Diabetes exacerbates the loss of basilar dendritic spines after ischemic stroke

by

Andrew Sweetnam Holmes
B.Sc., University of Guelph, 2010

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

MASTER OF SCIENCE

in the Division of Medical Sciences (Neuroscience)

Supervisory Committee

Diabetes exacerbates the loss of dendritic spines after ischemic stroke

by

Andrew Sweetnam Holmes
B.Sc., University of Guelph, 2010

Supervisory Committee

Dr. Craig Brown, Division of Medical Sciences
Supervisor

Dr. Leigh Anne Swayne, Division of Medical Sciences
Departmental Member

Dr. Kerry Delaney, Department of Biology
Outside Member

Abstract

Supervisory Committee
Dr. Craig Brown, Division of Medical Sciences
Supervisor
Dr. Leigh Ann Swayne, Division of Medical Sciences
Departmental Member
Dr. Kerry Delaney, Department of Biology
Outside Member

Most stroke survivors recover some degree of lost function after an ischemic event. Recovery however, is negatively affected by comorbid conditions such as diabetes. Successful recovery is dependent on the ability of adjacent surviving cortical tissue and functionally related areas to take over functions lost by the stroke. Recently our lab has shown that diabetes interferes with the remapping of sensory function to peri-infarct areas after photothrombotic stroke. Given this result, it is crucial to understand how diabetes affects the structure of neurons following stroke, particularly at the level of dendritic spines, which receive the vast majority of excitatory synaptic inputs. Type I diabetes was pharmacologically induced in transgenic mice expressing yellow fluorescent protein (YFP) in a subset of cortical neurons 4 weeks prior to receiving unilateral photothrombotic stroke in the forelimb area of the primary somatosensory cortex (FLS1). Spine density measurements were made on the apical and basilar dendrites of layer-5 pyramidal neurons at 1 and 6 weeks after stroke. Our analysis indicated that diabetes was associated with fewer apical and basilar dendritic spines in the peri-infarct region 1 week after stroke. At 6 weeks of recovery, peri-infarct dendritic spine density in both control and diabetic animals returned to baseline levels. These changes were specific to the peri-infarct cortex, as spine density in distant cortical areas such as the forelimb sensorimotor region of the contralateral hemisphere, were not affected by stroke. In order to relate changes in spine density to the recovery of forepaw function, we re-analyzed data from a previous study that employed the forepaw adhesive-tape-removal test. This analysis revealed that diabetes significantly increased the latency of tape removal from the impaired forepaw (when normalized to the unaffected paw) at 1 but not 6 weeks of recovery. Collectively, these findings indicate that diabetes exacerbates forepaw impairments and basilar spine loss initially after stroke, but does not affect the ability of the brain to replace lost spines over weeks of recovery.

Table of Contents

List of Tables

List of Figures

Abbreviation

2-DG	2-deoxyglucose
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid
BBB	Blood-brain barrier
CCD	A charge-coupled device
CNS	Central nervous system
CONTRA	Contralateral
FLS1	Forelimb area of the primary somatosensory cortex
FLS2	Forelimb area of the secondary somatosensory cortex
fMRI	Functional magnetic resonance
HV	High Voltage
LTP	Long-term potentiation
MCAo	Middle cerebral artery occlusion
MMP-9	Matrix metalloprotease-9
mNSS	Modified neurological severity score
NIH	National Institutes of Health
NMDA	*N*-Methyl-D-aspartate
nNOS	Neuronal nitric oxide synthases
NO	Nitric oxide
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
ROS	Reactive oxygen species
STZ	Streptozotocin
VSD	Voltage sensitive dye
YFP	Yellow fluorescent protein
VC	Visual cortex

Acknowledgements

This research project would not have been possible without the support of many people. Foremost, I would like to express my gratitude to my supervisor Dr. Craig Brown who has offered invaluable assistance, support and guidance. Dr. Brown's guidance helped me learn the ins and outs of research and the motivation that inspired me to stay engaged through the most arduous parts of this work. Additionally, I would like to thank my committee members, Dr. Delaney and Dr. Swayne, for their invaluable input, and suggestions. Also, thanks goes to Dr. Adam Krawitz for taking the time to be on my committee and for his forthcoming input which will be much appreciated. I would especially like to thank Dr. Kelly Tennant for her abundant guidance, mentorship, and our morning meetings, whereby I learned more about science than anywhere else. Also, I would like to thank past/present members of Brown Lab—Angela Seto, Patrick Reeson, Abdul Shehata, Akram Zamani, Josh Wang and Danielle Sweetnam—for their friendship and support.

None of this research could have been possible without the exemplary and diligent work of the ACC staff, particular the work of Raymond Norris-Jones and Matt Gordon who made the basement of MSB my favourite spot to work. I would like to also take the time to acknowledge the outstanding work of Karen Myers, and thank her for her candid advice and never failing support, without which I would never have met any of the deadlines.

This research has been made possible in part by Mark Walle and Danielle Sweetnam who contributed their time and effort running the adhesive tape removal task. Lastly, I would like to extend my appreciation to Jarrett Moore and Pryia Roseenberg for their initial difficult work on spine counting.

Introduction

Stroke

The brain is an elegant tangle of neurons, glia and vasculature. As an organ it comprises only 2% of the total body weight, yet accounts for 20% of the body's oxygen consumption and 25% of glucose utilization. The brain's vascular system must adequately distribute blood flow to meet these high metabolic demands. Interruptions to the flow of oxygen and nutrients to the brain results in tissue damage and neurological dysfunction commonly referred to as stroke. The signs and symptoms of a stroke can include a sudden inability to speak, confusion, loss of language comprehension, weakness in the limbs and/or face, loss of muscle control, impaired vision, headache and dizziness.

In Canada, more than 50,000 individuals are diagnosed with stroke each year (Public Health Agency of Canada, 2009). This equates to one stroke every 10 min. Stroke is the third leading cause of death and the leading cause of long-term disability (Public Health Agency of Canada, 2009). Stroke significantly impacts personal relationships, stresses the resources of stroke support groups and caretakers, and creates a large economic burden due to the cost of providing lifelong healthcare to stroke survivors with major disabilities.

The two forms of stroke are haemorrhagic, comprising 20% of incidents, and ischemic, comprising 80%. Haemorrhagic stroke results from a rupture in the cerebrovasculature causing a release of blood into perivascular spaces damaging brain tissue (Wu et al., 2011). Ischemic stroke occurs when intracranial vessel(s) become blocked, significantly reducing blood flow resulting in tissue death. The recovery of individual stroke survivors varies greatly, ranging from permanent disability to recovery of a high level of function. The severity and type of neurological dysfunction depends on several factors, such as the size and location of the damaged tissue, the type of stroke, and the presence of pre-existing disease states like diabetes.

In the diabetic, the vascular endothelium is constantly exposed to abnormally high blood sugar levels. Not surprisingly, diabetics are more likely to suffer co-morbid conditions such as coronary heart disease, renal failure, blindness and stroke due to dysfunction of blood vessels within these organs. The trigger for this dysfunction is likely due to the fact that chronic hyperglycemia alters metabolic processes in the endothelium (Fatehi-Hassanabad et al., 2010), which leads to excess production of reactive oxygen species (eg. superoxide) and advanced glycation end-products (AGE). As endothelial dysfunction progresses, the endothelial wall becomes thicker, tight junction proteins are lost and vaso-motor responsiveness is impaired (Vinik and Flemmer, 2002). Dysfunctional endothelium leads to the release of tumour necrosis factor α (TNFα) (Fatehi-Hassanabad et al., 2010) which stimulates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB increases transcription of inflammatory cytokines, such as IL-1 and IL-6 which stimulates the production of adhesion molecules (ICAM and VCAM) in the endothelium, as well as the recruitment of monocytes and platelets (Kent et al., 2001; Martini and Kent, 2007). The end result is a highly pro-atherosclerotic environment with a narrowing of blood vessels, deposition of platelets and white blood cells against the vessel wall, and an increase in the likelihood of vessel blockage and hence ischemia. Therefore, this thesis will focus on ischemic stroke, since it is often associated with diabetes. Additionally ischemic stroke is the most common form and tends to be more localized, and more survivable than haemorrhagic stroke (Carmichael et al., 2005). Changes in the endothelium lead to the release cause a pro-atherosclerotic environment due to increased expression of adhesion molecules (ICAM-1 and VCAM-1), monocytes, and incre0ased platelet aggregation (Yorek and

Dunlap, 2002; Ferreira et al., 2006; Reagan., 2012). Furthermore, inflammatory responses in the diabetic vasculature become exaggerated due to the release

Functional cortical plasticity underlying stroke recovery

The process of recovery after a stroke can be divided into three distinct phases (Carmichael, 2003). Admittedly, this is an over-simplification of a complex and dynamic process, but it helps to frame the process of recovery. In the first few minutes to hours after stroke, there is activation of cellular repair mechanisms such as microglia which phagocytise dying cells and recruit phagocytic immune cells to the site of injury. Concomitant with microglial activation, astrocytes act to restore ion, water and glutamate homeostasis (Dirnagl et al., 1999), which, if left unregulated exacerbates ischemic damage. The second phase during the first week following stroke involves changes in neuronal excitability and altered activation of pre-existing pathways in an effort to compensate for the loss of functions carried by dying cortical circuits (Witte et al., 2000; Dijkhuizen et al., 2001, 2003). The last phase of recovery is the generation and stabilization of short- and long-range synaptic connections in the peri-infarct cortex and more distant but functionally related areas. This protracted phase likely underlies the progressive re-emergence of new functional brain activity patterns and improvements in sensory, motor or cognitive function that occur weeks to months after stroke (Stroemer et al., 1995; Brown et al., 2009).

Changes in the function of the stroke-affected cortex can be observed by a number of means. Functional magnetic resonance, 2-deoxyglucose (fMRI and 2-DG, respectively) and voltage sensitive dye (VSD) imaging in rodents have all shown that a unilateral ischemic stroke of the somatosensory cortex leads to an initial loss of cortical responsiveness in peri-infarct regions (Jablonka et al., 2007; Winship and Murphy, 2008; Brown et al., 2009; Van Meer et al., 2010). At the same time, sensory-evoked patterns of brain activity are increased in the secondary somatosensory cortex (Sweetnam et al., 2012) and more remote areas, such as the contralateral somatosensory cortex (Dijkhuizen et al., 2003). Over several weeks, rodent limb function appears to recover, although this is rarely a “full” recovery, similar to what has been observed in human patients. Behavioural tests have been designed to assess the specific aspects of recovery and quantify them. Examples of some of the behavioural tests used currently include: the pasta handling test to assess fine motor movement of the digits in mice (Tennant et al., 2010), ladder rung walking test for evaluation of skilled limb placement (Metz and Whishaw, 2002), cylinder test for measuring postural and paw use asymmetries (Shanina et al., 2006) and the adhesive tape removal task to assess sensory function and neglect (Sweetnam et al., 2012).

After stroke to the forepaw somatosensory cortex, mice show impairments in their ability to detect and remove tape from the affected forepaw or traverse a horizontal ladder (Sweetnam et al., 2012). Several human and animal studies have shown that the restitution of brain activity patterns in the damaged hemisphere correlates best with functional recovery (Nudo et al., 1996; Cramer, 2008; Sweetnam et al., 2012). Furthermore, the re-activation or re-mapping of sensory functions to peri-infarct and functionally related regions appears to play a causal role in behavioural recovery since ablating or silencing these areas leads to the re-appearance of behavioural deficits (Abo et al., 2001; Dijkhuizen et al., 2003; Zepeda et al., 2004; Sweetnam et al., 2012). Remodelling of dendritic spines is thought to underlie these changes in functional remapping and actually precedes map reorganization (Kleim et al., 2004; Wilbrecht et al., 2010).

Plasticity of cortical dendritic structure

The adult cortex retains the ability to alter neuronal connectivity through modifications of axonal projections and dendritic processes. Dendrites, particularly at the level of dendritic spines, have caught the eye of neuroscientists for more than century, starting with Ramon y Cajal who in 1888 observed dendritic spines using standard light microscopy and hypothesized about their function (Ramon y Cajal, 1899; Yuste, 2011). Now it is known that 90 % of excitatory connections in the cortex are synapses on to dendritic spines in the mammalian brain (Nimchinsky et al., 2002). Structurally, dendritic spines are specialized actin-rich protrusions. Actin filaments provide the backbone for spines and has been shown to influence structural features such as size, shape, motility and stability of dendritic spines as well as impact their synaptic strength (Cingolani and Goda, 2008). The actin present within dendritic spines is highly reactive and turns over in less than a minute (Star et al., 2002), thereby quickly facilitating changes to spine morphology. There are several actin-regulating pathways that have been identified, which regulate spine formation and disassembly. The Rho family of GTPases are the primary regulators of the actin cytoskeleton within dendritic spines. The Rho GTPase Ras, Rac1, Rnd1 and CDC42 have been implicated in spine formation and growth. Conversely, RhoA and Rap cause spine disassembly (Tada and Sheng, 2006a; Yoshihara et al., 2009). Rac and RhoA are suggested to regulate spine density and shape, RhoA causes a reduction in spine density and length, whereas Rac1 produces increased numbers of small spines (Yoshihara et al., 2009). These proteins need to be activated by guanine nucleotide exchange factors (GEFs) to switch between the inactive GDP form to the active GTP form, in order to act on downstream effector proteins (Tashiro and Yuste, 2008). The actin cytoskeleton also plays a role in organizing the post-synaptic density, anchoring post-synaptic receptors and shuttling synaptic machinery to the synapse (Sheng and Hoogenraad, 2007; Hotulainen and Hoogenraad, 2010; Renner et al., 2010). Disruption in the regulation of the actin cytoskeleton can lead to memory disorders, mental retardation and psychiatric disorders (Cruz-Martín et al., 2010).

Spines can be classified based on morphology into stubby, thin or mushroom, with each spine type varying in size, shape and length (Figure 1). Stubby spines are short with no neck, thin spines have a small head with a long slender neck, and mushroom spines have large bulbous shaped heads with a thin neck (Peters, Alan Kaiserman-Abramof, 1970; Harris et al., 1992). The typical length of a spine is 0.3–2 μm with a spine head volume ranging from 0.01 to 0.8 μm^3 (Harris and Kater, 1994). The geometry of spines makes them ideal for compartmentalizing biochemical processes. The presence of a long thin neck may act in some capacity to filter electrical signals, and limit calcium diffusion (Yuste and Majewska, 2001; Yuste, 2011). Studies have shown a direct relationship between the size of the spine's head and the size of the post synaptic density; as well as the number of AMPA receptors (2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid receptors) at the post-synaptic density (Nusser et al., 1998; Majewska et al., 2000a, 2000b; Matsuzaki et al., 2001). The size of the head therefore, correlates with synaptic strength and the length of the neck can act to regulate calcium diffusion from the spine head to the shaft (Arellano et al., 2007). Smaller spines are thought to be more susceptible to activity-dependent synaptic modifications, whereas larger spines are thought to be more stable (Matsuzaki et al., 2004). Therefore, there is a high degree of structural variability at the level of dendritic spines, which can impact the function and stability of a neuronal circuit.

