Assessing Motor Impairments in a Mouse Model of Perinatal Stroke through Brain Mapping and Behaviour

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Abstract

Perinatal stroke, which occurs before or shortly after birth, may result in both beneficial and maladaptive plasticity in surviving tissue. However, current preclinical and clinical work have an unclear understanding on the relationship between functional outcome and neurophysiology. This thesis aims to dually characterize and correlate behaviour with cortical motor representations in a mouse model of perinatal stroke. On postnatal day 7, a unilateral photothrombotic stroke was produced in the primary motor cortex of Thy1-ChR2 mice. Sensorimotor function was evaluated in adulthood with a battery of behavioural tests. Subsequently, a transcranial window was implanted, and motor maps were created through optogenetic point stimulation. To evaluate the impact of skilled motor training on cortical reorganization, mapping was conducted before and after training on the single pellet reaching task. P7 stroke caused functional impairments across a battery of motor tasks, while both motor map size and movement latency were bilaterally impacted. Spontaneous limb use was positively correlated with map size of both hemispheres, but single pellet performance was only positively correlated with map size in the injured hemisphere. Following skilled motor training, both map size reductions and delayed latency was partially restored. Additionally, significant correlations between map size expansion and movement latency reduction following skilled motor training not only demonstrate that training-induced plasticity was beneficial, but also primarily mediated by the uninjured hemisphere. As the first study to conduct within-animal optogenetic motor mapping following perinatal stroke, we show that 1) perinatal stroke bilaterally impacts both cortical and descending aspects of the motor system, 2) the remaining movement sites in both the uninjured and injured hemispheres have a positive impact on functional outcome, and 3) skilled forelimb training can partially restore cortical and descending motor neurophysiology.

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List of Abbreviations

AHA	Assisting Hand Assessment
CBF	Cerebral Blood Flow
CFA	Caudal Forelimb Area
CIMT	Constraint-Induced Movement Therapy
CST	Corticospinal Tract
GMFCS	Gross Motor Function Classification System
H-I	Hypoxia-Ischemia
ICMS	Intracortical Microstimulation
IHC	Immunohistochemistry
IP	Intraperitoneal
LDF	Laser Doppler Flowmetry
M1	Primary Motor Cortex
MCA	Middle Cerebral Artery
MCAo	Middle Cerebral Artery Occlusion
NIBS	Non-Invasive Brain Stimulation
PBS	Phosphate-Buffered Saline
PT	Photothrombosis
RFA	Rostral Forelimb Area
RMT	Resting Motor Threshold
RST	Rubrospinal Tract
RtST	Reticulospinal Tract
S1	Primary Sensory Cortex
SD	Standard Deviation
SEM	Standard Error of the Mean
TMS	Transcranial Magnetic Stimulation
tPA	Tissue Plasminogen Activator

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1 – Introduction

1.1. Perinatal Stroke

Stroke is a debilitating neurological condition, and is currently one of the leading causes of death in Canada (1). Although predominantly affecting older populations (>65 years old) (2), it can occur at any timepoint in a person's life, including early infancy. In fact, the risk for stroke is highest amongst newborn infants compared to any other stage of pediatric or adolescent development (3,4). Perinatal stroke, defined as a cerebrovascular event occurring between 20 weeks in utero to 28 days after birth, is a particularly important cause of chronic morbidity in children (5). Currently, it is estimated that the incidence of perinatal stroke is approximately 1:1600 to 1:2300 live births (6).

1.2. Adult vs. Perinatal Stroke

Historically, the prevailing view has been that younger nervous systems have greater capacity for recovery following brain injury. Early evidence for this arose in the 1930s, with seminal studies conducted by Margaret Kennard in non-human primates. In these studies, younger animals displayed faster and more complete recovery of motor performance after cortical ablations compared to their adult counterparts (7,8). The concept that improving capacity for neurological recovery corresponds with decreasing age, commonly cited as the "Kennard Principle", served as a fundamental principle for understanding developmental brain injury in subsequent years. However, since these experiments, work in other animal models has shown limited generality to the Kennard Principle. For example, large bodies of developmental injury work in rodents show that improved recovery in neonatal animals is limited to a specific window of time (i.e. 2nd week of life) and dependent on the specific behaviour being tested (ex. cognitive vs. motor vs. species-typical tasks) (9). Similarly, studies in the feline motor system have shown that injuries during a "critical period" of development could lead to chronic anatomical and functional abnormalities (10–12). In the context of human stroke, perinatal stroke often presents with worse motor impairments compared to those which occur later in life (such as during childhood or adolescence) (13–15). As such, it is not accurate to assume that a perinatal injury will result in more complete recovery than an adult injury, particularly where it concerns stroke. In fact, the aforementioned evidence presents the possibility that perinatal stroke can similarly cause long-term neurological deficit. It also introduces the concept that there are distinct physiological factors which separate

the perinatal period from other age groups. Due to the overwhelming focus of stroke research on adult populations, greater effort must be made to differentiate perinatal stroke from adult stroke. For example, one difference lies in etiology and risk factors. In adults, one of the primary risk factors arises from poor cardiovascular health, which can be modified with lifestyle choices (16,17). However, the few risk factors that have been identified in perinatal stroke patients, such as congenital defects of the heart, vessels, or blood (5,14), are difficult to address, especially when the stroke occurs in utero. Without the ability to consistently identify and/or address risk factors to prevent perinatal strokes, solutions must instead be found in acute treatment and chronic management after cases have occurred.

Unfortunately, there are also unique challenges within this realm. While symptoms of most adult strokes can be identified acutely (18), patients with perinatal stroke are often not diagnosed until secondary symptoms appear, such as seizures, missed motor milestones, or hemiparesis, often taking up to 4-6 months to manifest (19–21). Latency in clinical diagnosis proves to be a significant challenge when considering treatments, since time-sensitive thrombolytic agents such as tissue plasminogen activator (tPA, currently the only approved pharmacological treatment for adult stroke) cannot be administered. Even amongst patients with timely diagnoses, the use of tPA is currently only experimental in neonates, with little evidence on its efficacy (16). Beyond acute treatments, there are also challenges with well-established chronic-stage treatments. Exercise and bimanual therapies are likely difficult to implement until perinatal stroke survivors have developed basic ambulatory/fine motor skills (on average 12 months and 6-18 months, respectively (22,23)). In addition, cognitive limitations of young infants can limit the patient's ability to follow a therapist's instructions (24). While these approaches have merit once a child reaches these developmental milestones, a more effective alternative may be to administer therapies within the first 6-12 months of life. In this time period, treatments may be able to redirect and/or harness the ongoing activity-dependent development of the descending motor pathways (covered more in detail in section 1.3.1). Evidence from studies in developing cats have shown the potential of both behavioural (ex. constraint-induced movement therapy, reach training) and biological (ex. electrical stimulation) interventions to steer corticospinal tract development towards a more normal pattern to improve recovery after cortical silencing (25–28). This preclinical evidence has led to the beginnings of early-implementation therapies such as constraint-induced movement therapy (whereby the uninjured limb is constrained to promote use of the injured limb, CIMT) (29),

home-based therapy (i.e. exercises that can be carried out at home) (30), stem cell transplantation (31), and non-invasive brain stimulation (NIBS) (32). Unfortunately, none have yet been formally adopted at the clinical level (16). With the current paucity of standardized treatment for these young patients, it is estimated that 57-75% of perinatal stroke survivors will develop some form of long-term disability (5,20). Of this proportion, motor deficits are one of the most common sequelae of impairments, impacting between 25%-68% of individuals (6,14,16,33). In fact, perinatal stroke is one of the leading causes of hemiparetic cerebral palsy, a permanent movement disorder that is characterized by weakness/paralysis and motor deficits on one side of the body (5,6).

Although motor deficits are also commonly present after adult stroke, perinatal stroke survivors must live with these impairments for the remainder of their lives (up to 60-70 years, (34)), resulting in an increased cumulative burden compared to adult strokes. For example, one direct cost analysis from northern California found that 5-year healthcare costs of a patient with perinatal stroke were already increased 15-fold when compared to age-matched healthy controls (35). Considering these results were only an analysis of direct costs within the first few years of life, the financial burden of perinatal stroke is likely to be even more substantial due to longitudinal factors such as lost productivity from parents and a child who matures into a disabled adult. Thus, although age-adjusted incidence of perinatal stroke falls vastly below adult stroke incidence, lifelong accumulation of such costs paired with increasing global prevalence (36) results in a significant and chronic burden on the healthcare system.

Overall, when considering the management of perinatal stroke, it is clear that there are important differences compared to adult stroke populations at multiple levels, including etiology, physiology, treatment strategies, and societal burden. These key differences highlight the importance of finding and validating effective treatments for the perinatal stroke population specifically.

1.3. Neurobiology of developmental injury

In order to develop new therapies and strengthen existing ones, there first needs to be an understanding of how the unique biology and physiology of the young brain impacts recovery after injury. In particular, the coincidence of the stroke with ongoing development of the nervous system provides a uniquely plastic environment within which recovery can occur. In turn, this

environment can potentially provide greater opportunity for beneficial recovery compared to adults, such as compensatory rerouting of extrapyramidal tracts (37) or re-establishment of normal connections to spinal cord motor neuron pools (24,28,38). However, it may also promote maladaptive changes, such as aberrant strengthening of the ipsilateral tract from the uninjured hemisphere (39). It is worth noting that each of these factors may be alternately beneficial or maladaptive to recovery depending on the individual. Unfortunately, the conditions under which one or the other occurs currently remains unclear and warrants further study.

1.3.1. Descending motor tracts

One important component to motor functioning and recovery are the descending motor tracts. In humans, the primary tract mediating movement is the corticospinal tract (CST). From cortical origins, such as the primary motor cortex and premotor areas, the CST descends through the medullary pyramids and terminates in the spinal grey areas of the spinal cord. Early in development, there is the presence of a bilateral innervation patterns from each hemisphere (i.e. both ipsilateral/uncrossed and contralateral/crossed tracts) onto distal musculature. However, with activity-dependent experience and maturation, contralateral tracts are gradually strengthened, and ipsilateral tracts are pruned away and retracted (23,40). By young adulthood, the motor system is a primarily contralateral system, with only 10-15% of tracts remaining as ipsilateral projections (41).

Supplementing the CST are the descending midbrain/brainstem pathways, often referred to as the indirect or extrapyramidal tracts since they pass through a variety of other areas outside of the medullary pyramids. These tracts comprise of four main pathways: 1) the rubrospinal tract (RST) originating from the red nucleus in the midbrain, 2) the tectospinal tract originating from the superior colliculus, 3) the vestibulospinal tract from the vestibular nuclei, and 4) the reticulospinal tract (RtST) originating in the reticular formation. In the human, the RST and RtST are particular sources of interest. The RST mediates voluntary movements of the distal muscles of the arms (including the hands) and legs so that more fine-tuned muscles are in a position to carry out their movements (42). The RtST has more of a primary role in innervating proximal muscles important for controlling posture and locomotion. However, there has also been evidence showing RtST innervation as distal as the wrist and hand muscles (43,44). Although the majority of post-stroke recovery is often focused on the CST, the remaining extrapyramidal tracts are all key to normal motor functioning and present potential avenues of post-stroke plasticity.

Following a unilateral perinatal brain injury, the CST refinement process is disrupted. With the sudden loss of functional brain tissue, descending projections are also consequently weakened. Through studies in both animal models and human patients, it is known that disruption of the normal refinement period can lead to abnormal innervation patterns. In kittens and rodents, aberrant ipsilateral projections from the uninjured hemisphere onto the paretic side are maintained after cortical injury, instead of being pruned and retracted away (11,45–48). Similar findings have been confirmed following perinatal stroke in humans, where bilateral responses can be evoked from the uninjured hemisphere (23,40,49). Notably, this bilateral innervation pattern is not found in adult patients with unilateral injuries, indicating that these changes in CST wiring are specific to the timing of perinatal strokes (23,40). However, it is unclear whether these aberrant projections contribute beneficially or maladaptively to functional performance (27,50–54). Both hemispheres likely have variable positive and/or negative contributions depending on the individual (55). Thus, it is important to concretely establish how rewiring occurs following perinatal stroke, and the individual roles of each cortex on functional outcome, before expanding upon directed therapeutic efforts which target individual cortices.

1.3.2. Cortical control of movement

As descending motor tracts have been shown to be plastic after stroke, ostensibly the cortical origins of these tracts may also be altered, particularly if the main cortical centre (i.e. the primary motor cortex, M1) is impacted. Indeed, with the popularization of various cortical stimulation techniques, the effect of stroke on cortical motor centres in adults has been described at length. However, the technique of cortical mapping has yet to be as extensively explored in perinatal injury models.

Due to the invasive nature of the experiments, human reports of cortical motor control in adults were first limited to stimulation during neurosurgical procedures. From those first studies in the 1900s, it was discovered that motor control is somatotopically organized across the cortex (56). That is, control of specific body parts can be associated with explicit and distinct areas, or "motor maps". Since the advent and popularization of transcranial magnetic stimulation (TMS) in the 1990s, motor maps can now be conducted non-invasively and safely in human subjects. In animal models, a similar evolution from invasive to non-invasive techniques has occurred. Traditionally, animal motor mapping has been carried out with the technique of intracortical microstimulation (ICMS), whereby motor neurons are stimulated with inserted electrodes. With

the establishment of optogenetic rodent lines in the early 2000s, cortical stimulation can now also be achieved non-invasively with laser light. However, due to the relatively recent advent of optogenetic mapping as well as the low spatial resolution of TMS mapping, the majority of the motor mapping knowledge base has been, and continues to be, carried out using the ICMS technique (57,58).

From this evidence base, one of the first major findings from ICMS studies was that adult motor maps can be reorganized with motor experience. This relationship was first shown by Nudo and Milliken in 1996, whereby healthy non-human primates underwent digit vs. forearm training, then mapped for their respective representations using ICMS. The authors found that with digit training, finger representations expanded at the expense of other forearm areas, whereas in forearm training, the opposite was seen (59). Since then, the inverse has also been shown, where restriction of movement leads to reduction in map size and a decrease in excitability (60,61). Subsequent experiments in rodents have also upheld similar tenets (62–67). These studies not only demonstrated experience-dependent plasticity, but also provided evidence that motor maps represent the capacity to produce and acquire skilled movement. Further support for this can be found in mapping studies in injury models, where focal ablation to motor maps impacted specifically skilled motor learning rather than overall movement (65,68). Building from these studies, it has also been demonstrated that the motor map ablation/reorganization that occurs with injury can be restored with motor rehabilitation, such as skilled forelimb training in animals, or bimanual therapy/CIMT in humans (52,69–73). Notably, a prevailing finding amongst both animal and human studies is that skilled and repetitive training is specifically required to induce lasting changes in map size, both in healthy and injured subjects (52,62,67,69,74).

Beyond areal expansion/retraction, mapping techniques can also yield other measures. For example, one useful measure is the amplitude of the induced movement, commonly quantified as the peak to peak sum of a movement trace. Typically, in healthy brains, contralateral movement sites have large amplitudes, whereas ipsilateral sites (if any) display smaller peaks (23,63,75,76). Another related measure is movement latencies, which can give insight to conduction of descending pathways. Depending on the latencies, direct vs. indirect pathways or monosynaptic vs. polysynaptic pathways can be derived. For example, in healthy brains, movement sites in the hemisphere contralateral to the recorded limb will have shorter latencies due to the faster-

conducting contralateral CST pathways, whereas ipsilateral movement sites will have longer latencies (40).

1.3.3. Motor map reorganization after developmental injury

Unilateral injury during the perinatal period can disrupt map development, resulting in similar consequences to developmental CST damage. Not only is the injured hemisphere explicitly ablated, but there are also changes in map organization in the uninjured hemisphere. Notably, rodents with perinatal injuries displayed significant increases in both excitability and map size of the injured forelimb representation in the uninjured hemisphere, whereas the adult injury and naïve control counterparts did not (46,75,77,78). This has since been corroborated in human patients as well (52,79–81). To support these physiological findings, direct anatomical findings from other rodent studies have described aberrant ipsilateral projections from the uninjured hemisphere onto the paretic side (45,47,82,83). Overall, evidence suggests that much like the CST, disruption of motor map development causes robust bilateral reorganization, an effect which is not as common after adult injury. However, similar with CST rewiring studies, clinical reports of correlations between map reorganization and behavioural outcomes are mixed (52,79,81). Animal studies may help clarify this relationship by limiting variability within the sample population. Unfortunately, there have yet to be any such preclinical perinatal injury studies to explicitly correlate motor map changes and functional outcome. As such, it is still unclear whether these map changes are beneficial or maladaptive.

Beyond describing motor map reorganization after the perinatal injury, it would also be valuable to observe whether these changes can be further modulated with motor training. After all, one key finding from the adult motor mapping field is that skilled forelimb training can partially "restore" maps after injury (68,84). Surprisingly, there are only a few perinatal injury studies which have recapitulated these results. These few studies have shown a variety of interventions to enhance map size after perinatal cortical ablation, such as environmental enrichment, skilled reaching, and CIMT (28,71). Altogether, this evidence suggests promise for the capacity to further modulate maps after developmental injury. Hearkening back to adult injury studies, skilled motor training could be a particularly effective approach. However, it would be beneficial to further increase the evidence base, especially with other forms of injury such as stroke. If there is indeed strong evidence for motor-training induced map reorganization after perinatal injury specifically,

then this could provide some level of biological basis for the bimanual/motor therapies currently being used in these young patients at the clinical level.

From these results, it is clear that there are still significant gaps in both clinical and preclinical literature on the exact role of maps and motor training on recovery after perinatal injury. While both clinical and preclinical approaches are necessary to fill this knowledge gap, animal models, and specifically rodent models, are an important first step as they can control for salient clinical challenges such as patient heterogeneity and limited spatial resolution of human motor mapping techniques. In the next section, I will explore the existing rodent models of perinatal injury, and how they can be used to address questions such as CST rewiring and motor map reorganization.

1.4. Rodent models of developmental injury

1.4.1. Comparison between rodents and human motor development

One important factor when considering the use of rodent models is the translational capacity between the human and murine nervous systems. In the case of the motor system, there are a few key similarities in favour of using rodents as a model organism. First, there are similarities at the anatomical level of the motor system. At maturity, descending corticospinal tracts in the rodent are also primarily decussated/contralateral, with an additional presence of a small subset of uncrossed/ipsilateral fibres (85–88). More importantly to perinatal stroke, there are also similarities in the development of the motor system. Specifically, CST organization is initially bilateral in the rodent, followed by refinement to a contralateral pattern by maturity. By postnatal day (P) 14, pruning of ipsilateral fibres begins in rodents (equivalent to ~1 month in humans), and reaches a peak by P21 (~2 yrs in humans) (85,89). In this manner, most rodent models can accessibly mimic the disruptive effect of perinatal injuries on motor system development by targeting the injuries to the P7-P14 timepoint.

Second, there are also parallels in cortical motor representation organization. As in humans, motor control can be mapped somatotopically onto the rodent cortex, with distinct, identifiable regions for body parts ranging from limbs to whiskers (90,91). More relevant to perinatal stroke is the presence of clear forelimb representations in the rodent. Not only do the forelimbs present an accessible parallel to the upper limb impairments common after perinatal neurological injury, but the representations can also be manipulated with forelimb training/motor experience. Within these

forelimb motor maps, there are further similarities in organization. Specifically, homologous regions to the human M1 and premotor areas can be found in the caudal forelimb area (CFA) and rostral forelimb area (RFA) of the rodent, respectively. Like the human/primate M1, the CFA acts as the origin point for the majority of descending CST projections towards areas such as the ventromedial thalamic nucleus and the spinal cord (88,92,93). In contrast, the RFA has fewer descending projections, a larger role in movement preparation, and modulatory effects on the CFA, much like the premotor areas in primates and humans (88,94). These similarities provide a particularly accessible avenue for studying the effects of injury or motor experience on map plasticity in the rodent.

Of course, it is also important to recognize the limitations of this model. Perhaps the most notable difference is the lack of direct corticomotoneuronal connections in rodents (95). In humans and certain primate species, these direct connections from the cortex to spinal cord motoneurons are key for providing fractionated and refined control of distal musculature (42). In rodents, motor control from the cortex is instead oligosynaptic, where descending projections from the brain synapse onto segmental interneurons and reticulospinal neurons before innervating motoneurons (95,96). Indeed, contrary to human motor system anatomy, the role of RtST is more emphasized in rodents (95,97). Particularly in mice, it has been suggested that reticulospinal circuits are the primary mediator of forelimb motor control, with the CST taking on a secondary role (97). Thus, not only is the anatomical basis of skilled distal limb movement different, but skilled distal/digit use itself is more limited compared to humans and primates (98). Another important disparity lies in the organization of cortical premotor centres; humans (and non-human primates) have six areas compared to the one in rodents (99). Although the majority of descending projections are still mediated by M1 in both species, the increased number of premotor areas in primates and humans increases the complexity of inter- and intrahemispheric interactions compared to rodents (94,100). This is particularly important when considering the potential for compensation or diaschisis of remaining motor areas after focal lesions. Despite these differences, there is still merit in using rodent models for studying perinatal stroke recovery. Behaviourally, while dextrous forelimb manipulations may be more limited in comparison to humans and primates, it is still present and amenable to qualitative movement analysis (101,102). More importantly, it is arguable that questions such as CST rewiring, hemispheric interactions, and cortical reorganization after

development injury should first be fully probed in "lower-order" model organisms before proceeding with expensive and resource-intensive primate/human experiments.

