MODULATION OF INTERHEMISPHERIC INHIBITION AND ITS EFFECT ON STROKE RECOVERY IN THE RAT

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ABSTRACT

Rationale: Residual disability following stroke is a significant challenge for patients and the healthcare system, both in New Zealand and worldwide. Poor recovery from motor cortex (M1) stroke has been associated with an increase in interhemispheric inhibition (IHI) initiated by the unlesioned cortex (Murase, Duque, Mazzocchio, & Cohen, 2004). Previously we showed using intracellular recordings from single neurons that intermittent theta-burst stimulation (iTBS; bursts of three pulses at 50 Hz at 5 Hz) delivered to the contralateral M1 acutely reduced IHI in normal rats (Barry, Boddington, Igelström, et al., 2014). Additionally, this stimulation improved motor recovery sub acutely following stroke (Boddington, Gray, Schulz, & Reynolds, 2020). In the present study we aimed to record IHI changes in the freely moving, stroke affected rat and measure any association with behavioural impairment. We also aimed to determine if iTBS could modify IHI and improve behaviour.

Method: A method of measuring IHI in rats using EEG recording techniques was first developed. Second, to study effects of stroke and iTBS, lesions were induced by injecting endothelin-1 into M1 and dorsal sub-cortical regions and electrodes implanted to measure IHI in freely moving animals. Sham stimulation or iTBS was delivered at low intensity to the contralesional cortex for 15 days, followed by three weeks of no stimulation. Behaviour was recorded throughout, using grid walking and pasta handling tasks.

Results: Baseline IHI was 19.1% ± 4.4 and was increased to 34.3% ± 5.5 one week following lesion induction (mean difference: 15.2 ± 6.0, $p=0.033$). In addition, increased IHI was weakly associated with worsened motor performance (R^2: 0.11, $p<0.005$). Comparing iTBS and sham stimulated animals, in the final week of the study, iTBS animals showed a 54% ± 8.7 functional improvement in foot faults from their post stroke grid walking impairment, compared to only 15% ± 11 in the sham group; mixed effects model: $p<0.05$. The pasta handling task also revealed significantly greater improvement following iTBS (improvement of 56% ± 17) compared to sham (14% ± 7.4): $p<0.05$. In addition, delivery of iTBS reduced IHI to a greater degree than the spontaneous reduction observed in the sham group (linear mixed model: $p<0.05$), both in the weeks during and following stimulation.

Conclusion: Our results show an association between IHI and behavioural recovery in the early stages of stroke recovery. They also show that IHI is reduced, and behavioural recovery improved by low intensity, contralesional iTBS.

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GLOSSARY OF ACRONYMS

°C	Degrees Celsius
ΔIHI	Change in interhemispheric inhibition
µA	Microamp
µl	Microlitre
µm	Micrometre
μm^3	Micrometre cubed
AEC	Animal Ethics Committee (refers to protocols approved by this committee)
a.m.	Ante meridiem
ANOVA	Analysis of variance
AP	Anterior posterior
CB1	Cannabinoid receptor type one
cm	Centimetre
CSD	Current source density
cTBS	Continuous theta burst stimulation
DV	Dorsal ventral
EEG	Electroencephalogram
eES	Epidural electrical stimulation
F	F-ratio (of variance between two group means)
g	Gram
GABA $_{(A \text{ or } B)}$	Gamma aminobutyric acid (A or B)
Hz	Hertz
i.p.	Intraperitoneal
IHI	Interhemispheric inhibition
IPG	Implantable pulse generator
ISI	Interstimulus interval
iSP	Ipsilateral silent period
iSTART	Implanted Stimulators To Augment Rehabilitation Therapy
iTBS	Intermittent theta burst stimulation
kg	Kilograms
kHz	Kilohertz
LIHI	Long latency interhemispheric inhibition
M	Molar
M1	Primary motor cortex
mA	Milliamp
MEP	Motor evoked potential
mg	Milligrams
min	Minute
mL	Millilitre

ML	Medial lateral
mm	Millimetre
mm^3	Millimetre cubed
ms	Millisecond
mV	Millivolt
n	Number
nL	Nanolitre
NMDA	N-Methyl-D-aspartate
P or p	Probability value
p.m.	Post meridiem
PMC	Pre-motor cortex
pmol	Picomole
PSP	Post synaptic potential
r	Pearson correlation coefficient
R^2	Coefficient of determination
ROUT	Robust regression and outlier removal
s.c.	Subcutaneous
SEM	Standard error of the mean
SICI	Short latency intracortical inhibition
SIHI	Short latency interhemispheric inhibition
SMA	Supplementary motor area
t	Student's t-test
TBS	Theta burst stimulation
tDCS	Transcranial direct current stimulation
TMS	Transcranial magnetic stimulation

1 INTRODUCTION AND STATEMENT OF THE PROBLEM

This thesis describes investigations of stroke induction and a subsequent protocol of electrical stimulation on the neurophysiological and behavioural measures of recovery in rats. In this chapter, the relevance of this project will be outlined, and the study aims will be presented.

Stroke, also known as cerebrovascular disease, is a leading contributor to the global burden of disease (Lopez, Mathers, Ezzati, Jamison, & Murray, 2006). In 2010, strokes resulted in 113 million 'disability adjusted life years' lost worldwide (Feigin et al., 2014; Feigin et al., 2015), with this burden increasing over time. In New Zealand, stroke causes over 30,000 lost disability adjusted life years, with the burden of morbidity and mortality disproportionately affecting Māori and Pacific people, and those from lower socioeconomic groups (Krishnamurthi et al., 2018).

Strategies for reducing the global and national burden of stroke morbidity and mortality generally have one of three aims: firstly, to prevent stroke through modification of the underlying risk factors (for example, by treating hypertension); secondly, to reduce the damage done in the acute phase of stroke (for example, by administering drugs to unblock the artery causing an ischaemic stroke); finally, to provide rehabilitation to people who have had a stroke to enable them to regain or relearn lost functions. This final strategy will be the focus of this thesis.

Clear evidence-based guidelines exist as to the amount and type of rehabilitation that should be given in order to confer the maximum possible benefit following stroke (Stroke Foundation, 2017; Stroke Foundation of New Zealand & New Zealand Guidelines Group, 2010). Unfortunately, often these are not able to be followed as closely as would be preferred, with insufficient staffing, lack of staff education and delay in transfer to rehabilitation some of the reasons for this in New Zealand (McNaughton, McRae, Green, Abernethy, & Gommans, 2014). Even when optimal rehabilitation is given, many stroke survivors continue to experience difficulty with returning to the level of function they enjoyed prior to stroke, and report lower quality of life (Broeks, Lankhorst, Rumping, & Prevo, 1999; Clarke & Black, 2005; Kong & Yang, 2006; Langhorne, Coupar, & Pollock, 2009). Loss of upper-limb function is one of the impairments which results in the greatest difficulty with performing 'activities of daily living' (Nouri & Lincoln, 1987). This may not be completely addressed with rehabilitation alone, with physiotherapists describing a 'ceiling of recovery' beyond which any improvements in function are very hard to achieve (Broeks et al., 1999; Hendricks, van Limbeek, Geurts, & Zwarts, 2002; Langhorne et al., 2009; Schaechter, 2004).

One proposed reason for this plateau is that it is due to innate mechanisms of recovery that occur in the brain following stroke, which lead to an imbalance of excitation and inhibition across the stroke lesioned and un-lesioned hemispheres (Cicinelli et al., 2003; Huynh, Vucic, Krishnan, Lin, & Kiernan, 2016). Particularly implicated is a source of inhibition known as interhemispheric inhibition, which in the healthy brain mediates unimanual movements and bimanual coordination via connections crossing the corpus callosum (Beaulé, Tremblay, & Théoret, 2012). In the stroke-affected brain, an excess of interhemispheric inhibition arising from the contralesional hemisphere develops, and has been shown in some instances to be associated with poor post stroke recovery (Du et al., 2018; Duque et al., 2005; Murase et al.,

2004). If this excess inhibition were to be reduced, the lesioned hemisphere may be better able to respond to rehabilitation to regain lost functions. The contralesional hemisphere has therefore been a target for interventions which aim to reduce inhibition and thus promote recovery of the lesioned hemisphere.

Multiple techniques have been developed to artificially modulate the processes of excitation, inhibition and plasticity within the brain. These include the non-invasive techniques of transcranial magnetic stimulation and transcranial direct current stimulation. These techniques can be delivered by a skilled operator without the need for invasive surgery, or in the case of transcranial direct current stimulation, can be applied by the patient themselves. While initial studies showed promise for these techniques in promoting stroke recovery, evidence for long term efficacy is lacking (Hao, Wang, Zeng, & Liu, 2013; Hsu, Cheng, Liao, Lee, & Lin, 2012). In addition, it is difficult with these techniques to target the stimulation to a specific area of the brain, and the mechanisms underlying their ability to induce plasticity or alter excitability are not well understood.

More invasive neuromodulation techniques have also been trialled. Epidural electrical stimulation involves surgically implanting a stimulating electrode, which can deliver targeted stimulation to a specific brain area. Investigations of this technique for promoting stroke recovery by targeting the lesioned hemisphere have shown promise in the past (Brown, Lutsep, Weinand, & Cramer, 2006; M. Huang et al., 2008; R.M. Levy et al., 2016; R. M. Levy et al., 2008) and more recently have been adapted to target the contralesional hemisphere. Barry et al. showed that delivery of a pattern of electrical stimulation known as intermittent theta burst stimulation delivered at low intensity to an electrode in the cortex of the rat could acutely

reduce interhemispheric inhibition from that hemisphere (Barry, Boddington, Igelström, et al., 2014), and trials of this stimulation paradigm in a rat model of stroke showed that it improved behavioural recovery (Boddington et al., 2020).

1.1 STUDY AIMS

In this project we had the following objectives:

Objective 1: To investigate the use of a minimally invasive technique for the measurement of interhemispheric inhibition in the freely moving animal.

Objective 2: To determine whether deficits after stroke are associated with an alteration of interhemispheric inhibition.

Objective 3: To determine whether, in an animal model of stroke, low intensity intermittent theta burst stimulation to the contralesional hemisphere can reduce interhemispheric inhibition and improve behavioural recovery.

This thesis therefore aims to determine if there is a link between interhemispheric inhibition and behavioural recovery, and any role application of intermittent theta burst stimulation may play in this. This project took place in the context of a concurrently running human trial of low intensity contralesional iTBS: the "Implanted Stimulators To Augment Rehabilitation Therapy" trial. It is our hope that these two trials will aid the development of contralesional

iTBS as a novel therapeutic approach to promote stroke recovery. The following chapter presents a review of the literature relating to this topic in order to guide the reader's understanding of the results presented later in the thesis.

1.2 SUMMARY OF iSTART TRIAL PROTOCOL AND RELATIONSHIP TO THIS THESIS

In addition to the research described in this thesis, it should be noted that a human trial of this intervention was underway at the time of writing. The "Implanted Stimulators To Augment Rehabilitation Therapy" (iSTART) project builds on the work of Barry, Boddington, Igelström, et al. (2014), Boddington et al. (2020) and R.M. Levy et al. (2016) in a neuromodulation trial aiming to maximise the potential for clinically meaningful benefit following stoke.

Only patients who are shown to have intact corticospinal pathways and motor responses to affected hemisphere stimulation were included, ensuring that any effect on the rebalancing of hemispheric excitability is able to have a functional benefit as well (R.M. Levy et al., 2016). In addition, a protocol of low-intensity iTBS over the affected hemisphere was used, in accordance with the observed results from rats (Barry, Boddington, Igelström, et al., 2014; Boddington et al., 2020), and so that the electrode was placed over a healthy area of the cortex which has the potential for plasticity. Thirdly, the stimulation took place in conjunction with rehabilitation, so that any plasticity which is enhanced by stimulation was associated with rehabilitation tasks (Harvey & Winstein, 2009; Takeuchi & Izumi, 2015). The trial itself is a safety and feasibility trial, and so is not powered to detect clinically meaningful endpoints. It is hoped that the design of this trial will inform a larger multicentre trial in future.

2 REVIEW OF LITERATURE

This review will begin with a description of the normal anatomy and functions of the motor cortex. Following this, the pathophysiology of stroke will be considered, including the processes of brain plasticity and recovery that follow, as well as a description of how this pathology has been modelled within experimental animals. Next, will be a description of interhemispheric inhibition and how it is affected following stroke. Finally, target pathways for intervention will be considered, as well as a review of the literature surrounding the efficacy of contemporary neuromodulation techniques.

2.1 MOTOR CORTEX

As early as the 18th century it was recognised that the functions of the brain were localised to anatomical regions. This principle now forms one of the pillars of modern neuroscience. Motor functions are subserved for the most part by the primary motor cortex (M1), which is located immediately anterior to the central sulcus, in the precentral gyrus (Figure 2.1). Motor control of the muscles of the body is mapped onto the motor cortex in an arrangement that roughly reflects their anatomical arrangement in the body itself, a concept known as somatotopy. This is often represented visually in a 'motor homunculus' diagram, which illustrates the part of M1 that corresponds to control of movement of each body part (Figure 2.1). Note that the size of the motor representation reflects the degree of control that is possible for that body part, so that for instance the hands have a larger representation than the feet. This arrangement is largely

conserved across individuals and to a certain extent between species, reflecting its importance to brain function.

Most of the output tracts from the motor cortex cross the midline within the brainstem, meaning that the left side of the brain primarily controls the movements of the right side of the body. There are a smaller number of uncrossed fibres, about 10%, which control movements of the same side as the hemisphere from which they originate (Kandel, Schwartz, & Jessel, 2000). These uncrossed fibres usually control proximal muscles in the midline, for example the trunk muscles that help with posture.

The functions and outputs of M1 are in part controlled by inputs from secondary motor areas e.g. the premotor cortex and supplementary motor area, which assist with planning and initiation of movements, as well as suppressing unwanted movements. The motor cortex also receives inputs from the opposite hemisphere via the corpus callosum, the large white matter tract that connects the two hemispheres of the brain. The connection between opposite motor cortices has been mapped to the posterior third and anterior fourth segments of the body of the corpus callosum (Figure 2.1) (Meyer, Röricht, & Woiciechowsky, 1998; Wahl et al., 2007).

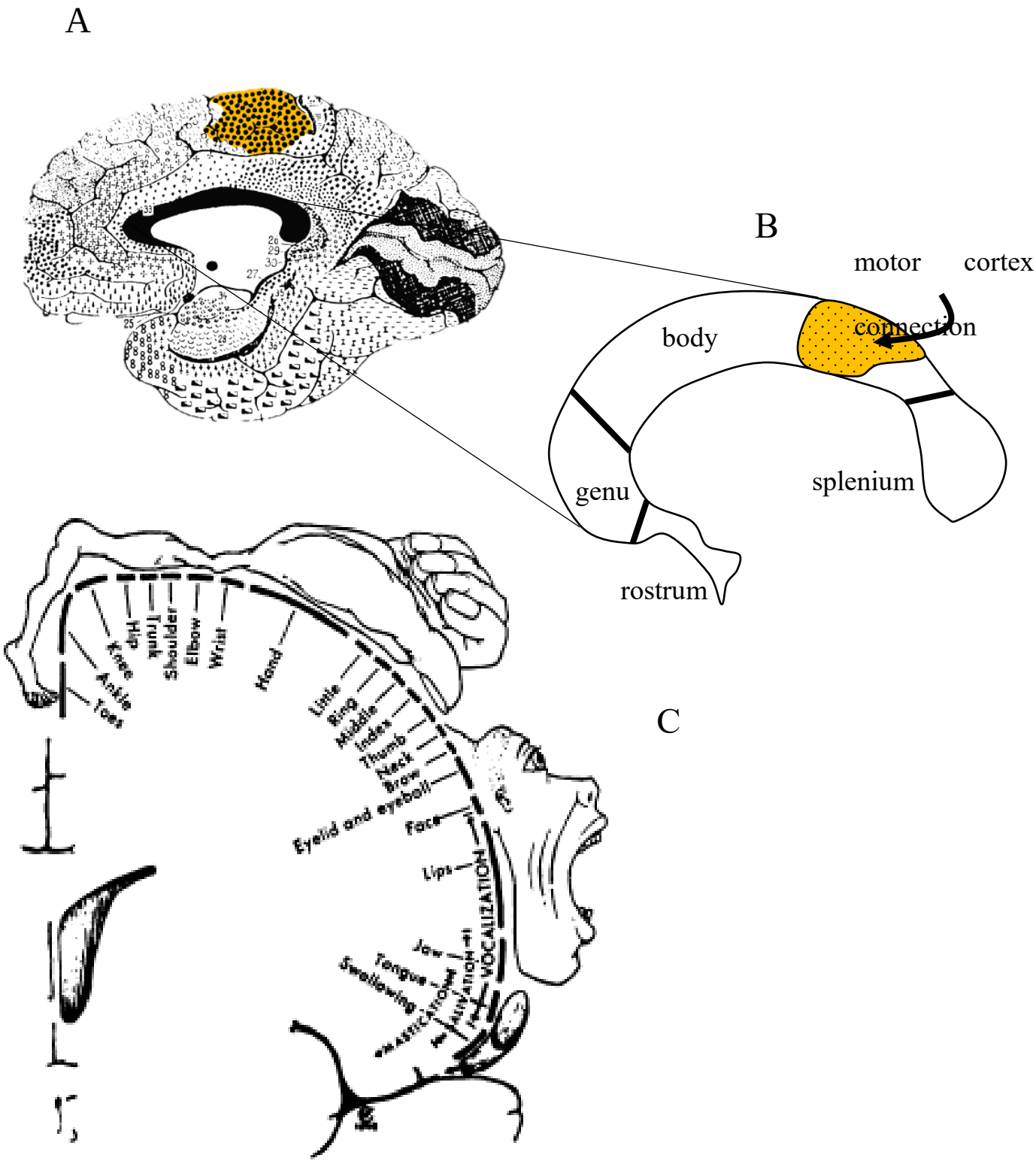

Figure 2.1 A: Location of the motor cortex (orange), with insert showing medial surface and corpus callosum location. B: Anatomy of the corpus callosum, highlighting the connection between motor cortices. C: Motor homunculus diagram illustrating the somatotopic representation of motor function. Adapted from Brodmann (1903); Schott (1993).

The primary motor cortex is arranged microscopically, with different types of neurons forming layers according to function. The two main types of neuron found in the cortex are the pyramid-shaped projection neurons, the ‘pyramidal’ neurons, which have outputs to remote areas of the brain or body, and the more heterogenous interneurons, which are typically inhibitory in nature and form local connections within the cortex. Across the cortex there are generally six layers, labelled I through to VI from superficial to deep (

Figure 2.2). The two layers which are of greatest importance to the motor cortex are layer II/III, and layer V. Layer II/III receives inputs from the corpus callosum (Gonchar, Johnson, & Weinberg, 1995; Palmer et al., 2012), and contains those pyramidal projection neurons that

themselves cross the corpus callosum (DeFelipe & Farinas, 1992). Layer V contains the large pyramidal neurons that send outputs to the motor units of the peripheral nervous system. Layer V neurons have a network of dendrites within the more superficial layers, which receive inputs from interneurons within the motor cortex and connections from further afield.

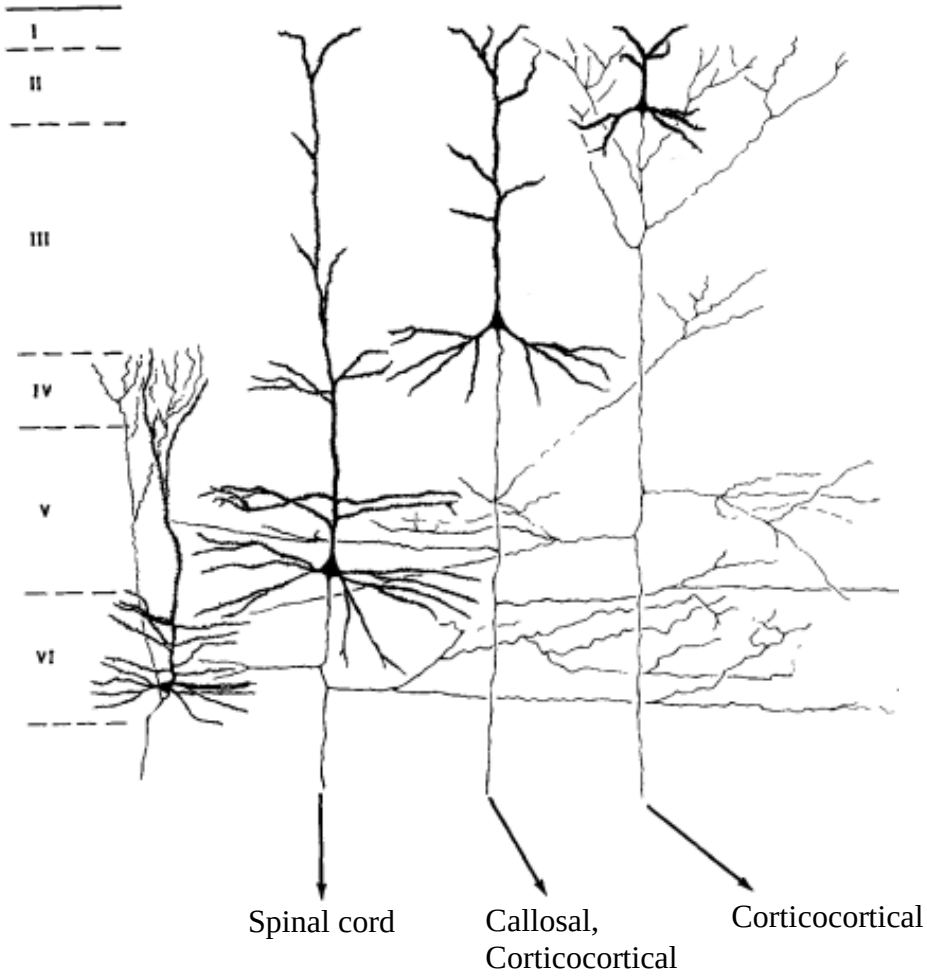

Figure 2.2 Layered arrangement of the motor cortex, with corticospinal output from layer V and callosal and corticocortical connections from layers II/III. Adapted from DeFelipe and Farinas (1992).

Interneurons themselves are a diverse group of cell types, however they most commonly utilise the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). This neurotransmitter can have either short or long-term effects, depending on the receptor to which it binds post-synaptically. The first of the two types are $GABA_A$ receptors, which are simple ion channels, and so respond very quickly (<5 ms). The second type of receptor, $GABA_B$, act on the post synaptic cell via G-protein coupled receptors, so have a slower (approximately 50 ms) effect (Connors, Malenka, & Silva, 1988; Kuriyama, Hirouchi, & Nakayasu, 1993).

2.2 PATHOPHYSIOLOGY OF STROKE

This review will now turn to a discussion of how the normal functions of the brain are deranged by stroke. It will also consider the pathological processes that drive these changes. The term 'stroke' refers to a collection of conditions which occur when the blood supply to the brain is interrupted. This can happen in several ways, most commonly through the blockage of one of the vessels supplying the brain (referred to as an ischaemic stroke), or through a bleed into the brain (haemorrhagic stroke). There are also a number of other less common pathologies (Krishnamurthi et al., 2018). The ischaemic strokes can be further divided by the cause of ischaemia, with large strokes due to emboli from the heart or large-artery atherosclerosis making up 45% in total, and smaller strokes from small vessel occlusion a further 21% (Cheng et al., 2017; Krishnamurthi et al., 2018).

At a basic level, the components predicting functional outcome following stroke are determined by the brain structures that are damaged (M. Corbetta et al., 2015; Forkert et al., 2015). When a specific area is damaged, there is a predictable syndrome of functional

impairment that results. Strokes typically affect subcortical structures, or a combination of cortical and subcortical structures, with purely cortical lesions being less common (M. Corbetta et al., 2015).

2.2.1 TIMELINE OF STROKE PATHOPHYSIOLOGY

The loss of blood supply to an area of the brain results in death of neurons in that area (Hughes et al., 2003). In addition, the release of the chemical contents of the dying neurons and a spread of a depolarising current causes the area surrounding the initial insult to become damaged as well (Sims & Muyderman, 2010). The region affected by spreading damage is known as the 'penumbra' and is often only partly damaged. In both rats and humans these processes of damage occur over approximately the first 72 hours, as imaged on MRI, at which time the process of gliosis (scarring) begins (Hughes et al., 2003; Saggu, 2013; Schwamm et al., 1998). This period of initial damage is typically referred to as the acute phase of stroke.

Following the initial damage, during the sub-acute phase of recovery, reorganisation occurs where the brain takes on the task of restructuring to support recovery of the lost functions. This period is from approximately one to three months after stroke in humans, and about nine days to three weeks in rodents (Vallone et al., 2016; Zeiler & Krakauer, 2013). One of the defining features of neural tissue is its ability to adjust structurally in response to new information, a process known as plasticity. The brain responds on an anatomical level to external stimuli and experiences, altering the strength of those pathways that are used more frequently (M. E. Diamond, Huang, & Ebner, 1994; Hebb, 1949). This process underlies the recovery following stroke, occurring when undamaged areas take on lost functions (Ward, 2005). This concept is

harnessed with rehabilitation, which aims to encourage formation of these new pathways, for example by repetitive task training with the paretic arm.

This principle has been demonstrated in rats; Lake et al. (2017) found that three weeks after an initial disruption to somatotopy, field potentials measured in the area surrounding the lesion became larger. This suggests a process of functional remodelling around the centre of the lesion. Starkey et al. (2012) showed that in lesioned adult rats, surviving neurons changed their spinal cord targets to provide afferents for those areas that had lost brain inputs, and that the success of this process correlated with better functional recovery.

The effects of rehabilitation can also be seen experimentally. In individuals that preferentially use the non-paretic limb following stroke damage, the ability to use the paretic limb is unlearned, and synapses targeting the 'healthy' limb are increased and strengthened, so-called 'maladaptive plasticity' (Jones, 2017; Starkey et al., 2012; Takeuchi & Izumi, 2012). On the other hand, if rehabilitation is used to train the paretic limb, the neural architecture supporting movement of this limb is formed and strengthened (Adkins, Voorhies, & Jones, 2004; Jones, 2017; Nishibe, Urban, Barbay, & Nudo, 2015; Ling Wang, Conner, Nagahara, & Tuszynski, 2016). The importance of targeting rehabilitation to support the paretic upper limb and avoid maladaptive plasticity from preferential use of the unaffected limb is reflected in current clinical guidelines. These recommend a program of physiotherapy involving constraining the unaffected limb during rehabilitation and during the day, known as constraint induced movement therapy (Langhorne et al., 2009; Stroke Foundation, 2017).

The final phase of stroke recovery is the 'chronic phase', which occurs months to years after stroke onset. There is some evidence to suggest that rehabilitation has the potential to be clinically useful well into the chronic phase of recovery (Lee et al., 1999; Massie et al., 2016; Pellegrino et al., 2011). This hints at the continued role of plasticity that is occurring throughout the life of the adult brain. It is this potential that modern neurorehabilitation techniques hope to harness. See the below table for a summary of the information in this section Table 2. 1.

Phase of stroke recovery	Humans	Rats
Acute - cell death followed by gliosis	72 hours (cell death), and then up to 1 month	72 hours (cell death), and then up to 9 days
Subacute – structural reorganisation	1 month to 3 months	9 days to 3 weeks
Chronic – consolidation of recovery	3 months onwards	3 weeks onwards

Table 2. 1 Estimated timelines of stroke recovery for humans and rats.

2.3 ANIMAL MODELS OF STROKE

Some of the research discussed thus far has achieved gains from study of models of stroke, most commonly in rats. It is important to consider the validity of these models before moving on to discussion of the specific pathway studied within this project. Ischaemic stroke is the most common cause of stroke pathology among human stroke survivors; therefore, it is the aim of most animal models of stroke to mimic this process as closely as possible. One method for achieving this, and the method employed in this study, is the use of the vasoconstrictive peptide endothelin-1. We will also briefly mention the photothrombotic model of lesion induction, to enable comparison between the current study and previous work in this field. Another model which warrants mentioning is the middle cerebral artery occlusion model. In this model, the large middle cerebral artery of one hemisphere is occluded, using a variety of techniques, producing a large and often heterogenous lesion encompassing almost an entire brain

hemisphere. For a comparison of the advantages and disadvantages of multiple stroke models, both Fluri, Schuhmann, and Kleinschnitz (2015) and (Conn, 2017) have a comprehensive review.

2.3.1 ENDOTHELIN-1 MODELS

Endothelin-1 is the most potent of the human vasoconstrictors. It binds irreversibly to blood vessels, causing a long-lasting but temporary constriction (Davenport et al., 2016). Local injection of endothelin-1 into the rat brain was proposed as a model for focal ischaemia in 1991 (Agnati et al.), with additional work done to develop the methodology and validate the model in the following decades (Fuxe et al., 1997; Windle et al., 2006). The strength of this model is that it closely mimics the natural pathology of ischaemic stroke damage as would be observed in human patients (Hughes et al., 2003; Saggu, 2013; Sommer, 2017). Crucially, it involves a period of ischaemia caused by vasoconstriction with subsequent vasodilation and reperfusion of the ischaemic area (Gilmour, Iversen, O'Neill, & Bannerman, 2004). This is significant, as reperfusion is an important component of the natural course of stroke. Reperfusion can have a negative effect by causing haemorrhage into the damaged area (Khatri, McKinney, Swenson, & Janardhan, 2012). However, reperfusion may ultimately be beneficial by limiting the size of the damaged area and halting the spread of the penumbra. This is the logic behind administration of drugs to dissolve the clot that is causing ischaemia, and conversely the reason why great care is taken to avoid bleeding when using these drugs (Wardlaw et al., 2012).

The endothelin-1 model is also useful for creating lesions in a targeted location, making it possible to focus on small lesions, specifically to the primary motor cortex and subcortex. This

model tends to produce heterogenous lesions, presenting a challenge for researchers, but mimicking more closely the heterogeneity in the naturally occurring lesions in human patients than other models (Fluri et al., 2015; Gilmour et al., 2004). Note also that endothelin-1 can also be used as a model of large volume stroke lesions, by infusion of the drug into the middle cerebral artery.

2.3.2 PHOTOTHROMBOTIC MODEL

The photothrombotic stroke model utilises a systemic infusion of light sensitive dye (Rose Bengal), coupled with application of a light source over the lesion target, to cause endothelial disruption and thrombus formation (Labat-gest & Tomasi, 2013; Watson, Dietrich, Busto, Wachtel, & Ginsberg, 1985). This methodology creates a more reproducible lesion, with low mortality rates, approximately 10% lower mortality than the endothelin-1 injection model (Labat-gest & Tomasi, 2013; Windle et al., 2006). However, the lesion pathology less closely mimics the processes of cerebral ischaemia seen in humans. Owing to the multiple foci of endothelial disruption caused during the lesioning process, this model results in a large degree of cerebral oedema at the lesion site, with little to no penumbral region (Fluri et al., 2015).

2.3.3 BEHAVIOURAL DETERMINANTS OF STROKE RECOVERY IN ANIMAL MODELS

Alongside the multiple models of stroke induction in animals are a variety of methods for determining behavioural deficit and recovery from stroke. Each method attempts to probe an aspect of function in order to assess the validity of the stroke model used and to evaluate the efficacy of interventions. The most useful methods will be ones which hold up to comparison with functional assessments of disability following stroke in human patients, as this gives

researchers an indication of whether the results will have meaningful application to the human population.

Assessments of behavioural recovery in animals began to be used when it was recognised that in human research, behavioural outcome was more important than lesion volume (Aronowski, Samways, Strong, Rhoades, & Grotta, 1996). However unfortunately, there appears to be no published comparison in the literature between rat behavioural outcomes following stroke and the human equivalent. Conclusions have been made about the functional domains each task is assessing, and these will be discussed below (Kleim, Boychuk, & Adkins, 2007; Schaar, Brenneman, & Savitz, 2010). However, there are no data, that correlate the degree of functional impairment measured by these tasks with the equivalent degree of impairment in humans (Jickling & Sharp, 2015; Turner, Jickling, & Sharp, 2011). The best conclusions that can be made therefore are by comparison with other animal work, and extrapolation of this to humans when designing translational studies.

One of the most common methods, and one of the two employed in this study, is the grid walk assessment. In this task, first described by Aronowski et al. (1996), the rat walks across a wire grid, and the number of times the feet are misplaced or fall through the grid are counted. This task assesses sensorimotor function and coordination and is good at assessing whether lesioning has been successful. However it has a low sensitivity to lesion size and is less robust beyond sixty days post lesion (Karthikeyan, Jeffers, Carter, & Corbett, 2018; Kleim et al., 2007; Schaar et al., 2010). This task has been validated for use with the local endothelin-1 injection model of lesion and has been shown to demonstrate improvement following interventions such as those trialled in this project (Adkins et al., 2004; Barry, Boddington,

Igelström, et al., 2014; Boddington et al., 2020; Gilmour et al., 2004; Kleim et al., 2007). From the perspective of human stroke recovery, this task is a proxy for the recovery of coordinated gross motor tasks.

Another component of functional recovery is the performance of coordinated fine motor tasks. In this study we chose the pasta handling task to measure this, a task which involves recording the adjustments made with the forepaws of a rat as it eats a piece of spaghetti pasta. This task requires skilled movements of the forepaws and digits, and is considered a valid model of assessing manual dexterity and fine motor skills (Schaar et al., 2010; Tennant et al., 2010). It also allows some qualitative analysis of impairment following stroke, by observing abnormal movements that are unique to lesioned rats (Allred et al., 2008; Tennant et al., 2010). This method is less commonly used than the grid-walking task, so fewer detailed data are available about sensitivity to lesion size and length of validity post stroke induction. In addition, it is more time consuming, and the need to pre-train the rats introduces an element of inter-experimenter variability (Tennant et al., 2010).

This model closely reflects the type of fine motor coordinated upper limb tasks necessary for performance of activities of daily living in human stroke patients, such as feeding oneself, and involves a more naturally occurring behaviour in rats compared to other techniques such as the Montoya staircase (Nouri & Lincoln, 1987). For our purposes, this model had the advantage of assessing a different modality of recovery compared to the grid walking task, however had a smaller training phase compared to other techniques for assessing fine motor function such as pellet reaching. Another advantage was that there was no need to food restrict the rats prior to testing, which was important given the short period of time between electrode implant

surgery and baseline behavioural testing. This task has been used previously with the topical endothelin-1 model of focal lesioning and represents a valid methodology for assessing this component of recovery (Allred et al., 2008).

2.4 INTERHEMISPHERIC INHIBITION

As mentioned in the introduction to this project, the circuit that will be the focus of this research is the transcallosal interhemispheric inhibition (IHI) pathway. This section details the research that has characterised its anatomical and physiological components.

The response across the corpus callosum, the transcallosal response, was first measured in humans by Cracco, Amassian, Maccabee, and Cracco (1989) using single magnetic and electrical pulses. Results from this study showed that stimulation in one hemisphere evoked transcallosal responses with high focal specificity, suggesting a direct transcallosal connection between homologous areas. Specifically, the two motor cortices are connected by fibres running through the posterior part of the body of the corpus callosum (Meyer et al., 1998; Wahl et al., 2007).

The nature of interhemispheric interaction via the corpus callosum was comprehensively characterised in humans by Ferbert et al. (1992). They described IHI in a cohort of healthy human participants as the tendency of one area of the cortex to inhibit the homologous area of the opposite cortex via the corpus callosum (Figure 2.3, A). They measured this using two different applications of transcranial magnetic stimulation (TMS). The first, and the method that will be employed in this study, was to use two pulses: the ‘bilateral paired-pulse protocol’. The two pulses were delivered sequentially by two magnetic coils, one positioned over each hemisphere. A ‘test’ pulse is delivered to one cortex (the ipsilateral cortex) and the typical response is established by taking an electromyography recording in a corresponding muscle. This is known as the motor evoked potential (MEP) and is used as an indicator of brain

excitability. In a second recording, a 'conditioning' pulse is delivered to the contralateral cortex in the area homologous to the test pulse, prior to the ipsilateral test pulse, and the resulting MEP is compared with the original 'unconditioned' response. This allows the effect of the ipsilateral cortex on the contralateral to be measured. When the conditioning pulse (contralateral) was given at a certain time point prior to the test pulse (ipsilateral), an inhibition of the response was seen. In this and other studies, the latency of this inhibitory response was found to be approximately 6-10 ms, which is consistent with transcallosal conduction (R. Chen, Yung, & Li, 2003; Di Lazzaro et al., 1999; Ferbert et al., 1992).

The second method used by Ferbert involves the application of a single magnetic pulse from one coil to elicit an ipsilateral silent period (iSP), which is measured from the reduction in amplitude of the electromyography recording in a sustained contraction following stimulation of the ipsilateral hemisphere (i.e. the hemisphere not corresponding to the active muscle). While both iSP and bilateral paired pulse IHI represent transcallosal inhibition, they describe different functions (R. Chen et al., 2003). This will be discussed in more detail later in the review.

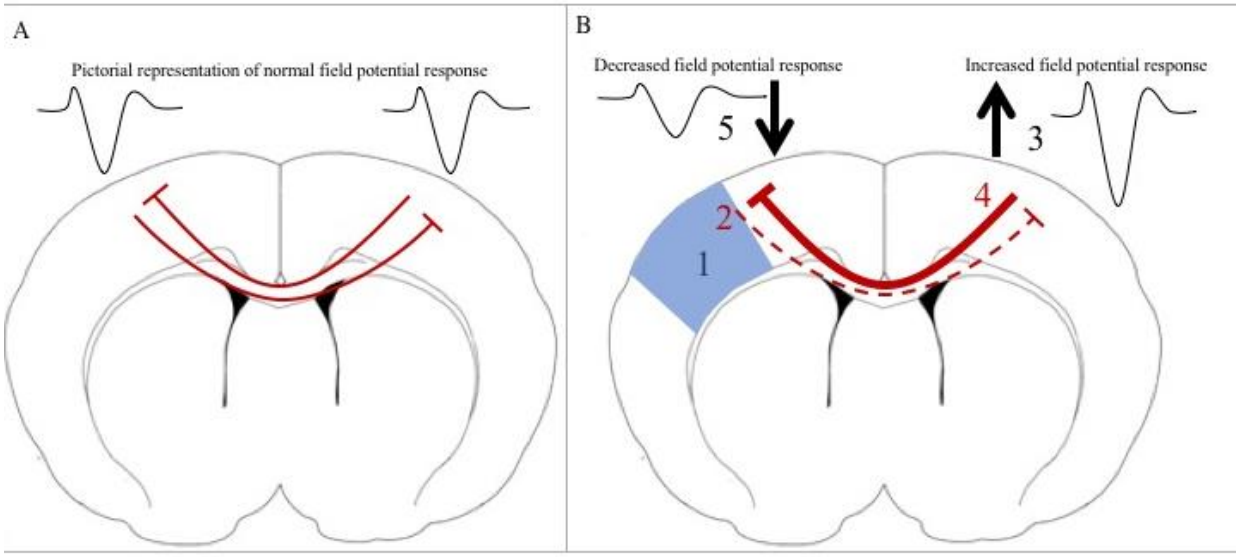

Figure 2.3 A: Interhemispheric inhibition in the healthy brain, demonstrated using a cartoon of a coronal section of a rat brain. B: The interhemispheric competition model of stroke; 1. Stroke lesion. 2. Decreased inhibition from lesioned side. 3. Disinhibition and increased excitability of contralesional side. 4. Increased inhibition from contralesional side. 5. Increased inhibition and decreased excitability of perilesional area. Brain diagram adapted from Paxinos and Watson (2007).