Filopodia are another prevalent dendritic protrusion. Filopodia are typically long thin protrusions that are highly dynamic in nature, particularly during development (Figure 1B). Filopodia undergo rapid extension and retraction within minutes to hours (Yoshihara et al., 2009), likely sampling the neuropil for appropriate sites for synapse formation (Knott et al., 2006). Electron microscopy studies have shown that filopodia can usually be distinguished from classical spine types by the absence of a synapse. Although not all filopodia form mature spines, they are generally considered to be a precursor to spines (Knott et al., 2006).

Before we can understand how stroke affects dendritic structure, we must first describe how dendritic spines behave in the intact brain. Longitudinal quantitative analysis of spine turnover can be undertaken *in vivo* using two-photon imaging of fluorescently labeled apical dendritic tufts of layer-5 and layer-2/3 neurons (Grutzendler et al., 2002; Trachtenberg et al., 2002). The first two studies on dendritic spine turnover came up with different values for the proportion of stable spines in the neocortex. Grutzendler et al. (2002) examined the visual cortex (at 3–5 months of age) and determined that spines were quite stable with approximately 4% turnover per month (Grutzendler et al. 2002). The 2nd study examined the somatosensory cortex in 6–10-week-old mice and observed only ~50% of the spine population persisted over 1 month, suggesting that the majority of spines were stable for only a few days (Trachtenberg et al., 2002). Subsequent studies have shown for the most part that spines are, in fact, relatively stable structures in adult mice (Holtmaat et al., 2005; Zuo et al., 2005b; Majewska et al., 2006). The relatively high spine turnover rate observed in the Trachtenberg et al. (2002) study may have depended on the cortical area studied and the age of the mice. Spine turnover is greatest during development but persists, to a lesser extent, in the adult brain (Figure 1B). Holtmaat et al. (2008) compared spine turnover rates in the visual and somatosensory cortex and found higher spine turnover rates in the primary somatosensory cortex (turnover rate S1, 15.4%) compared to the visual cortex (turnover rate VC, 5.7%) (Holtmaat et al., 2008). During development, for the first ~1.5 months, there is a substantial over-production of dendritic spines, which results in a subsequent high level of spine elimination ('pruning') and a net loss of spines (Holtmaat et al., 2005; Zuo et al., 2005a). Later in life, spine formation and elimination are about equal and density stays constant (Holtmaat et al., 2005; Knott and Holtmaat, 2008). Interestingly, recently published data have shown that animals on the far end of the spectrum (>20 months) have reduced size and stability of spines but density was unaffected (Mostany et al., 2013).

Recent studies have examined spine dynamics in relation to specific learning tasks, such as skilled reaching or fear conditioning (Xu et al., 2009; Yang et al., 2009). After skilled reach training, there was a rapid increase in spine formation that began within an hour of the first training session. Behavioral improvements after learning were associated with spine remodeling in task-specific cortical areas (Yang et al., 2009), a finding that was verified with unbiased stereological measurements and electron microscopy (Kleim et al., 2002). However, spines that were formed during learning persisted at the expense of spines that existed prior to learning, which had been pruned (Xu et al., 2009). A fear-conditioning experiment by Lai et al. (2012) demonstrated that spine formation in frontal cortical neurons decreased during fear learning and increased during extinction (Lai et al., 2012). Activity-dependent forms of synaptic plasticity that are thought to play a role in learning, such as long-term potentiation (LTP), can increase the stability and number of dendritic spines by increasing the recruitment of PSD-95 (De Roo et al., 2008b; Yoshihara et al., 2009). These results suggest that structural changes at the level of dendritic spines can lead to alterations in functional connectivity and synaptic strength (Ivanco et al., 2000; Monfils and Teskey, 2004).

Alterations in dendritic structure may not always be beneficial. Aberrant spine morphology has been associated with psychiatric disorders such as depression, anxiety, and addiction, and contributes to the pathogenesis of many neurological diseases. In Fragile X syndrome, spines display an immature phenotype and occur at a higher density than age-matched controls, whereas individuals with Down’s syndrome generally have a lower spine density

(Cruz-Martín et al., 2010). Addiction to stimulants such as amphetamine and cocaine are associated with an increase in both dendritic branching and spine density, particularly in the nucleus accumbens and the prefrontal cortex (Robinson and Kolb, 2004). Together these studies demonstrate that remodelling of dendritic spine number and shape has been associated with adaptive behaviours such as learning and negative ones such as drug addiction.

Figure 1: Spine dynamics and morphology

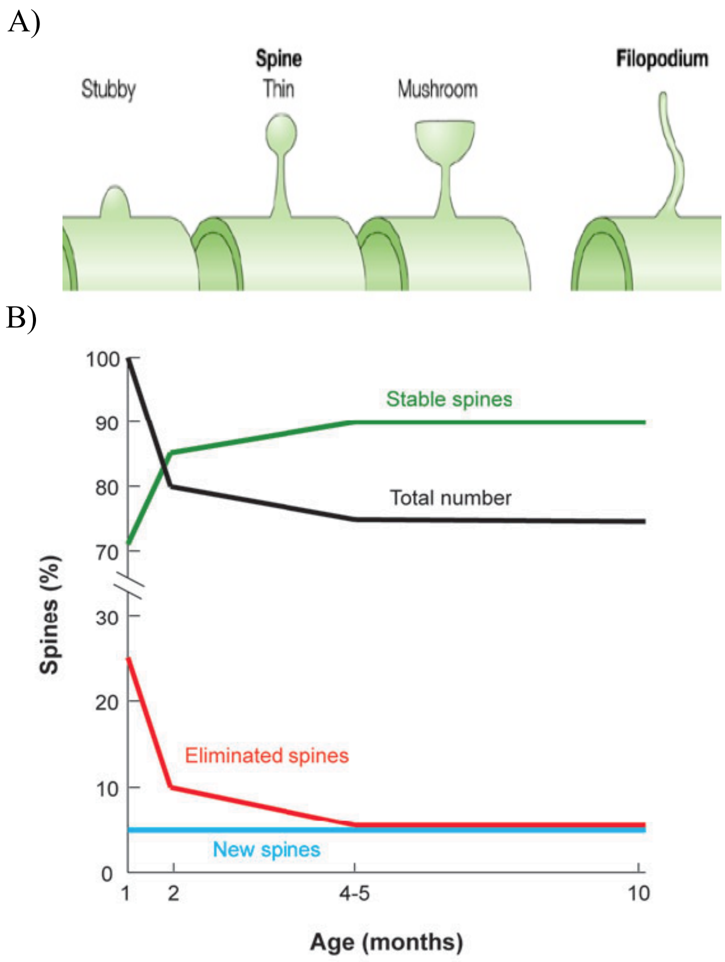


Figure 1: (A) Dendritic spine morphologies. Picture adapted from (Yuste and Bonhoeffer, 2004). (B) The rate of spine formation and elimination changes throughout normal development. The graph shows, in the first months of development, that the rate of spine elimination (red) exceeds the rate of formation (blue) by 3 months of age, while spine elimination declines to reach rates matching those of spine formation, resulting in no net change in spine density. Subsequently, there is an increase in the number of stable spines (green) and a decrease in the total number of spines (black) during this period (model based on data from Grutzendler et al. 2002, Holtmaat et al. 2005, Zuo et al.2005). Modified from (Alvarez and Sabatini, 2007).

Dendritic plasticity after stroke

Several studies from the Murphy laboratory and others have characterized dendritic changes during the first few minutes to weeks after a permanent or transient ischemic episode (Zhang et al., 2005; Zhang and Murphy, 2007; Brown et al., 2008, 2010; Li and Murphy, 2008; Murphy et al., 2008). In the healthy brain, synapses are normally found at a maximum of ~14 µm from a flowing vessel. After photothrombotic stroke, the maximum distance a neuron was found from a flowing vessel and still survived was approximately ~80 µm. This suggests that 80 µm is the limit to which sufficient O_2 and glucose can diffuse through the neuropil (Zhang and Murphy, 2007) to keep dendritic structures intact. This limit gives the cerebrovascular system some redundancies for the supply of O_2 and nutrients, perhaps explaining why dendrites show resistance to mild ischemia. Dendrites are highly sensitive to severe bouts of ischemia where blood flow is reduced to ≤80% of normal. Within 10 min of severe ischemia, signs of swelling, beading, loss of spines and even loss of dendritic arbours occur (Zhang et al., 2005). Amazingly, these same dendrites are not completely lost if reperfusion occurs within 60 min of occlusion, as some damage is reversible (Li and Murphy, 2008). The resilience of dendrites in response to injury has been documented in other conditions, such as re-warming of hypothermic tissue (Kirov et al., 2004) or the application of NMDA (*N*-Methyl-D-aspartate) antagonists to beaded dendrites (Ikegaya et al., 2001).

For neurons subjected to less severe restrictions in blood flow, as is the case in the peri-infarct or “penumbral” region, retaining some semblance of normal synaptic connectivity is critical. Zhang et al. (2005) and Brown et al. (2008) showed that peri-infarct neurons lose up to 40% of spines on apical dendritic arbors in the 24 hours following ischemic insult. Ultrastructural analyses of the ischemic penumbra confirmed that spine loss was associated with a loss of pre-synaptic boutons and putative synapses (Ito et al., 2006). Longitudinal *in vivo* imaging of spines has shown that the initial period of spine loss continues for 1 week followed by a sustained period of spine formation for up to 6 weeks after stroke (Brown et al., 2009; Mostany et al., 2010). Correlated with changes in spine turnover, spine density measurements showed a progressive return to normal density by 6 weeks of recovery (Brown et al., 2007). Of note, the extensive remodelling of dendritic spines occurs primarily in the peri-infarct cortex and not in homotopic regions of the contralateral cortex (Mostany et al., 2010; Johnston et al., 2012). The extent to which dendrites remodel over several days to weeks after stroke also appears to correlate with the level of blood flow in the peri-infarct area (Mostany et al., 2010).

Pathology of diabetes

Diabetes has become a serious health concern in recent years, with approximately 285 million people worldwide affected by the metabolic disorder (Public Health Agency of Canada and Ottawa, 2011). The defining feature of diabetes is uncontrolled high blood sugar, or hyperglycaemia. Diabetes is characterized by excessive urination (polyuria), excessive thirst (polydipsia), extreme weight loss and blurred vision (American Diabetes Association, 2010)

There are three types of diabetes, distinguishable by the body’s interaction with insulin: Type I (insulin-dependent), Type II (insulin-independent or insensitive), and gestational diabetes (a form of Type II diabetes that occurs during pregnancy). Although Type II diabetes is the most common (~90% of cases), my experiments utilized an inducible model of diabetes that is similar to Type I diabetes in humans. This literature review is therefore focused on Type I diabetes, which is an autoimmune disease that selectively destroys the insulin-secreting β cells in the pancreas. Type I diabetes can be induced in animal models by injecting streptozotocin (STZ), a β cell-selective toxin that mimics the autoimmune onset and disease pathology of Type I diabetes

(Etuk, 2010). These animals have symptoms similar to what is seen clinically, such as uncontrolled hyperglycaemia, polyuria, polyphagia, polydipsia, and weight loss.

The onset of Type I diabetes in humans is quick and commonly starts during puberty. The disease progresses with age, worsening if blood glucose levels are not carefully regulated. Prolonged uncontrolled severe hyperglycaemia can cause adverse effects such as ketoacidosis, coma and death. Individuals with Type I diabetes depend on exogenous insulin injections daily to regulate and maintain blood glucose at near normal levels. Insulin therapy significantly enhances the quality and duration of life for diabetics; however, it is not the cure-all, but a method to manage and cope with this disease. Insulin-treated patients, however, still face an assortment of health issues including problems with circulation in the eye, kidney and heart, that can ultimately lead to blindness, kidney failure and coronary artery disease, respectively (Baird et al., 2003). Diabetes is also correlated with an increased incidence of vascular dementia, Alzheimer’s disease and depression (Luchsinger et al., 2001; Forbes and Cooper, 2013). The diverse array of complications suggests that diabetes affects all organs in the body, including the brain.

The effect of diabetes on the central nervous system

Recently, an abundance of evidence has demonstrated that the deleterious effects of chronic diabetes extend to the central nervous system (CNS). Clinical and pre-clinical studies of Type I and II diabetes have revealed impairments in cognitive function, cognitive flexibility, synaptic plasticity, neurogenesis, vascular function and angiogenesis (Brands et al., 2005; Stranahan and Mattson, 2008; Zhang et al., 2008; Ergul et al., 2009; Reijmer et al., 2011).

Long-term uncontrolled hyperglycaemia in animals can lead to both behavioural and morphological changes in the brain (Magariños and McEwen, 2000; Martínez-Tellez et al., 2005; Sweetnam et al., 2012). Chronic hyperglycaemia in diabetic rats leads to impaired long-term spatial memory and a reduction in dendritic branching and spine density in hippocampal CA1 neurons (Malone et al., 2008). Insulin and corticosterone therapies were both shown to protect against the simplification of hippocampal dendritic arbours, and loss of spines in the hippocampus of Type I diabetic rodents (Magariños et al., 2001; Stranahan et al., 2008). Recently, it was found that diabetes can cause maladaptive dendritic spine remodelling in the spinal cord (Tan et al., 2012), resulting in the loss of mushroom-shaped spines and increased the numbers of thin spine types. Similar results were observed in the neocortex of diabetic rats (Martínez-Tellez et al., 2005). Chronic uncontrolled diabetes resulted in a reduction in branch length and spine density of layer-2/3 and 4 neurons in the parietal and occipital cortices (Martínez-Tellez et al., 2005). The observed alterations in dendritic structure throughout the CNS suggested that dendrites are sensitive to diabetes-induced damage.

Diabetes exacerbates the effects of stroke

A strong connection exists between diabetes, hyperglycaemia and stroke that has not been well examined or explained. Diabetes is considered a major risk factor for stroke, second only to hypertension; equal to lifelong smoking and coronary artery disease (Canadian Stroke Network, 2011). The Copenhagen Stroke Study (Jorgensen et al., 1994) found that diabetes did not affect the initial severity but it did affect the speed of recovery from stroke. It also lowers the age of incidence for a first stroke and increases mortality in human patients (Jorgensen et al., 1994). Diabetes was linked to increased severity of impairment and poorer recovery of function (Capes et al., 2001; Lindsberg and Roine, 2004). In humans, poor stroke recovery is reflected in the increased length of hospital stays, long-term disability and loss of independence. A large European clinical study found that diabetics who suffered a disability as a result of stroke had a reduced ability to recover (Megherbi et al., 2003). Inconsistent findings in the literature fail to reconcile whether diabetes is linked to an increase in mortality (Jorgensen et al., 1994; Hamidon and Raymond, 2003; Megherbi et al., 2003; Nannetti et al., 2009).

Vascular complications are a hallmark of diabetes and are further exacerbated by ischemic damage to the cerebrovasculature. Cerebral ischemia disrupts blood–brain barrier (BBB) integrity and results in edema and the extravasation of vascular contents. Diabetics show greater levels of blood plasma extravasation, edema and neuronal tissue damage after stroke compared to non-diabetics (Luitse et al., 2012; Ning et al., 2012). Even in the absence of ischemia, diabetic animals show increased vessel tortuosity and vascular remodelling compared to non-diabetic animals (Ergul et al., 2009; Elgebaly et al., 2011). Matrix metalloprotease-9 is suspected to play a role in vascular dysfunction since MMP-9 inhibition reduces aberrant vascular remodelling and the disruption of the BBB. Given the fact that diabetes disrupts the integrity of the vascular system and its response to stroke, it is conceivable that peri-infarct dendritic structure and plasticity may also be compromised.

