Evaluating the Functional Role of Enhancing Progenitor Cell Survival Following Stroke Recovery

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Abstract

Stroke is the leading cause of long-term neurological disability worldwide, signifying the need for viable therapeutic options. Pre-clinical and post-mortem stroke studies have demonstrated that stroke increases the number of newborn progenitor cells (PCs) in the adult brain that can migrate to the site of injury. While there is a positive correlation between increasing neurogenesis and improvements in stroke recovery, methods used to increase PCs and neurogenesis also alter many other forms of plasticity, making it difficult to determine the function of PCs *per se*. To investigate whether specifically enhancing PC survival is sufficient to improve recovery, the iBax transgenic mouse model was used to remove the pro-apoptotic gene *Bax* inducibly from nestin-expressing PCs either before or after focal strokes induced by photothrombosis. Increasing PC survival before or after stroke in the iBax mice increased the number of PCs in the peri-infarct region. Interestingly, the majority of the cells that migrated to the peri-infarct region expressed the glial fibrillary acidic protein (GFAP) which is found in astrocytes when *Bax* was removed prior to stroke, yet when *Bax* was removed after stroke the majority of the cells expressed doublecortin (DCX) which is expressed in neuroblasts. Irrespective of this significant increase in the different populations of surviving PCs following stroke, there was no change in long-term behavioural deficits on the adhesive removal, horizontal ladder, and cylinder tasks up to 90 days post stroke. Additionally, enhancing PC survival before or after stroke resulted in a significant increase in adult-generated neurons within the dentate gyrus, which was associated with a modest change in spatial learning on the Barnes maze. Together, these experiments suggest strategies that enhance the survival of the PCs by preventing cell death will, by themselves, be insufficient to promote sensorimotor recovery following stroke.

Table of Contents

List of Figures

Chapter 1: Introduction

Chapter 3: Sensorimotor Deficits Following Cortical Strokes Induced by Photothrombosis

Chapter 4: Functional Role of Enhancing Progenitor Cell Survival Following Stroke

Chapter 5: Functional Role of Enhancing Progenitor Cell Survival Before Stroke

List of Tables

List of Abbreviations

AC3	Activated caspases 3
ANOVA	Analysis of variance
A-P	Anterior-Posterior
AR	Adhesive Removal
ATP	Adenosine triphosphate
Bax	Bcl-2 –associated X protein
Bcl-2	B-cell lymphoma-2
BDNF	Brain-Derived Growth Factor
BLBP	Brain lipid-binding protein
BrdU	Bromodeoxyuridine
CYL	Cylinder
DCX	Doublecortin
DCX-TK	Doublecortin-thymidine kinase
EGF	Epidermal growth factor
EPO	Erythropoietin
ET-1	Endothelin-1
FACS	Fluorescence-Activated Cell Sorting
GCV	Ganciclovir
GF	Grip Force
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate-aspartate transporter
GS	Grip Strength
HL	Horizontal Ladder
IHC	Immunohistochemistry
IP	Intraperitoneal injections
ITI	Intertrial interval
Mash1	Mammalian achaete-scute homolog-1
MCA	Middle Cerebral Artery

MCAo	Middle cerebral artery occlusion
M-L	Medial-Lateral
MWM	Morris Water Maze
Nestin-TK	Nestin-thymidine kinase
OB	Olfactory Bulb
PBS	Phosphate-buffered saline
PCs	Progenitor Cells
PSD	Post-stroke depression
PT	Photothrombosis
RMA	Rostral migratory stream
RR	Rotarod
SBR	Spontaneous Biological Recovery
SC	Staircase
SGZ	Subgranular zone
SVZ	Subventricular zone
TAM	Tamoxifen
Tbr2	T-box transcription factor
TBS	Tris-buffered saline
t-PA	Tissue plasminogen activator
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
VEGF	Vascular Endothelial Growth Factor
wps	Weeks post-stroke
WT	Wild-type
YFP	Yellow Fluorescent Protein

Authorizations

Figure 1.1. Adult Neurogenesis in the Naïve Brain.
The **Figure** from Ming and Song (2011) was adapted with permission under the license # 4026010724559 from Elsevier for the reuse in this thesis.

Figure 1.2. PC Response Following Stroke.
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Chapter 1: Introduction

1.1: Stroke

Stroke is the leading cause of long-term neurological disability worldwide (Lindsay et al., 2008; Moskowitz et al., 2010; Carmichael, 2016). In Canada, 405,000 people live with the disabling effects of stroke and this number is projected to rise to 726,000 by 2038 (Krueger et al., 2015; Cameron et al., 2016). Due to these high prevalence rates, the economic costs to take care of patients living with stroke-related disability are astronomical, costing nearly three billion dollars per year (Mittmann et al., 2012). These facts underscore the critical need for the development of therapies for stroke recovery.

A stroke is categorized as hemorrhagic or ischemic (Moskowitz et al., 2010). Hemorrhagic strokes are defined by a ruptured blood vessel, account for 15% of all strokes, and are associated with higher mortality rates compared to ischemic strokes (Andersen et al., 2009). Ischemic strokes in comparison are due to an embolism or thrombosis that affects the entire brain (global ischemia) or a distinct sub-region of the brain (focal ischemia) and comprise of the majority of strokes (Back and Schuler, 2004; Hossmann, 2012). In both global and focal ischemia, a decline or complete loss of blood deprives neurons of essential nutrients and metabolic substrates leading to cell death. Correspondingly, neurological dysfunction can occur within seconds to minutes. However, the evolution of injury continues for days, translating into sensorimotor and cognitive disabilities in patients (Hossmann, 2006; Zhang and Murphy, 2007; Moskowitz et al., 2010).

1.2 Impairments Following Ischemic Stroke

Impairments in patients after a stroke can vary from mild to severe depending on the age of the patient, previous health history, as well as the size and location of the infarct (Moskowitz et al., 2010; Feigin et al., 2015). The majority of ischemic strokes are the result of a blockage in the middle cerebral artery (MCA), which supplies blood to the motor and sensorimotor cortical regions

(Ng et al., 2007). The lack of blood flow to these regions and resulting cell loss most commonly leads to unilateral paralysis and/or impairments in movement control, affecting functions associated with the limbs, face and throat (Purves D, et al, 2001; Ng et al., 2007). Stroke patients with one-sided weakness (hemiparesis) have difficulty with everyday activities such as walking or grasping objects, and have a higher risk of falling after stroke with almost 60% of patients experiencing a fall that leaves some with serious injuries (Weerdesteyn et al., 2008). Furthermore, upper limb impairments are one of the most common and persistent forms of motor impairments, leaving 60% of patients with impaired upper limb dexterity at six months following stroke (Nakayama et al., 1994; Lai et al., 2002). In addition to motor impairments, some stroke patients also lose their ability to feel touch or pain. These sensory deficits may hinder the ability of patients to recognize that they are holding objects (Kessner et al., 2016). Moreover, damage to brain regions that control face and throat muscles leads to difficulties in swallowing (Rofes et al., 2013). Altogether, these sensory and motor impairments severely affect the quality of life.

In addition to motor function, additional non-motor deficits occur in many patients, including in language, mood, and cognition. Stroke is the leading cause of aphasia, with nearly 25% of stroke survivors experiencing language impairments such as in their ability to speak, write or understand language (Cumming et al., 2013). There is also a bi-directional relationship between stroke and depression, with 20-50% of patients having a co-morbid diagnosis of post-stroke depression (PSD) that can often persist for years following a stroke (Burvill et al., 1997; Kotila et al., 1998; Whyte and Mulsant, 2002; Paolucci et al., 2006). Cognitive dysfunction is also particularly prevalent, affecting up to 40% of patients following a stroke, and can include deficits in attention (focusing or dividing attention on a particular task), learning and memory (recognition of visual and/or verbal information), as well as executive functioning (planning and organizing

thoughts) (Pohjasvaara et al., 2002; Cumming et al., 2013; Douiri et al., 2013). Reducing post-stroke cognitive impairments is an important goal due to its association with higher rates of mortality and institutionalization. Furthermore, reduced ability to understand tasks, plan and execute activities and solve problems is linked to both PSD and to the rate of recovery of motor function.

1.3 Treatments For Stroke

1.3.1 Stroke Treatments in the Hyper-Acute Phase

Hyper-acute stroke treatments are aimed at preserving as much brain tissue as possible to improve functional recovery. As hyper-acute treatments are time-dependent (within the first 24 hours), strategies are focused on the reperfusion of the occluded vessels through intravenous or intra-arterial thrombolysis, or in some cases endovascular thrombectomy.

Tissue plasminogen activator (t-PA) is the only drug approved for acute stroke treatment. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study (NINDS) group performed the seminal clinical trial of t-PA in 1995, where they demonstrated that intravenous administration of t-PA within the first three hours of stroke onset significantly improved functional outcomes of patients. Specifically, t-PA treatment was associated with a 30% likelihood of minimal to no disabilities for patients at three months post-stroke (NINDS, 1995). Whether t-PA should be administered via intravenously or intra-arterially continues to be debated, with recent meta-analysis studies supporting either no differences (Wardlaw et al., 2013; Nam et al., 2015), intra-arterial (Ma et al., 2015), or intravenous (Mullen et al., 2012) administration. Despite being very effective to reduce the long-term disabilities associated with ischemic stroke, both routes of administration are limited by a restricted time window of administration (within three hours of stroke onset), which reduces t-PA's use to under 10% of the population that suffer a stroke

(Iadecola and Anrather, 2011b, a; Corbett et al., 2015; Demaerschalk et al., 2016). This limitation has, in part, been addressed by a recent study by the European Cooperative Acute Stroke Study III (ECASS III) group that has extended the three-hour critical window of t-PA administration to 4.5 hours (Hacke et al., 2008).

Due to the narrow time window and low recanalization rates of t-PA, surgical procedures aimed at either repairing blood vessels or removing clots have been explored. Recently, endovascular therapy has shown promise due to significantly improving recovery and reducing mortality rates following ischemic stroke (Pan et al., 2016). Similar to t-PA, endovascular therapy is most effective prior to major irreversible damage that has occurred due to the stroke (< 12 hours after the onset of symptoms). In support of this, a multi-site clinical trial called the ESCAPE trial (Canadian Endovascular treatment for Small Core and Anterior circulation Proximal Occlusion with Emphasis on minimizing computerized axial tomography to recanalization times), performed in Canada, United States, Ireland and South Korea, studied 316 patients who were diagnosed with a major ischemic stroke. They performed a procedure where a thin catheter is inserted through the groin and guided to the blood clot in the brain using x-ray imaging where a stent is used to remove the clot. This resulted in a 50% reduction in the death rate and showed improved behavioural outcomes for patients of this study that were selected based on size and location of lesions (Goyal et al., 2015). These findings were supported by other endovascular therapy clinical trials, all identifying similar benefits following stroke (Berkhemer et al., 2015; Campbell et al., 2015; Jovin et al., 2015; Saver et al., 2015).

In addition to methods aimed at preserving brain integrity, recommended by many guidelines, is the early mobilization (<24 hours) of patients, which is suggested to contribute to stroke recovery (Indredavik et al., 1999; Govan et al., 2007). Against these guidelines, a recent multi-center clinical trial AVERT (A Very Early Rehabilitation Trial), showed no differences in

recovery of patients that were mobilized early to those that obtained standard care (n=1050) (AVERT et al., 2015).

Together, treatment with t-PA and endovascular therapies in the hyper-acute phase of stroke reduce the mortality rates following stroke, with an unintended paradoxical increase in those living with disabilities. This suggests that more people are living with the long-term consequences of a stroke (Carmichael, 2016) and, hence, there is a need for new treatments to reduce disabilities.

1.3.2 Stroke Recovery

There is no medical treatment specifically dedicated to stroke recovery, but current Canadian stroke best practices guidelines recommend addressing physical, cognitive and emotional task-specific activities that will allow patients to return to normal life (Heart and Stroke Canada, 2015). Given that strokes generate various types of deficits, rehabilitation requires a multidisciplinary team that includes physical therapists, speech and language pathologists and occupational therapists to maximize recovery and improve the quality of life. Many of the gains due to rehabilitation occur in the early (7 days - 3 months) and late sub-acute (3-6 months) phases of recovery aligning with many of the endogenous repair mechanisms (discussed in greater detail below). However, after the first few months following a stroke (chronic phase), recovery slows down or is stalled. For example, with the exception of patients that have suffered a small cortical stroke, ~50% of patients report persistent upper limb impairments sustained for many years after stroke (Kwakkel et al., 2003; Teasell et al., 2014). Thus, in spite of rehabilitation, most patients remain impaired hence the need to identify adjunctive therapies to promote recovery through basic and clinical research.

1.4 Animal Models of Focal Stroke

In order maximize translation of preclinical findings to the clinic, it is essential that animal stroke models mimic the manifestations of human stroke to include proportionality in size, targeted locations and behavioural deficits (Casals et al., 2011; Fluri et al., 2015; Kumar et al., 2016). The poor reliability of models for translation into the clinical setting for stroke recovery was highlighted by preclinical studies that have found drugs that were reportedly neuroprotective in animal models of stroke but subsequently failed in clinical trials, as animal models of stroke did not account for the heterogeneity of human strokes and many other factors (Carmichael, 2005; O'Collins et al., 2006). Common to most models of stroke is the cascade of mechanisms that lead to neuronal loss including excitotoxicity, mitochondrial response, protein mis-folding, and inflammation (George and Steinberg, 2015). Mechanisms of neuronal loss are distinct in the two regions of the ischemic stroke: the core and peri-infarct. The necrotic core is a region of irreversibly damaged tissue that receives less than 20% of the blood flow compared to baseline function (Felberg et al., 2000; Lo, 2008; Moskowitz et al., 2010) whereas the ischemic peri-infarct is a region surrounding the core that has significantly reduced tissue perfusion with minimal support of adenosine triphosphate (ATP) levels and oxygen metabolism. This region is at risk of death but is salvageable if perfusion is improved or the tissue is made resistant to injury (Moskowitz et al., 2010). Animal models of ischemic stroke attempt to capture the pathophysiology of the stroke, however, each models has advantages and disadvantages. There are many extensive reviews on the different types of models for ischemic stroke (Fluri et al., 2015; Kumar et al., 2016; Sommer, 2017), thus this thesis will highlight only the three of the most common models in rodents: intraluminal suture middle cerebral artery occlusion (MCAo), endothelin-1 (ET-1), and photothrombosis (PT).

The intraluminal suture middle cerebral artery occlusion (MCAo) model is often considered as the "gold standard" for ischemic strokes as branches of the MCA are commonly affected in human stroke (Kumar et al., 2016). In animals, strokes are induced via a suture that is introduced into the internal carotid artery and advanced until blood supply is interrupted at the branch of the anterior cerebral artery. This method allows for permanent or transient ischemia with reperfusion (Fluri et al., 2015). The advantages of this model include that it mimics human strokes in which there is reperfusion and a penumbra. Thus, it is commonly used model to test for neuroprotective drugs. Despite its popularity, strokes generated following MCAo are large in size and are often considered as a malignant infarction, since most patients do not survive or there is little or no chance of recovery (Kumar et al., 2016). The large size of these strokes also result in effects in many brain regions such as the hypothalamus and hippocampus, that are not typically affected in human stroke (Kumar et al., 2016).

Due to these concerns, there has been an increase in the usage of the ET-1 and PT stroke models as they produce infarcts that are more comparable in size to ones seen in humans. The ET-1 stroke is induced through the stereotaxic injection of ET-1, a potent, long-acting vasoconstrictive peptide, which results in a rapid decline in cerebral blood flow (~70-90%) followed by gradual reperfusion over several hours (Yanagisawa et al., 1988). The advantages of the ET-1 model are: (1) it can target any region in the brain, (2) is followed by reperfusion and (3) display low mortality rates. The primary disadvantages of this model are that it has strain-dependent effects in mice, and requires one, if not two, stereotaxic ET-1 injections into the brain (Horie et al., 2008; Fluri et al., 2015). Comparatively, the PT model induces an ischemic stroke through a photosensitive dye that is injected via intraperitoneal/intravenous injection followed by illumination of a beam of light to produce a stroke. In this case, the light produces singlet oxygen species, endothelial damage,

platelet activation and triggers clotting cascades (Watson et al., 1985). Similar to the ET-1 model, the PT model has low mortality rates, provides a method for targeted ischemia, and has the added advantage of shorter surgery duration, which is less invasive as it induces strokes through the intact skull in the mouse. However, the disadvantages of the PT model include that it does not target subcortical regions in the absence of fiber optic implantation, and there is minimal or no reperfusion which is often occurs in patients.

Differences in lesion size and locations of stroke models exhibit various levels of behavioural deficits following stroke. Additionally, behavioural tests differ in their sensitivity and magnitude to capture the deficits, which is dependent on the stroke model used. While there are a variety of behavioural tests, the most commonly used in rodent models of stroke include rotarod, cylinder, gait analysis, and staircase just to name a few (Balkaya et al., 2013b). Models such as permanent MCAo strokes that induce lesions that affect most of a hemisphere are likely to produce sustained deficits on many of the tests mentioned above. Comparatively, models with more restricted regions of focal ischemia such as the distal MCAo, ET-1, and PT are less likely to exhibit the same level of impairments, however, there are behavioural tests that generate long-term impairments with those models of ischemia. For example, the cylinder and adhesive removal tests are sensitive to detect long-term deficits on PT strokes (Overman et al., 2012; Clarkson et al., 2013). Interestingly, models of focal ischemia generally exhibit spontaneous recovery that occurs in rodents, similar to what is seen in humans even without any interventions.

1.5 Spontaneous Biological Recovery

Spontaneous biological recovery (SBR) has been shown to occur often in human stroke and in animal models of strokes, albeit within different time frames (Kwakkel et al., 2004; Cramer, 2008; Byblow et al., 2015). In humans, SBR is most likely to occur in the first three months after

stroke onset, and patients that suffered a mild stroke recover faster than those that experienced more severe strokes. For example, patients suffering hemiplegic strokes were reported in one study to have initial voluntary movements that ranged as early as six days, to as late as 33 days after stroke onset (Twitchell, 1951). Another study that recorded upper extremity function determined that most improvements in function occurred in the first three weeks, however, patients with severe paresis exhibited maximal improvements between six and 11 weeks after stroke onset (Nakayama et al., 1994). Despite these differences in primary studies, on average the majority of SBR in motor function is usually stated to occur within the first three months of stroke onset (Wade et al., 1983; Duncan et al., 1992; Duncan et al., 1994). Furthermore, if there is any spontaneous recovery that occurs beyond the first three months, it is most likely to occur in cognitive function. For example, Desmond et al. (1996) performed a battery of neuropsychological tests at three months, one year and two years after stroke and identified 35.9% of patients recovered after the first three months and generally stabilized between one and two years after a stroke.

In animal models, although the time course for SBR is dependent on the type of model, the critical (or sensitive) period of innate recovery generally occurs on a shorter timescale, usually within the first month of stroke onset (Krakauer et al., 2012). In support of this idea, animals exposed to an enriched environment in combination with rehabilitation between five and 14 days showed significant gains in functional recovery, however, delayed rehabilitation (later than 30 days) failed to produce any recovery (Biernaskie et al., 2004).

1.5.1 Mechanisms Underlying SBR

Insights into the underlying cellular, molecular and physiological mechanisms regulating recovery have come from clinical imaging techniques and preclinical models of stroke. Studies investigating the innate mechanisms of repair suggest that mechanisms include cortical

reorganization, angiogenesis and neurogenesis (Murphy and Corbett, 2009). As the study of neurogenesis is one of the main topic of this thesis, cortical reorganization and angiogenesis are briefly summarized below, followed by a more encompassing review of the contribution of the function of neurogenesis and SBR.

Diaschisis, which is defined by the depression of brain function at distal sites from stroke and vicariation and the ability of the neighbouring healthy tissue to take over the function of damaged stroke tissue, are two concepts that have guided studies in cortical remapping following stroke (Silasi and Murphy, 2014). Nudo and colleagues first identified that functional reorganization of damaged neural circuits by the recruitment of non-damaged regions was correlated with behavioural recovery (Nudo et al., 1996). In this study, monkeys were induced with strokes that injured part of the digit representation, which resulted in the depression of remaining digit areas and leading to functional impairment of hand use. Retraining monkeys on a skilled reaching task following stroke resulted in reorganization of the digit representation to regions that normally control the elbow and shoulder. Advances in technology allowed Brown et al. (2009) to show that eight weeks after a stroke, the surviving tissue in the peri-infarct region of the somatosensory cortex relayed signals to the neighbouring motor cortex, which resulted in remapping of sensory function. Cortical remapping following stroke is linked with structural changes caused by axonal sprouting and turnover of dendritic spines (Dancause et al., 2005; Brown et al., 2009; Overman et al., 2012). The mechanisms that regulate this process remain unclear, however, it is hypothesized that genetic programs activated following injury may contribute to this process (Silasi and Murphy, 2014).

Angiogenesis, defined as the development of new blood vessels from existing ones, is an additional process suggested to contribute to SBR. In post-mortem tissue from stroke patients, the

peri-infarct region showed an increase in angiogenesis (Krupinski et al., 1993; Szpak et al., 1999; Ergul et al., 2012). Similarly, following strokes in animals, genes promoting angiogenesis are upregulated within minutes, however, how this response leads to the development of new blood vessels remains to be elucidated (Krupinski et al., 1997; Hayashi et al., 2003). Additionally, there remain many different hypotheses about how a proangiogenic state is functionally beneficial for SBR. For example, one group demonstrated that there is only a transient presence of angiogenesis and thus hypothesized that the development of new vessels is part of the "clean-up" process after stroke, as opposed to the restoration of nutrient supply to the at-risk neurons (Manoonkitiwongsa et al., 2001). Other studies suggest that enhancing angiogenesis is important for restoration of function, for example by promoting the generation and migration of neural stem cells along blood vessels that are formed post stroke (Li et al., 2006; Petraglia et al., 2010). Proangiogenic factors, such as Vascular Endothelial Growth Factor (VEGF), have been investigated and suggested to promote recovery, although how this occurs remains to be determined (Liman and Endres, 2012). Furthermore, angiogenesis is shown to alter expression of growth factors and induces proliferation of stem cells contributing to other mechanisms that together may alter SBR.

1.6 Adult Neurogenesis in the Naïve Brain

Adult neurogenesis is often cited to be a mechanism that contributes to SBR and neural replacement and repair after injury. In the naïve adult human brain, the discovery of adult-generated neurons by Eriksson et al. (1998b) overturned the traditional view that neurons cannot form after embryonic development and raised great hope that these cells could be used for regeneration. The process of adult neurogenesis refers to the proliferation, differentiation, migration and integration of stem-like or progenitor cells (referred to as PCs from here on) into new functional neurons (Goncalves et al., 2016). This process is well characterized in two regions

of the adult brain: (1) the subgranular zone (SGZ) which gives rise to new neurons in the dentate gyrus subfield of the hippocampus and (2) the subventricular zone (SVZ), which gives rise to new neurons in the olfactory bulb (OB) **(Figure 1.1A).** More recently, active neurogenesis is suggested to occur in other regions such as the hypothalamus, however, the levels of neurogenesis in such regions are low compared to the well-established SVZ and SGZ niches (Sousa-Ferreira et al., 2014; Lim and Alvarez-Buylla, 2016).

In the last two decades, methodological enhancements to label and isolate PCs through the use of Bromodeoxyuridine (BrdU), Fluorescence-Activated Cell Sorting (FACS), inducible transgenic mouse models and viral methodologies have allowed visualization of PCs and identification of the different stages of cell maturation, as well as the cellular and molecular mechanisms that are important for the development of adult-generated neurons. Although there is no doubt that stroke elicits a strong response from adult-generated cells in the brain, the mechanisms that mediate this response and the functional significance of this response are not completely understood (Marlier et al., 2015). Prior to summarizing the literature on stroke and adult neurogenesis, a brief summary of adult neurogenesis in the naïve brain is given in order to provide context for the discussion of what happens after a stroke. Given that the process of adult neurogenesis is not identical within the SGZ and SVZ, each area is described first, followed by a review on the role of adult neurogenesis in both these areas in the context of stroke recovery.

1.6.1 The Process and Functional Roles of Adult Neurogenesis in SGZ in a Naïve Brain

As shown in **Figure 1.1B**, the dividing PCs in the hippocampus originate from "type-1 cells" and are often referred to as "radial glia stem-like" cells. These cells can be characterized by their expression of glial fibrillary acidic protein (GFAP), nestin and Sox2, as well as their distinct triangular shaped soma with a bushy arbour that projects into the granule cell layer

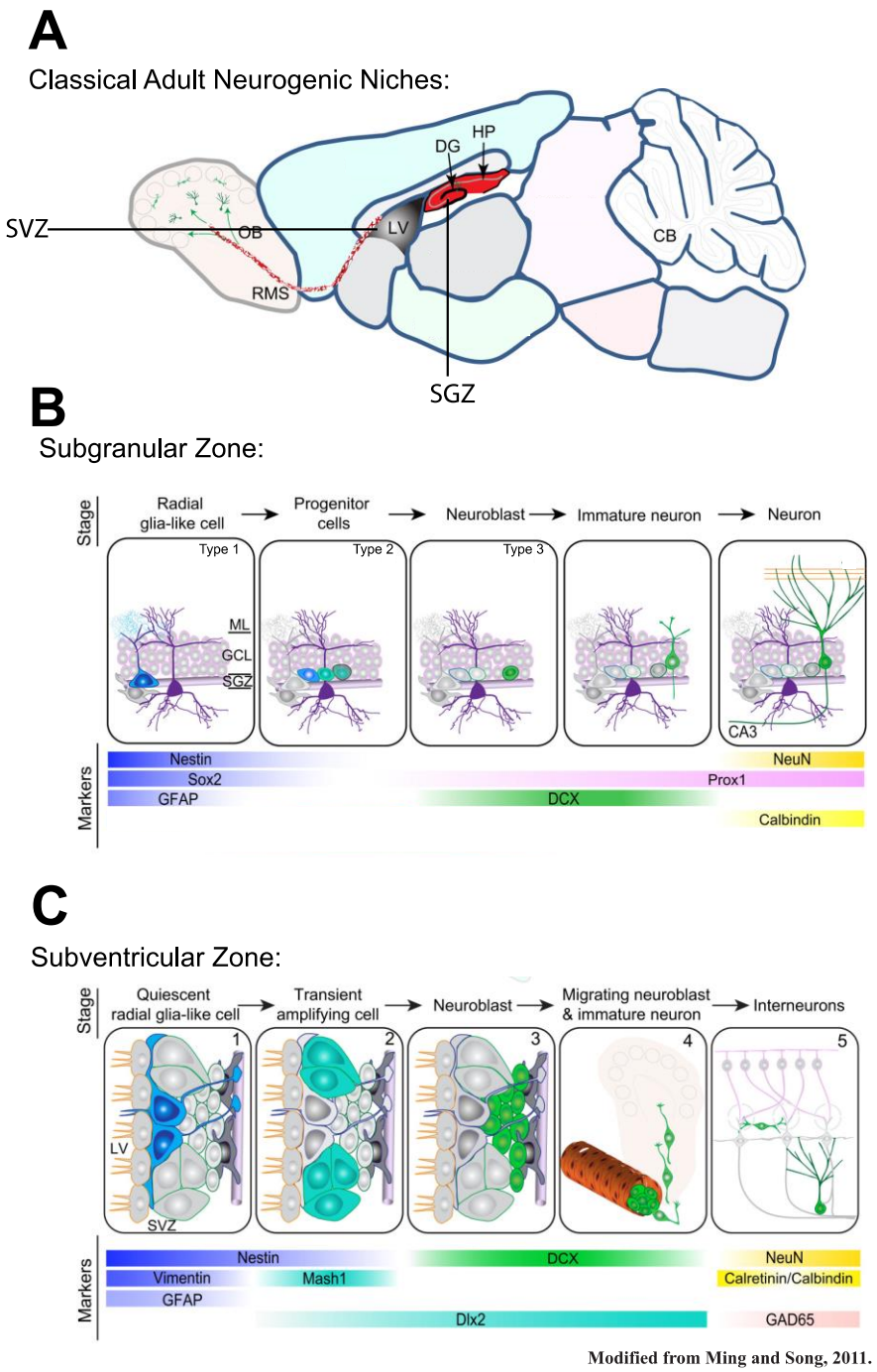


Figure 1.1 Neurogenesis in the Adult Naive Brain. (A) Rodent brain depicting the two neurogenic niches: (1) subgranular zone (SGZ) in the dentate gyrus (DG) subfield of the hippocampus where mature neurons integrate into the granule cell layer of DG and (2) subventricular zone (SVZ) of the lateral ventricles (LV) from where progenitor cells (PCs) migrate along the rostral migratory stream (RMS, red) and integrate as mature neurons in the olfactory bulb (OB). The developmental process of stem cells to mature neurons in the (B) SGZ and (C) SVZ including the morphological changes and common histological markers used to identify cells at the various developmental stages.

(Filippov, 2003; Moss et al., 2016). The terms "stem-like" is used because while these cells possess properties of multi-potency, whether they are truly stem cells that do not exhaust is an unresolved debate (Reynolds, 1992; Palmer et al., 1997). This debate is fuelled by variability in methods to isolate the cells for *in vitro* analysis first established by seminal work by Reynolds and Weiss (Reynolds, 1992; Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005). Similarly, *in vivo* studies have two opposing models on the self-renewability of stem cells. One report using *in vivo* clonal analysis performed by Bonaguidi et al. (2011) proposed that stem cells have the ability to continuously self-renew and differentiate to generate neurons and astrocytes. Contradicting this finding, population analysis performed by Encinas et al. (2011) proposed that stem cells are depleted after multiple rounds of division and terminal differentiation into astrocytes. These differences in stem cell models could be due to labelling active versus quiescent stem cells and supports for heterogeneity in the stem cell populations (Bond et al., 2015).

Type-1 cells give rise to Type-2 PCs, which are called "transiently amplifying" or "lineage determined progenitors." These cells rapidly divide and highly outnumber their predecessors (Kronenberg 2003). Additionally, these cells are often characterized by their expression of mammalian achaete scute homolog-1 (Mash1), T-box transcription factor (Tbr2) and doublecortin (DCX). Indeed, the absence and presence of the immature neuronal marker doublecortin (DCX) is also used to further classify the type-2 cells into type-2a and type-2b, respectively, with the type-2b PCs being the first to express this neuronal lineage commitment.

The type-2 cells subsequently develop into type-3 cells or "neuroblasts" (Kempermann et al., 2004). The type-3 cells no longer express nestin, however, can still proliferate within this stage of maturation and then become post-mitotic. Cells that are at an early post-mitotic stage of

development can be identified by their expression of calretinin and their change in morphology from a rounded to a triangular soma with an apical dendrite.

The maturation of type-1 to post-mitotic mature neurons usually takes ~2-6 weeks, during which the majority of the cell undergo apoptotic cells death and only a few survive to develop into mature neurons that reside in the granule cell layer of the dentate gyrus. Early studies examining cell death in the SGZ used apoptotic markers such as activated caspase 3 (AC3) or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to identify this process and suggested that PCs at the immature neuronal stage (type 2b, type 3) are most susceptible to apoptosis (Kuhn et al., 2005; Tashiro et al., 2006). In contrast, more recent work by Sierra et al. (2010) utilized a BrdU-pulsing paradigm to suggested that cell death occurs in two waves, an early (1-4 days) and late (2-4 weeks) stage of maturation, resulting in the overall loss of ~80% of PCs. Therefore, studies aimed at elucidating the factors that regulate the survival of these PCs became important, especially for future studies aimed to harness the potential of these cells in neurodegenerative diseases. To this end, our recent work used inducible transgenic mice to examine the role of anti-apoptotic protein, B-cell lymphoma-2 (Bcl-2), in adult hippocampal neurogenesis. We identified that removal of Bcl-2 from rapidly dividing PCs resulted in a complete loss of newborn neurons (Ceizar et al., 2016). Furthermore, inducible removal of pro-apoptotic protein, Bax (Bcl-2-associated X protein), resulted in a nearly 4-fold increase in the number of surviving neurons by eight weeks after removal of Bax (Sahay et al., 2011b). Together, these results add to the growing field of studies that suggest apoptotic mechanisms regulate the survival of PCs.

Due to the location of the newborn neurons in the hippocampus, the obvious proposed function for adult neurogenesis was in learning and or memory. Therefore methods that reduce or

ablate newborn neurons were used to test the contribution of these cells on hippocampal-dependent learning and memory tasks such as the Morris water maze (MWM) and the fear conditioning test (Deng et al., 2010). In many cases, spatial learning and/or memory as well as associative memory was affected (Snyder et al., 2005; Farioli-Vecchioli et al., 2008; Jessberger et al., 2009). There were also reports of contradictory findings, which suggested no requirements for neurogenesis in learning and memory (Shors et al., 2002; Meshi et al., 2006). One discrepancy in the findings may be the timing of behavioural assessment as adult-born neurons are suggested to particularly be impactful between four to six weeks following mitosis, when they become hyper-excitable (Gu et al., 2012; Goncalves et al., 2016). Other factors contributing to these contradictory finding may include the heterogeneity in methodology such as behaviour protocols, strains of animals, as well as PC ablation methods. A meta-analysis study by Groves et al. (2013) tested the functional role of adult neurogenesis in anxiety, spatial learning and memory, and associative memory suggested no significant role of newborn neurons in these tasks.

Recently, emerging data suggested a role for adult-born neurons in only certain types of learning and memory function, such as the ability to differentiate between two similar contexts, known as pattern separation (Deng et al., 2010; Sahay et al., 2011a; Yassa and Stark, 2011). For example, models that reduced neurogenesis in mice lead to impaired performance on a navigational radial arm maze test and on an operant chamber (Clelland et al., 2009; Nakashiba et al., 2012). Similarly, mice that had increased neurogenesis following removal of the *Bax* gene were able to distinguish between two similar contexts but performed comparable to control mice on spatial learning and memory and contextual fear conditioning tasks (Sahay et al., 2011b). In addition, a recent study by Danielson et al. (2016) used *in vivo* calcium imaging to record the activity of newborn neurons and demonstrated a direct involvement of newly born neurons in

context encoding and discrimination. Computational models have also suggested that newborn neurons should result in the loss of previously acquired memories through increased inhibition of mature neurons or due to competitive rewiring of the dynamic dentate gyrus circuit (Weisz and Argibay, 2012). In support of this, Akers et al. (2014) identified that increasing neurogenesis following the formation of a memory leads to memory loss. Thus, there is growing evidence for the role of newborn neurons in certain types of learning and memory.

1.6.2 Process and Functional Role of Adult neurogenesis in SVZ in the Naïve Brain

In addition to neurogenesis within the SGZ, a larger population of stem and PCs in the adult rodent brain are localized within the SVZ that lies adjacent to the lateral ventricles. The SVZ is separated from the ventricle through a layer of ependymal cells, known as type E cells. Analogous to the type 1,2 and 3 cells in the SGZ, there are type B, C and A cells present in the SVZ as shown in **Figure 1.1C** (Ming and Song, 2011; Lim and Alvarez-Buylla, 2016).

Slowly proliferating type B cells have characteristics that are similar to astrocytes and express the histological markers GFAP, glutamate aspartate transporter (GLAST) and brain lipid-binding protein (BLBP). Morphologically, type B cells have primary cilium that contacts with the ventricle, the soma that touches type C and A cells, and a basal process with end feet touching blood vessels. The quiescent versus active type B cells can be distinguished by their expression of nestin. The activated, nestin-expressing type B cells give rise to type C cells (Lim and Alvarez-Buylla, 2016).

Type C cells also called “transient amplifying progenitors”, rapidly proliferate, and continue to express nestin as well as Mash1 and Dlx2. Type C cells divide symmetrically ~3 times prior to differentiating into type A cells (Ponti et al., 2013).

Type A cells, also called "neuroblasts", begin to express the immature neuronal marker DCX. Type A cells aggregate with one another in chains and migrate to the OB along the rostral migratory stream (RMS) (Lois and Alvarez-Buylla, 1994). In the OB, these cells migrate radially and differentiate into granule cell or periglomerular interneurons and integrate into the neuronal network (Carleton et al., 2003). Similar to the SGZ, only a fraction (~40%) of the Type A cells survive to migrate to the OB, while the majority undergo apoptosis (Petreanu and Alvarez-Buylla, 2002).

The organization of the SVZ in the adult human brain is different from that seen in rodents. There are only a few cells that express the marker DCX, and the SVZ in the humans has a large gap layer that consists of astrocytes and ependymal cells (Sanai et al., 2004; Quinones-Hinojosa et al., 2006). It is hypothesized that the astrocytes in this gap layer have properties of stem cells that are quiescent and that migration to the OB is rare, however, this remains to be elucidated. More recent studies using ^{14}C to birth-date cells suggests that the cells in the SVZ migrate into the striatum and integrate as interneurons in humans (Ernst et al., 2014).

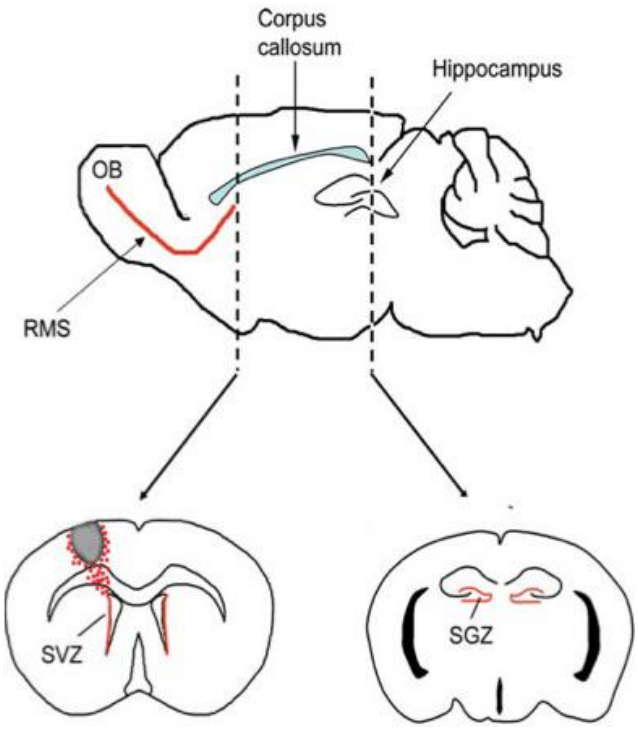
Studies have largely focused on understanding the functional role of neurogenesis in the hippocampus and less so on the SVZ-RMS-OB, partly owing to the smaller number of neurons, if any, that are born in the human OB (Kempermann, 2013). One function of newborn neurons is similar to that of the SGZ in odour discrimination (Lledo and Valley, 2016). Particularly, there is reduced apoptosis of newly-generated neurons in animals that learn to discriminate between two odorants (Petreanu and Alvarez-Buylla, 2002; Yamaguchi and Mori, 2005). Furthermore, several attempts have been made using genetic and chemical models to ablate PCs to determine if adult OB neurogenesis is required for olfaction (Imayoshi et al., 2008; Sultan et al., 2010; Sakamoto et al., 2011). These studies failed to provide a unifying role of OB adult neurogenesis. More recently,

a study showed that OB neurons were activated during an odour discrimination task suggesting a link between OB learning and newborn neurons (Alonso et al., 2012).

1.7 Stroke-Induced Responses of Adult PCs

1.7.1 PC Response in the SGZ Following Ischemia

It is well known that ischemia significantly increases SGZ neurogenesis in the adult rodent brain. After a stroke, there is an enhancement in the proliferation of PCs **(Figure 1.2)** (Zhang et al., 2004b; Zhang et al., 2007a). Liu and colleagues first reported this event in their seminal findings showing a 10-fold increase in the number of proliferating PCs in the dentate gyrus following transient global ischemia in adult gerbils (Liu et al., 1998). Since this time, many have shown that the proliferative response peaks between seven and 14 days post-stroke (Marlier et al., 2015). Similar to PCs in the naïve conditions, the majority of PCs in the SGZ die after stroke (Arvidsson 2002). The surviving PCs develop into newborn neurons in the SGZ and have characteristics of dentate granule cells in the absence of any ectopic migration (Tanaka et al., 2004). Recently, the laboratory of Christoph Redecker further characterized the PC response in the SGZ following focal strokes. In their 2010 report, using BrdU labelling paradigm and nestin reporter mice, they suggested that PCs dividing prior to PT stroke contribute to the enhanced survival of post-stroke neurogenesis (Keiner et al., 2010). In a 2012 study, using retroviruses following PT and MCAo strokes, Niv et al. (2012) found that remarkably, stroke stimulates a small percentage of newborn neurons (~5% in the PT model and ~10% in the MCAo model) to exhibit a bipolar morphology that is uncharacteristic for dentate granule cells and suggest that these aberrant neurons may contribute to functional impairments. Finally, to examine the recruitment of functionally integrated newborn neurons, Geibig et al. (2012) used BrdU-labeling immediately following stroke and then tested mice at eight weeks post-stroke on the MWM task (to measure of



Modified from Luo, 2011.

Figure 1.2 Progenitor Cell (PC) Response Following Stroke. Following stroke, there is an increase in the proliferation of PCs in the subventricular zone (SVZ) and subgranular zone (SGZ). Also, PCs from the SVZ in naive conditions normally migrate to the olfactory bulb (red, top image). Following stroke, PCs from the SVZ ectropically migrate to the stroke lesion (red dots, bottom left image).

spatiotemporal activation) and the ladder rung task (to measure sensorimotor activation) for seven days prior to sacrifice. The activated newborn network, witnessed through colocalization of BrdU and immediate early gene cfos, showed that a sensorimotor task activates more new neurons than a spatiotemporal one. Together, these studies provide a better understanding of the complex mechanisms that are regulating SGZ neurogenesis following a stroke.

1.7.2 PC Response in the SVZ Following Stroke

A variety of rodent ischemic injury models have shown that similar to the SGZ, a rapid increase in PC proliferation also occurs in the SVZ between one and two weeks following stroke (Jin et al., 2001; Arvidsson et al., 2002; Zhang et al., 2004b; Zhang et al., 2004a; Lichtenwalner and Parent, 2006; Kernie and Parent, 2010; Luo, 2011). This increase in the PCs population has been hypothesized to arise due to an increase in the type B cells lining the SVZ (Zhang et al., 2004b). While this proliferative response returns to baseline by six weeks following a stroke, the pool of type B PCs is expanded even up to 16 weeks (Thored et al., 2006). This is largely due to the caspase-mediated cell death that reduces the neuroblast population (Thored et al., 2006). In addition to an increase in type B cells, there is also evidence to suggest that the ependymal cells lining the ventricle (*i.e.*, type E cells), can increase following a stroke (Zhang et al., 2007b). Overall these studies suggest that an increase in the number of type B cells, as well as other populations of stem-like cells, results in an overall increase in the number of PCs in the SVZ after a stroke.

In the last two decades, there is strong evidence to support that, following a stroke, type A cells ectopically migrate from the SVZ to stroke-injured regions **(Figure 1.2)** (Lois et al., 1996; Arvidsson et al., 2002; Jin et al., 2003; Gotts and Chesselet, 2005; Thored et al., 2006; Yoshikawa et al., 2010). This was first identified through birth dating studies, using BrdU to label and track PCs (Arvidsson et al., 2002; Parent et al., 2002a). This phenomenon was also shown by others

who used viral approaches and transgenic mouse models to visualize the migration of PCs following stroke (Burns et al., 2007; Li et al., 2010). While some have suggested that the migration of PCs is transient, occurring within days after insult and declining within a few weeks following stroke (Gotts and Chesselet, 2005; Hua et al., 2008; Kreuzberg et al., 2010), Osman and colleagues (2011) demonstrated persistent migration of DCX-positive PCs to the peri-infarct up to a year following PT strokes. Interestingly, although not well studied, at least one report suggests that the migration of cells to the infarct region may occur at the expense of generation of OB neurons (Ohab et al., 2006).