1.4.2. Aspiration models

Considering these strengths and shortcomings, rodent models still present as an accessible and worthwhile animal model for studying human neurophysiology and pathophysiology. From the perspective of developmental injury, this is certainly true. There is a rich history of rodent models of perinatal brain injury, many of which have shown the limitations of the Kennard principle in addition to the behavioural, physiological, and anatomical consequences of developmental injury (as detailed in section 1.2 and 1.3). However, the vast majority of these findings were discovered in the aspiration model, whereby cortical injury was induced through opening the skull and surgically removing tissue using a suctioning pipette. While these findings provide important context and background evidence for how recovery proceeds after perinatal brain injury, the aspiration technique is not a fully representative model of clinical ischemia injuries. After all, surgical procedures which open the skull to acutely remove large amounts of brain tissue are typically used as an intervention for intractable epilepsy (103), and never as a result of ischemia itself. Instead, the lesions that result from stroke are due to a complex interaction of neurotoxicity, cell death cascades, reperfusion, etc. As such, it would be beneficial to reproduce/repeat these experiments with less invasive means of inducing cortical injury, such as using a stroke model. In this way, with increased accessibility to creating a perinatal brain injury, the evidence base for rewiring during development can also be correspondingly expanded.

1.4.3. Hypoxia-Ischemia Models

Where it concerns perinatal ischemia specifically, one of the most common rodent models of developmental stroke is the hypoxia-ischemia (H-I) model, which combines carotid artery occlusion with systemic hypoxia (104–106). However, similar to how clinical instances of perinatal stroke differ from adult stroke in a few key areas, there are also distinct differences between ischemia-alone vs. H-I injuries. One large difference first lies in the higher incidence and mortality rate in H-I compared to stroke. As mentioned earlier, perinatal stroke incidence is currently estimated to be 1 in 1600-2300, with 3% of this population resulting in death (16,107). In contrast, H-I is estimated to occur at 1.5 per 1000 (or 1 in ~667) (108), with 20-50% of individuals expiring during the newborn period (109). Additionally, H-I injuries tend to be diffuse due to the combination of damage from both ischemia and asphyxia, with majority of injuries

affecting not only multiple areas in the brain, but also systemically throughout the body (109–111). Perinatal stroke, on the other hand, is typically a neurological injury, and in the majority of cases limited to the MCA perfusion bed rather than globally throughout the brain (6,112). Finally, while both neurological injuries can present with encephalopathy or seizures, H-I patients tend to do so acutely, whereas it can takes 12-72 hrs for these symptoms to appear in perinatal stroke patients (6,109). Oftentimes stroke patients do not even present with overt symptoms within this period, instead only being identified once pathological handedness is shown or motor milestones are missed (up to 4-6 months) (6). Due to the immediate presentation of symptoms in H-I, this allows timely diagnosis and treatment, whereas stroke often does not have this quick clinical identification. Indeed, hypothermia remains the one clinically approved treatment for H-I (113), whereas perinatal stroke has no approved treatments (6). Consequently, while both forms of injury occur during the perinatal neonatal period, ultimately H-I and ischemia-alone are separate disorders.

1.4.4. Stroke Models

In the past 30 years, there have been several ventures into ischemia-only models during development. The first was an adaptation of the H-I model, where carotid artery ligation/transient filament occlusion (i.e. middle cerebral artery occlusion, MCAo) was used to induce stroke but in normoxic conditions (106,114). Similarly induced at P7, these models are advantageous because they closely mimic certain characteristics of clinical strokes, such as the presence of a penumbra of salvageable tissue and targeting of lesion to the MCA bed. Unfortunately, there are a few important considerations with this technique. First, MCAo injuries tend to be quite diffuse, with injuries beginning as subcortical lesions and only reaching the cortex with prolonged occlusion times (115). While this progression is useful for modelling subcortical strokes which occur clinically (16), cortical lesions may be more appropriate for the purpose of studying motor map reorganization. Currently, there is limited evidence for how subcortical lesions impact motor map reorganization (65,116). Thus, before understanding how subcortical and/or MCA bed damage at the perinatal timepoint affects motor maps, it may be more appropriate to first ensure reorganization with a focal injury to M1. Second, MCAo is a technically challenging method. Insertion of a filament into the carotid artery of neonatal rat pups requires a high level of mastery in surgical skills, otherwise resulting in high mortality rates (105,106,114). In mice, the technique is even more challenging, as mouse pups are one quarter the size of rat pups (117).

Considering these caveats, a more feasible technique in the mouse is photothrombosis (PT). First introduced in 1985, this technique produces emboli through laser irradiation. Specifically, the animal is first injected with a photoactive dye (Rose Bengal or erythrosine B), then the cortex is illuminated with a laser, whereupon the dye reacts and causes oxidative damage to vascular endothelium and platelet aggregation (118,119). Without both laser irradiation and dye injection, neither component on its own is sufficient to cause perfusion deficits (118). As such, both location and size of lesion can be easily modulated with changes in placement, power, and duration of laser irradiation. Another advantage is the relatively non-invasive nature of the procedure in young rodents (i.e. P0-P7), as surgeries require only incisions to the scalp, rather than the neck and arteries as in MCAo protocols. Like other techniques, PT does not mimic all aspects of the stroke, such as the peri-infarct penumbra of salvageable tissue or post-ischemia reperfusion (119). However, advantages such as low invasiveness and high degree of spatial control position it to be an especially accessible model for perinatal rodent ischemia. Despite these advantages, there has been relatively little use of PT in the developmental period since its introduction. The few studies that do exist have primarily focused on feasibility and lesion volume quantification, with little characterization of motor deficits, and no measurement of cortical reorganization (117,120,121).

Behavioural descriptions are important in order to align rodent impairments to clinical phenotypes. Brima et al. showed that perinatal PT caused long-term deficits on the rotarod, a test used primarily to test coordination and gross motor function (121). However, further quantification of behaviour across a battery of motor tests would give a more complete and accurate description of motor impairment. After all, deficits in patients are rarely relegated to only one task. Within this battery, a specific description of upper limb function would also be beneficial, as impairment of the upper limb is a significant and common disability amongst perinatal stroke patients (6,122). The forelimb also provides an accessible avenue for studying motor training-induced map plasticity, as this has yet to be robustly reproduced in perinatal stroke models, much less using the PT technique. A second importance of clear behavioural characterization is to provide a variety of motor functions with which map changes and individual hemispheric contributions can be correlated. With both mapping and robust behavioural characterization in a relatively non-invasive model of perinatal stroke, questions of how CST, motor map, and hemispheric plasticity correspond to functional outcome can be more accessibly probed.

1.5. Optogenetic mapping

With functional outcome better described with a battery of motor tests, the other half of this thesis concerns motor mapping. Thus far, all animal model mapping studies (adult vs. perinatal and healthy vs. injury) described in the previous sections have used ICMS. However, like any technique, ICMS presents with certain challenges. In this next section, I will cover in detail the most relevant shortcomings of the technique, and how optogenetics can circumvent them.

The first challenge posed by ICMS is the inability to target specific neuronal subtypes. Movement is not only evoked by direct electrical stimulation of neurons (which in itself will spread to a certain degree through the tissue), but also indirectly through activation of trans-synaptic connections (123). These effects may slightly confound the interpretation that movements are evoked from motor neurons only, as this trans-synaptic spread may result in stimulation of non-motor neurons as well. A second challenge is the time commitment of experiments. ICMS mapping is an arduous processes, often taking 2-5 hrs to map one animal (124). The primary issue is that animal anaesthetic is typically delivered in discrete boli and consciousness may fluctuate multiple times within such extended experimental sessions. Map reliability thus becomes an issue as movement amplitude and cortical responsiveness are heavily dependent on anaesthetic plane. A secondary issue from long experiments is the challenge of collecting multiple maps within the same session. This is compounded by the invasive nature of the technique as well, whereby a craniotomy is required for direct insertion of electrodes into cortical tissue. While there are reports of repeat sampling within the same animal (68,72,125–128), the health of the animals and integrity of cortical tissue is compromised by both the craniotomy and the permanent electrode tracks created in the cortical tissue (129,130). As such, ICMS studies are often constrained to between-subject experimental designs, presenting a limitation in understanding the longitudinal impact of injury and/or intervention on maps. In the context of perinatal stroke, this is especially salient, as there have been no reports of repeat mapping thus far. As such, having the ability to map at multiple timepoints within the same animal would add significantly to the field, particularly within the context of how motor training modulates motor map reorganization after perinatal stroke,.

With optogenetics, these caveats can be overcome. First, light-sensitive channels are only expressed in specific cell types, preventing non-discriminate stimulation of other cell types. Furthermore, bihemispheric mapping sessions can be completed within 10 minutes (129,130), contrasting the hours-long experiments in ICMS. Finally, optogenetic mapping is non-invasive,

and thus lends itself well to repeat sampling within the same animal over time. Particularly with the introduction of intact-skull cortical windows, motor maps can be collected over the course of several months without loss in map fidelity (131). Beyond these advantages, optogenetic mapping can also still quantify standard ICMS mapping outcomes, such as map size and movement latency, while also having the added measure of movement amplitude. Surprisingly, while the optogenetic technique has been used in a few adult studies, it has yet to be used in developmental models. Thus, with this novel tool at our disposal, questions of CST rewiring and motor map plasticity after perinatal stroke and motor experience can be directly targeted, and eventually related back to functional outcome.

1.6. Conclusion

In light of this background, there remain a few gaps in the literature which my thesis will be addressing. First, there is not only a need for a focal, perinatal stroke rodent model, but also a robust characterization of motor impairments within this model. This not only allows for alignment with clinical motor deficits, but also provides measures of functional outcome with which to correlate cortical reorganization. Currently, there is a lack of both these elements, as existing models either reproduce other forms of developmental injury (ex. H-I, surgical ablation) or have sparse characterization of behavioural deficits.

Second, motor map and corresponding CST projection changes have yet to be characterized in a rodent model of perinatal stroke. In adult stroke studies, there has been extensive work conducted in animals using ICMS showing varying detrimental effects of stroke on map size, output, and latencies. In developmental models, ICMS work also exists, although much less in-depth than in adult stroke. To date, all existing mapping studies in developmental models have been conducted after aspiration lesions, with none in perinatal stroke itself. As such, there has yet to be a perinatal stroke model which combines both behavioural outcomes with cortical motor map changes. In my thesis, I will be characterizing both, as well as probing the relationship between the two in order to elucidate the various roles of the injured and uninjured hemispheres in plasticity after perinatal stroke.

Third, the effect of motor experience on map plasticity after perinatal stroke has yet to be explored in depth. As mentioned above, skilled and repetitive training has been shown to modulate map outcomes in adult animals. These findings have been repeated in perinatal injury models such

as aspiration or pyramidotomy; however, there have been none in stroke models. Additionally, due to the invasive nature of the ICMS technique, these aforementioned perinatal injury studies have been unable to observe the direct effects of training at multiple timepoints within the same animal. By using optogenetic mapping techniques, my thesis will directly compare maps before and after a skilled forelimb training paradigm after perinatal stroke with a within-subject experimental design.

Thus, using a combination of behavioural analyses and optogenetics, my thesis will characterize how focal stroke at the perinatal timepoint affects motor function, cortical motor representations, and motor experience-induced plasticity in maps.

2 – Materials & Methods

2.1. Animals

2.1.1. General procedures and timeline

Animal care and research procedures were carried out in accordance with the Guidelines of the Canadian Council on Animal Care. Experimental protocols were approved by the Ethics Committee for Animal Research at the University of Ottawa. Male and female Thy1-ChR2-YFP breeders and their litters (B6.Cg-Tg(Thy1-COP4/EYFP)18Gfng/J; 007612, Jackson Laboratories, Jackson, NY, USA) were housed in a 12-hour light/12-hour dark cycle and provided food and water ad libitum, excepting single pellet training periods. During these periods, food was provided by the experimenter in order to maintain body weight at 80% baseline.

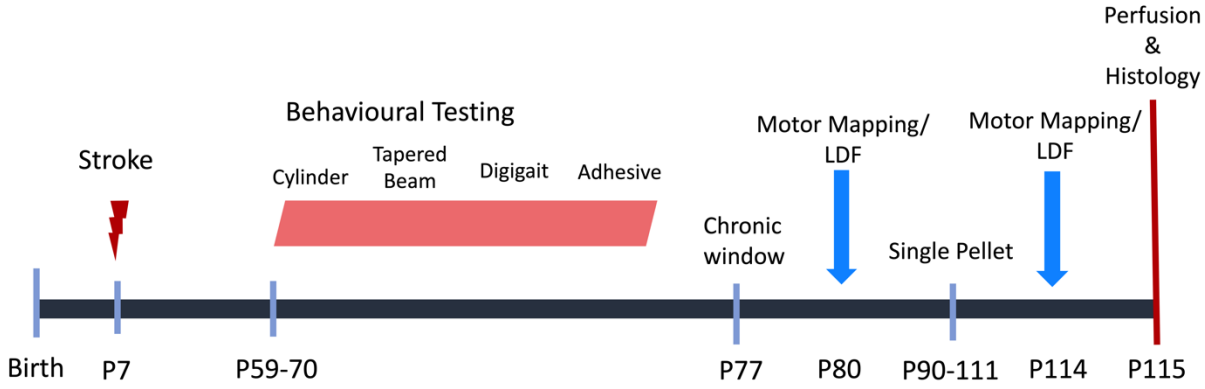

Figure 1. Experimental timeline. Full timeline of experiments for each animal.

Following induction of stroke at P7 (see section 2.2.1), animals underwent a series of consecutive behavioural and cortical imaging procedures. Sensorimotor behavioural tests were conducted over the course of two weeks, starting at age P59 (see section 2.3 for detailed descriptions of tests). The choice of this timepoint was to ensure that any behavioural deficits observed would correspond to persistent impairments, similar to those seen at the clinical level (5). Following behavioural characterization, chronic windows were surgically implanted onto the skulls of each animal at P77. These windows allowed non-invasive and longitudinal stimulation/imaging of the cortex. These experiments were conducted to measure sensory stimulation-evoked changes in cerebral blood flow (CBF) and forelimb motor representations, respectively. Both LDF and motor mapping were carried out at two timepoints: pre- (P80) and post-single pellet training (P114). The single pellet training task was used to not only characterize

skilled forelimb reaching performance after stroke (for more detail, refer to section 2.3.5), but to also provide an avenue of forelimb training for motor experience-mediated motor map reorganization. Due to the potential of the single pellet task to modulate cortical organization, the task was conducted separately from the other behavioural tests. In this way, the first motor map/LDF session measured cortical reorganization after perinatal stroke only, whereas the second session probed further changes following skilled forelimb training. At the end of the experimental protocol, animals were perfused, and brains were sectioned for lesion quantification.

2.1.2. Excluded animals

A total of 59 mice were used in the experiments for this thesis. From this initial total, mice were excluded at various points of the experimental timeline due to experimental complications. For behavioural test (i.e. cylinder, adhesive, tapered beam, and Digigait) outcome measures and corresponding motor map correlations, we excluded 20 animals from analysis due to: death within 48 hrs of perinatal PT surgery (n=18), incorrect genotype (n=1), and unexpected death prior to motor mapping (n=1). This resulted in a final sample size of sham: n=19, stroke: n=20. For single pellet outcome measures, correlations, and single pellet-induced motor map analyses, a further 11 animals were excluded from analysis due to failure to acquire skilled reaching behaviours (n=8), and unexpected death prior to the last timepoint of mapping (n=3). This resulted in a sample size of sham: n=15, stroke: n=13. Lesion volumes were quantified and presented for all animals which underwent pre-single pellet motor mapping (n=39), with 1 animal excluded due to seizure-induced tissue damage (n=1). This resulted in a sample size of sham: n=18, stroke: n=20.

Table 1. Summary of experimental numbers

Experiment	Sample Sizes (# excluded)	Reason for exclusion
P7 PT stroke	59 (18)	Unexpected death within 48 hrs post-stroke (n=18)
Behavioural Tests	41 (0)	-
Motor Maps/LDF	39 (2)	Incorrect genotype (n=1), unexpected death post-behaviour (n=1)
Final Sample Size: Sham: n=19, Stroke: n=20		
Single Pellet Task	28 (11)	Unexpected death post-mapping (n=3), absence of reaching behaviours (n=8)
Post-Reaching Maps/LDF	28 (0)	-
Final Sample Size:		

Sham: n=15, Stroke: n=13		
Lesion Volume	38 (1)	Seizure-induced tissue damage (n=1)
Final Sample Size: Sham: n=18, Stroke: n=20		

2.2. Experimental surgery

Surgery was used for both induction of stroke, as well as chronic window implantation. During such procedures, mice were anaesthetized by inhalation of isofluorane (induction 4%; maintained at 2%) with oxygen flowing at 0.5 L/min and secured in a stereotaxic frame. After surgery, mice were placed within an incubator until they were conscious and mobile, then returned to the home cage.

2.2.1. Photothrombosis (PT) stroke

On day P7 (day of birth was P1), mouse pups were separated from the dam to undergo photothrombotic surgery (117,118). Pups were first placed in a container that was maintained at 37˚C in an incubator and removed one at a time to be anaesthetized with isofluorane. Animals were placed on a warming blanket (37˚C), and a small incision was made mid-sagitally to expose the primary motor cortex (M1) area of the skull underneath. Bregma was marked, and a green laser (532 nm, 1.5 mm diameter, 20 mW power) was positioned 5 cm above the skull and aligned to M1 using stereotaxic coordinates relative to bregma (Fig. 2A; coordinates: right hemisphere, AP: 1.5 mm, ML: 1.0 mm). Craniotomy prior to irradiation was not necessary as the P7 skull is translucent to light. The photoactive dye rose Bengal was used to induce stroke and was prepared the day of surgery by dissolving dye powder (#330000, Sigma Aldrich) into phosphate buffered saline (PBS) at a concentration of 0.01g/mL. Prior to irradiation, mice were injected with the dye (0.1 mL/10g body weight i.p. injection), and allowed to circulate throughout the body for 2 min. The laser was then turned on and irradiated for 6 min. Sham animals underwent identical surgical procedures, with laser irradiation preceding rose Bengal injection. After photothrombosis, the incision was sutured with 3-0 nylon thread.

2.2.2. Chronic window implantation

Following behavioural testing (P77), chronic transcranial windows were prepared as previously described (131). Briefly, the animal was first anaesthetized, injected with meloxicam (5 mg/kg s.c.), and surgically prepped (i.e. shaved scalp, eyes treated with lubricant, secured in stereotaxic frame). Once secured, an incision along the midline of the scalp was made, and the

skin was cut away to expose the surface of the skull from lambda to approximately 3 mm anterior to bregma. The skull was then cleaned of hair and fascia, then affixed with a circular glass coverslip (Fig. 2B; Ted Parker, Inc, Redding, CA, USA; Product # 260368, 10 mm diameter) using the clear-drying C&B-Metabond dental cement (Parkell, Edgewood, NY, USA; Product: C&B Metabond, SKU: S380). A small screw (McMaster-Carr, Los Angeles, CA, USA; Product #94355A216) for head-fixation during cortical stimulation/imaging procedures was then placed on the surface of the skull with additional dental cement (Fig. 2B, AP: -5.0 mm, ML: 0.0 mm). Once the dental cement solidified (~20 min), animals were removed from the surgical set-up and allowed to recover in a warmed cage until fully mobile.

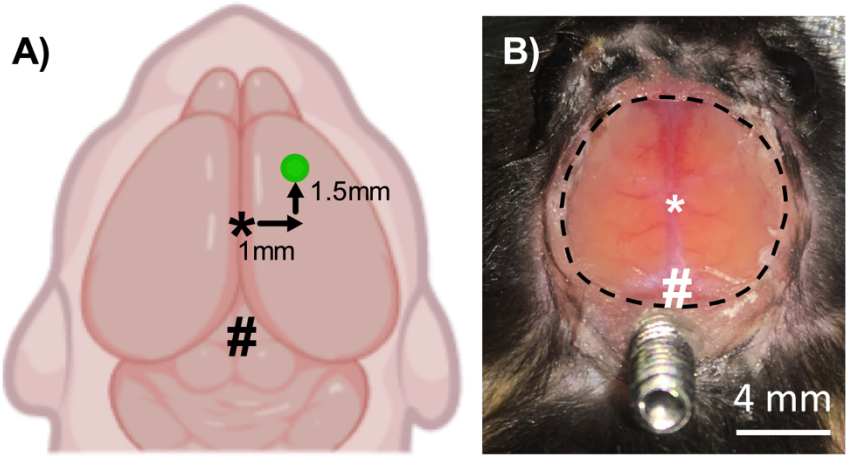

Figure 2. Surgical coordinates and set up. A) At the P7 timepoint, a green laser (green circle, 532 nm) was positioned to the stereotaxic coordinates of AP: 1.5 mm, ML: 1 mm (relative to bregma, *). Following 2 min of rose Bengal dye circulation, the laser was turned on and irradiated for 6 min. **B)** Representative image of sham animal with glass coverslip (black dashed circle) following chronic window implantation. A set screw was placed behind lambda (#).

2.3. Behavioural tests

Once animals reached P59, they were trained on a battery of sensorimotor/motor behavioural tests. Tests were conducted in the following order: cylinder test (P59), tapered beam (P59-63), Digigait (P60), adhesive tape test (P66-70). Both the cylinder and Digigait tests were measured on one day, whereas the tapered beam and adhesive tape tests were conducted over 5 days.

