The Functional Role of Enhancing the Survival and Activity of Progenitor Cells During Stroke Recovery

Sébastien Denizé

Thesis submitted to the Faculty of Graduate and
Postdoctoral Studies in partial fulfillment of the requirements for the
Master of Science in Neuroscience

Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

Abstract

Following a stroke, there is a significant increase in the number of precursor cells (PCs) that migrate to the site of injury. Their functional significance, based on studies that ablate PCs, remains controversial. This thesis aims to determine if enhancing the survival of PCs, and/or optogenetically stimulating PCs, is sufficient to improve stroke recovery. After photothrombosis-induced stroke, tamoxifen was used to recombine the nestin-expressing PCs and their progeny in iBax-ChR2 mice: inducible Nestin CreER$_{T2}$ mice that have a floxed *Bax*, pro-apoptotic gene, and a floxed channelrhodopsin (ChR2-YFP) which allows for the modulation of neural activity. Removal of the *Bax* gene results in a significantly increased number of PCs with a neuronal phenotype surrounding the infarct, in the absence of altering behavioral recovery. To test if enhancing the activation of PCs around the stroke site could alter recovery, iBax-ChR2 mice received daily optogenetic stimulation for 5 weeks. Interestingly, stimulation of PCs around the stroke site resulted in an increased rate of neuronal differentiation in WT-ChR2 mice but no behavioural improvements after stroke. These findings suggest that substantially increasing the neurogenic response following a stroke has no impact on behavioural recovery. Moreover, contrary to an increasing number of studies stimulating the cortex post-stroke, specifically activating PCs in the ipsilesional cortex does not alter recovery. The implications of these data are becoming increasingly relevant as stroke researchers are beginning to experiment with non-invasive stimulation and stem cell therapies for stroke survivors.

Table of Contents

List of Figures

List of Abbreviations

ABC	Avidin-Biotin Complex
ANOVA	Analysis of Variance
BrdU	Bromodeoxyuridine
C-14	Radioactive Carbon
ChR2	Channelrhodopsin
CIMT	Constraint-induced movement therapy
DAB	Diaminobenzidine
DAPI	4',6-diamidino- 2-phenylindole, dihydrochloride
DCX	Doublecortin
DPX	Distyrene, a Plasticizer, and Xylene
DREADD	Designer receptors exclusively activated by designer drugs
FACS	Fluorescence-activated cell sorting
fMRI	function Magnetic Resonance Imaging
GFP	Green Fluorescent Protein
iBax	inducible Bax transgene
IEG	Immediate Early Gene
IHC	Immunohistochemistry
iM1	ipsilesional Primary Motor Cortex
IP	Intraperitoneal injections
KO	Knockout
MCAo	Middle Cerebral Artery occlusion
MEP	Motor-evoked potential

NDS	Normal Donkey Serum
NSC	Neural Stem Cells
OB	Olfactory Bulb
PBS	Phosphate buffered saline
PC	Precursor Cell
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PT	Photothrombosis
RFP	Red Fluorescent Protein
SBR	Spontaneous biological recovery
SEM	Standard error of the mean
SGZ	Subgranular zone
SVZ	Subventricular zone
TAM	Tamoxifen
tDCS	transcranial Direct Current Stimulation
tMCAo	transient Middle Cerebral Artery occlusion
TMS	Transcranial Magnetic Stimulation
WPS	weeks post-stroke
WT	Wild-Type

Acknowledgements

Coming from an exercise physiology background into the field of Neuroscience was no easy task. First and foremost, without the love and support of my friends and family I definitely would not have been able to complete this degree. All of the members of the Lagace lab (past and present) have made the journey all the more worth it: Maheen Ceizar, Marc Vani, Timal Kannangara, Catherine Gnyra, Yingben Xue, Alëna Kalinina, Véronique LaFrance, Patricia Barra De La Tremblaye, Mikaël Ladouceur, Aisha Hufnagel, Damian Chwastek and, of course Dr. Diane Lagace. Whether it be distracting me from the stress of courses and deadlines or helping me with various techniques within the lab, their presence has made the last couple years fun and engaging.

Although we unfortunately never had the chance to work together, this project could not have been possible without the tireless efforts of Maheen Ceizar who laid much of the groundwork for my project. I would also like to take a moment to thank our lab technician, Yingben, who never hesitated to help with planned or unplanned experiments on top of genotyping all of the mice in the lab including my own.

I would also like to thank my thesis advisory committee, Greg Silasi and Jean-Claude Béïque, for giving me invaluable advice and being available for questions outside of our regular meeting times.

Finally, I would like to thank Diane Lagace. Thank you for believing in me even when I did not believe in myself. Throughout the course of my degree you have worked tirelessly day and night to help me become a better researcher and person and words cannot describe the appreciation I have for you as a mentor.

Introduction

1.1 Stroke

Stroke is the leading cause of adult long-term disability and results from the interruption of blood supply to the brain (Krueger et al., 2015). There are two major types of strokes, ischemic and hemorrhagic (Carmichael, 2016). Hemorrhagic strokes are less common compared to ischemic stroke and result from a ruptured blood vessel. In contrast, the more common ischemic strokes occur when a vessel is obstructed. In both instances, this reduction in blood supply results in the death of brain tissue that is not sufficiently perfused. Depending on the length of time without reperfusion, as well as the area of damage, this can result in a variety of cognitive and physical disabilities in stroke survivors (Chrichton, et al., 2016).

Largely due to advances in medical interventions for acute stroke, the mortality rate from stroke has reduced. Indeed, a stroke review paper found that the mortality rate within North America is estimated at 9%, suggesting that the vast majority of patients survive after a stroke (Krueger et al., 2015). For example, in Canada in 2015, it was estimated there are nearly 405,000 stroke survivors. In the same review, it was estimated that the number of stroke survivors will increase to 725,000 by 2038 in Canada alone. Unfortunately, approximately 40% of these stroke survivors will be unable to perform activities of daily living 5 years after their stroke. Stroke survivors are often plagued by reduced quality of life and a variety of disabilities such as hemiparesis, reduced cognitive function, and depression. The only approved treatment for recovery is rehabilitation therapy, which can improve some of these disabilities, yet there are varying degrees of improvement and often survivors remain with long-term disability following stroke therapy (Krakauer, Carmichael & Wittenberg, 2012; Boyd et al., 2017).

1.2 Spontaneous Biological Recovery

Remarkably, most stroke survivors experience an innate recovery in function in the absence of any treatment, that is often referred to as spontaneous biological recovery (SBR) (Carmichael, 2016; Murphy & Corbett, 2009). Unfortunately, the SBR that occurs post-stroke is limited, since it only occurs during the first 3 months following a stroke in humans. This time period is often referred to as the "critical time window" for recovery, that results in a plateau of functional recovery following the 3 month early sub-acute period of stroke recovery (Bernhardt et al., 2017). Since rehabilitation paradigms are often unable to alter this critical window of recovery, this has led to the suggestion that there is a limit on how much recovery can occur.

Within the past decade, there has been an ongoing debate regarding the degree of recovery that can occur following a stroke. In 2008, a theory coined the "proportional recovery rule" was proposed, suggesting that patients with an intact corticospinal tract would achieve a 70% improvement in motor function within the first 3 months following their injury (Prabhakaran et al., 2008). This theory has since been observed robustly in patients with non-severe upper limb motor impairments (Coupar et al., 2012) and pre-clinical models of stroke (Jeffers, Karthikeyan, & Corbett, 2018). Recent work within the field of stroke recovery has suggested that there is a mathematical bias in this theory, leading to the suggestion that recovery from a stroke is in fact much more difficult to predict (Lam et al., 2018). Moreover, recovery in function is not homogeneous as there is significant variance in the recovery profiles of stroke patients. Although it may be too early to predict the degree of recovery from a stroke, the general consensus within the field is that recovery does occur following most strokes (Bernhardt et al., 2017; Langhorne, Bernhardt, & Kwakkel, 2011).

Given that SBR occurs in so many different clinical and preclinical animal models, a number of studies have directed efforts towards identifying and harnessing the mechanisms responsible for innate recovery. This was done with the hope of discovering new treatments to improve the quality of life for stroke survivors. These studies in general have shown that SBR is due to many forms of plasticity, including cortical reorganization, angiogenesis, and adult neurogenesis.

Gertes and Ward (2013) examined the extent of cortical reorganization post-stroke within stroke survivors using fMRI and non-invasive brain stimulation techniques. The results of this work found that early post-stroke, there is an increase in bilateral neural activity while performing unilateral tasks using the paretic limb. The experimenters clearly showed that increased activity in the contralesional hemisphere was associated with worse stroke outcomes. Moreover, this study found that chronic stroke survivors had less contralateral activity than acute stroke patients. In rodent stroke models, ischemic ablation of the forelimb sensorimotor cortex resulted in the recruitment of corticospinal neurons within the hindlimb sensorimotor region (Starkey et al., 2012). Non-invasive brain stimulation studies have also demonstrated that reducing the overactivity within the contralesional forelimb sensorimotor cortex can improve functional outcomes during SBR (Bradnam, Stinear, & Byblow, 2013). In addition to cortical reorganization, increased angiogenesis is significantly correlated with improved stroke recovery outcomes and the removal of angiogenic mechanisms is correlated with the worsening of stroke outcomes (Gertz et al., 2006). Importantly, angiogenesis is also recognized as a requirement for the neuronal remodeling that occurs post-stroke (Hatakeyama, Ninomiya, & Kanazawa, 2020). Specifically, the increased oxygenation that occurs as a result of neovascularization can enhance neurogenesis post-stroke by accelerating the neuronal

differentiation of these newborn cells. These studies all indicate that SBR is most likely the result of a multitude of different forms of plasticity that ultimately result in functional improvements.

1.3 The Generation of Adult-Born Cells in Response to Stroke

Within the naïve adult brain, the subventricular zone (SVZ) lining the lateral ventricles of the forebrain and the dentate gyrus of the hippocampus have been identified as neurogenic niches (Bond, Ming, & Song, 2015). Studies have also suggested that other neurogenic niches may exist in a variety of regions in the brain, but more evidence is needed to corroborate these findings (Lu, Manaenko, & Hu, 2017). A review paper by Lu and colleagues (2017) described the maturation of newborn cells within both neurogenic niches. Within the subgranular zone (SGZ) of the hippocampus, neural stem cells (NSCs) give rise to the precursor cells (PCs), which proliferate giving rise to neuroblasts. The neuroblasts develop into immature neurons that radially migrate into the granule cell layer to differentiate into mature dentate granule neurons. These adult-generated cells in the hippocampus in general have a role in learning and memory, including both spatial memory and pattern separation. In the SVZ of the lateral ventricle, there are also NSCs that give rise to amplifying PCs which give rise to neuroblasts. These cells migrate through the rostral migratory stream to the olfactory bulb (OB) where they differentiate into interneurons. Ablation studies have also shown that these adult-generated interneurons within the OB are involved in short term olfactory memory as well as olfactory discrimination tasks (Alonso et al., 2012).

The majority of experiments studying adult neurogenesis have been restricted to rodents due to the inability to visualize these cells in the live human brain. Studies of adult neurogenesis

in the human brain have thus been limited to post-mortem analysis, including recent controversial studies (Kumar, Pareek, Faiq, Ghosh, & Kumari, 2019). This included a study by Sorrells and colleagues (2018) which examined the levels of hippocampal neurogenesis in post-mortem tissue from healthy and epileptic patients that suggested neurogenesis occurs at elevated rates during gestation until the first few years of life when it begins to sharply decline to negligible levels by the age of 18. In contrast, Boldrini and colleagues (2018) examined levels of neurogenesis within the hippocampi of healthy subjects ranging from 14-79 years of age and suggested that neurogenesis decreases from gestational levels but remains prominent relatively consistently throughout life. The conflicting results of these studies likely stem from the source of post-mortem tissue samples, since the inclusion of brain samples from patients with neurological disorders could significantly alter relative levels of neurogenesis (Kempermann et al., 2018). Recently, another study by Moreno-Jiménez and colleagues (2019) provided support for this explanation by demonstrating a prominent level of neurogenesis in healthy adult brains compared to the brains of Alzheimer's patients. Thus, overall it seems that neurogenesis does occur in the healthy human brain and it will remain a future challenge to be able to visualize these cells using live imaging techniques.

Following a stroke, in both preclinical and post mortem human studies, a significant increase in the proliferation of NSCs and PCs has been observed. This has been characterized by preclinical studies that have consistently demonstrated that this increase occurs in both the SVZ and SGZ region, following a variety of stroke models. Additionally, it has been extensively characterized that the PCs from the SVZ can ectopically migrate to the peri-infarct region, which is the site surrounding the stroke-injured brain tissue (Gotts & Chesselet, 2005). Post mortem studies have also shown that there is an increase in the proliferation of PCs in the

adult brain post-stroke (Macas, Nern, Plate, & Momma, 2006), and a significant number of immature neurons surrounding the stroke site (Jin et al., 2006). Examination of brain tissue more than one month post-stroke has also shown that there is a significantly reduced proportion of newborn neurons within the cortex after stroke (Nakayama et al., 2010). However, work from our laboratory and others have demonstrated that the neurogenic response to stroke is fairly weak even in preclinical models, since the majority of migrating PCs do not survive within the peri-infarct (Kannangara et al., 2018).

Of the small percentage of the PCs that do survive within the peri-lesioned area, preclinical work has demonstrated that the majority of the cells are fated to become astrocytes with only a small proportion having a neuronal phenotype (Kannangara et al., 2018; Lindvall & Kokaia, 2015). Additionally, recent work from our lab has examined the integration of adult-generated cells that migrate to the cortex using a combination of immunohistochemical and electrophysiological analysis. This study used both the Nestin-GFP and NestinGFP/DoublecortinDsRed reporter mouse models, to show that a limited number of the adult-generated neurons expressing the immature neuronal marker, doublecortin (DCX) at the peri-infarct can fire an action potential (Kannangara et al., 2018). These data suggest that the cells sparsely integrate into the surrounding cortex. Additionally, the immature neurons were characterized to be excited by GABA and had properties of an interneuron. These results raise the interesting hypothesis that, SVZ-derived PCs deviate from their usual path to the OB, yet maybe their fate does not change and as they continue to differentiate into different subtypes of interneurons. Since this work was completed in reporter mice models, the analysis could only be done up to 4 weeks post stroke. Thus, the long-term function and integration of adult-

generated immature neurons that migrate to the site of stroke damage remains largely unknown.

1.4 The Function of Adult-Generated Cells During Stroke Recovery

To test the requirement of adult-generated cells during stroke recovery, A number of studies have used different knockout models of neurogenesis in combination with various preclinical stroke models to suggest that these cells are required for SBR (Raber et al., 2004; Jin et al., 2010; Wang et al., 2012; Sun et al., 2013). All of the studies described below have suggested that adult neurogenesis plays a role in recovery following stroke, albeit some suggest they are important for motor recovery, whereas others suggest they only have a role in cognitive recovery.

In 2004, Raber and colleagues were the first to hypothesize that neurogenesis was necessary for stroke recovery through examination of a conditional ablation of PCs in gerbils that had a bilateral common carotid artery occlusion. This ablation model did not impair motor recovery but resulted in significant deficits in spatial learning and memory compared to WTs. More recently, transgenic mouse models have been used to specifically ablate PCs. One of these models included the use of a DCX-TK mouse which allows for specific ablation of PCs expressing the immature neuronal marker doublecortin (DCX) (Jin et al., 2010; Wang et al., 2012). This work showed that following a middle cerebral artery occlusion (MCAO), the DCX knockout (KO) mice had significant motor impairments compared to littermate controls. A more robust Nestin-TK mouse model also resulted in no motor differences in mice post-stroke, following KO of nestin-expressing PCs, yet found significant differences in spatial learning and memory (Sun et al., 2013).

An important distinction that remains understated is that we are still unaware of the role of these PCs following their migration to the site of injury. Results from the studies using KO mouse models, have resulted in conclusions that "neurogenesis" plays an important role in stroke recovery. However, unless we know the extent of the integration of these newborn neurons within the stroke-injured cortex it may be too early to claim that they are required for recovery. The time it takes for these PCs to migrate and mature into functional neurons suggests that they may not in fact be involved in the initial recovery that is observed in the first month post-stroke. For example, the DCX-TK mice had very large lesion volumes, that are not representative of clinical populations and saw differences in the recovery of these mice at 2 days post-stroke (Jin et al., 2010) which is not even enough time for these PCs to migrate to the site of injury, let alone differentiate into immature neurons. Evidence outlined above stresses the importance of elucidating the specific role of the neurogenic response in improving stroke outcomes, whether this is through a direct or indirect effect of these cells.

Williamson and colleagues (2019) have recently argued in a review that there are four possible functions of PCs following stroke: cell replacement, cytoprotection, remodelling of residual tissue, and immunomodulation. Cell replacement refers to the capacity of PCs to replace tissue damage. As aforementioned, experiments within our lab and others have shown that very few of these PCs differentiate into functional neurons within the stroke-injured cortex 4 weeks post-stroke (Kannangara et al., 2018). The small percentage of cells that do have a neuronal phenotype receive very few synaptic inputs from their surroundings. These results indicate that PCs have sparse integration into the peri-infarct region. Indeed, no study has tested the behavioural contributions of PC activity on functional recovery. The cytoprotective role of PCs following stroke has also been supported by NSC transplantation and PC ablation studies

that suggest that both exogenous and endogenous PCs can have a neuroprotective role after a stroke (Chrostek et al., 2019; Sun et al., 2013). The reduction in lesion volume and improved stroke outcome in many cases is also associated with the factors being secreted by the PCs (Williamson et al., 2019). For example, the secretion of endocannabinoids by SVZ-derived PCs regulates glutamatergic tone within the striatum and thus are hypothesized to reduce tissue damage (Butti et al., 2012). More work is required to identify which factors secreted by endogenous PCs have neuroprotective properties. Reorganization of the connectivity of residual neurons surrounding the stroke site is another proposed role of the PCs post stroke. Migrating PCs are capable of secreting neurotrophic factors to facilitate the plasticity that occurs post-stroke. The secretion of factors such as, vascular endothelial growth factor and brain-derived neurotrophic factor, have been identified as improving stroke outcomes (Andres et al., 2011). Lastly, more recent work suggests that the NSCs and PCs can exert potent immunomodulatory effects post-stroke. Following a stroke, there is a large inflammatory response that is hypothesized to be at first beneficial by reducing tissue damage, yet over time becomes detrimental for recovery (Williamson et al., 2019). PC transplantation studies have resulted in a significant reduction in this inflammatory response within both cortical and subcortical areas (Bacigaluppi et al., 2009). Specifically, immunomodulatory effects are exerted by reducing T-cell infiltration and promoting the migration of neuroprotective microglia rather than more crude phenotypes (Williamson et al., 2019). Thus, all together it appears that the contribution of PCs to stroke recovery likely occurs through a multifaceted mechanism.