The mechanisms regulating the ectopic migration of PCs to the site of stroke injury are only partially understood (Lindvall and Kokaia, 2015). Angiogenesis is clearly one important in factor in both SBR post-ischemic injury and is linked to stroke-induced PC migration. Specifically, newly born endothelial cells were located adjacent to newborn migrating PCs (Ohab et al., 2006) and the neuroblasts migrated along blood vessels to the peri-infarct region (Marlier et al., 2015). Furthermore, it has also been shown that ablation of angiogenesis resulted in a 90% reduction in the number of PCs around the stroke. Additionally, the most well-established factor promoting the migration of PCs is the chemokine factor, SDF-1 and its receptor CXCR4. SDF-1 is upregulated and expressed in astrocytes and microglia around the stroke and CXCR4 is expressed in neuroblasts. Blocking CXCR4 significantly attenuated the migration of neuroblasts to the stroke suggesting that signalling between SDF-1 and CXCR4 regulates migration of PCs to the damaged cortex (Robin et al., 2006). A similar relation was also witnessed in the chemoattractant, MCP-1, which is upregulated in astrocytes and microglia and its receptor CCR2 expressed in newborn neuroblasts following stroke (Yan et al., 2007).

While the proliferation and migration of PCs in rodents is well established, whether this also occurs in humans is controversial. Macas et al. (2006) found markers that are indicative of proliferation, Ki67, are increased in the ipsilateral SVZ in post-mortem tissue of stroke patients. In support of this, Marti-Fabregas et al. (2010) also detected increased Ki67 in patients that died within two weeks after an ischemic stroke. There is also additional histological evidence identifying the presence of cells labelled with the immature neuronal marker DCX in the ischemic penumbra (Jin et al., 2006). Whether this response is significant was recently addressed in a study using ^{14}C to birth-date cells in people that suffered strokes. Huttner et al. (2014) found evidence of DNA fragmentation in the penumbra, however, the genomic DNA had ^{14}C concentrations corresponded to the time of birth and not stroke suggesting that a significant number of neurons are not generated in the human cortex following a stroke. However, the results of this ^{14}C study may be limited due to the sensitively of labelling, in addition to failing to capture a transient neurogenic response that may occur in humans.

1.7.3 Survival and Differentiation and Integration of PCs Following Stroke

While the innate response of the brain following stroke is to increase the pool of PCs, the survival of PC is limited due to PCs undergoing apoptotic cell death (Lindvall and Kokaia, 2015). This has also been suggested within the clinical population. For example, in a study of 30 stroke patients, it has been shown that there is a downregulation of anti-apoptotic proteins such as Bcl-2 from cerebrospinal fluid signifying increased cell death (Tarkowski et al., 1999). These findings have raised the challenge for research to determine factors that can promote survival of PCs through inhibiting apoptosis, which should translate into better stroke recovery.

Despite a lack of studies that specifically target adult-generated PCs following a stroke, several studies have shown promising correlations between reducing apoptosis, to promote PC

survival, and improved stroke outcomes. For example, Schneider et al. (2005) intravenously administered G-CSF in stroked rats, which in turn promoted PC survival and translated into better recovery as shown using the neurological deficit score. Furthermore, administration of G-CSF to cultured neurons *in vitro* resulted in decreased expression of caspases-3, demonstrating a correlation between decreasing apoptosis and stroke (Schneider et al., 2005; Solaroglu et al., 2006). More recently, Osman et al. (2016) administered a broad spectrum caspase inhibitor, Q-VD-OPh, in an effort to promote PC survival, but found that the inhibitor eliminated the stroke-induced proliferation response, leading to decreased migration of PCs. Thus, these findings appear contradictory on whether modification of the apoptotic pathway is beneficial or detrimental for the PC response post-stroke. In addition, the interpretation of these studies is limited as the methods used to alter apoptosis are not specific to PCs, which give rise to many confounding variables.

In addition to the challenges on how to promote PC survival, there is minimal support to claim that the PCs that survive and migrate to the infarct will differentiate into neurons and display successful integration into surrounding circuits. The majority of studies that have claimed to show neurogenesis in the peri-infarct regions have used the expression of mature neuronal markers, NeuN or MAP-2, which in different models have given different outcomes. For example, early reports in the field that induced MCAo strokes associated with striatal injury and PC migration into the striatum suggested that, of the adult-generated neurons expressing NeuN, ~42% also express the marker DARPP-32, a phosphoprotein found in striatal spiny neurons (Arvidsson et al., 2002; Parent et al., 2002b). More recent work by Li et al. (2010) and Osman et al. (2011) both identify that less than 5% and 2% of the labelled PCs that migrate from SVZ to the striatum and cortex actually become neurons, respectively. Indeed, the majority of studies suggest that the predominant fate of PCs labelled prior to stroke is to become astrocytes, not neurons (Li et al.,

2010; Faiz et al., 2015). Together, these findings raise the interesting question of whether these few neuronal cells, or increase in astrocytes, could be functionally relevant for recovery. One method to examine if the migrated neurons are functional has been to test for their electrophysiological properties. In this regard, work by Hou et al. (2008) and Lai et al. (2008) show that PCs that migrate to the striatum have electrophysiological properties of striatal interneurons. Thus overall, these findings give support to the idea that neurons can form, at least in the striatum, after stroke and that this response may in some way provide a mechanism for cell replacement and repair.

1.8 Impact of Neurogenesis in Stroke Recovery

1.8.1 Correlative Evidence Supporting Stroke-Induced "Neurogenesis" in Stroke Recovery

One mechanism suggested to contribute to SBR is adult neurogenesis (Liu et al., 1998; Lichtenwalner and Parent, 2006; Thored et al., 2007). Since a host of interventions, including physical exercise and growth factors, have shown to increase neurogenesis and improve behavioural outcomes, this suggests for a positive correlation between neurogenesis and stroke recovery (Lagace, 2012). For example, Luo et al. (2007) exposed mice to voluntary running wheels and identified an increase in hippocampal neurogenesis, which was associated with enhanced spatial learning and memory following stroke. More recently, Zheng et al. (2014) reported an increase in the number of PCs and neurons formed after exercise, which was correlated with reduced infarct size and improved functional recovery following an ischemic stroke. These studies thus all suggest that physical activity, which is a strong stimulus for adult neurogenesis (van Praag et al., 1999; Kronenberg et al., 2003; Eadie et al., 2005; Snyder et al., 2009), may be an effective treatment due to increasing neurogenesis (Dragert and Zehr, 2013; Shimodozono et al., 2013).

Similarly, there are a variety of studies that have shown that growth factors known to regulate neurogenesis are also associated with improved behavioural outcomes after stroke (Larpthaveesarp et al., 2015). For example, erythropoietin (EPO), Epidermal Growth Factor (EGF), Brain-Derived Growth Factor (BDNF), and Vascular Endothelial Growth Factor (VEGF), have been correlated to increase neurogenesis and promote stroke recovery. EPO and EGF administration was first shown to enhance migration of PCs, contribute to the regeneration of cortical tissue and associated with improved function on motor tasks in a model of devascularization (Kolb et al., 2007). BDNF treatment can also increase the migration of SVZ PCs to PT infarcts, increase SGZ neurogenesis (Keiner et al., 2009), and is associated with better functional outcomes following stroke (Schabitz et al., 2007). Lastly, a variety of studies propose that VEGF exhibits a mitogenic role on PCs and contributes to promoting a neurogenic response leading to stroke recovery. Sun et al. (2003) and Wang et al. (2007) utilized the MCAo model to test the role of intraventricular infusion of VEGF and overexpressing VEGF, respectively. Both groups identified an increase in the survival of PC through BrdU labelling and identify better function following stroke. Thus, all of these growth factor studies suggest a positive correlation between increasing neurogenesis and improving outcomes, which may also be due to affecting other mechanisms of plasticity that regulate spontaneous recovery (e.g. angiogenesis); they do not, however, directly test the role of PCs and neurogenesis in stroke recovery.

1.8.2 Causative Links between Progenitor Cells and Stroke Recovery

Only four studies have examined the causal role of PCs during stroke recovery. All of these studies have examined this effect by ablating PCs and testing functional recovery following either global or focal ischemia (Raber et al., 2004; Jin et al., 2010; Wang et al., 2012; Sun et al., 2013). The first study was performed by Raber et al. (2004) and used the global bilateral common carotid

artery occlusion model of ischemia two weeks following the irradiation of the PCs. The irradiation was successful in ablating the newborn mature neurons as examined by co-localization of the pulse-label marker, BrdU, and a marker of mature neurons, Tuj1. Interestingly, the gerbils with ischemia and irradiation demonstrated impaired learning on the Morris Water Maze (MWM) test compared to control groups, suggesting the PCs post-stroke may be functionally important in their ability to encode information.

Given that irradiation has been shown to have nonspecific side effects, Jin et al. (2010) utilized a more specific inducible transgenic mouse model approach to ablate the PCs following MCAo strokes. Briefly, they ablated DCX-expressing type A PCs, by creating a doublecortin-thymidine kinase (DCX-TK) inducible transgenic mice that allows for the specific removal of DCX cells following administration of the drug, ganciclovir (GCV). Surprisingly after stroke, the DCX-TK mice had a significant increase in infarct volume that was accompanied by a reduced performance on the motor tasks, rotarod, limb placing, and elevated body swing tasks at 24 hours after stroke. This finding leads to the suggestion that PCs may produce short-term effects through a paracrine signalling mechanism. The question about whether this ablation would produce long-term deficits was tested in a second publication by Wang et al. (2012). In this paper, the DCX-TK mice were induced with milder strokes using distal MCAo, which resulted in a significant increase in stroke volume and deficits on the beam walk test up to eight weeks following ischemia. Together, these reports suggest that neurogenesis is functionally important for motor recovery. However, they raise questions about whether this occurs through a lack of cell replacement mechanism.

Most recently, Sun and colleagues in (2013) used nestin thymidine kinase (Nestin-TK) mice to ablate PCs prior to distal MCAo strokes and saw a significant decrease in the total number

of DCX expressing PCs at both the SGZ and the SVZ. Examination of motor recovery showed no changes as measured by the ladder and catwalk tests. They showed, however, that the Nestin-TK mice performed worse on the Barnes maze during both the acquisition and memory phase, suggesting that following stroke, PCs contribute to cognitive function. These findings mirror those of Raber et al. (2004) and suggest that the PCs may have a role in cognitive function after a stroke, but not in motor recovery.

Together, all four of these studies suggest that PCs have a positive role in stroke recovery. These studies, however, raise questions that need to be addressed in order to determine the viability of modulating PCs as a strategy for enhancing regeneration. For example, many of these studies use models that produce global ischemia or large infarct that encroaches upon SVZ and impair regions, such as the hippocampus, that are not typically affected in human stroke thus raising the question of whether these findings generalize to recovery from cortical strokes. In addition, there is also no delineation between the effects of the SVZ and SGZ in their contribution, if any, to recovery and confounding results leaving it unknown whether these PCs are important in motor recovery, which is mediated by the PCs from the SVZ, or in cognitive function, which is mediated by the PCs from the SGZ, following stroke? Lastly, these are all loss of function studies, making it still unknown whether an increase in PCs and neurogenesis would be sufficient to promote stroke recovery.

My thesis specifically addresses these questions and **investigates the functional role of PCs and neurogenesis through the use of a gain-of-function inducible mouse model during a cortical stroke.**

1.9 Objective, Hypothesis and Aims

Objective:

The objective of this study is to determine if enhancing PC survival is sufficient to improve sensorimotor recovery and/or cognitive function following stroke using the inducible Bax transgenic mouse.

Hypothesis:

Increasing the survival of PCs will lead to generation of more astrocytes and neurons following a cortical stroke and thus contribute to enhanced sensorimotor recovery and cognitive function following stroke.

Aims:

1. Sensorimotor Deficits Following Cortical Strokes Induced by Photothrombosis
2. Functional Role of Enhancing Progenitor Cell Survival Following Stroke
3. Functional Role of Enhancing Progenitor Cell Survival Before Stroke

Rationale:

Our initial aim was to perform a comprehensive assessment of a battery of sensorimotor and cognitive tests following cortical strokes to determine which tasks are sensitive to detect deficits. These behavioural tests were then used to examine the functional role of enhancing PC survival in a clinically relevant paradigm where PCs were enhanced following a stroke. This resulted in similar sensorimotor recovery patterns in mice that had an increase in PCs survival compared to controls, however, led to a modest increase in the rate of acquisition of spatial learning. Therefore, we hypothesized that by enhancing PCs following a stroke, PCs were not targeted early enough to translate into functional sensorimotor recovery. To test this hypothesis, we increased PCs prior to stroke and tested the functional outcomes following stroke, which also resulted in similar outcomes.

Chapter 2: Materials and Methods

2.1: Animals and Genotyping

2.1.1 General Procedures

Animal procedures were conducted with the approval of the University of Ottawa's Animal Care Committee and in accordance with the Guidelines of the Canadian Council of Animal Care. All animals were maintained on a 12-hour light cycle (lights off at 7 pm) under standard laboratory conditions with water and food available *ad libitum* unless otherwise stated. Room temperature and humidity levels were maintained at 23°C and 30-40%, respectively.

2.1.2 C57/BL6 Mice

C57BL/6 mice were ordered from Charles River (Canada) to determine which behavioural tests that are sensitive to produce deficits in mice following stroke on a battery of tests (Chapter 3). Mice were allowed to acclimatize to the facility in individual housing conditions for two weeks prior to the start of any procedures.

2.1.3 iBax Transgenic Mouse Model

Nestin-inducible Bax knockout mice (iBax) were a gift from Dr. Amar Sahay and were maintained through breeding Nestin-CreER^{T2}, R26R-eYFP, and floxed Bax (fBax) mice. The breeding strategy consisted of breeding mice that were homozygous for Nestin-CreER^{T2} and heterozygous for fBax with mice that were homozygous for R26R-eYFP and heterozygous for fBax. The resulting progeny from these breeding pairs gave the experimental mice that were heterozygous for both Nestin-CreER^{T2} and R26R-eYFP, and either homozygous (iBax) or wild-type (WT) for fBax. Males and female littermates were used for all experiments and were produced in approximately equal proportions.

2.1.4 Genotyping

Animals were genotyped to determine the zygosity of each of the three transgenes. Ear clippings were taken from ~3 week-old mice. DNA was extracted using a HotSHOT DNA extraction method (Truett et al., 2000). Briefly, the ear clipping is incubated at 95°C for 30 minutes in 75 µl of Alkaline Lysis Buffer (25 mM NaOH and 0.2 mM Na_2EDTA) followed by addition of 75 µl of the Neutralization solution (40 mM Tris-HCl). The genotype of the mice was determined using Polymerase Chain Reaction (PCR) based on previously published protocols for floxed Bax, Cre (Sahay et al., 2011), and YFP (Sorlano et al., 1999). The PCR products were separated by size on a 2% agarose gel with ethidium bromide using electrophoresis and visualized using UV radiation. PCR product size was estimated based on comparison with a DNA ladder (100 bp ladder; DM001-R500M, Frogga Inc.).

2.2. Sham or Photothrombosis surgery

Focal cerebral ischemia was induced in mice using the photothrombosis stroke model, as previously described (Watson et al., 1985). Mice were anaesthetized by inhalation using 5% isoflurane with oxygen at 1%. During surgery, the isoflurane level was reduced to 1.5% while the oxygen level was maintained at 1%. Rose-Bengal (10mg/ml; Sigma, R3877-5G) was injected intraperitoneally five minutes prior to laser illumination (532 nm wavelength, ~20mW power, Beta Instruments). The laser was turned on for 10 minutes at a distance of three centimeters from the skull surface at 0.7 mm anterior-posterior and 2.0 mm medial-lateral from bregma to target the sensorimotor cortex. For sham surgeries, all steps except for laser illumination were performed. Throughout the surgery, body temperature was maintained between 36°C and 37.5°C using a rectal probe and feedback blanket (Harvard Apparatus). Mice received 2% transdermal bupivacaine as an analgesic immediately following and four hours following surgery.

2.3 Drug Treatments

Tamoxifen: The iBax mice were treated with tamoxifen (TAM, dissolved in 90% sunflower seed oil and 10% EtOH) either one-week after (Chapter 4) or one-week before (Chapter 5) stroke via intraperitoneal injections of 160mg/kg/day for 5 consecutive days as previously described by Lagace et al., (2007). This use of TAM activates the inducible system that promotes the survival of cells in the iBax mice.

5-bromo-2'-deoxyuridine (BrdU): To label dividing cells, mice were treated with TAM and then two days later administered intraperitoneal (IP) injections of 5-bromo-2'-deoxyuridine (BrdU, Roche, 10280879001) once a day for 10 consecutive days at 150mg/kg (10mg BrdU /1mL saline + 7uL 1N Sodium Hydroxide (NaOH), as previously described by Sahay et al. (2011b).

2.4. Behavioural Testing

Behavioural testing to assess all sensorimotor tasks (staircase, grip strength, rotarod, adhesive, ladder and cylinder) was performed with mice on a normal cycle (lights on 7am-7pm). For cognitive testing (Barnes maze and fear conditioning), mice were placed in a reverse cycle room (lights on 7pm-7am) and allowed to habituate to the room for at least two weeks prior to start of the test.

2.4.1 Staircase Test

The staircase test, a measure of skilled reaching, was performed based on previously published protocols (Montoya et al., 1991; Baird et al., 2001). Mice were placed in the staircase chamber (7.2 cm X 2.6 cm X 4.2 cm) that contains two stairs, with each set of stairs being only reachable by one forepaw. Three sugar pellets were placed on each of the seven stairs, with the pellets placed on the top stair being the easiest to retrieve, compared to the ones on the bottom of the stair. Each day the mice were habituated to the testing room for 30 minutes prior to the start

of the test. Mice are then placed in the staircase chamber for 30 minutes to obtain as many pellets as possible. Thirty minutes after completing the staircase test, mice were given access to food for ~four hours and then deprived of food daily for ~ 20 hours. Prior to stroke surgeries, mice are trained to retrieve the pellets and are considered trained when 12 pellets are retrieved. After nine days of training, almost all mice learned to retrieve at least 12 pellets. After stroke, mice were tested on the same staircase protocol for two days and the average number of pellets collected between the two days is calculated and reported.

2.4.2 Grip Strength Test

Grip strength, a measure of maximum muscle strength, was performed using an automated grip strength meter (Columbus Instruments) to measure grip force (GF) in mice before and after receiving a cortical stroke as per experimental timelines included in results. Mice were brought to the testing room 30 minutes prior to the start of testing. In order to test one paw at a time, the mouse was first restrained and a small piece of tape was placed on one paw immediately prior to testing. To start the test, the mouse was picked up by the base of its tail and lowered onto the grid until the paw without the tape was gripping the grid. Once the paw was on the grid, the mouse was gently pulled away from the grip meter until the mouse let go, as per previous publications (Soliman et al., 2015). This procedure was repeated for five measurements on each forepaw with a 5-10 second intertrial interval (ITI). The maximum and average GF was calculated from all trials performed prior to and following stroke.

2.4.3 Accelerating Rotarod Test

The rotarod apparatus (IITC Life Science) was utilized to test general motor coordination and motor learning as per previously reported (Lee et al., 2007). Mice are habituated to the room one hour prior to being placed on the rod that accelerated from one to 45 rotations per minute for

five minutes. If the mice were unable to maintain themselves on the rod for the 5 minutes, they would fall on the platform, and the time spent on the rod was recorded. Mice were tested consecutively for five trials each day for two days, both before and after stroke. The maximum time that mice could remain on the rod and the average time between five trials from both days was calculated.

2.4.4 Adhesive Removal Test

The adhesive removal test, a measure of tactile response and asymmetries, was performed based on a previously published protocol (Bouet et al., 2009). During the test, a mouse was allowed to acclimatize to a new testing cage for one minute before one experimenter restrained the mouse, while the second experimenter placed a 0.3 X 0.4 cm piece of sticky tape on both forelimbs. With the tape on its limbs, the mouse was placed back into the same testing cage and the time to contact and time to remove was independently recorded by both experimenters. Animals were excluded if there was no latency in time to contact and remove tape at one week post-stroke. For the experiments where TAM was administered after stroke (Chapter 4), three mice (2 WT and 1 iBax) were removed out of a total of 39 mice for no deficits on this task. For the experiments where TAM was administered before stroke (Chapter 5), no mice were removed as they all had deficits following stroke on the adhesive task. The excluded animals were also removed for horizontal ladder and cylinder test (described below).

2.4.5 Horizontal Ladder Test

Ladder rung test was performed based on previously published protocol (Farr et al., 2006). Mice were trained on the ladder prior to receiving a stroke, which was achieved through having the mice perform 4-5 trials consecutively over two days. Post-stroke testing consisted of allowing the mice to cross the ladder for three consecutive trials on one day. All trials were recorded using

a video recorder and the last two trials performed in which the animal crossed the ladder unassisted were measured. The videos from the ladder recording were hand scored for the number of correct placements on the bars, misses as defined by slips through the rungs, as well as cheats which were characterized as placement of paws on the wall of the ladder. Percent success was measured by the following formula: ((correct placement)/(correct placement + misses + cheats))*100.

2.4.6 Cylinder Test

Cylinder test is a measure of spontaneous forelimb function (Balkaya et al., 2013a). Mice were placed in a glass cylinder (10 cm diameter X 15 cm high) and a minimum of 20 rears were recorded through the Ethovision software. Time spent on each paw and the paw placement of each of 20 rears were hand scored using Ethovision video scoring based on previously published protocols (Baskin et al., 2003; Clarkson et al., 2010).

2.4.7 Barnes Maze Test

The Barnes maze test is a measure of spatial learning and memory and was performed based on previously published protocols (O'Leary and Brown, 2013; Sun et al., 2013; Rosenfeld and Ferguson, 2014). The maze is a circular platform (122 cm in diameter at 95 cm from the ground) that has 40 holes around its perimeter. There are there are four phases of the Barnes maze test: (1) Habituation, (2) Training, (3) Probe, and (4) Reversal. For all phases, the ethovision software was used to track and videotape the mice and calculate outcome measures.

Habituation to the maze occurs for two consecutive days prior to the training phase with no cues on the wall and using dim lighting conditions. During each trial (two trials per day) a mouse is placed in the center of the maze and is guided to a random escape box using an upside down 3L beaker placed over the mouse. If the mouse does not enter the escape box, they are

given a slight nudge until they get in and are left in the escape box for one minute prior to being placed back into the home cage.

During the training phase, the room has spatial cues placed on the wall and each mouse had two trials that are three minutes each per day with an ITI of 15-20 minutes. The mouse is brought to the testing room and placed under a bucket in the center of the maze. Simultaneously, bright lights and the loud sound from the white noise generator is turned on and the bucket is lifted off the mouse allowing the mouse to locate and enter the escape box. If the mouse did not enter the escape box within the trial (mainly occurred during the first few trials), the mouse was nudged into the box by the experimenter. Once the mouse entered the escape hole, either on their own or through help from the experimenter, the bright lights and sound were turned off and the mouse was kept in the box for one minute prior to being returned to their home cage. Between trials, the maze was rotated and cleaned to minimize any olfactory cues.

The probe phase consists of one trial for three minutes. The mouse was allowed to roam the maze that had the escape box removed for three minutes prior to being placed back into their home cage.

The reversal phase consisted of two trials that are three-minute each per day with an ITI of 15-20 minutes. The protocol was the same as training phase except the escape box was placed in the opposite location from where it was placed during training.

2.4.8 Fear Conditioning Test

The contextual fear conditioning test, a measure of associative memory, was performed based on previously published protocols (Frankland et al., 2004; Cancino et al., 2013) using the Ethovision system to quantify freezing. Briefly, on the first day, the mice were exposed to the chamber for 10 minutes in order to measure baseline freezing time of exposure to the context. On

the second day, the mice were placed back into the same chamber and five seconds later received a single two-second shock (0.5mA) prior to being removed from the box in one minute. On the third day, the mice were placed back into the same chamber for six minutes and the percent freezing was calculated to quantify the amount of time the mouse remained frozen.

2.4.9 Beam Break Test

The beam break test is a measure of general locomotor activity and measures spontaneous home-cage activity (Tatem et al., 2014). Each mouse is placed in an individual shoe-box style cage with food and water. The cage is then placed on a frame that has invisible infrared light beams to track the movement of the mouse (Omnitech Electronics). The Micromax analyzer software records the movements of the mice. After 60 minutes, the mice are moved back into their original home cage. The distance travelled data was exported from the Micromax software and used to measure general locomotor activity of mice in a novel cage.

2.5 Perfusions and Tissue Collection

Animals were anaesthetized with Euthanyl and transcardially perfused with cold phosphate-buffered saline (PBS, pH=7.4, 42ml) followed by 4% paraformaldehyde (PFA, pH=7.4, 70ml). Brains were removed and post-fixed for one hour in 4% PFA and then cryoprotected in 30% sucrose with 0.1% sodium azide (NaN_3) in PBS. Coronal sections (40µm) were generated on a freezing microtome, collected in nine serial sections, and stored in PBS with 0.01% NaN3 at 4°C.

2.6 Immunohistochemistry (IHC) and Histological Staining

2.6.1 Cresyl Violet

Serial tissue sections were mounted onto SuperFrost Plus charged slides and dried overnight. Slides were dehydrated (consecutively immersing slides into 70, 95 and 100% ethanol),

rehydrated (consecutively immersing slides into 100, 95 and 70% ethanol), and then stained with cresyl violet for 5-7 minutes. Slides were then rinsed with MilliQ water, dehydrated, and then placed in the Citrisolv clearing agent (Fisher Scientific, 22-143-975) prior to being coverslipped with DPX (mixture of Distyrene, Plasticizer, Xylene; 44581; Sigma) mounting media.

2.6.2 Slide Mounted YFP and BrdU IHC

Slide-mounted IHC was used to detect the total number of YFP-positive and BrdU-positive cells using previously published protocols (Lagace et al., 2007; Ceizar et al., 2016). Every ninth section was mounted onto charged slides and allowed to dry overnight. Slides were then pre-treated with 0.1M citric acid (pH 6.0) at approximately 85°C for 15 minutes for antigen retrieval. The sections were then permeabilized using 0.1% trypsin for 10 minutes, followed by DNA denaturation in 2N hydrochloric acid (HCl) for 30 minutes. To prevent non-specific binding, slides were incubated in 3% Normal Donkey Serum (NDS; 017-000-121; Jackson Immuno Research Laboratories Inc.) and 0.3% Triton X-100 in 1X tris-buffer saline (TBS) for one hour prior to being incubated overnight in the primary antibody solution (1:5000 Chicken anti-GFP or 1:300 Rat anti-BrdU in 3% NDS in 0.3% Tween20 and 1X TBS). The following day, slides were incubated in: 1) 1:200 biotinylated Donkey anti-chicken or anti-rat secondary antibody in 1.5% NDS in 1X TBS for 60 minutes; 2) 0.3% H_2O_2 in 1X TBS for 30 minutes to quench endogenous peroxidases; 3) Avidin-Biotin Complex Solution (ABC, PK-6100; Vector Laboratories) for 90 minutes; 4) metal enhanced 3,3'-Diaminobenzidine (DAB; 34065; Thermo Scientific, 1:10) for 15-30 minutes; and 5) fast red nuclear stain (H3403; Cedarlane) for counterstaining. Between all steps, with exception of after blocking with NDS, the slides were rinsed 2-3 times with 1X TBS. Following staining, slides were dehydrated by consecutively immersing slides in 95% and 100% ethanol for 20

seconds, followed by CitriSolv clearing agent (22-143-975; Fisher) for 20 seconds, 1 minute, and 5 minutes. Slides were coverslipped with DPX mounting medium.

2.6.3 Free-Floating Fluorescent IHC

Free-floating fluorescence immunohistochemistry was performed for all colocalization analysis based a previously published protocol (Ceizar et al., 2016). Tissue sections were selected and washed in petri-dishes three times for five minutes each using phosphate-buffer saline (PBS). IHC was performed on free-floating sections using antibodies for GFP (GFP-1020, Aves, 1:5000, used also to detect YFP), DCX (sc8066, Santa Cruz,1:500), GFAP (BD Biosciences, 556327, 1:500), and Olig2 (Millipore, AB9610, 1:500) in a carrier solution (0.1% Tween, 0.1% Triton-X in 1XPBS) shaking overnight at 4°C. The following day the sections were incubated with Cy2, Cy3, and Cy5 conjugated secondary antibodies (Jackson Immunoresearch) used at a dilution of 1:500 for one hour at RT followed by counterstaining with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; 11836170001, Roche, 1:10000, 5 min). Between all steps the sections were rinsed 2-3 times with 1X PBS. Sections were then mounted onto slides, dried for ~10 minutes prior to cover-slipping with Immuomount (Fisher, 2860060) mounting medium.

2.7 Quantification of Lesion Volume and Cell Counts

All quantification was performed by an observer blind to the experimental conditions.

2.7.1 Stereological Analysis of Lesion Volume

Images of each cresyl violet-stained section were taken at the Aperio Slide Scanner at 20x magnification and exported using the Scanscope software. All measurements for stroke volume were calculated using the pixel count function of ImageJ (NIH; 2012 pixels per mm on the 20x images). The edges of stroke lesions were outlined to calculate the area of damaged tissue in each section (Clarkson et al., 2013). The volume was quantified using the formula: sum lesion volume

for all sections analyzed multiplied by 9 and multiplied by 0.04, in order to account for quantifying the lesion volume of one in nine wells, and the 40µm section thickness, respectively. Animals that had no stroke or had strokes that encroached upon the ventricle, hence creating no area for PCs to migrate, were excluded from the study. For the experiments where TAM was administered after stroke (Chapter 4), two mice were excluded for having no strokes. Furthermore, four mice (1 WT and 3 iBax) were excluded for strokes that encroached upon the ventricle. For the experiments where TAM was administered before stroke (Chapter 5), all mice had strokes that were restricted to the cortex thus no mice were excluded.

2.7.2 Quantification of YFP-Positive Cells in the SVZ and Peri-Infarct Regions

Stereological counting, as previously described (Lagace et al., 2007), was performed using the optical fractionator probe of the Stereo Investigator software (MBF Bioscience) to provide unbiased estimates of the population of YFP-positive cells in the SVZ and the peri-infarct region. The section that included the largest lesion volume was used for quantification and a 100 µm x 100 µm grid was superimposed, and cells were counted within a 25 µm x 25 µm counting frame, using 2 µm upper and lower guard zones, as well as an optical dissector of height of 20 µm. The SVZ was traced at 10X magnification and the region was defined as being within 100 µm of the ventricle wall. The peri-infarct was traced at 10X magnification and the region was defined by the location of the PCs. Cells were counted using the 40X objective with an average of 20 sites counted per animal. Due to random variation in the size of both the SVZ and peri-infarct region between animals, cell densities were calculated by dividing total cell count estimates by the volume of the region traced.

2.7.3 Quantification of YFP-Positive Cells in the SGZ Region

YFP-positive PCs in the SGZ were exhaustively counted on one bregma-matched section using the Olympus BX51 microscope at 50X magnification.

2.7.4 Colocalization Analysis

In order to determine if cells were colabeled with different protein markers (YFP/DCX; YFP/GFAP; YFP/Olig2; YFP/NeuN/DAPI) images were acquired using a Zeiss LSM510-META confocal microscope with the 40X oil immersion objective at emission wavelength of 488,543 and 633. The optical z-plane sectioning was used to evaluate if the cells were colabeled using ZEN 2009 acquisition software from Zeiss as previously described (Ceizar et al., 2016). For every animal, a minimum of 20 cells were analyzed.

2.8 Statistical Analysis

Outcomes are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6. A two-tailed unpaired t-test was used for analysis of two groups, and a one or two-way analysis of variance (ANOVA) followed by Bonferroni posthoc was used for analysis of more than two groups. A repeated measure two-way ANOVA was performed for all behavioural analysis. Statistical significance was set *a priori* at $p<0.05$.

Chapter 3:

Sensorimotor Deficits Following Cortical Strokes Induced by Photothrombosis

The rodent PT stroke model has become increasingly popular in stroke recovery research due to the ability to noninvasively and consistently create localized strokes that are relatively similar in size to those commonly observed in humans (Carmichael, 2005; Fluri et al., 2015; Kumar et al., 2016). We decided to utilize the PT model and target the infarct to the sensory and motor cortex and assess stroke volume, as well as behavioural tests following strokes in C57/BL6 mice. Although other studies have created similar sized strokes using PT and reported motor/sensorimotor deficits in the adhesive removal, cylinder, ladder, rotarod and staircase tests (Brown et al., 2009; Clarkson et al., 2011; Sweetnam et al., 2012; Clarkson et al., 2013), there is no comprehensive assessment that examines the sensitivity of a battery of validated tests with small strokes that are representative of the lesion volume seen in humans.

3.1: Dynamic Changes in Lesion Volumes in PT Strokes over Time

In order to localize the stroke to the sensory and motor regions, we first surveyed the bregma-mapped studies that had behavioural deficits following PT in mice **(Table 3.1)**. The most common coordinates in these studies were 0 mm anterior-posterior and +1.5 mm medial-lateral from bregma, therefore our initial studies induced strokes at this location. Using these coordinates, we recognized that ~60% of our strokes encroached upon the corpus callosum (data not shown). Since future work was going to examine the migration of PCs to the cortex, we moved our PT location to +0.7mm anterior-posterior and 2.0mm medial-lateral from bregma **(Figure 3.1A).**

We observed a significant reduction (2-3 fold) in lesion volume between one and two weeks post-stroke (wps) **(Figure 3.1B)**. As shown in **Figure 3.1C**, cresyl violet staining clearly demarcates the infarcted core tissue from the surrounding region allowing for the lesion volume to be calculated. Furthermore, as seen in the cresyl images, the reduction in lesion volume is apparent between one and two wps. These results are similar to other reports that have examined

Table 3.1: Locations used to Produce Sensorimotor Deficits Following Photothrombosis (PT) Induced Strokes

Coordinates/Location Relation to Bregma	Sensitive Behavioural Tests Reported	Longest Time post-PT (Days)	Reference
A-P: 0 M-L: +1.5	Cylinder Grid walk	42	Clarkson et al., 2010
	Cylinder Grid walk Single Pellet	42	Clarkson et al., 2011
	Cylinder Grid walk	56	Overmann et al., 2012
	Cylinder Grid walk Single Pellet	42	Clarkson et al., 2013
	Adhesive Removal Ladder Rung	1	Hines et al., 2013
A-P: 0; M-L: +2.4	Rotorod Staircase	30	Lee et al., 2004
	Rotorod Staircase	28	Lee et al., 2007
A-P: 0; M-L: +2.7	Adhesive Removal Round Beam	28	Park et al., 2006
Based on Mapping of Vasculature	Cylinder	56	Brown et al., 2009
	Adhesive Removal Ladder Rung	70	Sweetnam et al., 2012
Area of 1.5mm in Diameter	Adhesive Removal Cylinder Hanging Wire	14	Li et al., 2014

(A-P= anterior-posterior; M-L= medial-lateral)

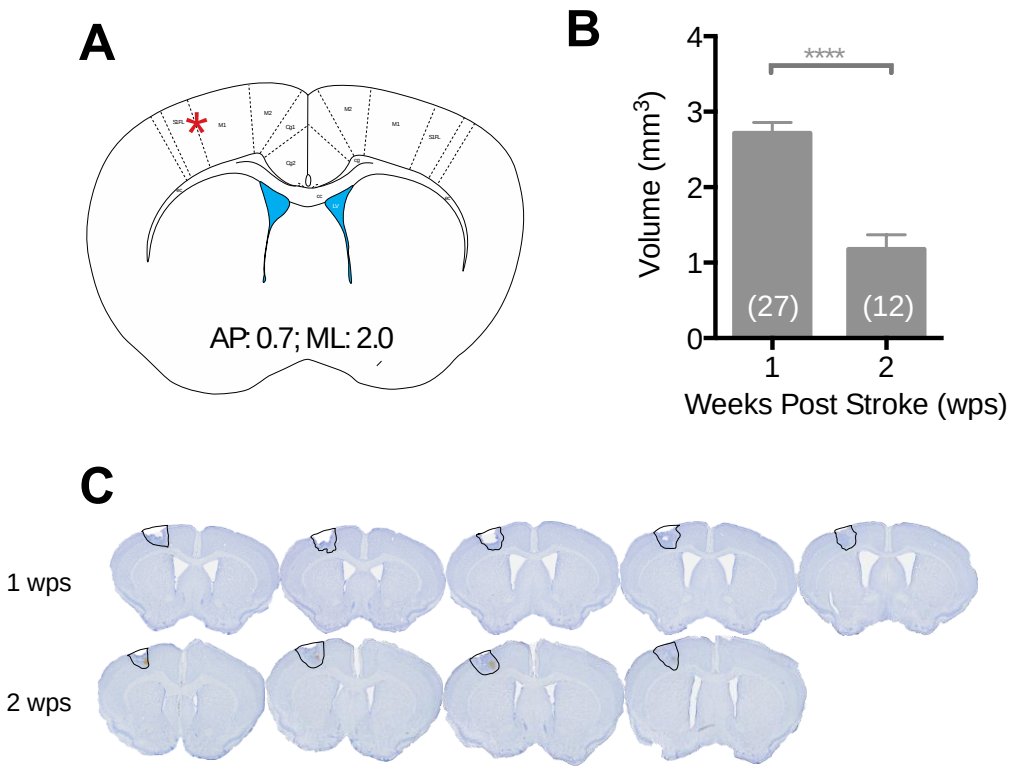


Figure 3.1 Photothrombosis Stroke Volume Significantly Decreases Over Time. (A) Schematic representation of the bregma location (∗) where photothrombosis-induced strokes were targettd in the sensory and motor cortex. (B) Quantification and (C) representative images showing a significant reduction in lesion volume between one and two weeks post-stroke. ****p<0.0001. Mean ± SEM.

PT stroke volumes in both rats and mice over time (Grome et al., 1988; Kuroiwa et al., 2009; Li et al., 2014).

3.2: Sensorimotor Deficits in Adhesive Removal, Horizontal Ladder, and Cylinder Tests Following PT Strokes

In different experiments, we tested sham and PT mice on the staircase **(Figure 3.3)**, grip strength **(Figure 3.4)**, rotarod **(Figure 3.5)**, adhesive removal **(Figure 3.7)**, horizontal ladder **(Figure 3.8)**, and cylinder test **(Figure 3.9)** to determine which behavioural tasks are the most sensitive at detecting deficits in mice following PT.

PT and sham mice were trained and then tested at one wps on the staircase, grip strength and rotarod tasks, in two separate experiments that were combined **(Figure 3.2)**. The staircase test measures long-term changes post-stroke by measuring asymmetry and forepaw dexterity (Montoya et al., 1991; Baird et al., 2001; Schaar et al., 2010). The mouse is placed in a plexi-glass container where two sets of staircases allow for reaching using each forepaw (**Figure 3.3A)**. There are a total of eight steps per side and each step is filled with three sugar pellets, permitting for a maximum of 24 pellets to be retrieved per forelimb. Reaching for the pellets on the upper steps is easier compared to pellets located on the lower steps. To perform this task, the mice were trained every day for nine days prior to PT strokes. As expected, there was a significant increase in the number of pellets retrieved during training **(Figure 3.3B;** $F_{(8,128)}=15.9$, $p<0.0001$**).** Additionally, prior to stroke, there was no difference in the number of pellets consumed using the right or left forelimb. To determine if PT impaired performance on the staircase task, the number of pellets retrieved was recorded following stroke. As expected, on the ipsilateral (unimpaired) side, sham and PT mice perform similarly, collecting on average ~10 pellets per side. However, there were no detectable differences between the sham and PT mice on the contralateral (impaired) forelimb

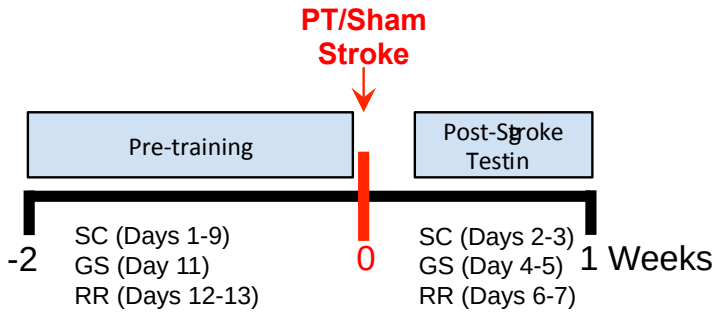


Figure 3.2 Experimental Timeline to Test Photothrombosis (PT) and Sham Mice on Staircase, Grip Strength, and Rotarod Tasks. Experimental timeline shows that mice were trained on the staircase (SC), grip strength (GS) and rotarod (RR) tests starting at two weeks prior to PT or sham surgery. Post-stroke testing was performed at one week post-stroke.

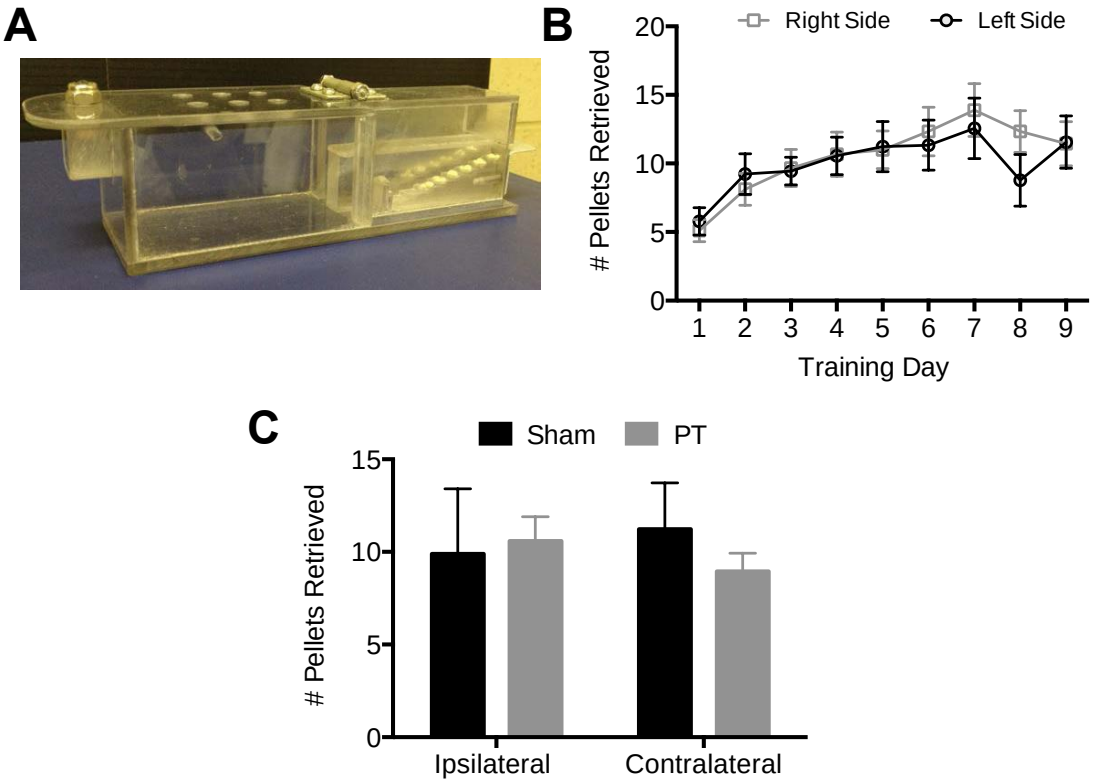


Figure 3.3 The Staircase Test Does Not Detect Deficits Following Photothrombosis (PT)-Induced Strokes. (A) Image of the staircase apparatus showing pellets on each stair used to measure skilled forelimb reaching. (B) Mice were pre-trained on the staircase test for nine days. (C) Number of pellets retrieved by sham and PT mice on the ipsilateral and contralateral side reveals no deficits following PT strokes. (n=3 Sham; n=12 PT). Mean ± SEM.

(Figure 3.3C), suggesting that the staircase test is not a sensitive measure to detect differences at one week following a cortical PT stroke. These findings contrast with reports that found deficits on the staircase test following PT; this may be attributed to the larger strokes induced in these studies (Lee et al., 2004; Lee et al., 2007). Furthermore, one report with similar lesion sizes as those produced in our model, had mice that elicited deficits on the staircase test following ET-1 strokes (Roome et al., 2014). Using their paradigm, we were still unable to produce deficits following PT strokes (data not shown). The sensitivity of the staircase test may be dependent on the depth of the stroke as Roome and colleagues identify stronger deficits on the staircase test correlated with deeper strokes particularly those that touch the corpus callosum. The majority of our PT strokes were restricted to the cortex and hence may not elicit a sensitive response on the staircase test.