2.3.1. Cylinder test

The cylinder test quantifies spontaneous, voluntary, forelimb usage and asymmetry (132). Mice were placed in a clear, hollow, plexiglass cylinder (10 cm diameter x 15 cm height) and

allowed to explore the cylinder until 25 rears occurred. The placement of paws during rearing and landing was filmed from below using a Raspberry Pi camera (Raspberry Pi Foundation, UK) and analyzed post-hoc. Contralesional paw preference during both rearing and landing behaviours was quantified with the following formula: [(contralesional paw contacts) / (contralesional paw contacts + ipsilesional paw contacts)] x 100%. This resulted in a number where >50% indicates a preference for the contralesional paw, whereas <50% indicates a preference for the ipsilesional paw.

2.3.2. Tapered beam test

The tapered beam tests chronic hindlimb function through analysis of foot faults (slips) during the traversal of a specially constructed beam (133). The length of the beam was 1 metre long, and tapered from a width of 3.5 cm to 0.5 cm. Animals were trained to walk along the top of the beam from the wide to narrow end; however, with motor impairments or tapering, they could make use of the depressed ledges (1 cm below the beam, 1 cm in width) on either side of the beam. Whenever an animal stepped off the main level onto these depressed ledges, a foot fault was recorded. The beam was marked in 1 cm intervals so that the distance of the first foot fault could also be recorded. The animals were trained/tested on the task over a period of five days, where each day consisted of three trials. An individual trial was defined as a traversal across the full length of the beam. On the first day of training, animals were habituated by allowing free exploration across the length of the beam without prompting from the experimenter. Over the next two days, animals were trained to walk the full length of the beam without stopping, falling, or turning around. Following this training period, beam performance was filmed and analyzed for number of foot faults and distance to first foot fault on days 4 and 5. Data was averaged across all six trials from both training days (days 4 and 5).

2.3.3. Digigait

The Digigait^{TM} treadmill (Mouse Specifics, Inc, Quincy, MA, USA) is an automated test for gait parameters during locomotion (134). Animals ran on the treadmill at a speed of 20 cm/s, 8˚ incline until a minimum of 3 seconds of steady and consistent gait (visual observation) was obtained. Videos were then analyzed with Digigait^{TM} software, which automatically identifies paw footprints. Manual alterations in the contrast of the images were made in the program, if necessary, to properly distinguish the footprints from background and other body parts (such as the tail). By analyzing the kinematics of the footprints frame-by-frame, the software automatically calculates

values for a total of 43 gait parameters. However, not all 43 gait parameters were deemed to be clinically relevant to perinatal stroke. Therefore, we chose to analyze a subset of the measures that have been previously shown to be affected after adult and perinatal stroke. Specifically, we analyzed the following measures: 1) swing time (i.e. the amount of time the limb is in the aerial phase of the stride cycle) and %swing/stride (i.e. the proportion of the stride cycle with which the limb is in the swing phase) (135), 2) Propel time (i.e. the amount of time the limb is propelling into the next step) and %propel/stride (136), 3) Stance time (i.e. the amount of time the limb is spent standing), and associated measures of %stance/stride and stance/swing ratio (i.e. the ratio between the stance and swing durations) (135), 4) Stride duration, or the amount of time required for a limb to undergo a full stride cycle (135), 5) Stride length (the equivalent of a step length in humans) (137), 6) stride frequency (or cadence) (135,137), 7) paw angle (otherwise known as the degree of external rotation of the paw during a step) and associated measure of paw angle variability (138,139).

2.3.4. Adhesive removal test

The adhesive removal test measures both sensory and motor asymmetry by quantifying the latency for the animal to sense/contact and remove an adhesive tape from its paws (133). Briefly, a small piece of adhesive tape (0.5 cm^2) was adhered on glabrous skin in the centre of both forepaws. Mice were then placed in a plexiglass cylinder (10 cm diameter x 15 cm height) and filmed until both pieces of tape were removed from the forepaws, or until the 2-min time limit was reached. The latency to both contact the forepaw with the mouth and remove the piece of tape from the paw was determined from video recordings. If the adhesive tape was not contacted or removed within the allotted time limit, then the measure was recorded as 120 seconds. All measures were averaged across five days of testing.

2.3.5. Single pellet reaching task

The single pellet reaching task is a quantitative measure of skilled forelimb functioning (102). Animals are trained to reach and grasp for a food pellet through a constrained reaching slot, in order to test skillful maneuvering of the forelimb and paw. In this experiment, mice underwent daily training on the task for 3 weeks (6 days/week) at P90, where training sessions involved 10-min trials or 30 reaches, whichever was achieved first. There was a 10-day period (P81-P90) between the first motor mapping/LDF session and training. The first three days were to prevent any potential confounding health complications from the ketamine/xylazine anaesthesia (P81-83),

which were then followed by 7 days of food deprivation (P84-P90). During the week of food deprivation, rodent chow and millet seeds/pellets were provided daily by the experimenter to achieve 80% baseline weight (as quantified as the average weight of the two days preceding the food deprivation period). Body weight was then maintained at this level for the duration of the training.

During the first two days of training, animals were placed in plexiglass testing boxes (19.5 cm length x 8 cm width x 20 cm height) and given free access to pellets on the chamber floor and the presentation shelf (1 cm length x 8 cm width) outside the box. In order to retrieve the pellets on the presentation shelf, animals would be required to lick or reach through a narrow, 1 cm-wide reaching slot in the middle of the front wall. Within these first two days, pellets were initially placed within licking distance of this slot. In subsequent days, pellets were gradually removed from the floor and placed farther away on the shelf until use of the contralesional limb was required to successfully retrieve the pellets (achieved by placing pellets in a shallow divot 1 cm from the reaching slot). Once animals were consistently reaching with the contralesional limb (5-7 days), they were trained to return to the back of the testing box between pellet presentations. This "walk-back" behaviour was reinforced by only presenting pellets on the shelf after the animals had first walked to the back of the chamber. Generally, walk-backs occurred spontaneously. However, in cases where animals did not consistently display this behaviour on their own, a millet seed would be placed at the back of the testing box to encourage the walk-backs. One reaching trial was then defined as a pellet contact followed by a walk-back. On the last three days of training, trials were filmed using a Raspberry Pi camera, then analyzed post-hoc.

Videos were analyzed first for individual reaches, then tallied for overall success rate and number of reaching attempts within successful reaches. Individual reaching trials were coded as: 1) success: mouse successfully grasped the pellet and brought it through the slot to its mouth, 2) knock: mouse contacted the pellet but knocked it from the divot, or 3) drop: mouse successfully grasped the pellet, but dropped it before reaching the mouth. Reaching attempts were quantified as the number of arm extensions through the reaching slot before contacting the pellet (through a success, knock, or drop). For example, if the animal made two arm extensions without contacting the pellet and only contacted the pellet on the third arm extension, the reaching trial would be coded as a success/knock/drop with three attempts. While reaching attempts were recorded across all reaching trials (i.e. success, knock, or drop), only the average number of attempts for the

successful reaches are reported, in order to clarify the quality of movement within this specific movement category. Reach attempts were calculated over the last three days of training as: (Σ number of attempts per successful reach) / (number of successful reaches). All animals that engaged with the task were able to achieve at least one successful reach by the last three days.

Overall reaching success was calculated in a similar manner. Over the last three days of testing, success rate was calculated as: (number of successful reaches / total number of reaching trials) x 100%.

2.4. Cortical imaging and stimulation

Mice underwent cortical imaging (LDF) and stimulation (motor mapping) experiments at two timepoints: pre-single pellet (P80) and post-single pellet (P114). There was a period of three days between chronic window implantation and the first session to prevent confounding effects of anesthesia and surgery. There were also three days between the single pellet training period and the second motor mapping/LDF session where animals were taken off food deprivation and body weight was allowed to return back to baseline. Mice were anaesthetized throughout both LDF and motor mapping experiments, which were conducted consecutively on the same day for each mouse. First, animals were briefly sedated with isofluorane (4%, 30s exposure), then injected subcutaneously with a ketamine (75 mg/kg) and xylazine (4 mg/kg) cocktail. Top-ups of the cocktail (ketamine: 35 mg/kg, xylazine: 2 mg/kg, s.c.) were provided if whisking movements were observed, which typically occurred 60 min after the initial injection. Sessions lasted between 50-120 min. Following optogenetic mapping, animals were then transferred to a warmed cage (30˚C) until they were conscious and mobile before being returned back to their homecage.

2.4.1. Laser Doppler Flowmetry

LDF was used to measure CBF during resting conditions and sensory stimulation. The experiments were conducted with a free-standing infrared laser and photodetector (MoorLDI2-IR; Moor Instruments, Axminster, Devon, UK). Mice were first placed on a heating pad and head-fixed under the system. Thereafter, an infrared laser (785 nm laser, 1.2 mm diameter, 2.5 mW power) was used to collect single-point readings of CBF. These measurements were used to quantify sensory stimulation-evoked changes in CBF in the primary sensory cortex (S1, as determined through stereotaxic coordinates: AP: 0 mm, ML: 2.0 mm; Fig. 3). Sensory stimulation was delivered through a vibrational stimulus to the paw. An external stimulator constructed from

a toothpick attached to an eccentric rotating mass vibration motor (Parallax, Rocklin, CA; Product ID: 28822) was attached to one forepaw at a time, taking care not to contact whiskers or other body parts (Fig. 3). The stimulator then vibrated the forepaw at 200Hz over 20 cycles, where each cycle consisted of 15s baseline (no stimulation), 3s stimulus, and 15s post-stimulus baseline. CBF was collected and averaged over all 20 cycles of stimulation, normalized to the pre-stimulus baseline, then quantified as the area under the curve during the 3 s of stimulation.

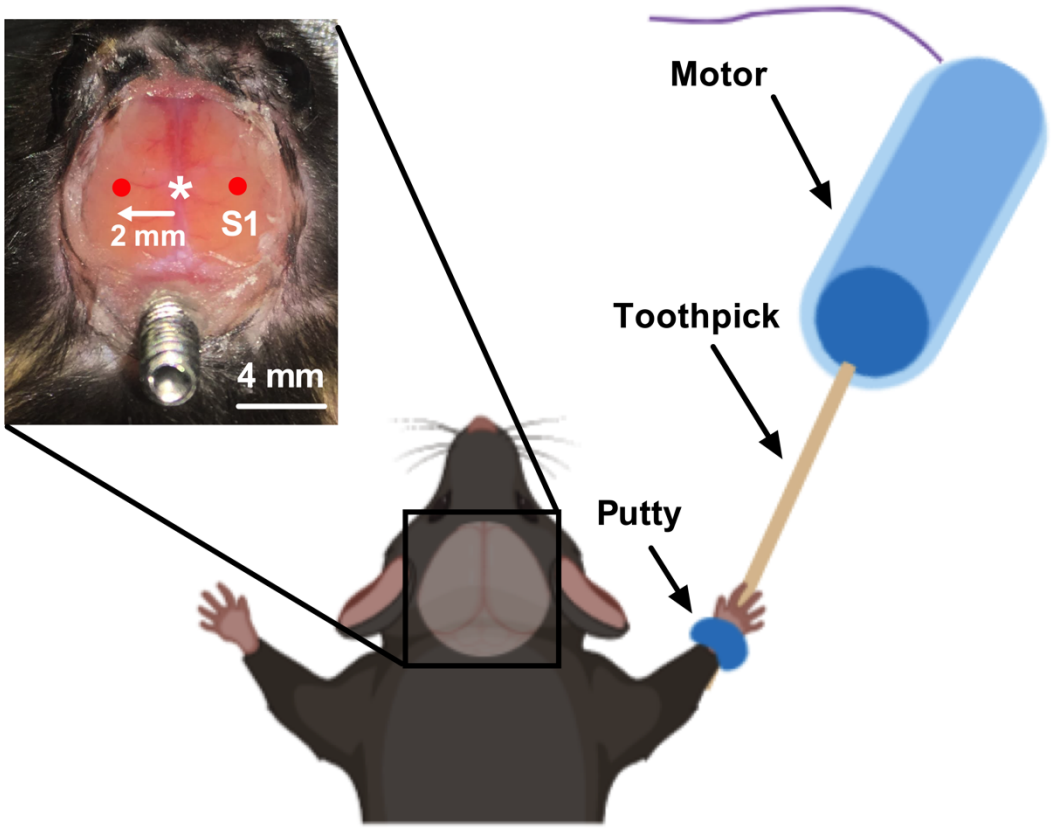

Figure 3. Laser doppler flowmetry set-up. Mice were set up on a heating pad and head-fixed underneath the LDF set up (not pictured). Vibrational sensory stimulation was delivered to the forepaw with a motor, which was attached to one paw at a time using putty and a toothpick. The inset image shows a representative chronic window of a sham animal, where the laser was directed to the S1 (red circle, as determined through stereotaxic coordinates, AP: 0 mm, ML: ± 2.0 mm) in the hemisphere contralateral to the stimulated paw.

2.4.2. Optogenetic motor mapping

Following CBF recordings from both forepaws and hemispheres, mice were transferred to the optogenetic rig (LabeoTech, Montreal, Quebec, Canada) to undergo optogenetic motor

mapping. Motor mapping was conducted in order to probe cortical motor representations of the forelimb, both after perinatal stroke and after skilled forelimb training.

Using the set screw, animals were head-fixed and positioned such that the forepaws were relaxed and hanging freely from the adjustable stage (Fig. 4A). Body temperature was maintained at 37˚C with a rectal probe and homeothermic heating pad. The angle and elevation of the chronic window was then adjusted such that the plane of the window was parallel to the stage and focused to a depth just below the major blood vessels visible through the macroscope camera (Fig. 4B). Three-axis accelerometers were affixed onto both wrists using wristbands to measure evoked forelimb movement in the x, y, and z axes (Fig. 4A).

In order to deliver optogenetic stimulation, a custom-made program (LabeoTech) was used to set laser power, position, and duration (473 nm laser, 50 um diameter). To evaluate the minimum laser power required to evoke a paw movement (i.e. resting motor threshold, RMT), laser duration was set to 5 ms, and starting at 20% maximum power (where the maximum was 12.5 mW), the laser power was increased at 5-10% intervals until movement was visually observed in the real-time accelerometer outputs of the program. Once movement was observed (as determined by a movement trace with similar amplitude above baseline noise consistently being evoked from a stimulation point), laser power was subsequently decreased by 2% intervals until the movement disappeared, and the RMT was set at the last interval which evoked movement. Following determination of RMT, a matrix of stimulation points was selected over the cortical surface (Fig. 4B, 300 μm spacing between each point, extending from ~AP ±3.5 mm to ~ML ±4.5 mm) and stimulated in a random order at the predetermined laser power with an interstimulus interval of 0.5 s. Thus, for a typical map of 25 x 28 stimulation points, one map would take ~6 min to collect. Stimulation of the grid was conducted a minimum of two times in each mouse in order to collect maps at both 120% and 150% RMT (in that order). Maps were re-collected/repeated based on the following conditions: 1) there were no, or few (i.e. < 5), evoked movements at any stimulation point, 2) there was sparse and non-clustered evoked movement points, 3) animals changed anaesthesia plane partway throughout the session (as determined by spontaneous movements and/or whisking movements). Factors #1 and #2 were often indicative of breathing artefacts and/or insufficient laser power. Clustering of movement points (i.e. factor #2) was especially important, as mouse motor maps should have clear CFA/RFA areas (90). On average, maps in the absence of these factors were collected within the first trial (120% RMT: 1.1 ± 0.043 trials, 150% RMT: 1.5

± 0.11 trials). Re-thresholding of RMT was conducted after each map to control for changes in anesthetic plane.

Evoked accelerometer signals were digitized through the custom-made data collection program (LightTrack, Labeotech), summed across the three axes of collection to quantify total movement, then exported into MatLab 2017a for offline analysis. Only responses with amplitude (i.e. peak-to-peak sums of the evoked movement traces) that exceeded nine times the SD of the 100ms pre-stimulus period were included in the mapping data, to control for breathing and movement artefacts. By marking bregma before data collection, the corresponding coordinates of this point was automatically assigned as (0, 0) in the data collection program, and all stimulation points were appropriately transformed relative to bregma. Generated maps marked bregma, and thus individual hemispheres were differentiated based on this landmark. From these maps, analysis was conducted in excel for map size, output, and movement latency of each hemisphere. Map size was calculated as the absolute number of active pixels (i.e. stimulation points which evoked movement). To calculate map output, movement amplitude was first normalized to the peak amplitude across the whole map, in order to control for anaesthetic effects on movement excitability. Then, the relative amplitudes were summed across each hemisphere to give total hemispheric output. Finally, movement latencies from each hemisphere in each treatment group (stroke vs. sham) were pooled together and plotted as cumulative frequency distributions. Changes in map size and latency were calculated as subtractions of each respective measure between the post- and pre-single pellet timepoints and are expressed as absolute values (i.e. map size: # of pixels, latency: ms).

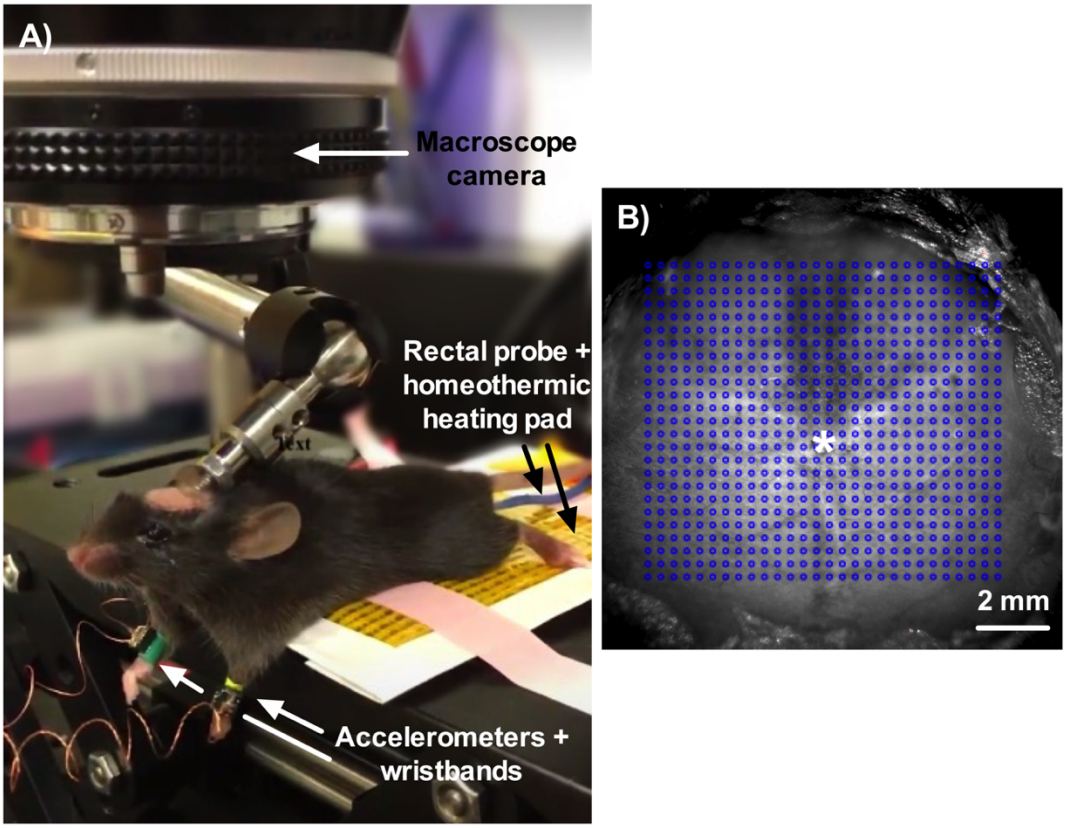

Figure 4. Optogenetic mapping set-up. A) Representative image of animal head-fixed and positioned in the optogenetic rig. The animal was anaesthetized, and body was positioned in a relaxed position such that the forelimbs were hanging freely off the stage. Body temperature was maintained with a rectal probe and homeothermic heating pad (pictured). Forelimb movements elicited by laser stimulation were measured with accelerometers affixed with small wristbands (pictured). **B)** Representative overhead image of a sham animal cortex taken with the macroscope camera. The stimulation grid extended from approximately AP: ±3.5 mm and ML: ±4.5 mm. With stimulation points evenly spaced at 300 μm (blue circles), this resulted in a grid of approximately 25 x 28 points. Bregma is denoted as (*).

2.5. Histology and infarct measurement

On the day following the last cortical imaging session, animals were deeply anaesthetized and trans-cardially perfused with PBS (7 mL/min for 6 min) and 10% neutral buffered formalin (7 mL/min for 10 min). Brains were removed and post-fixed at 4˚C for a minimum of 24 hrs in formalin, then cryoprotected in 30% sucrose in PBS until saturated. Saturation was determined through visual observation of buoyancy; once the brain was saturated, it would sink to the bottom of the solution. Subsequently, brains were frozen at -40˚C, then mounted and cryo-sectioned at 50 m in -22˚C. Every 2nd serial coronal section spanning from the base of the olfactory bulb (AP: +3.4 mm) to the back of the hippocampus (AP: -2.6 mm) was mounted on 1% gelatin-coated slides.

