Ablation of Progenitor Cells Does Not Impede Motor Recovery or Diminish Cognitive Function Following a Focal Cortical Stroke

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Abstract

Following a stroke there is a significant increase in the number and migration of progenitor cells (PCs) to the infarct, and positive correlations between neurogenesis and recovery. Loss-of-function studies have conflicting findings on whether the ablation of PCs impedes motor or cognitive function post-stroke. This thesis examines if neurogenesis *per se* is required for motor recovery and spatial learning and memory. PCs were ablated in an adult GFAP-TK rat model that allows for the inducible deletion of GFAP-expressing PCs in the brain. An endothelin-1 (ET-1) stroke was produced and assessment of motor function and spatial learning and memory revealed no differences between control and GFAP-TK rats in which PCs were ablated. This study is the first to use a focal cortical stroke model in a rat to study PCs and stroke recovery, and suggest that PCs and their progeny are dispensable for motor recovery and spatial learning and memory post-stroke.

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List of Abbreviations

AP	Anteroposterior
BCCAo	Bilateral common carotid artery occlusion
BrdU	5-bromo-2'-deoxyuridine
CCA	Common carotid artery
CC	Corpus callosum
Cx	Cortex
DAB	3,3'-Diaminobenzidine
DCX	Doublecortin
DG	Hippocampal dentate gyrus
dMCAo	Distal middle cerebral artery occlusion
DNA	Deoxyribonucleic acid
DV	Dorsoventral
ET-1	Endothelin-1
GCV	Ganciclovir
GCL	Granule cell layer
GFAP	Glial Fibrillary Acidic Protein
GTP	Guanine triphosphate
HSV-TK	Herpes Simplex Virus thymidine kinase
IHC	Immunohistochemistry
IL-2	Interleukin-2
IP	Intraperitoneal
L	Lesion
LE	Long-Evans hooded
LV	Lateral ventricles
MCA	Middle cerebral artery
MCAo	Middle cerebral artery occlusion
ML	Molecular layer
MLA	Mediolateral
MRI	Magnetic resonance imaging
NaN_3	Sodium azide
NaOH	Sodium hydroxide
NDS	Normal donkey serum
NeuN	Neuronal nuclear protein
NIH	National Institute of Health
OB	Olfactory bulb
PB	Peanut butter
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCs	Stem and progenitor cells
PFA	Paraformaldehyde
RMS	Rostral migratory stream

RT	Room temperature
SD	Sprague Dawley
SGZ	Subgranular zone
SVZ	Subventricular zone
TBS	Tris-buffered saline
TK	HSV-TK-positive rat
TK-v	HSV-TK-positive rat treated with valganciclovir
tPA	Tissue plasminogen activator
V	Ventricle
VGCV	Valganciclovir
WT	Wild-type rat
WT-v	Wild-type rat treated with valganciclovir

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Introduction

1.1 Stroke: What is it and how are Canadians affected?

A stroke is an abrupt loss of brain function that can evolve from a ruptured blood vessel in the case of a hemorrhagic stroke, or a disruption of blood flow to the brain due to a clot in the case of an ischemic stroke[1,2]. Whereas hemorrhagic strokes are associated with higher mortality risks[3], stroke patients most commonly survive an ischemic stroke, which accounts for 87% of all stroke incidences[4]. The ensuing damage to the tissues that the blood vessel feeds occurs because the brain, a highly oxygen-dependent organ, is deprived of oxygen and essential nutrients[5]. This deficiency disrupts normal cellular processes including oxidative metabolism resulting in an interruption of ATP production and a downstream cascade of events occur ultimately leading to cell death. Remarkably, it is estimated that almost two million neurons are destroyed each minute after a stroke[6,7].

In Canada, stroke is the third leading cause of death[8], and it is estimated to happen every 9 minutes, affecting 62,000 Canadians each year[6]. Despite improved outcomes for stroke patients, including a decline in death rates over the last 10 years, disability caused by stroke is a rising major public health issue in Canada. Approximately 83% of stroke victims survive, which means stroke debilitates more than it kills. As a result, about 405,000 Canadians are currently living with the disabling effects of a stroke – a number that is expected to nearly double in the next 20 years[6,9]. Consequently, stroke is the leading neurological condition for direct costs in Canada. In 2001, while only 10% ($67.6 million) of these costs were attributable to physician care and 3% ($17.8 million) were attributable to drugs, hospital care expenditures surmounted to 87% ($579.5 million) of the direct costs[1]. These facts highlight the desperate need to continue stroke research in order to develop effective treatments for stroke recovery. The consequences from stroke can vary widely

between patients from mild to profound disabilities. This variability in outcome depends on numerous determinants including individual characteristics of the patients such as such age and prior health status, as well as the location of infarct. For example, cerebellar strokes, which account for 1.5% of strokes[10], are considered the least disabling of all strokes and occur when one of the cerebellar arteries are blocked, restricting blood flow to the cerebellum[11]. While this can result in symptoms such as nausea and vomiting, they are not usually associated with major motor disabilities. The most common site of stroke occurs within the cortex due to occlusion of the middle cerebral artery (MCA) that can result in major disabilities[11]. The MCA mainly supplies blood to the cortex and the resulting cortical stroke often causes substantial cell loss within the primary motor and sensorimotor areas including those associated with areas of the upper limbs, face and throat[11,12]. Motor deficits are most commonly characterized by unilateral weakness in an arm or hand[13]. Additionally, other motor-related impairments such as having difficulty swallowing or speaking can severely affect quality of life.

Unfortunately, restrictions to motor functioning are not the only consequences of stroke, with over 50% of stroke patients also demonstrating impairments in learning and memory[14]. Furthermore, 30% of patients are diagnosed with depression at some point following stroke[15]. This is interesting since the brain structures responsible for these cognitive and psychosocial functions, such as the prefrontal cortex, entorhinal cortex, and hippocampus, rarely demonstrate neuronal loss by stroke[16]. These cognitive impairments pose additional challenges in promoting recovery since learning and memory are fundamental components of successful rehabilitation, for instance, through repetitive-task training[17]. Hence, the treatment of stroke recovery is complex and finding strategies to alleviate both motor and cognitive disabilities is the ultimate goal.

1.2 Stroke Treatment

Fortunately today more Canadians are surviving stroke than in the past due to advances in prevention and acute care[6]. This has in part been due to the establishment of *The Canadian Stroke Best Practice Recommendations*, which provides clear information on options and suggested recommendations for how to deal with the continuum of care required for stroke patients[18]. Briefly, these recommendations range from educating the public and raising awareness about recognizing the signs of stroke to helping stroke survivors reintegrate into their communities and deal with post-stroke mood, cognition and fatigue.

Due to advances in the area of prevention and acute care, more Canadians understand and recognize the signs of stroke[9]. However, it is remarkable that even with more efforts aimed at educating and raising awareness, only one-third of Canadians know all three signs: drooping face, inability to raise both arms, and slurred speech[6]. Knowing the signs of stroke and acting quickly are critical first steps towards providing the best quality of care for stroke patients. Failing to recognize these signs challenge the practice of acute care since all acute treatments have a limited effective time window associated with improved outcomes. For example, the most common current treatment for acute stroke is treatment with tissue plasminogen activator (tPA), which is a thrombolytic drug administered intravenously to break up the blood clot[19]. In order for tPA to be effective, however, it must to be administered within 4.5 hours from the initial onset of stroke. Similarly, there are a variety of surgical procedures that can be performed to repair blood vessels and/or remove accumulation of plaque, but these options need to be performed in a timely manner[19]. Recently, endovascular treatment has gained public attention and recognition since it was shown to reduce disability and death as highlighted in the ESCAPE (Canadian Endovascular treatment for Small Core and Anterior circulation Proximal Occlusion with Emphasis on

minimizing computerized axial tomography to recanalization times) Clinical Trial[20]. Briefly, in this procedure a thin tube is inserted through an artery in the patient's groin and guided towards the brain wherein a stent is used to remove the clot[6]. However, like tPA, endovascular treatment is most effective when performed before major damage has occurred. Unfortunately, for many stroke patients irreparable damage has taken place prior to receiving medical care, which limits the success of acute care. As a result, strategies to reverse the effects from stroke often take place during recovery.

Stroke recovery is a team effort and requires various experts including physiatrists, social workers and physiotherapists[21]. Depending on the patient's needs, there are often a number of other specialists that join the team to take on a multidisciplinary approach to address different impairments ranging from motor to psychosocial[17,21]. Since motor disabilities are the most common outcomes of stroke, about 60% of patients depend on rehabilitation therapy programs that are aimed at improving motor function[17]. It is recommended that rehabilitation includes an exercise component, which can be done at a rehabilitation center or at home[21], as well as training in task-specific and goal-oriented activities that are focused on helping patients return to their normal activities of daily living[18]. Patient outcomes from rehabilitation vary, although it is well known that the earlier a patient begins rehabilitation the better the patient's outcome[21]. Despite this knowledge, only 37% of patients with moderate to severe impairments receive standard rehabilitation in the weeks after stroke[4]. Additionally, significant gains during rehabilitation is limited for stroke survivors due to issues with access to resources and poor patient compliance[22]. Telestroke is one initiative that was launched to address these problems through using advances in technology to enable patients to receive direction and assistance in their rehabilitation therapy program outside of the hospital[6]. Despite these improvements, rehabilitation still remains a slow

and long process and does not meet rapidly growing demands. Hence, there remains a vital need to improve stroke recovery by developing therapeutic strategies that focus at the cellular level.

1.3 Innate Recovery

It is remarkable that patients can recover for many years following stroke even in the absence of treatment or therapies[23]. Known as "innate recovery", this natural improvement in function has been demonstrated in humans, as well as in animal models of ischemia. In stroke survivors, the most spontaneous recovery occurs within the first 3 months post-stroke, after which significant gains in motor recovery are rare[24]. Across patients, however, there is considerable heterogeneity in regards to when spontaneous recovery occurs. For instance, the first voluntary movements have been detected within the range of 6 to 33 days post-stroke in hemiplegic patients[25]. Using animal models, similar behavioural recovery profiles are demonstrated, but usually within a shorter time-course of recovery[26]. Nonetheless, animal models of stroke continue to provide insight and there remains hope to be able to harness these innate restorative effects to enhance recovery.

Various mechanisms have been shown to be involved in the innate recovery process following stroke[24]. These include angiogenesis, cortical reorganization, as well the generation of new neurons in the adult brain through the process of neurogenesis. Briefly, the involvement of angiogenesis, the birth of new blood vessels, in mediating functional recovery following stroke is correlated with an increase in number of endothelial progenitor cells and active angiogenesis within the ischemic penumbra[27]. This has been shown through use of Magnetic Resonance Imaging (MRI) in which recovery from stroke is correlated with heightened angiogenesis[28]. There are also mechanisms of neuroplasticity that have been correlated with stroke recovery[26]. For instance, cortical remapping, which is defined by new functional and structural circuits developing

between related cortical regions, has been shown to be positively associated with brain repair[26]. Using rodent models, studies have demonstrated that healthy neurons surrounding the infarct re-wire to process information previously performed by neurons lost to stroke[29,30]. Furthermore, the discovery of neuronal development within the adult brain through the process of adult neurogenesis has piqued the interest of stroke recovery research groups. Provided in more detail below, the post-stroke neurogenic response has raised the notion that newborn neurons could be involved in promoting innate recovery[31].

1.4 Adult Neurogenesis

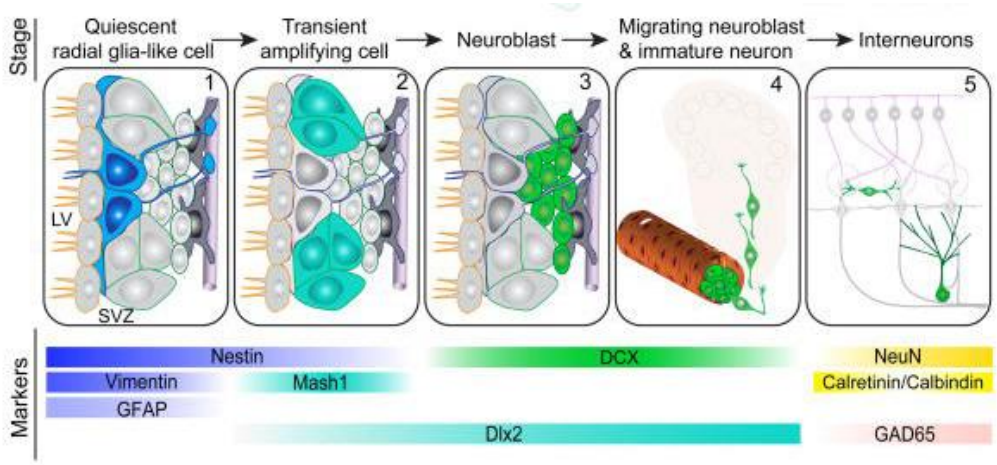
Adult neurogenesis was first discovered in humans in 1998[32] and laid the foundation for intense efforts at understanding how stem and progenitor cells (referred to as PCs from here on) form neurons in the adult brain in both physiological and pathological conditions. Initial efforts on studying the dynamics of adult neurogenesis were achieved through preclinical animal models and use of the synthetic thymidine analogue 5-bromo-2'-deoxyuridine (BrdU). BrdU incorporates into the DNA of proliferating cells allowing PCs to be birth dated and visualized through immunohistochemistry (IHC) methods[33]. More recently, birth dating studies have also been completed in post-mortem human tissue through the detection of carbon-14, which has extended our understanding of the process of neurogenesis in humans[34].

In both rodents and humans, dividing PCs are present in two restricted regions of the brain: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG)[35]. PCs from the SVZ migrate down the rostral migratory stream (RMS) towards the olfactory bulb (OB), where they integrate in the OB as interneurons. Unlike the migrating PCs that arise from the SVZ, dividing PCs from the SGZ establish local hippocampal granule neurons. Within both neurogenic niches, the development of the PCs into mature neurons

can be achieved through the detection of proteins, which provide markers of specific stages in cell development. As seen in Figure 1A, while GFAP and Nestin expression label the radial stem cells and PCs, doublecortin (DCX) is expressed and labels the differentiating and immature neurons in the SVZ[35]. This is similarly reflected in the SGZ (Figure 1B). Within a period of approximately 3 weeks, DCX-expressing immature neurons develop physiologically and morphologically into mature neurons, which express neuronal nuclear protein (NeuN), and then go on to develop electrophysiological properties similar to resident granule neurons by 6-8 weeks[36,37].

The discovery of new neurons being generated in the adult brain led to the question of what is the function of these neurons in physiological and pathological conditions. As reviewed by many[38-40], there remains no consensus on the physiological role of adult neurogenesis, although most suggest adult-generated neurons are important in learning and memory. This is not surprising given that neurogenesis occurs in the adult hippocampus, which is considered the center of emotion, learning and memory[41,42]. To date, a vast number of studies have examined the functional role of PCs and their progeny for cognitive abilities in the naïve brain[43,44]. Recently, a meta-analysis of these studies suggests that concerning spatial memory, contextual and cued fear conditioning, and anxiety, there are no significant effects of neurogenesis[38]. Importantly, however there was also a significant level of heterogeneity among these studies that was hypothesized to stem from differences in study methodology including differences in species, behavioral testing paradigms, and methods of ablation. There are also a number of recent studies that have suggested that the adult-generated neurons may have a more specific role in certain types of learning and memory, such as contextual discrimination[45,46]. In addition to this possible physiological role in learning and memory, a growing number of studies that have suggested the cells may be involved in the development or outcomes of a number of neurodegenerative diseases and brain injuries

A



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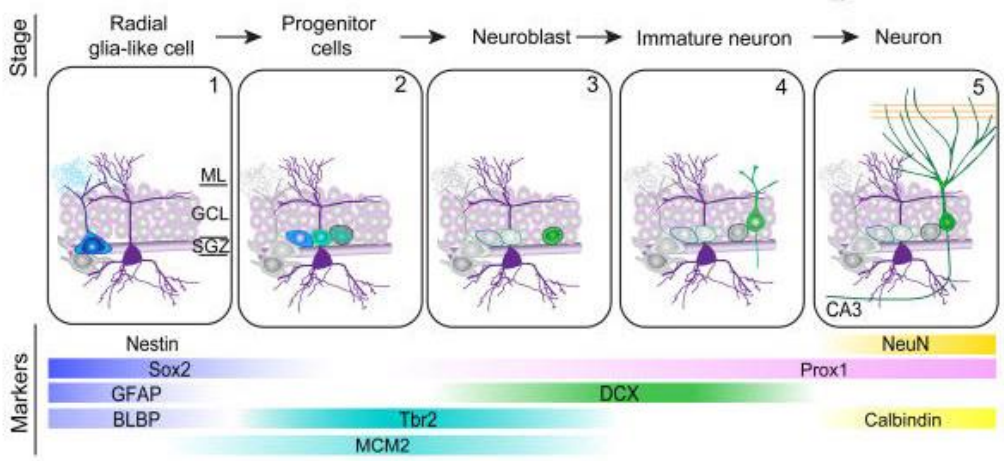


Figure 1. The maturation of PCs during adult neurogenesis. A) Summary of the developmental process of adult neurogenesis in the SVZ of the lateral ventricles and **B)** SGZ of the hippocampal dentate gyrus, including a time course of morphological development and commonly used histological markers that are used to label the various stages of cell development. LV, Lateral Ventricles; ML, Molecular Layer; GCL, Granule Cell Layer. Modified from: Ming, G.L. & Song, H. (2011).

such as stroke. Specifically, in the area of stroke research there have been great efforts in understanding the post-stroke neurogenic response in order to harness the therapeutic potential, if any, for stroke recovery.