The grip strength test measures the maximum force required to remove a rodent from a grid (**Figure 3.4A)** (Maurissen et al., 2003). Mice were tested prior to and following a stroke where the average **(Figure 3.4B)** and maximum **(Figure 3.4C)** GF to remove the mouse from the grid was determined from five trials performed by each mouse. As expected, the maximum and average GF on the ipsilateral side was similar in sham and PT mice. We were, however, unable to detect any deficits in PT mice on the contralateral side. This demonstrates the reason why the grip strength test is rarely used in mice after stroke. In fact, the lack of stroke-induced deficits is so pronounced that Ferrara et al. (2009) reported that a large MCAo stroke that affected both cortical and sub-cortical regions was unable to detect differences on the grip strength test between sham and stroked mice.

The rotarod is one of the most commonly used tests to measure gross motor function and motor learning (Brooks and Dunnett, 2009). As shown in **Figure 3.5A,** mice are placed on a rod

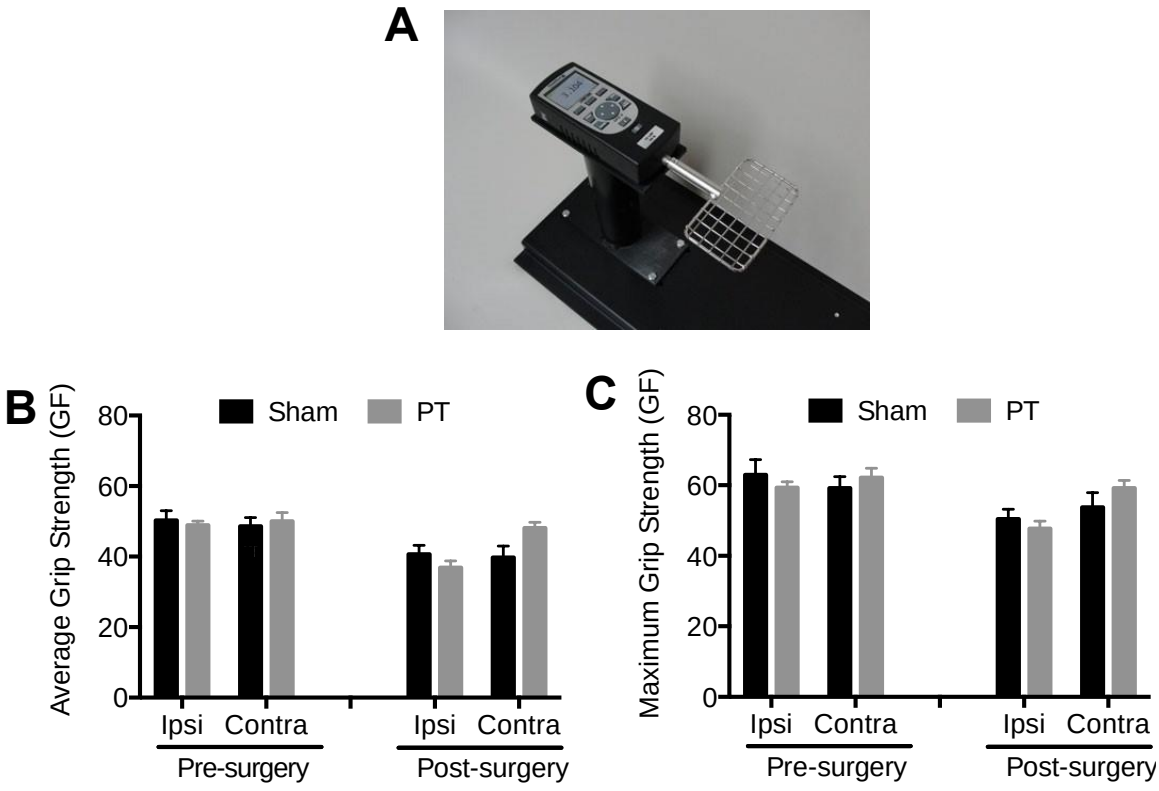


Figure 3.4 The Grip Strength Test Does Not Detect Deficits Following Photothrombosis(PT) -Induced Strokes. (A) Image of the grip strength apparatus. (B) Average and (C) maximum grip force is similar between sham and PT mice before versus after stroke, as well as between ipsilateral versus contralateral sides (n=11 Sham; n=17 PT). Mean ± SEM.

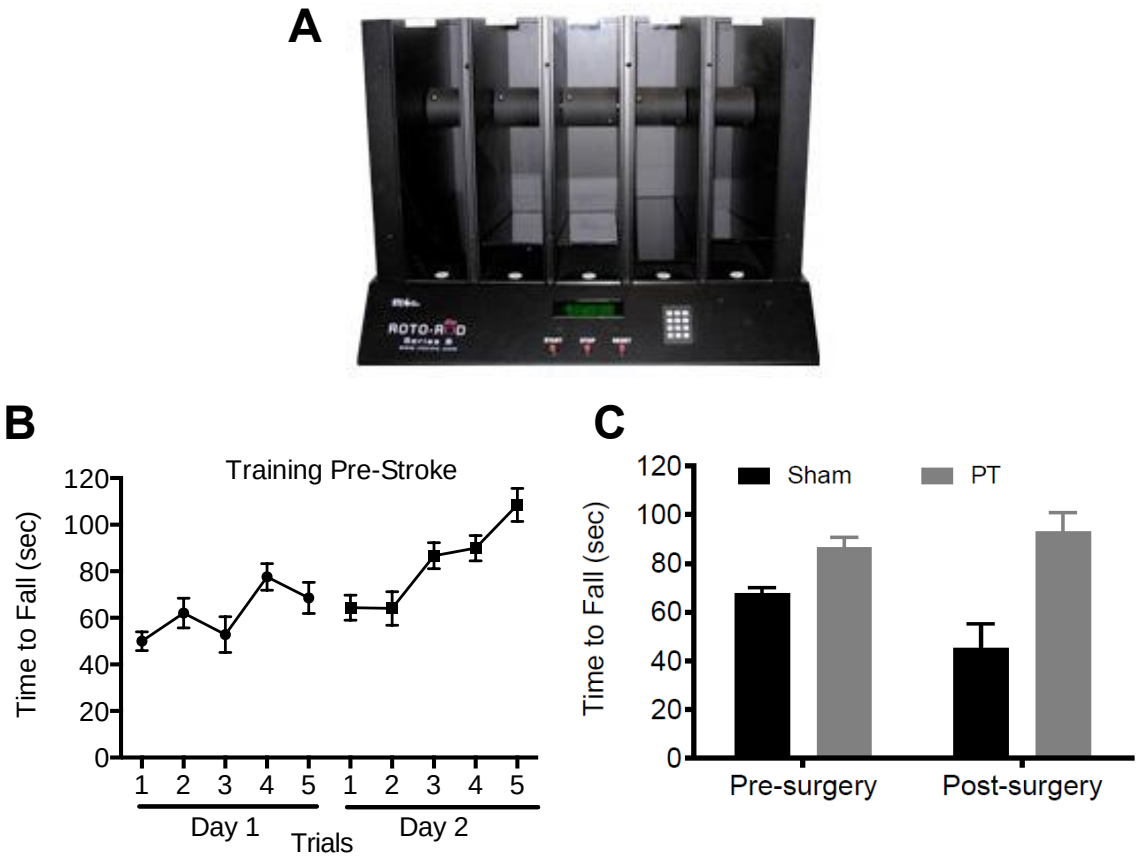


Figure 3.5 The Rotarod Test Does Not Detect Deficits Following Photothrombosis (PT)-Induced Strokes. (A) Image of the rotarod apparatus. (B) Pre-stroke performance shows mice are able to remain on the rod longer over the two day training period. (C) Sham and PT mice perform similarly on the Rotarod test before and after stroke. (n=3 Sham; n=12 PT). Mean ± SEM.

that rotates forcing the mice to remain on the moving rod as long as possible. The rod accelerates from zero to 45 rotations per minute for the duration of the test (5 minutes). Mice underwent five trials daily, and the maximum and average time a mouse remained on the rod was calculated. Pre-training data revealed that mice are able to remain on the rotating rod longer over the course of the two training days (**Figure 3.5B).** Compared to the pre-training performance, both sham and PT mice performed similarly at one wps. While the rotarod was used by others (Lee et al., 2004; Lee et al., 2007) following PT strokes in mice, it is unlikely to produce deficits following cortical PT strokes (Balkaya et al., 2013a).

All three of these behaviour tests did not demonstrate deficits at one wps, therefore mice from these behavioural experiments were sacrificed at two wps and the lesion volume was measured. Aligning with our initial measurement of lesion volume at two wps (**Figure 3.1)**, the PT mice had an average lesion volume of 1.17 mm^3 and predominately had strokes restricted to the cortex. In comparison to others that detected deficits on these behaviour tasks following PT, they had deeper strokes that were more caudal to the ones we produced (Lee et al., 2007).

In another experiment, sham and PT mice were tested on the adhesive removal and ladder rung test **(Figure 3.6)**. The adhesive removal test (i.e., sticky tape test) is a measure of bilateral tactile stimulation and tests the time it takes for the animal to contact and remove an adhesive from their paw to determine sensorimotor asymmetries (Bouet et al., 2009; Schaar et al., 2010). The time to contact and remove the tape has been suggested to correlate with sensory and motor deficits, respectively (Schaar et al., 2010). It is a commonly used test in PT strokes and detects long-term deficits by many groups (Diederich et al., 2012; Sweetnam et al., 2012; Hines and Haydon, 2013; Li et al., 2014). Mice were trained for five consecutive days and the time it took to contact **(Figure 3.7A)** and remove **(Figure 3.7B)** the tape was recorded. The five days of training

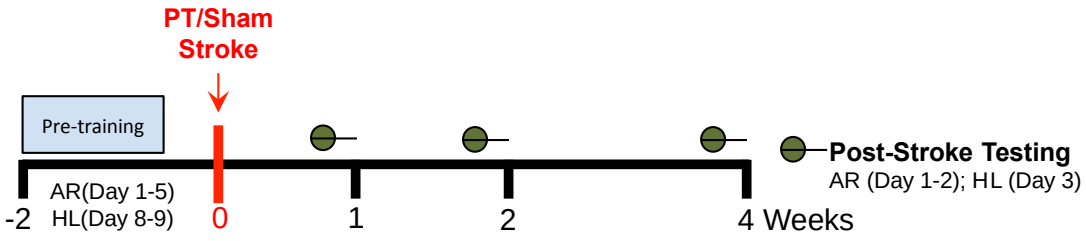


Figure 3.6 Experimental Timeline to Test Photothrombosis (PT) and Sham Mice on the Adhesive Removal and Horizontal Ladder Tasks. Experimental timeline showing mice were trained on the adhesive removal (AR) and horizontal ladder (HL) test starting two weeks prior to PT or sham surgery. Post-stroke testing was performed at one, two and four weeks post-stroke.

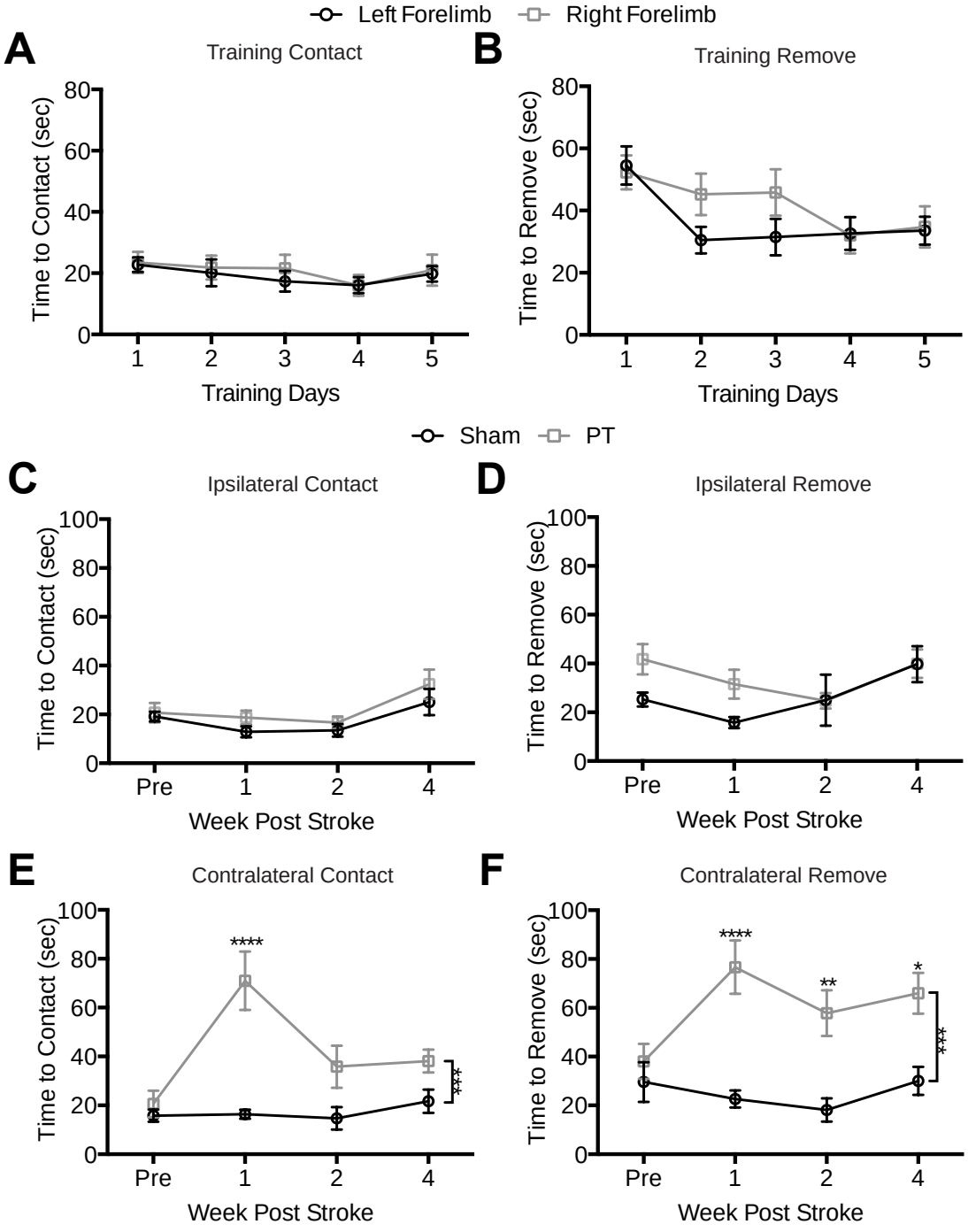


Figure 3.7 Significant Deficits on the Adhesive Removal Test Following Photothrombosis (PT)-Induced Strokes. Prior to Mice are trained for five days on the adhesive task and the (A) time to contact and (B) remove the tape is measured. (C) Time to contact and (D) remove the tape on the ipsilateral side is similar for sham and PT mice. (E) Time to contact and (F) remove the tape on the contralateral side is significantly reduced in PT mice compared to sham mice. (n=8 Sham; n=11 PT). Mean ± SEM.

revealed that the time to remove the tape declined significantly over the five days of training ($F_{(4, 260)}$= 3.9, p=0.003). Posthoc analysis revealed significant differences between one versus four (p=0.004) as well as one versus five (p=0.01) days of training, suggesting that there was a significant improvement in performance over time. Additionally, there was similar performance on time to contact and remove the adhesive on the left and right paw during the training phase. Following stroke, the sham and PT mice did not display deficits on the time to contact (**Figure 3.7C)** and remove **(Figure 3.7D)** the tape from the ipsilateral side compared to pre-stroke performance. On the contralateral side however, the time to contact the tape revealed differences over time, ($F_{(3,51)}$=5.2, p=0.003), between sham and PT mice, ($F_{(3,17)}$=15.9, p=0.0009) and a significant interaction between time and groups, ($F_{(3,51)}$=5.1, p<0.005). Posthoc results identified that PT mice had deficits in time to contact the tape at only one wps (p<0.0001) **(Figure 3.7E)**. Time to remove the tape revealed differences between sham and PT mice, ($F_{(1,17)}$=20.4, p=0.0003), as well as significant interaction between group and time, ($F_{(3,51)}$=3.4, p=0.02), with posthoc results showing a significant reduction in PT mice compared to sham mice at one (p<0.0001), two (p=0.004) and four (p=0.004) wps **(Figure 3.7F)**. Together, these results add to the growing body of literature that support that the adhesive test is sensitive to measure long-term deficits following PT strokes (Sweetnam et al., 2012; Hines and Haydon, 2013; Li et al., 2014).

The horizontal ladder assesses skilled walking, limb placement and coordination (Metz and Whishaw, 2002; Farr et al., 2006; Schaar et al., 2010). The strength of this test lies in its ability to measure both forelimb and hindlimb placement, and is commonly used to assess deficits following PT stroke (Antonow-Schlorke et al., 2013; Hines and Haydon, 2013). Mice traverse a horizontal ladder and the number of correct and incorrect steps were analyzed **(Figure 3.8A)**. A missed placement was scored when the paw completely missed the rung and slipped below the ladder.

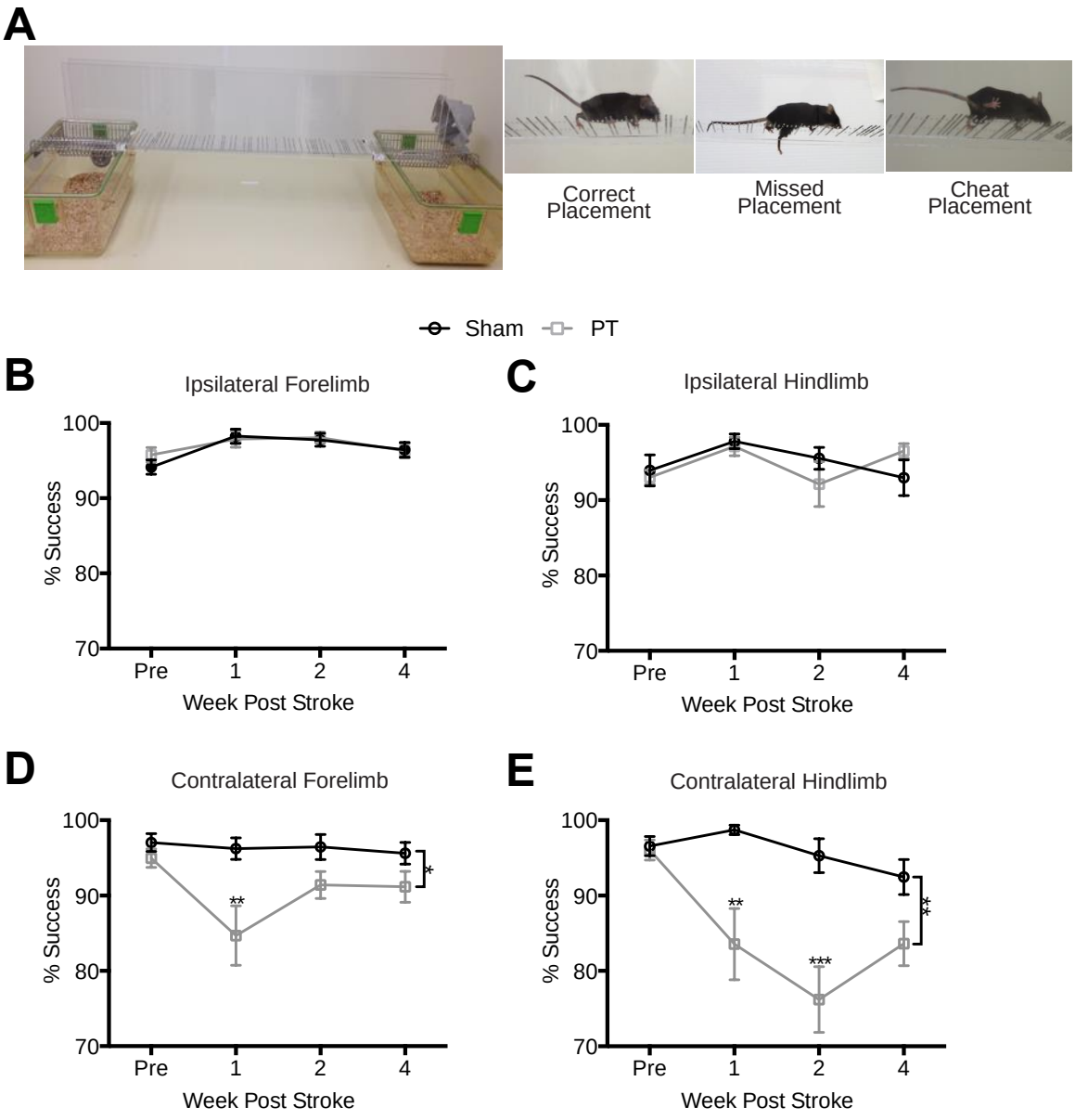


Figure 3.8 Significant Deficits on the Horizontal Ladder Test Following Photothrombosis (PT)-Induced Strokes. (A) Image showing the horizontal ladder and classifications for three different placements scored on the ladder: correct, missed, and cheat. There are no impairments on the (B) ipsilateral hindlimb and (C) forelimb function on the ladder test in sham and PT mice. PT mice have significant impairments on both the (D) contralateral forelimb and (E) hindlimb function on the ladder test. (n=8 Sham; n=11 PT). Mean ± SEM.

Additionally, we visualized a compensatory mechanism where mice placed their limb on the plexi-glass wall; we termed this as a cheat and categorized this movement as an error. Mice rarely cheated using their forelimb, however, the most common error on the hindlimb was cheating. Specifically, on the contralateral hindlimb at four wps, sham mice had ~1 and PT mice had ~3 cheats per trial. The percent success was computed by the number of correct steps over total steps taken and then multiplied by 100. On the ipsilateral side, sham and PT mice performed similarly on the forelimb **(Figure 3.8B)** and hindlimb **(Figure 3.8C)** with an average of 90-100% success rate. On the contralateral forelimb, there was a significant difference in PT versus sham mice ($F_{(1,18)}$=7.8, p=0.01). Posthoc results revealed a significant reduction to ~80% success at one wps (p=0.0029) in the PT compared to sham mice **(Figure 3.8D)**. On the contralateral hindlimb, there were also significant differences between PT and sham mice, ($F_{(1,18)}$=11.6, p=0.003), as well as a significant effect of time, ($F_{(3,54)}$=5.4, p=0.002), and an interaction between group and time, ($F_{(3,52)}$=4.2, p=0.009). Furthermore, there was a decrease in the ability of PT mice to correctly place their hindlimb at one (p=0.007) and two (p=0.0005) wps **(Figure 3.8E)**. These results confirm that in our hands that the horizontal ladder test is a sensitive measure of PT-induced sensorimotor deficits, consistent with previous reports (Sweetnam et al., 2012; Hines and Haydon, 2013).

Lastly, we tested mice on the cylinder test, a measure of spontaneous forelimb function (Brooks and Dunnett, 2009; Schaar et al., 2010; Balkaya et al., 2013a). In this test, animals are placed in a cylinder and the number of placements on the cylinder wall with the left and right forepaw is measured **(Figure 3.9A)** (Brooks and Dunnett, 2009; Balkaya et al., 2013a). Review of the literature using the cylinder test in mice revealed differences in analysis of the cylinder test, with outcome measures such as percent use of impaired forelimb, asymmetry index of forelimb

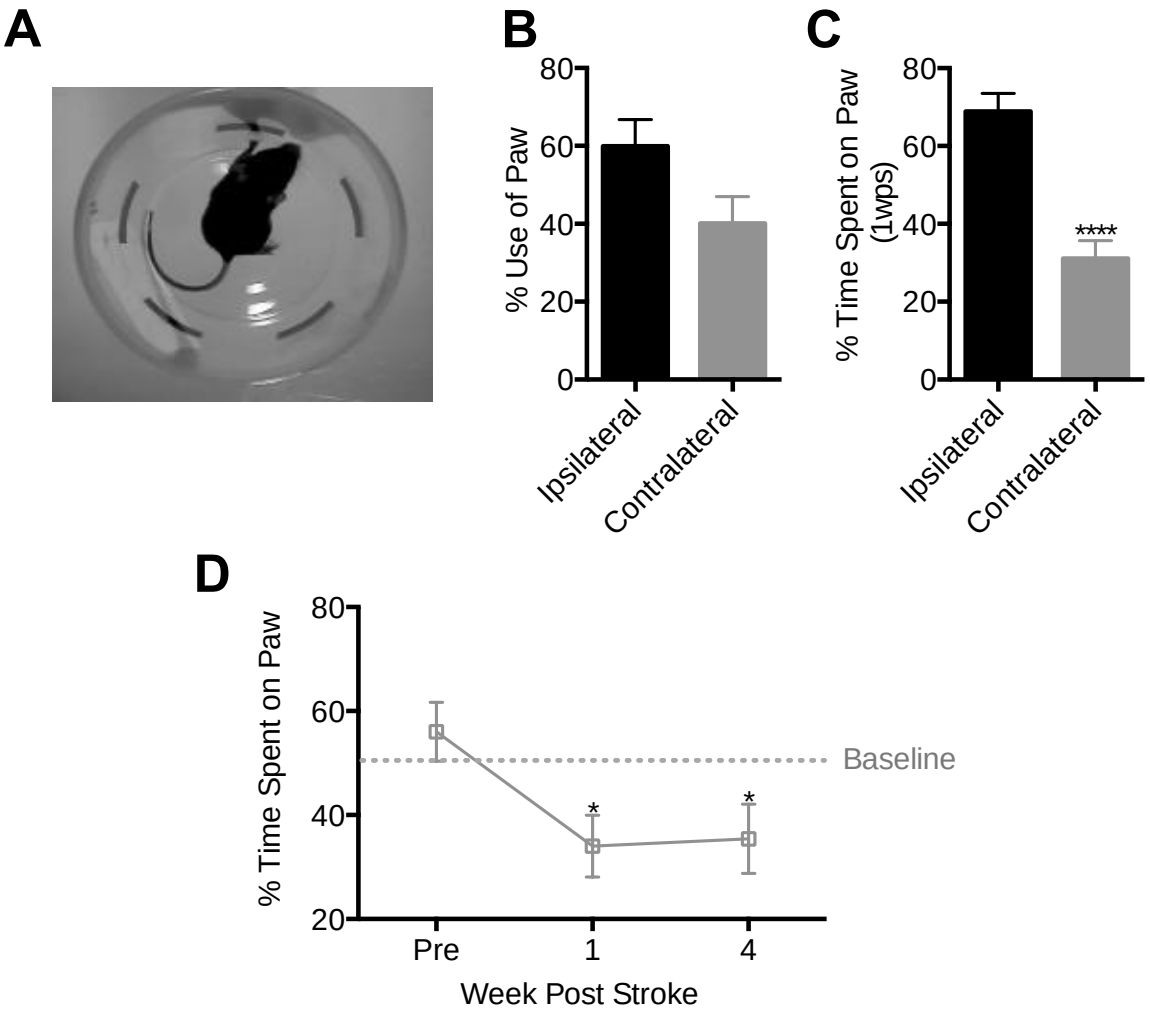


Figure 3.9 Significant Deficits on the Cylinder Test Following Photothrombosis (PT)-Induced Stroke. (A) Image of a mouse rearing on the wall of the cylinder. Mice were induced with PT strokes and were tested at one and four wps. Analysis of (B) percent use of impaired paw or (C) percent time spent rearing on the impaired paw revealed only the later had a significant reduction in time spent on contralaeral paw at one wps. (D) Long-term analysis of PT mice in the cylinder test revealed significantly less was time spent on the contralateral paw up to four weeks post-stroke. (n=14 PT). Mean ± SEM.

usage, and asymmetry index of percent time spent on the forelimbs (Clarkson et al., 2013; Li et al., 2014). Using the outcome measure of percent use of forelimb, we were unable to identify differences in the use of ipsilateral versus contralateral forepaw at one wps **(Figure 3.9B)**. Therefore, we initially arrived at the conclusion that the cylinder test is not sensitive to measure deficits following PT strokes. Contrary to our work, the laboratory of Thomas Carmichael reliably showed deficits on the cylinder test following focal PT strokes (Clarkson et al., 2010; Clarkson et al., 2011; Overman et al., 2012). Their method, however, differed from others in that they calculated the time the mice spent on each forepaw, instead of the number of forepaw placements. Calculation of the time spent on the forepaw revealed significant differences on the contralateral side at one wps ($p<0.001$) **(Figure 3.9C)**. With a separate cohort of mice, we measured if these deficits were sustained for four wps and indeed measuring the duration of time spent on the contralateral paw showed deficits at four wps ($p=0.0392$) **(Figure 3.9D)**. The cylinder test, along with adhesive is one of the most common sensitive measure used by others following PT, however, based on our findings the sensitivity of the cylinder test is largely dependent on the method of analysis (Clarkson et al., 2010; Clarkson et al., 2011; Overman et al., 2012; Clarkson et al., 2013; Li et al., 2014).

Combined, these results suggest the adhesive removal, horizontal ladder, and cylinder tests produce prolonged deficits following small cortical PT strokes allowing us to choose a model that will be used for assessing recovery after an intervention.

3.3: Cognitive Function is Not Altered as Shown on the Barnes Maze Test Following PT Strokes

Preclinical stroke models that damage the hippocampus, such as the intraluminal suture MCAo models, are often associated with spatial learning and memory deficits, which do not occur

in cortical models (Corbett et al., 2015). Although the PT strokes targeting the sensorimotor region were not hypothesized to induce deficits in cognitive function, we tested spatial learning and memory on the Barnes maze post-stroke given that our future work would be testing the effect of enhancing PCs survival, which also occurs in the hippocampus. C57/BL6 mice underwent sham or PT surgery and were tested on the Barnes maze at six weeks post-stroke **(Figure 3.10A).** Mice were placed in a room that had a circular platform with 40 holes around the perimeter and spatial cues on the wall to find their escape box located under one of the 40 holes during training. Three outcome measures for the training phase were analyzed including time to escape to the hole **(Figure 3.10B)**, the average distance traveled to reach the escape hole **(Figure 3.10C)**, and the average velocity when traversing the maze **(Figure 3.10D)**. On the first day of training, sham and PT mice took on average 175 seconds to find the escape hole, whereas by the eighth day the majority of mice were able to find the escape hole in less than 50 seconds. Although there was a significant reduction in time to find the escape hole during the training ($F_{(7,105)}$=55.3, $p<0.001$), there were no differences between sham and PT mice, suggesting that cortical strokes do not affect the ability to learn the location of the escape hole **(Figure 3.10B)**. Analysis of both distance travelled (**Figure 3.10C;** $F_{(7,105)}$=39.3, $p<0.001$**)** and average velocity (**Figure 3.10D;** $F_{(7,105)}$=5.2, $p<0.0001$**)** was also similar to the time taken to find the escape hole over the learning phase.

Following training, the ability of the mice to remember the location of the escape hole was tested during the probe trial. During this trial, mice were placed on the circular maze without an escape box and the percent of time spent in the target quadrant, which used to have the escape box was compared with the percent of time spent in the other three quadrants. This test presumes that if mice learned the task, they will spend more than 25% (*i.e.,* chance) of their time in the target quadrant. When compared to sham mice, PT mice did not have any significant differences in any

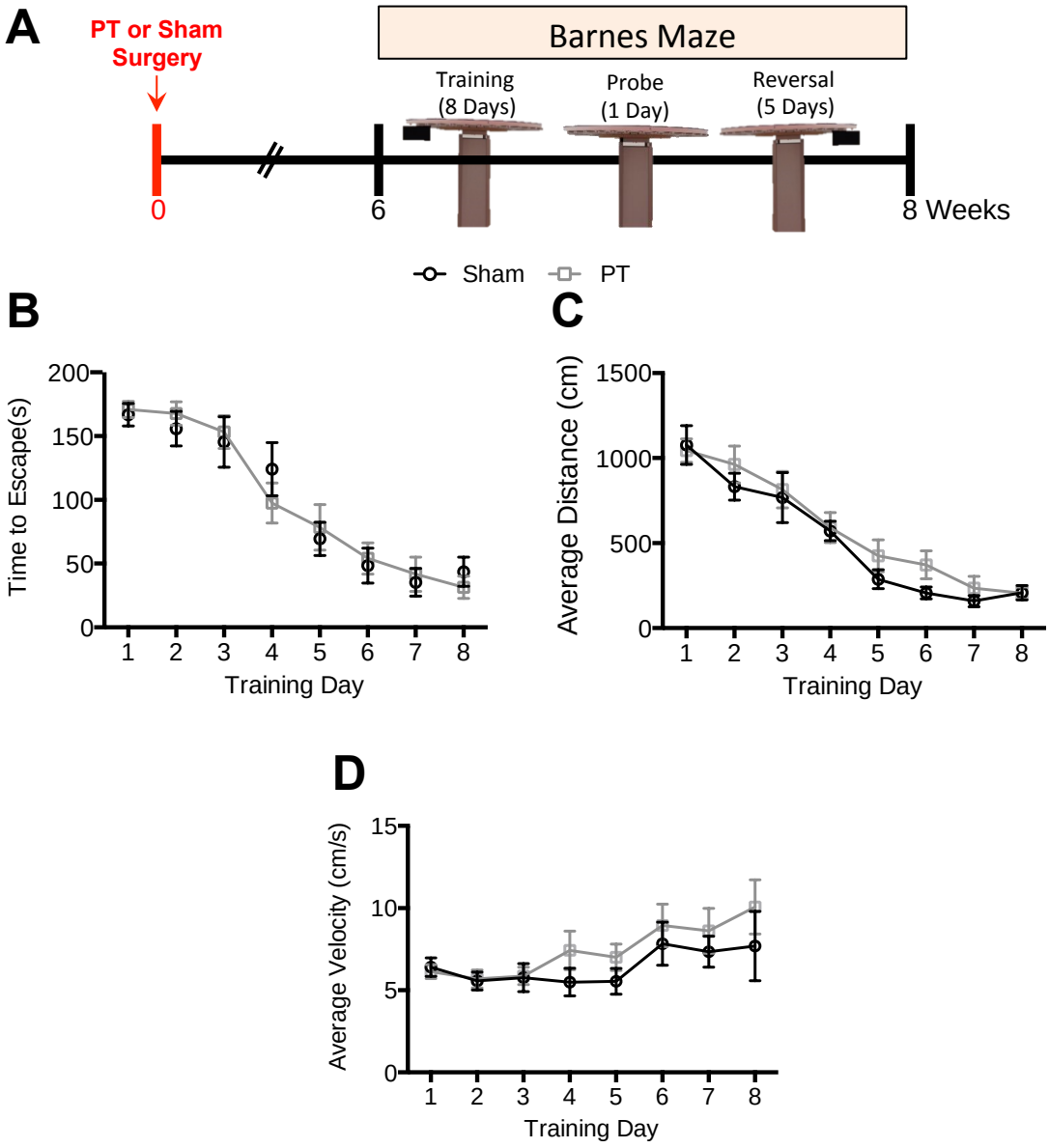


Figure 3.10 Photothrombosis (PT) and Sham Mice Have Similar Spatial Learning Abilities on the Barnes Maze. (A) Experimental timeline showing that mice were tested at six weeks following either PT or sham surgeries. Barnes maze protocol included training mice for eight days, probe test for one day and then reversal training for five days. During the eight days of Barnes maze training, the mice (B) enter the escape box faster, (C) travel less distance to find the escape box, and (D) have increased velocity with no differences between sham and PT mice. (n=8 Sham; n=9 PT). Mean ± SEM.

outcomes from the probe trial **(Figure 3.11A-C).** Both groups spent approximately ~40% of their time in the target quadrant, suggesting both groups successfully learned the location of the escape hole (**Figure 3.11A).**

In the final phase of the test, called reversal testing, the ability of the mice to re-learn a new location for the escape hole was tested by placing the box opposite to its initial location during the training phase. The re-learning (or reversal) phase was performed over five days and, as expected, less time was required to find the new location of the escape hole. Notably, PT mice had a non-significant trend towards performing better on the first couple of days compared to sham mice when measuring time to escape **(Figure 3.12A)**. Additional analysis of the distance travelled **(Figure 3.12B)** and average velocity **(Figure 3.12C)** identified similar performances between sham and PT mice. Together, these results suggest that PT-induced cortical strokes are not associated with a reduction in spatial learning or memory. These findings are thus in alignment with our hypothesis that sensorimotor cortical stroke do not elicit a spatial learning outcome, at least when measuring spatial learning, memory and re-learning on the Barnes maze.

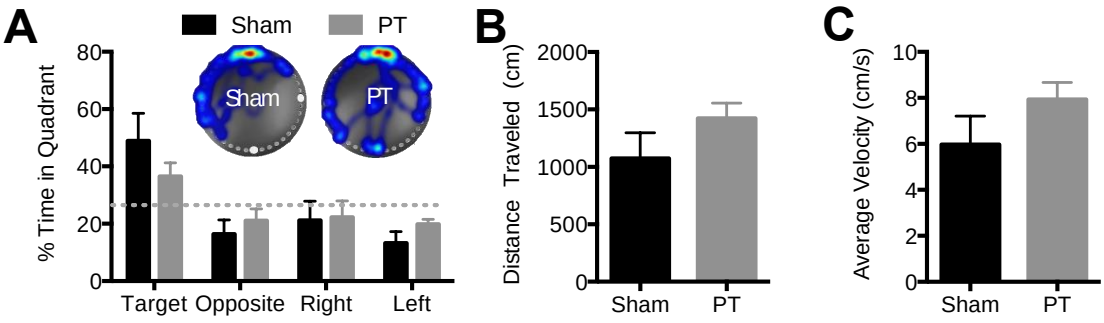


Figure 3.11 Photothrombosis (PT) and Sham Mice Have Similar Spatial Memory Abilities on the Barnes Maze. (A) PT and sham mice spent ~40% of their time in the target quadrant suggesting they learned the location of the escape box. PT and sham mice (B) travel similar distances and (D) have similar velocities on the probe trial. (n=8 Sham; n=9 PT). Mean ± SEM.

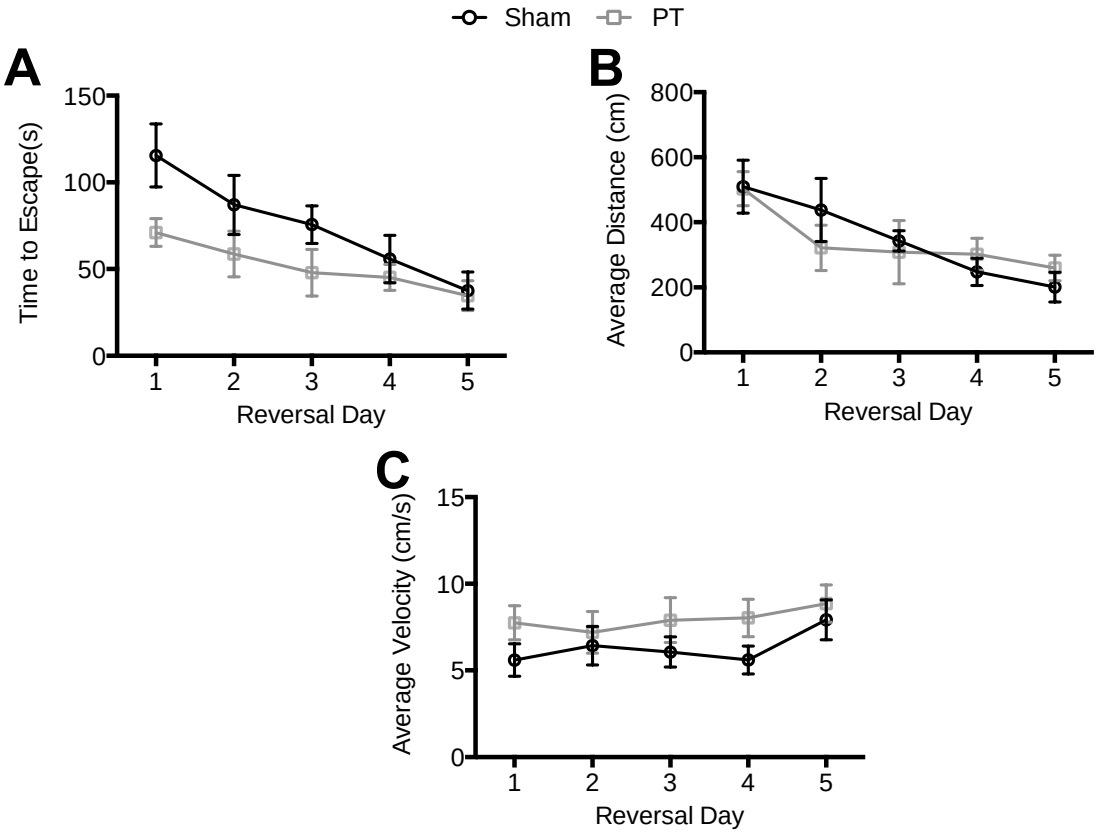


Figure 3.12 Photothrombosis (PT) and Sham Mice Perform Similarly on the Reversal Phase of the Barnes maze. (A) PT and sham mice have no significant differences in their ability to learn a new location of the esacape box during Barnes maze reversal testing. There was also no significant differences in the (C) distance travelled and (D) average velocities between PT and sham mice. (n=8 Sham; n=9 PT). Mean ± SEM.

Chapter 4:

Evaluate the Potential Benefits of Enhancing Progenitor Cells Survival Following Stroke

To augment the survival of PCs this project used the iBax mouse, which was previously published by Sahay et al. (2011b). Animals were generated by breeding an inducible Nestin-CreER^{T2} mouse, R26R-YFP reporter, and fBax knockout mouse **(Figure 4.1A)**. In this model, treatment of the iBax mice with tamoxifen (TAM) allows recombination to occur only in nestin-expressing cells and their progeny. This recombination allows for the expression of the yellow fluorescent protein (YFP) and removal of the *Bax* gene **(Figure 4.1B)**, resulting in the survival of PCs and ultimately increased amounts of neurogenesis (Sahay et al., 2011b). To test if recombination was dependent on TAM, we compared iBax mice given TAM with those that were not. As expected, only mice that received TAM displayed YFP expression **(Figure 4.1C)**.

To test the reliability of this model, we first performed a BrdU pulse experiment to label dividing cells following TAM in the iBax and wild-type (WT) control littermates and examined the number of surviving cells at four weeks post-labeling (**Figure 4.2A)**. WT littermate controls mice had both nestin-CreER^{T2} and R26R-YFP genes but still contained the Bax gene. The iBax mice have significantly more BrdU-labeled cells in the SGZ compared to WT mice ($p<0.0001$) (**Figure 4.2 B-C).** Furthermore, the iBax mice qualitatively had a significant increase in the BrdU-labeled PCs in the SVZ (**Figure 4.2D)**, RMS and OB (data not shown)**.** These findings replicated the results of Sahay et al. (2011b) and support that the iBax mice specifically increase the survival of dividing PCs, and also confirms the viability of this model to determine if increasing PC survival following stroke alters behavioural recovery.