Sections were then dehydrated (consecutively immersing slides into 70, 95, and 100% ethanol), rehydrated (consecutively immersing slides into 100, 95, 70% ethanol, and dH_2O), and then stained with Cresyl violet for 10 min. Thereafter, slides were rinsed with MilliQ water, dehydrated, and placed in Citrisolv clearing agent prior to being coverslipped with DPX mounting media (Sigma-Aldrich).

Cresyl violet-stained sections were scanned and imaged on a scanner at a resolution of 1200 dpi (Canon 9000F MKII Flatbed Scanner). In each section, the area of intact tissue of both hemispheres was manually traced and measured using ImageJ. The total infarct volume was calculated as: (tissue area of contralesional hemisphere – tissue area of ipsilesional hemisphere) x (distance between sections) x (thickness of sections).

2.6. Statistical analysis

Behavioural, histological, and mapping data were expressed as mean ± standard error of the mean (SEM). All analyses were performed using SPSS (IBM Corp., Armonk, USA) and GraphPad Prism 6 (San Diego, CA, USA).

Unpaired t-tests for sham vs. stroke groups were used for the following analyses: 1) cylinder test – paw preference during landing and rearing, 2) adhesive removal test – contact and removal times for both limbs, and 3) tapered beam – distance to first foot fault. A mixed repeated-measures analysis of variance (RM ANOVA) was used for the remainder of the behavioural test measures (tapered beam and Digigait), motor map measures (size/output and pre- vs. post-single pellet analysis), and laser doppler flowmetry (area under the curves). If interactions between factors were significant, then post-hoc comparisons were carried out with Sidak's test. For all of the above RM ANOVA analyses, the between-subject factor was treatment (stroke vs. sham). The within-subject factors for Digigait, tapered beam (number of foot faults), map size/output, laser doppler area under the curves, and pre- vs. post-single pelleting reaching maps were: 1) contralesional/ipsilesional side and forelimb/hindlimb, 2) contralesional/ipsilesional side, 3&4) hemisphere (uninjured vs. injured), and 5) time (pre vs. post), respectively.

Movement latency distributions were analyzed using a Kruskal-Wallis one-way ANOVA, with a Dunn's post-hoc adjustment for multiple comparisons. Latencies were compared across both hemispheres (uninjured/ipsilateral vs. injured/contralateral), treatments (stroke vs. sham), and timepoints (pre- vs post-single pellet).

3 – Results

al3.1. Perinatal PT stroke results in large and variable lesion volumes

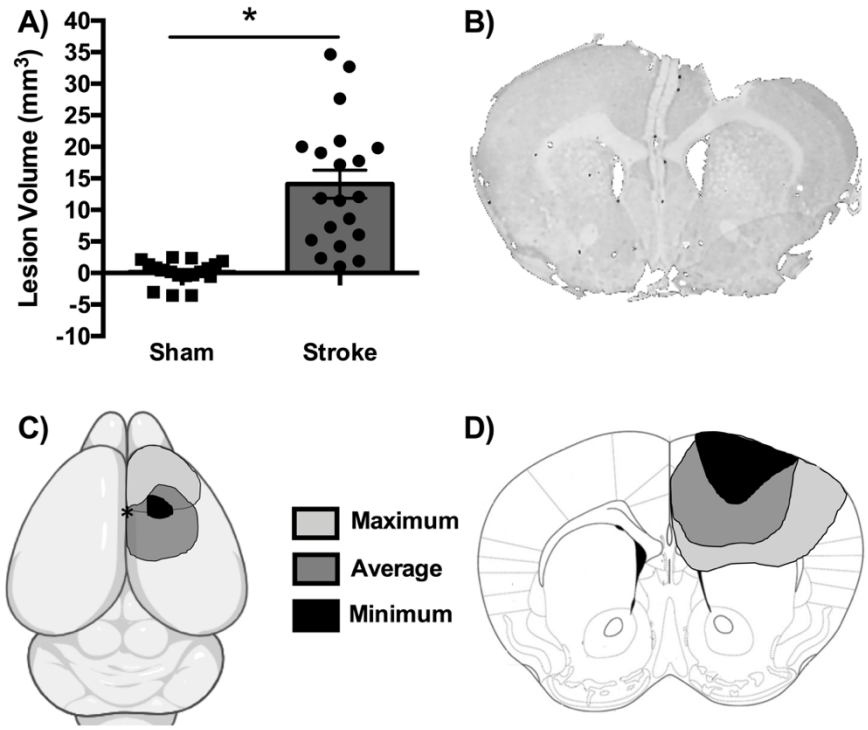

Figure 5. Stroke lesion volumes. A) Lesion volumes for both sham (n=18) and stroke groups (n=20). Data is presented as mean ± SEM**)**, with significance of $p < 0.05$ denoted by an asterisk (*). **B)** Representative coronal cross-section of a P7 stroke animal with the smallest lesion volume. Representative transverse **(C)** and coronal **(D)** images of the largest (light gray), mean (dark grey), and smallest (black) lesion volumes after P7 PT stroke. Bregma is denoted with an asterisk (*).

Our perinatal PT stroke protocol generated a wide range of lesion sizes (Fig. 5A). Mean lesion volumes in the sham and stroke animals were 0.13 ± 0.44 mm^3 and 14.1 ± 2.2 mm^3, respectively. An unpaired t-test between sham vs. stroke groups revealed a significant difference in lesion volume ($t(36) = 5.82$, $p < 0.0001$). Visual inspection of histological sections confirmed that even the smallest lesion volume extended through all cortical layers (Fig. 5B). However, in 15/20 stroke animals, damage also extended past the corpus callosum into the striatum (Fig. 5C-D). Sham animals attained non-zero stroke volumes due to infarcts being calculated based on differences in the volume of intact tissue between hemispheres. From these results, we see that although our protocol induced a spread of lesion volumes, our injuries were relatively severe (i.e. extending through all cortical layers).

3.2. Perinatal PT stroke induced longitudinal behavioural deficits

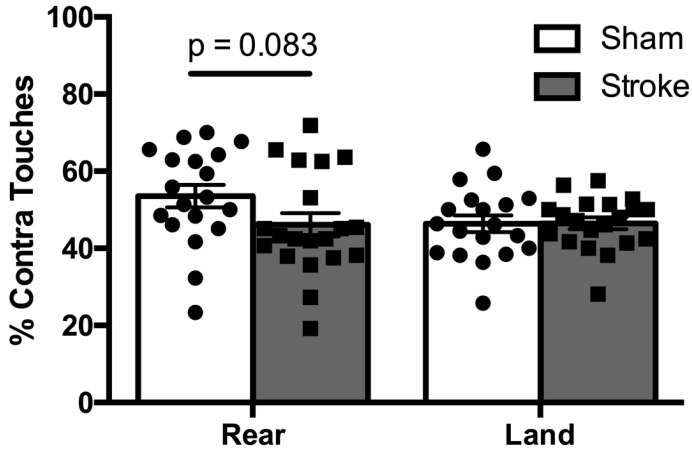

Figure 6. Cylinder outcome measures. Contralesional paw preference was calculated for paw use during exploratory rears (Rear) and landing after rears (Land). Data is presented as mean ± SEM for sham (white, n=19) and stroke (grey, n=20) animals.

Parameter	Contralesional Side		Ipsilesional Side		Significance
	Sham	Stroke	Sham	Stroke	(p-value)
Swing (s)	0.12±0.004	0.12±0.004	0.11±0.003	0.12±0.003	0.267
% Swing/Stride (ratio)	37.45±0.59	39.16±0.82	36.54±0.42*	39.15±0.58*	0.001
Propel (s)	0.13±0.003	0.12±0.002	0.12±0.002*	0.11±0.003*	0.007
% Propel/Stride (ratio)	41.56±0.94	40.24±0.81	40.58±0.71*	37.90±0.70*	0.007
Stance (s)	0.19±0.004	0.18±0.003	0.20±0.004*	0.18±0.003*	0.019
% Stance/Stride (ratio)	62.55±0.59	60.84±0.82	63.46±0.42*	60.85±0.58*	0.001
% Stance/Swing (ratio)	1.71±0.05	1.59±0.05	1.78±0.03*	1.57±0.04*	0.003
Stride (s)	0.31±0.01	0.30±0.01	0.31±0.01	0.30±0.01	0.416
Stride Length (cm)	6.21±0.15	6.05±0.12	6.20±0.13	6.05±0.12	0.261
Stride Frequency (stride/second)	3.33±0.09	3.40±0.07	3.34±0.08	3.39±0.07	0.318
	Forelimb		Hindlimb		
	Sham	Stroke	Sham	Stroke	
Paw Angle (˚)	12.22±0.78	12.46±0.83	10.93±0.83	11.38±1.00	0.227
Paw Angle Variability	5.68±0.26*	6.68±0.40*	6.92±0.39	7.52±0.41	0.038

Table 2. Digigait parameters. Digigait output from sham (n=19) and stroke (n=20) groups for contralesional/ipsilesional limbs and fore/hindlimbs. Data is presented as mean ± SEM, significance of $p < 0.05$ between sham vs. stroke groups is denoted with an asterisk (*).

At P59, mice underwent a battery of five behavioural tests, including cylinder, adhesive removal test, tapered beam, Digigait, and the single pellet task. Of these five sensorimotor tests,

the stroke group showed significant deficits in two (Digigait and single pellet). In the cylinder task, there was no significant decrease in preference for the contralesional limb in stroke animals during rearing behaviours (Fig. 6; sham: 53.5 ± 2.9%, stroke: 46.1 ± 3.0%; $t(37) = 1.78$, $p = 0.083$, unpaired t-test) or upon landing (Fig. 6; sham: 46.4 ± 2.2%, stroke: 46.5 ± 1.5%; $t(37) = 0.056$, $p = 0.96$, unpaired t-test). In the adhesive test, there was no significant difference in time to contact (Fig. S1A; sham: 22.7 ± 4.9 s, stroke: 27.1 ± 4.9 s; $t(37) = 0.63$, $p = 0.53$, unpaired t-test) or remove the adhesive tape on the contralesional paw (Fig. S1B; sham: 30.3 ± 5.3 s, stroke: 44.3 ± 5.9 s; $t(37) = 1.76$, $p = 0.088$, unpaired t-test). In the tapered beam test, sham and stroke groups were comparable in the distance to first foot fault (Fig. S1C; sham: 47.5 ± 4.47 cm, stroke: 41.8 ± 4.11 cm; $t(37) = 0.93$, $p = 0.36$, unpaired t-test). Additionally, there was no significant difference in the number of foot faults on either the contra- (sham: 9.12 ± 1.91, stroke: 7.89 ± 1.86) or ipsilesional side (sham: 5.47 ± 1.02, stroke: 6.93 ± 0.99) (Fig. S1D; $F(1,37) = 0.89$, $p = 0.352$, RM ANOVA). Finally, in the Digigait test, a RM ANOVA test was conducted across predetermined gait parameters (results and statistical values presented in Table 2). These results show that the stroke animals spent more time with the ipsilesional side of the body in the swing phase (i.e. in the air), less time propelling, and less time standing on the ipsilesional side. Stroke animals also showed a significantly increased paw angle variability in the forelimbs compared to the shams ($p = 0.038$, the variability in degree of external rotation of the paw to the midline axis of the body).

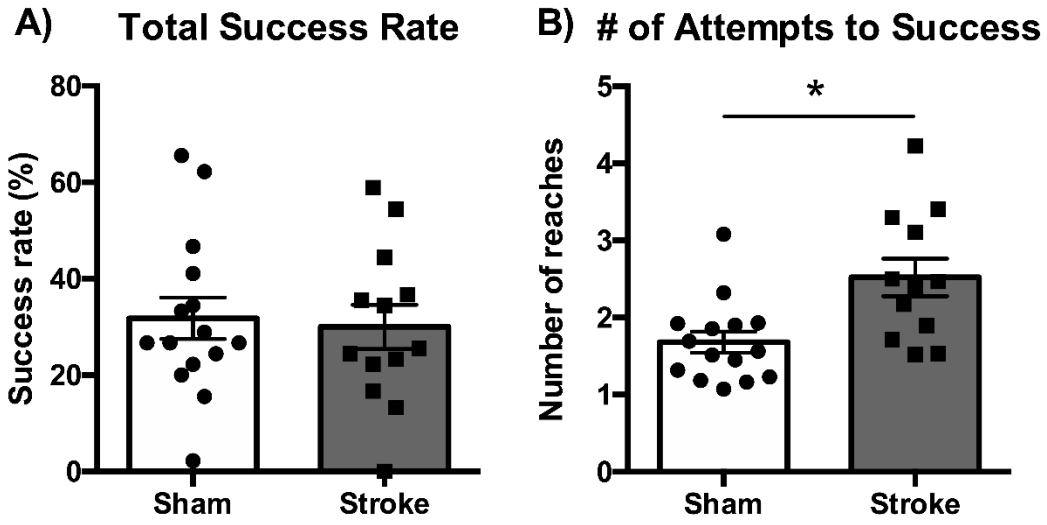

Figure 7. Single pellet reaching task outcomes. A) Total success rate and **B)** number of attempts to a successful reach for sham vs. P7 stroke groups were calculated and averaged across the last three days of testing. All data is presented as mean ± SEM for sham (n=15) and stroke (n=13) animals. * depicts significance of $p < 0.05$.

Figure 7A first shows no significant difference between the sham or stroke groups in overall success rate (sham: 31.8 ± 4.31%, stroke: 30.0 ± 4.56%; $t(26) = 0.283$, $p = 0.78$, unpaired t-test). However, there was a significant increase in reaching attempts in the stroke animals (2.52 ± 0.243) vs. sham animals (1.68 ± 1.36) (Fig. 7B, $t(26) = 3.17$, $p = 0.0040$, unpaired t-test).

Overall, our P7 perinatal stroke protocol was able to induce long-term behavioural deficits across a battery of behavioural tests, most notably in a test of skilled forelimb reaching function.

3.3. Perinatal PT stroke bilaterally impacted cortical motor maps

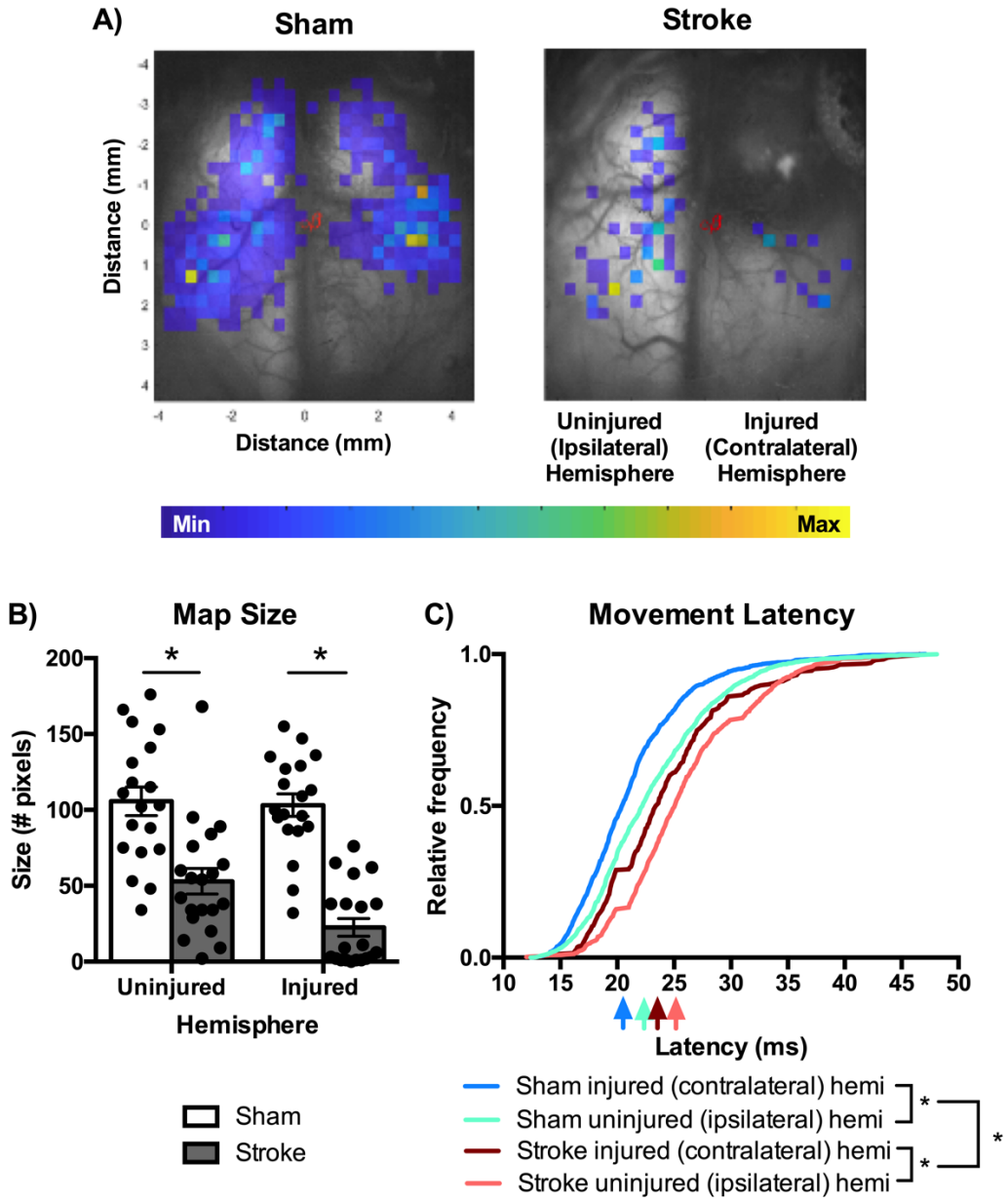

Figure 8. Motor mapping outcomes. A) Cortical motor maps of the contralesional forelimb from a representative sham (left) and stroke (right) animal. Coloured pixels depict stimulation points which evoked forelimb movement, with brighter pixels depicting larger movements. Red ß marks Bregma, and axes depict distances in the coronal and sagittal plane relative to bregma (0,0). **B)** Quantification of map size in each hemisphere of the contralesional forelimb representation. Data is presented as mean ± SEM for shams (white bars, n = 19) and stroke groups (grey bars, n = 20). **C)** Cumulative distributions of movement latencies of the contralesional forelimb from the injured (contralateral) and uninjured (ipsilateral) hemisphere. Data is pooled across all sham (blue, turquoise; n = 3734 data points over 19 mice) and stroke (red, pink; n = 1239 data points over 20 mice) animals. Coloured arrows depict corresponding medians. * depicts significance of $p < 0.05$.

At P80, sham and stroke animals underwent the first timepoint of optogenetic motor mapping. Heat maps of movement representations were generated from combining the stimulation matrix with evoked accelerometer signals following laser light stimulation (Fig. 8A). A two-way RM ANOVA analysis showed a main effect of stroke on motor map size ($F(1,37) = 10.7$, $p = 0.002$). Sidak post-hoc tests further showed that this effect was held over both the injured ($p < 0.0001$) and uninjured hemisphere ($p = 0.00018$). In the injured hemisphere, map size was reduced by 78% in the stroke vs. sham animals (Fig. 8B; stroke: 22.6 ± 5.83 pixels**,** sham: 103.1 ± 7.46 pixels). In the opposite, uninjured hemisphere, map size was reduced by 50% (Fig. 8B; stroke: 53.0 ± 8.45 pixels**,** sham: 105.7 ± 9.46 pixels).

Movement latencies were also examined due to the robustly bilateral movement representations from the sham animals (Fig. 8B; $p = 0.681$, Sidak's test). Thus, the latency comparisons may offer insight into whether the contralateral movements differed from ipsilaterally-evoked movements (since the motor system is typically thought as a crossed/contralaterally-organized system). In the sham group, movements evoked from the injured hemisphere (i.e. contralateral hemisphere) displayed shorter latencies (median latency: 20.5 ms) than those from the uninjured hemisphere (median latency: 22.3 ms) ($H(1,3) = 477.8$, $p < 0.0001$, Kruskal-Wallis one-way ANOVA), implying a more direct and/or faster-conducting motor pathway. In the stroke group, injured hemisphere latencies (Fig. 8C; median latency: 23.5 ms) were also significantly shorter than uninjured latencies (Fig. 8C; median latency: 25.1 ms; $p < 0.0001$, Dunn's Test). However, both uninjured and injured hemisphere latency distributions were significantly longer/slower compared to those of the sham group ($p < 0.0001$, Dunn's test).

Overall, perinatal PT stroke bilaterally impacted both cortical motor representations and descending motor pathways, with significant reductions in map size and delays in movement latencies.