1.5 Enhancing the Survival of Adult-Generated PCs Post Stroke Using iBax Mouse

It has been well characterized that a variety of drugs, such as antidepressants, and treatments such as exercise are capable of enhancing neurogenesis (Dranovsky & Hen, 2006). Given the hope that targeting neurogenesis would improve stroke recovery, a number of these methods have been applied to increase neurogenesis and examine its effect on stroke recovery. Although most of these studies show that enhancing neurogenesis through these indirect methods improves stroke recovery, they are all correlation studies (Lagace, 2012). Thus, they do not prove causation, meaning that it remains unknown if specifically increasing the number of adult-generated cells would be sufficient to improve stroke recovery.

Over the last few years our lab has directly tested whether enhancing the survival of adult-generated cells after stroke would improve functional recovery. This work formed the basis of a published PhD thesis (Ceizar, 2017) that utilized an inducible Bax KO (iBax) transgenic mouse model, that specifically promotes the survival of adult-generated PCs, in order to test their effect on behavioural recovery from a stroke (Figure 1). The iBax mouse model was first published by Sahay and colleagues (2011) and uses the Nestin-CreER$_{T2}$ transgene that allows for expression of Cre in PCs that express nestin (Figure 1A). This model also contains a floxed-STOP YFP transgene, and the floxed Bax gene, which allows for the expression of YFP and the removal of the pro-apoptotic Bax protein (Figure 1A). This results in an inducible mouse model in which the administration of tamoxifen (TAM) following stroke allows for the expression of YFP and the specific removal of Bax from PCs, to prevent the death of adult-generated cells post stroke (Figure 1B). In order to test if this model would have enhanced survival, a photothrombotic (PT) stroke was induced in the motor/sensorimotor cortex and then the animals were sacrificed at 4, 8 or 12 weeks post stroke (Figure 1C).

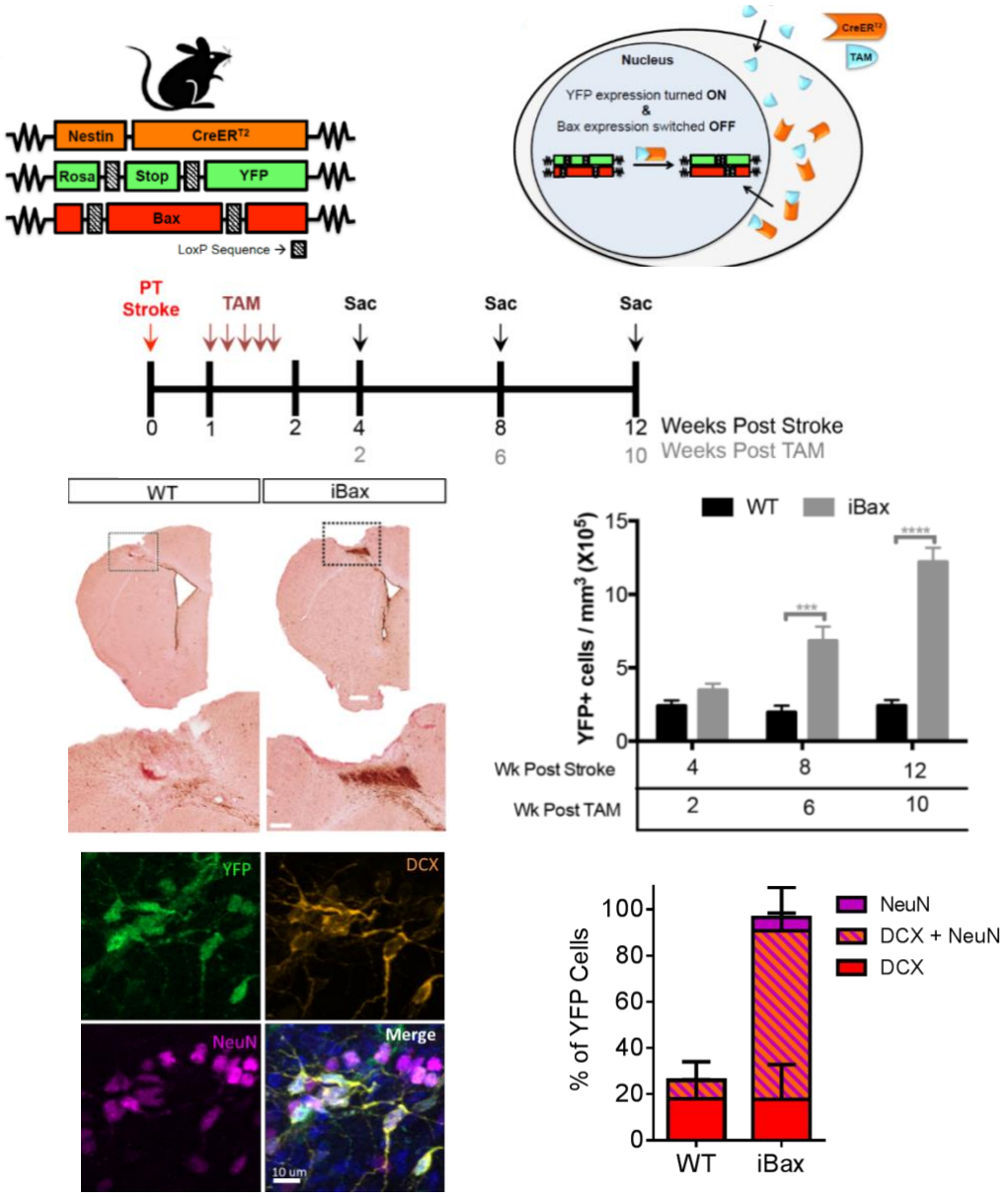

Figure 1. Tamoxifen-induced recombination of iBax mice results in an increased density of progenitor cells with a neuronal phenotype in the peri-infarct. (A) A triple transgenic iBax mousseline was generated by breeding Nestin-CreER^{T2} mouse, R26R-YFP reporter mouse and the floxed Bax mouse. (B) Administration of Tamoxifen (TAM) induced recombination in nestin-expressing PCs and resulted in the expression of YFP and removal of the *Bax* gene. (C) Experimental timeline showing iBax and WT mice were induced with photothrombosis (PT) strokes followed by TAM administration at one week post PT and then sacrificed at either 4, 8 or 12 wps. (D) Representative images and (E) quantification of YFP-positive PCs at the peri-infarct showing a significant increase in PCs in iBax mice at eight and 12 wps. (n=3 per group) (E) Immunohistochemical (IHC) stain illustrating expression of YFP, doublecortin (DCX) and NeuN. (F) Quantification of % co-localization of DCX and NeuN with YFP indicating increased neuronal differentiation in iBax mice. Scale Bar = 500 um (2.5); 200um (10x). *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001represents significance from WT.

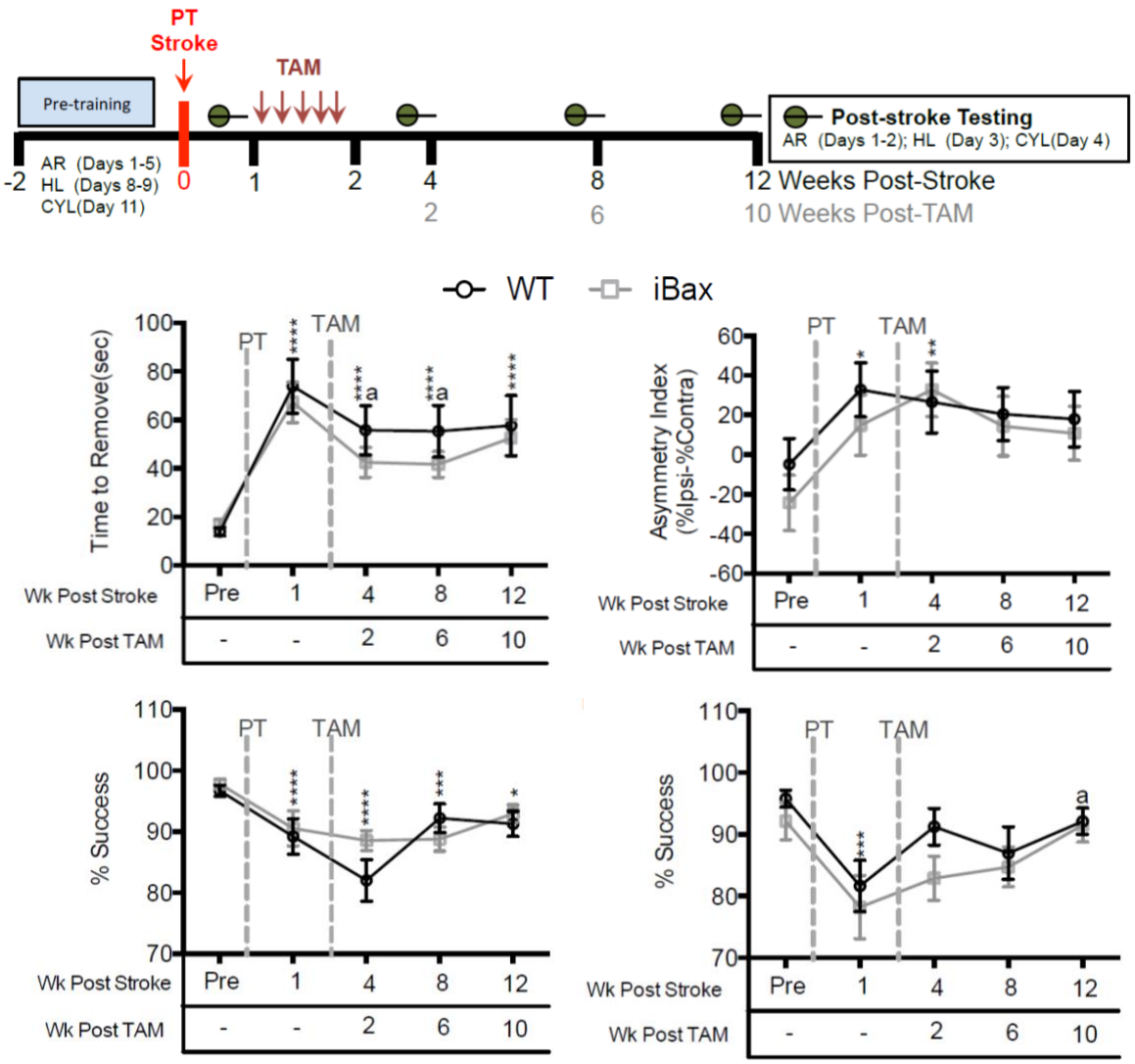

Figure 2. Behavioural testing revealed no significant difference in recovery from a stroke between iBax and WT mice. (A) Experimental timeline for WT (n=13) and iBax (n=17) mice which were trained on the adhesive removal (AR), horizontal ladder (HL) and cylinder (CYL) tests at 8 weeks old followed by PT strokes at 10 weeks. Following stroke, baseline deficits were recorded prior to tamoxifen (TAM) injections and post-stroke testing at 4, 8 and 12 weeks post-stroke. (B) Time to remove the tape on the contralateral side was significantly increased following stroke, with no differences between iBax and WT mice. (C) Percentage of time spent rearing with contralateral paw revealed significant stroke deficits but no group effect. There were significant contralateral (D) forelimb and (E) hind limb deficits with no differences between iBax mice and WT mice. (D) *p<0.005, **p<0.01, ***p<0.001, and ****p<0.0001 represents significance from pre-stroke performance. **a** = significance from one wps.

As expected, there was a robust and significant increase in the number of cells migrating to the peri-infarct region at 4, 8 and 12 weeks post-stroke in the iBax mice when compared to wild type (WT) controls (Figure 1D). Indeed, there was a more than 3-fold increase in PC migration to the peri-infarct region in the iBax mice compared to WT control mice at 12 weeks post stroke (Figure 1E). As described above, although other studies have established that the majority of migrating cells become astrocytes, this model revealed that when allowed to survive, the majority of the cells migrating to the stroke site expressed immature neuronal markers, such as DCX and the more mature neuronal marker, NeuN (Figure 1F,G). We hypothesized that the increased rate of neuronal differentiation in this model was due to the induction of recombination after stroke (i.e. treatment with TAM after stroke), as well as allowing the cell to survive. This hypothesis was developed since, previously, all other inducible mice models had induced recombination before stroke. In support of this hypothesis, inducing recombination in the same mouse model before stroke resulted in increased differentiation of the PC population into astrocytes. Ongoing work within the lab is aiming to determine why labeling the nestin-expressing PC population before stroke results in the majority of cells surrounding the lesion having an astrocyte fate, while labelling after stroke results in the majority of the cells having a neuronal fate.

Since the iBax model was able to allow for the enhanced survival of PCs after stroke that had a neuronal phenotype, this model was next utilized to test how increased survival of PCs would alter stroke recovery through a battery of behavioural assessments (Figure 2A). This was accomplished through use of a battery of sensorimotor tests that included: the adhesive test, cylinder test and ladder test, that all showed significant behavioral deficits at 1 week following PT-induced stroke (Figure 2B-2E) Examination of recovery on these tasks at 4, 8 and 12 weeks

post-stroke, surprisingly yielded no significant change in behavioural recovery between iBax mice and WT controls. Thus, this thesis concluded that promoting the survival of migrating adult generated neurons alone was not sufficient to promote stroke recovery.

1.6 Why Does Promoting Survival of Neuronal-Fated PCs Fail to Improve Recovery following Stroke?

The focus of this thesis was to work towards understanding why a significant increase in the number of cells located in the peri-infarct of iBax mice did not result in any behavioural improvements. **I hypothesized that surviving cells within the peri-infarct were unable to improve behavioural recovery from stroke due to a lack of integration and/or activation**. This hypothesis was based on earlier published work from our lab indicating that by 4 weeks post-stroke PCs are sparsely integrated into the cortex (Kannangara et al., 2018). This experiment revealed that a very small percentage of dividing PCs differentiate into GABAergic interneurons that are capable of producing action potentials but receive very few synaptic inputs. Since this work was completed in a Nestin/DCX reporter mouse, a small pilot experiment was performed to prove that PCs within the iBax model are phenotypically similar. Further support for my hypothesis that the surviving cells required more integration and/or activation, came from the Steinberg lab, which used optogenetics to stimulate transplanted NSCs within the stroke site. Stimulation of the transplanted NSCs resulted in improved functional recovery and a reduced inflammatory response (Daadi et al., 2016).

An alternative, but not mutually exclusive hypothesis, is that the cells may have sparse integration because they do not receive enough activation around the infarcted tissue. Midway through the acquisition of data for this thesis, an additional study by Liang and colleagues

(2019) provided further support for this hypothesis, by displaying that the extent and density of synaptic connections surrounding the peri-infarct is activity dependent. These results were proven using artificial activation with designer receptors exclusively activated by designer drugs (DREADDs), as well as through using constraint-induced movement therapy (CIMT) protocol to incite forelimb overuse. Both these results supported that activating the newborn cells resulted in improved recovery from a PT stroke.

A variety of models now exist to specifically activate cell types, making it possible to test in a preclinical model, if increased activation of adult-generated cells would improve recovery. This initially became possible with the introduction of optogenetics, which is the process of using light to directly control the activity of opsin-expressing cells (Butti et al., 2012; Deubner, Coulon, & Diester, 2019). Opsins are light-gated proteins which respond to light stimulation at a specific wavelength. In the case of channelrhodopsin (ChR2), stimulation of light between 470 - 540 nm will allow the resting membrane potential of neurons expressing this channel to be depolarized, resulting in an increased likelihood of firing an action potential. Within the fields of adult neurogenesis and stroke recovery, optogenetic stimulation has been utilized as a means of elucidating the role of various cell populations within the brain.

1.7 Optogenetic Stimulation of Cells Generated in the Adult Brain

A growing body of literature has examined the behavioral outcomes of *in vivo* optogenetic stimulation of adult-born cells. In 2016, Zhuo & Han (2016) were the first group to stimulate newborn cells within the hippocampus. In this study, young adult-born neurons in the hippocampus were silenced, resulting in significantly reduced performance on a location discrimination task, accompanied by significant changes in neural activity of the contralateral

hippocampus. Danielson and Kheirbek (2016) also used optogenetics to silence adult-generated cells in the dentate and showed that this produced significantly more freezing during a fear conditioning task. Interestingly, when the cells were transiently inhibited, this yielded no differences in performance during the conditioning context, as opposed to significantly impaired discrimination in the non-conditioned context. Together, these studies have suggested that the activation of adult-generated cells can improve function during pattern separation within ambiguous contexts in naïve animals. The results also suggest that newborn cells are capable of altering behavior in response to stimulation.

There is also a body of literature that has examined the effect of *in vivo* stimulation of SVZ-derived PCs. The first experiment to stimulate the SVZ-derived PC population was performed by Bardy and colleagues (2010). They demonstrated that the synaptic activity of new neurons in the olfactory bulb (OB) could be optically controlled and that the young interneurons were capable of releasing inhibitory GABA signals to their respective postsynaptic targets. They also reported that the stimulation of adult-born neurons resulted in improved integration of these cells into the OB with synaptic connections that modified the activities of principal cell types and of local interneurons. Alonso and colleagues (2012) added to these findings when they showed that optogenetic stimulation of the adult-born neurons in the olfactory bulb accelerated learning in an olfactory discrimination-learning task and improved olfactory memory. Interestingly, this effect only manifested itself when the newborn neurons were activated, at a high frequency (40 Hz), and in concurrence with the presentation of the odors, but failed when presentation of the odors was delayed. This suggests that SVZ-derived PCs respond best to high-frequency stimulation when paired with a relevant behavioral task.

1.8 Optogenetics and Stroke Recovery

There is also a growing body of literature that has tested the effects of optogenetic stimulation to enhance stroke recovery. This work was initiated due to the increasing use of transcranial magnetic stimulation (TMS) and transcranial direct current stimulation (tDCS) for stroke recovery within clinical populations (Dionísio, Duarte, Patrício, & Castelo-Branco, 2018). The rationale for this stimulation treatment post stroke was based on the need to regulate the imbalance in neuronal activity between the hemispheres in the brain after stroke (Spalletti et al., 2017). Specifically, function MRI (fMRI) studies have clearly shown increased activity in the contralateral hemisphere of stroke patients performing a unilateral task with their impaired limbs (Fisicaro et al., 2019). Consequently, stimulation of the ipsilesional hemisphere and inhibition of the contralesional hemisphere have resulted in improved stroke outcomes (Grefkes & Ward, 2019). However, current metareviews suggest that the findings are promising, albeit have inconsistent effects (Fisicaro et al., 2019). Moreover, since these treatments stimulate a large number of cells, it is very difficult to determine the mechanisms through which they are exerting their effects.

