

One potential function of sleep may be to expedite the removal of metabolic waste and toxic substances that build up in the nervous system, including those associated with neurodegenerative diseases such as Alzheimer's disease (Xie et al., 2013). In the brain, the glymphatic system is responsible for the removal of metabolic waste products. This system creates a convective flow of cerebrospinal fluid (CSF) along the perivascular spaces (PVS) of arteries, and across the brain tissue. As the CSF flows between brain cells, metabolic waste

products are carried away for removal from the brain via the PVS around veins (Iliff et al., 2012). The CSF-filled PVS around the blood vessels of the brain is completely surrounded by astrocyte endfeet (Mathiisen et al., 2010) and CSF is propelled along the PVS by arterial pulsation (Iliff et al., 2013). The perivascular endfeet of astrocytes contain an extremely high concentration of the water channel aquaporin-4 (Nagelhus et al., 2004) and it is this water channel that allows CSF to move from the PVS, across the astrocytic endfeet, and flush across the brain tissue.

The rate at which CSF flows into the brain is about 20x higher during sleep compared to wake (Xie et al., 2013), and the symptoms associated with sleep restriction may be the result of giving the system responsible for removing waste from the brain insufficient time to complete its task. Compromising the effectiveness of the glymphatic system also has serious long-term implications for health. For example, amyloid beta, the protein which comprises the plaques found in brains of people with Alzheimer's disease, is removed from the brain via the glymphatic system. Concentrations of extracellular amyloid beta in the brains of both humans and mice are known to rise during wake and fall during sleep. When mice predisposed to develop amyloid plaques are sleep deprived, they develop more extensive amyloid plaques (Kang et al., 2009).

1.3 Angiogenesis in response to increased neural activity

As the glymphatic system relies on CSF flowing between blood vessels, a change in the number of blood vessels in a region could impact the efficiency of waste removal. As discussed below, heightened levels of neural activity in a brain region can alter local blood vessel morphology by inducing angiogenesis, the growth of new blood vessels from existing vessels. For example, rats that were exposed to a complex environments for 30 days increased the density of blood vessels in the visual cortex by about 10-20% (Black et al., 1987; Sirevaag et al., 1988; Bengoetxea et al., 2008). A similar increase in blood vessel density was seen in the cerebellum (Black et al., 1990; Isaacs et al., 1992) and motor cortex (Swain et al., 2003) when animals were provided access to a running wheel for 30 days. A week of whisker stimulation for just 15 minutes per day increased blood vessel density in the barrel cortex by 20%, while whisker plucking to decrease neural activity in this region decreases blood vessel density by the same amount (Lacoste et al., 2014). Following stroke, whisker stimulation increased the number of vessels in the barrel cortex by 40% (Whitaker et al., 2007). Contrary to the results of these studies using adult rodents, one study has found that early in development increased neural activity can have an anti-angiogenic effect; chronic auditory stimulation, whisker stimulation and exercise disrupted normal angiogenesis in the auditory, barrel and motor cortices (Whiteus et al., 2014).

Many brain regions show distinct levels of neuronal activity during wake and sleep. For example, in the prefrontal cortex (PFC), compared to sleep, a brief

period of wake is associated with increased expression of immediate early genes, which are used as a marker of neuronal activity. 30 minutes of wake leads to increased expression of c-Fos protein and mRNA, as well as nerve growth factor inducible protein A (NGFI-A) protein and mRNA (Pompeiano et al., 1994). Similarly, periods of sleep deprivation ranging from 3 to 12 hours are associated with large increases in NGFI-A mRNA levels (Pompeiano et al., 1997). Observations of cerebral blood flow (Braun et al., 1997) and cerebral glucose metabolism (Nofzinger et al., 2002) show that metabolic demands in the PFC are much higher during wake compared to sleep.

Contrary to the findings in the PFC, neuronal activity is either decreased or unchanged in the dentate gyrus of the hippocampus during wake. 30 minutes of wakefulness is associated with a small decrease in NGFI-A mRNA expression with no change in c-Fos (Pompeiano et al., 1994), and periods of sleep deprivation ranging from 3 to 24 hours are associated with decreased NGFI-A mRNA expression (Pompeiano et al., 1997). Observations of cerebral blood flow showed that, compared to wake, metabolism was slightly elevated during NREM sleep, and much higher during REM sleep (Braun et al., 1997). Similarly, cerebral glucose metabolism in the hippocampus was observed to be elevated during NREM sleep compared to wake (Nofzinger et al., 2002). These findings are in accordance with the contemporary theory of memory consolidation which states that declarative memories are initially generated by high frequency signals from the cerebral cortex to the hippocampus during wake. During NREM sleep, high

frequency signals transfer information from the hippocampus to the cerebral cortex where memory storage is more permanent (Raichle et al., 2016).

As discussed above, elevated neuronal activity in a region of the brain due to a variety of stimuli has been found to cause angiogenesis. CSR would subject brain regions, such as the PFC, that show higher neuronal activity during wake and sleep deprivation than during sleep, to elevated neuronal activity for a prolonged period of time. It is, therefore, possible that CSR has angiogenic consequences in such regions.

1.4 Mechanisms of angiogenesis

All blood vessels in the body are lined by squamous epithelial cells known as endothelial cells, and in the adult brain endothelial cells normally maintain a quiescent phenotype, where the cells form junctions with neighboring cells and only a very tiny fraction are undergoing cell division (Kliche et al., 2011). Quiescent endothelial cells are attached to each other by vascular endothelial cadherin. When the endothelial Tie-2 receptor is activated by Angiopoietin-1 (Ang-1), which is expressed by pericytes and smooth muscle cells, Tie2 forms a complex with Tie-2 from an adjacent endothelial cell, reinforcing the connections between cells (Koh, 2013). For angiogenesis to occur, endothelial cells must be stimulated to proliferate, loosen connections with neighboring cells and migrate toward angiogenic signals.

Hypoxia inducible factor 1α (HIF1α) is consistently expressed by cells throughout the body, but under normal conditions is rapidly degraded (Park et al., 2011). When HIF1α is allowed to accumulate, expression of vascular endothelial growth factor A (VEGF-A), the most important proangiogenic signal, is upregulated (Jeltsch et al., 2013). In the brain, VEGF is expressed by astrocytes and neurons (Heine et al., 2005). When VEGF-A binds to its receptor VEGFR2 on endothelial cells, vascular endothelial cadherin is internalized via endocytosis. Endothelial cells also begin to express angiopoietin-2 (Ang-2) which antagonizes the Tie-2 complexes, further weakening blood vessel integrity (Koh, 2013). VEGFR2 activation also leads to phosphorylation of the transcription factor forkhead box protein O1 (FOXO1) which stimulates endothelial cell growth and proliferation (Betsholtz, 2016; Wilhelm et al., 2016).

As the name implies, HIF1α is regulated by hypoxia. This protein is rapidly degraded when exposed to normal physiological levels of oxygen. Prolyl hydroxylases hydroxylate HIF1α proteins and this change provides binding sites for von Hippel Lindau protein (pVHL) which tags HIF1α with ubiquitin, marking it for proteasomal degradation. Hydroxylation of HIF1α is prevented by low oxygen levels, leading to stabilization of HIF1α and angiogenesis (Metzen et al., 2003).

In the brain, hypoxia can induce angiogenesis and this is seen following stroke (Cunningham et al., 2012), but hypoxia is not the only driver of angiogenesis in the brain. Neurovascular coupling ensures that increased neuronal activity in a region is accompanied by vasodilation and increased blood flow (Attwell et al., 2011). It is therefore unlikely that increased neuronal activity

could stimulate angiogenesis via hypoxia. Accordingly, HIF1α stabilization in the brain can be mediated by nitric oxide (NO) independent of oxygen concentration. NO derived from neuronal nitric oxide synthase (nNOS) can prevent degradation of HIF1α by S-nitrosylation of pVHL, thereby initiating angiogenesis (Li et al., 2007; Tsui et al., 2011, 2014). nNOS is critical to the vasodilation component of neurovascular coupling. Inhibition of nNOS leads to a 70% reduction in the change in cerebral blood flow following neuronal activation (Stefanovic et al., 2007).

The study that showed that increased neuronal activity early in life could have an antiangiogenic effect in the associated brain region also tested whether NO was responsible for this effect (Whiteus et al., 2014). The antiangiogenic effect was blocked completely by a broad-spectrum NOS inhibitor or by loss of function mutations to nNOS or inducible nitric oxide synthase (iNOS), both of which are activated during prolonged neuronal activity. The contradictory findings of pro- and antiangiogenic effects of NO may be due to differences in NO concentrations between experiments. Increasing concentrations of NO have larger proangiogenic effects to a point, after which increased concentrations actually have the opposite effect (Jones et al., 2004).

In addition to NO, epoxyeicosatrienoic acid (EET) is another proangiogenic signal involved in vasodilation in neurovascular coupling. In the presence of NO, arachidonic acid in the endfeet of astrocytes is metabolized by cytochrome P450 (CYP) enzymes to produce EET, which activates large-conductance calcium-activated K+ channels in vascular smooth muscle, resulting

in vasodilation (Gordon et al., 2007; Sudhahar et al., 2010). In addition to its role in vasodilation, EET promotes angiogenesis by inducing endothelial cell proliferation and acts as a second messenger in VEGF-induced angiogenesis (Webler et al., 2008).

During angiogenesis, endothelial cells exit their quiescent state and take on phenotypes of “tip” and “stalk” cells. Tip cells are the front cells that direct migration of a blood vessel sprout. These cells have many filopodia which are sensitive to VEGF-A and will elongate toward regions of higher VEGF-A concentration. In this way, sprout growth is directed toward angiogenic VEGF-A signals (Gerhardt et al., 2003). Behind the tip cell are stalk cells which do not express filopodia. In these cells, exposure to high VEGF-A concentrations stimulates cell proliferation, leading to lengthening of the vessel sprout. When rat neovessels were grown in 3d culture, vessels elongated at an average rate of 5.3 μm/h. Vessels also retracted, and did so at a rate of 10.4 μm/h (Utzinger et al., 2015).

During angiogenesis, newly generated endothelial cells do not simply form a line and move in unison, but rather, they start, stop, advance at different speeds or move in reverse (Arima et al., 2011). New endothelial cells are in constant competition to reach the front of the sprout and become the tip cell. A feedback loop involving VEGF, Notch and Delta-like 4 (Dll4) ensures that when a stalk cell overtakes the tip cell, it becomes the tip cell and the former tip cell joins the stalk (Jakobsson et al., 2010). Cells high in VEGFR-2 and low in VEGFR-1 are most likely to take lead position in the sprout. The Notch ligand Dll4

expression in a cell is dictated by the relative VEGFR levels of that cell and adjacent cells. Maintaining a high Dll4 expression in stalk cells increases expression of VEGFR-1 and decreases expression of VEGFR-2 thereby inhibiting the tip cell phenotype (Arima et al., 2011).

1.5 Role of the vasculature in providing nutrients to the brain

The brain constantly requires large amounts of oxygen and glucose to function properly. Many of the functions necessary for information processing in the brain, including maintenance of membrane potentials and synaptic transmission are very demanding in terms of energy expenditure. At rest, the human brain accounts for about 20% of the body's oxygen (Masamoto and Tanishita, 2009) and glucose consumption (Mergenthaler et al., 2013). The cells of the brain rely very heavily on oxygen and glucose to produce the adenosine triphosphate (ATP) needed to fuel cellular processes. Glucose and oxygen are consumed to generate ATP, water and carbon dioxide. Oxygen is carried in the blood by hemoglobin found on red blood cells. Oxygen can freely diffuse across the blood brain barrier towards areas of lower oxygen concentration. Glucose is able to travel across membranes by facilitated diffusion. It crosses the blood-brain barrier via glucose transporter 1 (GLUT1) channels in endothelial cells, then is taken up by glia via GLUT1 and by neurons via glucose transporter 3 (GLUT3) which has a much higher transport capacity than GLUT1 (Mergenthaler et al., 2013).

Other energy molecules used in the periphery are not used in large amounts in the brain. Glycogen is stored in astrocytes only at very low concentrations, ketone bodies only supply energy to the brain during periods of prolonged fasting, and fatty acids are not used for energy in the brain, likely because fatty acid metabolism would increase oxygen consumption and generate superoxides (Schönfeld and Reiser, 2013). It has been recently shown that lactate, generated from glucose, can be used as an energy source for neurons. Lactate travels down its concentration gradient from astrocytes to neurons and may represent a small, quickly consumed energy reserve for neurons (Mächler et al., 2016).

Restricting the delivery of either oxygen or glucose to a region of the brain results very quickly in irreversible cellular damage. If blood flow to an area of the brain is blocked, the process of glutamate excitotoxicity is set into motion. Ion pumps which rely on ATP become compromised, leading to depolarization and calcium flow into neurons. Astrocytic reuptake of glutamate and its conversion to glutamine become impaired, and glutamate reuptake can be reversed, causing release of glutamate. High levels of extracellular glutamate lead to further depolarization, neuronal swelling and free radical production. When mitochondria become damaged, apoptotic cascades are initiated. Neuronal death releases massive amounts of glutamate which intensifies this process for nearby cells (Dirnagl et al., 1999; Bonde et al., 2005).