3.4. Motor map reorganization correlated with behavioural impairment, but not lesion volume

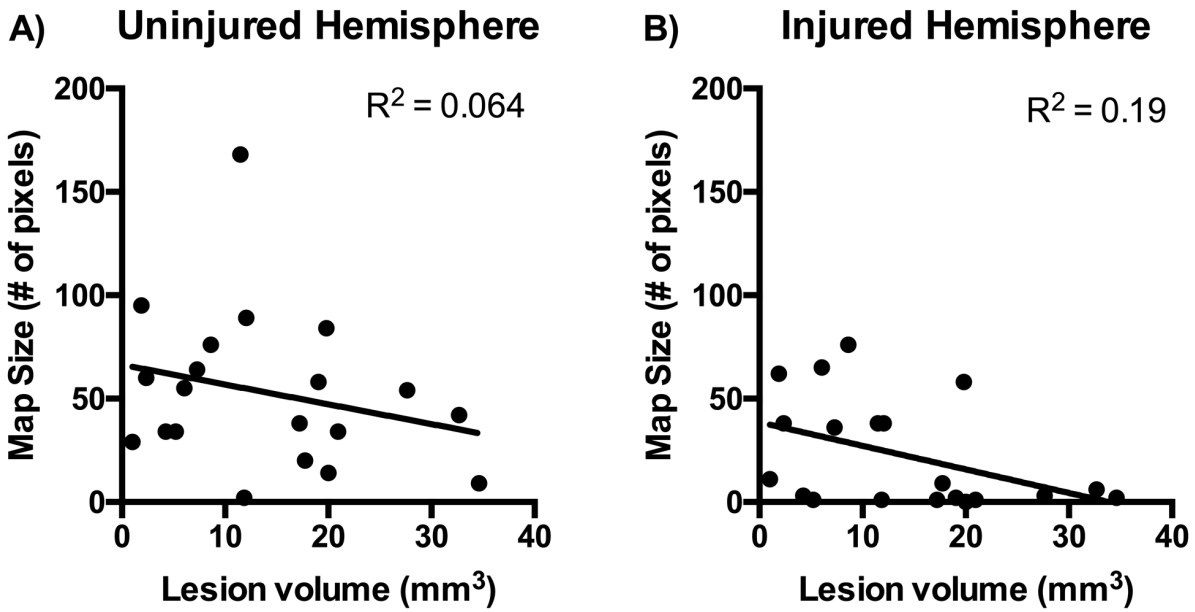

Figure 9. Correlations between map size and lesion volume. Correlations between contralesional forelimb map size in the uninjured **(A)** and injured **(B)** hemisphere and lesion volume in stroke animals (n = 20).

With our large and variable lesions, we were interested in whether post-stroke map size was related to the degree of injury (as quantified through lesion volume). There was no significant correlation in either the uninjured (Fig. 9A; $R^2 = 0.064$, $p = 0.28$) or the injured hemisphere (Fig. 9B; $R^2 = 0.19$, $p = 0.054$).

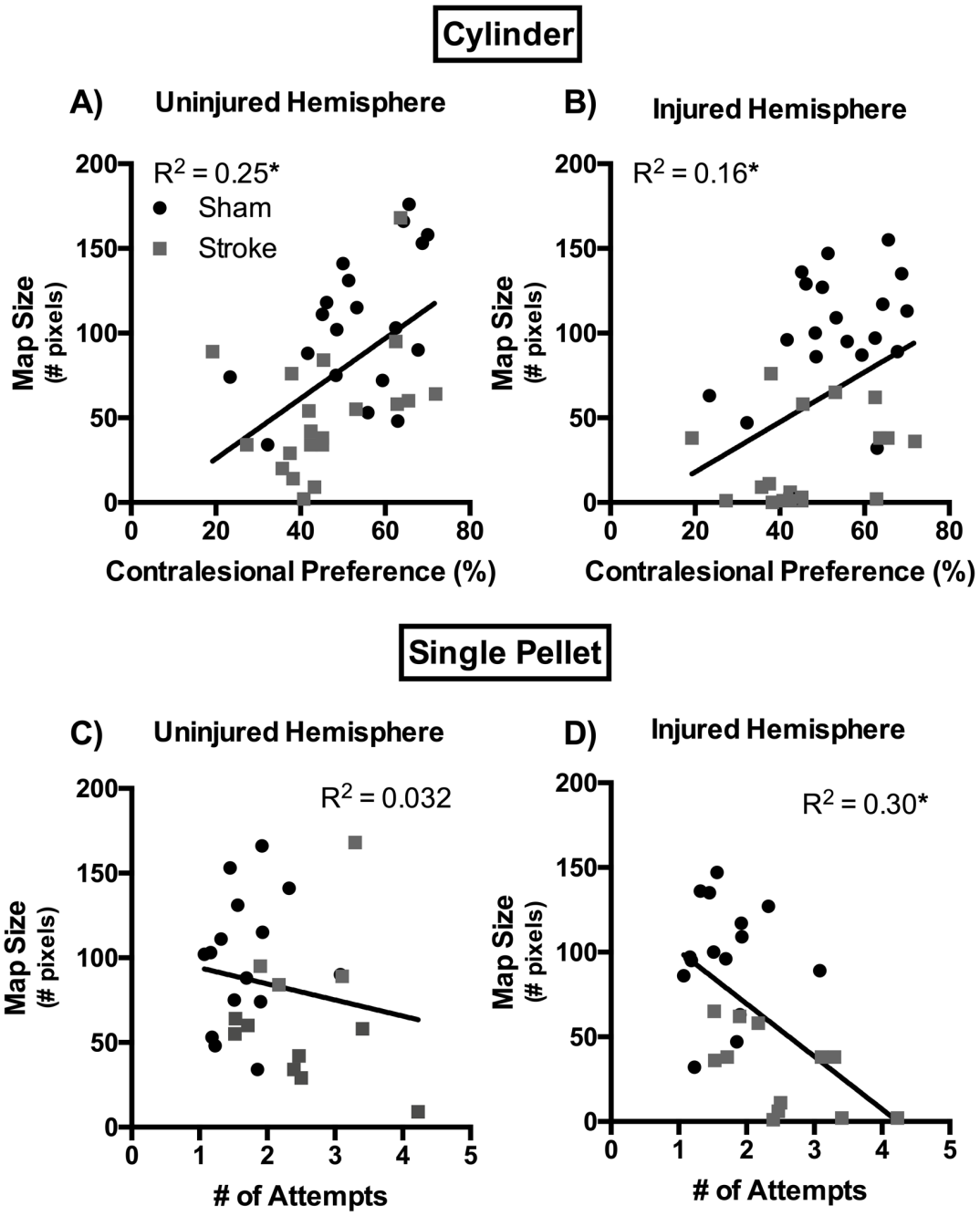

Figure 10. Correlations between behavioural outcomes and map size. A & B) Correlations between contralesional limb preference during cylinder test rearing behaviour and map size in the uninjured **(A)** and injured **(B)** hemispheres. Data is presented as sham (black circles, n=19) and stroke (grey squares, n=20). **C & D)** Correlations between motor map size and number of attempts to a successful reach (from the single pellet task) for the uninjured **(C)** and injured **(D)** hemispheres. Data is presented from stroke (black circles, n=15) and stroke (grey squares, n=13) animals. Sample sizes for the single pellet correlations differ from those in the cylinder task due to exclusion of animals which failed to acquire skilled reaching behaviours (total excluded: n=11). Significant correlations are denoted with asterisks (*).

There was no significant correlation between motor map size and any outcome of adhesive or tapered beam (Table S1). For Digigait, there was one significant correlation between stride time and map size in the injured hemisphere ($R^2 = 0.10$, $p = 0.048$), with the remaining parameters showing no significant correlation with either the uninjured or injured hemisphere (Table S2). In the cylinder task, there was a significant positive correlation between map size of both hemispheres and contralesional preference during spontaneous rears (Fig. 10A, B; uninjured hemisphere: $R^2 = 0.25$, $p = 0.0011$, injured hemisphere: $R^2 = 0.16$, $p = 0.013$). In the single pellet task, there was a significant negative correlation with map size in the injured (Fig. 10D; $R^2 = 0.30$, $p = 0.0031$), but not uninjured hemisphere (Fig. 10C, $R^2 = 0.032$, $p = 0.38$). Further, there was no significant correlation between single pellet success rate with either hemisphere (Table S1).

These correlations show that while perinatal stroke bilaterally reduced movement points, the remaining motor sites were beneficially associated with functional outcome across two types of motor tasks. However, this positive correlation across hemispheres was task dependent.

3.5. Single pellet forelimb training further modulated map organization following perinatal stroke

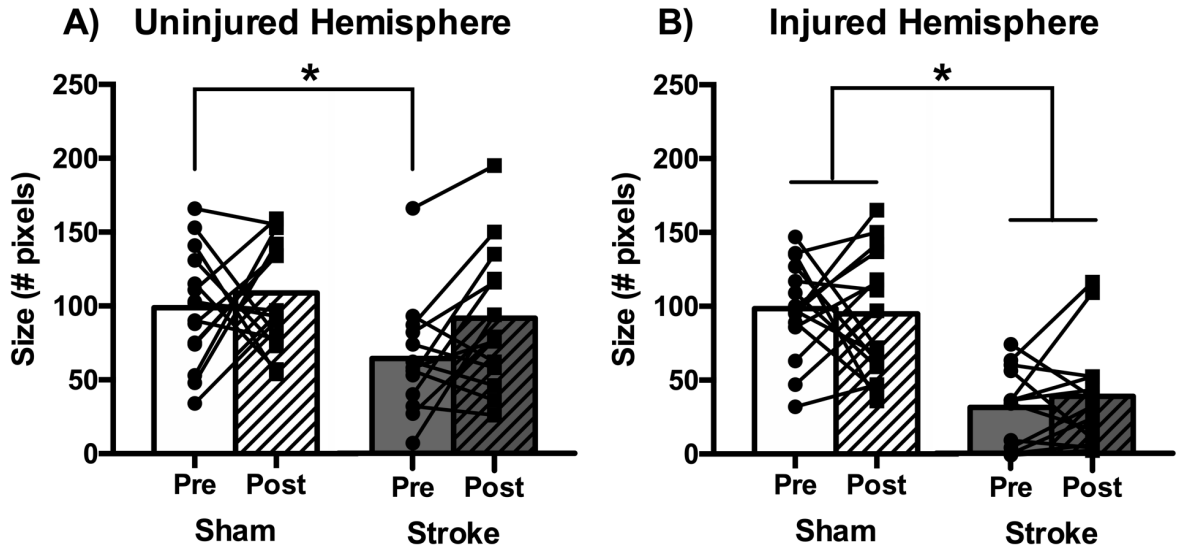

Figure 11. Map size pre- and post-single pellet reach training. A & B) Contralesional forelimb map size from the uninjured **(A)** and injured **(B)** hemispheres. Data is reported as means, with individual animals pre- (solid bars) and post-single pellet (dashed bars) connected by lines. Sample sizes consist of (n=15) sham animals (white bars) and (n=13) stroke animals (grey bars) due to exclusions of animals which failed to acquire skilled reaching behaviours (total excluded: n=11). Significance of $p < 0.05$ is denoted by an asterisk (*).

Within this subset of animals (see Methods for exclusion criteria), map size was still significantly reduced in stroke animals in both the uninjured (Fig. 11A; sham: 98.9 ± 10.1 pixels, stroke: 66.4 ± 10.8 pixels; $p = 0.028$, Sidak's test), and injured (Fig. 11B; sham: 98.4 ± 7.69 pixels, stroke: 33.3 ± 8.34 pixels; $p = 0.015$, Sidak's test) hemispheres at the pre-single pellet timepoint. Following single pellet, there was a differential effect on map size between hemispheres. In the injured hemisphere, there was no significant effect of time in either the sham or the stroke animals (Fig. 11B; sham: 94.9 ± 10.3 pixels, stroke: 40.8 ± 11.1 pixels; $F(1,26) = 0.41$, $p = 0.529$, mixed RM ANOVA). In the uninjured hemisphere, where map size reduction was less severe, there was also no significant interaction between time and treatment ($F(1,26) = 0.736$, $p = 0.40$, two-way RM ANOVA). However, post-hoc tests showed that while there was a significant difference between sham vs. stroke at the pre-single pellet timepoint ($p = 0.037$, Sidak's test), this statistical effect disappeared at the post timepoint (sham: 109 ± 11.1 pixels, stroke: 93.6 ± 12.0; $p = 0.359$, Sidak's test). Overall, these results show that single pellet training was able to subtly/partially restore map size following perinatal stroke.

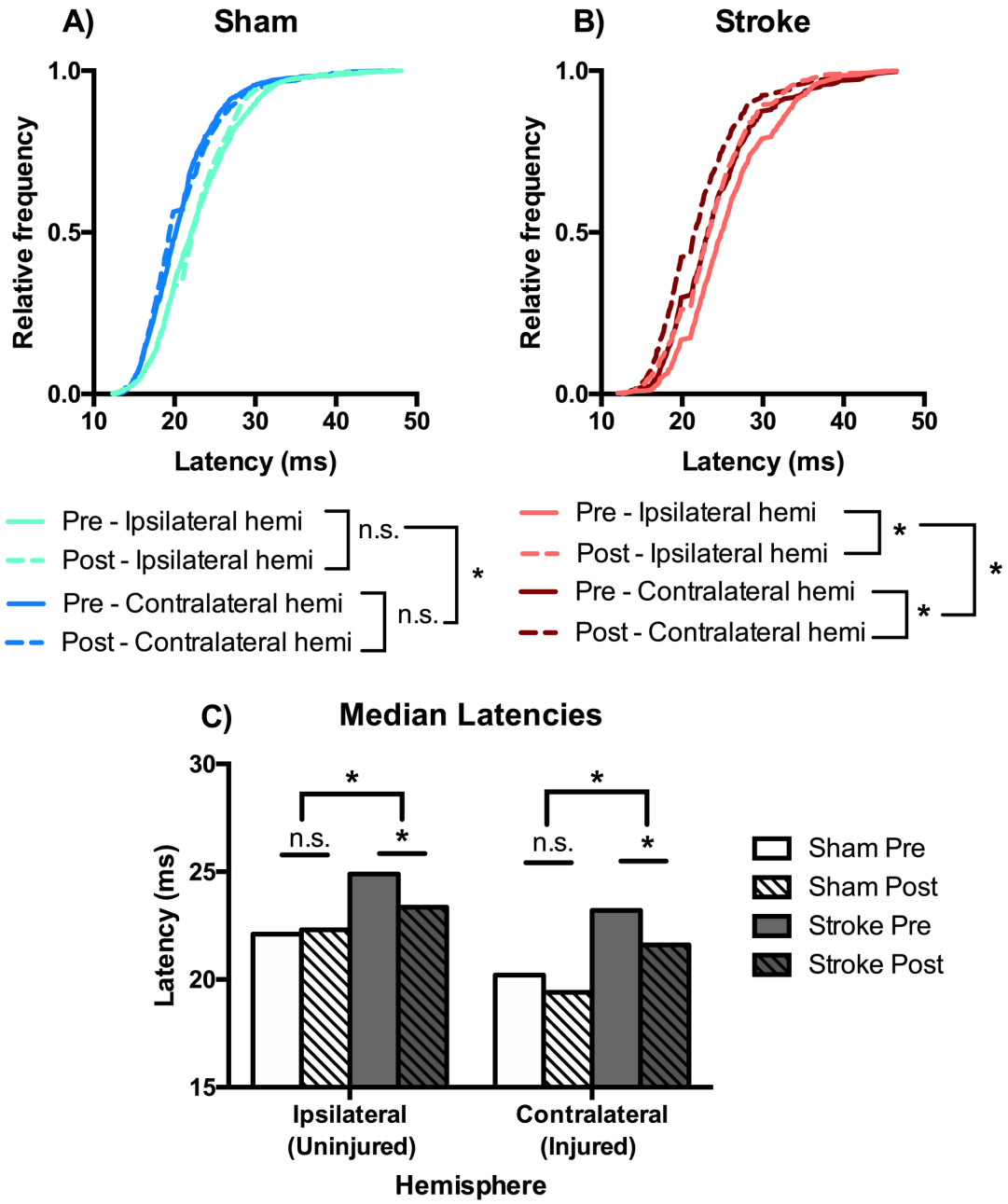

Figure 12. Latency distributions and medians pre- and post-single pellet. A & B) Cumulative distributions of movement latencies for the contralesional forelimb were derived from pooling all movement points from each treatment group. Distributions are plotted for both sham **(A)** vs. stroke **(B)**, ipsilateral (uninjured, turquoise and pink lines) vs. contralateral (injured, blue and maroon lines) hemispheres, and pre- (solid lines) vs. post-single pellet maps (dashed lines). For shams, curves consist of n = 3300-4300 points from 15 animals, whereas stroke sample size is n = 4000-5500 points from 13 animals. **C)** Medians of each trace in (A) and (B) are plotted in order to facilitate visualization of stroke vs. sham comparisons. Significance of $p < 0.05$ is denoted with an asterisk (*), whereas non-significance is denoted as n.s.

Unlike map size, single pellet training had a bilateral effect on movement latencies. Importantly, this effect was only seen in the stroke group and not in shams. In the latter group, median latencies for the uninjured (ipsilateral) hemisphere pre vs. post were 22.1 and 22.3 ms, whereas those for the injured (contralateral) hemisphere pre- vs. post- were 20.2 and 19.4 ms (Fig. 12A, C). A Kruskal-Wallis one-way ANOVA showed that there was a significant difference between at least one pair of distributions ($H(1,7) = 686.3$, $p < 0.0001$). Upon further analysis with a Dunn's post-hoc test, there was no significant difference between the pre vs. post timepoints in either hemisphere ($p > 0.99$ for the pre vs. post comparison in both the injured and uninjured hemisphere). However, there was a difference between the injured vs. uninjured hemisphere at both timepoints ($p < 0.0001$ for the injured vs. uninjured comparison at both pre and post timepoints).

In the stroke group, a Dunn's post-hoc test showed that contralateral latencies (i.e. injured hemisphere) were also consistently shorter than ipsilateral latencies (i.e. uninjured hemisphere) in both the pre- and post-single pellet timepoints (Fig. 12B, C; $p < 0.0001$ for injured vs. uninjured comparisons in both pre and post). In the pre timepoint, injured vs. uninjured median latencies were 23.2 and 24.9 ms, respectively. At the post timepoint, median latencies of the injured vs. uninjured hemisphere were 21.6 and 23.4 ms, respectively.

In Figure 12C, the median latencies of each curve were plotted in order to visualize the explicit comparison between the sham vs. stroke groups. In both hemispheres, comparison of sham vs. stroke groups showed first that pre-single pellet training latencies of the stroke animals were longer than those of the sham animal ($p < 0.0001$ for sham vs. stroke comparison in both the injured and uninjured hemispheres, Dunn's test), as previously shown in Figure 8C. At the post-single pellet timepoint, latencies were significantly reduced in the stroke animals compared to the pre timepoint; however, they were still significantly longer than sham animals. This effect was conserved across both the injured and uninjured hemispheres ($p < 0.0001$ for sham vs. stroke comparison in either injured or uninjured hemisphere, Dunn's test).

Overall, these data show that single pellet training had a stroke-specific effect on movement latencies. However, although movement latencies in the stroke group were significantly reduced/faster, they were only partially restored to sham latencies.

3.6. Change in map size and latency reduction correlated in the uninjured hemisphere following single pellet

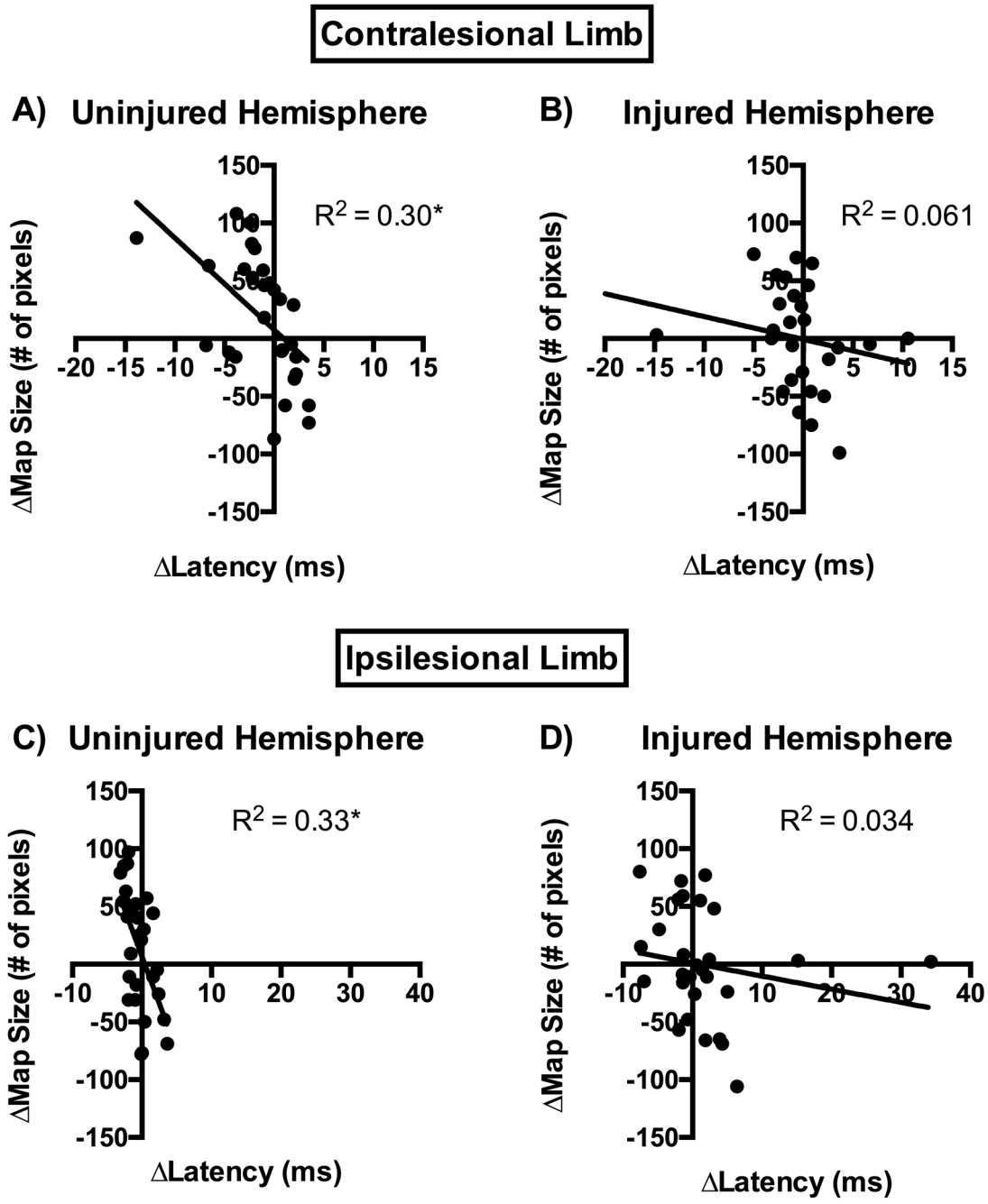

Figure 13. Correlations between change in map size and latency. Change in latency and map size pre- and post-single pellet were correlated in both hemispheres for the contralesional limb (A & B) and ipsilesional limb (C & D). Sample size was (n=15) sham animals and (n=13) stroke animals. Significance of $p < 0.05$ is denoted with an asterisk (*).