4.1: iBax Mice have Enhanced PC Survival Following PT Stroke at the SVZ and Peri-Infarct Regions

To assess the PC response in the iBax mice following stroke, 10-week old iBax and WT mice were induced with PT strokes followed by TAM at one wps, and then sacrificed either four,

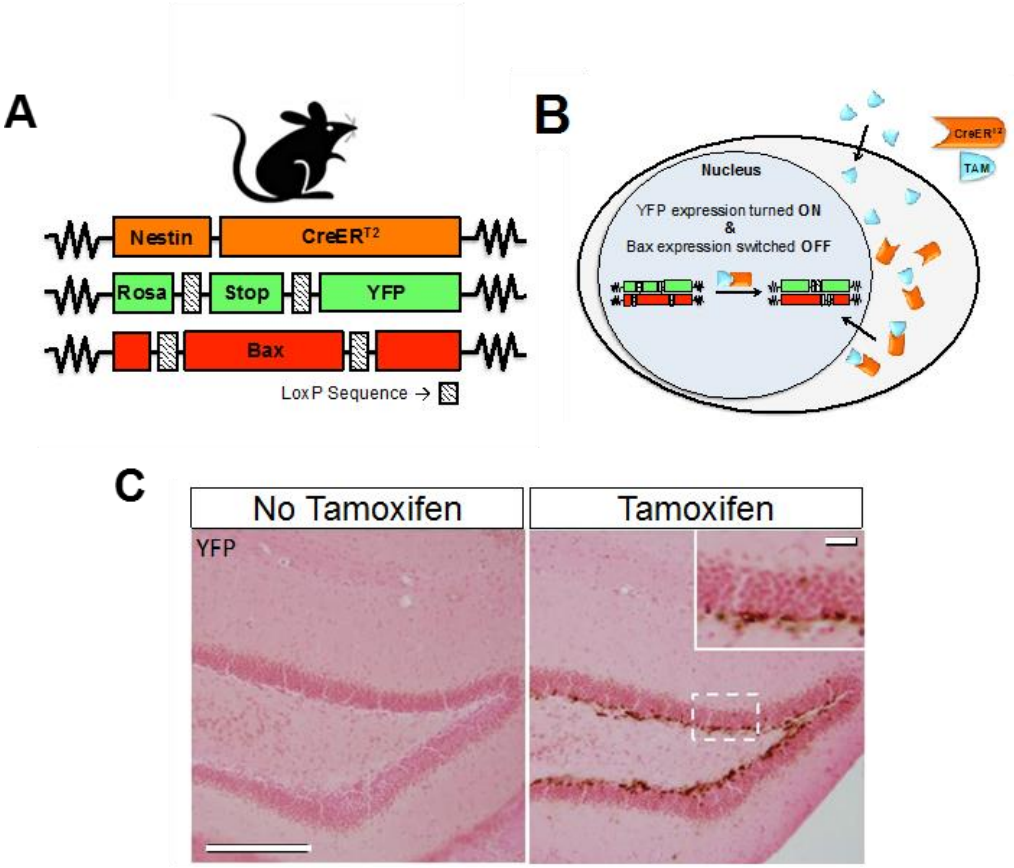


Figure 4.1 Tamoxifen-Induced Recombination in the iBax Mice. (A) A triple transgenic iBax mouseline was generated by breeding the inducible Nestin-CreER^{T2} mouse, R26R-YFP reporter mouse and the floxed Bax mouse. (B) Administration of Tamoxifen (TAM) induced recombination in nestin-expressing PCs and resulted in the expression of YFP and removal of the *Bax* gene. (C) Recombination as shown by YFP-positive cells occurred after the administration of TAM and was specific to the SGZ of the hippocampus. Scale Bar= 200μm. Inset Scale Bar =25 μm.

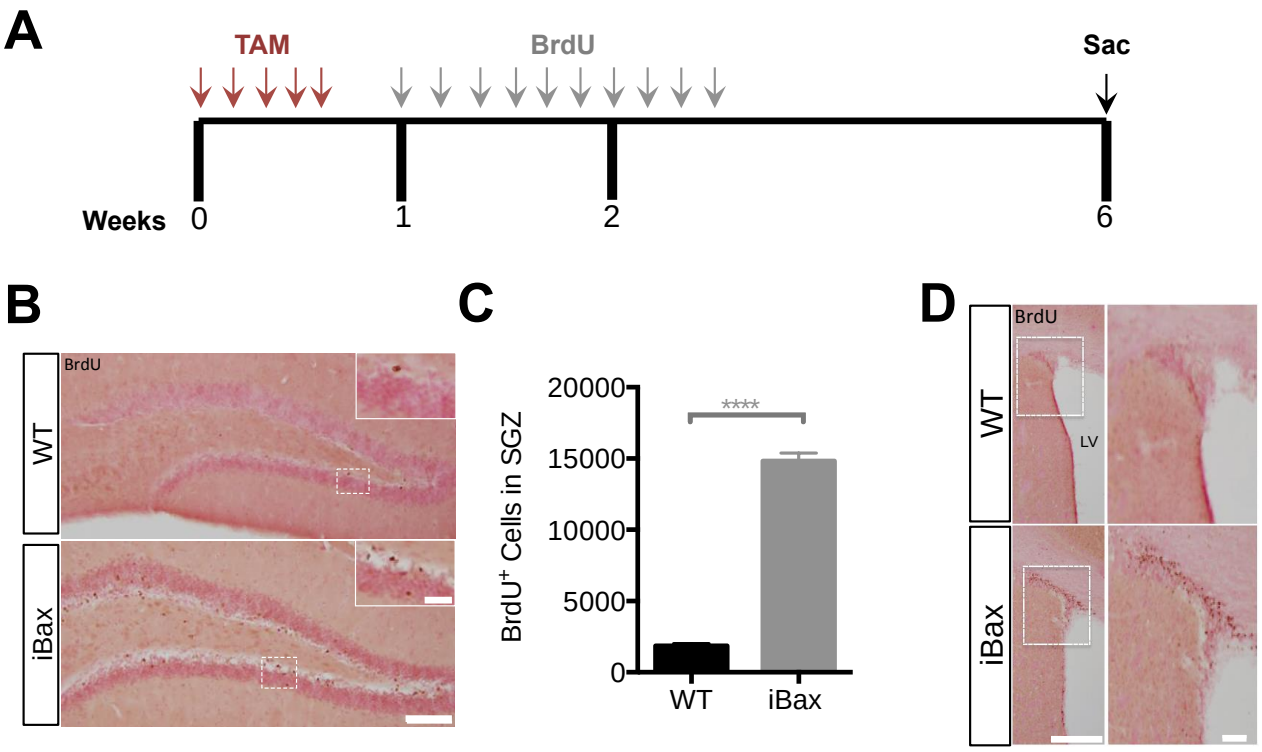


Figure 4.2 The iBax Mouse Enhances the Survial of Dividing Progenitor Cells (PCs). (A) Experimental timeline showing iBax and WT mice that were administred TAM for five days prior to 10 days of BrdU injections. These mice were then sacrificed four weeks after BrdU treatment. (B) Representative images and (C) quantification of BrdU-positive PCs in the SGZ of the WT and iBax mice shows a significant increase in the surviving PCs in the iBax mice (n=5 WT; n=3 iBax). (D) Representative images of the SVZ showing a increase in BrdU-positive PCs in the iBax mice. Scale Bar=100μm; Inset Scale Bar=25μm. ****P<0.0001. Mean ± SEM.

eight or 12 wps **(Figure 4.3A)**. The number of PCs at the SVZ **(Figure 4.3B, C)** and peri-infarct (**Figure 4.3 D, E)** regions was quantified. As expected between 4-12 wps ($F_{(2,12)}$=7.9, p=0.007), there was a significant increase in number of YFP-positive PCs in the iBax mice compared to control mice ($F_{(2,12)}$=27.4, p=0.0002) in the SVZ (**Figure 4.3C)**. Specifically, starting at eight (p=0.0109) and continuing to 12 (p=0.0004) wps there was a significant increase in number of PCs in the iBax mice. Similarly, at the peri-infarct region, there was a robust increase in PCs from 4-12 wps ($F_{(2,12)}$=23.5, p<0.0001) between iBax and WT mice ($F_{(2,12)}$=97.5, p<0.0001) and an overall interaction between time and genotype ($F_{(1,12)}$=22.6, P<0.0001). Furthermore, there was a significant increase in PCs in the iBax mice at eight (p=0.0006) and 12 (p<0.0001) wps.

4.2: Majority of PCs are Fated to Becomes DCX+ Neuroblasts Following PT Strokes

Previous studies have shown that newborn PCs at the peri-infarct regions are fated to become neurons, astrocytes and oligodendrocytes (Li et al., 2010; Li et al., 2014). Therefore, to determine the fate of the PCs following stroke, YFP-positive PCs in WT and iBax were colocalized with markers that delineate the different phenotypes of PCs. At 12 wps, there were no significant differences in the fate of the YFP-positive PCs in the iBax versus control mice **(Figure 4.4)** after stroke. On average ~60% of YFP cells co-expressed with the neuroblast marker doublecortin (DCX) (**Figure 4.4A)**; ~10% expressed the marker, GFAP (**Figure 4.4B)**; and no cells colabeled with the oligodendrocyte marker, Olig2 (data not shown). These results suggest that the iBax mice have a significant increase in the number of PCs that predominantly adopt a neuronal lineage, which will allow us to test whether these cells translate to enhanced sensorimotor function following stroke.

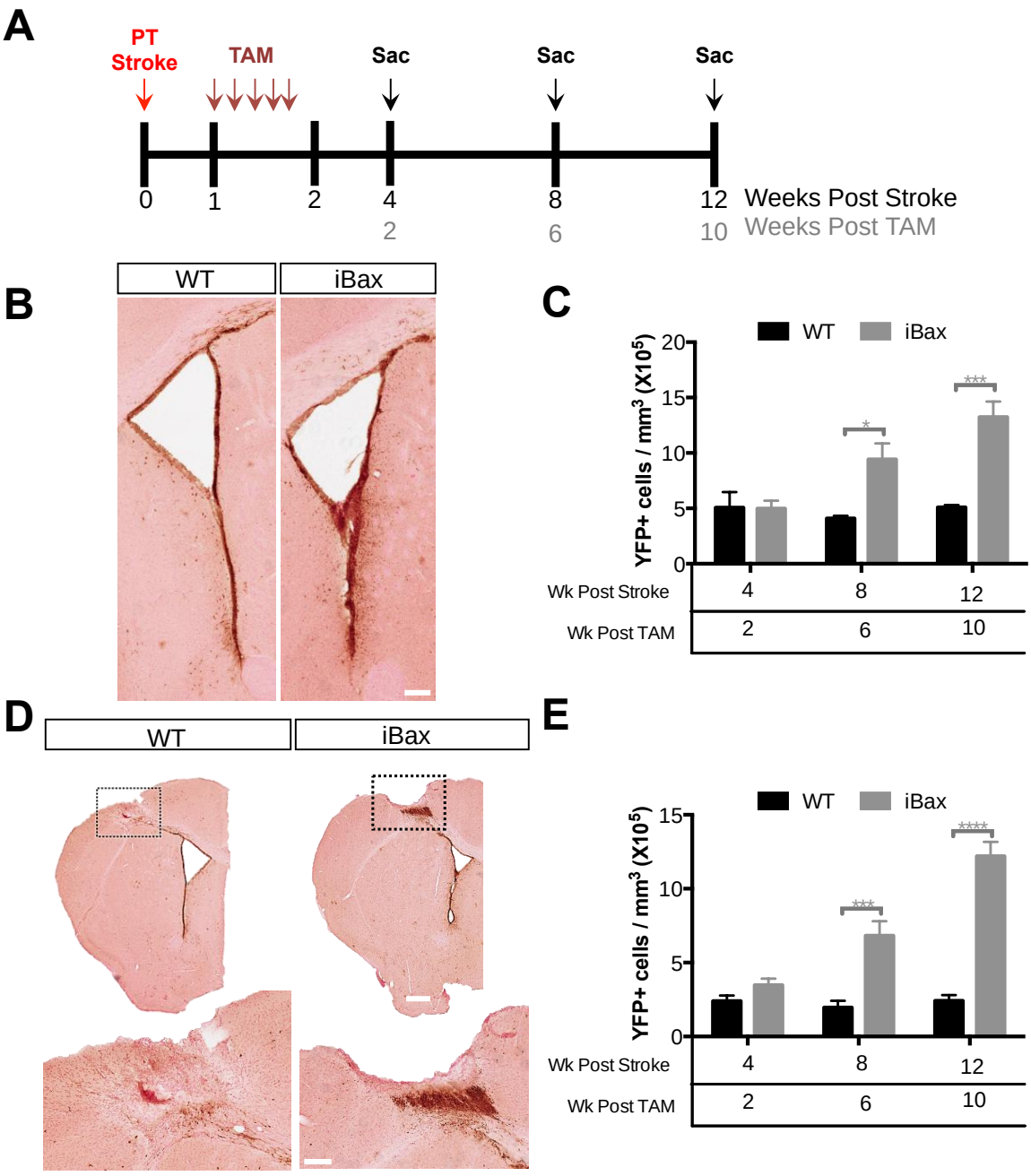


Figure 4.3 iBax Mice Have More Progenitor Cells at the SVZ and Peri-Infarct Regions. (A) Experimental timeline showng iBax and WT mice were induced with photothrombosis (PT) strokes followed by TAM administration at one week post PT and then sacrificed at either four, eight or 12 wps. (B) Representative images and (C) quantification of YFP-positive PCs at the SVZ shows a significant increase in the surviving PCs in the iBax mice at eight and 12 wps. Scale Bar = 200µm.(D) Representative images and (E) quantification of YFP-positive PCs at the peri-infarct also shows a significant increase in the surviving PCs in the iBax mice at eight and 12 wps. (n=3 per group) Scale Bar =500µm (low magnification); 200µm (inset) *p<0.05, ***p<0.001 and ****p<0.0001. Mean ± SEM.

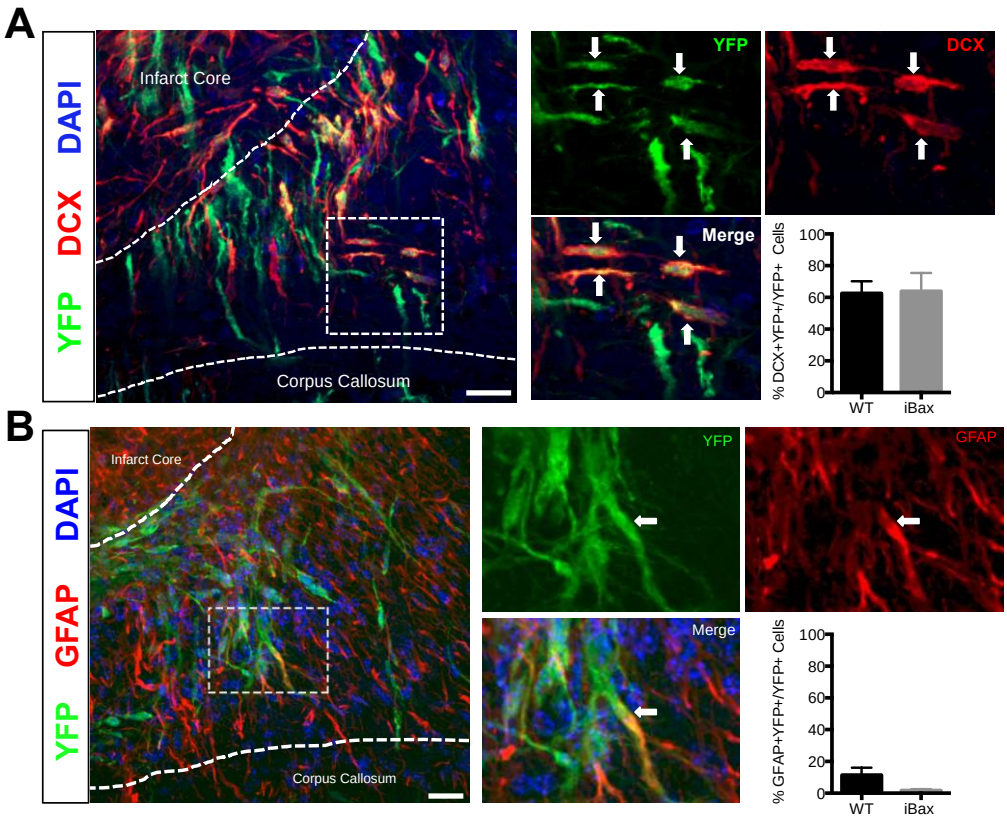


Figure 4.4 Progenitor Cells in the Peri-Infarct Region of iBax and WT Mice are Predominantly Neuroblasts at 12 Weeks Post-Stroke. (A) Representative image and quantification showing that recombined YFP-positive PCs and neuroblast marker, DCX, co-localize in the majority of PCs. (n=6 per group). (B) Representative image and quantification of recombined YFP-positive PCs and a marker found in astrocytes, GFAP co-localization showing less than 10% of YFP-positive PCs become astrocytes. (n=3 per group). Scale Bar= 50μm. Mean ± SEM.

4.3: Enhancing PCs Survival is Insufficient to Improve Sensorimotor Function Following Stroke

To test the implication of PCs survival post-stroke, eight-week-old iBax and WT littermate mice were trained on standardized motor/sensorimotor tests, including the adhesive removal, horizontal ladder, and cylinder tests prior to PT strokes at 10-weeks old. Within two to five days after stroke, all three behavioural tests were performed in order to establish baseline deficits. TAM was injected at one wps, which is the time point that corresponds to the peak in proliferation and migration following PT-induced cortical strokes (Zhang et al., 2001; Osman et al., 2011). Long-term behavioural measurements were completed at four, eight, and 12 wps, corresponding to two, six and 10 weeks post-TAM **(Figure 4.5)**. Post-stroke behavioural testing included the adhesive (**Figure 4.6**), horizontal ladder (**Figure 4.7)** and cylinder (**Figure 4.8)** tasks. These experiments were completed in two experimental cohorts and since there were no significant differences in outcomes, the experiments were combined.

For the adhesive test, mice were trained for five days' time, during which they got significantly faster at contacting ($F_{(4,240)}$=11.8, p<0.0001), and removing the tape ($F_{(4,240)}$=17.6, p<0.0001). On the first day of training, mice took on average ~23s to contact **(Figure 4.6A)** and ~30s to remove (**Figure 4.6B)** the tape. After five days of training, the time to contact and remove was reduced on average to ~11 and ~15 seconds, respectively. As expected following a stroke, performance on the ipsilateral side revealed no deficits in the time taken to contact **(Figure 4.6C)** or remove **(Figure 4.6D)** the tape, nor any differences between iBax and controls. On the contralateral side, there were significant deficits in the time taken to contact ($F_{(4,116)}$=14.2, p<0.0001) and remove ($F_{(4,116)}$=21.3, p<0.0001) the tape post-stroke, however, no differences were

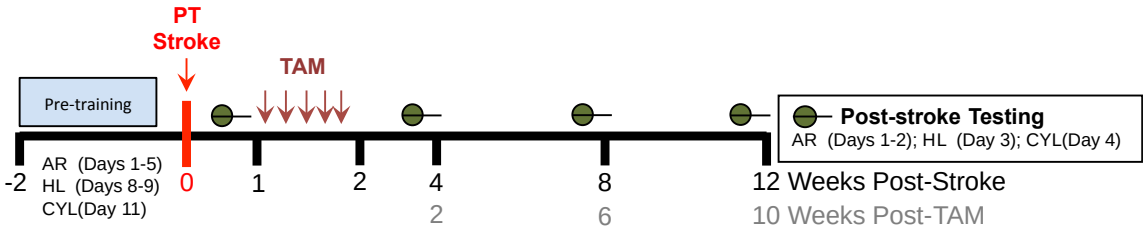


Figure 4.5 Experimental Timeline to Determine if Increasing Progenitor Cells Enhances Sensorimotor Recovery. WT (n=14) and iBax (n=17) mice were trained on the adhesive removal (AR), horizontal ladder (HL) and cylinder (CYL) tests at eight weeks old followed by Photothrombosis (PT) strokes at 10 weeks old. Following stroke, baseline deficits were recorded prior to Tamoxifen (TAM) injections at one wps. Recovery was measured by behavioural testing at four, eight and 12 weeks post-stroke, which corresponds to two, six and 10 weeks post-T AM.

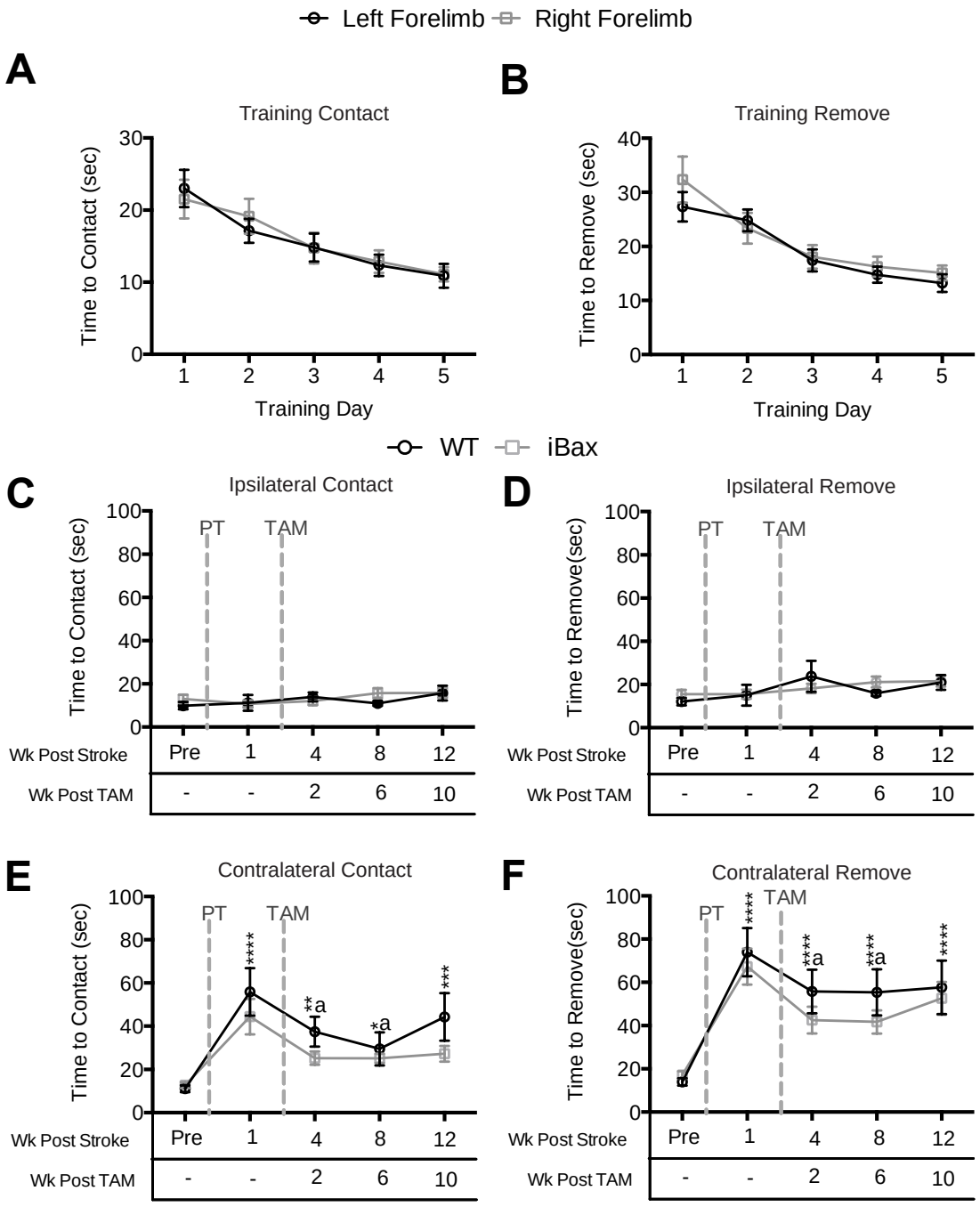


Figure 4.6 iBax and WT Mice Have Similar Contralateral Deficits on the Adhesive Removal Test After Stroke. (A)Mice learn to contact and (B) remove the adhesive tape faster over the course of the five training days . (C)Time to contact and (D) remove the tape was comparable to pre-stroke function on the ipsilateral side in iBax and WT mice. (E) Time to contact and (F) remove the tape on the contralateral side was significantly increased following stroke, with no differences between iBax and WT mice. *p<0.005, **p<0.01, ***p<0.001, and ****p<0.0001 represents significance from pre-stroke performance. **a** = significance from one wps. Mean ± SEM.

observed between iBax and controls. Specifically, mice took longer to contact the tape at one ($p<0.0001$), four ($p=0.005$), eight ($p=0.04$), and 12 ($p=0.0002$) wps (**Figure 4.6E)**. Similarly, mice took significantly longer to remove the adhesive tape at one, four, eight, and 12 ($p<0.0001$ for all time points) wps (**Figure 4.6F.)** Furthermore, posthoc analysis also suggests that SBR occurs after stroke since the time to contact and remove tape significantly improved between one and four/eight wps, suggesting both iBax and control mice equally improved in their ability to perform this test.

Similar to the adhesive task, the ladder test revealed significant unilateral deficits after stroke, in the absence of any differences between iBax and WT mice **(Figure 4.7).** Both iBax and WT mice perform differently over time on the forelimb (**Figure 4.7A;** $F_{(4,116)}=3.3$, $p=0.01$**)** and hindlimb (**Figure 4.7B;** $F_{(4,116)}=3.9$, $p=0.005$**)** function on the ipsilateral side. There was no impairments on the ipsilateral side at one wps but rather altered function between one and four wps ($p=0.04$) on the forelimb and one and four ($p=0.02$) and eight ($p=0.01$) wps on the hindlimb paw. On the contralateral side, there were significant forelimb (**Figure 4.7C;** $F_{(4,116)}=15.6$, $p<0.0001$**)** and hindlimb (**Figure 4.7D;** $F_{(4,116)}=6.3$, $p=0.0001$**)** deficits after stroke. The deficits on the contralateral forelimb persisted to one ($p<0.0001$), four ($p<0.0001$), eight ($p=0.0001$) and 12 ($p=0.0121$) wps, whereas the hindlimb deficits declined to levels close to the pre-surgery success rate by four wps. Mice, however, recovered between one and 12 wps ($p=0.002$) on the contralateral hindlimb, which is suggestive of SBR.

The cylinder test also revealed deficits after stroke, but performance was similar between the iBax and WT mice. Mice spent less time on their impaired paw compared to their pre-stroke performance ($F_{(4,76)}=4.1$, $p=0.004$), with posthoc analysis revealing significant deficits between pre-stroke performance and one ($p=0.02$) and four ($p=0.004$) wps **(Figure 4.8A)**. These results are mimicked by the asymmetry index **(Figure 4.8B)** as calculated by the percent time spent on the

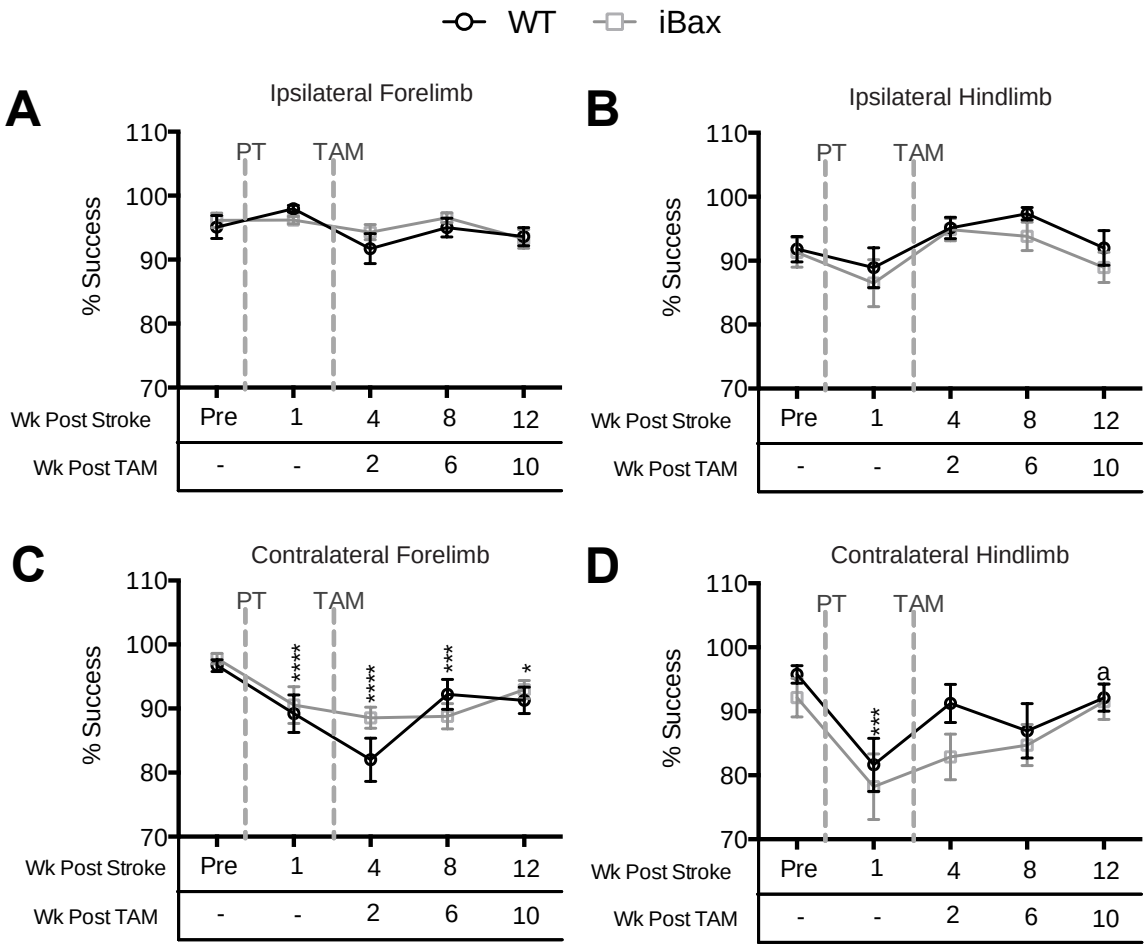


Figure 4.7 iBax and WT Mice Have Similar Contralateral Deficits on the Horizontal Ladder Test After Stroke. Ipsilateral (A)forelimb and (B) hindlimb performance was not different before versus after stroke and was similar for iBax and WT mice. There were significant contralateral (C)forelimb and (D) hindlimb deficits with no differences between iBax mice and WT mice. *p<0.05, ***p<0.001, and ****p<0.0001 represents significance from pre-stroke performance. **a** = significance from one wps. Mean ± SEM.

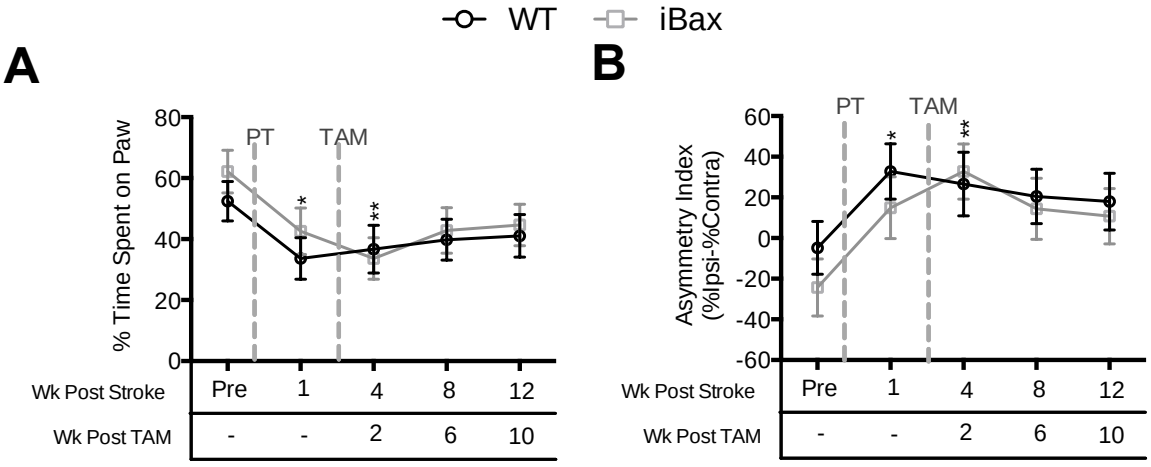


Figure 4.8 iBax and WT Mice Have Similar Reduced Usage of their Impaired Paw on the Cylinder Test Following Stroke. Cylinder data using either (A) percentage of time on paw or (B) asymmetry index both reveal iBax and WT mice have similar usage prior to stroke and a preference to use their ipsilateral paw more following stroke. *p<0.005, and **p<0.01 represents significance from pre-stroke performance. Mean ± SEM.

impaired side subtracted from the unimpaired side (Clarkson et al., 2010; Clarkson et al., 2011; Overman et al., 2012), suggesting that the cylinder test detects deficits in PT mice.

4.4: iBax Mice Have a Modest Improvement in Spatial Learning Following PT Strokes

In addition to sensorimotor tasks, we also tested whether the iBax mice would have significant improvements in learning post-stroke. We hypothesized that iBax mice would have an improvement due to the growing body of literature suggesting that neurogenesis in the SGZ has a functional role in learning and memory (Deng et al., 2010; Yau et al., 2015). Furthermore, previous work has suggested that ablation of PCs worsened the spatial acquisition and memory retention in the Barnes maze, in the absence of altering motor function following a cortical stroke (Sun et al., 2013).

A time course analysis of the number of YFP-positive PCs in the dentate gyrus revealed a striking increase in number of cells between 4-12 wps ($F_{(2,22)}$=4.1, p=0.03) in the iBax compared to WT littermates ($F_{(4,22)}$=28.8, p<0.0001) as well as an interaction between time and genotype ($F_{(2,22)}$ =4.703, p=0.02) (**Figure 4.9A-B)**. Posthoc analysis revealed a significant 5-fold increase in the number of cells at 12 wps in the iBax compared to WT mice (p<0.0001). At 12 wps, equating to 10 weeks after recombining the nestin-expressing PCs and their progeny, we hypothesized that the majority of PCs would be fated to become mature neurons. Phenotypic analysis of YFP-positive PCs with the mature neuronal marker NeuN revealed that the iBax mice had over 95% YFP+NeuN+ cells. This proportion was significantly higher than WT littermates that had only ~75% of the cells colocalized (p=0.001) **(Figure 4.9C, D)**. This difference was not unexpected and can be attributed to the forced survival of PCs in the iBax mice, allowing for the accumulation of mature neurons in the dentate gyrus.

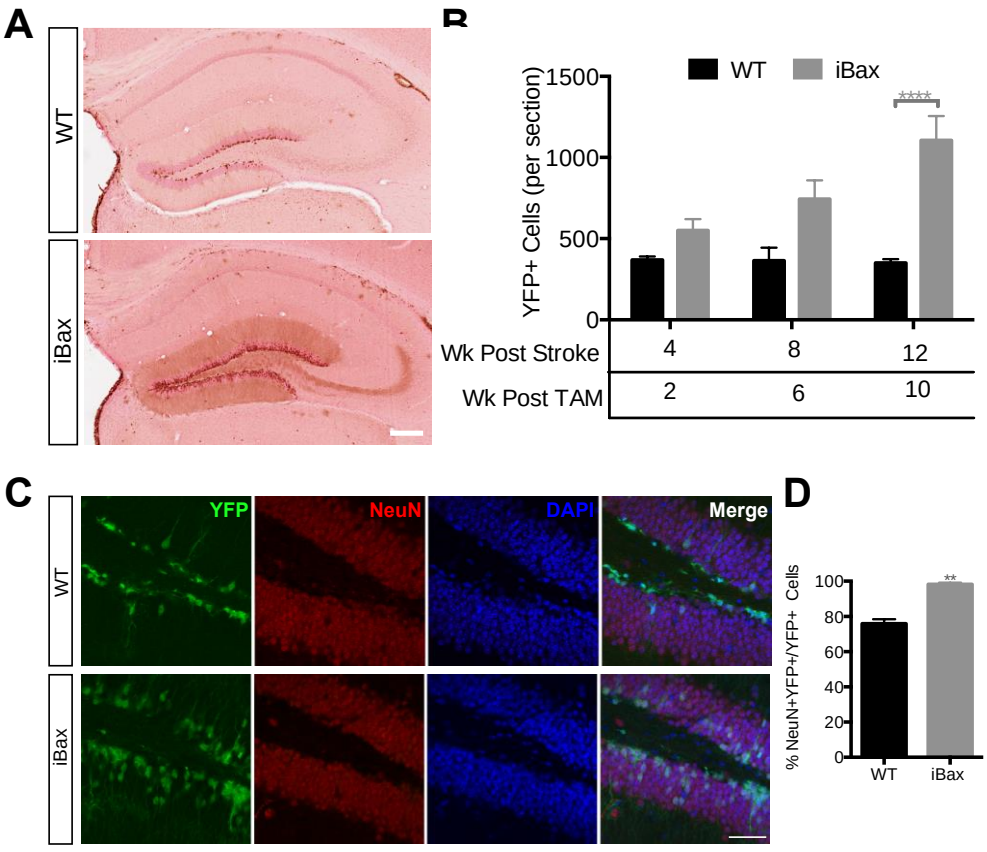


Figure 4.9 iBax Mice Have Significantly More Progenitor Cells at the SGZ that are Fated to be Neurons After Stroke. (A)Representative images and (B) quantification showing a significant increase in the number of YFP-expressing recombined PCs at the SGZ in the iBax mouse at 12 wps. Scale Bar=200μm. (C)Representative images and (D) quantification of YFP-positive PCs that co-localized with the mature neuronal marker,NeuN. iBax mice had significantly more YFP-positive, NeuN-positive PCs compared to WT mice. Scale Bar=50μm. Minimum n=3 per group. **p<0.01 and ****p<0.0001. Mean ± SEM.

Spatial and associative learning and memory function was tested using the Barnes maze (**Figure 4.10-4.15),** a task that was unaffected by the PT as shown in Chapter 3 **(Figure 3.8-3.10)**. To first determine if enhancing neurogenesis in the iBax mouse alone (independent of stroke) was sufficient to improve Barnes maze performance, 10-week-old naïve iBax and WT mice were treated with TAM and then tested on the Barnes Maze six weeks following treatment **(Figure 4.10A)**. The Barnes maze testing protocol consisted of training mice for six days to learn the location of the escape hole using the cues on the wall, followed by a probe trial to test their memory of the location of the escape hole, and then four days of reversal training to test their ability to re-learn a new spatial location. Over the six-day training period, the mice took significantly less time to locate and enter the escape box ($F_{(5,90)}$=3.5, p=0.006), with no differences between the iBax and WT littermate mice (**Figure 4.10B)**. Similarly, both groups travelled less distance ($F_{(5,90)}$=8.5, p<0.0001) at a similar velocity over the course of the six training days (**Figure 4.10C,D)**. On the probe trial, there was also no differences between iBax or WT mice, as they spent majority of their time (~50%) in the target quadrant compared to other quadrants ($F_{(3,54)}$=9.8, p<0.0001) searching for the escape hole **(Figure 4.11A)**. During the probe trial, both groups again travelled similar distances **(Figure 4.11B)** and used similar velocities **(Figure 4.11C)**. On the reversal phase, the iBax mice performed similarly to WT littermates in 1) their ability to relearn a new location for the escape hole as measured by the time taken to enter the target hole **(Figure 4.12A)**, 2) their distance travelled **(Figure 4.12B)**, and 3) average velocity **(Figure 4.12C).** Overall these results suggest that iBax mice have comparable spatial learning and memory abilities, which is consistent with previous reports demonstrating comparable MWM performance in these mice (Sahay et al., 2011b).

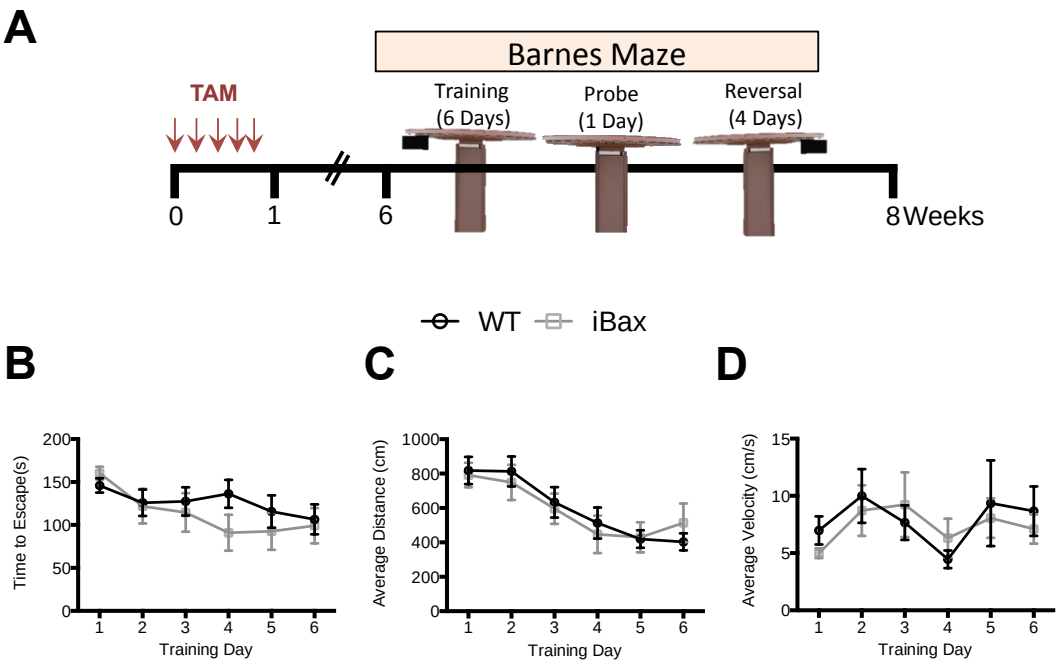


Figure 4.10 Naive iBax and WT Mice Have Similar Spatial Acquisition Abilities on the Barnes Maze. (A) 10 week-old iBax and WT mice received TAM treatment and were tested on the Barnes maze at six weeks post-TAM. iBax and WT mice spent (B) similar time, (C) travelled similar distance and (D) used similar velocities on the Barnes maze over the course of six training days. Mean ± SEM.

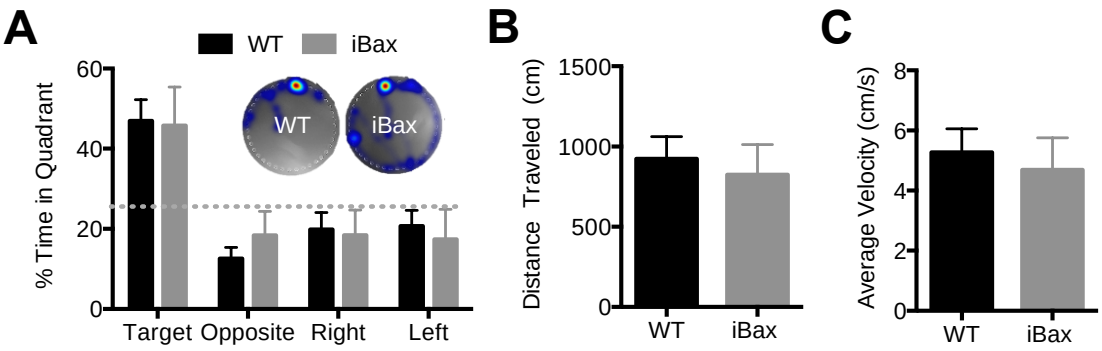


Figure 4.11 Naive iBax and WT Mice Have Similar Spatial Memory Abilities on the Barnes Maze. (A) Assessment of the probe trial revealed significantly more time was spent in the target quadrant compared to all other quadrants for both iBax and WT mice. Inset showing representative heat maps of the movement of iBax and WT mice during the three minute probe trial. iBax and WT mice (B) traveled similar distances and (C) had similar velocities on the Barnes maze during the pr obe trial. Mean ± SEM.

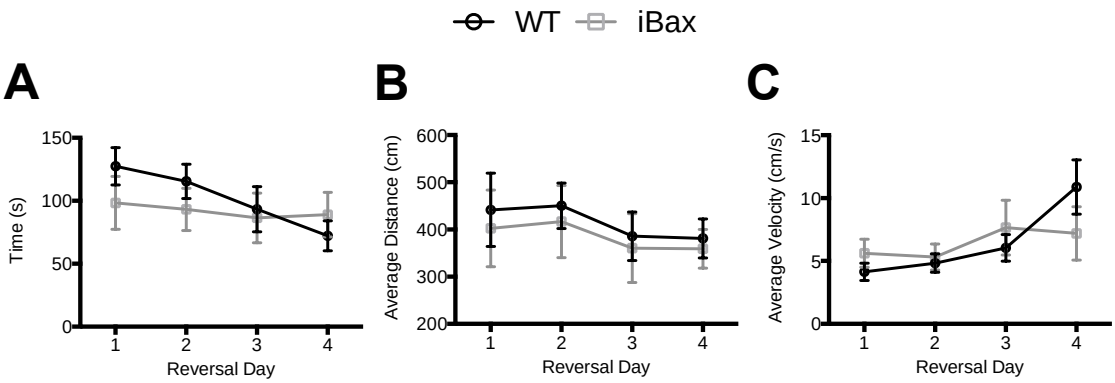


Figure 4.12 Naive iBax and WT Mice Have Similar Performance on the Reversal Phase of the Barnes Maze. (A) iBax and WT mice have no significant differences in their ability to learn a new location of the esacape box during the Barnes maze reversal testing. There were also no significant differences in the (C) distance travelled and (D) average velocities between iBax and WT mice. Mean ± SEM.