Rationale

Changes in dendritic spine number, shape and turnover rates following stroke is well characterized. However, these studies for the most part, have characterized the response to stroke in young healthy animals, which obviously does not closely reflect clinical realities. Diabetes is a major risk factor for ischemic stroke and it has been associated with greater severity of long-term functional disability. Previously, we have shown that an ischemic stroke in diabetic mice led to persistent deficits in forelimb sensorimotor function and interfered with the brain's ability to remap lost sensory functions onto the intact peri-infarct cortex (see Figure 2). The main goal of this study was to investigate whether poor recovery after stroke in diabetic mice was associated with changes in dendritic spine density in the peri-infarct cortex and other relevant regions, such as the homotopic region of the contralateral hemisphere. I hypothesized that diabetes will either exacerbate the initial loss of spines after stroke or limit the progressive re-population of lost spines.

Figure 2: VSD imaging shows that diabetes impairs re-mapping of the forelimb sensory representation after stroke

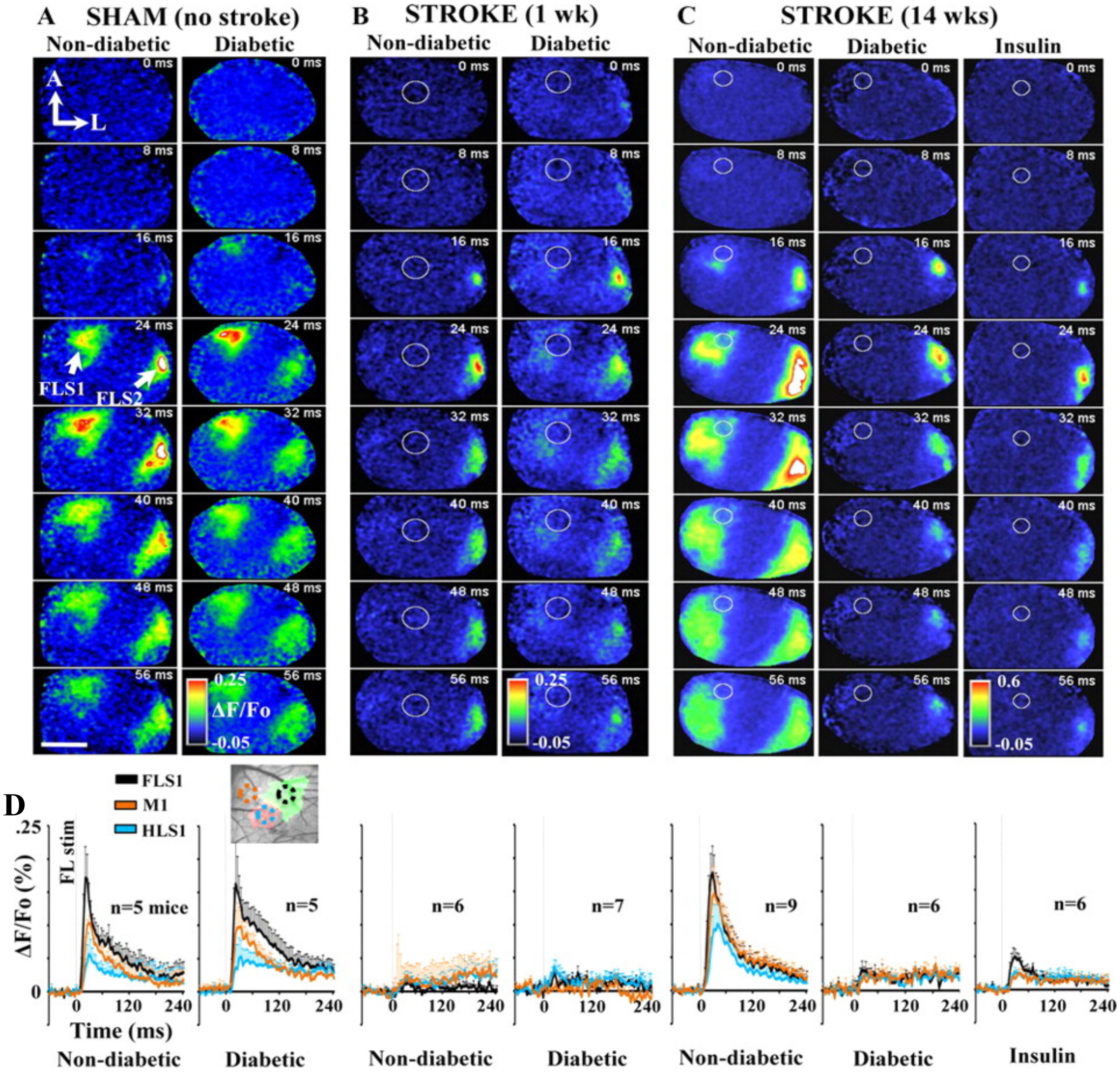


Figure 2. VSD imaging shows that diabetes impaired the remapping of sensorimotor function in the intact peri-infarct cortex. In figures *A–C*, the VSD imaging allowed for the spatiotemporal dynamics of cortical responses to forelimb stimulation to be visualized. Below each montage, the Δ*F*/*F*$_0$ plots average cortical responses to forelimb stimulation. Cortical responses in the forelimb cortex are shown in black, the orange line represents motor cortex, and the teal line is the hind limb primary sensory cortex. (A) In sham stroke, cortical responses to forelimb stimulation are similar between non-diabetic and diabetic mice. (B) One week after stroke (the white circle denotes the infarct region), the peri-infarct cortex shows little response to forelimb stimulation. (C) Fourteen weeks after stroke, forelimb-evoked depolarizations in the non-diabetic group was remapped in the peri-infarct cortex; however, the diabetic or insulin-treated mice did not show significant remapping. *Scale bar,* 2 mm modified from (Sweetnam et al., 2012)

Materials and Methods

Experimental overview

Two to three month old male YFP positive mice on a C57BL/6 background (YFP-H line; (Feng et al., 2000) were selected for the study. The transgenic YFP-H mouse line is well suited for the study of dendritic spines as it expresses YFP in the cytoplasm of a subset of cortical neurons, providing "Golgi staining-like" detail of dendritic morphology. All experiments were conducted according to the guidelines laid out by the Canadian Council for Animal Care and approved by the University of Victoria Animal Care Committee. Mice were group housed on ventilated racks on 12-hour light/dark cycles. Food and water was provided *ad libitum*. For this study, diabetic and non-diabetic mice were killed at 1 and 6 weeks recovery from photothrombotic stroke (1 week: diabetic n=6, non-diabetic n=7; 6 weeks: diabetic n=5, non-diabetic n=6). Time points were based on previous work analyzing dendritic spine turnover rates and densities in non-diabetic mice subjected to ischemic stroke (Brown et al., 2007, 2009). Sham stroke surgery was performed on six diabetic and five non-diabetic (n=5) mice, which were killed 1 week after surgery.

Induction of Type I diabetes and monitoring of blood glucose.

Intraperitoneal injections of 75 mg/kg streptozotocin (STZ) were given once daily for two consecutive days to induce Type I diabetes (Wu and Huan, 2008). Prior to injection, mice were food deprived for 4 hours. Immediately after injection, food was replaced and 5% sucrose solution was provided to prevent post-injection hypoglycaemia, while water was replaced 24 hours following the second injection.

The induction of diabetes was confirmed 3 days after the second STZ injection. A hand-held blood glucose meter (Accu-Chek, Aviva, Roche) was used to obtain glucose measurements from a small sample of fresh tail blood from fasted mice. Mice with blood glucose levels ≥14 mmol/L were considered diabetic. Blood glucose measurements were taken again prior to stroke and before tissue collection. Animals had to have met hyperglycaemic criteria (≥14 mmol/ml glucose) at all blood glucose sampling time points to be considered diabetic for the purposes of this study. Controls (i.e. non-diabetic mice) consisted of mice that were injected with only the vehicle solution or those that did not develop hyperglycemia after STZ injection.

Stroke induction

Focal ischemic stroke of the right forelimb somatosensory cortex was induced using the photothrombotic method (Watson et al., 1985; Sweetnam et al., 2012). This method is useful because of the highly targeted and reproducible nature of the stroke. Surgical procedures were performed in a fume hood using isoflurane anaesthesia (2% induction and 1.25–1.5% maintenance, in oxygen) and sterile techniques. The mouse was placed on a surgical stage with its head secured by atraumatic ear bars and an incisor bracket. Body temperature was regulated and maintained at 37°C with a heating pad and digital rectal thermometer. The skull over the forelimb cortex was exposed via a small incision over the midline followed by removal of connective tissue. Using stereotactic coordinates with Bregma as a reference, the skull above the forelimb somatosensory cortex was identified and thinned until transparent using a high-speed dental drill. Mice received an intraperitoneal injection of 1% rose bengal dye (110 mg/kg, i.p.) dissolved in 0.9% saline. Photothrombosis was initiated by directing a collimated green (532 nm) laser beam on the thinned area of the skull for 15 min (laser power = 17 mW; ~1.25 mm diameter). For sham surgery, mice received either a rose bengal injection or laser exposure, but not both. The incision was sutured and the mouse was allowed to recover under a heating lamp.

Tissue processing

After 1 or 6 weeks of recovery, mice were overdosed with sodium pentobarbital and transcardially perfused with 9 ml of phosphate buffered saline (PBS) followed by 9 ml of 4% paraformaldehyde (PFA) in PBS. Whole brains were submerged in 4% PFA and post-fixed for 24 hours, then transferred to a 30% sucrose solution. Brain sections were cut at 50 or 100 μm on a vibratome in the coronal plane and stored in 6 well trays in PBS with 0.2% sodium azide. The 100-μm-thick sections were used for quantification of basilar dendrites as it allowed for more of the dendritic tree to remain intact in order to trace dendrites back to their soma of origin. Serial sections (every 6th section) were then mounted onto glass slides and coverslipped with fluorescent mounting media (Fluoromount-G, Southern Biotech). Another set of brain sections was mounted onto glass slides and stained with cresyl violet for analysis of infarct volume.

Confocal imaging of dendritic spines

Brain sections were imaged with a 10x objective (NA= 0.40) and 60x oil objective (NA = 1.35) using an Olympus confocal microscope controlled by Fluoview software. Low (10x) magnification images were taken first to identify the infarct border. The infarct border was designated by of the loss of YFP-labeled neurons in the infarct, sharply delineating the border between intact and damaged tissue. The 300-μm section of tissue flanking the infarct was considered to be “peri-infarct” tissue (Zhang et al., 2005). We chose to only analyze the 300-μm area medial to the infarct because this is the typical location of cortical rewiring and remapping after FLS1 stroke (Winship and Murphy, 2008; Brown et al., 2009, Sweetnam et al., 2012). Within this area, basilar and apical dendrites (within layer 2/3) of layer-5 YFP-labelled neurons were identified and targeted for higher magnification imaging. High-resolution 1024 × 1024 image stacks (0.103 µm/pixel) were collected using a 515-nm laser to excite YFP. Image stacks were collected in 0.4–0.5-µm z-steps for basilar and apical dendrites respectively. Laser power was manually adjusted during imaging to maintain maximal saturation of pixel intensity at the dendritic shaft. Spine counting was performed manually with the cell counter plug-in using NIH (National Institute of Health) ImageJ software (http://rsbweb.nih.gov/ij/) by an experimenter blind to experimental conditions. In order to normalize highly variable fluorescence intensities between different neurons, we set the grey scale of each image to 40% of the maximum pixel intensity of the dendritic shaft. Only spines that protruded laterally were counted, and no morphological assessment of spine shape was done because our imaging technique had insufficient resolution to make such distinctions. For a protrusion to be counted as a spine, it must have been clearly visible, projecting at least 0.5 µm (5 pixels) from the dendritic shaft. Only apical dendrites with a shaft width between 1.5-4 um were selected for analysis. We could not definitively distinguish filopodia from the long, thin spine type, so all types of dendritic protrusions were considered to be spines. The total number of spines counted and the total length of dendritic segments were recorded for each animal. Spine density (the number of spines per 10 µm of dendrite) was calculated by dividing the total number of laterally oriented spines by the length of the dendrite segment and multiplying by 10.

Quantification of infarct volume

Images of cresyl violet stained brain sections were captured using a 4x objective with a 12-bit CCD (charged coupled device) camera and Image Capture software. Infarct analysis was preformed using NIH ImageJ software by measuring the area of the infarct in each brain section. All measurements were conducted blind to experimental conditions. Each section was measured three times to ensure consistency. Estimates of volume were calculated by summing the infarct areas for each section and multiplying by the distance between each section (Shih et al., 2005).

Statistical analysis

Statistical analysis of the data was conducted in SPSS 20 (SPSS Inc., Chicago, IL). Two-way analysis of variance (ANOVA) was performed to compare the mean spine density between groups at different time points. Significant main effects from the ANOVA were followed up with independent-sample *t*-tests. Paired t-tests were used to examine the effect of stroke on spine density between the ipsilateral and contralateral hemispheres. All p values ≤ 0.05 were considered statistically significant. Data are presented as mean ± SEM.

Results

To investigate the effects of Type I diabetes on functional recovery and dendritic spine density after focal ischemia, we assessed behavioural recovery on sensorimotor tasks and measured changes in spine density of layer-5 pyramidal neurons using confocal microscopy. We also assessed infarct volume to determine whether diabetes was associated with larger infarct volumes after stroke. The experimental outline is summarized in Figure 3A. Diabetic mice had significantly higher blood glucose levels than non-diabetic controls prior to euthanasia at 1 ($t_{(24)}=21.91$, $p<0.01$) and 6 weeks recovery ($t_{(10)}=9.29$, $p<0.01$; Figure 3B).

