INVESTIGATING LESION VOLUME AND LOCALIZATION EFFECTS ON DEVELOPMENTAL AND ADULT MOTOR IMPAIRMENTS IN A MOUSE MODEL OF PERINATAL STROKE.

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ABSTRACT:

Perinatal stroke is a subtype of stroke causing substantial lifelong morbidity in children often presenting as neurodevelopmental delays and sensorimotor impairments. Rodent models that present long-lasting motor impairments after perinatal stroke can be used to evaluate the relationship between injury parameters (size and location) and behavioural function. On postnatal day 7, a unilateral photothrombotic stroke was induced in the primary motor cortex of mice. To evaluate sensorimotor function during early development and adulthood, we used a series of behavioural tests. We hypothesized that pre-weaning behavioural tests will provide a direct measure of sensorimotor deficits induced by the stroke, while behavioural tests in adulthood will be influenced by endogenous plasticity and recovery processes. Interestingly, we found that both pre-weaning and adult behavioural assays revealed impairments. In the pre-weaning tests, impairments were observed in grip strength and hindlimb suspension tests. During adulthood, stroke animals demonstrated significant impairments in the contralesional limb in both the grid walk and the cylinder tests. Furthermore, performance on the grid walk test had a significant positive correlation between the number of miss-steps by the impaired forelimb with lesion volume. Finally, using a semi-automated lesion localization workflow, we were able to characterize the extent of injury with precision to understand its impact on various brain regions and its correlation to functional outcomes. The severity of injury in different brain regions proved to be significant for outcomes observed in the grid walk test. Overall, our perinatal stroke model produced long-lasting impairments that can be detected using both pre-weaning and adult behavioural assays. By performing precise characterization of the lesion, we were able to demonstrate that lesion size and location can be used as relevant predictors of perinatal stroke outcome.

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LIST OF ABBREVIATIONS:

ACC – Anterior cingulate cortex

ANOVA – Analysis of variance

AMBA – Allen mouse brain atlas

CIMT - Constraint-induced movement therapy

CP – Cerebral palsy

CST – Corticospinal tract

HASRA – Home-cage skilled reaching apparatus

HI – Hypoxia-ischemia

MCAo – Middle cerebral artery occlusion

MRI – Magnetic resonance imaging

M1 – Primary motor cortex

M2 – Secondary motor cortex

PT – Photothrombosis

RFID – Radio frequency identification

SEM – Standard error of the mean

TMS – Transcranial magnetic stimulation

t-PA - Tissue-plasminogen activator

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INTRODUCTION:

1.1 Perinatal stroke

Perinatal stroke is defined as a stroke occurring between 20 weeks of fetal life to 28 post-natal days of life. It is estimated that it occurs in 1:1600-1:2300 live births, presenting a high risk of occurrence (Dunbar & Kirton, 2019). Perinatal stroke encompasses a range of subtypes of stroke with focal injury, which are further classified according to the mechanism that induced the stroke (ischemic or hemorrhagic), the time of occurrence (pre-natal or neonatal), and when symptoms were first observed. When symptoms present acutely (within days after birth), the injury is classified as acute perinatal stroke. On the other hand, patients presenting symptoms later in infancy (in the first year of life or later) fall into the presumed perinatal stroke category.

1.2 The evolution of the concept of brain plasticity in the immature brain

There exists a historical notion in developmental injury studies that younger brains have a greater capability to recover from an injury due to their highly dynamic nature at a developmental stage. This principle originated from a study led by Margaret Kennard in 1936, where she investigated factors affecting recovery of motor function measured with behavioural observations following pre-motor and motor brain lesions in monkeys (Kennard, 1936). In this study, factors such as age, lesion size, site and effect of the time interval between operations were revealed to affect recovery following injury. Particularly, Kennard noted that young monkeys recovered motor function at a much faster rate than adult monkeys, but if given enough time, adult monkeys could also attain similar recovery. However, the scientific community focused on the finding that age heavily influenced the overall recovery and speed of recovery of motor function in primates and dubbed this the Kennard Principle. This proposition suggested that the concept of brain plasticity played a critical role in outcome after neonatal brain injury, and established a foundation for quantifying injury-induced plasticity in psychology and neuroscience studies. However, evidence challenging this notion came to light following the original Kennard publication from 1936. In 1971, Goldman investigated the effects of dorsolateral or orbital prefrontal cortex lesions in infant and adolescent monkeys and found that age did not always enhance recovery (Goldman, 1971). Notably, when comparing same age-group monkeys with different lesion location (orbital versus dorsolateral), they found that monkeys with orbital lesions had significantly better recovery outcome over time than monkeys

with dorsolateral lesions. This emphasizes the important role that lesion location may have in predicting the potential of overall recovery. Another study to consider was the work done by Murphy and Stewart in 1974, where they observed no significant effects of age in the performance of adult and infant rabbits on a visual discrimination task following lesions in the primary visual cortex (Murphy & Stewart, 1974). Specifically within the framework of perinatal stroke, evidence against this principle is also present. In fact, it has been observed that brain injury occurring during the perinatal time frame causes worse morbidities compared to stroke occurring later in childhood (Kirton & Deveber, 2013; Lansing et al., 2004).It could be argued that this is due to the higher susceptibility of the developing brain to oxidative stress and neuronal apoptosis making it more vulnerable to injuries, as demonstrated by numerous studies in recent years (Bayir et al., 2006; Blomgren & Hagberg, 2006; Ikonomidou & Kaindl, 2011; Potts et al., 2006). Developmental injuries are thus believed to be the most damaging, as they often result in significant long-term neuropsychological and sensorimotor impairments (Kirton & Deveber, 2013). Given the heavy focus of research in adult stroke models in the field, it is critical to give further attention to the differences between adult and perinatal stroke to better assess perinatal stroke.

1.3 Differences between perinatal and adult stroke

1.3.1 Process of diagnosis

To begin with, adult and perinatal stroke have divergent processes of diagnosis. In adults, it is often the case that symptoms manifest immediately or shortly after stroke, in the form of sudden numbness or weakness in the face, arm, or leg — especially on one side of the body. Other symptoms include sudden dizziness, loss of balance or coordination (*Stroke*, 2023). In contrast, it is significantly more challenging to diagnose perinatal stroke as symptoms are often more difficult to detect. In acute perinatal stroke patients, symptoms may manifest in the form of focal motor seizures or encephalopathy (Basu, 2014). However, it can be difficult to distinguish between small seizures and newborn body movements. If there is a lack of clinical suspicion and Magnetic Resonance Imaging (MRI) testing availability, the risk of a perinatal stroke going undiagnosed becomes more significant. In presumed perinatal stroke cases, symptoms only present months following stroke, manifesting in the form of delayed developmental milestones

(rolling over, crawling, etc.), pathological early hand-preference or seizures (Ferriero et al., 2019). Such symptoms may also be difficult to detect, as they can be mild. In some cases, patients may be asymptomatic, and are only able to find out about the injury post MRI testing. Given the variability in the timing and presentation of symptoms in patients with perinatal stroke, it quickly becomes a daunting challenge to obtain a timely diagnosis of the disease.

1.3.2 Post-stroke treatment options in adults following stroke

Established treatments for adult stroke underscore the critical importance of prompt intervention to minimize negative outcomes. In contrast, the delay in diagnosis for children with perinatal stroke prevents the application of immediate interventions, which further limits the potential of applicable therapies (Basu, 2014). In adult cases, treatments for ischemic stroke include acute and non-acute therapies and prevention strategies (Campbell et al., 2019; Campbell & Khatri, 2020). The most often employed treatment acutely is the use of thrombolytic drugs, such as tissue-plasminogen activator (t-PA), which treats the infarct by dissolving the blood clot responsible for the blockage of blood flow to the brain (Ferriero et al., 2019). Another established treatment is the thrombectomy method, where blood flow is restored by the removal of the blood clot from an artery or vein (Ferriero et al., 2019). There are two types of thrombectomy, one is surgical (surgeons need to make an incision to get to the obstructed blood vessel before cutting it open to remove the blood clot), while the other one is less invasive (percutaneous procedure, special devices can be inserted through catheter to suction out clots from within blood vessels) (Karthikesalingam et al., 2011). As for treatments in the chronic phase, many focus on the promotion of neuroplasticity to encourage brain remodeling to enhance impaired functions. This is done via rehabilitation therapies, which often consist of repetitive stimulation by engaging in therapeutic exercises that help survivors reduce or keep morbidities in control to prevent worsening of outcome (Basu, 2014). Such therapies include constraint-induced movement therapy (CIMT), robotic-assisted therapy and activities that promote muscular endurance (Gordon et al., 2005). Finally, preventive strategies include the use of antithrombotic drugs that prevent the formation of new blood clots which could potentially block blood flow and cause stroke (Ferriero et al., 2019).

1.3.3 Post-stroke treatment options for perinatal stroke survivors

Due to the limitation of acute treatment or prevention strategies, current available therapies for perinatal stroke survivors heavily focus on supportive care and neuroprotection following stroke. Following perinatal stroke, the most common outcome presents in the form of life-long neurologic and motor impairments, which poses a direct risk to a patient's future independence (Dunbar & Kirton, 2019). Notably, perinatal stroke remains the main cause of cerebral palsy (CP) in children (Kirton & Deveber, 2013). Cerebral palsy is predominantly characterized by movement impairments, poor balance and sensory deficits (Vitrikas et al., 2020). Most rehabilitation strategies are thus focused on physical rehabilitation, with the two currently most efficient ones being CIMT bimanual training (Sakzewski et al., 2014). CIMT consists in immobilization of the less-affected arm with a cast to force patients to practice use of the injured limb to perform tasks such as grasping or picking up objects. This technique is recommended for infants aged 18 months and above, however it can also be applied in infants with 3-8 months of age (Baby-CIMT) (Eliasson et al., 2018). In bimanual training, patients are engaged in skilled tasks requiring coordination of the two hands, such as twisting bottle caps, placing coins in a coin bank, and so on. Application of such strategies has been proven to be effective, even more so in high doses (Gordon, 2011). In fact, a study led by Andrew Gordon (2011) investigated the effects of intensity in sessions of CIMT and bimanual training with sufficient repetitions over many hours of training and revealed that 90 hours of training had greater outcomes in recovery compared to 60 hours of training.

There are also difficulties associated with employing such time-intensive therapies in survivors of perinatal stroke. To begin with, the activities must be accommodated to work with their capabilities, and must be motivating enough for children to actively participate in such time-intensive strategies (Gordon et al., 2005, 2007; Majnemer et al., 2010). Due to the repetitive nature of training sessions and the time it takes to create a consistent routine, going into a clinic for rehabilitative training can easily become challenging for perinatal stroke survivors and their caretakers. Although studies have shown that extending the exercises at home in the form of playing or day-to-day activities is beneficial for recovery (Novak et al., 2009; Schnackers et al., 2018), it can also become a stressful task for parents, as most put pressure on themselves in the pursuit of becoming the best therapist to their child at home, without affecting their role as

parents. Efficiency of training is also limited by the cognitive development of the patient, which is often affected by the stroke, and can significantly hinder their ability to understand tasks and instructions from therapists or caretakers (Martin et al., 2011). Moreover, such motor training requires patients to have developed certain fine motor skills (being able to lift one's wrist, active movement of fingers for grasping exercises, minimum strength in limbs) which could be further delayed due to the suffered injury. Other recent advancements have put forward other techniques including non-invasive brain stimulation like transcranial magnetic stimulation (TMS) in children (Hilderley et al., 2019; Kirton, 2017; Rajapakse & Kirton, 2013). Notably, a recent study led by Hsing-Ching Kuo demonstrated it is possible and safe to perform TMS mapping in children (Kuo et al., 2022). Moreover, although remaining at a preclinical stage, stem cell therapy in a rat model of neonatal stroke showed that the transplantation of mesenchymal stem cells significantly reduced infarct volume (Kim et al., 2012).

1.3.4 Life after perinatal stroke

It is undeniable that the outcomes of stroke cause a terrible burden on the lives of both adults and infants affected by stroke at different times of their lives. Children affected by perinatal stroke must grow and learn to live for a substantially greater number of years with deficits compared to an adult affected by stroke later in life. They must learn how to develop new abilities, including speech and language, as well as physical motor skills, while being limited by the outcomes of perinatal stroke (Kirton & Deveber, 2013). The challenges in this are not only limited to motor function, but also to the learning in social interactions and relationships. With that said, perinatal stroke also has a direct impact on the mental health of the family, especially the mother, as it is unfortunately common for misplaced guilt to take place (Kirton & Deveber, 2013).

Perinatal stroke is a substantially difficult disease to work with. Not only are there no prevention strategies available, but there are considerable challenges in the overall diagnosis and treatment processes. With such limitations, it is estimated that 55% of children affected by perinatal stroke end up with life-long morbidities including behavioural and cognitive deficits (Golomb et al., 2001). In the long-term, this is a costly disease that affects an entire family and often affects the mental health of parents (Dunbar & Kirton, 2019; Kirton & Deveber, 2013). The need for intensive therapy treatments, as well as constant worry about the child's prospective

future lifestyle can be difficult to navigate throughout so many years. It is thus crucial for research efforts to focus on better understanding such models of developmental injury.

1.4 Developmental brain injury

There is evidence that the developmental brain responds differently to injury compared to a mature brain (Babikian et al., 2010; Blomgren et al., 2007; Claus et al., 2010). Thus, understanding how the developmental brain responds to early injury is of great importance as a first step towards improving outcomes from perinatal stroke.

One of the most important developmental processes that can get disrupted by a unilateral perinatal brain injury is the development of descending motor pathways that constitute the corticospinal tract (CST) (Martin et al., 2011). The CST plays an important role in voluntary motor control as it connects the cortex through origins in the primary motor, primary somatosensory, pre-motor cortices, and supplemental motor area to the spinal cord to conduct movement of distal extremities. It influences the activity of motor neurons in the spinal cord directly or indirectly through an intrinsic network of relay nuclei and spinal interneurons (Moreno-Lopez et al., 2021). In humans, the CST travels down to the brainstem and the bundle of tracts compact before reaching the medulla, where a great majority of fibers decussate to the contralateral side, with only a small proportion of fibers remaining ipsilateral (Lohia & McKenzie, 2024). During embryonic development, many of the axons previously incorporated into the CST get removed or pruned as the CST develops (Natali et al., 2024). Notably, the refinement of the CST is not complete until after birth as motor control develops (Jang, 2014). By the end of maturation, the motor system becomes an almost exclusively contralateral system.