In order to prevent these catastrophic consequences which result when inadequate blood flow reaches an area of the brain, the cerebrovasculature has a

number of unique adaptive mechanisms which ensure that adequate quantities of nutrients are continuously delivered. For instance, blood is supplied to the brain by the vertebral and internal carotid arteries and blood from these arteries arrives at a structure at the base of the brain known as the circle of Willis. This circular structure allows blood to be delivered to all connected arteries, even if one portion of the circle becomes compromised. This is very important, as the cerebral arteries connected to the circle of Willis deliver blood throughout the brain via a branching structure of arteries and capillaries which is remarkably extensive. The average distance between capillaries in the human brain is only 40 μm. This short distance from the vasculature to any point in the brain allows diffusion of nutrients to happen very quickly. For example, in the rat somatosensory cortex, diffusion of oxygen to a tissue where oxygen has been depleted by neuronal activity is estimated to take 1.2 seconds (Masamoto and Tanishita, 2009).

As previously mentioned, neurovascular coupling allows blood flow throughout the brain to rise and fall to match the energy needs of nearby neurons. Smooth muscle cells control the diameter of larger vessels and pericytes perform this function for capillaries. Both smooth muscles and pericytes respond to a number of signals released by neurons and astrocytes following neurotransmission. For example, following glutamate transmission, nNOS in the postsynaptic neuron can release NO causing vasodilation. Glutamate also activates glutamate receptors on astrocytes causing arachidonic acid to be generated. At lower oxygen concentrations, arachidonic acid is converted to

EETs and prostaglandins, both of which cause vasodilation. When higher concentrations of oxygen are available, arachidonic acid is converted to 20-hydroxyeicosatreaenoic acid (20-HETE) which causes vasoconstriction. Synthesis of 20-HETE requires oxygen, and this allows astrocytes to induce constriction or dilation based on local oxygen requirements (Attwell et al., 2011).

A drop in blood pressure, leading to reduced cerebral blood flow could have devastating consequences. Similarly, very high blood pressure damages vessels and can lead to stroke. To prevent these outcomes, blood pressure is maintained by cerebral autoregulation, whereby rates of cerebral blood flow are held constant at a wide range of blood pressures. Blood vessels constrict or dilate in response to changes in shear stress and pressure from blood flow (Peterson et al., 2011). This autoregulation maintains cerebral blood flow at blood pressures between about 60 – 160 mmHg. When blood pressure drops below this range, or during hypoxia, cerebral blood flow increases significantly. Hypoxia results in vasodilation by stimulating NO production and reducing ATP concentration, which opens K+ channels on smooth muscle cells (Cipolla, 2009).

1.6 The goal, hypothesis and significance of the study

Based on the findings that angiogenesis occurs in brain regions with elevated neuronal activity (see Section 1.3), this study investigated the possibility that CSR could initiate angiogenesis in regions of the brain that are most active during wake. The prefrontal cortex, which shows increased neuronal activity

during wakefulness and sleep deprivation relative to sleep (Pompeiano et al., 1994, 1997; Braun et al., 1997; Nofzinger et al., 2002), and is known to be sensitive to sleep loss (Muzur et al., 2002), was hypothesized to show angiogenesis in response to CSR. On the other hand, an angiogenic response was not expected in the hippocampus, which is not known to have increased neuronal activity during wakefulness or sleep deprivation compared to sleep (Pompeiano et al., 1994, 1997; Braun et al., 1997; Nofzinger et al., 2002).

To assess the impact of CSR on blood vessel density and possible angiogenesis, brain sections were stained for tomato lectin, nestin and 5-bromo-2'-deoxyuridine (BrdU). Tomato lectin binds to hyaluronic acid, a component of the extracellular matrix secreted by endothelial cells, and is commonly used as a marker for blood vessels (Mazzetti et al., 2004). Nestin is a filament protein expressed in proliferating cells and is expressed in endothelial cells which have recently divided. This expression is lost as the endothelial cells mature (Suzuki et al., 2010). This expression makes nestin a useful marker protein to identify the growth of new blood vessels during angiogenesis (Mokrý et al., 2004, 2008) and it has been used as such a marker in the adult brain (Ohlin, et al, 2011; Boldrini et al., 2012). Double labelling with lectin and nestin identifies recently divided endothelial cells which likely represent newly grown vessel segments. BrdU is structurally similar to thymidine and if present during S-phase of the cell cycle is incorporated into DNA. Injections of BrdU are commonly used to detect cell proliferation (Junek et al., 2010) and neurogenesis in the brain.

This experiment used sleep restricted (SR) animals subjected to 1 or 4 four-day cycles of CSR in rotating wheels. Two different durations of CSR were used to assess the effect of CSR duration. In addition to SR animals, two control groups were used: locked wheel (LW) controls kept in wheels which never rotate, and exercise controls (EC) which could spontaneously exercise. It was hypothesized that higher blood vessel density, as shown by increased tomato lectin staining, would be observed in the PFC, but not the hippocampus, in rats subjected to CSR compared to control animals. In addition, increased blood vessel density was expected to be accompanied by markers of angiogenesis; more newly grown blood vessels labeled with nestin and more BrdU stained nuclei colocalized with vessels were expected in the PFC following CSR. Animals subjected to four cycles of CSR were expected to have larger increases in blood vessel density and markers of angiogenesis compared to animals subjected to only one cycle of CSR.

Cerebral angiogenesis due to CSR would be a physiologically significant event because in addition to the role of blood vessels in the delivery of oxygen and nutrients to cells throughout the brain, they also facilitate the glymphatic system, which removes toxic cellular metabolites from the brain. The glymphatic system flushes CSF across the brain tissue from the PVS of arteries toward the PVS of veins (see Section 1.2). Increasing the density of blood vessels in a region of brain tissue would likely increase the efficiency of this process by increasing the amount of CSF entering a region and decreasing the distance between arteries and veins. Given the fact that the glymphatic system has been

found to function 20 times more efficiently during sleep than wake (Xie et al., 2013), sleep restricted individuals will take longer to remove toxic substances including amyloid-beta from the brain. If CSR initiated angiogenesis and enhanced the efficiency of the glymphatic system, this would provide a compensatory mechanism to reduce the negative impacts of reduced sleep time and the associated increase in toxic substances in the brain.

2: METHODS

2.1 Animals and chronic sleep restriction protocol

2.1.1 Animals

This study used adult male Wistar rats with body weights of 301-380g at the beginning of the experiment. Throughout the experiment food and water were freely available and lights were on from 7am to 7pm. Body weights were recorded at the beginning of habituation and before and after each cycle of sleep restriction (see section 2.1.2 below). The one-cycle experiment included animals who underwent CSR (n = 7) and LW controls (n = 6). The four-cycle experiment included animals who underwent CSR (n = 6) and LW controls (n = 6). A third experiment involved rats who underwent four cycles of CSR (n = 3) and EC animals (n = 2).

2.1.2 3/1 CSR protocol

Rats were housed individually in motorized wheels (Model 80860, Lafayette Instrument, Lafayette, IN: Figure 1) which were controlled via AWM software (Lafayette). During four days of habituation, the wheels were activated for 5, 10, 15 and 20 minutes, respectively, allowing the rats to become accustomed to wheel rotation.

This study used the 3/1 sleep restriction protocol (Figure 2) developed by Deurveilher et al. (2012). This protocol mimics the polyphasic nature of rat sleep

by alternating 3 hour periods of sleep deprivation and 1 hour periods of sleep opportunity. In this experiment, activity wheels rotated at 2.5 m/min, as this speed was found to be the minimum necessary to prevent consolidated sleep periods (Deurveilher et al., 2012). One “cycle” of sleep restriction lasted for four days, consisting of 24 repetitions of 3 hours sleep restriction and 1 hour of sleep opportunity. One group of rats was subjected to one cycle of sleep restriction, and a second group was subjected to four cycles of sleep restriction. Rats which underwent four cycles were transferred into plastic cages for two days of rest between cycles. All rats were perfused at 10am following an additional 3 hour sleep restriction period after four days of sleep restriction.

2.1.3 BrdU injections

Some of the rats which were subjected to one cycle of CSR (n = 2) or locked wheel control conditions (n = 2) were given injections of BrdU (50 mg/kg, i.p.) to label mitotic cells. BrdU solutions were prepared fresh daily and given at 7am and 3pm on each day of sleep restriction with a final injection at 7am on the day of perfusion, for a total of 9 injections.

2.1.4 Perfusing and brain sectioning

Rats were deeply anesthetized with ketamine (90 mg/kg), xylazine (4.8 mg/kg) and acepromazine (0.9 mg/kg) injected intraperitoneally, and transcardially perfused with 0.1M phosphate buffered saline followed by 4%

paraformaldehyde in 0.1M phosphate buffer. Brains were postfixed in the same fixative for 2 hours, then transferred to 20% sucrose solution in 0.1M phosphate buffer. 40 µm coronal sections were cut on a freezing microtome in five sets. Sections were selected approximately 2.7 to 3.0 mm anterior to bregma, containing the prelimbic cortex within the prefrontal cortex, and approximately 2.7 to 3.2 mm posterior to bregma containing the hippocampus, where the shape of the dorsal hippocampus remained relatively constant. Three sections were selected for each region for each animal.

2.2 Immunohistochemistry

2.2.1 Double labeling for tomato lectin and nestin

Sections were incubated overnight at room temperature in a mouse anti-nestin antibody (BD Pharmingen 556309, 1:1000) in 0.05 M Tris-buffered saline (TBS) containing 0.01% sodium azide, 2% normal donkey serum and 0.3% Triton-X. The following day, the sections were rinsed three times for 10 minutes each in 0.05 M TBS, then incubated for one hour, at room temperature in Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch 715-165-150, 1:400) in 0.05 M TBS containing 2% normal donkey serum and 0.3% Triton-X. Sections were rinsed three times, then incubated for two hours in fluorescein isothiocyanate (FITC) -conjugated tomato lectin (Sigma L0401, 20 µg/ml) in 0.05 M TBS containing 1% bovine albumin. Sections were then rinsed three times and mounted on slides. The following day, the slides were coverslipped using

cytoseal 60. Slides were coded, allowing microscopy and image analysis to be performed blind to treatment group.

2.2.2 Triple labeling for tomato lectin, nestin and BrdU

BrdU immunohistochemistry requires antigen retrieval pretreatment which exposes the BrdU epitope, facilitating antibody binding (Junek et al., 2010). Sections were incubated in 50% formamide in saline (0.3 M) sodium citrate (0.03 M) for 2 hours at 65°C, then washed twice for 10 minutes in saline sodium citrate. Sections were then incubated in 2N HCl for 30 minutes at 37°C before being neutralized in sodium borate buffer (pH 8.5) for 10 minutes. Sections were rinsed in 0.05M TBS twice for ten minutes each.

Sections were incubated overnight at room temperature in a sheep antibody against BrdU (Fitzgerald 20R-BS001, 1:2000) and a mouse anti-nestin antibody (BD pharmingen 556309, 1:1000) in 0.05 M TBS containing 0.01% sodium azide, 2% normal donkey serum and 0.3% Triton-X. The following day, sections were rinsed three times for 10 minutes each in 0.05 M TBS, then incubated a Cy3-conjugated donkey anti-mouse IgG (Jackson 715-165-150, 1:400) and a biotinylated donkey anti-sheep IgG (Jackson 713-065-147, 1:400) in 0.05 M Tris-buffered saline (TBS) containing 2% normal donkey serum and 0.3% Triton-X for one hour. Sections were again rinsed three times and incubated in Cy5 streptavidin (Jackson 016-170-084, 1:1800) for one hour. Sections were rinsed three times, and incubated in FITC-conjugated tomato lectin (Sigma

L0401, 20µg/ml) in 0.05 M TBS containing 1% bovine albumin for 2 hours. Sections were then rinsed three times and mounted on slides. The following day, the slides were coverslipped using cytoseal 60. Slides were coded, allowing microscopy and image analysis to be performed blind to treatment group.

2.3 Microscopy

Fluorescent sections were imaged using a Zeiss LSM 510 upright laser scanning confocal microscope (25x oil immersion objective, 0.8 numerical aperture) using 488, 543 and 633 nm excitation. Images (336µm x 336µm) were acquired for the entire thickness of the sections (z-interval = 0.77µm).

Eight images were taken for each brain region in each animal. For each region, two images were taken from the left and right hemispheres of two sections. If there was a defect in the tissue such as a tear, a third section was used. For the prelimbic cortex, the images were centered between the midline and the forceps minor of the corpus callosum, with the top of the first frame lined up to the most dorsal aspect of the forceps minor. A second image was taken 300µm ventral to the first. For the dentate gyrus of the hippocampus, the first image was taken at the most medial point where the dentate gyrus was wide enough that the entire frame of the image included only the granule and polymorph layers of the hippocampus. A second image was taken 300µm lateral to the first image. The placement of the image boxes in the prefrontal cortex and hippocampus are shown in Figure 3.