Figure 3: Experimental outline and blood glucose levels

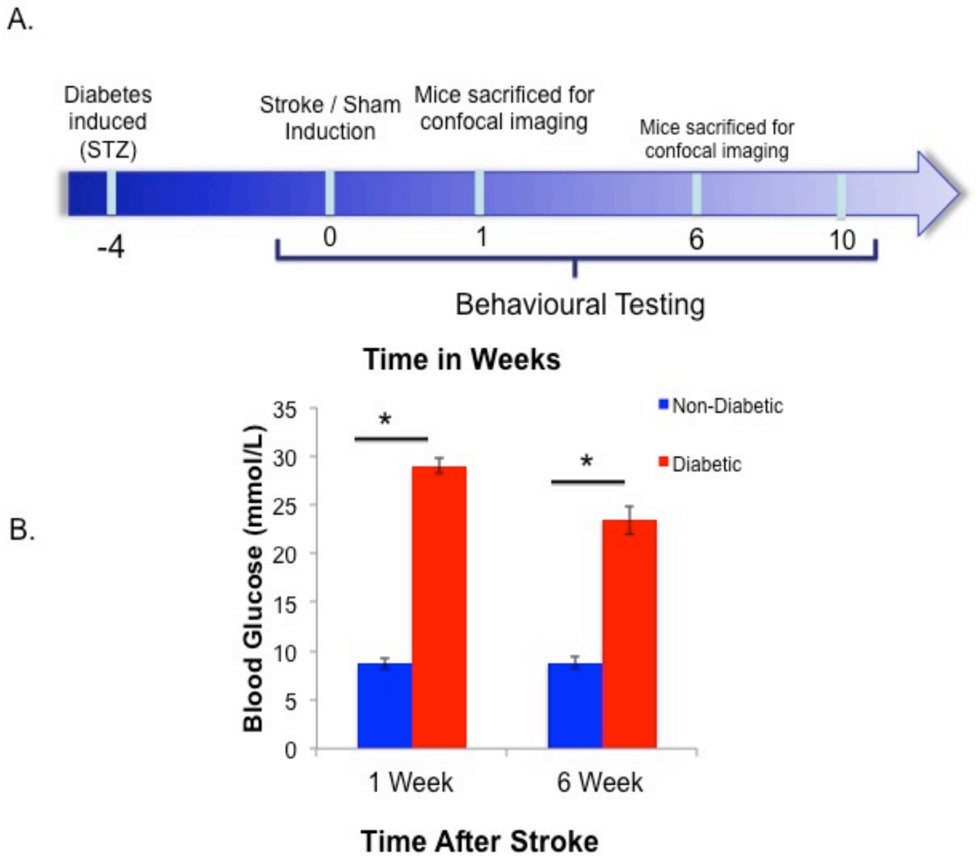


Figure 3. (A): Experimental outline. Type I diabetes was induced 4 weeks prior to stroke by STZ injection. Mice were given photothrombotic or sham stroke at time 0 and euthanized for histology and confocal imaging at either 1 or 6 weeks recovery. (B): Blood glucose measurements were taken just prior to euthanizing animals. Diabetic animals had significantly higher blood glucose levels (mmol/L) at 1 and 6 weeks post-stroke than non-diabetic mice. *p<0.01.

Diabetes alters functional recovery from ischemic stroke

The adhesive tape removal test has been shown to be highly sensitive in detecting sensorimotor deficits caused by ischemia in the primary sensorimotor cortex (Tennant and Jones, 2009; Sweetnam et al., 2012). Although our previous study showed that tape removal latencies were significantly longer in diabetic mice at later stages of stroke recovery (i.e. 6–10 weeks), there was considerable variability in the data, which may have prevented the detection of deficits at earlier time points. One way to reduce inter-animal variability and control for individual differences in anxiety and fear (indicated by freezing behaviour), which can confound latency scores, is to normalize tape removal measurements. This can be done by taking the difference in latency to remove tape from the unaffected right paw relative to the affected paw. Our re-analysis of an existing data set (Sweetnam et al., 2012) indicated there was no effect of diabetes on the normalized score prior to stroke ($t_{(23)}$=0.95, p=0.35). During the first 1–2 weeks after stroke (Fig. 4A), diabetic mice showed significantly higher difference scores compared to non-diabetic mice ($t_{(22)}$=2.98, p<0.01). There were no differences between diabetic and non-diabetic mice at weeks 3–4 ($t_{(23)}$=0.82, p=0.42) and 5–6 weeks recovery ($t_{(23)}$=0.52, p=0.61). However, at the 7–8 week ($t_{(16)}$=2.95, p<0.01) and 9–10 week ($t_{(12)}$=1.76, p=0.05) time period, diabetic mice had significantly higher difference scores than non-diabetic mice. In summary, we show that difference scores are significantly greater in diabetic mice at early (1–2 weeks) and later (>6 weeks) stroke recovery periods.

Infarct volume was unaffected by diabetes

The extent of stroke-induced cortical damage could affect the degree of recovery in diabetic animals. Therefore, we analyzed the infarct volume of diabetic and non-diabetic animals at 1 and 6 weeks post stroke. As shown in Figure 4B and C, there were no significant differences in infarct volume between groups at either 1 ($t_{(10)}$=0.10, p=0.92) or 6 weeks after stroke ($t_{(10)}$=1.06, p=0.31). These results were consistent with previous study from our lab (Sweetnam et al., 2012). Therefore, differences in behavioural recovery patterns between diabetic and non-diabetic animals cannot be sufficiently explained cortical infarct volume.

Figure 4: Sensorimotor assessment and histological infarct size analysis

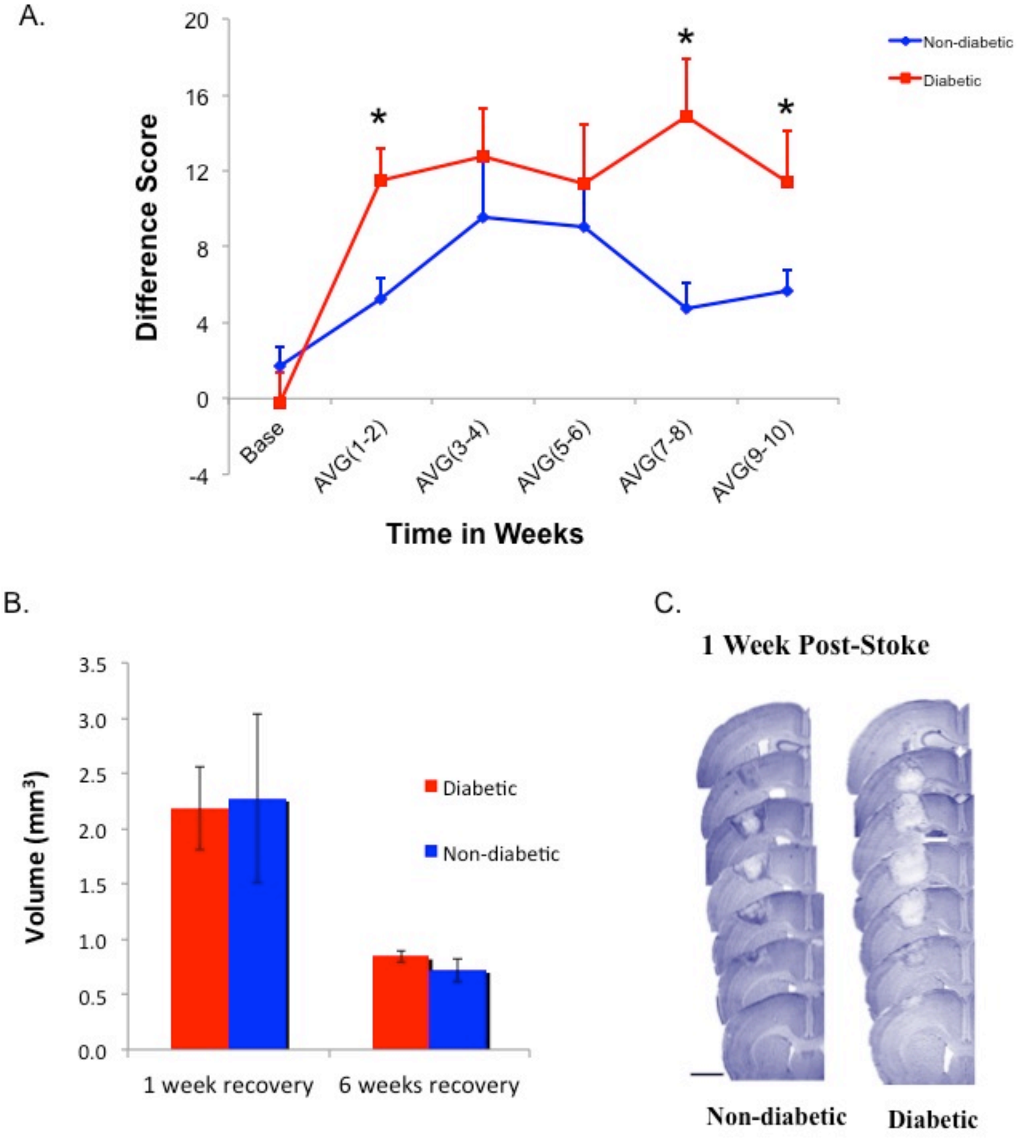
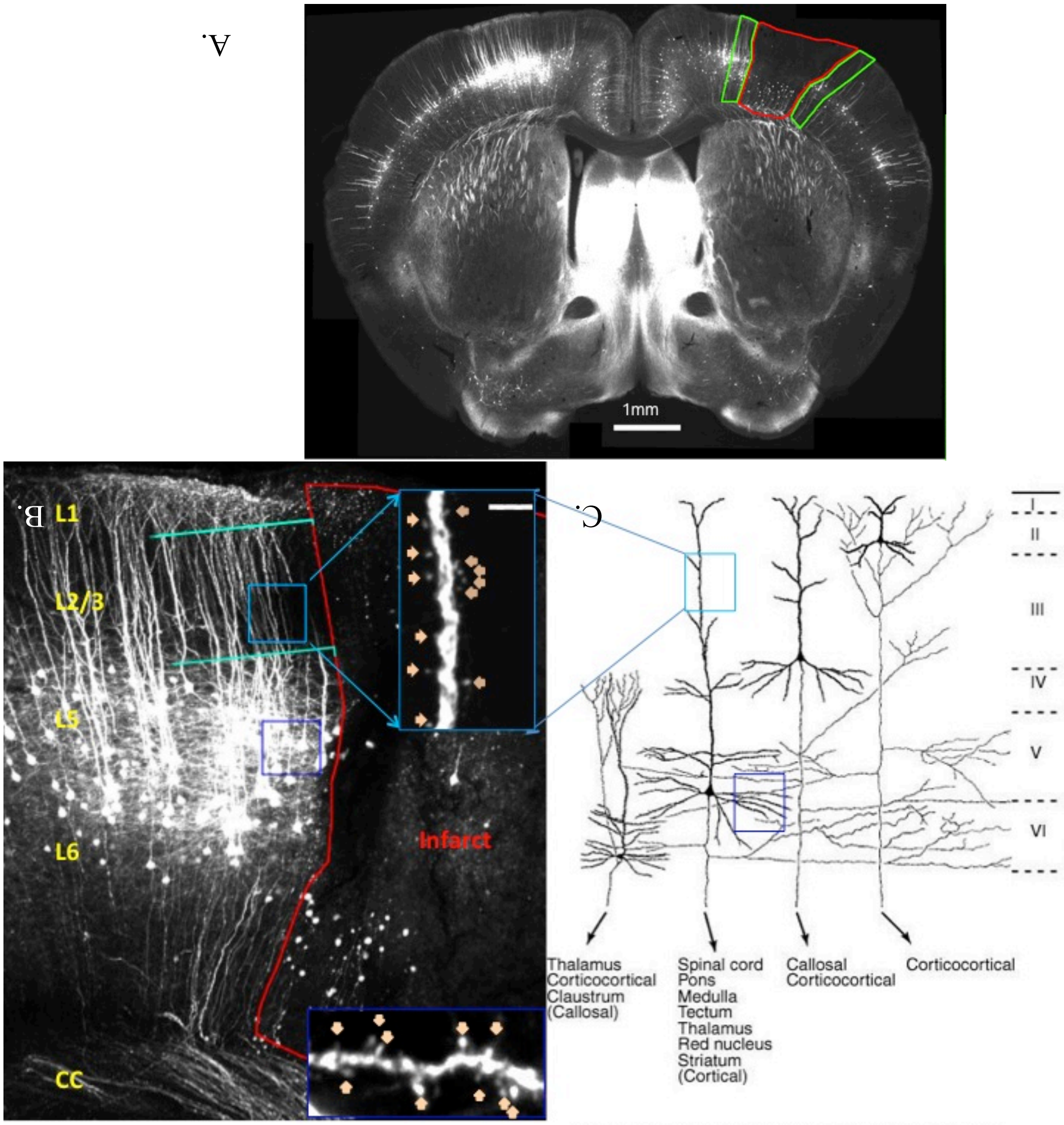


Figure 4: Diabetes was associated with greater sensorimotor deficits initially after stroke but did not affect cortical infarct volume. (A) Plot showing the average difference score (time to remove tape from affected paw–unaffected paw) binned into 2-week averages. Stroke increased difference scores in the tape removal task for both diabetic and non-diabetic animals. (*p≤0.05 significant difference between non-diabetic and diabetic animals.) (B) Analysis of infarct volume demonstrates that there was no difference between groups at 1 or 6 weeks (C) Cresyl violet staining of the stroke-affected hemisphere 1 week after photothrombotic stroke in diabetic and non-diabetic animals. Scale bar = 1 mm

Lower apical dendritic spine density in diabetic mice

A recent *in vivo* voltage-sensitive-dye-imaging study has shown that diabetes impairs the re-mapping of sensory function to peri-infarct regions after stroke (Sweetnam et al., 2012). Since voltage-sensitive-dye imaging reports change in the membrane potential of dendrites and cell bodies in the superficial layers of the cortex (Ferezou et al., 2006), we hypothesized that defects in functional cortical plasticity may be associated with changes in dendritic spine density in layers 2/3. Dendritic spine density was assessed on the primary apical dendrites of layer-5 pyramidal neurons that extended into layer 2/3 of the cortex. However, layer-1 apical tufts were not assessed because the density of YFP labelling is too high to adequately separate one dendrite's spines from another. Furthermore, the apical tufts are often truncated during tissue processing, making it very difficult to determine the origin of the layer of the parent soma. Patterns of YFP expression in the sensorimotor cortex can be seen in Figure 5.

Figure 5:YFP$^+$ expression and spine density within the peri-infarct tissue



Kandel ER. *Principles of Neural Science, 4th ed.* McGraw-Hill, New York.

Figure 5: YFP labeling of layer 5 neurons in the sensorimotor cortex (A) Coronal section of a YFP$^+$ mouse 1 week after stroke. The section shows the expression of YFP labeled neurons, typical stroke size and location. The green box highlights the peri-infarct zones and the red box outlines the infarct core. (B) Magnified view of the peri-infarct cortex. Teal lines measured 300 µm perpendicular from the infarct border. All measurements of apical and basilar dendrites were taken within this 300µm zone. (C)Anatomical drawings depict the projections of neurons within each cortical layer and the blue boxes illustrate the area of the dendritic arbor imaged for spine density counts.

A total of 66,859 spines were analyzed: 34,844 spines for basilar and 32,015 spines for apical compartments (Table 1). The peri-infarct cortex after photothrombotic stroke has previously been defined as cortical tissue within ~300 µm of the infarct border (Zhang and Murphy, 2007). We binned spine density measurements in 100-µm increments from the infarct border (eg. 0–100, 101–200, 201–300 µm) in both diabetic and non-diabetic mice, and found that density measurements did not change significantly within the 300-µm span of peri-infarct cortex for each group (non-diabetic: $F_{(2,13)}=0.34$, p=0.7; diabetic: $F_{(2,9)}=1.90$, p=0.20). Therefore, spine density measurements within 300 µm of the infarct border were classified as "peri-infarct" cortex. The average distance from the infarct border where peri-infarct spine density measurements were taken from was not significantly different between groups at either 1 (t(10)=1.26, p=0.23) or 6 weeks recovery (t(9)=0.50, p=0.63).