1.5 The Progenitor Cell Response Following a Stroke

As shown in Figure 2, following a stroke there is a robust increase in the proliferation of PCs and their ectopic migration to the site of damage[47]. Specifically, it is estimated that there is a 1.5-3 fold increase in the number of PCs within the SVZ and a 5-12 fold increase in the SGZ following stroke[48]. While a peak in proliferation occurs at 1 week post-stroke[49], survival of PCs is low with only an estimated 20% of these cells surviving by six weeks post-stroke[50]. Remarkably, the surviving PCs have been found to ectopically migrate to the site of tissue damage[47]. Of the cells that do survive and migrate, rodent studies suggest that a small fraction of dead neurons are replaced by new neurons after stroke[48,51]. This finding has also been supported by analyses of post-mortem human brain tissue, which has confirmed the presence of PCs and adult-generated neurons within the ischemic penumbra[52]. Together, these findings have raised questions about the therapeutic potential of PCs or their progeny for restoration of brain function following stroke, and whether increasing the survival or integration of neurons would improve recovery[31]. In order to address these questions, a number of studies have examined the correlations between neurogenesis and stroke recovery, or directly tested the requirement of neurogenesis for stroke recovery.

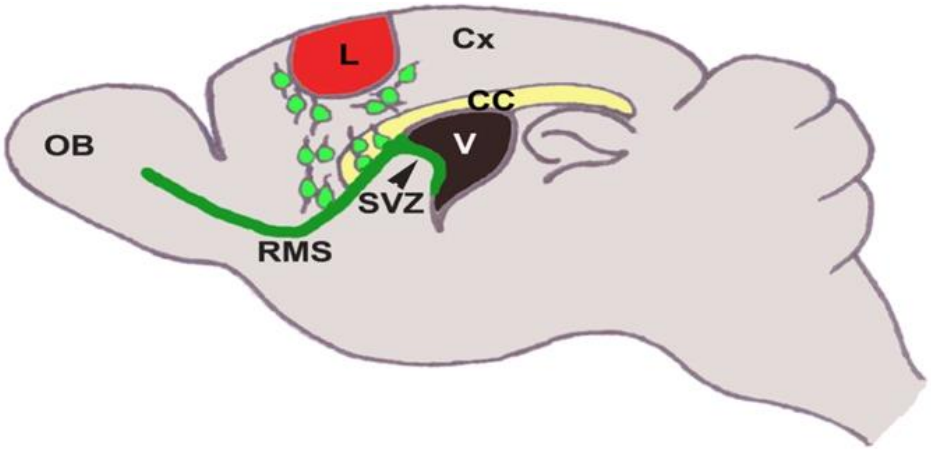


Figure 2. Ectopic migration of PCs from the SVZ to the infarct. During basal levels of adult neurogenesis, PCs from the SVZ normally migrate down the RMS to the OB to integrate as OB interneurons. Following a stroke, PCs from the SVZ migrate to the stroke lesion. Cx, Cortex; CC, Corpus Callosum; V, Ventricle; L, Lesion. Modified from: Saha et al. (2012).

1.6 A Correlative Link: Increasing "Neurogenesis" Improves Stroke Recovery

As recently reviewed by many preclinical studies, adult neurogenesis is a contributor for innate stroke recovery in various species[48,53,54]. These studies show that an increase in neurogenesis by different means improve behavioural response following stroke, thus suggesting a positive correlation between the neurogenic response and stroke recovery[31]. For instance, by allowing rodents to exercise through free access to a running wheel, there is an improvement in both motor and cognitive behavioural performance tasks following stroke[55,56]. Improved stroke recovery has also been observed by other interventions that also enhance neurogenesis, such as treatment with neurotrophic factors. Although these findings are suggestive of a relationship between neurogenesis and stroke recovery, the observed changes in behavioural outcomes in these studies cannot be directly proven to be attributed to adult-generated neurons, or "neurogenesis" *per se*[31]. For example, in the case of improvements associated with running, while physical activity promotes cell proliferation and neurogenesis, it also has other various effects such as enhancing neurotrophin levels and gene expression[57]. Consequently, the observed improvements in stroke recovery could be a result of other factors in the microenvironment and not necessarily due to neurogenesis. Therefore, it is necessary to study a causal role for adult neurogenesis in order to determine if there is a functional role for PCs and their progeny in stroke recovery.

1.7 A Causative Link: Ablation of PCs Impedes Stroke Recovery

To date, three research teams have ablated PCs using a variety of knockout approaches to examine the requirement of PCs for behavioural recovery, which has resulted in four publication titles all suggesting adult neurogenesis *per se* is required for stroke recvery[58-61]. The first study was published in 2004 by Raber and colleagues who used X-ray irradiation to conditionally ablate PCs in gerbils and assessed motor, as well as spatial learning and memory tasks following a

bilateral common carotid artery occlusion (BCCAo). They reported that ablation of PCs did not hinder motor performance, but was associated with a significant deficit in spatial learning and memory. Since these results could be attributed to non-specific effects of X-ray irradiation to ablate the dividing PCs, recent studies have used transgenic rodent models to specifically target PCs. This includes a transgenic DCX-TK mouse model used by one group who published two papers, the first in 2010 and the second in 2012, which allowed for the specific ablation of PCs through treatment of the antiviral drug, ganciclovir (GCV). Assessment of sensorimotor function following middle cerebral artery occlusion (MCAo) and distal middle cerebral artery occlusion (dMCAo) demonstrated a hindrance of post-stroke motor recovery in DCX-TK mice compared to littermate controls up to 8 weeks post-stroke. In contrast to these findings, Sun et al. in 2013 used a Nestin-TK mouse model and found no differences in post-stroke motor recovery, but significant deficits in spatial learning and memory following dMCAo combined with occluding the common carotid artery (CCA). Together, these findings suggest that adult neurogenesis is required for stroke recovery since all of these studies suggest that either motor or cognitive function declines in the absence of the PCs during stroke recovery.

Careful examination of these studies raises many questions regarding the role of PCs. For instance, the following still remain unknown: Whether the adult-generated cells functionally integrate as neurons during recovery and whether the PCs or adult-generated neurons are required for motor and/or cognitive functioning during recovery. All four studies ablated PCs prior to stroke and have titles suggesting that neurogenesis is required for recovery. However, the data supporting these conclusions may be premature given the models and techniques utilized in these studies. For example, in the DCX-TK mouse study the volume of tissue damage was extensive and larger than the average human infarct[58], which makes it difficult to assess whether their findings would

translate when tested with more clinically relevant stroke models. Recovery in this model was also observed within two days post-stroke[59]. This period of time is too short to attribute motor recovery to new fully developed neurons, which take weeks to mature and integrate. Furthermore treatment of GCV was discontinued after stroke, which enables the proliferation of PCs to occur post-stroke. This makes it difficult to achieve an indisputable assessment of the requirement of PCs for post-stroke function. In addition to these specific issues associated with the DCX-TK studies, two of the four studies do not include the sham surgery controls[60,61], which raises the question of whether the observed diminished cognitive functioning post-stroke was associated with the ablation of PCs alone or the combination of PC ablation and stroke. In order to further elucidate the role of PCs in stroke recovery, this work was designed to clarify the requirement of PCs and their progeny for stroke recovery.

Objective and Hypothesis

Objective:

The objective of this study was to determine the *requirement* of PCs for behavioural recovery following a focal cortical stroke in the forelimb motor cortex using a GFAP-TK transgenic rat. The following two aims were investigated:

Aim 1: Determine the *requirement* of PCs for motor recovery following stroke using behavioural tests of forelimb and hindlimb motor function.

Aim 2: Determine the *requirement* of PCs for post-stroke cognitive function using behavioural tests assessing spatial learning and memory.

Hypothesis:

Based on the findings of numerous correlative studies, as well as previous ablation studies, I hypothesized that ablation of PCs in TK-v rats would impede post-stroke motor recovery and cognitive function compared to littermate controls.

If my hypothesis is correct, this work would strengthen the need to develop novel therapies to protect PCs as a potential treatment for promoting stroke recovery.

Materials and Methods

2.1 Animals

General Procedures

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

GFAP-TK Transgenic Rat

We obtained the GFAP-TK transgenic rat line that was first described at Society for Neuroscience in 2011 by Snyder et al. with a material transfer agreement with Dr. Heather Cameron at the National Institute of Health (NIH (MD, USA). With a Long-Evans hooded (LE) rat background strain, this rat allows for the inducible ablation of glial fibrillary acidic protein (GFAP) expressing radial stem cells that give rise to the dividing PCs[62]. In this model, the Herpes Simplex Virus thymidine kinase (HSV-TK) is under expression of the GFAP promoter (Figure 3A). GFAP is endogenously expressed in radial-glial stem cells, as well as in mature astrocytes [63]. Ablation of PCs does not occur with the integration of the HSV-TK transgene alone in the GFAP-expressing cells, but requires administration of an antiviral drug, valganciclovir (VGCV), which is converted to ganciclovir (GVC) after ingestion (Figure 3B). In the presence of HSV-TK, GCV is phosphorylated to become an analogue guanine monophosphate, which is phosphorylated by other cellular kinases to an analogue of guanine triphosphate (GTP).

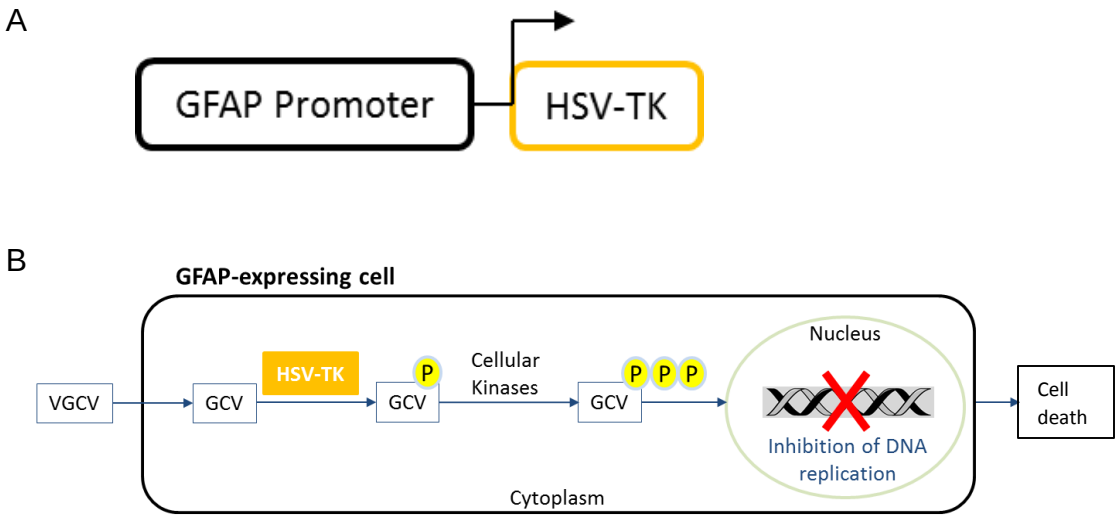


Figure 3. Transgenic GFAP-TK rat allows for the inducible ablation of GFAP-expressing stem and progenitor cells. A) HSV-TK is driven by the GFAP promoter. **B)** Treatment with VGCV inhibits DNA replication in dividing GFAP-expressing cells containing the HSV-TK transgene. P represents a phosphate group.

Phosphorylated GCV acts as a competitive inhibitor of deoxyribonucleic acid (DNA) polymerase by mimicking GTP, resulting in the disruption of DNA replication during cell division[64]. Thus, the requirement of cell division selectively targets dividing PCs for cell death. This model is ideal to test the requirement of PCs in stroke recovery because it is specific to only the dividing GFAP stem and PCs, as well as inducible at any stage through treatment with VGCV.

Breeding

Two female GFAP-TK were obtained from Dr. Heather Cameron's laboratory at the NIH (MD, USA). These breeders were paired with age-matched male LE rats obtained from Charles River Laboratories (Massachusetts, USA). This cross produced male and female wild-type (WT) and HSV-TK positive (TK) offspring that established our colony. The colony was maintained by breeding female TK rats with male LE rats from Charles River for 40 crosses. Female breeders were retired from breeding at 7 months of age due to hindlimb paresis. Experimental rats were obtained from F25, F26 and F34 (motor study) and F28, F29, F30 and F31 (cognitive study).

2.2 Valganciclovir Administration

To ablate PCs, rats were treated with VGCV (02413825; Teva-Valganciclovir,). VGCV is the valyl ester prodrug of GCV and has been shown to provide increased bioavailability in comparison to GCV when administered in oral form[65]. VGCV was given to the animals in the form of a peanut butter ball (PB) that was consumed voluntarily. Each 0.5g peanut butter ball consisted of standard rodent chow mixed with Skippy® PB in a 1:1 ratio by volume, combined with 0.4% VGCV by mass. Control balls consisted of the PB and rat chow vehicle without VGCV. At 7 weeks of age, rats were habituated to PB by eating one control ball every day for 3 days. At 8 weeks of age, all

drug treated rats were administered one PB ball with VGCV twice weekly (Mondays and Fridays) while rats not receiving drug were given one control ball.

2.3 Genotyping

At 3 weeks of age, ear samples were collected for genotyping to distinguish between WT and TK rats. To isolate DNA from the ear samples, the HotSHOT extraction method was performed. Standard polymerase chain reaction (PCR) was used to detect the HSV-TK transgene using the following primers:

Forward: 5' – CTG GGC TGG GCG CAG TGG CGC ACA ACT GTA ATT – 3'

Reverse: 5' – CTG CAG CCT CAA CCT CCC AGC CTT AAG TGA TCC C – 3'

WT animals were distinguished using the control primers for the Interleukin-2 (IL-2) gene:

Forward: 5' – CTA GGC CAC AGA ATT GAA AGA TCT – 3'

Reverse: 5' – GTA GGT GGA AAT TCT AGC ATC ATC C – 3'

PCR amplicons were electrophoresed on a 2% agarose gel, stained with ethidium bromide and subsequently visualized by ultraviolet illumination. The HSV-TK band was detected at 218 base pairs and the IL-2 band was detected at 324 base pairs.

2.4 5-bromo-2'-deoxyuridine Injections

To label dividing cells, rats were administered an intraperitoneal (IP) injection of BrdU at 150mg/kg (10mg BrdU /1mL saline + 7uL 1N sodium hydroxide (NaOH)) using a 23 gauge (0.6 mm diameter) needle that was ¾ of an inch long (19 mm) (305143; BD PrecisionGlide™).

2.5 Endothelin-1 Stroke Surgeries

Focal cerebral ischemia was induced using the Endothelin-1 (ET-1) focal cortical stroke model, as previously described [66]. The surgeries were performed by laboratory technicians, Matthew Jeffers

and Anthony Carter, along with my assistance. Rats were on a full-fast overnight prior to surgery and first anesthetised by inhalation using 4% isoflurane with oxygen maintained at 1.5%. During surgery the isoflurane level was reduced to 2% while the oxygen level was maintained at 1.5%. Using stereotaxic surgery, 2µl of ET-1 (400 pmol/µl sterile H_2O, human, porcine, ab120471; abcamBiochemicals®,) was injected at 2 locations within the motor cortex using the following anteroposterior (AP), mediolateral (MLA) and dorsoventral (DV) coordinates relative to Bregma:

Injection 1: AP = 2 mm, MLA = 2.5 mm, and DV = -1.7 mm
Injection 2: AP = 0 mm, MLA = 2.5 mm, and DV = -1.7 mm

Throughout the surgery, core body temperatures were monitored and maintained between 36°C and 37.5°C using a rectal probe. Following surgery the rats received 2% transdermal bupivacaine (Chiron) in order to minimize pain associated with the procedure and food was available again *ad libitum.*

2.6 Behaviour Testing

General Procedures

All behavioural tests were performed between 8am and 5pm. Rats were transported to the testing room and were left undisturbed for 30 minutes to habituate to the room in which a radio was turned on to generate white noise. The motor studies included 2 cohorts and were 11-12 weeks old at the beginning of pre-stroke training. Motor testing was conducted in the following order: staircase, beam walk, and cylinder test. Similarly, the cognitive studies included 2 cohorts and were 15-16 weeks old at the start of post-stroke testing and performed Barnes Maze followed by fear conditioning. All testing was performed blinded to experimental group.