2.4 Image analysis

Using ImageJ, maximum intensity projections were created for the entire thickness of the tissue. Blood vessels were manually traced in these 2D images using the simple neurite trace plugin (Longair et al., 2011). Blood vessel length, number of blood vessel segments, nestin-positive blood vessel length and number of nestin positive segments were recorded for each image. Due to the fact that vessels which overlap in a z-stack may appear to be branching in a 2D z-projection, all blood vessel branch points were verified by referring to the original z-stack image. An example of this image analysis is shown in Figure 4.

2.5 Statistics

For analysis of blood vessel length, segments, nestin-positive blood vessel length and nestin-positive branches, the eight values for each region for each animal were averaged. Two-way ANOVAs were used to analyze the effect of treatment and number of cycles on each of the four independent variables in each region as well as for the body weight data. Three-way ANOVAs were carried out using brain regions, treatment groups and the number of CSR cycles as factors. The Sidak multiple comparison test was used to determine significance. Mann-whiney U tests were used to compare blood vessel data in the exercise control experiment. Probabilities <0.05 were considered statistically significant.

3: RESULTS

3.1 The effect of CSR on blood vessel density

Effects of CSR on blood vessel density were assessed using the following measures: density of blood vessels in general, density of nestin-positive blood vessels, and the number of BrdU-positive endothelial cells in each image (336 μm x 336 μm). Blood vessel density further was quantified using two indices: the *total blood vessel length* and the *number of blood vessel segments* in each image. Blood vessel length was traced as shown in Figure 4. For counting blood vessel segments, a segment was defined as the part of a blood vessel that is delimited by two closest branching points. Thus, any point from which a blood vessel led in three directions was noted as the point of origin for three blood vessel segments. The total length and number of nestin-positive blood vessel segments were similarly obtained. BrdU-positive nuclei which colocalized with the tomato lectin blood vessel label were counted as BrdU-positive endothelial cells. Example of immunohistochemical staining for nestin and BrdU and are shown in Figures 5 and 6, respectively.

3.1.1 Correlations between two blood vessel indices as analyzed for blood vessels in general and for nestin-positive blood vessels

The two blood vessel density indices used, i.e., total blood vessel length and the number of blood vessel segments were highly correlated with each other when all blood vessels in each image were analyzed regardless of nestin

immunoreactivity ($r = 0.894$, $p < 0.01$, Figure 7A). An even stronger correlation was observed when analysis was restricted to nestin-positive blood vessels ($r = 0.981$, $p < 0.01$, Figure 7B). These high correlation coefficients between total length and number of segments suggests that when there is an increase in the total length of blood vessels, this is due to increased branching, rather than elongation of existing vessels. The high correlations also indicate that both variables are accurate indicators of blood vessel density for both the entire population of blood vessels and those with nestin immunoreactivity. A stronger correlation in the length and number of nestin-positive, as opposed to total, vessels indicates that there is less variability in the length of newly formed vessel segments compared to mature vessels.

3.1.2 Effect of CSR on blood vessel density in the PFC

Based on findings that neural activity is increased in the PFC during wakefulness and numerous studies showing that increased neural activity in a brain region leads to angiogenesis in that region (Black et al., 1987; Lacoste et al., 2014, also see Section 1.3), it was hypothesized that CSR would promote angiogenesis in the PFC.

No increase in blood vessel density was observed following either one cycle or four cycles of CSR, as measured by either blood vessel length (Figure 8A left) or the number of segments (Figure 8B left). This was true after both one and four cycles of CSR. When nestin-positive vessels were specifically analyzed

after one cycle of CSR, there were increases in the total nestin-positive blood vessel length ($F_{1,20}$ = 7.59, p = 0.01 for SR/LW treatment x number of cycles; p < 0.01 post-hoc; Figure 8C left) and the number of nestin-positive blood vessel segments ($F_{1,20}$ = 7.46, p = 0.01 for SR/LW treatment x number of cycles; p < 0.01 post-hoc; Figure 8D left), indicating a significant increase in nestin expression in blood vessels, which suggests the growth of new blood vessels. Nestin-positive blood vessels represented approximately 1.5% of the total blood vessel length and 1.4% of the blood vessel segments in the PFC after one cycle of CSR. This increase in nestin expression was selective to one cycle and was not observed following four cycles of CSR (Figure 8C,D right).

3.1.3 Effect of CSR on blood vessel density in the hippocampus

It was hypothesized that in the hippocampus, an area which does not show an increase in neural activity during wakefulness, CSR would not promote angiogenesis. In support of this hypothesis, no differences in blood vessel density (Figure 9A,B) or nestin expression (Figure 9C,D) were observed between the sleep restricted and control rats after either one or four cycles of CSR.

3.1.4 Assessment of angiogenesis using BrdU

As an additional method to assess angiogenesis, injection of BrdU was used in conjunction with one cycle of CSR. BrdU is structurally similar to thymidine and is inserted into a cell's DNA if present during the S phase of the

cell cycle. This allows BrdU to label cells which are to divide for a period of several hours following each injection (Junek et al., 2010) In the present experiment, rats received a series of nine injections over the course of 4 days of CSR. An example of immunohistochemical labeling using BrdU in the hippocampus is shown in Figure 6, which shows many BrdU-positive nuclei outside of blood vessels.

Analysis of BrdU staining (Figure 10) revealed cells that were BrdU-positive, indicating that these cells became post-mitotic during the four days of the sleep restriction period. Newly generated endothelial cells would be identified by the presence of BrdU immunoreactive nuclei in lectin-labeled blood vessels. If angiogenesis was seen to result from CSR, more BrdU immunoreactive nuclei will be seen in blood vessels following CSR compared to the control condition. In fact, very few BrdU-positive cells were seen in tomato lectin-labeled blood vessels in either treatment group. Very small numbers of such cells were seen in the hippocampus and zero were seen in the prefrontal cortex. These results indicate that endothelial cells in these brain regions were of a quiescent phenotype, regardless of sleep condition, which is consistent with the result that blood vessel density did not increase in either the PFC (Figure 8A,B) or the hippocampus (Figure 9A,B) after CSR. However, these results oppose the findings of increased incidence of nestin-positive blood vessels in the PFC that were seen after one cycle of CSR (Figure 8C,D).

Interestingly, when quantifying BrdU-positive cells that were not associated with blood vessels, there was a trend to see more mitotic cells in

sleep restricted rats compared to locked wheel controls in both the PFC and hippocampus. While the sample size of this BrdU experiment was small, with only two sleep restricted and two locked wheel rats, this finding is intriguing and warrants further observations to determine the types of cells which are proliferating. For example, the effect of CSR on adult hippocampal neurogenesis has not been established and these results suggest that CSR may increase the generation of new neurons.

3.2 Assessment of the possible role of exercise in CSR-induced angiogenesis

In the present study, sleep restricted rats were forced to walk 2.7 km per day, while control rats were quite sedentary, as they were kept in locked wheels. Unpublished work in our lab by Madjou Bah et al. has shown that the 2.7 km our rats were forced to walk is not significantly different from the distance that rats will run in these wheels if they are allowed to spin freely. This raises the question of whether any effect of CSR, might be due to the difference in exercise amount between the two treatment groups.

Previous studies have shown that exercise stimulates angiogenesis in the dentate gyrus of the hippocampus. For example, providing young mice with access to a running wheel for one month enhanced angiogenesis in the dentate gyrus; the number of vessels was unchanged while measures of blood vessel perimeter and surface area increased (van Praag et al., 2005). A long period of

exercise was not necessary to induce this effect; mice with access to running wheels for either 3 or 10 days increased density of blood vessels in the dentate gyrus (Van der Borght et al., 2009). The angiogenic effects in both studies were very short-lasting and blood vessel density returned to baseline levels after only 1 day without access to the running wheel. In both these studies, the effect of exercise was fairly large, with a 25-30% increase in blood vessel density.

These findings would suggest that several days of exercise in activity wheels could initiate angiogenesis in the dentate gyrus. However, this was not seen in CSR rats that spent 75% of their time walking in activity wheels or adjusting postures. One possible explanation is that that CSR somehow blocked the angiogenic effect of exercise in the dentate gyrus. This hypothesis, can be tested by using an additional control group, i.e., rats that were allowed to exercise spontaneously without being subjected to CSR and comparing their results with the results of standard CSR animals.

Thus an additional experiment was conducted using rats receiving four cycles of CSR using the 3/1 protocol (n = 3) and exercise control (EC) rats (n = 3). The EC rats were allowed to freely turn their wheels during the four day CSR cycles, and were moved to plastic cages for two days between cycles similarly to the SR animals. One EC animal had to be removed from the analysis due to inadequate perfusion of the brain tissue, resulting in n = 2 for this group. Blood vessel density and nestin immunoreactivity were compared between the two groups. The results from this experiment are shown in Figures 11 and 12 for the PFC and hippocampus respectively.

Mann-Whitney U tests revealed no significant effect for all four variables in either brain region. Blood vessel density values were within the ranges of values that were seen in the previous four cycle SR and LW animals (Fig 11A,B left). There was no evidence for the strong angiogenic effect of exercise in the dentate gyrus as described by van Praag et al. (2005) and Van der Borght et al. (2009).

For measures of nestin-positive blood vessels, two animals had high values in both the PFC (Figure 11C,D right) and hippocampus (Figure 12C,D right) which would be seen as outliers when compared to previous data from four cycle animals. Contrary to the hypothesis that exercise induces angiogenesis in the hippocampus, one animal with very high nestin expression was from the EC group and the other from the SR group. It is not clear what caused these two animals to express such high quantities of nestin as they did not have higher blood vessel density than the other four cycle animals with much lower nestin expression.

3.3 Reduced blood vessel density after four cycles regardless of sleep condition

Regardless of whether the rats were subjected to CSR, blood vessel density for blood vessels irrespective of nestin-stain was reduced in both the PFC and hippocampus after four cycles. In the PFC, 10% fewer blood vessel segments were observed after four cycles ($F_{1,21} = 4.92$, $p = 0.04$ for number of cycles; Figure 8B). In the hippocampus, blood vessel length was reduced by 11% ($F_{1,21} = 4.36$, $p < .05$ for number of cycles; Figure 9A) and number of segments

was reduced by 13% ($F_{1,21}$ = 6.51, p = 0.02 for number of cycles; Figure 9B) after four cycles.

3.4 Regional differences: Comparing blood vessels in the PFC and hippocampus

Blood vessel density was found to be much higher in the PFC compared to the hippocampus. Blood vessel length was 42% higher in the PFC compared to the hippocampus ($F_{1,24}$ = 79.39, p < 0.001 for region; Figures 8A, 9A) and segments were 41% more common in the PFC ($F_{1,24}$ = 65.06, p < 0.001 for region; Figures 8B, 9B), regardless of the treatment condition. In the hippocampus, nestin expression was considerably more common than in the PFC. Measures of nestin-positive blood vessel length ($F_{1,24}$ = 8.17), p < 0.001 for region; Figures 8C, 9C) and nestin-positive blood vessel segments ($F_{1,24}$ = 8.19, p < 0.001 for region; Figure 8D, 9D) were both 4.2 times higher in the hippocampus. The average length of a blood vessel segment, calculated as the total length divided by number of segments, was not significantly different between these two regions.

3.5 Effects of chronic sleep restriction on body weight

The body weights of rats subjected to four cycles of CSR and their locked wheel controls are shown in Figure 13, both as actual weights (Figure 13A) and as changes in body weight during each CSR or corresponding control period of

the experiment (Figure 13B). Rats housed in locked wheels gained weight at a relatively constant rate throughout the experiment. Sleep restricted rats lost approximately 8% of their body weight during the first four days of sleep restriction ($F_{1,5} = 35.14$, $p < 0.01$). In subsequent cycles, they appeared to have adapted to the sleep restriction and regained their weight at a similar rate as the locked wheel controls. This rebound was not complete, however, and the rate of weight gain declined over the next three cycles, and was significantly less than control during the fourth cycle ($F_{1,5} = 12.07$, $p < 0.01$). It should be noted that rats in locked wheels had very minimal physical activity.

4: DISCUSSION

It was hypothesized that due to elevated levels of neuronal activity during wakefulness, blood vessel density would increase in the PFC following CSR. This was a topic of interest because of the possible impact of increased blood vessel density on the glymphatic system. While there was a small increase in nestin-positive vessels in this region following one cycle of CSR, no increase in overall blood vessel density was observed and BrdU staining revealed no proliferating endothelial cells in this region. In the hippocampus, where angiogenesis was not expected, CSR had no impact on blood vessel density, nestin expression or BrdU-positive endothelial cells. These results suggest that CSR under the current experimental conditions had no major impact on blood vessel density in the brain, and, therefore, on the efficiency of the glymphatic system.

