Diabetes Impairs Cortical Map Plasticity and Functional Recovery Following Ischemic Stroke

by

Danielle Sweetnam-Holmes
BSc, University of Victoria, 2009

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Supervisory Committee

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Abstract

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One of the most common risk factors for stroke is diabetes. Diabetics are 2 to 4 times more likely to have a stroke and are also significantly more likely to show poor functional recovery. In order to determine why diabetes is associated with poor stroke recovery, we tested the hypotheses that diabetes either exacerbates initial stroke damage, or inhibits neuronal circuit plasticity in surviving brain regions that is crucial for successful recovery. Type 1 diabetes was chemically induced in mice four weeks before receiving a targeted photothrombotic stroke in the right forelimb somatosensory cortex to model a chronic diabetic condition. Following stroke, a subset of diabetic mice were treated with insulin to determine if controlling blood glucose levels could improve stroke recovery. Consistent with previous studies, one behavioural test revealed a progressive improvement in sensory function of the forepaw in non-diabetic mice after stroke. By contrast, diabetic mice treated with and without insulin showed persistent deficits in sensori-motor forepaw function. To determine whether these different patterns of stroke recovery correlated with changes in functional brain activation, forepaw evoked responses in the somatosensory cortex were imaged using voltage sensitive dyes at 1 and 14 weeks after stroke. In both diabetic and non-diabetic mice that did not have a stroke, brief mechanical stimulation of the forepaw evoked a robust and near simultaneous depolarization in the primary (FLS1) and secondary somatosensory (FLS2) cortex. One week after stroke, forepaw-evoked responses had not been remapped in the peri-infarct cortex in both diabetic and non-diabetic mice. Fourteen weeks after stroke, forepaw evoked responses in non-diabetic mice re-emerged in the peri-infarct cortex whereas diabetic mice showed very little activation, reminiscent of the 1 week recovery group. Moreover, controlling hyperglycemia using insulin therapy failed to restore sensory evoked responses in the peri-infarct cortex. In addition to these differences in peri-infarct

responsiveness, we discovered that stroke was associated with increased responsiveness in FLS2 of non-diabetic, but not diabetic or insulin treated mice. To determine the importance of FLS2 in stroke recovery, we silenced the FLS2 cortex and found that it re-instated behavioural impairments in stroke recovered mice, significantly more so than naïve mice that still had a functioning FLS1. Collectively, these results indicate that both diabetes and the secondary somatosensory cortex play an important role in determining the extent of functional recovery after ischemic cortical stroke. Furthermore, the fact that insulin therapy after stroke did not normalize functional recovery, suggests that prolonged hyperglycemia (before stroke) may induce pathological changes in the brain's circulation or nervous system that cannot be easily reversed.

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List of Abbreviations

ACSF	Artificial Cerebral Spinal Fluid
ACU	Animal Care Unit
ADA	American Diabetes Association
BG	Blood Glucose
CDA	Canadian Diabetes Association
ERG-1	Early Growth Responce-1
FL	Forelimb
FLS1	Primary Forelimb Somatosensory Cortex
GABA	γ-Aminobutyric Acid
GLUT2	Glucose Transporter 2
HL	Hindlimb
HLS1	Primary Hindlimb Somatosensory Cortex
IP	Intraperitoneal
M1	Primary Motor Cortex
MCAO	Middle Cerebral Artery Occlusion
MMP	Matrix Metalloproteinases
MTP	Mitochondrial Transitional Pore
NF-κβ	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
NOD	Non-obese Diabetic
PBS	Phosphate Buffered Saline
PFA	paraformaldehyde
PVDF	Polyvinylidene Fluoride
ROS	Reactive Oxygen Species
SOP	Standard Operating Procedure
STZ	Streptozotocin
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TF	Tissue Factor
VEGF	Vascular Endothelial Growth Factor
VSD	Voltage Sensitive Dye
VPL	Ventral Posterior Lateral Nucleus

Professional Recognition

This thesis has been adapted from a manuscript that has been submitted to the Journal of Neuroscience:

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The contributing authors of this paper deserve special recognition, as they were instrumental in its completion.

Craig Brown: Principal Investigator.
Charles Wong: Setup the surgical areas and VSD rig in the Brown lab, summer 2009; assisted in collecting data from initial craniotomies in the summer of 2010.
Paul Jones: Collected the pilot data for this study from 2009-2010
Mark Walle: Collected data for adhesive tape removal test from 2009-2011.
Andrew Holmes: Aided with data collection and analysis on horizontal ladder experiments from 2010-2011.

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I am forever grateful for my friends and family who have supported me, but a special thanks goes out to my Aunt Dorise who has read every paper I have written, and listened to every talk I have given, and who still gets excited for the next one, thank you for caring.

To Tyler McKay, I can never sum up the support and love you have provided me. What else can I say? You are the best.

Finally, thank you to all the mice that gave their lives there are no words for my appreciation. To all these people and many more, thank you.

Dedication

This thesis is dedicated in honor of my parents,
Carol J. Sweetnam & E.R. Clare Holmes,
who could not be here, but who throughout my childhood encouraged my inquisitive mind and taught me the value of a hard days work. You taught me what dedication is.

The price of success is hard
work, dedication to the job at
hand, and the determination
that whether we win or lose,
we have applied the best of
ourselves.
-Vince Lombardi

1. Introduction

Rationale

According to the Canadian Diabetes Association one of the primary risk factors for stroke is diabetes (Public Health Agency of Canada, 2011). Diabetics are 2-4 times more likely to suffer an ischemic stroke and are prone to a poor functional recovery (Public Health Agency of Canada, 2011). Poor stroke recovery could be due to greater stroke damage or impaired brain plasticity in the weeks to months following a stroke. We also investigated if controlling the blood glucose after stroke with insulin therapy would improve stroke recovery. In order to investigate this issue, I induced an ischemic stroke in forelimb region of the somatosensory cortex in three separate groups of mice; the control group, which had normal blood glucose levels (euglycmeia), the diabetic group, which had elevated blood glucose levels (hyperglycemia) for 4 weeks prior to stroke, and the insulin therapy group, which had elevated blood glucose for 4 weeks before stroke and then had their blood glucose controlled after stroke. I specifically examined the extent to which diabetes 1) impairs functional recovery using behavioural measures 2) affects acute ischemic stroke damage and 3) affects the ability of the surviving brain regions to form and strengthen new and existing sensory circuits.

1.1 The Etiology of Type 1 Diabetes

Diabetes mellitus, commonly referred to as diabetes, is a chronic metabolic disorder that is characterized by hyperglycemia. Hyperglycemia, or high blood glucose occurs in diabetes because the body either does not produce enough insulin or is

insensitive to it. Without insulin the glucose in the blood is unable to be stored in the muscle, liver, and fat cells, or used in the other cells of the body. Insulin triggers the up-regulation of the GLUT transporters on target cells, which in turn allow for the transport of glucose across the lipid membrane. There have been over 12 GLUT transporters classified but the most relevant ones are GLUT1-4. The GLUT2 and 4 transporters mediate the majority of glucose uptake in the body. The GLUT 2 receptor allows glucose to flow into the cell during glycolysis and out during gluconeogenesis. In the brain, glucose transport is mediated by two GLUT transporters. GLUT 1 is found on erythrocytes and endothelial cells that comprise the blood brain barrier and GLUT 3 is expressed exclusively on neurons. Thus even without insulin, glucose in the blood is able to cross the blood brain barrier. Since glucose uptake in the brain is insulin independent, this means that the diabetic brain is constantly bathed in a high glucose medium.

In humans, hyperglycemia is defined as a blood glucose concentration greater then 8.1mM/L (**Table 1**). Diabetes mellitus is the most common cause of hyperglycemia in our society. There are three types of diabetes: Type 1 (TID), Type 2 (T2D) and gestational diabetes. Type 1, known as insulin dependent diabetes, results from the body's failure to produce insulin, and individuals affected require injections of exogenous insulin to survive. Type 2, often called non-insulin dependent diabetes, is where insulin is produced yet the cells are insensitive to it. Type 2 diabetes usually develops slowly over time and the risk for developing it increases with a low level of activity, poor diet, and excess body weight around the waist. A person with Type 2 diabetes will have higher levels of insulin in their blood than a non-diabetic person. This is because the liver, muscle and fat cells become insensitive to the insulin. Gestational diabetes is a form of

type 2 diabetes, which occurs during pregnancy and may precede T2D. Pre-diabetes (**Table 1**) is a relatively new term referring to blood glucose (BG) values that are higher than normal but below that of diabetic blood glucose levels. Approximately 25% of people with pre-diabetes will develop diabetes within 3–5 years (Public Health Agency of Canada, 2011). Ninety percent of individuals with diabetes have type 2 diabetes, yet type 1 diabetics shares many of the same symptoms and complications.

Type 1 diabetes is a complex metabolic disorder caused by the autoimmune destruction of the β-cells in the pancreas; thus, the body produces little to no insulin. Type 1 diabetes often develops early in life and is a chronic condition that cannot be cured, but is effectively managed with daily doses of exogenous insulin. In 1922, after the discovery of insulin, insulin was considered a miracle drug able to "cure" diabetes. However, over the years it has become apparent that insulin treatment is not effective against a myriad of health issues that diabetics face including problems in the circulatory and nervous systems of the feet, eyes and kidney, as well as depression, weight loss and frequent urination (Baird et al., 2002). New research has also demonstrated that even during the pre-diabetic condition, damage to the circulatory and peripheral nervous systems is already occurring (DeFronzo and Abdul-Ghani, 2011). Though less well understood, there is evidence to suggest that cerebral structure and function are affected in long-term diabetics (Biessels et al., 1994, Biessels and Gispen, 2005). Progressive changes to the central nervous systems leads to a condition referred to as diabetic encephalopathy, characterized by a slowing of mental processing and flexibility, as well as diminished learning and memory (Brands et al., 2005).

Condition	Human Blood Glucose mMol/L	Human Fasting Blood Glucose mMol/L	Mouse Blood Glucose mMol/L
Normal	<7.8	<6.1	<10
Pre-diabetic	≥8.1	≥6.1– <7.0	≥10.1– <14.9
Diabetes Mellitus	≥8.1	≥7.0	≥15.0

Table 1. Outlines the levels of glucose in the blood used to determine the prediabetes/diabetes condition. The World Health Organization has set these parameters. The fasting blood glucose measurement is taken after a 5 hour fast and if the preferable means to measure the blood glucose in the hospital. The last column refers to the blood glucose parameters for mice that were used to define the experimental conditions.

Diabetes and Our Society

According to the World Health Organization (WHO) in January 2011, more than 220 million people worldwide have been diagnosed with diabetes (type 1 and type 2) and this number is expected to rise dramatically in the next few decades. The Canadian Diabetes Associations (CDA) estimated that in 2000, 1.4 million Canadians had diabetes, and that this number will increase to 2.4 million in 2016 (Ohinmaa et al., 2004). Furthermore, many cases of diabetes are completely uncontrolled, given that a third of diabetics are unaware of their condition (American Diabetes Association). In North America, 1 in 4 people are diabetic or pre- diabetic, making diabetes a pandemic.

Diabetes is not only a personal burden; it is also a burden to the Canadian health care system. The nationwide cost of diabetes in 2010 was $12.2 billion, which is double what was spent in 2000 (CDA), and is expected to increase to 16.9 billion in 2020. This is largely due to the plethora of complications diabetics suffer from. For example in Canada, diabetes is the leading cause of blindness and it accounts for 70% of non-traumatic limb amputations. Perhaps the most troubling statistic is that 80% of people with diabetes will die from a heart attack or stroke (CDA) (Public Health Agency of Canada, 2011). Of the 50,000 individuals who have a stroke in Canada each year, 8% have diabetes (Public Health Agency of Canada, 2011). Thus with the number of diabetics worldwide increasing dramatically, the research community must focus on finding new ways to minimize complications and maximize the quality of life for the diabetic population.

1.2 Stroke and Diabetes

A stroke is a disturbance in the blood supply to a region of the brain, resulting in an infarct, which is a region of tissue that has undergone necrosis due to the lack of oxygen. This results in a loss of neurological function in that region, which often manifests itself through sensory, motor and cognitive impairments. Some of these symptoms become permanent disabilities, making stroke the leading cause of adult disability in high-income countries (Heart and Stroke Foundation, 2008). Eighty percent of all strokes are ischemic, in which blood flow to a brain region is reduced or stopped for a period of time due to a clot in the blood vessel (American Heart Association, 2011). This is further subdivided based on the etiology of the clot—cardioembolic, artery-to-artery, embolism, so-called large vessel, or lacunar stroke (Public Health Agency of Canada, 2011, Adams et al., 2007). The other 20% are hemorrhagic, where a blood vessel bursts thereby disrupting the flow of oxygen and nutrients to brain tissue.

The effects of diabetes on the brain is reflected by the alarming statistic that diabetics are significantly more likely to suffer a stroke; men are 2-4 times more likely whereas women are 3-6.5 times more likely (Iemolo et al., 2002, Laing et al., 2003). Furthermore, epidemiological studies have clearly shown that diabetes is strongly correlated with poor neurological outcome and loss of functional independence after stroke (Toni et al., 1994, Kruyt et al., 2010, Wei et al., 2010). The combination of increased prevalence of stroke and a reduced prognosis for recovery makes stroke the leading cause of death and disability for diabetics (CDA and American Diabetes Association). One of the most popular explanations for poor stroke recovery is that diabetes exacerbates initial stroke damage by altering the activation of apoptotic and

inflammatory signalling pathways (Muranyi et al., 2003, Kumari et al., 2007). However, clinical and experimental studies have not reached a consensus on this issue (MacDougall and Muir, 2011), as some reports have shown that diabetes can increase infarct volume (Nedergaard and Diemer, 1987, Duverger and MacKenzie, 1988), decrease it (Ergul et al., 2007, Li et al., 2010b) or have no effect at all (Mankovsky et al., 1996). Another explanation that has not been tested in depth is that diabetes may limit the brain's ability to initiate vascular and neuronal adaptations that are enacted over the months following stroke and are crucial to an improved functional outcome.

Hyperglycemia and Ischemic Injury

One hypothesis for the impaired recovery experienced by most diabetics is that high blood glucose levels exacerbate ischemic damage (Li et al., 2000, Baird et al., 2003, Anderson et al., 1999, Garg et al., 2006). During an ischemic stroke, the cells within the infarct core rapidly deplete the oxygen and glucose stores that are necessary for ATP production (Parsons et al., 2002). An ischemic infarct can be divided into two regions; the "core" is the region with the most extreme ischemic conditions and, where cells die within the first minutes of the ischemic event. The "penumbra" lies between the healthy brain tissue and the ischemic core. The penumbra is electrically silent, with limited blood flow and partial energy metabolism for approximately the first 24 hours after stroke (Murphy and Corbett, 2009). Even in non-diabetic stroke cases, the survival of cells within this region is dubious. Therefore challenging these already compromised cells with high levels of glucose (as would be expected in diabetes), may further compromise their ability to survive.

Elevated levels of blood glucose could affect the mechanisms of ischemic damage such as: excitotoxicity due to ion imbalances, reactive oxygen species (ROS), inflammation and limiting reperfusion of blood to the penumbra (Kawai et al., 1998, Li et al., 2000, Baird et al., 2003, Garg et al., 2006, Johnston and Parsons, 2010)(**Fig. 1**). Immediately following an obstruction of blood flow, brain cells lose in the ischemic core stop producing ATP (energy), which is required to maintain ion gradients across cell membranes. The disruption of ion gradients causes neurons and glial cells to depolarize, subsequently releasing excitatory neurotransmitters such as glutamate. Glutamate found in the extra-cellular space is normally modulated via glutamate reuptake mechanisms, but this process is also energy-dependant and thus the glutamate accumulates in the extracellular space. Excess glutamate activates ionotropic NMDA glutamate receptors, causing an influx of Ca^{++} into the cell. (Li et al., 2000) (**Fig. 1**).