Staircase

Beginning the day before training, rats were food deprived (12g rodent chow/rat/day) for this reward-based test for the duration of testing. Mildly food deprived rats were individually placed in separate Plexiglas staircase testing boxes for 15 minutes. Each box contained a set of stairs (7 steps) descending from both sides (one for each forelimb) filled with sugar pellets (3 pellets/step). Rats were trained to retrieve pellets twice daily for at least 10 days, or until a plateau in learning was achieved on the last three days, obtaining an average of at least 15 pellets with a standard deviation of less than 2.5 pellets. Forelimb dexterity was measured by comparing the number of pellets eaten with those that are left behind or dropped. Rats were trained and tested in the same boxes and boxes were not cleaned unless necessary.

Beam walk

Rats were placed on the end of a tapered beam (6 cm at widest, 1.5 cm at narrowest, 160 cm long) and trained for one day (4-6 trials) to learn to cross the beam towards a darkened goal box with sugar pellets. The beam has two levels in which the bottom level was the same length as the top level and present on both the right and left sides of the top level with a width of 2 cm. A mirror placed horizontally allowed for viewing of limbs on the other side of the beam during recording with a video camera. The number of slips (foot faults) off the top level onto the bottom level was counted during playback of video recordings. The test requires one day of training prior to recording 4 trials per animal (inter-trial interval of 15 seconds) in one day in order to obtain baseline results. Post-stroke testing on the beam walk was also based on one day of testing with 4 trials per animal.

Cylinder

Rats were placed inside a small upright cylinder (20 cm in diameter, 35 cm tall). Video recordings are taken from below the cylinder in which rats freely explored the cylinder. As a measurement of spontaneous forelimb placement, contact with the cylinder wall during a rear (rat stands up on hindlimbs) using the right, left or both forelimbs was recorded. The test is over when the rat makes 20 rears. The cylinder test does not require pre-exposure/training and only one trial is required for baseline performance, as well as post stroke testing.

Barnes Maze

The maze (125 cm diameter) consists of 18 holes (9 cm diameter) and is located in a room with different spatial cues on each wall (diagonal stripes, vertical stripes, a large triangle, and the fourth wall had no cue). Using an online random number generator (www.random.org) each rat was assigned a spatial cue where the goal box was located. The rats were encouraged to find the dark, enclosed goal box through use of bright lights (600 lux) and white noise (75 dB) within the testing room, which were mildly aversive stimuli[67,68]. The goal of the test was for rats to locate and enter the target goal box using the spatial cues on the wall.

The rats were placed in the center of a maze under a start chamber in a randomized direction. After the researcher left the testing room, the chamber was lifted and the rat freely explored the maze for a maximum of 3 minutes. The rat's movement on the maze was visualized and recorded using EthoVision XT 10.0 (Noldus) software. The Barnes Maze protocol consisted of five phases in the following order: habituation (1 day), training (8 days), probe trial (1 day), reversal training (5 days), and reversal probe trial (1 day) using similar protocols with modifications to those previously described[68,69]. For all phases except habituation, each rat had 2 trials with an inter-trial

interval of 20 minutes. Within each 3 minute trial, rats always located the goal box. Before a rat started a trial, the maze was rotated in a randomized fashion to minimize learning the task using olfactory cues.

Fear Conditioning

The fear conditioning test consisted of 3 days for a total of 8 minutes per day as previously described with some modifications[38,70]. On Day 1, rats were conditioned to associate context and tone to a foot shock. On Day 2, rats were tested for contextual conditioned fear response. On Day 3, rats were tested for cue conditioned fear response. Fear conditioning was performed using PhenoTyper boxes (Noldus) and the movement of the rat was recorded using EthoVision XT 10.0 (Noldus) software under normal lighting conditions unless otherwise specified.

On Day 1, the rat's baseline freezing in the novel context was recorded during the first 2 minutes followed by exposure to a 20s tone co-terminated with a 1 second foot shock (0.6mA). This was repeated 3 more times 1 minute apart for a total of 4 pairings. For the last two minutes, the rat remained in the box with no tone or foot shock.

On Day 2, to test contextual fear conditioning the rat was placed into the same environment as on Day 1 and freezing behaviour was recorded for 8 minutes (no tone or shock).

On Day 3, to test cued fear conditioning the rat was placed into a novel environment, which was a different PhenoTyper box with inserts in order to change the shape of the box. Additional modifications included a vanilla scented room, red lighting and yellow rubber gloves for handling. During the first 4 minutes of testing, the freezing behaviour was recorded in order to confirm the novelty of the context. During the final 4 minutes, the tone from Day 1 was played.

2.7 Tissue Preparation

Perfusion & Cutting

Rats were perfused as previously described [71] in order to allow for histological assessments of stroke lesion volume and confirmation of the ablation of PCs. The rats were first anesthetized with sodium pentobarbital, which was administered by IP injection at a dosage of 750mg/kg. Rats were then transcardially perfused at a rate of 10 ml/min with cold 1X phosphate-buffered saline (PBS) (pH 7.4) for 12.5 minutes followed by cold 4% paraformaldehyde (PFA) in 1X PBS (pH 7.4) for 15 minutes. Following perfusion, the brains were removed and post-fixed in 4% PFA for 24 hours and then transferred to 30% sucrose in 1X PBS with 0.1% sodium azide (NaN_3) for cryoprotection. The brains were sectioned coronally at a width of 40µm using a cryostat (Leica CM1850). The brain sections were collected serially in 9 wells and stored in 1X PBS with 0.1% NaN_3 at 4^{o}C to prevent microbial growth.

Cresyl Violet Staining to Visualize Infarcts

All infarcts were measured using a sampling method as previously described[66]. Tissue sections with visible stroke damage were laid out in order in a petri dish containing 1x PBS. Depending on the number of sections with stroke, a mounting factor (i.e. every section, every other section, every third section, or every fourth section) was selected in order to mount at least 8 sections with stroke plus book-ends on each side. Sections were mounted onto SuperFrost Plus charged slides and allowed to dry overnight. The following day, the slides were loaded onto a slide holder and dehydrated, rehydrated, and stained with cresyl violet (0.25% cresyl violet dissolved in Acetate Buffer (.5% sodium acetate, 0.003% acetic acid, 1.4% ethanol in distilled H20) and pH to 4.0) followed by clearing using the Citrisolv (22-143-975; Fisher) clearing agent.

Stroke Volume Quantification

Pictures of each cresyl violet-stained section were taken on a dissecting scope (Zeiss Stereo Discovery V20) at 10x magnification and opened with ImageJ software (NIH) for stroke volume quantification. The stroke lesion volume of each animal was calculated as previously described[72]. Briefly, this analysis includes measurement of structures in both hemispheres in order to account for any edema or compression that may have arisen in the brain as a result of the stroke. All measurements were calculated using the pixel count function of ImageJ (98.44 pixels per mm on the 10x images). First, the surface area of the intact tissue of each hemisphere (lesioned and non-lesioned), as well as their corresponding ventricles were measured. The mounting factor represents how many sections lie between two of the sections that were mounted for analysis. For instance, if every section in a well was mounted, then the mounting factor would be nine. If every other section was mounted, then the mounting factor would be 18.

$$Stroke\ volume = Total\ surface\ area\ of\ stroke * 0.04\ mm * mounting\ factor$$

Immunohistochemistry for BrdU

Slide-mounted IHC was used to detect the total number of BrdU positive cells in the SVZ and SGZ using previously published protocols[73,74]. Every ninth section through the SVZ and every 18th section through the SGZ 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 by incubating them at room temperature (RT) in 0.1% trypsin for 10 minutes, followed by DNA denaturation in 2N hydrochloric acid for 30 minutes at RT. To prevent non-specific binding, slides were incubated in 3% Normal Donkey Serum (NDS; 017-000-121; Jackson Immuno Research Laboratories Inc.) and 0.3% Triton X-100

in 1X tris-buffered saline (TBS) for 60 minutes prior to being incubated overnight in the primary antibody solution (1:300 (0.2%) Rat anti-BrdU in 3% NDS in 0.3% Tween20 and 1X TBS). The following day, slides were incubated at RT in: 1) 1:200 biotinylated Donkey anti-rat secondary antibody in 1.5% NDS in 1X TBS for 60 minutes; 2) 0.3% H2O2 in 1X TBS for 30 minutes to quench endogenous peroxidases; 3) Avidin-Biotin Complex Solution (ABC, PK-6100; Vector Laboratories) for 90 minutes; 4) metal enhanced 3,3'-Diaminobenzidine (DAB; 34065; Thermo Scientific, 1:10) for 5 to 15 minutes; and 5) fast red nuclear stain (H3403; Cedarlane) for counterstaining. Between all steps, with the exception of post-blocking with NDS, the slides were rinsed 2-3 times with 1X TBS. Following staining, slides were dehydrated by consecutively immersing slides in 95% and 100% ethanol for 20 seconds, followed by CitriSolv clearing agent for 20 seconds, 1 minutes, and 5 minutes. Slides were cover-slipped with DPX mounting medium (mixture of Distyrene, Plasticizer, Xylene; 44581; Sigma).

2.8 Statistical Analyses

General

All data were analyzed and presented using GraphPad Prism 6 and IBM SPSS Statistics 23. Analysis of performance overtime, between groups and side was completed using a two-way ANOVA with a Bonferroni post-hoc analysis. Single-variable data, such as stroke volume between groups was analyzed using a two-tailed two-sample student's t-test. For all analyses, a significance level of 0.05 was used.

Excluded animals/Outliers

Rats achieving greater than 80% success on the staircase test at 1 week post-stroke (n= 9/55) or demonstrating less than 5% success on the staircase test at 1 week post-stroke in addition to having

a stroke volume greater than 100mm^3 (n=3/55) were excluded from the study. These exclusion criteria were set prior to the study in order to make fair assessments between rats with significant deficits in which measuring recovery was also possible.

Results

3.1 VGCV treatment ablates PCs in the GFAP-TK rat following stroke

To test the requirement of PCs for stroke recovery we obtained a line of transgenic GFAP-TK rats, created by our collaborator, Dr. Heather Cameron, at the National Institute of Health. In 2011, her post-doctoral fellow, Dr. Jason Snyder, first presented this model as a poster at the Society for Neuroscience Meeting where he showed a complete ablation of PCs after 4 weeks of VGCV treatment in naïve TK rats[62]. To confirm that this transgenic rat line would provide a model of ablated PCs following stroke, 8 week-old WT and TK rats were treated with VGCV (WT-v and TK-v, respectively) for 6 weeks, after which ET-1 stroke was induced in the forelimb motor cortex (Figure 4A). One week post-stroke, rats were injected intraperitoneally with BrdU and sacrificed 2 hours post-injection in order to label dividing PCs. IHC analysis of BrdU positive cells was performed in both the SGZ and SVZ. As expected, PCs were present in both the SGZ and the SVZ of WT-v rats (Figure 4B). In striking contrast, the TK-v rats showed very few cells in both the SGZ the SVZ. These findings reveal an ablation of PCs in TK-v rats following stroke, suggesting that GFAP-TK rats were an ideal model to test whether the removal of PCs would impede stroke recovery.

3.2 VGCV does not alter performance on sensorimotor tests

The prodrug of VGCV, GCV, has been used in numerous preclinical studies, including work using rodent models that take advantage of the HSV-TK transgene to ablate adult neurogenesis[38,59-61]. Among this literature, there are a number of studies that suggest GCV can produce a variety of secondary effects, including inactivity[75]. In order to test if VGCV alters sensorimotor behavioural outcomes in our rats, a pilot study was performed to compare WT rats

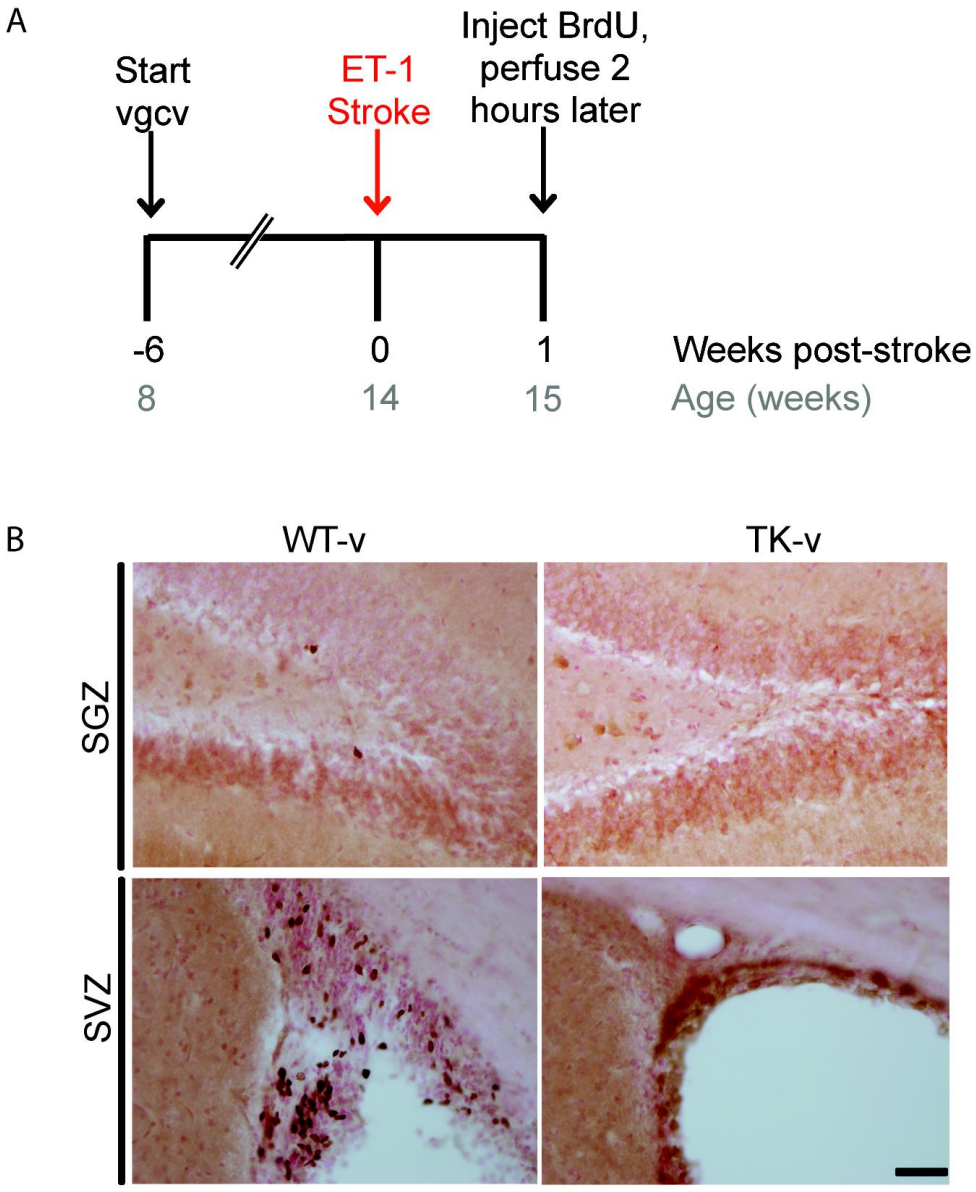


Figure 4. VGCV treatment ablates PCs in the GFAP-TK rat following stroke. A) WT-v and TK-v rats were treated with VGCV continuously until sacrifice. After 6 weeks of VGCV treatment, rats were given an ET-1 stroke. At 1 week post-stroke, rats were administered 1 injection of BrdU to labelling dividing cells and sacrificed 2 hours later. **B)** BrdU-stained coronal sections shows BrdU-labeled PCs in the SGZ and SVZ of WT-v rats, but not in TK-v rats. Scale bar = 200 μm.

(from the same litter as TK rats, but lack the transgene) that were administered the peanut butter vehicle (WT, control) to WT-v littermates. As shown in Figure 5, pre-stroke training on the staircase and cylinder tests began after 4 weeks of vehicle or VGCV treatment followed by ET-1 surgery. Post-stroke testing was performed up to 4 weeks after which rats were sacrificed.

The staircase test was used to assess independent use of forelimbs in skilled reaching and has been shown to be very sensitive in detecting forelimb deficits post-stroke[76]. As shown in Figure 6A, for this test the rat is positioned facing forward in a Plexiglas box that has two sets of staircases, one for each forelimb. Each of the 7 steps contains 3 pellets permitting the rat to retrieve a maximum of 21 pellets per forelimb. In order to perform this task, prior to stroke the rats are habituated to the box and are trained twice daily for up to 2 weeks in order to learn how to grasp, lift, and consume retrieved pellets from the steps. As expected, there was a significant increase in the number of pellets retrieved over the training period (Figure 6B). Additionally, there was no difference in training performance between WT and WT-v rats, suggesting that VGCV treatment does not affect staircase performance prior to stroke.