A disruption to the development of the CST can cause atypical motor system connections between the developing CST and spinal motor circuits. In 1970, Hicks and D'Amato observed the presence of an aberrant uncrossed corticospinal tract in infants, but not in adults, following hemispherectomy in rats (Hicks & D'Amato, 1970). In this study, rats hemispherectomized as adults presented impairment in forelimb stride during running, while the infant group did not. This suggested that the uncrossed CST fibers resulting from the developmental injury may have provided the bilateral corticospinal innervation required for normal stride for the infant rats, suggesting compensation in rats injured as infants. Similarly, following surgical ablation of the contralateral CST in rats, Castro observed the formation of abnormal ipsilateral corticospinal

projection, which was not present in control animals with lesions occurring during adulthood (Castro, 1975). Whether such plasticity and re-organization induced by interruption of developmental processes is beneficial or maladaptive remains elusive. In the context of perinatal stroke, damage to the developing CST can result in CP. Notably, clinical studies also found the presence of abnormal motor pathways in the form of aberrant ipsilateral corticospinal axons in patients with CP (Carr et al., 1993; Eyre et al., 2001, 2007). In particular, studies showed that in subjects sustaining unilateral brain damage early in development, low intensity transcranial magnetic stimulation (TMS) of the intact cortex elicited large motor responses in ipsilateral muscles with short latencies - suggesting the presence of significant fast-conducting ipsilateral corticomotoneuronal projections (Benecke et al., 1991; Carr et al., 1993). However, the same aberrant projections are not found in adult patients with unilateral brain injuries (Eyre et al., 2001), providing further evidence for this abnormal reorganization of descending motor pathways to be specific for injuries occurring during the perinatal period. As post-stroke reorganization of the CST appears to be distinct between developmental and adult injuries, we could anticipate differences in motor cortex reorganization following injury depending on the timing of the injury. As briefly described, the descending motor pathways from the CST originate from cortical regions, including the sensorimotor cortex. If the cerebral regions from which the tracts originate from (including the motor cortex, relevant for most stroke cases) are impacted by a lesion during development, it is plausible to anticipate how this could affect the subsequent development of the CST.

In adult studies, non-invasive techniques such as TMS have been used to probe and identify brain regions implicated in control of specific body parts, to create what are called “motor maps”. With motor mapping, we can identify the cerebral regions that could be implicated in recovery so the latter can be targeted for stimulation therapies. In fact, when applied repetitively in a controlled location, TMS has been demonstrated to promote positive recovery as it modulates cortical excitability (Blumberger et al., 2018; W. Huang et al., 2022). In animal models, motor mapping has been achieved by intracortical microstimulation (ICMS). In this technique, electrodes are inserted into deep layers of the motor cortex to generate maps of movement representation (Asanuma & Rosén, 1972; Li & Waters, 1991; Nudo et al., 1990). Importantly, studies using ICMS have found that motor map ablation can be restored with motor rehabilitation such as skilled forelimb training in rodents (Boychuk et al., 2011; Williams et al.,

2006), which supports the notion of experience-dependent plasticity. Since perinatal stroke outcome commonly involves motor impairments requiring intensive rehabilitation training (as detailed in section 1.3.3), it would be interesting to investigate whether map size can be enhanced with similar motor training in perinatal cases. This would help support the benefits of intensive and long-term motor rehabilitation therapies for survivors of perinatal stroke.

Considering these findings and available techniques, it is then important to pursue further research with relevant animal models in a preclinical setting to elucidate how the brain responds to developmental injury. This would considerably help in best utilizing potential innate brain responses and develop more appropriate therapeutic strategies for perinatal stroke cases.

1.5 Rodent models of perinatal stroke

To investigate mechanisms underlying pathology and potential therapeutic strategies for perinatal stroke, different animal models have been developed and employed over the years. The most frequently used are rodent models, as they share important similarities to the human brain from a developmental viewpoint. In fact, like in humans, the corticospinal tract in rodents originates in the cerebral cortex and projects down to the medullary pyramid before CST axons decussate to the contralateral side. Similarly, once mature, a great majority of axons descending from the CST are decussated, with only a small proportion of axons remaining ipsilateral. In the context of the motor system, there are also similarities between humans and rodents. Additionally, in terms of development, it has been established that the post-natal day (PND) 7-10 rodent brain development is closely equivalent to that of a near term human (Hagberg et al., 1997). This facilitates the goal of inducing injury at an approximate timepoint to that of most perinatal stroke injuries. It is also important to consider relevant differences between the two species. To begin with, the extent of ipsilateral CST is quite different between the two species. In humans, around 10-15% of tracts remain uncrossed (Lohia & McKenzie, 2024), while in rodents, such non-decussated tracts only make up around 2-4% out of all tracts (Rouiller et al., 1991). Considering all its benefits and limitations, rodent models remain the most reliable and accessible model and are used extensively in the field of perinatal stroke.

1.5.1 The Rice-Vannucci model and hypoxia-ischemia injuries

The most popular rodent model for neonatal stroke was introduced by Rice and Vannucci in the early 1980s. This hypoxia-ischemia (HI) model combines unilateral common carotid artery ligation with subsequent hypoxia in post-natal day 7 rats to induce unilateral brain damage ipsilateral to the ligated artery (Rice et al., 1981). Given that the major etiological factors of CP are HI and that brain damage in this model resembles that observed in CP cases, this model has greatly contributed to CP research. However, due to the need for systemic hypoxia to create infarction, this model presents some limitations for studies interested in perinatal stroke induced by ischemia alone. In fact, hypoxic conditions can trigger pathological events that would not be induced by ischemia alone, such as precipitated autophagy leading to enhanced levels of cell death (Adhami et al., 2006; Levine & Klionsky, 2004). Additionally, HI injuries from the Rice-Vannucci model are quite diffuse, and have been shown to affect multiple brain regions (Rice et al., 1981). It is essential to note that although perinatal stroke and HI injuries share a predominant outcome that is CP, important differences remain. Most importantly, ischemic strokes are characterized by a focal ischemic core and penumbra, whereas HI injuries produce patchy cell death. Furthermore, HI injuries have a significantly higher mortality rate, with 20-50% of HI encephalopathy-affected individuals passing away as newborns (Vannucci, 2000), compared to perinatal stroke, with a mortality rate of 3% (Lynch & Nelson, 2001).

Following the classic Rice-Vannucci model, several optimizations of the latter were consecutively developed to study different types of injuries within HI related conditions. For instance, to investigate global hypoxia-ischemia, investigators have opted for bilateral ligation of carotid arteries with (Hattori et al., 1989) and without (Uehara et al., 1999) subsequent hypoxia to model global hypoxia-ischemia. Notably, Ashwal and their group developed a reversible middle cerebral artery occlusion (MCAo) model using a filament to transiently occlude the MCA without the need for craniectomy or subsequent hypoxia (Ashwal et al., 1995). However, the coefficient of variation of lesions by MCAo is large (70%), suggesting difficulty in reproducibility (Qian et al., 2016). It is also important to consider the difficulty alone of performing MCAo procedures in small pups (ranging from P7-12), as it requires high precision and technical skill.

1.5.2 The photothrombotic stroke model

The development of the photothrombotic (PT) technique by Watson et. al. (1985) set the basis for the growth of new methods within the perinatal stroke field. This technique was based on the concept of photothrombosis, where the activation of a light-activated dye induces a coagulation cascade resulting in thrombosis creating a focal cortical infarction. This method has been well-established in adult animal models of ischemic stroke. It is a non-invasive technique that is considerably simpler to execute, with no craniectomy required (Labat-gest & Tomasi, 2013). In the context of perinatal stroke studies, the technique was first attempted in post-natal day 7 rodents where they targeted the medial frontal cortex and the primary somatosensory cortex in a study led by Maxwell and Dyck in 2005. To control the shape and size of the lesion, they used masks to control exposure area and different light irradiation durations (45, 60 and 80 seconds). In this study, variability in infarct volume between groups was observed, with increased laser durations positively correlating with larger lesion volume. In 2013, Brima et. al. used a perinatal PT stroke model to investigate the extent of motor impairments in rats in two ischemic lesions of different size (30 sec vs 5 min of light irradiation). In this study, they found that 60 days after stroke, behavioural performance was significantly worse in stroke mice compared to the control groups (Brima et al., 2013). The behavioural tests used in this study included the rotarod test, forelimb and hindlimb bar holding, inclined grid and ladder rung walking tests. Additionally, their results were also consistent with previous findings suggesting that lesion size significantly impacts motor outcomes after stroke (further discussed in section 1.6), with larger lesions resulting in worse sensorimotor impairments. However, the characterization of the induced lesions was limited to infarct volume. In fact, although the study estimates the location of the produced lesions using observations from Nissl-stained coronal brain sections, a precise identification of cerebral regions affected by the lesion is lacking. It would be interesting to address this limitation to have a better estimate of the impact of the lesion in different areas of the brain, and how this could further affect behavioural outcomes.

Focal ischemic stroke is characterized by the presence of a penumbra, which is an area around the ischemic core that is salvageable given timely reperfusion. The penumbra is then the major target for neuroprotective therapy, as it can be rescued and thus reduce further expansion of the infarct. However, there is discourse about the ability of the photothrombotic induced

stroke models to allow for penumbra formation. There is a prevalent understanding that an infarct caused by a PT model has little to no ischemic penumbra or regions of collateral blood flow or reperfusion at the targeted area (Carmichael, 2005; C. J. Sommer, 2017; Uzdensky, 2018). Given this issue, a modified version of PT stroke model where they occlude the proximal MCA model using photothrombosis, and visualized the penumbra surrounding and surprisingly, inside the lesions using noninvasive MRI (Qian et al., 2016). However, considering the substantially lower level of invasiveness and difficulty in performing PT stroke surgeries, it makes it a more favorable technique for ischemic stroke studies. Although this technique has been widely established in adults, only a few have applied it to longitudinal developmental brain injury studies.

1.5.3 Behavioural assessment in perinatal stroke models

Besides establishing models that produce lesions comparable to the stroke of interest, it's also important to assess whether behavioural outcomes from such lesions are relevant to that observed in humans. As previously detailed in section 1.3, perinatal stroke survivors' most prominent impairments are long-lasting motor deficits. Since therapeutic efforts are focused on motor rehabilitation therapy following perinatal stroke (as described in sections 1.3.3 and 1.3.4), it is also important that we observe long-lasting motor impairments in perinatal stroke models. Notably, work done by Shanina et.al. demonstrated in an adult model of PT stroke that rats with small lesions (with intact subcortical structures) presented sensorimotor impairments only detectable during the first two weeks after stroke (Shanina et al., 2006). It remains to be demonstrated whether the perinatal PT stroke model can result in long-lasting motor impairments for different ranges of infarct sizes.

It is relevant to note that the onset of adulthood in mice is considered to begin at P60 (~2 months of age), as the post-pubertal adolescence period ends (Brust et al., 2015). Moreover, most mouse studies use animals up until 5-6 months of age for adulthood timepoints, as older mice may present age-related changes (Flurkey et al., 2007).

Particularly, for the PT perinatal stroke rodent model, few have performed longitudinal assessment of motor function. In a study led by Brima, motor performance was evaluated in a rat model of PT stroke induced at P7 using a series of motor tests (Brima et al., 2013). They conducted motor tests including the rotarod, bar holding and inclined grid tests 60 days (~2

months) after surgery. Additionally, Zhang et. al. investigated functional impairments on a PT perinatal mouse model with a battery of motor tests including the cylinder test, tapered beam test, Digigait, the single pellet reaching task and adhesive removal test between P59-P70 (~2 months) (Zhang et al., 2021). In this study, stroke animals only demonstrated motor deficits in the single pellet reaching task and the Digigait test at 2 months of age. Considering 2 months of age is an early timepoint in adulthood, it remains to be demonstrated whether the PT perinatal stroke model can produce persistent motor impairments in adult mice.

For a more comprehensive understanding of the impact on motor development and behavioural outcomes following perinatal stroke, it is also important to assess acute motor deficits after injury. Researchers have established methods to acutely assess motor performance and development in neonatal mice at a pre-weaning time point (Feather-Schussler & Ferguson, 2016; W. M. Fox, 1965; Hill et al., 2008). In a neonatal mouse model of CP, 48 hours after injury, pups demonstrated motor deficits in the following tests: ambulation, hindlimb angle, hindlimb suspension, four limb grip strength, and grasping reflex (Feather-Schussler & Ferguson, 2016). To the best of our knowledge, there have been no studies looking into behavioural outcomes acutely in a PT perinatal stroke model.

1.6 Lesion volume and location effects in stroke outcomes

In the context of PT stroke model, studies have demonstrated that larger infarct volumes can be produced with longer light irradiation durations (Labat-gest & Tomasi, 2013; Maxwell & Dyck, 2005; Uzdensky, 2018). Additionally, extensive research has established that lesion size can act as a good predictor for functional outcome after stroke, with bigger lesions resulting in greater sensorimotor impairments (Saunders et al., 1995; Schiemanck et al., 2005; Vogt et al., 2012). Logically, it is implied that with bigger infarcts, there is a potentially greater number of brain areas being impacted by the lesion. A study led by Chen et.al. demonstrated that motor outcomes after stroke in humans importantly correlated with the primary areas affected by the lesion more than just the lesion size itself (Chen et al., 2000). Similarly, studies focusing on infants with neonatal arterial ischemic stroke showed that the localization of the infarct was critical in the prediction of functional outcomes, particularly for motor outcomes such as CP (Dinomais et al., 2015; Wiedemann et al., 2020). However, the specific relationship between affected brain areas and behavioural outcomes have not been well investigated for rodent models

of perinatal stroke. This is important to consider since as previously discussed, the potential of plasticity and adaptation processes in developmental injuries remains elusive. With a better understanding of how affected brain areas directly impact the behavioural outcomes of perinatal stroke both acutely and later in adulthood, this could help better understand how the PT model is progressing and how well it mirrors outcomes observed in human survivors of perinatal stroke.

1.7 Conclusion

With a high risk of occurrence, no means of prevention, and being the cause of lifelong morbidities in survivors, perinatal stroke remains a complex subtype of stroke representing a global burden to numerous families. Following perinatal stroke, survivors are left with time intensive and heavy therapies focusing on motor rehabilitation, as functional impairments are the most common outcome of such a type of stroke. To best target neuroprotective and motor rehabilitation therapies, it is important to establish a perinatal stroke model that presents persisting motor impairments reflective of what is seen in perinatal stroke survivors. It is also crucial to investigate the relationship between lesion volume and location on the impairments observed to establish grounds for future interventional plasticity therapies that consider any effects of infarct volume and location.

Given this background, our aim is to lead a comprehensive longitudinal study that will characterize the behavioural outcomes following a perinatal stroke induced by photothrombosis in neonate mice. We aim to understand the relationship between the characteristics of the lesion, including volume and location, and the behavioural outcomes observed early in development and later throughout adulthood. This work will shed light on the characterization of the PT stroke model for perinatal stroke, which will serve as the foundation for further studies. A better understanding of the time-course and persistence of motor deficits will help establish a foundation for future studies to use this model to optimize therapies that reduce morbidities following perinatal stroke.

2 - MATERIALS AND METHODS:

2.1. Animals and experimental design

Animal care and research procedures were carried out in accordance with the University of Ottawa's Animal Care Committee's regulations. This study used male and female Thy1-ChR2-YFP transgenic mice (The Jackson Laboratory, stock #007612) that were born in our vivarium. We chose to use this strain with the purpose of continuity for previous work done in our laboratory using the same model (Zhang et al. 2021). On post-natal (P) day 7, mice were randomly assigned into stroke (n=22) and sham (n=14) groups. Stroke induction was achieved by photothrombosis (PT) (see section 2.2.1).