An increase in intercellular Ca^{++} initiates a cascade of cellular processes that activate proteolytic enzymes that degrade major components of the cytoskeleton such as actin and spectrin. Additionally, the high intracellular levels of Ca^{++}, Na^{+} and ADP stimulate the mitochondria, specifically the phospholipase A2 and the cyclooxygenase to produce an excessive amount of reactive oxygen species (ROS) (Anderson et al., 1999, Li et al., 2000, Garg et al., 2006)(**Fig. 1**). Reactive oxygen species are chemically reactive molecules containing oxygen with one reactive electron that can disrupt other molecules, especially the double bonds. These reactive oxygen species cause damage to other molecules such as lipids, proteins and nucleic acids. Normally antioxidants such as superoxide dismutase, catalase, glutathione, alpha-tocophenol and ascorbic acid, would

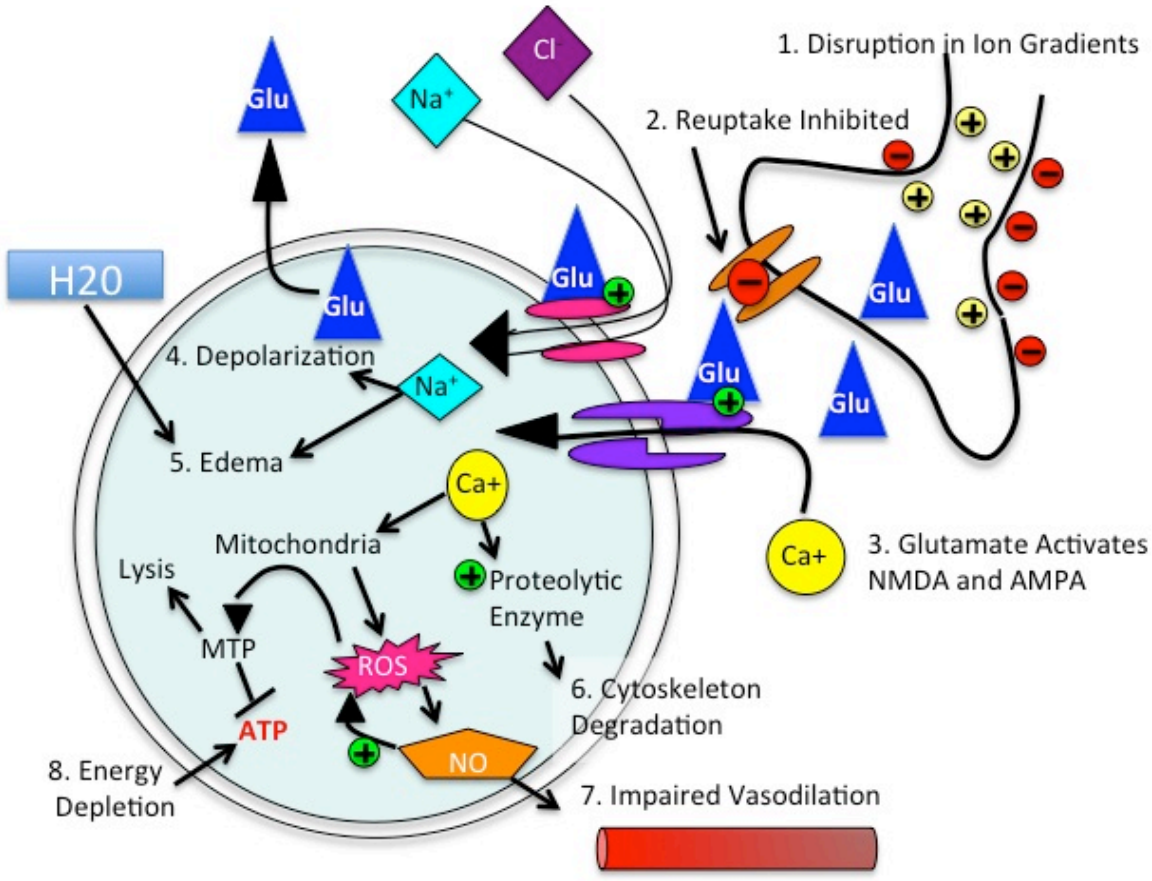

Figure 1. The cellular processes behind ischemic damage in the penumbra. This figure was adapted from U. Dirnagl (1999). **1.**Without oxygen and glucose the cells can not produce the energy needed to maintain the ion gradients across the cell membrane thus the neuron depolarizes releasing glutamate into the system. **2.** Glutamates collects in the extracellular space because the reuptake is an energy dependent process. **3.** The excess glutamate then stimulates NMDA (purple) and AMPA (pink) receptors, which allow the influx of Ca^{++} and Na^{+}/Cl^{-} into the cell respectively. **4.** The influx of the ions results in the depolarization of the cell and the release of more glutamate. **5.** As more ions are flowing into the cell then out, thus excess water flows passively into the cell resulting in edema. **6.** Ca^{++} is a universal second messenger and it initiates many cellular cascades. For example it activates proteolytic enzymes that degrades cytoskeleton proteins and extracellular proteins. **7.** The high intercellular levels of Ca^{++} stimulate the mitochondria, specifically the phospholipase A2 and the cyclooxyenase to produce an excessive amount of reactive oxygen speices. These reactive oxygen species react with nitric oxide (NO) and form peroxynitrites a very volatile reactive oxygen species. This reduces the endogenous levels of nitric oxygen and inhibits vasodilation. **8.** The increase in reactive oxygen species disrupts the inner mitochondria membrane, creating a mitochondrial transition pore (MTP) and thus preventing the creation of ATP, further depleting the cells energy stores.

counteract the ROS. However, in animals that are hyperglycemic, endogenous levels of antioxidants are significantly reduced which may impair the cell's natural defense mechanisms against ROS (Murakami et al., 1998). These reactive oxygen species alter innocuous molecules such as nitric oxide. When nitric oxide reacts with a ROS it produces peroxynitrite, a highly reactive ROS, which causes more tissue damage (Iadecola and Ross, 1997) (**Fig. 1**). Additionally reperfusion into the penumbra is promoted by NO as it triggers the CO_2-induced cerebral vasodilation (Dandona et al., 1978). Diabetics are known to have inhibited production of endothelial NO even prior to stroke (Weih et al., 1999, Toth et al., 2008). Thus increase of ROS after the stroke will decrease the basal level of NO even more.

Reactive oxygen species generated by ischemia also disrupt the inner mitochondrial membrane by creating a mitochondrial transition pore. This pore disrupts the proton gradient and prevents more ATP from being formed in the electron transport chain, creating a catastrophic loss in cellular energy production, mitochondrial swelling and eventual mitochondrial lysis (**Fig. 1**). The lysis of the mitochondria releases cytochrome C that was part of the mitochondria transport chain and is a trigger for cell death.

Another mechanism through which hyperglycemia could aggravate tissue damage after stroke is by compromising the inflammatory responses of microglial, astrocytes and macrophages (Kumari et al., 2007). For example, it has been shown that the release of interkuekin-1β by macrophages and microglia after ischemia (which normally occurs within minutes) is delayed by up to 6 hours in diabetic animals (Herx and Yong, 2001). Interkuekin-1β is important in activating astrocytes, which help with the repair and

scarring process in the brain (Herx and Yong, 2001). Without a proper coordinated release of cytokines and recruitment of immune cells, the inflammation response in diabetics is not effective, leading to greater cellular damage. Hyperglycemia will also cause an increase in the production of NF-κβ even prior to a cerebral insult (Barnes and Karin, 1997). NF-κβ is a nuclear factor that is responsible for the production of pro-inflammatory cytokines, and chemokines such as tumour necrosis factor-α and monocyte chemoattractant protein, which attracts a greater number to leukocytes to the area. Under normal conditions NF-κβ is found in the cytoplasm bound to an inhibitory factor. Post-ischemic attack, the inhibitory factor is ubiquitinated and thus releases the NF-κβ. This will eventually attract macrophages, monocytes and neutrophils in such great numbers that 5 to 7 days post stroke these immune cells become the predominate cells in the penumbra (Weih et al., 1999).

Hyperglycemia leads to enhanced production of activator protein-1 (AP-1), which regulates the transcription of matrix metalloproteinases (MMPs). MMPs are enzymes that degrade the extracellular matrix. The increase in MMP-9 disrupts the integrity of the blood brain barrier by reducing the prevalence of structural proteins such as laminin, endothelial barrier antigen, and zona occludens (Garg et al., 2006). In addition, hyperglycemia leads to abnormal and sometimes excessive production of vascular endothelial growth factor (VEGF). Although VEGF can stimulate new blood vessel growth or angiogenesis, it is also known to degrade the blood brain barrier (Zhang et al., 2000). Therefore, changes in the production of MMP's or VEGF in diabetics could lead to disruption of the blood brain barrier, thereby contributing to cerebral edema after stroke.

Insulin Therapy and Ischemic Injury

Considering that hyperglycemia can profoundly affect brain function, researchers hypothesized that lowering blood glucose after ischemic injury would help alleviate some of the problems diabetics face. In the late 1980s and early 1990s, many animal studies demonstrated that insulin could reduce the negative effects of hyperglycemia during acute focal ischemia (Fukuoka and Scheele, 1989, Voll and Auer, 1991, Hamilton et al., 1995). The Hamilton study (1995) used insulin to reduce blood glucose levels within the physiological range prior to stroke, and found reduced ischemic damage following middle cerebral artery occlusion (MCAO). This work appeared so promising that tight glycemic control was implemented as standard practice in intensive care units (ICUs) across America (Adams et al., 2007). This launched several large human case studies examining the effect of insulin infusions on stroke recovery. Unfortunately, human trials so far have not shown any clear changes in infarct size or improvements in prognosis after stroke (Gray et al., 2007, McCormick et al., 2010).

Neuroplasticity and Functional Recovery

Stroke is the leading cause of morbidity and disability in our society. Although a number of neuroprotectants have been developed in the laboratory, they have uniformly been a disappointment when tested in clinical trials. Even in the absence of therapeutic interventions, there is some degree of spontaneous recovery during the weeks to months after stroke. Ultimately, if we are to facilitate recovery after stroke, we need a better understanding of the mechanisms of brain plasticity that underlie spontaneous recovery.

Recovery from stroke is defined as an improvement in the health or function of an affected individual. Stroke recovery can be divided into three parts: resolution of acute tissue damage, compensation and neuroplasticity (Carmichael, 2003). In resolution of acute tissue damage, the body needs time for the edema, inflammation and ischemic damage associated with the infarct to subside. Second is behavioural compensation where strategies are adopted to counteract the functional disability. The third stage involves complex progressive changes in neuronal and vascular circuitry in surviving brain regions (Carmichael et al., 2005). Indeed, a number of studies have shown that successful recovery from ischemic stroke correlates with extensive structural and functional remodelling of neural and vascular circuits within surviving regions surrounding the stroke, known as the peri-infarct cortex (Carmichael, 2003, Carmichael et al., 2005, Brown et al., 2009, Murphy and Corbett, 2009). For example, recent work using in vivo voltage-sensitive dye imaging demonstrated that behavioural recovery from forelimb cortex stroke was associated with new routes of forelimb related sensory processing in the peri-infarct cortex (Brown et al., 2009). This finding was supported by other imaging studies, which showed that recovery of forepaw function was associated wth a redistribution of forepaw evoked sensory responses in peri-infarct and homotopic regions of the opposite hemisphere at 3 and 14 days after MCAO stroke in rats (Dijkhuizen et al., 2001). Similar changes in brain activity are seen in human patients that are recovering motor function after cortical stroke (Ward et al., 2003, Cramer, 2008). Immediately after cortical injury, functional responsiveness is reduced in the damaged cortex that is accompanied by an increase in the contralateral (ie. unaffected) hemisphere's activity (Carmichael, 2003, Ward et al., 2003, Cramer and Riley, 2008). In later stages, a return

of functional responsiveness to sites adjacent to stroke damage has been correlated with better behavioural recovery (Ward et al., 2003). Therefore, there is extensive evidence showing that unilateral stroke causes an initial shift in cortical responsiveness to the undamaged hemisphere, which is followed by the progressive restoration of brain responses in surviving regions of the damaged hemisphere.

To explain the new activation patterns and plastic changes observed in the stroke affected hemisphere, two mechanisms have been proposed. The first is that existing neural pathways in functionally relevant brain regions are reinforced via dis-inhibition and or potentiation. A second mechanism is that new neuronal circuitry is formed via synaptogenesis and axonal sprouting (Dijkhuizen et al., 2001). Electrophysiological recordings within the peri-infarct zone support the idea that existing neural circuits become more excitable and possibly strengthened after stroke. For example, recordings in layer V neurons of the peri-infarct region revealed an increase in the baseline-firing rate and plasticity (ie. long-term potentiation) of these neurons after stroke (Schiene et al., 1996, Hagemann et al., 1998) (**Fig. 2)**. Furthermore, inhibitory postsynaptic potentials were diminished in these peri-infarct neurons. The proposed mechanism behind these changes in excitability is that stroke augments the expression of glutamate and GABA receptors. Supporting this, autoradiography labeling studies showed there was a decrease

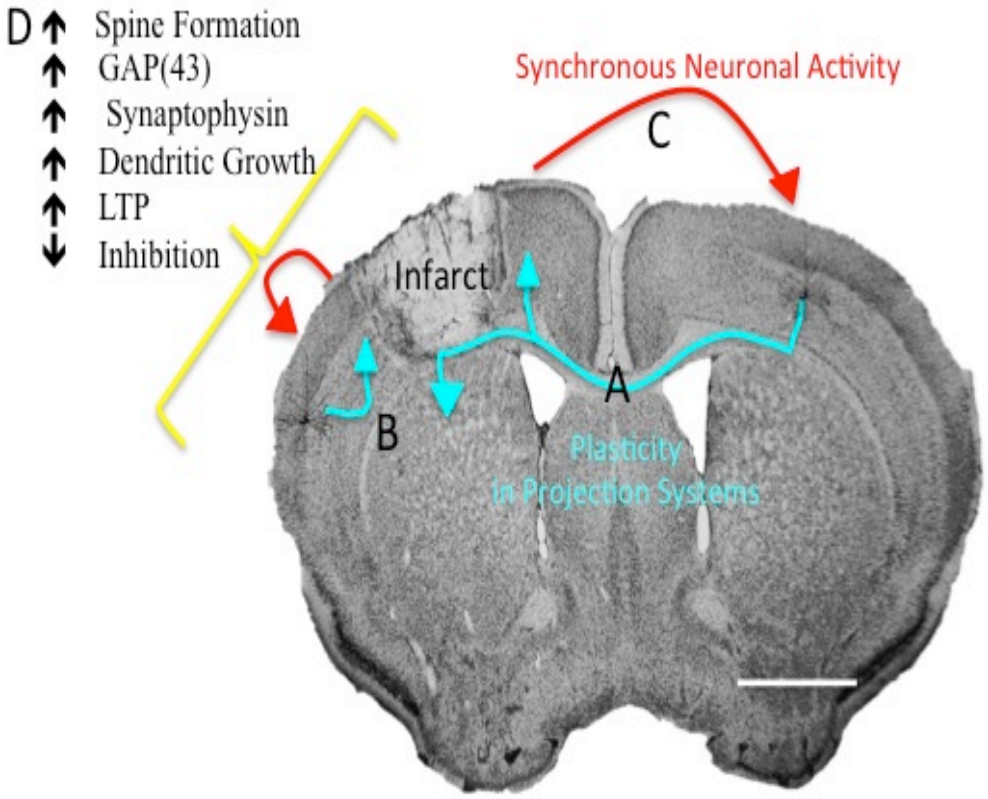

Figure 2. This figure demonstrates an overview of the physiological and anatomical changes that occur after stroke. This figure has been adapted from Carmichael 2003 paper. **A)** The teal line represents the axonal projections from the contralateral hemisphere along the corticostriatal tract. **B)** The teal line represents the projections to the peri-infarct region. **C)** Red line indicates the origin of the synchronous neuronal activity, and its region of effect. **D)** Outlines physical changes that happen within the peri-infarct cortex following stroke. Scale bar = 1mm

in $GABA_A$ binding sites and an increase in NMDA receptors in peri-infarct regions (Schiene et al., 1996, Redecker et al., 2002). Together these results show that stroke alters the excitability of peri-infarct connections, which may facilitate synaptic plasticity and functional re-arrangements of circuitry.