4.1 CSR and angiogenesis

4.1.1 The PFC

It has been shown in many brain regions that angiogenesis occurs following a period of elevated neuronal activity (Black et al., 1987, 1990; Swain et al., 2003; Lacoste et al., 2014). As the PFC is known to show higher levels of neural activity during wake, it was hypothesized that CSR would lead to angiogenesis in the PFC. The glymphatic system is known to remove waste only very slowly during wakefulness, and accelerate the flow rate by 20 times during sleep. Because of this, the restorative function of sleep is likely the result of

clearing toxic substances that accumulate in the brain during wake (Xie et al., 2013). If a strong angiogenic effect were found in the PFC following CSR, it would have significant implications for the functioning of the glymphatic system in sleep restricted individuals, likely improving clearance both by increasing the rate that CSF can flow into a brain region and by decreasing the distance between arteries and veins. The results of blood vessel density analysis, however, showed no increase in the total length or number of blood vessels in the PFC following CSR.

As a second approach to quantify changes in the vasculature, nestin was used as a marker for angiogenesis. This filament protein is expressed in cells which have recently divided and is lost as cells mature (Mokrý et al., 2008; Suzuki et al., 2010). Quantifying nestin-positive blood vessels allows the differentiation of blood vessels composed of mature endothelial cells from those composed of recently generated cells. Increases in the length and number of nestin-positive blood vessels were observed in the PFC following one cycle of CSR. While this effect was statistically significant ($p < 0.01$), these new vessels accounted for only a small fraction of the total vascular network in that region. Specifically, nestin-positive blood vessels made up 1.5% of total length and 1.4% of the blood vessel segments that were observed in the PFC following one cycle of CSR. Thus, the contribution of new vessels was small and when angiogenesis was quantified as an increase in the blood vessel density in this region, there was no difference between CSR and control animals. A potential explanation for the finding of increased nestin expression without an increase in blood vessel

density is that CSR initially causes the growth of new vessels and that this is offset by a reduction in the survival of mature vessels.

As explained in the Introduction (see section 1.4), a possible mechanism for CSR to induce the growth of this small amount of new blood vessels involves NO and VEGF signalling. Increased neuronal activity in the PFC during CSR increases local energy demands and cerebral blood flow to this region increases by means of neurovascular coupling (Attwell et al., 2010). The NO released from neurons, which induces vasodilation, also s-nitrosylates pVHL (Li et al., 2007) which prevents the degradation of HIF1α, leading to increased expression of VEGF-A (Jeltsch et al., 2013). When VEGF-A binds to VEGFR2 on endothelial cells, connections with adjacent cells are weakened by internalization of vascular endothelial cadherin and the release of Ang-2 to disrupt Tie-2 complexes (Koh, 2013). VEGF-A signalling also phosphorylates FOXO1 leading to proliferation of endothelial cells (Betsholtz, 2011).

Contrary to the initial hypothesis, the increase in nestin-positive blood vessels observed after one cycle of CSR was not seen following four cycles of CSR, implying that an adaptation might have occurred to prevent the continual growth of blood vessels for weeks at a time in response to CSR. Such adaptation with four cycles of CSR has been observed for autonomic measures (Bah et al., unpublished observations). Indeed, continuous blood vessel growth for weeks at a time could be problematic, likely leading to increased intracranial pressure. However, the 1.5% of the total blood vessels in the hippocampus found to be expressing nestin is well below the findings of other angiogenesis studies which

routinely find increases of blood vessel density of 10-20% (Black et al., 1987, 1990; Sirevaag et al., 1988; Isaacs et al., 1992; Swain et al., 2003; Bengoetxea et al., 2008; Lacoste et al., 2014).

A third approach to quantify angiogenesis involved a series of injections of BrdU over one cycle of CSR, which labels cells that exit the cell cycle after BrdU incorporation and final mitosis (Junek et al., 2010). In the PFC, no BrdU-positive nuclei colocalized with the tomato lectin blood vessel label in any LW or SR animal after one cycle of CSR. This does not agree with the finding of increased nestin expression in blood vessels of the PFC following one cycle of CSR. This discrepancy may be explained by the possibility that BrdU might not have labelled all cells that became postmitotic during the four days of CSR. BrdU bioavailability for incorporation during S-phase lasts about two hours (Junek et al., 2010). With two injections per day at 7am and 3pm, BrdU would be available for two hours each in the morning and the afternoon each day. As the cell cycle typically has a period of approximately 24 hours, and is controlled by circadian regulators (Chaix et al., 2016), it is possible that the timing of BrdU injections did not entirely match the S-phase of proliferating endothelial cells, whereas all proliferating endothelial cells would be labeled with nestin.

In summary, while CSR did stimulate the growth of a small number of new blood vessels in the PFC, these vessels did not significantly increase the blood vessel density in this region. The effect was also short-lived, or was subject to adaptation, as it was absent in animals which underwent four cycles of sleep restriction. No recently divided endothelial cells were found using BrdU in this

region following one cycle of CSR. Based on these results it is unlikely that the effect of CSR on the blood vessels of the PFC would have a major effect on the efficiency of the glymphatic system, at least under the current experimental conditions.

4.1.2 CSR and angiogenesis in the hippocampus

Levels of neuronal activity in the hippocampus are similar during sleep compared to wake in rats (Pompeiano et al., 1994, 1997). In humans, metabolism in this region is at its highest level during slow-wave sleep (Braun et al., 1997; Nofzinger et al., 2002). For these reasons, the hippocampus was not expected to undergo angiogenesis in response to CSR, and was included as a control region that could be compared to the PFC. In agreement with this prediction, CSR was not associated with any change in blood vessel density, nestin-positive blood vessels or BrdU-positive blood vessels in the hippocampus.

4.2 Exercise did not affect blood vessel density in the PFC and Hippocampus

Previous studies have shown that the hippocampus can have a large angiogenic response to exercise (van Praag et al., 2005; Van der Borght et al., 2009), while in the present study neither EC nor SR animals showed increased blood vessel density compared to LW animals in either the PFC or hippocampus. Similarly, with the exception of one-cycle of CSR in the PFC, nestin expression

did not favour EC or SR animals over LW animals in this experiment. One possible explanation for the lack of effects of exercise or physical activity is that the EC and SR rats in this experiment did not exercise enough to induce angiogenesis in the dentate gyrus. Mice in the van Praag et al. study ran 4.9 km each day while those in the Van der Borght et al. study gradually increased their distance from 6 to 13 km each day, much farther than the 2.7 km traveled by the EC and SR rats in this study.

4.3 Stress and angiogenesis

One possible explanation for the small angiogenic effect of CSR is that stress signals, such as corticosterone, that could be associated with CSR, had angiostatic effects that counteracted any angiogenic effect provided by prolonged wakefulness. Sleep restriction for just a few hours caused an increase in plasma corticosterone levels (Hairston et al., 2001) which remained elevated for the duration of 2 days of sleep deprivation or 7 days of CSR (Meerlo et al., 2002). However, sleep deprivation studies where animals were continuously sleep deprived for 6 to 48 hours (Junek et al., 2010) or 96 hours (Guzmán-Marín et al., 2003) did not find higher levels of corticosterone in sleep deprived animals compared to controls. Corticosterone levels were not measured in the present study, and as these previous findings are inconsistent, it is unclear what effect of the 3/1 CSR protocol has on corticosterone levels. However, it is interesting to note that many studies have found that stress and associated corticosterone have a strong angiostatic effect. For example, continuous corticosterone

treatment for 48 hours reduces VEGFR2 levels in the PFC by approximately half (Howell et al., 2011) and inhibits endothelial cell proliferation in both the PFC and hippocampus (Ekstrand et al., 2008). In addition, an unpredictable stress paradigm, known to increase corticosterone levels, reduces VEGFR2 in the hippocampus (Heine et al., 2005). The angiostatic effects of corticosterone are likely due to reduced endothelial cell proliferation resulting from elevated FOXO1 levels and reduced VEGF levels, as well as reduced production of matrix metalloproteinase-2 which is necessary to break down collagen IV in the basement membrane (Shikatani et al., 2012).

Contrary to the role of corticosterone in angiostasis, it is possible for stress to promote angiogenesis via alternative pathways. It has been shown that chronic stress from restraint for 6 hours each day for 21 days increases both VEGF and VEGFR2 in the prefrontal cortex (Elfving et al., 2015). While chronic restraint does increase corticosterone levels (Tuli et al., 1995), this effect may be mediated by another factor such as adrenocorticotropic hormone (ACTH) which is greatly elevated during restraint but not strongly affected by sleep restriction (Meerlo et al., 2002).

As corticosterone was not measured in this study, it is impossible to estimate its contribution to the finding that only a very small increase of nestin-positive vessels occurs in CSR with no increase in overall blood vessel density.

4.4 Reductions in blood vessel density after four cycles of CSR and control condition

Blood vessel density was observed to decrease by 8-13% after the four-cycle period compared to the one-cycle period regardless of whether the animals were subjected to the CSR treatment. Two possible explanations are discussed below.

4.4.1 Angiostatic stress signals

One possibility is that this general decrease could be due to the angiostatic effect of corticosterone that might have been released during the experimental protocol. Namely, the length of experiment (27 days) may have negatively impacted blood vessel density because animals in all treatment groups could have been subjected to a variety of stressors including noise, isolation and a lack of environmental enrichment, as discussed below.

In this experiment, the rats were constantly exposed to white noise, a potentially stressful stimulus, from fans which circulated air through their cages. It is known that chronic exposure to loud white noise can induce stress responses which is subsequently maintained for at least 30 days, including elevated levels of norepinephrine and heat shock protein in the brain as well as plasma corticosterone (Samson et al., 2007).

At the beginning of this experiment, the rats were taken from the animal facility where they were housed in pairs. For the experiments they were placed

individually into activity wheels. The animals remained isolated from the other rats for the remainder of the experiment. Compared to rats housed in groups, socially isolated rats are more susceptible to the effects of stress, showing larger increases in corticosterone after administration of corticotropin releasing factor and smaller decreases after dexamethasone administration (Serra et al., 2005). Isolated rats show greater anxiety-like behaviour, and these behaviours are correlated to the animals' corticosterone levels (Butler et al., 2014).

The animals in the present study spent the entire experiment in an environment that can be described as dull and unstimulating. There were no objects inside the activity wheels other than the food pellets and water spout. Between cycles of sleep restriction, the animals were moved to home cages which only contained saw dust and a hard plastic tube. Environmental enrichment in the form of toys for rats to chew or material with which to build nests has been shown to reduce the concentrations of stress hormones corticosterone and ACTH in rats (Belz et al., 2003). When rats were housed in a large cage with toys, tunnels, swings, running wheels and ten animals per cage, baseline levels of corticosterone increased, but corticosterone responses to stressful stimuli were reduced (Moncek et al., 2004). Accordingly, animals housed in enriched environments had improved performances on behavioural tests of psychological stress including the Morris water maze and open field (Larsson et al., 2002).

As levels of stress hormones were not measured in this study, it is impossible to know the extent of their involvement in the loss of blood vessel

density observed in the four-cycle animals. Future research to measure stress signals including corticosterone and ACTH would help to clarify the involvement of stress. Knowing the extent that stress hormones rise during the 3/1 protocol may also be valuable for the interpretation of other experiments using this protocol.

4.4.2 Changes in cerebral blood vessel density during rat brain development

Another potential explanation for the reduction in blood vessel density seen in four-cycle CSR and control rats is that this is a normal event during rat brain development. Reductions in blood vessel density due to aging have been reported in both rats and humans in many brain regions including the hippocampus and frontal cortex (Brown and Thore, 2011). These studies were concerned with changes in the anatomy of the brain during aging as it relates to various pathologies such as Alzheimer's disease. For that reason, they compared young adult brains to old brains. For example, in rats, blood vessel density is reduced in both the hippocampus and cerebral cortex at 27.5 months compared to 18 months (Jucker et al., 1990) as well at 24 months compared to 12 months (Amenta et al., 1995). Changes in blood vessel density have also been observed very early in life. Blood vessel density in the rat brain more than doubles from 3 days to 24 days of age. At this point density becomes stable until at least 33 days of age. (Ogunshola et al., 2000).

During the period between early postnatal development and middle age, changes in blood vessel density have not been a focus of research. In the four-cycle rats in the present study, blood vessel density decreased in both the PFC and hippocampus over a period from approximately 8 to 12 weeks of age. It is unknown what type of changes in cerebral blood vessels are typical at this point in a rat's development. The rats used in this study would be considered young adults and given that blood vessel density appears to have plateaued after 24 days of age, it seems unlikely that aging could contribute to a significant loss in blood vessel density during the four weeks of this experiment.

4.5 Increased BrdU staining of non-endothelial cells following one cycle of CSR

4.5.1 The PFC

In the PFC, no BrdU-positive endothelial cells were seen in any animal. There was, however, an increase in BrdU positive non-endothelial cells in sleep restricted, compared to control, animals. This is an interesting finding that merits further study to increase sample size and to identify the types of cells which proliferate in response to CSR using additional immunohistochemical markers. For example, possibilities that these cells could be glial cells such as astrocytes or microglia should be considered.

A previous study measured BrdU staining in adult male Wistar rats in the PFC and hippocampus (Czéh et al., 2007). When rats were injected with BrdU

three weeks before sacrifice, approximately 3000 cells were labeled in the PFC. It was observed that in the PFC of control animals, about 75% of BrdU-positive cells were neural/glial antigen 2 (NG2) -positive glial cells, 20% were endothelial and a small amount were unidentified. As endothelial cells were not found to be proliferating the PFC in the present study, the most likely candidates to account for increased proliferation after CSR are NG2-derived glia, which would include astrocytes, microglia and oligodendrocytes.