Optogenetics allows for increased spatiotemporal control of neuronal activity and thus can help elucidate the specific contributions of distinct cell populations for stroke recovery. As recently reviewed, preclinical work using optogenetic stimulation to improve stroke recovery has shown several effective target circuits that have the potential for neuromodulatory intervention in the brain after stroke (Lu et al., 2017). These targets include the ipsilesional primary motor cortex (iM1), the efferent corticospinal circuits, and the afferent (thalamocortical) circuits to the motor area. In 2015, Gary Steinberg's lab first revealed that stimulation of Thy1-expressing neurons of the ipsilesional motor cortex was sufficient to

promote functional recovery (Cheng et al., 2015). Additionally, other groups have demonstrated that stimulation of the intact spinal cord combined with rehabilitation can improve motor function through reestablishment of cortical microcircuits (Wahl et al., 2017). An important note about this experiment is that results were most significant when stimulation was paired with a rehabilitation paradigm. Finally, optogenetic stimulation of thalamocortical circuits resulted in significant improvements in the affected forelimb (Tennant, Taylor, White & Brown, 2017). Notably, optogenetic stimulation of this circuit promoted the rewiring of thalamocortical circuits by inducing the branching and retraction of axonal boutons. Altogether, these results have begun to identify how stimulation can improve recovery and have begun to identify the functional outcome of stimulating specific groups of cells during stroke recovery.

1.9 The Effect of Stimulation on PCs and the Neurogenic Niche during Stroke Recovery

Another study from the Steinberg lab used optogenetics to activate transplanted neural stem cells within the stroke site of an MCAO stroke in rats, leading to improved sensorimotor function and increased forelimb use on the contralateral side compared to controls (Daadi et al., 2016). The following year, Song et al (2017) demonstrated that optogenetic stimulation to the striatum of Thy1-ChR2 mice subjected to focal ischemic stroke had significant effects on the migrating PC population. Specifically, stimulation promoted the proliferation and migration of SVZ-derived neuroblasts into the peri-infarct cortex, increased neuronal differentiation and improved long-term functional recovery. Shortly hereafter, an experiment by Lu and colleagues (2017) advanced the field again by inhibiting neuronal activity within the striatum following the transplantation of NSCs into the stroke site after MCAO surgery.

This work demonstrated that inhibition of the striatum following NSC transplantation resulted in a significant reduction in stroke volume, as well as improved functional outcome compared to striatal excitation. Additionally, an experiment by Liang and colleagues (2019) attempted to increase neuronal activity in the motor cortex surrounding the stroke site to replicate the effects of clinical rehabilitation. In order to do this, a chemogenetic approach was utilized to show that activating the excitatory neuronal population resulted in increased migration of neuroblasts to the infarct and improved stroke outcomes. Additionally, stimulation of the ipsilesional hemisphere has been associated with increased neurogenesis and migration to the site of injury. No published work has ever optically activated endogenous PCs that have migrated within the peri-infarct region. **These papers provide a rationale for optogenetically stimulating surviving adult-born neurons in the peri-infarct region of adult mice following a stroke to examine the effect this has on behaviour and the fate of the adult-born cell population.**

Objective:

Determine if stimulation of adult born neurons in the peri-infarct region promotes functional recovery in the iBax-ChR2 mouse model.

Hypothesis:

iBax-ChR2 mice that receive optogenetic stimulation post stroke will have enhanced behavioural recovery when compared to control WT mice.

2 - Materials and Methods

2.1 Animals

2.1.1 General Procedures

Animal procedures were conducted with approval from the University of Ottawa's Animal Care Committee and in accordance with the Guidelines of the Canadian Council of Animal Care. Mice were housed on a 12:12 hour light-dark cycle to maintain consistent circadian rhythm. Behavioral testing and optogenetic stimulation was conducted during the light cycle between 7am and 7pm. Water and food was available to the animals *ad libitum*. Room temperature and humidity levels were maintained at 23 degrees C and 30-40% respectively.

2.1.2 Mouse model

A triple transgenic iBax-ChR2 mouse model was created through combining two transgenic mouse lines: 1) an iBax mouse which has a nestin-CreER$_{T2}$ transgene, that allows for specific targeting of PCs which express nestin; as well as the floxed Bax transgene to allow inducible deletion of the pro-apoptotic protein, bax and prevent the death of the adult-generated cells (Sahay et al., 2011); and 2) a floxed ChR2 mouse line, which allows for the inducible expression ChR2 (Lerner, Ye & Deisseroth, 2016; Deisseroth, 2011). The mice were bred to create both WT-ChR2 and iBax-ChR2 littermate mice. As shown in Figure 3A, the WT-ChR2 mice expressed nestin-ChR2 (heterozygous) WT BAX (homozygous), and floxed ChR2 (homozygous). The iBax-ChR2 mice expressed nestin-CreERT2 (heterozygous), floxed iBax (homozygous) and floxed ChR2 (homozygous).

2.1.3 Excluded Animals

There were 67 mice used in the experiments for this thesis. From the 75 mice, 34 (45%) mice were excluded due to predetermined endpoints. The reasons for exclusions included: loss of implants (n=16), absence of baseline stroke deficits (n=12), and unexpected death following the surgery or TAM treatment (n=6). Resulting in a final sample size of 41 animals.

2.1.4 Sex

Out of the final sample size of 41 animals, there were 20 male and 21 female mice with relatively similar distribution between the experimental groups (WT-ChR2 (M=5, F=3); Stimulated WT-ChR2 (M=9, F=10); Stimulated iBax-ChR2 (M=6, F=8)).

2.2 Genotyping

At 3 weeks of age, ear samples were collected for genotyping to distinguish between WT-ChR2 and iBax-ChR2 mice. DNA was isolated from the ear samples using an Extraction Solution composed of a base that breaks down cells and a Tissue Preparation solution containing protease enzymes that degrade proteins (Sigma). This is followed by a polymerase chain reaction (PCR) using primers ordered from Integrated DNA Technologies, to detect the respective transgenes. PCR amplicons were electrophoresed on a 2% agarose gel, stained with ethidium bromide and subsequently visualized by ultraviolet illumination.

2.3 Experimental Surgery

Mice were anaesthetized by inhalation using 5% isoflurane with oxygen flowing at 1.5 L/min. During surgery, the isoflurane level was reduced to 1.5% while the oxygen level was maintained at 1.5L/min. Surgery was used for both to induction of a stroke, as well as cannula implantation for optogenetic stimulation. After surgery, mice were allowed to recover within

an incubator after being given a transdermal injection Buprenorphine and topical application of Bupivacaine at the surgical site.

2.3.1 Photothrombosis (PT) Stroke

Focal cortical strokes were induced in the forelimb motor area using the PT stroke model (Watson et al, 1985). Specifically, the infarct was targeted at bregma -0.7 mm AP, +2.0 mm ML (Kannangara et al., 2018). Briefly, the mice were given an intraperitoneal (IP) injection of a photosensitive dye, Rose Bengal. At 5 minutes after injection of the dye, a green laser (532 nm) located 2.5 cm above the scull, was turned on for 10 minutes to focally irradiate the region of the primary cortex responsible for forelimb movement (Tennant et al., 2011)

2.3.2 Cannula Implantation

Conical ceramic ferrules that were 6.4 mm long with a 127-131 um bore were purchased from Precision Fiber Products and used to create the cannula implants. A 100-um fiber optic wire was stripped of its insulation and cleaned with 100% ethanol prior to being measured and precisely cut with a scribe. This optic fiber was then placed inside the ferrule with the flattest end of the fiber slightly extending past the larger opening of the ferrule. An epoxy glue was used to fix the fiber in place within the ferrule and allowed to dry for at least 24 hours. Once dry, the end of the ferrule with the smaller opening was polished on 4 separate textured fabrics of progressively increasing smoothness. After preparation, implant power was measured using a power meter (OPHIR Photonics). Only implants with measured power over 1.5 mW/mm^2 were implanted into animals within this study.

Cortical strokes were immediately followed by stereotaxic implantation of the fiber optic implant precisely at the stroke site, 0.8 mm deep from the dura in order to allow for light stimulation post stroke. In order to maintain consistency, a skull marker is used to mark the

location of the stroke and a small hole is drilled in this location. The tip of the fiber optic implant is then brought to the dura and lowered another 0.8 mm where it is held in place while allowing the Metabond glue formula to dry. Once the glue has been crystallized, the animal is taken off anesthesia and allowed to recover in a warm incubator before being returned to their home cage.

2.4 Tamoxifen Treatment

The mice were treated with TAM dissolved in 90% sunflower seed oil and 10% ethanol (EtOH) at one-week after stroke via IP injections of 160mg/kg/day for 5 consecutive days, as previously described (Lagace et al., 2007).

2.5 Behavioral Tests

Two weeks after implantation, the mice were trained on sensorimotor/motor behavioral tests within the University of Ottawa Behavioural Core Facility. Testing was completed between 8am and 5pm. In this order of testing, the standardized tests used within this study include the: cylinder test, adhesive removal test, and the horizontal ladder test.

2.5.1 Adhesive Removal Test

The adhesive test measures forelimb sensorimotor behaviour by quantifying the amount of time it takes for the mouse to contact and remove an adhesive strip on its paws (Bouet et al., 2009). The time to contact is a measure of the animal's sensory function, whereas time to remove the adhesive strip is indicative of motor function. The animals were trained for 5 days to establish a pre-stroke baseline. Contact and removal times were averaged for two days of testing prior to stroke and then again at each post-stroke time point.

2.5.2 Cylinder Test

The cylinder test quantifies spontaneous voluntary forelimb usage and asymmetry (Balkaya et al., 2013). Animals were placed in a clear, hollow cylinder and 20 rears that occurred spontaneously were analyzed. The spontaneous placement of their paws on the walls of the cylinder were quantified using the Ethovision software to determine asymmetrical forelimb use (Clarkson et al., 2010). The asymmetry index was calculated by subtracting the percent time spent on the impaired side from the unimpaired side in order to determine a bias in forelimb use. The formula for this calculation is based on the time spent on each paw: ((right paw contacts – left paw contracts) / (right paw contacts + left paw contracts + both paw contacts)) multiplied by 100%. This results in a positive number indicating that mice use the right paw predominantly and negative number indicating that mice use the left paw predominantly during spontaneous rears.

2.5.3 Horizontal Ladder Test

The horizontal ladder test is used to quantify both forelimb and hindlimb deficits through analysis of the number of foot faults (Metz & Whishaw, 2009). This test involves exposing the mouse to a ladder with unevenly-spaced rungs and measuring the number of errors in paw placements as they traverse from one side to the other. Mice typically display significant motor deficits in the use of their contralesional forelimb and hindlimb, with generally worse performance specifically in the hindlimb post-stroke (Ceizar, 2017; Metz & Whishaw, 2009).

2.6 Stimulation Paradigm

To induce activation of the ChR2-expressing adult-generated neurons, mice underwent a daily optogenetic stimulation paradigm. Stimulation was administered using conical and symmetrical light propagation through a 100-μm optical fiber to deliver daily stimulation. The

pattern of the stimulation was 3 pulses of 90-second light exposure; 2-minute inter-exposure interval, using a 495-nm blue laser at 40 Hz for 5 ms light pulses. This paradigm was used since it has been shown to be effective for ChR2 stimulation in the motor cortex (Alonso et al., 2012; Bardy et al., 2010; Daadi et al., 2016). The approximate depth of the fiber tip was 0.8 mm from the skull, which corresponds to an approximate depth of 300 μm in the cortex. Initial power density is reduced exponentially with distance in brain tissue. Based on previous studies that have analyzed light penetrance, 473nm LED light power density is reduced to 10% of initial power density 500 μm into brain tissue with a light spread of roughly 1 mm diameter (Yizhar et al., 2011). LED light power from the fiber tip was measured as ranging from 1.5 to 3.0 mW/mm2 meaning that 500 μm into brain tissue this is reduced to 150 to 300 mW/mm2. According to the aforementioned study, power density below 0.9 mW/mm2 is lower than the activation threshold, which suggests that the light stimulation in this study should have only been capable of activating PCs surrounding the infarct.

2.7 Perfusion and Sectioning

Animals were anaesthetized and trans-cardially perfused with chilled phosphate-buffered saline (PBS), pH=7.4, 7 ml/min for 6 minutes) followed by 4% paraformaldehyde (PFA, pH=7.4, 7 ml/min for 10 minutes). Brains were removed and post-fixed for one hour in 4% PFA and then cryoprotected in 30% sucrose with 0.1% sodium azide (NaN3) in PBS. Whole-brains were mounted with dry ice and coronal sections (35μm) were generated on a microtome, collected in nine serial sections, and stored in PBS with 0.01% NaN3 at 4°C.

2.8 Histology

2.8.1 DCX Slide Mounted Immunohistochemistry (IHC)

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; Jackson Immuno Research Laboratories Inc.) and 0.3% Triton X-100 in 1X PBS for one hour prior to being incubated overnight in the primary antibody solution (1:5000 Goat anti-DCX a in 3% NDS in 0.3% Tween-20 and 1X PBS). The following day, slides were incubated in: 1) 1:200 biotinylated Donkey anti-goat secondary antibody (112941; Jackson Immuno Research Laboratories) in 1.5% NDS in 1X TBS for 60 minutes; 2) 0.3% Hydrogen Peroxide (H2O2) in 1X TBS for 30 minutes to quench endogenous peroxidases; 3) Avidin-Biotin Complex Solution (ABC, ZD-0510/ZD0511; Fisher Brand) 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 (ZC0707; Vector) 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 (172230; Decon Laboratories) for 20 seconds, 1 minute, and 5 minutes. Slides were cover-slipped with DPX (which is a mixture of Distyrene, a Plasticizer, and Xylene) mounting medium (BCBH4393V; Sigma).

2.8.2 Fluorescence Free-Floating Immunohistochemistry

Free-floating fluorescence immunohistochemistry was performed for all colocalization analysis based on previously published protocols (Ceizar et al., 2016; Kannangara et al., 2018). Tissue sections were selected and washed in petri-dishes 3 times for 5 minutes each using PBS. IHC was performed on free-floating sections using antibodies for Green Fluorescent Protein (GFP) (697986, Aves, 1:1000, used also to detect YFP), DCX (10314, Santa Cruz,1:500) and NeuN (BD Biosciences, 556327, 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 1 hour at RT followed by counterstaining with 4',6-diamidino- 2-phenylindole, dihydrochloride (DAPI; 70231722, Roche, 1:10000, 5 min). Between all steps the sections were rinsed 2-3 times with 1X PBS. Sections were mounted onto slides, dried for ~10 minutes prior to cover-slipping with Immu-mount (Thermoscientific, 448575) mounting medium.

2.8.3 Stereological Counts

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 peri- infarct region. The section with the largest lesion 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 height of 20 μm. 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 the peri-infarct region between animals, cell densities were calculated by dividing total cell count estimates by the volume of the region traced.

2.8.4 Analysis of Colocalization

In order to determine if cells were colabeled with different protein markers, 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 co-labeled using ZEN 2009 acquisition software from Zeiss as previously described (Ceizar et al., 2016). For every animal, a minimum of 50 cells were analyzed.

2.8.5 Lesion Volumes

Every 9th serial tissue section throughout the visible lesion, and 2 extra sections on each side 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 (BCBH4393V; Sigma) mounting media.

2.9 Statistical Analyses

Behavioural and histological data are all expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6. A one-way ANOVA was used to analyze stereological counts and lesion volumes between the 3 genotypes. A 2-way Analysis of Variance (ANOVA) followed by Tukey's post hoc was used for analysis of colocalization with 2 neuronal markers between the 3 genotypes. A repeated measure two-

way ANOVA was performed for all behavioural analysis. Statistical significance was set at $p<0.05$.

3 - Results

3.1 Creation of iBax–ChR2 Mice and Experimental Design

In order to test the impact of the survival and activity of PCs for stroke recovery, we created an iBax-ChR2 transgenic mouse model by crossing the iBax mouse model (Sahay et al., 2011) with the floxed ChR2-eYFP model (Deisseroth, 2011). In this model, as shown in Figure 3A, treatment of the mice with TAM, allows for removal of the loxP sites resulting in the removal of the pro-apoptotic *Bax* gene and the expression of ChR2-eYFP specifically in nestin-expressing PCs.

As shown in the timeline on Figure 3B, young adult mice were singly-housed and performed baseline behavioral testing at 8 weeks. At 10 weeks of age, all of the mice received surgery in order to induce a PT stroke to the sensorimotor forelimb region and implant an optogenetic cannula. At one-week post stroke the mice were tested on the same 3 behavioural tasks in order to quantify behavioral deficits post stroke. After behavioural testing, the mice received 5 days of TAM treatment to induce recombination, followed by one-week recovery in the home cage.

The WT-ChR2 mice were segregated to received stimulation or not receive stimulation based on their baseline and post stroke performance on the adhesive test. This was done to ensure equal baseline deficits between the groups prior to stimulation. Figure 3C graphically depicts the three experimental groups. Both WT- and iBax-ChR2 mice received the same optogenetic stimulation paradigm. Optogenetic stimulation was administered daily within the home cage for 5 weeks from 3 to 8 weeks after stroke using a 495-nm blue laser at 40 Hz for a total

duration of 11 minutes. To assess recovery after stroke, all the mice had behavior outcomes assessed at both 4 and 8 weeks after stroke. As depicted in Figure 3C and described in the methods, three experiments were performed to enable a final sample size of 41 mice (WT-ChR2 n=8, WT-ChR2 (Stim): n=19, iBax-ChR2 (Stim): n=14).