In addition, structural plasticity of axonal connections and dendritic spines likely contribute to functional plasticity after stroke. For instance, growth associated protein (GAP) 43 is responsible for triggering new axonal growth cones and is ubiquitously found in humans and animals (Carmichael, 2006). This protein is elevated in the peri-infarct cortex as early as 1 day post stroke and remains elevated for several weeks (Stroemer et al., 1995). Approximately 3 weeks following stroke, GAP43 expression decreases followed by an increase in the synaptophysin protein, which is associated with the formation of mature synapses (Stroemer et al., 1995) (**Fig. 2)**. Tract tracing studies have also provided evidence that new cortical connections form in the region around the stroke. Recently, Brown et al., (2009) showed that long-term stroke recovery correlated with the formation of new cortical connections from peri-infarct cortex to regions associated with motor functions such as the retrosplenial cortex and striatum. This was supported by work done in a primate model that showed axonal sprouting extending to the peri-infarct region from the premotor region (Carmichael ST, 2001). This shift in inter-hemisphere projections is so robust that some axonal projections shift as much as 180° from their normal targets (Carmichael ST, 2001) (**Fig. 2)**. Although it is not entirely clear what triggers these extensive changes in axonal patterning, Carmichael and Chesselet (2002) showed that synchronous neuronal activity, arising from the peri-infarct

regions likely plays a role, especially considering that silencing synchronous activity prevented sprouting (Carmichael and Chesselet, 2002) (**Fig. 2**). Of note, slow wave synchronous neuronal activity is also seen the developing nervous system, during the initial period of axonal growth and synaptogenesis (Carmichael, 2003).

Changes in dendritic arbours and spines could support new patterns of cortical connectivity after stroke. Jones (1994) work demonstrated that there was an increase in dendritic branching and spines in both the contralateral hemisphere and peri infarct region after stroke (Jones and Schallert, 1992, 1994, Jones et al., 1996). This increase in branching spiked at day 18, and by day 30, there was a reduction in branching compared to day 18 but the volume was still above baseline (Jones and Schallert, 1994, Jones et al., 1996). Given that dendritic spines are the post-synaptic target of most excitatory synapses, examining changes in the rate of spine formation/elimination provides an indication of synapse turnover during stroke recovery. Using in vivo two-photon microscopy, Brown et al., (2007, 2009) showed that immediately after stroke, there was a considerable increase in dendritic spine turnover, which remained above baseline for 5 weeks (Brown et al., 2009). Further, this finding was limited to the region directly next to the stroke, within ~700-800um (Brown et al., 2009) (**Fig. 2)** suggesting that the primary site of cortical plasticity occurs in the peri-infarct region. More recently, Mostany et al. (2010) examined dendritic spine plasticity in relation to blood flow in the peri-infarct tissue for 3 months post stroke (Mostany et al., 2010). However, in this study, they used a MCAO stroke model, which produces a larger peri-infarct zone with graded levels of blood flow. They found that the degree of local perfusion determined the rate, magnitude and mode of synaptic turnover in the peri-infarct region (Mostany et al., 2010). More

specifically, dendrites in cortical regions with preserved blood flow post stroke (>70% blood flow) recovered faster by adding a greater quantity of spines that were relatively short lived. By contrast, regions with reduced blood flow (<70% blood flow) managed to slowly increase spine numbers by reducing the rate of spine elimination (Mostany et al., 2010). Thus, it appears that the rate/means by which the dendrites recover lost spines is partially attributed to their access to local blood supply.

Functional recovery correlates with the ability to remap cortical functions, and this remapping can only occur with the coordination of many molecular mechanisms. Thus it is reasonable to hypothesize that poor stroke recovery in diabetics could be related to impaired neuroplasticity. Indeed, numerous studies have shown that diabetic humans and animal models of type 1 and 2 diabetes have impairments in cognitive function, synaptic transmission, synaptic plasticity (such as long-term potentiation), neurogenesis and synaptogenesis (Manschot et al., 2003, Brands et al., 2005, Biessels et al., 2006, Stranahan et al., 2008a). There have also been documented changes in the morphology of neurons in diabetic mice/rats such as dendritic atrophy in the hippocampus and a decrease in axonal length in the prefrontal cortex (Martinez-Tellez et al., 2005, Toth et al., 2006). Although there is evidence to suggest the health of the diabetic brain and neurons are compromised, there has yet to be a connection made between the poor prognosis for stroke recovery and impaired neuroplasticity in diabetics.

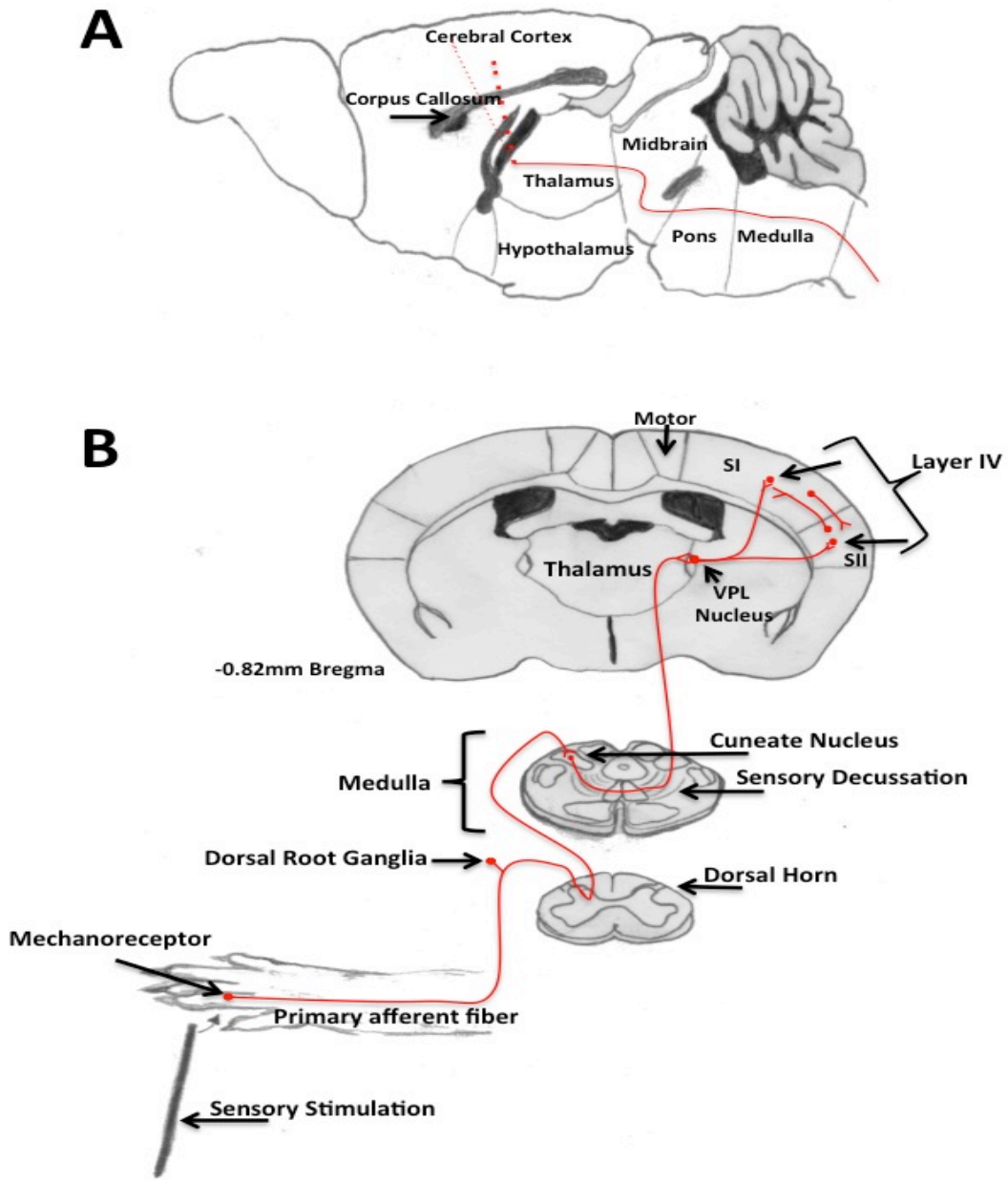

Figure 3. The somatosensory pathway. A) A sagittal view of the mouse brain and the path the somatosensory information travels**.** The thin red dotted line is the primary somatosensory projection from the VPL in the thalamus. The thick red dotted line is the secondary somatosensory projection from the VPL in the thalamus. **B)** When the forepaw is touched, mechanoreceptors in the skin depolarize. This generates action potentials that travel up to the dorsal horn of the spinal cord. In the spinal cord, the sensory nerve travels up to the cuneate nucleus in the medulla. The medulla is where sensory information decussates and then ascends to the VPL nucleus of the thalamus along the medial lemniscus tract. The thalamus processes, filters, and relays all sensory information to the cortex. The VPL transmits sensory information to layer IV of primary and secondary somatosensory cortex

1.3 Anatomy of the Somatosensory Cortex

In order to understand the neuroplasticity and functional recovery after ischemic damage to the somatosensory cortex, it is important to understand both the anatomy and physiology of the somatosensory system. Even the simplest action such as detecting a touch on your hand and responding with a movement requires the integration and communication of many nuclei and cortical regions along with the peripheral receptors. There are several different types of mechanoreceptors embedded in the skin that detect tactile sensations. Meissner and Pacinian mechanoreceptors adapt rapidly to stimulation and are responsible for encoding edges/flutter and vibration. By contrast, slowly adapting receptors such as the Merkel and Ruffini receptors report textures and stretching of the skin or muscles (Vallbo and Johansson, 1984). Therefore, the activation of these receptors in different combinations can represent many aspects of tactile stimuli.

When the forepaw is touched, mechanoreceptors in the skin depolarize which generates action potentials in the afferent sensory nerves that convey this information from the paw to the dorsal horn of the spinal cord. In the spinal cord, the sensory nerve travels up to the cuneate nucleus. The cuneate nucleus is located in the caudal medulla and here the information decussates and then ascends to the ventral posterior lateral (VPL) nucleus of the thalamus along the medial lemniscus tract (**Fig. 3)**.

The thalamus processes and conveys all afferent sensory information to the cerebral cortex. It also filters the incoming information depending on the state of the animal. For example information processed in the thalamus is subject to brain stem modification via the serotonergic or adrenergic systems, inhibitory feedback from the

reticular nucleus of the thalamus, and excitatory feedback from the neocortex. Axons from the cells in the thalamus project to the cerebral cortex through, the internal capsule, a large fibre bundle that contains nearly all the thalamic projections that innervate the cortex.

There has been a long-standing debate on whether the thalamocortical somatosensory area is organized into a hierarchical or serial processing system. Previously it was thought that S1 and S2 processed information in a hierarchical manner, because after the S1 region was surgically ablated, there was no response in the S2 region (Garraghty et al., 1990, Felleman and Van Essen, 1991). This suggested that the sole input to S2, in these primates, came from intracortical connections from the S1.

More recently, however, there has been overwhelming evidence that supports the serial method of processing. Firstly, there are direct thalamic projections to both regions, S1 and S2. Secondly, sensory evoked activation in both S1 and S2 cortex are independent of each other, which was elegantly demonstrated by the Rowe group, who reversibly inactivated the S1 cortex and examined responsiveness in the S2 cortex (Turman et al., 1992, Turman et al., 1995). What they found was that only 20% of the neurons in S2 cortex had reduced activity after the loss of the S1. The 20% neurons that did demonstrate a reduction in activity most likely had intercortical connections from S1 to S2, which modulated their activity (Ghosh et al., 1992, Turman et al., 1992, Turman et al., 1995).

Once sensory information reaches layer 4 of the somatosensory cortex, it is then relayed vertically into layers 2/3. From there, sensory information can propagate in many directions, such as layers 5 and 6, other cortical regions and even back to the thalamus. It is important to note that FLS1 and S2 have extensive connections with one another (Burton and Fabri, 1995, Krubitzer et al., 1995). The major purpose of the somatosensory system is to process sensory inputs and integrate this information with other sensory or motor regions, for example to determine if a movement needs to be modified when touching an object. Thus, once sensory information is processed in the primary and secondary somatosensory cortex, signals are sent to the premotor regions and then to the primary motor region. It is here that an action is decided upon and the motor cortex can provide an output signal. To do this, neurons in Layer V of the primary motor cortex project their axons directly to the ventral horn of the spinal cord. These axons travel along the cortiospinal tract which consists of approximately 1 million axons that descend through the mid and hindbrain at the medulla, where 90% of the inputs cross over. Some of axons terminate directly at motor neurons and control specific movements, others form synapses with interneurons and co-ordinate large groups of muscles. All motor information relayed in the corticospinal tract is significantly modulated by the somatosensory system and, thus, these tracts continuously send information to make voluntary movement accurate.

1.4 Background on Methodology

Induction of Type I Diabetes Mellitus by Streptozocin.

Animal models are crucial in helping the scientific community better understand the etiology of a disease and in improving the quality of life for those who suffer from it. Type 1 diabetes murine models can be divided into two major groups: the genetic models and the diabetogenic chemical models. The most common genetic model is the non-obese diabetic (NOD) mouse, which has no endogenous insulin due to an autoimmune attack on the β-cells. However, this type 1 diabetes is not genetically homologous to the human form, as the NOD model does not cause deafness or the loss of a C5 compliment, as in humans (Atkinson and Leiter, 1999).

For the diabetogenic chemical models, the most prominent chemical utilized by the research community is streptozotocin (STZ) (Etuk, 2010). By varying the dose of STZ, the severity of the disease can be controlled. STZ is a glucose analogue that is transported into the insulin secreting β-cells of the pancreas via the GLUT2 transporters. These transporters, which are found in the liver, hypothalamus, in the renal tubular cells of the kidneys and the small intestine, are transmembrane carrier proteins that passively transport glucose across the membrane. Although the highest concentrations of GLUT2 transporters are found on the pancreatic β-cells, which, explains their dose-dependent selective toxicity, STZ can cause damage to the liver and kidneys when particularly high doses are administered (Wang and Gleichmann, 1998, Lenzen, 2008b). By selectively destroying the β-cells of the pancreas, diabetogenic chemicals induce classical symptoms of type 1 diabetes, including increased water and food intake, loss of weight, frequent

urination, retinopathy and kidney disease (Wong and Tzeng, 1993, Montanari et al., 2005).

STZ is a potent alkylating agent that consists of an *M*-methyl-*N*-nitrosourea moiety linked to a deoxyglucose molecule (Karunanayake et al., 1976, Tjalve et al., 1976, Yamamoto et al., 1981, Lenzen, 2008a). Once inside the cell, the *M*-methyl-*N*-nitrosourea moiety is cleaved from the glucose moiety and then alkylates and cleaves the cell's DNA (Yamamoto et al., 1981, Lenzen, 2008b). These breaks in the DNA lead to the depletion of the cell's energy stores, which prevents vital cellular processes such as DNA, RNA, protein synthesis, and finally, cellular necrosis (Pieper et al., 1999, Lenzen, 2008a). The result of pancreatic β-cell necrosis is an acute release of insulin, resulting in temporary hypoglycaemia followed by permanent hyperglycemia as no further insulin is produced, thus mimicking the effects and clinical presentation of type 1 diabetes (Lenzen, 2008a).