To determine if VGCV treatment affects staircase performance following stroke, rats in both groups were tested on their ability to retrieve pellets at 1, 3 and 4 weeks post-stroke. For both the ipsilateral and contralateral forelimbs, the number of pellets retrieved was divided by the maximum number of pellets, 21, and multiplied by 100% to determine the % success at each time point. Since stroke impairments occur contralateral to the lesioned hemisphere, we expected contralateral forelimb deficits in the absence of ipsilateral forelimb deficits. At 1 week post-stroke, both WT and WT-v rats demonstrated ipsilateral and contralateral forelimb impairments compared to baseline (time point at -1) (Figure 6C, D). The ipsilateral deficits however did not persist, whereas significant contralateral forelimb deficits were demonstrated at 1, 3 and 4 weeks

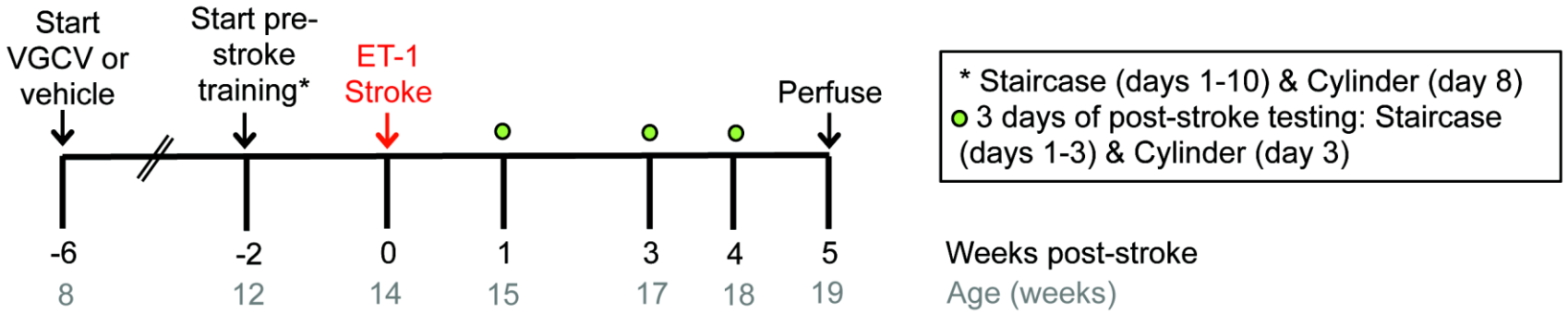


Figure 5. Timeline of experiment to determine the effect of VGCV on sensorimotor tests. WT rats were treated with vehicle or VGCV starting at 8 weeks of age. After 4 weeks of treatment, pre-stroke performance on the staircase and cylinder tests was collected over a 2 week training period followed by ET-1 stroke at 14 weeks of age. Post-stroke testing was performed at 1, 3 and 4 weeks post-stroke for the staircase test and at 3 and 4 weeks post-stroke for the cylinder test.

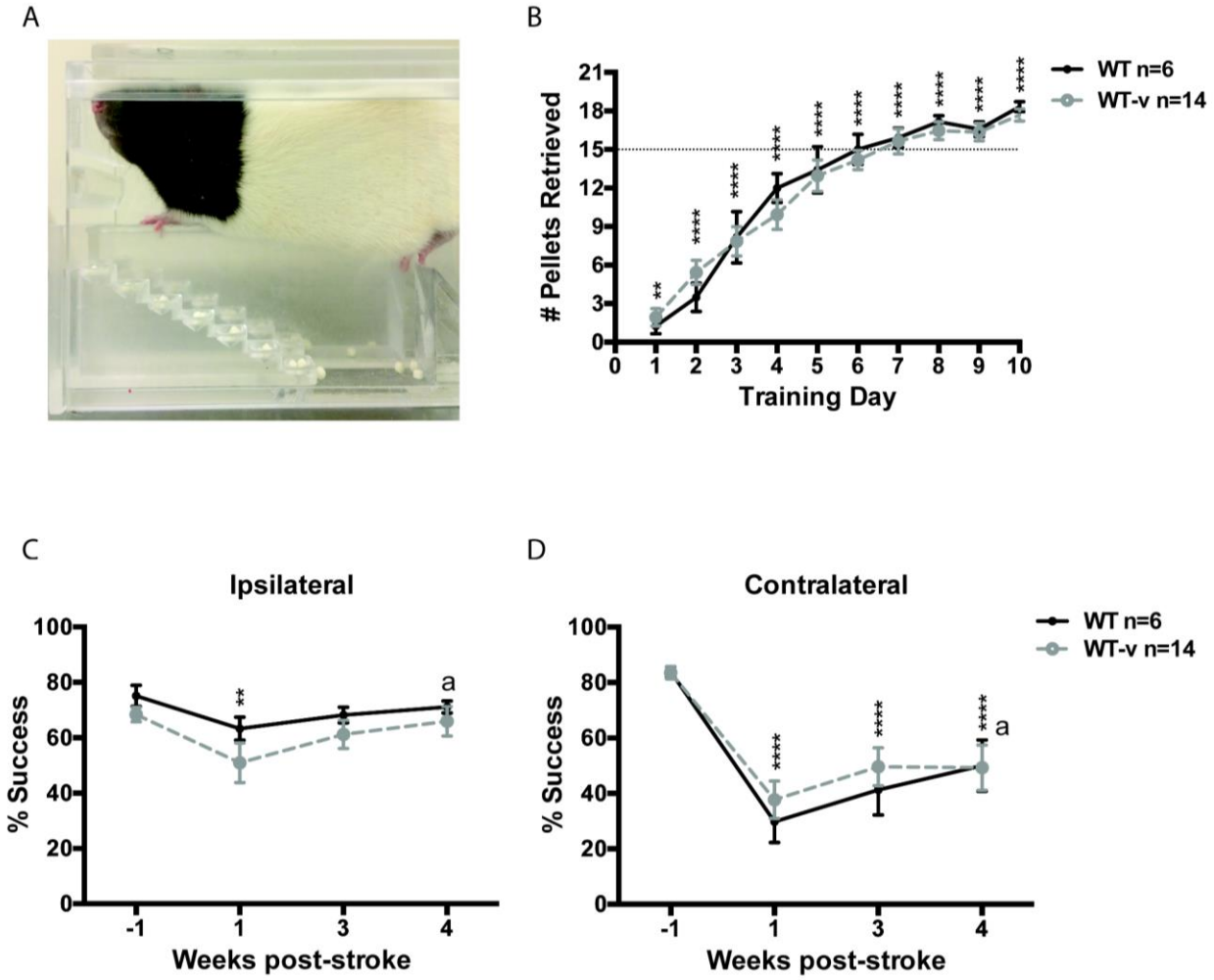


Figure 6. VGCV does not alter performance on the staircase test. A) Picture of a rat in the staircase apparatus that allows for individual assessment of forelimbs in skilled reaching of sugar pellets. **B)** WT and WT-v rats show similarly significant improvement in number of pellets retrieved during training ($F_{(9, 162)}$=78.3, p<0.0001). The dotted line at 15 pellets represents the mean number of pellets over 3 days that must be retrieved to be considered having successfully learned the task, which is achieved by day 8 for both groups. **C)** Assessment of the ipsilateral forelimb reveals a difference in performance over time ($F_{(3, 42)}$=5.4, p<0.005) and no difference between groups. Post-hoc analysis comparing baseline performance to post-stroke time points reveals a significant decline in performance at 1 week post-stroke compared to baseline, as well as a significant increase between 1 and 4 weeks post-stroke (a p<0.05). **D)** Assessment of the contralateral forelimb reveals a difference in performance over time ($F_{(3, 42)}$=41.1, p<0.0001) and no difference between groups. Post-hoc analysis comparing baseline performance to post-stroke time points reveals a significant decrease at 1, 3, and 4 weeks post-stroke, as well as a significant increase between 1 and 4 weeks post-stroke (a p<0.05). Error bars are SEM. (** p<0.01; *** p<0.005; **** p<0.001).

post-stroke compared to baseline (Figure 6D). There was no significant difference in performance between groups over time, suggesting that VGCV does not affect sensorimotor performance on the staircase test. Furthermore, a comparison of performances between 1 and 4 weeks post-stroke reveals significant improvement in both groups suggesting the occurrence of innate recovery.

As an additional assessment of forelimb sensorimotor function, rats performed the cylinder test (Figure 7A), which provides a measurement of spontaneous forelimb usage of the left or right forelimbs upon contact with the vertical Plexiglas enclosure[77]. As expected, there was no significant difference in use of the left or right forelimbs prior to stroke, with rats in both groups used each forelimb approximately 50% of the time (Figure 7B). At 3 weeks post-stroke, both WT and WT-v rats demonstrate a similar significant reduction in the use of their impaired forelimb, which persists up to 4 weeks post-stroke. These findings parallel the staircase test results and suggest that VGCV treatment does not alter behavioural outcome pre- and post-stroke.

3.3 Conditional ablation of PCs prior to stroke and continual suppression of PCs following stroke does not impede motor function

To test the requirement of PCs for motor function following stroke, WT-v and TK-v rats were treated with VGCV starting at 8 weeks of age and started training on three sensorimotor tests at 12 weeks of age (Figure 8). At 14 weeks old, rats underwent ET-1 surgery and post-stroke testing was conducted at 1, 3, 5 and 7 weeks post-stroke.

During staircase training, both WT-v and TK-v rats demonstrated a significant increase in the number of pellets retrieved over time (Figure 9A). This suggests that although naïve TK-v

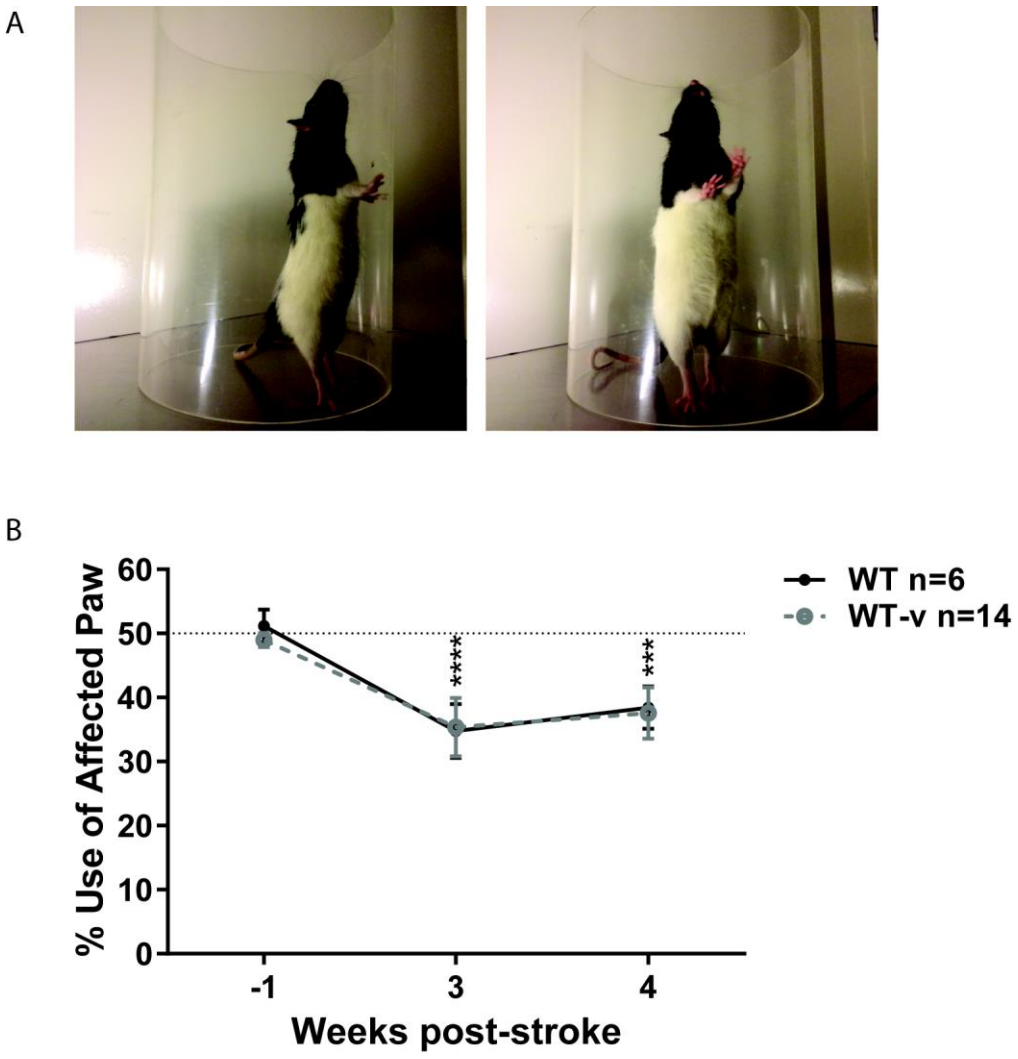


Figure 7. VGCV does not alter performance on the cylinder test. A) Example picture of a rat in the cylinder apparatus that allows a measure of spontaneous forelimb usage. **B)** Assessment of the percent use of affected forelimb reveals a difference in performance over time ($F_{(2,36)}$=16.4, p<0.0001) and no difference between groups. Post-hoc analysis comparing baseline performance to post-stroke time points reveals a significant decrease at 3 and 4 weeks post-stroke. Error bars are SEM. (*** p<0.001; **** p<0.0001).

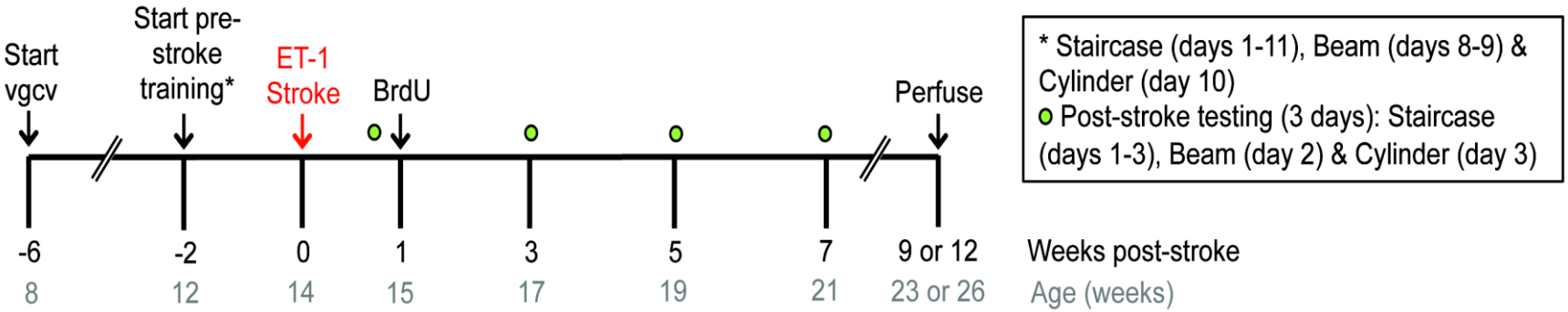


Figure 8. Timeline of experiment to determine if ablation of PCs impedes motor recovery. WT-v and TK-v rats were treated with VGCV starting at 8 weeks of age. After 4 weeks of treatment, pre-stroke performance on the staircase, cylinder and beam walk tests was collected over a 2 week training period followed by ET-1 stroke at 14 weeks of age. Post-stroke testing was performed at 1, 3, 5 and 7 weeks post-stroke. At 1 week post-stroke, rats were injected with BrdU and sacrificed at 9 or 12 weeks post-stroke.