Two-way analysis of variance (ANOVA) demonstrated that there was a significant main effect of Diabetes ($F_{(1,19)} = 7.63$, $p = 0.01$) and a main effect of Time post-stroke ($F_{(1,19)} = 18.5$, $p < 0.001$) on the mean spine density and no significant interaction between time and condition ($F_{(1,19)} = 0.65$, $p = 0.65$). For mice subjected to sham stroke procedure, there was a trend towards lower apical spine densities in the diabetic group but this was not statistically significant (Fig. 6A; $t_{(9)}=1.67$, p=0.06). As expected, photothrombotic stroke reduced spine densities in the peri-infarct zone in both non-diabetic ($t_{(9)}=2.37$, p<0.05) and diabetic ($t_{(10)}=3.76$, p<0.01) mice at 1 week recovery (Fig. 6A). Spine density in the contralateral hemisphere (at 1 week recovery) was not affected by stroke in either group (p>0.05). After 6 weeks recovery from stroke, apical spine density returned to sham control levels for both non-diabetic ($t_{(9)}=0.57$, p=0.29) and diabetic animals ($t_{(10)}=1.35$, p=0.10).

Figure 6: Spine density on the apical dendrites of layer-5 neurons within cortical layer 2/3 after photothrombotic stroke

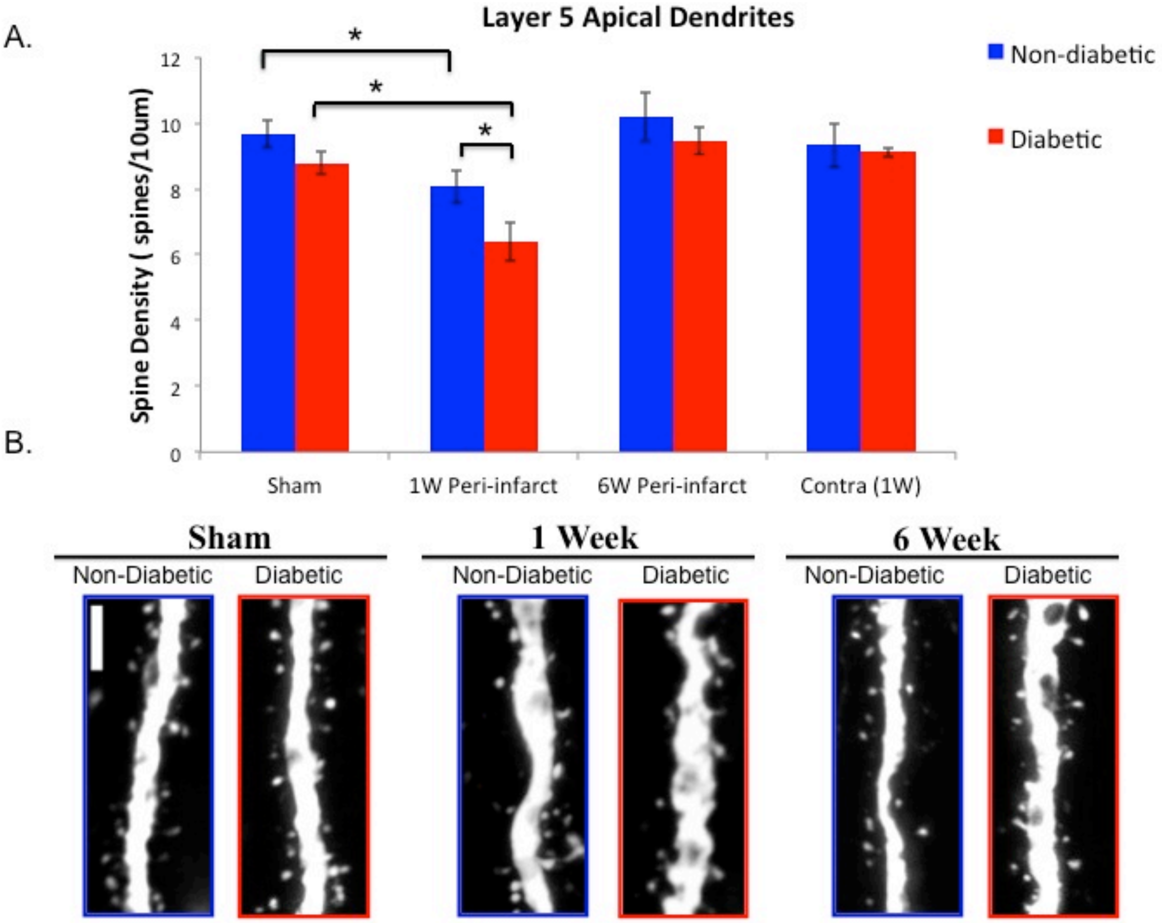


Figure 6: Diabetes increases spine loss on apical dendrites in peri-infarct cortex. (A) Histogram shows spine density in the sham-operated mice, contralateral and peri-infarct cortex at 1 and 6 weeks recovery. (B) Representative confocal images of the primary apical dendrite in each group. Scale bar= 5µm $^*p \leq 0.05$.

Diabetes exacerbates spine loss on basilar dendrites after stroke

Basilar dendritic spines were assessed to determine whether the pattern of spine loss on apical dendrites extended into deeper cortical layers. This allowed us to determine whether there was a global loss of spines on layer-5 neurons or whether this was a loss of layer-specific inputs. A two-way ANOVA revealed a significant main effect of Diabetes ($F_{(1,19)} = 6.87, p = .016$) and Time after stroke ($F_{(2,19)} = 54.7, p = <.001$) with no significant interaction between variables ($F_{(2,19)} = 6.82, p = 0.02$). Spine densities on the basilar dendrites of layer-5 neurons were not significantly different between non-diabetic and diabetic sham operates (Fig. 7A,B; $t_{(9)}$=0.01, p=0.49). One week after stroke (Fig. 7A,B), peri-infarct spine density on basilar dendrites was significantly reduced for both non-diabetic ($t_{(9)}$=4.03, p<0.01) and diabetic mice ($t_{(10)}$=6.37, p<0.01). Dendritic spine loss at 1 week was greater in the diabetic mice ($t_{(10)}$=4.18, p<0.01) than the non-diabetic. In the contralateral hemisphere at 1 week recovery, stroke did not affect basilar spine density in either non-diabetic ($t_{(10)}$=1.14, p=0.20) or diabetic ($t_{(10)}$=0.42, p=0.14) mice. Similar to that observed for apical dendrites, spine density levels recovered to sham-operate levels for both diabetic ($t_{(8)}$=1.95, p=0.09) and non-diabetic ($t_{(6)}$=1.99, p=0.09) mice. Collectively, these results show that diabetes exacerbates the loss of spines from the basilar and apical dendrites of layer-5 neurons in the peri-infarct cortex, but does not affect the recovery of spine numbers.

Figure 7: Spine density on the basal dendrites of layer-5 neurons during recovery

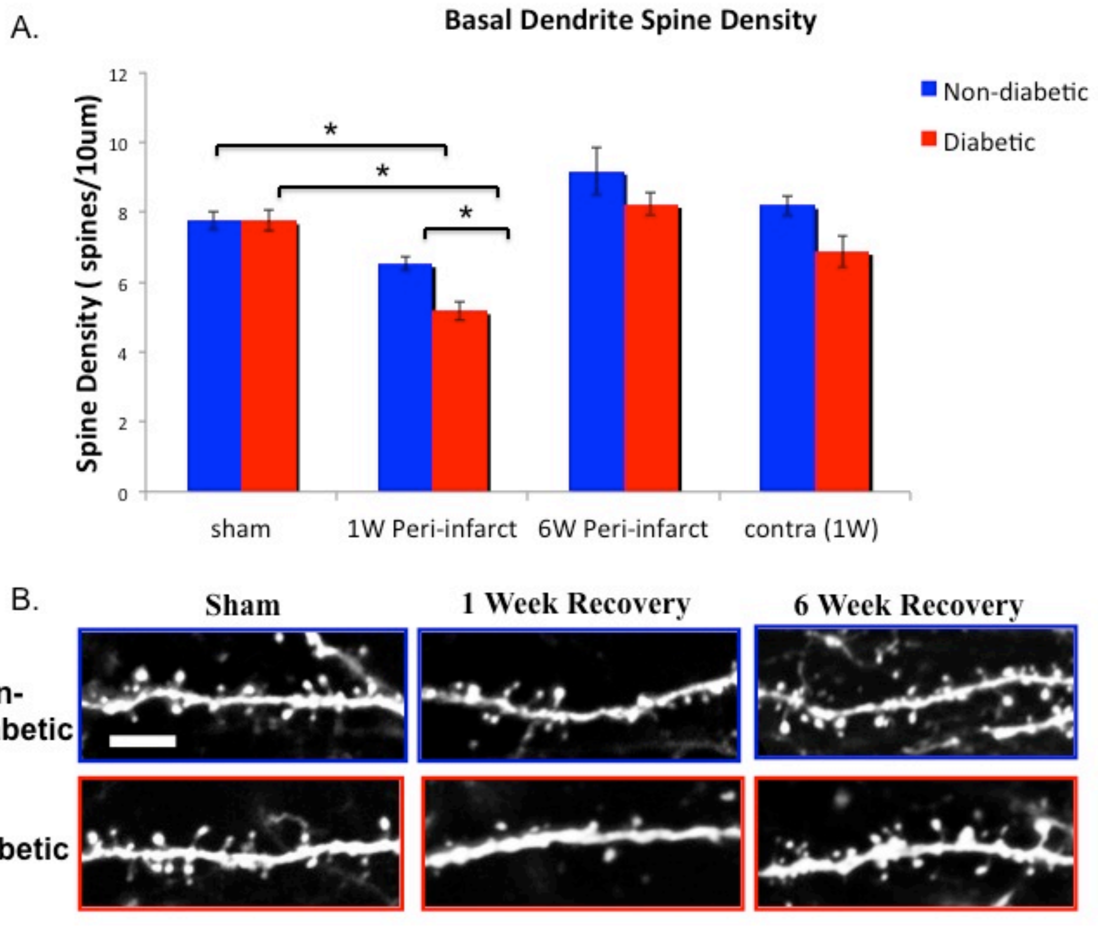


Figure 7: Diabetes enhances spine loss on basilar dendrites after stroke in the peri-infarct cortex. (A) Histogram shows spine density in the sham-operated mice, contralateral and peri-infarct cortex at 1 and 6 weeks recovery. (B) High magnification confocal images of dendritic spines on basilar dendrite in each group. Scale bar= 5 µm

Discussion

Summary

In this study, we investigated how diabetes affects the density of dendritic spines in layer-5 cortical neurons following focal unilateral ischemic stroke in mice. Diabetes was induced by STZ injection 4 weeks prior to stroke and blood glucose was uncontrolled for the duration of the experiment. In the first 2 weeks after stroke, diabetic mice showed more severe sensory deficits in the adhesive tape removal task than non-diabetics. By 6 weeks after stroke, both diabetic and non-diabetic mice were equally impaired in the tape removal test. From 7 weeks onward, non-diabetic mice showed a slight but significant improvement in performance relative to diabetic mice. The greater initial behavioural deficits in diabetic mice could not simply be attributed to greater cortical damage, as infarct volumes 1 week after the stroke were indistinguishable between groups. Analysis of dendritic spines indicated that diabetes was associated with fewer apical dendritic spines and a greater loss of spines on basilar dendrites of layer-5 pyramidal neurons in the peri-infarct cortex at 1 week post-stroke. Unexpectedly, diabetics showed a resurgence of spine density in peri-infarct cortex by 6 weeks recovery. These results indicate that poor recovery of function in diabetic animals correlates well with the early loss of fine synaptic structure rather than the volume of the infarct core. Following the initial loss of spines, diabetes does not interfere with the progressive re-generation and/or maintenance of new dendritic spines in peri-infarct tissue.

Diabetes worsens functional recovery after stroke

Ischemic stroke to the forelimb area of the somatosensory cortex in the rodent brain induces sustained sensorimotor impairments with variable degrees of spontaneous behavioural recovery. Successful recovery of behavioural function is mediated by both structural and functional changes in brain circuits in the peri-infarct cortex and other connected areas (Murphy and Corbett, 2009). Many clinical studies have shown that diabetes was associated with greater brain injury and a worse outcome following cerebral ischemia; however the neurobiological mechanisms underlying poor functional outcome in diabetics following stroke is unclear. In our study, diabetic animals exhibited more severe behavioural impairments than non-diabetics when assessed 1–2 weeks and >6 weeks after stroke. Both groups showed persistent sensory deficits for the entirety of the 10-week testing period. Full recovery of sensorimotor function is unlikely given the massive number of lost neurons with highly specific functions (Murphy and Corbett, 2009). The persistence of behavioral deficits after stroke, especially in diabetic mice, is consistent with recently published work from our lab using the tape removal and horizontal ladder tests (Sweetnam et al., 2012). Other research groups have also found poor behavioral outcome after stroke in diabetic animals at acute time points (24 hours) after ischemic injury. For example, both Type I and Type II diabetic mice have shown significantly poorer performance in the tape removal test, grid walking test, and increased scores on the modified neurological severity score (mNSS) 1 day after stroke compared to non-diabetic mice (Chen et al., 2011; Ning et al., 2012). There are only few studies that have shown the effect of diabetes on long-term recovery. With respect to long term recovery, two studies reported more persistent deficits after stroke in a rat model of type II diabetes (Moreira et al., 2007; Langdon et al., 2011). Furthermore, the degree of behavioural recovery in Type II diabetic rats was negatively correlated with the duration of diabetes prior to stroke (Langdon et al., 2011). These findings demonstrate that diabetes exacerbates the severity of behavioural deficits initially after stroke and reduces the extent of long-term (10 weeks) recovery.

Greater behavioural deficits in diabetic mice are not due to larger infarct size

A popular theory for explaining why diabetes worsens functional impairments after stroke (eg. hand or paw use) is that diabetes increases the volume of the cerebral infarction. Our analysis of infarct size at 1 and 6 weeks after stroke showed no difference in the volume of damaged cortical tissue in diabetic and non-diabetic mice at either time point. These data replicate the findings of a previous study from our lab (Sweetnam et al., 2012). However, this finding likely does not extend to all other models of diabetes or stroke given the wealth of information showing that the effects of hyperglycaemia and diabetes on infarct size after stroke are highly variable (for review see MacDougall and Muir, 2011). This inconsistency may be related to the type of stroke model, post-stroke treatment with insulin, severity of hyperglycaemia and amount of reperfusion, all of which have been shown to affect infarct volume (Mankovsky and Metzger, 1996; Kent et al., 2001; Garg et al., 2006; Ning et al., 2012). In the present study, we utilized a method of stroke that induces a severe restriction of blood flow in a relatively small ischemic core with limited reperfusion in penumbral areas. We chose not to provide insulin to these animals as our previous work indicated that insulin administration immediately after stroke did not rescue diabetes-related deficits in either functional recovery, or in cortical map plasticity (Sweetnam et al., 2012). It is worth noting that diabetes could enhance ischemic cell loss using other models of stroke, such as middle cerebral artery occlusion (MCAo) where reperfusion is greater, the infarct border is less well defined, and the volume of tissue damage is more extensive and may explain some of the variation seen in the diabetic group.