Photothrombotic Stroke

Photothrombosis is an effective method for creating a targeted and reproducible ischemic stroke (Watson et al., 1985a). The infarct produced by the photothrombotic method is typically small, as it affects 5–15% of the hemisphere, and thus is comparable to a survivable human stroke (Brown et al., 2009). Photothrombosis uses a photosensitizing dye together with an excitatory wavelength of light to generate platelet activation and microvascular occlusion. Commonly used dyes include fluorescein isothiocyante (FITC), Photofrin, and Rose Bengal (Herrmann, 1983, Watson et al., 1985a, Ishikawa et al., 2002, Schroeter et al., 2002). Rose Bengal is a potent

photosensitizing dye that, upon irradiation by a 532-nm (green) light, generates singlet oxygen species. Singlet oxygen causes cell-surface lipid peroxidation on endothelial cells (Herrmann, 1983, Watson and Ginsberg, 1989, Inamo et al., 1996)(Herrmann, 1983, Watson and Ginsberg, 1989, Inamo et al., 1996). As a result, the aggravated endothelial cells initiate a normal thrombogenic (clot promoting) response (Watson and Ginsberg, 1989). The clot formation is limited to the region of the brain exposed to the 532-nm light.

Voltage Sensitive Dye (VSD) Imaging

Over the last few decades there has been an emergence of many powerful functional imaging techniques. In the present study, voltage sensitive dye (VSD) imaging was chosen to image functional recovery after stroke because it directly reports changes in membrane voltage (on a millisecond scale), unlike conventional functional imaging approaches that detect very slow changes in metabolism (on the order of seconds). VSD imaging allows one to visualize large populations of neurons as well as their connections between discrete cortical regions (Grinvald and Hildesheim, 2004, Berger et al., 2007). Thus, *in vivo* VSD imaging is ideal for studying how stroke recovery affects the spatiotemporal dynamics of cortical processing (Ferezou et al., 2007, Brown et al., 2009). The VSD molecules bind indiscriminately onto the external surface membranes of all the cell types in the brain without altering the normal functioning of these cells (Grinvald and Hildesheim, 2004, Chemla and Chavane, 2010). VSD acts to transform changes in membrane potential into an optical signal. Even though the change in trans-membrane electric field is large (10^7–10^8 mV/m), if the dye wasn't touching the membrane it would

not be able to report the depolarization; therefore, dye that is not bound will not fluoresce (Hirase et al., 2002). Since the dyes acquisition into the membrane is so critical to its function, it is important to mention that the dye cannot bind to any myelinated region (Chemla and Chavane, 2010). Thus, the signal primarily originates primarily from the soma, dendrites and non-myelinated axons of superficial cortical layers *in vivo*.

2. Materials and Methods

2.1 Animals

All experiments were conducted according to the guidelines laid out by the Canadian Council of Animal Care and the University of Victoria Animal Care Committee. Two month old wild-type or GFP-M line mice with a C57BL/6 background (Feng et al., 2000) were used in the present study. Mice were group housed in a temperature controlled room at 21° ± 2°C under a 12 hour light/dark cycle. Animals were provided with environmental enrichment devices and given free access to water and standard laboratory diet. Animals displaying signs of illness or unreasonable pain following any procedure were sacrificed according to UVic animal care SOPs (UVIC, 2010).

2.2 Induction of Type I Diabetes and Monitoring of Blood Glucose

To induce type 1 diabetes, 2 month old mice were deprived of food for 4 hours, then given a single intraperitoneal (IP) injection of Streptozotocin (STZ) at a dose of 140mg/kg dissolved in buffer (Yamamoto et al., 1981, Lenzen, 2008a). Controls were food deprived and given an injection solely consisting of buffer. The citrate buffer (50mM) was made by dissolving 1.47 of sodium citrate in 50ml of dH_20. The pH of the buffer was reduced to 5.0 by the drop-wise addition of 12N hydrochloric acid and tested using a pH meter. The STZ was dissolved in the sodium citrate buffer to a concentration of 35 mg/mL. The solution was then filtered through a sterile 0.45µm syringe filter. Post-injection, mice were supplied with a 5% glucose/dH_2O solution to prevent acute hypoglycemia, which occurs in the first few hours after injection of STZ (Lenzen,

2008a). The mice were passively monitored for signs of diabetes such as frequent urination and their blood glucose was taken 3 days post injection. Blood glucose levels were checked using an Aviva™ Accu-Chek® blood glucose meter. Blood glucose levels were measured weekly or every other week by fasting mice for 2-3 hours and then withdrawing a drop of blood from the tail vein. Control mice had normal blood glucose levels (~8-10 ± 2 mM/L), and the diabetic group had elevated blood glucose levels (15 to 33 mM/L) (**Fig. 4C**).

2.3 Targeted Photothrombotic Stroke

Focal ischemic stroke of the right forelimb somatosensory cortex was induced using the photothrombotic method (Watson et al., 1985b, Brown et al., 2007). Briefly, mice were anesthetized using 1.5-1.8% isoflurane mixed with oxygen. Each mouse was kept on a heating pad during surgery to stabilize body temperature at 37°C, which was measured with a rectal thermoprobe and temperature feedback regulator. Opthalmic gel was applied to ensure adequate lubrication of the eyes, and a local anesthetic (Lidocaine, 0.02ml) was injected subcutaneously to the scalp to block local pain responses during surgery. Scissors were used to trim excess hair on the scalp, and a midline incision (~2mm) was made using a stainless steel surgical scalpel (#11). The scalp was retracted with small clamps. The skull was thinned to 50% of its original thickness over a 1.25mm by 1.25mm region 2mm lateral from bregma. The skull was thinned using a high-speed dental drill and then moistened using artificial cerebral spinal fluid (ACSF). Then a 1.3% agarose low melt agarose solution (37°C -39°C; type 3A Sigma; a9793) dissolved in a HEPES-buffered artificial cerebral spinal fluid was placed over the region of interest and a coverslip was placed over it.

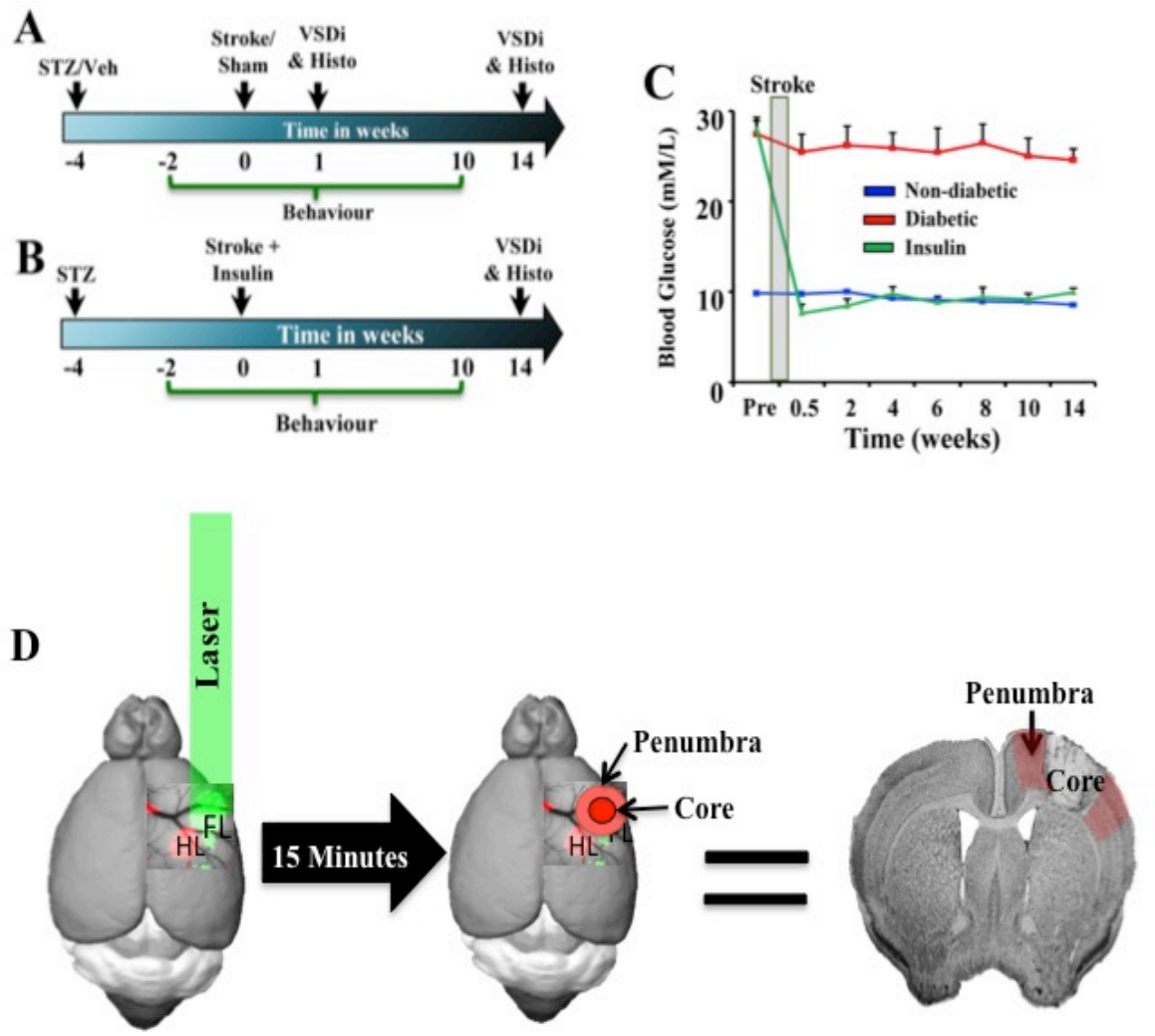

Figure 4. Diagram summarizing experiments used to investigate the effect of diabetes and chronic insulin treatment on stroke recovery. A) Timeline of the experiment expressed in weeks relative to the induction of stroke. Four weeks prior to the stroke diabetes was induced in young adult mice with a single dose of STZ (140 mg/kg), whereas non-diabetic mice received vehicle injection or STZ but did not develop hyperglycemia. Behavioural tests of sensori-motor function of the forepaw were conducted on a weekly basis for 2 weeks before, and 10 weeks after photothrombotic stroke (induced at time 0). Mice were imaged and then sacrificed for histological assessment at 1 and 14 weeks to determine if diabetes affected acute stroke damage or long-term recovery. **B**) To determine whether insulin treatment can normalize stroke recovery, diabetic mice were subjected to photothrombotic stroke and then had slow release insulin pellets subcutaneously implanted. Similar to that described above, forepaw function was tested at weekly intervals and at the end of the 14-week recovery period, cortical responses to forepaw stimulation were imaged. **C)** Average fasting blood glucose levels (mM/L) for each group. The insulin implants were inserted after the stroke. **D)** A schematic of the photothrombotic stroke model. The animal is injected (IP) with Rose Bengal dye then the primary forelimb somatosensory cortex is exposed to a green laser for 15 min. This produces a reproducible infarct, localized within the primary forelimb somatosensory cortex. The infarct consists of two regions; the core, where the ischemic conditions are the most severe and the loss of the blood supply results in the necrosis of the cells in this region; the penumbra which is electrically silent, with limited blood flow, and partial energy metabolism.

In order to precisely target the stroke to the forelimb sensory cortex, mice were first prepared for intrinsic signal optical signalling. The cortical surface and vasculature were photographed using white light filtered through a GFP filter on an Olympus microscope equipped with an XLfluor 2X (NA 0.14) objective. The plane of focus was set to 300μm below the surface cortex to reduce the interference from the surface vessels. The brain was illuminated with a red LED (light emitting diodes) of 635 ηm. Red light was used to detect forelimb-evoked changes in light reflectance caused by an increase in levels of deoxyhemoglobin within the cortex (Frosting et al., 1990). Acquisition was performed using a MiCAM02 HR high-speed camera coupled to Brainvision imaging software 8.19. Each imaging session consisted of 20-40 forelimb or hindlimb stimulation trials subtracted by null stimulation trials. During each trial, 100 image frames were collected over 3s. Contralateral fore- or hindlimb stimulation was delivered 1.5s into each trial using piezoelectric device at 100Hz for 1s. To generate maps of the forelimb and hindlimb areas, trials for each limb were first summed and mean filtered (radius=3) using NIH image J software. Responsive areas were then identified by dividing all frames taken 1.5s after stimulation, by those taken before stimulation (Brown et al., 2009). The responsive areas were then assigned a particular colour and merged onto the image of the cortical surface vasculature. Consistent with previous research on cortical primary somatosensory maps, the region for the forelimb was anterior and lateral to the hindlimb representation at a 45° angle.

For the induction of photothrombotic stroke, a collimated green laser (532 nm, 17 mW; ~1.25 mm diameter) was positioned over the forelimb cortex for 15 minutes after injecting 1% Rose Bengal dye (i.p. 110 mg/kg dissolved in 0.9% saline) (**Fig. 4D)**. The

scalp was sutured together (braided silk, reverse cutting 45cm sterile, Ethicon 62G) and then knots were fortified with cryanoacrylate glue. The mice were allowed to recover under a heating lamp and then returned to their cages. Sham operated mice were exposed to all parts of the experiment except were given either the rose bengal dye or the green laser.

2.4 Insulin Implants

In order to control blood glucose levels in diabetic mice, slow release insulin pellets were inserted subcutaneously into mice (Linshin Canada Inc.) within the first hour after stroke **(Fig. 4C)**. To do this, the fur was shaved from a 2cm square patch of skin between the scapulae. The skin was disinfected with 70% ethanol, and a small incision was made. The pellets were inserted under the skin with a trocar and then the incision was closed with a single suture (braided silk, reverse cutting 45cm sterile, Ethicon 62G). The dosage is outlined by Linshin Canada Inc.: two pellets for the first 20g of mouse and 1 pellet for every 5g after that. Blood glucose levels were measured (Accu-Chek, Aviva, Roche) weekly by fasting mice for 3-4 hours and then withdrawing a drop of blood from the tail vein. If blood glucose levels ≥14mM/L, mice were lightly anesthetized and 1 pellet was inserted subcutaneously in the mid dorsal region using a trocar. Mice were allowed to recover and blood glucose was re-assessed the following day after food deprivation.

2.5 Quantification of Infarct Volume

1 week or 14 weeks after stroke, mice were euthanized following the University of Victoria animal care unit (ACU) SOP for pento-barbiturate overdose, Euthasol (240 mg/mL) (UVIC, 2010). The mice were then perfused transcardially with 10 mL of 0.1M phosphate buffered saline (PBS), and then 10mL of 4% paraformaldehyde (PFA). The brains were removed and then stored in 4%PFA over night and then in PBS until they were sectioned. A Leica vibrotome was used to section the brains at 50µm in the coronal plane. Every 6^{th} section was stained using cresyl violet, and mounted onto charged glass slides. Serial sections were imaged with a 4X objective under bright field illumination using a 12-bit CCD Photometrics™ CoolSnap HQ camera, and Image Capture software (**Fig. 8)**. The images were then quantified blind using Image J software (version 1.44). The area of infarction was measured in each section three times and an estimate of volume was calculated by summing up the infarct area for each section (s) multiplied by the distance (d) between each section (volume = s1d1 + s2d2 + s3d3 + s4d4) (Shih et al., 2005).

2.6 Behavioural Assessment of Forepaw Sensory-Motor Function

The adhesive tape removal and horizontal ladder test has been previously used to measure sensory neglect and sensori-motor function, respectively after stroke (Schallert, 2006, Shanina et al., 2006). These tests were administered at weekly intervals for 2 weeks before stroke and 10 weeks afterwards. For the tape removal test, a circular piece of tape (5 mm diameter) was placed on the palm of each forepaw (**Fig. 5E)**. Mice were then placed in a glass cylinder and filmed for 60 seconds (**Fig. 5D)**. This was repeated 3 times

per testing session with the time taken to remove the tape from each paw was scored by an observer blind to the condition. Sensori-motor function of the forepaw was assessed by videotaping mice as they walked across an elevated, 70 cm long horizontal ladder with it's rungs (1 mm diameter) randomly spaced at 1 or 2 cm apart (**Fig. 5A)**. Forepaw grasping of the rungs was scored on a frame-by-frame basis using similar criteria to previous work (Farr et al., 2006). Forepaw placements were scored as: i) "correct" (**Fig. 5B**, forepaw placement centered on the rung), ii) "partial" (**Fig. 5C** forepaw partially grasping rung or required a correction of the placement), or iii) "slip/miss". Due to inherent variability in behavioural measurements, data for each mouse was averaged in 2-week bins.