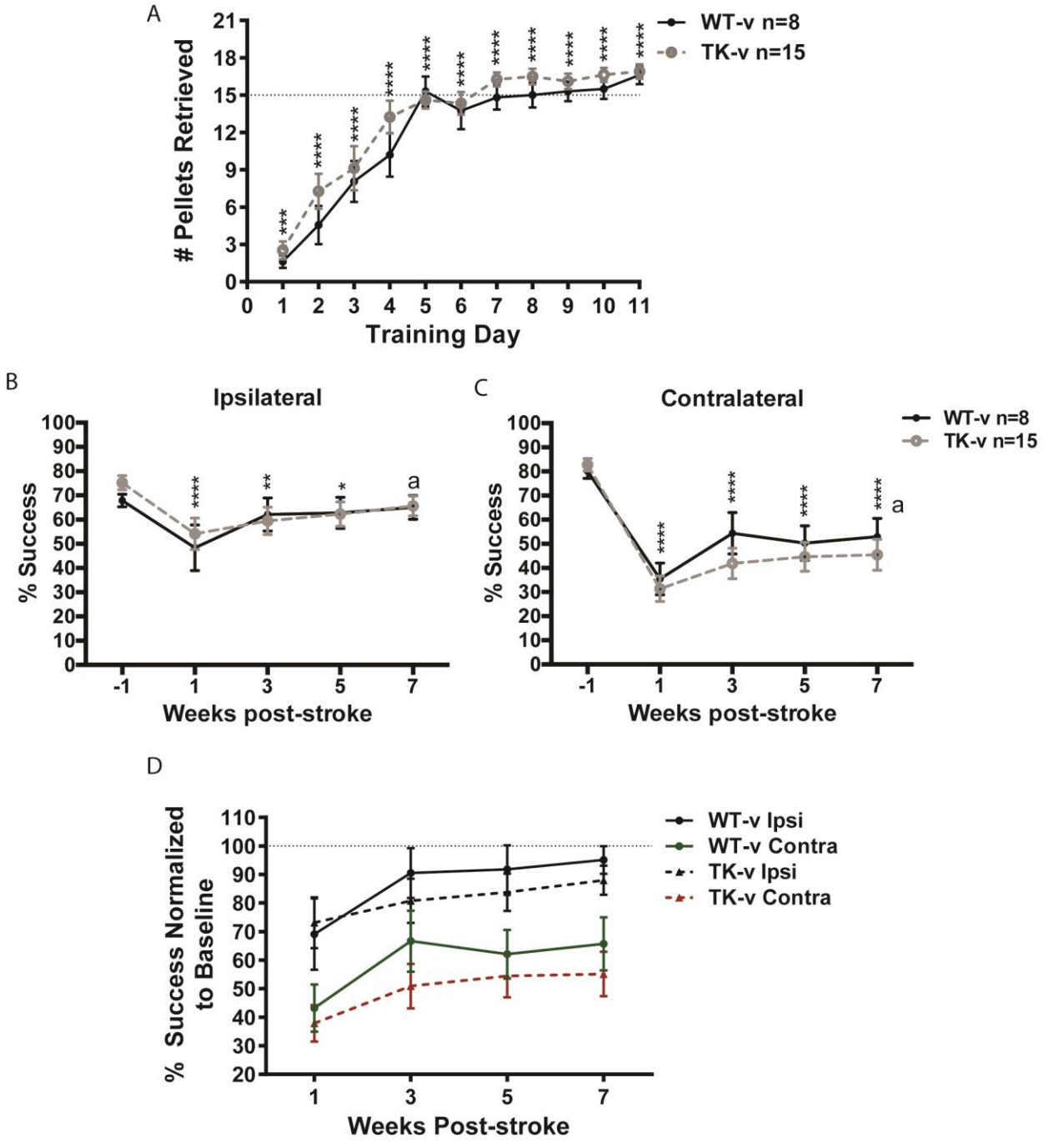

A
Pellets Retrieved
Training Day
WT-v n=8
TK-v n=15
B
Ipsilateral
% Success
Weeks post-stroke
C
Contralateral
% Success
Weeks post-stroke
WT-v n=8
TK-v n=15
D
% Success Normalized to Baseline
Weeks Post-stroke
WT-v Ipsi
WT-v Contra
TK-v Ipsi
TK-v Contra

Figure 9. Ablation of PCs does not impede skilled reaching on the staircase test. A) WT-v and TK-v rats show similarly significant improvement in number of pellets retrieved during training ($F_{(10, 210)}$=44.6, p<0.0001). The dotted line at 15 pellets represents the mean number of pellets over 3 days that must be retrieved to be considered having successfully learned the task, which is achieved by day 9 for both groups. **B)** Assessment of the ipsilateral forelimb reveals a difference in performance over time ($F_{(4, 84)}$=9.3, p<0.0001) and no difference between groups. Post-hoc analysis comparing baseline performance to post-stroke time points reveals a significant decline in performance at 1, 3, and 5 weeks post-stroke, as well as a significant increase between 1 and 7 weeks post-stroke (a p<0.01). **C)** Assessment of the contralateral forelimb reveals a difference in performance over time ($F_{(4, 84)}$=45.2, p<0.0001) and no difference between groups. Post-hoc analysis comparing baseline performance to post-stroke time points reveals a significant decrease at 1, 3, 5, and 7 weeks post-stroke, as well as a significant increase between 1 and 7 weeks post-stroke (a p<0.001). **D)** Assessment of the ipsilateral and contralateral forelimbs normalized to baseline reveals a difference between sides ($F_{(1, 42)}$=13.6, p<0.001). Error bars are SEM. (* p<0.05; ** p<0.01; ****p<0.0001).

rats have been reported to show an anhedonic phenotype when tested on the sucrose preference test[62], ablation of PCs does not affect ability to learn the staircase test compared to controls. At 1, 3 and 5 weeks post-stroke, the staircase test revealed significant deficits on the ipsilateral (Figure 9B) and contralateral (Figure 9C) forelimbs when compared to baseline performance for both WT-v and TK-v rats**.** However, by 7 weeks post-stroke, the deficits on the ipsilateral forelimb returned to baseline levels, whereas the contralateral forelimb deficits remained significantly different from baseline levels. In order to compare ipsilateral and contralateral forelimb performances, individual post-stroke performances were normalized to their respective baseline performances, as shown in Figure 9D. This analysis revealed significantly greater contralateral deficits for both groups and again no difference in performance between WT-v and TK-v rats. Together these results suggest that PCs are not required for skilled reaching following ET-1 stroke in the forelimb motor cortex.

Similar to performance on the staircase test, at 1-week post-stroke on the cylinder test both groups used the affected forelimb significantly less, which sustained up to 7 weeks post-stroke (Figure 10). Interestingly, a comparison of performances at 1 and 7 weeks post-stroke revealed no significant increase in the percent use of the impaired forelimb for either group, suggesting that innate recovery was not demonstrated on this specific test. Nevertheless, there was no difference in performance between WT-v and TK-v rats over time, demonstrating that PCs are not required for spontaneous forelimb usage following stroke.

In addition to the staircase and cylinder tests which provide measurements of forelimb sensorimotor function, the beam walk test was included to allow for assessments of both hindlimb and forelimb motor functions[26,77]. As illustrated in Figure 11A, the number of hits and slips provided measures of correct and incorrect forelimb placements, respectively. At 1 week

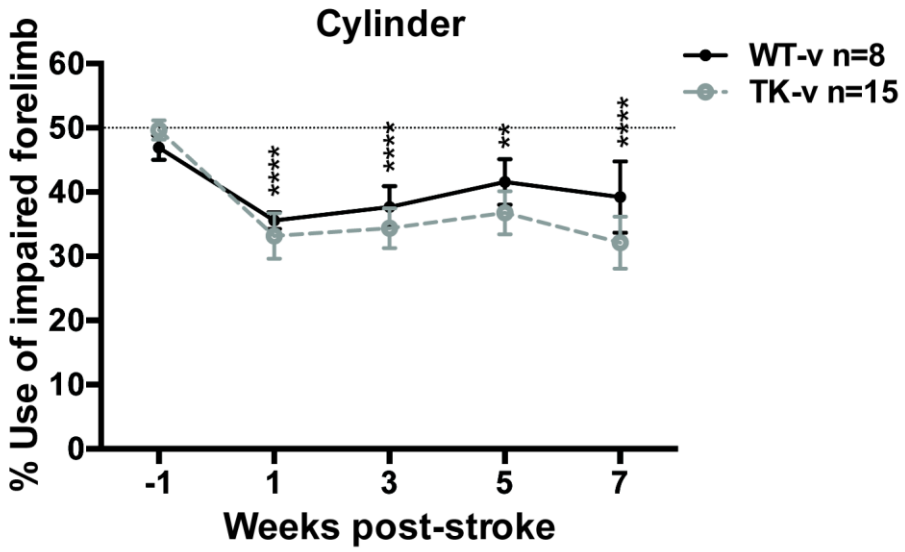


Figure 10. Ablation of PCs does not impede spontaneous forelimb use on the cylinder test. Assessment of the percent use of affected forelimb reveals a difference in performance over time ($F_{(4, 84)}$=7.6, p<0.0001) and no difference between groups. Post-hoc analysis comparing baseline performance to post-stroke time points reveals a significant decrease at 1, 3, 5, and 7 weeks post-stroke. Error bars are SEM. (** p<0.01; **** p<0.0001).

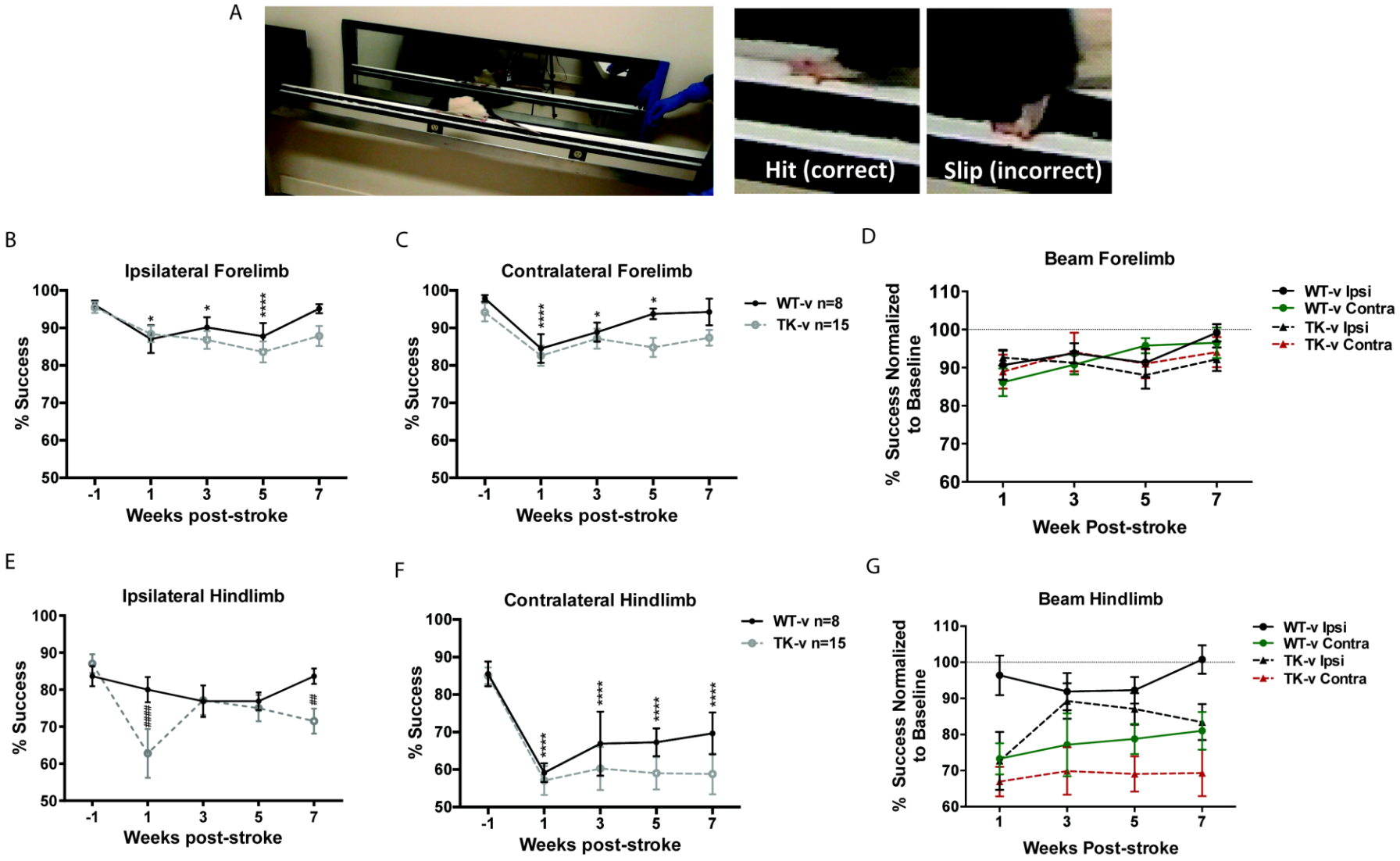

A
Hit (correct)
Slip (incorrect)
B
Ipsilateral Forelimb
% Success
Weeks post-stroke
C
Contralateral Forelimb
WT-v n=8
TK-v n=15
D
Beam Forelimb
% Success Normalized to Baseline
Week Post-stroke
WT-v Ipsi
WT-v Contra
TK-v Ipsi
TK-v Contra
E
Ipsilateral Hindlimb
F
Contralateral Hindlimb
G
Beam Hindlimb

Figure 11. Ablation of PCs does not impede forelimb and hindlimb motor coordination on the beam walk test. A) Pictures of a rat on the beam apparatus that allows for assessment of both forelimb and hindlimb motor function by recording number of hits or slips. **B)** Assessment of the ipsilateral forelimb reveals a difference in performance over time ($F_{(4, 84)}$=5.4, p<0.005) and no difference between groups. Post-hoc analysis comparing baseline performance to post-stroke time points reveals a significant decrease at 1, 3, and 5 weeks post-stroke. **C)** Assessment of the contralateral forelimb reveals a difference in performance over time ($F_{(4, 84)}$=5.7, p<0.001) and a difference between groups ($F_{(1, 21)}$=5.0, p<0.05), which was not significant when corrected for multiple comparisons. Post-hoc analysis comparing baseline performance to post-stroke time points reveals a significant decrease at 1, 3, and 5 weeks post-stroke **D)** Assessment of the ipsilateral and contralateral forelimbs normalized to baseline reveals no difference between sides. **E)** Assessment of the ipsilateral hindlimb reveals a difference in performance over time ($F_{(4, 84)}$=3.4 p< 0.05), no difference between groups, and an interaction between time and group ($F_{(4, 84)}$=2.6, p<0.05). Post-hoc analysis comparing baseline performance to post-stroke time points reveals a significant decrease for only TK-v rats at 1 and 7 weeks post-stroke (## p<0.05, #### p<0.0001). **F)** Assessment of the contralateral hindlimb reveals a difference in performance over time ($F_{(4, 84)}$=19.5, p<0.0001) and no difference between groups. Post-hoc analysis comparing baseline performance to post-stroke time points reveals a significant decrease at 1, 3, 5, and 7 weeks post-stroke. **G)** Assessment of the ipsilateral and contralateral hindlimbs normalized to baseline reveals a difference between sides ($F_{(1, 42)}$=11.4, p<0.01). Error bars are SEM. (* p<0.05; **** p<0.0001).

post-stroke, both groups demonstrated significant impairments on the ipsilateral forelimb, which persisted up to 5 weeks post-stroke (Figure 11B). This was also demonstrated by the contralateral forelimb in which significant deficits were observed at 1-week post-stroke and sustained up to 5 weeks post-stroke (Figure 11C). Although at 5 weeks post-stroke WT-v rats performed significantly better than TK-v rats on the contralateral forelimb, this effect was not significant when corrected for multiple comparisons. By 7-weeks post-stroke, both control and TK-v rats returned to baseline performance showing no differences between groups over time. In order to compare ipsilateral and contralateral forelimb performances on the beam walk test, individual post-stroke performances were normalized to their respective baseline performances, which revealed no difference between ipsilateral and contralateral forelimbs (Figure 11D).

Assessment of the ipsilateral hindlimbs revealed a significant interaction between performance of the WT-v and TK-v rats over the different time points tested. The WT-v rats showed no significant ipsilateral hindlimb deficits post-stroke, whereas the TK-v rats demonstrated significant deficits at 1 and 7 weeks post-stroke. In contrast, there were significant deficits at all post-stroke time points for the contralateral hindlimbs of both WT-v and TK-v rats (Figure 11F). The additional assessment of individual post-stroke performances, normalized to their respective baseline performances, revealed significant asymmetry with greater deficits on the contralateral limb (Figure 11G). Thus, overall the beam walk test revealed no differences in performance between TK-v rats and WT-v controls. Together with our findings from the staircase and cylinder tests, these results demonstrated ablation of PCs does not impede motor recovery following stroke.

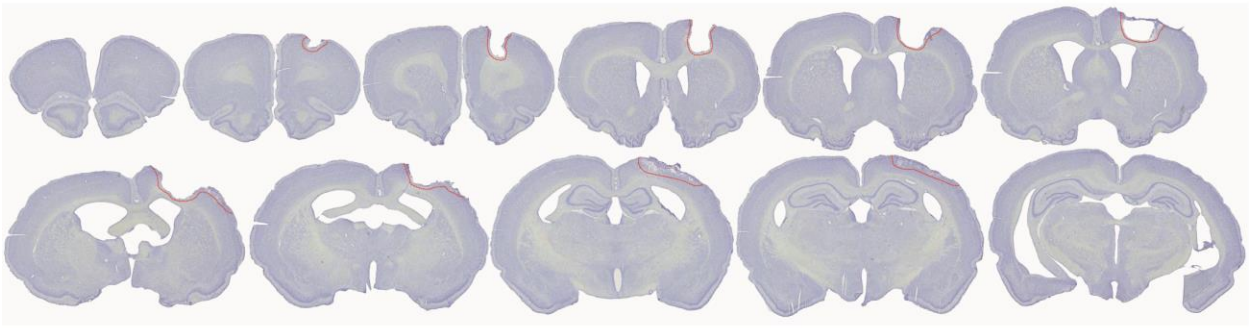
3.4 Ablation of PCs does not alter infarct volume following motor testing

Previous studies examining the requirement of PCs during stroke recovery have reported that ablation of PCs increases stroke volume and worsens motor outcome, suggesting that PCs are involved in stroke recovery[59,60]. Given that the TK-v rats did not demonstrate any significant differences in motor performance, we hypothesized that they would also have similar stroke volumes. Stroke volume was measured and most infarcts showed unilateral damage with obvious infarcts spanning the sensorimotor cortex, which is shown in Figure 12A. In agreement with our hypothesis quantification of stroke volume revealed no significant differences in average hemispheric lesion volume between WT-v and TK-v rats, with mean stroke volumes of 65mm^3 and 69mm^3, respectively at approximately 6 months post-stroke. (Figure 12B).