A study by Shannon Hall, another graduate student in our lab, found that colocalization of the microglial marker ionized calcium-binding adaptor molecule 1 (Iba1) with BrdU in the medial PFC after one cycle of CSR was extremely rare. This study used the same animals as the present study, leading us to believe that any rise in cell division taking place during CSR may not involve microglia.

To obtain definitive answers, immunohistochemical analysis including glial fibrillary acid protein (GFAP), myelin basic protein (MBP), and NG2 would allow analysis of BrdU colocalization with some of the most common glial cell types. GFAP is a component of the cytoskeleton present in most astrocytes (Oberheim et al., 2009). MBP is present in oligodendrocytes, cells that insulate axons in the brain (Xu et al., 2015). NG2 is found on the plasma membrane of glial progenitor cells. Expression of this protein is lost as cells differentiate and mature (Moshrefi-Ravasdjani et al., 2016). The PFC is particularly sensitive to sleep loss (Muzur et al., 2002) and identifying the proliferating cells may contribute to our understanding of the events leading to the decline in mood and cognitive functions resulting from CSR.

4.5.2 The hippocampus

In the hippocampus, the vast majority of BrdU-positive cells were seen in the granule cell layer, and most of them are likely neuronal progenitors, as this is the site of adult neurogenesis (Van der Borght et al., 2009). Adult hippocampal neurogenesis has been shown to be affected by sleep deprivation. For example, several studies have found a decrease in proliferation in the adult dentate gyrus (Guzmán-Marín et al., 2003; Mirescu et al., 2006; Mueller et al., 2008). When Brdu injections were given two hours before the end of sleep deprivations of various lengths, a biphasic effect on cell proliferation was observed. Proliferation increased after 12 hours of deprivation and decreased after 48 hours of deprivation (Junek et al., 2010). 30 days of CSR was not found to change rates of proliferation in the dentate gyrus (Novati et al., 2011). While preliminary, the results from the present study suggest that hippocampal neurogenesis may be increased during four days of CSR. As these rats were injected nine times throughout the four days of sleep restriction, the time course of the effects on neurogenesis over those four days is not clear and would require multiple treatment groups to determine.

An interesting follow-up study could include markers such as polysialylated neuronal cell adhesion molecule (PSA-NCAM), doublecortin and neuronal nuclear antigen (NeuN), markers of immature, more mature and mature neurons respectively. These markers would show whether the increased numbers of BrdU-positive cells seen following sleep restriction survive to become mature neurons.

4.6 Conclusions

This experiment set out to test the hypothesis that due to increased neuronal activity during wakefulness, CSR would lead to significant angiogenesis in the PFC. If this were the case, it would have important implications for the glymphatic system, which is thought to perform most of its waste removal function during sleep. Specifically, a higher density of blood vessels after CSR would increase the efficiency of glymphatic function and would provide a compensatory mechanism to offset the reduced time given to this system to work. The results of this study, however, did not support the hypothesized increase in angiogenesis. Although a small increase in newly grown blood vessels was found in the PFC after one cycle of CSR, this increase did not have an impact on overall blood vessel density, and it is unlikely that this CSR-induced angiogenesis has any appreciable consequence on the rate of glymphatic clearance in the brain.

This study emphasizes the importance of getting a good night's sleep. Toxic metabolites accumulate in the extracellular spaces of the brain during wake and must be removed by the glymphatic system. CSR, as conducted in the current study, does not induce structural changes to blood vessels which would make sleep more efficient for removal of metabolic waste.

4.7 Future Directions

The only significant effect of CSR on blood vessel density was a small increase in nestin-positive vessels after one cycle of sleep restriction in the PFC. This effect appears to be short-lived and was not observed after four cycles of CSR. It would be interesting to look at shorter time points such as one day as it seems that this effect might be stronger early during CSR. It is also possible that changes to blood vessels may occur after CSR has ended. Allowing a period of rest between CSR and sacrifice would make it possible to assess this possibility.

There is substantial variability in both the measures of blood vessel density and the measures of nestin-positive blood vessels used in this study. There are also large individual differences in sleep requirements (Orzeł-Gryglewska, 2010). Animals which are identified with behavioural tests, such as a physchomotor vigilance task, to be strongly affected by CSR may show a stronger correlation between CSR and measures of blood vessel density and nestin expression.

As previously mentioned, measuring the effect of the 3/1 CSR protocol on stress hormones such as corticosterone and ACTH would be helpful for interpretation of data, and would be useful knowledge for future studies making use of this protocol. A very interesting finding from this study was the increased BrdU staining observed in non-endothelial cells in both the PFC and hippocampus. The experiments proposed in section 4.5 could determine if this finding is valid by increasing the sample size and identifying the types of cell that are proliferating with additional immunohistochemical markers.

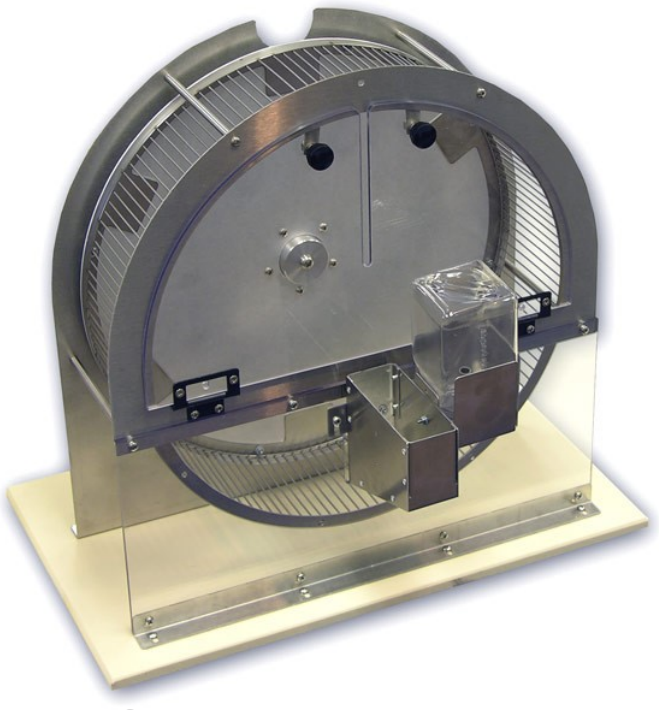

Figure 1: Activity wheels used for sleep deprivation in CSR. Rats were housed individually in these wheels for each four-day cycle of sleep restriction. These wheels are programmed to rotate at 2.5 m/min during the 3-hour sleep deprivation phases of the experimental protocol (see figure 2). Each wheel measured 35.5 cm in diameter and 11 cm in width. Image from lafayetteneuroscience.com.

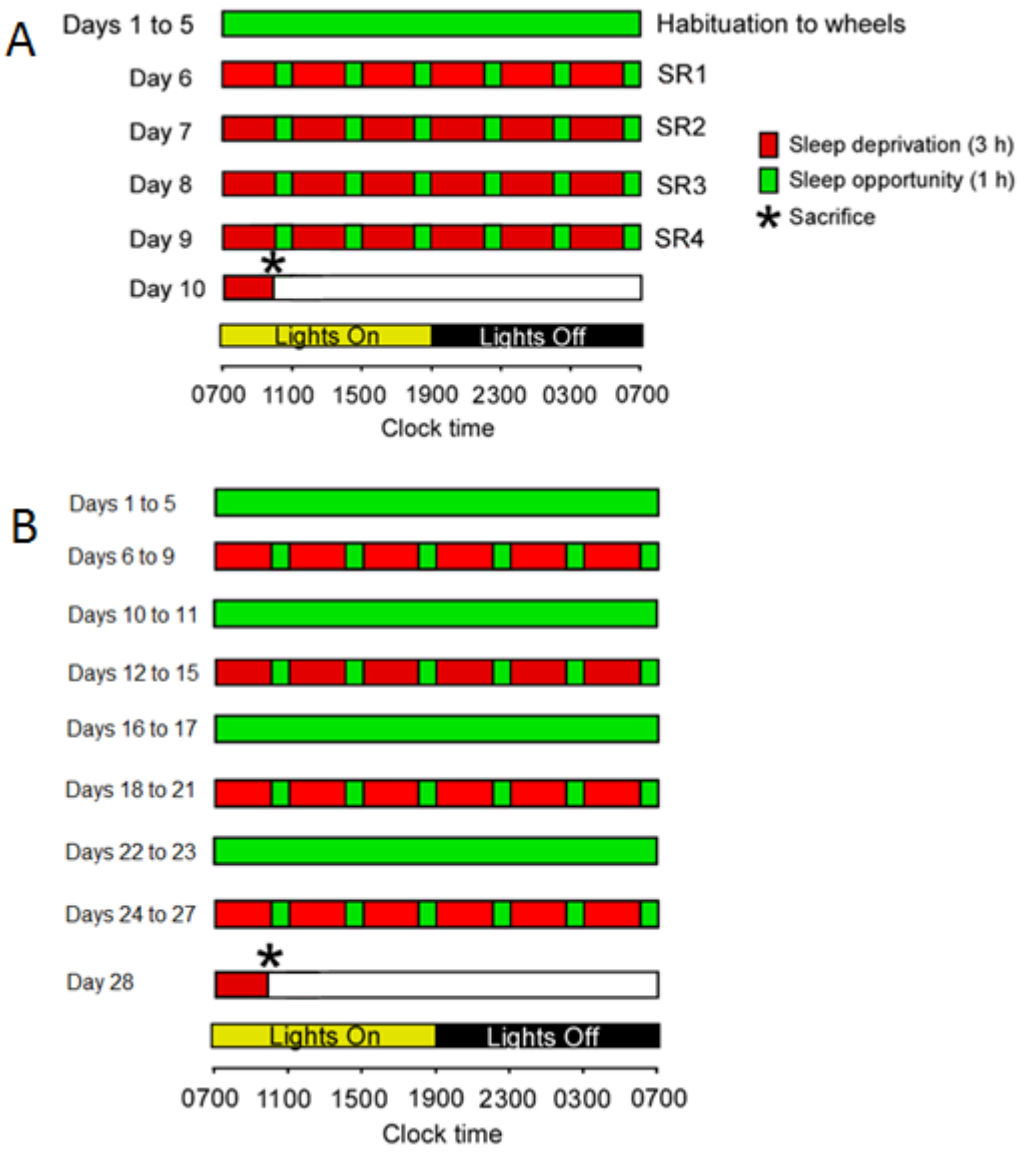

Figure 2: 3/1 protocol in one-cycle and four-cycle paradigms. A: One cycle of CSR. After 5 days of habituation, rats undergo 4 days of alternating 3 hour sleep deprivation periods and 1 hour sleep opportunities. One additional 3 hour sleep deprivation period was given on the fifth day of CSR prior to sacrifice. **B:** Four cycles of CSR. After the four-day cycle of sleep restriction, the wheels stop at 7am and rats are transferred into plastic home cages for 2 days. This process is repeated four times and one additional 3 hour sleep deprivation period is given on the final day prior to sacrifice.

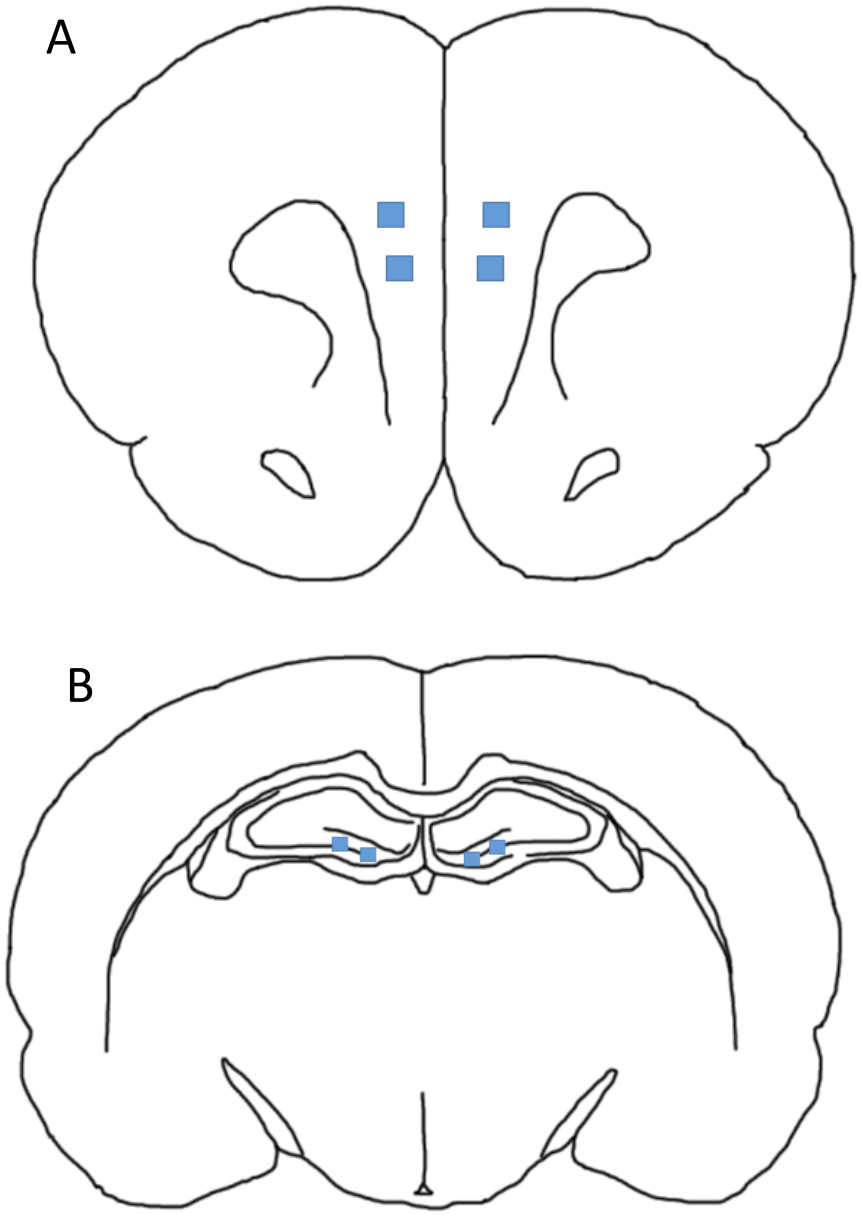

Figure 3: Regions of analysis in the prefrontal cortex (A) and the hippocampus (B). A: A diagram showing the size and positioning of image boxes in the prelimbic area of the prefrontal cortex, each centered between the midline of the brain and the forceps minor of the corpus callosum. Top and bottom boxes are approximately 300μm apart. **B:** Images were taken in the dentate gyrus of the hippocampus at the most medial point where the dentate gyrus was wide enough to accommodate the entire image box. An additional image is taken approximately 300μm lateral to the medial box. Adapted from Paxinos and Watson, 2006.