2.7 Voltage Sensitive Dye Imaging

The mice were anesthetized with isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane), at a concentration of 2% for induction and 1-1.5% for maintenance throughout the procedure, with a consistent oxygen flow rate of 0.7L/min. Paw withdrawal, whisker movement and eye blink reflexes were absent throughout the procedure. Mice were fitted into a stereotaxic frame, whereupon the eyes were moistened with antibiotic ointment (pentamycetin) and body temperature was maintained at 37° C with a rectal thermo-probe and temperature feedback regulator. For every 2 hours of anaesthesia, mice were given 0.15mL of 20mM glucose dissolved in buffer to maintain proper hydration and glucose levels. The skin was pulled back over the cranium and the

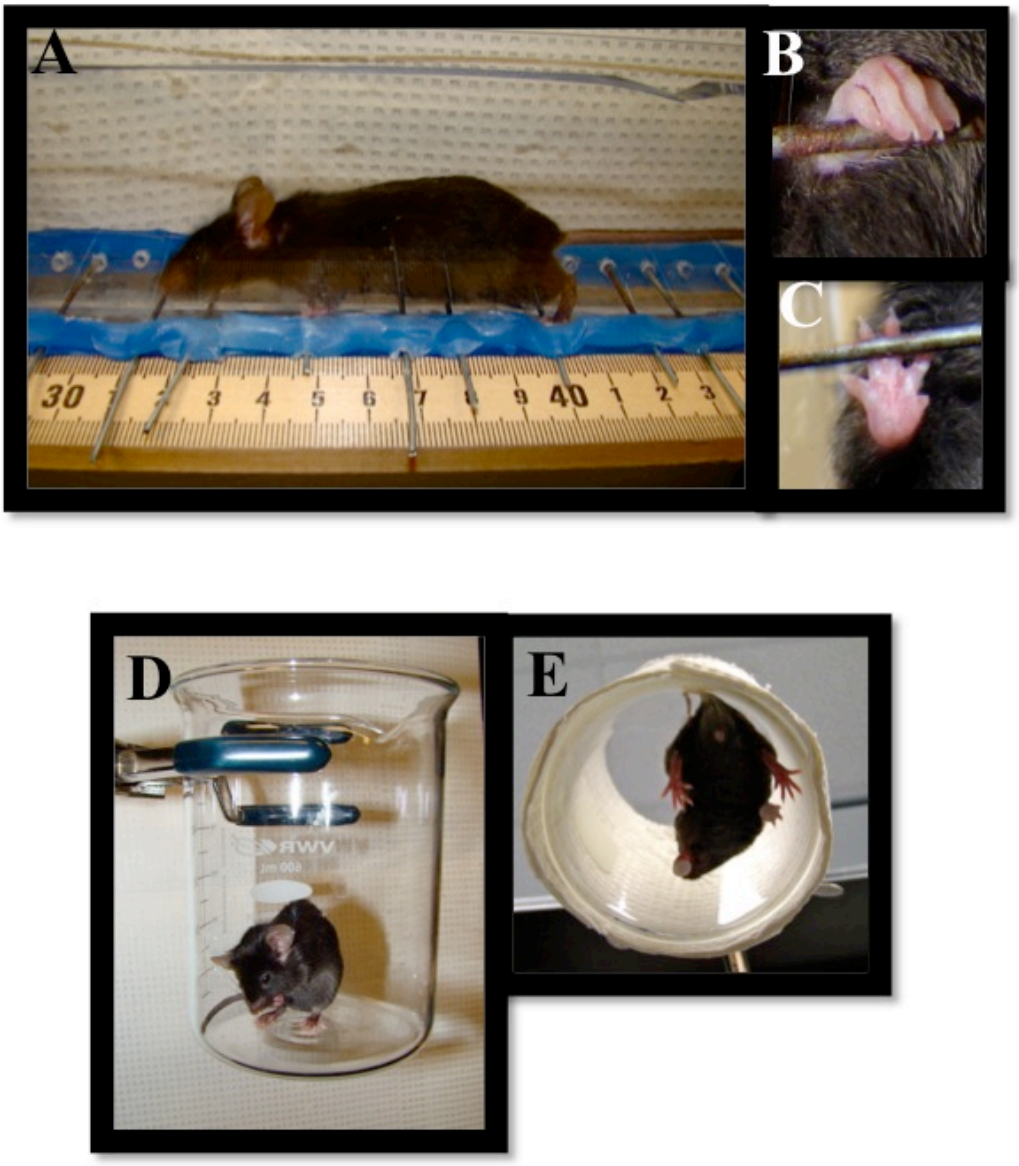

Figure 5. The adhesive tape removal and horizontal ladder test, which were used to measure sensory neglect and sensori-motor function after stroke. A) The horizontal ladder test assessed mice as the crossed 70cm long ladder with rungs that were 1 mm in diameter and space randomly at 1 or 2 cm apart. Forepaw grasping of the rungs was scored on a frame-by-frame basis. Forepaw placements were scored as: **B)** Correct, forepaw placement centered on the rung. **C)** partial, forepaw partially grasping rung. **D)** The setup used for the tape removal test, here the mouse is removing a piece of tape affixed to the forepaw. **E)** Demonstrates the angle from which the tape removal test was filmed. You can see the mouse still has both pieces of tape affixed to its paws.

muscle over the squamosal bone was pulled back to allow access to the secondary somatosensory region. To prevent any movement during imaging, the skull was secured to a metal plate using cyanoacrylate glue and dental cement, which was fastened to the surgery stage. A large (~6.5 x 5mm) region of the skull overlying the right cerebral hemisphere was drilled and the skull and dura were carefully removed. Gel foam soaked in HEPES buffered artificial cerebral spinal fluid (ACSF) was used to keep the brain moist throughout the surgical procedure. The exposed brain was bathed in RH1692 VSD (1 mg/ml passed through 0.22µm Polyvinylidene Fluoride (PVDF) syringe filter) dissolved in HEPES-buffered ACSF for 90 minutes. After the incubation period, the brain was washed thoroughly with brain buffer, covered with 1.3% low-melt agarose dissolved in a HEPES-buffered ACSF and sealed with a glass cover slip. The surgery stage was then mounted underneath an upright Olympus BX51 microscope for imaging.

For VSD imaging, 12-bit image frames (184x124 pixels) were captured every 4ms using a MiCAM02 HR high speed camera coupled to Brain Vision imaging software version 8.19. The dye was excited with Luxeon K2 red LED (627nm, ~20mW at back aperture) that was passed through a Cy5 filter cube (exciter: 605-650nm, emitter: 670-720nm). Red light was focused 200-300µm below the cortical surface using an Olympus XFluor 2X objective (NA=0.14). Mechanical stimulation of the forepaw was achieved by gluing a pencil lead to the paw, which was connected to a piezoelectric wafer (Q220-AY-203YB, Piezo Systems; ~300µm deflection in the caudal-rostral plane). During each trial, images were collected 250ms before a single 5ms deflection of the forepaw (or not for null stimulation trials) and then 550ms afterwards. This process was repeated 12-24 times with approximately 10-second interval between trials. To correct for dye bleaching,

stimulation trials were divided by null stimulation trials. VSD images are presented as the percent change in VSD fluorescence (ΔF/Fo) by dividing frames collected after stimulation by the average of those taken 100ms before stimulation. Montages of cortical responses were generated by mean filtering ΔF/Fo image stacks (radius=2) and then binning 2 frames in time (hence 8ms between each image). The amplitudes of cortical depolarizations were expressed as the percentage change in VSD signal (ΔF/Fo) relative to baseline fluorescence. Using Image J software, forelimb-evoked cortical responses were quantified by placing a circular region of interest (500μm diameter) over the center of the FLS1, HLS1, M1 or S2 cortex (**Fig. 7A**). After stroke, the FLS1 was defined as the remaining piece of forelimb cortex that showed the shortest latency to respond next to the stroke, typically immediately below or above the center of the original FLS1 region. The peak amplitude (ΔF/Fo), time to peak amplitude and half-width (ie. duration) of VSD signals in the first 250ms after stimulation were measured with Clampfit 9.0 software (Molecular Devices).

2.8 Reversible Inactivation of S2 Cortex

In order to determine whether S2 cortex plays a role in stroke recovery, we inactivated the S2 cortex in the right hemisphere of stroke recovered (ie at 11 weeks recovery) or sham operated mice by injecting muscimol, a GABA-A receptor agonist. Mice were anesthetized with isoflurane and a small hole was drilled through the skull approximately 1.5mm posterior and 4mm lateral of bregma. Intrinsic optical imaging maps generated initially to target the stroke to the forelimb cortex were also used to precisely identify the location of S2 cortex. A stainless steel Hamilton syringe with a 33 gauge needle was lowered 1.3mm deep into the brain. Muscimol hydrobromide (5μg/μL;

Sigma G019) dissolved in ACSF with 1% Texas Red dextran was slowly pressure injected (0.2μL) over a 5-minute period. Inclusion of Texas Red dextran (70kDa, Invitrogen, D-1864) allowed us to verify injection placement (**Fig. 10A)**. As a control, several mice were injected an equivalent volume of ACSF, which, had no effect on tape removal latency (**Fig. 10B**). Approximately 4 hours after muscimol injection, mice were administered the adhesive tape removal test.

2.9 Statistics

Statistical comparisons for the effect of diabetes on stroke recovery were made using an ANOVA with post-hoc student t-tests. Paired t-tests were used to examine the effect of muscimol injection on tape removal latency. All p values ≤ 0.05 were considered statistically significant. All data are presented as the mean ± standard error of the mean.

3. Results

3.1 Induction of Diabetes and Stroke

Figure 4A and B summarize the two primary experiments described in the present study. First, in order to understand why diabetes is associated with poor stroke recovery in humans, we imaged changes in the function of the somatosensory cortex using *in vivo* voltage sensitive dye (VSD) imaging in diabetic mice subjected to ischemic stroke in the right forelimb somatosensory cortex. In tandem with functional brain imaging, recovery of forepaw function after stroke was assessed weekly using the adhesive tape removal and horizontal ladder tests (**Fig. 4A**). Our second aim was to determine whether any diabetes related deficits in cortical plasticity or functional recovery could be reversed by regulating blood glucose levels with long-term insulin treatment (**Fig. 4B**).

To induce diabetes, 2 month old C57BL/6 male mice were given a single injection of 140mg/kg streptozotocin (STZ, i.p.) or vehicle As shown in **Figure 4C**, blood glucose levels in STZ injected ("diabetic" group) mice were chronically elevated relative to vehicle injected controls ("non-diabetic" group) or diabetic mice implanted with slow release insulin pellets ("insulin" group). We should note that some of the mice in the non-diabetic group consisted of STZ injected mice that did not develop hyperglycemia (blood glucose levels < 12mM/L). Four weeks after STZ injection, mice had a focal ischemic stroke induced by laser-targeted photothrombosis to the right forelimb primary somatosensory cortex (FLS1) (**Fig. 4D)**. This method of stroke consistently produced a cerebral infarction that extended through all cortical layers, but left the white matter

intact (Watson et al., 1985b, Brown et al., 2007). Mice subjected to sham stroke were exposed to either the laser or the photosensitive dye Rose Bengal but not both. Sham stroke mice showed no signs of ischemic damage.

3.2 Diabetes is Associated with Poor Recovery of Sensory Function After Stroke

The adhesive tape removal test is considered a sensitive measure of sensory neglect (ie. loss of attention to the stroke affected limb) that is commonly found after stroke in the right somatosensory cortex (Schallert, 2006, Tennant and Jones, 2009). In all 3 groups tested, stroke increased the time it took for mice to remove tape from the left (affected) paw in the first couple weeks after stroke (**Fig. 6A**).

Interestingly, there was no difference between diabetics and controls in tape removal latency in the first 1-2 weeks after stroke ($t_{(23)}$=0.74, p=0.23). However, several weeks after stroke, both diabetic and insulin treated mice were significantly more impaired on the tape removal test than non-diabetic mice (**Fig. 6A**; Main effect of Diabetes: $F_{(2,182)}$=5.53, p=<0.01; 7-8wks Non-diabetic vs. Diabetic: $t_{(22)}$=1.83, p<0.05 vs. Insulin: $t_{(18)}$=2.91, p<0.01; 9-10wks Non-diabetic vs. Diabetic, $t_{(20)}$=1.76, p<0.05 vs. Insulin $t_{(20)}$=1.77, p<0.05). For the right (unaffected) paw, there were no significant differences between groups in latencies at any time point (**Fig. 6B**, $F_{(2,182)}$=1.23, p=0.29). This data suggests that persistent impairments in attention to the left forepaw in diabetic or insulin treated mice after stroke could not be attributed to any global effects of diabetes on sensory function.

Sensori-motor function of the left (impaired) forepaw was also examined using the horizontal ladder test. Consistent with previous studies (Clarkson et al., 2010), stroke degraded performance on this test reflected by a significant increase in the incidence of partial forepaw placements on the ladder rungs (Main effect of Stroke: $F_{(5,113)}=50.3$, $p<0.001$) rather than correct forepaw placements which dominate before stroke (**Fig. 6C**). We should note that even after stroke, more obvious errors such as slips or complete misses were very infrequent and did not differ between groups. However, unlike the tape removal test where group differences became quite obvious, all 3 groups displayed relatively similar deficits in ladder performance (**Fig. 6C**; Main effect of Diabetes: $F_{(2,113)}=0.53$, $p=0.58$). One interesting trend was that performance, particularly in the very late stages of recovery, was worse in diabetic mice (compare blue and red bars at 7-8 and 9-10 weeks in **Fig. 6C).** Collectively, these results suggest that diabetes inhibits the progressive improvement on the adhesive tape removal test, but has only minor impact on more stubborn sensori-motor deficits such as those observed on the horizontal ladder test.