Determinants of interhemispheric inhibition have also been made through measuring the effect of transcallosal inhibitory circuits on the size of monosynaptic synaptic responses in rodents using intracellular recording techniques, and are very comparable to human measurements (Barry, Boddington, Igelström, et al., 2014; Boddington, 2016; Palmer et al., 2012; Spalletti et al., 2017).

2.4.1 FUNCTIONAL ROLE OF IHI

Interhemispheric inhibitory circuitry has a significant functional role, however this role is not fully understood. Interhemispheric inhibition appears to be implicated in bimanual coordination, where it is involved in suppressing unwanted movements. Mirror movements occur when one limb simultaneously mimics the intended movement made with the opposite limb. They occur due to the sharing of excitatory information across the corpus callosum (van der Knaap & van der Ham, 2011). Prior to the myelination of the corpus callosum, children tend to perform mirror movements. After development, the inhibitory connections within the corpus callosum play a role in suppressing unwanted mirror movements, and allow asymmetry of movement (Beaulé et al., 2012; Koerte et al., 2010; Mayston, Harrison, & Stephens, 1999).

It has been found that between individuals, greater IHI is associated with suppression of motor activity from the opposite hemisphere and fewer mirror movements. This has the converse effect of reducing the precision of force generation in bimanual tasks. For example, a shotput thrower who requires a large amount of force for a unimanual movement might benefit from high IHI, whereas a pianist who needs to precisely control both hands simultaneously might require lower IHI (Fling & Seidler, 2012). In fact, it has been found that IHI is plastic and can be altered with training, providing further evidence for its functional role in movements. For

example, professional musicians (such as pianists) who had been training since childhood showed less IHI and a more developed corpus callosum than other individuals (de Manzano & Ullen, 2017; Ridding, Brouwer, & Nordstrom, 2000). This is theorised to be the response to training in bimanual hand coordination. This balance of bimanual and unimanual task performance demonstrates the interconnected functional roles of the callosal pathway.

Studies measuring IHI in humans immediately prior to movement onset can be used to further explore the role of IHI in the generation of movements. Immediately prior to movement onset, the influence of the opposite hemisphere on the hemisphere corresponding to the moving finger changes from inhibitory to faciliatory (Figure 2.4, A) to allow the movement to take place (Duque et al., 2007; Liuzzi et al., 2010; Murase et al., 2004). This phenomenon has been observed primarily in movements of the dominant hand (Duque et al., 2007; Lewis & Perreault, 2007). In contrast, the active hemisphere exerts a constant inhibitory influence on the resting hemisphere, resulting in asymmetry of inhibition prior to movement onset, preventing unintended movement. This asymmetry is likely necessary for bimanual coordination, and probably contributes to differences in coordination between dominant and non-dominant hands (Beaulé et al., 2012).

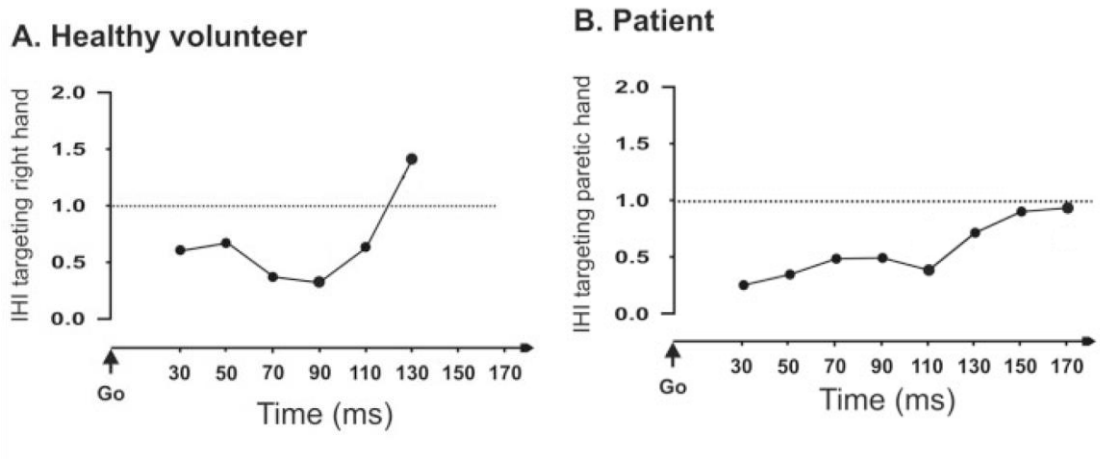

Figure 2.4 A: Interhemispheric inhibition in the healthy brain. Demonstrates inhibition targeting the active hemisphere prior to movement initiation, with interhemispheric facilitation immediately prior to movement onset. B: The same measure in a stroke affected patient onto the affected hemisphere prior to movement of the paretic limb, with interhemispheric inhibition present throughout. Adapted from Murase et al. (2004).

2.4.2 ANATOMICAL COMPONENTS OF INTERHEMISPHERIC INHIBITION

The anatomy of interhemispheric connections has also been considered extensively. In a study by Boroojerdi, Diefenbach, and Ferbert (1996), the nature of IHI in stroke patients was investigated. This study aimed to confirm the cortical origin of IHI by comparing stroke patients with purely subcortical lesions, to patients with mixed cortical-subcortical lesions. This revealed that patients with purely subcortical lesions, having no effect on transcallosal fibres, had IHI comparable with healthy subjects. However, subjects with mixed lesions had a partial reduction in IHI from the lesioned to non-lesioned hemisphere. Since only patients with some cortical involvement showed an alteration of IHI, the inhibition is thought to have a predominantly cortical origin (Boroojerdi et al., 1996; Di Lazzaro et al., 1999; Ferbert et al., 1992). This was further corroborated with the rat studies of Palmer et al. (2012).

The corpus callosum is the other anatomical component of the IHI pathway. A studies of patients with agenesis of the corpus callosum or following callosectomy showed that where the posterior part of the body of the corpus callosum was absent, IHI could not be elicited, confirming the transcallosal nature of this phenomenon (Brus-Ramer, Carmel, & Martin, 2009; Genç, Ocklenburg, Singer, & Güntürkün, 2015; Li, Lai, & Chen, 2013; Meyer, Röricht, von Einsiedel, Kruggel, & Weindl, 1995).

The microscopic structure of the corpus callosum has been described in great detail, both in humans and in rats (Conti & Manzoni, 1994; Gonchar et al., 1995; Meyer et al., 1998; Wahl et al., 2007). Comparisons of the structure of the corpus callosum between species has found that larger, more intellectually developed species tend to have a smaller corpus callosum, perhaps

reflecting the development of lateralised functions (Olivares, Montiel, & Aboitiz, 2001). One other key difference is that humans have a lower proportion of unmyelinated fibres than rats, which contributes to increased speed of information transfer across the hemispheres (Olivares et al., 2001). Small interspecies differences in primates are hypothesised to contribute to differences in coordinated task performance (Phillips, Kapfenberger, & Hopkins, 2009), so the same could likely be said for the larger differences between rat and human corpora callosa. This has potential implications for the interpretation of results in this study, especially when considering whether the grid walking or pasta handling tasks are robust measures in comparison to human outcomes. It would have been of value to this project to have a more detailed understanding in the literature of these differences.

Researchers have found a predominance of excitatory, pyramidal neurons within the corpus callosum of rats, with only 1-6% of neurons shown to be inhibitory in nature (Conti & Manzoni, 1994; Gonchar et al., 1995). This second group of neurons, GABAergic in nature, may have a diffuse inhibitory influence over deep areas of the contralateral cortex (Gonchar et al., 1995). However, this pathway is not considered to be primarily responsible for interhemispheric inhibition.

The pathway mediating interhemispheric inhibition is instead considered to be di-synaptic, made up of a callosal excitatory neuron forming a synapse with an inhibitory interneuron in the opposite cortex (Conti & Manzoni, 1994; Gonchar et al., 1995; Kawaguchi, 1992). This theory was developed from recordings of the response to callosal stimulation in rats, which consist of an excitatory post synaptic potential, followed by an inhibitory post synaptic potential, at latencies consistent with a two synapse pathway (Kawaguchi, 1992). Palmer et al.

(2012) aimed to more comprehensively describe this pathway in rats and showed that there was an inhibitory interneuron involved in interhemispheric inhibition, mediated by $GABA_B$. This experiment was later replicated in mice by Spalletti et al. (2017). This interneuron in cortical layer II/III receives transcallosal input and projects to layer V where it has its inhibitory action (Figure 2.5). Note however that the type of IHI explored in the Palmer and Spalletti studies was a long latency response, as opposed to the shorter latency IHI response first described by Ferbert, the focus of the present study.

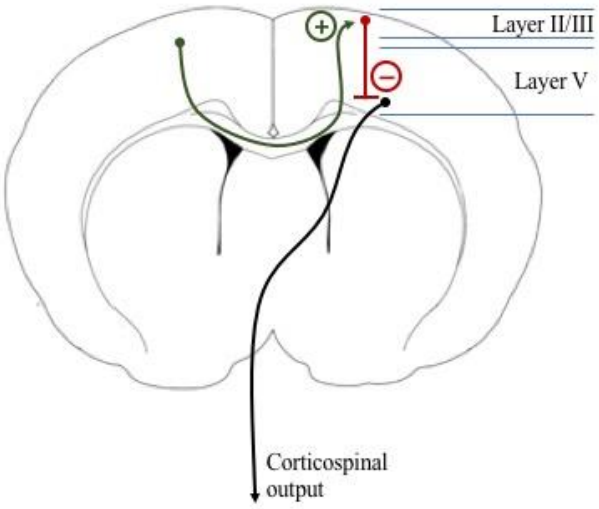

Figure 2.5 Proposed interhemispheric inhibition circuit, based primarily on rat experiments. Neuron one, in green, is an excitatory transcallosal neuron which synapses in layer II/III onto neuron two, in red. Neuron two, an inhibitory interneuron, inhibits corticospinal output at the layer V pyramidal neurons, probably using $GABA_A$. Brain diagram adapted from Paxinos and Watson (2007).

2.4.3 SHORT AND LONG LATENCY INTERHEMISPHERIC INHIBITION

The present study focuses entirely on short-latency interhemispheric inhibition (SIHI), however it is useful to consider the relationship between this and long-latency interhemispheric inhibition (LIHI) to help understand their function and mechanisms. Short latency IHI occurs at latencies of 6-15ms, and LIHI occurs between 40-100ms, depending on the species in which it is measured (Avanzino, Teo, & Rothwell, 2007; R. Chen et al., 2003; Ghosh et al., 2013; Irlbacher, Brocke, Mechow, & Brandt, 2007; Ni et al., 2009; Reis et al., 2008; Uehara, Morishita, Kubota, Hirano, & Funase, 2014).

From a functional perspective, SIHI can be seen to increase dynamically with limb movement, and is related to bilateral paired pulse measurements (R. Chen et al., 2003; Uehara et al., 2014). Bilateral paired pulse measurements of SIHI are thought to demonstrate timed inhibition of corticospinal outputs, such as during the initiation of movement within the opposite cortex. Long interval IHI is not shown to be related to performance of a unilateral task and is linked to iSP measurements. Functionally, this means that iSP measurements of LIHI represent a fluctuation in the tonic activation of the corticospinal output and are not tied to movement generation. While they represent different processes and functions, they can interact with inputs to the corticospinal tract as a final common pathway, which can be measured using motor evoked potentials (Ghosh et al., 2013). The difference between these two measures becomes significant when considering how they are differently affected after stroke. A potential model for how these two pathways interact and are measured is outlined in Figure 2.6, and is based on the work of Daskalakis-Zafiris, Christensen-Bruce, Fitzgerald-Paul, Roshan, and Chen (2004); Ferbert et al. (1992); Kukaswadia, Wagle-Shukla, Morgante, Gunraj, and Chen (2005); and Reis et al. (2008).

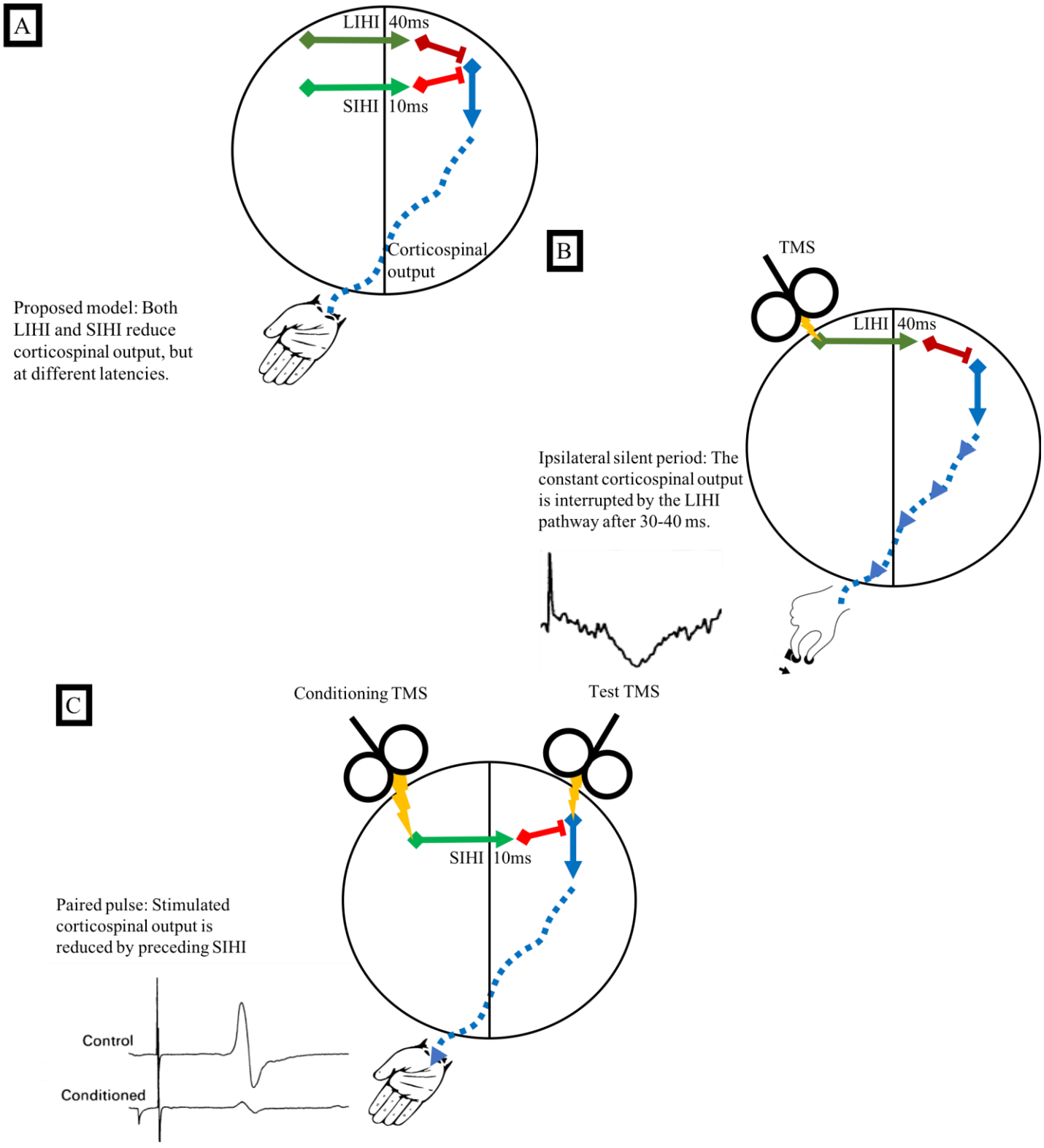

Figure 2.6 A: Proposed model of interaction between SIHI and LIHI. B: Demonstration of this model in the context of iSP measurements. C: This model in the context of bilateral paired pulse recordings of IHI. Data shown are from Ferbert et al. (1992).

While LIHI has been shown to be $GABA_B$ mediated (Chowdhury, Kawashima, Konishi, & Matsunami, 1996; Daskalakis-Zafiris et al., 2004; Irlbacher et al., 2007; Palmer et al., 2012; Spalletti et al., 2017), the mechanism of SIHI remains largely unknown. There is some indication for a role of $GABA_A$ in SIHI, as the $GABA_A$ antagonist bicuculine has been shown to reduce SIHI responses in cat and rat models (Chowdhury et al., 1996; Mansoori et al., 2014). This gap in the research presents a challenge for the present research, as the pathway under investigation is not yet fully understood.

2.5 ALTERATIONS OF INTERHEMISPHERIC INHIBITION FOLLOWING STROKE

Interhemispheric inhibition has also been studied in stroke patients and this research forms part of the understanding of the mechanisms of recovery following stroke. The levels of IHI across the two hemispheres become imbalanced following stroke, with greater inhibition onto the lesioned side, and this has been hypothesised to contribute to poor functional recovery (Figure 2.3, B).

Boroojerdi et al. (1996) were some of the first to measure IHI in stroke patients, however as discussed earlier, this study aimed to investigate IHI rather than stroke recovery. Subsequent examination of clinical cases led to the development of the theory that IHI was implicated in stroke recovery. In these cases, patients with a stroke causing a particular functional impairment experienced dramatic recovery of that function after a corresponding stroke to the opposite hemisphere (Sauerbrei & Liepert, 2012; Vuilleumier, Hester, Assal, & Regli, 1996). From this, the conclusion made is that the second stroke removed the inhibitory influence of

the previously non-lesioned hemisphere on the analogous area of the lesioned hemisphere, allowing that hemisphere to rapidly recover lost functions.

The balance of inhibition and excitability following stroke has also been measured. *In vitro* recordings from an animal model of stroke found that the area opposite the lesion becomes disinhibited (Buchkremer-Ratzmann & Witte, 1997; Mohajerani, Aminoltejari, & Murphy, 2011). There are also increases in immunohistochemical measures of excitability appearing in the opposite cortex within days after lesioning rats with endothelin-1 (Adkins et al., 2004).

This disinhibition has also been found in human patients, with TMS investigations showing that there is hyperexcitability within the contralesional hemisphere following stroke. It is hypothesised that this is due to disruption of the inhibitory influence of the lesioned hemisphere on to the non-lesioned hemisphere (Shimizu et al., 2002). This theory is supported by functional magnetic resonance imaging research showing that after stroke, movements of the paretic hand have a greater tendency to produce mirror movements in the unaffected hand, reflecting a reduced inhibitory influence from that hemisphere (Kim et al., 2003). The lesioned side is unable to increase the level of IHI in order to suppress activation of the other hemisphere, an effect that is more pronounced if the dominant hemisphere was affected (Lewis & Perreault, 2007).

Less excitability in the ipsilesional hemisphere has been shown to correlate with worse hand performance (Du et al., 2018). In patients with small stroke volumes and good functional recovery there is an initial decrease in excitability shown using the measure of short-interval intra-cortical inhibition (SICI), in both lesioned and non-lesioned hemispheres (Bütefisch,

Weβling, Netz, Seitz, & Hömberg, 2008; Huynh et al., 2016). Over time, this SICI remains reduced in the ipsilesional hemisphere, however has a tendency to return towards normal in the contralesional hemisphere, creating an imbalance between the hemispheres (Calabro et al., 2019; Cicinelli et al., 2003; Huynh et al., 2016). In addition, following stroke SICI does not reduce during movement initiation on the stroke affected side, a facilitatory effect that is seen in healthy individuals (Hummel et al., 2009).

Murase et al. (2004) measured IHI at multiple time points prior to movement initiation in stroke patients, allowing the function of IHI to be explored along with describing the effect of stroke on IHI. As with Duque et al. (2007) and Xu et al. (2019), this study found that in a healthy participant, IHI targeting the hemisphere corresponding to the moving hand first increased, and then reduced, switching to interhemispheric facilitation immediately prior to movement onset. However, in a stroke-affected patient, the interhemispheric influence from the non-lesioned to the lesioned hemisphere had a purely inhibitory effect (Figure 2.4, B) (Murase et al., 2004; Xu et al., 2019). The abnormally high IHI did reduce as movement onset approached, however it never became interhemispheric facilitation. This was similar to the lack of facilitatory effect following stroke found by Hummel et al. (2009) for local inhibition (SICI). Crucially, a higher level of IHI from the intact to lesioned hemisphere was seen to correlate with poorer functional recovery from stroke (Du et al., 2018; Duque et al., 2005; Murase et al., 2004).

Using iSP measures of transcallosal inhibition produces more variable results, with some researchers finding an increase in iSP from the unaffected hemisphere (Takechi et al., 2014) and others finding no increase (Bütefisch et al., 2008; Stinear, Petoe, & Byblow, 2015). The iSP measure used in these studies, as described earlier, represents a component of transcallosal

inhibition different to that measured by bilateral paired pulses. The bilateral paired pulse technique is related to SIHI and can study the effect of inhibition on the corticospinal system at rest, prior to or during activation, and thus can be used to assess the effect on movement initiation. Ipsilateral silent period is related to LIHI and looks at the effect on ongoing voluntary motor activity, so cannot be used to assess the initiation of movement, which is the function most likely affected after stroke (R. Chen et al., 2003; Murase et al., 2004).

2.6 MODELS OF POST STROKE RECOVERY

It is thought that not all patients experience imbalanced IHI after stroke, due to the different processes involved in the recovery of function after stroke with different sized lesions. This can also explain, in part, the heterogeneity of results seen when assessing IHI imbalances after stroke. The area that takes on lost functions after stroke depends on the amount of 'structural reserve' that exists, i.e. the volume of functional brain tissue remaining in the lesioned hemisphere following the lesion. This has been explained using two models of stroke recovery: the 'vicariation' model and the 'interhemispheric-competition' model (Di Pino et al., 2014).

2.6.1 VICARIATION MODEL

In patients with a large amount of damage from stroke and therefore a less reserve within the lesioned hemisphere, recovery can be predicted by the vicariation model (Di Pino et al., 2014). This model describes a recovery process whereby the contralesional hemisphere takes on some of the functions of the lost areas of the lesioned hemisphere by utilising those 10% of fibres that supply proximal muscle groups and do not cross the midline (Cunningham et al., 2015). This is shown in rats, where in those with larger lesions, disruption of the unaffected

hemisphere leads to a greater decline in function as compared to rats with smaller lesions (Biernaskie, Szymanska, Windle, & Corbett, 2005). Adkins et al. (2004) showed markers of synaptic plasticity and dendritic reorganisation in the contralesional hemisphere in the two weeks following lesioning in rats, indicating a tendency for this hemisphere to functionally reorganise in response to damage. In humans, it has been shown using fMRI that the contralesional hemisphere reorganises, presumably to support recovery of the affected hand (Bestmann et al., 2010; Rehme, Fink, von Cramon, & Grefkes, 2011).

In patients with larger lesions, functions lateralise to the unaffected hemisphere, with the stroke affected hemisphere playing a smaller role in recovery. Cramer (2004) reported that in the first six months after stroke, the lateralisation of functions between the hemispheres altered, with the 'healthy' hemisphere being recruited for tasks usually performed by the lesioned hemisphere. This is associated with lower subjective reports of disability, but is not necessarily associated with better functional recovery, which is more closely associated with corticospinal tract integrity (Cunningham et al., 2015).

In this model, it is less relevant to consider imbalances of interhemispheric inhibition as a target for intervention. The lesioned hemisphere is not contributing significantly to recovery, with the healthy hemisphere playing the central role. When the contralesional hemisphere was inhibited in a group of patients with larger lesions, MEP measures of excitability of the paretic hand failed to increase. Conversely, facilitation of the unaffected hemisphere did increase excitability of the paretic hand (Sankarasubramanian et al., 2017). This model also presumably plays a role in maladaptive plasticity, where the contralesional hemisphere reorganises in order

to promote function of the non-paretic limb over and above the paretic limb (Jones, 2017; Takeuchi & Izumi, 2012).

2.6.2 INTERHEMISPHERIC COMPETITION MODEL

In patients with a large amount of functional tissue remaining around the lesion site, i.e. high structural reserve in the lesioned hemisphere, the areas surrounding the infarct are able to take on the lost functions, to the point where complete recovery of function is theoretically possible (Moon, Alaverdashvili, Cross, & Whishaw, 2009). This reveals the significance of the penumbral region, which if spared, can perform this role. In this second model, the 'interhemispheric-competition' model, the imbalance of excitability and resulting inhibition of the hemispheres becomes more significant.

After stroke, inhibitory connections from the lesioned to the non-lesioned hemisphere are reduced, while those from the contralesional hemisphere remain. The interhemispheric competition model predicts that this ultimately results in an excess of inhibition on to the lesioned hemisphere, which limits the ability for that area to recover (Figure 2.3, B) (Kirton, Deveber, Gunraj, & Chen, 2010; Takechi et al., 2014). The suggested mechanism is supported by a dose response relationship between the level of IHI acting on the lesioned hemisphere and functional recovery (Duque et al., 2005; Murase et al., 2004).

Other experimental evidence also exists. An excess of inhibition to the perilesional area is shown to reduce plasticity and the recovery of function in a mouse model of small stroke, where antagonising $GABA_B$ related perilesional inhibition results in improved functional recovery

(Spalletti et al., 2017). In other animal models of stroke, $GABA_A$ antagonists can improve behaviour while reducing SIHI responses (Chowdhury et al., 1996; Clarkson, Huang, MacIsaac, Mody, & Carmichael, 2010; Mansoori et al., 2014). While unsuccessful at producing alterations of SIHI, midazolam (a $GABA_A$ agonist) did cause stroke deficits to transiently re-emerge in human patients (Irlbacher et al., 2007; Lazar et al., 2010). Interventions to produce inhibition of the contralesional hemisphere in patients with small lesions tends to result in increased MEP size and functional benefit, whereas it produces no effect in those with larger lesions. (Bertolucci, Chisari, & Fregni, 2018; Sankarasubramanian et al., 2017). The proposed mechanism for this recovery is a structural reorganization of function in the areas surrounding the lesion (Ackerley et al., 2015; Favre et al., 2014; Touvykine et al., 2016).

Therefore, interventions to disinhibit the ipsilesional cortex and encourage perilesional vicariation of function should be offered to patients with smaller lesions. Patients to whom this second model applies will be the focus of the present research project, as they have the potential to benefit from modulation of IHI. In large lesions, the reorganisation of the contralesional hemisphere plays a larger role in driving recovery, (Ackerley et al., 2015; Biernaskie et al., 2005), thus interventions to inhibit the contralesional side would not be helpful in these patients.

The heterogeneity of results seen when studying changes in IHI following stroke, may in part be explained by these differences in recovery models, and stratification by severity and anatomical location of stroke may be helpful in explaining differences in results and determining the best course of treatment. For example, R.M. Levy et al. (2016), found that recovery using their neuromodulation technique was dependent on the presence of intact

corticospinal tracts, and other studies use behavioural measures such as Fugl-Meyer Assessment or physiological measures such as TMS evoked motor responses to include or exclude patients.

2.7 NEUROMODULATION TECHNIQUES FOR PROMOTING STROKE RECOVERY

Neuromodulation is a growing field in stroke rehabilitation. The technique aims to target the neural mechanisms of plasticity to bring about or support those changes that best promote recovery (Takeuchi & Izumi, 2015). Various techniques have been trialled, many with the aim of increasing functional recovery by increasing ipsilesional excitability or decreasing contralesional excitability. These trials have had varying degrees of success. The main neuromodulation techniques that have been investigated are TMS, and electrical stimulation delivered either as transcranial direct current stimulation (tDCS) or epidural electrical stimulation (eES).

2.7.1 PRACTICAL CONSIDERATIONS OF NEUROSTIMULATION TECHNIQUES

When considering how neuromodulation is delivered, it is important to consider the patient context. For example, TMS requires patients to visit a specialised user, and is thus less convenient for a patient long term than an implanted stimulator which can be turned off and on by the patient themselves. The advantages of TMS and tDCS are that they are non-invasive, and therefore potentially more acceptable to patients. In contrast, eES is a more invasive technique, which requires surgery to implant an electrical stimulator. Regardless of practical differences, there is also controversy surrounding the efficacy of these techniques. Studies

utilising these techniques to enhance recovery are heterogeneous, with differences in stimulation method, site, pattern and intensity, and with or without simultaneous rehabilitation.

2.7.2 TRANSCRANIAL MAGNETIC STIMULATION

The main neuromodulation technique which has been researched is TMS, the application of which is intended to correct interhemispheric imbalance by either decreasing excitability in the unaffected hemisphere or increasing the excitability in the affected hemisphere. A meta-analysis (Hsu et al., 2012) of the effects of repetitive TMS on upper limb motor function found that repetitive TMS did alter cortical excitability, as measured by motor threshold, but this effect was non-significant overall. However, the effect on functional improvement was both statistically and clinically significant. In contrast to this, a later Cochrane review (Hao et al., 2013) found that the benefit to motor function was not statistically significant. Both reviews also found that most of the trials ended less than a month after treatment onset, so that effects on long term outcome could not be assessed. Secondly, many of the trials included were assessed to be of poor quality in the Cochrane review. Together, these reviews show that there is no conclusive clinical benefit to repetitive TMS, and while its potential continues to be explored, other techniques must also be considered.

From our perspective, there was also the added challenge that TMS application is very challenging in rats and mice. The scaling down in size of human coils is often inaccurate in terms of the dose that is delivered, and the limited focality which challenges human TMS delivery is only compounded with the smaller skull size of rodents. In addition, training rats to be stationary while TMS is applied is very challenging and requires a significant amount of rat

training, which would be further compounded in our study by the presence of the EEG recording electrodes and IHI recording electrodes. These challenges and more are summarised in a paper by leading researchers in this field: M. T. Wilson et al. (2018).

2.7.3 THETA BURST STIMULATION

The Hsu review showed that theta burst stimulation (TBS) when given intermittently over the affected hemisphere can be beneficial (Hsu et al., 2012). The theta burst stimulation protocol is based on a naturally occurring pattern of activity from the hippocampus that can be recorded during learning and exploring behaviours (Winson, 1974). It has been shown experimentally to have long lasting effects on cortical excitability (D. M. Diamond, Dunwiddie, & Rose, 1988; Y. Z. Huang, Edwards, Rounis, Bhatia, & Rothwell, 2005). The protocol is generally delivered in either a continuous manner (cTBS), which has a tendency to decrease measures of excitability, or intermittently (iTBS), which tends to increase excitability (Di Lazzaro et al., 2008; Gamboa et al., 2011; Y. Z. Huang et al., 2005).

2.7.4 TRANSCRANIAL DIRECT CURRENT STIMULATION

Transcranial direct current stimulation has been trialled with two different approaches. The first uses anodal stimulation to increase the excitability of the ipsilesional cortex, and the second uses cathodal stimulation to decrease the excitability of the contralesional cortex (Schlaug, Renga, & Nair, 2008). Two recent systematic reviews and meta-analyses of tDCS reported a tentative benefit to stroke patients, of low clinical significance (Elsner, Kugler, Pohl, & Mehrholz, 2016; Kang, Summers, & Cauraugh, 2015; Solomons & Shanmugasundaram, 2019). However this was not shown to persist more than twenty-four weeks in any of the studies

included (Kang et al., 2015). The technique has not been proven to produce a clinically significant benefit, nor is the mechanism fully understood, therefore this technique needs further investigation to ascertain its utility for clinical application (Ward, 2015).

2.7.5 EPIDURAL ELECTRICAL STIMULATION

The technique that is to be explored in this project approximates eES, where in human participants the stimulating electrode is laid directly on the patient's dura. Note that, in the present study, due to the need to maintain a stable electrode position in a rat and the more delicate nature of rat dura, the electrode penetrates the dura and is secured superficially into the cortex.

Electrical stimulation in rat trials has been proven to be effective in inducing plasticity (Adkins-Muir & Jones, 2003; Jahanshahi et al., 2013), and approaches aimed at increasing perilesional excitability have shown promise in animal models of stroke (Adkins-Muir & Jones, 2003; Adkins, Hsu, & Jones, 2008; S. K. Moon et al., 2009). Some evidence also exists as to the feasibility and efficacy of eES in humans. A case study in a patient with a stimulator implanted for chronic post-stroke pain over the stroke site found that the stimulation also produced a significant improvement in their Broca's aphasia. This benefit was reversible after six years; when the stimulator battery ran out the aphasia worsened, and improved when the battery was replaced (Balossier, Etard, Descat, Vivien, & Emery, 2012, 2013).

Two safety and feasibility studies in stroke patients showed promising efficacy of eES over the lesion site in augmenting rehabilitation (Brown et al., 2006; M. Huang et al., 2008; R. M. Levy

et al., 2008). Subsequently, a larger multicentre randomised controlled trial was also completed (Harvey & Winstein, 2009). The approach taken in the Harvey trial was to implant the electrode over the affected cortex and deliver high frequency (50 Hz) repetitive stimulation at a subthreshold amplitude, a protocol designed to increase cortical excitability. The stimulation was given in combination with rehabilitation. This study did not meet its primary endpoints for efficacy, however in a subgroup analysis including only those patients in which the stimulator could induce a motor response (i.e. those who were likely to have an intact corticospinal tract pathway), a clinically significant improvement was seen in 69% of participants, as compared to 29% of the control group (R.M. Levy et al., 2016).

In continuing to explore the potential for eES to produce clinically meaningful improvements in stroke rehabilitation outcome, a variety of stimulation parameters have been trialled. A study by Barry, Boddington, Igelström, et al. (2014), found that TBS had the potential to be clinically useful through the application of electrical stimulation, albeit applied via a superficially implanted intracortical electrode and not extradurally. It was found that iTBS, a stimulation parameter typically associated with increasing excitability, when applied at a low intensity with an implanted electrode to the unaffected hemisphere, reduced IHI onto the affected hemisphere. By contrast, continuous TBS increased IHI and high intensity iTBS had no effect.

Behavioural studies designed following these findings showed lasting improvements in forelimb function of stroke affected rats that were treated with an iTBS protocol through an implanted electrode within the unaffected hemisphere, as compared with control (Boddington et al., 2020). Rats receiving cTBS showed worsening of function compared with control.

Boddington hypothesised that the behavioural recovery observed following iTBS of stroke lesioned animals was due to the reduction of IHI onto the lesioned cortex in these animals.

However intracellular recordings made at the termination of Boddington et al. (2020)'s experiments were unable to demonstrate any alteration in IHI that could explain this recovery. What was found in these experiments was a tangential link, showing that in those iTBS treated animals with maximal functional recovery, ipsilesional excitability was also increased. There was a second strong relationship between ipsilesional excitability and reduced IHI. This could describe a mechanism by which iTBS reduces IHI, and the subsequent increase in ipsilesional excitability improves functional recovery.

In Boddington's experiments, owing to the invasive nature of intracellular recording techniques, IHI prior to iTBS was unable to be recorded and compared with post stimulation inhibition. In addition, there is reason to believe that IHI measured using the bilateral paired pulse technique under anaesthesia is different to the dynamic measurement of IHI made during movement initiation, which is shown to be affected during stroke. There is a lack of research demonstrating whether the mechanism behind improved behavioural recovery following iTBS is due to a reduction in IHI, and this question informed the development of this thesis.

2.8 STUDY AIMS AND HYPOTHESES

The aim of the present study was to extend and consolidate the results of Barry and Boddington by measuring the effects of intermittent theta burst stimulation on both behavioural recovery and interhemispheric inhibition. It was hypothesised that application of iTBS would result in a reduction of interhemispheric inhibition while simultaneously enhancing the behavioural recovery of stroke lesioned rats. The overall aim of this study was therefore to ascertain whether, following low intensity contralesional iTBS, behavioural recovery in an animal model of stroke occurs alongside a reduction in IHI. In addition, the effects of stroke on interhemispheric inhibition will be investigated, in order to help address some of the controversy in this area. This will be achieved through the development of minimally invasive techniques for measurement of IHI, so that multiple measurements of IHI can be made within the same experimental animal. This will allow chronological changes in IHI to be tracked, to see the within-animal effects of both stroke lesion and iTBS.

2.8.1 OBJECTIVE ONE

The first objective relates to the development of the IHI measurement technique:

Objective 1: To investigate the use of a minimally invasive technique for the measurement of interhemispheric inhibition in the freely moving animal.

We used implanted electroencephalogram electrodes to record interhemispheric inhibition using the bilateral paired pulse technique in anaesthetised rats as a minimally invasive, high

yield alternative to intracellular recording. Within these experiments we set a few smaller objectives and corresponding hypotheses which will be explored in detail within Chapter 4.

2.8.2 OBJECTIVE TWO

The second objective relates to the need for further clarity of the effect of stroke lesion on interhemispheric inhibition.

Objective 2: To determine whether deficits after stroke are associated with an alteration of interhemispheric inhibition.

This was investigated in rats using the techniques developed in the first objective. Recording and stimulating electrodes were implanted to measure IHI in the freely moving animal. A stroke lesion was induced using the endothelin-1 injection model. Measurements of IHI and behavioural impairment on the grid walk and pasta handling tasks were taken before and after stroke induction, see Figure 2. 7. Hypotheses relating to the expected changes in interhemispheric inhibition and behaviour will be outlined in Chapter 5.

2.8.3 OBJECTIVE THREE

The final objective addresses the focus of the study;

Objective 3: To determine whether, in an animal model of stroke, low intensity intermittent theta burst stimulation to the contralesional hemisphere can reduce interhemispheric inhibition and improve behavioural recovery.