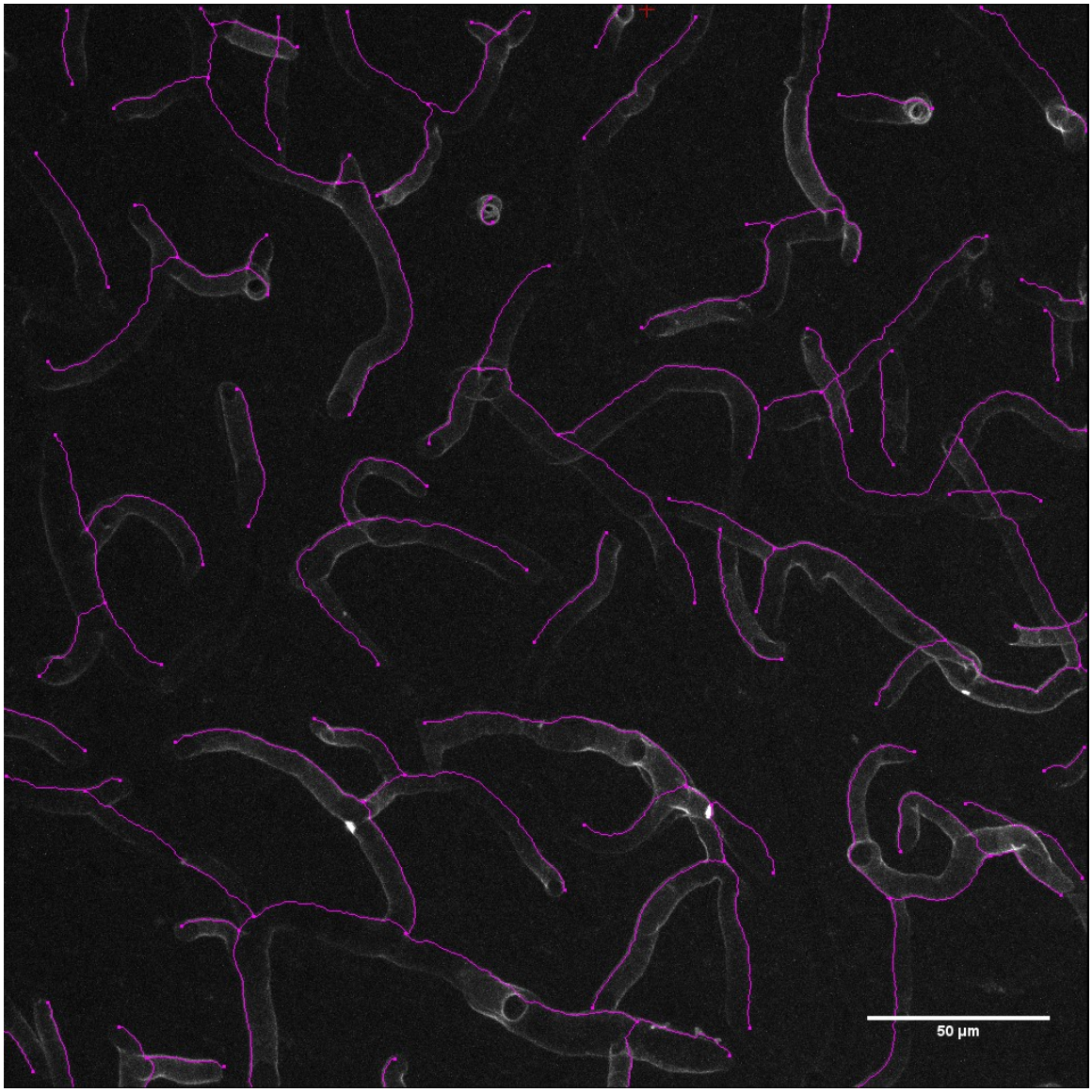

Figure 4: Blood vessel analysis using the simple neurite tracer ImageJ plugin. Manual tracings, shown in purple, allow quantification of the blood vessel length and the number of blood vessel segments in each image.

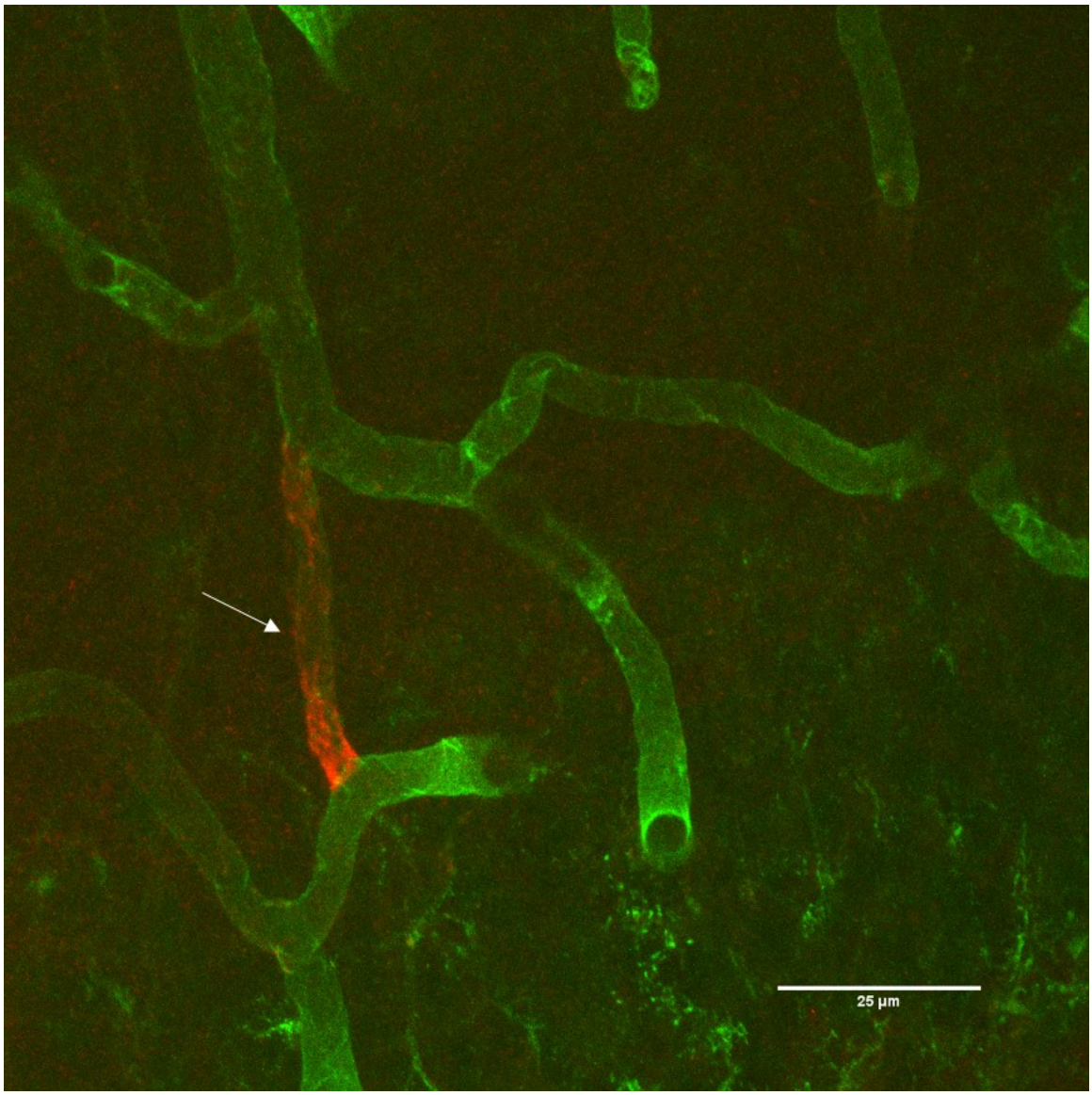

Figure 5: Identifying mature and newly generated blood vessels using tomato lectin and nestin. An example confocal microscope image at 63x magnification from rat hippocampus. Blood vessels are labeled in green with tomato lectin. Double-labeling with lectin in green and nestin in red identifies a blood vessel segment containing newly generated endothelial cells (arrow).

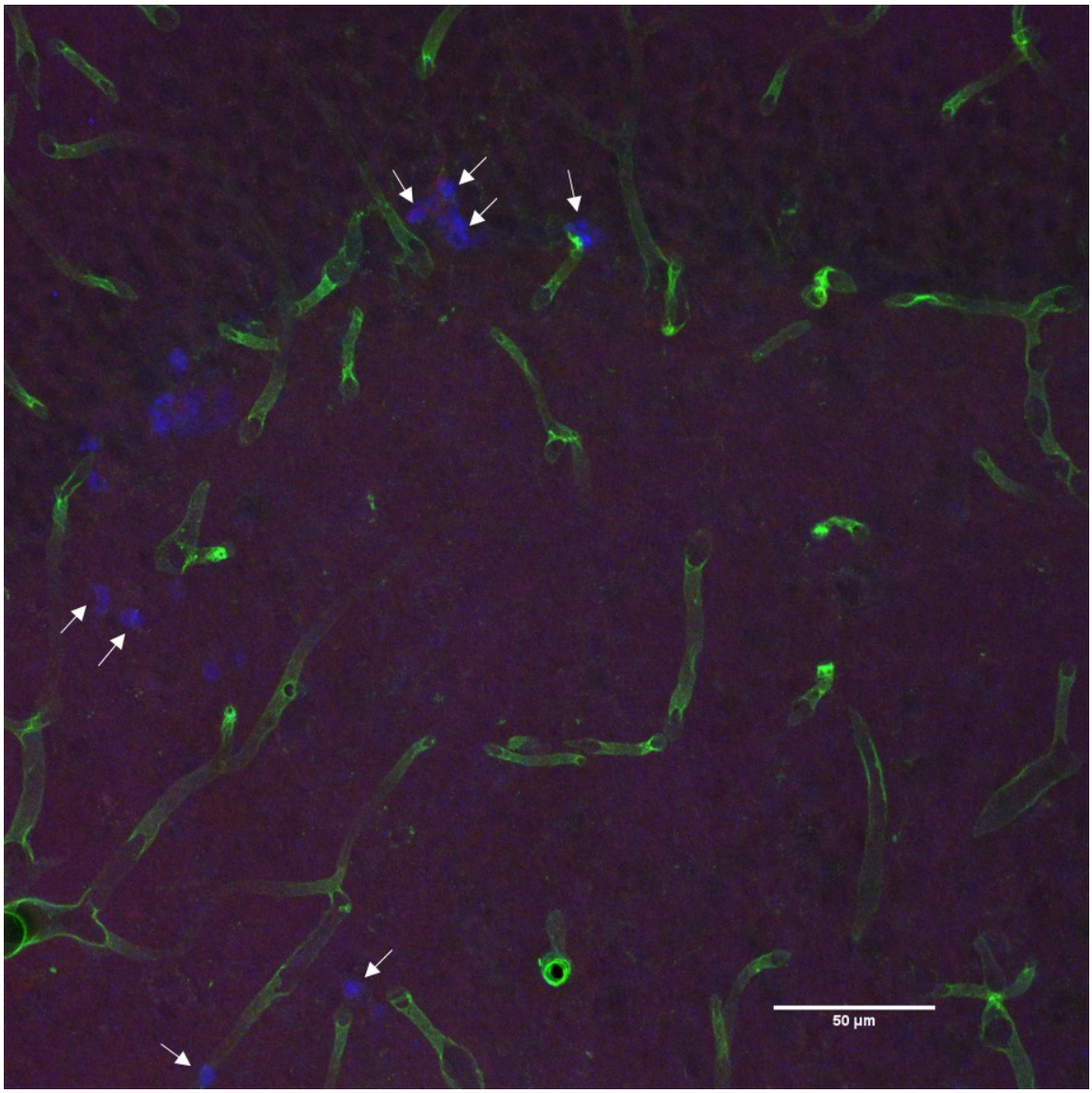

Figure 6: Example of a section of rat hippocampus labeled with tomato lectin (green) and BrdU (blue). In this image from a sleep restricted rat, none of the BrdU nuclei are colocalized with the tomato lectin blood vessel label. Most of the BrdU-positive cells are likely immature neurons. Arrows show examples of BrdU-positive nuclei.