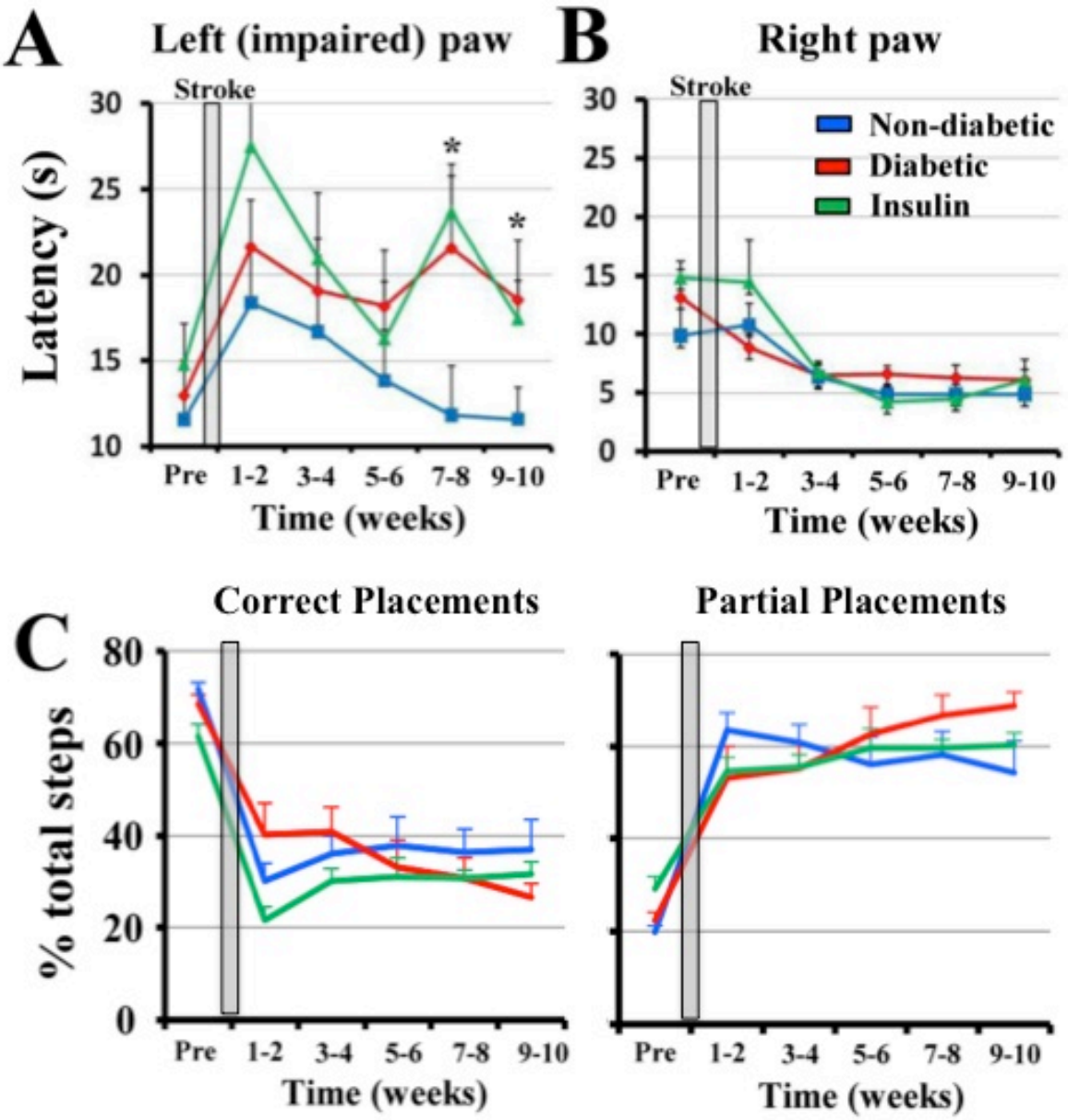

Figure 6. Diabetes impedes the recovery of sensory and motor function after stroke. A) Plot showing the average time it took mice to remove adhesive tape from the left (impaired) forepaw. Stroke increased tape removal latencies for the left paw which appeared to slowly recover in non-diabetic mice at 7-8 and 9-10 weeks, but not diabetic or insulin treated mice. **B**) Latencies for the right forepaw were unaffected by stroke or diabetes. **C**) Plotsshowing summary of left forepaw placements on the horizontal ladder rung test. Before stroke, the majority (~70%) of forepaw steps on the ladder rung test were considered correct placements, meaning the forepaw was firmly placed and centered over the rung. After stroke, the percentage of partial or incorrect forepaw placements on the rung increased dramatically. There was a slight, but non-significant trend towards improved performance in non-diabetic mice at later stages of recovery. *$p<0.05$ relative to non-diabetic controls.

3.3 Diabetes Impairs the Remapping of Sensory Function in Somatosensory Cortex

Recovery of forepaw function is thought to be mediated by adaptive changes in the neural circuitry of peri-infarct and more distant but functionally relevant cortical regions (Dancause et al., 2005, Brown et al., 2009). Accordingly, we investigated whether poorer behavioural outcome in diabetic mice was associated with any impairment in cortical plasticity. To do this, we imaged forepaw-evoked patterns of brain activity across a large portion of the right cerebral hemisphere (6.5 x 5 mm field of view) in mice that had recovered for either 1 or 14 weeks after right forelimb cortex stroke. This large imaging window allowed us to visualize forelimb responses in both the primary (FLS1) and secondary somatosensory cortex (S2), which previous studies have not examined. Furthermore, by examining mice after either short or long periods of recovery (**Fig. 8A,B**), we could determine whether diabetes affected the initial size of the infarct (1 week) or rather, affected plasticity associated with remapping of sensory responses to peri-infarct regions (14 weeks). Consistent with previous *in vivo* VSD imaging data (Ferezou et al., 2007, Brown et al., 2009), brief mechanical stimulation of the forepaw (one 5ms tap) in sham-operated mice produced a strong depolarization in the FLS1 which peaked on average 22-25ms after stimulation (**Fig. 7A,Table 2 and 3** for peak amplitude and time to peak averages) and then decayed thereafter. As expected, depolarizations that originated in FLS1 cortex spread medially into primary motor (M1) cortex and posterior-medial into hindlimb primary somatosensory cortex (HLS1). In addition to the FLS1 response, a robust depolarization was also observed laterally in the S2 cortex (**Fig. 7A**).

The average peak amplitude and latency of FLS1 and S2 responses were very similar between sham operated non-diabetic and diabetic mice (**Fig. 7A, D, Table 2 and 3**). These results indicate that diabetes alone does not significantly alter the spatio-temporal dynamics of forelimb evoked sensory responses. One week after focal stroke (stroke denoted by white circle in **Fig. 7B**), stimulation of the forepaw failed to elicit distinct responses in peri-infarct cortex for both non-diabetic and diabetic mice (**Fig. 7B; Table 3**). Of note, cortical responses to the forepaw were preserved in S2 cortex demonstrating that its responses are not fully dependent on feed forward connections from FLS1 cortex.

Since diabetes did not noticeably affect cortical responsiveness 1 week after stroke, we then examined cortical responses 14 weeks after stroke when new sensory maps would have already formed (Brown et al., 2009). In agreement with previous studies examining non-diabetic mice (Brown et al., 2009), forepaw-evoked responses re-emerged in the remaining fragment of S1FL cortex which then spread to adjacent peri-infarct HLS1 and M1 cortex regions (**Fig. 7C**). Quantitative analysis of peak cortical depolarizations in non-diabetic mice indicated that cortical responses dropped in the first week after stroke and then increased significantly in FLS1 ($t_{(8)}$=3.72, $p<0.005$), HLS1 ($t_{(10)}$=1.94, $p<0.05$) and M1 ($t_{(10)}$=2.37, $p<0.05$) by 14 weeks recovery (compare blue bars at 1 and 14 weeks in **Fig. 7D**). In stark contrast, diabetic mice showed no signs of cortical remapping as forepaw-evoked depolarizations were barely detectable in peri-infarct cortex (**Fig. 7C**). Similarly, treating diabetic mice with insulin for 3 months after stroke failed to rescue these defects in cortical remapping (**Fig. 7C)**. The absence of cortical remapping in these groups can also be noted in **Figure 7D**, where peak amplitudes drop 1

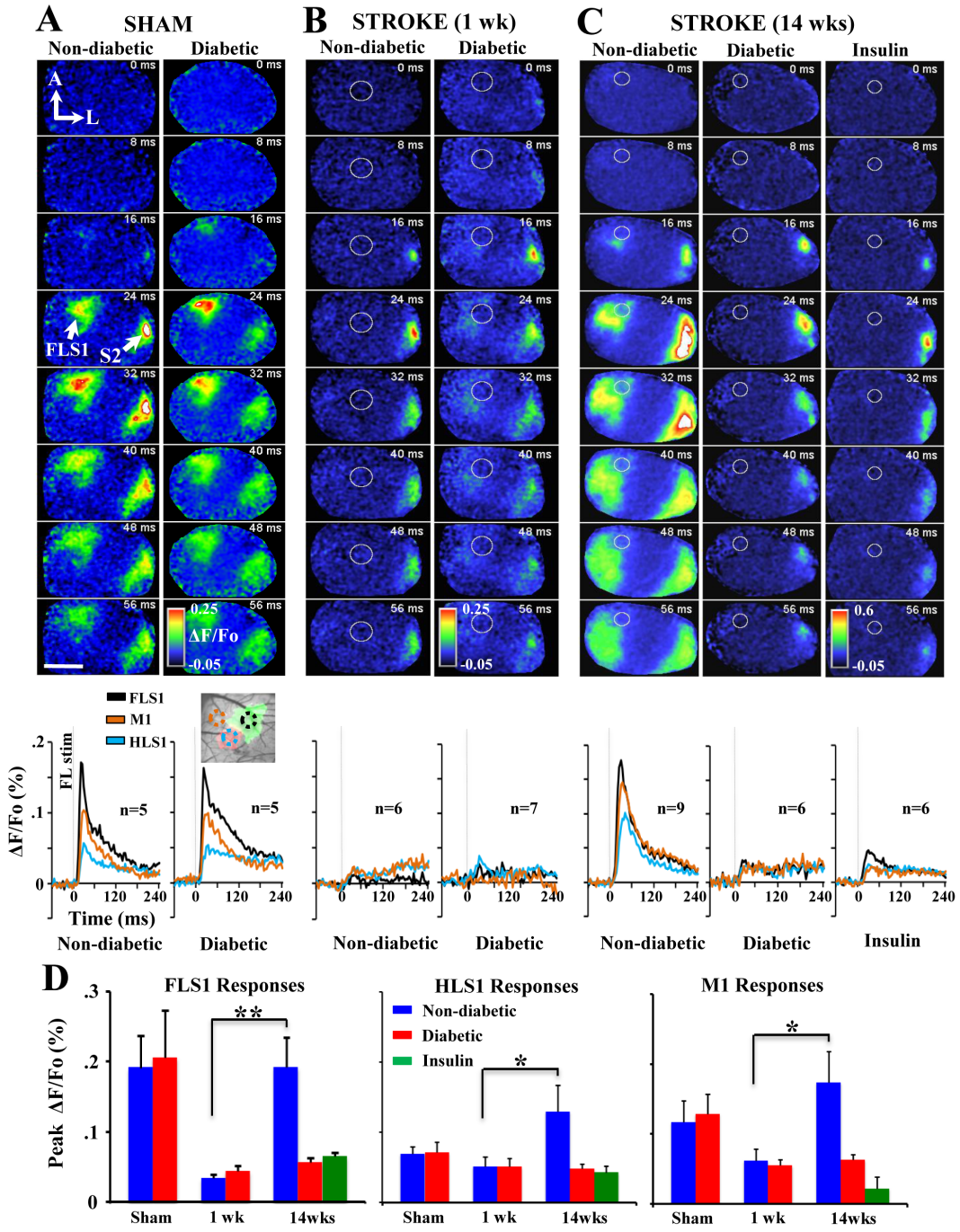

Figure 7. VSD imaging shows that diabetes impairs re-mapping of the forelimb sensory representation after stroke. (**A-C**) Montages showing the spatio-temporal dynamics of cortical responses to forepaw stimulation. Below each montage are corresponding ΔF/Fo plots showing average responses to forepaw stimulation in primary forelimb cortex (FLS1, black line), motor cortex (M1, orange line) and primary hindlimb cortex (HLS1, teal line). Inset with dashed circles indicates the location for where responses were measured in each region. After stroke, FLS1 responses were measured where short latency responses first emerged, typically in cortex directly above or below the infarct. In the absence of stroke damage **A**), forelimb-evoked cortical depolarizations were similar between non-diabetic and diabetic mice. One week after stroke **B),** white circle denotes infarct), the peri-infarct cortex shows little, if any response to forepaw stimulation, while responses are preserved in S2 cortex. Fourteen weeks after stroke **C**), forelimb-evoked depolarizations re-emerge in peri-infarct cortex of non-diabetic mice, but not in diabetic or insulin treated mice. The S2 cortex also appears more responsive to forepaw touch in non-diabetic mice. **D**) Quantification of forepaw-evoked responses in each cortical region before and after stroke. *p<0.05, **p<0.01. Scale bar=2mm.

	Sham Stroke		Stroke (1 week)		Stroke (14 weeks)		
	Non-diabetic	Diabetic	Non-diabetic	Diabetic	Non-diabetic	Diabetic	Insulin
FLS1	22.4±0.98	24.8±2.0	137.3±35.5	130.3±37.9	28.5±2.4	82±28.4	42.9±7.9
HLS1	74.2±23.2	69.3±36.9	123.9±35.9	86.3±36.2	75.1±18.6	124.6±26.6	113.7±29.7
M1	29.9±2.3	32.3±2.7	127.8±34.0	126.3±34.9	35.6±2.8	142.5±23.9	121.7±25.9
S2	24.5±2.0	25.3±2.4	24.2±2.2	21.1±0.8	24.4±0.7	23.5±1.2	23.4±1.0

Table 2: Average time to peak forelimb-evoked cortical response (ms) in each cortical region

	Sham Stroke		Stroke (1 week)		Stroke (14 weeks)		
Region	**Non-diabetic**	**Diabetic**	**Non-diabetic**	**Diabetic**	**Non-diabetic**	**Diabetic**	**Insulin**
FLS1	0.19± 0.05	0.21± 0.07	0.03± 0.005	0.04± 0.008	0.19± 0.04	0.06± 0.006	0.07± 0.01
HLS1	0.07± 0.01	0.07± 0.02	0.05± 0.01	0.05± 0.01	0.13± 0.04	0.05± 0.007	0.04± 0.009
M1	0.12± 0.03	0.13± 0.03	0.06± 0.02	0.06± 0.008	0.17± 0.04	0.06± 0.007	0.02± 0.02
S2	0.25± 0.07	0.24± 0.08	0.33± 0.07	0.21± 0.03	0.49± 0.05	0.36± 0.07	0.34± 0.05

Table 3: Average peak amplitude of forelimb-evoked responses (peak % ΔF/Fo) in each cortical region

week after stroke and never recover. Importantly, S2 responses were clearly detectable in all groups examined which suggests that the absence of peri-infarct responses in diabetic and insulin treated mice was not caused by a global loss of cortical responsiveness due to anesthesia or inability to tolerate the surgical/imaging procedure. Furthermore, differences in anesthetic depth were unlikely given that there were no significant differences between groups in heart rate ($F_{(2,9)}$=0.28, p=0.75), breath rate ($F_{(2,9)}$=2.62, p=0.12) or O_2 saturation ($F_{(2,9)}$=2.45, p=0.14) during VSD imaging.

One obvious explanation for the absence of cortical remapping in diabetics is that they may have suffered a much larger stroke than non-diabetics. Accordingly 1 and 14 weeks after stroke, diabetic and non-diabetic mouse brains were sectioned, stained for cresyl violet and imaged to assess infarct volume (**Fig. 8A,B**). Our analysis indicated that cortical infarct volumes in the two groups were nearly identical at both 1 week (Non-diabetic = 2.03±0.26 mm^3 vs. Diabetic = 2.04±0.43 mm^3; $t_{(14)}$=0.01, p=0.49) and 14 weeks recovery (Non-diabetic = 1.69±0.22 mm^3 vs. Diabetic = 1.71±0.28 mm^3; $t_{(14)}$=0.05, p=0.48). In light of this data, it would appear that impairments in cortical remapping in diabetics cannot be explained simply by having a more severe cortical infarction.