3.5 Conditional ablation of PCs does not impede spatial learning or memory on the Barnes Maze

Recently Sun et al. (2013) demonstrated that ablation of PCs using a Nestin-TK mouse model does not alter motor function post-stroke, but significantly impedes spatial learning and memory as shown by the Barnes Maze. This finding suggests that PCs within the SGZ may contribute to stroke recovery and supports the notion that SGZ neurogenesis is required for hippocampal-dependent learning and memory[31,58,61]. Since our TK-v model also had an ablation of PCs in the SGZ, we investigated whether the TK-v model would reveal an impediment in learning and memory post-stroke. To compare our results to Sun et al. (2013), spatial learning and memory was assessed using the Barnes Maze followed by an additional test for contextual and cue based memory using the fear conditioning test (Figure 13).

A



B

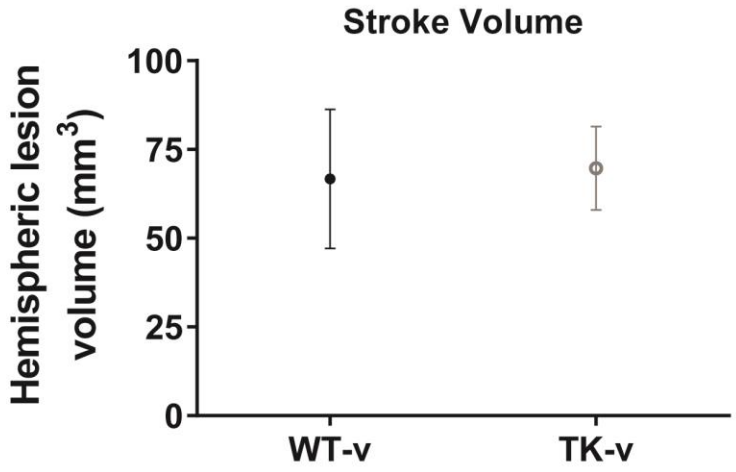


Figure 12. Ablation of PCs does not increase infarct size in motor cohorts. A) Representative images of **c**resyl violet stained brain sections from one of the rats in the study showing unilateral damage spanning the sensorimotor cortex and an infarct volume of 64.88mm^3). Red border outlines damaged tissue. **B)** Quantification of hemispheric lesion volume reveals no difference between WT-v and TK-v rats. Error bars are SEM.

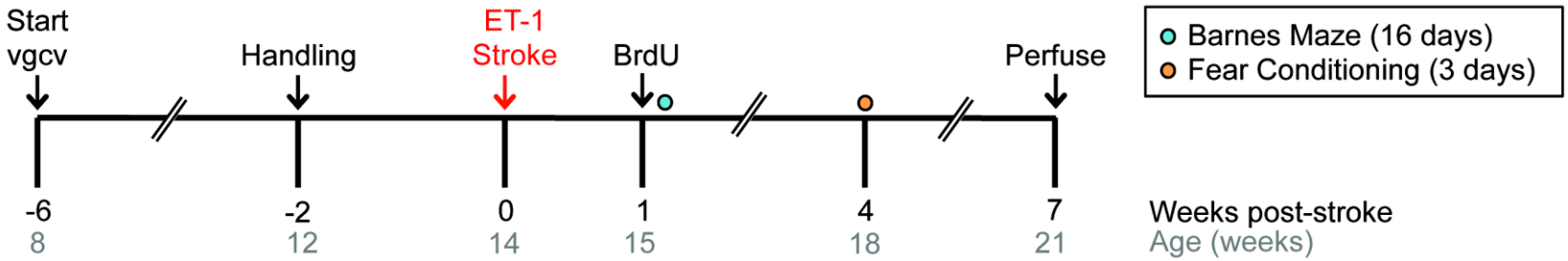


Figure 13. Timeline of experiment to determine if ablation of PCs impedes cognitive function post-stroke. WT-v and TK-v rats were treated with VGCV starting at 8 weeks of age. After 4 weeks of treatment, rats were handled to become habituated to the researcher followed by ET-1 stroke at 14 weeks of age. At 1 week post-stroke, rats were injected with BrdU and performed Barnes Maze testing the day after injection. At 4 weeks post-stroke, rats performed the fear conditioning tests followed by sacrifice at 7 weeks post-stroke.

The first phase of the Barnes Maze test assesses spatial learning by examining the ability of the rat to locate a target box using spatial wall cues (Figure 14A). During daily training for eight days, spatial learning was assessed by three measures: number of errors made, latency to enter the goal box, as well as distance taken to locate and enter the goal box[67]. Examination of the number of error made by the rats, as defined by the number of visits made to non-target holes combined with the number of visits made to the target hole without entering the goal box, revealed a significant reduction in the average number of errors made over eight training days for TK-v and WT-v rats (Figure 14B). Similarly, assessment of latency to enter the goal box was significantly decreased over time for both groups revealing no difference between groups (Figure 14C). Lastly, in agreement with the rats learning the spatial task, there was a significant reduction in the distance taken to locate and enter the goal box, once again in the absence of any group differences (Figure 14D). These results demonstrate that TK-v and control rats were equally able to learn the location of the target box, suggesting that PCs are not required for post-stroke spatial learning.

One day following the completion of training, rats were tested on a three-minute probe trial to assess the requirement of PCs for spatial memory retention. During the probe trial, the target goal box was removed from the maze. The rat was able to freely explore the maze and the location of the rat was defined by its location within six zones that were predefined and exclude the inner circle (Figure 15A). As expected, on average both WT-v and TK-v rats spent significantly more time in the target zone than the opposite zone (Figure 15B). Both groups also spent more time than chance in the target zone, as defined by 16.7% (1/6), suggesting that they learned the task. Additionally, heat maps of rat movement qualitatively demonstrate similar patterns of

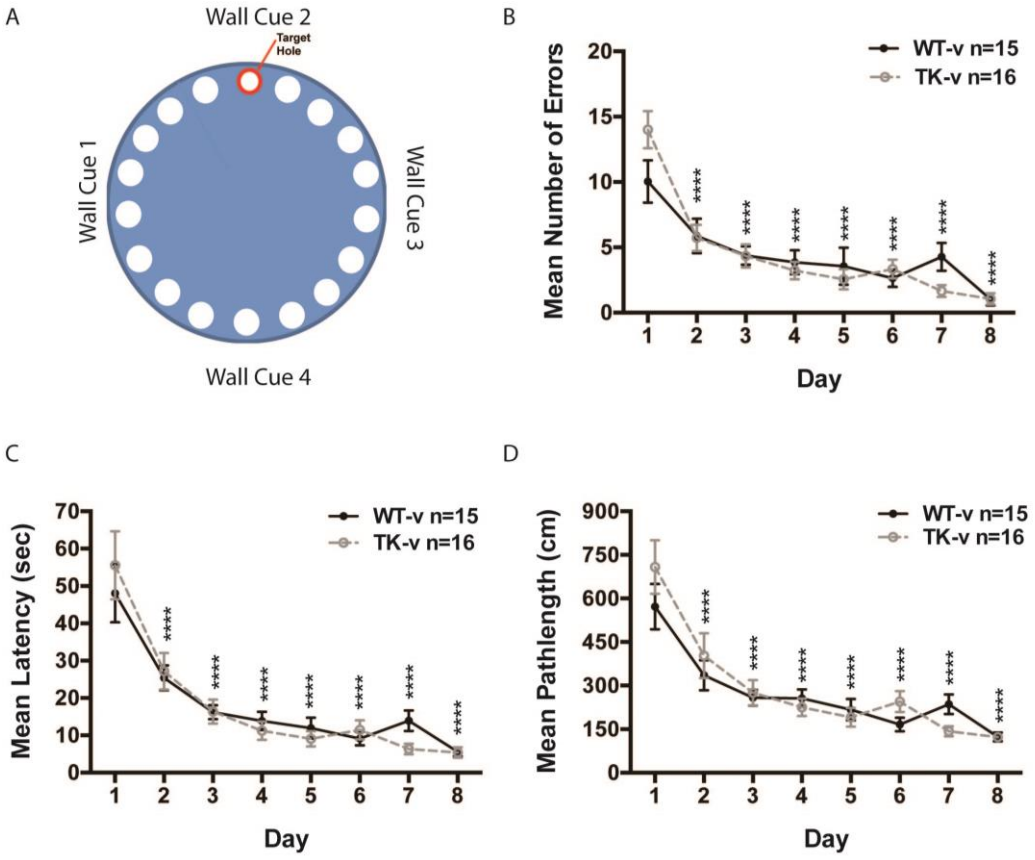


Figure 14. Ablation of PCs does not impede spatial learning during training on the Barnes Maze test. A) Schematic of the Barnes Maze arena consisting of 18 holes surrounded by four walls, each with a different cue. Under one of the holes (outlined in red) exists a target escape box. **B)** Assessment of mean number of errors reveals a difference in performance over time ($F_{(7, 203)}$=24.9, p<0.0001). There is no difference between groups with both WT-v and TK-v rats achieving close to zero errors by day 8, which is significantly fewer errors compared to day 1. **C)** Assessment of mean latency to enter the goal box reveals a difference in performance over time ($F_{(7, 196)}$=30.8, p<0.0001) and no difference between groups. WT-v and TK-v rats enter the goal box in under 10 seconds on average by day 8, which is significantly less time compared to day 1. **D)** Assessment of mean pathlength taken to enter the goal box reveals a difference in performance over time ($F_{(7, 196)}$=24.2, p<0.0001) and no difference between groups. WT-v and TK-v rats enter the goal box in under 150 cm of distance on average by day 8, which is significantly less distance compared to day 1. Error bars are SEM. (**** p<0.001, compared to day 1).

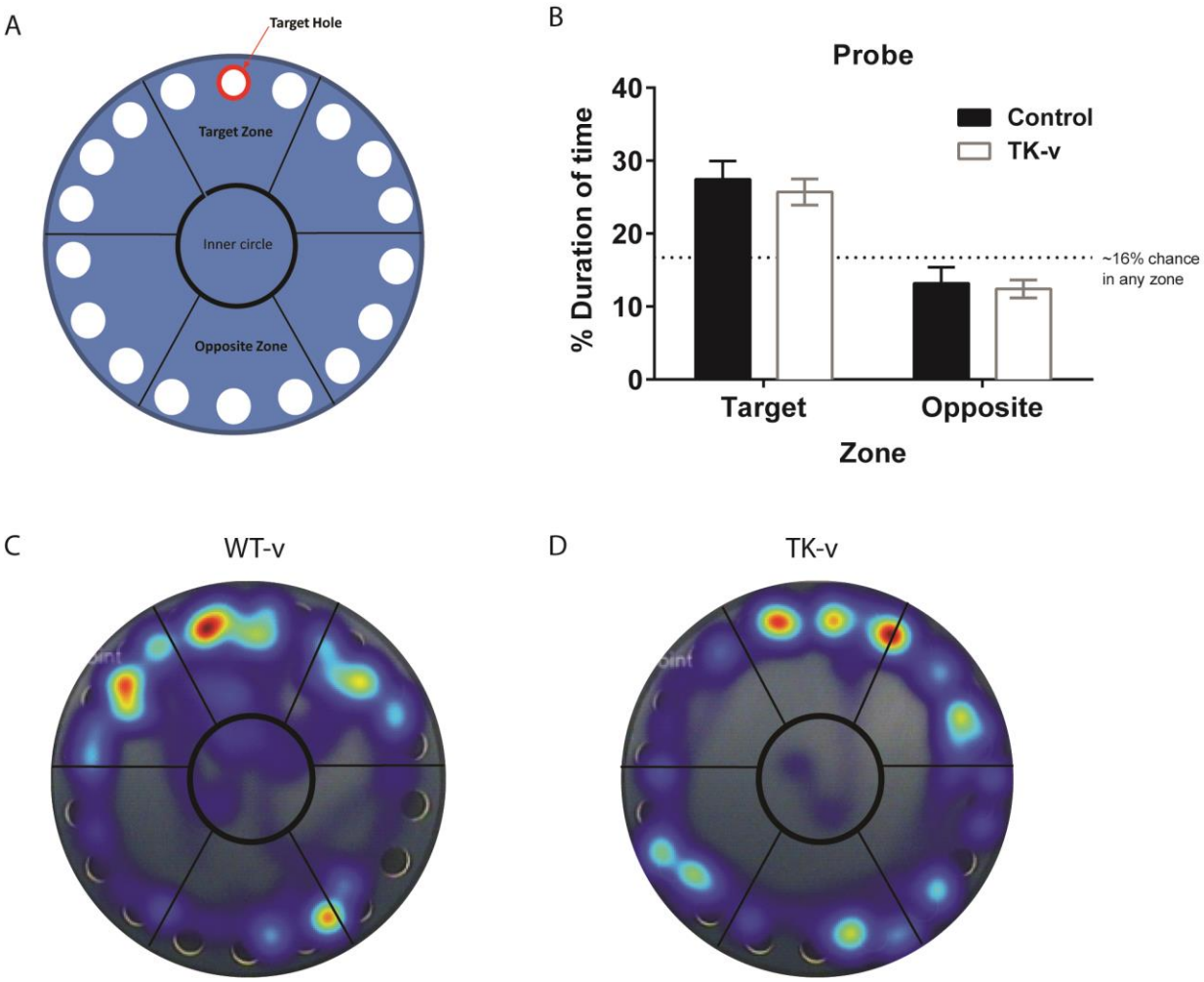


Figure 15. Ablation of PCs does not impede spatial memory retention during the probe trial of the Barnes Maze test. A) Schematic of the Barnes Maze arena for the probe trial during which the goal box is removed and the location of the rat is defined by six zones, including Target and Opposite Zones, and excluding the Inner Circle. **B)** Assessment of the probe trial reveals significant differences in between time spent in the zones ($F_{(1, 58)}$=49.0, p< 0.0001), with both WT-v and TK-v spending more time in the Target Zone compared to the Opposite Zone. **C)** Heat map showing one example of a WT-v rat during the probe trial spending the majority of its time (red) near the target hole. **D)** This is similar to a heat map during the probe trial of a representative TK-v rat spending the majority of its time (red) near the target hole. Error bars are SEM.

exploration during the three-minute trial between WT-v (Figure 15C) and TK-v (Figure 15D) rats. Among these measures there was no significant difference between WT-v and TK-v rats suggesting an equal capacity for spatial memory recall between TK-v and control animals and no requirement of PCs for spatial memory retention following stroke. Following the probe trial the rats started a five-day reversal test in order to test their ability to re-learn the spatial task, which is a measure of executive functioning[78]. During reversal testing, the target goal box is inserted back into the maze directly opposite of its original location during training (Figure 16A). Assessment of the average number of errors showed a significant reduction in the number of errors over time, with TK-v and WT-v rats achieving on average less than 2 errors after five days of testing (Figure 16B). Similarly, over the 5 days there was a significant decrease in time taken to enter the goal box (Figure 16C)**,** as well as a significant decrease in mean pathlength to reach the goal box (Figure 16D). For all three of these measures there was no significant difference between TK-v and control rats suggesting that PCs are not required for re-learning a spatial task following stroke.

The final assessment performed on the Barnes Maze was completed the day after reversal training and consisted of a second three-minute probe trial using the newly defined target goal box during reversal. Both groups spent more percent time than chance (>16.7%) in the target zone and a significantly greater duration of time in the target zone than the opposite zone, thereby confirming that they had re-learned the task (Figure 16E). There was no significant difference between the WT-v and TK-v rats. Together these results are in agreement with our first probe trial following initial training and suggest that PCs are not required for spatial memory retention post-stroke.