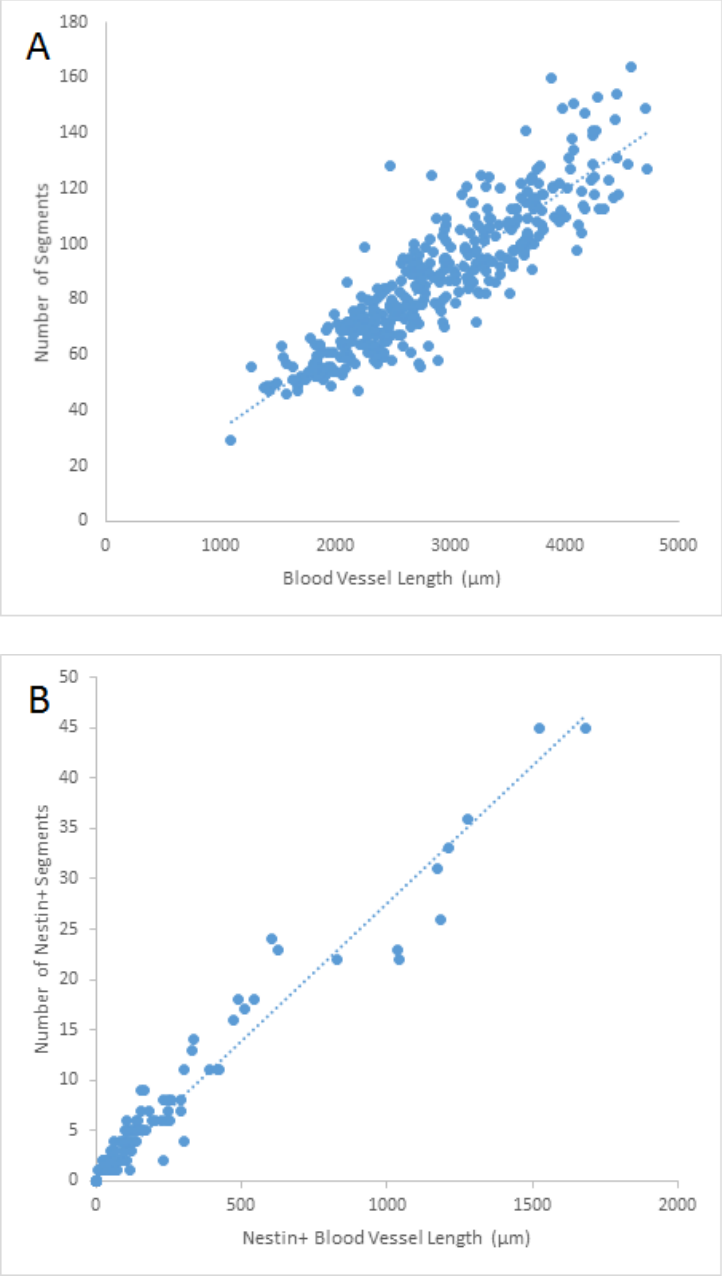

Figure 7: Correlations between two measures of blood vessel density and two measures of blood vessel growth. Each data point represents one image. Both correlations include data from both the PFC and hippocampus from sleep restricted (n = 12) and control (n = 13) animals. **A:** Correlation between two measures of blood vessel density, i.e., blood vessel length and number of segments (n = 400, r = 0.894, $p < 0.01$). **B:** Correlation between two measures of new blood vessel growth, i.e., nestin-positive blood vessel length and nestin-positive blood vessel segments (n = 400, r = 0.981, $p < 0.01$).

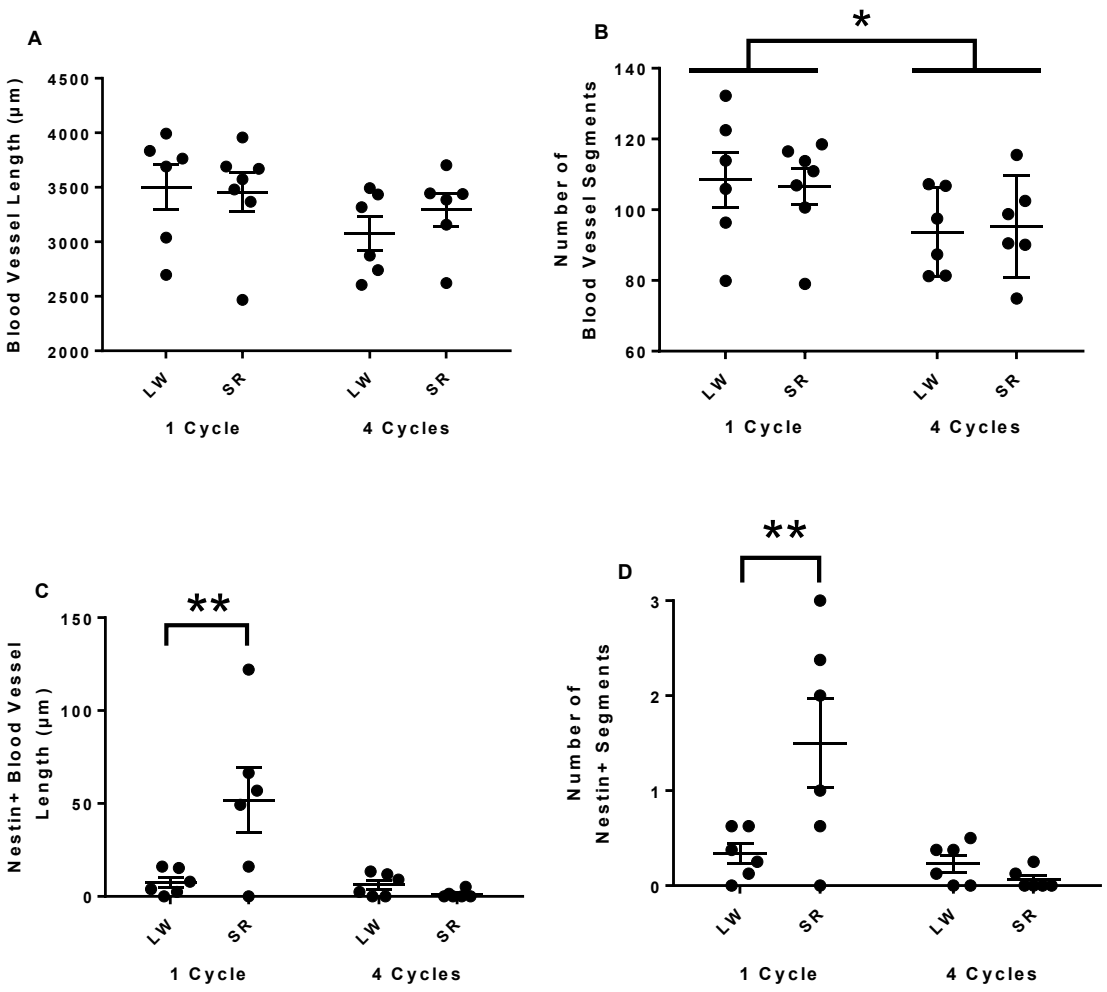

Figure 8: The effect of CSR on blood vessel density in the PFC. A: Total blood vessel length shows no significant difference between locked wheel (LW) and sleep restricted (SR) animals after one or four cycles of CSR. n = 6-7 for each treatment condition. **B:** Number of blood vessel segments did not differ between LW and SR animals after either one or four cycles of CSR. However, the number of segments was decreased after four cycles compared to one cycle irrespective of the treatment condition ($p = 0.04$) n = 6-7 **C:** Total nestin-positive blood vessel length was significantly increased in SR animals compared to controls after one cycle of CSR ($p < 0.01$). This effect was not observed after four cycles of CSR. n = 6 **D:** The number of nestin-positive blood vessel segments was significantly increased in SR animals after one cycle of CSR ($p < 0.01$). This effect was not observed following four cycles of CSR. n = 6

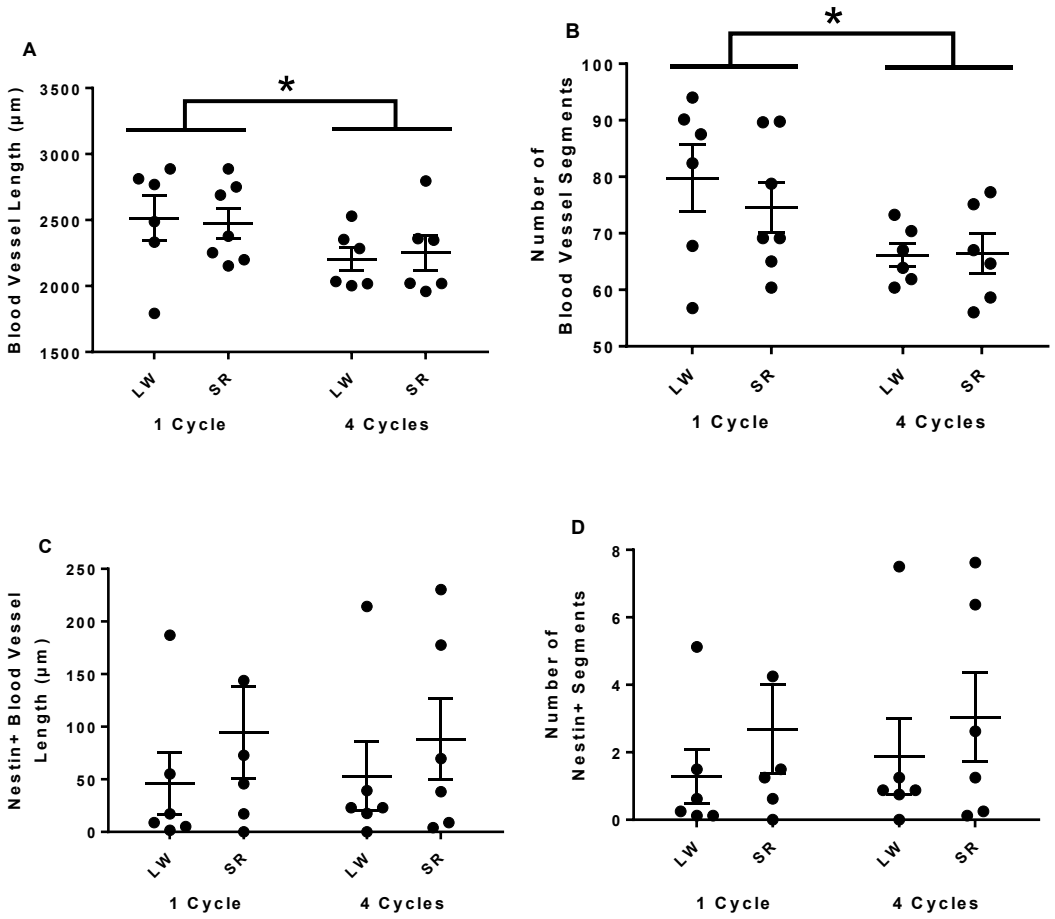

Figure 9: The effect of CSR on blood vessel density in the hippocampus. A: Total blood vessel length did not differ between the LW and SR groups. However, it was decreased after four cycles compared to one cycles regardless of the SR or LW condition ($p = 0.049$). n = 6-7 for each treatment condition. **B:** The number of blood vessel segments did not differ between the LW and SR groups after either one or four cycles of CSR. However, it was reduced after four cycles compared to one cycle ($p = 0.02$) n = 6-7. **C:** Nestin-positive blood vessel length did not differ between LW and SR animals after one or four cycles of CSR. n = 6. **D:** No significant difference was found in the number of nestin-positive blood vessel segments between LW and SR animals after either one or four cycles of CSR. n = 6.