To determine if enhancing PC survival following stroke would enhance Barnes maze performance, the same protocol was completed on the iBax and WT mice after they completed sensorimotor testing at 14 weeks post-stroke (**Figure 4.13A)**. During the five days of training, the mice took less time to find the escape hole ($F_{(4,108)}$=17.3, p<0.0001) and there was a non-significant trend for the iBax mice to learn faster than WT littermates **(Figure 4.13B)**. Analysis of the distance travelled during training also showed all mice travelled shorter distances over the training days ($F_{(4,108)}$=16.9, p<0.0001), however, iBax mice travelled less to enter the escape hole compared to WT mice **(Figure 4.13C;** $F_{(1,27)}$=13.7, p=0.001**)**. This finding aligns with the Sun et al. (2013) reporting a significant increase in path length on the Barnes maze in mice that had PCs ablated. Mice had an increase in the average velocity ($F_{(4,108)}$=5.5, p=0.0004) over the course of the five training days. Notably, the iBax mice, at each time point, consistently had a lower velocity compared to the WT mice **(Figure 4.13D)**.

Since the iBax mice had a shorter path length compared to WT mice during training, we hypothesized that the iBax mice were more likely using different spatial strategies to enter the escape hole compared to the WT mice. Mice predominately use a random search strategy on the first day of training, however, over time, they significantly reduced the use of this strategy **(Figure 4.13E,** $F_{(4,108)}$=8.4, p<0.0001**)**. Simultaneously, as expected, iBax and WT mice transitioned into using either a serial or spatial/sub-spatial strategies **(Figure 4.13F, G)**. Analysis of the precentage of animals that used each of these strategies did not reveal any differences between the iBax and WT mice, which is against our hypothesis as it did not appear that a spatial strategy was more often used by the iBax mice.

Analysis of the probe trial revealed the iBax and WT mice spent greater than 25% of their time in the target quadrant that previously contained the escape box, which suggests that they all

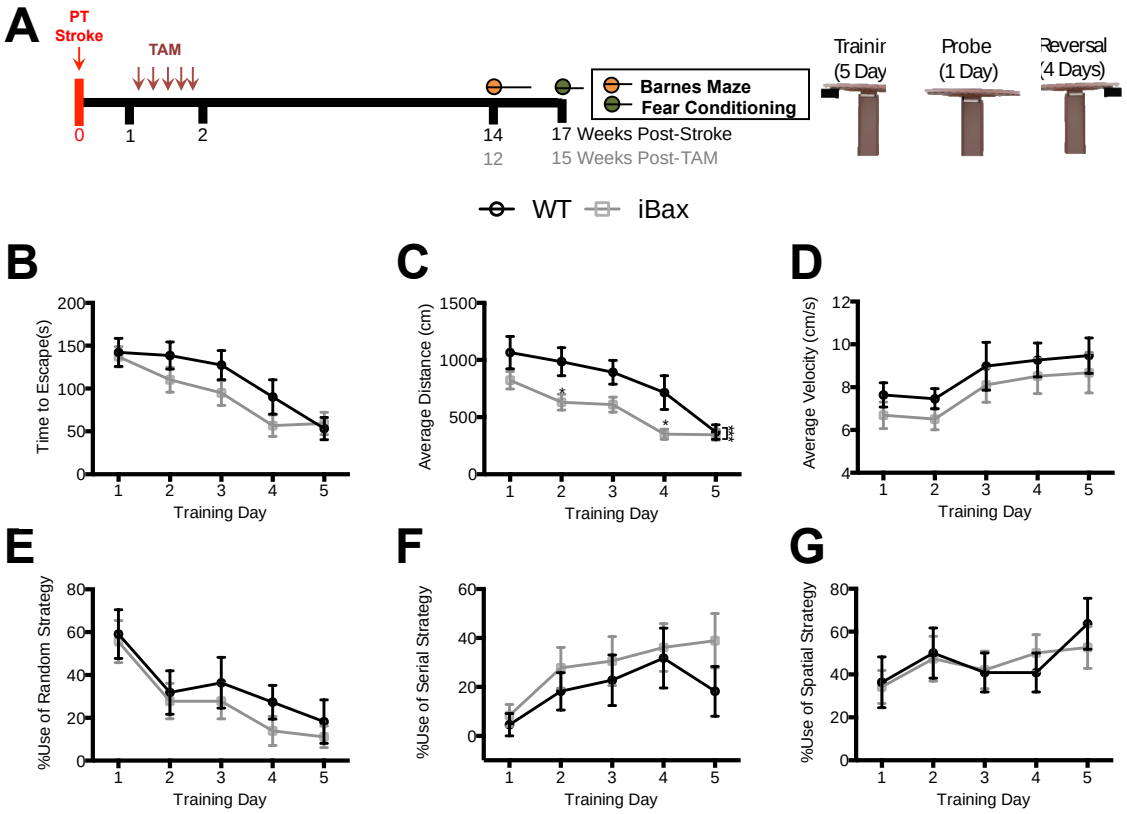


Figure 4.13 iBax Mice Have a Modest Improvement in Spatial Learning on the Barnes Maze Following Stroke. (A) Experimental timeline showing 10 week-old untreated iBax and WT mice received PT surgery followed by TAM treatment at one wps. Barnes maze testing started at 14 wps. Barnes maze protocol included training mice for five days, probe test for one day and then reversal training for four days. (B) During the five days of training, iBax mice had a trend to locate the escape box faster than WT controls. (C) iBax mice travelled significantly shorter distances (D) at the same speed to locate the escape box compared to WT littermates. Over the training days, iBax and WT mice use the (E) random, (F) serial and (G) spatial strategies similarly. (n=11 WT; n=18 iBax). *p<0.05 and ***p<0.001.

remembered the location of the escape box **(Figure 4.14A;** $F_{(3,81)}$=13.2, p<0.0001**)**. WT mice, however, performed significantly better than iBax littermates as revealed by an overall interaction between quadrant and genotype ($F_{(3,81)}$=2.6, p=0.05) as well as posthoc analysis suggesting that WT mice spent more time in the target quadrant (p=0.02). This effect was accompanied by no changes between the iBax and WT mice in the distance travelled **(Figure 4.14B)** or average velocity **(Figure 4.14C)** during the probe test.

Finally, on the reversal phase, both groups relearned the task equally well, taking significantly less time to find the new location of the escape hole (**Figure 4.15A;** $F_{(3,81)}$=9.6, p<0.0001**)**, traveling less distance (**Figure 4.15B;** $F_{(3,75)}$=7.1, p=0.0003**)**, and having a similar average velocity (**Figure 4.15C)**. Additional analysis of the search strategy revealed no significant differences between the groups in the strategy they used to relearn the new location of the escape whole. At the beginning of the reversal phase, most mice used either random **(Figure 4.15D)** or serial strategy **(Figure 4.15E)**. This was different by the end of the reversal training, where mice rarely used the random strategy and transitioned into using either serial or spatial strategies **(Figure 4.15F)**. Together, the Barnes maze data suggests that, after a stroke, the iBax mice perform slightly better compared to WT mice in spatial learning, worse in spatial memory, and have similar abilities to relearn the task which is a measure of executive function. These results are puzzling given that we would have predicted that more cells at the SGZ would either have no effect or in general improve performance on learning and memory.

Since neurogenesis is associated with increased ability for learning and memory, we additionally conducted contextual fear conditioning testing, which measures hippocampal-dependent associative learning and memory (Frankland et al., 2004; Cancino et al., 2013) at 17 wps (seven-month-old mice) in the same cohort that completed the Barnes maze. As shown in

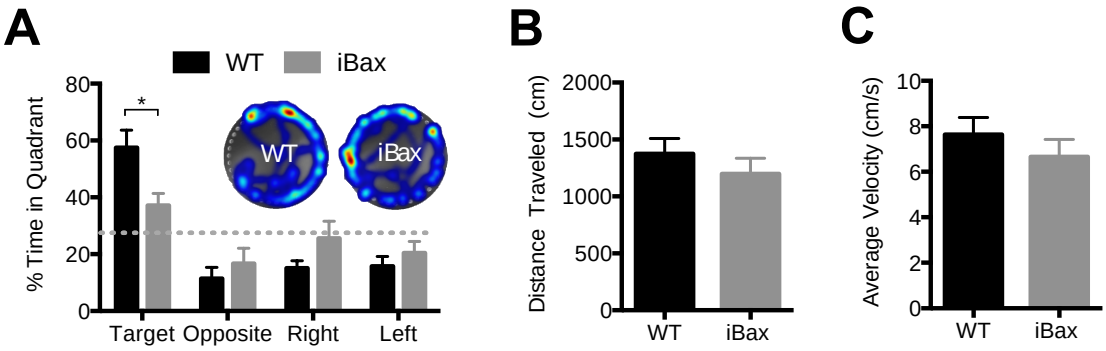


Figure 4.14 WT Mice Have Enhanced Spatial Memory Abilities on the Barnes Maze Following Stroke. (A)Assessment of the probe trial reveals iBax and WT mice spent more time in the target quadrant compared to all other quadrants. Inset showing representative heat maps of the movement of iBax and WT mice during the three minute probe trial. WT mice spent longer in the target quadrant compared to iBax mice as shown by representative heat maps. iBax and WT mice (B) travel similar distances and have (C) similar velocities on the probe trial of the Barnes maze. *$p<0.05$. Mean ± S EM.

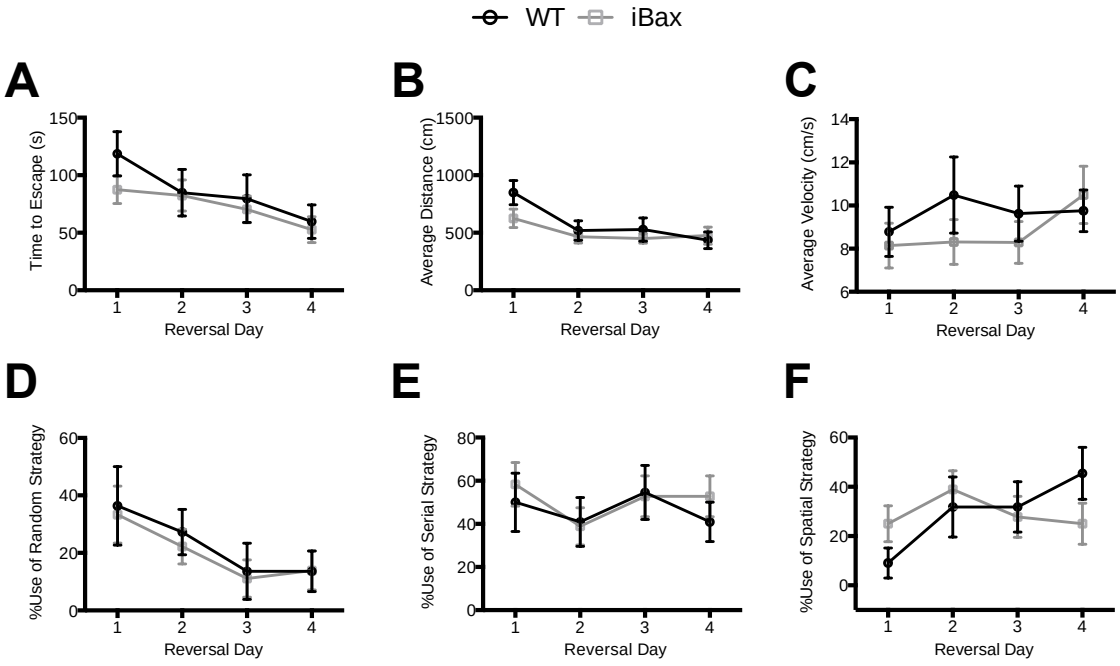


Figure 4.15 iBax And WT Mice Have Similar Performance on the Reversal Phase of the Barnes Maze Following Stroke. (A) iBax and WT mice have no significant differences in their ability to learn a new location of the esacape box during Barnes ma ze reversal testing. There was also no significant differences in the (B) distance travelled and (C) average velocities between iBax and WT mice following stroke. iBax and WT mice use the (D) random, (E) serial and (F) spatial strategies similarly over the four reversal days. Mean ± SEM.

Figure 4.16A, all mice were pre-exposed to the conditioning box on day one which has been suggested to make the task harder and more likely to detect differences in mice with altered neurogenesis (Cancino et al., 2013). The day following pre-exposure, mice were placed in the same box and were given a 0.5 mA shock to induce a fear memory within the context of the box. One day later, on the third day of testing, mice were placed back into the context and percent freezing was measured as an outcome measure of contextual memory. During the pre-exposure to the context on the first day, both the iBax and WT mice performed similarly and froze ~20% of the time (**Figure 4.16B)**. In comparison, one day after exposure to the shock both the iBax and WT mice on average froze ~50% of the time with no differences between the groups (**Figure 4.16C**). This data suggests that iBax and WT mice have similar hippocampal-dependent associative memory abilities.

Finally, to confirm that behavioural performance of iBax and WT mice was not altered due to general locomotor activity, movement was measured for one hour using a photo-beam break test at 16 wps **(Figure 4.17A)**. As expected, when placed in a novel cage, similar to their regular home cage, both the iBax and WT had reduced activity within the cage over the hour **(Figure 4.17B;** $F_{(11,231)}=12.2$, $p<0.0001$**)** suggesting that the iBax and WT mice have similar acclimatization patterns to a novel environment.

4.5: Enhancing PCs Survival Does Not Alter Lesion Volume

Given that we observed no difference in sensorimotor deficits post-stroke and only a mild effect on the Barnes maze, we predicted that there would be no differences in lesion volumes between WT and iBax mice. As shown in **Figure 4.18A,** the minimum, average and maximum lesion locations obtained following PT-induced strokes demonstrated that the strokes were localized between 1.18 mm to 0.26 mm in reference to bregma and induced infarcts within

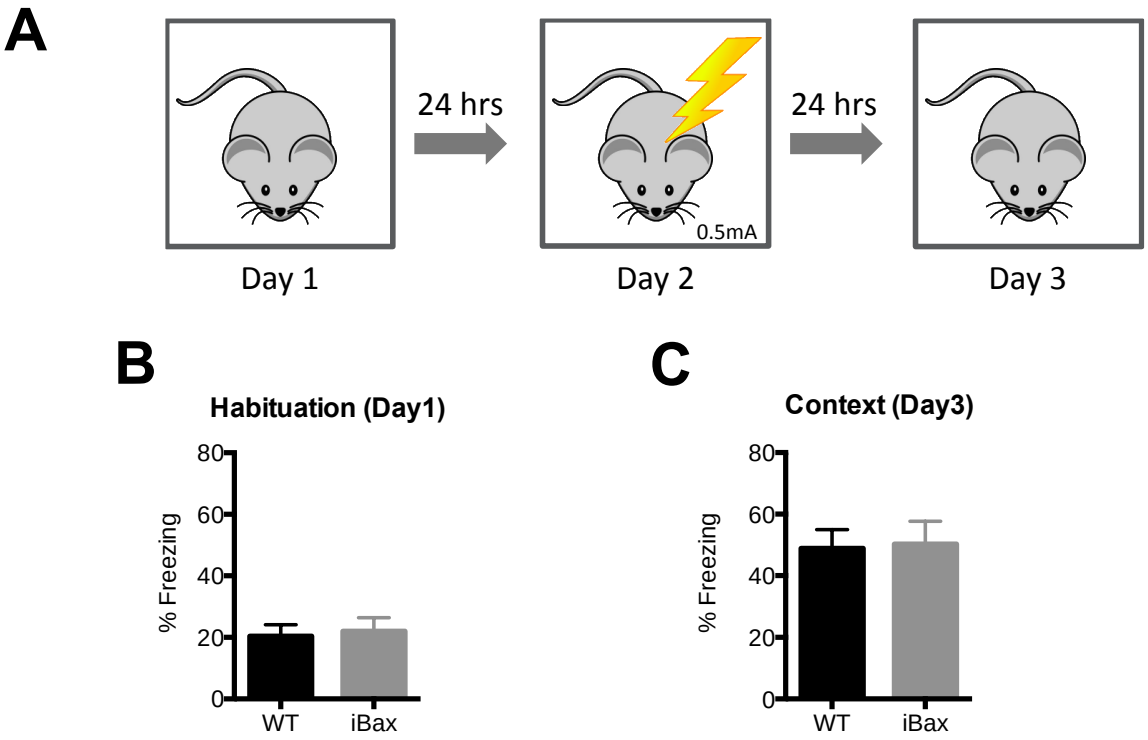


Figure 4.16 iBax and WT Mice Have Similar Abilities on the Fear Conditioning Test Following Stroke. (A) Schematic of the three day fear conditioning protocol showing day 1 allows for habituation and pre-exposure to the context as well as providing baseline freezing values; Day 2 allows for conditioning mice to associate the context with a fear memory; Day 3 determines their association of the context with a fear memory. (B) On day one, WT and iBax mice have similar baseline activity in the box. (C) Assessment of contextual fear conditioning reveals that WT and iBax mice have similar context-dependent freezing. Mean ± SEM.

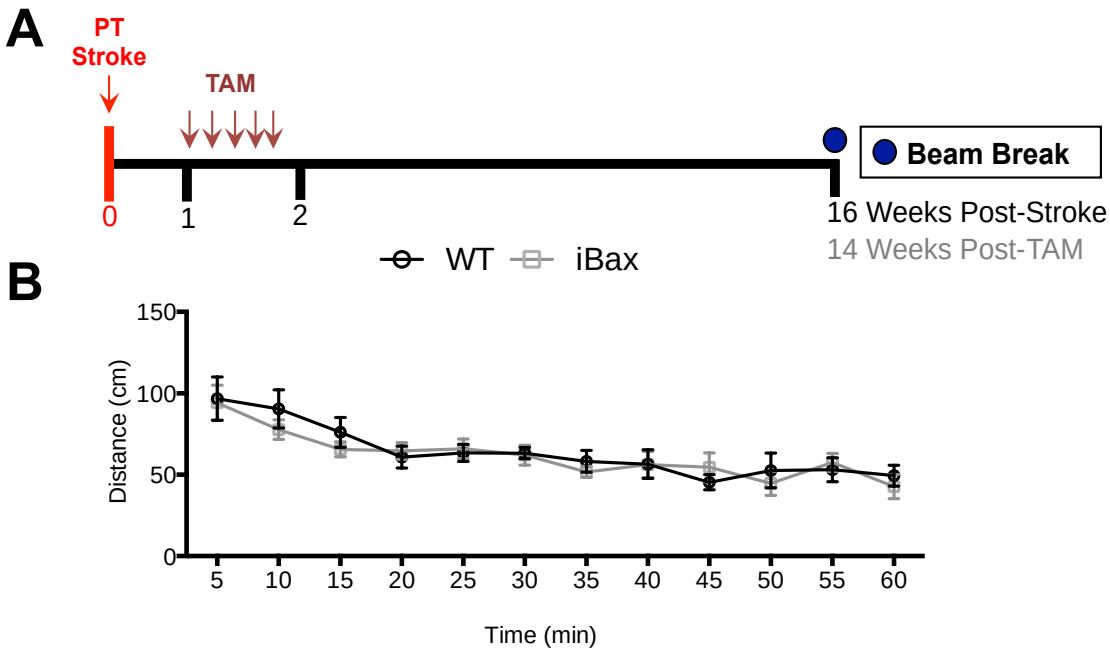


Figure 4.17 The Amount of General Locomotor Activity is the Same for iBax and WT Mice Following Stroke. (A) Experimental timeline showing iBax and WT mice were induced with strokes at 10-weeks of age followed by TAM at one wps. General locomotor activity was measured using beam break test at 16 wps. (B) iBax and WT mice have similar locomotor activity in the first 60 minutes in a novel cage. Mean ± SEM.

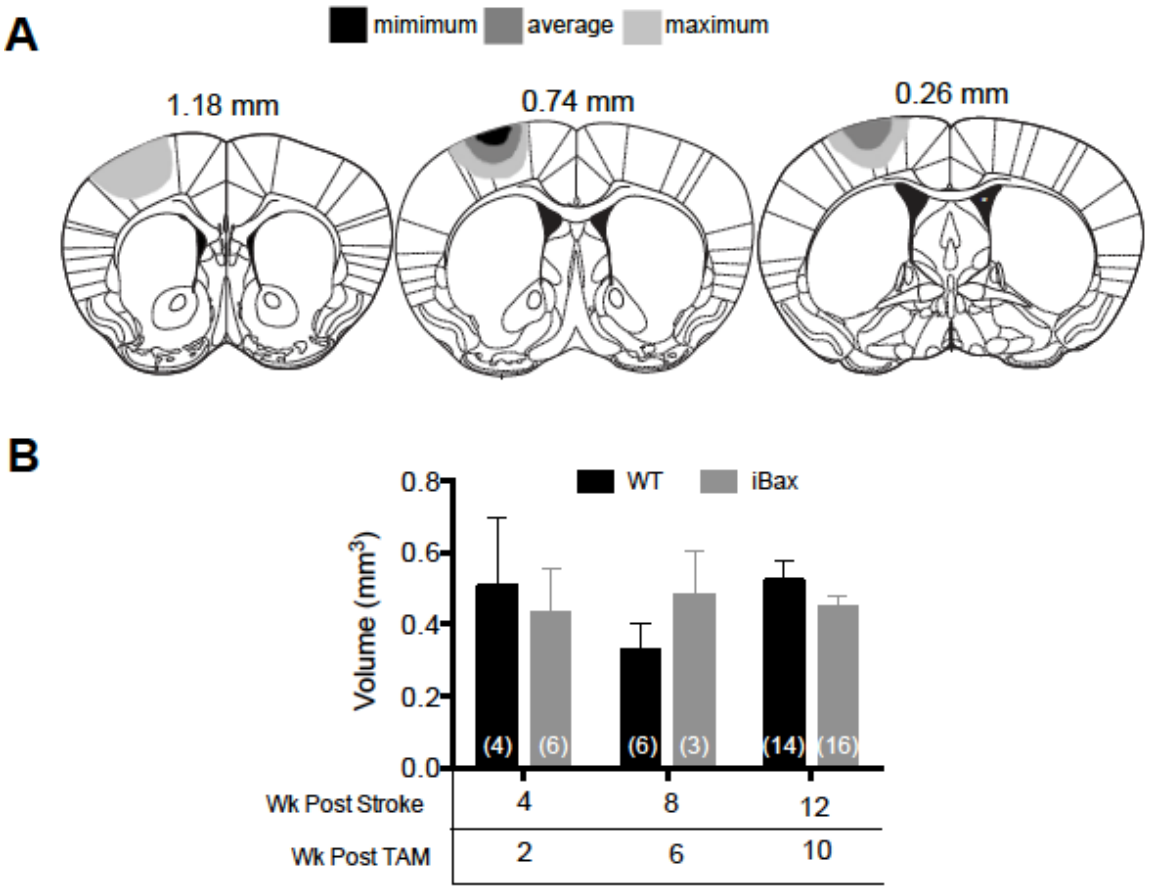


Figure 4.18 iBax and WT Mice Have Similar Lesion Volumes Following Photothrombosis. (A)Schematic diagram of coronal mouse brain sections showing minimum, average and maximum lesion volumes created following PT strokes in iBax and WT mice. (B) iBax and WT mice have similar lesion volumes at four, eight and 12 wps. Mean ± SEM.

the primary motor and primary somatosensory cortical regions. Analysis of stroke volume also demonstrated similar lesion volumes between iBax and WT mice at all time points when behaviour was measured (**Figure 4.18B)**. Notably, the stroke lesions were relatively small (~0.5 mm^3), did not encroach on the corpus callosum, and yet were associated with significant sensorimotor deficits.

Chapter 5: Functional Role of Enhancing Progenitor Cell Survival Before Stroke

5.1: Removal of *Bax* Prior to Stroke Enhances PC Survival at the SVZ and Peri-Infarct Regions

As shown in Chapter 4, enhancing PC survival after stroke was not sufficient to improve sensorimotor/motor outcomes. One possible explanation for this outcome could be due to the enhancement in PCs not occurring early enough during the "critical window" for stroke recovery (Murphy and Corbett, 2009). Therefore, we tested whether removing *Bax* prior to stroke altered sensorimotor and cognitive function. To assess the PC response at the SVZ and peri-infarct regions, iBax and WT mice were treated with TAM two weeks prior to PT strokes and sacrificed at four, eight and 12 wps, corresponding to six, 10, and 12 weeks post-TAM **(Figure 5.1A)**. As expected, at the SVZ there was a significant increase in the number of YFP-positive PCs in the iBax mice compared to WT controls ($F_{(1,14)}$=15.9, p=0.0001) with posthoc results identifying a trend at eight wps (p=0.08) and a significant difference at 12 wps (p=0.02) **(Figure 5.1 B,C).** In agreement with this data, in the peri-infarct region there was also a significant increase in number of cells between the iBax and WT mice ($F_{(1,14)}$=24.7, p=0.0002), an increase over time ($F_{(2,14)}$=20.5, p<0.0001), and an overall interaction effect ($F_{(2,14)}$=5.8, p=0.01 **(Figure 5.1 D,E**). Posthoc results showed iBax mice had a trend for more PCs at eight wps and significantly more PCs at 12 wps compared to WT littermates (p=0.0003).

5.2: Majority of PCs Express the Marker GFAP+ Following Stroke

Previous studies have suggested that PCs labelled prior to stroke are predominately fated to be astrocytes (Li et al., 2010; Faiz et al., 2015), therefore we determined the proportion of YFP cells that expressed GFAP, a marker known to be expressed in astrocytes, at 12 wps. We found that approximately 80% of YFP-positive PCs expressed GFAP in both iBax and WT mice, suggesting that the majority of dividing recombined PCs that migrate to the infarct area may be

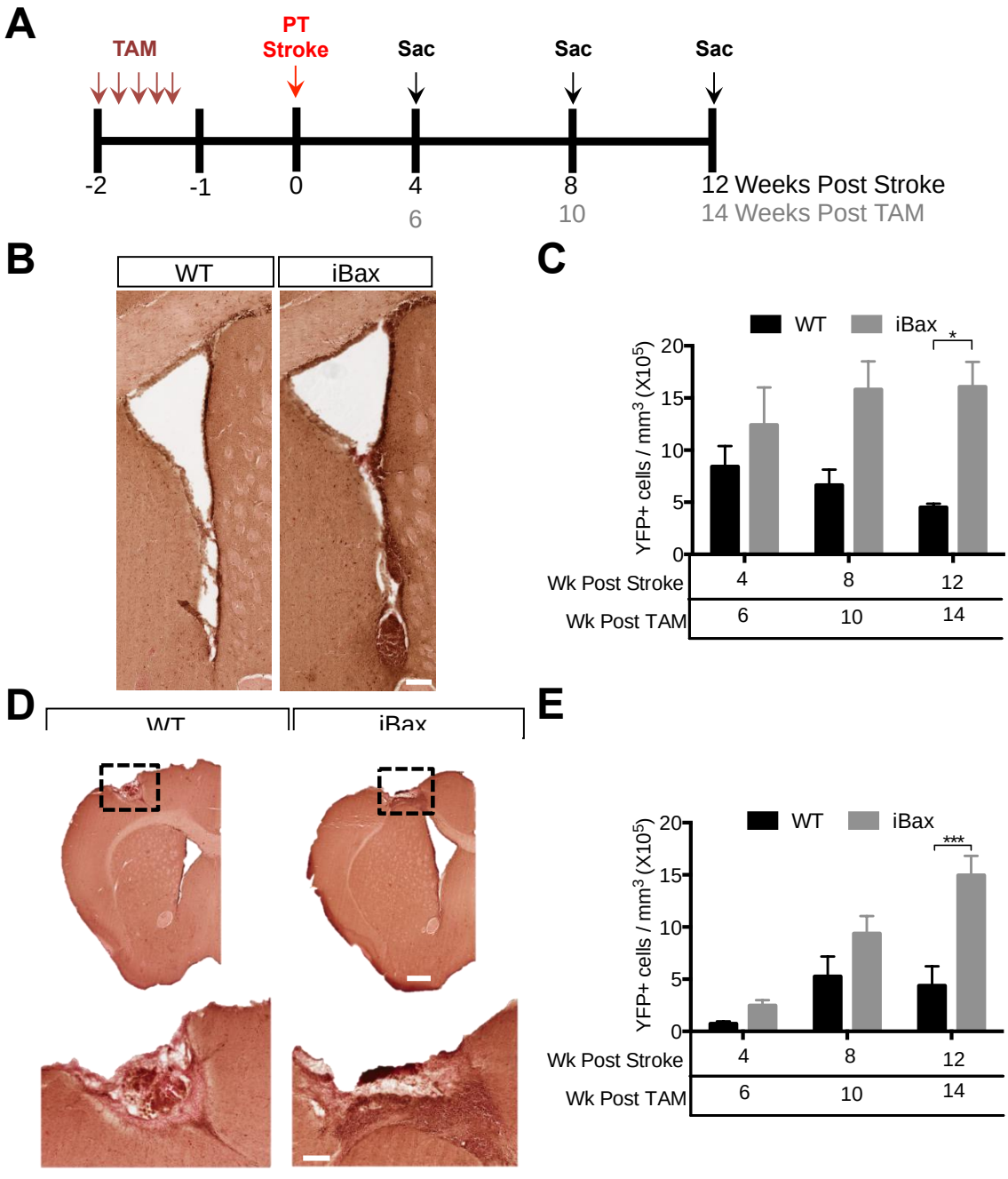


Figure 5.1 iBax Mice Have More Progenitor Cells at the SVZ and Peri-Infarct Regions. (A) Experimental timeline showing iBax and WT mice were administred TAM two weeks prior to being induced with Photothrombosis (PT) strokes and then sacrificed at either four, eight or12 wps. (B) Representative images and (C) quantification of YFP-positive PCs at the SVZ shows a significant increase in the surviving PCs in the iBax mice at 12 wps. Scale Bar= 200μm. (D) Representative images and (E) quantification of YFP-positive PCs at the peri-infarct shows a significant increase in the surviving PCs in the iBax mice at 12 wps. (Minimum n=3 per group). Scale Bar=500μm (low magnification); 200μm (inset). *P<0.05, and ***P<0.001. Mean ± SEM.

fated to become astrocytes (**Figure 5.2A, B)**. Additional work on other astrocytic markers, such as Ald1I1, would confirm these findings.

This is very interesting since this finding is in striking contrast to our previous results that found the majority of the PC were neuroblasts when PCs were recombined to remove *Bax* after the stroke **(Figure 4.4)**. This result suggests that nestin-expressing PCs have a differential fate if they are labelled and recombined either before or after the stroke. To our knowledge, this project is the first to label PCs post-stroke, since all other inducible studies in stroke recovery have labeled PCs prior to stroke. Thus our results suggest that PCs labeled before versus after stroke have different fates.

5.3: Removal of *Bax* Prior to Stroke Does Not Alter Sensorimotor Function

To test the functional role of removing *Bax* prior to stroke, mice were trained at six weeks old on the adhesive, ladder and cylinder tasks, which are the same behaviour tasks as described in Chapter 4. TAM was administered to eight-week-old mice followed by PT strokes at 10 weeks **(Figure 5.3)**. Long-term behavioural testing was performed at one, four, eight and 12 wps, which corresponds to three, six, 10 and 14 weeks post-TAM**.**

On the adhesive task, mice learned to contact **(Figure 5.4A;** $F_{(3, 186)}=4.6$, $p=0.004$**)**, and remove **(Figure 5.4B;** $F_{(3,186)}=7.8$, $p<0.0001$**)** the tape faster over the four training days. Specifically, there was a significant improvement in time to contact the tape between one and three ($p=0.005$), as well as one and four ($p=0.03$) training days. Similarly, mice removed the tape faster between one and three ($p=0.001$) as well as one and four ($p=0.0005$) training days. Following a stroke, on the ipsilateral side, there were significant differences in time taken to contact **(Figure 5.4C;** $F_{(4, 120)}=2.9$, $p=0.02$**)** and remove **(Figure 5.4D;** $F_{(3, 120)}=4.0$, $p=0.004$**)** the tape in the absence of any differences between iBax and WT mice. On the contralateral side, both groups had

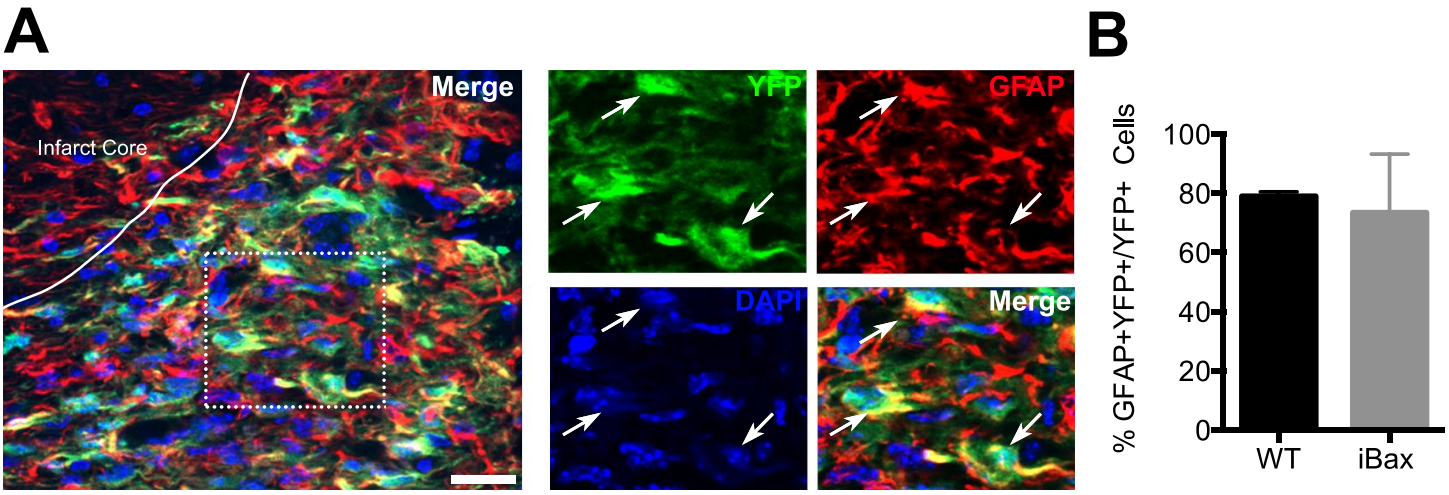


Figure 5.2 Progenitor Cells in the Peri-Infarct Region of iBax and WT Mice Predominantly Express GFAP, a Marker of Astrocytes, at 12 Weeks Post-Stroke. (A) Representative image and (B) quantification showing recombined YFP-positive PCs and astrocyte marker GFAP co-localize in the majority of the PCs. (n=3 per group) Scale Bar= 50μm. Mean ± SEM.

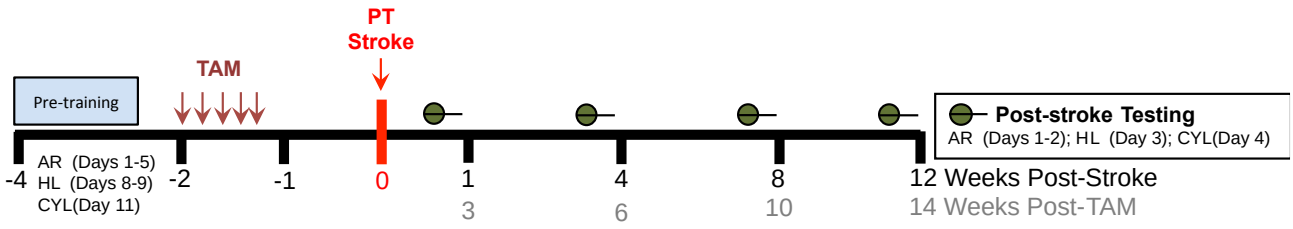


Figure 5.3 Experimental Timeline to Determine if Increasing Progenitor Cells Enhances Sensorimotor Recovery. WT (n=19) and iBax (n=11) mice were trained on the adhesive removal (AR), horizontal ladder (HL) and cylinder (CYL)tests at six weeks old followed TAM treatment at 8-weeks old. PT strokes were induced to TAM treated mice at 10 weeks old. Recovery was measured by behavioural testing at one, four, eight and 12 wps, which corresponds to three, six, 10 and 14 weeks post-TAM.

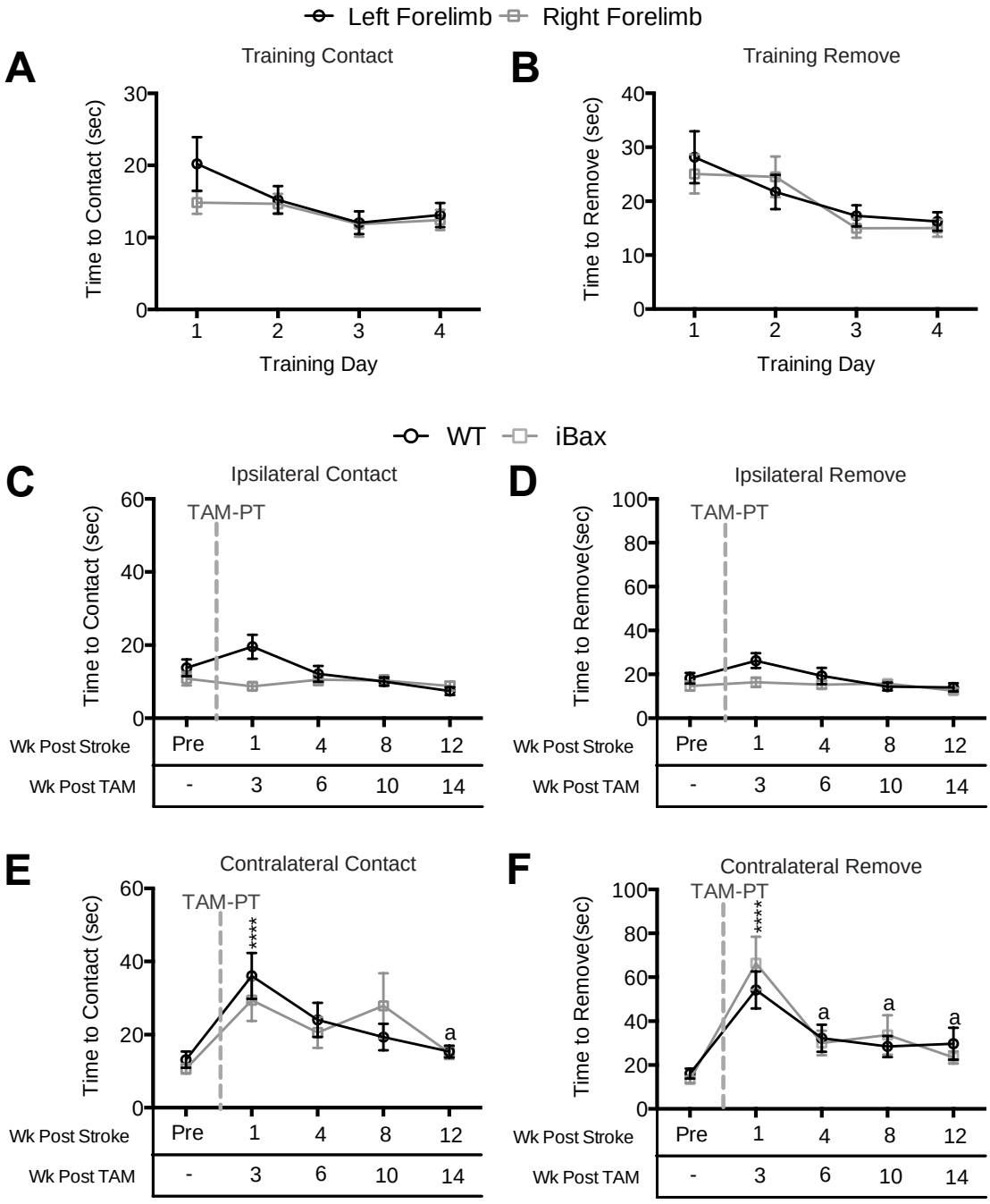


Figure 5.4 iBax and WT Mice Have Similar Contralateral Deficits on the Adhesive Removal Test After Stroke when Bax is Removed Prior to Stroke. (A)Mice learn to contact and (B) remove the adhesive tape faster over the course of the four training days. (C)T ime to contact and (D)remove the tape was comparable to pre-stroke function on the ipsilateral side. (E)Time to contact and (F) remove on the tape on the contralateral side was significantly increased following stroke with no differences between iBax and WT mice. ****p<0.0001 represents significance from pre-stroke performance. **a** = significance from one wps. Mean ± SEM.

significant deficits in the time taken to contact (**Figure 5.4E;** $F_{(4,120)}$=7.9, p<0.0001**)** and remove (**Figure 5.4F;** $F_{(4,120)}$=16.1, p<0.0001**)** the tape in the absence of any differences between the group. Mice took longer to contact (p<0.0001) and remove (p<0.0001) the tape at one wps compare to pre-stroke performance. Furthermore, there was a significant improvement in the ability of mice to contact the tape between one and 12 wps (p=0.0001) and to remove the tape between one wps compared to either four, eight, and 12 (p<0.0001) wps, which is suggestive of SBR.

Similar to the adhesive test, the ladder test did not reveal any differences between iBax and WT mice. On the ipsilateral side, it was surprising to find significant forelimb **(Figure 5.5A;** $F_{(4,80)}$=20.1, p<0.0001**)** and hindlimb **(Figure 5.5B;** $F_{(4,80)}$=5.5, p=0.0006) impairments following stroke. Posthoc results on the forelimb and hindlimb revealed a non-significant trend for deficits at one wps with no differences between iBax and WT mice. Analysis of the contralateral forelimb revealed significant impairments in performance over time **(Figure 5.5C;** $F_{(4,80)}$=20.1, p<0.0001**)**. Lastly, on the contralateral hindlimb, there were robust deficits **(Figure 5.5D;** $F_{(4,80)}$=10.9, p<0.0001**)** with no differences between the groups. These deficits were significant, with approximately 20% more errors made after stroke and were sustained at one (p<0.0001), four (p=0.0003), eight (p<0.0001), and 12 (p<0.0001) wps.