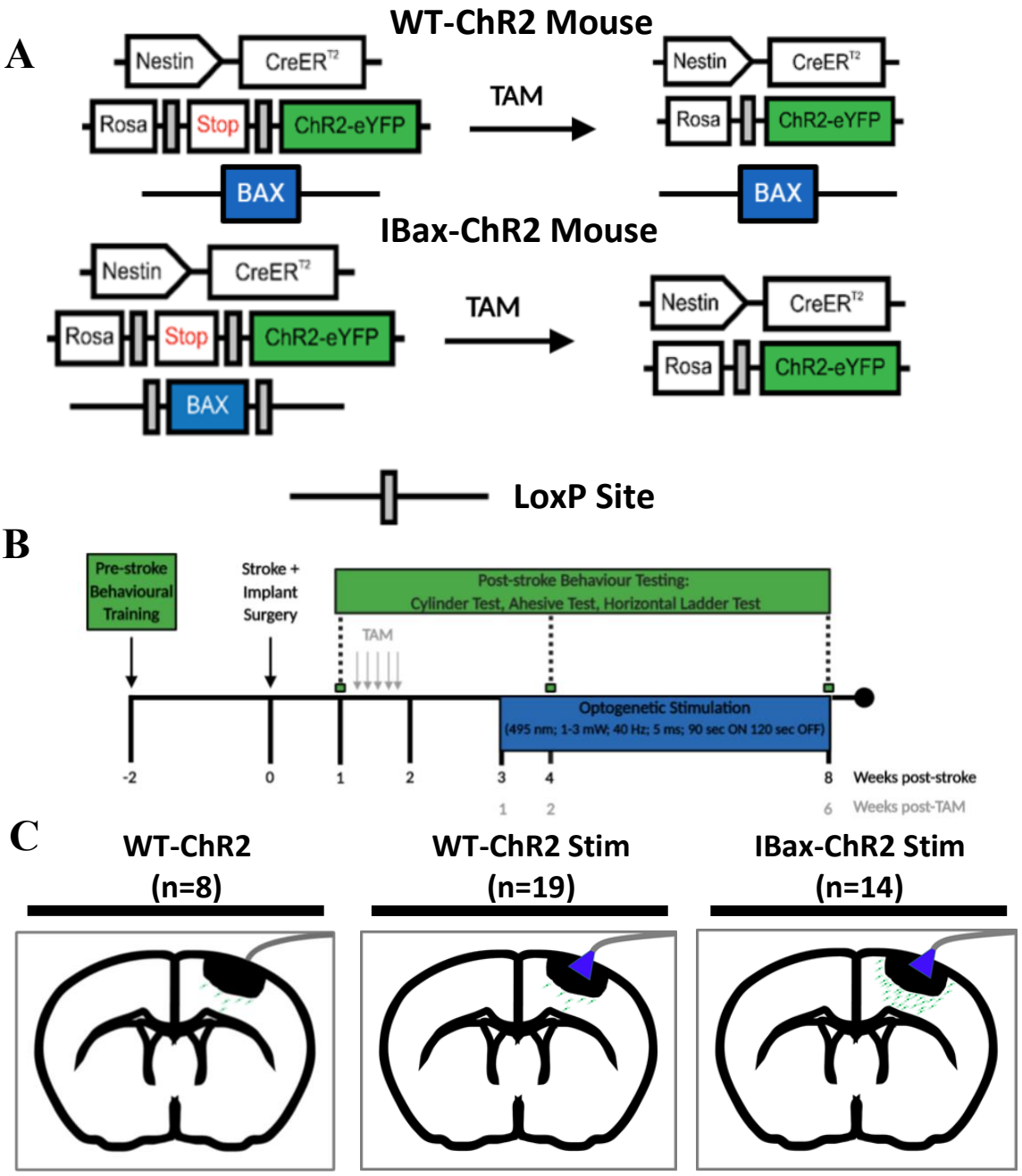

Figure 3. IBax-ChR2 Mouse model and experimental plan. A) Tamoxifen (TAM) -induced recombination in iBax-ChR2 mice results in the removal of *Bax* and expression of ChR2-eYFP in all nestin-expressing cells. In contrast in the WT-CHR2 (WT) mice TAM-induced recombination results in expression of ChR2-YFP without removal of *Bax*. (B) Experimental timeline for the 10-week study design, including 2 weeks prior to stroke and 8 weeks after stroke. (C) Graphical representation of the three experimental groups and final sample sizes. Additionally, we hypothesized that there will be a low number of YFP-expressing PCs (shown as green cells) migrating to the infarct site (black area of cortex) in the WT compared to iBax mice. Non-stimulated mice will receive same stereotaxic fiber optic implant surgery but will remain un-tethered in their home cage during stimulation period.

3.2 Optogenetic stimulation of PCs in iBax and WT mice did not alter stroke recovery

Stroke recovery was assessed using three behavioral tasks: the adhesive, cylinder and horizontal ladder test. Mice were trained on the adhesive test on the right and left limb prior to stroke and displayed a stereotyped learning curve over the 5 training days (Figure 4). As expected, over time there was a significant improvement in time to contact (Figure 4A, $F_{4,344}$= 8.908, $p<0.0001$) and remove (Figure 4B, $F_{4,344}$ =8.097, $p<0.0001$) the tape. A plateau in performance occurred on the last 2 days of training, which was confirmed by post-hoc analysis which found no differences in performance between days 4 and 5 on both contact and removal. Due to this plateau, the average of day 4 and 5 of training was used as the baseline pre-stroke value for contact and removal prior to stroke.

Comparison of performance on the contralateral forelimb before and after stroke also revealed a significant difference in performance over time for both contact (Figure 3C, $F_{3,114}$= 7.2, p= 0.0002) and removal (Figure 4D, $F_{3, 114}$= 17.3, $p < 0.0001$) with no overall differences between the three groups. Post-hoc analysis revealed that, following the stroke, all three groups took significantly longer to contact and remove the adhesive strip at 1 week post stroke when compared to baseline performance. To assess recovery, post-hoc analysis was also used to compare performance at 1 week post stroke, compared to 4 and 8 weeks post stroke. This analysis revealed no differences in recovery when examining time to contact the tape, but a significant reduction in the time to remove the adhesive strip at 4 and 8 week post stroke, compared to 1 week post stroke.

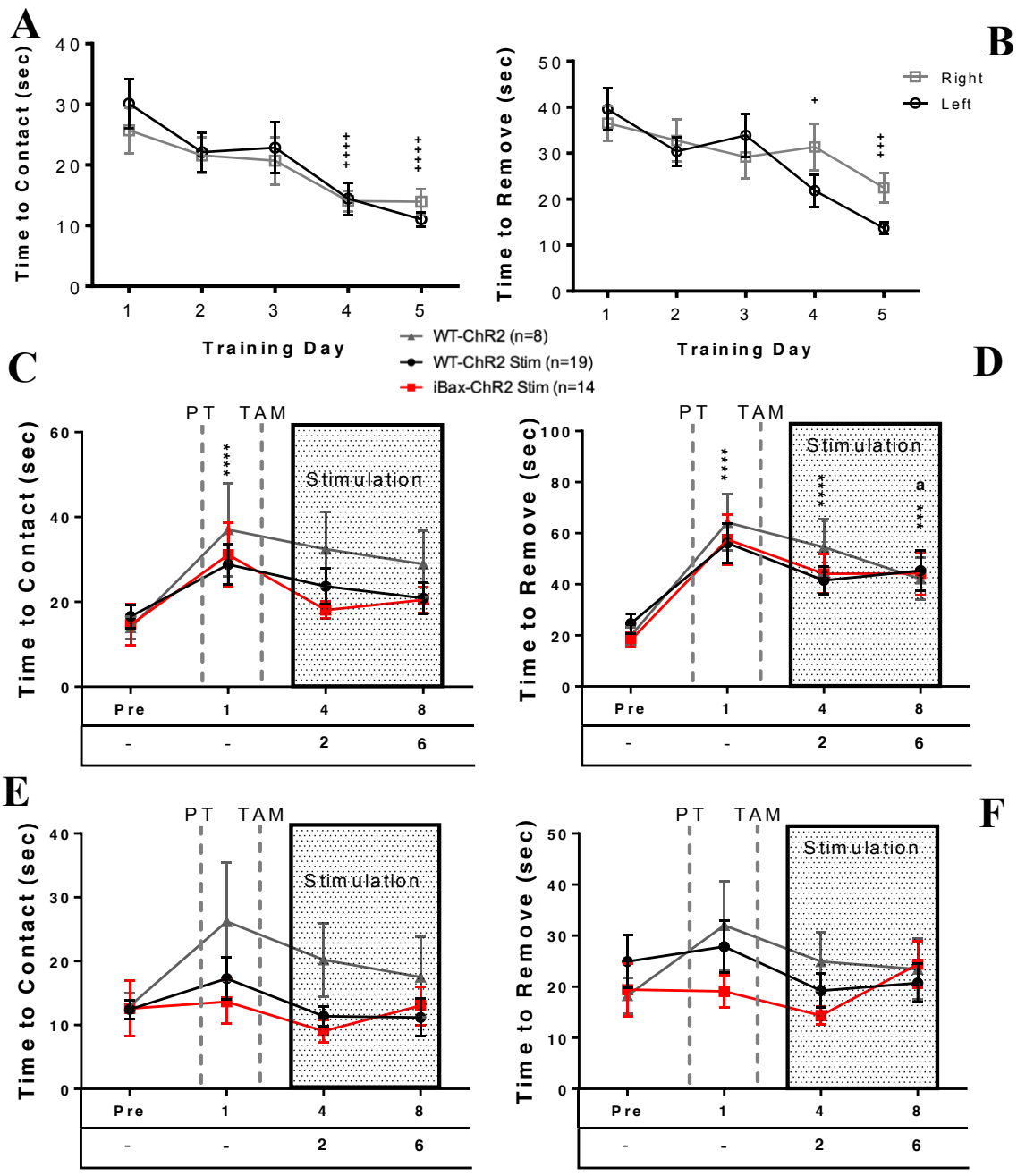

Figure 4. Adhesive contact and removal times for contralateral paw in all three behavioural groups. (A) Contact and (B) removal times for all mice in all three behavioural groups displaying progressive improvement throughout the 5 days of training. Contralateral (C) contact and (D) removal times for all behavioural groups displaying a significant effect of time. Significant difference was observed in adhesive contact between pre and 1wps ($p<0.0001$). Relative to pre-stroke there was a significant difference in removal times at 1 ($p<0.0001$), 4 ($p<0.0001$) and 8wps ($p=0.0001$). Time to remove the adhesive was significantly lower at 8wps relative to removal times at 1wps ($p=0.0394$). *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$ represents significance from pre-stroke. **+** = significance from Day 1. **a** = significance from 1wps.

These findings suggest that the mice recovered their ability to remove the tape, as described extensively in the literature. Nevertheless, there were no significant differences in behavior between the three experimental groups over time, suggesting that neither stimulation, nor enhancing PC survival altered recovery. As expected after a unilateral stroke, there were no significant differences over time or between mice, in their ipsilateral forelimb performance on the task (Figure 4E, 4F). This supports the assumption that the deficits observed on the contralateral limb were likely due to the stroke, and not a general effect of motivation or surgery.

The cylinder test assesses forelimb asymmetry during spontaneous rears and was first analyzed by measuring the amount of time spent with the contralateral forepaw on the walls of the cylinder (Figure 5). Comparison of the time spent rearing with the contralateral paw revealed a significant effect of time (Figure 5A $F_{3,120}= 7.4$ $p= 0.0001$). However, similar to the adhesive results, this difference occurred in the absence of any significant differences between the stimulated and non-stimulated WT-ChR2 mice, and the iBax-ChR2 stim mice. Prior to the stroke, mice used their contralateral forepaw to rear around 50% of the time. By 1 week after surgery, all 3 groups of mice used their contralateral forepaws significantly less and had significant unilateral deficits. By 8 weeks post-stroke, the mice had recovered and used their contralateral forepaw significantly more as they approached equal use of the contralateral and ipsilateral forelimb.

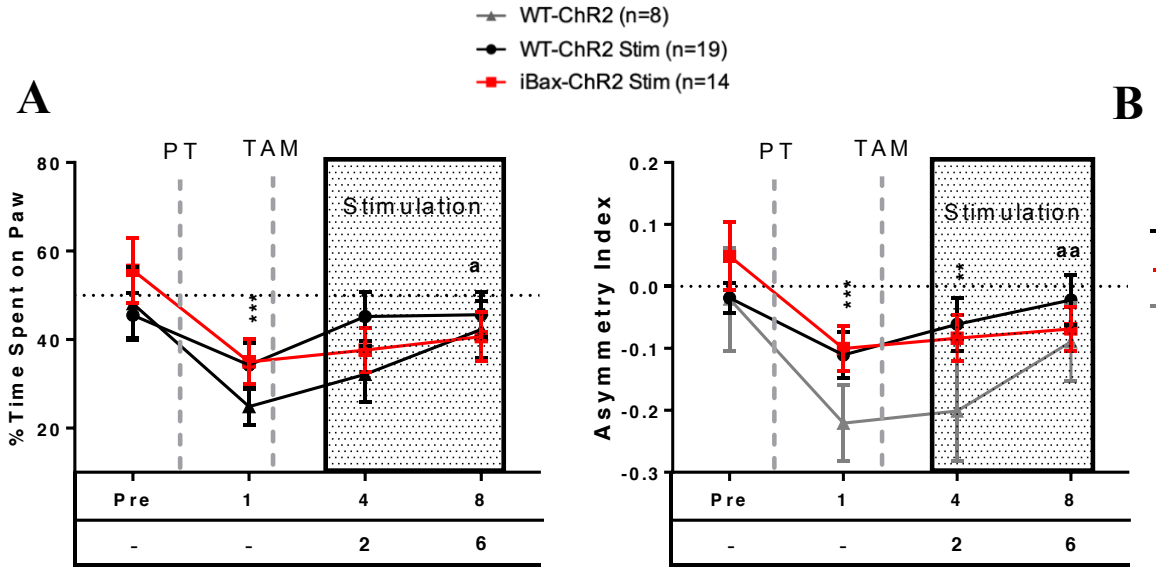

Figure 5. Cylinder data for all three behavioural groups. (A) % time spent on contralateral paw at pre-stroke and each post-stroke time point for all experimental groups. Compared to the pre-stroke baseline, a significant reduction in time spent using the contralateral paw was observed at 1wps (p=0.0001). Significant recovery was observed between 1 and 8 wps (p=0.0293). (B) Asymmetry index indicating unilateral deficits on the contralateral paws post-stroke. Significant deficits were present at 1 (p=0.0001) and 4 wps (p=0.0051). Behavioural recovery was observed at 8 wps compared to 1 wps (p=0.0491). *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 represents significance from pre-stroke. **a** = significance from one wps.

The asymmetry index of mice on the cylinder test was also calculated since this measure gives a clearer indication of limb preference. Similar to results obtained from analyzing time on forepaw, the asymmetry index showed a significant difference in performance at time points assessed before and after stroke (Figure 5B, $F_{3,120} = 8.4$, $p < 0.0001$). There were significant deficits at 1 and 4 weeks post stroke followed by a significant improvement in forelimb use by 8 weeks post stroke. Analysis of the 3 groups also showed no significant differences in performance. Overall, these results suggest that there were significant deficits in cylinder performance after stroke and that stimulation did not alter recovery in performance. Importantly, both methods of analyzing cylinder performance revealed significant recovery in function by 8 weeks post stroke.

The horizontal ladder test was used to assess forelimb and hindlimb performance by measuring the percentage of correct limb placements. Similar to the adhesive task, the mice were pre-trained on the ladder for two days, with the results from the second day of testing being used for the pre-stroke measure of performance. Analysis of contralateral forelimb performance between pre-stroke and post stroke revealed a significant effect of time ($F_{3,114}= 11.4$, $p < 0.0001$) with no differences between the between the WT-ChR2, stimulated WT-ChR2, and the stimulated iBax-ChR2 mice (Figure 6A). All 3 groups displayed significant deficits at 1 week post stroke compared to pre-stroke performance. Interestingly, post-hoc analysis revealed no significant difference in successful paw placements between 1 week post stroke and either 4 or 8 weeks post stroke, suggesting that mice did not recover over time on this forelimb task. Unexpectedly, analysis of forelimb placements using the ipsilateral limb also suggest a significant effect of time (Figure 6C, $F_{3,114}= 7.7$ $P < 0.0001$). However, post-hoc analysis revealed no difference in ladder performance at 1 week post stroke compared to the

pre-stroke baseline. Since there were no deficits immediately post-stroke and no other test found any significant effects in the ipsilateral limb, this delayed reduction in performance on the ladder task does not appear to be a general effect of motivation due to task or effect of surgery.

Unlike all the other behavioral measures, analysis of the contralateral hindlimb did not reveal any significant deficits in performance between time points assessed, nor any significant differences between the 3 groups (Figure 6B). As expected, there was also no significant effect of group or time in the ipsilateral hind limb (Figure 6D). The lack of deficits in the contralateral hind limb was unexpected since previous work using iBax mice had demonstrated significant deficits on this outcome measure (Ceizar, 2017). However, unlike previous work, there appears to be more variability within the groups, as well as between groups on the hind limb, that may have contributed to this result.

Overall, analysis of the 3 behavioural tests supports that there are significant deficits following PT stroke in the absence of any differences in the recovery profiles of iBax mice that received optogenetic stimulation compared to the control groups.

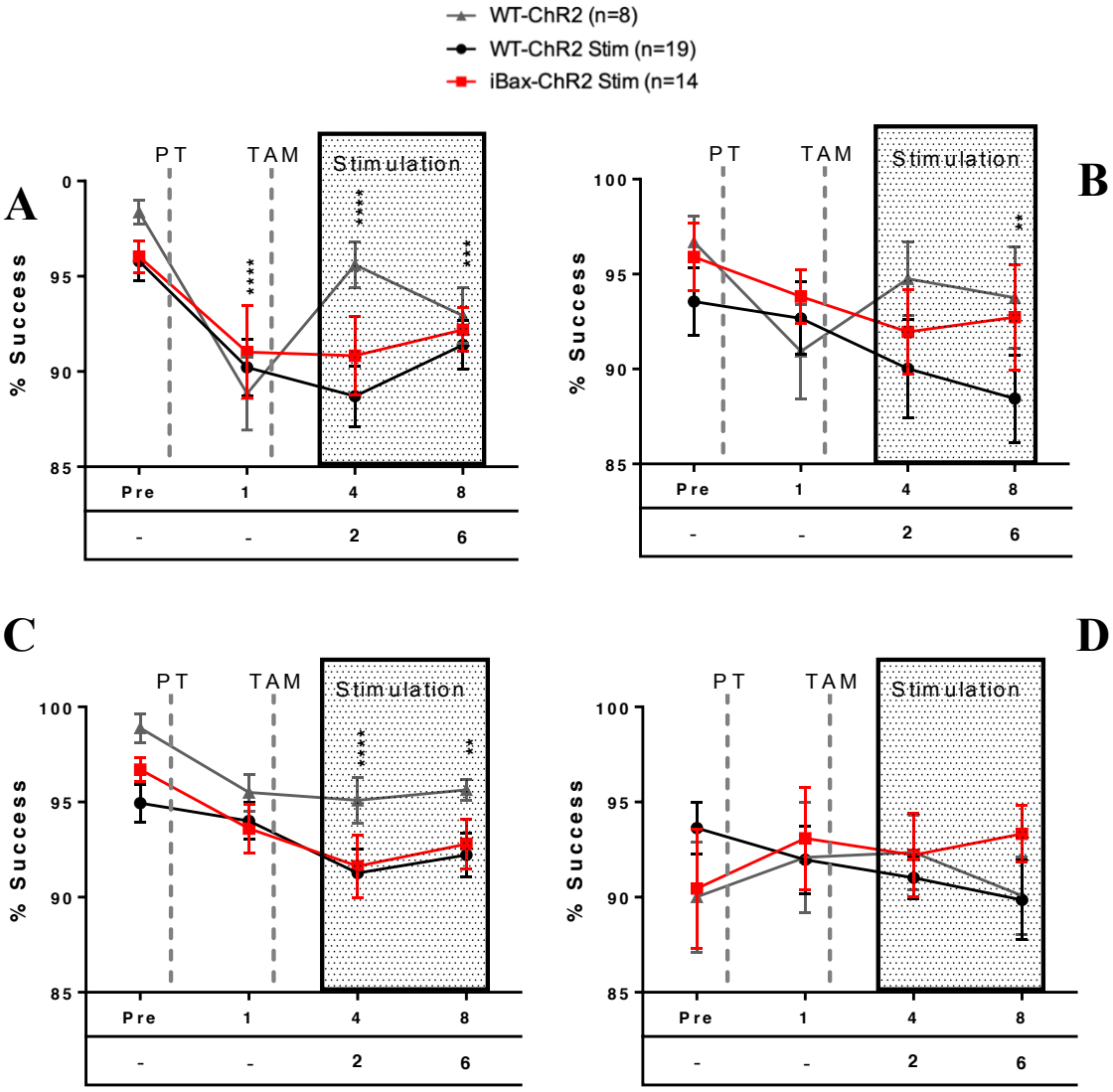

Figure 6. Horizontal ladder data for all three behavioural groups. % Successful paw placements with the contralateral (A) forepaw displaying significantly worse performance at 1 ($p<0.0001$), 4 ($p<0.0001$) and 8 wps ($p=0.0009$).(B) Contralateral hindlimb performance on horizontal ladder showing significant reduction in % successful paw placements at 8 wps compared to pre-stroke ($p=0.0283$). (C) % Successful paw placements with the ipsilateral forepaw displaying significantly worse performance at 4 ($p<0.0001$) and 8 wps ($p=0.0015$) compared to pre-stroke. (D) Ipsilateral hindlimb performance on horizontal ladder revealed no significant effects. *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$ represents significance from pre-stroke performance.