To further evaluate whether the latency reductions and map size changes following single pellet were beneficial or maladaptive, we correlated the two with each other for movements/representations from both limbs. In the contralesional limb representations, there was a significant correlation in the uninjured/contralateral hemisphere (Fig. 13A; $R^2 = 0.30$, $p = 0.0027$), whereas the injured/ipsilateral hemisphere was non-significant (Fig. 13B; $R^2 = 0.061$, $p = 0.20$). This effect was paralleled in the ipsilesional limb representations, where significance was found in only the correlation in the uninjured/ipsilateral hemisphere (Fig. 13C; $R^2 = 0.33$, $p = 0.0013$), but not the injured/contralateral hemisphere (Fig. 13D; $R^2 = 0.034$, $p = 0.35$). This significant correlation in the uninjured hemisphere across both limbs suggests that these motor system changes are true and related.

4 – Discussion

4.1. Perinatal PT stroke induces behavioural deficits in skilled reaching and gait

In order to address the paucity of longitudinal behavioural characterization in the perinatal PT literature, we measured outcomes from a battery of sensorimotor behavioural tests. The goal of this was threefold: 1) to align our mouse model with clinical impairments, 2) to provide functional outcome measures with which to correlate motor map changes, and 3) to provide a means of skilled forelimb motor training with which to test further capacity for map modulation post-stroke.

Following stroke, we showed that impairments were present in Digigait and the single pellet task. To contextualize our lesion volumes and behavioural results to clinical measures, our lesions comprised on average 3.3% of total brain volume. At the clinical level, patients that developed cerebral palsy following perinatal stroke had an average lesion volume of 6.5% total brain volume (140). In this study, the majority of patients (14/16) displayed relatively good motor function, including unassisted walking and running, with limitations in speed, balance, and coordination (as quantified as Gross Motor Function Classification System (GMFCS, (141)) Level I). In another cohort study, the cut-off for developing cerebral palsy was only 3.3% total brain volume (142). This study had a smaller proportion of mildly impaired patients (9/14; GMFCS Level I), with the remainder requiring mobility assistance devices (GMFCS Levels II/III). In comparison to these results, the level of motor disability in our mice was relatively similar to these clinical descriptions. Our animals displayed normal motor behaviours on the majority of sensorimotor tests (i.e. cylinder, adhesive removal, and tapered beam tasks), with impairments in skilled reaching and a few clusters of gait parameters. Specifically, we saw differences between the stroke and sham group in the duration of different phases of the gait cycle, such as the swing phase (where the limb is airborne), the propulsion phase (where the limb is actively accelerating to push off into the next stride), and the stance phase (where the limb is contacting the ground). Clinically, these differences have also been observed in perinatal stroke patients, most notably in those with hemiparetic cerebral palsy (135,136,138,139). It is interesting to note that despite the gait impairments shown with the Digigait test, the deficits in our model are not as severe as those seen clinically. As mentioned above, there is a significant portion of patients whose mobility deficits are severe enough to require walking assistance devices. Whereas with our animals, mobility was intact enough for the animals to be able to walk and even run without difficulty on

the Digigait treadmill. One potential explanation for this discrepancy is that in animals where our PT stroke protocol induced more severe deficits, additional comorbidities (such as seizures) may have resulted in early mortality. After all, with our protocol, 18/59 animals died due to unknown factors in the first three weeks post-stroke. However, it is also possible that maternal care could be a cause for these premature deaths. Dam behaviour and care of litters is sensitive to both external (such as housing conditions, environmental stress, etc.) as well as internal factors (such as pup health). As we did not monitor post-surgical maternal care, we cannot entirely rule out this factor as an explanation for the post-surgical mortality.

Outside of our motor impairments, it is also important to acknowledge that we did not measure all common impairments of perinatal stroke, such as post-neonatal seizures, epilepsy, or muscle tone/spasticity (5,14,16). Without these data, we cannot claim that our model is a perfect recapitulation of perinatal stroke. However, it is arguable that with the significant differences in physiology between humans vs. rodents (ex. lack of corticomotoneuronal connections, differing cerebral anatomy, etc.), this may not be possible. As such, where it concerns our primary focus of motor impairments and cortical representations, we opted not to measure these other clinical characteristics. For our intents and purposes, our perinatal stroke protocol was sufficient to induce upper limb deficits, as commonly seen amongst perinatal stroke patients (14), while also providing neural substrate with which to conduct motor mapping. To compare to clinical reports of upper limb function, one study using a robotic upper limb movement apparatus (KINARM) showed kinematic abnormalities specifically related to the trajectory of the contralesional arm during reaching movements (143). Other studies using the box and block test (perhaps a more direct clinical analog to our single pellet reaching task) describe deficits in the number of blocks moved (implicating deficits in manual dexterity) (50,144,145). Although our model did not show a deficit in overall reaching success (which could be interpreted as the parallel outcome measure to the box and blocks test), the increase in reaching attempts may mirror the kinematic deficits found in the KINARM study.

From the large body of perinatal aspiration lesion work from Whishaw and Kolb, we see a few similar themes in single pellet reaching findings. For example, one study using bilateral P4 injuries showed a presence of motor deficits amongst certain tasks (such as the elevated beam, swimming test, and tongue extension test), but absence in others (such as food pellet manipulation or latch manipulation) (146). In conjunction with our findings, these results further emphasize the

task-specificity of impairments and the need for a battery of motor tests. In another study, animals with early (P0) unilateral injuries showed a similar increase in reaching attempts compared to controls (47). Interestingly, from this body of work, unilateral lesions often also affect overall reaching success rate (71,147,148). It is not entirely clear why we would fail to see a similar impairment in this measure of single pellet. One potential explanation is a species-specific difference in skilled reaching capacity. For example, even in our sham mice, overall success rate was ~30%, whereas the aforementioned studies showed a control performance of ~50-60% in rats (71,147,148). There has been precedent for this in the past, where a direct comparison of rat vs. mouse reaching performance showed a significant difference in endpoint success rate between species (149). Specifically, it was mentioned explicitly in this study that while "rats showed a consistent improvement with training…mice were somewhat inconsistent" (149). As such, perhaps the mouse model itself already introduces a ceiling of sensitivity with which to detect skilled forelimb impairments. Of course, the main motivation behind choosing to use mice over rats was the ability to optogenetically stimulate the mouse brain, as currently there are no established layer 5 motor cortex-specific optogenetic lines for the rat. Additionally, it is also worth mentioning that despite imperfect replication of reaching results from the Whishaw and Kolb aspiration studies, we nevertheless were able to induce longitudinal reaching deficits in the present study.

4.2. Bilateral map size changes following perinatal PT stroke

Predictably, our perinatal PT strokes ablate forelimb representations in the injured hemisphere. Interestingly, the loss of movement sites was not only restricted to areas of tissue loss (within the core of the lesion), but also seemingly in intact perilesional tissue as well. This could be due to these remaining areas functioning as non-motor areas; however, stimulating these corresponding sites in the opposite hemisphere yielded movement. Indeed, ICMS mapping studies in intact animals have shown that forelimb motor maps extend caudally as far back as 2.0 mm from bregma (90). A second explanation may stem from an interesting observation from the cresyl-violet stained histological sections, where a large portion of animals (12/20 quantified animals) had lesions extending posteriorly underneath a layer of intact cortical tissue. As such, in many instances, the lesion actually extended further back than what was seen from an overhead view, and thus tissue in this area potentially lacked the axonal projections necessary to stimulate movement. Unfortunately, detailed analysis beyond visual observation of the exact extent of the

lesion was not conducted in this study (for further discussion on this, see section 5.1.3). When we correlated lesion volume with map size, we found no significant relationship in either hemisphere. This suggests that the perilesional ablation we observe is dependent on other factors besides lesion volume. So, barring the lack of projecting axons from these large lesions, a third reason for the observed ablation in the injured hemisphere may be due to "crowding" or overlap of motor and/or sensory representations. Initial reports of forelimb representations after stroke in adult non-human primates showed expansion of proximal representations at the expense of distal representations (68). In 2013, Harrison et al. built from these findings, showing that sensory area strokes also disrupted motor map representations (150). Feasibly, with our large lesion volumes, disruption of non-CFA areas (ex. premotor or sensory representations) could cause them to shift into areas previously controlled by forelimb motor maps.

In the uninjured hemisphere, we also observed a similar reduction in movement sites/map size. At a gross histological level, the tissue in the uninjured hemisphere displayed no overt damage (Fig. 5). However, communication between the hemispheres was more than likely affected, as the corpus callosum was almost completely transected at the core of the lesion in 15/20 of the animals in the injured hemisphere. With this important interhemispheric "highway" interrupted, remote functional depression (or diaschisis) is a likely candidate for the ablation in the uninjured hemisphere. One explicit study observing the role of the corpus callosum in motor representations described a significant increase in power threshold required to elicit movements from the ipsilateral hemisphere (75). In the context of these results, our movement representation ablations in the uninjured/ipsilateral hemisphere makes sense considering we dually disrupted both the cortex and the corpus callosum of the opposite, injured hemisphere. Outside of this one study, there exists other evidence for robust interhemispheric interactions as well. For example, in one study quantifying cortical connectivity using optogenetic stimulation and voltage sensitive dye, they described strong connectivity between not only homotopic regions in the two hemispheres (such as motor-motor or sensory-sensory interactions), but also between non-homotopic regions (including areas such as the anterior cingulate cortex and parietal association areas) (151). Another study measured hemispheric interactions using paired pulse ICMS stimuli (94). By delivering two ICMS pulses at various intervals, evoked movement was either inhibited or facilitated. Here, Touvykine et al. showed that between hemispheres, there was a strong facilitatory effect of the RFA on the CFA, whereas CFA-CFA interactions are equally inhibitory and facilitatory. As such,

with large lesions that likely ablate both the RFA and CFA (although specific borders between the two were unable to be quantified in this study), this loss of facilitatory modulation from the injured hemisphere could explain for a parallel loss of excitability in the uninjured hemisphere.

4.2.1. Lesion volume was not correlated with map size

As there was significant variation in our lesion volumes, we decided to correlate the degree of injury with map size. This could offer potential explanations as to whether the ablations we observed were due to only the degree of injury, or with other factors (as explored above). Because we did not observe a significant correlation in either hemisphere, the ablation caused by perinatal stroke does seem to be multifactorial outside of simply the degree of tissue loss. This is interesting to note, as one current theory at the clinical level in both adult and perinatal stroke fields is increased viability of the injured hemisphere with smaller injuries, and vice versa (55,152). With our lack of significance however, our model suggests that smaller lesions do not result in greater potential for sparing. In fact, they may be just as disruptive as the larger injuries. This may be due to the unique timing of perinatal strokes, as these injuries coincide with 1) heightened sensitivity to oxidative damage (13,153), and 2) active development of descending motor tracts. As a result, it is possible that perhaps even the smaller injuries can have significant downstream/chronic effects.

Another explanation could stem from the subcortical damage that the majority of our animals sustained. As mentioned earlier, the effect of subcortical strokes on motor maps has yet to be systematically explored in a perinatal model. However, in an adult rodent model of stroke, it has been shown that injuries with combined cortical and subcortical damage resulted in the greatest degree of functional deficits (154). Moreover, their data suggested that striatal damage specifically was an important predictor of stroke recovery, separate from lesion volume. In this way, this could potentially explain our lack of correlation with motor maps as well, where even small lesions could disrupt function/maps if they extended into the striatum. With explicit experiments subdividing groups of animals into purely cortical, combined cortical + subcortical, and purely subcortical lesions (as done in the aforementioned study), the exact role of perinatal subcortical damage on motor maps could be more clearly delineated.

4.3. Delayed movement latencies following perinatal PT stroke

One notable feature of our optogenetic motor maps is the robust bilateral distribution of movement sites. Specifically, contralesional limb movements were evoked from not only the

contralateral hemisphere (i.e. the injured hemisphere), but also the ipsilateral hemisphere (i.e. uninjured). From an anatomical standpoint, the motor system is a primarily contralaterally-organized fibre pattern. Classically, when using techniques such as TMS in adult humans and ICMS in adult rodents, evoked motor maps reflect this uni/contralateral anatomical organization, where maps are primarily relegated to the contralateral hemisphere of the forelimb of interest. One consideration, however, is that many such studies either do not collect maps from both hemispheres due to technical limitations, or do not explicitly report the results. Additionally, although not as widely reported, there have been reports of ipsilateral responses using both TMS and ICMS (46,75,78,155,156). Clearly, ipsilateral motor control exists and can be elicited through brain stimulation techniques; however, they are often small and sparse representations. Contrastingly, one optogenetics mapping study which also quantified ipsilateral representations found robust bilateral representations for one limb, similar to our findings (157). Because we were able to replicate such bilateral maps, and in a perinatal stroke model at that, it is possible that the technique of optogenetic mapping itself may be capable of unmasking a larger amount of ipsilateral movement sites that are not accessible using TMS or ICMS.

The bilateral nature of these maps is salient to the question of hemispheric output after perinatal stroke. As mentioned previously, one common theory is that after both adult and perinatal stroke, decreased interhemispheric inhibition from the injured hemisphere results in an increase in hemispheric output from the uninjured hemisphere. Unfortunately, with our bilateral maps, conclusions surrounding specific hemispheric output is difficult to ascertain from just map size, since at baseline, the uninjured hemisphere is already involved. So, as a means of targeting individual hemispheric contributions, we opted to quantify movement latency as well. It has been shown that amongst individuals from which ipsilateral movements can be elicited (including young infants and children), contralateral movements display faster latencies due to the more direct/faster-conducting contralateral CST (23,40,155). In our study, the sham animals did indeed display faster movement latencies in the hemisphere contralateral to the contralesional forelimb (i.e. the “injured” hemisphere) compared to the opposite hemisphere. In this way, despite equal distribution in number of movement sites across both hemispheres, the contralateral hemisphere could still be differentiated through faster latencies.

In the stroke animals, beyond bilateral ablation of movement sites, there were also changes to the movement latencies in both hemispheres. First, contralateral movement sites still displayed

faster latencies compared to ipsilateral movements, suggesting that normal hemispheric balance was maintained, although more detailed experiments (such as paired pulse measures) are required to confirm this. Second, despite the maintenance of the contralateral-ipsilateral latency relationship, latencies from both hemispheres were significantly slower than those of the sham. This indicated that either the integrity of descending pathways as a whole were impacted, or that there was some degree of rewiring from the remaining cortical/subcortical motor centres. Although we did not conduct explicit experiments to test one hypothesis over the other, there are a few potential explanations for this delay in movement latencies.

First, it has been shown previously that the primary descending tracts in both the adult and neonatal mouse comprise not only of the CST, but also the reticulospinal tract (RtST) (97,158). In the former study, Alstermark & Ogawa found that the CST mediated medium- to long-latency movements, while the RtST mediated short-latency movements (97). As such, with the incidence of the perinatal stroke, perhaps these short-latency RtST circuits are disproportionately disrupted, resulting in a greater reliance on the longer-latency CST circuits. To build upon this evidence, another study in 2014 showed the importance of reticular formation nuclei in skilled reaching in particular, which may explain the skilled reaching deficits that we observed as well (159).

Second, it may also be possible that the delay is due to rewiring of the cortical areas to lesser-used pathways with multiple intercalated synapses, such as the rubrospinal or corticopontine tracts. Although these aforementioned circuits have not been shown to have a significant role in movement execution in naïve, adult mice, it is possible that the uniquely plastic neural environment of the developmental brain could support growth of this otherwise weaker circuit. Precedent for these circuits has been found in early developmental injury studies, where anatomical tracing experiments showed an increase in the number of projections in the red nucleus and pontine nucleus on the injured side (i.e. atypical side) (37,82,160). Because these circuits are not typically implicated in skilled forelimb control in healthy animals, it is possible that factors such as fibre diameter or myelination levels may not be optimized for fast conduction like the RtST or CST, which may explain the delayed latencies.

Of course, neither of these first two postulations entirely explain why latencies in the opposite/ipsilateral hemisphere are also further delayed. One potential reason for why these latencies are further delayed despite no overt cortical damage may be damage to the overall integrity of the descending tracts, ipsilateral and contralateral alike. This may stem from

widespread demyelination of descending motor tracts. White matter damage is an important consequence after ischemia (161), and it has been shown that stroke has the capacity to bilaterally affect oligodendrocyte health (162). At the perinatal timepoint, this damage may be further exacerbated, as oligodendrocyte progenitor cells are particularly susceptible to oxidative damage (13,153,161,163).

4.4. Functional outcome correlates with map size

When we correlated post-perinatal stroke map size and functional outcome, we found that the relationship between the two was not only task-dependent, but also hemisphere dependent. It is interesting to note first the bilateral correlation between a spontaneous, "gross" motor functioning task such as the cylinder, whereas a task testing more "fine-tuned" motor skills such as the single pellet task correlated with map size in only the injured hemisphere. Although speculative, these results could suggest differing hemispheric control between different types of movement (ex. proximal vs. distal). After all, it is known that in both rodents and humans, proximal musculature often displays more bilateral innervation patterns, whereas distal musculature tends to be unilateral (42,96,98,156). In the mouse specifically, anatomical and physiological studies have implicated the RtST as a large component of distal/forelimb motor control, with some proximal/axial innervation (97,158,164). Moreover, the majority of these projections are unilateral. Contrasting this, RST projections in the mouse primarily project down to non-forelimb motor areas in the spinal cord, with demonstrated ipsilateral fibres (165). These two different innervation patterns between the RST and RtST could provide additional evidence for differing hemispheric involvement in proximal vs. distal motor control.

Within the relationship between the single pellet task and map size, our results match the camp of evidence positing the importance of the injured hemisphere on upper limb performance post-stroke (55,126,166). However, as mentioned in a timely review of post-stroke reorganization and neuromodulation, the true relation between hemispheric contribution and outcome is complicated and likely patient-specific (152). As such, the specific contribution our results provide to this evidence base are those in the context of large, unilateral lesions extending into subcortical areas induced at the perinatal timepoint.

The positive, bilateral correlations between map size and spontaneous forelimb use in the cylinder task are a little more difficult to interpret, as there are few preclinical studies which

corelate this specific task with map characteristics. One study using a dopamine depletion injury in the striatum similarly described a positive correlation between forelimb map size in the injured hemisphere with contralesional paw use {Formatting Citation}. Unfortunately, this study did not collect maps in the opposite hemisphere, and thus does not offer any evidence for the role of the opposite hemisphere. From a clinical perspective, we can indirectly compare with a study which correlated map threshold (i.e. the amount of TMS power required to elicit movement) and the Assisting Hand Assessment (AHA). As the AHA task measures the degree of spontaneous hand use of the contralesional limb (where the higher the score, the more the contralesional limb is being used), it is a more direct clinical comparison to the cylinder task rather than the goal-oriented single pellet reaching task. In this study they found a negative correlation between motor threshold and AHA scores, meaning that the greater the use of the contralesional hand, the more responsive the injured hemisphere (81). Interestingly, while they did measure both motor threshold and map size in the uninjured hemisphere, there was no significant correlation with AHA scores. Again, perhaps the discrepancy between our results and these lie in the severity of the injury, where our results represent those from the severe end of the spectrum, whereas this study contained a wide range of lesion severities.

More generally, these correlations can comment on beneficial vs. maladaptive role of the two hemispheres following stroke, as currently the classical mindset is that control from the uninjured hemisphere becomes maladaptive following perinatal stroke, whereas control from the injured hemisphere is viewed as beneficial (10,20,24,32,152). With respect to this theory, our significant correlations in the injured hemisphere are in agreement, where increased output from this hemisphere corresponded with better functional outcome. As the positive relationship was observed across both a “gross” and “fine” motor task, this suggests that the injured hemisphere is more important compared to the uninjured hemisphere in our model. However, our findings in the uninjured hemisphere provide contradictory results to the classical theory. The relationship between the uninjured hemisphere and functional outcome is at most beneficial (as shown with the positive correlation with cylinder), and at least neutral (as shown with the non-significant correlations in single pellet and other behavioural tasks). This may stem from a few discrepancies. First, it is important to note that many of these such studies which showed a maladaptive role of the uninjured hemisphere did so with group comparisons, rather than correlations. It is possible that while within an averaged cohort, an overall effect may be seen, but when controlled for various

factors (such as lesion volume, age of presentation, etc.), these results are no longer observed. Indeed, one explanation for our lack of "interhemispheric interference" from the uninjured hemisphere may be due to the near-complete disruption of the corpus callosum, thus eliminating a significant avenue for interhemispheric communication. As such, perhaps the uninjured hemisphere is indeed beneficial, but on the more severe end of the spectrum of injuries. Support for this can be found in the adult stroke field, where entire reviews have been dedicated to the potential of the uninjured hemisphere following large strokes (55,58,167).