Peri-infarct spine loss is exacerbated by diabetes

Previous studies have demonstrated that prolonged (8–16 weeks) uncontrolled diabetes leads to a 20–60% reduction in spine density, as well as a truncation and simplification of pyramidal dendrites in the hippocampus and various regions of the neocortex (Martínez-Tellez et al., 2005; Malone et al., 2008). Here, we did not observe this phenomenon in our sham-operated diabetic mice, nor in the contralateral hemisphere of the 1-week recovery group, which were hyperglycemic for 5 weeks. Even in mice that had uncontrolled diabetes for 10 weeks before histological processing, (6-week recovery group), spine densities in both apical and basilar dendrites were similar to those of non-diabetic mice. Several possibilities could explain the discrepancy in the findings. For one, both of the previous studies were done on rats, who may be less tolerant to the detrimental effects of prolonged hyperglycemia. Second, the duration of hyperglycemia in the previous studies was longer. Therefore, it is possible that 5-10 weeks of hyperglycemia used in the present study was not sufficient to induce considerable spine loss in mice. Third, previous studies used Golgi-Cox staining of dendrites, which has been known to result in a significant underestimation (and presumably loss) in the number of spines due to the fact that the brains are not fixed with formaldehyde. Therefore, previously reported differences may be an artifact of the staining method used to label spines. Lastly, previous studies examined basilar dendrites in layer-2/3 and 4 pyramidal neurons. It is possible that chronic hyperglycemia may selectively damage those neurons while sparing layer-5 neurons that are the focus of our study. With respect to diabetes-related changes in dendrite length, our study was not designed to measure this parameter as brains were cut 50 μm thick, thereby truncating the majority of dendritic arbours that are typically ~300 μm in length. Future research using longitudinal *in vivo* imaging to measure changes in dendritic length, incorporating the nature of dendritic spine formation and elimination in the diabetic animal after stroke, is warranted.

Previous studies of non-diabetic mice subjected to stroke have shown that spine formation is elevated in the peri-infarct cortex for several weeks after stroke (Brown et al., 2009;

Mostany et al., 2010). Moreover, apical dendritic spine densities in the peri-infarct cortex returned to control levels by 6 weeks recovery, which correlated with the partial recovery of forepaw function (Brown et al., 2009; Mostany et al., 2010). Our examination of non-diabetic mice confirmed and extended previous findings by showing that both apical and basilar spine densities returned to control levels by 6 weeks recovery. Contrary to initial expectations, we found that spine density in the peri-infarct cortex of diabetic animals was not significantly different from non-diabetics when assessed at 6 weeks after stroke. Furthermore, at this time point, spine density was slightly elevated in both groups compared to shams. This overshoot may be due to the "relearning" of the sensory task after stroke, as increases in spine density have previously been shown to occur during the acquisition phase of learning followed by spine pruning in the refinement phase (Xu et al., 2009).

Interestingly, regaining pre-stroke spine density levels in the peri-infarct cortex was not associated with substantial improvements in forepaw sensory function in diabetic or non-diabetic mice. There are several possible explanations for why the recovery of spine density at 6 weeks would not be correlated with improved behavioural function. For one, the behavioural data was not collected from same the mice that spine density measurements were made from. Therefore, drawing correlations between the two variables is somewhat problematic, especially when one considers the highly variable nature of behavioural recovery. Second, the existence of new spines does not necessarily imply the existence of functional synapses. Although we can use spine density measurements to estimate the number of synaptic inputs, it does not provide information on the strength of each synaptic input nor does it provide information about the structural properties of spines, such as turnover, stability and morphology that can influence synaptic function. After stroke, spine formation increases to replace lost spines (Brown et al., 2008;

Mostany et al., 2010). New spines generally have a thin spine morphology and electron microscopy has demonstrated that not all new spines initially express a post-synaptic density (PSD). Newly formed spines need to stabilize before being functionally integrated into the local neuronal circuitry (De Roo et al., 2008a). The stabilization of a spine involves the recruitment of post-synaptic density proteins and adhesion molecules (Kasai et al., 2003). It is conceivable that there was a surge in the formation of immature spines at 6 weeks recovery that later became stabilized and functional in non-diabetic mice whereas they were lost in diabetic mice. As mentioned previously, certain neurological disorders such as Fragile X syndrome illustrate the point that reduced synaptic stability could have a negative impact on cognitive function. *In vivo* longitudinal imaging could be used to investigate this possibility by measuring spine turnover rates and the persistence of spines. Additionally, whole cell recordings in tandem with glutamate uncaging could be used to estimate the strength of synaptic inputs onto layer 5 dendritic spines to determine if despite their being relatively normal numbers of spines, a significant number are relatively weak. Furthermore detailed assessment of spine morphologies by electron microscopy could identify difference in spine morphology.

One limitation of the present study was that dendritic spines were analyzed in only layer 5 pyramidal neurons. These layer 5 neurons comprise the main efferent projection from the cortex to subcortical structures and the spinal cord, and layer-5 apical dendrites sample all of the cortical layers above it. Focusing on these neurons in the context of stroke recovery was based on the assumption that structural changes on the dendrites of these output neurons would likely correlate with changes in sensorimotor function. However, being able to predict recovery from spine density on layer 5 neurons is a highly simplistic expectation considering that sensory-motor information processing is distributed across multiple cortical areas, layers and distinct types of cortical neurons. Sampling spine densities in a broader set of neurons, for example in layer 2/3 might lead to stronger correlations with behavioural recovery patterns. At least by looking at both apical and basilar compartments, we were able to discern whether or not the effect on spine density was input specific. The apical dendrite of layer-5 neurons receives input primarily from cortical-cortical projections from neurons in layer 2/3, while the basal compartment receives intra-cortical projections from within column layer 2/3 and 5 neurons, as well as other cortical layers and regions (Douglas and Martin, 2004). If spine loss was restricted to layer 2/3, it would suggest that stroke induces local de-afferentation of layer 2/3 intra-cortical projections onto the apical dendrite. However, our data show a trend towards spine loss in both compartments implying that ischemia was globally affecting spines on peri-infarct neurons. The influence of the contralateral homotopic cortex in stroke recovery is not well understood. Some groups have shown significant dendritic modification of layer-5 neurons after stroke, while others have not detected any changes (Jones and Schallert, 1992; Jones and Schallert, 1994; Biernaskie et al., 2004; Gonzalez et al., 2004). Jones and Schallert (1994) suggest that damage to the sensorimotor cortex leads to an increase in the size of dendritic arbors of layer-5 pyramidal neurons in the contralateral hemisphere. Further, the expansion of the dendritic arbour was correlated with the greater behavioural recovery. Johnston et al. (2012) used *in vivo* longitudinal imaging to examine this issue and found no change in spine density or branching of dendritic tufts in layer-5 cortical neurons in the contralateral cortex after stroke. One caveat to the study was that their technique was limited to only imaging changes in layer 1 of the cortex (Johnston et al., 2012). In the present study, we were unable to observe any changes in the density of spines in apical or basilar dendrites of the contralateral homotopic hemisphere; therefore, neither stroke, nor diabetes, seemed to affect the spine density of layer-5 neurons in the contralateral hemisphere.

Possible mechanisms underlying greater spine loss in the diabetic brain after stroke

This is the first study to show that diabetes exacerbates the loss of dendritic spines in the peri-infarct cortex after stroke. Dendritic arbour damage after ischemia is due to a variety of mechanisms, such as calcium dysfunction, excitotoxicity, free radical production and spreading depolarization (Dirnagl et al., 1999; Moskowitz et al., 2010; Risher et al., 2010). How diabetes interacts with these mechanisms to produce excessive spine loss is unclear. Diabetic animals have shown greater astrocytic injury and swelling (Muranyi et al., 2006) in response to ischemia, which could possibly lead to increased spine loss. Astrocytes can play a role in dendritic spine development, maturation and help to regulate spine numbers. Astrocytes secrete multiple signaling molecules, such as cholesterol, TNF-α, and activity-dependent neurotrophic factor (ADNF) (Barker and Ullian, 2010) and thrombospondins (Christopherson et al., 2005), which promote spine formation in dendrites. Therefore, greater disruption of astrocytic function in diabetic animals may contribute to more extensive spine loss after stroke.

Since diabetes is known to increase oxidative stress in the vascular endothelium (Vinik and Flemmer, 2002), a loss of spines could be a consequence of a dysfunctional vascular system. Ongoing research in our lab has shown that diabetes increases the permeability of the blood-brain barrier in the peri-infarct area (unpublished work). The extravasation of potentially toxic elements of blood plasma such as glutamate could account for increased dendritic spine loss in the first week after stroke. Increased extracellular glutamate has been observed in obsereved after stroke in diabetes (Li et al., 2000). Furthermore, our lab described microvascular dynamics in the peri-infarct zone of diabetic mice (unpublished work) and demonstrated that diabetes leads to an abnormal increase in peri-infarct microvessel blood flow and lumen diameter. While the functional significance of these changes in not yet clear, higher blood flow velocities could reduce the efficiency of oxygen transport from red blood cells to the brain or could increase the probability for hemorrhage in microvessels.

Retraction of spines may also be related to the loss of pre-synaptic terminals. It is known that ischemic stroke in the somatosensory cortex leads to the loss of thalamic and intracortical projections within peri-infarct regions (Carmichael et al., 2001). It was recently demonstrated that intracortical axonal remodeling is impaired in diabetics after stroke (Yan et al., 2012). If thalamic and cortical axons in diabetic mice were intrinsically less resilient to ischemia due ongoing oxidative stress or if they lacked sufficient glial or vascular support to survive, one might expect greater loss of pre-synaptic terminals which would likely be reflected in a loss of post-syanptic spines.

Diabetes could affect other molecular pathways involved in the regulation of dendritic spines; for example, some diabetic complications have been associated with alterations in the Rho family of GTPases. Rho GTPase regulates cellular adherence, migration, proliferation and apoptosis through control of the actin cytoskeleton and are known to regulate dendritic spine morphology, assembly and disassembly (Yong-jun and Zhou, 2010). Tan et al (2012) showed that Rac1 (a GTPase) regulated morphological changes from mushroom to thin spine types in the dorsal horn of the spinal cord in a diabetic model of chronic pain (Tan and Waxman, 2012; Tan et al., 2012). Furthermore, an up-regulation of protein phosphatase 1 (PP1) has been shown in the brain tissue of diabetic rats (Takizawa et al., 1994), which interacts with the RhoGEF (Lfc) nuerabin, spinophilin and actin, leading to reduced spine size (Tada and Sheng, 2006). Matrix metalloproteinase-9 (MMP-9) are proteases that break down the extracellular matrix resulting in increased structural instability and likely contribute to spine disassembly. MMP-9 has been shown to be abnormally up-regulated in diabetic rats exposed to ischemia (Signorelli et al., 2005; Ergul et al., 2007; Kamada et al., 2007). MMP-9 causes a significant elongation and thinning of dendritic spines, as well as a loss of mushroom-shaped spines. However, it does not seem to affect spine density, at least in the hippocampus (Michaluk et al., 2011). Additionally, overproduction of ROS in diabetic animals has been shown to disrupt the production of nitric oxide (NO) (Arrick et al., 2007). Under normal physiological conditions, NO signalling regulates spine development on pyramidal neurons and modifies spine morphology by increasing the complexity of the post-synaptic density (Audesirk et al., 2003; Nikonenko et al., 2008; Steinert et al., 2008). NO was suggested to be a retrograde signal between newly forming spines and presynaptic targets since blocking neuronal nitric oxide syntheses (nNOS) interferes with synaptic formation and results in a loss of spines (Nikonenko et al., 2008). Future studies should determine the role of these molecular mechanisms on spine stability in the diabetic stroke-affected brain.

Conclusion

The central aim of this project was to characterize how diabetes affects the fine synaptic structure of layer-5 neurons in brain regions implicated in stroke recovery. Behavioural testing of forepaw sensation indicated that diabetic mice were significantly more impaired for the first 2 weeks after stroke. Consistent with previous work (Sweetnam et al., 2012), poor sensory function in diabetic animals could not be explained by larger cerebral infarcts. Our analysis of layer-5 dendritic spines revealed that diabetic mice had significantly greater basilar dendritic spine loss in the peri-infarct cortex as compared to non-diabetic animals. Interestingly, after 6 weeks of recovery, the spine density in both diabetic and non-diabetic mice had returned to sham control levels. Furthermore, changes in spine density were not observed in any of the distant cortical areas that have previously been implicated in stroke recovery. Overall, our findings suggest that diabetes contributes to a selective and robust loss of basilar dendritic spines in the peri-infarct cortex at 1 week, which could explain more severe behavioural deficits in diabetic mice in the acute, but not chronic stages of stroke recovery.

Table 1: Total spine numbers, total length of dendrites, and average length of dendritic segments

Apical dendrites	Total number of spines counted	Total length of dendritic segments (µm)	Average length of dendritic segments (µm)
Sham diabetic (n =5)	3,553	4,144	85.1
Sham non-diabetic (n =6)	3,321	3,829.1	83
Contralateral diabetic (n=5)	3,294	3,423	78.4
Contralateral non-diabetic (n=5)	4,675	4,907.3	85.6
1 week diabetic (n=6)	3,486	4,048.9	75.3
1 week non-diabetic (n=6)	5,685	8,107.1	90.4
6 week diabetic (n=5)	4,886	5,896.1	93.3
6 week non-diabetic (n=6)	3,115	3,142.5	93.3
TOTALS	**32,015**	**37,498**	**85.55**

Table 1.1 Apical dendrites: Column 1) Total number of individual dendritic spines counted on the apical dendrites per group. Column 2) Cumulative length of the dendritic shafts measured on the apical dendrites. Column 3) The average length of dendritic segments counted for apical dendrites per group.

Basal Dendrite	# spines counted	Total length of dendritic segments (µm)	Average length of dendritic segments (µm)
Sham diabetic (n =6)	6,370	7,946.5	61.8
Sham non-diabetic (n =5)	3,774	4,815.2	54.5
Contralateral diabetic (n=6)	4,069	5,122	59.7
Contralateral non-diabetic (n=7)	4,938	6,213.2	55.7
1 week diabetic (n=6)	3,256	5,624.9	56.3
1 week non-diabetic (n=6)	3,975	6,034	58.8
6 week diabetic (n=4)	4,695	5,445.1	60.5
6 week non-diabetic (n=6)	3,230	39,78.2	54
TOTALS	**34,307**	**45,179.1**	**57.6625**

Table 1.2 Basal dendrites: Column 1) Total number of individual dendritic spines counted on the basal dendrites per group. Column 2) Cumulative length of the dendritic shafts measured on the basal dendrites. Column 3) The average length of dendritic segments counted per group.

Bibliography

Abo M, Chen Z, Lai LJ, Reese T, Bjelke B (2001) Functional recovery after brain lesion--contralateral neuromodulation: an fMRI study. Neuroreport 12:1543–1547.

Alvarez V, Sabatini BL (2007) Anatomical and physiological plasticity of dendritic spines. Annual Review of Neuroscience 30:79–97.

American Diabetes Assocaition (2010) Diagnosis and classification of diabetes mellitus. Diabetes Care 33 Suppl 1:S62–9.