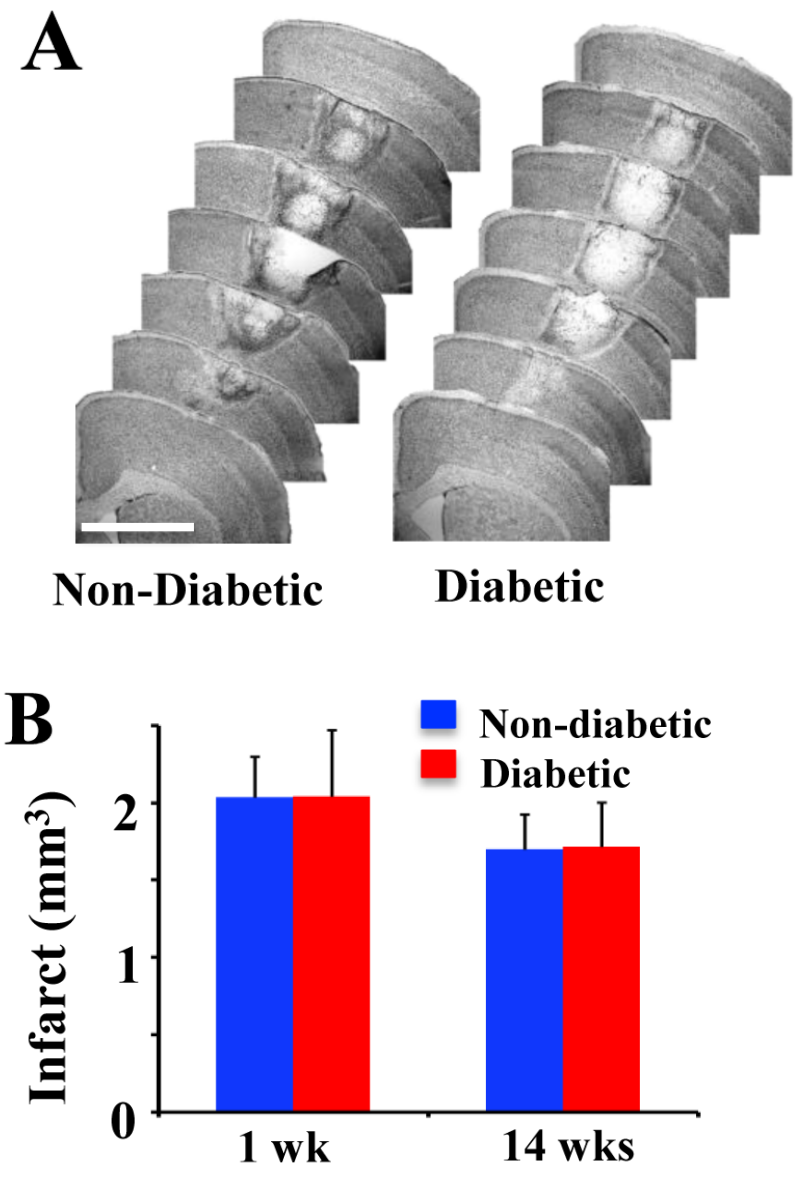

Figure 8. No effect of diabetes on infarct volume at 1 or 14 weeks recovery. A) Representative Nissl stained coronal sections of the stroke affected hemisphere in non-diabetic and diabetic mice one week post-stroke. **B**) Histogram shows that diabetes has no effect on infarct volume at 1 or 14 weeks recovery (n=8 mice per group). Scale bar=2mm.

3.4 Local Inactivation of S2 Cortex Re-Instates Functional Impairments

Although the focus of this study was on diabetes and its effect on stroke recovery and peri-infarct cortical plasticity, we noted that cortical responsiveness in S2 cortex increased dramatically in stroke-recovered mice (**Fig. 7C**). Indeed the amplitude of S2 responses in non-diabetic mice 14 weeks after stroke was almost double that of sham operated controls (**Fig. 9A,C and Table 2;** $t_{(12)}$=2.79, p<0.01). A similar trend was observed in diabetics recovering from stroke (~50% increase, **Fig. 9B,C and Table 3)** although this did not reach statistical significance ($t_{(9)}$=1.13, p=0.14). The timing of peak forelimb evoked responses in S2 cortex was not affected by stroke or diabetes (**Table 2**).

In order to elucidate what role S2 cortex might be playing in stroke recovery, we micro-injected 0.2µL of the GABA agonist muscimol (1µg total) into the right S2 cortex of sham stroke control mice and those that had recovered for 10 weeks (**Fig. 10A**; 7 non-diabetic and 2 diabetic mice at 10 weeks recovery). Previous studies have shown that muscimol potently inhibits neuronal activity for up to 12 hours after injection(Martin and Ghez, 1999). We hypothesized that if S2 cortex participated in functional recovery, then silencing it should re-instate functional deficits in stroke recovered mice. Approximately 4 hours after muscimol injection, mice were administered the tape removal test. We chose not to examine mice on the horizontal ladder given that spontaneous recovery, at least on this specific test, was relatively minor. Inactivating S2 cortex in sham stroke controls increased tape removal latencies relative un-injected (sham) or vehicle injected (sham + veh) mice but this did not reach statistical significance ($t_{(10)}$=1.54, p=0.07; **Fig. 10B**). However, when S2 cortex was silenced in stroke recovered mice, tape removal latencies increased by ~300% relative to performance at 9-10 weeks recovery (see grey

bars in **Fig. 10B**; $t_{(8)}=3.92$, $p<0.005$). Furthermore, this increase was significantly greater than that observed for sham stroke controls injected with muscimol ($t_{(18)}=2.62$, $p<0.01$). Collectively, these data suggest that the S2 cortex may play a greater role in forepaw sensation/attention when S1 cortex is damaged by stroke.

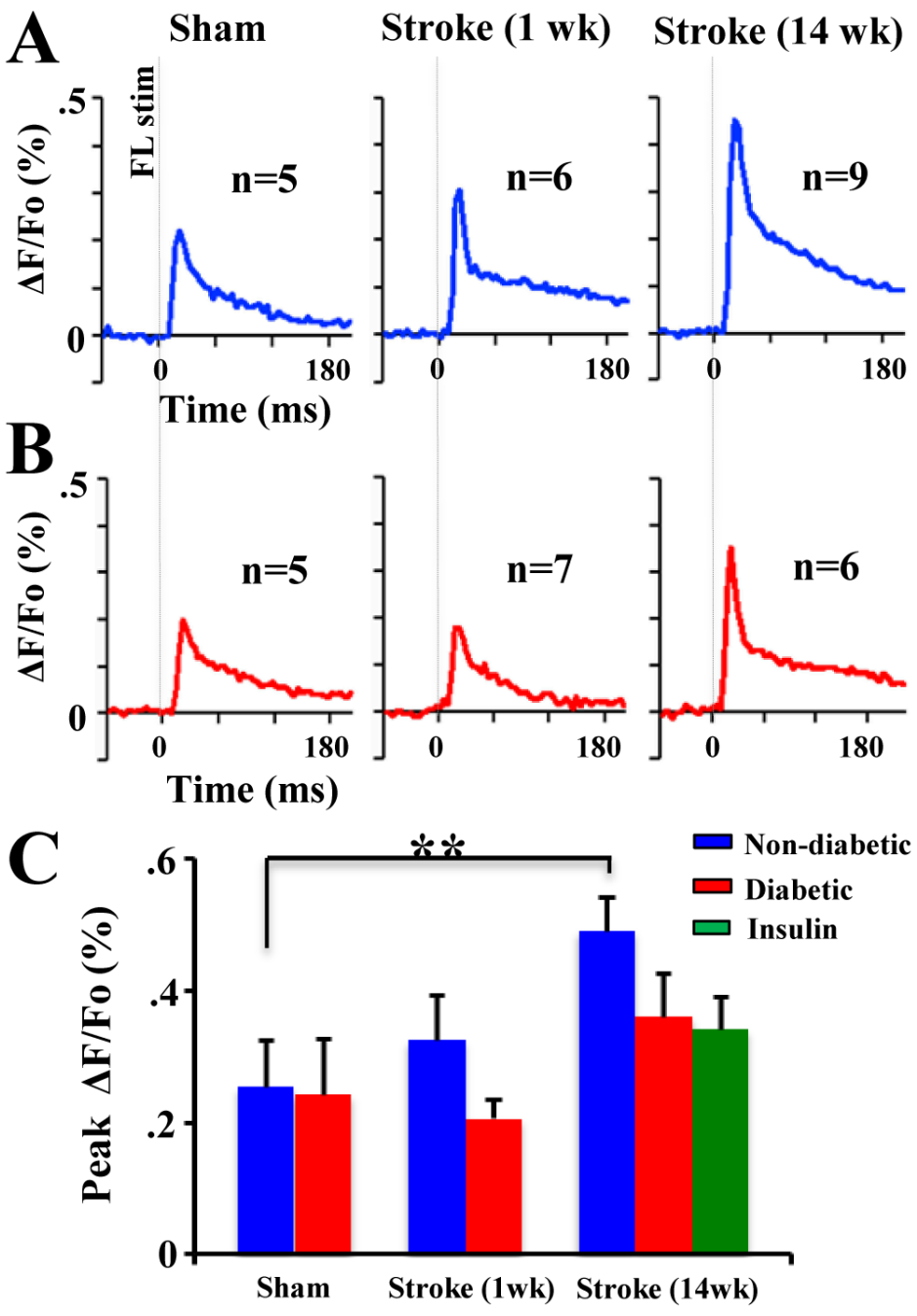

Figure 9. S2 cortex becomes more responsive to forepaw stimulation after stroke. (**A-B**) Plots showing average forepaw-evoked responses (% ΔF/Fo) in S2 cortex in non-diabetic (blue trace in A) and diabetic mice (red trace in B). **C**) The peak amplitude of responses in S2 cortex increased significantly in non-diabetic mice after stroke. There was a non-significant trend towards increased responsiveness in diabetic mice. **p<0.01.

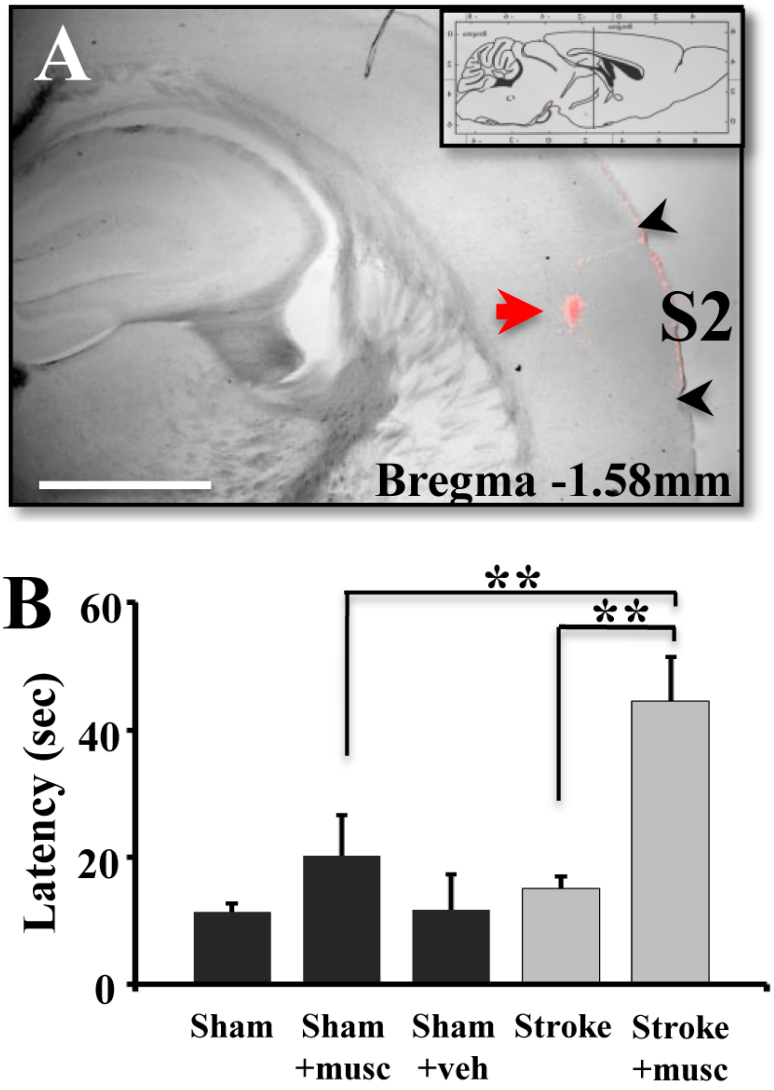

Figure 10. Inactivating S2 cortex re-instates functional impairments. A) Photomicrograph showing the location of forelimb related S2 cortex in a coronal section. The black arrowheads demarcate S2 cortex, as defined by stereotaxic co-ordinates and VSD imaging experiments. In order to verify the location of each injection, 1% Texas Red dextran (see red arrow) was added to muscimol solution and imaged in post-mortem brain sections. B) Histogram shows the average time it took mice to remove tape from the left forepaw in sham stroke (black bars) and stroke recovered mice (grey bars, 7 non-diabetic and 2 diabetic) following muscimol (musc) or vehicle (veh) injection in the right S2 cortex. Muscimol injection significantly increased tape removal latencies in stroke recovered mice (Stroke + musc) relative to baseline levels of performance (Stroke) or sham operate mice injected with muscimol (Sham + musc). Scale bar = 1mm. **$p<0.01$.

4. Discussion

Here we employed *in vivo* VSD imaging and behavioural tests of sensory-motor function to examine the impact of diabetes (a common co-morbidity) on stroke recovery. Although diabetes did not significantly affect cortical responsiveness in the first week after stroke or the volume of cortical infarction, it did however, prevent the re-emergence of the forelimb sensory representation onto peri-infarct regions and limited stroke-induced changes in the functional responsiveness of the S2 cortex (see **Fig. 11** for summary). Furthermore, lowering blood glucose levels with chronic insulin treatment did not rescue defects in cortical plasticity or forepaw function. These results provide new insights into the neurobiological mechanisms underlying poor functional outcome in diabetics and suggest that simply reducing blood glucose levels after stroke, may not be enough to reverse this phenomenon.

4.1 Effect of Diabetes on Stroke Recovery

The cerebral cortex is a dynamic structure that can respond to insult by adapting both structurally and functionally. Even though an individual's recovery from stroke is notoriously variable and difficult to predict some common principles have emerged. One is that successful recovery from stroke correlates with the brain's ability to remap sensory and motor functions to unaffected regions (Cramer, 2008, van Meer et al., 2010). Supporting this, numerous studies have shown that spontaneous recovery of hand or paw use after stroke is associated with new patterns of brain activation in cortical regions linked to the damaged zone (Dijkhuizen et al., 2001, Jablonka et al., 2010, van Meer et al., 2010). A second common principle is that certain co-morbidities or risk factors are

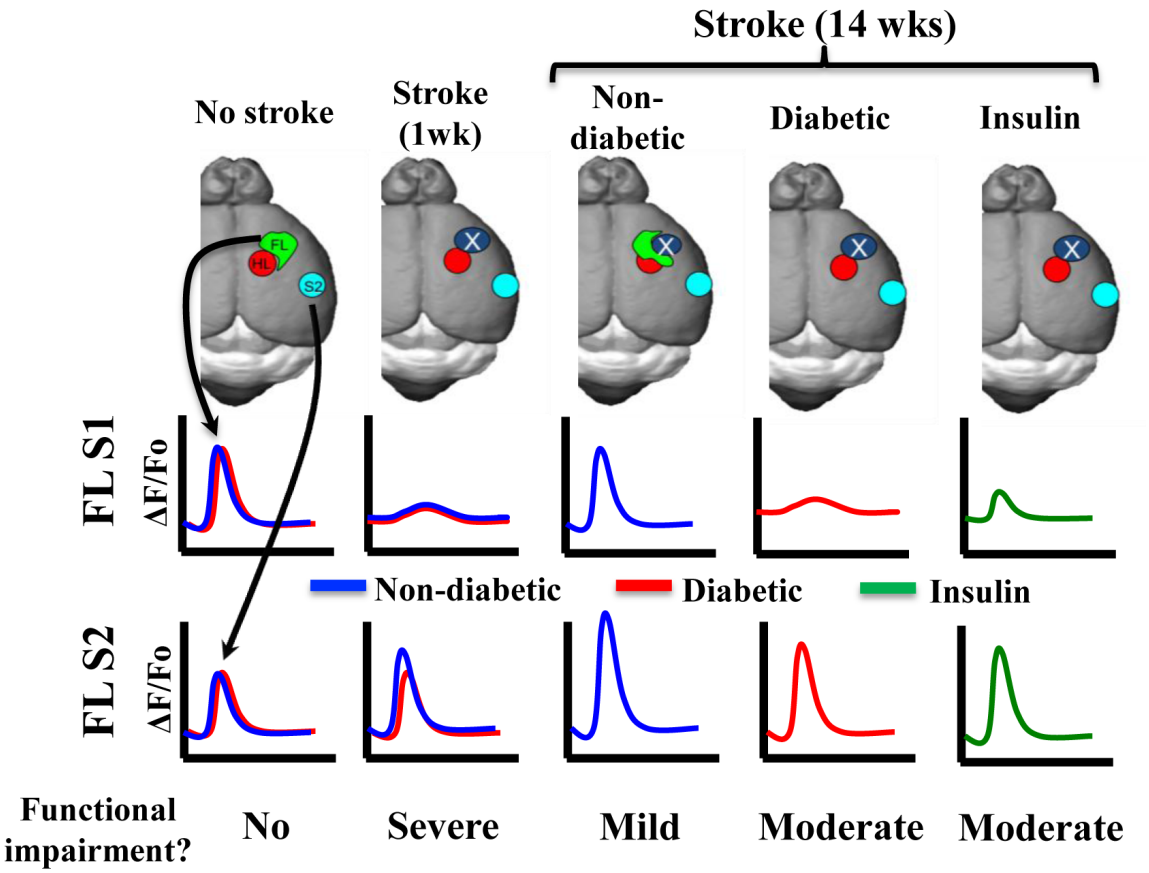

Figure 11. Summary of functional imaging and behavioural data. Before stroke, forepaw evoked depolarizations in FLS1 and S2 are similar between groups. In the acute stage of stroke recovery (1 week), peri-infarct responses are diminished and mice show severe impairments in spatial attention and forepaw sensori-motor function. After several weeks recovery, forepaw evoked responses re-map onto peri-infarct cortex and become stronger in S2 cortex in non-diabetic mice, who show the greatest improvements in forepaw function. By contrast, cortical plasticity and functional recovery are stunted in both diabetic and insulin treated mice.

strongly associated with poor neurological outcome. In particular, clinical studies have shown that diabetes increases their risk of ischemic stroke and is associated with slower or impaired recovery of function/independence (Clavier et al., 1994, Jorgensen et al., 1994, Baird et al., 2002, Hankey et al., 2007), even when adjusting for confounding factors such as age and stroke severity (Wei et al., 2010).