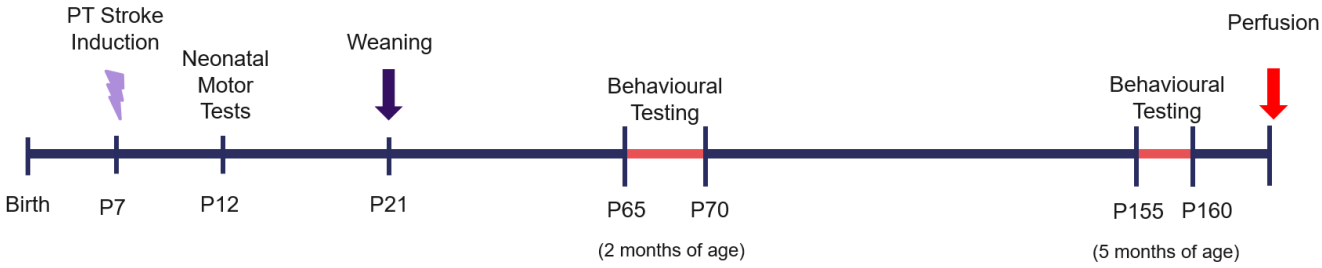

Figure 1. **Experimental timeline.** Complete timeline of experiments.

Following stroke induction, animals underwent a series of sensorimotor tests at P12 prior to weaning at P21. The choice of this earlier development timepoint was to assess stroke-induced sensorimotor deficits as early as possible prior to the further development of compensatory strategies or extensive plasticity. Five days following stroke induction was the earliest time we found reasonable to avoid issues with survivability (dam rejecting or cannibalizing pups) given the disturbance of the nest with the handling of pups for the tests acutely after the injury. After weaning, mice completed a battery of sensorimotor behavioral tests from P65-P70 (2 months of age), and then from P155 and P159 (5 months of age). We chose these two adulthood timepoints to certify that any behavioural impairments observed correspond to persistent deficits. Animals were perfused for histological analysis at P160 (5 months of age).

2.1.1 Excluded animals

We used a total of 36 mice in this study (N=36; 16 female, 20 male, stroke = 22, sham=14). Out of the 36 animals, one litter of 7 pups (sham = 4, stroke=3) had pre-weaning tests that could not be evaluated as recordings were not properly recorded. Out of the 22 mice in the stroke group, one was excluded from behavioural testing during adulthood and histological analysis due to bilateral injury, leaving 21 mice in total. Additionally, one animal passed away unexpectedly before reaching 5 months of age, so the total number of stroke mice included in the second adult behavioural timepoint was 20.

2.2. Experimental surgery

2.2.1 Photothrombotic ischemic stroke model

On post-natal day 7, (day of birth being P0) approximately half the pups from the litter were separated from the dam and prepared for photothrombotic surgery (Maxwell & Dyck, 2005). We chose to never take litters as a whole, but in batches, so that parents are not left without any mouse pups. The pups were placed in a container with some of the nest bedding before being taken to the surgery room. This measure was taken to prevent contaminating the pups with foreign scents during the period of separation from the dam. The container was placed inside an incubator maintaining a temperature of 37°C, from where pups were individually removed one at a time for surgery.

Pups were placed in an induction chamber and anesthetized by inhalation of isoflurane (4%) for 7 minutes with an oxygen flow rate of 0.5L/min. Once induced, the animal was placed on a warming blanket (37°C) on top of a stereotaxic frame and maintained at 2-3% isoflurane. Animals received a small midline scalp incision midsagitally to expose the skull and mark bregma position. At P7, the mouse skull is highly translucent to light, so a craniotomy was not necessary. Rose Bengal dye (0.01g/ml in saline) was administrated by intraperitoneal injection (0.1 ml/10 g body weight), and two minutes was allowed for it to circulate systematically and reach the brain. Following circulation, the laser used for photothrombotic stroke (532 nm, 1.5 mm diameter, 20 mW power) was positioned 5 cm above the exposed skull and aligned to the primary motor cortex (M1) over the right hemisphere using stereotaxic coordinates relative to bregma (coordinates: AP: 1.5 mm; ML: 1.0 mm) (Fig. 2B). The laser was then turned on and

illumination lasted 6 minutes (Fig. 2A). Finally, the incision was sutured with a vicryl thread (6-0 gauge). Sham animals underwent the same procedure but with light illumination preceding the injection of the Rose Bengal dye, avoiding blood coagulation. Additionally, sham animals received an ear notch in their left ear to distinguish between stroke and sham groups. Finally, pups would be placed back in the warmed incubator to recover before being returned to the home cage as soon as possible. Pups were separated from the dam for approximately 35 minutes for the totality of the surgery procedure.

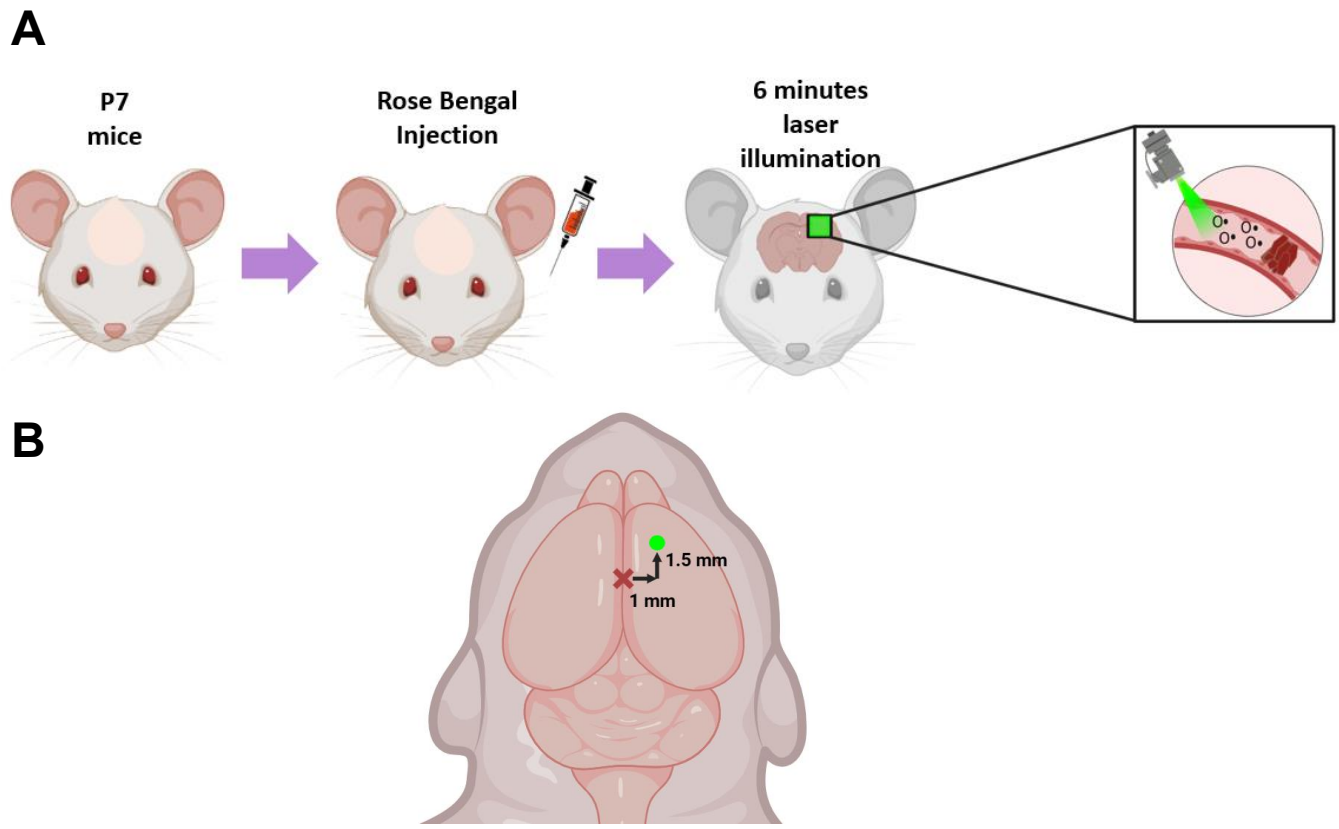

Figure 2. Photothrombotic stroke procedure. **(A)** At P7, an incision is made to the midline of the scalp, Rose Bengal dye is injected and circulates through the brain vasculature for 2 mins. A 520 nm laser illuminates the target stereotaxic coordinates for 6 minutes to induce a perinatal photothrombotic stroke. **(B)** Representative image of stereotaxic coordinates used to induce stroke (AP: 1.5 mm; ML: 1.0 mm), where the red cross represents the bregma point, and the target stroke site is represented by the green circle.

2.3. Behavioural testing

Following surgery, mice underwent a series of behavioural tests at three timepoints: one at P12, and two in adulthood (2 months of age and 5 months of age).

2.3.1 Pre-weaning tests

A series of neonatal motor tests were used to evaluate general motor function and natural reflexes developing in young mice. We followed Feather-Schussler & Ferguson's methods to run four motor tests and evaluate impairments following stroke during the pre-weaning period (2016). Each test was repeated thrice, with at least 3 minutes of rest between each test. The results are the average of three trials. To reduce time away from the dam (approximately 35 minutes), pups were removed individually from the home cage and returned as soon as testing was complete.

2.3.1.1 Pre-weaning: Hindlimb suspension test

For the hindlimb suspension test, mice were suspended upside down by the hindlimb on the rim of a falcon tube padded with gauze. We noted the latency to fall and the positioning of the hind paws and tail as a measure of impairment using the scoring system by Feather-Schussler & Ferguson. The scoring is based on the positioning of the hindlimb paws and tail during the trial (Table 1).

Score	Hindlimbs position	Clasping paws	Tail position
4	Not touching, normal separation	No	Raised
3	Closer together, seldom touching each other (<=3 times)	No	Raised
2	Close and often touching each other (>=4 times)	No	Raised
1	Almost always touching (>=2 times do not touch)	Yes	Raised
0	Always touching	Yes	Lowered

Table 1. Hindlimb suspension scoring system used in the pre-weaning hindlimb suspension test.

2.3.1.2 Pre-weaning: Forelimb suspension test

The forelimb suspension test was used to test the forelimb strength of the pups at P12. We positioned the pups so that they were allowed to grasp with both paws a wire strung across two metal poles. Underneath the wire, the drop area was padded with gauze. We recorded the latency to fall as a measure of forelimb strength.

2.3.1.3 Pre-weaning: Grip strength test

The grip strength test measured the paw strength of all four paws simultaneously. Pups were placed on top of a (dimensions) wire screen and were given 30 seconds to adjust to the environment. A protractor was fixed to the wall behind the testing area, with its center aligned with the bottom left side of the mesh grid, so the angle of the screen could be recorded. Next, we slowly rotated the grid from a horizontal to a vertical position. As the angle increases, the gravitational force exerted on the mouse does as well, requiring the mice to sustain muscle tension in all four limbs. We recorded the angle of the screen at which the pup would lose strength and release from the grid. The fall zone was padded with gauze.

2.3.2 Adulthood tests

2.3.2.1 Adulthood: Cylinder test

The cylinder test quantifies spontaneous forelimb use and asymmetry during exploration (Gharbawie et al., 2004). Mice were placed in a clear and hollow Plexiglas cylinder (10 cm diameter, 15 cm height) and were allowed to explore the walls until 25 rears were reached. Trials were recorded from below using a Raspberry Pi v2 camera (Raspberry Pi Foundation). In our scoring, we quantified the paw(s) used for push-off, landing, first contact and total contacts on the cylinder wall.

Contralesional paw preference during rearing behaviour was quantified with the following formula:

$$Contralesional\ paw\ preference = \frac{Contra + \frac{1}{2}Both}{(Contra\ +\ Ipsi + Both)} * 100$$

where *contra* is the total number of contralesional paw contacts, *ipsi* is the total number of ipsilesional paw contacts, and *both* is the total number of contacts using both paws simultaneously.

2.3.2.2 Adulthood: Grid walk test

The grid walk test evaluates sensorimotor coordination of the four limbs by counting the number of missed steps with the stroke-affected forelimb over the total number of steps during locomotion on a horizontal grid (Metz & Whishaw, 2002). Mice were placed on a 21 by 33 cm grid and allowed to explore for 5 minutes. Trials were recorded by a Raspberry Pi v2 camera placed under the grid.

2.4. Histology

Following the completion of the last behavioural testing in adulthood, mice were euthanized using euthanyl and perfused (3.8 mL/min) transcardially with phosphate buffered saline (pH 7.4) followed by 4% paraformaldehyde. Brains were extracted, photographed dorsally, then sectioned coronally on a vibratome at 100µm thickness. and mounted on 0.5% gelatin-coated slides. To quantify lesion volume and location, tissue was stained with cresyl violet (C. Sommer, 2016). and cover-slipped using permount mounting media (Sigma-Aldrich).

2.4.1 Lesion volume quantification

Following cresyl violet staining, sections were imaged at 2.5X magnification using a Zeiss microscope (Zeiss Axio Imager M2). We used *NIH ImageJ* (Schneider et al., 2012) to outline and measure the area of damaged stroke tissue. To obtain the total infarct volume, we used the following equation, where the tissue volume between each section was equal to $100\mu m^3$:

$$\Sigma(area\ of\ damage\ for\ each\ section) * tissue\ volume\ between\ each\ section$$

2.4.2 Semi-automated lesion localization workflow

To determine how the lesion affected various brain regions, we used open-source software from the semi-automated QUINT workflow (Yates et al., 2019). We utilized the previous outline of the damaged tissue area from the lesion volume analysis done on *NIH ImageJ* to create a mask for the lesion in each section (Fig. 3A). The sections with the lesion overlaid were then registered to the Allen Mouse Brain Atlas (AMBA, Allen Reference Atlas – Mouse Brain [brain atlas]. Available from atlas.brain-map.org.) using *QuickNII* v2.2 (RRID:SCR_016854, (Puchades et al., 2019)) (Fig.3B). *QuickNII* is a three-dimensional tool that allows us to superimpose our original

section images to the reference AMBA. By adjusting the opacity of the atlas, we were able to align our sections to the correct position in the brain atlas by using anatomical landmarks such as the anterior commissures, the corpus callosum, the lateral ventricles, and Ammon's horn. Another utility from *QuickNII* allowed us to rotate, stretch, compress, and adjust the cutting angle of the atlas to match our original sections.

Once our sections were aligned to the AMBA, we exported the registered custom maps from *QuickNII* and imported them into *VisuAlign* v0.8 (RRID:SCR_017978, (Puchades et al., 2019)). *VisuAlign* allowed us to further optimize the initial alignment by performing non-linear transformations to the reference atlas and compensate for any brain deformations (Fig. 3C). We first started by setting anchor points on the contour of the reference section to fit it to our original section, then applied more points on the inside, to maintain anatomical regions in place using the landmarks previously described. The registration results were stored in a new JSON file and the PNG images of the atlas sections were exported.