This was investigated in the same implanted, stroke-lesioned rats as described in Objective 2. Rats received fifteen doses of iTBS over three weeks, or the same duration of sham stimulation. Prior to the first dose of stimulation, IHI was measured, and the grid walk, and pasta handling tasks were performed. For each week of stimulation, and for a further three weeks, IHI and behaviour were measured, see Figure 2. 7. Hypotheses and results relating to this objective are contained within Chapter 6.

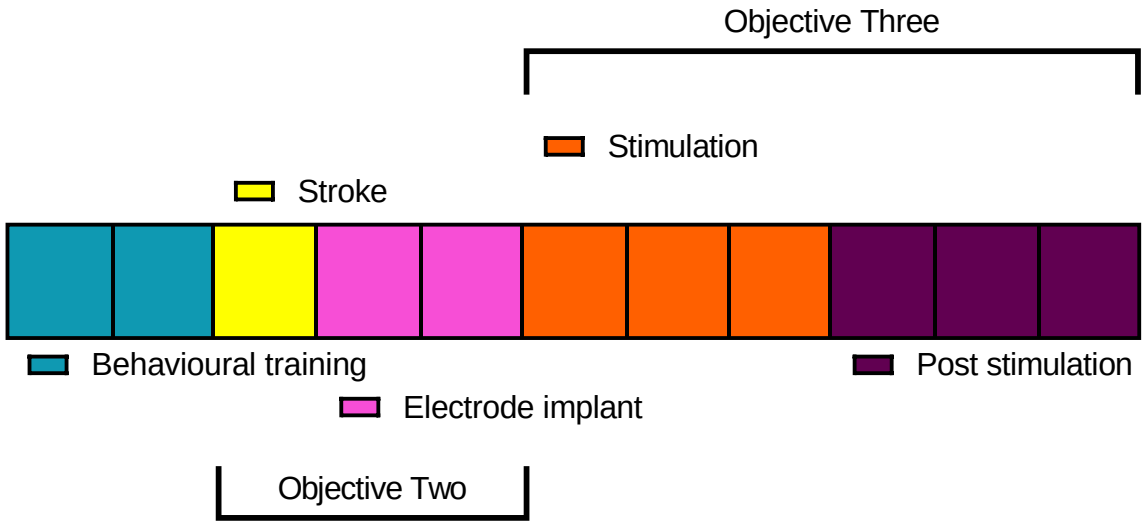

Figure 2. 7 Visual timeline for experiments addressing Objectives Two and Three.

3 GENERAL METHODS

3.1 INTRODUCTION

The focus of this project is the measurement of interhemispheric inhibition under a variety of conditions. The present study employs the bilateral paired-pulse technique which was described in Chapter 2 to measure IHI. This technique is thought to provide a dynamic measure of the functional component of IHI (R. Chen et al., 2003; Murase et al., 2004). The bilateral paired-pulse technique involves two separate measurements. Firstly, the baseline response to an ipsilateral electrical stimulus in the test cortex is measured. Secondly, the response to the same ipsilateral stimulus is measured in the presence of a preceding contralateral stimulus. The degree to which the response to the ipsilateral pulse is diminished by the contralateral pulse is thought to represent interhemispheric inhibition. The two pulses can be delivered at a range of intensities, and at a range of inter-stimulus intervals (ISIs) - the amount of time between the delivery of the contralateral conditioning pulse and the ipsilateral pulse.

This technique was employed both in anaesthetised rats, and in freely moving animals. Measurements of IHI were compared, within individual animals, before and after stroke and in the context of an intervention specifically designed to target IHI. This is mentioned here to highlight the novelty of the subsequent methods: the development of a minimally invasive technique for the measurement of IHI and the measurement of IHI in the awake animal. Excluding the possibility that publications were overlooked in the review of literature, this methodology represents a unique approach to measure changes in IHI after stroke.

This chapter will begin with a description of techniques that are the same or similar across all experiments. This will include animal welfare details, and methodologies for the three anaesthetics employed. The next section will describe the methods for acute animal experiments, including surgical and recording techniques. The chronic animal experiments will then be explained, including surgical methods, anaesthetised and awake recording techniques, the iTBS protocol and behavioural methods. The chapter will close with brief discussion of euthanasia and histology protocols, electrophysiological analysis techniques and statistical methods.

3.2 ANIMAL DETAILS

Animal experimentation took place under institutional ethical approval, granted by the University of Otago Animal Ethics Committee (AEC), under AEC numbers 75/14, 06/16, 78/16 and 92/17. A total of 160 male outbred Wistar rats were used, which were 200 – 400 g at the time of entry into the study and were five weeks or older at the date of arrival into the housing unit. Of the 160 rats:

- 73 underwent acute non-survival surgeries under AECs 75/14 and 92/17, of which 53 contributed to the results described in this thesis.
- 3 were used under 06/16 to pilot stroke induction techniques.
- 84 were used in the chronic intervention study and were manipulated under AEC 78/16. Of these, 60 were included in analysis. The remaining 24 rats were euthanised due to complications arising from surgery, part way through the study due to complications

(such as wound infection, or electrode dislodgement), or were excluded from analysis for other reasons, which will be described in detail in the results chapters.

- See Figure 3. 1 for an overall visual summary of animal numbers.

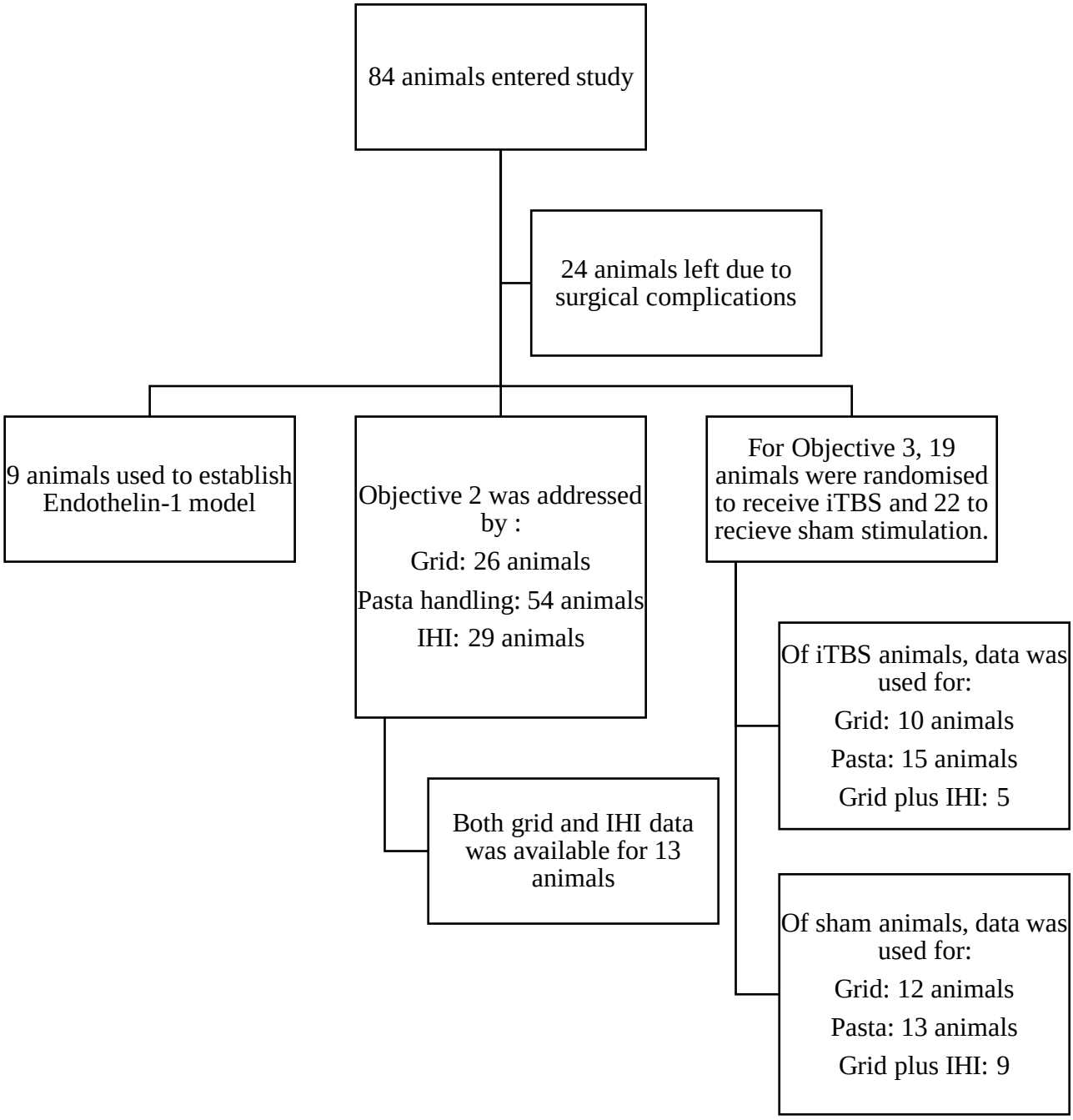

Figure 3. 1 Flow chart indicating animal numbers in each section of the study.

3.2.1 RAT HOUSING

Rats were housed in individually ventilated cages, in a temperature (19-21 °C) and humidity-controlled environment, with a reverse day-night cycle, so that dark (active) periods were during the workday. They were group housed prior to surgery, and individually housed thereafter, within sight and sound of other rats. Individual housing was preferred so that companion rats did not disturb the recovering surgical sites or dislodge the electrodes. Each rat was provided free access to food and water. Additionally, the cages were equipped with a corn cob flooring, paper wool, a plastic tunnel and a wooden chew block.

3.3 ANAESTHESIA

3.3.1 URETHANE

Three anaesthetics were used for the experiments described in this thesis, each for a different purpose. Urethane allows for a long duration of anaesthetic effect, and can be topped up throughout surgery to maintain an appropriate plane of anaesthesia (Bazin, Constantin, & Gindre, 2004). Urethane was the anaesthetic of choice for acute, non-survival surgeries, which often lasted over four hours and could last up to 10 hours. However, urethane is not considered appropriate for survival surgery (Bazin et al., 2004), so different choices were made for chronic animal experiments.

A 200 mg/mL urethane solution was freshly prepared prior to each surgery using urethane crystals (Sigma-Aldrich Inc., St Louis, USA) and heated saline. An intraperitoneal (i.p.) induction dose of urethane was given at a dose of 1400 mg/kg. A further 1 mL dose (200 mg)

was given i.p. 15 minutes later, generally with onset of a surgical plane of anaesthesia 30 minutes later. Depth of anaesthesia was checked using the absence of pedal withdrawal in both hind paws as the mark of a surgical level of anaesthesia. If this was equivocal, the corneal blink reflex was checked using a small piece of cotton touched against the cornea. If the dosage administered was insufficient to achieve anaesthesia, an extra 0.2-0.3 mL (40-60 mg, i.p.) dose was given every 15 minutes until anaesthesia was achieved. The same was done during surgery via an i.p. catheter to maintain surgical levels of anaesthesia. The electroencephalogram (EEG) trace of a well-anaesthetised animal showed oscillations of approximately 0.8 to 1 Hz, indicating cell synchrony at delta frequency (Musizza et al., 2007). The loss of surgical anaesthesia was identified by the desynchronization of the EEG signal, which occurred long before the return of the pedal withdrawal reflex. Such top-ups were typically given every 2-4 hours, approximately 1-3 times per surgery. A further 1-2 mL (2-300 mg, i.p.) of urethane was administered at the close of surgery for euthanasia.

3.3.2 KETAMINE-DOMITOR

Ketamine-domitor is a mixture of an anaesthetic (ketamine) and a smaller amount of a sedative and analgesic medication (domitor). It is useful for shorter periods of anaesthesia (two to three hours), and is used in veterinary medicine and for some survival surgeries at this institution (Bazin et al., 2004; Nevalainen, Pyhala, Voipio, & Virtanen, 1989). This anaesthetic was used for electrode implant surgeries where the recording apparatus made inhaled anaesthetics impractical.

An induction dose of ketamine (75 mg/kg, subcutaneous/s.c., Phoenix Pharm Distributors Ltd, NZ) and domitor (0.5 mg/kg, s.c., Orion Pharma Pfizer, Finland) mixture was given, followed 30 minutes later by an additional 10% of this dose of ketamine alone (12 mg/kg, s.c.); this supplemental dose was repeated 15 minutes later where necessary. Depth of anaesthesia was checked as above. Surgery was in most cases short enough that top-up doses were unnecessary, however when required, a top up of ketamine-domitor (0.25 mg/kg, s.c. & 0.17 mg/kg respectively) was given after three hours. The effect of domitor was reversed using antiseden (2.5 mg/kg, s.c., Orion Pharma, Pfizer, Finland) at the end of all surgical procedures. Rats typically awoke within 15-30 minutes to 2-3 hours, and were bright, alert and responsive.

3.3.3 ISOFLURANE

Isoflurane is often the anaesthetic of choice for many animal researchers and veterinarians (Bazin et al., 2004), owing to the short induction and recovery times and the ability to vary the amount of inhaled anaesthetic on a moment-to-moment basis. This anaesthetic was used for stroke induction surgeries, and for short procedures that required anaesthesia such as replacing a broken suture.

The rat was placed in the induction chamber of the table-top animal anaesthesia system (V-1, Vet Equip, USA) which was filled with 5% isoflurane (Merial, New Zealand Ltd) delivered at a rate of 500 mL/min until the rat appeared to fall asleep. The rat was quickly moved to the stereotaxic frame (Kopf, Germany) and nose-cone (Vet Equip, USA) where the oxygen/isoflurane mix was again delivered at 500 mL/min, and once it was checked that the rat was in a surgical plane of anaesthesia (as above) the isoflurane percentage was reduced to

2.5-3%. During surgery, depth of anaesthesia was checked periodically, and isoflurane dose adjusted accordingly. At the conclusion of surgery, the rat was returned to the induction chamber and the rat allowed to return to consciousness with oxygen only (0% isoflurane). Rats typically awoke within five minutes.

3.4 ACUTE EXPERIMENT METHODS

Acute experiments were conducted under urethane anaesthesia with 73 animals. These experiments are referred to as ‘acute’ because they took place over a single day, and animals were euthanised at the conclusion of the day’s experiment. Several experiments were performed with this group, all of which are related to Objective 1:

Objective 1: To investigate the use of a minimally invasive technique for the measurement of interhemispheric inhibition in the freely moving animal.

3.4.1 OBJECTIVES RELATING TO ACUTE ANIMAL EXPERIMENTS

Objective 1 was separated into smaller objectives, which are listed here along with a brief description of the experiment conducted to meet this objective. The rationale for each, as well as corresponding hypotheses, is discussed in Chapter 4.

Objective 1.1 To measure characteristics of single pulse ipsilaterally-evoked field responses measured using single wire electroencephalogram recording.

Objective 1.2 To measure characteristics of single pulse field responses to contralateral stimulation using single wire EEG recording.

Baseline ipsilateral and contralateral responses were measured in all animals undergoing acute procedures while under urethane anaesthesia. These were analysed and compared with similar recordings in the literature.

Objective 1.3 To record interhemispheric inhibition using paired ipsilateral and contralateral pulses.

Objective 1.4 To measure the effect of varying the intensity of contralateral conditioning stimulation on the degree of interhemispheric inhibition measured.

The bilateral paired pulse technique that was described earlier was used to measure interhemispheric inhibition. Variations to this protocol were made, for example altering the intensity of a conditioning stimulus, in order to further explore the characteristics of the cortico-cortical pathway.

Objective 1.5 To measure the acute effect of intermittent TBS and continuous TBS on IHI in a naïve, anaesthetized animal, using minimally invasive recordings.

In a subset (n=25) of animals, the Prodigy IPG (St Jude, Minnesota, USA) was used to deliver iTBS or cTBS, to determine the effect of this on transcallosal inhibition. In these animals the

Prodigy IPG was also used to deliver the contralateral conditioning stimulus. A description of how the Prodigy IPG was employed in this manner is given in Appendix 1 (page 264), along with results from a pilot investigation of this method.

Objective 1.6 To compare interhemispheric inhibition measured under urethane anaesthesia with that measured under ketamine-domitor anaesthesia.

Using data gathered in the course of other experiments, the IHI was compared in this group of urethane anesthetised animals with that measured under ketamine-domitor anaesthesia as part of chronic, survival experiments.

The timeline for acute experiments depended on which research question that experiment was designed to address, but each followed approximately the same timeline, given below:

1. Rat anaesthetised using freshly prepared urethane *(3.3.1)*
2. Rat positioned, and craniotomy performed *(3.4.2, 3.4.3)*
3. Ipsilateral and contralateral electrodes implanted *(3.4.4)*
4. Depth profile recordings were obtained for both ipsilateral and contralateral stimuli – not completed for all animals *(3.4.7)*
5. EEG implanted over the dura *(3.4.5)* and the following recordings made:
    a. Input-output curves for ipsilateral and contralateral electrodes *(3.4.8, 3.4.9)*
    b. Interhemispheric inhibition recordings obtained *(3.4.10)*
        i. IHI recordings were obtained using a range of inter-stimulus intervals
        ii. IHI recordings were obtained using a range of conditioning pulse intensities – not completed for all animals

iii. IHI recordings were obtained using either the in-house stimulating equipment, or using the Prodigy internal pulse generator (IPG, St Jude, Minnesota, USA)

6. Stimulation protocol – not completed for all animals *(3.4.11)*
    a. A stimulation protocol was delivered (sham, intermittent theta burst, or continuous theta burst stimulation) using the IPG
    b. A repeat IHI measurement was obtained, using the same parameters as the first measurements
7. The animal was euthanised, and the brain removed and stored for later processing *(3.6)*
8. All processes took place within the same day.

3.4.2 POSITIONING OF RAT AND PREPARATION OF THE SKULL

Once anaesthesia was achieved the rat was prepared for surgery. The scalp was shaved and the local anaesthetic bupivacaine (2.5 mg/kg, s.c., Astra-Zeneca, NZ) injected into the scalp wound site and at least 10 minutes allowed for it to take effect. An intraperitoneal catheter was inserted and secured using tape to allow delivery of saline and urethane. A rectal probe was inserted and used with a heat pad to maintain internal temperature at 36.5 °C. Non-survival ear bars were inserted to secure the skull in the stereotaxic frame (Narishige, Japan), the front incisors held in the incisor bar and nose clamp lowered. These were adjusted so that no movement could take place and the rat was positioned in the centre of the stereotaxic frame. A midline scalp incision was made with a scalpel, the skin retracted, and connective tissue cleared away from the bone. Any bleeding was controlled with ‘gelfoam’ (Pfizer, USA) or cautery (Jorgensen Laboratories, USA). The surface of the bone was abraded with a scalpel to improve adherence of dental cement to the bone.

3.4.3 CRANIOTOMY

A craniotomy was performed using a small (0.7 mm) burr to remove areas of the skull over the sites for implantation of electrodes and electroencephalogram wire. The areas used were anterior posterior (AP) 0, medial lateral (ML) -2.5 mm and AP 0, ML +2.5 mm from bregma for implantation of the electrodes superficially into the primary motor cortex, and -2.5 ML, AP 2.0 from bregma for placement of the EEG wire anterior to the electrode implanted on the left (Figure 3.2). Coordinates were based on previous experiments done in this lab to explore IHI (Barry, Boddington, Igelström, et al., 2014), and correspond to the primary motor cortices (Paxinos & Watson, 2007).

3.4.4 IMPLANTATION OF ELECTRODES

The dura was cleared from the craniotomy sites in which the electrodes were to be implanted. Two bipolar twisted electrodes (Plastics One, USA) were implanted superficially at a depth of 1.5 mm below the surface of the skull on each side, at the previously mentioned coordinates (Figure 3.2). Sometimes coordinates were changed slightly (within 1 mm of the original) to avoid blood vessels. The two electrodes were fixed in place with dental cement (Vertex-Dental, The Netherlands). The electrode in the left cortex, which was closest to the EEG recording electrode, was labelled the ipsilateral or test electrode. The right electrode acted as the contralateral, or conditioning electrode.

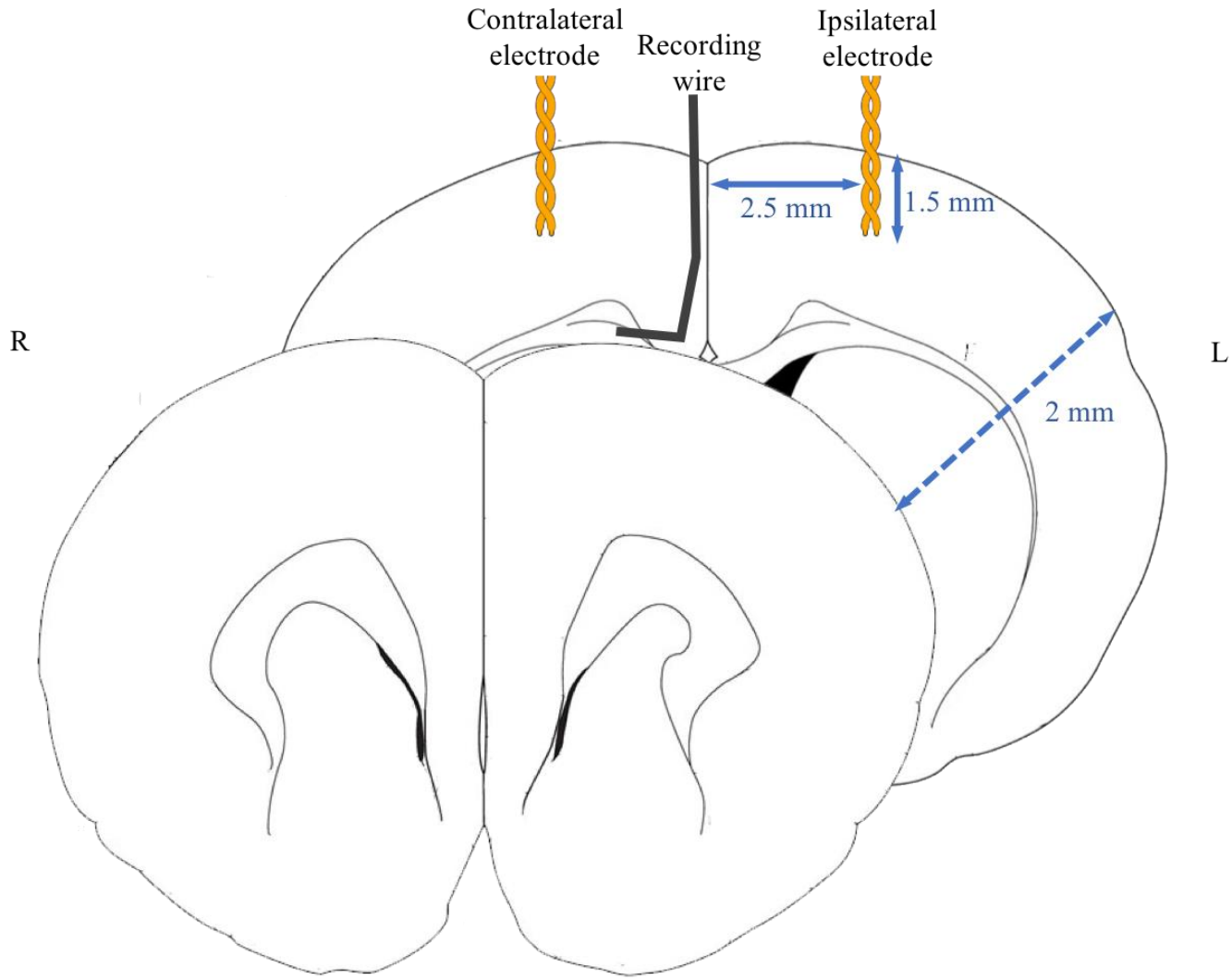

Figure 3.2 Acute non-survival experiment setup. Dimensions are not to scale. Image adapted from Paxinos and Watson (2007).

3.4.5 ELECTROENCEPHALAGRAM RECORDING

A silver, Teflon coated EEG wire (0.38 mm, AT15-T, Science Products, Germany) was bent so that a length approximately 1.5 mm could be placed onto the dura of the anterior craniotomy site. The Teflon was cleared from the tip, and the wire fixed in place with dental cement, with the help of an oscilloscope to verify that sufficient contact had been made with the dura. A reference electrode was positioned subcutaneously in the animal's back and connected to recording earth. Recordings were then made using this electrode, 10 recordings made under each condition, with a gap of at least 10 seconds between each repeat recording unless specified otherwise.

3.4.6 ANAESTHETISED RECORDING AND STIMULATION SETUP

In the anaesthetised rat, an *in-vivo* recording rig was used to make extradural electroencephalogram and local field potential recordings. The recording electrode was connected to a 1.0 x Axoclamp 900 A Headstage (Digimeter, Molecular Devices, USA), and an Axoclamp 900 A Microelectrode Amplifier (Digimeter, Molecular Devices, USA). The output was digitised using a DigiData 1322 A (Axon Instruments, Molecular Devices, USA) at a sampling rate of 10 kHz, and collected using a pClamp software package (Molecular Devices, USA). This signal was also diverted to be read on a digital storage oscilloscope (TPS2000B, Textronix, USA), so that the EEG could be monitored for signs of desynchronization.

A custom software package and digital-to-analog converter (custom build, Prof. J. Reynolds, University of Otago) were used to deliver timed signals to the STG4002 2-channel stimulus

generator (Multichannel Systems, Germany), which could be programmed to deliver pulses of specific amplitude and width to the implanted electrode components.

3.4.7 DEPTH PROFILE RECORDINGS

In order to investigate the transcallosal response, local field potential recordings were also made in some non-survival animals, as part of a depth-profile analysis of the contralateral cortex. These were made using the previously described recording setup but instead of a bipolar twisted electrode utilised a fine silver wire electrode (0.08 mm, AT15-T, Science Products, Germany), which was lowered into the ipsilateral cortex using the Inchworm Microdrive System (LSS-8000, Atlas Electronics Resources, Singapore).

Firstly, the dura was cleared from the recording site, and the wire lowered onto the cortical surface until the pressure of the wire was seen to just dimple the surface. This was set as the control 'zero' depth. The Microdrive was then programmed to lower the wire in one step of -200 μm, to allow the wire to penetrate the cortex. If cortical penetration was unsuccessful, then this process was repeated. Once the cortex had been penetrated, the wire was raised to a depth of 50 μm below the surface to begin recordings.

Depth profile recordings were made with a uniform stimulus intensity of 400 μA delivered to the contralateral cortical electrode as a 500 μs monophasic pulse. This stimulus intensity was high enough to reliably elicit a contralateral field potential, while remaining in the range of tested contralateral conditioning pulse intensities. At each depth, four recordings were made of the response to contralateral stimulations, with an inter trial interval of five seconds. The

depths assessed were 50 μm, 100 μm, 150 μm etc. up to 1000 μm. In order to allow measurement of IHI, the fine wire was withdrawn completely at the end of these measurements and a thicker EEG wire implanted over the surrounding dura.

3.4.8 IPSILATERAL INPUT-OUTPUT CURVE

Ipsilateral and contralateral input-output curves were recorded for all animals using the following parameters. A 500 μs wide monophasic square pulse was delivered to implanted bipolar ipsilateral electrodes at sequentially higher amplitudes. Amplitudes used were 200 μA, 400 μA, 600 μA, 800 μA and 1000 μA. The amplitude producing the largest peak to peak amplitude of response was identified. Test intensity for IHI measurement was set as 65-75% of the amplitude required to produce the maximum field potential response, so that the conditioning pulse could modulate the size of this response. The values were determined online, by visually comparing the peak-to-peak amplitudes of the different responses. The response amplitude was limited to 1100 μA to avoid damaging the cortical tissue.

3.4.9 CONTRALATERAL INPUT-OUTPUT CURVE

A 500 μs monophasic square pulse was delivered to the bipolar contralateral electrode at sequentially higher amplitudes. Firstly, an amplitude of 50 μA was used, and then sequential 50 μA intervals above this were tested to identify the threshold intensity; the intensity shown to produce a contralateral field potential response in at least 50% of trials. Next, amplitudes at 100 μA intervals were recorded up to a maximum of 1000 μA to construct the remainder of the curve. Once the threshold intensity was identified, the conditioning stimulus intensities could be calculated. The amplitudes chosen for use as conditioning stimuli during IHI

measurement were equivalent to 50% of threshold (subthreshold), threshold itself, 150% of threshold (supra-threshold), and 300% of threshold (high supra-threshold). Stimulation intensity for delivery of TBS was set to 50% of the threshold value.

3.4.10 RECORDINGS OF INTERHEMISPHERIC INHIBITION

The pulses of stimulation used for measurement of interhemispheric inhibition were delivered either by the Prodigy implantable pulse generator (St. Jude, USA), or using the MC_Stimulus control system. The method used with the IPG is described in Appendix 1 (page 264), as it does not contribute to the conclusions made during the course of this thesis. The use of small TMS coils designed for use in rats was also piloted but was ultimately unsuccessful. This pilot experiment is described in Appendix 2 (page 269).

For the measurement of IHI, recordings were made in blocks of four, each block of four having its own ISI and conditioning pulse intensity. Interstimulus intervals were varied at random from 10 ms to 1 ms in 1 ms intervals – so that a total of 10 ISIs were trialled. Non-conditioned trials, with ipsilateral stimulation alone, were also randomly interspersed. The process was repeated three times to give a total of 12 trials for each condition, with 10 seconds between each trial.

3.4.11 MEASUREMENTS OF INTERHEMISPHERIC INHIBITION IN RELATION TO THETA BURST STIMULATION

In some animals, the effect of a programme of sham, cTBS or iTBS on IHI was assessed. The IHI measurement using IPG conditioning stimulation described in Appendix 1 (page 264) was

first used to gain data on baseline IHI. Following 10 minutes of no stimulation, the chosen protocol was delivered, up to a maximum of 600 pulses, or 2 minutes of no stimulation, consistent with the protocol used by Barry, Boddington, Igelström, et al. (2014) and Boddington et al. (2020). Following a further 10 minutes of no stimulation, the same IHI measurements were repeated.

The burst stimulation used was a theta burst stimulation protocol (Figure 3.3). It was delivered using a Prodigy internal pulse generator (St Jude, USA). The basic theta burst pattern consisted of three pulses of 0.5 ms width delivered at 50 Hz, the bursts delivered at a rate of 5 Hz. Continuous theta burst stimulation was delivered as described, continuously until 600 pulses had been given. Intermittent theta burst stimulation was given as described, for two seconds, then off for eight seconds, then on again for two seconds etc. until 600 pulses had been delivered.

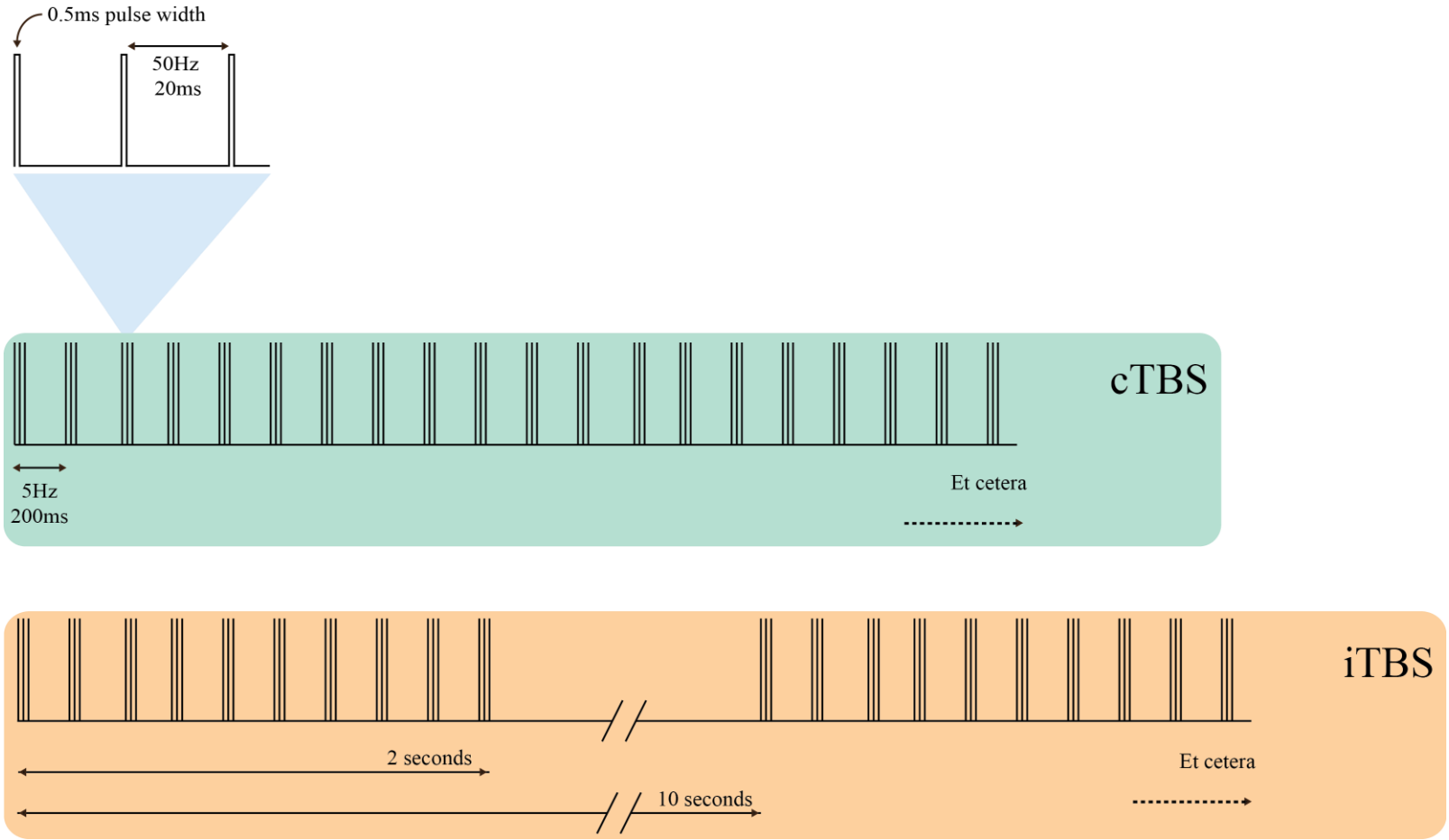

Figure 3.3 Continuous and intermittent theta burst protocols and their parameters.

The IPG was limited in that it could only deliver theta burst stimulation in increments of 50 μA, and individual pulses of stimulation at increments of 100 μA. Stimulation was given at 50% of the previously determined contralateral electrical threshold - that is the threshold that was previously determined to produce a field potential response when delivered to the contralateral side 50% of the time. This was in accordance with previous work which showed that subthreshold stimulus intensities produced a reduction in interhemispheric inhibition in the anaesthetised animal (Barry, Boddington, Igelström, et al., 2014). Note that in cases where the threshold was lower than 100 μA, the threshold was set at 100 μA and stimulation intensity to 50 μA as default.

3.5 CHRONIC EXPERIMENT METHODS

Chronic experiments were performed over 11 weeks, and were designed to meet the second and third study objectives:

Objective 2: To determine whether deficits after stroke are associated with an alteration of interhemispheric inhibition.

Objective 3: To determine whether, in an animal model of stroke, low intensity intermittent theta burst stimulation to the contralesional hemisphere can reduce interhemispheric inhibition and improve behavioural recovery.

Each experiment was designed to maximise the amount of information that could be obtained while reducing the number of animals used, so each animal underwent several different

processes. Results from baseline, post stroke week one and week two, are used to meet Objective 2. Both behavioural and electrophysiological data were measured before and after stroke induction to determine the effect of stroke on these measures at two time points. Results relating to these experiments are in Chapter 5. Results from weeks two to seven, during and after stimulation, are used to address Objective 3, and described in Chapter 6.

In order to understand how the following processes relate to each animal individually, detailed timelines for the acute and chronic experiments are presented below (Table 3.1). Each of the eleven weeks can be broken into days 1-5. Because of practical limitations, occasionally timings were altered by up to 2 days. The processes mentioned in Table 3.1 will be described in detail in chronological order in this section.

Timeline - detailed		
Week -3	*Gentling*	
	Days 1-5	Animal handled and given pasta pieces
Week -2	*Behavioural training*	
	Days 1-5	Animal trained in pasta behaviour in testing environment
Week -1	*Electrode surgery/Baseline*	
	Day 1	Electrode implantation surgery under ketamine-domitor anaesthesia, anaesthetised input-output curves and IHI baseline
	Day 2	Post-operative recovery check
	Day 3	Post-operative recovery check
	Day 4	Post-operative recovery check (morning, a.m.), input-output curve and IHI measurement in freely moving animal (afternoon, p.m.)
	Day 5	Post-operative recovery check (a.m.), behaviour baseline - pasta handling and grid walk (p.m.)
Week 0	*Stroke surgery/Post stroke week 1*	
	Day 1	Stroke induction surgery under isoflurane anaesthesia
	Day 2	Post-operative recovery check
	Day 3	Post-operative recovery check
	Day 4	Post-operative recovery check (a.m.), input-output curve and IHI measurement (p.m.)
	Day 5	Post-operative recovery check (a.m.), pasta handling and grid walk behaviour (p.m.)
Week 1	*Post stroke week 2*	
	Day 4	IHI measurement, randomisation to iTBS or sham, and then motor threshold assessment for intervention animals
	Day 5	Pasta handling and grid walk behaviour
Week 2	*Stimulation week 1*	
	Day 1	Stimulation and grid walking task
	Day 2	Stimulation and grid
	Day 3	Stimulation and grid
	Day 4	Stimulation, grid and IHI measurement
	Day 5	Stimulation, grid and pasta handling task
Week 3	*Stimulation week 2* – AS ABOVE	
Week 4	*Stimulation week 3* – AS ABOVE	
Week 5	*Post stimulation week 1*	
	Day 4	IHI measurement
	Day 5	grid and pasta handling task
Week 6	*Post stimulation week 2* – AS ABOVE	
Week 7	*Post stimulation week 3*	
	Day 4	IHI measurement
	Day 5	Grid walk, pasta handling task, pentobarbital injection for euthanasia, remove brain for histology

Table 3.1 Detailed timeline for chronic animal experiments. This timeline applies to each individual animal, not to the whole study.