The reasons why diabetics are pre-disposed to poor stroke recovery are not well understood. There is empirical support for the notion that diabetes, specifically the hyperglycaemia and the low insulin levels, aggravates initial stroke damage. In animal models, diabetes increases the size of cerebral infarction and edema (Nedergaard and Diemer, 1987, Vannucci et al., 2001, Langdon et al., 2011), the number of necrotic or apoptotic cells (Muranyi et al., 2003, Moreira et al., 2007) and inhibits the brain's inflammatory response to injury (Kumari et al., 2007). In the present study, we found virtually identical infarct volumes between diabetic and control mice at 1 week recovery. Furthermore, our behavioural data suggested that diabetics were no more impaired, at least initially, than non-diabetics in tests of sensori-motor function. This finding is not unprecedented as there are several reports stating that diabetes had either no effect on infarct volume or actually reduced ischemic damage (Mankovsky et al., 1996, Ergul et al., 2007, Li et al., 2010b). Perhaps some of these differences could be attributed to the fact that certain types of stroke may be more affected by glycemic status. For example, middle cerebral artery occlusion (MCAO) tends to produce a more slowly evolving infarction (Carmichael, 2005) with a larger "penumbral" region of vulnerable, hypo-perfused tissue around the infarct core (Nedergaard, 1988, Dirnagl et al., 1999), than is found after photothrombotic stroke, which typically has a very sharp infarct border and

limited re-perfusion (Carmichael et al., 2005, Zhang and Murphy, 2007). If diabetes exacerbates the spread of damage into the penumbra then one would likely expect a greater effect of diabetes on infarct volume in MCAO models of stroke.

The other confounding factor in the literature is the duration of diabetes prior to stroke. For example in the study by Bomonts (1995), diabetic rats were induced with STZ only 4 days prior to MCAO, whereas the insulin group's blood glucose was controlled 2 days prior to MCAO. What they found was that diabetes increased the size of the cortical infarct and insulin prevented this increase in infarct size (Bomonts et al., 1995). However, one might criticize the clinical relevance of this study since a chronic state of diabetes is required for their increased incidence of stroke. Interestingly, the effect of longer periods of uncontrolled diabetes on infarct size has been overlooked by the research community. A recent meta-analysis of studies by MacDougall et al., (2011) revealed that only 2 studies have looked at the effects of chronic diabetes on stroke size. Both of the studies that looked at chronic diabetes demonstrated an increase in infarct size after a MCAO stroke. Collectively, these studies suggest that our null effect of diabetes on infarct size may be related to differences in how long animals were diabetic before stroke, or possibly the model of stroke used in the present study.

Another possible explanation for our infarct volume results could be related to the type of diabetes modelled. In two studies where diabetes reduced the size of ischemic damage, the Goto-Kakizaki rats were used (Ergul et al., 2007, Li et al., 2010). These are a non-obese Wistar sub-strain rats that develop type 2 diabetes early in life. As previously mentioned, insulin is a neuroprotectant and these animals still have insulin circulating throughout their bodies, which might affect the evolution of the infarct after stroke. Thus,

the literature has not reached a consensus on whether diabetes exacerbates infarct size, because there is no conformity among the studies to provide a definitive answer.

When examining long-term recovery from stroke, we found that diabetes significantly impeded the recovery of sensory and motor function. To date, there have been relatively few studies that have examined the impact of diabetes on recovery of limb use/function in the weeks to months after stroke, and no study to our knowledge, that has imaged cortical map plasticity. Two studies reported greater, more persistent functional impairments in forepaw function after cortical injury in rat models of type 2 diabetes(Langdon et al., 2011, Moreira et al., 2007)). These findings are consistent with the present study showing that STZ induced diabetic mice performed significantly worse on tests of spatial attention in the later stages of stroke recovery. Although our data show that motor recovery was somewhat limited in all groups, this likely reflects the difficulty of the ladder rung test (using randomly spaced rungs) and in our opinion, does not mean that animals showed absolutely no improvements in motor function. We should point out that progressive improvement in spatial attention or forepaw sensori-motor function after stroke has been widely reported in the animal literature (Napieralski et al., 1998, Gonzalez and Kolb, 2003, Shanina et al., 2006, Tennant and Jones, 2009). Furthermore, human studies have shown that spatial neglect is very common after damage to the right or non-dominant hemisphere that usually shows some form of recovery (Blennerhassett et al., 2007, Cramer, 2008). The neural mechanisms underlying the recovery of sensori-motor function or spatial attention are not clear, but our data suggest that functional changes in peri-infarct and S2 cortex likely play a role. This idea is consistent with MRI imaging studies showing that the re-balancing of brain activation patterns in the stroke

affected hemisphere correlate with improved neurological outcome (Dijkhuizen et al., 2001, Corbetta et al., 2005, van Meer et al., 2010). The fact that diabetes suppressed functional plasticity in peri-infarct and S2 cortex points to a common theme that diabetes impedes stroke recovery by impairing progressive, adaptive changes in functional brain connectivity.

In the hospital, a diabetic that presents with a confirmed ischemic stroke immediately undergoes strict glucose regulation with insulin treatment (Adams 2007). Although there is a growing body of data suggesting that intensive insulin therapy reduces the absolute risk of stroke in diabetics (Nathan et al., 2005) and possibly acute stroke damage (Auer, 1998), there is still little evidence showing that strict glycemic control can improve long-term stroke outcome (Kruyt et al., 2010, MacDougall and Muir, 2011). Intriguingly, our data show that diabetic mice chronically treated with insulin after stroke did not fare significantly better, in terms of functional recovery and cortical plasticity, than their untreated counterparts. We should note that insulin was still beneficial in a general sense as these mice appeared healthier in terms of body weight, appearance and mortality rates which were below that of diabetic mice (mortality rate over 14 week recovery period was 26% and 12.5% for diabetic and insulin treated mice, respectively). The fact that mice were hyperglycemic for at least 4 weeks before the induction of stroke suggest that some pathological processes must have already been in place by the time blood glucose levels were regulated. Although the mechanisms behind these pathological processes are not understood, chronic hyperglycemia could irreversibly compromise the vascular system's integrity ("leakiness") or its ability to make adaptive changes after stroke such as re-distributing blood flow through collateral

vessels or changing vascular tone (Shih et al., 2009). It should be noted that our study is not the first to show that normalizing blood glucose levels in diabetic animals (after several weeks of uncontrolled blood glucose levels) does not rescue diabetes related neuropathology. For example, sensory neuron loss in the dorsal root ganglia of STZ injected mice could not be prevented even if mice spontaneously regained islet function and euglycemia (Kennedy and Zochodne, 2005a). Similarly, diabetes-related deficits in spatial learning and hippocampal long-term potentiation could not be reversed by insulin treatment (Biessels et al., 1998). One other plausible explanation for our data could be that exogenous insulin infusion was not perfect in keeping blood glucose levels within the normal range. Indeed, insulin treated mice would occasionally show higher than normal blood glucose levels when the insulin pellet had completely dissolved and needed to be replaced. This of course, reflects the challenge that human diabetics face in that exogenously delivered insulin will never control blood glucose levels as well as normally functioning pancreas. In summary, these findings suggest that even 4 weeks of uncontrolled diabetes can compromise the brain's ability to recover from stroke.

4.2 Mechanisms for Impaired Cortical Plasticity

The cellular and molecular mechanisms that underlie the progressive re-emergence and remapping of cortical areas have been extensively studied in non-diabetic subjects. Studies have shown that stroke transiently upregulates the production of growth associated proteins (Carmichael et al., 2005), dendritic spines (Brown et al., 2009, Mostany et al., 2010), axonal sprouting (Carmichael et al., 2001, Dancause et al., 2005, Li et al., 2010a) and angiogenesis (Zhang and Chopp, 2009). Deficits in any one aspect of

this co-ordinated response could explain the impaired cortical plasticity and recovery in diabetics. Indeed, it has been shown that hyperglycemia can impair synaptic plasticity (eg. long-term potentiation) and reduce dendritic spine density in the hippocampus and cerebral cortex (Biessels et al., 1996, Gispen and Biessels, 2000, Malone et al., 2008, Stranahan et al., 2008a, Stranahan et al., 2008b). Diabetes is also known to have a detrimental effect on the regenerative capacity of peripheral nerves (Kennedy and Zochodne, 2005b, Duran-Jimenez et al., 2009, Polydefkis et al., 2011). It is conceivable that after stroke, thalamic axons may not be able to re-innervate their target neurons in the remaining fragment of the forelimb cortex or peri-infarct region, which could explain why forelimb-evoked responses do not re-emerge in diabetics.

Considering that diabetes is almost invariably linked with macrovascular disease such as stroke, myocardial infarction and peripheral arterial disease (Vinik and Flemmer, 2002), deficits in cortical plasticity could be attributed to widespread vascular dysfunction in the cerebral cortex. This notion is supported by several reports showing that diabetes in both humans and rodent models is associated with reduced blood flow and increased vessel leakage in the hippocampus and cortex, particularly after ischemia (Dietrich et al., 1993, Hawkins et al., 2007, Kruyt et al., 2010). Chronic hyperglycemia can also lead to the generation of reactive oxygen species and glyceation end products that damage the endothelial cells lining blood vessels (Toth et al., 2008). This damage to the endothelium would limit vascular responsiveness and enhance plaque formation, thereby limiting blood flow. Furthermore, diabetes augments the generation of new blood vessels and collaterals which is a primary cause of pathology in the retina leading to blindness (Antonetti et al., 1999). Whether the cerebral cortex is similarly affected has

received little attention although a recent paper has shown that type 2 diabetes increases cerebrovascular density and the number of collateral arteries between anterior and middle cerebral arteries (Li et al., 2010b). In summary, diabetes could limit the recovery and re-wiring of spared neuronal circuits as a consequence of poor blood flow or impairments in the ability to re-route blood supply through changes in vascular tone or angiogenesis.

4.3 Role of S2 Cortex in Functional Recovery

It is well established, that recovery from stroke is supported by functionally related cortical regions (Cramer, 2008, Murphy and Corbett, 2009). For example, ablating or disrupting neuronal activity in peri-infarct cortex or homotopic regions in the opposite hemisphere can re-instate functional impairments in the affected hand or forepaw (Castro-Alamancos and Borrel, 1995, Werhahn et al., 2003, Biernaskie et al., 2005). In the present study, we noted that forepaw-evoked responses in the S2 cortex progressively increased after stroke. Our findings are in agreement with fMRI imaging data showing that improved clinical outcome in stroke patients was paralleled by increased activation volume in S2 cortex (Carey et al., 2002, Nhan et al., 2004). However, our data contributed to these important clinical observations by showing that inactivation of the S2 cortex with $GABA_A$ agonist muscimol is sufficient to unmasked profound deficits in sensory function that had appeared to recover. Intuitively, the notion that the S2 cortex participates in the recovery of sensory functions such as spatial attention is appealing. As anatomical and imaging studies (including our own) demonstrate, the S2 cortex is highly interconnected with primary somatosensory cortex (Krubitzer et al., 1997, Chakrabarti and Alloway, 2006) and readily responds in a parallel manner to forepaw touch (Brett-

Green et al., 2004, Benison et al., 2007). Furthermore the S2 region can be independently activated without S1 input through the VPL connection. Previous studies on the inactivation of the S1 cortex found that the responsiveness within the S2 cortex was reduced by 20-70%, depending on the animal model (Turman et al., 1992, Zhang et al., 2001). These previous experiments used temporary thermal or chemical inactivation and recorded single neurons immediately after cortical inactivation. Conversely, our study examined the long-term effects of S1 damage on the S2 cortex and found that there was an increase in the responsiveness of the S2 cortex. This increase was only statically significant in the non-diabetic group, suggesting again that the pathophysiology of diabetes impairs plasticity. Regrettably I can add little concrete data as to mechanism behind this increase in S2 activation, yet I can speculate a mechanism. The increase is most likely due to a strengthening of the intact VPL thalamic projections. These projections innervate the S1 and S2 cortex, thus it is reasonable to expect following the loss of the S1 region the remaining projections within the VPL would convey more of the sensory information to the S2. What is truly novel is that our data suggests that this region plays a more dominant role in forepaw function after stroke than it normally would (**Fig. 10B**). In conclusion, our data strongly suggest that S2 cortex plays a role in recovery of function after stroke in S1 cortex. Future areas of research could look at enhancing stroke recovery by increasing functional responsiveness in the S2 cortex through transcranial stimulation or opto-genetics. This could be especially significant in vulnerable populations, such as diabetics.

5. General Conclusions

The primary goal of the present study was to determine the effect of diabetes on recovery from ischemic stroke. To do this we induced an ischemic photothrombotic stroke in three groups: a) non-diabetic mice, b) diabetic mice with uncontrolled blood glucose levels and c) diabetic mice that received insulin therapy to control blood glucose levels after stroke. Our behavioural experiments indicated that diabetic mice, regardless of how well blood glucose levels were controlled after stroke, showed poorer recovery of forepaw sensory function relative to non-diabetic mice. Correlating with the poor recovery of forepaw sensorimotor function, diabetic and insulin treated mice did not show re-mapping of forepaw evoked responses into the peri-infarct cortex, typically found in non-diabetic mice. Of note, these differences in behavioural recovery and cortical remapping could not be explained by larger cerebral infarcts. These results indicate that diabetes has a profound effect on the brain's ability to change its circuitry after stroke, which correlates with poorer recovery of forepaw sensori-motor function. Furthermore, the fact that treating diabetic mice with insulin did not normalize stroke recovery suggests that prolonged periods of hyperglycemia (ie. 4 weeks in our study) induces pathological changes in the brain that cannot be easily reversed.

One unexpected but intriguing finding was that forepaw evoked responses in FLS2 increased after ischemic stroke in FLS1. This increase was only found in non-diabetic mice. To determine the role of FLS2 in stroke recovery, we discovered that silencing S2 cortex in stroke recovered mice re-instated functional impairments of the forepaw. This finding strongly suggests that when the FLS1 is damaged by stroke, the

FLS2 plays a more important role in sensory functions of the forepaw. In summary, these findings provide new insights into the role of diabetes and the secondary somatosensory cortex in recovery of function following ischemic stroke.

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