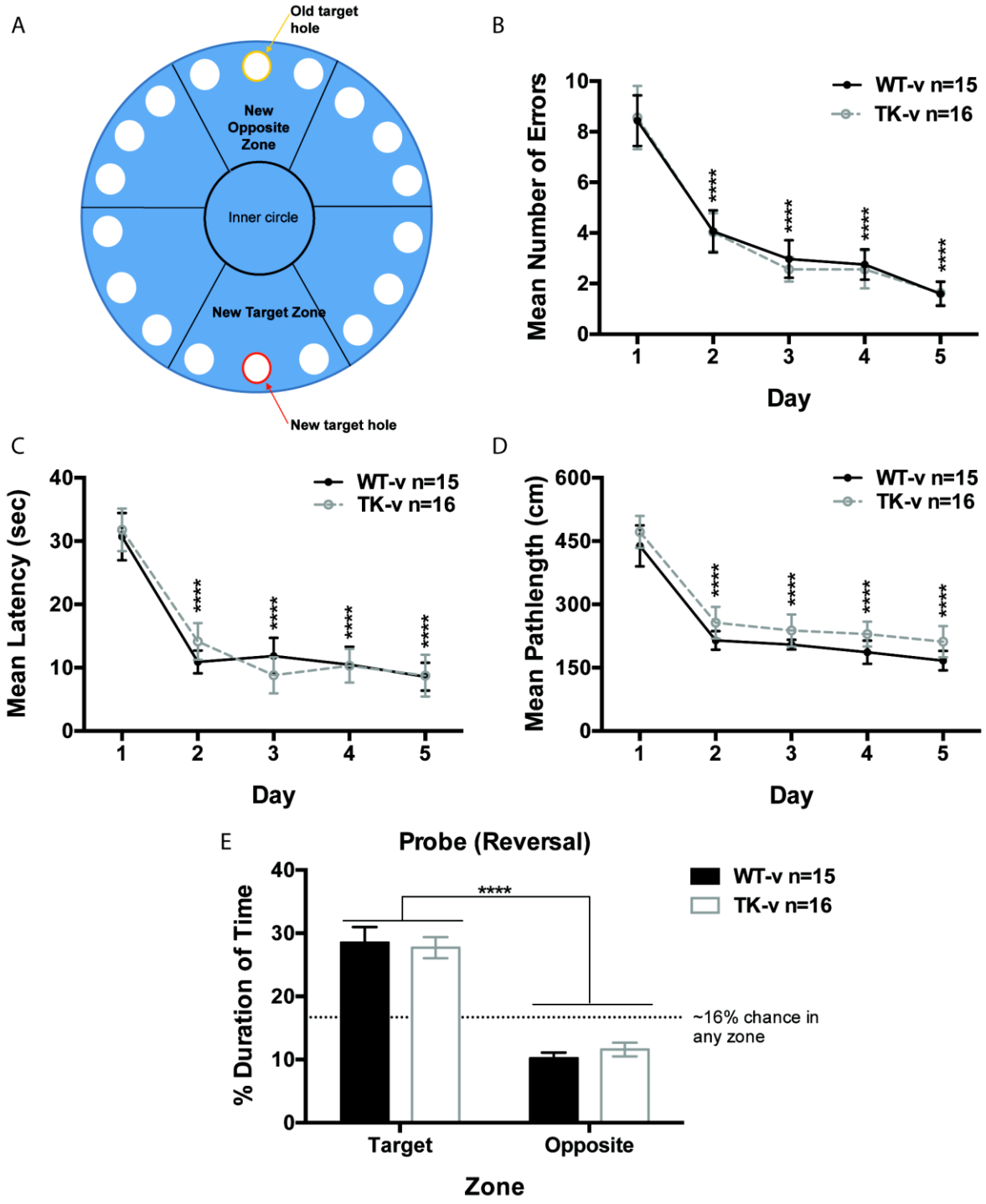

A
Old target hole
New Opposite Zone
Inner circle
New Target Zone
New target hole
B
Mean Number of Errors
WT-v n=15
TK-v n=16
Day
C
Mean Latency (sec)
WT-v n=15
TK-v n=16
Day
D
Mean Pathlength (cm)
WT-v n=15
TK-v n=16
Day
E
Probe (Reversal)
% Duration of Time
WT-v n=15
TK-v n=16
~16% chance in any zone
Target
Opposite
Zone

Figure 16. Ablation of PCs does not impede spatial re-learning during reversal training or spatial memory retention during the reversal probe trial on the Barnes Maze test. A) Schematic of the Barnes Maze arena during reversal training in which the new target hole (outlined in red) is directly opposite of its original location (outlined in yellow) during training. New target and opposite zones are defined. **B)** Assessment of mean number of errors reveals a difference in performance over time ($F_{(4, 116)}$=29.6, p<0.0001) and no difference between groups. WT-v and TK-v rats achieve less than 2 errors on average by day 5, which is significantly fewer errors compared to day 1. **C)** Assessment of mean latency to enter the goal box reveals a difference in performance over time ($F_{(4, 112)}$=34.7, p<0.0001). WT-v and TK-v rats enter the goal box in about 10 seconds on average by day 5, which is significantly less time compared to day 1. **D)** Assessment of mean pathlength taken to enter the goal box reveals a difference in performance over time ($F_{(4, 112)}$=30.8, p<0.0001) and no difference between groups. WT-v and TK-v rats enter the goal box in under 300 cm of distance on average by day 2, which is significantly less distance compared to day 1. **E)** Assessment of the probe trial reveals significantly more time spent in the Target Zone compared to the Opposite Zone for both WT-v and TK-v rats ($F_{(1, 58)}$=111.2, p<0.0001), with no difference between groups. There is no difference between WT-v and TK-v rats in the percent duration of time spent in the Target Zone. Error bars are SEM. (**** p<0.001).

3.6 Conditional ablation of PCs does not impede contextual or cued fear conditioning

Three weeks following the completion of Barnes Maze testing, animals were tested for fear conditioning as an additional assessment of learning and memory (Figure 13A). The percent time spent freezing, a natural demonstration of fear behaviour in animals, was recorded as a quantitative measure of memory associated with the aversive stimulus, which was a mild foot shock. During the first day of the test the rats were conditioned to associate both the environment (context) and tone (cue) to aversive foot shocks by receiving 4 tone-shock pairings (Figure 17A). On day 2, hippocampal-dependant memory was tested by examining the amount of time spent freezing in the same context as during training. This was followed by day 3 of testing that examined both hippocampal- and amygdala-dependent memory by examining freezing in a novel environment in the presence of the same tone[79].

On Day 1, during pre-conditioning both WT-v and TK-V rats spent on average a low percentage (<10%) of time spent freezing (Figure 17B). In contrast, on Day 2 during context test, both WT-v and TK-v rats spent approximately 35% of their time freezing (Figure 17B), which was significantly more than baseline levels of freezing prior to conditioning. On Day 3, the cue test was performed in which the rat was placed into a novel environment in order to test freezing associated with the tone. In agreement with the rats not recognizing the environment, both groups showed low levels of freezing prior to the tone being played, which significantly increased once the tone was played (Figure 17C). Similar to the results of the Barnes Maze, all fear conditioning outcome measures demonstrated no significant differences between the WT-v and TK-v rats. Thus, these results suggest that PCs are not required for learning and memory following stroke.

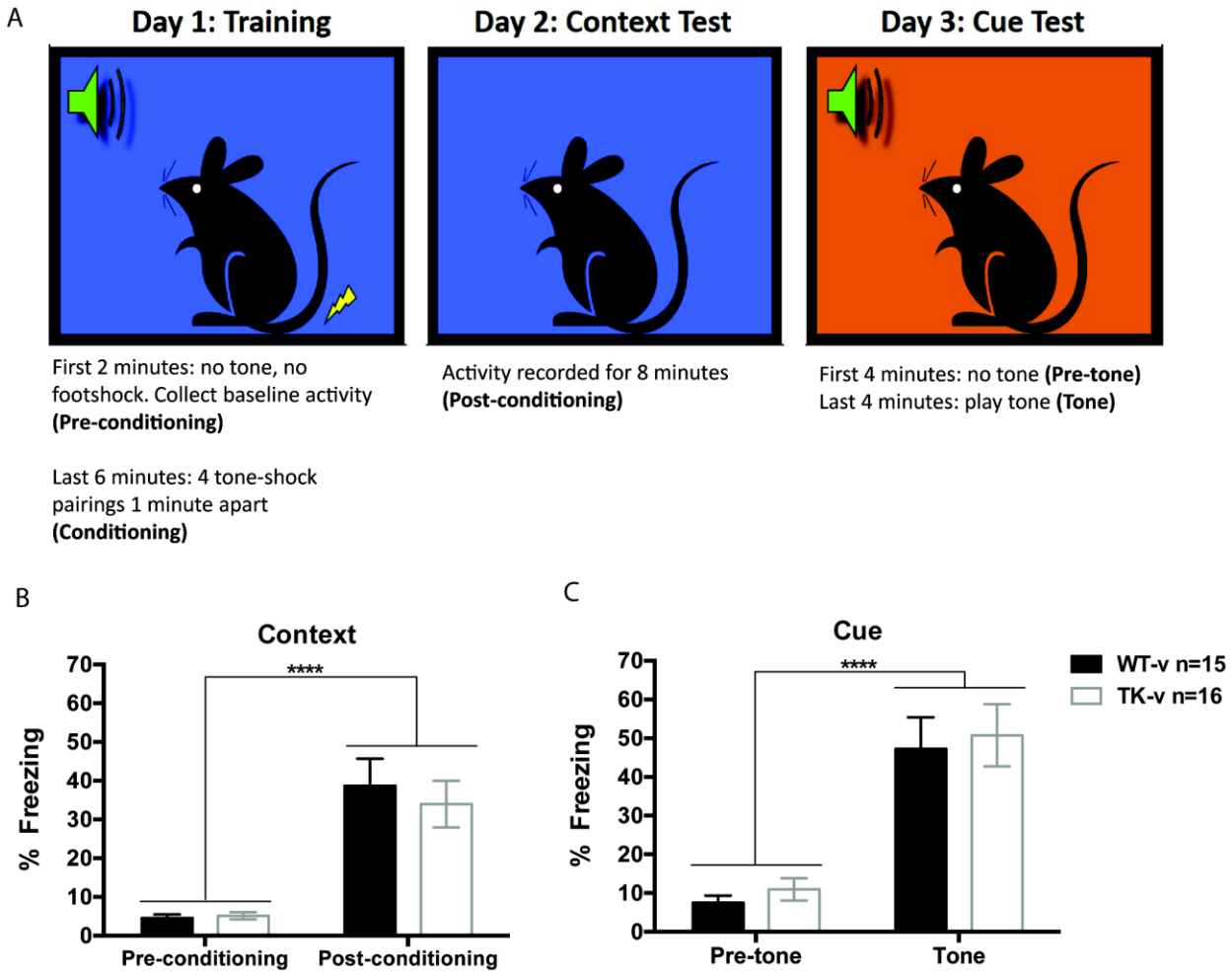


Figure 17. Ablation of PCs does not impede learning and memory during contextual or cued fear conditioning. A) Schematic of the 3-day fear conditioning protocol. On Day 1, baseline activity of each rat is initially recorded for 2 minutes during Pre-conditioning, followed by Conditioning during which each rat is exposed to 4 tone-shock pairings 1 minute apart. On Day 2, Post-conditioning tests contextual fear conditioning. The rats are placed back into the same environment for 8 minutes and freezing is recorded. Day 3 tests cued fear conditioning. The rats are placed into a novel context and activity is recorded for 4 minutes in the absence of tone during Pre-tone. This is followed by the tone being played for 4 minutes during which freezing is recorded. **B)** Assessment of contextual fear conditioning reveals no differences between WT-v and TK-v rats. As expected, there was significantly more freezing during Post-conditioning compared to Pre-conditioning ($F_{(1, 53)}$=43.4, p<0.0001). **C)** Similarly, assessment of cued fear conditioning reveals no group differences and significantly more freezing during Tone compared to Pre-tone ($F_{(1, 56)}$=44.0, p< 0.0001), as expected. Error bars are SEM.

3.7 Ablation of PCs does not alter infarct volume following cognitive testing during stroke recovery

Stroke volume was quantified for all animals in our cognitive study (Figure 13A) except one rat whose tissue was difficult to measure accurately due to tissue quality. Quantification of hemispheric lesion volume demonstrated no differences in infarct volume between the WT-v and TK-v rats with an average stroke volume of 43mm^3 and 44mm^3, respectively (Figure 18). This analysis confirms that an ablation of PCs does not alter infarct volume, which is in agreement with no difference in stroke size in our motor cohorts, as well as the most recent published study examining PCs in learning and memory post-stroke[61].

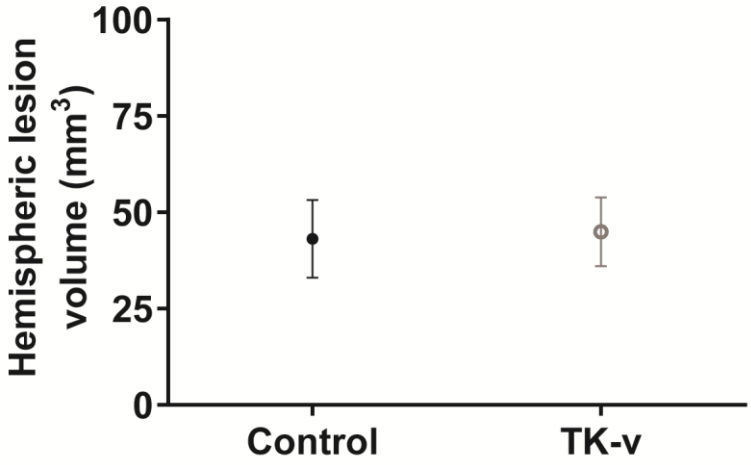


Figure 18. Ablation of PCs does not increase infarct size in cognitive cohorts. Quantification of hemispheric lesion volume reveals no difference between WT-v and TK-v rats. Error bars are SEM.

Discussion

In order to determine if PCs are required for post-stroke motor and/or cognitive functions, we quantified recovery from a well-characterized focal cortical stroke in the inducible transgenic GFAP-TK rat model that ablates PCs and neurogenesis. Our findings demonstrate PCs are dispensable for motor recovery and spatial learning and memory. Specifically, the TK-v rats in which PCs were ablated showed significant deficits in post-stroke sensorimotor skills and stroke volumes analogous to WT-v littermate controls. Likewise, the ablation of PCs did not diminish post-stroke spatial learning and memory in TK-v rats compared to littermate controls. These findings do not support the initial hypothesis that TK-v rats would demonstrate impediments in post-stroke recovery. These results also contrast previous studies supporting a positive correlation between neurogenesis and recovery, and the results of three different loss-of-function models that have also suggested a requirement of PCs in motor or cognitive recovery[58-61]. Our results oppose the current dogma and suggest innate recovery can occur independent of PCs. Therefore, our findings raise caution towards the notion that adult neurogenesis has a significant functional role in innate recovery following stroke.

4.1 Motor Function

In order to investigate the requirement of PCs for motor function following stroke, an infarct was made in the forelimb motor cortex with ET-1. This stroke model produced significant contralateral motor impairments on the staircase, cylinder and beam walk tests in our TK-v and controls rats. The observed deficits in our rats, which have a LE background strain, were similar to those reported in Sprague Dawley (SD) rats subjected to ET-1[80,81]. To our knowledge this is the

first time cortical injections of ET-1 have been used in LE rats to produce a cortical stroke and these findings demonstrate the robustness of this stroke model within different rat strains.

At 1 week post-stroke, there were significant deficits in all three motor tests for both WT-v and TK-v rats with no differences between groups. This is expected since it is generally assumed that the migration of PCs to the stroke site occurs 2-3 weeks post-stroke in the rat[82]. Thus, if PCs at the site of injury were involved in motor recovery, we do not expect differences between WT-v and TK-v rats at 1 week post-stroke. Any acute effects of PCs observed post-stroke is more suggestive of the PCs having a neuroprotective effect that is independent of the migration and integration of PCs surrounding or within the infarct, as was found in the DCX-TK mouse model[59]. Specifically, Jin et al. (2010) reported that DCX-TK mice at 24 hours post-stroke had 50% larger lesion volumes and corresponding exacerbated impairments in behavioural performance, suggesting that PCs prior to stroke provide neuroprotection. In contrast to this finding and in agreement with our findings, ablation of nestin-expressing PCs in the study by Sun et al. (2013) did not alter infarct size or motor recovery. Additionally, other studies that have either increased or decreased neurogenesis and examined recovery from stroke have reported no effect on stroke size[83]. It is possible that neuroprotection was only observed in the DCX-TK mouse model since this model ablates the DCX cells. However, this seems unlikely because by ablating GFAP-expressing PCs, as done in this study, or the nestin-expressing PCs as done by Sun et al. (2013), the DCX cells are also inhibited since they arise from the GFAP- and nestin-expressing PCs. In the TK-v rat, the ablation of DCX cells was shown in the initial characterization of the GFAP-TK rat[62] and we also re-confirmed this in one of our pilot studies (data not shown). Another, maybe more likely explanation for the neuroprotection observed in the DCX-TK study could be due to their use of a permanent MCAO stroke model. The permanent MCAO model does not allow for

reperfusion, whereas the ET-1 model used in this study and the dMCAO model combined with transient occlusion of the CCA used in the nestin-TK mouse model do permit reperfusion. This is important to consider since a substantial degree of reperfusion occurs in human stroke[84]. Thus, at least for stroke models that allow for reperfusion, our findings combined with the work of others[83] support that endogenous PCs do not provide a neuroprotective role in stroke.