3.5.1 BEHAVIOUR

Behavioural tasks were performed either by the candidate or by other members of the laboratory team. They took place in a darkened room illuminated with sodium lighting, during the hours of the 'dark' cycle.

GRID WALK

The grid walk apparatus consisted of a table 70 cm high topped with a wire grid (area 40 x 40 cm, mesh size 2.5 x 2.5 cm, wire thickness 1.5 mm, Emtech, University of Otago). The rats were contained within this area within a clear Perspex enclosure. A camera (IR Board Camera, EZSpyCam, USA) was positioned under the grid to film the entire area under the Perspex container. A red lamp illuminated the area for ease of analysis later. Rats were assessed on the grid for two minutes each time.

Analysis was performed blind and post-hoc by the candidate. Results were not unblinded until the conclusion of analysis to avoid bias based on knowledge of the animal's treatment allocation or lesion status. Blinded videos were watched at one-third speed and all steps with the left and right forepaw recorded, including steps where the paw was lifted and replaced in the same position. In addition, foot faults with the right and left forepaw were recorded. Faults were defined in accordance with previous literature (Chao, Pum, Li, & Huston, 2011; Schaar et al., 2010) and previous work in this lab (Boddington, 2016). A fault was counted when:

1. The foot was not placed on the grid, but slipped through the grid entirely,

2. The paw was correctly placed on the grid, but slipped through the grid during weight bearing,
3. The paw was incorrectly positioned, with the wrist rather than the pad of the paw contacting the grid.

PASTA HANDLING TASK

Methods for this task were based on the first descriptions of this technique by Allred et al. (2008) and Tennant et al. (2010). The test is designed as a measure of fine motor skill and manual dexterity (Allred et al., 2008; Schaar et al., 2010; Tennant et al., 2010).

Pasta pieces were made from spaghetti (San Remo, Australia) cut into 7 cm lengths and dipped for 1 minute in a 1:3 sugar: water solution. The sweetened pasta was then dried overnight. Sweetened pasta was used as it was found that it made training more efficient without altering behavioural performance. Animals were trained for two weeks prior to baseline task measurement. For the first week they were handled daily in order to familiarise them with the researcher. Whole or broken pasta pieces were placed in the group cage environment each day to familiarise the animals with the taste and shape of the sweetened pasta.

In the second week, animals were familiarised with the testing environment, either a clear, square cage, or an open top round glass tube (20 cm diameter, 30 cm height). The cage was preferred as it seemed to cause the animal less stress and they were unable to escape from it. Rats were placed in this environment for 10-20 minutes each day and given free access to pasta

pieces. Animals that were more reluctant to eat or appeared more stressed in the testing chamber were given extra time each day.

Testing took place on a weekly basis. Rats were placed in the testing chamber and a camera (DCR-SX44E, Sony, Japan) set up to film the environment. Rats were presented with individual lengths of pasta. Pasta eating behaviour has been characterised by previous researchers as follows. The typical rat eating a long piece of pasta commences with a certain paw at the base of the pasta and the other paw closer to the mouth. The base paw is labelled the 'grasp' paw, and the paw closer to the mouth labelled the 'guide' paw. Later in eating, when the piece is short, the paws move closer together and the digits interlock or 'interdigitate' (Tennant et al., 2010).

Movements of each paw were recorded live by means of two click counters. Movements were only counted while the pasta was in the rat mouth, and movements of the left and right paw were counted separately. A movement was counted if it involved a release and re-grasp action of the paw, not if the pasta simply slid through the paw. A movement was counted even if it did not advance the pasta further into the mouth. Movements were also counted when the pasta remained in contact with the paws, but the grip was adjusted. The 'grasp paw' for each piece was recorded at the time of testing. In some cases, there was not a clear 'grasp paw' at the base, and the paws were recorded as being together at the start of eating. In addition, other abnormal movements were recorded either at the time or by analysis of the video later.

Abnormal movements were as follows:

- Together: Paws positioned together at the start of eating
- Break: If the rat broke the piece, the piece was removed because the handling of short pieces is different to long pieces.
- One paw only: the rat handled the pasta with one paw only, the other paw was typically placed on the floor of the cage
- Mouth only: the pasta was eaten by pulling with the mouth only
- Switching paws: the position of the left and right paws was switched part way through eating
- Drop: the pasta was dropped after eating had commenced

During each administration of the task, movements of each paw were counted in real time, and behaviour was videoed for the eating of five individual pieces. The piece was repeated if broken partway through. Additional analysis took place blind and offline and consisted of double-checking abnormal behaviours on video copies, and applying the following rules:

1. Data from pieces that were broken or not completed by the rat were ignored.
2. Grasp paw preference was determined at the start of eating, unless the piece was dropped and grasp paw preference changed or paws were used 'together', in which case 'no preference' was recorded.
3. Following this, the overall grasp paw preference for each testing session (of five pieces eaten) was determined, with any uses of the left paw or right paw without the use of the opposite paw ('one paw only') included in ambiguous cases. If this was still unclear, 'no preference' was recorded for that testing session.

3.5.2 GENERAL SURGICAL METHODS FOR SURVIVAL SURGERIES

Methods were similar to those described for non-survival animals, however with significant changes to ensure animal welfare and sterile surgical conditions. Techniques were in accordance with this institution's guidelines and the experimenter was assessed to ensure the methods met the standards for animal welfare.

All tools were sterilised either by autoclave or by using a glass-bead steriliser between animals (Germinator, CellPoint Scientific, USA). The stereotaxic frame and surrounding area were sprayed and cleaned between each surgery with 70% ethanol. All surgical swabs cotton etc. were autoclaved before use. The outsides of drug containers were sterilised using 70% ethanol before drugs were drawn up and all needles were used once only.

The scalp was shaved and bupivacaine (2.5 mg/kg, s.c.) given as above, however before injection the site was prepared with several applications of betadine antiseptic (Sanofi-Aventis, Australia). Amphoprim (trimethoprim & sulphamethazine, 12 mg/animal, Virbac, France), an animal specific, pyrimidine-type antibiotic was used for pre-emptive infection control.

Eyes were moistened using 'refresh' eye ointment (Allergan, Ireland), a paraffin based, non-medicated eye lubricant. The rat was placed in non-traumatic ear bars, which were first lubricated with 2% Xylocaine Jelly (Astra-Zeneca, NZ). The incisor bar and nose clamp were also used to secure the skull. The temperature of the animal was maintained during longer surgeries, e.g. electrode implant surgeries, by using reusable 'recrystallisation' hand warmer packs (Kathmandu, NZ). The packs were activated before surgery, wrapped so that the skin

was not warmed excessively, and placed under the rat. The surgical field was isolated by covering the scalp with ‘glad press-and-seal’ (Glad, USA), which was pressed against the stereotaxic frame to secure it for the duration of surgery. The whole area was then sprayed with ethanol, and gloves changed to sterile surgical gloves.

A midline scalp incision was made, and the skin retracted with loops of suture. The connective tissue was cleared from the bone surface, and bleeding controlled using gelfoam. The surface of the bone was abraded with a scalpel to assist with adherence of dental cement.

3.5.3 ELECTRODE IMPLANTATION SURGERY

Electrode implantation surgery took place under ketamine-domitor anaesthesia. Five components were implanted: two stimulating electrodes, two recording electrodes, and a ground electrode (Figure 3.4 and Figure 3.5). The stimulating electrodes used were a custom product from Plastics One, Virginia, US, (via BioScientific, Australia) consisting of bipolar twisted wire electrodes, to which two gold pins could be attached. The recording electrodes were made in-house using silver wire (0.25 mm, Teflon coated, AG15-T, Science Products, Germany), with a gold pin at one end, and a bent portion with 1 mm exposed wire at the other. The reference (earth) electrode was identical to the recording electrodes, with a 3 mm bone screw (J & G Hardware, China) attached to the exposed portion of wire. In addition, 5-6 extra 3 mm bone screws were used to improve adherence of the components to the skull.

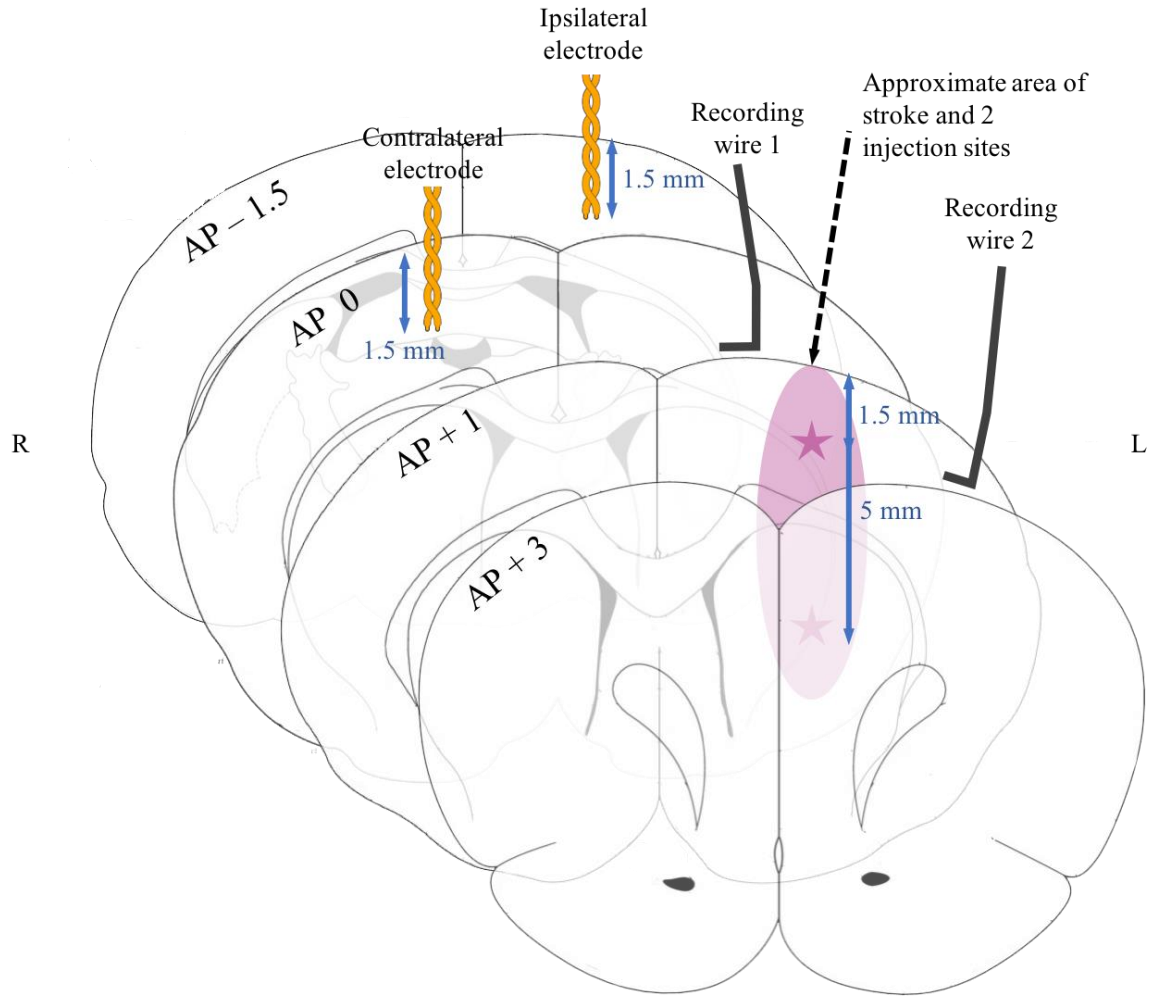

Figure 3.4 Chronic experiment setup. Dimensions are not to scale. Image adapted from Paxinos and Watson (2007).

CRANIOTOMY

A craniotomy was performed to remove areas of the skull over the sites for implantation of electrodes and electroencephalogram wires. A narrow channel was also drilled in the skull for later stroke induction. The exact locations used are given in Table 3.2.

	AP (mm)	**ML (mm)**	**Depth (mm)**
Contralateral stimulating electrode	0	-2.5	-1.5
Ipsilateral stimulating electrode	-1.5	+2.5	-1.5
Recording electrode site 1	+1	+1	Dura surface
Recording electrode site 2	Approximately +3	Approximately +2.5	Dura surface
Stroke induction	+1	+2.7	-1.5, -5

Table 3.2 Coordinates used for chronic lesion experiments.

Coordinates were based on previous experiments done in this lab to explore IHI (Barry, Boddington, Igelström, et al., 2014), and correspond to the primary motor cortices (Paxinos & Watson, 2007). Some of the placements were adjusted from the optimal location due to the practical difficulties in implanting multiple electrodes very close together Figure 3.4 and Figure 3.5). Skull screws were screwed into narrow drill bores surrounding the craniotomy sites.

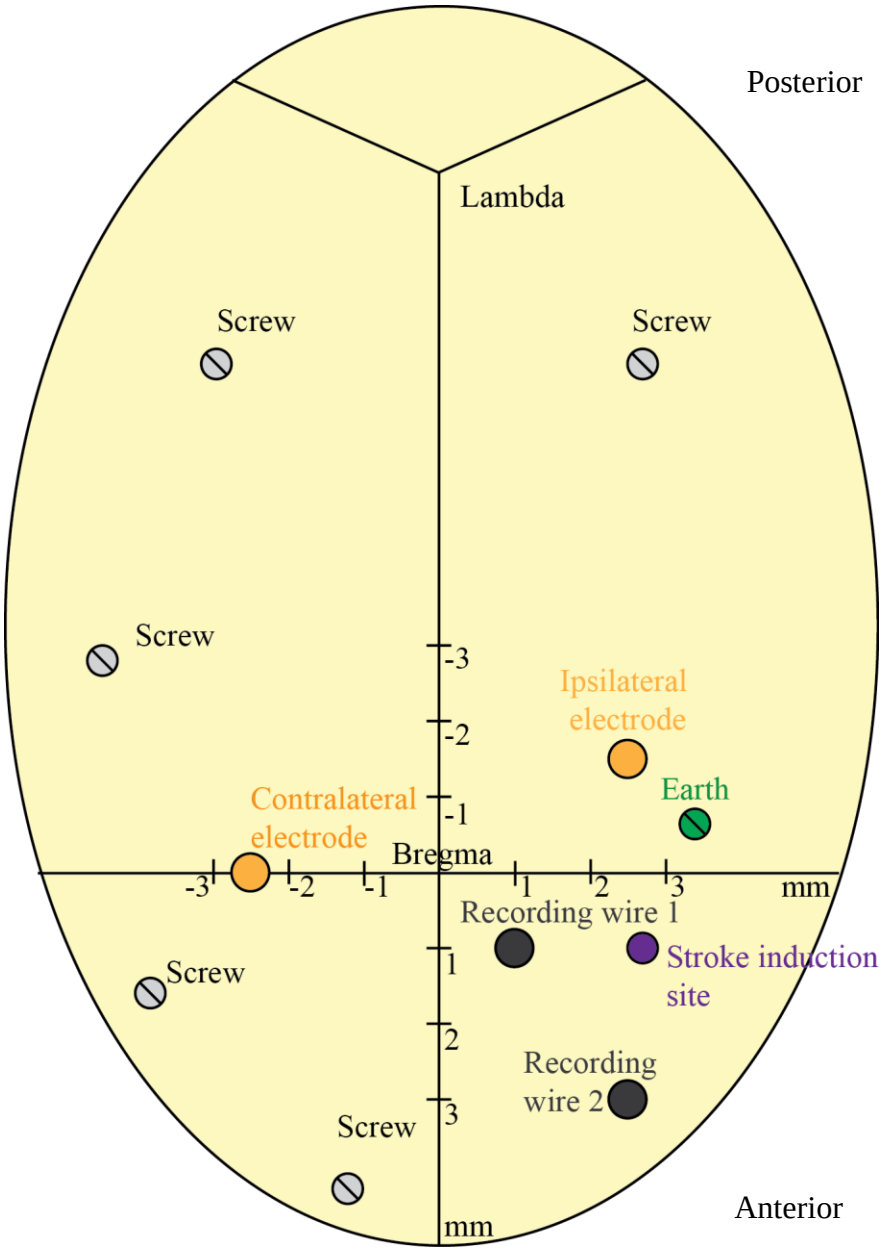

Figure 3.5 Locations for implantation of recording and stimulation components, superior view.

Implantation of stimulating electrodes occurred as described for non-survival procedures. Two EEG recording wires were placed, as described for non-survival procedures *(3.4.5)*, against the exposed dura of the two anterior-most craniotomy sites. The wires were fixed in place with dental cement. The reference (earth) electrode was screwed laterally to the ipsilateral stimulating electrode (Figure 3.4). At this time, baseline recordings were made using the previously described electrophysiology rig *(3.4.6)*.

RECORDINGS MADE AT THE TIME OF ELECTRODE IMPLANT

Recordings of ipsilateral and contralateral responses were made in an identical manner to that described in *3.4.8* and *3.4.9* for non-survival animals to determine conditioning and test amplitudes. For the measurement of IHI, recordings were made in blocks of 6, each block having its own ISI. Interstimulus intervals were varied at random from 10 ms to 4 ms in 1 ms intervals – so that a total of seven ISIs were trialled. Non-conditioned trials, with ipsilateral stimulation alone, were also randomly interspersed. Inter-trial intervals were at least five seconds. This condensed methodology was used, both because these recordings were not a focus of our analysis, and because of the need to use the shorter lasting ketamine-domitor anaesthetic in these animals.

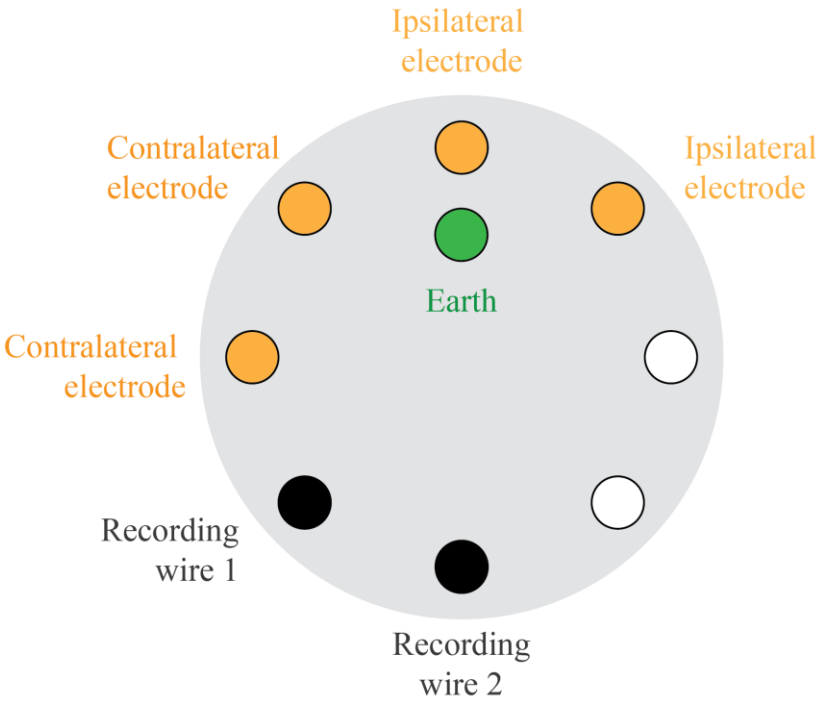

Figure 3.6 Key for arrangement of electrical components within plastic socket on rat's head for alignment with head stage (superior view of the socket).

PLACEMENT OF ELECTRODE PLUG

The electrical components were inserted into a 9-hole plastic plug (Ginder Scientific, Canada) as shown in Figure 3.6. This arrangement was determined by the design of the corresponding head stage, which allowed specific pins to perform specific functions such as recording or stimulation. A dummy 9-pin head stage socket (Ginder Scientific, Canada) was used to ensure alignment, so that once awake the rat could be attached to the head stage with minimum effort. The wires were twisted together, and dental cement was used to enclose all the electrode wires and secure the plug against the skull. Care was taken not to block the site for later introduction of the stroke induction needle. The dental cement was smoothed into a mound around the base of the plastic plug, with no exposed wires, allowing the skin to be sutured around the base without causing irritation of the rat's skin.

3.5.4 STROKE INDUCTION

The stroke induction protocol was based on studies by Windle et al. (2006). The two injection regimes in that paper that formed the basis for that employed in the experiments described in this thesis were:

A: Two cortical injections of 800 pmol endothelin-1, totalling 1600 pmol, to stereotaxic coordinates of AP 0.0 mm, +2.3 mm, ML +2.5 mm, DV −2.3 mm (for both injections).

And B: Three injections of 400 pmol endothelin-1, two cortical and one subcortical, totalling 1200 pmol. Cortical injections were to the same coordinates as A, plus AP +0.7 mm, ML +3.8 mm, DV −7.0 mm for the striatal injection.

Coordinates were changed from Windle et al. for two reasons. Firstly, only one burr hole could be drilled because of the number of other components attached to the skull, so an AP coordinate between the two chosen by Windle was used alone. Secondly, the ML coordinate was shifted 2mm to the right, and the DV coordinates raised by 0.8 and 2mm respectively, owing to a small number (but high proportion) of animal deaths early in the study when the endothelin-1 caused bleeding into the ventricles. Therefore, the coordinates used in this study were:

C: Two injections of 800 pmol endothelin 1, one cortical and one subcortical, totalling 1600 pmol. To stereotaxic coordinates of AP +1.0 mm, ML +2.7 mm, DV −1.5 and -5 mm.

Stroke induction took place under isoflurane anaesthesia. This surgery was very simple, because it was only necessary to re-open the scalp sutures and introduce the syringe into the site pre-drilled during electrode surgery. In most cases surgery took less than 30 minutes.

Endothelin-1 (human and porcine, Sigma-Aldrich, USA) was shipped frozen and stored at negative 20 ℃ in lyophilized form until needed. Once hydrated, the endothelin-1 was stored at -20 ℃ for a maximum of 2 weeks, according to manufacturer recommendations. The endothelin-1 was hydrated using a 9% acetic acid solution, using 1 μL acetic acid solution per μg endothelin 1, to create a 400 nmol/L solution. The endothelin-1 was mixed using a vortex mixer to ensure complete incorporation of the powder.

The animal was anaesthetised, and scalp prepared as described above. If not pre-prepared, a craniotomy site was drilled at AP +1, +ML 2.7 mm (Table 3.2, Figure 3.4). A 10 μL Hamilton

syringe was filled with the prepared endothelin-1 solution and lowered through the craniotomy site to a depth of 1.5 mm below the dura, and after 2 minutes 2 μL endothelin-1 was injected at a rate of 450 nL/minute, using a syringe pump (UltraMicroPump with SYS Micro4 Controller, Coherent Scientific, Australia). After 2 minutes, the needle was lowered to a depth of 5 mm and after a further 2 minutes a second 2 μL injection was delivered. Thus, two injections of 800 pmol were given. The depth of the dura was occasionally hard to visualise, so the depth at which cerebrospinal fluid was seen to escape from the punctured dura was taken as the reference depth. The volume of liquid in the Hamilton syringe was manually checked to ensure the correct volume was given with each injection, and where needed a top-up injection was given. After a further 2 minutes, the needle was withdrawn slowly, and the incision sutured.

3.5.5 SURGICAL RECOVERY

Once either implantation or lesioning surgery was completed, the retracting sutures were removed, and the incision was sutured using an interrupted suture technique. The suture used was SofSilk, 3-0 silk, black braided C-23 cutting (Covidien, Ireland). The sutures were used to secure the skin around the base of the plastic cap containing the electrodes, leaving only the socket and screw thread exposed.

Recovery from survival surgery depended on the type of surgery (electrode implant or stroke induction), duration of surgery, and anaesthesia type. Rats anaesthetised under isoflurane awoke quickly, often within five minutes, and showed faster overall recovery. Surgeries under

ketamine-domitor anaesthesia had longer recovery times, with rats re-awakening in 30 minutes to an hour. In general, longer surgeries had longer re-awakening and recovery times.

The animal was left to recover from anaesthesia in a warm environment. They were provided with bedding, soft food and access to a water bottle. The rats were kept under observation by the experimenter until they had awoken from anaesthesia, and then were transferred to an individual cage, within sight and sound of other rats. Post-surgical checks were performed twice daily, for four days following surgery, according to this institution's guidelines.

3.5.6 AWAKE RECORDING AND STIMULATION SETUP

After surgery to implant the 9-pin plastic socket, the rat was able to undergo electrophysiology stimulation and recording while freely moving. The stimulation took place in an operant chamber (Med Associates, USA) in a dark, sodium lit behavioural room. The rat was familiarised with the stimulation environment for several minutes prior to the first time it was attached to the head stage. To attach the head stage, the rat was held close to the researcher's body in one hand, while the other hand was used to connect the headstage to the implanted plug.

The head stage was custom build by the University of Otago, Department of Psychology. It was attached via a 12-pin commutator (Pinnacle Tech, USA) to a custom battery source also built in the Otago Psychology Department. This battery source enabled the head stage to be attached to two different bipolar stimulation inputs, two recording outputs, and a reference earth.

For iTBS, the stimulation input corresponding to the right, contralateral electrode was attached to the output of a Neurolog stimulator (Digitimer, United Kingdom). If sham stimulation was required, this connection was left open. For investigation of IHI, the two stimulation inputs were connected to the two outputs of a PHM-152/2 Dual Programmable ICSS Stimulator (with Monitor Output) (Med Associates, USA). These stimulators were controlled by means of a Med Associates software package (Med-PC IV), which interfaced with the stimulators and operant chamber using a Med Associates Interface ("PCIe Operating Package for Two Compartment CPP", Med Associates, USA).

The two recording outputs were connected to a Neurolog amplifier module: first to the NL106 AC-DC Amp, then through the NL104 A.C (Digitimer, United Kingdom) preamp module which subtracted one input from the other to create a recording differential, and finally filtered through the NL125/126 filter with mains notch on. The recording output was digitised at a rate of 1 kHz via a 2 channel MiniDigi 2-Channel Digitiser (Molecular devices, San Jose, USA) to enable collection of recordings with the ClampEx software package (Molecular devices, San Jose, USA). The second channel of the MiniDigi was used to collect output recordings from the stimulator in use at the time.

3.5.7 RECORDINGS MADE IN THE AWAKE ANIMAL

The ipsilateral "test" amplitude to be used was first determined. A universal amplitude of 400 µA was used unless:

1. No response to this amplitude was recorded - in which case 600 µA was used.

2. This amplitude produced a motor twitch from the rat - in which case 300 µA (n=2) or 250 µA (n=1) intensities were used - whichever was low enough to not produce a motor twitch.

An intensity of 400 µA was chosen as default, as it was the amplitude determined to be 70% of the amplitude at which maximum response was seen in the anaesthetised animal (in most animals). Owing to the difficulty in conducting online analysis of the input-output curve with this setup, the intensity required to determine maximal response was not re-established in the moving animal.

Interhemispheric inhibition was measured weekly in awake animals - and testing conditions were optimised for efficiency owing to the need to test many animals on the same day. In the first three weeks, and in the final week of measurement, IHI measurements were made for conditioned-test ISIs of four through ten milliseconds, as well as measurements of the unconditioned ipsilateral response, and the contralateral response. Following analysis of these recordings, four or five ISIs which produced the largest degree of inhibition were identified, and these were the only intervals tested on the other testing days, in addition to the unconditioned ipsilateral and contralateral responses. The amplitudes of stimuli were determined as described above. The responses were recorded in blocks of ten, with a five second inter trial interval. The order of trials was varied at random.

3.5.8 MED-PC PROGRAMMING FOR THE MEASUREMENT OF INTERHEMISPHERIC INHIBITION

The Med-PC software, with ICSS package included, was used to programme the delivery of conditioning and test stimuli for IHI measurement in the freely moving animal. The complete programme written for this task is supplied in Appendix 3 (page 272). The ICSS stimulators were able to deliver square biphasic pulses at a pre-set width, amplitude and inter-pulse interval. To achieve bilateral paired-pulse measurement of IHI, the first phase of the biphasic pulse was set to an amplitude of zero, and the width of this pulse used to time the delivery of the second phase, allowing the interval between pulses to be precisely varied. The second phase was set to the predetermined conditioning or test amplitude and delivered at a width of 500 μs. The inter-trial interval was set as five seconds, up to a maximum of 10 pulses. A second programme was written as a simplification of the first to enable the contralateral and ipsilateral pulses to be delivered on their own to measure input-output curves and non-conditioned ipsilateral responses.

It should be noted that while the stimuli given were confirmed to be accurate using a digital oscilloscope, there was an additional artefact delivered by these stimulators. This consisted of two 500 ms second long, low amplitude pulses. This artefact was unable to be removed, even following overhaul of the device by the manufacturer. It did not seem to contribute to the responses measured, owing to its low amplitude and wide pulse width. In addition, the effect of this pulse could be eliminated using a 30 Hz High Pass filter, or through offline techniques. Appendix 4 (page 278) shows a representative example where offline filtering is used to demonstrate that the addition of a filter did not alter the level of IHI measured.

3.5.9 STIMULATION PROTOCOL

The stimulation used was a theta burst stimulation protocol, developed during previous research (Barry, Boddington, Igelström, et al., 2014; Boddington, 2016; Hsu et al., 2012; Y. Z. Huang et al., 2005). It was delivered five of seven days for three weeks using a Neurolog stimulator system (Digitimer, United Kingdom). The theta burst pattern is described earlier (see Figure 3.3). The Neurolog unit was used for stimulation of awake animals and was attached via the implanted electrode plug.

Animals were randomly assigned to receive sham or iTBS using a random number generator. The stimulation intensity was based on two measurements, the electrical threshold as determined under surgical anaesthesia, and the motor threshold. The motor threshold was determined for those animals which were assigned to receive stimulation the week prior to commencing stimulation (Table 3.1). Intermittent TBS was delivered to the animal at increasing intensities until a motor twitch was observed in the left fore or hind paw, during the period at which the iTBS was 'on'. The intensity was then lowered to determine the lowest threshold at which a twitch could be seen. This was labelled the motor threshold. The stimulation intensity was then set as 50% of motor threshold plus electrical threshold. This method was consistent with previous work (Boddington et al., 2020).

3.6 EUTHANASIA

Euthanasia was performed using an overdose of urethane or pentobarbital. Once delivered, the animal typically died within 30 minutes. Death was verified through complete absence of pulse

and respirations. The brain of the rat was then removed and preserved in paraformaldehyde solution until being prepared for histology.

3.7 HISTOLOGY

Brains were cut into 100 μm sections, mounted on gelatine coated slides and stained using cresyl violet stain. See Appendix 5 (page 280) for details of this protocol. Slices were examined to assess lesion extent and to confirm the positioning of electrodes. Analysis of histology was conducted using high powered photographs at 20x magnification made using the Aperio Digital Slide Scanning System (Leica Biosystems, Germany) and analysed using Aperio Image Scope software (Leica Biosystems, Germany).

Mediolateral and dorsoventral electrode placement was determined using the slice at which the electrode was deepest. Electrode position coordinates were estimated based on relative positions of surrounding structures, using the Rat Brain Atlas (Paxinos & Watson, 2007) as a guide. Lesion extent was calculated based on the lesion area calculated from each slide. An area of A μm^2 on one slide was equivalent to 100A μm^3, and by summing each of these volumes for every slice, an estimate of lesion volume was obtained. Analysis was done with the analyser blinded to the experimental outcome for each animal.

3.8 ELECTROPHYSIOLOGICAL ANALYSIS

Electrophysiological analysis of EEG stimulus response curves was performed offline using AxoGraph software. Data files were first grouped according to animal and testing condition.

Once like trials had been combined, they were aligned to the stimulus artefact. Any trials which exhibited no response to stimulus, or a response in the opposite direction to the remainder of trials, were excluded from analysis. The trials were then aligned to baseline and the mean response curve was calculated.

Analysis focused on the second peak of the response, which was generally the largest part of the signal. The two variables investigated were the maximum slope of this curve, and the peak to peak amplitude (see Figure 4.1). To account for baseline levels of inhibition in anaesthetised animals, IHI results were normalised to a linear regression of the five central data points. Throughout Chapter 4, it is clearly identified which data was treated in this manner.

3.8.1 CURRENT SOURCE DENSITY ANALYSIS

Current source density analysis was performed on depth profile responses using methods previously employed in this laboratory, adapted from Aroniadou and Keller (1993) and Mitzdorf (1985). The equation given below was applied to groups of five responses at successive depths 'a-e', with the result representing the current-source density for depth 'c'. This was done for each group of five traces.

$$C(c) = 2(a + e) - (b + d) - 2c$$

$$Where\ a - e\ are\ values\ at\ successive\ depths, and\ C = Current\ source\ density$$

3.8.2 ANALYSIS OF AWAKE RECORDINGS

Awake animal responses were analysed in the same way as anaesthetised recordings. Responses obtained resembled those obtained during anaesthetised experiments and described in Chapter 4, except for being recorded at a lower sampling rate. To ensure that the responses were being analysed identically, the portion of the response curve that was analysed was determined using the latencies found during the anaesthetised animal experiments. Appendix 4 (page 278) gives an example of this analysis.

In most recordings, the crossed contralateral response which was measured in the milliseconds before the test response altered the baseline of the subsequent test response. To control for this, a recording of the contralateral response alone was subtracted from the final recording. This technique was adapted from Boulogne, Andre-Obadia, Kimiskidis, Ryvlin, and Rheims (2016).

3.9 STATISTICS

The main statistical techniques employed in this thesis are paired and unpaired t-tests, one and two-way repeated measures analyses of variance (ANOVAs), correlation analyses and a linear mixed effects model. The overall level of significance was $p<0.05$. On two occasions the 10% level of significance was considered when post-hoc analyses revealed an important trend. Additional analyses that were infrequently used are described with their relevant results. All analyses were conducted using Prism statistical and graphing software (GraphPad Software, California, USA).

3.9.1 PAIRED AND UNPAIRED T-TESTS

Unpaired t-tests were used to compare the IHI measured in different populations, for example to determine adequacy of randomisation. Paired t-tests were used to determine whether IHI was present and to compare IHI following IPG stimulation with baseline IHI within the same population of anaesthetised animals.

3.9.2 ONE AND TWO-WAY REPEATED MEASURES ANOVA

A repeated measure analysis of variance (ANOVA) was used when the groups of data to be compared were from the same population at multiple timepoints. One- and two-way ANOVAs were used when there were one or two samples from different populations to be included respectively. One-way repeated measures ANOVAs were used in these experiments to analyse if changes in behaviour or IHI following stroke were significant. The two-way repeated measures ANOVA was used when changes over time in sham versus iTBS groups were to be analysed.

3.9.3 ANALYSIS OF CORRELATIONS

When two different groups of data relating to the same animals were being assessed for correlation, an assessment of the significance of correlation was made. If correlation was significant at the 5% level, a linear regression model was fitted, and the degree of variability explained by the linear regression assessed using Pearson's r and R^2 values.

3.9.4 LINEAR MIXED EFFECTS MODEL

A linear mixed effects model was employed when there were missing data for individual animals, and where these animals could not simply be removed without severely restricting the size of the data set. A linear mixed model is designed to account for missing data in cases where there are multiple relationships within the data set – for example the IHI within the same animal on two different days will have a relationship, as will the IHI across two different animals in the same treatment group at the same timepoint within the study. This hierarchy of 'non-independence', as well as the predicted degree of random effect, is considered within the model, and missing data accounted for appropriately.

3.10 RESULTS

All results are given as mean ± standard error of the mean (SEM), in order that both the standard deviation of the data and the sample size is captured in the error value. Data are given to as many decimal places as the accuracy of the raw data, typically one decimal place. P values and other statistical measures are given to two significant figures.

4 RESULTS: INTERHEMISPHERIC INHIBITION IN THE ANAESTHETISED RAT

4.1 INTRODUCTION

The overall aim of this thesis is to measure changes in interhemispheric inhibition before and after stroke and during application of iTBS, to determine its association with stroke recovery. Previously, IHI in rats has been measured using intracellular recordings. However, this technique gives a one-off measure, as rats are euthanised at its completion. It was therefore necessary to develop a comparable but less invasive technique of IHI measurement.

The technique to be used has been described in Chapter 3, and modifies the TMS based bilateral paired pulse technique first employed by Ferbert et al. (1992). Rather than transcranial magnetic stimulation, electrical stimulation is used to elicit the pulses, a technique employed by Barry et al. (2014). However, where Barry used intracellular recording, here extradural electroencephalogram (EEG) recording will be used. This has the dual advantages of being compatible with a freely moving animal recording apparatus and being potentially translatable to measures used in human applications of this research. The nature of recordings made in this manner is poorly understood, so the main objective of this chapter is to demonstrate in an anaesthetised animal some properties of these recordings that make them appropriate for use

in the freely moving animal experiments reported later in this thesis. The results in this chapter relate to Objective 1:

Objective 1: To investigate the use of a minimally invasive technique for the measurement of interhemispheric inhibition in the freely moving animal.

By way of introduction, this chapter will start with a description of local field potential recordings. This will be followed by the hypotheses relating to each of the chapter's objectives, the corresponding results and a discussion of their significance in relation to the rest of the thesis.

4.2 PROPERTIES OF LOCAL FIELD POTENTIAL RECORDINGS

The recording technique with the greatest similarity to the extradural recording used in the present study is the local field potential. A useful way of thinking about the local field potential is to consider it as the extracellular component of the circuit formed by the dendrite at one end of the neuron and the synaptic terminal of the axon at the other. When considering a system with a single neuron receiving a current input at the dendrite, recordings near the dendrite will show a negative deflection, representing an output from the dendrite to the rest of the cell. When describing local field potentials, outputs such as these are known as current sources. Compare this with a similar neuron recorded near the soma. Here the local field potential will show a positive deflection, representing the input from the dendrite to the cell body. Inputs such as this are termed current sinks. This is shown in pictorial format in Figure 4.1 and Figure 4.2.

This simple model is complicated by the fact that the local field potential is a record of many such circuits from the whole area surrounding the recording site. However, as the neurons of the cortex are arranged in parallel, the majority of currents are flowing in the same direction, and summate to create the local field potential response, see Figure 4.2.

Extrapolating from the single neuron to the cortical slice, experimenters investigated the properties of the layered cortex by taking recordings from sequentially deeper locations within the cortex. The local field potentials from these layers can then be analysed using techniques developed by Mitzdorf (1985), in order to detect the locations of sinks and sources at various depths, known as current source density (CSD) analysis. This reveals the functional properties of each layer. Aroniadou and Keller (1993) describe results from such experiments in cat visual cortices in detail. An example of their findings is shown in Figure 4.3 as an illustration of this technique.