3.3 WT Stimulated mice had larger lesion volume compared to Stimulated iBax or non-Stimulated WT mice

Analysis of lesion volumes surprisingly revealed a significant variation in stroke sizes between groups. This is illustrated by the representative cresyl violet stained sections (Figure 6A), as well as a graphical representation of the minimum, average and maximum lesion volumes from matched sections at 0.86 mm A/P from bregma (Figure 6B). Quantification of the infarct volume revealed that the stimulated WT-ChR2 mice had significantly larger strokes compared to the WT-ChR2 and stimulated iBax-ChR2 mice ($F_{2,42}$= 7.2, p= 0.002). This was further confirmed by analyzing the number of sections that had a lesion, which revealed that : WT-ChR2 mice had minimum lesions spanning 2 sections from 1.10 to 0.86 mm, average lesions spanning 3 sections from 1.70 to 0.74 mm, and maximum lesions spanning 4 sections from 1.94 to 0.74 mm; WT-ChR2 stimulated mice had minimum lesions spanning 2 sections from 1.42 to 1.10 mm, average lesions spanning 4 sections from 2.10 to 0.86 mm and maximum lesions spanning 5 sections from 1.70 to 0.02 mm; iBax-ChR2 stimulated mice had minimum lesions spanning 2 sections from 1.34 to 0.74 mm, average lesions spanning from 1.34 to 0.50 mm and maximum lesions spanning from 1.98 to 0.86 mm.

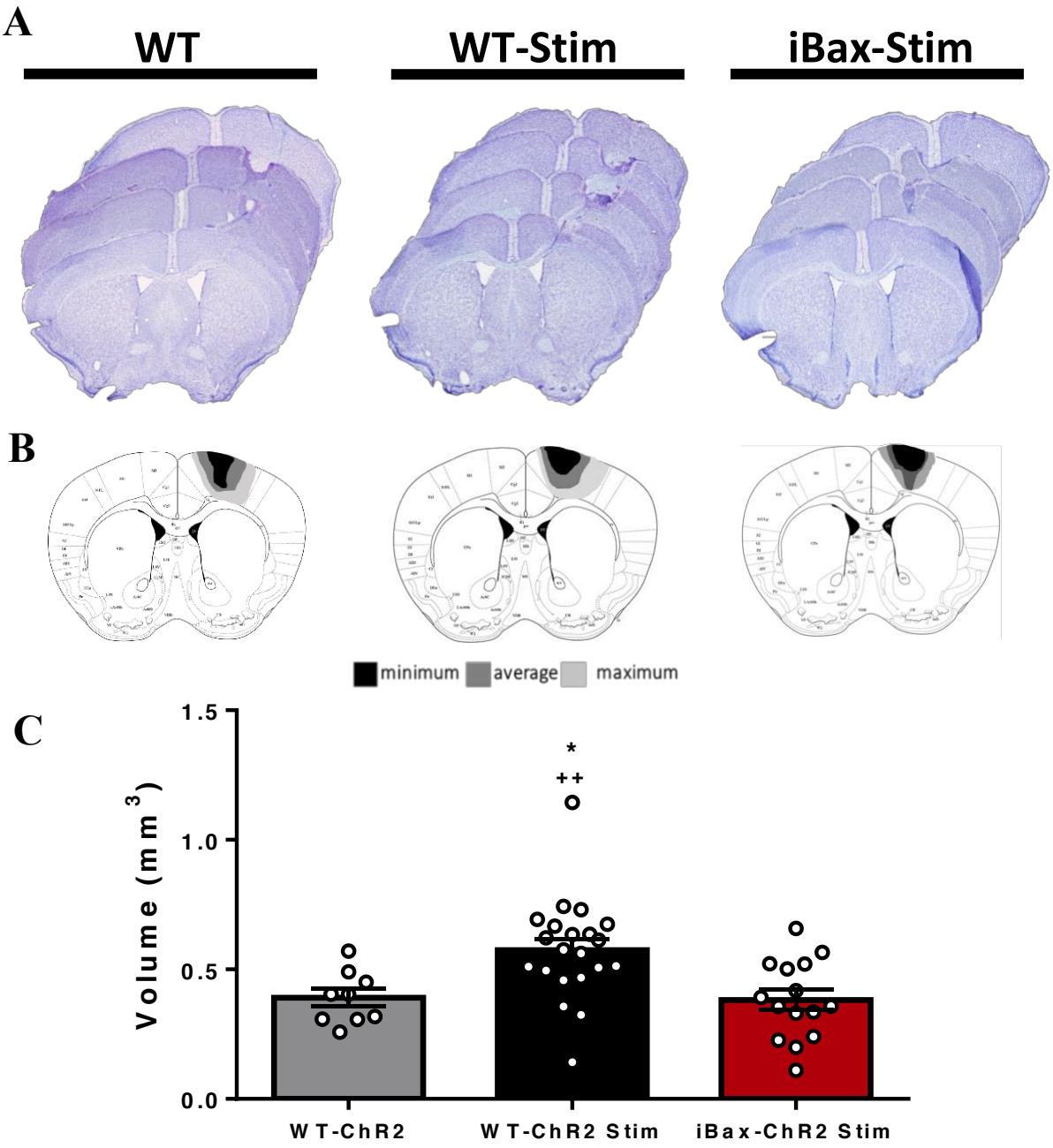

Figure 7. Stroke lesion volumes measured using Cresyl Violet staining. (A) Representative cresyl violet stained brain slices for each experimental group displaying the stroke lesions. Cut through tissue indicates the non-stroked hemisphere of the brain. (B) Representative images of largest (light gray), average (dark grey) and smallest (black) lesion volumes for each experimental group. (C) Bar graph representing average lesion volumes for each experimental group with individual lesion volumes super-imposed. Compared to the stimulated WT-ChR2 mice, stimulated iBax-ChR2 ($p=0.0040$) and non-stimulated WT-ChR2 ($p=0.0216$) mice had significantly smaller stroke volumes. *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$ represents significance from non-stimulated WT. + = significance from stimulated iBax-ChR2.

3.4 Optogenetic stimulation and removal of *Bax* from the progenitor cells increases the number of immature neurons surrounding the infarct

Previously, our lab demonstrated that iBax mice had a significant increase in the number of PCs that expressed the immature neuronal marker, doublecortin (DCX), and migrated to surround the infarct region compared to WT control mice (Ceizar, 2017). Examination of the stimulated and non-stimulated WT-ChR2 and stimulated iBax-ChR2 mice demonstrated, as expected, a qualitative increase in the number of DCX-expressing cells in the peri-infarct of iBax-ChR2 mice compared to both stimulated and non-stimulated control mice (Figure 7a). Interestingly, we observed a slightly larger neurogenic response in stimulated WT-ChR2 mice compared to non-stimulated controls. Stereological and statistical analysis of the density of the DCX-expressing cells within the peri-infarct revealed a significant difference between the WT-ChR2, WT-ChR2-stim and the iBax-ChR2-stim mice (Figure 7B, $F_{2,8}= 29.5$, $p= 0.0002$). Post-hoc analysis found a significant increase in the DCX density of iBax-ChR2-stim mice compared to both the WT-ChR2 and WT-ChR2-stim mice. Stereological counts of the peri-infarct region show a trend towards increased DCX density in stimulated WT-ChR2 compared to non-stimulated controls, although this was not significant. These results therefore extend previous findings from our lab, confirming that stimulation of PCs that had the *Bax* gene removed, results in a massively (3-fold) increased neurogenic response surrounding the infarct. These results also offer new evidence to support the hypothesis that stimulation of PCs may enhance the neurogenic response to stroke. However, in order to test this directly, future work would need to compare the stimulated iBax-ChR2 group to an additional control group of iBax-ChR2 mice that were not stimulated.

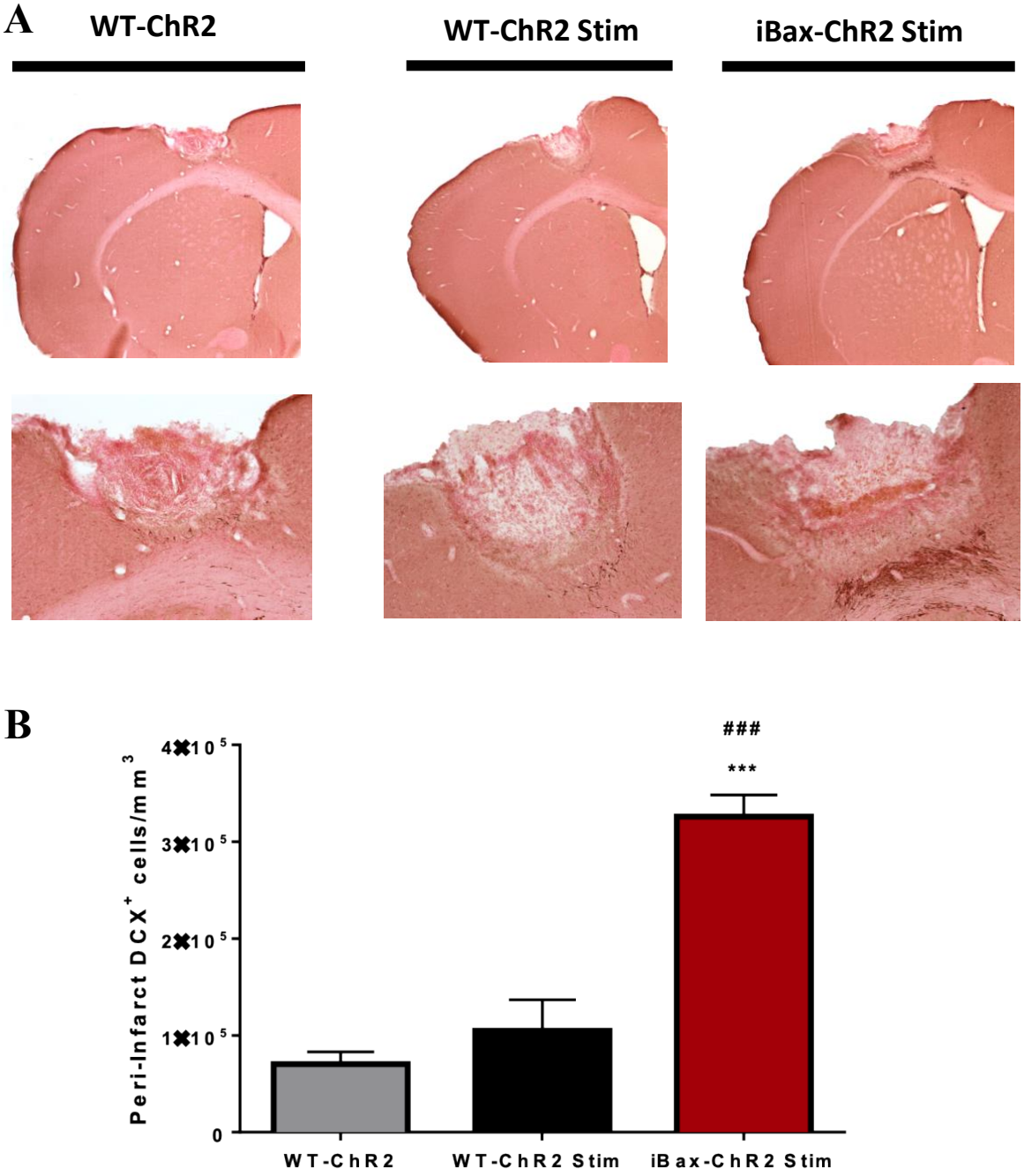

Figure 8. DCX staining counterstained with Nuclear Fast Red. (A) Representative images of DCX expressing cells (dark brown) at 2.5X and 10X magnification for each experimental group. (B) Stereological counts of the density of DCX+ cells surrounding the stroke site. Stimulated iBax-ChR2 mice had a significantly increased density of DCX compared to both stimulated (p=0.0006) and non-stimulated (p=0.0002) WT-ChR2 mice. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 represents significance from non-stimulated WT. **#** = significance from stimulated WT-ChR2.

3.5 Optogenetic stimulation enhanced the rate of neuronal differentiation of PCs that migrated to the infarct

In order to determine whether stimulation of PCs resulted in an increased rate of neuronal differentiation, IHC was utilized to quantify the proportion of ChR2-YFP+ cells that expressed DCX, and the mature neuronal marker, NeuN. As shown in Figure 9 and 10, there was a qualitative increase in the density of ChR2-YFP expressing PCs in iBax-Stim mice compared to both control groups. Quantifying the percentage of the ChR2-YFP+ cells that colocalized with either DCX or NeuN revealed significant differences between the groups ($F_{2,8}$= 12.58, P= 0.0034). As expected, within the peri-infarct of iBax-Stim mice there was a significant increase in the ChR2-YFP-expressing cells that were co-labelled with a neuronal marker, with nearly 80% of all ChR2-YFP+ cells colocalizing with either DCX or NeuN. There was also a 2-fold non-significant increase in the colocalization of neuronal markers in stimulated WT-ChR2 mice compared to WT non-stimulated controls. Specifically, WT-ChR2 mice had 17% of ChR2-YFP cells colocalize with either neuronal marker compared to WT-ChR2 stimulated mice, which had approximately 40% colocalization.

As shown in Figure 11B, the data was also segregated to examine, for each group, the percentage of colocalization of ChR2-YFP with either DCX, NeuN or both of these neuronal markers. The results of this data indicate that there is a significant group effect ($F_{2,24}$= 14.4, $p < 0.0001$) as well as a significant difference in the proportion of colocalization with DCX, DCX+NeuN and NeuN for all groups ($F_{2,24}$= 17.7, $p < 0.0001$). This is due to the increased proportion of cells expressing DCX within the 2 stimulation mice, compared to the WT-ChR2 control mice. Specifically, there was a significant difference in the percentage of cells that expressed both DCX and NeuN in the iBax-stim mice compared to the other 2 experimental

groups. Moreover, WT-ChR2-stim mice had a significantly larger proportion of PCs expressing only DCX+ compared to both stimulated iBax-ChR2 and non-stimulated WT-ChR2 mice. These results suggest that stimulation is associated with more PCs expressing immature or mature neuronal markers.

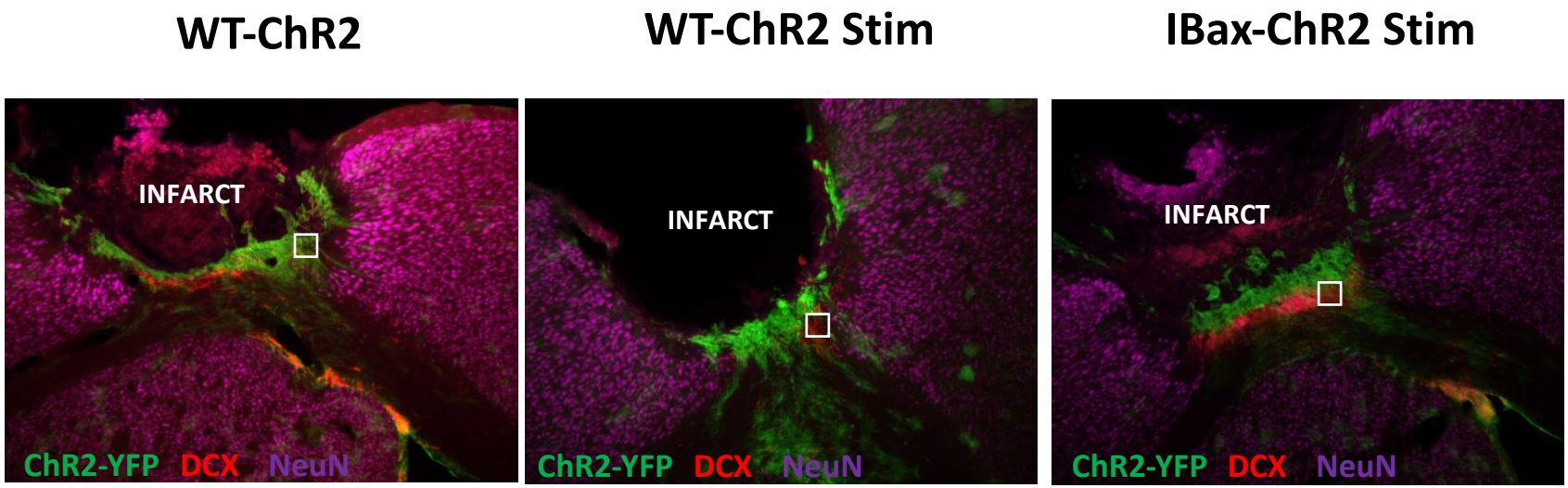

Figure 9. Channelrhodopsin (ChR2-YFP), Doublecortin (DCX) and NeuN expression in peri-infarct. Representative images for each experimental group taken with a 10x objective. White square indicates region of interest for higher magnification image.