Similar to the adhesive and ladder test, the cylinder test revealed deficits after stroke that were not statistically different between the iBax and WT mice. Prior to stroke iBax and WT mice spent ~50% of their time on the contralateral paw, however, following stroke both groups spent ~20% less time on their impaired paw (**Figure 5.6A;** $F_{(4,120)}$=4.2, p=0.003**)**. Posthoc results revealed deficits between pre-stroke performance and one (p=0.0005), and four (p=0.02) wps. Analysis of the asymmetry index (calculated by the percent time spent on the impaired side subtracted from the unimpaired side) revealed similar but stronger deficits ($F_{(4,120)}$=9.9, p=0.0001)

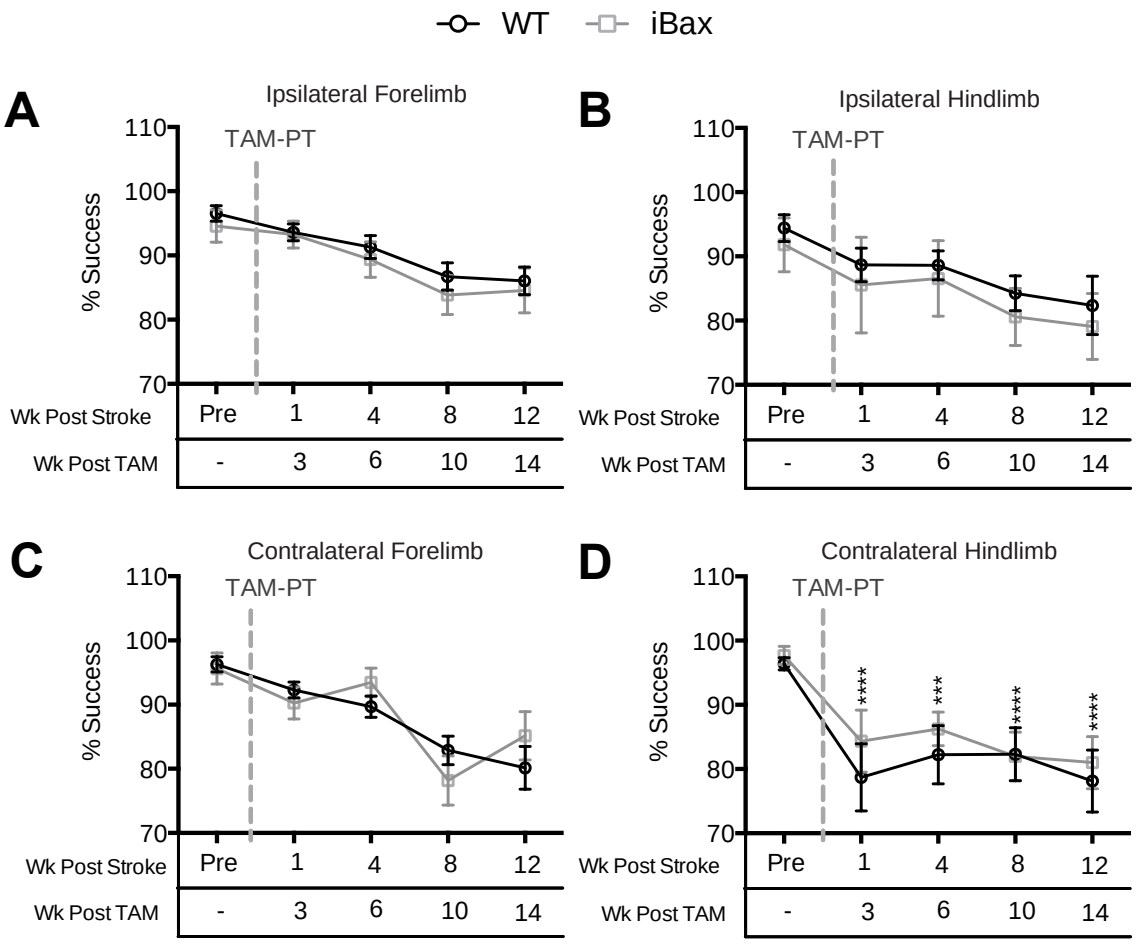


Figure 5.5 iBax and WT Mice Have Similar Contralateral Hindlimb Deficits on the Horizontal Ladder Test After Stroke when Bax is Removed Prior to Stroke. Ipsilateral (A)forelimb and (B) hindlimb performance is similar for both the iBax and WT mice following stroke. (C) There are no deficits following stroke on the contralateral forelimb. (D) There are significant contralateral hindlimb deficits with no differences between iBax and WT mice. ***p<0.001 and ****p<0.0001 represents significance from pre-stroke performance. Mean ± SEM.

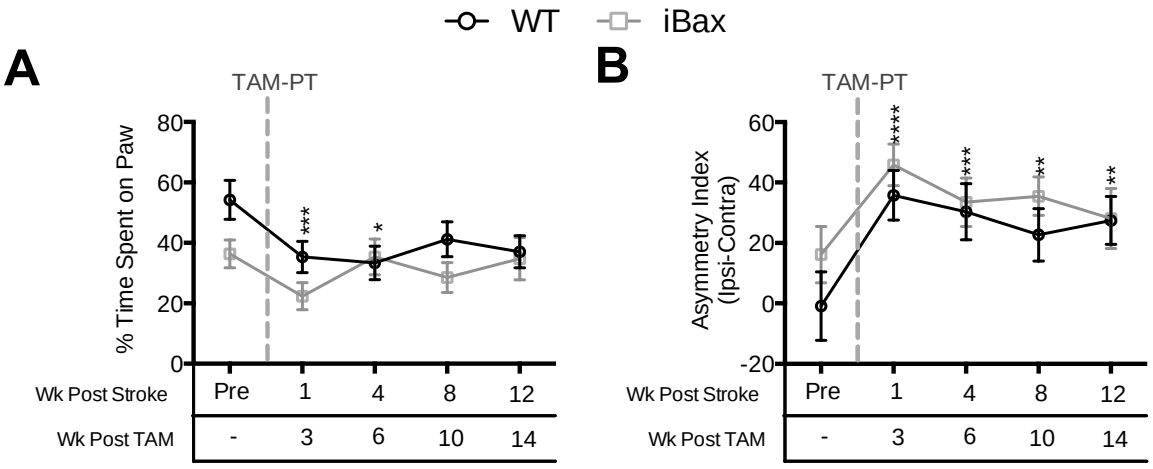


Figure 5.6 iBax and WT Mice Have Similar Reduced Usage of their Impaired Paw on the Cylinder Test Following Stroke when Bax is Removed Prior to Stroke. Cylinder data using either (A) percentage of time on paw or (B) asymmetry index both reveal iBax and WT mice have similar usage prior to stroke and a preference to use their ipsilateral paw more following stroke. *p<0.005, **p<0.01, ***p<0.001, and ****p<0.0001 represents significance from pre-stroke performance. Mean ± SEM.

that were sustained at one ($p<0.0001$), four ($p=0.0002$), eight ($p=0.002$) and 12 ($p=0.002$) wps (**Figure 5.6B)**. Combined with the other behavioural results, these results suggest that the iBax mice perform similarly to WT mice on sensorimotor tasks and suggest that increasing PC survival prior to stroke is also not sufficient to improve recovery.

5.4: iBax Mice Have a Modest Improvement in Spatial Learning and Re-Learning Abilities Following PT Strokes

Given that we had previously shown an increase in the survival of PC after stroke in the iBax mice in the SGZ **(Figure 4.3)** as well as the ability of iBax mice to learn faster on the Barnes maze **(Figure 4.13)**, we wanted to test whether there was a similar effect in the iBax mice when we increased survival of PCs prior to stroke. Quantification of the YFP-positive PCs in the SGZ revealed a striking increase and significant interaction between the number of cells in the iBax mice compared to WT mice between 4-12 wps **(Figure 5.7 A, B;** $F_{(2,34)}=6.0$, $p=0.006$**)**. Posthoc analysis revealed iBax mice had significantly more PCs at four ($p=0.02$), eight ($p=0.001$) and 12 ($p<0.0001$) wps (**Figure 5.7B)**. Phenotyping the YFP-positive PCs that expressed the neuronal marker, NeuN, in SGZ also revealed that iBax mice had nearly all PCs that were NeuN-positive whereas WT littermates had ~75% PCs that expressed NeuN ($p=0.001$) **(Figure 5.7C, D)**. This was expected since the iBax mice allow for the accumulation of mature neurons because of increased survival due to the removal of *Bax* gene.

To determine if the increase in PC survival correlated to enhanced associative and spatial learning and memory function, the Barnes maze and contextual fear conditioning was performed as shown in the timeline in **Figure 5.8A** which was similar to that used in Chapter 4. In these experiments, mice received TAM two weeks prior to stroke and were tested on the Barnes maze at 14 wps and fear conditioning at 17 wps.

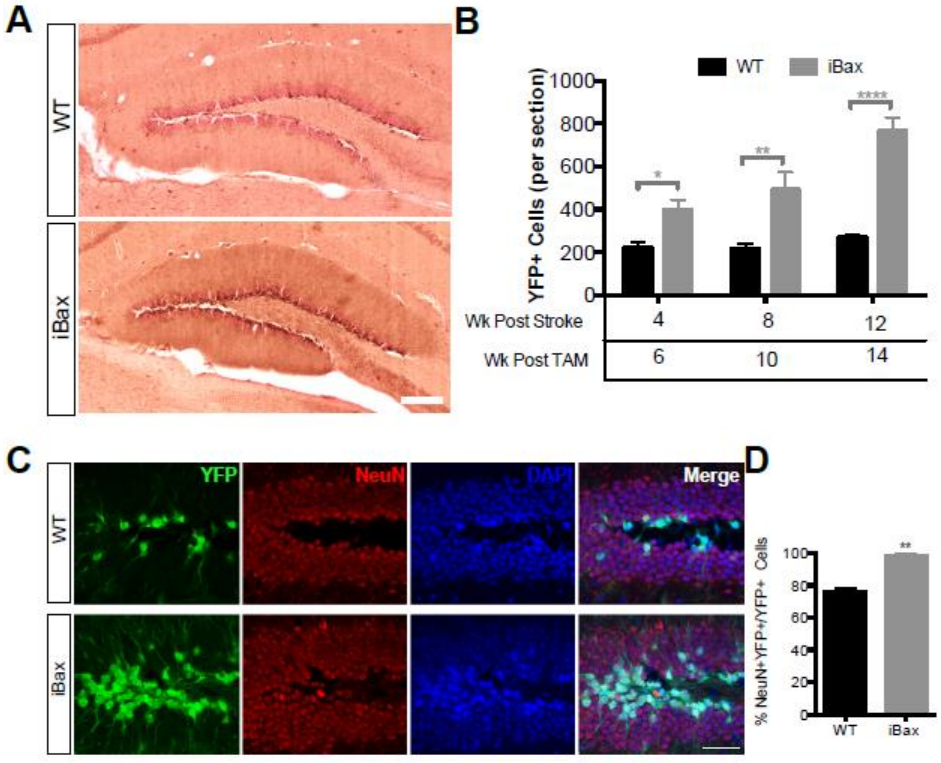


Figure 5.7 iBax Mice Have Significantly More Progenitor Cells at the SGZ that are Fated to be Neurons. After Stroke when Bax is Removed Prior to Stroke. (A)Representative images and (B) quantification showing a significant increase in the number of YFP-expressing recombined PCs at the SGZ in the iBax mice at four, eight and 12 wps. Scale Bar=200μm (C)Representative images and (D) quantificaiton of YFP-postive PCs that co-localized with mature neuronal marker, NeuN. iBax mice had significantly more YFP-positive, NeuN-positive PCs compared to WT mice. Scale Bar=50μm. (n=3-6 per group). *P<0.05, **P<0.01 and ****P<0.0001. Mean ± SEM.

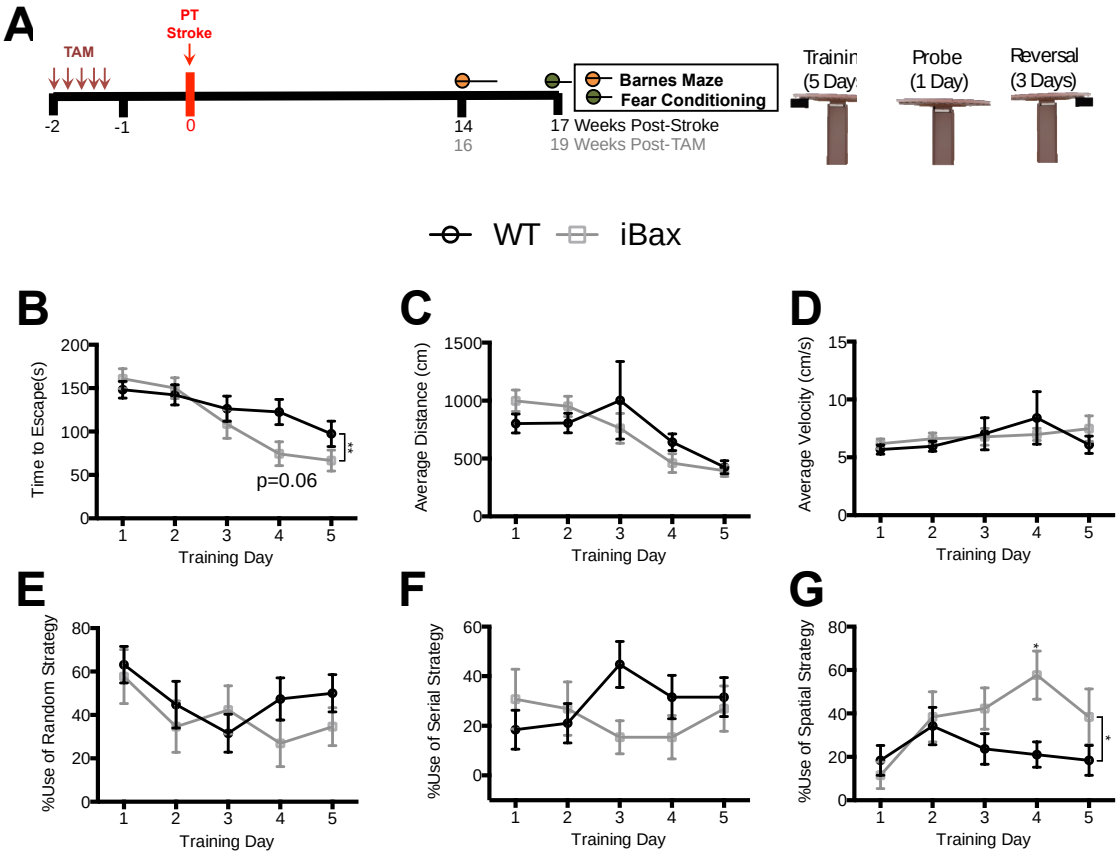


Figure 5.8 iBax Mice Have a Modest Improvement in Spatial Learning on the Barnes Maze Following Stroke when Bax is Removed Prior to Stroke. (A) Experimental timeline showing eight week-old iBax and WT mice were treated with TAM followed by PT surgery at 10 week-old. They were tested on the Barnes maze at 14 weeks post-stroke. Barnes maze protocol included training mice for five days, porbe test for one day and then reversal training for three days. (B) During the five days of training, iBax mice located the escape box faster than WT controls. iBax and WT mice (C) travelled similar distances and (D) used the same velocities during the training phase. iBax and WT mice use the (E) random and (F) serial strategies similarly over the five training days. (G) Over the course of five training days, iBax mice use the spatial strategy more frequently than WT mice. (n=19 WT; n=11 iBax) *P<0.05 and **P<0.01. Mean ± SEM.

Over the five training days, iBax and WT mice learned the location of the escape box faster **(Figure 5.8B;** $F_{(4,120)}$=20.15, p<0.0001). Furthermore, there was an interaction between genotype and time during the training phase ($F_{(4,120)}$=3.501, p=0.009). The posthoc results reveal a trend for iBax mice to locate the escape hole faster on the fourth training day. Analysis of the distance travelled revealed both groups travelled less distance over the training days ($F_{(4,120)}$=5.417, p=0.0005) with no differences between iBax and WT mice **(Figure 5.8C)**. There were also no differences in the average velocity between iBax and WT mice **(Figure 5.8D)**. Analysis of search strategies used to find the goal box revealed no differences in WT and iBax mice when they used the random **(Figure 5.8E)** and serial **(Figure 5.8F)** strategies. Both groups used the spatial strategy more frequently over the training days ($F_{(4,120)}$=2.5, p=0.4) and there was a striking difference with the iBax mice using the spatial strategy significantly more than WT mice **(Figure 5.8G;** $F_{(4,120)}$=6.6, p=0.02**)**. The proportion of iBax mice that used the spatial strategy was significantly increased on day four of training (p=0.02). These outcomes are similar as the improvements in learning that we observed when the iBax mice were given TAM post-stroke **(Figure 4.13)** and suggest that iBax mice have a slightly improved ability to acquire a spatial learning task following a stroke.

On the probe test, the iBax and WT mice spent more time in the target quadrant compared to all other quadrants ($F_{(3,90)}$=9.9, p<0.0001) **(Figure 5.9A)**. Specifically, iBax mice spent 40% of their time in the target quadrant compared to the opposite (p=0.0001), right (p<0.0001) and left (p=0.005) quadrants with no differences between WT and iBax mice. Both groups also travel similar distances **(Figure 5.9B)** and had similar velocities **(Figure 5.9C)** on the probe test after stroke suggesting spatial memory is not altered following stroke.

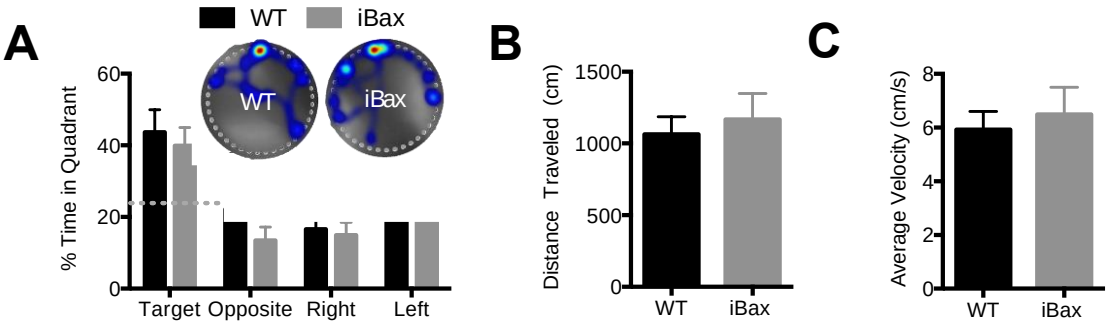


Figure 5.9 iBax and WT Mice Have Similar Spatial Memory Abilities on the Barnes Maze Following Stroke when Bax is Removed Prior to Stroke. (A) Assessment of the probe trial reveals iBax and WT mice spent more time in the target quandrant compared to all other quadrants. Inset showing representive heat maps of the movement of iBax and WT mice during the three minutes probe trial. iBax and WT mice (B)travelled similar distances and (C) used similar velocities on t he probe trial after stroke. Mean ± SEM.

When testing executive function by examining outcomes on reversal testing, the iBax mice learn the location of the new escape hole slightly faster than WT littermates **(Figure 5.10A** $F_{(1,30)}$=5.9, p=0.02**)**, suggesting the iBax may have better executive functioning when *Bax* is removed prior to stroke. This increased ability to find the new location of the goal box was not due to differences in the distances the mice moved (**Figure 5.10B)** or associated with any changes in their velocity **(Figure 5.10C)** over the reversal days. Analysis of search strategies also revealed over time ($F_{(2,60)}$=3.2, p=0.04) both WT and iBax mice utilize the random strategy less frequently **(Figure 5.10D)**. Although not significant, this was accompanied by a slight increase in the use of the serial **(Figure 5.10E)** and spatial **(Figure 5.10F)** strategies, with no differences in the iBax ad WT mice. Together, the Barnes maze data suggests that removal of *Bax* prior to stroke improves the acquisition of a spatial task, as well as re-learning a new spatial location for iBax mice compared to WT littermates. These results are puzzling because they do not align with the results we obtained from the Barnes maze when *Bax* was removed and survival of PCs was increased after stroke **(Figure 4.15)**. While removal of *Bax* either before or after stroke leads to better spatial learning, removal of *Bax* after stroke leads to poorer spatial memory and removal of *Bax* before corresponds to better relearning. The differences are unclear considering similar cellular results were seen in both paradigms.

To test associative learning, the fear conditioning test was utilized. As shown on **Figure 5.11A,** mice were placed in a context to measure habituation on day one, followed by a 0.5mA shock on day two in the same context, and finally placed back into the box on day three to measure freezing associated with the context**.** On day one, both WT and iBax mice freeze ~30% of the time when exposed to the context **(Figure 5.11B).** On day three, both groups freeze close to 60% of the time suggesting they similarly associate the context with a fear memory **(Figure 5.11C).** These

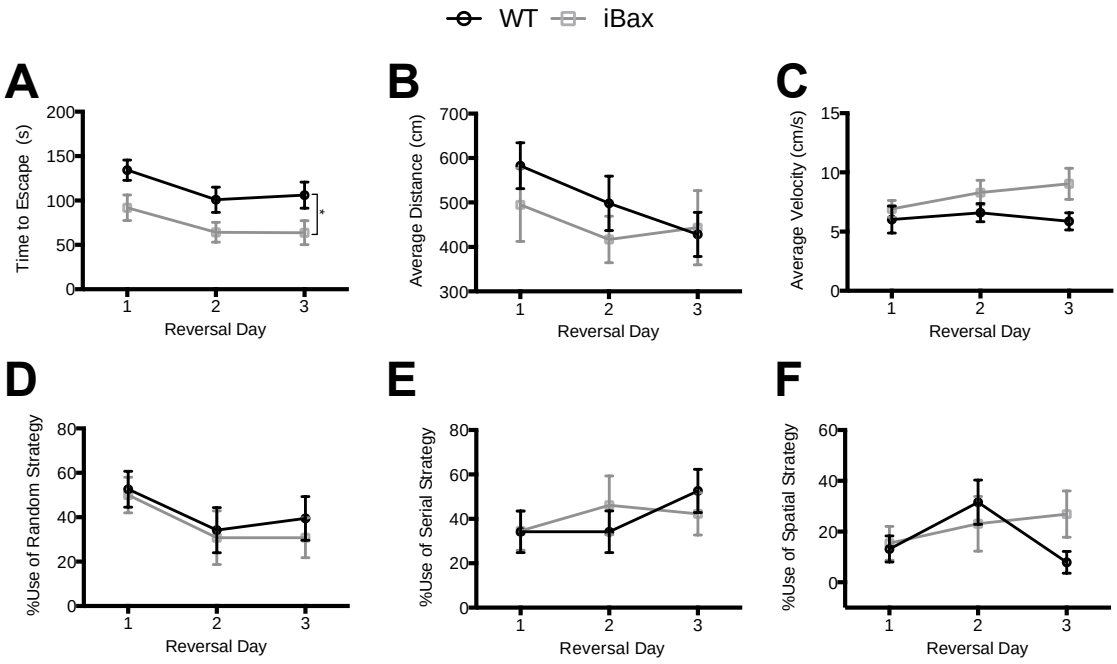


Figure 5.10 iBax Mice Have a Modest Improvement in Re-learning Abilities on the Barnes Maze Following Stroke when Bax is Removed Prior to Stroke. (A) During the three days of reversal testing, iBax mice are able to acquire the new escape box faster than WT mice. Both groups (B) travelled similar distances and (C) used same velocities on the reversal phase. Both groups also use the (D) random, (E) serial and (F) spatial strategies similarly over the three reversal days. Mean ± SEM.

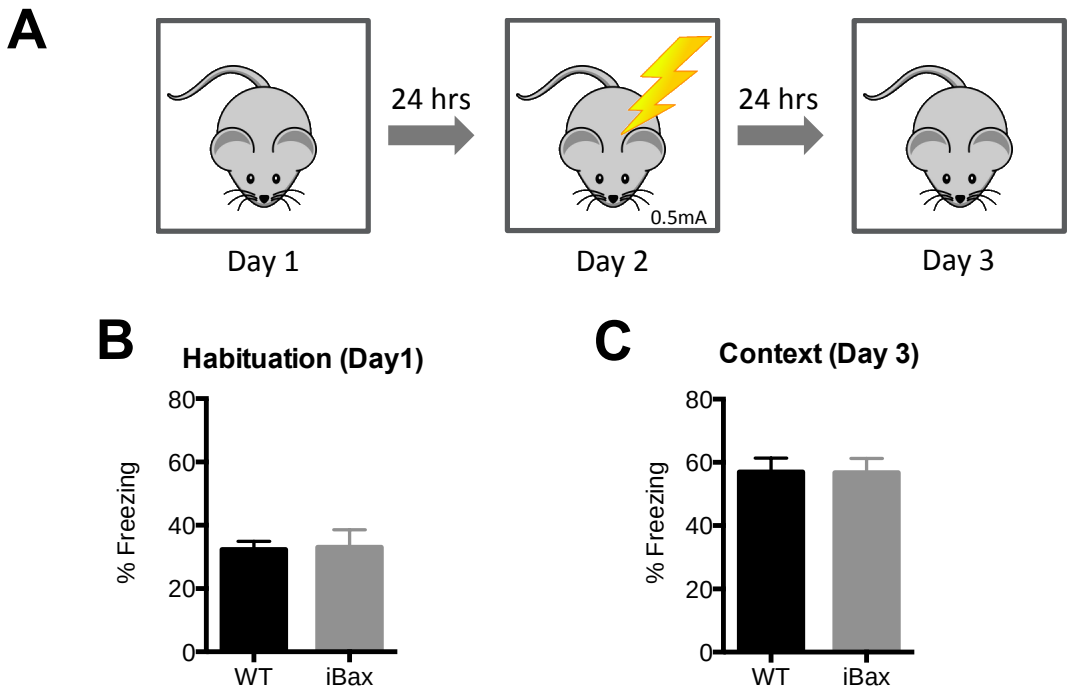


Figure 5.11 iBax and WT Mice Have Similar Abilities on the Fear Conditioning Test Following Stroke when Bax is Removed Prior to Stroke. (A) Schematic of the three day fear conditioning protocol showing day 1 allows for habituation and pre-exposure to the context as well as providing baseline freezing values; Day 2 allows for conditioning mice to associate the context with a fear memory; Day 3 determines their association of the context with a fear memory. (B) On day one, WT and iBax have similar baseline activity in the box. (C) Assessment of contextual fear conditioning reveals that WT and iBax mice have similar context-dependent freez ing. Mean ± SEM.

results are in alignment to those obtained when Bax was removed after stroke and suggest that enhancing the survival of PCs has no effects on the ability of the mice to perform associative learning tests after a cortical stroke.

Finally, to confirm there was no difference in general locomotor activity between iBax and WT mice that could confound behavioural testing, the beam break test was performed for an hour in a novel cage at 16 wps **(Figure 5.12A)**. As expected, both iBax and WT mice had reduced activity in the novel cage over the hour ($F_{(11,220)}$=18.5, $p<0.0001$), suggesting both groups acclimatize to a novel context similarly.

5.5: Enhancing PCs Survival Does Not Alter Lesion Volume

Since we did not identify any differences in sensorimotor recovery in the iBax mice compared to WT littermates, we hypothesized that there would also be no differences in lesion volume between the iBax and WT mice. As shown in **Figure 5.13A**, the minimum, average and maximum lesion volumes were located between 1.18mm to 0.14mm in reference to bregma. Quantifying lesion volumes at four, eight and 12 wps also revealed strokes that were relatively small (~0.5 mm^3) and did not encroach on the corpus callosum, yet produced sensorimotor deficits.

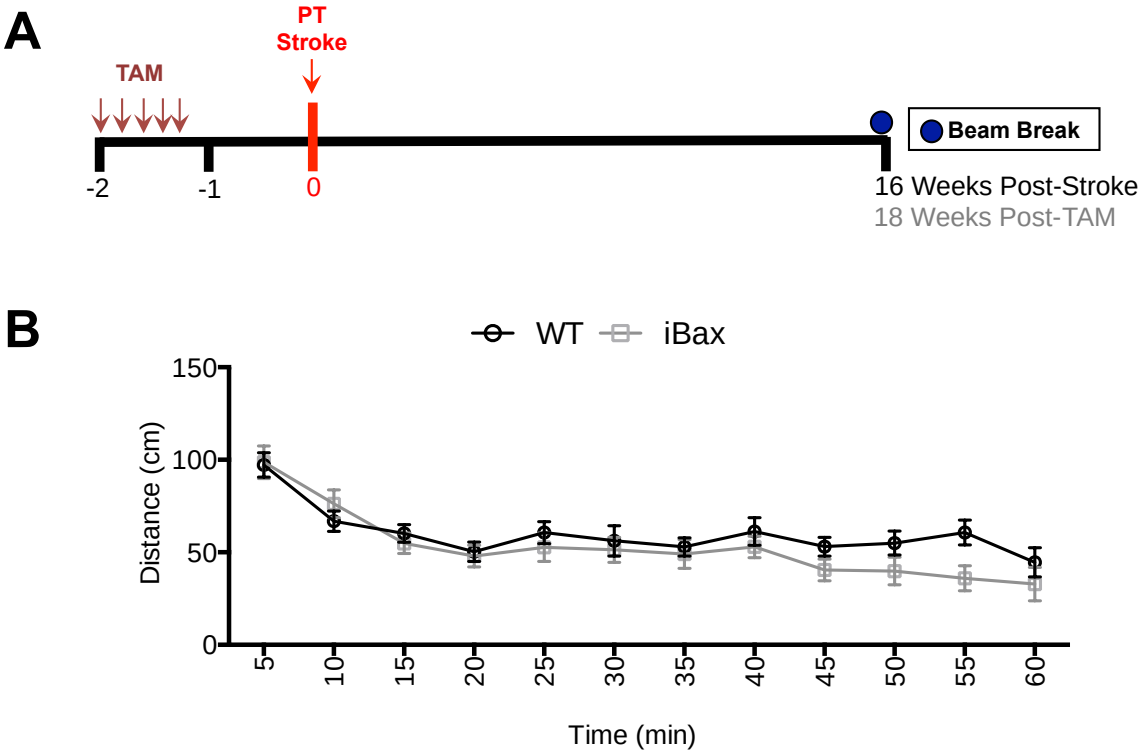


Figure 5.12 The Amount of General Locomotor Activity is the Same for iBax and WT Mice Following Stroke when Bax is Removed Prior to Stroke. (A) Experimental timeline showing iBax and WT mice were administered with TAM at eight weeks of age followed by PT strokes at 10-weeks of age. General locomotor activity was measured using beam break test at 16 weeks post-stroke. (B) iBax and WT mice have similar locomotor activity in the first 60 minutes in a novel cage. Mean ± SEM.

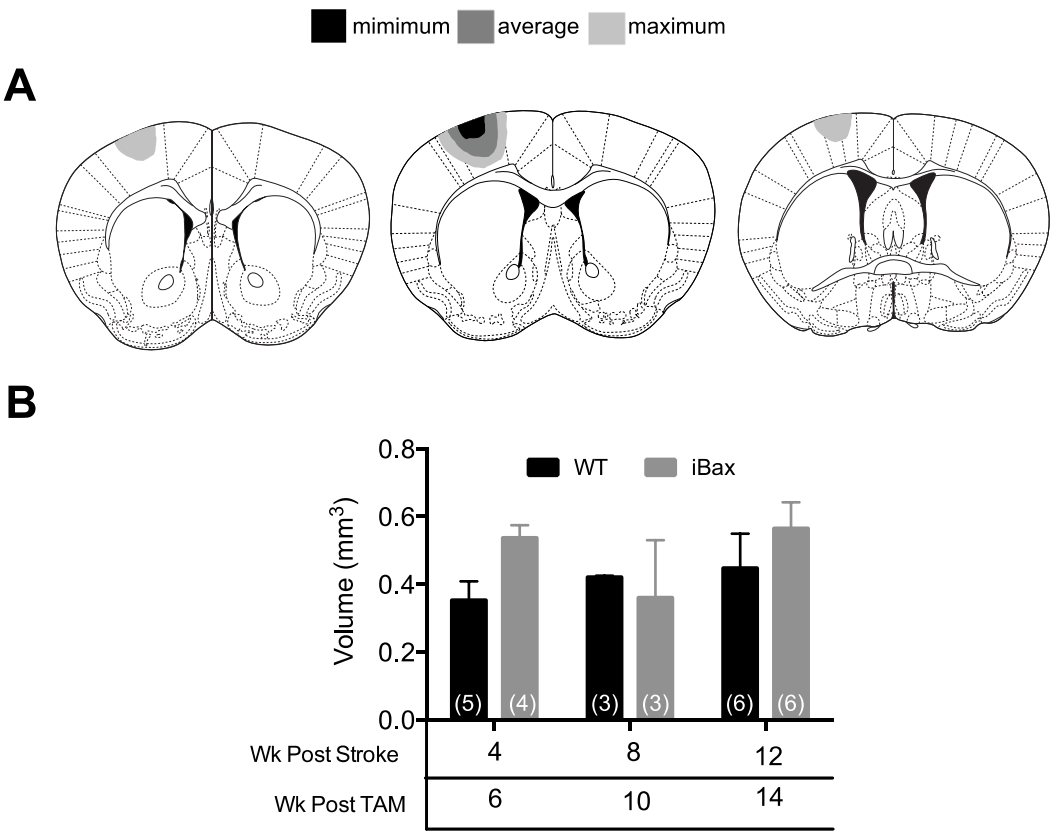


Figure 5.13 iBax and WT Mice Have Similar Lesion Volumes Following Photothrombosis when Bax is Removed Prior to Stroke. (A)Schematic diagram of coronal mouse brain sections showing minimum, average and maximum lesion volumes created following PT strokes in iBax and WT mice. (B) iBax and WT mice have similar lesion volumes at four, eight and 12 wps. Mean ± SEM.

Chapter 6: Discussion

The hypothesis that adult neurogenesis and/or the increase in the number of PCs promotes stroke recovery is mainly based on studies that have ablated PCs and reported reduced recovery following stroke (Raber et al., 2004; Jin et al., 2010; Wang et al., 2012; Sun et al., 2013). In order to determine if promoting PC survival is a viable option for novel therapeutic strategies in stroke recovery, the studies presented in this thesis directly increased the survival of PCs generated either before or after a stroke. To accomplish this goal, PC survival was specifically enhanced using the iBax triple transgenic mouse model (Sahay et al., 2011b) and PT strokes were created so that they did not encroach on neurogenic niches while still producing sustained behavioural deficits. Our model demonstrated that enhancing PC survival either following or prior to, resulted in more PCs in the cortical peri-infarct area that developed into over a 5-fold increase in newly generated neuroblasts a 3-fold increase in newly generated GFAP-expressing PCs and, respectively. Despite this robust response, there were no changes in lesion volume and long-term sensorimotor recovery. Within the SGZ, enhancing PC survival before and after stroke resulted in an increase in PCs that were fated to be mature neurons. This increase in PCs at the SGZ translated into modest change in spatial memories. Overall, these findings suggest that promoting the survival of PCs may allow for a small increase spatial learning, in the absence of altering sensorimotor function.

6.1 Recovery Following Cortical Photothrombotic Strokes in Mice

Prior to examining the role of PCs in stroke recovery, we sought to develop a stroke model in mice with two criteria: 1) the stroke model should produce infarcts clinically relevant in size, and 2) the stroke model should generate long-term sensorimotor deficits. The first criterion was necessary due to the failure of preclinical studies to translate their findings to clinical trials; this failure has been at least partially attributed to the difference in infarct size generated in animal stroke models, versus those observed in stroke patients (O'Collins et al., 2006; Carmichael, 2016).

For example, infarcts in human strokes range from 28-80 mm^3, which is only 4-14% of the ipsilateral hemisphere (Brott et al., 1989; Lyden et al., 1994; Nopoulos et al., 2000; Sowell et al., 2003). This is unlike many of the larger focal models of stroke, such as the intraluminal suture MCAo model, which creates infarcts that are often the size of almost an entire hemisphere, making it unlikely for a human to survive or recover from such a massive injury. Additionally, stroke models that produce large infarcts are also associated with damages to regions such as the hypothalamus and the neurogenic regions, that are not normally affected in human strokes (Carmichael, 2005). Thus, this work utilized the PT method to produce small cortical strokes using similar methodologies as those utilized by others (Overman et al., 2012; Clarkson et al., 2013). Importantly, the strokes did not encroach upon the SGZ or SVZ neurogenic niches, which was essential for our subsequent work identifying the role of PCs in stroke recovery.

The strokes in the PT model showed significant decline in volume between one and two weeks following injury, resulting in the final infarct size of ~0.5 mm^3. The reduction in infarct size is similar to the decrease found in patients and other preclinical models. For example, Schwamm *et al.* (1998) report that the maximum lesion volume occurred at 70 hours post-stroke and that the final lesion volume, which was measured at a minimum of 42 days after the initial magnetic resonance imaging (MRI) scan, was smaller in over 70% of patients. Other preclinical animal models of stroke such as the MCAo and ET-1, have also identified the maximum lesion volume occurs 24-48 hours after stroke onset (Liu et al., 2009a; Liu and McCullough, 2011; Li et al., 2014; Nguemeni et al., 2015). Moreover, in support of our PT model, Li *et al.* (2014) reported that the PT strokes produces a maximum lesion volume within a couple of days after stroke induction, which then gradually declined up to two wps. While the PT model, a relatively non-invasive procedure, has the ability to induce reproducible strokes that are small, a disadvantage of this

model is the small penumbra (Fluri et al., 2015). Indeed, as seen through the migration of PCs in our small PT model, we create a relatively small penumbra compared to the larger ones seen in other models of focal ischemia.

The second criterion, that our stroke model should produce long-term deficits, was necessary to correctly mirror the long-term impairments of stroke patients. To this end, we wanted to identify behavioural tasks that were (1) sensitive to detect deficits following strokes and, if sensitive, (2) examine the extent of innate recovery that occurs on these tasks. Therefore, we performed a battery of sensorimotor behavioural tests. The staircase, grip strength, and rotarod tests were not sensitive to detect impairments following PT strokes, while the adhesive removal, horizontal ladder and cylinder tests, produced long-lasting deficits in mice following stroke. On the adhesive test, Li *et al.* (2014) identified impairments in PT mice that were sustained only to four days post-stroke even with larger lesions than those employed in our study. In alignment with our finding, Sweetnam *et al.* (2012) detected long-term deficits on the adhesive test that were present at six wps in PT mice with infarct volumes of ~2mm^3. On the ladder rung test, we were able to detect deficits that were present to four wps similar to previously reported impairments observed on the grid-walking test (Clarkson et al., 2010; Clarkson et al., 2011; Overman et al., 2012; Clarkson et al., 2013). Finally, the cylinder test revealed sustained impairments to four wps and is a measure commonly used to demonstrate deficits up to two months post-stroke (Overman et al., 2012; Clarkson et al., 2013).

In addition to testing whether the sensorimotor tests could detect deficits post-stroke, we additionally examined the extent of recovery on these outcome measures given that adult neurogenesis is hypothesized to contribute to SBR (Marlier et al., 2015). Although not often explicitly studied in previous mouse models, we assessed SBR by comparing whether there was a

significant improvement in recovery over time following the initial impairment post-stroke. Using these criteria, the behavioural tests following PT strokes had varied levels of SBR. For example, the time to contact the tape on the adhesive removal test, as well as forelimb function on the ladder test had significant improvements between one and two wps. Furthermore, SBR was also significant, but to a lesser extent, in time to remove the tape and hindlimb performance on the ladder test. These findings support the work of others that have also shown that the PT model robustly exhibits SBR on the adhesive (Diederich et al., 2012; Sweetnam et al., 2012; Li et al., 2014) and ladder (Antonow-Schlorke et al., 2013) tests. In comparison, the cylinder test did not detect any SBR, which was present in studies by Li et al. (2014) and Clarkson et al. (2013). The lack of SBR in the cylinder test could be attributed to differences in the location of stroke, such that, our strokes target a region that specifically impairs spontaneous forelimb as measured on the cylinder task. All together, we selected three tests that were able to detect deficits following small PT strokes of which two, were associated with SBR.

In addition to assessing sensorimotor function, we tested spatial learning and memory on the Barnes maze, which revealed no deficits following PT strokes. Our findings were in alignment with our hypothesis that there would be no significant difference in cognitive function between sham and PT mice, as the strokes target the sensorimotor cortex. This also supports our previous work inducing a cortical ET-1 stroke in a rat model that also reported no cognitive deficits (Lee et al., 2017, Appendix A). Our findings contrast those that report cognitive impairment following stroke, such as the intraluminal suture MCAo strokes that extensively damage many regions including the hippocampus (Liu et al., 2007; Zhao et al., 2009). Furthermore, the apparent deficits following the MWM test seen in these studies are difficult to dissociate from the sensorimotor

dysfunction. Thus, our stroke model specifically targets the sensorimotor cortical region, and produces deficits on sensorimotor tasks, but not spatial learning and memory tasks.

6.2 What is the Population Labelled that Migrate to the Peri-Infarct Regions after Stroke?

One of the most exciting and unexpected findings of this thesis was how PCs localized to the peri-infarct regions had a different phenotype depending on when they were labeled. When TAM was administered following a stroke (detailed in Chapter 4), PCs predominantly expressed the neuroblast marker DCX, suggestive of a neuronal fate. When TAM was administered before a stroke (detailed in Chapter 5), the majority of the PCs expressed a marker commonly present in astrocytes, GFAP. Interestingly, this difference occurred irrespective of the genotype suggesting this different in phenotype was not due to enhanced survival *per se*. This finding is unexpected and could be called a serendipitous result of using the same model to labeled PCs before versus after stroke and examined the phenotype of the cells surrounding a cortical infarct.

Early studies examining PCs and stroke identified that the dividing PCs could migrate to the site of striatal injury post-stroke through the use of birth-dating markers such as BrdU. These studies showed that PCs labeled following a stroke migrated to the infarct and expressed markers of mature neurons, such as PSA-NCAM, Map2 and NeuN in the peri-infarct region (Arvidsson et al., 2002; Parent et al., 2002b). This idea was further supported by more recent studies that either labeled PCs using BrdU following stroke, or examined the endogenous migrating cells from the SVZ to a cortical infarct, and identified migrating DCX expressing neuroblasts, in alignment with our findings (Keiner et al., 2009; Osman et al., 2011). For example, Osman et al. (2011) identified a continuous stream of migrating DCX-expressing cells up to two years after a PT stroke. Together, these studies lead to a hypothesis that neuronal replacement in the cortex may occur following stroke. One limitation is that, while studies have shown the presence of histological markers of

neuronal fate, only a small proportion of PCs become neurons (Li et al., 2010; Osman et al., 2011). While our model doesn't alter the proportion of PCs that adopt a neuronal fate, it does improve PC survival, thus increasing the population of DCX-expressing PCs in the peri-infarct region. Despite this manipulation, sensorimotor function is not improved following stroke. One explanation for the lack of improvement may be that the DCX-expressing PCs are unable to successfully integrate as mature cortical neurons and communicate with the surrounding cells to promote recovery.

Recent work has challenged the hypothesis that PCs become neurons and have suggested that the PCs, in a majority of cases, become astrocytes. This has been shown using the Nestin-CreER^{T2} inducible mouse models, which suggest that the majority of PCs become astrocytes as determined by their expression of GFAP (Li et al., 2010; Benner et al., 2013; Faiz et al., 2015). Thus these findings are in alignment with our methodology that showed PCs express GFAP when we label the PCs prior to stroke. Similar to our work, others that have reported that the PCs become astrocytes in studies in which they also labelled the PCs prior to stroke and then tracked the migration and fate of the PCs. For example, Benner and colleagues (2013) tracked PCs from the SVZ that were labeled prior to PT stroke and found the production of SVZ-derived astrocytes that was important in the formation of the glial scar. Inhibition of this response lead to a defective glial scar and microvascular hemorrhaging. In 2015, Faiz and colleagues demonstrated that 10 days after a cortical ET-1-induced stroke, nearly all the PCs from the SVZ become reactive astrocytes at the infarct. These studies highlight that SVZ derived PCs can produce astrocytes following stroke. In our study, we observed a significant increase in the number of GFAP-expressing cells, derived from the SVZ in the iBax mouse yet there was no alterations in sensorimotor recovery, suggesting that increasing the astrocytic response alone is insufficient to promote functional recovery.

Our findings suggest that PCs labeled before a stroke produce astrocytes and after a stroke produce neurons, which raises many questions for future studies. For example, it is important to determine whether these results are specific to the PT mouse model and PCs that migrate to the cortex, or if they apply to other stroke models. Faiz et al. (2015) and Benner et al. (2013) use the ET-1 and PT model, respectively, to label PCs prior to stroke and identify that the PCs became astrocytes. Also using the PT model, Keiner et al. (2009) labeled PCs using BrdU for 14 consecutive days following stroke, and show that ~1200 BrdU+ cells/mm^3 expressed DCX and since they identified a total of ~2000 BrdU+ cells/mm^3, this suggests that ~60% of BrdU+ cells were neuroblasts around the stroke, a finding that is similar to ours. Whether similar results would translate in a subcortical stroke model remains unknown since all these results come from studies that have used a cortical stroke model. Another issue is whether these findings are specific to the Nestin-$CreER^{T2}$ model. All studies that identify that PCs are fated to become astrocytes use a nestin-inducible transgenic mouse model to label and track PCs prior to stroke leaving it unknown if the same effect would hold in other models that label the stem and PCs in the SVZ. In order to address these issues, future work is required to test whether our results generalize to subcortical stroke models and occur in other non-nestin inducible transgenic mouse models that label the PCs and their progeny.