One last consideration is the distinction between correlation and causation. In order to truly establish a causal relationship between cortical motor maps and function, behaviour must be observed after some measure of map disruption (beyond the stroke itself). As covered in a recent review by Bundy and Nudo, there have been a few ventures into map silencing following adult stroke (57). For example, one study which injected the protein-synthesis inhibitor anisomycin into the reorganized CFA showed reinstated disruption of skilled reaching performance following initial recovery after stroke (168). In another study in healthy animals, motor map silencing through cholinergic input blockade disrupted map plasticity, which in turn prevented acquisition of skilled reaching (65). Through these two examples, it's clear that it is possible to delineate a causative relationship between either baseline motor function or rehabilitation-mediated recovery.

4.5. Skilled motor training is sufficient to further modulate map characteristics after stroke

Using the optogenetic technique, we had the advantage of non-invasively collecting motor maps at two timepoints: pre- and post-single pellet training. These multi-timepoint maps showed that there was a specific interaction of the stroke with our protocol of motor training, as map changes were observed post-training across two separate measures in only the stroke group. However, it is interesting to note that depending on the motor map measure, there was a differing effect of the single pellet training between hemispheres. Specifically, map size was restored (albeit partially) in only the uninjured hemisphere, whereas partial restoration of latencies was observed across both hemispheres. This suggests that single pellet training has differential effects on cortical vs. descending motor control.

4.5.1. Single pellet training induces subtle changes in map size

Following single pellet training, we found that map size in the injured hemisphere was not changed (relative to pre) in either the sham or stroke groups. As such, the significant reduction of

map size in the stroke group was conserved at both timepoints. In the uninjured hemisphere, there was a subtle effect of single pellet training between shams vs. strokes. Specifically, at the pre-single pellet timepoint, there was a significant reduction of map size in the stroke compared to the sham animals. However, after single pellet, this reduction was no longer significantly different. Interestingly, when observing the changes in pre vs. post in the stroke group only, map size increase was not statistically significant. Thus, while single pellet did increase map size in this hemisphere, the effect was small. It is possible that with an increased sample size, the statistical significance could be strengthened. However, in light of the robust latency effects, it may be more likely that our training protocol was not intensive enough to induce stronger changes in the cortical representations (to be explored in more detail in 4.5.2).

An alternative explanation for the relatively small effect size is the fact that the change was in the uninjured hemisphere – that is, the hemisphere ipsilateral to the trained limb. With the anatomical organization of the motor system, intuitively it would make more sense that the effects of training would be primarily relegated to the injured/contralateral hemisphere. However, there is evidence that skilled forelimb training is capable of reorganizing representations in the ipsilateral hemisphere of naïve, adult animals (63). With the incidence of a stroke, it is possible that injury could dampen the strength of this training-induced effect on the ipsilateral hemisphere. Alternatively, perhaps it was a dosage/duration issue with our training. In the aforementioned study, the ipsilateral map reorganization was not only transient, but also only demonstrated after 3 months of training. As such, potentially a more robust increase in the ipsilateral representations could be demonstrated in our model with prolonged training. Further support for this has been shown in a classic study by Kleim et al., where motor map reorganization (albeit in the contralateral hemisphere) did not change until after single pellet performance had plateaued (62). In contrast, our training protocol was carried out for a predetermined amount of time (i.e. 3 weeks) rather than based on a "plateau" in performance.

Moving from the uninjured hemisphere, the effects that we observed in the injured hemisphere are more straightforward to contextualize with other adult rehabilitation/stroke mapping studies due to the extensive reports of training-induced perilesional reorganization. However, these comparisons should be interpreted with caution. First, there are distinct differences between adult and perinatal neurophysiology at the time of injury and throughout recovery. Second, even within the adult stroke field, there is a lack of clear consensus on how post-stroke maps

further reorganize after motor training. This partially stems from large variation in training protocols, lesion volumes, animal model, etc. For example, in comparison to the classical mapping study conducted by Nudo et al. in 1996, our lack of map size expansion in the injured hemisphere matches what they observed, where individual representations increased or decreased, but overall map size remained the same following skilled forelimb training (59). As our mapping experiments quantified overall forelimb movement rather than distinguishing between distal vs. proximal representations, these results fit neatly into the exemplar set by Nudo and colleagues. However, Okabe et al. described an increase in RFA map size in the injured hemisphere following rehabilitative training (84). Correspondingly, our lack of overall map size increase (which encapsulates both the RFA and CFA, although we did not explicitly differentiate between the two due to a lack of non-responsive border between the areas in our maps) does not align with these findings. One notable difference between our model and this study is the size of lesion induced. After all, the majority of our strokes almost completely ablated M1, with the majority of lesions extending into subcortical areas as well. It is possible that with the pure extent of ablation in this hemisphere, there simply is not enough tissue to support further reorganization following training. Overall, while the adult stroke literature can provide important context and background for findings in perinatal stroke experiments, overt comparisons should not be interpreted too critically. Ultimately, the perinatal stroke field displays its own set of neurophysiological characteristics and should be interpreted independently.

So, how exactly do our results compare to those in the perinatal stroke/developmental injury field specifically then? Unfortunately, there is a relative paucity of training-induced motor map reorganization in the developmental injury field. Moreover, key differences between our study and those that do exist limit the direct comparison of results. In one preclinical study, rats which sustained aspiration lesions in the prefrontal cortex at P10 displayed map expansion in the injured hemisphere after skilled reach training (71). The major difference between this study and ours is similar to the issue of large vs. small lesions as above. Specifically, while the prefrontal cortex lesions did distally reduce M1 representations, ultimately the motor cortex tissue was still intact (71). Because our perinatal model also shows training-induced ipsilesional reorganization after small/distal lesions, our findings supplement the idea of a minimum amount of required tissue to support further motor map plasticity. A clinical TMS mapping study in perinatal stroke patients echoed these results. In this study, Friel et al. described map expansion in both the uninjured and

injured hemisphere following skilled hand training (52). Notably, investigators only gathered maps from one hemisphere in each patient, where individuals who could not elicit injured/contralateral hemisphere movements were instead measured for uninjured/ipsilateral hemisphere maps. On the one hand, it's difficult to definitively conclude whether our results match with these considering the lack of bilateral maps. On the other though, in those without contralateral maps, training was still not able to restore representations in this hemisphere. In other words, individuals with very "reduced" injured hemisphere motor maps showed no training-induced expansion, whereas those with viable tissue (in the form of TMS-receptive tissue) did. As such, this further supports the idea that our model recapitulated findings from a specific subset of the clinical population – i.e. those with larger/more severe lesions.

4.5.2. Single pellet training partially restores delays in movement latencies

Unlike map size, single pellet training had a much larger effect size on movement latencies. Specifically, while contralaterally-evoked movements maintained faster latencies than those in the ipsilateral hemisphere following training, both hemispheres displayed a significant reduction in latency, indicating faster conduction times with training. Importantly, this reduction was not observed in the sham animals, indicating a stroke-specific effect.

First, it is interesting to note the robust and bilateral effects in latency in comparison the relatively small increase in map size (and only within the uninjured hemisphere at that). As mentioned above, we speculate that perhaps our training was not intensive enough to trigger changes in map area, especially considering our animals were only engaged in the task for 10 minutes a day for three weeks. From a clinical perspective, 10 minutes of on-task rehabilitation is most likely insufficient to result in meaningful functional recovery (169). Functional performance aside however, with these results, it is clear that even this limited amount of training was able to change some aspect of the descending motor conduction pathways. As such, perhaps the effect we observed was a "priming" of the motor system, upon which more intensive training can build to result in persisting cortical motor reorganization and/or actual improvement in behavioural performance. Alternatively, the disparity between latencies vs. map size may also be a result of the physiological means with which these changes were achieved. Ostensibly, if these latency reductions were a result of anatomical changes (such as increased fibre diameter or improved myelination), the single pellet-induced effects may be more persistent than those seen in the more dynamic synaptic environment of the cortex. After all, it has been previously shown that motor

maps have the capacity to change very rapidly, and require continuous maintenance (ex. through experience-dependent input or protein synthesis) to maintain its organization (123,170–172).

Building off of the idea that these reductions in latencies were achieved through anatomical means, there are a few candidates for which descending pathways mediated these changes. First, there is the possibility that the single pellet training strengthened the normal (though initially ablated) descending pathways prior to the single pellet training. In both adult and perinatal human stroke patients, the importance of the CST to functional recovery has been shown with both anatomical (ex. MRI imaging) and physiological evidence (ex. TMS) (49,155,173–177). Indeed, one of the more explored pharmacological treatments is Anti-Nogo immunotherapy, which putatively aids corticospinal growth and improves functional outcome through suppression of the Nogo-A protein (178–180). In the neonatal rat, Monfils et al. found that administration of fibroblast growth factor (FGF-2) not only restored stroke-induced delays in movement latency from the injured hemisphere, but that there was also increased CST sprouting from this hemisphere. In face of the extensive precedence from human studies and rat models, it is tempting to attribute any spontaneous, rehabilitation, and treatment-induced recovery in mouse models to the CST as well. However, it is also important to consider the role of the RtST, especially considering its putative role in movement circuits in mouse forelimb motor control (97). As such, instead of reinforcing only the CST, it may be more likely that our single pellet training protocol strengthened the RtST as well. Considering that the RtST mediates short latency descending volleys, our partial restoration of stroke latencies to sham speeds could provide evidence for this hypothesis. Additionally, there have been extensive reports of RtST involvement in both human and rat models of stroke and spinal cord injury, where the RtST has a comparatively lesser role relative to the mouse (181–185).

A second possibility is the reinforcement of lesser-used circuits, such as the rubrospinal tract (RST), corticopontine tracts, or tectospinal tracts. With respect to the RST, this extrapyramidal tract was initially an attractive candidate for rewiring after stroke in humans due to its complementary functional role in unilaterally innervating distal muscles (98). Interestingly, while there is extensive anatomical evidence for novel rubrospinal tract wiring after perinatal stroke itself, there is a dearth of similar data following rehabilitation, even in adult stroke models. That said, there have been a few reports of increased rubrospinal tract sprouting after Anti-Nogo treatment in rat models of stroke (186,187). Thus, it is possible that although activity-dependent

interventions may preferentially stimulate RtST rewiring, other treatments (such as pharmacology, or even brain stimulation) may still encourage RST rerouting. Similarly, there is also evidence for plasticity in the corticopontine and tectospinal tracts, where increased fibre density has been observed in the pontine nucleus and superior colliculus following developmental neurological injury including, but not limited to, aspiration lesions, strokes, hemidecortications, pyramidotomies (37,82,188). Thus, although not yet concretely implicated in recovery after rehabilitation/training, these tracts all clearly show the capacity for plasticity.

4.5.3. Training-induced map and latency plasticity correlate in the uninjured hemisphere

After the stroke itself, we showed a significant positive correlation in the injured hemisphere in both the cylinder and single pellet tasks. These results suggested that without intervention, contribution from the injured hemisphere may be more important to functional outcome after stroke compared to the uninjured hemisphere. However, we wanted to observe whether this relationship held after motor training, particularly since between-timepoint change in latencies and map size could comment more concretely on plasticity, rather than just impairment as shown in the one-timepoint correlations from earlier. Interestingly, when correlating the change in map size and latency pre- vs. post-single pellet, we found that an expansion in contralesional map size corresponded with latency reductions in only the uninjured hemisphere. However, perhaps this positive correlation could be construed as a "contralateral hemisphere" effect, rather than a specific beneficial role of the uninjured hemisphere. As such, we compared the changes in map size and latency in the ipsilesional limb representations as well. Here, we found the same significant relationship. Importantly, this effect was still relegated to the uninjured hemisphere. This interrelation of changes in two separate aspects of the motor system, regardless of limb, suggests that the uninjured hemisphere has an increased potential to mediate plasticity after training. This differs from the prior correlations before single pellet training, where the degree of impairment in the injured hemisphere correlated more strongly with functional outcome. One implication is that hemispheric contribution may actually differ between spontaneous vs. training-induced recovery. At the very least, our findings provide evidence for the idea that interhemispheric balance and output is complicated, where one theory cannot explain all manners of recovery post-stroke.

5 – Conclusion

5.1. Significance

Currently, there is an unclear understanding of how perinatal stroke affects cortical neurophysiology, and how this relates to functional recovery. In our study, we aimed to clarify both of these aspects individually, as well as the relationship between the two. As a result, we were the first to characterize 1) behaviours across a battery of sensorimotor tests, 2) cortical motor representations in a preclinical focal model of perinatal stroke, and 3) the effect of further motor training on stroke-induced map changes.

Within our behavioural descriptions, we showed longitudinal gait and upper limb impairments similar to those seen at the clinical level. Furthermore, within the motor mapping portion, our use of optogenetic motor mapping allowed the first within-subject measure of motor representations following skilled motor training following developmental injury. The importance of these results is twofold. First, we provided an important point of replication for other developmental aspiration lesion experiments. In this manner, we echoed other aspiration mapping findings in that perinatal stroke also disrupted perilesional maps and induced descending cortical pathway changes. Interestingly, we have also shown that our stroke protocol also caused similar disruptions in the uninjured hemisphere as well, implicating a widespread depressive effect of the perinatal injury. Second, we also demonstrated capacity for further modulation of stroke-induced map/latency changes with a single pellet training regimen. These results not only confirm the capacity for recovery of cortical neurophysiology and descending motor pathways at the chronic phase but may also offer a potential mechanism for the bimanual/motor therapies currently implemented at the clinical level.

The last question we aimed to address in this study was hemispheric contribution after perinatal stroke. Our findings first described a significantly beneficial role for the injured hemisphere, in addition to either a neutral or beneficial involvement of the uninjured hemisphere with functional outcome. These results suggest that the theory of maladaptive control from the uninjured hemisphere may not be as robust as previously posited. Secondly, we showed that further map changes post-motor training was contrastingly mediated more strongly by the uninjured hemisphere. Overall, in line with sentiments echoed by other researchers, the present study suggests a more nuanced relationship between hemispheric output and functional outcome that may depend on various factors such as task, location of lesion, and training.

5.2. Limitations & Future Directions

As touched upon briefly in the discussion, there remain a few key limitations and future directions for this project.

5.2.1. Lesion location modulations

On average, the lesions induced with our PT stroke protocol were quite variable. However, the majority of cases ablated cortical tissue down to the corpus callosum at the centre of the infarct and disrupted the white matter tracts into the striatum. It is possible that our combination of cortical and subcortical lesions is contributing the robust bilateral map and latency effects (both pre- and post-training). Unfortunately, the present study did not systematically explore the effect of perinatal cortical vs. subcortical injury on motor maps and behaviour. As such, one viable future direction is to have explicit experimental groups to pursue this question, particularly since subcortical strokes make up a significant portion of clinical perinatal stroke cases (though not as prevalent as MCA-bed/cortical injuries) (16).

5.2.2. Kinematic Behavioural Analysis

Kinematics is an important follow-up to our current skilled forelimb reaching results. First, the field of clinical stroke recovery as a whole is moving towards kinematic analysis (189). Second, qualitative assessment of movement can further clarify the impairments that we have already described in the single pellet reaching task. Specifically, information such as endpoint variation, trajectory lengths, and paw velocity could inform why we saw differences in reaching attempts but not overall success rate. A recent kinematic analysis of reaching behaviour in healthy adult mice showed that late-phase kinematics (including both position and velocity trajectories) correlated more strongly with reaching success compared to early-phase adjustments (190). As such, it would be interesting to see whether this relationship also holds in our perinatal stroke model. With the use of automated computer programs that can estimate body positions and kinematics with both marker and marker-less tracking (191–193), all these aforementioned aspects of reaching can be addressed.

5.2.3. Motor Mapping

One shortcoming of our study is the relatively limited number of timepoints for sampling motor map reorganization. After all, one of the most powerful advantages of the optogenetic technique and chronic window preparation is the ability to non-invasively map at a multitude of

timepoints. Particularly with our observation of bilateral reduction after perinatal stroke, it would be interesting to see how this effect evolves at more acute and chronic timepoints. Although there is a theoretical age minimum for chronic window implantation (as animals may "outgrow" windows), this has not yet been definitively tested in our lab. Thus, it could be worthwhile to determine the exact minimum age/size such that window integrity is not compromised with head growth. With windows at an earlier age, we could not only measure the change of perinatal stroke-induced maps, but also the development of naïve maps in general (as there are only currently a few such descriptions).

A second avenue of exploration would be to elaborate upon our bilateral motor map ablation findings. To test for diaschisis effects specifically, we could pursue *ex vivo* electrophysiological techniques to test the synaptic connectivity of this tissue. Alternatively, *in vivo* paired pulse protocols (as used by (94)) may be a promising avenue to measure both inter- and intrahemispheric connectivity. Following these descriptive measures, more mechanistic avenues could be explored, where we could test for a causal relationship of various neurotransmitter systems that have been previously implicated in motor map reorganization (ex. GABA, acetylcholine, dopamine) (65,116,194).

5.2.4. Single Pellet

As mentioned in the methods section, a total of 8 animals were excluded due to the inability to acquire reaching behaviours. One potential explanation for this phenomenon was the "forced" use of the contralesional limb across all animals. Typically, in adult single pellet reaching studies, the animal is tested for limb preference in the early phases of shaping. In our study, we instead designated the reaching limb to be the contralesional limb from the beginning of the protocol. Moreover, we actively discouraged reaching with the other limb by taking away pellets when this occurred. It is possible that within the animals which did not reach, the contralesional limb was not the preferred limb of use. One reason for choosing the same limb within all animals was first to maintain the exact same conditions between treatment groups, excepting the actual induction of the lesion. A second reason is that for this study, we were particularly interested in how motor training would affect the injured hemisphere, as the majority of existing adult stroke studies primarily focus on perilesional map organization. Within adult stroke studies, lesions are often induced after a baseline training period, and thus can be modulated appropriately to the preferred limb. In a perinatal stroke model however, this is not feasible, as only the most basic of motor

behaviours have been established by P7. As such, with the lesioned hemisphere pre-determined, we opted to train only the contralesional limb in order to maximize the effect of our reaching protocol.

It is possible that training the ipsilesional limb could still have yielded perilesional reorganization. It has been shown previously that reach training is capable of modulating map representations in not only the contralateral hemisphere, but also the ipsilateral hemisphere (63,195). However, there is also evidence within adult stroke studies that training of the ipsilesional/non-injured limb detrimentally affects not only behaviour, but also perilesional neurophysiology (such as synaptic density, fosB activation, cortical motor representations) (195–198). Although these interactions have not yet been explored in a perinatal stroke model, we deemed it more pertinent to first observe how contralesional limb training affected perilesional representations, before varying limb use during single pellet training.

With these excluded animals, it was an option to include them as a non-reaching control. However, we did not pursue this option as we could not definitively explain why these animals failed to reach. Without a concrete explanation, it was difficult to ascertain whether map differences between reaching vs. non-reaching animals were truly due to a lack of reaching, or instead due to another confounding factor. With this in mind, our lack of non-reaching control is certainly a limitation. In our experimental design, it is arguable that having sham animals similarly undergo this reaching protocol (and display no changes) partially mitigates this weakness. Additionally, there is evidence that in naïve, adult animals at least, maps remain stable between weeks to months (150,157). However, we recognize that without a true non-reaching control, our conclusions about our single pellet-induced map changes may be diluted somewhat.

5.2.5. Anatomy

Following our latency and map size measurements after both stroke and single pellet training, an obvious follow-up question is how either of these changes are being mediated. Particularly with the latency measurements, our present experiments can only position us to speculate which descending pathways may be causing the delays/restorations in movement latencies from either hemisphere. For future directions, perhaps the most straight-forward follow-up experiments would be to employ tracing or immunohistochemistry (IHC) techniques. For example, IHC staining for delta-fosB proteins could help identify whether certain cortical areas or midbrain/brainstem nuclei were more involved over others (ex. red nucleus, reticular formation,

pontine nucleus). From these results, we could then further target more involved/time-intensive tracer experiments.

5.2.6. Post-single pellet

In addition to further anatomical experiments, it would also be worthwhile to add a measure (or multiple) of behavioural outcome following the single pellet training. As mentioned in the discussion, while we observed these physiological changes with our motor training protocol, it is unknown whether they would translate to functional improvement in other motor domains. In adult stroke studies, functional recovery is often quantified as the change in performance pre- vs. post-stroke. In a perinatal model, this is clearly not possible due to developmental limitations at the time of stroke induction. In our model, behaviour could instead be measured as pre- vs. post-single pellet. Within this option, recovery could be addressed by reconducting the battery of behavioural tests already used pre-single pellet (ex. adhesive removal, tapered beam, Digigait). However, our deficits observed in these tests were already subtle, so it is possible that we would not see any significant effect size with rehabilitation training. Additionally, it has been shown that recovery is specific to the task chosen for rehabilitation (69,84,199). Perhaps a better alternative is to instead quantify performance on another skilled forelimb reaching task pre- and post-single pellet (such as the pasta handling or Montoya staircase task). With functional outcome concretely tested after the single pellet task, we could more definitively comment on whether our physiological changes were actually functionally relevant.