In addition to examining acute deficits post-stroke, this study was designed to determine if PCs are required for long-term innate recovery, which was assessed by examining both contralateral and ipsilateral deficits, as well as assessing multiple time points between 1 and 7 weeks post-stroke. We found no difference in performance between WT-v and TK-v rats at any time points tested on the staircase, cylinder or beam walk tests. Inclusion of comparing contralateral versus ipsilateral performance in this study additionally allowed for the assessment of deficits related to surgical effects or diaschisis, rather than strictly unilateral deficits associated with the infarct. In the staircase test, there was a difference between contralateral and ipsilateral forelimb performance for both WT-v and TK-v rats. Ipsilateral deficits were present only at 1 week post-stroke, whereas contralateral deficits were sustained up to 7 weeks post-stroke. Therefore, the staircase test suggests that tissue damage contralateral to the stroked hemisphere occurred, which was confirmed by cresyl violet staining (data not shown), but was minimal.

On the beam walk test both contralateral and ipsilateral forelimb deficits were sustained up to 5 weeks post-stroke, whereas contralateral hindlimb deficits persisted until 7 weeks post-stroke. The relatively larger magnitude of deficits in the hindlimb compared to the forelimb were not surprising since the beam walk test has been shown to be a more sensitive measure of hindlimb function[77]. However, the sustained deficits in hindlimb performance were unexpected and we hypothesize may be attributed to the gait of this strain of rats when they traversed the beam. Our

LE rats show obvious differences in gait while crossing the beam compared to SD rats. While SD rats generally cross by walking step by step, one paw in front of the other, LE rats appeared to traverse the beam in a hop-like fashion, with hindlimbs often being placed on the beam's lower level and thus, defined as a slip or incorrect placement. Strain differences between LE and SD rats have been previously reported, in which SD rats perform better than LE rats on the beam walk test[85]. Additionally, the rats in this study usually ran across the beam as opposed to walking as demonstrated by SD rats, and thus the increased speed likely increased their chances of making a slip. Since slips did not result in negative consequences for the rats, there was no motivation to correct slips during training. Indeed, following training during baseline assessment, our rats demonstrated correct hindlimb placements approximately 85% of the time, which is lower than the 95% that is often observed for hindlimb placements SD rats[86]. However, despite these observed deficits in baseline performances in the GFAP-TK rat model, there were significant long-lasting contralateral impairments for both the WT-v and TK-v rats in this study, suggesting that the beam walk test was a sensitive measure of hindlimb coordination.

By assessing multiple time points during the post-stroke recovery period, this study also allowed for the measurement of innate recovery. Both WT-v and TK-v rats had significant improvements on the staircase test during recovery. For this test, pellet retrieval performance at 7 weeks post-stroke was significantly greater than at 1 week post-stroke, suggesting innate recovery on this task. In contrast, despite some mild improvements in the cylinder and beam walk tests post-stroke, significant deficits remained at 7 weeks post-stroke with no significant difference compared to 1 week post-stroke. Innate recovery may have only been observed on the staircase test as a result of our exclusion criteria. Due to the inherent variability of stroke damage such as size and location produced by ET-1 between rats, this study, as well as other studies[87,88], utilized exclusion criteria

to ensure injuries alike and stroke profiles are being compared. For example, rats (n=2/10 WT-v and n=4/20 TK-v) from this study were excluded since they had greater than 80% success on the staircase test at 1 week post-stroke. These rats were excluded because they were unlikely to demonstrate recovery since they showed mild initial deficits, which were not suitable for the testing of innate recovery. In contrast, one of the twenty rats in the TK-v group was excluded since this rat demonstrated less than 5% success on the staircase test at 1 week post-stroke and had a stroke volume greater than $100mm^3$. In this case, there was no possibility for innate recovery since the rat had an infarct volume approximately twice the volume of the average lesion size and displayed large deficits that did not improve over time (data not shown). Thus, while these criteria help to examine motor recovery by ensuring robust post-stroke deficits with the possibility of improvement, they do not allow for the generalization of our findings to stroke injuries that are not amendable by innate recovery

Examination of innate recovery following the ablation of PCs has been previously reported once by assessing a time course of recovery in DCX-TK mice[60]. The results from this study contradict our work by suggesting that the ablation of PCs impedes innate stroke recovery. Specifically, Wang et al. (2012) show a transient and modest impediment in recovery in the DCX-TK mice compared to controls up to 8 weeks following stroke. For example, on the beam walk test, the effect observed in the DCX-TK mice was a difference of one hindlimb slip more than controls at 4 and 8 weeks post-stroke. Moreover, by 8 weeks post-stroke, DCX-TK mice and controls demonstrated on average a total of one and almost zero slips, respectively, on the beam walk test. In our hindlimb assessment on the beam walk, by 7 weeks post-stroke WT-v and TK-v rats did not show a significant difference compared to 1 week post-stroke. Thus, one major difference between our findings is the magnitude of behavioural improvement over time, in which

the GFAP-TK rats show longer-lasting significant deficits when compared to the deficits in the DCX-TK mice. Additionally, as noted above, the DCX-TK mice also had larger (50% greater) stroke volumes compared to controls. Thus, it is possible that some of the mild effects observed may be more greatly attributed to neuroprotection, rather than innate recovery. Lastly, while ablation of PCs was continuous in our model with VGCV starting 6 weeks prior to stroke, in the DCX-TK mouse study GCV administration was discontinued after stroke. This is important to note since it raises the possibility that the restoration of post-stroke neurogenesis could have allowed for equalized performance during recovery, as demonstrated by their beam walk test results at 12 weeks post-stroke. Therefore, although our findings obtained from GFAP-TK rats contrast the findings obtained from DCX-TK mice, the differences in magnitude of behavioural responses, demonstration of possible neuroprotection, and discontinued ablation of PCs following stroke in their work may all contribute to the differences between our findings.

In support of our finding demonstrating no requirement of PCs for motor recovery, the nestin-TK mouse model study by Sun et al. (2013) reported no difference in motor recovery in the absence of PCs at 8 weeks post-stroke[61]. Although recovery in the nestin-TK mice was not examined by performing a time course analysis, our studies combined share important parallels in study methodology including continuous ablation of PCs and comparing similar infarct sizes between groups. Therefore, in the case of ablating PCs prior to stroke onset and continuing ablation during recovery, our findings using the GFAP-TK rats and the work in the nestin-TK mice by Sun et al. (2013) both support PCs are dispensable for long-term motor recovery.

4.2 Cognitive Function

We found no significant differences in cognitive function post-stroke between WT-v and TK-v rats. These results are in contrast to the findings by two groups that suggest PCs are required for post-stroke learning and memory[58,61]. One major difference between our work and these two previous studies is that we tested for cognitive function at 1 week following stroke. This is much earlier than the testing done by Raber et al. (2004) and Sun et al. (2013) at 4 and 8 weeks post-stroke, respectively. It may be possible that cognitive effects would only be observed many weeks after a stroke due to evidence suggesting that PCs need to be at least 6-8 weeks old in order to contribute to behavioural outcomes[89]. In order to address this concern, we performed an additional Barnes Maze test at 9 weeks post-stroke at the end of a motor study cohort and did not observe a difference between groups (Appendix A). Our findings suggest that timing of testing post-stroke did not contribute to the lack of difference observed between TK-v and control rats.

An alternative and more likely possibility as to why no differences in cognition were detected between WT-v and TK-v rats could be because ET-1 strokes were produced in the motor cortex. Raber et al. (2004) used a model of cerebral global ischemia by occluding the bilateral common carotid artery, which restricts blood flow to the whole brain and is similar to what occurs after cardiac arrest[90]. Thus, it is possible that we did not observe effects on spatial learning and memory as reported in their irradiated gerbils because our ET-1 stroke does not induce ischemic injury in areas of the brain important for cognitive functioning such as the hippocampus. While it is important to use different stroke models to ensure outcomes are not model specific, models of global ischemia as opposed to focal ischemia challenge the translation of findings to human stroke patients since the resulting damage is larger than the average human infarct[90]. Furthermore, the method of cranial X-ray irradiation used to suppress neurogenesis in the gerbils did not solely

inhibit neurogenesis, making it difficult to assess the role of adult neurogenesis *per se*. Thus, although Raber et al. (2004) were the first to suggest a causal role of adult neurogenesis for spatial learning and memory following a stroke, the application of a global ischemic model and use of irradiation to induce ablation might be important factors contributing to their outcome.

More recently, Sun et al. (2013) also reported cognitive deficits in the nestin-TK mice that underwent stroke produced by dMCAO combined with occlusion of the CCA. This study did not show representative images of their infarcts, however, as a focal stroke model, this model generally produces injury restricted to the cerebral hemisphere, avoiding hippocampal damage[91]. Thus, differences in our findings are likely not attributable to stroke location or size. Additionally, it is not possible to assess whether ablation alone or a combination of PC ablation and stroke in the nestin-TK mice resulted in diminished ability for spatial learning and memory since a non-stroke control group was not included. In our model, we verified that the ET-1 stroke itself did not produce deficits on the Barnes Maze test. Furthermore, using a second behaviour paradigm testing for both contextual and cued fear conditioning we found no effect of ablating PCs on learning and memory post-stroke. Therefore, through two different assessments, our work is the first to suggest that in the case of focal cortical strokes, PCs are not required for spatial learning and memory. The lack of effect on cognition in the context of stroke recovery following ablating PCs may not be surprising considering that the role of PCs and neurogenesis in the adult brain for learning and memory has been controversial and is now thought to be more important for specific types of learning and memory such as context discrimination[45,46]

4.3 Summary

Overall, our work demonstrates that PCs are not required for motor recovery and spatial learning and memory following ischemia. This contradicts the take-home messages from three other groups that suggest ablation of PCs hinders stroke recovery. A lack of unanimity between our results and those previously reported are likely attributed to heterogeneity in study methodologies and design, including differences in species, stroke model, method of ablation and behavioural testing paradigm, as well as interpretation of results. By using the GFAP-TK rat and ET-1 stroke model we also addressed the question of whether PCs are required for motor recovery or spatial learning and memory following ischemia in a number of novel ways compared to previous work in that: 1) This is the first time a rat model is used to study the requirement of PCs for stroke recovery, which may be seen as advantageous since rats offer a robust behavioral phenotype[92] ; 2) This is the first time the ET-1 stroke model is used to study the requirement of PCs for stroke, which allowed for significant deficits and innate recovery to be measured; 3) This is the first time that both motor and cognitive function has been tested at multiple time points allowing for a long-term assessment of innate recovery; and 4) This is the first study that has included both male and female rodents. Although stroke affects both men and women, all four previous studies have excluded female subjects. Interestingly, previous work studying neurogenesis using rodents have shown a marked preference for males as test subjects due to the notion that the data acquired from female rodents is more variable because of their hormonal cycle[93]. Unlike previous causal studies ablating PCs and measuring recovery[58-61], we included both male and female test subjects to further enable the reliability of translating our findings to human stroke patients. Since we found no differences between sexes, we combined the sexes to show WT

versus TK rats. Taken together, this study advances our understanding of the role of PCs during stroke recovery.

Importantly, this work also continues to raise future research questions. Since it is estimated that 80% of adult-generated neurons die post-stroke[50], it may not be "neurogenesis" *per se* that is involved in stroke recovery, but other cellular processes in the ablation studies that suggest neurogenesis is required for recovery. Our study highlights the need to identify the fate of surviving PCs at the site of stroke damage in spite of not observing behavioural changes. It also remains highly interesting and important to examine the effects of ablating or promoting the survival of PCs after a stroke as opposed to prior to stroke injury. This would improve the translation of preclinical stroke work since realistically strategies to promote stroke recovery could only be achieved post-injury. In order to address this question, our laboratory is currently testing if promoting survival of PCs following a stroke could enhance recovery. Despite allowing a significantly increased number of PCs to survive and migrate to the site of stroke injury, there is no change in recovery suggesting that enhancing cell survival alone appears insufficient to improve recovery[94]. Combined with the results of this work, this has led our team to now question whether the additional manipulation of surviving PCs to improve their integration might be required to enhance recovery.

Given the debilitating effects that stroke has on survivors and our society at large, continuing our efforts in stroke recovery are imperative. The prevalence of stroke is projected to rise as our demographics transitions to an aging population and stroke is beginning to affect an increasing number of young adults[6,9]. Although life expectancy has improved, disability is more prevalent and we remain challenged by stroke at all ages, for both men and women. Additionally, the majority of patients have comorbidities and suffer from at least one other chronic condition

such as diabetes. As a result, stroke patient profiles have changed and treating the present day stroke survivor is more complex[9]. This has raised the additional need for future preclinical animal work to consider using models with comorbidities to better model human stroke patients. As efforts in stroke recovery using preclinical models continue, it is essential that we remain mindful of the translation of this work for the real patients, that is, the millions of people affected by stroke worldwide.

Conclusion

Use of an inducible transgenic GFAP-TK rat to ablate PCs, the well-validated ET-1 stroke model, and three robust behavioural tests of motor functions provided results strongly suggesting that PCs and their progeny are not neuroprotective and are not required for innate motor recovery. Moreover, the PCs and their progeny appear to be dispensable for spatial learning and memory following a focal cortical stroke using two separate tests of cognitive function. These findings add to a growing body of work that has begun to question the functional significance of the neurogenic response after a stroke. Although PCs appear to not be required, this leaves open the question of whether manipulating the survival and/or integration of the PCs may allow for enhanced neurogenesis to improve the lives of millions of people affected by stroke worldwide.

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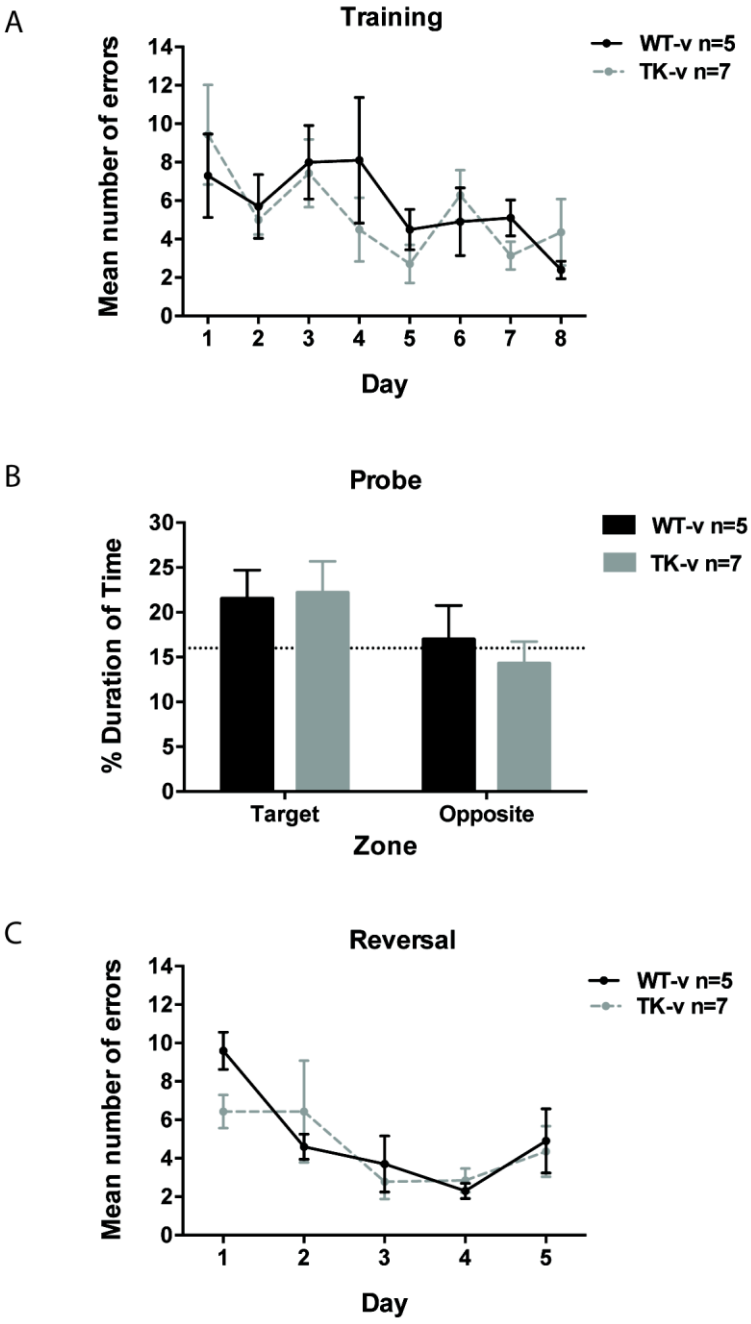
Appendix



Appendix A. Ablation of PCs does not impede spatial learning and memory on the Barnes Maze test 9 weeks post-stroke. A) Assessment of spatial learning during training reveals no difference in mean number of errors between groups. **B)** Assessment of spatial memory retention during the probe trial reveals no difference in percent duration of time spent in the target zone between groups. **C)** Assessment of spatial re-learning during reversal training reveals no difference in mean number of errors between groups. Errors bars are SEM.