Next, we created segmentation images of the lesion area in each section using *NIH ImageJ* (Fig. 3D). These binary segmentation images outlined and filled the lesion area in black, while the rest of the image was masked in white. To achieve this, we employed a batch processing macro script in *NIH ImageJ* that modified image brightness and contrast settings.

We then created lateralization masks with *NIH ImageJ* to cover the injured hemisphere in white by drawing a white polygon shape over the latter (Fig. 3D). Similar to the method used in the segmentation images, we used a macro script to apply a transformation that would apply a black mask over the healthy hemisphere in an automated manner. The segmentation images and lateralization masks were necessary to limit the lesion localization analysis to the injured hemisphere. Finally, we used the software *Nutil* (RRID: SCR_017183, (Puchades et al., 2019)) to input the refined atlas maps, the lateralization masks and segmentation images to perform the quantification of damage in select brain regions (Fig. 3E). The anatomical regions of interest included: the primary motor cortex (M1), the secondary motor cortex (M2), the somatosensory areas (SS), the anterior cingulate cortex (ACC) ventral (vACC) and dorsal (dACC) areas, with, the corpus callosum, and the ventricular systems.

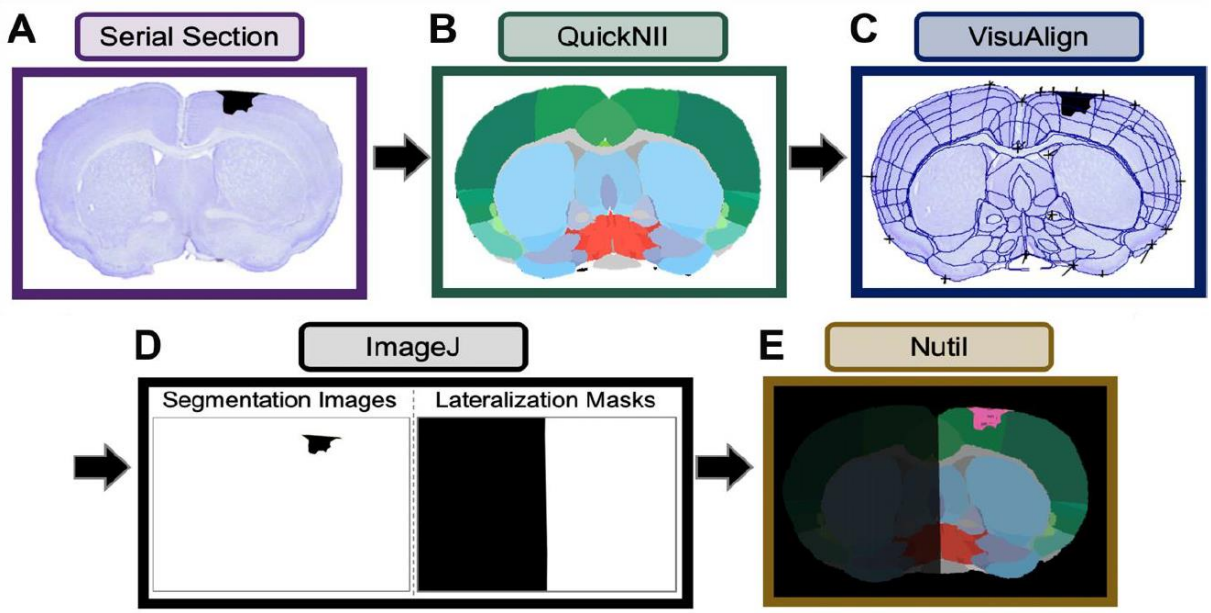

Figure 3: **Lesion localization workflow. (A)** Coronal section with the lesion outlined and filled in black. **(B)** Alignment of a section to the reference AMBA using *QuickNII*. **(C)** Refinement of previous alignment using anchor points to perform non-linear transformations in *VisuAlign*. **(D)** Creation of binary segmentation images and lateralization masks, where we contrast the lesion area and injured hemisphere using *NIH ImageJ*. **(E)** Quantification of lesion in each anatomical region delineated by the AMBA using *Nutil* software.

2.5. Statistical analysis

All behavioural, histological and lesion localization analyses were expressed as mean + standard error of the mean (SEM). Statistical analyses were performed with GraphPad Prism 10 (San Diego, CA, USA).

Unpaired t-tests for sham vs stroke groups were used for the pre-weaning tests (latency to fall for both forelimb and hindlimb, average of inverted fall angle) and the cylinder test (paw preference for limb used for push-off, first wall contacts, total wall contacts and landing). The Mann-Whitney test was used to compare medians of the hindlimb suspension score between stroke and sham groups for the hindlimb suspension pre-weaning test.

Two-way analysis of variance (ANOVA) was used to compare between subject factors (stroke versus sham group) for the following analyses: (1) grid walk – percentage of missed steps for impaired and non-impaired limb, (2) cylinder test - paw preference for first wall contact and

total wall contacts. The grid walk test analysis was followed by Sidak's multiple comparisons test to compare the mean between groups. For the cylinder test, two-way ANOVA was followed by Sidak's multiple comparisons test with a single pooled variance.

Pearson's bivariate correlation was used for all correlation analysis between lesion volume and localization results with the behavioural results.

3 – RESULTS

3.1. Perinatal PT stroke resulted in variable lesion volumes

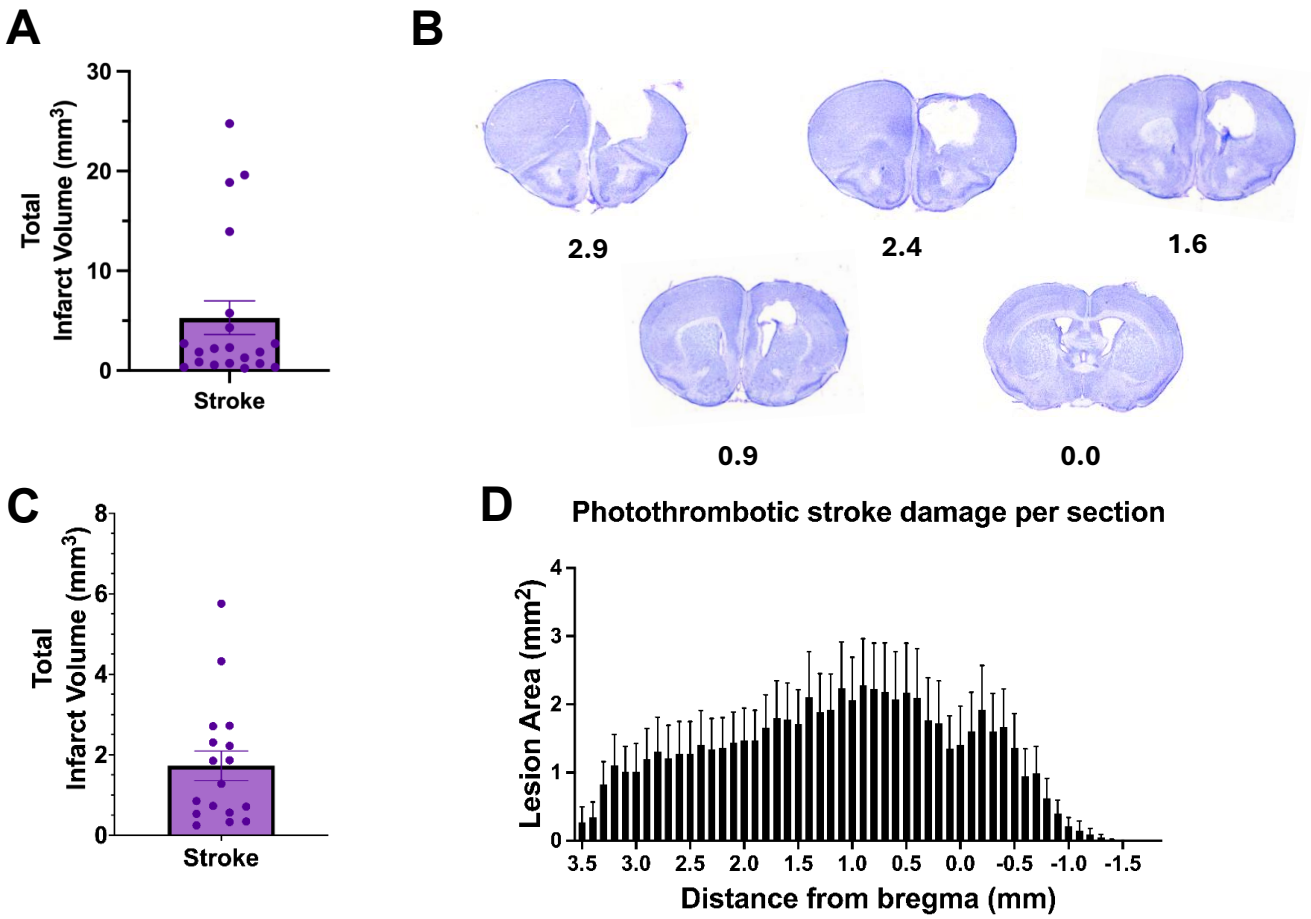

Figure 4. Lesion volume and impact of stroke. (A) Mean ± SEM and individual infarct volumes in mm^3 (n=21). **(B)** Cresyl violet series from a stroke animal with a lesion volume of 5.75 mm^3. Sections correspond to the following AP coordinates: 2.9, 2.4, 1.6, 0.9, and 0.0 mm. **(C)** Mean ± SEM and individual infarct volumes in mm^3, excluding 4 largest lesions (>10mm^3) (n=17). **(D)** Photothrombotic stroke lesion distribution (n=17; 4 largest lesions excluded).

Our perinatal PT stroke surgeries resulted in a mean lesion volume of 5.072 ± (1.615) mm^3 (Fig. 4A). Amongst the 21 stroke animals analyzed, 4 had considerably larger lesion volumes (>10 mm^3) and were categorized as the large lesion group. From these 4 animals, only two (with lesion volume of 18.865 mm^3 and 24.762 mm^3) were from the same litter (Table 2). Excluding the 4 largest lesion volume animals, the mean lesion volume was of 1.726 ± (0.371) mm^3 (Fig. 4C). Across most stroke animals, the maximum range of injury extended from 3.5 to -1.4 mm distance from bregma (Fig. 4D). Only 2 of the largest injured brains had injuries extending to the striatum.

Mouse ID	Cohort Number	Total Infarct Volume (mm3)
251901_1	1	13.9426
251902_1	1	2.3079
254459_1	2	0.2451
254459_2	2	0.7299
254460_1	2	4.3245
254460_2	2	0.8511
254460_3	2	1.8516
254460_4	2	0.3425
255011_1	3	18.8653
255012_1	3	24.7621
256206_1	4	0.5646
256206_2	4	19.6048
256207_1	4	5.7558
256212_1	4	0.3309
256953_1	5	2.7088
256953_2	5	2.2216
256953_3	5	2.7218
256954_3	5	0.7104
259119_1	6	1.2802
259120_1	6	1.8656
267795_3	7	0.5341
Average Lesion volume (mm3)		5.0724

Table 2. Lesion volume measurements for the total of 21 stroke mice used in this study, and their corresponding cohort numbers.

3.2. Perinatal PT stroke lesion localization was not limited to the targeted primary motor cortex

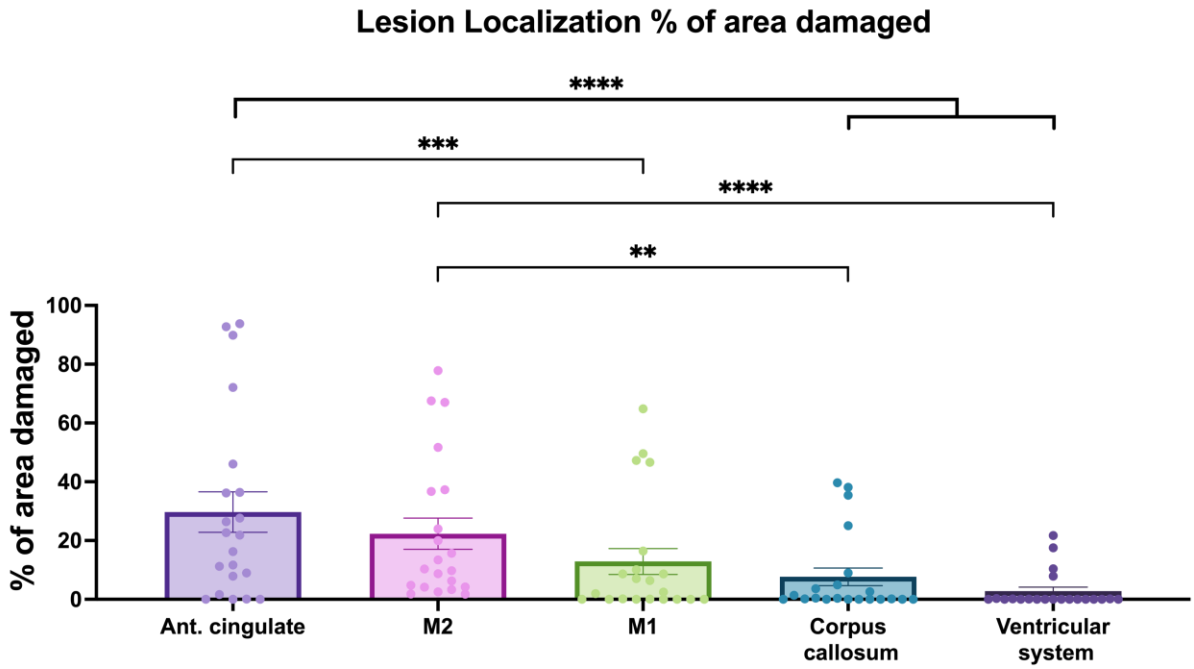

Figure 5. Lesion localization to the Allen Mouse Brain Atlas. Mean percentage of anatomical region damaged by lesion + SEM for all stroke animals.

We compared the regions damaged among all stroke animals with a two-way ANOVA. The most affected brain regions by the lesion were the anterior cingulate cortex, the secondary motor cortex, the primary cortex, the corpus callosum and the ventricular system with an average percentage of area damaged of 29.68 ± 6.90 %, 22.32 ± 5.31%, 12.87 + 4.42%, 7.66 + 3.01 % and 2.77 ± 1.37 % respectively (Fig. 5).

3.3. Perinatal PT stroke mice presented longitudinal motor impairments

We observed longitudinal motor impairments in our perinatal PT stroke model. First, during the earlier timepoint before weaning, then persisting throughout the two adulthood timepoints.