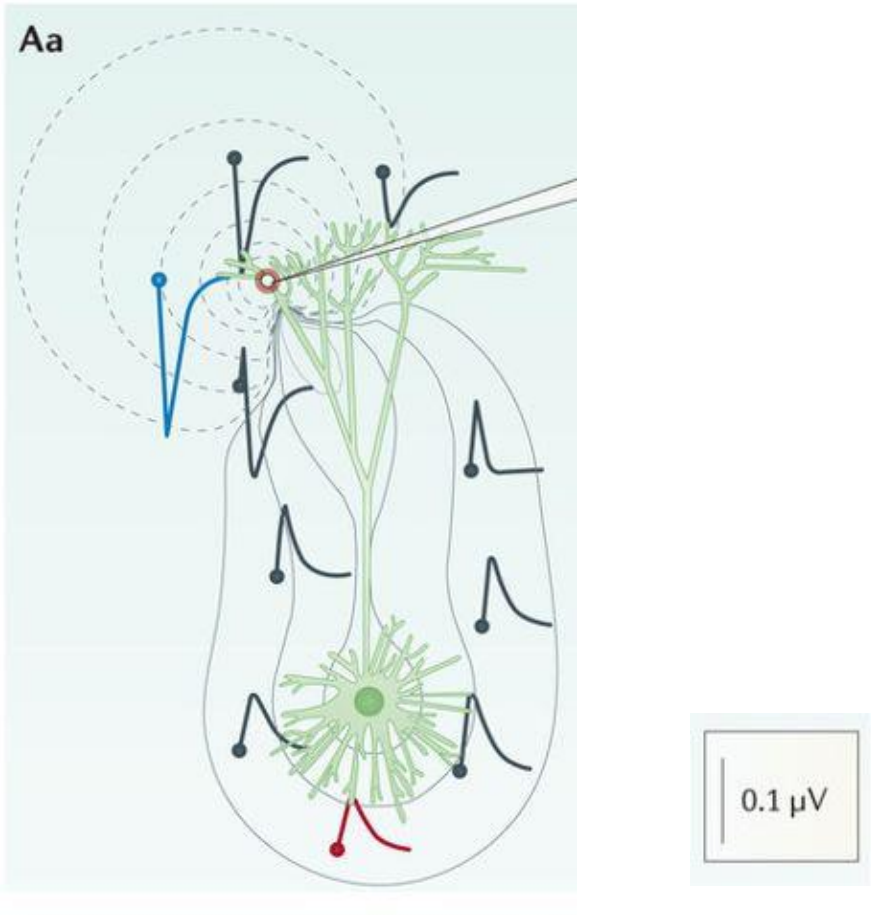

Figure 4.1 Schematic demonstrating representative local field potentials at different parts of the neuron. Adapted from Einevoll et al. (2013).

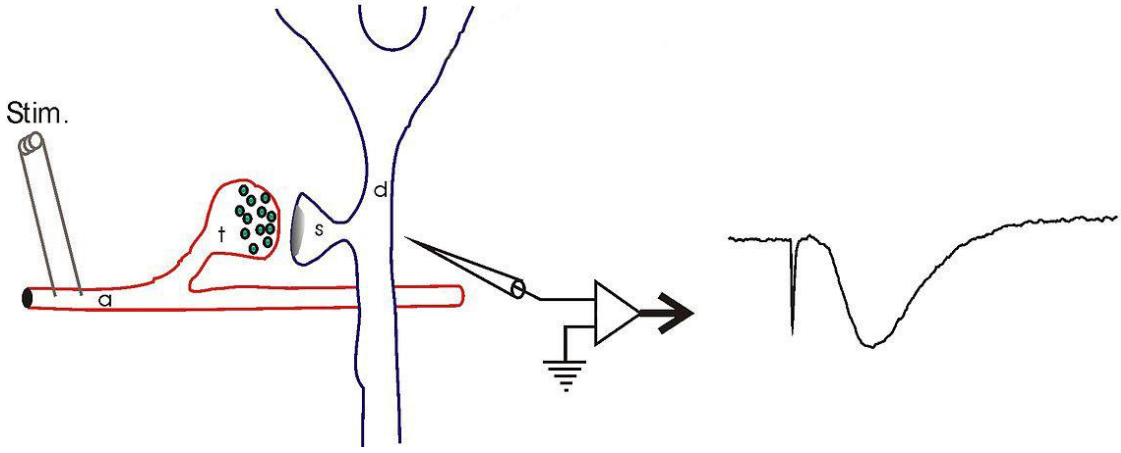

Figure 4.2 Schematic of local field potential recording from the rat hippocampus showing recording near dendrites revealing a current sink. The neuron drawn on the left symbolises a larger group of neurons with similar morphologies. Labels refer to: a=axon, t=axon terminal, s=synaptic bouton, d=dendrite. Adapted from Synaptitude (2005).

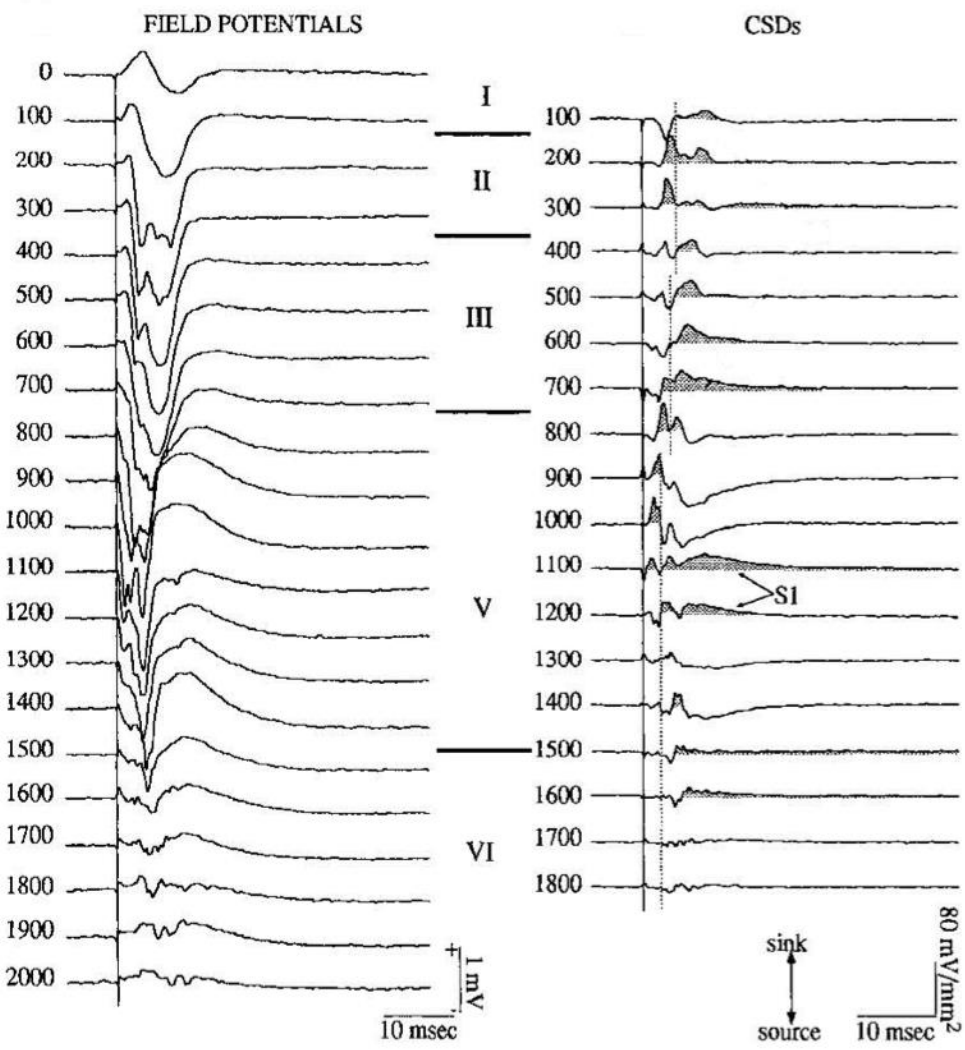

Figure 4.3 Example field potentials and corresponding CSD analysis recorded in response to adjacent layer V stimulation. A sink is marked S1, and corresponding current sources are seen in the two layers superficial to this. Adapted from Aroniadou and Keller (1993).

4.3 PROPERTIES OF THE IPSILATERALLY EVOKED RESPONSE

Based on the literature described above, the properties of ipsilaterally-evoked local field potentials within this study were predicted.

Objective 1.1 To measure characteristics of single pulse ipsilaterally-evoked field responses measured using single wire electroencephalogram recording.

- **Hypothesis 1.1** That recorded responses will demonstrate similar morphological characteristics to those measured using intracortical field potential recording techniques.

The ipsilateral electroencephalogram response exhibited a characteristic waveform shape, with minimal variation between animals (see Figure 4.4). The response itself was comparable morphologically to local field potential responses measured intracortically that have been reported in the literature previously. In particular it bore resemblance to recordings made in layer I, which is closest to the recording site (Aizenman, Kirkwood, & Bear, 1996; Aroniadou & Keller, 1993). Onset of this response was at a latency of 1.67 ms ± 0.11, n=18. Three peaks, a positive, negative and a final positive peak, occurred at 3.02 ± 0.32 ms; n=15, 9.31 ± 0.16 ms; n=18 and 31.1 ± 1.30 ms; n=18 respectively. There was some variation between responses due to the baseline up and down states that are characteristic of the deep plane of anaesthesia used. They represent the synchronised subthreshold changes in membrane potential that occur during sleep or anaesthesia (C. Wilson, 2008). As they are a function of the anaesthetised recordings, they did not occur in the recordings of contralateral responses in awake animals,

and so are not significant in the analysis of IHI in awake animals later chapters. This variation was not notable at the higher amplitudes used for test stimuli.

The measures used to determine IHI were determined once results from several bilateral paired pulse recordings had been analysed offline. Interhemispheric inhibition was most prominently seen when measuring the maximum slope of the second peak of the test field potential response. These measures were used to determine the maximum IHI and will be used in this report. Examples where peak to peak amplitude is measured are given in Appendix 7 (page 282). An example ipsilateral response demonstrating the maximum slope measure is shown in Figure 4.4.

The ipsilateral response was measured at sequentially higher amplitudes to obtain a current voltage curve. These results showed a linear relationship between current input and voltage response measured at the maximum slope of the second peak of the response across animals. This means that any increase in voltage response size seen corresponds to an increase in the input current to that area. Test intensity was set to 70% of that which produced the maximum response size. This was determined online. The average intensity at which the maximum response was seen was 940 $\pm$ 50 µA; n=18, and the average test intensity 660 $\pm$ 40 µA, n=18. Amplitudes used for each animal are given in Appendix 6 (page 281).

Hypothesis 1.1 was accepted. This ipsilateral response will be used as a baseline to determine the inhibitory effect of contralateral stimulation throughout this thesis. Knowing that this measure has characteristics like those previously reported allows us to compare work later in this thesis with the pre-existing body of literature on this topic.

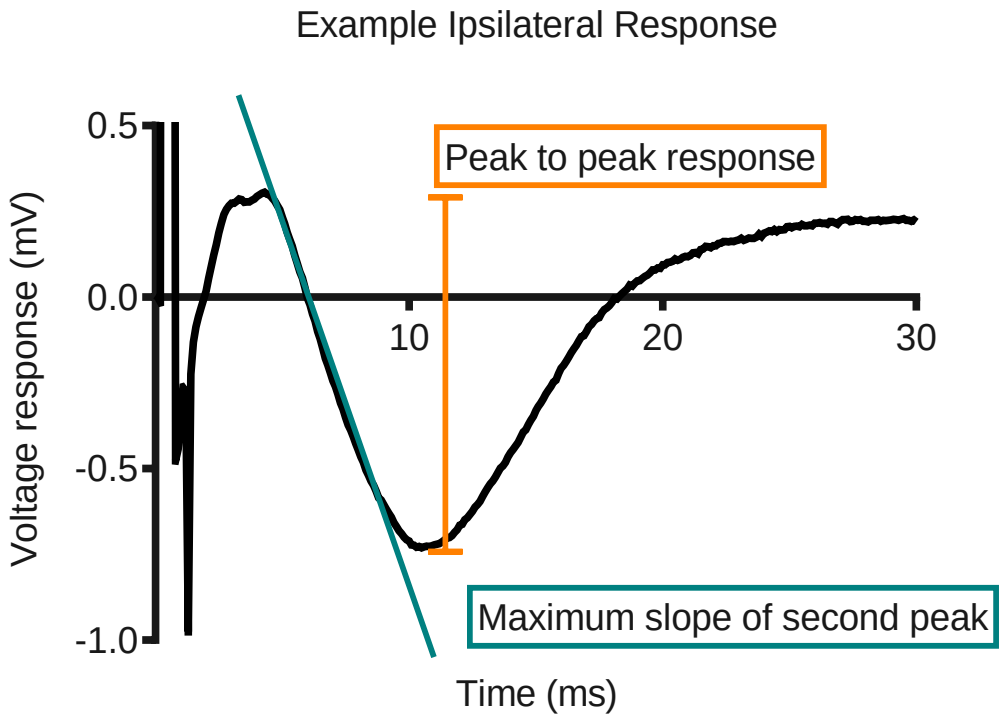

Figure 4.4 Example of 10 averaged ipsilateral responses at test intensity (800 µA) in a single animal. Peak to peak amplitude is represented by the orange line and maximum slope of second peak by the blue line. The initial positive deflection with larger negative deflection is characteristic of responses recorded in this thesis and in the literature describing local field potentials in layer I.

4.4 PROPERTIES OF THE CONTRALATERALLY EVOKED RESPONSE

Bilateral paired pulse recordings of interhemispheric inhibition involve both an ipsilateral 'test' pulse, and a contralateral 'conditioning' pulse. The characteristics of the contralateral pulse measured extracellularly are different to those of the ipsilateral. Recordings of the contralateral pulse have a delayed onset, with a longer, and potentially multi-synapse pathway of current through the corpus callosum. The latency of the contralateral response in the rat has been reported as 4.34 ms ± 1.28 using a comparable technique and animal population to that described in this thesis (Boddington, 2016). The nature of the transcallosal pathway is not fully understood, with different opinions as to whether one or two synapses are involved, and whether these synapses are inhibitory or excitatory in nature (Conti & Manzoni, 1994; Toyama, Matsunami, Ohno, & Tokashiki, 1974). It has been shown that increasing the stimulus intensity delivered to the cortex directly contralateral to the site of recording results in progressively reduced onset latencies (Boddington, 2016; Chang, 1953). This has been hypothesised to be due to the role of multiple synapses within the pathway and/or recruitment of neurons with faster conduction velocities at higher amplitudes.

Anatomical studies of the corpus callosum provide some additional information. Conti and Manzoni (1994) describe the anatomy of the corpus callosum in detail, including the fact that the inputs to the cortex from the contralateral hemisphere are located primarily in layers II/III, with a smaller input to layer V. This hypothesis will be investigated in this chapter, with CSD analyses of the contralaterally-evoked potential used to locate current inputs from the contralateral hemisphere.

The approach to investigation of the contralaterally-evoked response, taking into account the previous literature, is summarised by the following objective and hypotheses:

Objective 1.2 To measure characteristics of single pulse field responses to contralateral stimulation using single wire EEG recording.

- **Hypothesis 1.2.1** That contralaterally evoked responses will demonstrate similar characteristics to those previously reported.
- **Hypothesis 1.2.2** That responses measured from stimulation of the contralateral hemisphere will be observed as a current input to the ipsilateral cortex in layer II/III.

4.4.1 CONTRALATERAL FIELD POTENTIAL RESPONSE

Animals displayed internally consistent responses to contralateral conditioning stimuli. An example of a representative response is given in Figure 4.5. The contralateral field potential showed characteristic waveform characteristics, with some variation between the responses seen in up states versus down states of the synchronized EEG. When averaged across both up and down states, the average latency of onset (for the positive deflection) was 4.21 ± 0.38 ms, n=20. This latency is comparable to that reported previously: a contralaterally evoked response latency of 4.34 ± 1.28 ms was measured in response to 200 μA stimulation using a comparable technique and animal population (Boddington, 2016).

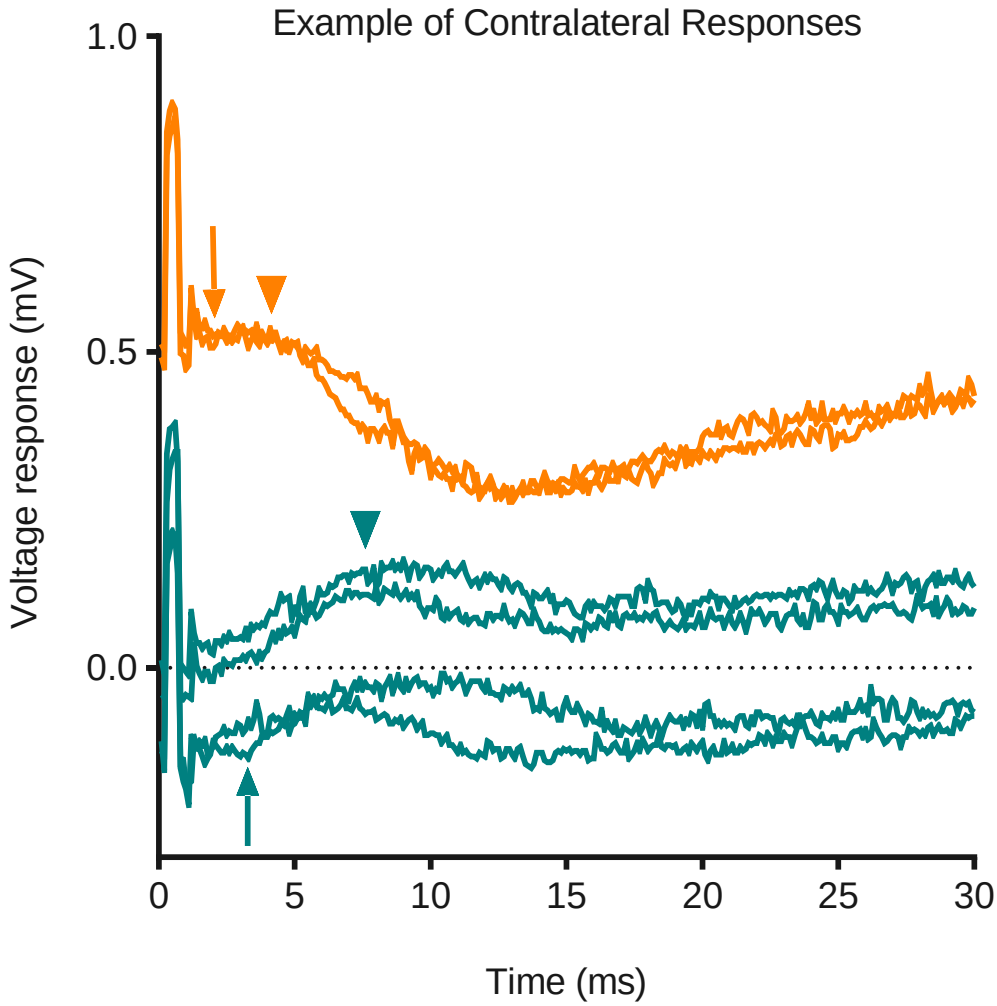

Figure 4.5 Example of six contralateral responses at threshold intensity (150 µA) in a single animal. Characteristic responses are seen for up and down states and are representative of those seen in other animals. Orange arrow shows latency of small positive going response in up states followed by an orange arrowhead showing the latency of the larger negative going response. Teal arrow shows the latency of a large positive going response in down states, with the teal arrowhead showing the longer latency of the negative going response.

Similar to Boddington (2016) and Chang (1953), when stimulation amplitudes were increased, there was a corresponding decrease in onset latency (Figure 4.6). While the significance of this relationship is not understood, it does demonstrate that these experiments are measuring a similar pathway to that previously described.

4.4.2 CONTRALATERAL THRESHOLDS

There was difficulty in determining the threshold level in some cases, owing to noise in the signal, which made small responses difficult to distinguish online. Overall, the threshold at which the first response was seen varied from 50 µA to 300 µA, with the mean intensity being 130 ± 20 µA, n=18. The threshold was most commonly found at 100 µA (6 of 18 animals). This is less than previously reported threshold amplitudes, with prior work finding an average threshold at a stimulation intensity of 215 µA ± 20 (Boddington, 2016). One potential explanation for this is the different anaesthetics employed, with urethane anaesthesia used in the results described here and ketamine-domitor in the Boddington study. The role of different anaesthetics on electrophysiological recordings is explored later in the chapter.

With the exception of minor variations in threshold, the results obtained with electroencephalogram and local field potential recording were similar to those from previous literature. This allows us to accept the hypothesis 1.2.1, “that contralaterally evoked responses will demonstrate similar characteristics to those previously reported.”

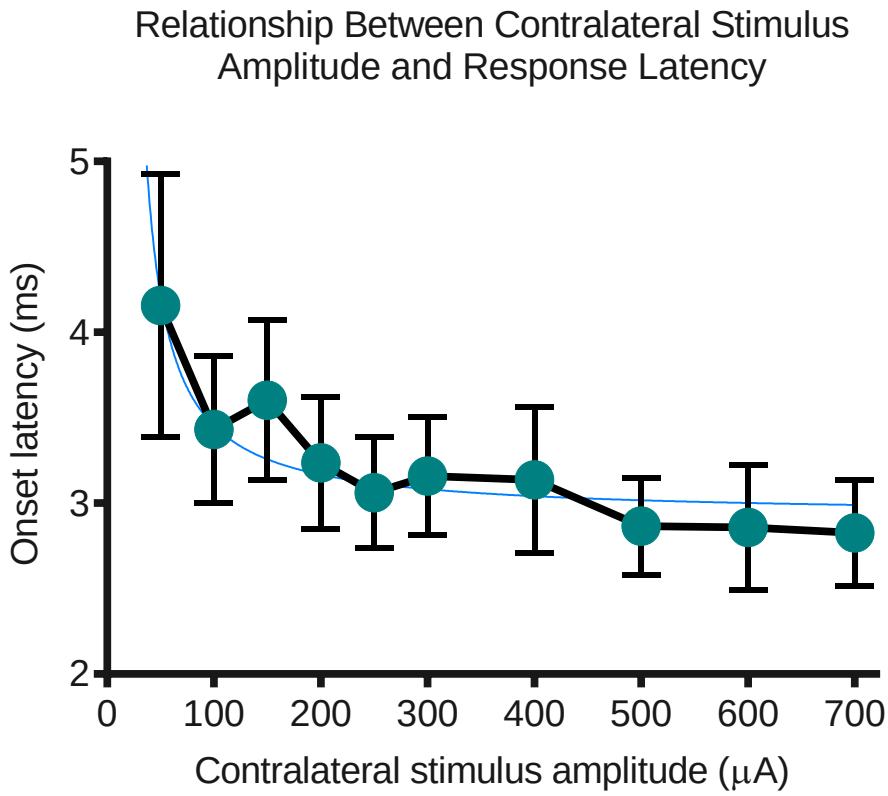

Figure 4.6 Onset latency at different contralateral stimulation intensities. Blue line is a non-linear (hyperbola) line of best fit. Error bars show standard error of the mean. (n=18)

4.4.3 CONTRALATERAL CURRENT SOURCE DENSITY ANALYSIS

Measuring the response to stimulation at increasing depths and performing current source density (CSD) analysis calculations can reveal information about inputs and outputs (termed sinks and sources) to areas of the cortex (Aizenman et al., 1996; Einevoll et al., 2013). This analysis was performed on 13 animals with stimulation at a depth of 1500 μm adjacent to the recording electrode. Results from these experiments are displayed in Figure 4.7. Stimulation of the contralateral side revealed a current sink at a depth of 300 μm, potentially representing contralateral inputs to the dendrites of layers II/III at a latency of 4-5 ms following stimulation. Sources are seen at various deeper depths, with latencies similar to or later than those of the sink at 300 μm depth, which could be presumed to represent responses and outputs following the input to layer II/III. There is also a sink seen at a depth of 1300 μm, potentially representing a smaller callosal input to layer V. These findings correlate with the anatomical descriptions of the corpus callosum by Conti et al. (1994), who describe the predominant inputs from the corpus callosum as entering layer II/III, with a smaller input to layer V. We can therefore accept hypothesis 1.2.2, “that responses measured from stimulation of the contralateral hemisphere will be observed as a current input to the ipsilateral cortex in layer II/III.”

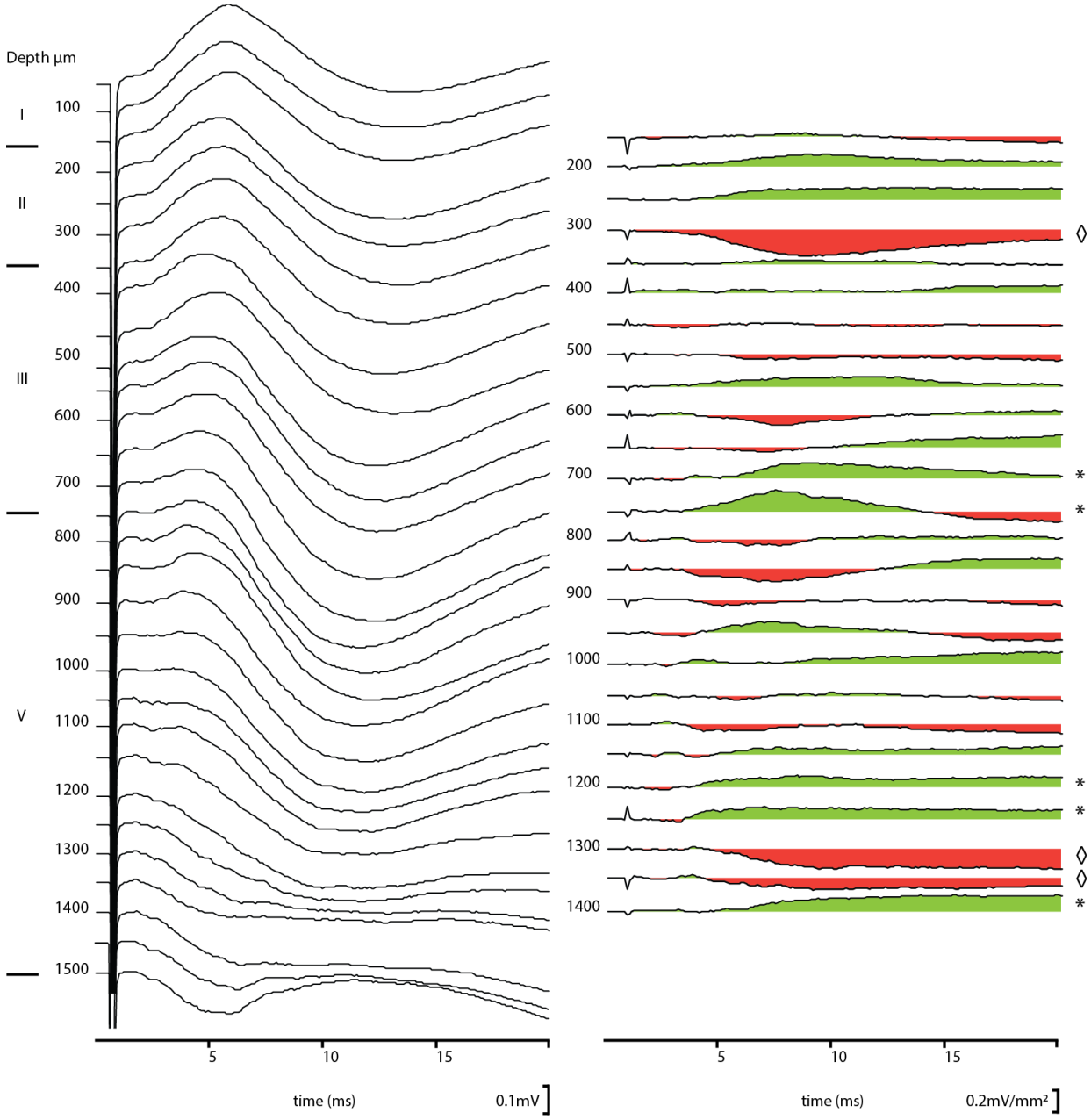

*Figure 4.7 Left: Field potential recordings in response to contralateral stimulation averaged across 13 animals. Right: CSD analysis for the same animals. Layer depths are taken from Aroniadou and Keller (1993). Green areas represent sources and red areas sinks. Sources referred to in the text are marked with *, and sinks mentioned are marked ◊.*

This thesis was limited to electrophysiological studies in intact rat brains and does not include anatomical studies of the corpus callosum. Neither are experiments included in which the corpus callosum is cut or inhibited with GABA inhibitors. We therefore cannot be certain that the inhibition that is measured in this and subsequent chapters is due to a callosal pathway, or for which GABA receptors are primarily responsible. The current source density analysis reported in this chapter do show that the contralateral stimulus activates areas of the cortex corresponding to callosal inputs, at latencies consistent with previously published work on the callosal response. This lends support to the assumption that the contralateral response recorded is callosally mediated.

4.5 PROPERTIES OF IHI IN THE RAT

When contralateral and ipsilateral pulses are paired, investigations of IHI can be made. Previous investigations of IHI in the rat using intracellular recordings have identified certain characteristics. The latency of inhibition has been reported to occur (in rats) at interstimulus intervals of between 3.8 and 12.8 ms (Barry, Boddington, Igelström, et al., 2014; Boddington, 2016), see Figure 4.8. The degree of inhibition reported is fairly similar across reports of intracellularly recorded IHI in rats, one study reporting 17.2% ± 1.5; n=12 (Barry, Boddington, Igelström, et al., 2014) and another 15.3% ± 2.1; n=8 (Boddington, 2016). One key observation of Barry, Boddington, Igelström, et al. (2014) was that only low intensity conditioning stimuli were able to elicit IHI. High intensity conditioning pulses resulted in either facilitation or no effect on the test response. Thus, two objectives and hypotheses were designed, aiming to replicate the results of these earlier studies with minimally invasive techniques.

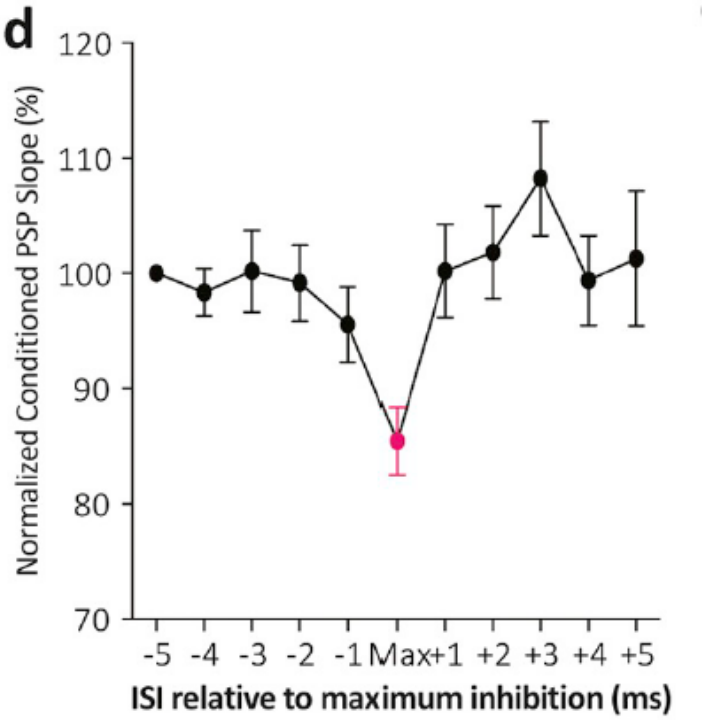

Figure 4.8 Interhemispheric inhibition measured with normalized conditioned post synaptic potential slope (%) for all sham treated cells with inter-stimulus intervals aligned to the interval where the normalized slope was most reduced. From Boddington et al. (2020)

Objective 1.3 To record interhemispheric inhibition using paired ipsilateral and contralateral pulses.

- **Hypothesis 1.3:** That a conditioning pulse delivered to the contralateral hemisphere prior to the measurement of the response to an ipsilateral 'test' pulse will reduce the amplitude of that response at a latency consistent with transcallosal inhibition, and to a degree consistent with previously reported literature.

Objective 1.4 To measure the effect of varying the intensity of contralateral conditioning stimulation on the degree of interhemispheric inhibition measured.

- **Hypothesis 1.4:** That conditioning pulses of low intensities will reduce the size of the test response to a greater degree than high intensity conditioning pulses.

4.5.1 MEASUREMENT OF INTERHEMISPHERIC INHIBITION

Interhemispheric inhibition was measured by pairing a contralateral stimulus with an ipsilateral stimulus delivered at specific interstimulus intervals and comparing the response to that of ipsilateral stimulation alone, thus, determining the effect of the contralateral hemisphere on the response in the ipsilateral recording hemisphere. Most animals showed an inhibition of the ipsilateral response when conditioning intensities at or below threshold were applied prior. Inhibition was not seen when higher than threshold conditioning intensities were used. In general, at threshold conditioning had an overall inhibitory effect, whereas suprathreshold stimulation had an overall faciliatory effect. In most animals, an interstimulus interval (ISI)

where this effect was more pronounced could be identified, and this was labelled as the point of maximum inhibition. An example from a single animal is shown in Figure 4.9.

Several intensities were selected for investigating the effects of changing conditioning pulse intensities on the crossed cortico-cortical pathway. These were equivalent to 50%, 100%, 150% and 300% of the threshold intensity. These four intensities are referred to as subthreshold, threshold, suprathreshold and high intensity conditioning stimuli, respectively. These were chosen to give a range of intensities above and below a set level (threshold) so that effects could be more easily compared between animals. In this chapter, results with threshold and suprathreshold conditioning stimuli are given, with the remainder reported in Appendix 8 (page 285). The stimulation intensities used for each animal are given in Appendix 6 (page 281).

4.5.2 LATENCY OF INHIBITION

As the latency of the contralaterally-evoked response was on average 4.21 ± 0.38 ms, it is unlikely that any inhibition measured with conditioning stimulation at an ISI of less than 4 ms was due to the conditioning stimulation itself. Therefore, only responses inhibited by stimuli at ISIs of 4 ms or greater were used for measurement of the maximum IHI for that animal. This can be summarised by saying that the point of maximum inhibition for each animal must be consistent with the expected latency of a crossed response: ≥4 ms. Similar criteria have been applied in previous work (Boddington et al., 2020).

Inhibition was very time sensitive and tended to only occur at one or two interstimulus intervals per animal. The average ISI (≥4 ms) at which the maximum inhibitory effect occurred was 7.2

± 0.3 ms, n=17, determined from aligning the point of maximum inhibition of the slope of the response; Figure 4.9 and Figure 4.10. This represents the latency of interhemispheric inhibition, and is consistent with the range of ISIs reported in previous work measuring IHI using intracellular recording techniques in the rat (Barry, Boddington, Igelström, et al., 2014; Boddington, 2016).

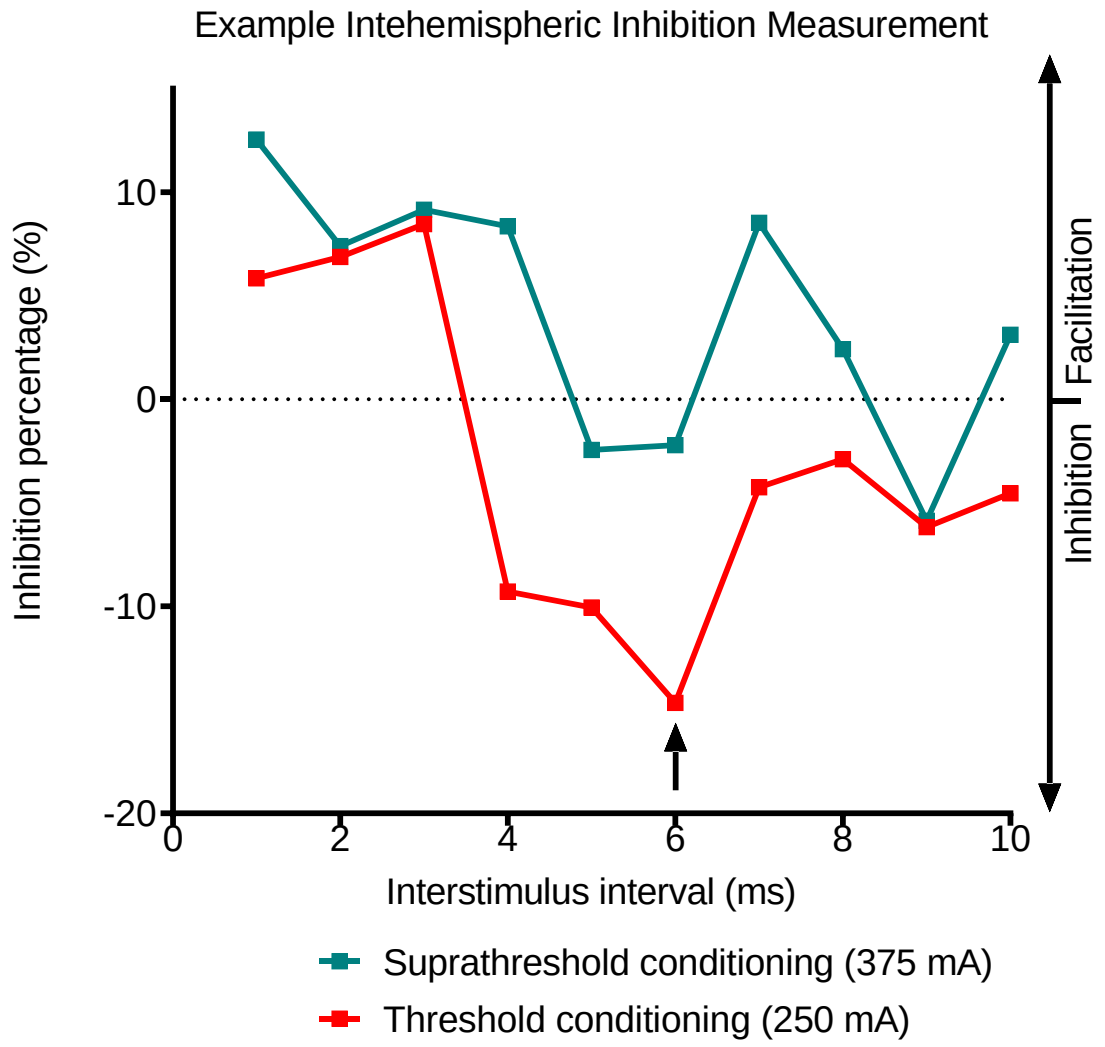

Figure 4.9 Maximum slope of the second peak of the local field potential, at four conditioning intensities, expressed as a percentage of the mean non-conditioned response. The conditioning pulse was delivered using MC_Stimulus. Example interhemispheric inhibition in a single animal. There was high inter-experiment variation in results, however this experiment is representative of the effects seen in the group as a whole. The maximum inhibitory effect can be seen at an interstimulus interval (ISI) of 6 ms (arrow). No inhibition is seen with suprathreshold conditioning.