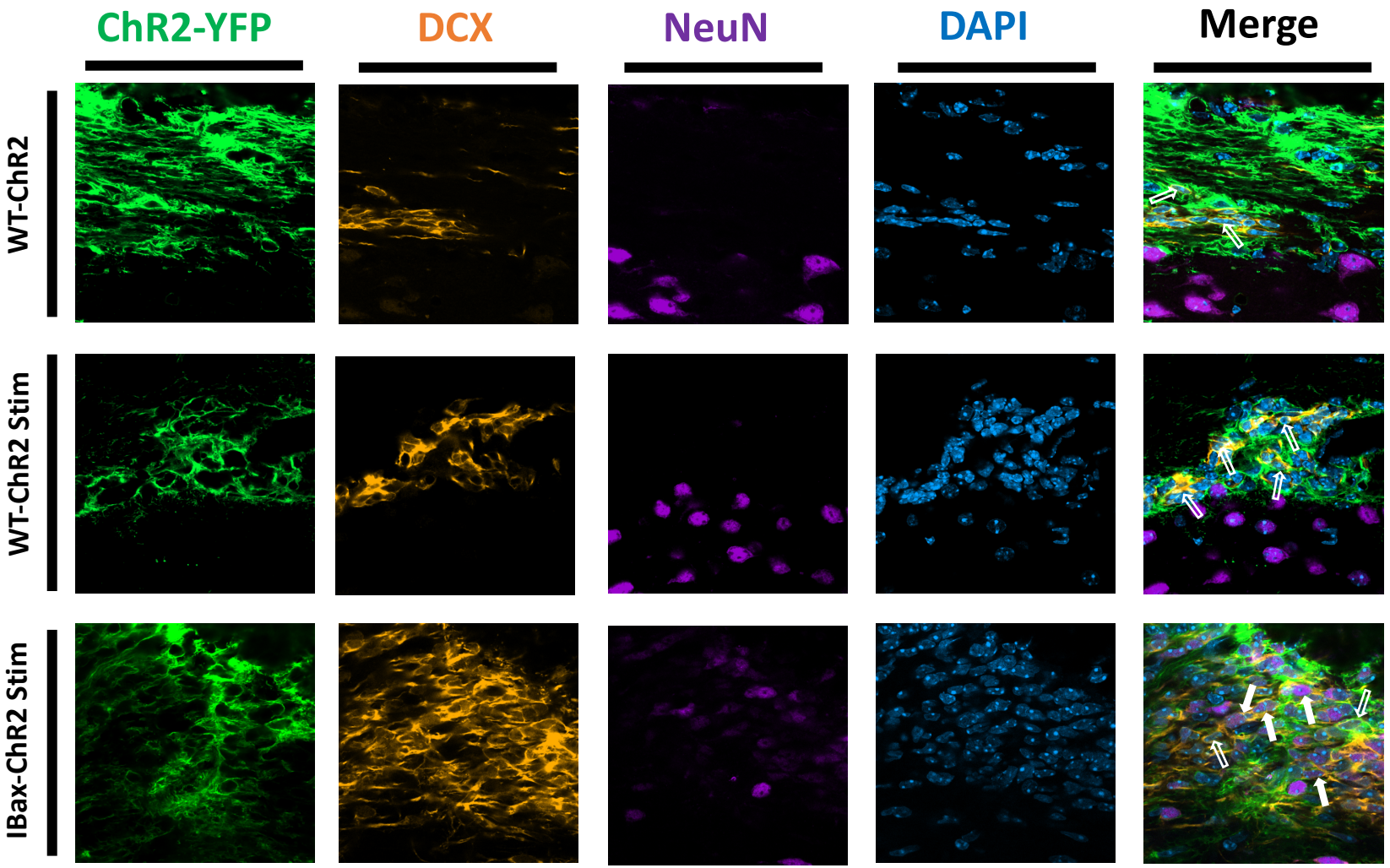

Figure 10. Channelrhodopsin (ChR2-YFP), Doublecortin (DCX) and NeuN counterstained with DAPI. Representative confocal images for each experimental group taken with a 63x oil objective. Open arrows show ChR2-YFP+ cells co-localized with DCX+ and filled arrows show ChR2-YFP+ cells that are co-localized with both DCX and NeuN.

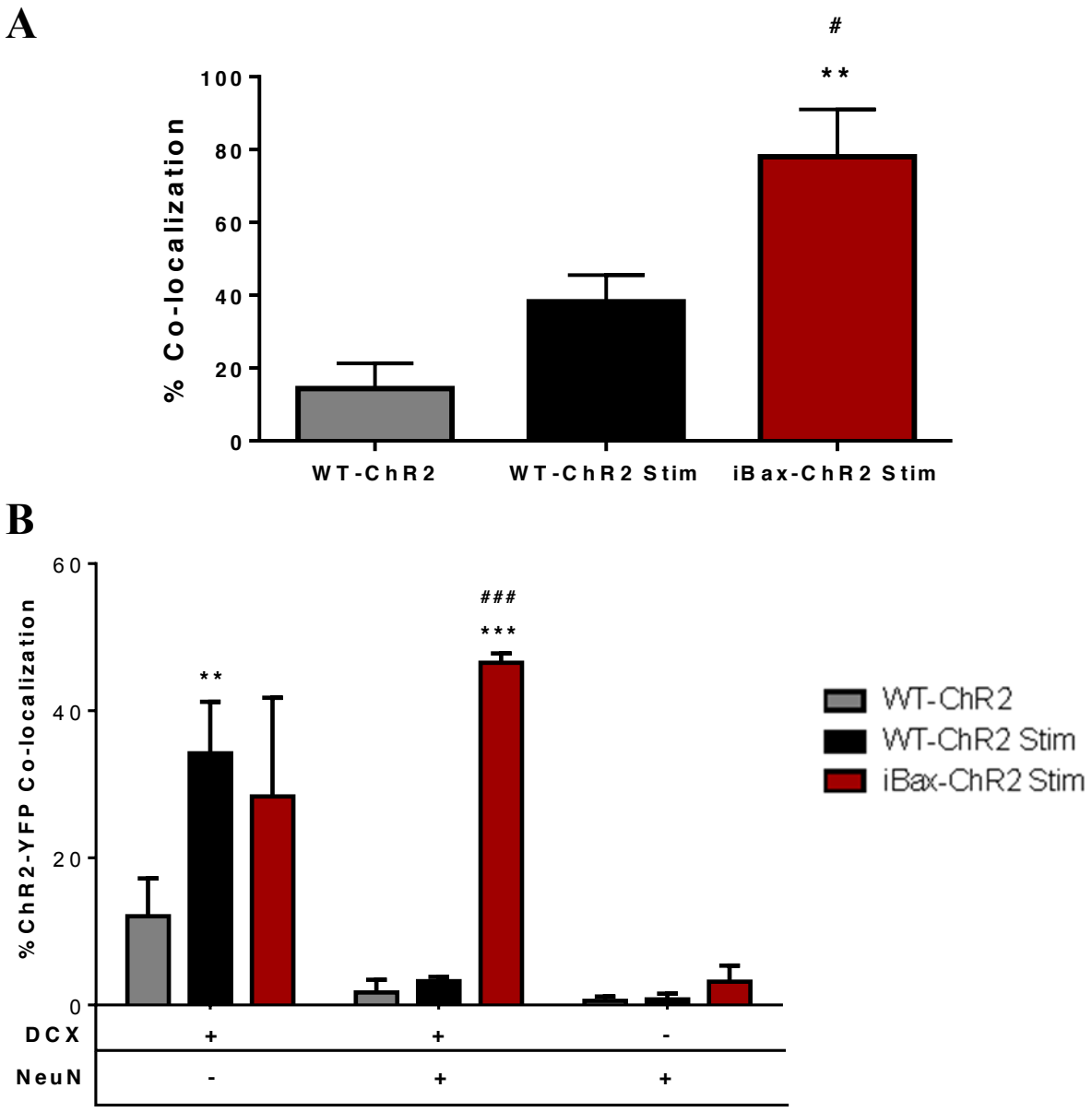

Figure 11 Quantification of Channelrhodopsin (ChR2-YFP) co-localization with neuronal markers. (A) Bar graph displaying the proportion of ChR2-YFP+ cells that co-localize with neuronal markers around the stroke site. Stimulated iBax-ChR2 mice had significantly higher co-localization with neuronal markers compared to both stimulated (p=0.0336) and non-stimulated (0.0027) WT-ChR2 mice. (B) Graphical representation of % co-localization of ChR2-YFP with only DCX, both DCX and NeuN and only NeuN. PCs within the stimulated WT-ChR2 group had significantly higher co-localization with DCX compared to WT mice (p=0.0069). Stimulated iBax-ChR2 mice had significantly higher co-localization with both neuronal markers compared to both stimulated (p<0.0001) and non-stimulated (p<0.0001) WT mice. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 represents significance from non-stimulated WT. **#** = significance from stimulated WT-ChR2.

4 – Discussion

As acute stroke interventions continue to improve and the average age of the population continues to increase, there is a significant increase in the number of stroke survivors in Canada. So far, even with rehabilitation therapies, many are left with life-long disabilities and there is increasing need to find novel methods to improve recovery. As a result, there has been a lot of hope in the ability to harness and enhance the neurogenic contributions to SBR processes in order to improve stroke outcomes. Our lab recently used the iBax mouse model to increase the survival of PCs that migrate to the stroke site and showed that, despite a large number of cells surviving and having a neuronal phenotype, there was no improvement in recovery (Ceizar, 2017). Additionally, we have published data showing that within the peri-infarct region, adult-generated immature neurons receive very few inputs from their surrounding environments (Kannangara et al., 2018). This thesis followed up on these results by testing if enhancing the survival of the PCs combined with stimulating the adult-generated neurons surrounding the infarct would be sufficient to improve recovery. Contrary to our hypothesis, optogenetic stimulation of the PCs was insufficient to improve recovery in the deficits that were observed in the battery sensorimotor tasks. Despite the lack of behavioural improvement, stimulation of the PCs increased the proportion of cells in both WT- and iBax-ChR2 mice that expressed mature neuronal markers. In fact, the iBax-ChR2 mice had the highest proportion of cells expressing NeuN. These results therefore raise the need for future studies to identify and further test if some experimental factors such as the optogenetic stimulation paradigm, stroke model, experimental timeline, and use of this iBax-ChR2 mouse line, may have contributed to the lack of improvement in recovery.

4.1 Optogenetic Stimulation Paradigm

Deciding which optogenetic stimulation paradigm to use for this study was difficult since there were no previously published studies that had stimulated endogenous PCs surrounding the stroke site. However, as noted in the introduction, there are a variety of studies that have stimulated PCs in the naïve brain, or in the peri-infarct cortex that helped to rationalize this study design. Specifically, *in vivo* studies have demonstrated that adult-generated cells in the RMS and OB respond best to high frequency (i.e. 40Hz) compared to lower (10 & 5 Hz) frequency stimulation (Alonso et al., 2012). Among the stroke recovery studies that have simulated the ipsilesional cortex there is little published work that has directly compared lower versus higher frequency. However, the majority of these studies have been done by Steinberg's group that uses the 10 Hz stimulation paradigm and find improvements in functional recovery (Cheng et al., 2014). Notably, this 10 Hz paradigm was also used to stimulate transplanted ChR2-YFP-expressing NSCs, derived from embryonic stem cells, which resulted in improvements in functional recovery (Daadi et al., 2016). Thus, based on this existing literature, a pilot study was conducted to compare the efficacy of 40 Hz or 10 Hz stimulation paradigms.

My preliminary pilot experiment was not included in the results section but was conducted to compare WT-ChR2 mice that received either 40 Hz or 10 Hz stimulation paradigms compared to mice that did not receive any simulation. The 40 Hz mice received the same stimulation paradigm (i.e. 40 Hz, 5 ms pulse width, 90 sec ON, 120 sec OFF) as was done when previous work stimulated *in vivo* SVZ-derived PCs in the OB (Alonso et al., 2012). The 10 Hz stimulation group received a stimulation paradigm that mirrored work from Steinberg's lab stimulating transplanted NSCs as well as Thy1-expressing neurons within the sensorimotor

cortex (i.e. 10 Hz, 20 ms pulse width, 90 sec ON, 120 sec OFF) (Cheng et al., 2014; Daadi et al., 2013). Although this pilot data was not quantified, qualitatively it appeared as though there was an increase in the expression of the immediate early gene (IEG), cFos, within the ipsilesional cortex of mice that received stimulation at 40 Hz compared to both 10 Hz and non-stimulated mice. This result suggested that more cells were activated using the 40 Hz paradigm, therefore we decided to proceed with this stimulation paradigm for the remainder of the project. As such, all stimulated mice within this manuscript received the same 40 Hz stimulation paradigm.

One of the most unique characteristics of this study is the specificity of the stimulation. This is the first study that has used optogenetics to specifically stimulate the endogenous SVZ-derived PCs in the peri-infarct region. As such, it was important to ensure that only PCs that have migrated to the stroke site could be activated by the optogenetic stimulation. In our study, implants were lowered 0.8 mm from the skull surface, meaning they extended on average 450 μm into the center of brain tissue (Kawakami & Yamamura, 2008). Recently, a Nature Methods paper measured the penetrance of light stimulation through brain tissue and demonstrated that from the end of the optical fiber, the power level of light is exponentially reduced as it penetrates through brain tissue falling to below 10% of its initial intensity by 500 μm. Within the field of optogenetics, the activation threshold (i.e. the minimum power level of light required to induce an action potential) for ChR2 is 1mW/mm^2. Given that the initial power intensity from the fiber tip ranged from 1.5mW to 3.0mW between all the mice, basic calculation suggests that our stimulation paradigm was well-targeted to only reach the PCs surrounding the stroke site. Together these data suggest that PCs only within 250 μm from the stroke site in this study were activated by light stimulation. This therefore eliminates the

possibility of activating PCs that are within the corpus callosum (>1 mm from the cortex) or outside of the peri-infarct region.

Together, this work provides the rationale for our stimulation paradigm and suggests that PCs within the peri-infarct region were activated by the daily optogenetic stimulation. This claim is supported by the results of this work, which showed increased neuronal differentiation in stimulated WT-ChR2 mice compared to non-stimulated controls. Nevertheless, we have yet to prove the responsiveness of PCs to the stimulation paradigm described in this study. Ongoing studies are examining the relative densities of *c-fos* expression between the experimental groups in this study. I hypothesize that there will be a significant increase in IEG expression within the peri-infarct of stimulated compared to non-stimulated mice. Moreover, I expect to observe increased activation in stimulated iBax-ChR2 compared to stimulated WT-ChR2 mice, as a result of the increased neurogenic response observed following the removal of *Bax*.

4.2 No significant improvements in functional recovery

4.2.1 Stroke model: Did we use the right stroke model?

It is possible that both iBax mice alone as shown previously (Ceizar, 2017), and the stimulated iBax-ChR2 mice in this study did not have significant improvements in part due to the use of the PT stroke model to induce strokes within the forelimb sensorimotor region. Indeed, virtually all preclinical studies performing optogenetic stimulation following stroke have only used tMCAO models (Lu et al., 2017). Compared to the PT model used in this study, the tMCAO model results in a short-lived significant disruption in blood flow due to a subsequent reperfusion step. Thus, it may be that using the tMCAO stroke model in stimulated iBax-ChR2

mice may produce more improvements. In support of this hypothesis, there is growing number of studies showing the importance of vascular support and angiogenesis for both stroke recovery and the growing links between neurogenesis, angiogenesis, and stroke recovery. Since 1993 it has become accepted that there is an angiogenic response to stroke after finding increases in angiogenesis and blood vessel density within the penumbra (Paciaroni, Caso & Agnelli, 2009). Indeed, the definition of penumbra is the perilesional area that receives perfusion within the threshold of physiological impairment yet importantly is capable of recovering with improved perfusion (Gauberti et al., 2016). Importantly, the perilesional area surrounding a PT-induced infarct cannot be referred to as the penumbra since targeted blood vessels within this model are permanently occluded (Watson et al., 1985). Within both clinical and animal populations, increased angiogenesis has been significantly correlated with improvements in stroke recovery (Lindvall & Kokaia, 2015). For example, exercise studies using animal models of stroke have demonstrated that exercise is associated with an upregulation of angiogenesis correlated with improved outcomes (Zhang et al., 2013). Additionally, others have shown that the abolition of angiogenesis nullified the beneficial effects of exercise post-stroke (Gertz et al., 2006). Thus, it is possible that the lack of improvements in function could be due to a lack of angiogenesis in iBax-ChR2 mice. In order to address this question, future work could replicate this experiment in a reperfusion model of stroke.

4.2.2 Experimental Timeline: Did the stimulation occur too late to alter recovery?

A limitation of this project design that could have also contributed to the lack of improvements is the timing of the optogenetic stimulation. No study demonstrating behavioural

improvements has commenced optogenetic stimulation later than 1 week post stroke (Lu et al., 2017). Largely due to the constraints of this experimental design, mice in this study first received optogenetic stimulation at the 3 weeks post stroke time point. As shown in Figure 3B, following the induction of the PT stroke, mice recovered for 3 days before beginning sensorimotor testing for 5 days to measure the baseline deficits from the stroke. Tamoxifen treatment then began on the last day of behavioural testing and was administered for 5 consecutive days in order to hinder pro-apoptotic mechanisms and allow expression of ChR2-YFP in the PC population. Finally, one week after TAM treatment, stimulation was administered to the respective experimental groups. Studies examining the neurogenic response to stroke have described that, although it depends on the distance from the site of injury, PCs take around 7 days to migrate to the site of injury and about 2-3 weeks before starting to express the immature neuronal marker, DCX (Lindvall, & Kokaia, 2015). Thus, due to the time it takes cells to migrate and differentiate into the stroke-injured cortex, we were unable to start stimulation earlier than 3 weeks after stroke.

Unfortunately, this may already be past the critical time window of neuroplasticity that occurs within the brain post-stroke. Although, clinical studies suggest that SBR occurs for 3 months following a stroke this process is accelerated in mice, resulting in the majority of behavioural improvements occurring within the first month post-stroke (Nakayama et al., 2010). Indeed, all of the pre-clinical studies have stimulated between 1 and 5 weeks following stroke induction (Lu et al., 2017). These studies were able to begin the stimulation earlier since they had either transplanted the cells into the site of injury or stimulated resident neuronal populations.

Although these studies have shown promise, none of them have ever activated endogenous SVZ-derived PCs following a stroke. In order to specifically address this question, ongoing work from our laboratory is aimed at dissecting the SVZ and isolating PCs, using fluorescence-activated cell sorting (FACS), in order to transplant these cells into the stroke site of another animal immediately following PT surgery. This methodology will allow us to specifically stimulate SVZ-derived PCs immediately following a stroke since we will no longer have to wait for their migration to the stroke site.

Several clinical studies testing stimulation treatment in chronic stroke patient populations (i.e. more than 3 months post-stroke) have resulted in promising improvements in behavioural recovery (Dionísio et al., 2018). This provides evidence contrary to the theorized "critical time window" for recovery from a stroke, however it seems unlikely that these results would be due to adult-generated cells that have migrated to the infarct region since post-mortem studies of neurogenesis in humans have demonstrated that by 3 months post-stroke, there is a negligible number of newborn cells within the peri-lesioned cortex.