3.3.1. Pre-weaning deficits

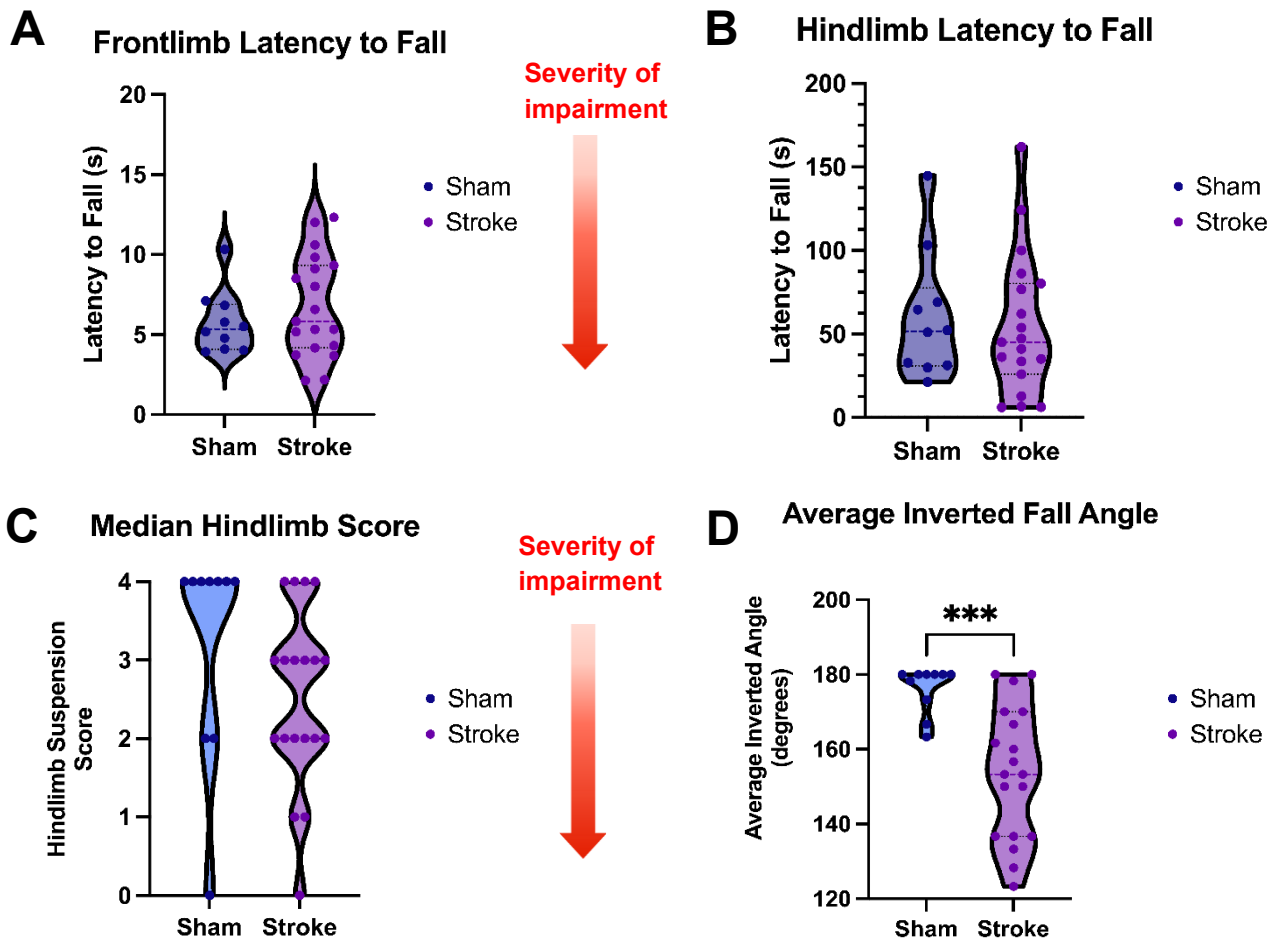

Figure 6. Pre-weaning behavioural outcomes (sham = 10, stroke = 19). **(A) Forelimb suspension**: Latency to fall not significant (p=0.3775) **(B) Hindlimb suspension:** Latency to fall not significant (p=0.7463). **(C)** Although not statistically significant (Mann-Whitney test, p=0.0815), stroke mice had more variation in the scoring for the hindlimb suspension test, with a greater number of low scores indicating impairment. **(D) Grip strength:** Stroke mice had a significantly lower average in the inverted fall angle than sham. Unpaired t-test, p=0.0007, ***p <0.002.

Our pre-weaning test showed that injured mice were impaired on some of the behavioural tasks. During forelimb suspension (Fig. 6A, stroke: 6.742 ±, sham: 5.752 ±, p = 0.3775, unpaired t-test) and hindlimb suspension (Fig. 6B, stroke: 54.79 ±, sham: 60 ±, p = 0.7463, unpaired t-test) tests, there was no significant difference in the latency to fall between groups. Although not

statistically significant, stroke mice had lower scores in the hindlimb suspension score, whereas the sham mice mostly scored 4, indicating more impairment in stroke animals compared to the sham group (Fig. 6C, median stroke: 3 ±, sham: 4 ±, p = 0.0815, Mann-Whitney test). In the grip strength test, stroke pups fell at a significantly lower angle compared to sham animals across three trials (Fig. 6D, stroke: 153.3 ±, sham: 176.2 ±, p = 0.0007, unpaired t-test).

3.3.2. Adulthood deficits: 2 and 5 months of age

2 months of age

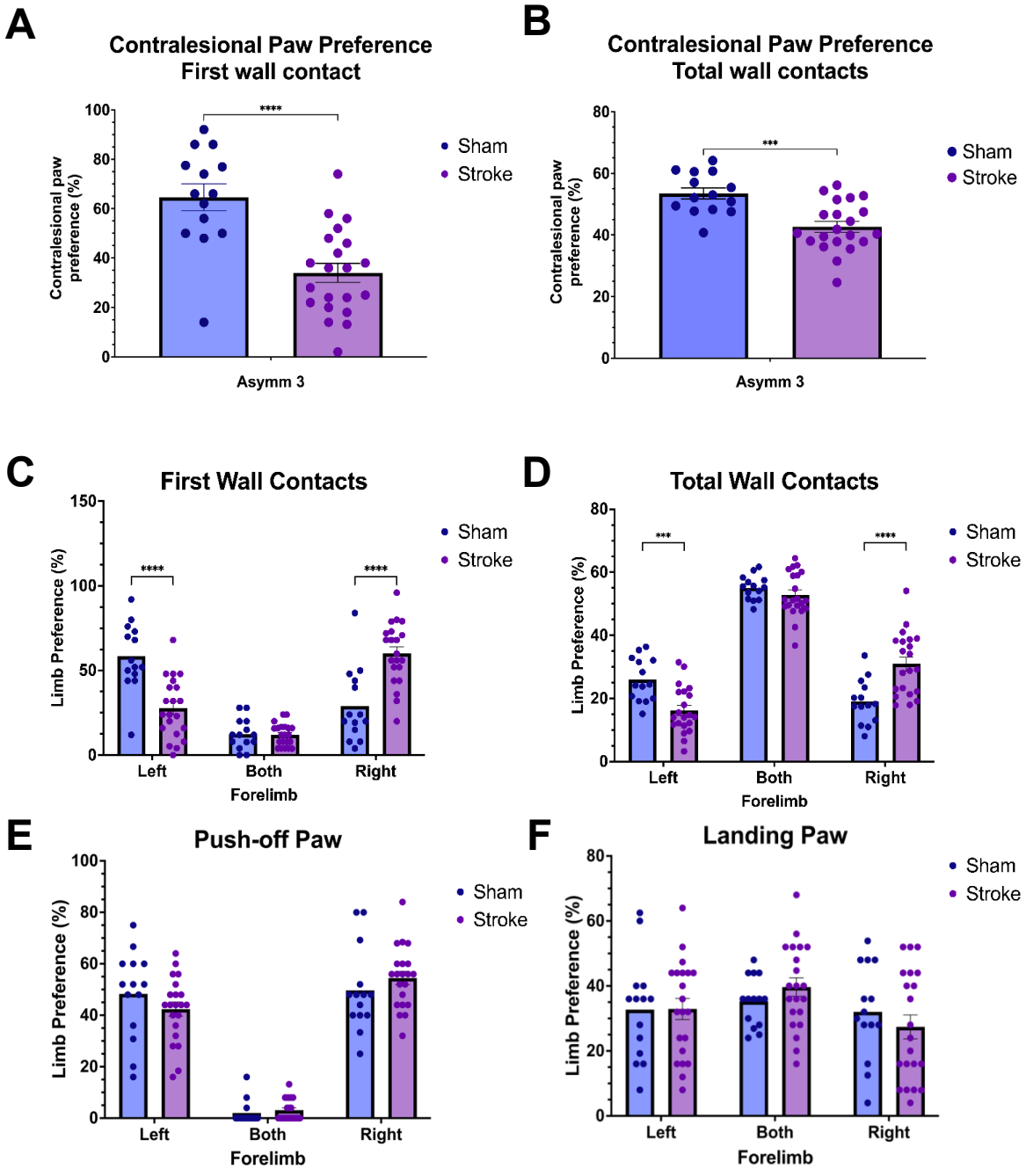

Figure 7. Cylinder test behavioural outcomes for stroke (n=21) and sham (n=14) groups at 2 months of age. Contralesional paw preference was measured for different behaviours. **(A)** Contralesional paw preference for first paw used in the cylinder test. Unpaired t-test ****p<0.0001. **(B)** Contralesional paw preference for total paw contacts used in the cylinder test. Unpaired t-test ***p<0.0002. **(C)** Percentage of limb preference for first paw used to contact wall in the cylinder test. Two-way ANOVA with Sidak's multiple comparison. ****p<0.0001. **(D)** Percentage of limb preference for paw used for total wall contacts in the cylinder test. Two-way ANOVA with Sidak's multiple comparison. ***p<0.0002 and ****p<0.0001. **(E)** Percentage of limb preference for paw used for push-off paw in the cylinder test. Two-way ANOVA with Sidak's multiple comparison. **(F)** Percentage of limb preference for paw used as landing paw in the cylinder test. Two-way ANOVA with Sidak's multiple comparison.

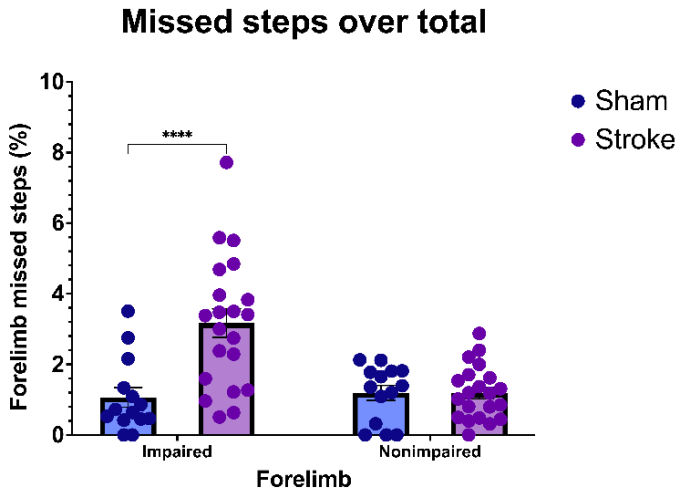

Figure 8. Grid walk test behavioural outcomes for stroke (n=21) and sham (n=14) groups at 2 months of age. Percentage of forelimb missed steps for impaired and nonimpaired paw. Two-way ANOVA, ****p<0.0001.

The cylinder test revealed that stroke mice had a significantly lower contralesional (left) paw preference for initial wall contact (Fig. 7A; sham = 64.60 ± 5.45 %; stroke 34.01 ± 3.84%; ****p<0.0001; unpaired t-test), as well as in the total wall contacts (Fig. 7B; sham 53.48 = ± 1.77 %; stroke 42.65 ± % 1.75; ***p<0.0002; unpaired t-test) compared to the sham group. Additionally, stroke mice had a significantly higher ipsilesional (right) paw preference compared to the sham group on the first wall contacts (Fig. 7C; sham = 29.16 ± 5.83 %; stroke 60.1 ± 3.99

%; ****p<0.0001; Sidak's multiple comparisons test) and total wall contacts (Fig. 7D; sham = 18.9 ± 1.87%; stroke 30.94 ± 2.17%; ****p<0.0001; Sidak's multiple comparisons test). No significant differences were found between sham and stroke mice in preference for limb used during push-off and landing (Fig. 7E, 7F).

In the grid walk test, stroke animals had a significantly higher percentage of missed steps with the impaired limb over the total number of steps taken by the impaired limb than sham animals (sham = 1.068 ± 0.279%; stroke = 3.168 ± 0.404%; p****<0.0001, uncorrected Fisher's LSD; Fig. 8)

5 months of age

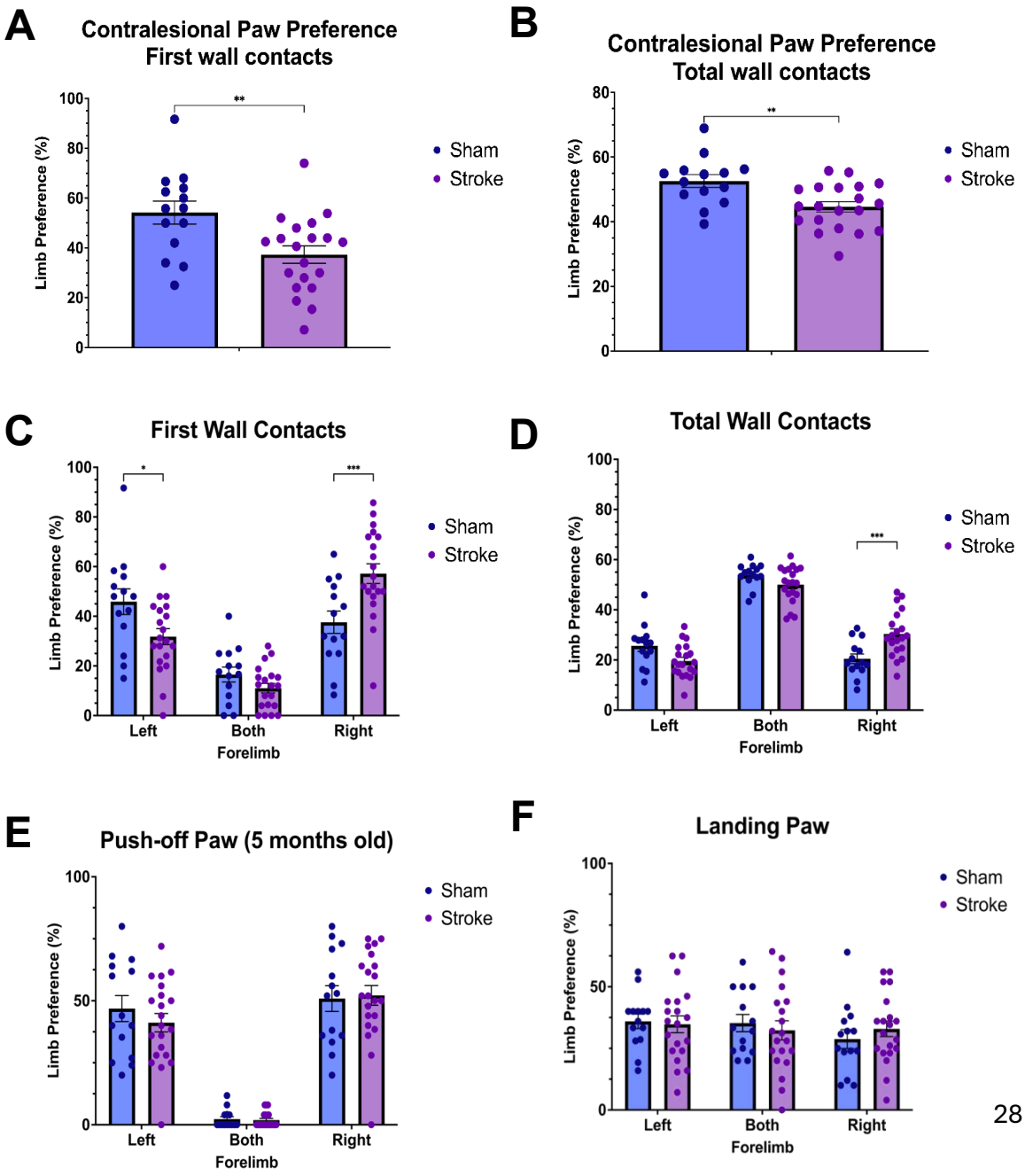

Figure 9. **Cylinder test behavioural outcomes for stroke (n=20) and sham (n=14) groups at 5 months of age.** Contralesional paw preference was measured for different behaviours. **(A)** Contralesional paw preference for first paw used in the cylinder test. Unpaired t-test **p<0.0021. **(B)** Contralesional paw preference for total paw contacts used in the cylinder test. Unpaired t-test **p<0.0021. **(C)** Percentage of limb preference for first paw used to contact wall in the cylinder test. Two-way ANOVA with Sidak's multiple comparison, *p<0.0332 and ***p<0.0002. **(D)** Percentage of limb preference for paw used for total wall contacts in the cylinder test. Two-way ANOVA with Sidak's multiple comparison. ***p<0.0002. **(E)** Percentage of limb preference for paw used for push-off paw in the cylinder test. Two-way ANOVA with Sidak's multiple comparison. **(F)** Percentage of limb preference for paw used as landing paw in the cylinder test. Two-way ANOVA with Sidak's multiple comparison.