4.5.3 MEASURING MAXIMUM SLOPE TO DETERMINE INHIBITION

At threshold conditioning intensity, at the point of maximum inhibition for each animal, the maximum slope of the test response was inhibited by 9.2% ± 2.6; n=17 as compared with the non-conditioned response. This was after normalising to a linear regression fitted to the five data points central to the interstimulus interval where there was maximum inhibition. Table 4.1 shows both raw and normalised values. At suprathreshold conditioning intensity (at mean 155% of threshold), the conditioning stimulus induced no inhibition of the ipsilateral response: 0.5 ± 4.4%, n=16, post normalisation. The normalised results are displayed in Figure 4.10.

A paired t-test was used to compare the effect of threshold and suprathreshold conditioning stimulation on the maximum inhibition of the test response. The raw values were used, not those normalised to linear regression, because the normalisation protocol was applied to the averaged results, not to each animal individually. There was a significant difference in the effect of threshold stimulation and suprathreshold stimulation $t(15) = 3.7$, $p = 0.002$. See Table 4.1 for a full list of results. Similar results were seen when peak to peak amplitude was used to measure inhibition. These results are given in Appendix 7 (page 282). Results from additional conditioning intensities are given in Appendix 8 (page 285).

	Threshold	Suprathreshold
Change in the maximum slope - raw result (%)	Inhibition: 3.1 ± 2.6, n=17 *	Facilitation: 9.2 ± 4.4, n=16 *
Change in the maximum slope - normalised result (%)	Inhibition: 9.2 ± 2.6, n=17	Facilitation: 0.5 ± 4.4, n=16

*Table 4.1 IHI measured with bilateral paired pulse investigations using the MC_Stimulus system. Shaded boxes indicate that the test response was inhibited. A paired t test was conducted between the results marked * and revealed a statistically significant difference.*

The different effect of high and low conditioning pulses in eliciting inhibition was characterised by Barry, Boddington, Igelström, et al. (2014), and this chapter demonstrates that this result is able to be replicated using the minimally invasive techniques developed for this thesis. These results relate to hypothesis 1.4, "that conditioning pulses of low intensities will reduce the size of the test response to a greater degree than high intensity conditioning pulses." This also indicates that the inhibitory pathway activated by the contralateral conditioning stimulation is of a similar low threshold to that previously described. This becomes important in subsequent chapters when this threshold level is used to determine the optimal amplitude for stimulation of the awake, stroke lesioned animal.

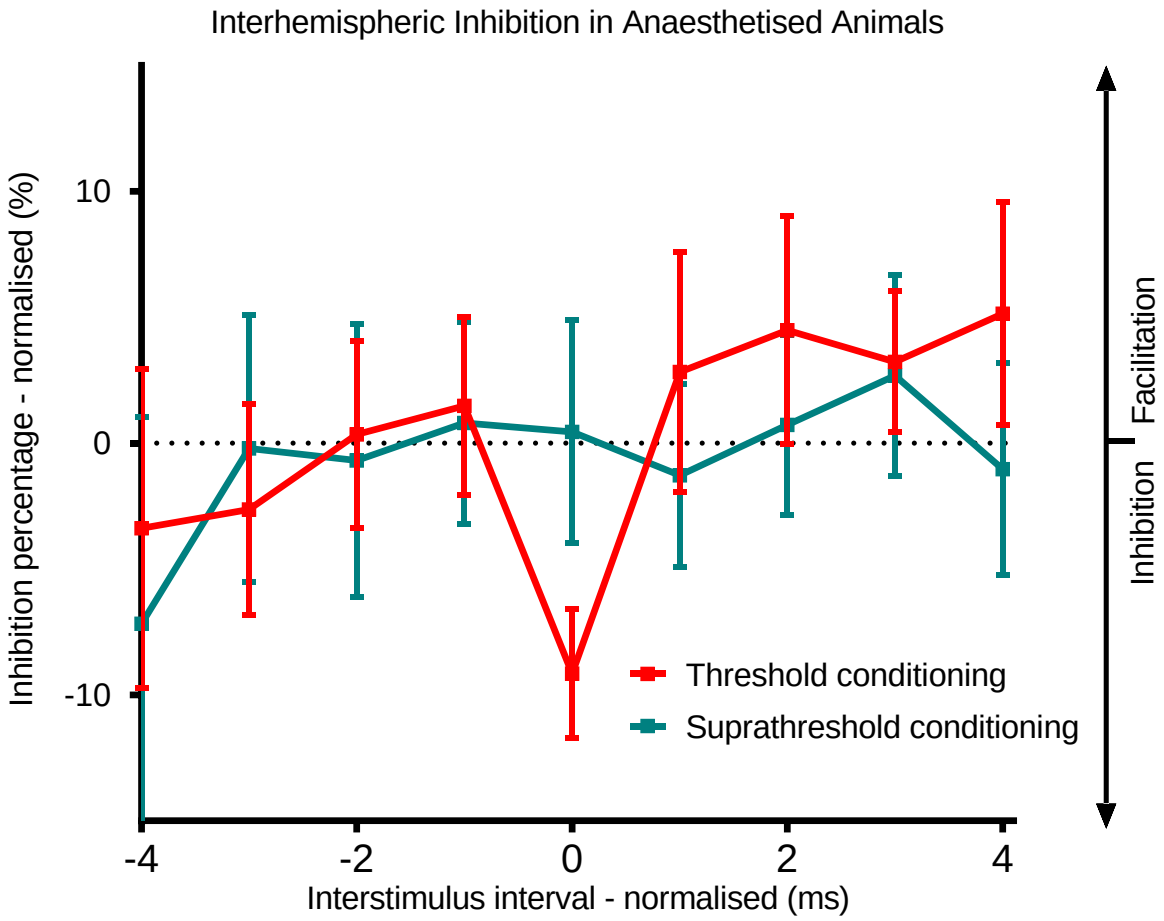

Figure 4.10 Mean values of the maximum slope of the second peak of the local field potential, at threshold (n=17) and suprathreshold (n=16) conditioning intensities, expressed as a percentage of the mean non-conditioned response. Data are aligned at x=0 to the interstimulus interval (ISI) corresponding to the maximum inhibitory effect for each animal, where inhibition is seen with threshold, but not suprathreshold conditioning. Data are normalised to a linear regression of the central data points. The conditioning pulse was delivered using MC_Stimulus. Error bars display standard error of the mean.

4.5.4 NOTE ON INTERPRETATION OF INTERHEMISPHERIC INHIBITION MEASUREMENTS

Unfortunately, there is no anatomical or pharmacological evidence of the precise neuronal pathway that is being stimulated in any of these experiments, nor in those conducted by Barry, Boddington, Igelström, et al. (2014) and Boddington et al. (2020). There is therefore no certainty that each is measuring the callosal interhemispheric inhibitory pathway. It should be pointed out therefore, that where the term interhemispheric inhibition is used in this thesis, this should be cautiously interpreted as 'putative interhemispheric inhibition'.

4.6 MODULATION OF INTERHEMISPHERIC INHIBITION USING THETA BURST STIMULATION

As has been discussed in Chapter 2, Barry, Boddington, Igelström, et al. (2014) have previously shown that low intensity intermittent theta burst stimulation eliminates interhemispheric inhibition in the anaesthetised rat. Additional work by Boddington (2016) showed that a low intensity dose of continuous TBS (cTBS) delivered chronically to stroke lesioned rats increased interhemispheric inhibition as compared with sham or iTBS. It was therefore hypothesised that delivering iTBS to an anaesthetised rat would result in a decrease in IHI, and that cTBS would result in an increase.

Objective 1.5 To measure the acute effect of intermittent TBS and continuous TBS on IHI in a naïve, anaesthetized animal, using minimally invasive recordings.

- **Hypothesis 1.5:** That iTBS will acutely reduce, and cTBS acutely increase IHI.

In 25 animals, following recording of IHI, the Prodigy IPG was used to deliver either sham (n=8), iTBS (n=8) or cTBS (n=9) stimulation to urethane anaesthetised animals. The full description of this process can be found in the methods. Once stimulation had been delivered and IHI measured again, no discernible effects on IHI could be detected, either by measuring the maximum slope of the response, or by measuring the peak to peak amplitude. Results are displayed raw in Figure 4.11. Raw results are reported, i.e. not aligned to the linear regression, as alignment did not reveal any previously masked changes.

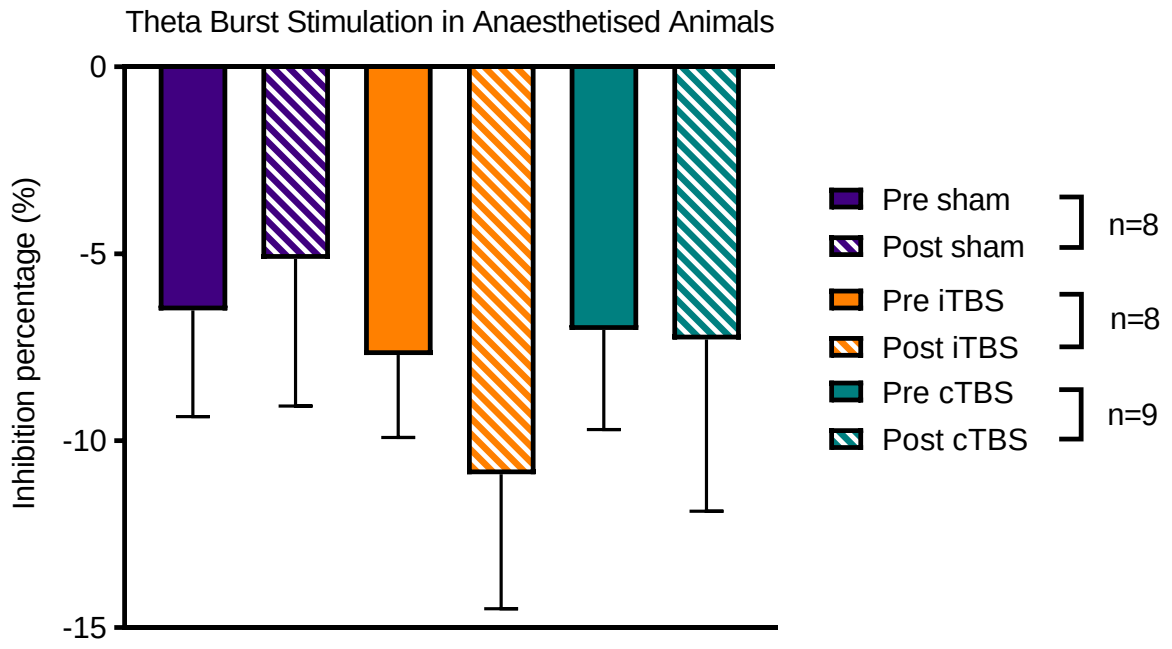

Figure 4.11 Percentage inhibition of the maximum slope of the second peak of the ipsilateral response, both before and after three types of stimulation (sham, iTBS and cTBS), raw results are given. No significant differences are seen. Error bars display standard error of the mean.

Hypothesis 1.5 was not borne out by the experiments reported here. This is in contrast with initial studies by Barry, Boddington, Igelström, et al. (2014) who showed that iTBS could eliminate IHI in the anaesthetised animal. One possible explanation for this discrepancy is that the intensity of stimulation used in this study was lower than that used by Barry et al, where a 'stimulation intensity just below that which induced a post synaptic potential result' was used. Owing to the limitations of the Prodigy IPG, the TBS in the experiments conducted here could only be delivered in amplitudes at 50 μA increments, which meant that 'just below threshold' was 50 μA lower than threshold. It is plausible that the intensity of stimulation delivered during the experiments reported in this chapter was too low to produce the modulatory effect on IHI observed by Barry et al. Further investigation is therefore needed to determine the optimal stimulation intensity for alteration of IHI, particularly in the anaesthetised animal.

A second explanation is that the higher variability of the extradural recordings used in these experiments meant that small variations in IHI were unable to be detected. In later chapters, experiments in awake animals are reported where the delivery of theta burst stimulation was not made using the IPG and where changes over time within individual animals receiving stimulation were recorded, increasing the accuracy and sensitivity of the methods.

4.7 ALTERATIONS OF THE LOCAL FIELD POTENTIAL UNDER ANAESTHESIA

The different effects of various anaesthetics on neuronal responses have been reported previously in multiple publications (Hara & Harris, 2002; Hayton, Kriss, & Muller, 1999; L.

Huang, Bai, Zhao, & Xiao, 2013). Urethane has pleiotropic effects, with small effects on multiple neurotransmitter gated ion channels (Hara & Harris, 2002). This means that recordings made under urethane anaesthesia are theoretically closer to physiological normal as compared with an anaesthetic agent with a large effect on a single neurotransmitter. Compare this to ketamine, with its major action against N-methyl-D-aspartate (NMDA) receptors. This has a marked effect on recordings of neuronal responses, for example ketamine has been shown to increase response latencies as compared with urethane or other anaesthetics (Hayton et al., 1999; L. Huang et al., 2013). It is rarely used for electrophysiological studies owing to its short duration of effect and its effects on the neurons being recorded.

It would not be unreasonable to assume that IHI would be altered based on the type of anaesthesia used. However, with no way to predict what the alteration to IHI would be, given the limited research into short latency IHI in rats, the null hypothesis is given.

Objective 1.6 To compare interhemispheric inhibition measured under urethane anaesthesia with that measured under ketamine-domitor anaesthesia.

- **Hypothesis 1.6** That there will be no difference between IHI measured under urethane and that measured under ketamine-domitor anaesthesia.

4.7.1 INTERHEMISPHERIC INHIBITION RESULTS UNDER ANAESTHESIA

The inability to detect changes in interhemispheric inhibition during TBS with the described invasive recording techniques could be due to the role of anaesthesia. To examine the potential

effect of anaesthesia on measured interhemispheric inhibition, a series of IHI measurements were made under two types of anaesthesia.

The previously reported measurements of IHI made under urethane anaesthesia, were compared with measurements made at the time of electrode implantation in animals that would later undergo stroke induction surgery. This second group was anaesthetised using a ketamine-domitor mix. Raw results showed a greater degree of inhibition in the ketamine-domitor anaesthetised animals: compare 24.1 ± 4.3%, n=41 inhibition with 1.8 ± 2.8%, n=15 inhibition in the urethane anaesthetised group, see Figure 4.12. Once normalised to a linear regression, this difference reduced, with inhibition of 15.0 ± 4.3%, n=41 % in the ketamine-domitor group and 8.3 ± 2.8%, n=15 % in the urethane anaesthetised group. An unpaired t-test comparison of the raw level of inhibition measured under the two types of anaesthesia was made, using Welch's correction to account for unequal variances. The difference between the groups was statistically significant, $t(56) = 4.2$, $p < 0.0001$, however the normalised difference was non-significant, $t(56) = 0.84$, $p = 0.40$, see Figure 4.12 and consistent with the given null hypothesis.

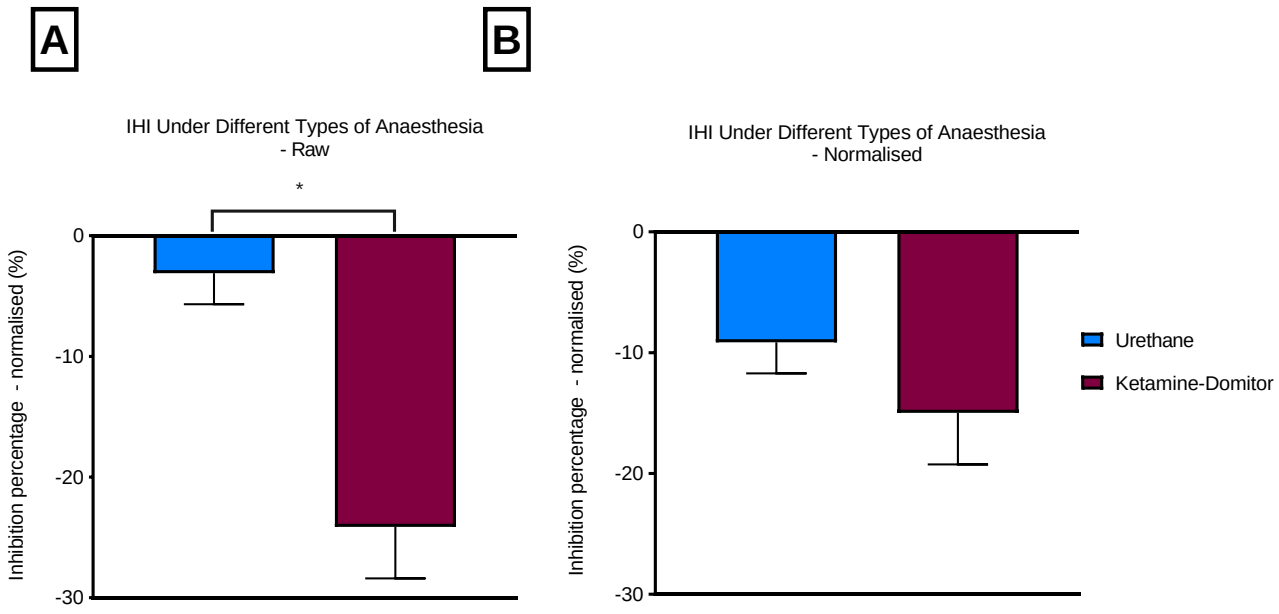

Figure 4.12 Interhemispheric inhibition under either urethane or ketamine-domitor anaesthesia. A: Raw results demonstrating a significantly higher level of inhibition in the ketamine anaesthetised group, B: Results normalised to a linear regression, where there is no statistically significant difference. Error bars display standard error of the mean.

Given that urethane typically increases inhibitory responses, it is somewhat unexpected that IHI was detected at a greater level in ketamine anaesthetised rats (L. Huang et al., 2013). This may be due to the multiple inhibitory mechanisms of action of urethane (as opposed to the NMDA antagonist action of ketamine), which may inhibit the neural responses to such an extent that any additional inhibition from the transcallosal pathway is minimal. It will therefore be prudent to consider results obtained under different types of anaesthesia (or awake) in isolation. The results reported in awake animals in subsequent chapters will likely be a better reflection on the 'normal' levels of IHI operating within the rat, as the modulatory effect of anaesthesia is not present.

4.8 MEASURING CHANGES IN INTERHEMISPHERIC INHIBITION DUE TO STROKE

In Chapter 2, the evidence for and against the presence of an enhanced interhemispheric inhibitory effect following stroke is considered. It is hypothesised that the recovery process will involve unbalanced interhemispheric competition, and rats will therefore demonstrate an increased IHI following stroke lesion. As a preliminary investigation, IHI was compared in two separate groups of animals, one group before stroke lesioning and another group measured one week after lesioning. In these animals IHI was measured under anaesthesia, as opposed to the measurements of IHI in awake animals that are in Chapter 5.

Comparing the raw inhibition percentage in these two groups showed a non-significant difference in inhibition: 24.1 ± 4.3%, n=41 in the group measured prior to stroke and 9.7 ± 5.9%, n=17 in the group measured after stroke, see Figure 4.13. There was no significant

difference between the groups when investigated with an unpaired t-test; t(56)= 1.88, p=0.065. Following alignment to linear regression, the level of inhibition in the two groups becomes similar, with 15.0% in the pre-stroke group, and 14.0% in the post stroke group.

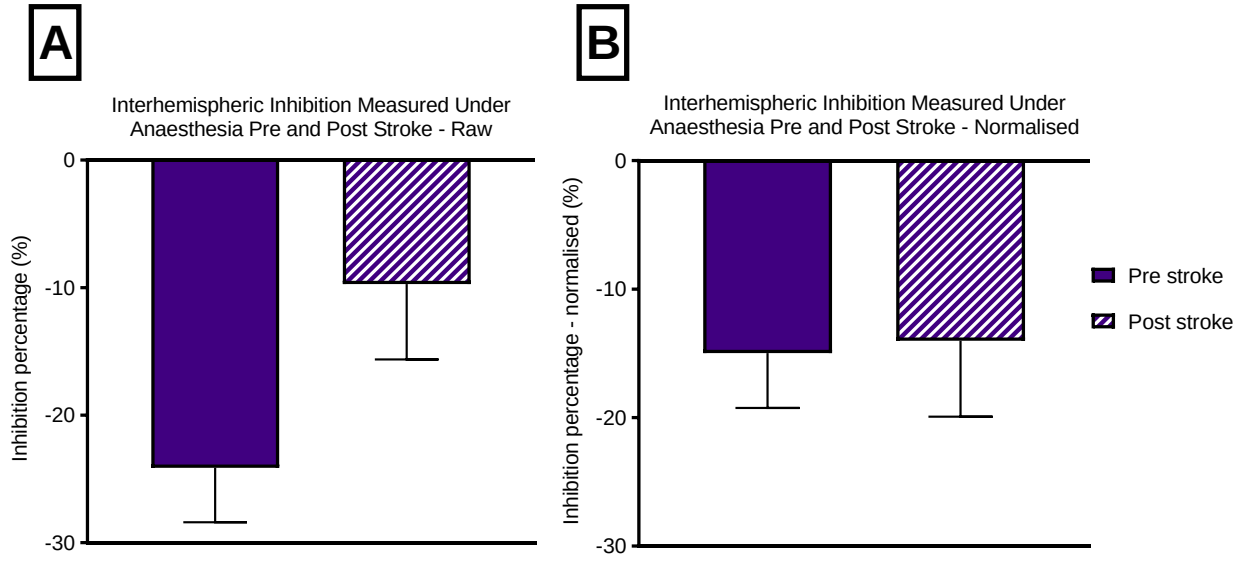

Figure 4.13 Interhemispheric inhibition in two groups of animals, before and after stroke induction, and measured under ketamine-domitor anaesthesia. A:Raw results, B: results normalised to a linear regression. There is no statistically significant difference between the two groups Error bars display standard error of the mean.

As mentioned earlier, ketamine-domitor is not the most reliable anaesthetic for conducting electrophysiological recordings, so perhaps this could explain this lack of measurable effect from stroke. In addition, IHI was measured in two different groups of animals, with inter-individual differences in baseline IHI. This weakness in experimental design also exists within the current body of literature investigating IHI following stroke, as there is no way to predict who will have a stroke and to measure IHI prior to this event. Previous animal work has used non-survival intracellular recordings, so again experiments were not able to be repeated with the same animal. These factors are addressed in the experiments described in the next chapter.

A final explanation is that in human trials where an increase in interhemispheric inhibition was demonstrated following stroke, IHI was measured during movement initiation with the studied limb (Duque et al., 2005; Murase et al., 2004). When measured in the limb at rest or under sustained contraction, alterations in IHI following stroke were not always found (Bütefisch et al., 2008; Stinear et al., 2015). The recordings reported here took place with the rat's limb at rest, under anaesthesia. In Chapter 5, pre-movement IHI was not measured, but IHI was measured in a freely moving animal, which should be more physiologically relevant than IHI measured under anaesthesia.

4.9 SUMMARY AND CONCLUSIONS

The results outlined in this chapter demonstrate that the techniques of minimally invasive recording of interhemispheric inhibition outlined in Chapter 3 produce results comparable to those previously reported in the literature. There are also internal consistencies within the

results, for example when comparing the trends seen when using the MC_stimulus system with those gained by using the Prodigy IPG in Appendix 1 (page 264).

The results reported in this chapter satisfy Objective 1, by demonstrating that a method of measuring IHI using minimally invasive techniques can produce results consistent with those previously reported in related literature. Now that this has been demonstrated, these techniques will be further adapted in the subsequent two chapters for use in the awake, freely moving animal. Our results regarding iTBS and cTBS in the anaesthetised animal and the effects of anaesthesia itself on IHI are areas to be followed up in the ensuing experiments in awake animals. We also found that comparison of separate populations of animals before and after stroke does not reveal changes in IHI, hence the need to measure within the same individual animals to determine any effect of stroke on IHI.

5 RESULTS: EFFECTS OF ENDOTHELIN-1 LESION ON INTERHEMISPHERIC INHIBITION AND BEHAVIOUR IN RATS

5.1 INTRODUCTION

In the previous chapter a method for measuring interhemispheric inhibition in a minimally invasive fashion was evaluated. In this chapter, results obtained from using this method in the context of a small volume stroke lesion will be outlined. These results will be supplemented with behavioural and histological results from the same animals.

Interhemispheric inhibition in stroke has been previously investigated in both animals and humans, however changes over time have been more challenging to explore in animals owing to the use of one-time-only intracellular recording, or the absence of a pre stroke baseline in human participants. In this chapter the main objective to be addressed is Objective 2.

Objective 2: To determine whether behavioural deficits after stroke are associated with an alteration of interhemispheric inhibition.

This will be explored using data relating to four different measures: interhemispheric inhibition, grid walking performance, pasta handling characteristics and histological analysis of lesion size.

5.1.1 REVIEW OF LITERATURE PERTINENT TO THE CHAPTER

To place the following results in the body of existing literature, the relevant prior research will be discussed in brief, directing attention to those studies directly related to the methods employed in this chapter. The specific objectives and hypotheses which relate the literature to the main objective, Objective 2, will also be given. The relevant results will then be given, followed by discussion of the findings.

5.1.2 ENDOTHELIN-1 INDUCED STROKE LESION

The method of stroke induction employed in this study is the endothelin-1 model. The reasons for choosing this model are given in Chapter 2. In brief, these relate to the similarity between the mechanism by which endothelin-1 produces ischaemia (namely vasoconstriction) and the common pathogenesis of stroke in human patients (Hughes et al., 2003; Saggu, 2013; Sommer, 2017). The main drawback from using this technique is the heterogeneity of the lesions produced (Fluri et al., 2015; Gilmour et al., 2004; Gilmour et al., 2005; Windle et al., 2006), although it should be noted that this is consistent with the heterogeneity seen in stroke survivors.

This technique was described in detail in 2006 (Windle et al.), and the method used in this study was largely adapted from their work. The methodology is described in Chapter 3. It was designed to produce a small volume lesion targeting both primary motor cortex and subcortical structures. The injections used in this study were:

- Two injections of 800 pmol endothelin-1, one cortical and one subcortical, totalling 1600 pmol, at stereotaxic coordinates of AP +1.0 mm, ML +2.7 mm, DV −1.5 and -5 mm, to the left hemisphere.

Based on the results described by Windle et al. the lesions were expected to have a volume of approximately 86 to 96 mm^3, based on the lesion volumes produced by injections with comparable methodology to that employed here (see Table 1 from Windle et al. (2006) and Figure 3.4 in this thesis). Note that Windle reported a large degree of variability with this method. Other studies using smaller endothelin-1 volumes (120-400 pmol) reported smaller lesion volumes of between one and 12.5 mm^3, again with a large degree of variability both within and between papers (Adkins et al., 2004; Allred et al., 2008; Blasi, Whalen, & Ayata, 2015; Fang et al., 2010; Gilmour et al., 2004). One paper commented that the lesions were particularly focal (Blasi et al., 2015), and it seemed from interpretation of the studies that the number of injection locations had an effect on eventual lesion volume as well as the volume of endothelin-1 injected.

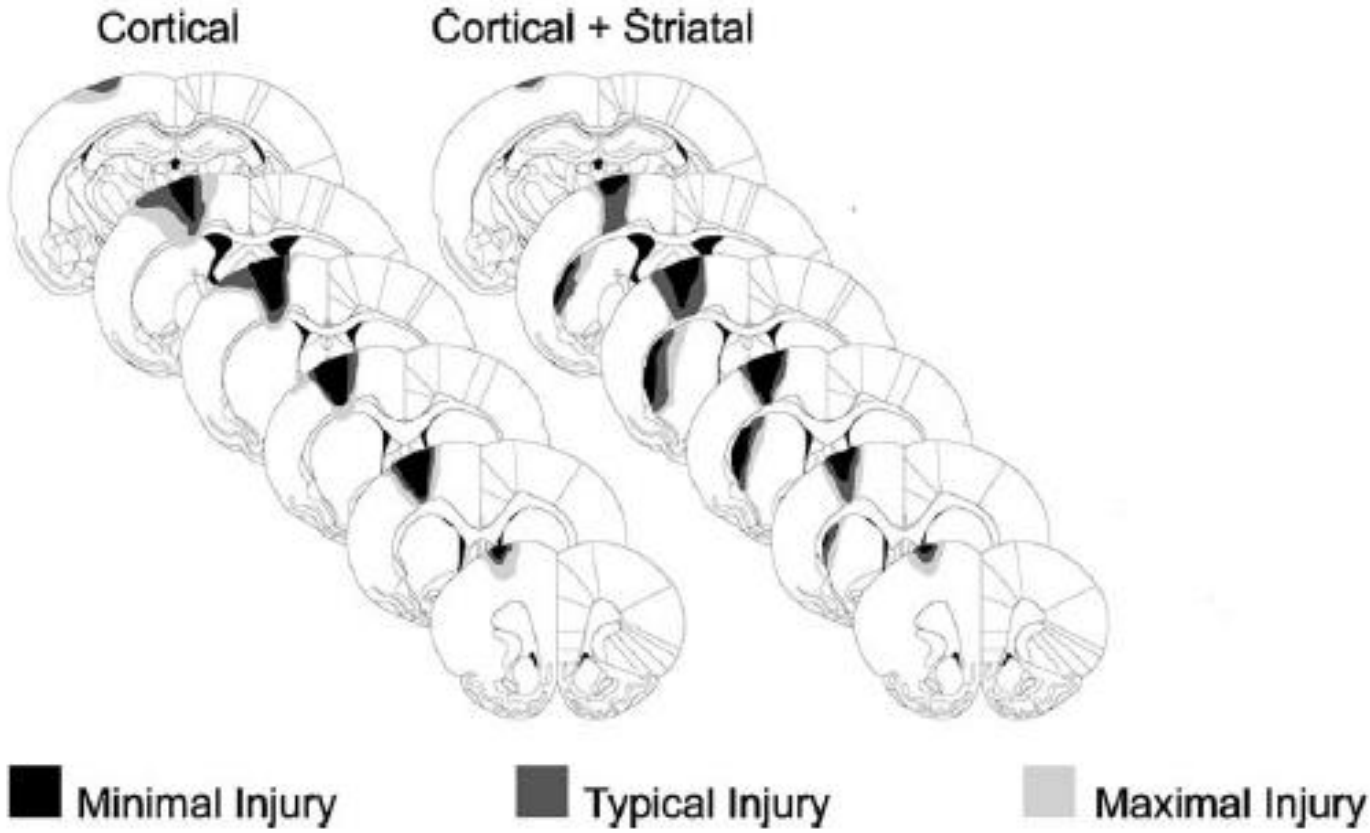

Figure 5.1 Representative illustrations of typical, minimal and maximal injury areas for two endothelin-1 injection methods. Adapted from Windle et al. (2006).

Windle et al. (2006) also reported the number of deaths and success rate with the endothelin-1 injection technique. In those animals lesioned with comparable parameters to those used here, they described a 70-75% survival rate, and 67-70% of survivors showed behavioural deficit. With the alteration of coordinates to avoid bleeding into ventricles, as described in Chapter 3, the survival rates were increased in this study.

Objective 2.1 To characterise lesions (volume and location) produced with endothelin 1.

- **Hypothesis 2.1** That lesion volumes and locations demonstrate consistency across animals within the study, and are consistent with that expected from pre-existing literature, with a higher survival rate.

5.1.3 GRID WALKING TASK

As a more modern method of stroke lesion induction, limited information exists as to the behavioural results that would be expected with different endothelin-1 lesion protocols. Four studies were found which used the grid walking task to assess performance following endothelin-1 lesion (Adkins et al., 2004; Blasi et al., 2015; Fang et al., 2010; Gilmour et al., 2005). Each used a smaller endothelin-1 volume than that reported by Windle et al. (120-400 pmol per animal). Where lesion volumes were reported, they were significantly smaller than those measured by Windle, and were estimated from the figures presented to be in the range of 1-12.5 mm^3 per animal (Adkins et al., 2004; Blasi et al., 2015; Fang et al., 2010; Fuxe et al., 1997; Gilmour et al., 2005). The lesion locations also varied between studies, targeting the cortex with topical administration (Adkins et al., 2004), targeting the cortex with local injections (Fang et al., 2010; Gilmour et al., 2005) or targeting the white matter with local

injections (Blasi et al., 2015). Studies which produced large lesion volumes by targeting the middle cerebral artery were not used as reference.

In addition to the heterogeneity of methodology in these studies, they also reported results differently, making it difficult to interpret the degree of impairment that can be expected in the present study. Taking the two studies that are more easily compared, one would predict an approximately 4-6% increase in foot faults in the contralesional limb in first week following stroke as a percentage of all steps taken (Blasi et al., 2015; Gilmour et al., 2005).

Criteria for determining impairment were also based loosely on that previously used in a photothrombotic lesion model by Boddington et al. (2020). However, owing to the intrinsically higher variability observed with the endothelin-1 model, criteria were less restrictive. In this thesis an 8% threshold, as used by (Barry, Boddington, Igelström, et al., 2014), rather than 9% as used by Boddington et al. was chosen.

Grid walking exclusion criteria:

1. Animals were excluded that showed baseline contralesional foot faults of ≥8 percent of all steps taken.
2. Animals were excluded that did not show a deficit of ≥8 percent contralesional foot faults following stroke.
3. Animals were excluded that did not show an increase in the number of foot faults from baseline, prior to randomisation to stimulation group.

Objective 2.2 To quantify grid-walking performance before and after stroke lesion induction.

- **Hypothesis 2.2** That animals will demonstrate a significant impairment in grid walking performance in the contralesional limb following stroke induction, and that this degree of impairment will be comparable to that reported previously using endothelin-1 lesions.

5.1.4 PASTA HANDLING TASK

The method for this task was described in detail in Chapter 3. In brief, rats were trained to eat lengths of spaghetti pasta and the number of adjustments made with each paw as the pasta was eaten was counted. In addition, the rat chose a "grasp" paw which was held at the base of the pasta, and a "guide" paw which was closer to the mouth, and this preference was noted. Finally, any abnormal movements, such as dropping the pasta, were recorded.

This task has been previously used in conjunction with the endothelin-1 model, but the literature contains only one detailed account of this (Allred et al., 2008). This paper describes the changes in pasta handling behaviour following small volume (11.5 ± 0.9 mm^3) lesions to the sensorimotor cortex using 200 pmol of topically applied endothelin-1. The figure in which these results were described is reproduced below (Figure 5.2).

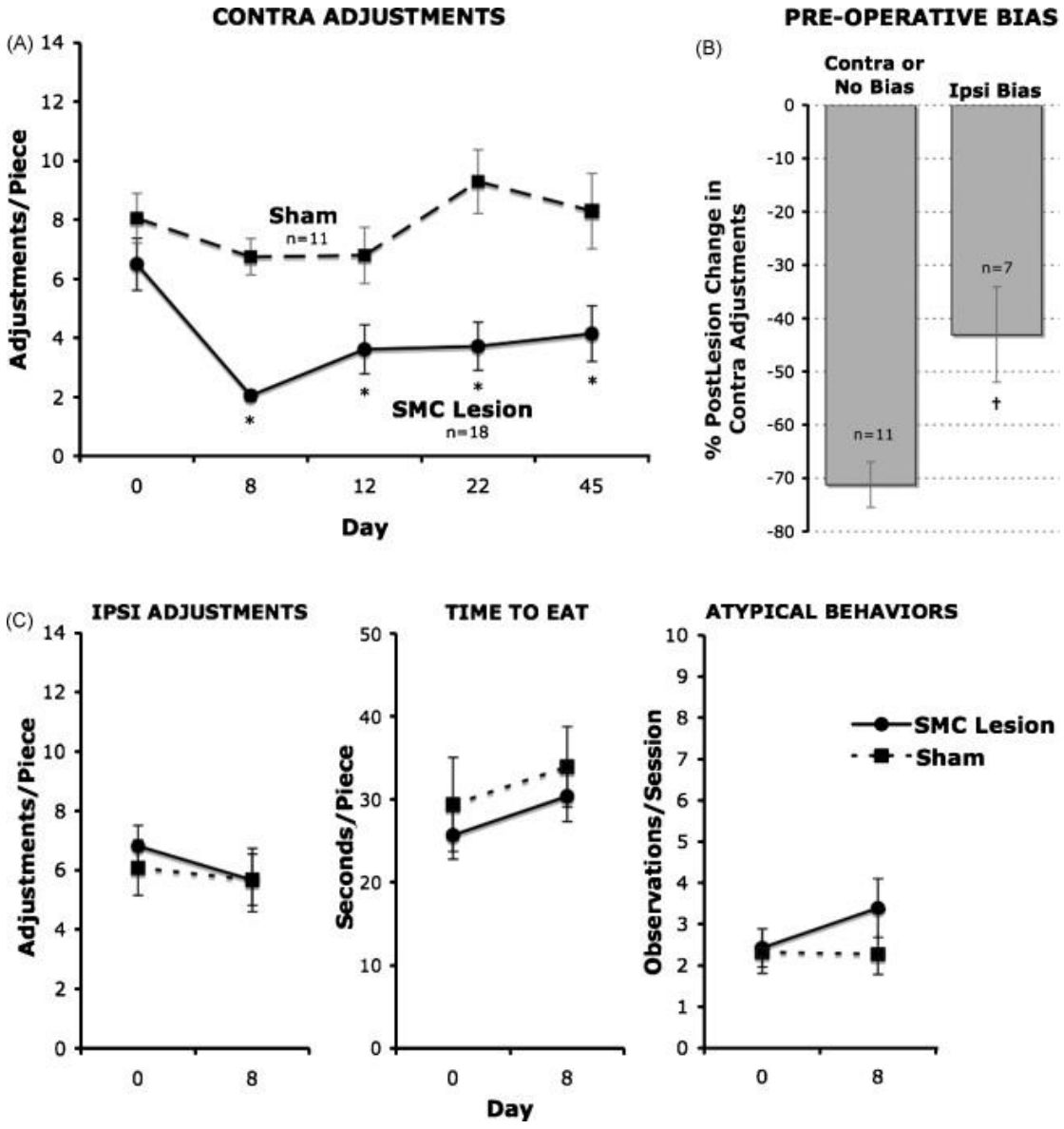

Figure 5.2 Ischemic sensorimotor cortex lesion effects on [pasta] handling. (A) Adjustments made with the contralateral forepaw decreased compared to sham-operated animals and this endured throughout the 45-day post-lesion period of testing. Day 0 is the pre-operative time point. $^{*}P < 0.05$ significantly different from sham. (B) The magnitude of the post-lesion change in adjustments varied with endogenous asymmetries. Lesion effects in contralateral adjustments were less sensitively detected in rats that preferred to use the unimpaired forelimb for adjustments prior to the lesion (ipsilateral bias subgroup). $^{\dagger}P < 0.05$ significantly different from contralateral/no bias subgroup. (C) Adjustments with the ipsilateral forelimb, time to eat and atypical behaviours were not significantly changed after the lesion. Copied without alteration from Allred et al. (2008)

In Allred's study, rats with endothelin-1 lesions showed a significant decline in the number of adjustments made with the contralesional (impaired) paw during eating compared to sham-operated animals, with no change in the number of ipsilateral movements. Grasp paw preference did not change following lesioning and neither did atypical behaviours.