5.2.7. Sensory mapping

Although the primary focus of this study was motor function and representations following perinatal stroke, it would also be interesting to measure how sensory function/representations change after perinatal stroke. From a clinical perspective, sensory impairments can also be present after perinatal stroke, though not as widely reported as motor dysfunction (200,201). These impairments can manifest in a variety of ways, including in proprioception, tactile discrimination, and stereognosis (200–203). In our study, we indirectly measured sensory function in the adhesive removal test but did not employ any other sensory-specific behavioural test. This decision was made partially due to the desire to align our results with the large clinical and preclinical evidence base on motor impairments and motor mapping. A secondary reason was the relative dearth of sensitive and reliable sensory tests, with the von Frey hairs test being one of the only robustly validated behavioural tests for mechanical sensation (204). Overall, it remains a challenge in the

preclinical field to test sensory behaviours, as clinical tests can rely on self-reporting, whereas animal research must find alternative sensitive, yet objective, means.

While sensory behavioural testing is not as feasible in a mouse model, there are means of testing sensory physiology. For example, sensory representations can be reliably measured by measuring blood flow changes following sensory stimulation. In our study, we achieved this through single-point laser flowmetry. With these measurements, we showed that the magnitude of sensory-evoked increase in CBF was reduced in stroke animals relative to sham. However, the scope of these measurements was limited to only the primary somatosensory cortex in one hemisphere at a time, primarily due to the low spatial resolution of the technique. In light of the bilateral reductions in motor maps, it would be more useful to be able to gather bihemispheric data on CBF responses during paw stimulation. Techniques such as intrinsic optical signaling or laser speckle contrast imaging could offer further insight into how sensory maps reorganize. Importantly, it could also shed light on whether sensory map encroachment into motor map areas is the cause for the perilesional ablation observed, as shown previously in adult stroke animals (150).

6 – Supplemental Figures and Data

Appendix A: Behaviour

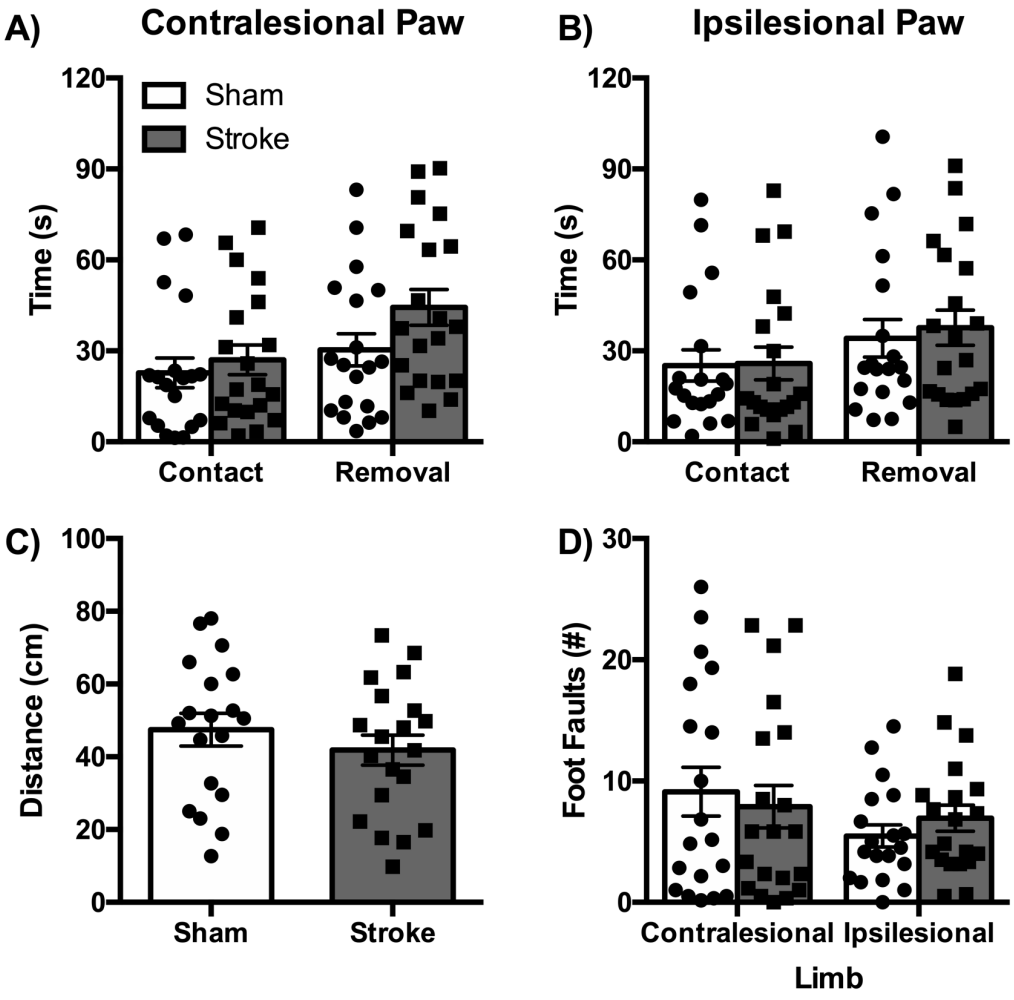

Figure S 1. Behavioural outcomes from battery of sensorimotor tests. A & B) Time to contact and remove adhesive tape during the adhesive removal test from the contralesional **(A)** and ipsilesional **(B)** paw. **C)** Distance to first foot fault (cm) during tapered beam task. **D)** Number of foot faults on the contralesional vs. ipsilesional side. All data is presented as mean ± SEM for sham (white, n=19) and stroke (grey, n=20) groups.

In addition to adhesive measures in the contralesional forelimb (see section 3.2), Figure S1B also shows time to contact and remove the adhesive tape from the ipsilesional forelimb. Mean time to contact the adhesive tape on the ipsilesional paw in sham and stroke animals was 25.2 ± 5.1 s and 25.9 ± 5.4 s, respectively. An unpaired t-test showed no significant difference between groups ($t(37) = 0.094$, $p = 0.93$). Similarly, there was also no significant difference in time to remove the tape (sham: 34.1 ± 6.2 s, stroke: 37.6 ± 5.8 s; $t(37) = 0.41$, $p = 0.68$, unpaired t-test).

Appendix B: Motor maps

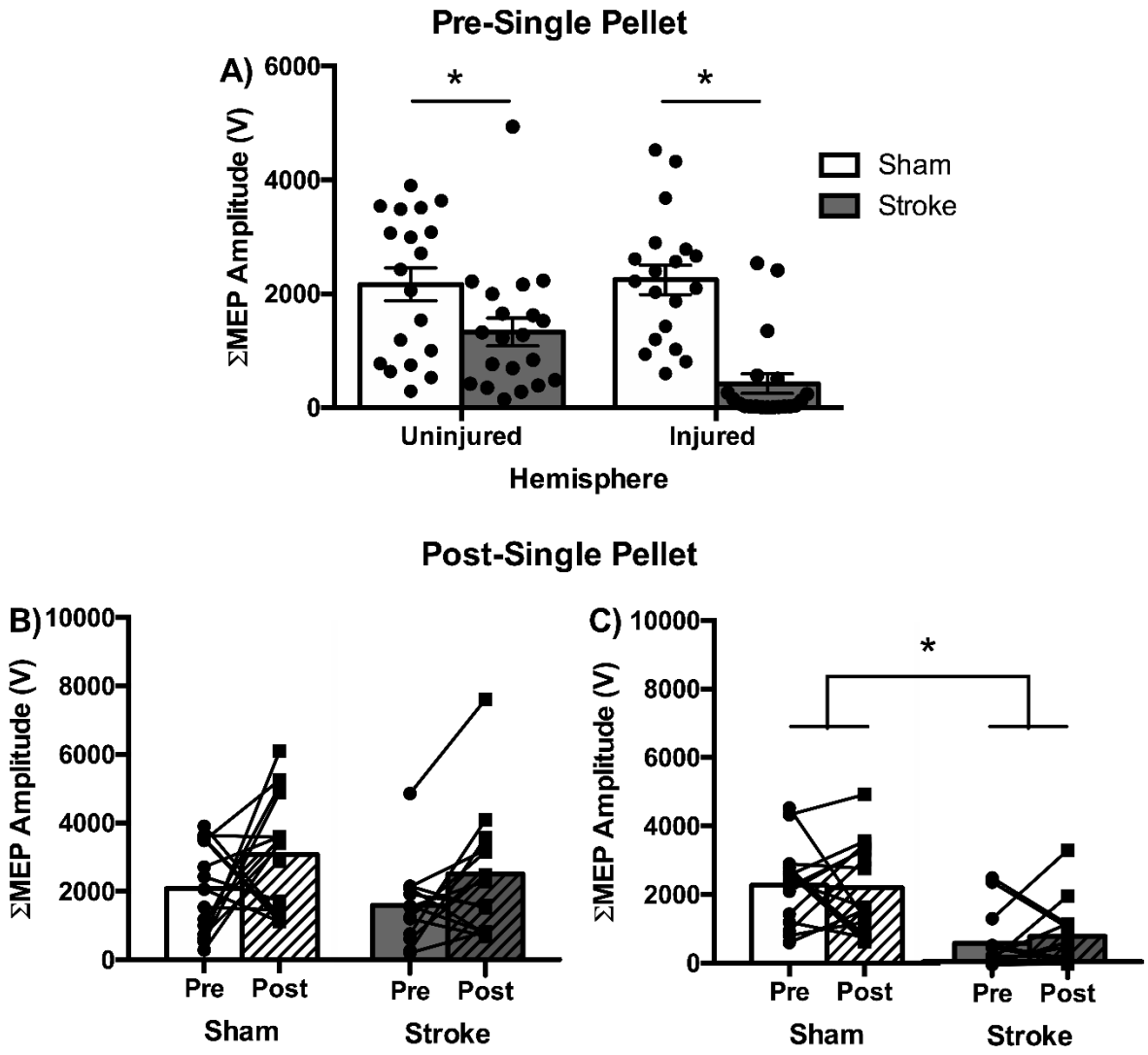

Figure S 2. Motor map output pre- and post-single pellet reaching task. A) Motor map output for the contralesional limb (as calculated as the sum of normalized movement amplitudes for either hemisphere) in sham (white bars, n=19) and stroke animals (grey bars, n = 20). **B & C)** Motor map output at both pre- (solid bars) and post-single pellet (dashed bars) timepoints for both the uninjured **(B)** and injured **(C)** hemispheres. Sample size is reduced from (A) due to exclusion of animals which failed to acquire reaching behaviours (sham: n = 15, stroke: n = 13; total excluded: n = 11). Data points for the same animal pre and post are connected by lines. All data is presented as mean ± SEM; significance of $p < 0.05$ is denoted by an asterisk (*).

At the pre-single pellet timepoint with the full sample size (Fig. S2A), there was a significant interaction between hemisphere and treatment (i.e. stroke vs. sham) ($F(1,37) = 7.65$, $p = 0.009$, RM ANOVA). A Sidak post-hoc test showed specifically that there was a reduction of

map output of stroke animals compared to shams in both the injured ($p = 0.00038$) and uninjured hemisphere ($p = 0.033$). Mean map output in the sham animals in the uninjured and injured hemispheres was 2164.2 ± 270.6 V and 2245.3 ± 220.0 V, respectively. In the stroke animals, uninjured vs. injured hemisphere map output was 1326.0 ± 263.8 V and 418.8 ± 214.5 V. Thus, similar to the effects seen in map size, map output was also bilaterally reduced following perinatal stroke.

In the reduced sample size for pre- and post-single pellet analysis, results slightly differ from map size changes. Firstly, in this subset of animals, there was no reduction in map output between the stroke vs. sham animals in the uninjured hemisphere (Fig. S2B; $p = 0.388$, Sidak's test). Furthermore, there was no effect of single pellet training, as means in both sham and stroke pre/post single pellet training did not differ (sham: $p = 0.088$; stroke: $p = 0.14$, Sidak's test). In the injured hemisphere, reduction of map output between shams vs. strokes was maintained at both the pre- and post-single pellet timepoint (post: $p = 0.005$, Sidak's test). However, again, there was no effect of single pellet training on either sham or stroke animals (sham: $p = 0.84$; stroke: $p = 0.61$, Sidak's test). These results match the effects seen in map size, where single pellet did not restore stroke-induced reductions in map size.

Appendix C: Behaviour-Map Correlations

Behavioural Test	Behavioural Outcome	Uninjured Hemisphere (R^2, p-value)	Injured Hemisphere (R^2, p-value)
Cylinder	% Contralesional Preference upon Landing	0.0076 (p = 0.29)	0.0017 (p = 0.80)
Adhesive	Contralesional Contact	0.0096 (p = 0.55)	7.65×10^{-6} (p = 0.97)
	Contralesional Removal	0.045 (p = 0.19)	0.025 (p = 0.33)
	Ipsilesional Contact	0.00023 (p = 0.93)	0.0046 (p = 0.68)
	Ipsilesional Removal	0.023 (p = 0.87)	0.0068 (p = 0.62)
Tapered Beam	Contralesional Foot Fault	0.00015 (p = 0.94)	0.021 (p = 0.77)
	Ipsilesional Foot Fault	0.0083 (p = 0.58)	0.0052 (p = 0.66)
	Distance to First Foot Fault	0.0089 (p = 0.57)	0.0056 (p = 0.65)
Single Pellet	Overall Success Rate	0.023 (p = 0.44)	0.0072 (p = 0.67)

Table S 1. Correlations of map size and behavioural test outcomes. Contralesional forelimb map size was correlated with behavioural outcomes from sham (n=19) and stroke groups (n=20) for the cylinder, adhesive, and tapered beam test. Single pellet overall success rate was derived from a reduced sample size due to failure to acquire reaching behaviours (sham: n = 15, stroke: n = 13).

Parameter Contralesional:Ipsilesional Ratio	Uninjured Hemisphere (R^2, p-value)	Injured Hemisphere (R^2, p-value)
Swing (s)	0.019 (p = 0.40)	0.083 (p = 0.075)
% Swing/Stride (ratio)	0.018 (p = 0.41)	0.082 (p = 0.077)
Propel (s)	0.0094 (p = 0.56)	0.052 (p =0.16)
% Propel/Stride (ratio)	0.013 (p = 0.49)	0.071 (p = 0.10)
Stance (s)	0.0055 (p = 0.65)	0.032 (p = 0.28)
% Stance/Stride (ratio)	0.018 (p = 0.41)	0.081 (p = 0.079)
% Stance/Swing (ratio)	0.0034 (p = 0.73)	0.012 (p = 0.51)
Stride (s)	0.032 (p = 0.28)	0.10* (p = 0.048)
Stride Length (cm)	0.0032 (p = 0.73)	0.014 (p = 0.47)
Stride Frequency (stride/second)	0.020 (p = 0.39)	0.040 (p = 0.23)
Forelimb:Hindlimb Ratio		
Paw Angle (˚)	0.019 (p = 0.41)	0.011 (p = 0.52)
Paw Angle Variability	0.036 (p = 0.25)	0.019 (p = 0.40)

Table S 2. Correlations of map size and Digigait outcomes. Contralesional forelimb map size was correlated with predetermined Digigait parameters for sham (n = 19) and stroke (n = 20) animals. Digigait data was first pooled across limbs (i.e. contra- vs. ipsilesional and fore- vs.

hindlimbs), since significance was found in these groups in the initial RM ANOVA. For ease of data presentation, the parameters were then correlated with map size as ratios between contra- vs. ipsilesional or fore- vs. hindlimb. Significance of $p < 0.05$ is denoted with an asterisk (*).

Appendix D: Laser Doppler Flowmetry

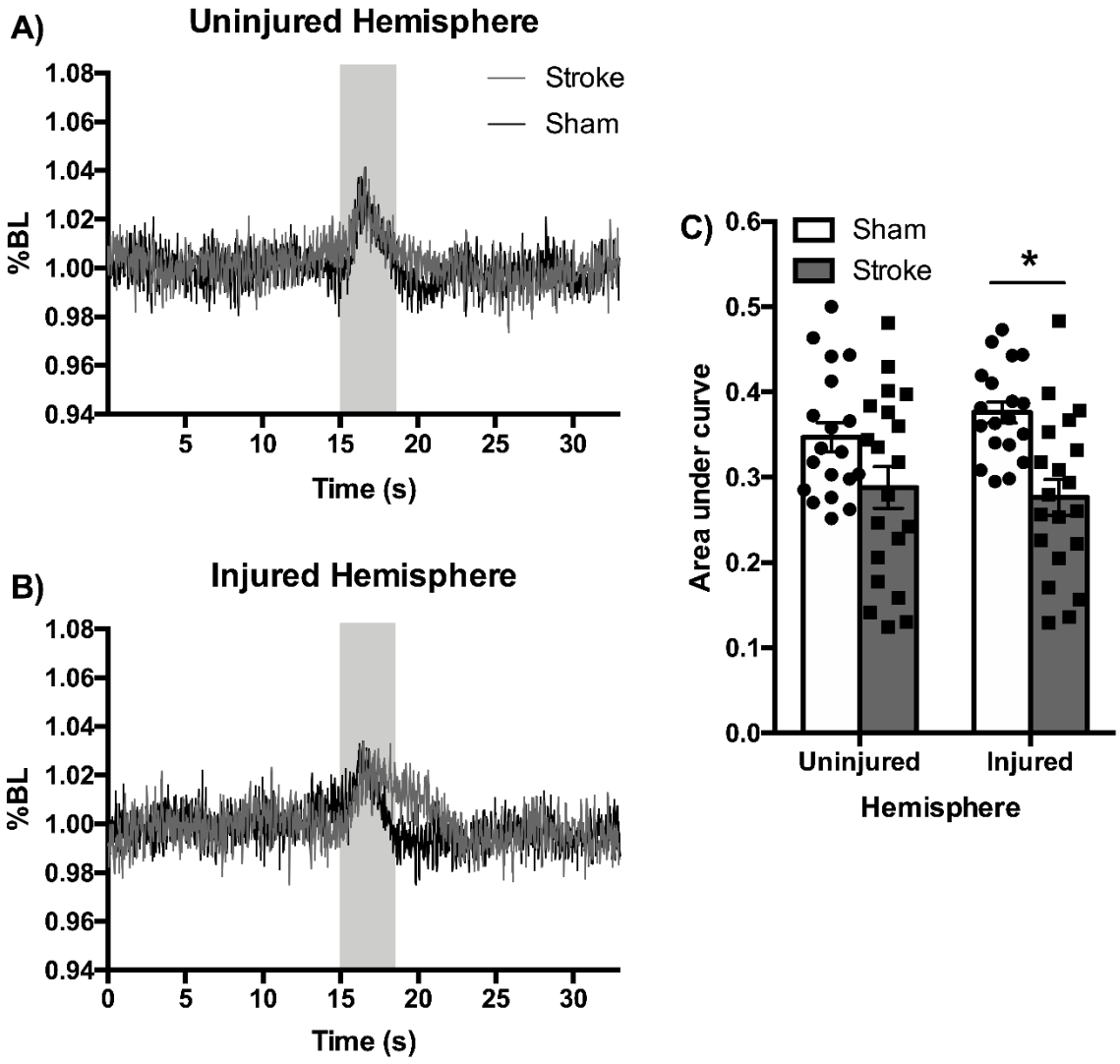

Figure S 3. Laser-Doppler flowmetry recordings during sensory stimulation. A & B) Single-point recordings of CBF were measured from the S1 contralateral to either the ipsilesional **(A)** or contralesional paw **(B)**. Recordings were measured over 20 cycles of stimulation (15s no stimulation baseline, 3s stimulation (shaded grey box), 15s post-stimulation baseline), then normalized to the first 15s of no-stim baseline. **C)** Quantification of the area under the curve during the 3s of stimulation, in order to compare the sensory stimulation-evoked increase in CBF in shams (grey bars/traces, n=19) vs. stroke (white bars/black traces, n=20). Data is presented as mean ± SEM, with significance of $p < 0.05$ denoted with an asterisk (*).

In addition to mapping the motor representations, a form of "sensory mapping" was also achieved using single-point laser doppler flowmetry of the S1 during sensory stimulation. Through visual observation, the averaged CBF traces from the uninjured hemisphere did not differ between

the stroke vs. sham groups (Fig. S3A). Contrasting this, the injured hemisphere seemingly has a delayed return to baseline CBF following stimulation (Fig. S3B). In order to concretely quantify the differences between groups in both hemispheres, the area under the curve of the CBF traces during the 3s of sensory stimulation was calculated and plotted for each individual animal (Fig. S3C). In the uninjured hemisphere, there was no significant reduction in the CBF peak (sham: 0.35 ± 0.02, stroke: 0.29 ± 0.02; $t(37) = 1.96$, $p = 0.058$, unpaired t-test). In the injured hemisphere, CBF was significantly decreased (sham: 0.38 ± 0.012, stroke: 0.28 ± 0.021; $t(37) = 4.04$, $p = 0.00026$, unpaired t-test). Thus, these results show that not only was there a delay in the sensory-evoked increase in CBF in the injured hemisphere, but also the absolute magnitude of this increase during the 3s of stimulation was smaller compared to sham animals.

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