4.2.4 Specificity of the iBax-CHR2 Mouse: Ectopic recombination?

After creating the iBax-ChR2 mice, pilot staining for the YFP+ recombined cells in the WT-ChR2 and iBax-ChR2 Stim mice revealed brightly labeled YFP+ cells in the SVZ with a stream of cells leading to site of stroke and specifically accumulating around the infarct. However more detailed analysis of YFP+ cells within all the brains after the study revealed there were some ectopic YFP+ cells. The ectopic cells looked morphologically different from the PCs and were labelled throughout the whole brain, with the exception of the infarct region. These cells were in WT and iBax mice regardless of whether they received stimulation. They

were also present in control WT-ChR2 and iBax-ChR2 mice that did not receive TAM treatment. However, these cells were not present in the original floxed ChR2-YFP mice, indicating that the mouse we have did not have leaky "off-target" expression, which has recently been identified by others (Prabhakar, Vujovic, Cui, Olson, & Luo, 2019). Indeed, the ectopic expression only occurred when the mice were crossed with the iBax mice that had the NestinCreER^{T2} gene (line 5.1) and floxed *Bax* gene (Sahay et al., 2011). Interestingly, this did not occur in previous studies when the same iBax NestinCreER^{T2} line 5.1 was crossed with a Rosa-YFP mouse, in the iBax mice (Ceizar, 2017). Therefore, this expression happens independent of recombination and appears to be specific to the NestinCreERT2 line 5.1 when crossed with the floxed CHR2-YFP mice. There are many different NestinCreER^{T2} drivers and direct comparison of a few of these lines has shown that the NestinCreER^{T2} (Line K: Lagace et al., 2007) has the greatest specificity (Sun et al., 2014). Thus, to evaluate if the ectopic expression was due to the NestinCreERT2 line 5.1, I made a new breeding of the NestinCreER^{T2} line K mice with the floxed iBax mice and ChR2-YFP mice. This newly created line had labelling that was restricted to the PCs, and there was no ectopic expression. However, as reported previously, the NestinCreER^{T2} line K mice did not appear to have as many labeled PCs, which was expected since although this line has a high degree of specificity, it does not have as much efficiency (Sun et al., 2014).

This ectopic expression of YFP+ cells in the mice used for this study raises the possibility that stimulation could have been occurring in the nestin-expressing PCs, as well as ectopic YFP+ cells. Although it is possible, it seems unlikely since ectopic YFP-ChR2+ cells look morphologically different from the labelled PCs and they were rarely found around the infarct. Thus, given that the stimulation was targeted around the infarct, it seems unlikely that they

would have an impact on our results, especially since we found no improvement in recovery. However, this possibility cannot be ruled out and thus, the newly bred iBax-ChR2 mice, that were created using the NestinCreERT2 (line K), could be used in future work to test the effect of stimulating these mice post stroke.

4.3 WT-Stim mice had the largest lesion volume

An unexpected and puzzling result of this study was that the stimulated WT-ChR2 group had the largest lesion volume compared to both the stimulated iBax-ChR2 mice and non-stimulated WT-ChR2 mice (Figure 7). This was surprising since there was no difference in stroke volume between iBax and WT mice 12 weeks after PT stroke (Ceizar, 2017), suggesting that enhancing the survival of PCs does not alter stroke size. Additionally, since the stimulated WT-ChR2 mice were the only group with significantly larger stroke size and the stimulated iBax-ChR2 received the same optogenetic stimulation paradigm, it does not appear that stimulation *per se* was associated with altering stroke size.

Our findings are also in contrast with existing literature suggesting that optogenetic stimulation can have a neuroprotective effect post-stroke, resulting in significant reductions in lesion volume (Bo et al., 2018). This difference, however, may be attributed to the acute nature of these studies, as well as differences in stimulation paradigms used. Thus, it remains unknown why there was a larger stroke in the stimulated WT-ChR2 group. Retrospectively, in order to understand more about why this occurred, the addition of MRI scans of the live post-stroke brain, would have helped to determine when this effect first occurs. Since without post-stroke imaging of the lesion volume it remains unknown if stimulation altered lesion volume since there is no measure of initial lesion volumes prior to stimulation.

It is important to also highlight that, on average, this significant difference was only 0.3 mm^3. This small value begs the question of whether such change in lesion volume is clinically relevant. Indeed, despite this difference in lesion volumes there were no behavioural differences between the three groups of mice. One could also argue that the difference in behaviour was not observed due to the lack of sensitivity of our tests when detecting these improvements. The battery of behavioural tests was specifically chosen for this thesis work, because they were capable of detecting robust deficits in mice up to 8 weeks post stroke previously in the iBax and WT mice (Ceizar, 2017). However, our results show some level of improvement from baseline in the 3 groups of mice as early as 4 weeks post stroke. Therefore, it is possible that a more robust method in measuring deficits post-stroke, or inducing larger strokes as discussed above, could have better allowed for detection of changes between the different groups of mice included in this study. In this regard, it has been exciting for me also to be involved with collaborations with other stroke labs in order to develop 3-dimensional kinematic assessment protocols for detecting stroke deficits in mice.

4.4 Effects of Optogenetic Stimulation on PC Migration and Neuronal Differentiation

As expected, the iBax-ChR2 stimulated mice had a significant increase in the number of cells that migrated to the peri-infarct region, as well as the proportion of cells that had a neuronal phenotype when compared to stimulated and non-stimulated WT-ChR2 mice. This was expected and mirrors the findings found previously when examining the same outcome measures in the iBax and WT mice (Ceizar, 2017). However, a comparison between the results presented in this thesis (Figure 8) and the previous data using iBax mice (Figure 1) shows differences. Specifically, stimulated iBax-ChR2 mice appear to have a lower average

percentage of YFP+ cells that colocalize with DCX and NeuN (Figure 11, 78+/-13%), compared to the iBax mice that were not stimulated (Figure 1, 95+/- 23%). However, this direct comparison should likely not be done since differences in colocalization can largely be the PC expression of ChR2-YFP protein in the current study, versus the YFP protein in previous work (Ceizar, 2017). These are not similar because ChR2-YFP is only localized within the cell membrane, whereas the YFP was localized within the cytosol. Due to this fact, colocalization of the ChR2-YFP+ cells were incredibly difficult in this study, since it was difficult to determine the location of the cell soma. This resulted in a number of neuronal markers that were visible, but could not be counted, suggesting that percentage of colocalization may have been underestimated for ChR2-YFP mice. This theory is further strengthened by the fact that the non-stimulated WT-ChR2 group in this stimulation study also had a lower % colocalization with neuronal markers (Figure 11, 14 +/-7%) compared to the WT mice in the previous iBax study (Figure 1, 26+/-12%). Unfortunately, the lack of clear cell somas in ChR2-YFP-expressing cells coupled with the large number of PCs in the iBax-ChR2-stim mice make it impossible to quantify the density of ChR2-YFP+ PCs in the peri-infarct region, as was done in the previously described iBax study (Ceizar, 2017). For this reason, DCX was used as a measure of the density of immature neurons surrounding the infarct. This could be overcome in future work through injecting the mice with Bromodeoxyuridine (BrdU) following PT-stroke and sacrificing the animals at a given time point to directly test the impact of stimulation and enhanced survival on PC migration to the stroke site, as was previously done by Sahay et al., (2011) in the iBax mice.

Despite these limitations, it was obvious that the iBax mice have a striking increase in the number of cells in the infarct zone with the majority of these cells having a neuronal

phenotype. However, since there was no iBax non-stimulated group included in this work, it is not possible to know if a similar effect would occur between stimulated and non-stimulated iBax mice.

Recently, an experiment by Liang and colleagues (2019) suggested that increased activity within the peri-lesional cortex is associated with increased migration of PCs to the stroke site, as well as enhanced neuronal differentiation. Increased activity was achieved through either stimulation of motor cortex, or increased forelimb use in a CIMT protocol, both resulting in significant improvements in recovery from a stroke. Similarly, in this thesis stimulated WT-ChR2 mice had significantly higher colocalization with the immature neuronal marker DCX, suggesting that stimulation of this PC population enhanced the rate of their neuronal differentiation. Thus, the results presented in this thesis and this recent paper are similar since both studies find enhanced neuronal differentiation with stimulation. However, this thesis finds no improvement in recovery, whereas their findings demonstrated improvements in stroke recovery.

4.5 Are the Neurogenic Cells in the Peri-infarct Cortex Being Integrated?

One hypothesis as to why the WT-ChR2 and iBax-ChR2 stimulation groups in this thesis did not have improvements in function could be that the adult-generated cells were not being integrated within the cortex. Previously, work within our lab, demonstrated using nestin-GFP and DCX-DsRed mice, that the majority of PCs that migrate to the site of injury do not survive and differentiate into immature, let alone mature neurons (Kannangara et al., 2018). Electrophysiological recordings of the few surviving cells that had a neuronal phenotype revealed that these cells receive limited synaptic inputs yet are capable of producing action

potentials. The result of this work concluded that adult-generated neurons have sparse integration within the stroke-injured cortex. Attempts to patch the PCs within the iBax model by a postdoctoral fellow in the lab, proved unfeasible due to the difficulty of isolating individual cells due to the overwhelming migration of PCs to the cortex in this model. Thus, future work could overcome this issue through using a red-fluorescent protein (RFP) retrovirus, which would make the cell much easier to identify to patch.

The previous work from the lab also demonstrated that the surviving PCs differentiated into subsets of GABAergic interneurons. Future work is required to determine if these cells remain interneurons (Kannangara et al., 2018). Since this work used reporter mice, no analysis could be done after 4 weeks, when the reporters were no longer active. Thus, ongoing work is using FACS sorting the YFP+ cells in iBax mice at 8 weeks post stroke in order to make mRNA of the isolated cells to test for expression of interneuron markers. Additionally, IHC staining for interneuron markers that were previously identified can be used on the tissue that has been collected for the iBax study, as well as the iBax-ChR2 animal generated in this study. Lastly, it would be of interest to use similar methods published by Liang and colleagues (2019) to determine what functional connections the PCs make within the stroke-injured cortex. This could be accomplished by staining these PCs with a pre-synaptic marker as well as post-synaptic markers in order to count the number of connections made between PCs and resident neurons surrounding the infarct as determined by colocalization. These studies are important since the functional role that PCs have following their migration to the site of injury remains unknown.

4.6 Cell-type Specific Targeting of Stimulation in the Peri-infarct Cortex

It is promising that non-invasive brain stimulation techniques are capable of increasing or reducing the activity of the brain. Both tDCS and rTMS stimulation have resulted in significant improvements in stroke outcomes within both clinical and pre-clinical populations (Gauberti et al., 2016; Boonzaier et al., 2018; Dionísio et al., 2018). The interhemispheric competition model suggests that the tissue death that occurs after stroke results in reduced activity within the ipsilesional hemisphere and increased trans-callosal inhibition from the contralesional hemisphere, resulting in significantly worse stroke outcomes. The rationale for the use of non-invasive brain stimulation is that cortical excitability can be modulated to increase plasticity and improve motor outcomes (Caglayan et al., 2019). Within the field of stroke recovery, non-invasive brain stimulation has typically been used as a means of either increasing activity within the damaged hemisphere or reducing activity in the less affected hemisphere. The majority of non-invasive brain stimulation studies with clinical populations have stimulated the contralesional hemisphere in chronic stroke survivors more than 6 months following stroke in order to avoid worsening stroke outcomes (Dionísio et al., 2018). Unfortunately, review studies have demonstrated that stimulation results in the greatest level of improvement when administered during the sub-acute period of stroke. Although the majority of stimulation studies have resulted in functional improvements in stroke patients, a systematic review of all stimulation studies by Dionísio and colleagues (2018) has revealed conflicting reports regarding the efficacy of tDCS compared to rTMS, and stimulation of the more affected compared to the less affected hemisphere. Although the results from recent non-invasive brain stimulation studies have been promising, fundamental limitations have made it difficult to determine exactly how stimulation exerts its effects (Polanía et al., 2018). Primarily, both

forms of non-invasive brain stimulation have very low spatial resolutions relative to pre-clinical stimulation techniques. Although both methods can have relatively precise regional specificity, they are unable to modulate the activity of specific cell populations making it difficult to determine their mechanism of action.

Preclinical studies using optogenetics provide the benefit of being able to use transgenic models and viral injections to specifically target distinct cell populations to help understand more about what cells are contributing to improvements in recovery (Cheng et al., 2014). Optogenetics has been used to modulate the activity of: Thy-1 expressing pyramidal neurons, excitatory glutamatergic neurons, GABAergic neurons, thalamocortical neurons and transplanted NSCs during stroke recovery. In this thesis we demonstrated that increased activation and survival of migrating PCs does not significantly impact behavioural recovery in spite of significantly enhanced neuronal differentiation. Thus, these results suggest that newborn neurons within the stroke site are not the target of non-invasive brain stimulation experiments that have resulted in improved stroke outcomes.

5 - Conclusions and Significance

Countless studies have suggested that a number of factors such as running, or antidepressant treatments improve stroke recovery as a result of correlated increases in neurogenesis. The results outlined in this manuscript provide considerable evidence contrary to the notion that enhanced neurogenesis is sufficient to improve stroke recovery. Indeed, the results suggest that enhancing the survival and differentiation of PCs into neurons, is insufficient to improve recovery. The implications of this work are increasingly relevant to clinical populations since the use of non-invasive brain stimulation has become more prevalent in stroke patients and suggest that the variabilities in these outcomes are likely not due to effects of stimulation of adult-generated cells. It remains important that these results are interpreted in light of the limitations previously described, given this is the first experiment to test the hypothesis that stimulation of PCs could alter recovery, there remains a lot more work to be done to elucidate the role of this cell population during stroke recovery. Thus, it will be the challenge of future work to determine if the potential of these endogenous cells can be harnessed to improve quality of life for the growing number of stroke survivors in Canada.

References

1. Alonso, M., Lepousez, G., Sebastien, W., Bardy, C., Gabellec, M.-M., Torquet, N., & Lledo, P.-M. (2012). Activation of adult-born neurons facilitates learning and memory. *Nature Neuroscience*, *15*(6), 897–904. https://doi.org/10.1038/nn.3108
2. Andres, R. H., Horie, N., Slikker, W., Keren-Gill, H., Zhan, K., Sun, G., … Steinberg, G. K. (2011). Human neural stem cells enhance structural plasticity and axonal transport in the ischaemic brain. *Brain: A Journal of Neurology*, *134*(Pt 6), 1777–1789. https://doi.org/10.1093/brain/awr094
3. Bacigaluppi, M., Pluchino, S., Peruzzotti-Jametti, L., Jametti, L. P., Kilic, E., Kilic, U., … Hermann, D. M. (2009). Delayed post-ischaemic neuroprotection following systemic neural stem cell transplantation involves multiple mechanisms. *Brain: A Journal of Neurology*, *132*(Pt 8), 2239–2251. https://doi.org/10.1093/brain/awp174
4. Balkaya, M., Kröber, J., Gertz, K., Peruzzaro, S., & Endres, M. (2013). Characterization of long-term functional outcome in a murine model of mild brain ischemia. *Journal of Neuroscience Methods*, *213*(2), 179–187. https://doi.org/10.1016/j.jneumeth.2012.12.021
5. Bernhardt, J., Hayward, K. S., Kwakkel, G., Ward, N. S., Wolf, S. L., Borschmann, K., … Cramer, S. C. (2017). Agreed definitions and a shared vision for new standards in stroke recovery research: The Stroke Recovery and Rehabilitation Roundtable taskforce: *International Journal of Stroke*. https://doi.org/10.1177/1747493017711816
6. Bo, B., Li, Y., Li, W., Wang, Y., & Tong, S. (2018). Optogenetic excitation of ipsilesional sensorimotor neurons is protective in acute ischemic stroke: A laser

speckle imaging study. *IEEE Transactions on Biomedical Engineering*, 1–1. https://doi.org/10.1109/TBME.2018.2872965

7. Boldrini, M., Fulmore, C. A., Tartt, A. N., Simeon, L. R., Pavlova, I., Poposka, V., … Mann, J. J. (2018). Human Hippocampal Neurogenesis Persists throughout Aging. *Cell Stem Cell*, *22*(4), 589-599.e5. https://doi.org/10.1016/j.stem.2018.03.015
8. Bond, A. M., Ming, G., & Song, H. (2015). Adult Mammalian Neural Stem Cells and Neurogenesis: Five Decades Later. *Cell Stem Cell*, *17*(4), 385–395. https://doi.org/10.1016/j.stem.2015.09.003
9. Boonzaier, J., van Tilborg, G. A. F., Neggers, S. F. W., & Dijkhuizen, R. M. (2018). Noninvasive Brain Stimulation to Enhance Functional Recovery After Stroke: Studies in Animal Models. *Neurorehabilitation and Neural Repair*, *32*(11), 927–940. https://doi.org/10.1177/1545968318804425
10. Bouet, V., Boulouard, M., Toutain, J., Divoux, D., Bernaudin, M., Schumann-Bard, P., & Freret, T. (2009). The adhesive removal test: A sensitive method to assess sensorimotor deficits in mice. *Nature Protocols*, *4*(10), 1560–1564. https://doi.org/10.1038/nprot.2009.125
11. Boyd, L. A., Hayward, K. S., Ward, N. S., Stinear, C. M., Rosso, C., Fisher, R. J., … Cramer, S. C. (2017). Biomarkers of stroke recovery: Consensus-based core recommendations from the Stroke Recovery and Rehabilitation Roundtable. *International Journal of Stroke: Official Journal of the International Stroke Society*, *12*(5), 480–493. https://doi.org/10.1177/1747493017714176

12. Bradnam, L. V., Stinear, C. M., & Byblow, W. D. (2013). Ipsilateral Motor Pathways after Stroke: Implications for Non-Invasive Brain Stimulation. *Frontiers in Human Neuroscience*, *7*. https://doi.org/10.3389/fnhum.2013.00184

13. Butti, E., Bacigaluppi, M., Rossi, S., Cambiaghi, M., Bari, M., Cebrian Silla, A., … Martino, G. (2012). Subventricular zone neural progenitors protect striatal neurons from glutamatergic excitotoxicity. *Brain: A Journal of Neurology*, *135*(Pt 11), 3320–3335. https://doi.org/10.1093/brain/aws194

14. Caglayan, A. B., Beker, M. C., Caglayan, B., Yalcin, E., Dilden, A., Yulug, B., … Kilic, E. (2019). Acute and post-acute neuromodulation induces stroke recovery by promoting survival signaling, neurogenesis and pyramidal tract plasticity. *Frontiers in Cellular Neuroscience*, *13*. https://doi.org/10.3389/fncel.2019.00144

15. Carmichael, S. T. (2016). Emergent properties of neural repair: Elemental biology to therapeutic concepts. *Annals of Neurology*, *79*(6), 895–906. https://doi.org/10.1002/ana.24653

16. Ceizar, M. (2017). *Evaluating the Functional Role of Enhancing Progenitor Cell Survival Following Stroke Recovery* (Thesis, Université d'Ottawa / University of Ottawa). http://dx.doi.org/10.20381/ruor-20426

17. Ceizar, M., Dhaliwal, J., Xi, Y., Smallwood, M., Kumar, K. L., & Lagace, D. C. (2016). Bcl-2 is required for the survival of doublecortin-expressing immature neurons. *Hippocampus*, *26*(2), 211–219. https://doi.org/10.1002/hipo.22504