Objective 2.3 To characterise pasta handling behaviour before and after stroke lesion induction and compare the alterations following stroke with that previously reported for similar lesions.

- **Hypothesis 2.3.1** That animals will show a significant reduction in the use of the contralesional limb to manipulate the pasta for eating following stroke, consistent with that previously reported.

- **Hypothesis 2.3.2** That animals will show a similar number of abnormal eating movements following lesioning as compared to prior lesioning, consistent with previously reported data for similar lesions.

5.1.5 THE EFFECT OF STROKE ON INTERHEMISPHERIC INHIBITION

The literature relating to the changes in interhemispheric inhibition following stroke are discussed in Chapter 2, with the conclusion that stroke lesions, especially those of small volume, tend to produce an increase in the level of inhibition from the 'healthy' to the lesioned hemisphere. The timeline for this change is not clear; studies that measure IHI from human stroke patients are predominantly in the chronic phase of stroke (Duque et al., 2005; Murase et al., 2004). The majority of rodent studies that show changes in excitability following stroke

tend to measure only within the sub-acute and early chronic phase (Adkins et al., 2008; Buchkremer-Ratzmann & Witte, 1997; Mohajerani et al., 2011; Shimizu et al., 2002; Vallone et al., 2016).

Spalletti et al. (2017) showed an increase in $GABA_B$ mediated transcallosal inhibition at both five and thirty days after stroke in mice. Literature comparing the timeline of pathology following stroke in rats with that in humans is sparse. This makes it difficult to predict when an increase in IHI would occur in rats, given that it tends to be measured in the subacute-chronic phase in humans, and that some changes suggesting interhemispheric imbalance have been detected in the acute and sub-acute phase in mice.

The results presented in this chapter aim to address this gap in the rodent literature. The hypothesis was made that IHI would increase in comparison to baseline when measured in the two weeks following stroke. This was based on a few assumptions. One can assume that rats recover from small stroke lesions in a similar way to humans, based on the interhemispheric competition model that is described in Chapter 2, and so will show an increase in contralesional inhibition when the competing inhibitory influence from the ipsilateral lesioned area is removed.

One can also assume that the timeline of stroke recovery is more rapidly occurring in rats than in humans. This is based on the degree of spontaneous motor recovery that occurs in rats within the first two weeks following stroke, in the absence of any rehabilitation training (Adkins et al., 2004; Gilmour et al., 2004; Hernandez & Schallert, 1988; Karthikeyan et al., 2018; Kleim et al., 2007; Schaar et al., 2010). In addition, electrophysiological and fMRI studies compared

between rodent and human work, show that the sub-acute period 9-23 days after stroke in rodents is somewhat equivalent to one to three months after stroke in humans (Kleim et al., 2007; Vallone et al., 2016; Ward, Brown, Thompson, & Frackowiak, 2003; Zeiler & Krakauer, 2013). Early changes in IHI are therefore predicted to be detectable with bilateral paired pulse recordings in the first fortnight following stroke in rats, in the subacute recovery phase (Spalletti et al., 2017; Vallone et al., 2016).

Objective 2.4 To measure interhemispheric inhibition in the awake rat prior to stroke induction and compare with the same measure at two timepoints one and two weeks after lesioning.

- **Hypothesis 2.4** That interhemispheric inhibition will increase at one- and two-weeks following stroke lesioning compared to that measured at baseline.

5.1.6 RELATIONSHIPS BETWEEN THE ABOVE MEASURES

As each of the measures described thus far in this chapter were all assessed in the same group of animals over the same three-week period, any potential relationships between the results can be investigated. Predictions as to what each of these relationships may show were made based on existing literature.

The relationship between interhemispheric inhibition and behavioural measures has been investigated previously in this and other labs. Interhemispheric inhibition has been shown to be linked with coordination, and people who train to perform coordinated skills have lesser

degrees of IHI overall (de Manzano & Ullen, 2017; Ridding et al., 2000). More recent data from our lab have shown a more direct link between electrophysiological changes and functional improvement in the context of stroke. Boddington et al. (2020) found in rats that increased IHI was independently associated with lower post synaptic potential (PSP) amplitude (a proxy for cellular excitability), measured with single cell recordings around the stroke area. They then found that lower PSP amplitude was associated with worse functional recovery from stroke, as measured with the grid walking task described in Chapter 3. When IHI and functional recovery were directly compared however, a significant association was not found. This was based on a single measure of IHI taken during a terminal recording experiment after the animal had completed all behavioural tasks. The work in this thesis was designed to address this missing link by measuring IHI throughout the behavioural experiment, and the prediction is that a greater degree of inhibition towards the paretic (right) limb would be reflected in an increased number of faults made by that limb.

Objective 2.5 To analyse the relationships between IHI, behavioural impairment and lesion volume.

- **Hypothesis 2.5.1** That greater interhemispheric inhibition onto the lesioned hemisphere will be associated with greater foot fault impairment.

The pasta handling task has never previously been used in the context of measurements of IHI, but predictions can be made by extrapolating from experiments involving related concepts. Typically, worse fine motor performance is thought to be associated with greater inhibition from the hemisphere opposite to that controlling the movement (Xu et al., 2019). One example of this is the difference in fine motor skill between the dominant and non-dominant hands. As

the pasta handling task is a measure of fine motor performance in the rat, greater inhibition could potentially result in greater impairment.

- **Hypothesis 2.5.2** That greater interhemispheric inhibition onto the lesioned hemisphere will be associated with greater preference of the non-paretic limb in making pasta adjustments during eating.

Lesion volume would seem to be an important predictor of behavioural deficit, and indeed this seems to be the case for human patients (M. Corbetta et al., 2015; Forkert et al., 2015). Importantly however, the volume of the lesion is less important than which structures are affected. In the rat, research from the traumatic brain injury field does suggest that greater lesion volume is associated with a greater number of foot faults (Talley Watts et al., 2014; Xiong et al., 2011). Other studies modelling foot faults following induction of a photothrombotic lesion have found no such link (Boddington et al., 2020). The traumatic brain injury literature is used as the basis for the following hypothesis:

- **Hypothesis 2.5.3** That greater lesion volume will be associated with greater foot fault impairment.

A relationship between lesion volume and pasta handling behaviour has not been shown in earlier work (Allred et al., 2008). This will act as the basis for the following hypothesis:

- **Hypothesis 2.5.4** That greater lesion volume will not be associated with a change in pasta handling asymmetry.

It is not possible to predict whether lesion volume will be associated with either higher or lower IHI based on the existing literature (or rather lack thereof). As all lesions induced in the present study are small volume, recovery via the interhemispheric competition model would be predicted for all animals, with no effect of small variations in lesion volume on inhibition.

- **Hypothesis 2.5.5** That greater lesion volume will not be associated with alteration in interhemispheric inhibition.

5.2 SUMMARY OF ANIMALS USED

Three animals were used under AEC protocol 06/16 to establish the endothelin-1 injection technique in this lab. Nine animals were used to establish the experimental model and endothelin-1 injection coordinates for this thesis, under AEC protocol 78/16. Three of these animals died immediately following stroke induction, with autopsy demonstrating bleeding into the lateral ventricles. When injection coordinates were shifted posterio-laterally (to AP +1.0 mm, ML +2.7 mm), and the striatal depth increased (to 5.0 mm), the rate of deaths reduced dramatically. These changes were made with the aim of avoiding the ventricles while still targeting M1 and the underlying striatum (Paxinos & Watson, 2007).

See Figure 3. 1 for a summary of animal numbers. Animal losses were due to stroke induction (four animals), and five other deaths occurred related to anaesthesia or surgery. Six additional animals exited the study at earlier timepoints than planned due to issues with the implanted electrode. See Figure 2. 7 for a visual timeline of experiments.

5.3 HISTOLOGY RESULTS

Histology was performed on animals from which behavioural or electrophysiological data was available (n=50). Analysis focussed only on those animals that were not excluded based on a lack of grid walk impairment (n=31). Average lesion volume was 9.5 ± 1.3 mm^3 and incorporated both a cortical and striatal component. Slices also showed an overall decrease in hemisphere volume and increase in ventricle volume which was not quantified. Two examples of typical lesions are shown in Figure 5.3.

Average electrode placement was 0.1 ± 0.1 mm medial and 0.3 ± 0.1 mm deep to its target on the right, and 0.2 ± 0.1 mm lateral and 0.3 ± 0.1 mm deep to its target on the left. Coordinates were often obscured by the presence of the stroke lesion on the left, so fewer data were available. Coordinates for individual animals are shown in Figure 5.4.

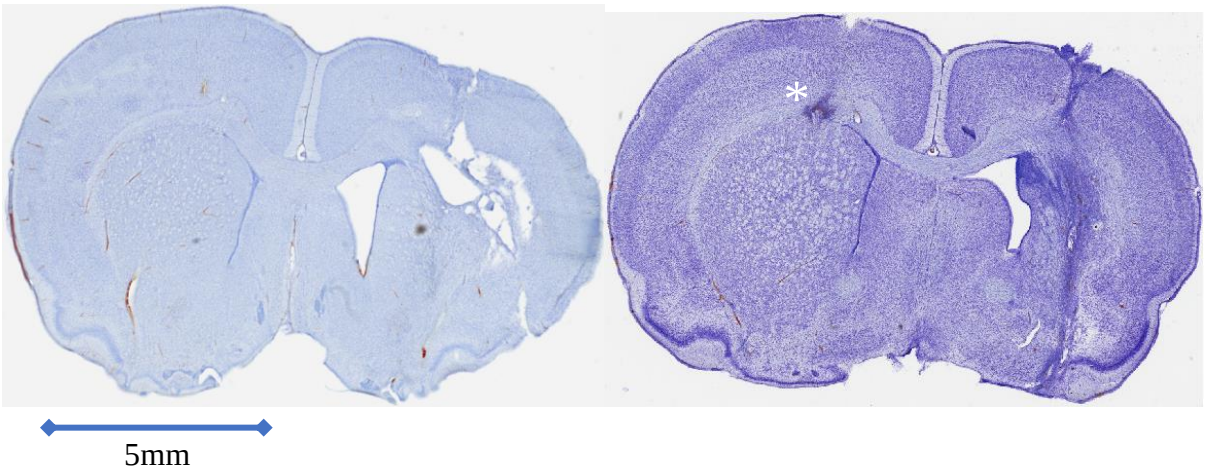

Figure 5.3 Examples of lesions from two experimental animals. The right side of the image corresponds to the left hemisphere and vice versa. Cortical and striatal components of the lesion are clearly seen in the left hemisphere of both examples. The image on the right also shows the location of the contralateral conditioning electrode within the right hemisphere (*).

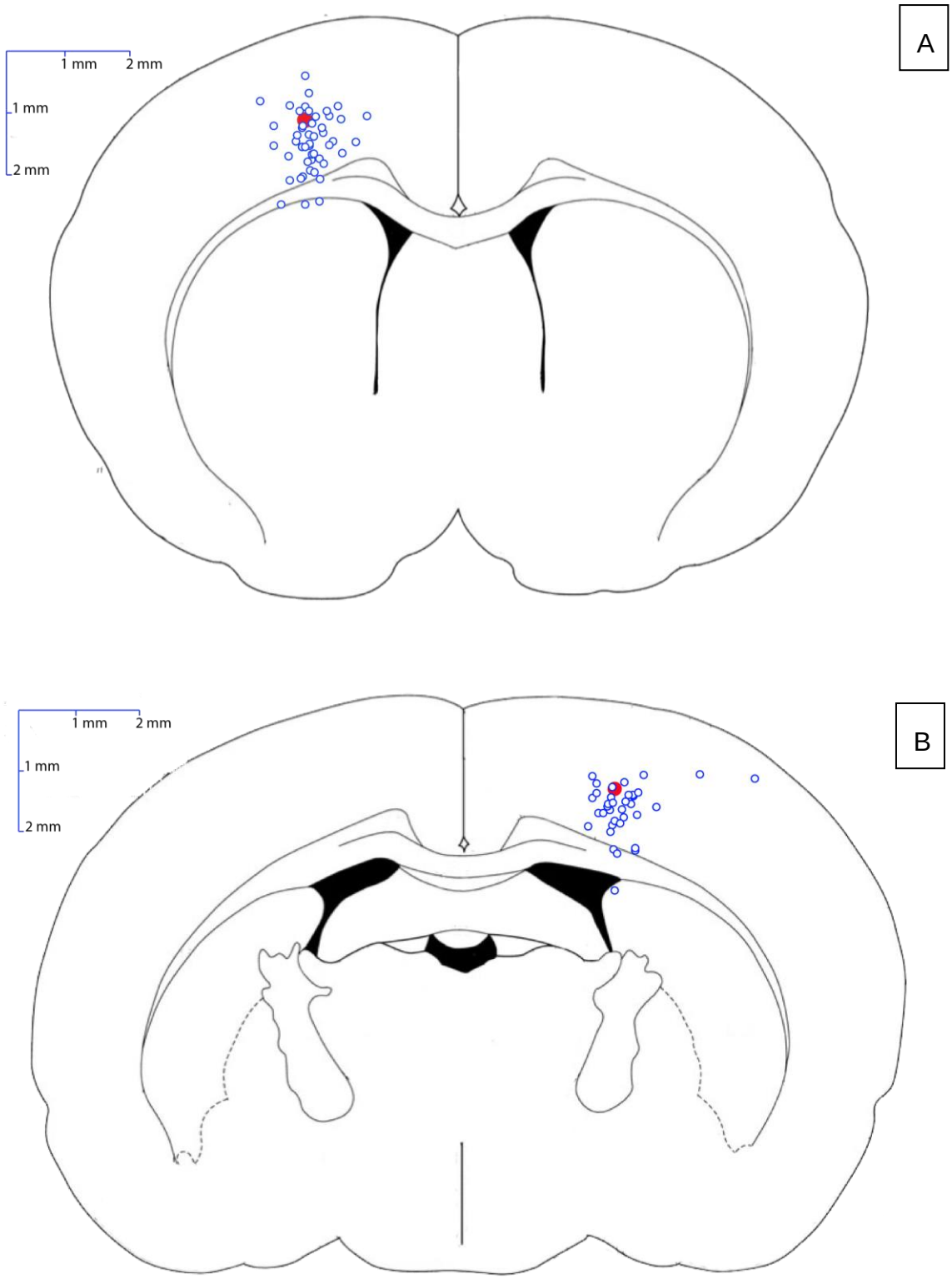

Figure 5.4 Coordinate locations for A: the right, conditioning electrodes on an image of the rat brain in the coronal plane at 0 mm from bregma and B: the left, test electrodes on an image of the rat brain in the coronal plane at 1.5 mm posterior to bregma. Red dots show target location. Background image from Paxinos and Watson (2007),

5.4 GRID WALK DEFICIT FOLLOWING STROKE

There were 60 animals for which grid walking measures were obtained before and after lesion induction. Animals were excluded if they were determined not to have had successful stroke induction, according to the criteria described in the introduction to this chapter. Fourteen were excluded for having a baseline foot fault rate >8%, eleven based on not meeting the 8% threshold for impairment, and four as they did not show an increase in foot faults prior to randomisation. Based on these criteria, 31 animals remained for analysis. A further five had missing data (defined as having baseline data missing), so were excluded from a one-way ANOVA, leaving a total of 26.

When results for these remaining animals were investigated with a one-way repeated measures ANOVA, a significant relationship between time and contralesional grid walking deficit was seen, [$F(1.6, 41) = 20$, $p<0.0001$]. Animals had a mean baseline percentage of foot faults in the right limb of 3.9% ± 0.4, which increased to 9.5% ± 1.0 in the first week following lesion induction and 7.0% ± 0.7 a week later. This is shown in Figure 5.5. The greatest degree of impairment was seen at week one following stroke, with some spontaneous recovery seen in week two. Even at week two, multiple comparisons analysis revealed a significant difference from baseline ($p=0.0001$). The average degree of impairment was 5.6 ± 1.0 more faults per 100 steps taken compared with baseline.

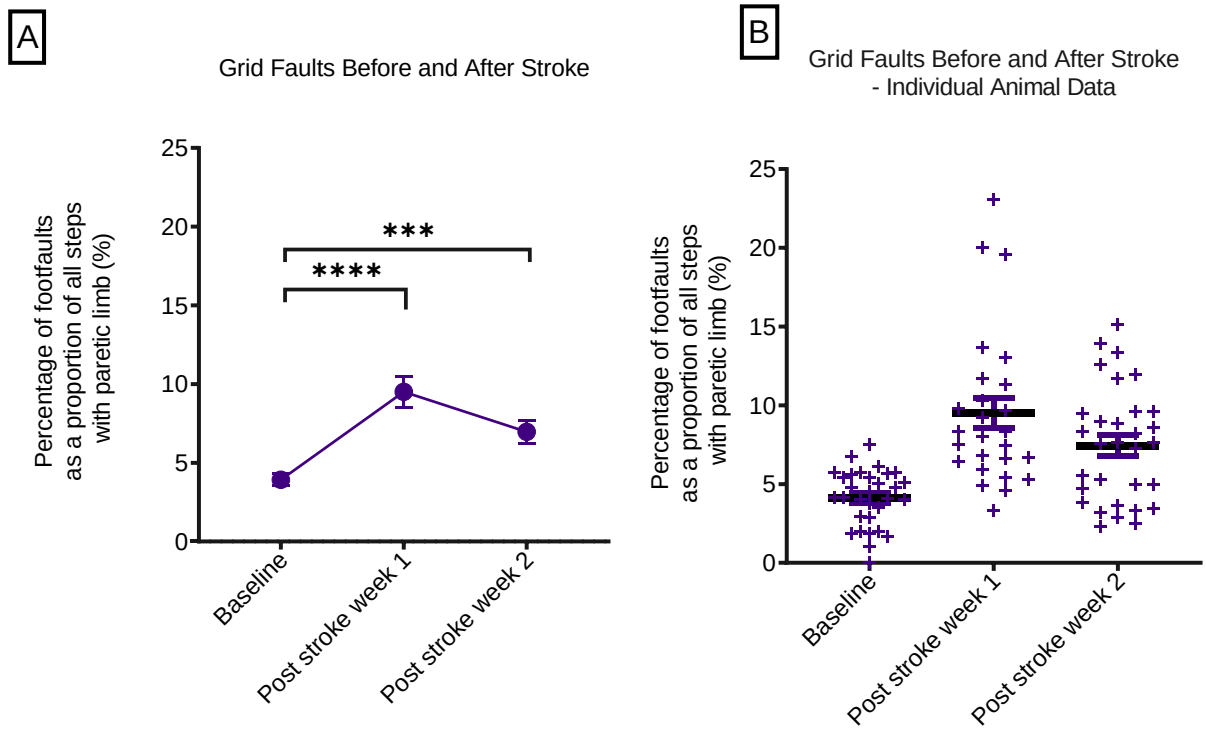

Figure 5.5 A: Percentage of foot faults as a proportion of all steps taken with the paretic limb, comparing baseline with post lesion week one and week two. Animals without complete data set excluded. Differences from baseline were statistically significant: $p<0.0001$ (****) and $p=0.0001$ (***). B: Data for individual animals, no exclusions for missing data (n=31). Error bars demonstrate mean ± SEM.

5.5 PASTA HANDLING BEHAVIOUR FOLLOWING STROKE

5.5.1 USE OF PARETIC PAW FOR ADJUSTING PASTA FOLLOWING STROKE

Fifty-four animals were observed and videoed eating pasta both before and after stroke induction. Data were missing in six animals, leaving 48 for analysis. As this task has not been as extensively studied in the past as the grid walk behavioural measure, and was new to this lab, no results were excluded based on suspected failure of lesion induction. Nevertheless, all but six animals showed a reduction in the use of the right paretic paw following lesioning on raw measures. Following calculation of the mean number of paw adjustments across all the pieces eaten on the day of testing, data are presented as a percentage of paw adjustments made with the contralesional, paretic paw. The result is normalised to zero by subtraction of 50, so that zero represents an equal number of adjustments made with the right and left paws, and any number greater than this shows a preference for the right, paretic paw. The following equation was used to calculate this result:

$$100\,\frac{mean\ number\ of\ adjustments\ made\ with\ contralesional\ paw}{mean\ total\ number\ of\ adjustments\ made\ with\ either\ paw} - 50$$

$$= normalised\ proportion\ of\ adjustments\ made\ with\ the\ contralesional\ paw$$

A one-way ANOVA of all 48 animals with a complete data set showed a significant relationship between time and use of the right paw, with a significant reduction in the use of the right paw following lesioning [$F(1.9, 91) = 5.1$, $p=0.0087$]. Animals had a mean baseline percentage of right limb use of 0.39% ± 1.7 (i.e. no preference). This decreased to negative 4.9% ± 2.0 in the first week following lesion induction and negative 4.5% ± 2.1 a week later, with negative values denoting unlesioned paw preference. See Figure 5.6 for graphic representation of these data.

Previous literature describing changes in pasta handling paw preference following stroke relied upon the absolute number of paretic paw adjustments and did not express this as a proportion of all paw movements made. The issue with using an absolute measure in the present study is the lack of an un-lesioned control, so that any effects of time alone cannot be evaluated, such as a task practice effect on total number of adjustments with either paw. For completeness, a graph showing the effect of stroke lesion on the number of paretic movements per piece eaten is given in Figure 5.7, where the same trends are seen.

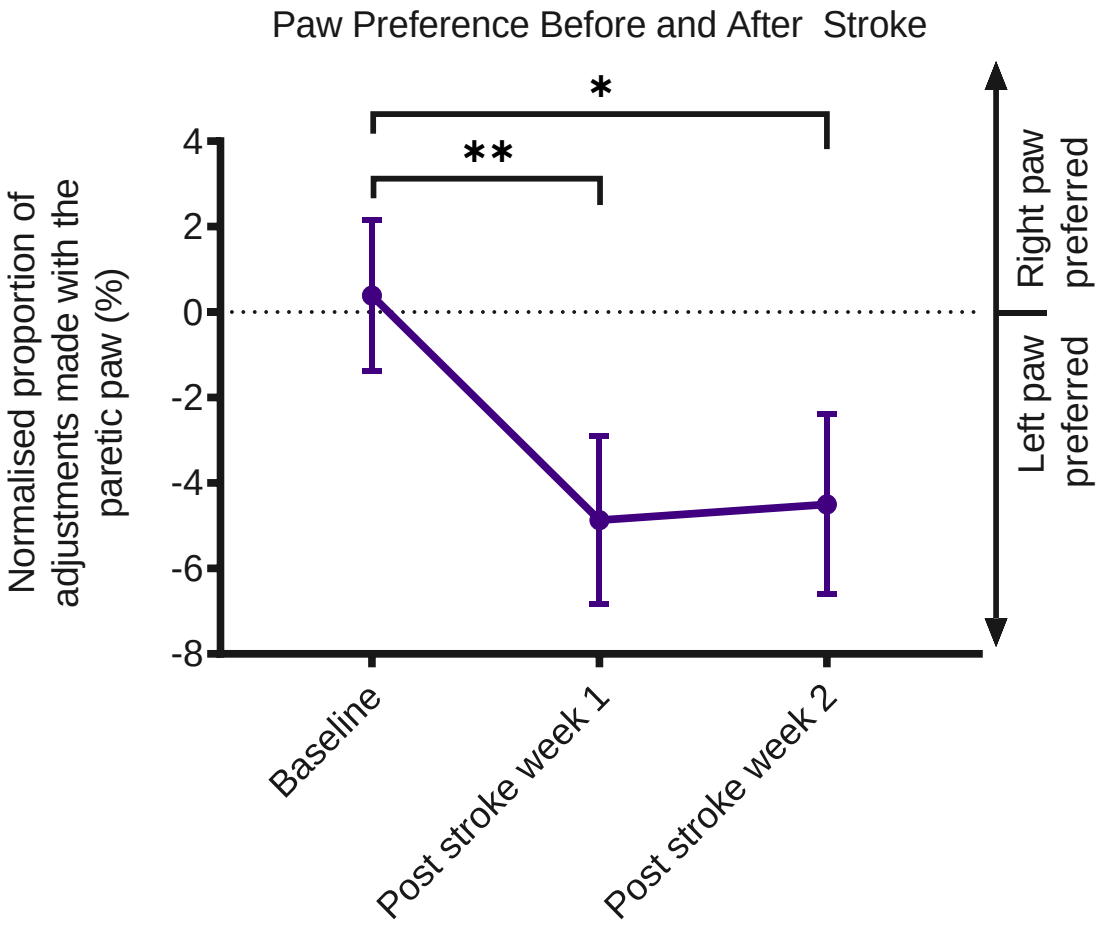

Figure 5.6 Normalised paretic paw preference following stroke induction, comparing baseline with post lesion week one and week two. Positive values represent right (paretic) paw preference and negative values represent left paw preference. Differences from baseline were statistically significant: p=0.0050 (**) and p=0.025 (*). Error bars demonstrate mean ± SEM.

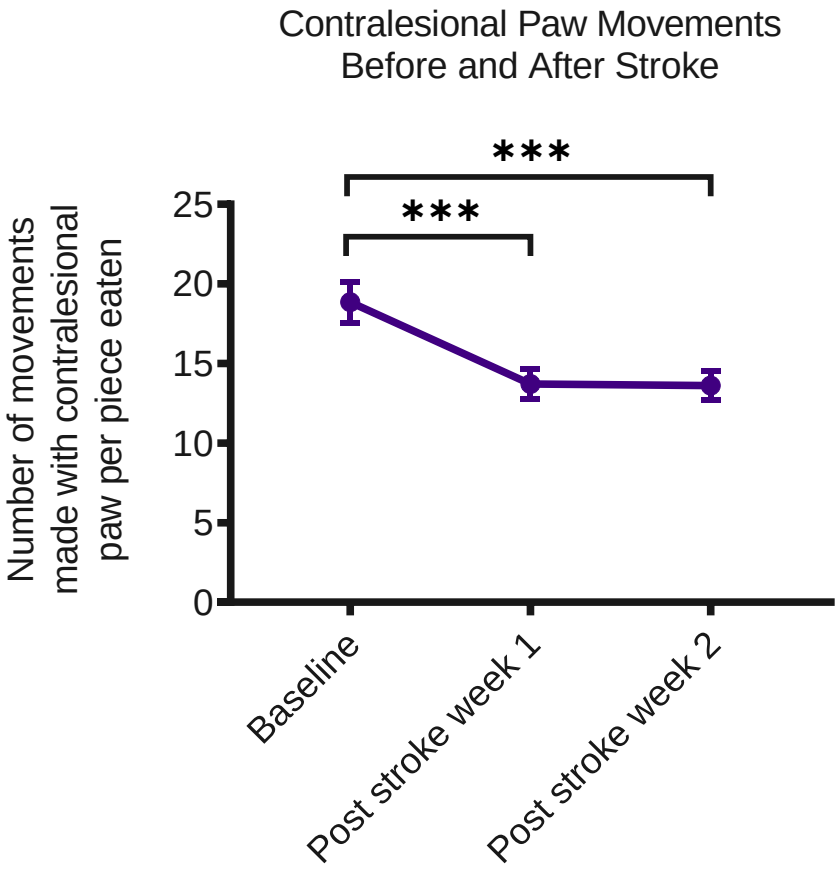

Figure 5.7 Contralateral (paretic) paw movements following stroke induction, comparing baseline with post lesion week one and week two. Differences from baseline were statistically significant: p=0.0002-0.0004 (***). A one-way ANOVA of contralateral movements over time was statistically significant [F (1.80, 85) =13, p<0.0001]. Error bars demonstrate mean ± SEM.

5.5.2 ABNORMAL MOVEMENTS DURING PASTA EATING FOLLOWING STROKE

Atypical movements were counted and categorised for each animal, and the total number of atypical movements made per individual piece eaten was calculated. This was expressed as a proportion of the number of atypical movements per five pieces eaten. For example, if a rat ate five pieces of pasta, and during this dropped one and used its mouth for eating during another, the number of atypical movements per five pieces eaten would be two. This can be expressed as:

$$5\ \frac{total\ number\ of\ atypical\ movements\ during\ all\ trials}{number\ of\ trials}$$

$$= atypical\ movements\ made\ per\ 5\ pieces\ eaten$$

Forty-seven animals had atypical movement analyses completed for the week prior to and both of the weeks following lesion induction. As baseline they showed an average of 3.4 ± 0.8 atypical movements per five pieces eaten, in other words, they showed an atypical movement in over half of all pieces eaten. In the two weeks following lesioning this did not change significantly, and a one-way repeated measures ANOVA of the proportion of atypical movements versus time showed no relationship, nor did comparisons between baseline and either of the post lesion week's results reveal significant differences (Figure 5.8).

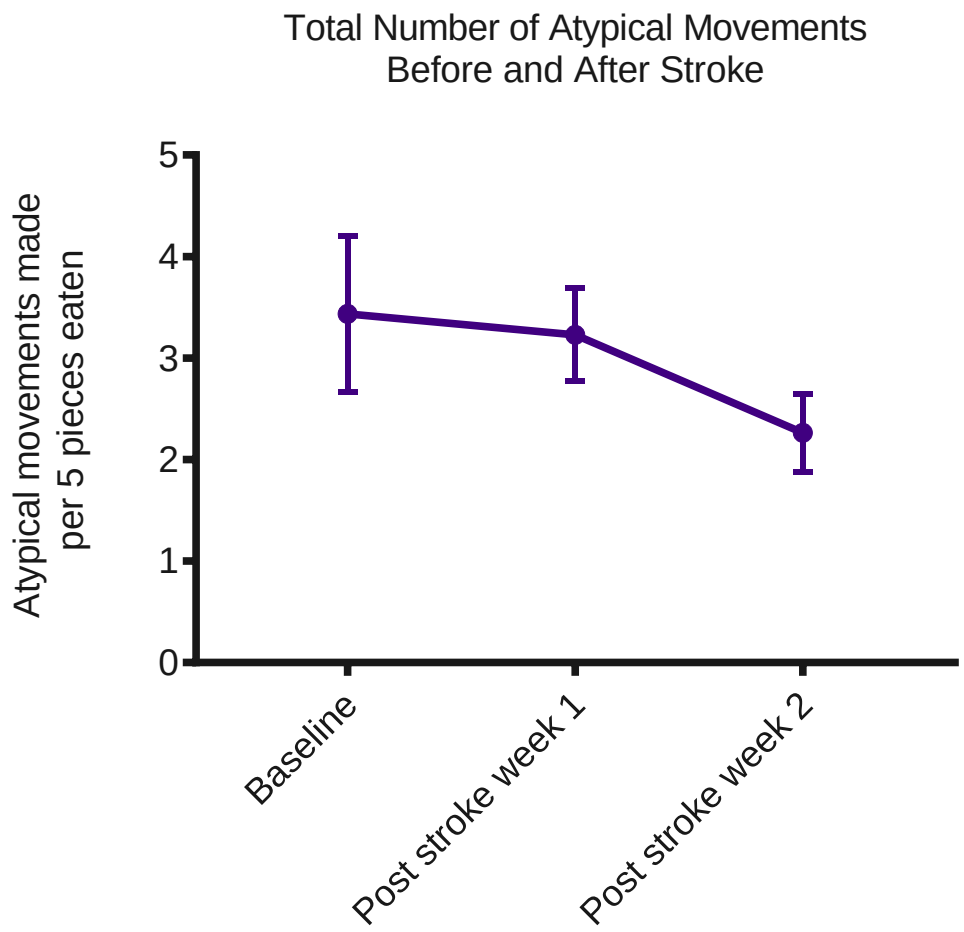

Figure 5.8 Atypical movements (combined) per five pieces eaten, comparing baseline with post lesion week one and week two. No statistically significant differences from baseline were found .

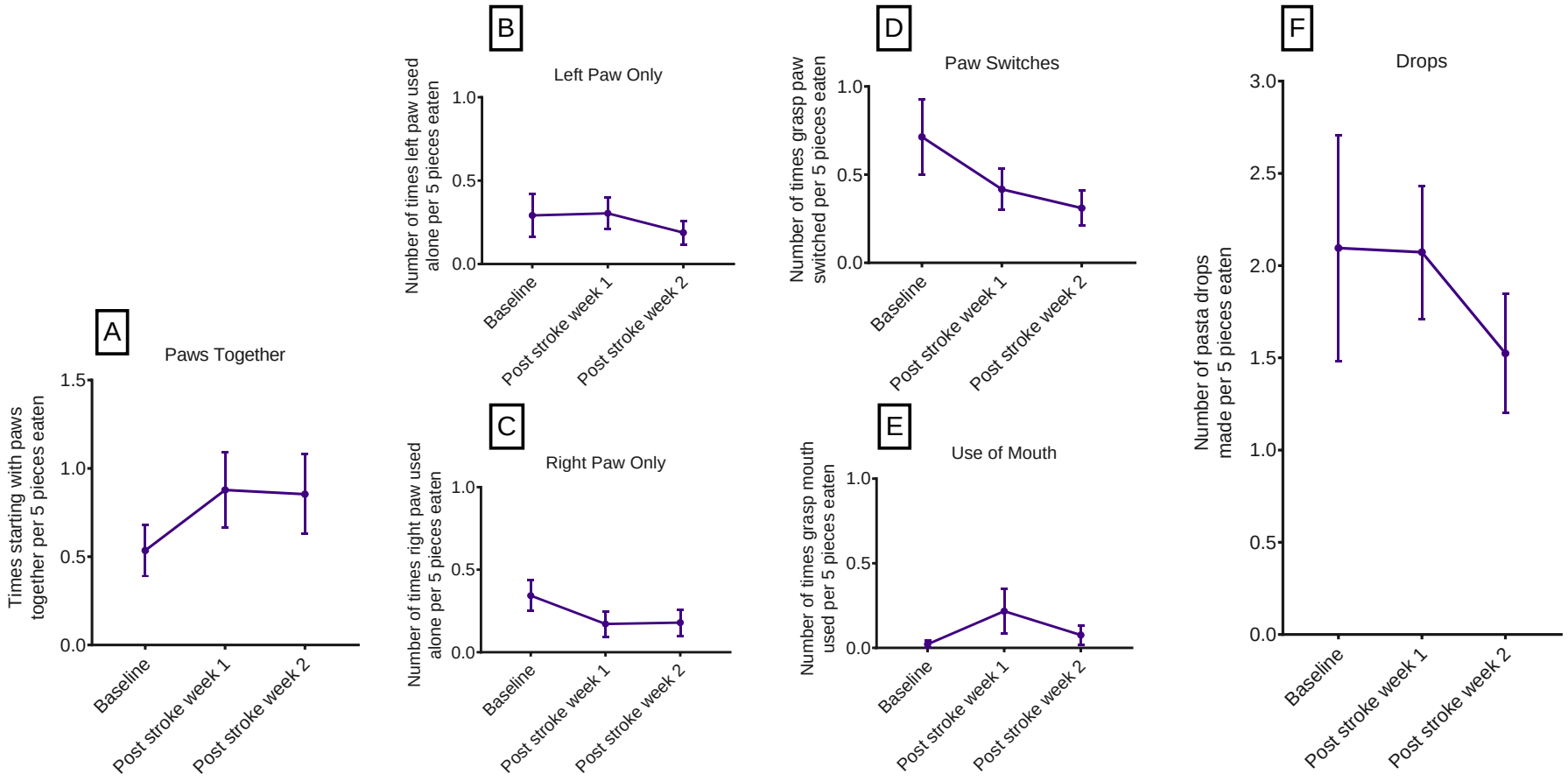

Figure 5.9 Atypical movements (by type) during pasta eating following stroke, per five pieces eaten, comparing baseline with post lesion week one and week two. A: Number of times paws were held together at the start of eating. B: Instances of use of the left paw only. C: Instances of use of the right paw only. D: grasp paw switches during eating. E: Number of times mouth was used for eating without paw contact. F: Number of drops of the pasta. No statistically significant differences from baseline were found for any of the measures.

When different atypical movements were analysed in isolation (e.g. only looking at the number of drops made during eating), no significant trends were found. Graphs for each of these movements are shown in Figure 5.9. The most common atypical movement was dropping the pasta, followed by holding the paws together at the start of eating rather than showing a grasp paw preference.

5.5.3 GRASP PAW PREFERENCE DURING PASTA EATING

During pasta eating, the paws are labelled the 'grasp' paw which supports the pasta, and the 'guide' paw which feeds it into the rat's mouth. Fifty-five animals had data collected on grasp versus guide paw preference at baseline, of which 23 preferred the left paw as the supporting grasp paw, 28 the right paw, and four did not show a preference. Of those 23 which began with the left as the preferred grasp paw, five switched to the right in the week following lesioning, and a further eight switched in the second week. In those 28 which began with preferring the right paw at baseline, a total of six switched to the left. In other words, a higher proportion of animals were using their right (paretic) paw as the grasp paw following lesioning. As another example of this, only 13 of the original 55 animals preferred their left paw as the grasp paw for both two post lesion weeks (including two which showed no preference at baseline). Compare this with 27 animals that preferred their right paw as the grasp paw for both the post lesion weeks.