Arellano JI, Benavides-Piccione R, Defelipe J, Yuste R (2007) Ultrastructure of dendritic spines: correlation between synaptic and spine morphologies. Frontiers in Neuroscience 1:131–143.

Arrick DM, Sharpe GM, Sun H, Mayhan WG (2007) nNOS-dependent reactivity of cerebral arterioles in Type 1 diabetes. Brain Research 1184:365–371.

Audesirk T, Cabell L, Kern M, Audesirk G (2003) Enhancement of dendritic branching in cultured hippocampal neurons by 17β-estradiol is mediated by nitric oxide. International Journal of Developmental Neuroscience 21:225–233.

Baird T a, Parsons MW, Phanh T, Butcher KS, Desmond PM, Tress BM, Colman PG, Chambers BR, Davis SM (2003) Persistent poststroke hyperglycemia is independently associated with infarct expansion and worse clinical outcome. Stroke; a Journal of Cerebral Circulation 34:2208–2214.

Barker AJ, Ullian EM (2010) Astrocytes and synaptic plasticity. The Neuroscientist; 16:40–50.

Biernaskie J, Chernenko G, Corbett D (2004) Efficacy of rehabilitative experience declines with time after focal ischemic brain injury. The Journal of Neuroscience 24:1245–1254.

Brands, Augustina M.A. Biessels, Geert Jan. De Haan, Edward H.F. Kappelle, L. Jaap. Kessels RPC (2005) The Effects of Type 1 Diabetes on Cognitive Performance. Diabetes Care 28:726–735.

Brown CE, Aminoltejari K, Erb H, Winship IR, Murphy TH (2009) In vivo voltage-sensitive dye imaging in adult mice reveals that somatosensory maps lost to stroke are replaced over weeks by new structural and functional circuits with prolonged modes of activation within both the peri-infarct zone and distant sites. The Journal of Neuroscience 29:1719–1734

Brown CE, Boyd JD, Murphy TH (2010) Longitudinal in vivo imaging reveals balanced and branch-specific remodeling of mature cortical pyramidal dendritic arbors after stroke. The Journal of Cerebral Blood Flow and Metabolism 30:783–791.

Brown CE, Li P, Boyd JD, Delaney KR, Murphy TH (2007) Extensive turnover of dendritic spines and vascular remodeling in cortical tissues recovering from stroke. The Journal of Neuroscience 27:4101–4109.

Brown CE, Wong C, Murphy TH (2008) Rapid morphologic plasticity of peri-infarct dendritic spines after focal ischemic stroke. Stroke; a Journal of Cerebral Circulation 39:1286–1291.

Canadian Stroke Network (2011) The Quality of Stroke Care in Canada Canadian Stroke Network.

Capes SE, Hunt D, Malmberg K, Pathak P, Gerstein HC (2001) Stress Hyperglycemia and Prognosis of Stroke in Nondiabetic and Diabetic Patients: A Systematic Overview. Stroke 32:2426–2432.

Carmichael ST (2003) Plasticity of Cortical Projections after Stroke. The Neuroscientist 9:64–75.

Carmichael ST, Archibeque I, Luke L, Nolan T, Momiy J, Li S (2005) Growth-associated gene expression after stroke: evidence for a growth-promoting region in peri-infarct cortex. Experimental Neurology 193:291–311

Carmichael ST, Wei L, Rovainen CM, Woolsey T a (2001) New patterns of intracortical projections after focal cortical stroke. Neurobiology of Disease 8:910–922.

Chen J, Cui X, Zacharek A, Cui Y, Roberts C, Chopp M (2011) White matter damage and the effect of matrix metalloproteinases in type 2 diabetic mice after stroke. Stroke; a Journal of Cerebral Circulation 42:445–452.

Christopherson KS, Ullian EM, Stokes CC a, Mullowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres B a (2005) Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell 120:421–433.

Cingolani L a, Goda Y (2008) Actin in action: the interplay between the actin cytoskeleton and synaptic efficacy. Nature Reviews Neuroscience 9:344–356.

Cramer SC (2008) Repairing the human brain after stroke: I. Mechanisms of spontaneous recovery. Annals of Neurology 63:272–287

Cruz-Martín A, Crespo M, Portera-Cailliau C (2010) Delayed stabilization of dendritic spines in fragile X mice. The Journal of Neuroscience 30:7793–7803.

De Roo M, Klauser P, Mendez P, Poglia L, Muller D (2008a) Activity-dependent PSD formation and stabilization of newly formed spines in hippocampal slice cultures. Cerebral Cortex (New York, NY : 1991) 18:151–161

De Roo M, Klauser P, Muller D (2008b) LTP promotes a selective long-term stabilization and clustering of dendritic spines. PLoS Biology 6:219-228.

Dijkhuizen RM, Ren J, Mandeville JB, Wu O, Ozdag FM, Moskowitz M a, Rosen BR, Finklestein SP (2001) Functional magnetic resonance imaging of reorganization in rat brain after stroke. Proceedings of the National Academy of Sciences of the United States of America 98:12766–12771

Dijkhuizen RM, Singhal AB, Mandeville JB, Wu O, Halpern EF, Finklestein SP, Rosen BR, Lo EH (2003) Correlation between brain reorganization, ischemic damage, and neurologic status after transient focal cerebral ischemia in rats: a functional magnetic resonance imaging study. The Journal of Neuroscience 23:510–517

Dirnagl U, Iadecola C, Moskowitz M a (1999) Pathobiology of ischaemic stroke: an integrated view. Trends in neurosciences 22:391–397.

Douglas RJ, Martin KC (2004) Neuronal circuits of the neocortex. Annual Review of Neuroscience 27:419–451.

Elgebaly MM, Ogbi S, Li W, Mezzetti EM, Prakash R, Johnson MH, Bruno A, Fagan SC, Ergul A (2011) Neurovascular injury in acute hyperglycemia and diabetes: A comparative analysis in experimental stroke. Translational Stroke Research 2:391–398.

Elgebaly MM, Prakash R, Li W, Ogbi S, Johnson MH, Mezzetti EM, Fagan SC, Ergul A (2010) Vascular protection in diabetic stroke: role of matrix metalloprotease-dependent vascular remodeling. The Journal of Cerebral Blood Flow and Metabolism 30:1928–1938.

Ergul A, Elgebaly MM, Middlemore M-L, Li W, Elewa H, Switzer J, Hall C, Kozak A, Fagan SC (2007) Increased hemorrhagic transformation and altered infarct size and localization after experimental stroke in a rat model type 2 diabetes. BMC Neurology 7:33-42.

Ergul A, Li W, Elgebaly MM, Bruno A, Fagan SC (2009) Hyperglycemia, diabetes and stroke: focus on the cerebrovasculature. Vascular Pharmacology 51:44–49.

Etuk E. (2010) Animals Models for Studying Diabetes Mellitus. Agric Biol JN Am 1:130–134.

Fatehi-Hassanabad Z, Chan CB, Furman BL (2010) Reactive oxygen species and endothelial function in diabetes. European Journal of Pharmacology 636:8–17

Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JM, Lichtman JW, Sanes JR (2000) Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron 28:41–51.

Ferezou I, Bolea S, Petersen CCH (2006) Visualizing the cortical representation of whisker touch: voltage-sensitive dye imaging in freely moving mice. Neuron 50:617–629.

Ferreira IA, Mocking AIM, Feijge M a H, Gorter G, van Haeften TW, Heemskerk JWM, Akkerman J-WN (2006) Platelet inhibition by insulin is absent in type 2 diabetes mellitus. Arteriosclerosis, Thrombosis, and Vascular. Biology 26:417–422

Forbes JM, Cooper ME (2013) Mechanisms of diabetic complications. Physiological Reviews 93:137–188.

Garg R, Chaudhuri A, Munschauer F, Dandona P (2006) Hyperglycemia, insulin, and acute ischemic stroke: a mechanistic justification for a trial of insulin infusion therapy. Stroke; a Journal of Cerebral Circulation 37:267–273.

Gonzalez CLR, Gharbawie O, Williams PT, Kleim J, Kolb B, Whishaw IQ (2004) Evidence for bilateral control of skilled movements: ipsilateral skilled forelimb reaching deficits and functional recovery in rats follow motor cortex and lateral frontal cortex lesions. The European Journal of Neuroscience 20:3442–3452.

Grutzendler J, Kasthuri N, Gan W (2002) Long-term dendritic spine stability in the adult cortex. Nature 420:812–816.

Hamidon B, Raymond A (2003) The Impact of Diabetes Mellitus on In-hospital Stroke Mortality Address for Correspondence : Journal of Postgraduate Medicine:307–310.

Harris KM, Jensen FE, Tsao B (1992) Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation. The Journal of Neuroscience 12:2685–2705.

Harris KM, Kater SB (1994) Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function. Annual Review of Neuroscience 17:341–371.

Holtmaat a, De Paola V, Wilbrecht L, Knott GW (2008) Imaging of experience-dependent structural plasticity in the mouse neocortex in vivo. Behavioural Brain Research 192:20–25

Holtmaat AJGD, Trachtenberg JT, Wilbrecht L, Shepherd GM, Zhang X, Knott GW, Svoboda K (2005) Transient and persistent dendritic spines in the neocortex in vivo. Neuron 45:279–291

Hotulainen P, Hoogenraad CC (2010) Actin in dendritic spines: connecting dynamics to function. The Journal of Cell Biology 189:619–629.

Ikegaya Y, Kim J a, Baba M, Iwatsubo T, Nishiyama N, Matsuki N (2001) Rapid and reversible changes in dendrite morphology and synaptic efficacy following NMDA receptor activation: implication for a cellular defense against excitotoxicity. Journal of Cell Science 114:4083–4093.

Ito U, Kuroiwa T, Nagasao J, Kawakami E, Oyanagi K (2006) Temporal profiles of axon terminals, synapses and spines in the ischemic penumbra of the cerebral cortex: ultrastructure of neuronal remodeling. Stroke; a Journal of Cerebral Circulation 37:2134–2139.

Ivanco TL, Racine RJ, Kolb B (2000) Morphology of Layer III Pyramidal Neurons Is Altered Following Induction of LTP in Sensorimotor Cortex. 22:16–22.

Jablonka J a, Witte OW, Kossut M (2007) Photothrombotic infarct impairs experience-dependent plasticity in neighboring cortex. Neuroreport 18:165–169

Johnston DG, Denizet M, Mostany R, Portera-Cailliau C (2012) Chronic In Vivo Imaging Shows No Evidence of Dendritic Plasticity or Functional Remapping in the Contralesional Cortex after Stroke. Cerebral cortex (New York, NY : 1991):751–762.

Jones T, Schallert T (1992) Overgrowth and pruning of dendrites in adult rats recovering from neocortical damage. Brain research 581:156–160.

Jones T, Schallert T (1994) Use-Dependent Damage Growth of Pyramidal Neurons after Neocortical Damage. The Journal of neuroscience 14:2140–2152.

Jorgensen H, Nakayama H, Raaschou HO, Olsen TS (1994) Stroke in patients with diabetes. The Copenhagen Stroke Study. Stroke 25:1977–1984.

Kamada H, Yu F, Nito C, Chan PH (2007) Influence of hyperglycemia on oxidative stress and matrix metalloproteinase-9 activation after focal cerebral ischemia/reperfusion in rats: relation to blood-brain barrier dysfunction. Stroke; a Journal of Cerebral Circulation 38:1044–1049.

Kandel ER, Schwartz JH, Jessell TM 2000. *Principles of Neural Science, 4th ed.* McGraw-Hill, New York. ISBN 0-8385-7701-6

Kasai H, Matsuzaki M, Noguchi J, Yasumatsu N, Nakahara H (2003) Structure-stability-function relationships of dendritic spines. Trends in Neurosciences 26:360–368.

Kent T a., Soukup VM, Fabian RH (2001) Heterogeneity Affecting Outcome From Acute Stroke Therapy: Making Reperfusion Worse. Stroke 32:2318–2327

Kirov SA, Petrak LJ, Fiala JC, Harris KM (2004) Dendritic spines disappear with chilling but proliferate excessively upon rewarming of mature hippocampus. Neuroscience 127:69–80.

Kleim J A, Barbay S, Cooper NR, Hogg TM, Reidel CN, Remple MS, Nudo RJ (2002) Motor learning-dependent synaptogenesis is localized to functionally reorganized motor cortex. Neurobiology of learning and memory 77:63–77.

Kleim J a, Hogg TM, VandenBerg PM, Cooper NR, Bruneau R, Remple M (2004) Cortical synaptogenesis and motor map reorganization occur during late, but not early, phase of motor skill learning. The Journal of Neuroscience 24:628–633.

Knott G, Holtmaat A (2008) Dendritic spine plasticity--current understanding from in vivo studies. Brain Research Reviews 58:282–289.

Knott GW, Holtmaat A, Wilbrecht L, Welker E, Svoboda K (2006) Spine growth precedes synapse formation in the adult neocortex in vivo. Nature Neuroscience 9:1117–1124.

Lai CSW, Franke TF, Gan W-B (2012) Opposite effects of fear conditioning and extinction on dendritic spine remodelling. Nature 483:87–91.

Langdon KD, Clarke J, Corbett D (2011) Long-term exposure to high fat diet is bad for your brain: exacerbation of focal ischemic brain injury. Neuroscience 182:82–87.

Li P A., Shuaib A., Miyashita H, He Q-P, Siesjo BK, Warner DS (2000) Hyperglycemia Enhances Extracellular Glutamate Accumulation in Rats Subjected to Forebrain Ischemia Editorial Comment. Stroke 31:183–192

Li P, Murphy TH (2008) Two-photon imaging during prolonged middle cerebral artery occlusion in mice reveals recovery of dendritic structure after reperfusion. The Journal of Neuroscience 28:11970–11979

Lindsberg PJ, Roine RO (2004) Hyperglycemia in acute stroke. Stroke; a Journal of Cerebral Circulation 35:363–364.

Luchsinger JA, Tang M, Stern Y, Shea S, Mayeux R (2001) Diabetes mellitus and risk of Alzheimer's disease and dementia with stroke in a multiethnic cohort. American Journal of Epidemiology 154:635–641.

Luitse MJ, Biessels GJ, Rutten GE, Kappelle LJ (2012) Diabetes, hyperglycaemia, and acute ischaemic stroke. Lancet Neurology 11:261–271.

MacDougall NJJ, Muir KW (2011) Hyperglycaemia and infarct size in animal models of middle cerebral artery occlusion: systematic review and meta-analysis. The Journal of Cerebral Blood Flow and Metabolism 31:807–818.

Magariños a M, McEwen BS (2000) Experimental diabetes in rats causes hippocampal dendritic and synaptic reorganization and increased glucocorticoid reactivity to stress. Proceedings of the National Academy of Sciences of the United States of America 97:11056–11061.

Magariños AM, Jain K, Blount ED, Reagan L, Smith BH, McEwen BS (2001) Peritoneal implantation of macroencapsulated porcine pancreatic islets in diabetic rats ameliorates severe hyperglycemia and prevents retraction and simplification of hippocampal dendrites. Brain Research 902:282–287.

Majewska A, Brown E, Ross J, Yuste R (2000a) Mechanisms of calcium decay kinetics in hippocampal spines: role of spine calcium pumps and calcium diffusion through the spine neck in biochemical compartmentalization. The Journal of Neuroscience 20:1722–1734.