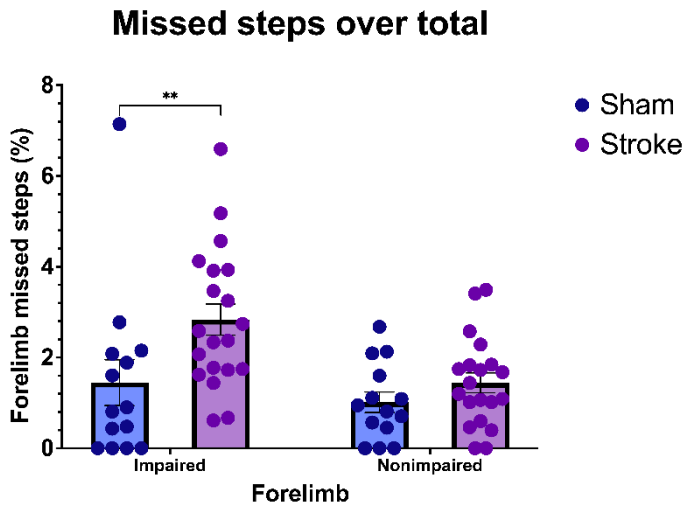

Figure 10. **Grid walk test behavioural outcomes for stroke (n=20) and sham (n=14) groups at 5 months of age.** Percentage of forelimb missed steps for impaired and nonimpaired paw. Two-way ANOVA, **p<0.0021.

At 5 months of age, similar behavioural outcomes were observed during the second timepoint in the cylinder test. Stroke mice maintained a significantly lower contralesional (left) paw preference during initial wall contact (Fig. 9A; sham = 54.15 ± 4.61%; stroke = 37.31 ± 3.48%; p=0.0036; unpaired t-test) and total wall contacts (Fig. 9B; sham = 52.58 ± 2%; stroke = 44.6 ± 1.57 %; p=0.0082; unpaired t-test) compared to the sham group. Moreover, stroke animals

had significantly higher preference for the ipsilesional (right) paw for the first wall contacts (Fig. 9C; sham = 37.59 ± 4.5%; stroke = 57.17 ± 3.96%; p=0.0009; Sidak's multiple comparisons test) and total wall contacts (Fig. 9D; sham = 20.48 ± 1.9%; stroke = 30.38 ± 2.03 %; p=0.0007; Sidak's multiple comparisons test) compared to the sham group. No significant differences were found between sham and stroke mice in preference for limb used during push-off and landing (Fig. 9E, 9F).

In the grid walk test, motor impairments observed at 2 months of age persisted at 5 months of age. Stroke mice had a significantly higher percentage of missed steps with the impaired limb over the total number of steps taken by the impaired limb than sham animals (sham = 1.447 ± 0.5%; stroke = 2.836 ± 0.344%; p =0.0048, uncorrected Fisher's LSD; Fig. 10).

To summarize our findings, testing in adulthood showed motor impairments in perinatal PT stroke mice in both the cylinder and grid walk tests. Although impairments persisted at 5 months of age, we noted that impairments were stronger at 2 months rather than at 5 months of age.

3.4. Lesion volume and localization correlated with impairments observed in the grid walk test at 2 months of age

3.4.1. Lesion volume correlation analysis

In our correlation analysis, we found that lesion volume only had significant correlation with the grid walk test, and not with the cylinder test at 2 months of age (Table 3). Lesion volume positively correlated with the severity of impairment measured by the grid walk test with the percentage of missed steps taken by the impaired limb over the total number of steps of the same limb (Pearson's r = 0.5257 and *p<0.0332; Fig. 11A, Table 3). However, at the same timepoint, we did not see significant correlation between lesion volume and deficits measured by the cylinder test. In fact, there were no significant correlations between lesion volume and contralesional paw preference for first wall contacts (Pearson's r=-0.03569 and p=0.8779; Fig. 11B) and total wall contacts (Pearson's r=-0.2489 and p=0.2778; Fig. 11C).

At 5 months of age, no significant correlations were observed between lesion volume and motor deficits observed in adult behavioural testing (Table 4). This was true for the contralesional paw preference for first wall contacts (Pearson's r=-0.1814; p=0.4441, Table 4) and total wall

contacts (Pearson's r=0.2176; p=0.3567) in the cylinder test. Similarly, no significant correlation was observed between lesion volume and grid walk behavioural outcome at 5 months of age (Pearson's r = -0.09; p = 0.703).

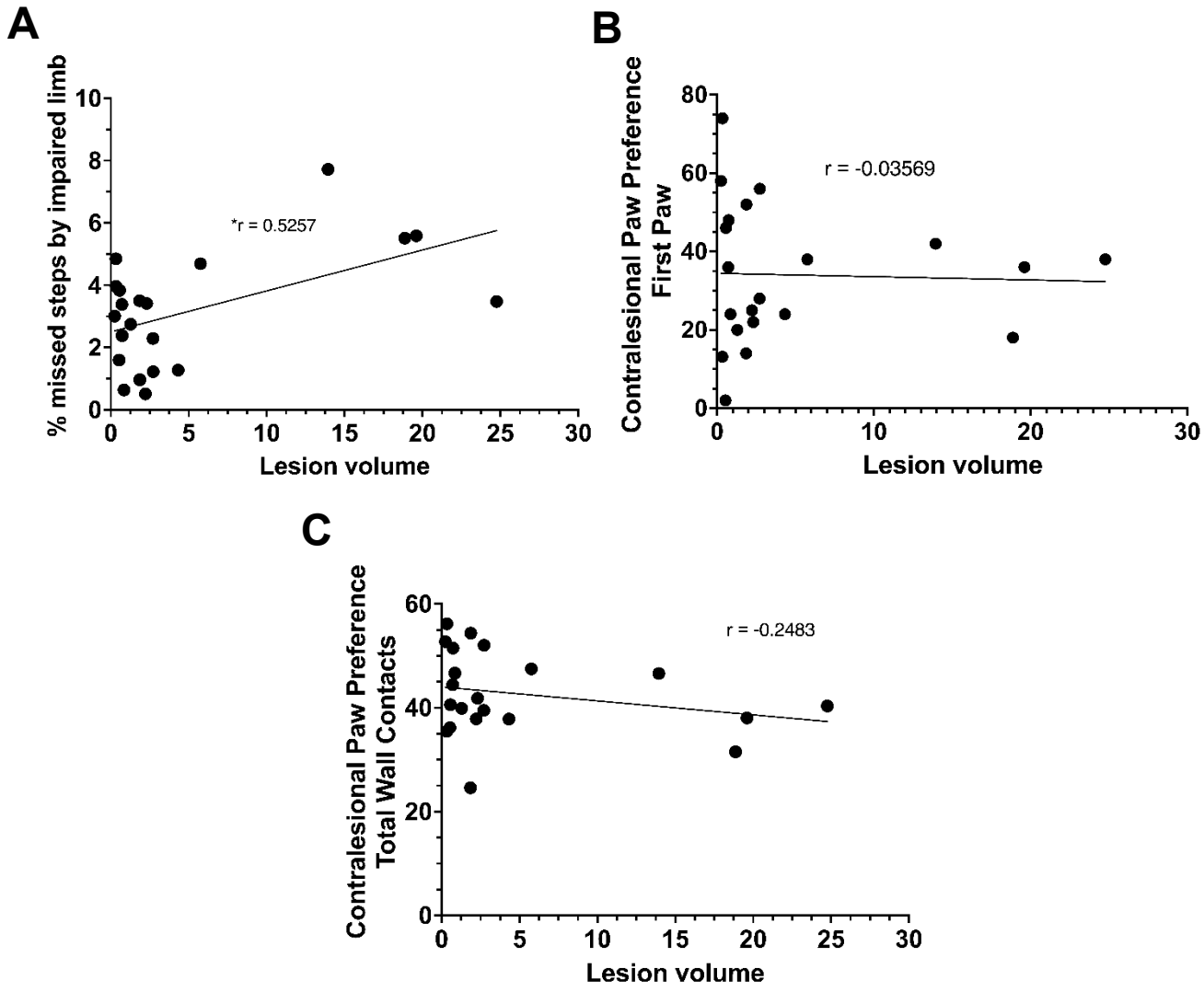

Figure 11. Correlation between lesion volume and behaviour outcomes in the cylinder and grid walk tests at 2 months of age. (A) Correlation between lesion volume and grid walk test outcome, percentage of missed steps by the impaired limb over the total number of steps taken with the impaired limb. Correlation between lesion volume and contralesional paw preference in the cylinder test for **(B)** first wall contacts and **(C)** total wall contacts.

Behaviour measurement	Contralesional Paw Preference First wall contact	Contralesional Paw Preference Total wall contacts	Impaired Missteps normalized
Pearson r	-0.03569	-0.2483	0.5257
95% confidence interval	-0.4603 to 0.4022	-0.6142 to 0.2054	0.1216 to 0.7803
R squared	0.001274	0.06165	0.2764
P value (two-tailed)	0.8779	0.2778	0.0144
P value summary	ns	ns	*
Significant? (alpha = 0.05)	No	No	Yes

Table 3: Correlation results for lesion volume and behavioural outcomes for the cylinder and grid walk test at 2 months of age.

Behaviour measurement	Contralesional Paw Preference First wall contact	Contralesional Paw Preference Total Wall contacts	Impaired Missteps normalized
Pearson r	-0.1814	0.2176	-0.09079
95% confidence interval	-0.5776 to 0.2839	-0.2488 to 0.6022	-0.5127 to 0.3665
R squared	0.0329	0.04737	0.008243
P value (two-tailed)	0.4441	0.3567	0.7034
P value summary	ns	ns	ns
Significant? (alpha = 0.05)	No	No	No

Table 4: Correlation results for lesion volume and behavioural outcomes for the cylinder and grid walk test at 5 months of age.

For the pre-weaning timepoint, we took the average lesion volume of each litter to test for correlation between lesion volume and behaviour performance in the battery of pre-weaning tests, but no significant correlations were observed, due to the variability in lesion volume within litters. For the hindlimb suspension test, we found no significant correlation between lesion volume and the hindlimb suspension score median (Pearson's r= -0.2401; p=0.6972). For the grip strength test, no significant correlation was found between lesion volume and the average inverted fall angle (Pearson's r=0.3053; p=0.6174).

3.4.2. Lesion localization correlation analysis

At 2 months of age, the percentage of area injured for various anatomical regions positively correlated with worse performance in the grid walk test for stroke animals (Table 5). The brain

areas that were significantly correlated with the latter were the primary motor cortex (Pearson's r=0.5535 and **p<0.0021; Fig. 12A), the secondary motor cortex (Pearson's r=0.4592 and *p<0.0032; Fig. 12B), the anterior cingulate cortex (ACC) (Pearson's r=0.4611 and *p<0.0032; Fig. 12C), ACC ventral area (Pearson's r=0.5315 and *p<0.0032; Fig. 12D), the corpus callosum (Pearson's r=0.5703 and **p<0.0021; Fig. 12E), and the ventricular system (Pearson's r=0.4931 and *p<0.0032; Fig. 12F).

Specifically for the layers of the motor cortices, only layer 6 from the secondary motor cortex did not positively correlate to deficits observed in grid walk test performance at 2 months of age. We found positive correlations between the performance in the grid walk test and the percentage of area injured for layers one (Pearson's r = 0.5606 and **p<0.0021; Table 6), two/three (Pearson's r = 0.5715 and **p<0.0021; Table 6), five (Pearson's r = 0.5092 and *p<0.0032; Table 6), and six (Pearson's r=0.5550 and **p<0.0021; Table 6) of the primary motor cortex and layers one (Pearson's r=0.4783 and *p<0.0032; Table 6), two/three (Pearson's r=0.4778 and *p<0.0032; Table 6) and five (Pearson's r=0.4436 and *p<0.0032; Table 6), of the secondary motor cortex.

Impaired limb missteps over total (%)	M1	M2	Somatosensory areas	S1	Ant. cingulate	Ant. cingulate cortex (ventral)	Corpus callosum	Ventricular system
Pearson r	0.5535	0.4592	0.3675	0.3660	0.4611	0.5315	0.5703	0.4931
95% confidence interval	0.1600 to 0.7952	0.03438 to 0.7435	-0.07626 to 0.6898	-0.07799 to 0.6889	0.03667 to 0.7446	0.1295 to 0.7834	0.1839 to 0.8041	0.07804 to 0.7625
R squared	0.3064	0.2109	0.1351	0.1340	0.2126	0.2825	0.3253	0.2432
P (two-tailed)	0.0092	0.03262	0.1012	0.1027	0.0354	0.0132	0.0069	0.0231
P value summary	**	*	ns	ns	*	*	**	*
Significant? (alpha = 0.05)	Yes	Yes	No	No	Yes	Yes	Yes	Yes

Table 5: Correlation results for lesion localization results with grid walk behaviour outcome at 2 months of age.