18. Chang, W. H., Kim, Y.-H., Bang, O. Y., Kim, S. T., Park, Y. H., & Lee, P. K. W. (2010, September). Long-Term Effects of rTMS on Motor Recovery in Patients After Subacute Stroke [Text]. https://doi.org/info:doi/10.2340/16501977-0590

19. Cheng, M. Y., Wang, E. H., Woodson, W. J., Wang, S., Sun, G., Lee, A. G., … Steinberg, G. K. (2014). Optogenetic neuronal stimulation promotes functional recovery after stroke. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(35), 12913–12918. https://doi.org/10.1073/pnas.1404109111

20. Chrostek, M. R., Fellows, E. G., Crane, A. T., Grande, A. W., & Low, W. C. (2019). Efficacy of stem cell-based therapies for stroke. *Brain Research*, *1722*, 146362. https://doi.org/10.1016/j.brainres.2019.146362

21. Clarkson, A. N., Huang, B. S., Macisaac, S. E., Mody, I., & Carmichael, S. T. (2010). Reducing excessive GABA-mediated tonic inhibition promotes functional recovery after stroke. *Nature*, *468*(7321), 305–309. https://doi.org/10.1038/nature09511

22. Coupar, F., Pollock, A., Rowe, P., Weir, C., & Langhorne, P. (2012). Predictors of upper limb recovery after stroke: A systematic review and meta-analysis. *Clinical Rehabilitation*, *26*(4), 291–313. https://doi.org/10.1177/0269215511420305

23. Crichton, S. L., Bray, B. D., McKevitt, C., Rudd, A. G., & Wolfe, C. D. A. (2016). Patient outcomes up to 15 years after stroke: Survival, disability, quality of life, cognition and mental health. *Journal of Neurology, Neurosurgery, and Psychiatry*, *87*(10), 1091–1098. https://doi.org/10.1136/jnnp-2016-313361

24. Daadi, M. M., Hu, S., Klausner, J., Li, Z., Sofilos, M., Sun, G., … Steinberg, G. K. (2013). Imaging neural stem cell graft-induced structural repair in stroke. *Cell Transplantation*, *22*(5), 881–892. https://doi.org/10.3727/096368912X656144

25. Deisseroth, K. (2015). Optogenetics: 10 years of microbial opsins in neuroscience. *Nature Neuroscience*, *18*(9), 1213–1225. https://doi.org/10.1038/nn.4091

26. Dennis, C. V., Suh, L. S., Rodriguez, M. L., Kril, J. J., & Sutherland, G. T. (2016). Human adult neurogenesis across the ages: An immunohistochemical study. *Neuropathology and Applied Neurobiology*, *42*(7), 621–638. https://doi.org/10.1111/nan.12337

27. Deubner, J., Coulon, P., & Diester, I. (2019). Optogenetic approaches to study the mammalian brain. *Current Opinion in Structural Biology*, *57*, 157–163. https://doi.org/10.1016/j.sbi.2019.04.003

28. Dionísio, A., Duarte, I. C., Patrício, M., & Castelo-Branco, M. (2018). The Use of Repetitive Transcranial Magnetic Stimulation for Stroke Rehabilitation: A Systematic Review. *Journal of Stroke and Cerebrovascular Diseases: The Official Journal of National Stroke Association*, *27*(1), 1–31. https://doi.org/10.1016/j.jstrokecerebrovasdis.2017.09.008

29. Dranovsky, A., & Hen, R. (2006). Hippocampal neurogenesis: Regulation by stress and antidepressants. *Biological Psychiatry*, *59*(12), 1136–1143. https://doi.org/10.1016/j.biopsych.2006.03.082

30. Ergul, A., Alhusban, A., & Fagan, S. C. (2012). Angiogenesis: A Harmonized Target for Recovery after Stroke. *Stroke; a Journal of Cerebral Circulation*, *43*(8), 2270–2274. https://doi.org/10.1161/STROKEAHA.111.642710

31. Fisicaro, F., Lanza, G., Grasso, A. A., Pennisi, G., Bella, R., Paulus, W., & Pennisi, M. (2019). Repetitive transcranial magnetic stimulation in stroke rehabilitation: Review of the current evidence and pitfalls. *Therapeutic Advances in Neurological Disorders*, *12*, 1756286419878317. https://doi.org/10.1177/1756286419878317

32. Gauberti, M., De Lizarrondo, S. M., & Vivien, D. (2016). The "inflammatory penumbra" in ischemic stroke: From clinical data to experimental evidence. *European Stroke Journal*, *1*(1), 20–27. https://doi.org/10.1177/2396987316630249

33. Gertz, K., Priller, J., Kronenberg, G., Fink, K. B., Winter, B., Schröck, H., … Endres, M. (2006). Physical activity improves long-term stroke outcome via endothelial nitric oxide synthase-dependent augmentation of neovascularization and cerebral blood flow. *Circulation Research*, *99*(10), 1132–1140. https://doi.org/10.1161/01.RES.0000250175.14861.77

34. Gotts, J. E., & Chesselet, M.-F. (2005). Migration and fate of newly born cells after focal cortical ischemia in adult rats. *Journal of Neuroscience Research*, *80*(2), 160–171. https://doi.org/10.1002/jnr.20434

35. Grefkes, C., & Ward, N. S. (2014). Cortical reorganization after stroke: How much and how functional? *The Neuroscientist: A Review Journal Bringing Neurobiology, Neurology and Psychiatry*, *20*(1), 56–70. https://doi.org/10.1177/1073858413491147

36. Hatakeyama, M., Ninomiya, I., & Kanazawa, M. (2020). Angiogenesis and neuronal remodeling after ischemic stroke. *Neural Regeneration Research*, *15*(1), 16. https://doi.org/10.4103/1673-5374.264442

37. Jeffers, M. S., Karthikeyan, S., & Corbett, D. (2018). Does Stroke Rehabilitation Really Matter? Part A: Proportional Stroke Recovery in the Rat. *Neurorehabilitation and Neural Repair*, *32*(1), 3–6. https://doi.org/10.1177/1545968317751210

38. Jin, K., Wang, X., Xie, L., Mao, X. O., & Greenberg, D. A. (2010). Transgenic ablation of doublecortin-expressing cells suppresses adult neurogenesis and worsens

stroke outcome in mice. *Proceedings of the National Academy of Sciences*, *107*(17), 7993–7998. https://doi.org/10.1073/pnas.1000154107

39. Jin, K., Wang, X., Xie, L., Mao, X. O., Zhu, W., Wang, Y., … Greenberg, D. A. (2006). Evidence for stroke-induced neurogenesis in the human brain. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(35), 13198–13202. https://doi.org/10.1073/pnas.0603512103

40. Jones, T. A. (2017). Motor compensation and its effects on neural reorganization after stroke. *Nature Reviews. Neuroscience*, *18*(5), 267–280. https://doi.org/10.1038/nrn.2017.26

41. Kannangara, T. S., Carter, A., Xue, Y., Dhaliwal, J. S., Béïque, J.-C., & Lagace, D. C. (2018). Excitable Adult-Generated GABAergic Neurons Acquire Functional Innervation in the Cortex after Stroke. *Stem Cell Reports*, *0*(0). https://doi.org/10.1016/j.stemcr.2018.10.011

42. Kawakami, M., & Yamamura, K. (2008). Cranial bone morphometric study among mouse strains. *BMC Evolutionary Biology*, *8*(1), 73. https://doi.org/10.1186/1471-2148-8-73

43. Kempermann, G., Gage, F. H., Aigner, L., Song, H., Curtis, M. A., Thuret, S., … Frisén, J. (2018). Human Adult Neurogenesis: Evidence and Remaining Questions. *Cell Stem Cell*, *23*(1), 25–30. https://doi.org/10.1016/j.stem.2018.04.004

44. Krakauer, J. W., Carmichael, S. T., Corbett, D., & Wittenberg, G. F. (2012). Getting neurorehabilitation right: What can be learned from animal models? *Neurorehabilitation and Neural Repair*, *26*(8), 923–931. https://doi.org/10.1177/1545968312440745

45. Krueger Hans, Koot Jacqueline, Hall Ruth E., O'Callaghan Christina, Bayley Mark, & Corbett Dale. (2015). Prevalence of Individuals Experiencing the Effects of Stroke in Canada. *Stroke*, *46*(8), 2226–2231. https://doi.org/10.1161/STROKEAHA.115.009616

46. Kumar, A., Pareek, V., Faiq, M. A., Ghosh, S. K., & Kumari, C. (2019). ADULT NEUROGENESIS IN HUMANS: A Review of Basic Concepts, History, Current Research, and Clinical Implications. *Innovations in Clinical Neuroscience*, *16*(5–6), 30–37.

47. Lagace, D. C. (2012). Does the endogenous neurogenic response alter behavioral recovery following stroke? *Behavioural Brain Research*, *227*(2), 426–432. https://doi.org/10.1016/j.bbr.2011.08.045

48. Lagace, D. C., Whitman, M. C., Noonan, M. A., Ables, J. L., DeCarolis, N. A., Arguello, A. A., … Eisch, A. J. (2007). Dynamic contribution of nestin-expressing stem cells to adult neurogenesis. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *27*(46), 12623–12629. https://doi.org/10.1523/JNEUROSCI.3812-07.2007

49. Lam, T. K., Binns, M. A., Honjo, K., Dawson, D. R., Ross, B., Stuss, D. T., … Chen, J. L. (2018). Variability in stroke motor outcome is explained by structural and functional integrity of the motor system. *Scientific Reports*, *8*(1), 1–11. https://doi.org/10.1038/s41598-018-27541-8

50. Langhorne, P., Bernhardt, J., & Kwakkel, G. (2011). Stroke rehabilitation. *The Lancet*, *377*(9778), 1693–1702. https://doi.org/10.1016/S0140-6736(11)60325-5

51. Lerner, T. N., Ye, L., & Deisseroth, K. (2016). Communication in Neural Circuits: Tools, Opportunities, and Challenges. *Cell*, *164*(6), 1136–1150. https://doi.org/10.1016/j.cell.2016.02.027

52. Liang, H., Zhao, H., Gleichman, A., Machnicki, M., Telang, S., Tang, S., … Carmichael, S. T. (2019). Region-specific and activity-dependent regulation of SVZ neurogenesis and recovery after stroke. *Proceedings of the National Academy of Sciences*, *116*(27), 13621–13630. https://doi.org/10.1073/pnas.1811825116

53. Lindvall, O., & Kokaia, Z. (2015). Neurogenesis following Stroke Affecting the Adult Brain. *Cold Spring Harbor Perspectives in Biology*, *7*(11). https://doi.org/10.1101/cshperspect.a019034

54. Lu, J., Manaenko, A., & Hu, Q. (2017). Targeting Adult Neurogenesis for Poststroke Therapy. *Stem Cells International*, *2017*. https://doi.org/10.1155/2017/5868632

55. Lu, Y., Jiang, L., Li, W., Qu, M., Song, Y., He, X., … Wang, Y. (2017). Optogenetic Inhibition of Striatal Neuronal Activity Improves the Survival of Transplanted Neural Stem Cells and Neurological Outcomes after Ischemic Stroke in Mice. *Stem Cells International*, *2017*, 4364302. https://doi.org/10.1155/2017/4364302

56. Macas, J., Nern, C., Plate, K. H., & Momma, S. (2006). Increased generation of neuronal progenitors after ischemic injury in the aged adult human forebrain. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *26*(50), 13114–13119. https://doi.org/10.1523/JNEUROSCI.4667-06.2006

57. Metz, G. A., & Whishaw, I. Q. (2009). The ladder rung walking task: A scoring system and its practical application. *Journal of Visualized Experiments: JoVE*, (28). https://doi.org/10.3791/1204

58. Moreno-Jiménez, E. P., Flor-García, M., Terreros-Roncal, J., Rábano, A., Cafini, F., Pallas-Bazarra, N., … Llorens-Martín, M. (2019). Adult hippocampal neurogenesis is abundant in neurologically healthy subjects and drops sharply in patients with Alzheimer's disease. *Nature Medicine*, *25*(4), 554–560. https://doi.org/10.1038/s41591-019-0375-9

59. Murphy, T. H., & Corbett, D. (2009). Plasticity during stroke recovery: From synapse to behaviour. *Nature Reviews. Neuroscience*, *10*(12), 861–872. https://doi.org/10.1038/nrn2735

60. Nakayama, D., Matsuyama, T., Ishibashi-Ueda, H., Nakagomi, T., Kasahara, Y., Hirose, H., … Taguchi, A. (2010). Injury-induced neural stem/progenitor cells in post-stroke human cerebral cortex. *European Journal of Neuroscience*, *31*(1), 90–98. https://doi.org/10.1111/j.1460-9568.2009.07043.x

61. Paciaroni, M., Caso, V., & Agnelli, G. (2009). The concept of ischemic penumbra in acute stroke and therapeutic opportunities. *European Neurology*, *61*(6), 321–330. https://doi.org/10.1159/000210544

62. Polanía, R., Nitsche, M. A., & Ruff, C. C. (2018). Studying and modifying brain function with non-invasive brain stimulation. *Nature Neuroscience*, *21*(2), 174–187. https://doi.org/10.1038/s41593-017-0054-4

63. Prabhakar, A., Vujovic, D., Cui, L., Olson, W., & Luo, W. (2019). Leaky expression of channelrhodopsin-2 (ChR2) in Ai32 mouse lines. *PLOS ONE*, *14*(3), e0213326. https://doi.org/10.1371/journal.pone.0213326

64. Prabhakaran, S., Zarahn, E., Riley, C., Speizer, A., Chong, J. Y., Lazar, R. M., … Krakauer, J. W. (2008). Inter-individual Variability in the Capacity for Motor

Recovery After Ischemic Stroke. *Neurorehabilitation and Neural Repair*, *22*(1), 64–71. https://doi.org/10.1177/1545968307305302

65. Raber, J., Fan, Y., Matsumori, Y., Liu, Z., Weinstein, P. R., Fike, J. R., & Liu, J. (2004). Irradiation attenuates neurogenesis and exacerbates ischemia-induced deficits. *Annals of Neurology*, *55*(3), 381–389. https://doi.org/10.1002/ana.10853

66. Sahay, A., Scobie, K. N., Hill, A. S., O'Carroll, C. M., Kheirbek, M. A., Burghardt, N. S., … Hen, R. (2011). Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. *Nature*, *472*(7344), 466–470. https://doi.org/10.1038/nature09817

67. Song, M., Yu, S. P., Mohamad, O., Cao, W., Wei, Z. Z., Gu, X., … Wei, L. (2017). Optogenetic stimulation of glutamatergic neuronal activity in the striatum enhances neurogenesis in the subventricular zone of normal and stroke mice. *Neurobiology of Disease*, *98*, 9–24. https://doi.org/10.1016/j.nbd.2016.11.005

68. Sorrells, S. F., Paredes, M. F., Cebrian-Silla, A., Sandoval, K., Qi, D., Kelley, K. W., … Alvarez-Buylla, A. (2018). Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. *Nature*, *555*(7696), 377–381. https://doi.org/10.1038/nature25975

69. Spalletti, C., Alia, C., Lai, S., Panarese, A., Conti, S., Micera, S., & Caleo, M. (2017). Combining robotic training and inactivation of the healthy hemisphere restores pre-stroke motor patterns in mice. *ELife*, *6*. https://doi.org/10.7554/eLife.28662

70. Sun, C., Sun, H., Wu, S., Lee, C. C., Akamatsu, Y., Wang, R. K., … Liu, J. (2013). Conditional ablation of neuroprogenitor cells in adult mice impedes recovery of poststroke cognitive function and reduces synaptic connectivity in the perforant

pathway. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *33*(44), 17314–17325. https://doi.org/10.1523/JNEUROSCI.2129-13.2013

71. Sun, M.-Y., Yetman, M. J., Lee, T.-C., Chen, Y., & Jankowsky, J. L. (2014). Specificity and efficiency of reporter expression in adult neural progenitors varies substantially among nestin-CreERT2 lines. *The Journal of Comparative Neurology*, *522*(5), 1191–1208. https://doi.org/10.1002/cne.23497

72. Tennant, K. A., Adkins, D. L., Donlan, N. A., Asay, A. L., Thomas, N., Kleim, J. A., & Jones, T. A. (2011). The Organization of the Forelimb Representation of the C57BL/6 Mouse Motor Cortex as Defined by Intracortical Microstimulation and Cytoarchitecture. *Cerebral Cortex (New York, NY)*, *21*(4), 865–876. https://doi.org/10.1093/cercor/bhq159

73. Tennant, K. A., Taylor, S. L., White, E. R., & Brown, C. E. (2017). Optogenetic rewiring of thalamocortical circuits to restore function in the stroke injured brain. *Nature Communications*, *8*(1), 1–14. https://doi.org/10.1038/ncomms15879

74. Wahl, A. S., Büchler, U., Brändli, A., Brattoli, B., Musall, S., Kasper, H., … Schwab, M. E. (2017). Optogenetically stimulating intact rat corticospinal tract post-stroke restores motor control through regionalized functional circuit formation. *Nature Communications*, *8*(1), 1187. https://doi.org/10.1038/s41467-017-01090-6

75. Wang, X., Mao, X., Xie, L., Sun, F., Greenberg, D. A., & Jin, K. (2012). Conditional depletion of neurogenesis inhibits long-term recovery after experimental stroke in mice. *PloS One*, *7*(6), e38932. https://doi.org/10.1371/journal.pone.0038932

76. Watson, B. D., Dietrich, W. D., Busto, R., Wachtel, M. S., & Ginsberg, M. D. (1985). Induction of reproducible brain infarction by photochemically initiated thrombosis. *Annals of Neurology*, *17*(5), 497–504. https://doi.org/10.1002/ana.410170513

77. Williamson, M. R., Jones, T. A., & Drew, M. R. (2019). Functions of subventricular zone neural precursor cells in stroke recovery. *Behavioural Brain Research*, *376*, 112209. https://doi.org/10.1016/j.bbr.2019.112209

78. Yizhar, O., Fenno, L. E., Davidson, T. J., Mogri, M., & Deisseroth, K. (2011). Optogenetics in Neural Systems. *Neuron*, *71*(1), 9–34. https://doi.org/10.1016/j.neuron.2011.06.004

79. Zhang, P., Yu, H., Zhou, N., Zhang, J., Wu, Y., Zhang, Y., … Hu, Y. (2013). Early exercise improves cerebral blood flow through increased angiogenesis in experimental stroke rat model. *Journal of NeuroEngineering and Rehabilitation*, *10*, 43. https://doi.org/10.1186/1743-0003-10-43

80. Zhuo, J.-M., Tseng, H.-A., Desai, M., Bucklin, M. E., Mohammed, A. I., Robinson, N. T., … Han, X. (2016). Young adult born neurons enhance hippocampal dependent performance via influences on bilateral networks. *ELife*, *5*. https://doi.org/10.7554/eLife.22429