Majewska a, Tashiro a, Yuste R (2000b) Regulation of spine calcium dynamics by rapid spine motility. The Journal of Neuroscience 20:8262–8268.

Majewska AK, Newton JR, Sur M (2006) Remodeling of synaptic structure in sensory cortical areas in vivo. The Journal of Neuroscience 26:3021–3029.

Malone JI, Hanna S, Saporta S, Mervis RF, Park CR, Chong L, Diamond DM (2008) Hyperglycemia not hypoglycemia alters neuronal dendrites and impairs spatial memory. Pediatric Diabetes 9:531–539.

Mankovsky B, Metzger B (1996) Cerebrovascular Disorders in Patients With Diabetes Mellitus. Journal Diabetes and Its Complications 8727:228–242.

Martínez-Tellez R, Gómez-Villalobos MDJ, Flores G (2005) Alteration in dendritic morphology of cortical neurons in rats with diabetes mellitus induced by streptozotocin. Brain Research 1048:108–115.

Martini SR, Kent T a (2007) Hyperglycemia in acute ischemic stroke: a vascular perspective. The Journal of Cerebral Blood Flow and Metabolism 27:435–451

Matsuzaki M, Ellis-Davies GC, Nemoto T, Miyashita Y, Iino M, Kasai H (2001) Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons. Nature neuroscience 4:1086–1092

Matsuzaki M, Honkura N, Ellis-Davies G, Kasai H (2004) Structural basis of long-term potentiation in single dendritic spines. Nature 429:761–766.

Megherbi S-E, Milan C, Minier D, Couvreur G, Osseby G-V, Tilling K, Di Carlo A, Inzitari D, Wolfe CD a, Moreau T, Giroud M (2003) Association between diabetes and stroke subtype on survival and functional outcome 3 months after stroke: data from the European BIOMED Stroke Project. Stroke; a Journal of Cerebral Circulation 34:688–694.

Metz G, Whishaw IQ (2002) Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: a new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. Journal of Neuroscience Methods 115:169–179.

Michaluk P, Wawrzyniak M, Alot P, Szczot M, Wyrembek P, Mercik K, Medvedev N, Wilczek E, De Roo M, Zuschratter W, Muller D, Wilczynski GM, Mozrzymas JW, Stewart MG, Kaczmarek L, Wlodarczyk J (2011) Influence of matrix metalloproteinase MMP-9 on dendritic spine morphology. Journal of Cell Science 124:3369–3380.

Monfils M-H, Teskey GC (2004) Skilled-learning-induced potentiation in rat sensorimotor cortex: a transient form of behavioural long-term potentiation. Neuroscience 125:329–336.

Moreira T, Cebers G, Pickering C, Ostenson C-G, Efendic S, Liljequist S (2007) Diabetic Goto-Kakizaki rats display pronounced hyperglycemia and longer-lasting cognitive impairments following ischemia induced by cortical compression. Neuroscience 144:1169–1185.

Moskowitz M, Lo EH, Iadecola C (2010) The science of stroke: mechanisms in search of treatments. Neuron 67:181–198.

Mostany R, Anstey JE, Crump KL, Maco B, Knott G, Portera-Cailliau C (2013) Altered Synaptic Dynamics during Normal Brain Aging. Journal of Neuroscience 33:4094–4104.

Mostany R, Chowdhury TG, Johnston DG, Portonovo S a, Carmichael ST, Portera-Cailliau C (2010) Local hemodynamics dictate long-term dendritic plasticity in peri-infarct cortex. The Journal of Neuroscience 30:14116–14126.

Muranyi M, Ding C, He Q, Lin Y, Li P-A (2006) Streptozotocin-induced diabetes causes astrocyte death after ischemia and reperfusion injury. Diabetes 55:349–355.

Murphy TH, Corbett D (2009) Plasticity during stroke recovery: from synapse to behaviour. Nature Reviews Neuroscience 10:861–872.

Murphy TH, Li P, Betts K, Liu R (2008) Two-photon imaging of stroke onset in vivo reveals that NMDA-receptor independent ischemic depolarization is the major cause of rapid reversible damage to dendrites and spines. The Journal of Neuroscience 28:1756–1772.

Nannetti L, Paci M, Baccini M, Rinaldi L a, Taiti PG (2009) Recovery from stroke in patients with diabetes mellitus. Journal of Diabetes and Its Complications 23:249–254.

Nikonenko I, Boda B, Steen S, Knott G, Welker E, Muller D (2008) PSD-95 promotes synaptogenesis and multiinnervated spine formation through nitric oxide signaling. The Journal of Cell Biology 183:1115–1127.

Nimchinsky E a, Sabatini BL, Svoboda K (2002) Structure and function of dendritic spines. Annual Review of Physiology 64:313–353.

Ning R, Chopp M, Yan T, Zacharek A, Zhang C, Roberts C, Cui X, Lu M, Chen J (2012) Tissue plasminogen activator treatment of stroke in type-1 diabetes rats. Neuroscience 222:326–332.

Nudo RJ, Wise BM, SiFuentes F, Milliken GW (1996) Neural substrates for the effects of rehabilitative training on motor recovery after ischemic infarct. Science (New York, NY) 272:1791–1794

Nusser Z, Lujan R, Laube G, Roberts JD, Molnar E, Somogyi P (1998) Cell type and pathway dependence of synaptic AMPA receptor number and variability in the hippocampus. Neuron 21:545–559.

Peters, Alan Kaiserman-Abramof IR (1970) The small pyramidal neuron of the rat cerebral cortex. The perikaryon, dendrites and spines. The American Journal of Anatomy 127:321–355.

Public Health Agency of Canada, Diabetes in Canada. (2011) Facts and figures from a public health perspective. Ottawa.

Ramo´ n y Cajal, S. (1899). La Textura del Sistema Nerviosa del Hombre y los Vertebrados (Madrid, Moya (Primera Edicion)).

Reagan LP (2012) Diabetes as a chronic metabolic stressor: causes, consequences and clinical complications. Experimental Neurology 233:68–78

Reijmer YD, van den Berg E, de Bresser J, Kessels RPC, Kappelle LJ, Algra A, Biessels GJ (2011) Accelerated cognitive decline in patients with type 2 diabetes: MRI correlates and risk factors. Diabetes and Metabolism Research and Reviews 27:195–202.

Renner M, Lacor PN, Velasco PT, Xu J, Contractor A, Klein WL, Triller A (2010) Deleterious effects of amyloid beta oligomers acting as an extracellular scaffold for mGluR5. Neuron 66:739–754.

Risher WC, Ard D, Yuan J, Kirov S (2010) Recurrent spontaneous spreading depolarizations facilitate acute dendritic injury in the ischemic penumbra. The Journal of Neuroscience 30:9859–9868.

Robinson TE, Kolb B (2004) Structural plasticity associated with exposure to drugs of abuse. Neuropharmacology 47 Suppl 1:33–46.

Shanina E V, Schallert T, Witte OW, Redecker C (2006) Behavioral recovery from unilateral photothrombotic infarcts of the forelimb sensorimotor cortex in rats: role of the contralateral cortex. Neuroscience 139:1495–1506.

Sheng M, Hoogenraad CC (2007) The postsynaptic architecture of excitatory synapses: a more quantitative view. Annual Review of Biochemistry 76:823–847.

Shih AY, Li P, Murphy TH (2005) A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia in vivo. The Journal of Neuroscience 25:10321–10335.

Signorelli SS, Malaponte G, Libra M, Di Pino L, Celotta G, Bevelacqua V, Petrina M, Nicotra GS, Indelicato M, Navolanic PM, Pennisi G, Mazzarino MC (2005) Plasma levels and zymographic activities of matrix metalloproteinases 2 and 9 in type II diabetics with peripheral arterial disease. Vascular Medicine 10:1–6.

Star EN, Kwiatkowski DJ, Murthy VN (2002) Rapid turnover of actin in dendritic spines and its regulation by activity. Nature Neuroscience 5:239–246.

Steinert JR, Kopp-Scheinpflug C, Baker C, Challiss R a J, Mistry R, Haustein MD, Griffin SJ, Tong H, Graham BP, Forsythe ID (2008) Nitric oxide is a volume transmitter regulating postsynaptic excitability at a glutamatergic synapse. Neuron 60:642–656.

Stranahan AM, Arumugam T V, Cutler RG, Lee K, Egan JM, Mattson MP (2008) Diabetes impairs hippocampal function through glucocorticoid-mediated effects on new and mature neurons. Nature Neuroscience 11:309–317.

Stranahan AM, Mattson MP (2008) Impact of energy intake and expenditure on neuronal plasticity. Neuromolecular Medicine 10:209–218.

Stroemer RP, Kent TA, Hulsebosch CE (1995) Neocortical Neural Sprouting, Synaptogenesis, and Behavioral Recovery After Neocortical Infarction in Rats. Stroke 26:2135–2144

Sweetnam D, Holmes A, Tennant K a, Zamani A, Walle M, Jones P, Wong C, Brown CE (2012) Diabetes impairs cortical plasticity and functional recovery following ischemic stroke. The Journal of Neuroscience 32:5132–5143.

Tada T, Sheng M (2006a) Molecular mechanisms of dendritic spine morphogenesis. Current Opinion in Neurobiology 16:95–101.

Takizawa N, Mizuno Y, Ito Y, Kikuchi K (1994) Tissue Distribution of Isoforms of Type-1 Protein Phosphatase PP1 in Mouse Tissues and Its Diabetic Alterations 1. Journal of Biochemistry 116:411–415.

Tan AM, Samad O a, Fischer TZ, Zhao P, Persson A-K, Waxman SG (2012) Maladaptive dendritic spine remodeling contributes to diabetic neuropathic pain. The Journal of Neuroscience 32:6795–6807.

Tan AM, Waxman SG (2012) Spinal cord injury, dendritic spine remodeling, and spinal memory mechanisms. Experimental Neurology 235:142–151.

Tashiro A, Yuste R (2008) Role of Rho GTPases in the morphogenesis and motility of dendritic spines. Methods in Enzymology 439:285–302.

Tennant K, Asay AL, Allred RP, Ozburn AR, Kleim J, Jones T (2010) The vermicelli and capellini handling tests: simple quantitative measures of dexterous forepaw function in rats and mice. Journal of visualized experiments : JoVE:1–5

Tennant K, Jones T (2009) Sensorimotor behavioral effects of endothelin-1 induced small cortical infarcts in C57BL/6 mice. Journal of Neuroscience Methods 181:18–26.

Trachtenberg JT, Chen BE, Knott GW, Feng G, Sanes JR, Welker E, Svoboda K (2002) Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. Nature 420:788–794.

Van Meer MP a, van der Marel K, Wang K, Otte WM, El Bouazati S, Roeling T a P, Viergever M a, Berkelbach van der Sprenkel JW, Dijkhuizen RM (2010) Recovery of sensorimotor function after experimental stroke correlates with restoration of resting-state interhemispheric functional connectivity. The Journal of Neuroscience 30:3964–3972.

Vinik A, Flemmer M (2002) Diabetes and macrovascular disease. Journal of diabetes and its complications 16:235–245

Watson BD, Dietrich WD, Busto R, Wachtel MS, Ginsberg MD (1985) Induction of reproducible brain infarction by photochemically initiated thrombosis. Annals of Neurology 17:497–504.

Wilbrecht L, Holtmaat A, Wright N, Fox K, Svoboda K (2010) Structural plasticity underlies experience-dependent functional plasticity of cortical circuits. The Journal of Neuroscience 30:4927–4932.

Winship IR, Murphy TH (2008) In vivo calcium imaging reveals functional rewiring of single somatosensory neurons after stroke. The Journal of Neuroscience 28:6592–6606

Witte OW, Bidmon HJ, Schiene K, Redecker C, Hagemann G (2000) Functional differentiation of multiple perilesional zones after focal cerebral ischemia. The Journal of Cerebral Blood Flow and Metabolism 20:1149–1165

Wu H, Wu T, Xu X, Wang J, Wang J (2011) Iron toxicity in mice with collagenase-induced intracerebral hemorrhage. The Journal of Cerebral Blood Flow and Metabolism 31:1243–1250.

Wu KK, Huan Y (2008) Streptozotocin-induced diabetic models in mice and rats. Current Protocols in Pharmacology / editorial board, SJ Enna (editor-in-chief) . [et al] Chapter 5:Unit 5.47.

Xu T, Yu X, Perlik AJ, Tobin WF, Zweig J a, Tennant K, Jones T, Zuo Y (2009) Rapid formation and selective stabilization of synapses for enduring motor memories. Nature 462:915–919.

Yan T, Chopp M, Ye X, Liu Z, Zacharek A, Cui Y, Roberts C, Buller B, Chen J (2012) Niaspan increases axonal remodeling after stroke in type 1 diabetes rats. Neurobiology of Disease 46:157–164

Yang G, Pan F, Gan W-B (2009) Stably maintained dendritic spines are associated with lifelong memories. Nature 462:920–924.

Yong-jun L, Zhou H (2010) RhoA/Rho kinase: a novel therapeutic target in diabetic complications. Chinese Medical Journal 123:2461–2466.

Yorek M A., Dunlap J A. (2002) Effect of increased concentration of D-glucose or L-fucose on monocyte adhesion to endothelial cell monolayers and activation of nuclear factor-[kappa]B. Metabolism 51:225–234.

Yoshihara Y, De Roo M, Muller D (2009) Dendritic spine formation and stabilization. Current Opinion in Neurobiology 19:146–153.

Yuste R (2011) Dendritic spines and distributed circuits. Neuron 71:772–781.

Yuste R, Bonhoeffer T (2004) Genesis of dendritic spines: insights from ultrastructural and imaging studies. Nature reviews Neuroscience 5:24–34.

Yuste R, Majewska a. (2001) Book Review: On the Function of Dendritic Spines. The Neuroscientist 7:387–395.

Zepeda A, Sengpiel F, Guagnelli MA, Vaca L, Arias C (2004) Functional reorganization of visual cortex maps after ischemic lesions is accompanied by changes in expression of cytoskeletal proteins and NMDA and GABA(A) receptor subunits. The Journal of Neuroscience 24:1812–1821.

Zhang S, Boyd J, Delaney K, Murphy TH (2005) Rapid reversible changes in dendritic spine structure in vivo gated by the degree of ischemia. The Journal of Neuroscience 25:5333–5338.

Zhang S, Murphy TH (2007) Imaging the impact of cortical microcirculation on synaptic structure and sensory-evoked hemodynamic responses in vivo. PLoS Biology 5:e119.

Zhang W-J, Tan Y-F, Yue JTY, Vranic M, Wojtowicz JM (2008) Impairment of hippocampal neurogenesis in streptozotocin-treated diabetic rats. Acta Neurologica Scandinavica 117:205–210.

Zuo Y, Lin A, Chang P, Gan W-B (2005a) Development of long-term dendritic spine stability in diverse regions of cerebral cortex. Neuron 46:181–189.

Zuo Y, Yang G, Kwon E, Gan W-B (2005b) Long-term sensory deprivation prevents dendritic spine loss in primary somatosensory cortex. Nature 436:261–265.