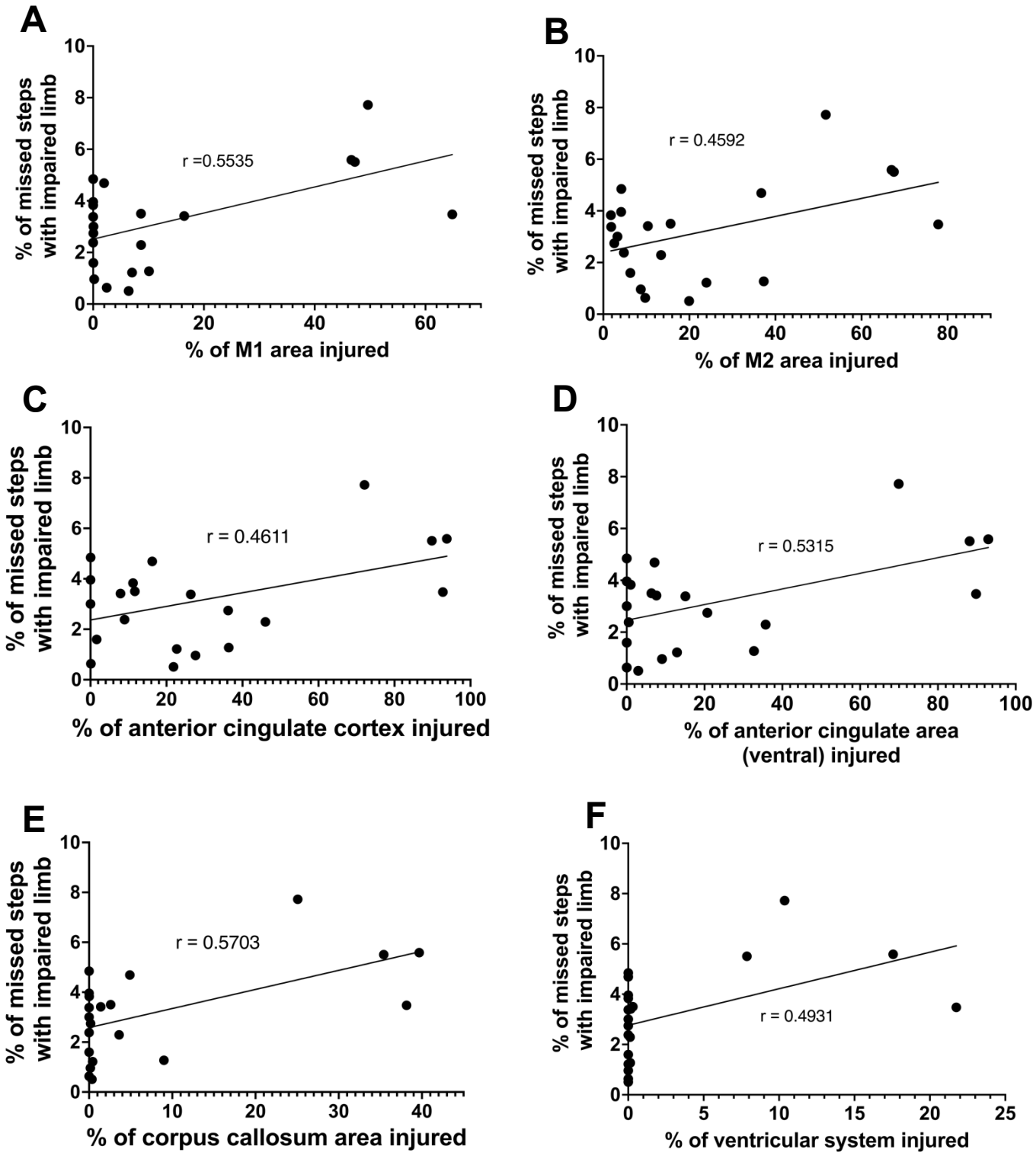

Figure 12. Correlation between percentage of area injured for an anatomical region and grid walk behaviour outcome at 2 months of age. (A) Correlation for primary motor cortex (M1)

(Pearson's r=0.5535 and **p<0.0021). **(B)** Correlation for secondary motor cortex (M2) (Pearson's r=0.4592 and *p<0.0032). **(C)** Correlation for anterior cingulate cortex (ACC) (Pearson's r=0.4611 and *p<0.0032). **(D)** Correlation for anterior cingulate cortex (ventral region). (Pearson's r=0.5315 and *p<0.0032). **(E)** Correlation for corpus callosum (Pearson's r=0.5703 and **p<0.0021). **(F)** Correlation for ventricular system areas (Pearson's r=0.4931 and *p<0.0032).

Impaired limb missteps over total (%)	M1 L1	M1 2/3	M1 L5	M1 L6	M2 L1	M2 L2/3	M2 L5	M2 L6
Pearson r	0.5606	0.5715	0.5092	0.555	0.4783	0.4778	0.4436	0.4272
95% confidence interval	0.1700 to 0.7989	0.1856 to 0.8047	0.09935 to 0.7713	0.1622 to 0.7960	0.05869 to 0.7542	0.05805 to 0.7540	0.01473 to 0.7346	-0.005553 to 0.7251
R squared	0.3142	0.3266	0.2593	0.308	0.2287	0.2283	0.1968	0.1825
P (two-tailed)	0.0082	0.0068	0.0184	0.009	0.0283	0.0285	0.044	0.0534
P value summary	**	**	*	**	*	*	*	ns
Significant? (alpha = 0.05)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No

Table 6: **Correlation results for lesion localization results within the layers of the motor cortices with grid walk behaviour outcome at 2 months of age.**

At 5 months of age, no areas of the brain had a significant correlation between the percentage of their injured area with performance in the grid walk test (Table 7). Similarly, in the cylinder test, contralesional paw preference for the first wall contacts or total wall contacts did not correlate to any of the areas we investigated at 2 months (Table 8 and 9) and 5 months of age (Table 10 and 11).

Impaired limb missteps over total (%)	M1	M2	Somatosensory areas	S1	Ant. cingulate	Ant. cingulate cortex (ventral)	Corpus callosum	Ventricular system
Pearson r	-0.0345	-0.1237	-0.0959	-0.09666	-0.08567	-0.03106	-0.05092	-0.1049
95% confidence interval	-0.4698 to 0.4143	-0.5368 to 0.3373	-0.5165 to 0.3620	-0.5171 to 0.3613	-0.5089 to 0.3709	-0.4672 to 0.4172	-0.4826 to 0.4006	-0.5231 to 0.3541
R squared	0.00119	0.0153	0.009198	0.009344	0.00734	0.0009649	0.002593	0.01099
P (two-tailed)	0.8852	0.6034	0.6875	0.6852	0.7195	0.8966	0.8312	0.66
P value summary	ns	ns	ns	ns	ns	ns	ns	ns
Significant? (alpha = 0.05)	No	No	No	No	No	No	No	No

Table 7: Correlation results for lesion localization results with grid walk behaviour outcome at 5 months of age.

Cylinder test - First wall contact	M1	M2	Somatosensory areas	S1	Ant. cingulate	Ant. cingulate (ventral)	Corpus callosum	Ventricular system
Pearson r	-0.0628	-0.06411	0.04811	0.0476	-0.05913	-0.0751	-0.05953	0.03092
95% confidence interval	-0.4814 to 0.3792	-0.4824 to 0.3780	-0.3917 to 0.4700	-0.3921 to 0.4696	-0.4786 to 0.3823	-0.4909 to 0.3685	-0.4789 to 0.3820	-0.4062 to 0.4565
R squared	0.003944	0.00411	0.002315	0.002266	0.003497	0.005641	0.003544	0.0009558
P value (two-tailed)	0.7868	0.7825	0.8359	0.8377	0.799	0.7463	0.7977	0.8942
P value summary	ns	ns	ns	ns	ns	ns	ns	ns
Significant? (alpha = 0.05)	No	No	No	No	No	No	No	No

Table 8: Correlation results for lesion localization results with cylinder test behaviour outcome (contralesional paw preference for first wall contact) at 2 months of age.

Cylinder test – Total wall contacts	M1	M2	Somatosensory areas	S1	Ant. cingulate	Ant cingulate cortex (ventral)	Corpus callosum	Ventricular system
Pearson R	-0.2716	-0.2681	-0.1127	-0.113	-0.2749	-0.2894	-0.2899	-0.1831
95% confidence interval	-0.6295 to 0.1813	-0.6272 to 0.1849	-0.5192 to 0.3353	-0.5193 to 0.3351	-0.6316 to 0.1779	-0.6410 to 0.1626	-0.6413 to 0.1621	-0.5698 to 0.2699
R squared	0.07377	0.0719	0.01271	0.01276	0.07557	0.08374	0.08402	0.03354
P (two-tailed)	0.2336	0.2399	0.6266	0.6259	0.2278	0.2033	0.2025	0.4268
P value summary	ns	ns	ns	ns	ns	ns	ns	ns
Significant? (alpha = 0.05)	No	No	No	No	No	No	No	No

Table 9: Correlation results for lesion localization results with cylinder test behaviour outcome (total wall contacts) at 2 months of age.

Cylinder test - First wall contact	M1	M2	Somatosensory areas	S1	Ant. cingulate	Ant. cingulate (ventral)	Corpus callosum	Ventricular system
Pearson r	-0.1795	-0.2166	-0.01279	-0.0125	-0.2451	-0.2088	-0.2007	-0.121
95% confidence interval	-0.5763 to 0.2857	-0.6015 to 0.2499	-0.4528 to 0.4322	-0.4525 to 0.4324	-0.6203 to 0.2215	-0.5963 to 0.2575	-0.5908 to 0.2654	-0.5349 to 0.3397
R squared	0.03224	0.04691	0.0001637	0.0001563	0.06006	0.04361	0.04029	0.01464
P value (two-tailed)	0.4488	0.359	0.9573	0.9583	0.2977	0.3769	0.3961	0.6114
P value summary	ns	ns	ns	ns	ns	ns	ns	ns
Significant? (alpha = 0.05)	No	No	No	No	No	No	No	No

Table 10: Correlation results for lesion localization results with cylinder test behaviour outcome (contralesional paw preference for first wall contact) at 5 months of age.

Cylinder test – Total wall contacts	M1	M2	Somatosensory areas	S1	Ant. cingulate	Ant cingulate cortex (ventral)	Corpus callosum	Ventricular system
Pearson R	-0.1921	-0.189	0.05524	0.05598	-0.226	-0.197	-0.2166	-0.1088
95% confidence interval	-0.5849 to 0.2737	-0.5828 to 0.2766	-0.3970 to 0.4859	-0.3964 to 0.4865	-0.6077 to 0.2406	-0.5883 to 0.2689	-0.6015 to 0.2499	-0.5260 to 0.3506
R squared	0.03689	0.03573	0.003051	0.003134	0.05108	0.03883	0.04692	0.01184
P (two-tailed)	0.4172	0.4248	0.8171	0.8147	0.338	0.405	0.359	0.648
P value summary	ns	ns	ns	ns	ns	ns	ns	ns
Significant? (alpha = 0.05)	No	No	No	No	No	No	No	No

Table 11: Correlation results for lesion localization results with cylinder test behaviour outcome (contralesional paw preference for total wall contacts) at 5 months of age.

We also performed the correlation analysis for the data excluding the four largest lesions and found no significant correlations between lesion volume and localization with behaviour performance in the two tests. It would be of interest to increase the sample size to further investigate the potential of lesion volume as a predictor for motor impairments following injury in the PT model.

4 – DISCUSSION

Perinatal stroke is a complicated subtype of stroke representing a global burden to numerous families. It has a high chance of occurrence, no means of prevention and causes lifelong morbidities in survivors. Following perinatal stroke, survivors are left with time intensive and heavy therapies focusing on motor rehabilitation, as functional impairments are the most common outcome of such a type of stroke. To best target neuroprotective and motor rehabilitation therapies, it is important to establish a perinatal stroke model that presents persisting motor impairments reflective of what is seen in perinatal stroke survivors. Previous studies have developed hypoxia-ischemia injury models to study perinatal stroke. However, the latter have proven to be challenging techniques that remain a model primarily of diffuse hemispheric injury rather than a focal occlusive injury. Over the past 25 years, new models producing reproducible focal ischemic lesions in neonate rodents by photothrombosis have been developed to address the limitations of the classic HI models. However, studies focusing on longitudinal assessment of behavioural impairments (from a pre-weaning timepoint to later in adulthood) following PT stroke in a perinatal mouse model have been limited. Additionally, further characterization of the lesion, such as precise localization analysis, has also been limited in perinatal PT stroke mouse models.

To address this, we used a photothrombotic model to induce a focal unilateral brain injury targeting the primary motor cortex and subjected animals to a battery of motor tests at three timepoints: one prior to weaning during early development, and the other two as adults at 2 and 5 months of age. Our perinatal PT stroke model produced long-lasting behavioural impairments. Motor impairments were observed at the pre-weaning timepoint with the grip strength test, while during adulthood, deficits were observed in both the cylinder and grid walk test. We found that the produced PT lesions resulted in infarcts with varying total infarct volume, which was a valuable opportunity to study the effects of lesion size on stroke outcome. We found that lesion volume was a good predictor of functional outcome for the grid walk test, but not for the cylinder test. Furthermore, using a semi-automated lesion localization analysis pipeline, we were able to further characterize the extent of the lesion by obtaining the percentage of damaged area across various brain regions. We found that the most impacted brain regions correlated with worse performance in the grid walk test, but not the cylinder test which provides further support for the grid walk test being a more sensitive assay to detect perinatal stroke deficits. The four largest

lesions were driving the observed correlations, increasing the sample size with larger lesions could help demonstrate such correlations. Our findings demonstrate the importance of understanding changes in motor deficits over time. Through understanding these changes, future studies can devise more optimal therapies following perinatal stroke.

4.1 Perinatal PT stroke model results in variable lesion size and location

Contrary to what has been previously defined in literature (Labat-gest & Tomasi, 2013; Maxwell & Dyck, 2005; Uzdensky, 2018; Watson et al., 1985), lesions produced by the PT stroke method did not result in consistent infarct volumes in our perinatal stroke model. In fact, we found considerable variability in total infarct volumes produced with a consistent duration of light irradiation during the photothrombotic procedure. Interestingly, this variability was present sometimes within the same litter (Fig. 4, Table 2). The variability of lesion size within cohorts was especially strong in cohorts 1, and 4, with the largest differences in lesion volume reaching 11 and 18.7 mm^3 respectively (Table 2). It is important to discuss the possibility of litter effects affecting findings in our study. A considerable number of studies have reported on the importance of litter-to-litter variation (litter effects) biasing findings in research relying on rodent models (Holson & Pearce, 1992; Hughes, 1979; Lazic & Essioux, 2013; Zorrilla, 1997). It has been established that animals from different litters are subjected to different environmental factors, such as dam-dependent care, potentially different litter sizes that impact competition for feeding behaviour, etc. Besides the environmental aspect, animals from the same litter are phenotypically more similar to each other than to mice from different litters of the same strain (Jiménez & Zylka, 2021). Unfortunately, in the present study, we did not track individuals within each litter as we had not previously established an identification system for neonates. For this project, we used multiple breeder cages (1 male and 1 female pair each), and cohorts were defined by the date of surgery. At times, the cages would synchronize in their breeding cycles, so multiple litters would undergo surgical procedure on the same day, forming a cohort. Since animals were P7 at time of surgery, it was not possible to determine their sex at that time, so we could not predict the total number of males and females to be weaned and grouped into separate cages in the future. Considering that we wanted to avoid single-housed animals to reduce possibility of behaviour bias, animals were then weaned together by animal care staff. In future projects, litters could be identified with ear notching or tail marking at the time of weaning, so litter effects can be investigated for the rest of the experiments.