Our findings also raise the important question about whether the change in phenotype of the PCs is due to the timing of when the PCs were labeled in relation to when the stroke occured. Our differences in phenotype of migrating PCs may have occurred due to the labeling of different stem and progenitor cell populations along the SVZ following stroke. In alignment with this hypothesis, Carlen et al. (2009) discovered that the Type E cells (ependymal cell layer) are quiescent under naïve conditions but are activated following stroke. One experiment to address this hypothesis

could therefore evaluate the short-term (within a few days) expression of PCs labeled along the SVZ before versus after stroke. This would allow us to determine if the labeled PCs co-localize with similar or different sub-populations of stem and progenitor cells.

6.3 Effects of PCs and Neurogenesis on Sensorimotor Recovery Following a Focal Stroke

Our studies are the first to determine that enhancing the survival of PCs is insufficient to improve sensorimotor recovery following a cortical stroke. Our first study examined the effect of enhancing PC survival after stroke. This was initially performed since interventions aimed at promoting survival of cells would only occur following the onset of stroke. To this end, we administered TAM to remove pro-apoptotic gene *Bax* at one week following stroke to align with the peak proliferative response that occurs following stroke (Jin et al., 2001; Arvidsson et al., 2002; Zhang et al., 2004b). Our second study tested the effect of enhancing the survival of PCs prior to stroke which had a timeline in alignment with our previous loss-of-function model (Lee et al., 2017, Appendix A), as well as the work of others that ablated the PCs prior to stroke (Jin et al., 2010; Wang et al., 2012; Sun et al., 2013). All previous work has ablated PCs prior to stroke since these models require multiple weeks to effectively, using pharmacological approaches, ablate the majority of the PCs. For example, our study using the GFAP-TK rat requires the administration of valglanciclovir for four weeks, leading to ablation of nearly all GFAP-expressing PCs and their progeny (Lee et al., 2017, Appendix A). We predicted that enhancing survival prior to stroke would have a better chance at improving recovery compared to enhancing survival after stroke for several reasons: (1) some, but not all studies show impairments when ablation occurred prior to stroke (Jin et al., 2010; Wang et al., 2012), and (2) it is possible that enhancing survival after stroke would result in us missing the critical time window when PCs could be most effective. Regardless,

we found no improvement in sensorimotor function, leading to the question of why enhancing the number of surviving PCs did not produce sensorimotor improvements.

It is possible that the iBax mice did not have improvements in recovery due to our model not altering the number of surviving PCs during the hyper-acute (i.e., <24 hours) and acute phase (i.e., 1-7 days) post-stroke. For example, when PCs were recombined in the iBax mice for three weeks prior to stroke, the iBax and WT mice had similar significant deficits in function compared to pre-surgery performance, as well as similar stroke volumes in the WT mice. It has been previously suggested that improvements in sensorimotor function were promoted by the neuroprotective properties of PCs. Specifically, Jin and colleagues reported reduced motor function and significantly larger stroke lesions at 24 hours following MCAo strokes in the DCX-TK ablation mouse model. This work was also supported by an *in vitro* study that overexpressed DCX and found significant reduction in the number of dying cells when the cells were exposed to oxygen and glucose deprivation (Santra et al., 2006). In contrast, findings from Sun et al. (2013) using the nestin-TK model and our GFAP-TK rat model (Lee et al., 2017, Appendix A), both find no differences in lesion volume or acute function in the absence of PCs. One could suggest that the neuroprotective role is specific to the role of the DCX cells, however, this is unlikely because the DCX population arises from the nestin or GFAP populations, which is also ablated in the Nestin-TK mouse and our GFAP-TK rat model before and after stroke onset (Sun et al., 2013; Lee et al., 2017). One major difference in the DCX-TK study is that they use the permanent MCAo model that nearly ablates an entire hemisphere (Jin et al., 2010). This is in contrast to the smaller strokes that are primarily located in the cortex that resulted from the PT model utilized in this thesis, the ET-1 model used to ablate PCs in the GFAP-TK rat (Lee et al., 2017, Appendix A), and the distal MCAo model used by Sun et al. (2013).

In agreement with the Stroke Therapy Academic Industry Roundtable (STAIR) guidelines, we performed a timeline to assess functional outcomes using a variety of behavioural measures that were assessed in the early and late sub-acute phases of stroke recovery (Fisher et al., 2009). We found no improvements in recovery in the iBax mice up to three months post-stroke and would predict that examining mice at longer time points post-stroke would show similar outcomes. Our work with the GFAP-TK rat and the work of others using the Nestin-TK mice model also support, using a variety of motor and sensorimotor tasks, that PCs are not required for sensorimotor recovery up six and seven weeks post-stroke, respectively (Sun et al., 2013; Lee et al., 2017). These results contradicted findings from a follow-up study that performed long-term assessment of the DCX-TK mouse, where DCX-expressing PCs were ablated for two weeks prior to distal MCAo strokes while led to the conclusion that PCs are required for long-term recovery from sensorimotor function. However, this interpretation is limited since: 1) in their study, they only ablated the PCs prior to stroke and do not continue to ablate the cells post-stroke; 2) they have transient and modest effects that only occurred on one behavioural test; and 3) similar to their acute study (Jin et al., 2010) there were larger stroke lesions for the DCX-TK mice and thus the behavioural results are likely more of a reflection of the neuroprotective effects. Thus, overall the evidence thus far, does not support for a role of PCs in stroke recovery.

We had utilized the PT model since it exhibits SBR and thus this model allowed us to observe if enhancing PC survival could promote SBR. One possibility as to why there were no changes in sensorimotor recovery, may be that the timing of the SBR and the timing of enhanced PC survival did not align and thus we may have missed the critical window where enhancing survival could modify SBR. Specifically, we observed SBR between one and four wps. Yet, removing *Bax* from the PCs either before or after stroke, resulted in no significant increase in PCs at the peri-infarct

region in the iBax mice compared to WT mice until eight wps. The delay in observing this enhanced survival is expected since it takes time for PCs to recombine, migrate to the peri-infarct region, and accumulate around the stroke. These results, therefore, suggest that increasing the survival of the PCs around the infarct is insufficient to modify recovery and support the hypothesis that modifications that occur after the critical window of SBR may not be beneficial (Biernaskie et al., 2004). These findings are also in agreement with a more general view supporting it is not neurogenesis *per se*, nor the cells that migrate to the peri-infarct area that can improve motor recovery (Zhang and Chopp, 2016a). This also does not disprove however, the PCs themselves could have a specific effect on SBR, and solely increasing the number of PCs may be sufficient to improve recovery.

6.4 Cognitive Function of Adult Neurogenesis in Stroke Recovery

Increasing PC survival in the iBax mice mildly enhanced spatial learning on the Barnes maze following stroke, which may support that adult neurogenesis may mediate cognitive function following stroke. Changes in spatial learning in our study were found when TAM was administered before the stroke, or after stroke, albeit on different outcome measures. For example, when TAM was administered before stroke, iBax mice displayed faster learning. When TAM was given after stroke, iBax mice travelled less distance to learn the task. These differences may be attributed to a relatively "milder" spatial learning improvement that does not meet the level of detection in all outcomes measured for the Barnes maze. Our findings are also in agreement with the significant, but again relatively mild, reduced spatial learning and memory observed post stroke in the nestin-TK transgenic PC-specific ablation mouse model (Sun et al., 2013).

This finding also supports that these improvements in spatial learning are most likely due to enhanced neurogenesis at the SGZ. Our quantification of cell numbers in the SGZ reveals a time-

dependent increase in cell survival in the dentate gyrus of iBax mice following stroke. In a small pilot study we also found that the increase in PC survival in the SGZ greater than what is usually seen in the naïve mice. For this pilot study, iBax mice were induced with PT strokes or underwent sham surgery, followed by TAM administration to determine if there were enhanced neurogenesis in iBax mice that had stroke. Indeed, there was an almost 2 fold increase in YFP-expressing cells in the iBax mice that had a stroke when compared to sham mice at 60 days post-stroke (sham iBax mice: 466.7 ± 29.01; PT iBax mice: 744.8 ± 114.6). Furthermore, we tested spatial learning and memory at 17 wps, and at this time there were significantly more cells in the iBax versus WT mice. Together, these results suggest that the effects seen in spatial learning may be attributed to the increase in PCs in SGZ.

Enhancing survival of PCs after and before stroke gave rise to neuroblasts and GFAP-expressing cells at the peri-infarct region. Whereas, regardless of time point of TAM administration, the majority of cells in the hippocampus were fated to become mature neurons as measured by colocalization of YFP with NeuN at 90 days post-stroke. Not surprisingly, nearly all (>95%) cells in the iBax mice expressed NeuN, which was significantly less than the 80% of the cells in WT littermates. This difference may be attributed to the accumulation of the mature NeuN expressing cells in the iBax mouse. Our findings support the findings of the ablation study by Sun et al. (2013) using the nestin-TK mouse and a distal MCAo strokes. They find that the ablation significantly depleted the number of cells in the Nestin-TK mice compared to WT mice in the SGZ. Correspondingly, similar to our finding, Sun et al. (2013) identify reduced spatial learning. Unlike our findings, they also identify reduced spatial memory in the nestin-TK mice. Furthermore, aligning with the fate of cells seen in the SGZ of our model, Sun et al. (2013) show that a majority of cells in the WT mice are fated to be neurons expressing either DCX or NeuN.

The modest improvement in spatial learning in the iBax mouse appears to be due to a combination of a significant increase in the number of neurons at the SGZ because of removing *Bax*, and the effects of the stroke. In support of this idea, there were no effects of spatial learning in naïve iBax mice. This is similar to Sahay et al. (2011b) who performed the MWM in naïve iBax and WT mice and identify no differences on this task in iBax versus WT mice. Furthermore, there was also no difference in spatial learning and memory in PT versus sham mice. The improvement in spatial learning was only seen in stroked iBax mice. Together, these findings suggest the existence of a threshold of neurogenesis that is required to promote learning and memory post-stroke.

These findings add to the recent debate about the role of adult neurogenesis in learning and memory as summarized by Aimone et al. (2014) and Lieberwirth et al. (2016). Many studies have utilized a variety of approaches to ablate and promote the survival of adult hippocampal neurogenesis and test how this modulates learning and memory. In general, these studies have found that the ablation of PC contributes to either impaired spatial learning and memory or no change in cognitive function (Saxe et al., 2006; Dupret et al., 2008; Deng et al., 2009). More recent work in computational models as well as knockdown approaches have provided strong evidence for a functional role of adult hippocampal neurogenesis in pattern separation such as fear context discrimination, and two-choice discrimination tasks (Clelland et al., 2009; Aimone et al., 2011; Nakashiba et al., 2012). It currently remains unknown if adult neurogenesis can modulate these behavioural tasks following stroke. However, since the most common cognitive impairments in patients after a stroke are slower information processing as well as executive function (Cumming et al., 2013), future studies measuring either spatial learning or memory or new studies measuring pattern separation may not be useful in the field of stroke recovery.

6.5 Future Directions

Enhancing the survival of PCs lead to a pronounced increase in number of neurogenic cells surrounding the infarct in the absence of changing stroke recovery in the iBax mice. Although this thesis demonstrated that the majority of PCs after stroke express DCX, it remains to be determined if these cells develop into functional neurons. This is one of the most crucial questions that remain in the field – can endogenous PCs develop into neurons in the cortex that will be able to incorporate and participate in motor function? One method to test this hypothesis is to use electrophysiological techniques to determine whether the cells receive inputs and what is their output. Hou et al. (2008) have addressed this question in part, when they studied the temporal development of retrovirally-labelled PCs that migrate into the striatum after a 30 minute transient MCAo stroke. The maturing PCs showed an increase in dendritic length and branch formation, and exhibited markers of mature neurons, NeuN and MAP2 as well as phosphosynapsin I, a marker of vesicle activation. Importantly, these newborn neurons at 6-8 wps were also able to fire action potentials. This work was further supported by similar work by Lai et al. (2008) that showed that eight weeks following permanent MCAo strokes, newborn neurons could integrate into the striatal networks. Given that, many of the cells within the striatum are spiny interneurons it remains unknown if following focal ischemia the same results would occur. It is possible that there may be differences between the striatum and cortex, given that motor cortex has a variety of cell types, includes ones that have to establish long axonal connections to the spinal cord and brain stem. In our laboratory we have begun to test if the PCs become functionally integrated following PT strokes. Our unpublished work has used immunohistochemistry and whole-cell electrophysiology to show that newborn PCs in the injured cortex are hyper-excitable and in some cases share features similar to granule cells and periglomerular cells from the olfactory bulb. This suggests that while PCs shift their migratory

pathway from the OB to the injured cortex following stroke, the cells do not appear to be forming cortical pyramidal neurons, but remained fated to become olfactory bulb neurons. These findings thus lead to the suggestion that in order to promote behavioural recovery through an endogenous "neuronal replacement" strategy, genetic modification to the cells or the niche could be required to make the PCs become functional cortical neurons.

Given the strong associations between angiogenesis and neurogenesis as summarized in the introduction, it will also be important for future work to examine the vascular system in the iBax model post-stroke to determine if the PCs in the peri-infarct area have microvascular support. Given that the iBax mouse had a significant increase in PCs around the stroke we have begun to also perform preliminary experiments to examine if the iBax mice also had an associated increase in vascular support in collaboration with the Laboratory of Baptiste Lacoste. Our preliminary findings suggest the iBax mice have a similar increase in vasculature post-stroke as occurs in WT mice, as assessed by measuring branch points and vessel density using the immunohistological marker, CD31 (Lacoste et al., 2014). These preliminary results therefore suggest that the iBax mice do not have the concurrent increase in vasculature support that may be required to functional support the PCs the migrate to the per-infarct region, and this may have contributed to the lack in improvements in behavioural recovery.

One large debate in the field of regenerative medicine for stroke recovery has focused on the value of using either endogenous or exogenous cell replacement strategies as reviewed by Sullivan et al. (2015). Exogenous cell therapy using neural stem cells, embryonic stem cells, induced pluripotent stem cells, and mesenchymal stem cells have all been proposed to have promise in the treatment of stroke recovery (Marlier et al., 2015). However, several essential questions must be addressed prior to entering the clinic, such as, what cells, at what doses, at what

time post-stroke and using what routes of administration would promote functional recovery following stroke. Alternatively, pre-clinical studies have also isolated endogenous stem cells, expanded them in culture, and transplanted them back into the ischemic brain. Specifically, in one study, rats that were transplanted with these endogenous cells during the MCAo strokes performed better on sensorimotor tasks such as adhesive removal and ladder rung up to 35 days post-stroke (Zhang et al., 2003). However, a primary challenge of using exogenous cell replacement approaches remains that the majority of transplanted cell die (Kelly et al., 2004; Bacigaluppi et al., 2009). Given that many transplanted cell do not survive, one approach that could be used to overcome this limitation could be to extract cells from the iBax model in naïve conditions, expand them in culture, and then transplant them back into the ischemic brain in order to test if this would be sufficient to alter behavioural outcomes. This method could also be used to treat the extracted PCs with factors *in vitro* to promote them to differentiate into cortical neurons. There are obvious issues with this approach when trying to directly translate this type of pre-clinical work into humans, but these studies would be an important step in determining if enhancing the number of regenerated cortical neurons would enhance recovery. This is especially important given the surge in work with induced pluripotent stem cells and the pre-clinical efforts to improve recovery through exogenous transplantation studies.

6.6 The Endogenous Response of PCs in Stroke Recovery in Humans

The goal of this pre-clinical work is to determine if enhancing survival of PCs is a viable mechanism to improve stroke recovery. The translation of the efforts studying endogenous PCs and neurogenesis in the context of stroke recovery relies on there being a PC response to stroke in humans. In support of this requirement, two clinical post-mortem studies completed over a decade ago first used immunohistochemistry techniques to identify PCs in tissue obtained from patients

that suffered a stroke prior to death. First, Macas et al. (2006) observed elevated number of cell in the ipsilateral SVZ of ischemic patients expressing Ki67, which is expressed in proliferating cells and PSA-NCAM, which is expressed in neuroblasts. Second, Jin et al. (2006) reported that the cortical region adjacent to the infarcted core contained cells expressing Ki67, as well as cells expressing both Ki67 and DCX, suggesting newborn neurons are present in the ischemic peri-infarct region following cortical strokes.

These post-mortem findings have been more recently questioned by the Frisen laboratory that have identified ^{14}C concentration in neuronal DNA and shown, within the cortex, a lack of adult neurogenesis after a cortical human stroke (Huttner et al., 2014). However, there are several possible explanations for why ^{14}C dating methodology may not have detected the adult-generated cells following a stroke (Lindvall and Kokaia, 2015; Carmichael, 2016). For example, it has been hypothesized that the ^{14}C study was not sensitive enough to detect the cells if they were very few in number. However, this technique was sensitive to detect adult neurogenesis in other regions such as the striatum and hippocampus (Ernst et al., 2014; Bergmann et al., 2015). It seems more likely that the ^{14}C study was not sensitive enough due to it being unable to capture a transient PC response that may occur in humans following a stroke. Indeed the rapid decline of endogenous PCs is occurring with aging (Galvan and Jin, 2007) and the robust apoptotic cell death that occurs in the newborn PCs post-stroke could have all contributed to the inability to detect the cells. Thus, although the findings of Huttner et al. (2014) do not support that there is adult neurogenesis in the human cortex post-stroke, clearly other studies are required to confirm this result. Ultimately the goal is to measure adult neurogenesis in the living human brain in order to establish its importance in stroke recovery. Although this is a huge challenge, this is the task for the neuroimaging research

and there continues to be progress within this area, so there is hope that this will be possible in the future (Ho et al., 2013).

6.7 Concluding Remarks

This thesis utilized the iBax mouse to successfully enhance the survival of adult born PCs following stroke. Furthermore, we tested whether enhancing survival of the PCs would be sufficient to improve sensorimotor and cognitive behavioural outcomes from a cortical PT-induced stroke model. The absence of any improvement in recovery post-stroke in the iBax mice was unexpected given the robust increase in PCs at the SVZ, and peri-infarct region. The lack of this effect also occurred when survival was increased before or after stroke, which surprisingly, led to astrocytes and neuroblasts in the peri-infarct region, respectively. This outcome gives caution to those that have, and continue to, suggest a positive correlation between increasing neurogenesis and improvements in sensorimotor recovery (Lindvall and Kokaia, 2015; Marlier et al., 2015). This finding also raises caution towards the development of any future therapeutic strategies that aim to enhance PCs alone in order to improve innate recovery following stroke. In contrast to the finding that iBax mice have no changes in sensorimotor recovery post-stroke, the iBax mice had some modest improvements in their ability to learn a spatial task which we hypothesize is related to the enhanced neurogenesis in the dentate gyrus. This finding also is in agreement with a previous model that ablate PCs and suggests that adult neurogenesis contributes to cognitive function (Sun et al., 2013).

Due to the rise in the number of patients surviving after a stroke because of advances in acute stroke care and the growth of our aging population, it is imperative to continue our efforts to provide a game-changing therapeutic option for stroke recovery. The discovery of neurogenesis in the human brain (Eriksson et al., 1998a) raised a lot of hope 19 years ago that this process would

be that game changer. Despite the innate ability of the adult brain to enhance the number of PCs post-stroke, this study does not suggest that a simple increase in PC number contributes to sensorimotor recovery. Indeed my work raises the need for more research to determine whether it is possible for additional interventions to allow the surviving PCs to integrate within the cortical network and improve function, which has implications for regenerative medicine strategies that are being tested using endogenous and exogenous cells. Given the complexity of stroke, I would predict that only modifying the neurogenic response to stroke will not be successful in being the sole mechanism that may translate into recovery and that a multi-faceted approach, including high intensity rehabilitation, is required. This approach, which could include targeting neurogenesis, is more likely to be the best strategy to improve functional recovery following stroke by activating complementary mechanisms involved in neuroplasticity and repair.

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Appendix A

Lee K, Ceizar M, Vani M, Carter A, Jeffers M, Cameron H, Corbett D, Lagace DC (2017). "Ablation of Neural Progenitor Cells Does Not Impede Motor Recovery or Reduce Cognition Following a Cortical Stroke." (In preparation)

Contribution of co-authors

Karah Lee: Performed sensorimotor and cognitive behavioural assessments, perfusing and sectioning all tissue, data analysis, and helped writing the manuscript.

Maheen Ceizar: Performed and analyzed all co-localization histology experiments, analyzed the data, and helped write the manuscript.

Marc Vani: Performed histological counts and analyzed lesion volumes.

Anthony Carter: Performed the ET-1 stroke surgeries and analyzed cylinder data.

Matthew Jeffers: Performed the ET-1 surgeries.

Heather Cameron: Gifted the GFAP-TK rats

Dale Corbett: Contributed to the design of the experiments.

Diane C. Lagace: Contributed to the design, analysis, interpretation of results and writing the manuscript.

Ablation of Neural Progenitor Cells Does Not Impede Motor Recovery or Reduce Cognition Following a Cortical Stroke

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Abbreviated Title: Adult Neurogenesis is not Required for Stroke Recovery

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Significance Statement

Stroke activates a neurogenic response that includes the robust proliferation, ectopic migration and differentiation of adult-generated progenitor cells. This neurogenic response has often been shown to correlate with improved recovery, yet its requirement for motor or cognitive function post-stroke remains controversial. Here we demonstrate that the conditional ablation of PCs and resulting decrease in neurogenesis in a transgenic GFAP-TK rat does not alter motor recovery or cognitive function up to seven weeks after a cortical stroke. These findings question the hypothesis that endogenous adult neurogenesis is required for stroke recovery.

Abstract

Following stroke there is a neurogenic response in the adult brain that consists of a significant increase in the number of progenitor cells (PCs), ectopic migration of PCs from the subventricular zone to the site of damage, and positive correlations between neurogenesis and enhanced recovery in rodent models. Loss-of-function studies have yielded conflicting results on whether the ablation of PCs impedes motor recovery or diminishes spatial learning and memory post-stroke. This study examines if adult neurogenesis is required for motor recovery and spatial learning and memory post-stroke using the transgenic GFAP-TK rat model that allows for the inducible deletion of PCs in the adult brain. Endothelin-1 (ET-1) injection into the forelimb motor cortex induced strokes in GFAP-TK rats that were of similar size and associated with similar contralateral motor deficits up to 7 weeks post-stroke, compared to control rats. Similarly, there was no difference between GFAP-TK and control rats post-stroke in performance on spatial or associative learning and memory. These findings suggest that PCs and their progeny are not required for recovery of motor or spatial and associative learning and memory functions following stroke.

Introduction

In preclinical models, stroke evokes a robust response from stem and rapidly dividing progenitor cells (PCs) in the adult brain (reviewed in: Saha et al., 2012; Gregoire et al., 2015; Lindvall and Kokaia, 2015; Marlier et al., 2015). This response includes a striking increase in the proliferation of PCs in the subventricular zone (SVZ) of the lateral ventricles, generating new cells that can ectopically migrate to the site of injury. There is also an increase in the proliferation of PCs in the subgranular zone (SGZ) of the hippocampal dentate gyrus. The PCs in the dentate gyrus survive and develop almost exclusively into neurons in the granule cell layer. In contrast, the PCs surrounding the site of injury have been reported to develop into astrocytes, oligodendrocytes, and neurons. This dynamic stroke-induced PC response within the adult brain has been well documented in numerous studies since the first reports of post-stroke neurogenesis (Liu et al., 1998; Jin et al., 2001; Arvidsson et al., 2002). Despite 20 years of preclinical work, the function of the PCs or the importance of the neurogenic response *per se* during stroke recovery remains unclear (Lindvall and Kokaia, 2015; Carmichael, 2016; Zhang and Chopp, 2016b).

Many preclinical studies have positively correlated the PC response and/or neurogenesis to motor and cognitive recovery in a variety of species and models (reviewed in:Lagace, 2012; Marlier et al., 2015), leading to the suggestion that adult neurogenesis is functionally important for stroke recovery. Three research groups have directly tested the functional requirement of neurogenesis for stroke by using loss-of-function models (Raber et al., 2004; Jin et al., 2010; Wang et al., 2012; Sun et al., 2013). Raber et al. (2004) first utilized a global ischemia model and ionized irradiation to ablate PCs in gerbils and found diminished spatial learning and memory in the Morris water maze post-stroke, independent of altering ischemia-induced cell death in CA1. Jin et al. (2010) created a transgenic doublecortin-thymidine kinase (DCX-TK) mouse model that allowed for the

specific deletion of DCX-expressing immature neurons and reported an increase in infarct size and reduced short- and long-term recovery (Jin et al., 2010; Wang et al., 2012). Most recently, Sun et al. (2013) tested the requirement of PCs for both motor and cognitive recovery following a focal cortical stroke using a transgenic nestin-TK inducible mouse. In contrast to the DCX-TK mouse studies, ablation of PCs prior to and during recovery from stroke did not alter stroke size or motor recovery. However, the nestin-TK model did show diminished post-stroke spatial learning and memory, consistent with the global ischemia study by Raber et al. (2004). Taken together, these conflicting findings confound interpretations about whether the PCs and neurogenesis contribute to infarct size, motor function, and/or cognitive function post-stroke.

We sought to perform a comprehensive study to investigate the requirement of PCs and their progeny for motor and cognitive behavioural tasks using our recently created transgenic GFAP-TK rat model to ablate PCs (Snyder et al., 2016). The rat is an ideal preclinical model to assess stroke recovery because the requirement of neurogenesis in stroke recovery has never been tested in rats, yet rat models represent the most robust well-characterized preclinical model with the requisite long-term functional deficits required for translational research (Corbett et al., 2015; Bernhardt et al., 2016). In this study, we ablate the PC response and perform behavioural testing to quantify recovery following endothelin-1 (ET-1)-induced focal cortical stroke in GFAP-TK rats. We performed a battery of sensorimotor tests up to 7 weeks post-stroke and measure the requirement of PCs for post-stroke cognitive function using the Barnes maze and the fear conditioning tests. We found that the GFAP-TK rats have an almost complete elimination of: 1) dividing PCs in the SGZ and SVZ, 2) migration of PCs and immature neurons from the SVZ to the peri-infarct region and 3) neurogenesis in the SGZ and peri-infarct region in response to stroke. Despite this robust ablation of the neurogenic response and robust deficits in behaviour following

cortical stroke, there is no significant change in infarct size or pattern of behavioural recovery. These findings suggest that adult neurogenesis is not functionally required for stroke recovery.

Materials and Methods

All procedures were performed in accordance with the [Author's University] Animal Care Committee.

Animals

Rats expressing HSV-TK under the human GFAP promoter (GFAP-TK) on a Long Evans background were generated at the National Institute of Health (Snyder et al., 2016). Heterozygous GFAP-TK female rats were bred with age-matched Long Evans male rats obtained from Charles River (Montreal, Canada). The male and female experimental animals were pair-housed and maintained on a reversed 12-hour light cycle with water and food available *ad libitum*. Separate experimental animals were used for behavioural analysis (n=25 WT; n=36 GFAP-TK) and histological assessment of PCs and neurogenesis (n=8 WT; n=8 GFAP-TK).

Treatments

Valganciclovir (VGCV): Rats were treated orally with valganciclovir hydrochloride (VGCV, 02413825; Teva-Valganciclovir) delivered in a 0.5 g peanut butter (PB) ball which consisted of standard rodent chow mixed with Skippy® PB in a 1:1 ratio by volume that was consumed voluntarily (Snyder et al., 2016). At 7 weeks of age, rats were habituated to the PB balls by eating a control ball every day for 3 days. At 8 weeks of age, all rats were administered one VGCV-containing PB ball twice weekly (Mondays and Fridays) and thus assigned to the v-TK or v-WT groups. Each rat was administered the PB ball in the cage by hand to ensure consistent dosing since they voluntarily consumed the ball upon presentation. Animals included in the behavioural and histological experiments both received PB ball treatment until perfusion.

5-bromo-2'-deoxyuridine (BrdU): To label cells in S-phase of the cell cycle, rats were administered an intraperitoneal (IP) injection of BrdU at 150 mg/kg at 1 week following stroke. Animals used for histological experiments were not used for behavioural analysis and randomly assigned to be sacrificed either 2 hours (n=4 v-WT, n=4 v-TK) or 6 weeks (n=4 v-WT, n=4 v-TK) following BrdU administration.

Endothelin-1 (ET-1) Stroke Surgeries

Forelimb motor cortex strokes were induced in GFAP-TK and WT rats at 14 weeks of age using the Endothelin-1 (ET-1) stroke model (Windle et al., 2006; Jeffers et al., 2014). Rats were fasted overnight prior to surgery and anesthetised by inhalation using 4% isoflurane with 1.5% oxygen. During surgery, the isoflurane level was reduced to 2%, while the oxygen level was maintained at 1.5%. Using stereotaxic surgery, 2 µl of ET-1 (400 pmol/µl sterile H_2O, human, porcine, ab120471; Abcam®) was injected in 2 locations within the motor cortex on either the right or left side of the brain based on staircase training results such that the dominant paw was targeted for

impairment, or in the case where the staircase test was not performed, targeted hemispheres were randomly assigned. ET-1 was delivered at a flow rate of 0.4 μl/min through a Hamilton syringe (26 gauge; Hamilton, Nevada, USA) using the following anteroposterior (AP), mediolateral (ML) and dorsoventral (DV) coordinates relative to bregma: Injection 1: AP = 2 mm, ML = 2.5 mm, and DV = -1.7 mm; Injection 2: AP = 0 mm, ML = 2.5 mm, and DV = -1.7 mm. Following injection the rats were left undisturbed for 1 minute prior to injection and 2 minutes prior to being retracted from the brain. The scalp was sutured and the incision site was treated with topical anaesthetic (2% Bupivacaine, 0.1 ml, Chiron, Ontario, Canada). Body temperature was monitored continuously and maintained at 37.0 ± 0.2 °C for the duration of surgery using a thermostat-controlled heating blanket (Harvard Apparatus). Following surgery, animals were placed into a 37 °C incubator until awake and active (approximately 30 minutes), then given a subcutaneous injection of buprenorphine (0.05 mg/kg; Chiron) and returned to their home cages. Mortality following surgery included 4/29 v-WT and 2/36 v-TK rats.

Behavioural Tests

All behavioural tests were performed between 8am and 5pm in behaviour testing rooms and separate experiments were performed for the motor (n=10 for v-WT; n= 20 for v-TK) and the cognitive (n=15 for v-WT; n=16 for v-TK) tests. Motor testing consisted of the staircase, beam walk, and cylinder tests (performed in that order). For the motor tests pre-training and baseline measurements were obtained 1 to 3 weeks prior to stroke and then post-stroke measurements were taken at 1, 3, 5, and 7 weeks post-stroke. Cognitive testing was performed in a separate cohort of rats starting 1 week following stroke and included testing for spatial learning and memory using the Barnes maze followed by testing for associative learning and memory using the fear conditioning test. For all behavioural tests the rats were transported to the testing room and were

left undisturbed for 30 minutes to habituate to the room. White noise was generated by a radio tuned to a vacant frequency to provide a consistent level of background noise for all tests except fear conditioning. An experimenter that was blind to treatment group completed all behaviour tests and analysis.

*Montoya Staircase Test***:** The staircase test was performed as previously described (Montoya et al., 1991). Briefly, rats were food-restricted for the duration of testing beginning the day before training (12 g rodent chow/rat/day). Mildly food deprived rats were individually placed in separate Plexiglas staircase testing boxes for 15 minutes. Each box contained a set of stairs (7 steps) descending from both sides (one for each forelimb) filled with sugar pellets (3 pellets/step). Rats were trained to retrieve pellets twice daily for at least 10 days, or until a plateau in learning was achieved on the last three days. A plateau was defined as the rats obtaining an average of at least 15 pellets with a standard deviation of less than 2.5 pellets with one forelimb. Rats were trained and tested within the same box and the box was not cleaned unless necessary. Forelimb dexterity was measured by comparing the number of pellets eaten with those that were left behind or dropped. Due to the inherent variability of stroke damage expected based on prior studies (Biernaskie and Corbett, 2001; Biernaskie et al., 2004), we excluded n=2/10 v-WT and n=4/20 v-TK rats based on our established *a priori* criteria that rats with greater than 80% success on the staircase test at 1 week post-stroke would be excluded because their mild initial deficits would result in spontaneous recovery.

Cylinder. Rats were placed inside a small upright cylinder (20 cm in diameter, 35 cm tall) under which a video camera recorded the rats as they freely explored the cylinder in order to assess spontaneous forelimb placement, as per previous publications (MacLellan et al., 2011). Each rat reared 20 times before it was removed from the cylinder to complete the one trial required for

baseline and each of the post-stroke assessments. The number of contacts the rat had with the cylinder wall during a rear (rat stands up on hindlimbs) using either the right, left or both forelimbs was recorded and analyzed.

Beam walk. Hindlimb performance was measured by testing rats on the beam walk and measuring foot faults (Schaar et al., 2010). Rats were placed on the end of a tapered beam (6 cm at widest, 1.5 cm at narrowest, 160 cm long) and were trained for one day (4-6 trials, inter-trial interval (ITI) of 15 seconds) to learn how to cross the beam towards a darkened goal box with sugar pellets. The beam had two levels with the bottom level having a consistent width of 2 cm on both sides. In order to allow viewing of all limbs during video recording, a mirror was placed behind the beam. Baseline (prior to stroke) and post-stroke measurements were obtained by recording 4 trials per rat with an ITI of 15 sec. The number of slips (foot faults) off the top level onto the bottom level were counted during playback of video recordings through blinded manual recording.

Barnes Maze. Spatial learning and memory was assessed using the Barnes maze (125 cm diameter) that consists of 18 holes (9 cm diameter) located in a room with 4 different spatial cues on each wall (diagonal stripes, vertical stripes, a large triangle, and the fourth wall had no cue). The goal of the test is to locate and enter the target goal box using the spatial cues on the wall. Using an online random number generator (www.random.org) each rat was assigned a spatial cue where the goal box was located. Aversive stimuli within the testing room including bright lights (600 lux) and white noise (75 dB) encouraged the rats to find the dark, enclosed goal box. The maze was rotated in a randomized fashion to minimize learning the task using olfactory cues between each trial. The protocol was a modification from published work (O'Leary and Brown, 2013) and consisted of four phases in the following order: habituation (1 day), training (8 days), probe trial (1 day), reversal training (5 days).

Habituation was performed in the absence of any visual spatial cues during four consecutive trials completed in one day. On the first trial the rats were placed directly in front of the target hole and goal box in the presence of the bright lights and white noise and the rat was permitted to explore the maze freely for two minutes, after which they were partially guided to the target goal box by a researcher if they did not enter the box on their own. Once the rat was in the target goal box, the lights and sound were then switched off. The same protocol was used for trial 2 and 3, except the rat was progressively placed further away from the hole when being placed on the maze. On the fourth trial the rat was placed in the center of the maze under a bucket and at the start of the trial the bucket was raised simultaneously with exposure to the light and noise. The rat was allowed to roam the platform for up to three minutes until the animals successfully located and entered the goal box. The training phase began 24 hours following habituation, and each rat underwent two trials per day with an ITI of 20 minutes. For each animal, the target goal box location was fixed at one of three randomized locations relative to the visual cues on the wall. Trials for the training phase were conducted as described above for the fourth habituation trial. The rats were placed on the maze facing a randomized direction in order to prevent self-orientation based on their starting position and between each trial, the maze was cleaned and rotated in a randomized fashion to reduce residual odor cues. The probe trial was conducted 24 hours following the last day of training and consisted of one trial. The testing parameters were identical to those used during training except that the target goal box was removed and the rats were permitted to roam the platform for a total of three minutes. The reversal phase began 24 hours following the probe trial and each rat underwent two trials per day with an ITI of 20 minutes. The reversal phase was conducted identically to the training phase except the target goal box was repositioned at 180° from its original position. For all phases of the testing the rat's movement on the maze was visualized,

recorded and analyzed using video camera and EthoVision XT 10.0 (Noldus) software. Total errors were measured manually during the trial by recording the number of visits made to non-target holes and the number of visits made to the target hole without entering the goal box.

Fear Conditioning. For the fear conditioning test rats were exposed to one trial (8 minutes) per day for three days within the PhenoTyper boxes (Noldus) that were connected to the EthoVision XT 10.0 (Noldus) software. On day one, the rat's baseline freezing in the novel context was recorded during the first 2 minutes followed by exposure to a 20 s tone co-terminating with a 1 s foot shock (0.6mA). This was repeated 3 more times 1 minute apart for a total of 4 pairings. For the last two minutes, the rat remained in the box with no tone or foot shock. On day two, contextual fear conditioning was tested by placing the rat into the same environment as on day one and recording freezing behavior for 8 minutes (no tone or shock). On day three, cued fear conditioning was tested in a novel environment. Modifications to the testing conditions included the experimenter using yellow rubber gloves for handling the rat instead of white latex gloves, the room being illuminated by red instead of white lights, changing the shape of the PhenoTyper boxes by adding plastic inserts, and adding a vanilla scent within the testing box. During cued fear testing, no tone was played for the first 4 minutes to test the novelty of the context, and the auditory cue paired on day one with the shock was played for the last 4 minutes to test freezing associated with the cue.

Tissue preparation

For histological experiments rats were perfused at 2 hours or 6 weeks following BrdU. Prior to perfusion the rats were anesthetised with sodium pentobarbital (Euthanyl, 750 mg/kg, IP). Rats were then transcardially perfused at a rate of 10 ml/min with cold 1X phosphate-buffered saline (PBS) (pH 7.4) for 12.5 minutes followed by cold 4% paraformaldehyde (PFA) in 1X PBS (pH

7.4) for 15 minutes. Following perfusion, the brains were removed and post-fixed in 4% PFA for 24 hours and then transferred to 30% sucrose in 1X PBS with 0.1% sodium azide (NaN_3) for cryoprotection. The brains were sectioned coronally at a thickness of 40µm using a cryostat (CM1850; Leica). The brain sections were collected serially in 9 wells and stored in 1X PBS with 0.1% NaN_3 at 4^{o}C to prevent microbial growth.

Immunohistochemistry

Slide-mounted IHC was used to detect the total number of BrdU-positive cells in the SVZ and SGZ, as well as DCX-positive cells in the SVZ. Serial sections were mounted onto charged Superfrost slides and dried over night. Slides for BrdU or DCX staining were immersed into 0.1M citric acid (Sigma; pH 6.0) at approximately 95°C for 15 minutes for antigen retrieval. Sections stained for BrdU were then permeabilized by incubating them at room temperature (RT) in 0.1% trypsin (Sigma) for 10 minutes, followed by DNA denaturation in 2N hydrochloric acid (Sigma) for 30 minutes at RT. To prevent non-specific binding, all slides were incubated in 3% Normal Donkey Serum (NDS; 017-000-121; Jackson Immuno Research Laboratories Inc.) and 0.3% Triton X-100 (Fisher Scientific) in 1X tris-buffered saline (TBS) for 60 minutes prior to being incubated overnight in the primary antibody solution of either Rat anti-BrdU (1:300, OBT0030; Accurate Chemical & Scientific Corporation) or Goat anti-DCX (1:300, SC8066; Santa Cruz) in 3% NDS in 0.3% Polyoxyethylene-20-sorbitan Monolaurate (Tween20; Sigma) and 1X TBS. The following day, slides were incubated at RT in biotinylated donkey anti-rat (1:200, 712-065-153; Cedarlane) or anti-goat (1:200, 705-065-147; Cedarlane) secondary antibody in 1.5% NDS in 1X TBS for 60 minutes followed by 0.3% H_2O_2 in 1X TBS for 30 minutes to quench endogenous peroxidases. Slides were then treated with Avidin-Biotin Complex Solution (ABC, PK-6100; Vector Laboratories) for 90 minutes and positive cells were visualized with metal enhanced 3,3'-

Diaminobenzidine (DAB; 34065; Thermo Scientific, 1:10) for 5 to 15 minutes. Tissue was then counterstained with fast red nuclear stain (H3403; Cedarlane). Between all steps, with the exception of post-blocking with NDS, the slides were rinsed 2-3 times with 1X TBS. Following staining, slides were dehydrated consecutively immersing slides in 95% and 100% ethanol for 20 seconds, followed by CitriSolv (22-143-975; Fisher Scientific) clearing agent for 20 seconds, 1 minutes, and 5 minutes. Slides were cover-slipped with DPX mounting medium (mixture of Distyrene, Plasticizer, Xylene; 44581; Sigma).

Free-floating IHC was used to detect BrdU-positive cells that colocalized with GFAP and neuronal nuclear protein (NeuN) through triple-labeling with BrdU, GFAP and NeuN antibodies. Briefly, sections were rinsed three times with 1X PBS, and placed into 12-well plates containing 2N HCl for 1 hour. Sections were then rinsed with 0.1 M sodium borate for 15 minutes followed with blocking in 3% NDS for 1hr at RT. Sections were then incubated in the primary antibody solution (1:500 rat anti-BrdU (OBT0030; Accurate Chemical & Scientific Corporation); 1:500 rabbit anti-GFAP (Z0334; DAKO); 1:500 mouse anti-NeuN (MAB377; Cedarlane) in 0.1% TritonX, 0.1% Tween20, 1xPBS) overnight on a shaker at 4°C. The following day, all sections were incubated in a 1:500 CY3 Donkey anti-rat antibody (712-585-153; Cedarlane) and 0.1% Tween20 for 1 hour followed by another 1 hour incubation in 1:500 CY2 donkey anti-mouse (715-546-150; Cedarlane), 1:500 CY5 donkey anti-rabbit (711-175-152; Cedarlane) antibodies and 0.1% Tween20 for 1 hour. Following secondary antibody incubations, all sections were counterstained with 1:10000 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI; 10236276001; Roche). Between all steps, with the exception of post-blocking with NDS, the slides were rinsed 2-3 times with 1X PBS. Sections were then mounted onto slides, dried for approximately five minutes, and coverslipped with Immu-mount (2860060; Fisher Scientific).

For infarct analysis, infarct sections were stained with Cresyl Violet. Briefly, tissue sections with visible stroke damage were laid out in order in a petri dish containing 1X PBS. Depending on the number of sections with stroke, a mounting factor was selected in order to mount at least 8 sections with stroke and one bookend that was anterior and posterior to the stroke. Sections were mounted onto slides and allowed to dry overnight. The following day, the slides were loaded onto a slide holder and dehydrated, rehydrated, and stained with Cresyl Violet (0.25% cresyl violet acetate powder (C5042-10G; Sigma) in acetate buffer) followed by Citrisolv.

Quantification

DAB-stained sections were visualized on an Olympus IX51 inverted microscope at 400x magnification. For histological experiments the number of BrdU cells were exhaustively quantified throughout the entire dentate on every 18th section. BrdU cells in the dorsolateral SVZ, as well as DCX cells in the dorsolateral SVZ were quantified exhaustively using one bregma-matched section. Colocalization analysis was performed on Zeiss LSM 510 META confocal microscope using a 40X oil immersion objective by an observer blind to the experimental conditions. A minimum of 20 BrdU+ cells per animal was evaluated for all co-labelling analysis.

Stereology: The Optical Fractionator probe of Stereo Investigator software (MBF Bioscience) was used to provide unbiased estimates of the population of BrdU+ cells in the peri-infarct using one coronal section per rat that had the largest infarct size. The peri-infarct was traced at 10X magnification and the region was defined as the area above the corpus callosum and between the midline of the brain to the middle of the infarct and did not include the infarct itself. A 200 µm x 200 µm grid was superimposed over the traced region, and cells were counted within a 200 µm x 200 µm counting frame at 40X (to count the entire region). Upper and lower guard zones of 2 µm were used for an optical dissector of height of 29 µm. Average number of sites counted was 85

sites and the number of cells per section was 37 cells.

Stroke Volume: Pictures of each cresyl violet-stained section were taken on a dissecting scope (Zeiss Stereo Discovery V20) at 10x magnification and opened with ImageJ software (NIH) for stroke volume quantification. The stroke lesion volume of each animal was calculated similar to published work (Biernaskie and Corbett, 2001; Biernaskie et al., 2004). Briefly, measurement of structures in both hemispheres was completed in order to account for any edema or compression that may have arisen in the brain as a result of the stroke. All measurements were calculated using the pixel count function of ImageJ (98.44 pixels per mm on the 10x images). The surface area of the intact tissue of each hemisphere (lesioned and non-lesioned), as well as their corresponding ventricles were measured by subtracting the intact area of the lesioned hemisphere area from the area of the non-lesioned hemisphere.