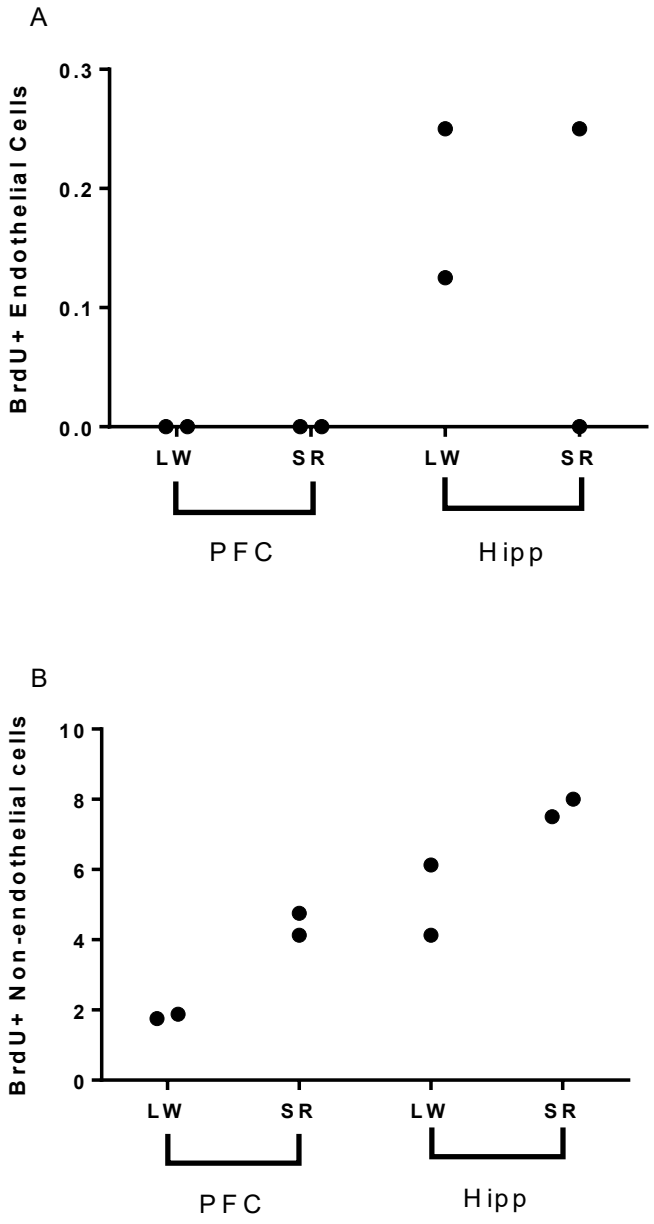

Figure 10: Counts (means of 8 images per animal) of BrdU-positive endothelial (A) and non-endothelial (B) cells following one cycle of CSR. A: BrdU-positive nuclei which colocalized with the tomato lectin blood vessel label were not seen in the PFC and were rarely seen in the hippocampus. CSR did not increase the prevalence of BrdU-positive endothelial cells. n = 2 **B:** BrdU-positive cells which were not associated with blood vessels were much more common. In both the PFC and hippocampus CSR was associated with higher numbers of non-endothelial BrdU staining. n = 2

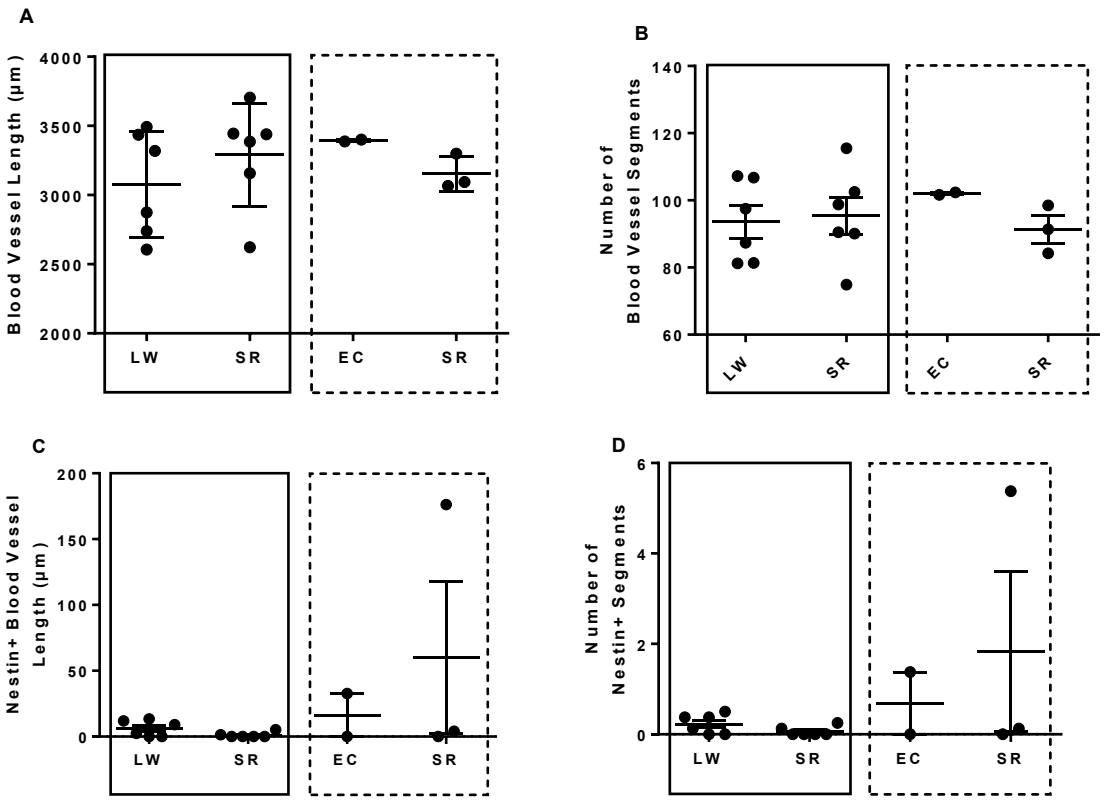

Figure 11: Blood vessel density data from the PFC of rats subjected to four cycles of CSR and two groups of controls. n = 6,6,2,3. LW and SR data in the solid boxes are the same as previously shown in figure 7. Exercise control (EC) and SR data are separated into the dashed boxes because these animals underwent the experimental treatments and tissue processing in a separate experiment conducted later. EC animals, which were able to exercise without being subjected to CSR, did not have significant differences in blood vessel length (A) or number of blood vessel segments (B) in the PFC compared to LW or SR animals. One EC animal and one SR animal had very high nestin expression, as measured by nestin-positive vessel length (C) or number of nestin-positive segments (D). These two animals did not have higher blood vessel density compared to other animals in their groups with much lower nestin expression. It is not clear why these high nestin values occurred in these animals, but the EC animals do not appear to be undergoing increased angiogenesis compared to the other groups.

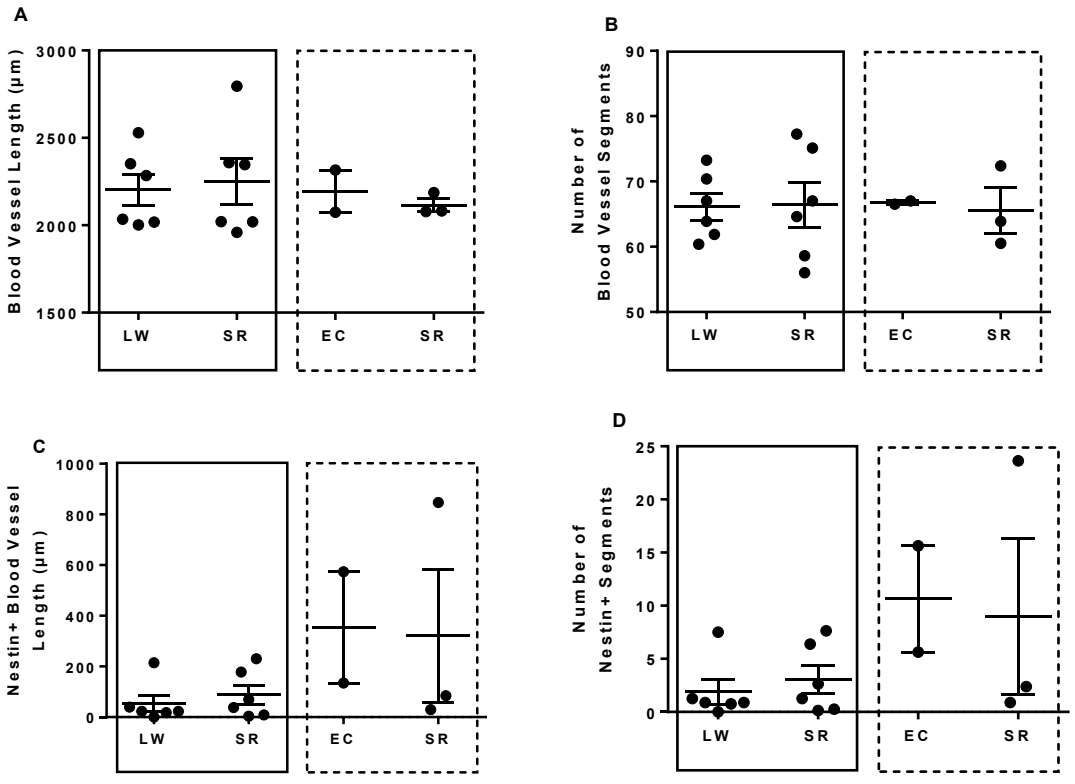

Figure 12: Blood vessel density data from the hippocampus of rats subjected to four cycles of CSR and two groups of controls. n = 6,6,2,3. LW and SR data in the solid boxes are the same as previously shown in figure 8. EC and SR data are separated into the dashed boxes because these animals underwent the experimental treatments and tissue processing separate from the LW and SR group. EC animals, which were able to exercise without being subjected to CSR, did not have increased blood vessel length (A) or increases in the number of blood vessel segments (B) in the hippocampus. As was the case in the PFC (see figure 11), one EC animal and one SR animal had very high nestin expression, as measured by nestin-positive vessel length (C) or number of nestin-positive segments (D). These are the same animals that have elevated nestin expression in the PFC. Elevated nestin expression in the hippocampi of these two animals is not associated with higher hippocampal blood vessel density compared to other animals in their groups.

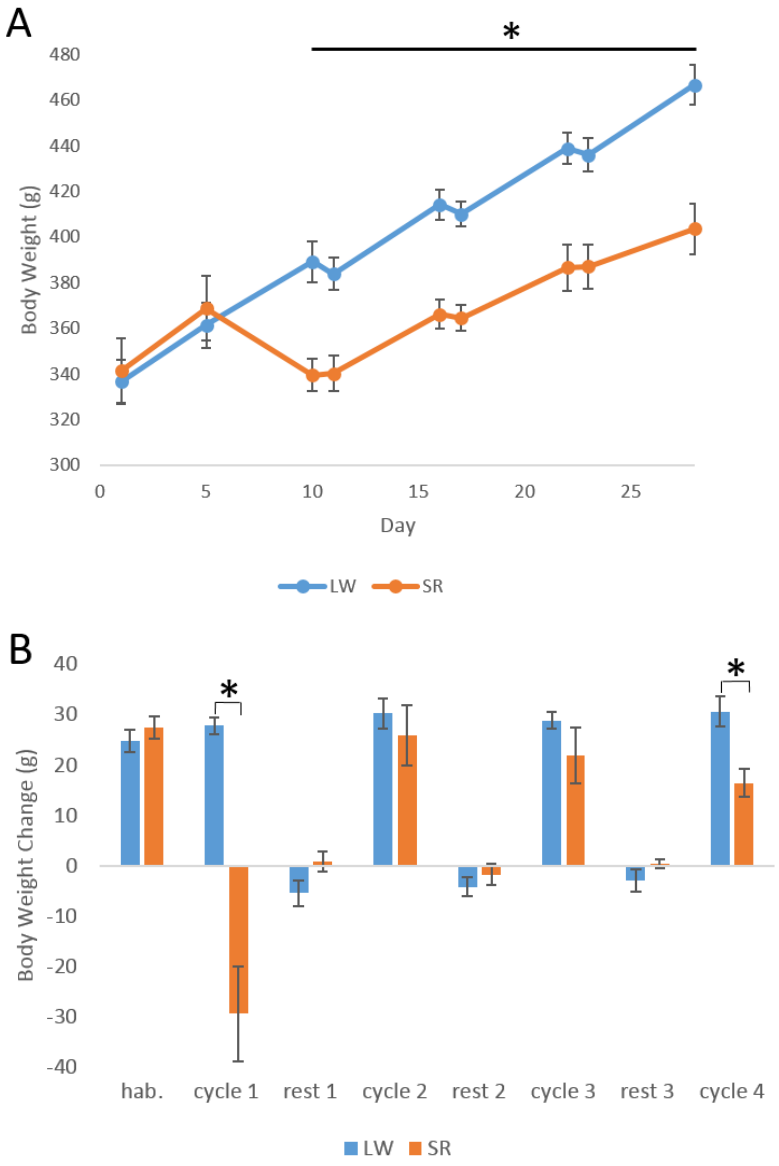

Figure 13: Rat body weights during four cycles of CSR treatment. n = 6 per group. **A:** Body weights for sleep restricted and locked wheel control rats were recorded at the start of habituation, and the beginning of each sleep restriction or rest period. **B:** Data from the same animals depicted as the change in body weight during each period of the experiment. Error bars show standard errors of the mean.

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