Using the semi-automated localization analysis workflow, we further characterized lesions to assess the extent of damage across various brain regions. We noted that the targeted primary motor cortex was affected by the stroke in all mice except for one. However, it was surprisingly not the brain area with the highest percentage of area damaged. In fact, the most affected areas were the anterior cingulate cortex, the secondary motor cortex, only then followed by the primary motor cortex and the corpus callosum. Additionally, we noted that the areas of infarcted tissue extended to subcortical areas of the brain, often reaching the corpus callosum and lateral ventricles. More precisely, out of the 21 infarcts produced, only 1 did not reach the anterior cingulate cortex, 5 did not reach the corpus callosum and 13 did not reach the lateral ventricles.

However, this variability in infarct volume and location presented a valuable opportunity to investigate the relationship between infarct characteristics and the severity of impairments, which we will go into more details in sections 4.4 and 4.5.

4.2 Perinatal PT stroke model induces behavioural deficits early in development

We were able to demonstrate that the perinatal PT stroke model can induce lesions with persistent sensorimotor impairments. In fact, stroke mice presented deficits at both pre-weaning and adulthood timepoints using a series of sensorimotor tests.

Consistent with the behavioural deficits observed in a study using a hypoxia-ischemia mouse model of CP (Feather-Schussler & Ferguson, 2016), we observed significant deficits in stroke mice in the grip strength test at a pre-weaning timepoint. Although we did not observe significant differences between stroke and sham mice for the forelimb and hindlimb suspension latencies to fall, we did observe differences, although not significant, in the spread of hindlimb suspension scores (Fig. 5C). Significant differences in the scoring would suggest an impairment in stroke mice compared to the sham group. Our results suggest that our perinatal PT stroke model produces deficits acutely, 5 days after stroke. As mentioned previously, one of the limitations in this timepoint is the fact that we could not make it earlier than five days following stroke to reduce mortality of litters. Due to the rapid rate at which developmental processes take place in the mouse brain, these five days following stroke is still a considerable amount of time for potential compensation processes or plasticity to affect observed behaviours. The ability to observe impairments early in development provides the opportunity for future work to investigate the

potential of early therapies on future stroke outcome, and whether the severity of symptoms at an earlier timepoint may be a good predictor for functional deficits outcome later in adulthood.

4.3 Perinatal PT stroke model induces persistent behavioural deficits in adulthood

Later in adulthood, stroke mice demonstrated persistency in the sensorimotor deficits observed in the adult behaviour tests they performed. Using the cylinder test, we were able to measure spontaneous forelimb use during exploratory behaviours in mice following stroke. Deficits in this test were characterized by differences in the preference for use of the contralesional paw for initial wall contacts and for overall exploration of the cylinder wall, as measured by the total wall contacts. These results corroborate with the observed asymmetries in forelimb use in neonatal (Chang et al., 2005; Grow et al., 2003) and adult (Encarnacion et al., 2011; Schallert et al., 2000) rodent models of stroke.

Additionally, we found deficits in motor function and limb coordination with the grid walking test. Stroke mice had significantly higher percentages of missed steps with the impaired limb compared to the sham group. This is consistent with findings of studies using the grid walk test in perinatal (Lubics et al., 2005) and adult (Bland et al., 2000; Encarnacion et al., 2011) rodent models of stroke. In fact, various models corroborate our findings in the production of measurable deficits in neonatal stroke models during adulthood. In a perinatal HI stroke model, Lubics et. al. (2005) observed deficits in the grid walking test at 2 and 4 weeks of age but did not investigate deficits further during adulthood. Similarly, a study using an aspiration model targeting the sensorimotor cortex observed impairments in the grid walk test and in forelimb placing tasks (head-on tasks) at 30 and 42 days post stroke (Barth & Stanfield, 1990). Additionally, in 2017, Gennaro et. al. showed that endothelin-1 (ET-1) model of focal ischemic stroke induced in P14 and P21 rats produced motor function and muscle strength impairments in the ladder climbing and grip test at P59, ~2 months of age (2017). In the same study, they also found that locomotor behaviour and skilled reaching performance demonstrated deficits in the two stroke groups (Gennaro et al., 2017). In 2017, Tsuji et. al. showed that a permanent MCAo model in neonate mouse pups produced sensorimotor impairments in the rotarod test 7 weeks after stroke (Tsuji et al., 2013).

In the context of PT stroke models, studies have also shown that lasting behavioural deficits in the cylinder and grid walk tests were produced in models targeting the motor cortex throughout

a period of 8 weeks following stroke (Clarkson et al., 2013). Notably, in 2013, Brima et. al. investigated behavioural impairments in a neonatal PT stroke rat model targeting the left sensorimotor cortex 60 days after stroke and found significant deficits in a series of behavioural tests. Using the rotarod test, they evaluated motor coordination and equilibrium and found significant deficits in stroke rats. Additionally, deficits in limb strength for both fore- and hind-limbs with the bar holding test were also demonstrated. Interestingly, they only observed deficits in locomotor and exploratory behaviour with the open field test in animals with larger lesions and not in the ones with smaller ones compared to control groups (Brima et al., 2013). These results indicate that sensorimotor impairments were in fact measurable in a PT stroke model at a similar timepoint during adulthood, at around 2 months of age. Additionally, Zhang et. al. used a perinatal PT stroke model and assessed behavioural impairments during adulthood. In this study, they demonstrated motor impairments in the single pellet reaching task at around 3 months of age, but not in the adhesive removal, and tapered beam tests. Interestingly, contrary to the forelimb asymmetry observed in our cylinder test outcomes, the same deficits were not observed in the study by Zhang et. al. (2021). In our study, we observed deficits in the cylinder test with stroke mice demonstrating significantly lower contralesional paw preference in first wall contacts and total wall contacts compared to sham animals at 2 and 5 months of age.

We were able to demonstrate persistency in the functional deficits observed during adulthood, as deficits remained at 5 months of age, or ~22 weeks after stroke. More specifically, the grid walking test was the most sensitive sensorimotor test compared to the cylinder test at the two adulthood timepoints.

Within the perinatal stroke context, it is essential to emphasize the relevance of the behavioural deficits observed with the set of behavioural tests chosen in the present study. While the grid walk test evaluates skilled locomotion and motor coordination, the cylinder test assesses spontaneous forelimb use. These are sensorimotor functions that are commonly impaired following perinatal stroke. As the leading cause of CP, perinatal stroke survivors with unilateral CP often struggle with daily activities requiring skilled bimanual function relying on muscle coordination and bilateral limb functionality (Marcroft et al., 2019). Additionally, it is important to stress the significance of being able to produce long-lasting motor impairments in a perinatal stroke model as this allows for testing of potential rehabilitation therapies. In this investigation, we observed persistency in sensorimotor impairments throughout adulthood, out to 5 months after

stroke in both cylinder and grid walk tests. In a clinical perspective, perinatal stroke survivors' most common therapies are time intensive training that focuses on motor rehabilitation, as previously detailed in section 1.3.3. Given this, it would be interesting to further explore the benefits of intensive versus non-intensive therapies in the perinatal PT stroke model for motor recovery using a skilled motor task such as the skilled reaching task. In recent years, our lab has developed a home-cage skilled reaching apparatus (HASRA) that could be used for this purpose (Salameh et al., 2020). The HASRA runs autonomously on 24 hr/7 days a week basis, allowing food-restricted animals to train as they wish. The software developed for the HASRA can track training parameters for individual mice identified by radio frequency identification (RFID) tags. Thus, a simple modification to the software would allow us to limit the number of training sessions for certain mice. With this, we could implement different training regimes for mice to create intensive and non-intensive therapy groups. In this project, cohorts 1, 5 and 6 (stroke = 9; sham = 12) had been put in the HASRA for skilled reaching training, but we were unfortunately unable to establish a successful training paradigm, so the data was excluded. It would be beneficial for future studies to follow up on establishing a training paradigm for the perinatal PT stroke model, so we can explore these questions.

4.4 Lesion volume correlated with behavioural deficits observed in the grid walk test.

In the present study, the variability observed in both lesion volume represented a valuable opportunity to investigate this relationship in the proposed perinatal PT stroke model. As previously discussed, it is currently suggested that larger lesions lead to worse outcomes following stroke (Bava et al., 2007; Long et al., 2011; Saunders et al., 1995; Wiedemann et al., 2020). More specifically within the perinatal stroke context, it has been suggested that lesion volume can be a valuable predictor for CP in perinatal stroke survivors (Dinomais et al., 2015; Wiedemann et al., 2020). In 2020, Wiedemann et. al. demonstrated this concept using acute diffusion weighted imaging to characterize lesion volume in 37 infants with neonatal arterial ischemic stroke (NAIS). They found that lesion volume is in fact associated with an increased risk of CP, and more specifically, that there was a cut-off at 3.3% stroke volume to divide children into high or low rates for risk of CP. Consistent with these findings, we found that larger lesions produced worse outcomes in sensory motor function measured with the grid walk test. In fact, lesion volume positively correlated with the percentage of missed steps by the impaired limb over the total

number of steps taken by that paw at 2 months of age (Fig. 9A). However, this correlation did not persist with the performance in the grid walk test at 5 months of age. Moreover, we did not observe correlation between lesion volume and the behavioural deficits observed in the cylinder test.

4.5 Lesion localization correlated with behavioural deficits observed in the grid walk test.

The same study discussed in 4.4 by Wiedemann et. al. also suggested that having smaller lesions in survivors with NAIS does not directly rule out the risk of CP. If lesions extend to susceptible brain areas, such as the CST and the thalamus, such injuries lead to CP even when they were below the 3.3% stroke volume (Wiedemann et al., 2020). This finding suggests the importance of lesion localization in functional outcome after stroke, which has been corroborated in other clinical studies (Chen et al., 2000; Dinomais et al., 2015). Due to the relative variation in lesion localization in the produced injuries in this study, we were able to investigate the relationship between lesion site and behavioural deficits in a perinatal PT stroke model. First, it is important to discuss the roles of the brain areas that were the most impacted by our stroke model, to understand how these could have affected the behavioural outcomes observed.

The most affected area was the anterior cingulate cortex (ACC). A considerable amount of literature has been published regarding the function and role of the ACC. Notably, anatomic studies have reported that the ACC has descending corticospinal projections in rats and monkeys (Gabbott et al., 2005; Galea & Darian-Smith, 1994). This suggests that the ACC can directly influence spinal cord neurons and subsequently impact motor function. Given this, we must consider the possibility of developmental injury to the ACC consequently affecting the development of such corticospinal projections, and therefore the observed functional outcomes.

Furthermore, the ACC is a crucial part of the prefrontal cortex in mice and is known to support a variety of cognitive functions such as those related to attention, planning and execution of movements, as well as reward guided decision making (Peeters et al., 2020; Rushworth & Behrens, 2008). Others have suggested the possibility of ACC being involved in other cognitive functions such as motivation. Within the perinatal stroke context, stroke survivors often end up with cognitive morbidities including attention and memory-related disorders, as well as impairments in planning and problem solving skills (Kirton & Deveber, 2013). Since the ACC was the mostly affected region by our lesions, it is possible that stroke mice had cognitive related deficits. Considering our attempt at training perinatal stroke animals in the single pellet reaching

task (as discussed in section 4.3), we could potentially explain the challenge in training them in the reaching task was due to injuries in the ACC. In fact, such lesions may have affected important cognitive functions for skilled motor training, such as motivation and reward guided decision making.

As detailed throughout this thesis, our study focused on assessment of motor impairments since our original target site was the primary motor cortex. Given this finding, it would then be interesting for future projects using the perinatal PT stroke model to add cognitive behavioural testing to the battery of tests to ensure an even more comprehensive characterization of the outcomes in this model. To address this, we could use the Morris water maze test, which has been widely used to evaluate long-term cognitive function in rodent models of ischemic stroke (Gibson & Murphy, 2004; Y. R. Kim et al., 2015). This test measures the latency for the rodent to find a hidden platform in a large circular pool, which is a measure of memory deficits. Besides the water maze test, there is a great variety of behavioural tests for cognitive assessment in mice after stroke that we could explore, such as the Y-maze task, and the novel object recognition test (Ruan & Yao, 2020).

The second and third most affected areas were the secondary and primary motor cortex. These regions are part of the somatomotor areas, which are fundamental in the transmission of signals to direct body movement, and muscle control. The primary motor cortex in particular has been described as the main region responsible for the planning and execution of motor commands (Ebbesen & Brecht, 2017, p.; Whishaw et al., 1986). Lesions targeting the motor cortex in preclinical studies have shown to result in measurable motor impairments, particularly for ischemic stroke models. In the present study, we showed that the perinatal PT stroke model produced long-lasting motor impairments that were measurable in a series of behavioural tests. Impairments were not only observed during adulthood, but also at a pre-weaning timepoint, earlier in development. This finding underlines the relevance of this perinatal PT stroke model and its ability to recapitulate perinatal stroke pathology for preclinical studies. However, we must acknowledge that we did not explore other potential neurological outcomes that are commonly associated with perinatal stroke, such as cognitive function impairments (Kirton & Deveber, 2013), which may bring limitations to this model.

Finally, another relevant brain area that was significantly affected by our model was the corpus callosum. The corpus callosum is a highly myelinated structure, composed of white matter

tracts that acts as a bridge connecting the left and right cerebral hemispheres, allowing for the transmission of information. It is well-known for its major role in movement control, as well as cognitive functions (memory and learning), and vision. When the corpus callosum is surgically severed or injured, communication between the two cerebral hemispheres is reduced or completely cut off, often resulting in muscle coordination and cognitive impairments (X. Huang et al., 2015). Since PT induced lesions often extended to the corpus callosum in our study, we should consider the potential impact this could have had on the behavioural outcomes observed. As discussed for the ACC, injuries in the corpus callosum could also be impacting cognitive functions, so assessing the latter with behavioural tests would be beneficial to better understand their involvement in the outcomes observed. Investigating possible correlations between cognitive test performance and the extent of lesion in the corpus callosum and ACC could result in interesting findings.

Finally, we need to acknowledge the limitation in the follow-up of animals from birth to endpoint in our study. Since we did not track mice throughout their development, we were not able to conduct correlation analysis between lesion volume and localization to the behavioural outcomes at the pre-weaning timepoint. In the future, we could tag animals using a neonate rodent tattoo identification system such as the one described by (Westmark et al., 2021).

5 – CONCLUSION

Overall, we showed that the perinatal PT stroke model produces long-lasting motor impairments that are measurable from an early developmental timepoint prior to weaning until later in adulthood. In our study, we were also able to characterize infarcts by performing lesion volume analysis and by using a semi-automated pipeline for lesion localization analysis. This detailed lesion characterization allowed us to comprehensively assess the extent of the lesion and identify the most affected brain areas - thus allowing for the investigation of their involvement in functional outcomes following stroke.

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