Statistical Analyses.

All data were analyzed and presented using Prism 6 (GraphPad). Outlier tests were run using the ROUT outlier test on all outcome measures. Using this test, one v-TK rat was excluded since this rat had a stroke volume greater than $100mm^3$, which is approximately twice the volume of the average lesion size and displayed large deficits that did not improve over time (data not shown). Stroke volume between the two groups (v-WT and v-TK) was analyzed using a two-tailed two-sample student's t-test. For all analyses, a significance level of 0.05 was used and all outcomes are provided in Table 1.

Results

Specific inhibition of stroke-induced neurogenesis in v-TK transgenic rats

VGCV treatment in naïve GFAP-TK rats induces a near-complete conditional ablation of adult neurogenesis within the hippocampus and olfactory bulb (Snyder et al., 2016). To test whether

proliferation and neurogenesis is similarly inhibited post-stroke, histological methods were utilized to examine the PCs and their progeny at 1 and 7 weeks following a ET-1 induced cortical stroke. This model was utilized according to best practice guidelines in order to produce an infarct of similar size to those that occur in humans (Carmichael, 2005), as well as to produce sustained sensorimotor deficits in order to improve the translational impact (Corbett et al., 2015; Bernhardt et al., 2016).

In order to examine the proliferating cells 1 week post-stroke, the v-TK and v-WT littermate control rats were injected with BrdU to pulse label the dividing cells. In the SVZ, v-TK rats had very few proliferating cells present, with a significant 93% reduction in BrdU-expressing (BrdU+) cells compared to v-WT rats (**Figure** 1A-B). In the SGZ, the v-TK rats had a complete ablation of BrdU+ cells (**Figure** 1C-D). In the peri-infarct zone, there were proliferating BrdU+ cells (**Figure** 1E). Unlike the significant reduction in number of proliferating cells in the SVZ and SGZ of the v-TK rats, there was no significant difference in number of proliferating BrdU+ cells in the peri-infarct zone between the v-TK and v-WT rats (**Figure** 1E-F). The dividing cells around the infarct were unlikely to arise from the SVZ given they were labeled 2 hours prior to sacrifice and very few cells in the v-TK rats were labeled with BrdU in the SVZ at 1 week post-stroke (**Figure** 1A-B). There was also minimal colocalization of the 2 hour-old BrdU-labeled cells and GFAP within either the v-WT (4.4±0.4%) or v-TK (1.3±1.3%) rats post-stroke which supports there are few dividing astrocytes at 1 week post ET-1 stroke in the peri-infarct region. This minimal amount of astrogenesis in v-WT and v-TK rats arising from progenitor cells in the cortex is similar to those reported after ET-1 models in mice (Faiz et al., 2015), but contrasts the large response following a stab injury that is ablated in the GFAP-TK mice (Bush et al., 1999). Together these results suggest v-TK rats have a near complete ablation of proliferation within the SVZ, a complete

ablation of proliferating cells in the SGZ, yet have proliferating non-GFAP-expressing cells within the peri-infarct region at 1 week post-stroke.

To examine the survival and fate of the dividing BrdU+ cells at 7 weeks following ET-1 induced cortical stroke, BrdU was injected 1 week post-stroke and the number of surviving 6-week-old BrdU+ cells was quantified. In the SVZ, the v-TK and v-WT rats had very few 6-week-old BrdU+ cells (<5 cells per section). This is expected since most of the dividing labeled BrdU+ cells would have migrated away from the SVZ or diluted the BrdU label 6 weeks following labeling. In the SGZ, the v-TK rats had no 6-week-old BrdU+ cells, while these cells could be easily detected in v-WT rats (**Figure** 2A-B). Similar to naïve v-WT rats (Snyder et al., 2016), over 90% of the surviving BrdU+ cells in the SGZ of the v-WT rats had a neuronal phenotype as determined by their expression of the neuronal marker NeuN (**Figure** 2C-D).

In the peri-infarct region, there were 6-week old BrdU+ cells present in the v-WT and v-TK rats with no significant differences in the number between v-WT and v-TK rats (**Figure** 3A-B). Phenotyping of these cells revealed that almost 60% of the surviving BrdU+ cells expressed GFAP in both the v-WT and v-TK rats (**Figure** 3C-D). These 6-week-old GFAP cells are not deleted in the v-TK rats since they are not dividing. Further evaluation of neurogenesis through quantification of BrdU and NeuN colocalization in the peri-infarct region revealed that <5% of 6-week-old BrdU cells had developed into neurons in the v-WT rats, with no BrdU+NeuN+ cells in the v-TK rats (**Figure** 3E-F). To test the neurogenic response to stroke in the rats and confirm if neurogenic cells were migrating from the SVZ to the peri-infarct region, independent of BrdU labeling, we analyzed the number of immature neurons expressing DCX. The v-TK rats had an 84% reduction in the number of DCX+ cells (**Figure** 3G-H). Together this data suggests that in

the weeks following a stroke, the v-TK rats had a significantly reduced migration response from the SVZ to the infarct and significant inhibition of neurogenesis.

Inducible ablation of PCs does not affect lesion size

As demonstrated in **Figure** 4A, the ET-1-induced infarcts in both the v-WT and v-TK rats spanned much of the sensorimotor and motor cortex and displayed heterogeneity in size as shown previously (Windle et al., 2006)**.** The infarcts extended from bregma 6.12 to -5.83 in the v-WT and bregma 6.12 to - 6.13 in the v-TK rats, with the majority (75% in v-WT, 73% v-TK) having exclusively a unilateral injury. There was also no significant difference in the average hemispheric lesion volume between the v-WT and v-TK rats, with an average volume of ~60mm^3 for both groups of rats (**Figure** 4B). These findings suggest that PCs are not neuroprotective.

Significant post-stroke deficits of sensorimotor function in v-TK and v-WT rats

We utilized the staircase, cylinder and beam behaviour tests to quantify recovery at 1, 3, 5, and 7 weeks post-stroke (**Figure** 5). These tests were used since they have been validated to be sensitive to detect long-term behavioural outcomes with the rat ET-1 stroke model (Biernaskie and Corbett, 2001; Pagnussat et al., 2012; Jeffers et al., 2014). Furthermore, utilizing these tests enabled comparisons to prior related studies that had performed similar tests in mouse conditional ablation stroke studies (Jin et al., 2010; Wang et al., 2012; Sun et al., 2013).

The Montoya Staircase Test (Montoya et al., 1991) was used to assess motor learning as well as forelimb reaching (Whishaw and Pellis, 1990; Biernaskie and Corbett, 2001). When training the rats on staircase prior to stroke, both v-WT and v-TK rats demonstrated similar abilities to learn to reach for the food pellets using both forelimbs (**Figure** 5A-B). After the stroke, both v-WT and v-TK rats exhibited significant contralateral deficits in the range of a 50% reduction in performance that was significantly sustained up to 7 weeks post-stroke when compared to baseline

(**Figure** 5C). In contrast, on the ipsilateral side both v-WT and v-TK rats had minor deficits in the range of <20% reduction in performance (**Figure** 5D). These ipsilateral deficits were transient and not significantly different by 7 weeks post-stroke compared to baseline. Both v-WT and v-TK rats also had significant improvements, suggestive of innate recovery on the staircase test as demonstrated by significant increases in pellet retrieval at 7 weeks post-stroke compared to 1 week post-stroke. Overall, on all outcomes measures for the staircase test, v-TK compared to v-WT rats showed no significant differences suggesting that PCs and neurogenesis do not influence recovery of post-stroke forelimb reaching abilities.

The cylinder test was used as a measure of spontaneous forelimb use and was quantified as the percent use of each forelimb (MacLellan et al., 2011). Prior to stroke both v-TK and v-WT rats displayed equal use of both forelimbs. After stroke the v-TK and v-WT rats had a significant reduction in use of the contralateral forelimb up to 7 weeks post-stroke that did not differ between genotypes (**Figure** 5E). This was accompanied by a similar relative increase in the use of the ipsilateral forelimb (**Figure** 5F). These results are in alignment with the staircase findings and suggest that post-stroke there is a significant reduction in use of the impaired forelimb, independent of whether adult neurogenesis is absent or present.

The beam walk test was used to assess hindlimb function and asymmetry through quantification of slips off the beam (Schallert et al., 2002). Both the v-WT and v-TK rats had significant contralateral hindlimb deficits post-stroke when compared to baseline performance and there was no significant difference between v-WT and v-TK rats (**Figure** 5G). On the ipsilateral side there were significant hindlimb deficits at 1-week post-stroke when compared to baseline (**Figure** 5H). The deficits in the ipsilateral side at 1 week post-stroke were variable, as shown by the SEM, however there was no statistical difference between the v-WT and v-TK rats at any time point.

Thus when combined together, these motor test results demonstrate that the v-TK and v-WT rats had robust significant contralateral motor impairments on all three motor tests. These deficits are of similar magnitude to those reported in Sprague Dawley rats subjected to ET-1 (Windle et al., 2006; Jeffers et al., 2014), suggesting that the ET-1 stroke model is consistent across rat strains. Additionally the lack of differences in impairment between the v-WT and v-TK rats suggests that ablation of neurogenesis does not alter sensorimotor or motor function during stroke recovery.

Inducible ablation of PCs does not modify cognitive function following a stroke

Sun et al. (2013) demonstrated that ablation of PCs using a nestin-TK mouse model does not alter motor function post-stroke, but significantly impedes spatial learning and memory on the Barnes maze. This finding suggests that PCs within the SGZ may contribute to stroke recovery and supports the notion that SGZ neurogenesis is required for hippocampal-dependent learning and memory post-stroke (Raber et al., 2004; Lagace, 2012; Sun et al., 2013). Since the v-TK rats have a complete loss of neurogenesis in the SGZ, we investigated whether the v-TK model would have impairments in learning and memory abilities post-stroke. Spatial learning and memory was assessed using the Barnes maze, as well as contextual and cue-based memory using the fear conditioning test.

Over the course of daily training for eight days on the Barnes maze, spatial learning was assessed by three outcome measures: number of errors made, and the distance and time taken to locate and enter the goal box (Barnes, 1979). Over the eight training days both v-TK and v-WT rats showed a significant reduction in the average number of errors made, without any differences between the groups (**Figure** 6A). Similarly, both v-TK and v-WT rats exhibited a significant reduction in the time and distance taken to locate and enter the goal box (**Figure** 6 B, C). These results demonstrate

that v-TK rats were able to learn the location of the target box, suggesting that PCs are not required for learning a spatial task post-stroke.

Following learning the Barnes maze task, the rats were tested on a probe trial to assess the requirement of PCs for spatial memory retention. As expected, both v-WT and v-TK rats spent significantly more time in the target zone than the opposite zone (**Figure** 6D) suggesting that both groups learned and can perform short-term memory recall. Additionally, heat maps of rat movement qualitatively demonstrated similar patterns of exploration between v-WT and v-TK rats during probe testing (**Figure** 6E). Following the testing day for the probe trial, rats were assessed on their ability to re-learn the task through reversal testing by locating the goal box opposite to its original location. Both v-WT and v-TK rats were equally able to acquire the new location of the goal box through 5 days of testing (**Figure** 6 F-H). These results suggest that unlike previous findings in the nestin-TK mice (Sun et al., 2013), rats with an almost complete ablation of neurogenesis displayed no significant deficits in performance on the Barnes maze.

We also examined associative memory using the fear conditioning test, in which the rats are conditioned to associate a context and cue (tone) to a foot shock on the initial training phase of the fear conditioning task (**Figure** 7). On day 1 of testing, prior to shock, baseline freezing in the novel context was not significantly different between the v-TK and v-WT rats (**Figure** 7A). On day 2, during the context test both v-TK and v-WT rats spent approximately 35% of their time immobile, which was more than baseline levels of freezing prior to conditioning (**Figure** 7B). On day 3, both the v-TK and v-WT rats did not recognize the environment as suggested by the relatively low levels of freezing prior to the tone being played at 4 minutes into the task (**Figure** 7C). During the tone there was a significant increase in freezing in both the v-TK and v-WT rats (**Figure** 7C). Thus, similar to the results of the Barnes maze, all fear conditioning outcome measures

demonstrated no significant differences between the v-WT and v-TK rats. Together these results suggest that PCs are not required for hippocampal or amygdala-based associative learning or spatial learning and memory following a stroke.

Discussion

The aim of our work was to clarify the function of adult neurogenesis in motor and/or cognitive function post-stroke. We found the absence of the neurogenic-response to stroke in the GFAP-TK rat did not modify infarct size or reduce recovery after a stroke. These results indicate that in the rat, innate motor recovery from a cortical stroke can occur independent of PCs arising from SGZ and SVZ, and the neurogenic response to stroke. Therefore our findings do not support the hypothesis that ablation of neurogenesis impairs functional outcome post-stroke (Raber et al., 2004; Jin et al., 2010; Wang et al., 2012; Sun et al., 2013).

The ablation obtained in the v-TK rat following a stroke was specific for the dividing PCs within the neurogenic niches of the SVZ and SGZ. The loss of the cells in the SVZ resulted in a dramatic reduction in immature DCX+ neurons and absence of surviving adult-generated neurons in the peri-infarct area in v-TK rats. In contrast, v-WT rats had immature neurons (DCX+) cells, and a relatively low percentage of cells survived, of which few (<5%) adopted a neuronal fate. The relatively few adult-generated neurons in the cortex found in this study in the v-WT rat is in agreement with our previous work, as well as studies by others, that have shown in a variety of stroke models that the cells migrating from the SVZ to the cortex or striatum usually die, and only a few, if any, differentiate into neurons (Grégoire et al., 2015; Lindvall and Kokaia, 2015; Marlier et al., 2015). It remains unknown if these migrating cells can fully integrate into the cortical circuitry post-stroke (Arvidsson et al., 2002; Liu et al., 2009b; Kreuzberg et al., 2010; Faiz et al., 2015). Clinical studies have examined this question, and post-mortem studies have identified cells

expressing immature neuronal markers surrounding cortical infarcts, yet, ^{14}C labeling studies have demonstrated a lack of adult–generated cells in the cortex (Jin et al., 2006; Huttner et al., 2014). The combination of these preclinical and clinical findings makes it unclear whether cortical adult neurogenesis occurs post-stroke. However, this body of work suggests that when it is observed, stroke-induced neurogenesis surrounding the infarct is relatively small in magnitude compared to the large number of neurons lost by stroke, and is thus unlikely to have a significant role in neuronal replacement to induce functional recovery.

One of the most striking differences between the v-WT and v-TK rats following a stroke was the complete ablation of neurogenesis within the dentate gyrus, which is in alignment with a similar effect found in naïve v-WT and v-TK rats previously reported (Snyder et al., 2016). A significant increase in bilateral hippocampal neurogenesis post-stroke has been hypothesized to be functionally important in hippocampal-dependent memory after global and focal ischemia (Raber et al., 2004; Lagace, 2012; Sun et al., 2013). In the complete absence of hippocampal neurogenesis during stroke recovery, we find no significant differences in contextual fear conditioning or spatial learning/reference memory on the Barnes maze tasks in the v-TK rats. These findings align with the lack of any spatial behavioural task differences that was found in an independently generated GFAP-TK rat in the absence of stroke (Groves et al., 2013). The debate regarding the role of hippocampal adult neurogenesis in learning and memory is ongoing with data conflicting from both computational models and behavioural outcomes with loss- and gain-of-function models under normal physiological conditions (Cameron and Glover, 2015; Hersman et al., 2015; Ramsaran and Frankland, 2016). A recent study using two-photon calcium imaging to monitor the activity of young adult-generated hippocampal granule cells *in vivo* has strengthened the hypothesis that adult-generated granule cells are involved in encoding of new contextual

information (Danielson et al., 2016). The cognitive tests utilized in this study were not designed to specifically examine context encoding and discrimination, or pattern separation, which might be more likely to reveal deficits. However, testing this hypothesis is likely a low priority for stroke recovery research, because slower information processing and diminished executive functioning are the two most predominant types of cognitive deficits post-stroke (Cumming et al., 2013).

The rise in the number of patients surviving after a stroke due to advances in acute stroke care and the growth of our aging population has led to a heightened critical need to discover treatments for stroke recovery (Corbett et al., 2015; Bernhardt et al., 2016). The discovery of ischemia-induced neurogenesis raised hope that the host stem and PCs are part of the mechanisms of plasticity that contribute to innate recovery and could be boosted and harnessed to develop treatments to promote recovery. This study, however, provides direct evidence that progenitor cells at the SVZ and SGZ, and the new neurons they generate, are not required for recovery in a rat focal cortical stroke model. This finding does not preclude that specifically enhancing neurogenesis through promoting the long-term survival, differentiation and integration of the PCs into the potential restrictive characteristics of the regenerative niche can promote recovery. Testing this hypothesis will be important for the translation of preclinical stroke recovery work since creating stroke-induced newborn neurons may make significant gains in enhancing stroke recovery.

Figure Legends

Figure 1. Treatment with VGCV in GFAP-TK rats ablates proliferating cells at the SVZ and SGZ without altering the number of cells within the peri-infarct region at 1 week post-stroke. BrdU was administered to 15-week-old rats, 1 week post-stroke and 2 hours prior to sacrifice to assess cell proliferation. BrdU+ cells are shown in the representative images of the **(A)** SVZ, **(C)** SGZ, and **(E)** peri-infarct regions. **(A-B)** In the SVZ, the number of BrdU+ cells was significantly diminished in the v-TK rats compared to v-WT controls (n=4 per group, scale bar = 200 μm, inset scale bar = 100μm). **(C-D)** In the SGZ, no BrdU+ cells were present in the v-TK rats (n=4 per group, scale bar=200μm). **(E-F)** In the peri-infarct region both the v-WT and v-TK rats have similar number of BrdU+ cells (n=2 v-WT; n=3 v-TK). (Scale bar = 200μm) LV = Lateral Ventricle; H = Hilus; CC = Corpus Callosum.

Figure 2. 6-week-old surviving BrdU labeled cells in the SGZ have a neuronal phenotype in the v-WT rats.

(A-B) Representative images and quantification of the number of 6-week-old BrdU+ cells in the SGZ in v-WT and v-TK littermate rats revealed a significant reduction in the v-TK rats (Scale bar= 50μm). **(C-D)** Representative confocal image and quantification of the number of triple-labeled (BrdU, NeuN, and DAPI) cells showing that almost all surviving cells in the SGZ of the v-WT rats have a neuronal phenotype (Scale bar = 100μm). (n=3-4 per group).

Figure 3. The v-TK rats have a reduced migration response from the SVZ and no neurogenesis in the peri-infarct region.

(A-B) Representative image and quantification of BrdU+ cells revealed both v-WT and v-TK have similar number of 6-week-old surviving BrdU cells within the peri-infarct region (Scale bar = 20 μm). **(C-D)** Representative confocal image of BrdU, GFAP and DAPI staining and analysis revealed that the majority of BrdU+ cells express the astrocyte marker GFAP in both the v-WT and v-TK rats (Scale bar = 50 μm). **(E-F)** Representative image of BrdU, NeuN, and DAPI staining and quantification revealed few 6-week-old BrdU cells in v-WT rats colocalized with NeuN compared to none in the v-TK rats (Scale bar = 10 μm). **(G-H)** Representative image and quantification of DCX expressing cells revealed a significant reduction in v-TK rats compared to v-WT littermates (Scale bar =100μm). (n=3-4 per group).

Figure 4. v-TK and v-WT rats have similar locations and volumes following a ET-1 induced cortical stroke.

(A) Representative schematic diagram of brain sections revealing the minimum, average, and maximum area of strokes. **B)** Quantification of hemispheric lesion volume reveals no difference between v-WT and v-TK rats (n=8 v-WT, n=15 v-TK).

Figure 5. v-TK and v-WT rats have significant long-lasting post-stroke sensorimotor deficits post stroke.

(A-B) During staircase training both the v-WT and v-TK rats show similar increases in number of pellets retrieved on both the contralateral and ipsilateral side during the 11 days. The dotted line

at 15 pellets represents the mean number of pellets over the last 3 days of training that must be retrieved to be classified as having learned the task prior to stroke. **(C)** Assessment of the contralateral forelimb staircase performance reveals significant deficits that are sustained up to 7 weeks post-stroke without any differences between v-WT and v-TK rats. **(D)** Assessment of the ipsilateral forelimb staircase performance reveals minor deficits that are not sustained to 7 weeks post-stroke. **(E)** Assessment of percent use of forelimb on the cylinder test reveals significantly reduced use of impaired paw with **(F)** a corresponding increased use of the unimpaired paw. **(G)** Assessment of contralateral hindlimb on the beam test reveals a significant and sustained decline in performance for both v-WT and v-TK rats. **(H)** Assessment of ipsilateral hindlimb on the beam test reveals a transient deficit in hindlimb errors that is significantly different from baseline at 1 week post-stroke. (n=8 v-WT; n=15 v-TK). B=Baseline; *Denotes significant difference from baseline.

Figure 6. v-TK and v-WT rats post-stroke have similar spatial learning and memory abilities as assessed by the Barnes maze.

In the training phase of the Barnes maze, assessment of **(A)** mean number of errors, **(B)** latency to escape and **(C)** pathlength reveal significant improvement in performance over time, in the absence of any difference between v-WT and v-TK rats. **(D)** In the probe trial both v-WT and v-TK rats spent significantly more time in the target zone compared to the opposite zone, as also shown by **(E)** the heat-map images. In the reversal phase of Barnes maze, assessment of **(F)** mean number of errors, **(G)** latency to escape and **(H)** pathlength revealed a difference in performance over time in the absence of any difference between v-WT and v-TK rats. (n=15 v-WT; n=16 v-TK).

Figure 7. v-TK and v-WT rats post-stroke have similar associative learning and memory abilities as assessed by the fear conditioning test.

(A) In the novel context on day 1 of the fear conditioning protocol, both v-WT and v-TK rats freeze <10% prior to the exposure to a foot shock. **(B)** On day 2 of the protocol both the v-WT and v-TK rats freeze equally in response to being exposed to the same context that was paired with the shock. (**C)** On day 3 of the protocol, in a novel environment, both the v-WT and v-TK rats had a significant increase in freezing in response to the cue (tone) that was associated with the shock (n=15 v-WT; n=16 v-TK).

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Figure 1

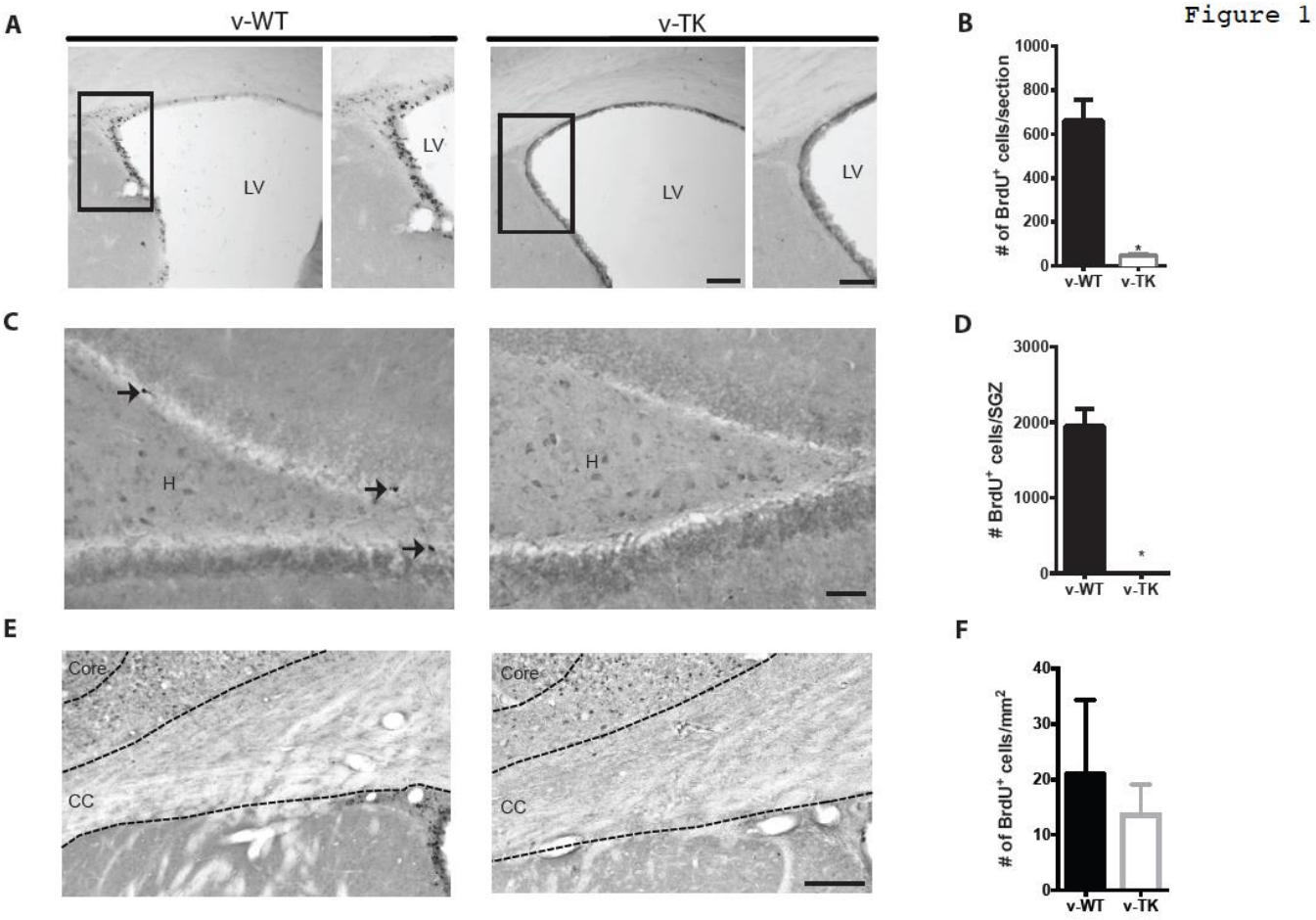
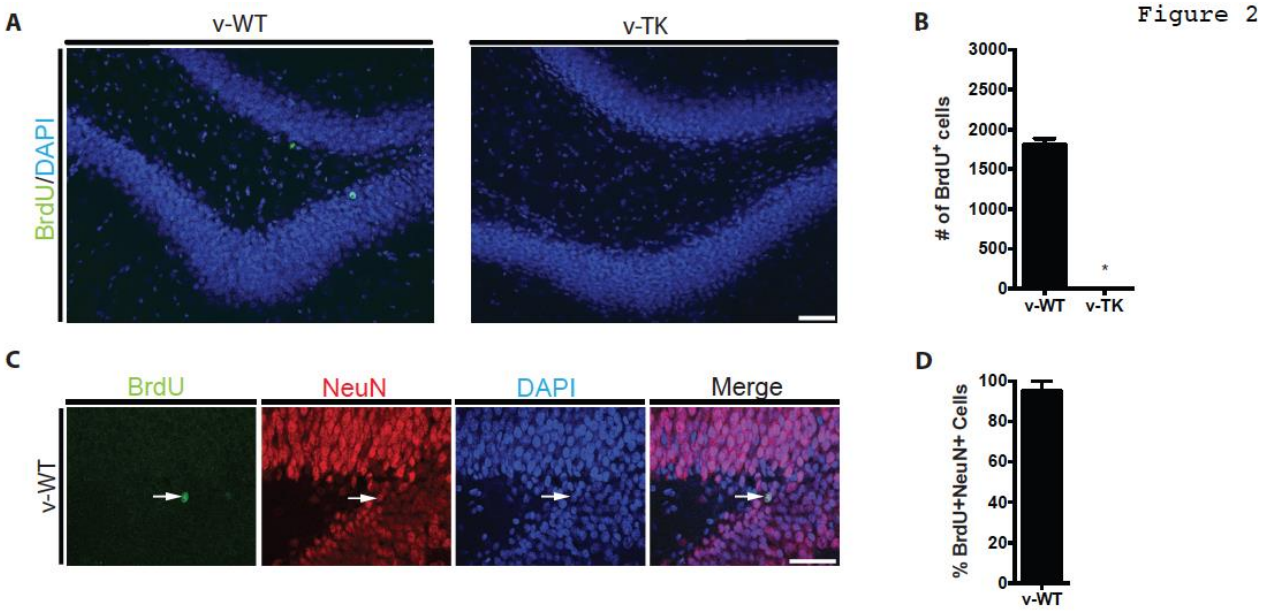
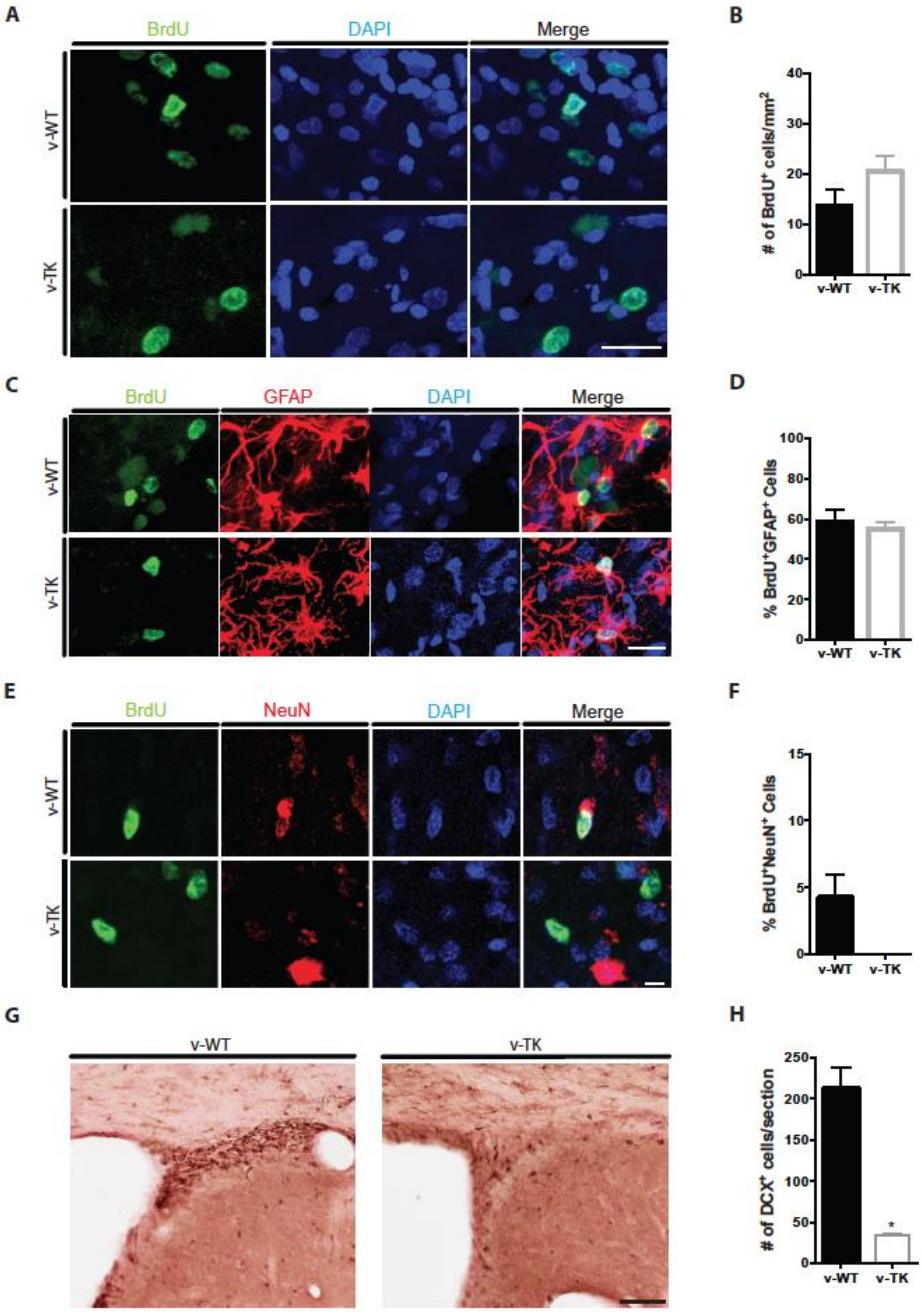

A
v-WT
v-TK
LV
LV
LV
LV
B
# of BrdU+ cells/section
1000
800
600
400
200
0
*
v-WT
v-TK
C
H
H
D
BrdU+ cells/SGZ
3000
2000
1000
0
*
v-WT
v-TK
E
Core
CC
Core
CC
F
# of BrdU+ cells/mm2
40
30
20
10
0
v-WT
v-TK

Figure 2


A
v-WT
v-TK
BrdU/DAPI
B
# of BrdU+ cells
3000
2500
2000
1500
1000
500
0
v-WT
v-TK
*
C
BrdU
NeuN
DAPI
Merge
v-WT
D
% BrdU+NeuN+ Cells
100
80
60
40
20
0
v-WT


A
BrdU
DAPI
Merge
v-WT
v-TK
B
# of BrdU+ cells/mm2
40
30
20
10
0
v-WT
v-TK
C
BrdU
GFAP
DAPI
Merge
v-WT
v-TK
D
% BrdU+GFAP+ Cells
100
80
60
40
20
0
v-WT
v-TK
E
BrdU
NeuN
DAPI
Merge
v-WT
v-TK
F
% BrdU+NeuN+ Cells
15
10
5
0
v-WT
v-TK
G
v-WT
v-TK
H
# of DCX+ cells/section
250
200
150
100
50
0
*
v-WT
v-TK

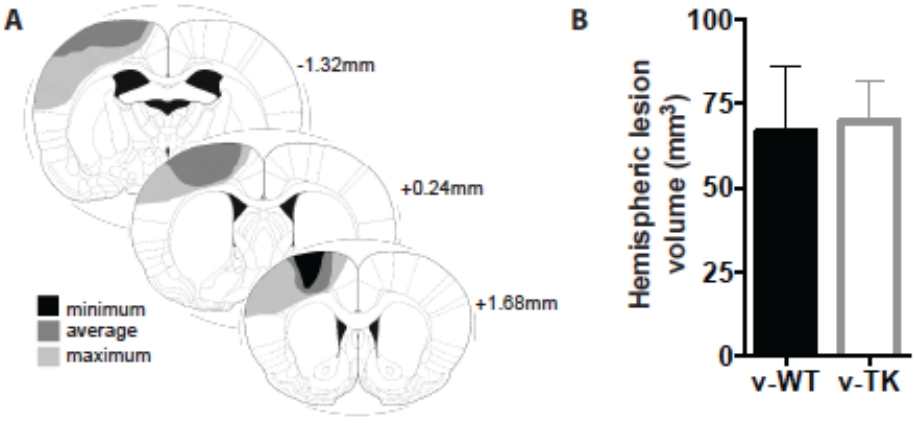

A
-1.32mm
+0.24mm
+1.68mm
minimum
average
maximum
B
Hemispheric lesion volume (mm³)
100
75
50
25
0
v-WT
v-TK

Figure 5

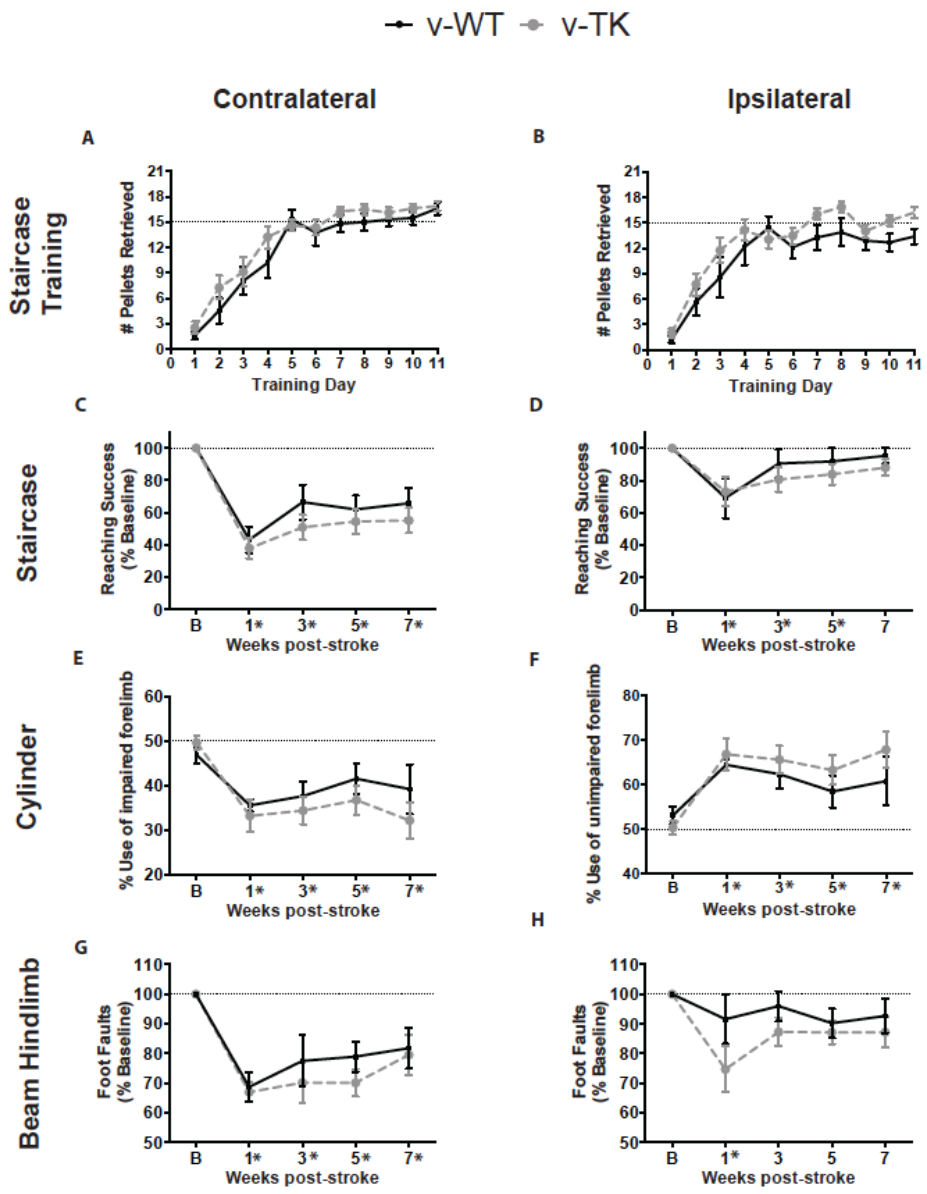

v-WT
v-TK
Contralateral
Ipsilateral
A
B
C
D
E
F
G
H
Staircase Training
Staircase
Cylinder
Beam Hindlimb
Pellets Retrieved
Training Day
Reaching Success (% Baseline)
% Use of impaired forelimb
% Use of unimpaired forelimb
Foot Faults (% Baseline)
Weeks post-stroke
B
1*
3*
5*
7*
7

Figure 6

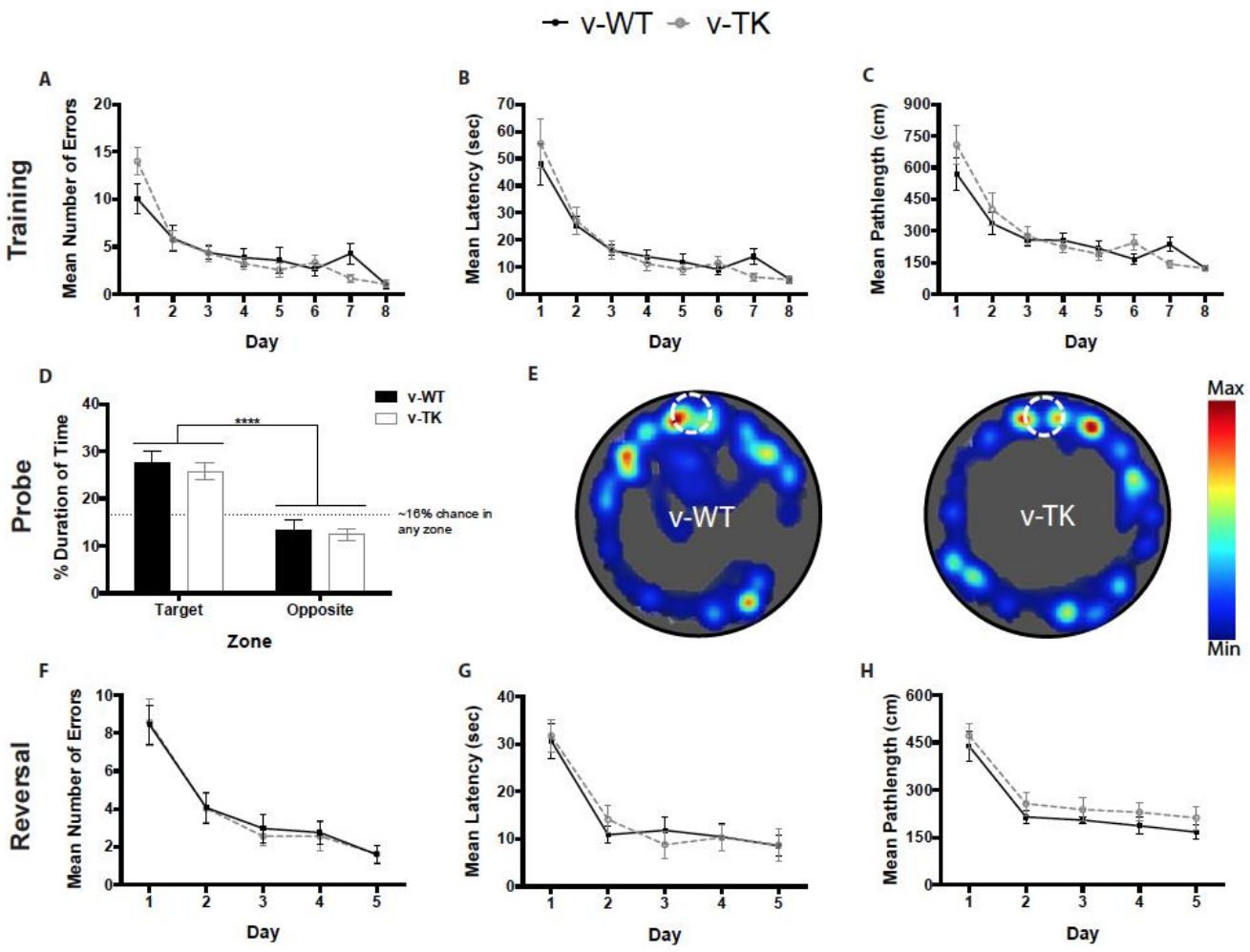
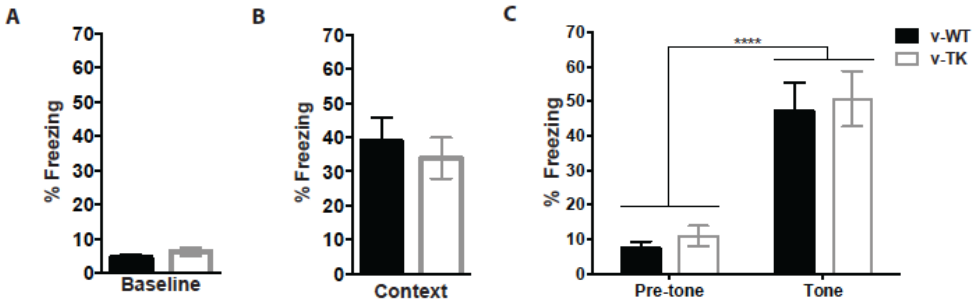

v-WT
v-TK
A
Training
Mean Number of Errors
Day
B
Mean Latency (sec)
Day
C
Mean Pathlength (cm)
Day
D
Probe
% Duration of Time
v-WT
v-TK
****
~16% chance in any zone
Target
Opposite
Zone
E
v-WT
v-TK
Max
Min
F
Reversal
Mean Number of Errors
Day
G
Mean Latency (sec)
Day
H
Mean Pathlength (cm)
Day

Figure 7


A
% Freezing
70
60
50
40
30
20
10
0
Baseline
B
% Freezing
70
60
50
40
30
20
10
0
Context
C
% Freezing
70
60
50
40
30
20
10
0
****
v-WT
v-TK
Pre-tone
Tone