This would seem to suggest a preference for having the paretic paw as the grasp paw and the left, healthy paw as the guiding paw following lesioning, and may reflect issues with using the paretic paw for the higher degree of dexterity required of the guide paw. Information about grasp and guide paw preference following lesioning has not been previously reported, and so

it was not known how to interrogate this finding statistically. This is a potential avenue of future investigation, to determine if grasp paw preference is a statistically valid measure of impairment following stroke.

5.6 THE EFFECT OF STROKE ON INTERHEMISPHERIC INHIBITION

There were 35 animals for which data on interhemispheric inhibition were obtained both before and after stroke induction. Data from two animals were excluded because they were outside two standard deviations of the mean, and a further four were excluded due to missing data. This left a total of 29 animals with IHI data prior to stroke induction, at one week (approximately day four) and two weeks (approximately day 11) following stroke induction. As mentioned in Chapter 3, responses in the awake animal resembled closely those obtained under anaesthesia and were analysed in a similar way. See Figure 4.4 for an example of the response obtained under anaesthesia, and Appendix 4 (page 278) for examples of traces from the awake animal.

Animals showed a mean baseline percentage inhibition of 19.1% ± 4.4, which increased to 34.3% ± 5.5 in the first week following lesion induction and 23.5% ± 5.4 a week later. These data are presented in Figure 5.10, A. A one-way ANOVA of the relationship between time and interhemispheric inhibition was not significant at the 5% level but showed a trend at the 10% level [$F(1.7, 49) = 2.9$, $p=0.071$]. Multiple comparisons analysis revealed a significant increase in inhibition in week one compared to baseline (mean difference: 15.2 ± 6.0, $p=0.033$) but the comparison with week two did not show a significant change. Figure 5.10, B shows individual animal data.

When only animals with grid walking impairment were included, leaving a sample of 13 rats, the changes in interhemispheric inhibition were qualitatively similar to those seen with the complete data set (Figure 5.11). A one-way ANOVA and corresponding comparisons with baseline also showed a statistically significant change at the 10% level [$F(1.8, 22) = 2.9$, $p = 0.083$].

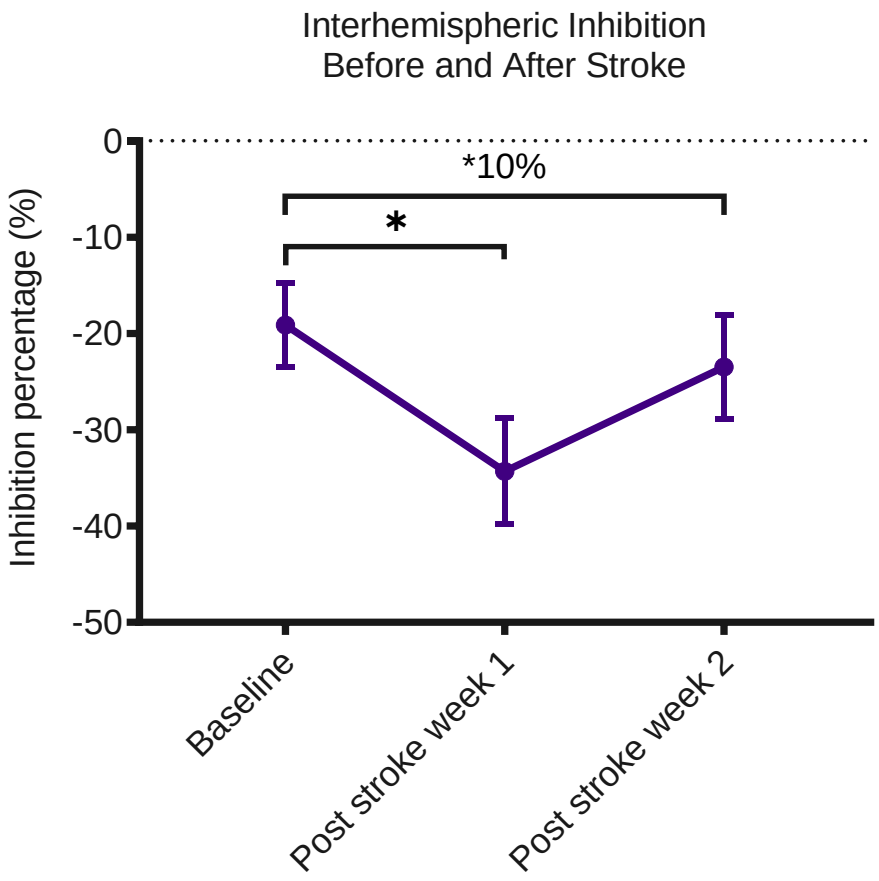

*Figure 5.10 A: Interhemispheric inhibition following stroke induction, comparing baseline with post lesion week one and week two. One-way repeated measured ANOVA was significant at the 10% level (*10%). Difference from baseline was statistically significant in week one: p=0.033 (*) but not week two: p=0.66. Error bars demonstrate mean ± SEM. B: Data from individual animals, showing the variation in development of IHI changes over time. In these graphs increasing positive values represent greater degrees of inhibition.*

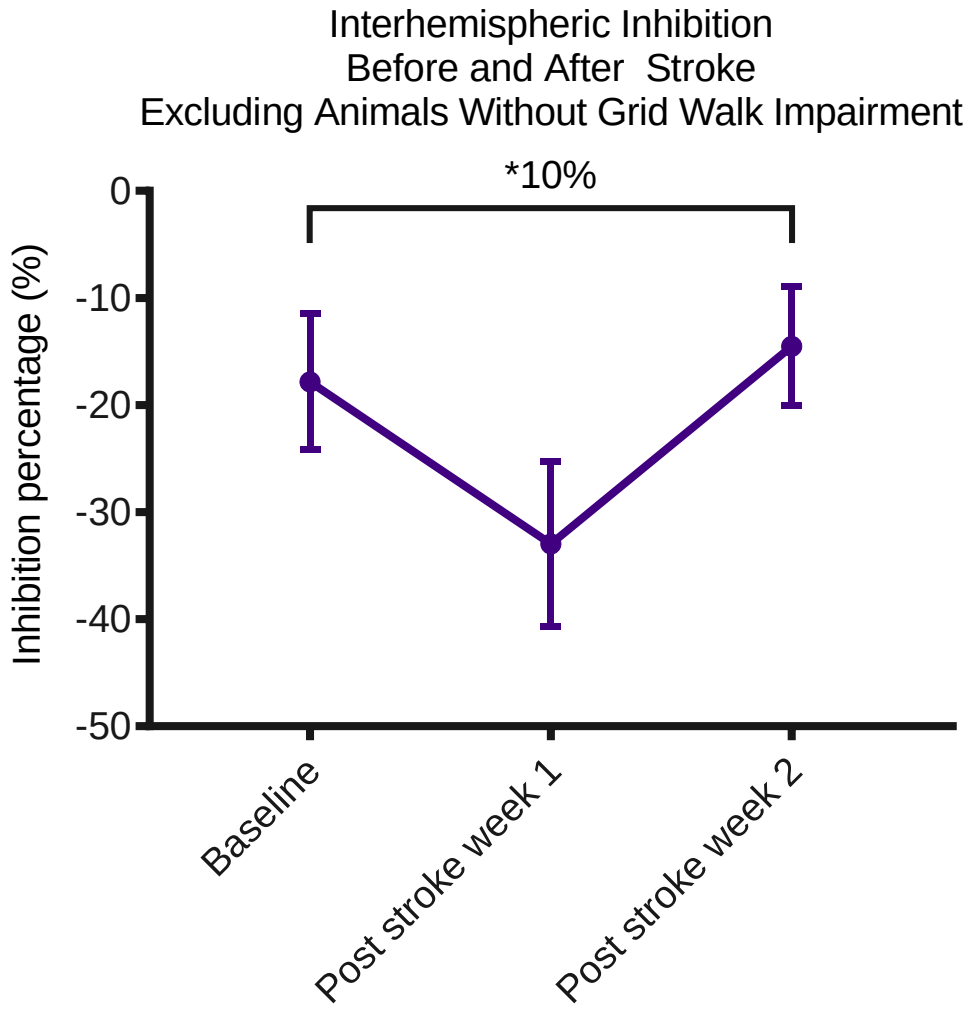

Figure 5.11 Interhemispheric inhibition following stroke induction in animals which met the inclusion criteria for grid walk impairment. One-way ANOVA of IHI over time was significant at the 10% level (*).

5.7 COMPARISONS OF DATA SETS

5.7.1 RELATIONSHIP BETWEEN BEHAVIOUR AND INTERHEMISPHERIC INHIBITION

When grid walking impairment was plotted against percentage inhibition for all the first three weeks of data collection (before stroke, one- and two-weeks post stroke), a weak association between higher impairment and greater inhibition was seen. The slope of the linear regression was significantly different from zero, with a p value of 0.0046 (Figure 5.12). The R^2 value representing goodness of fit was 0.11, meaning that 11% of the total variation in foot-faults was "explained" by the variation in IHI. Note that the interpretation of 'positive IHI' is that there was an inability to detect IHI during testing.

A similar relationship did not exist between pasta handling data and IHI. When percentage inhibition was plotted against pasta handling paw preference for all the first three weeks of data collection (before stroke, one- and two-weeks post stroke), no trend was seen (Figure 5.13). The slope of the linear regression was not significantly different from zero (p=0.68). This model can therefore be rejected as an explanation for variation in pasta handling behaviour.

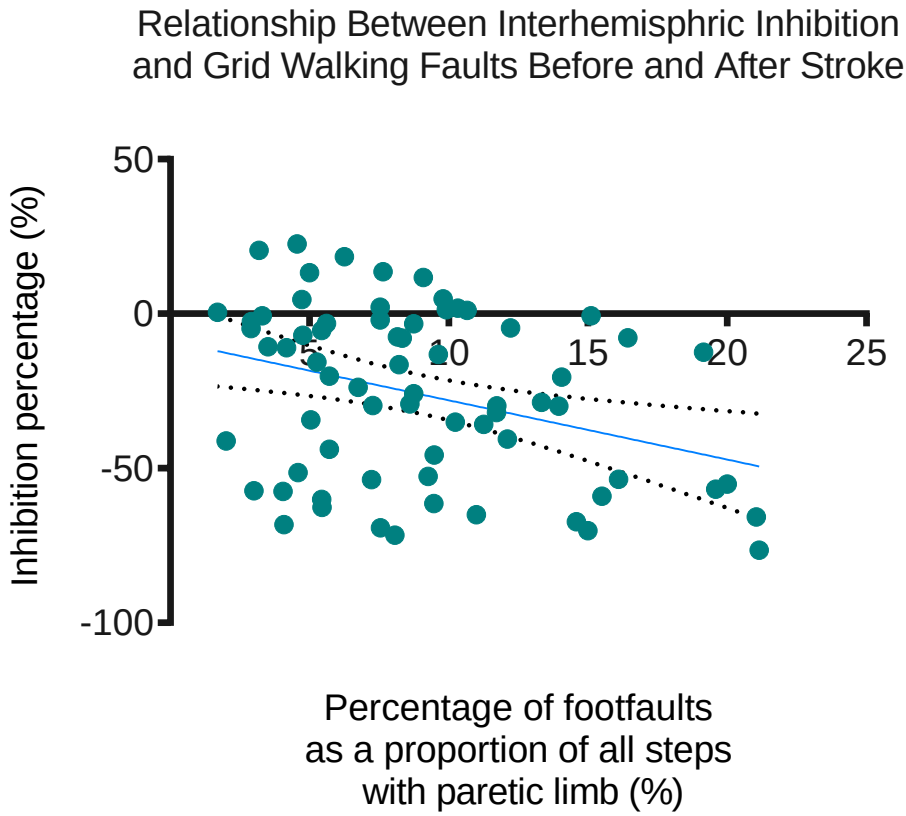

Figure 5.12 Interhemispheric inhibition versus foot fault impairment, with individual animal data shown for baseline and post stroke weeks one and two. Solid line shows linear regression of IHI versus foot faults, and dotted lines show 95% confidence intervals for this regression. $R^2=0.11$.

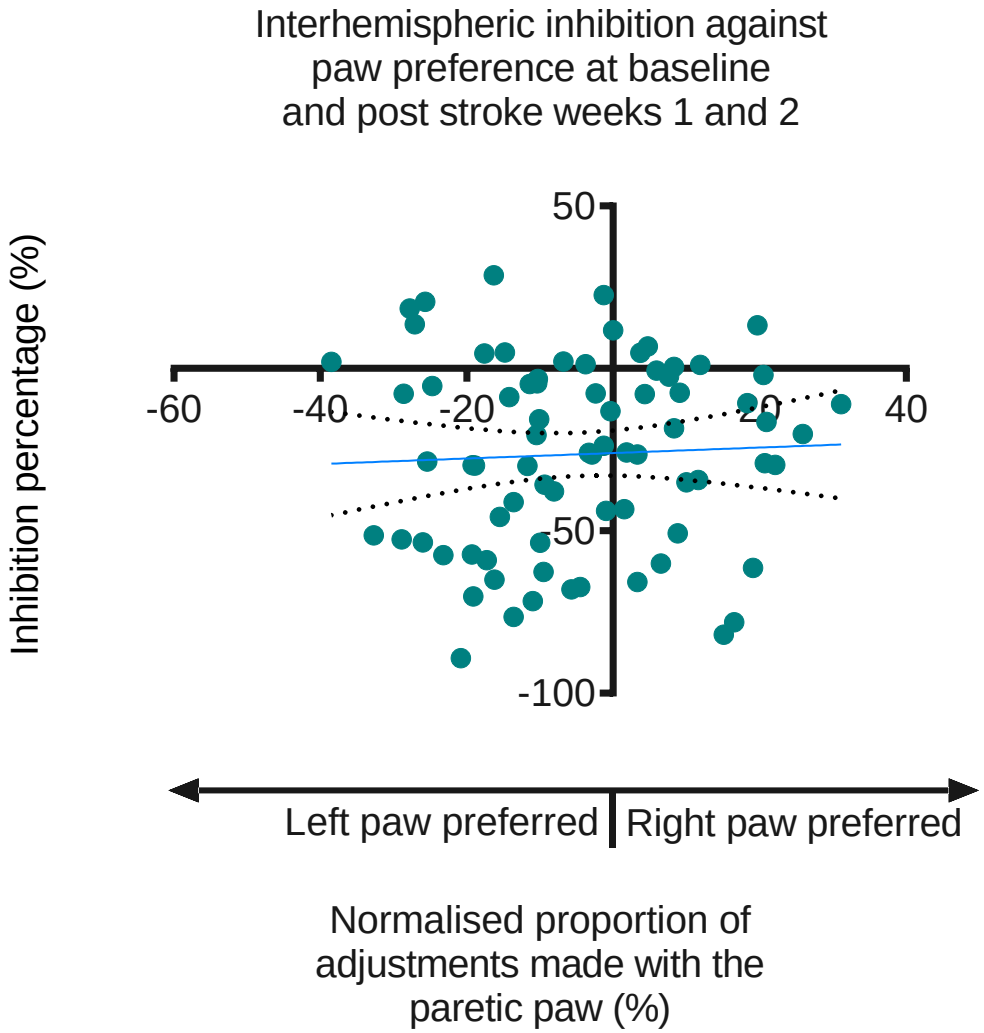

Figure 5.13 Interhemispheric inhibition versus paw preference, with individual animal data shown for baseline and post stroke weeks one and two. Solid line shows linear regression of IHI versus paw preference, and dotted lines show 95% confidence intervals for this regression. No relationship is seen between the two variables.

5.7.2 RELATIONSHIP BETWEEN BEHAVIOUR AND LESION VOLUME

When grid walking impairment was plotted against lesion volume at the time of maximum grid walk impairment (at any point in the eight weeks following lesion induction), an association between larger lesion size and higher impairment was present (Figure 5.14). The slope of the linear regression was significantly different from zero, with a p value of <0.0001. The R^2 value was 0.33, meaning that 33% of the variance in grid walk performance can be explained by lesion volume.

A similar, although weaker, association was present between lesion volume and pasta handling (Figure 5.14). The difference between paw preference at baseline, and at the time of least use of paretic paw was taken as the maximum pasta handling impairment. This was plotted against lesion volume for all animals with data for both measures (n=41). The slope of the linear regression was significantly different from zero, with a p value of 0.027. The R^2 value representing goodness of fit was 0.12, meaning that 12% of the variability in pasta handling impairment might be explained by lesion volume.

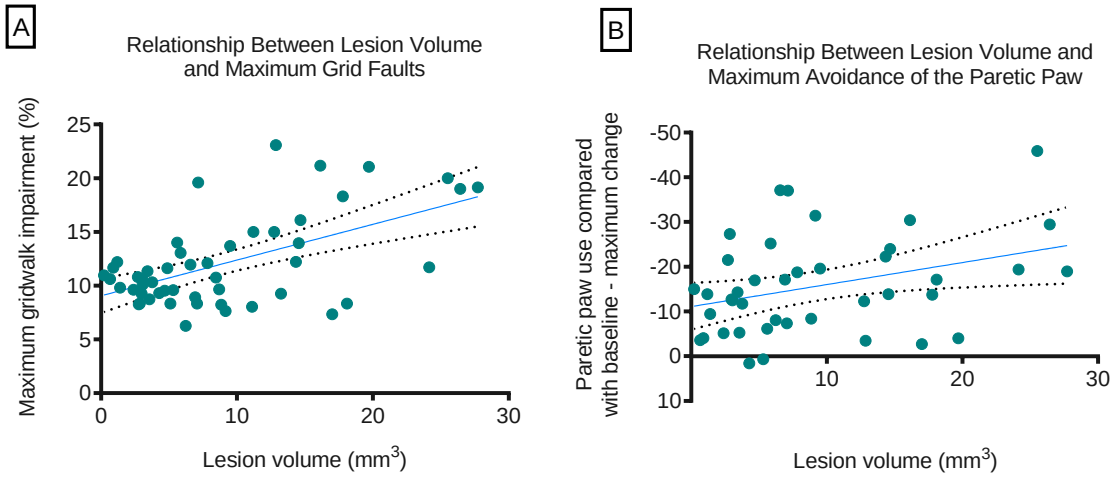

Figure 5.14 Relationships between lesion volume and maximum behavioural impairment on grid walk task (A: n=50) and pasta handling task (B: n=41). Individual animal data shown. Solid line shows linear regression of lesion volume versus the behavioural outcome, and dotted lines show 95% confidence intervals for this regression.

5.8 DISCUSSION OF RESULTS WITHIN THIS SECTION

This chapter outlines results relating to alterations of behaviour and interhemispheric inhibition, following endothelin-1 induced stroke lesion. The results obtained were overall in concordance with the hypotheses stated. Each section of results and its implications for the remainder of this thesis and the wider literature is discussed below.

5.8.1 HISTOLOGY FINDINGS

The endothelin-1-induced lesions in this study were smaller than expected based on comparable literature. Windle et al. reported lesion volumes of 86-96 mm^3 with similar volume endothelin-1 injections, and studies with smaller volumes of endothelin-1 reported lesion volumes of 1-12.5 mm^3 (Allred et al., 2008; Blasi et al., 2015; Fang et al., 2010; Fuxe et al., 1997; Gilmour et al., 2004; Gilmour et al., 2005; Windle et al., 2006). As reported, the lesion determined in this study was 9.5 ± 1.3 mm^3. What was clear from comparison of these studies is that the relationship between lesion volume and endothelin-1 volume is not linear. The reasons that lesion volumes in this study might have been smaller than anticipated are considered below.

One reason that must be considered is the quality of the endothelin-1 prepared for injection. Certain batches of endothelin-1 produced smaller lesions overall, indicating an effect of endothelin-1 quality on lesion volume. As referenced in the introduction, there was also a significant effect of injection number on lesion volume in previous studies. Protocols with a higher number of injections produced comparably larger lesions. This is presumably due to limitations of local diffusion of endothelin-1, restricting the lesion volume that can be achieved

at an individual injection site. In this study only two injection sites were used, compared with three with a comparable cortical-striatal lesion by Windle et al. In addition, the coordinates used here were vertically aligned, so that the anterior-posterior diameter of our lesions was less than reported by Windle et al. who were able to spread out their injection coordinates by using multiple craniotomy sites. This difference in methodology is likely to have reduced the lesion volumes able to be obtained in the present study.

One final explanation is that the longer time course animals spent in the present study, compared with those previously reported, was associated with a physiological reduction of lesion volume. Windle et al. reported that lesion volumes were greater at 48 hours following stroke as determined with magnetic resonance imaging as compared with the volume at sacrifice at 30 days post stroke. In addition, literature from human research describes a reduction in lesion volume in the first three months following stroke (Gaudinski et al., 2008; Windle et al., 2006). This is worth considering as it relates to the natural history of stroke lesions and their recovery, which is the focus of this study. Overall, a combination of the above factors is likely to explain the lesion volumes obtained.

For comparison, the strokes obtained in this thesis represent an average of 4% hemisphere volume, compared to human strokes producing 'moderate impairment' which are an average of 5% volume of the human brain (Conn, 2017). The model described by Windle et al. (2006) produces strokes of 30% hemisphere volume, greater than the 8% which is the average volume of all human strokes combined. Future studies could consider comparisons of small and large volume lesions e.g. with middle cerebral artery occlusion or attempt to create larger lesion volumes with a similar endothelin-1 technique.

The relevant hypothesis related to this is given below:

- **Hypothesis 2.1** That lesion volumes and locations demonstrate consistency across animals within the study, and are consistent with that expected from pre-existing literature, with a higher survival rate.

This hypothesis was borne out by experiments, with internal consistency in both volume and location of lesion. In our hands, animals did show a higher survival rate, an advantage obtained by the alteration of endothelin-1 injection coordinates. While the above histology results are important and interesting, in human research, behavioural impairment is used as the measure of stroke severity, rather than lesion volume. The subsequent section describes how these animals showed behavioural results comparable with studies using smaller endothelin-1 volumes and similar lesion volumes.

5.8.2 GRID WALK RESULTS

Grid walking impairments measured in this set of experiments provided measures of gross motor functions and coordination and revealed functional deficits. They also allowed us to distinguish those animals that may not have been adequately lesioned.

The hypothesis presented at the start of this chapter in relation to grid walk impairment is in two parts:

- **Hypothesis 2.2** That animals will demonstrate a significant impairment in grid walking performance in the paretic limb following stroke induction, and that this degree of impairment will be greater than that reported previously using smaller endothelin-1 injection volumes.

The first part has been borne out by experimental data: animals showed a significant impairment in grid walking performance, as measured by the proportion of foot faults, in weeks one and two following stroke lesioning. It should be noted that grid walking impairment was used to verify the adequacy of stroke lesion, with corresponding exclusion criteria. This means that this result is biased towards showing a relationship between stroke induction and impairment.

The second part was not adequately addressed, based on differing features of methodology (namely exclusion criteria). Across three previous studies with similar outcomes and lesion methodologies, a 4-6% greater impairment in foot faults was reported in the stroke affected group compared to sham at week one. Animals were excluded from the present study based on not meeting a threshold of 8% foot faults per steps taken in week one or two post stroke, a target set in part by examination of this literature as well as previous work in this lab (Barry, Boddington, Igelström, et al., 2014; Boddington, 2016). It is therefore not entirely valid to compare these results with those from previous studies which did not report their exclusion criteria.

Similar to previously described, this investigation found a baseline foot fault rate of 4.2 ± 0.3 (n=26), similar to that reported by Blasi et al. (2015). In addition, animals in the present study showed an increase in foot faults following lesioning, reaching 9.7 ± 1.0 (n=26) after four days. The 5.5 % (approximately) increase is similar to that seen by Gilmour et al. (2005), although as discussed, our results are affected by the inclusion criteria applied. The number of animals meeting the threshold for impairment in this study was comparable to that reported previously by Windle et al. during investigation of the endothelin-1 lesion model.

5.8.3 PASTA HANDLING RESULTS

Pasta handling behaviour was used to investigate impairments in fine motor and coordination skills. A previous paper by Allred et al. (2008) was used as the basis for two hypotheses relating to pasta handling behaviours:

- **Hypothesis 2.3.1** That animals will show a significant reduction in the use of the paretic limb to manipulate the pasta for eating following stroke, consistent with that previously reported.

- **Hypothesis 2.3.2** That animals will show a similar number of abnormal eating movements following lesioning as compared to prior lesioning, consistent with previously reported data for similar lesions.

Allred et al. reported raw data for contralateral movements, where the present study relies on measuring the use of the contralateral paw as a proportion of all movements to control for any unknown effects on total movement number. When contralateral movement total is studied in isolation some important differences exist between this and Allred's work. Animals in the present study made more movements of the contralateral paw overall at baseline. Allred reported baseline contralateral paw use of 6-8 adjustments per piece, whereas animals in this study made 18.9 ± 1.3 adjustments per piece with the contralateral paw (Allred et al., 2008). This could reflect a difference in the understanding of what is to be counted as an individual movement, or actual differences in behaviour in the two different rat populations studied.

Allred et al. (2008) demonstrated a decrease in pasta adjustments with the paretic paw of approximately four fewer movements per piece eaten on day eight following stroke. In this study a similar magnitude change was seen with just over five fewer movements per piece

eaten on days four and eleven following lesioning. Overall, the proportional change is lower, owing to the higher baseline number of adjustments in our hands. The similarity between this and Allred's result means that hypothesis 2.3.1 can be accepted. Abnormal handling behaviours did not alter significantly with lesioning induction in the population of animals studied in either these or the Allred experiments, confirming hypothesis 2.3.2.

Not only do these results assist in confirming the presence of impairment following endothelin-1 lesioning in the rats of this study, they also help to validate this method of motor testing following stroke. Other measures of fine motor control in the rat tend to be labour intensive, e.g. the single pellet reaching task (Schaar et al., 2010). The pasta handling task could represent a low budget, faster alternative in the stroke field. Note that one modification that would be suggested for future users of this technique, would be to lesion the side corresponding to the dominant limb, which would potentially increase the consistency of results.

5.8.4 INTERHEMISPHERIC INHIBITION RESULTS

The key result in this chapter was the observation that interhemispheric inhibition significantly increased in the week immediately following stroke. However, we could not prove that IHI remained elevated in the second week following stroke, which meant we failed to accept hypothesis 2.4:

- **Hypothesis 2.4** That interhemispheric inhibition will increase at one- and two-weeks following stroke lesioning compared to that measured at baseline.

This has implications for the field of research into the role of IHI after stroke, and the modelling of stroke pathology with rat models. It is clear that the timeline of artificially induced stroke in rats is different from the natural history of stroke in humans. Increases in IHI in humans and rats are generally measured in the chronic phase after stroke (Duque et al., 2005; Murase et al., 2004), while in this experiment an increase in IHI is observed in the acute phase, one week following lesioning. One experiment in this lab measuring IHI in rats found that at 31-51 days following lesion induction, there was no difference in IHI compared with non-lesioned animals from an earlier study (Barry, Boddington, Igelström, et al., 2014; Boddington et al., 2020).

There is no clearly established timeline for IHI changes in either rats or humans, and most conclusions are made by comparing disparate studies measuring IHI at different time points. Vallone et al. (2016) found imbalanced interhemispheric connectivity 16-23 days after stroke in mice, correlating with a 'late sub-acute period', however they did not measure IHI specifically. Spalletti et al. (2017) found an increase in $GABA_B$ mediated transcallosal inhibition at five and thirty days after mouse stroke but did not measure short latency IHI. The use of different animal models and different techniques for recording interhemispheric interactions makes it difficult to construct a clear picture of what changes in IHI would be expected in the acute, sub-acute and chronic phases in either rats or humans. Overall, the acute increase measured in the present study adds to our understanding of the timeline of IHI development following stroke.

5.8.5 RELATIONSHIPS BETWEEN POST STROKE RESULTS

- **Hypothesis 2.5.1** That greater interhemispheric inhibition onto the lesioned hemisphere will be associated with greater foot fault impairment.

- **Hypothesis 2.5.2** That greater interhemispheric inhibition onto the lesioned hemisphere will be associated with greater preference of the non-paretic limb in making pasta adjustments during eating.

As hypothesised, increased foot faults were associated with an increase in IHI. This was a small effect, with an increase of one percent foot fault impairment associated with a rise in IHI of 1.92% ± 0.65 (slope of linear regression ± standard error). This confirms that interhemispheric inhibition and grid walk impairment are linked, but the small effect size suggest that other factors must also be involved. The result adds to the previously reported findings of Boddington et al. (2020), by providing a more direct link between alterations in IHI and functional impairment. The implication is that interhemispheric inhibition remains a target to investigate means of improving recovery following stroke, however interhemispheric imbalance does not explain completely the residual functional impairment following stroke, hence other mechanisms must also be targeted. A similar finding was not made for pasta handling data, which was perhaps a reflection of the high degree of intrinsic variability associated with both IHI and pasta handling data. Alternatively this could reflect the fact that coordinated bimanual tasks involve IHI in a different way to gross motor actions, recalling that the suspected role of IHI in stroke impairment is excessive contralateral inhibition at the time of unimanual movement initiation (Ridding et al., 2000).

Lesion volume provides a snapshot of the pathology within the brain that is being examined by the techniques described. The lesion that is seen may or may not represent the full degree of functional damage done to the neuronal tissue. It is understandable therefore that lesion volume does not entirely explain behavioural impairment. A lesion that appears large, may in fact have undergone some functional remodelling, and lesions which appear small may not demonstrate the complete area of functionally damaged tissue. The maximum level behavioural

impairment was compared with estimated lesion volume, showing the correlation between increased volume and increased impairment shown in Figure 5.12. This finding also gives us some validation that the behavioural measures themselves relate to pathology within the brain. The hypotheses relating to this finding are:

- **Hypothesis 2.5.3** That greater lesion volume will be associated with greater foot fault impairment.
- **Hypothesis 2.5.4** That greater lesion volume will not be associated with a change in pasta handling asymmetry.

This first hypothesis was confirmed by our findings. Interestingly, the present study did show an association between lesion volume and pasta handling impairments whereas Allred et al. (2008), from which the null hypothesis 2.5.4 was derived, did not. This could be the effect of greater animal numbers in the present study, as Allred et al. reported a non-significant but positive correlation between lesion volume and asymmetry of adjustments.

- **Hypothesis 2.5.5** That greater lesion volume will not be associated with alteration in interhemispheric inhibition.

As predicted, there was no relationship between lesion volume and the maximum degree of interhemispheric inhibition measured ($p=0.60$). This means that any relationship between IHI and behaviour is independent of lesion volume. Thus, the association between grid walk impairment and IHI is unlikely to be confounded by lesion volume.

5.9 SUMMARY AND CONCLUSIONS

Endothelin-1 induced stroke lesions in the rats described in these experiments were associated with both functional impairment and increased levels of interhemispheric inhibition. Interhemispheric inhibition is therefore a potential target for modulation of functional recovery, either to return levels of IHI back to baseline and restore normal inhibitory balance, or to reduce IHI levels still further and improve the ability to initiate movements of the paretic arm. Chapters 2 and 4 introduced the idea of using intermittent theta burst stimulation at low intensity over the contralesional hemisphere as a potential intervention to reduce IHI. The following chapter describes how intermittent theta burst stimulation was used in these rats, and the subsequent effects on behaviour and interhemispheric inhibition.

6 RESULTS: EFFECTS OF INTERMITTENT THETA BURST STIMULATION ON BEHAVIOUR AND INTERHEMISPHERIC INHIBITION IN RATS

6.1 INTRODUCTION

In the previous chapter, the behavioural and neurophysiological effects of stroke on a group of rats were detailed. In this chapter, results pertaining to the same group of rats will be presented, this time following randomisation to receive either sham or intermittent theta burst stimulation (iTBS). As in the previous chapter, the literature relevant to understanding the results will be briefly discussed, prior to explanation of both behavioural and interhemispheric inhibition data. The results in this chapter relate to objective three:

Objective 3: To determine whether, in an animal model of stroke, low intensity intermittent theta burst stimulation to the contralesional hemisphere can reduce interhemispheric inhibition and improve behavioural recovery.

6.1.1 INTERMITTENT THETA BURST STIMULATION PROTOCOL

Two-weeks after stroke induction, rats were randomised to iTBS or sham groups. Following randomisation, motor threshold was measured in iTBS animals and iTBS intensity calculated as discussed in Chapter 3. Animals received 15 doses of stimulation over three weeks. Behavioural and IHI measurements were made weekly for six weeks following randomisation, including three weeks after stimulation had finished. They were then euthanised and their brains collected for histology. Refer to Chapter 5 for histological results.

This timeline was designed using previous experiments from this lab as a guide. Boddington (2016) investigated multiple regimens of iTBS delivery, including a group with a ten-day delay following stroke after which animals had been implanted with electrodes and then received five applications of 600 pulses of iTBS each day, five days in a week over a three-week period. This protocol had the 10 day 'delayed start' in rats to represent the time a person might wait following stroke before administration of iTBS via electrode implantation or non-invasive application could be commenced. This also correlates roughly with the 'early-sub-acute period' in both humans and rodents which is thought to be the time of highest post-stroke plasticity (Vallone et al., 2016; Zeiler & Krakauer, 2013). The intensive five times-a-day regimen was used to maximise the chance of detecting a functional improvement, however Boddington et al (2020) observed no advantage over a more modest application of one application of iTBS two-three times per week (Figure 6.1). This protocol was modified in the experiments described in this thesis to an 'intermediate' stimulation dose. Animals received one application of 600 pulses of iTBS, five days a week, over a three-week period, at a delay of 14 days after stroke induction.

As introduced in Chapter 2, theta stimulation is based on a naturally occurring theta rhythm that has been recorded in rats during learning behaviours (Winson, 1974). Since the description of this, the theta rhythm has been employed in numerous neuromodulation studies. The intermittent theta burst pattern that is employed in this study is typically associated with increasing excitability (Y. Z. Huang et al., 2005), however more recent data showed that at low intensities it could decrease interhemispheric inhibition to the contralateral hemisphere and improve behavioural outcomes following stroke in the rat (Barry, Boddington, Igelstrom, et al., 2014; Boddington et al., 2020). For a reminder of the parameters of iTBS, refer to Figure 3.3.

6.1.2 EFFECT OF INTERMITTENT THETA BURST STIMULATION ON RAT BEHAVIOUR

The hypotheses for how stroke lesioned rats will respond to iTBS are based primarily on previous work in this laboratory, given the novel nature of this protocol. The previous experiments done in this lab used grid walking performance to measure functional improvements associated with iTBS. Boddington (2016) showed that iTBS was associated with functional improvements on this task, but only when given immediately after stroke (Figure 6.1, B). In the experiments described in this thesis, the 'delayed-start, intensive dose' regimen from Boddington was used as a framework (results not shown), with greater animal numbers included. Given this, it is predicted that the animals receiving iTBS will show greater functional improvement than those receiving sham stimulation.

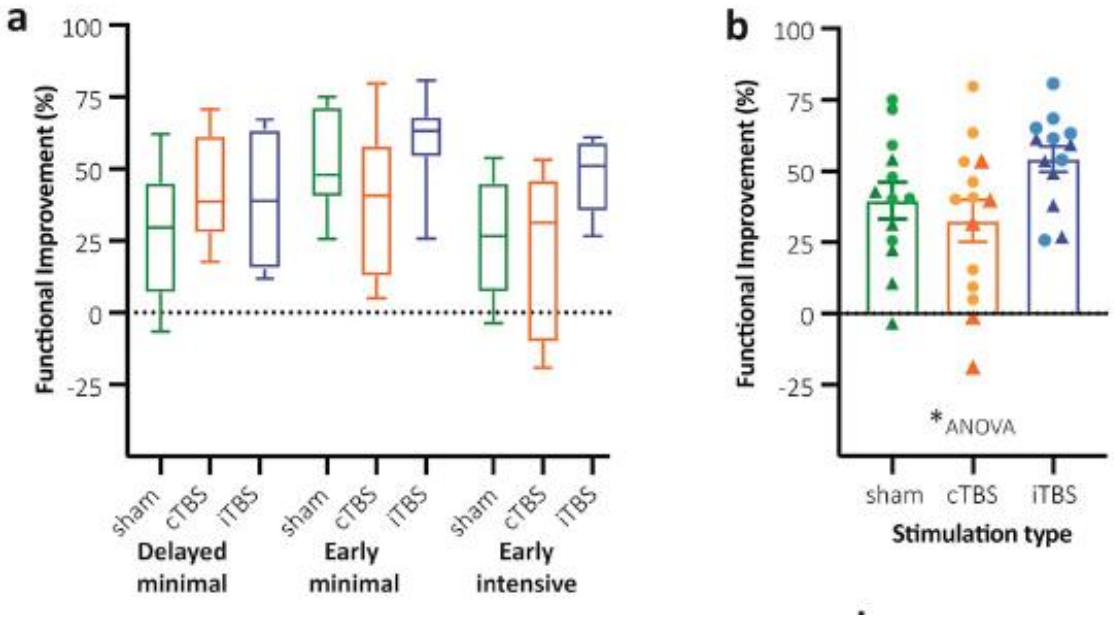

Figure 6.1 Theta burst stimulation influences functional recovery on the grid-walking task after stroke. (a) Box and whisker plots showing the percentage improvement of rats within each experimental group. (b) Functional improvement for the early stimulation groups of each intensity combined. Triangles indicate intensive; circles minimal stimulation. Copied without modification from Boddington et al. (2020).

The results for pasta handling performance are more difficult to predict. In Chapter 5, a significant change in pasta handling behaviour following stroke lesion was shown; rats showed a 5.3% decrease in the use of the paretic paw following lesioning. Two human studies showed improvements in fine motor skill in the corresponding limb following TMS delivery of iTBS, one in healthy participants (Butts, Kolar, & Newman-Norlund, 2014) and one in stroke patients with iTBS applied ipsilesionally (Y. J. Chen et al., 2019). If iTBS results in functional improvement in fine motor skill, which the pasta handling task purports to measure, a return toward equal paw preference would be predicted in the present study. A hypothesis for abnormal movements during the pasta task was not proposed, given that abnormal movements during pasta handling were not a valid measure of stroke impairment. The following objective and hypotheses relate to the effect of iTBS on behaviour.

Objective 3.1: To determine if rats undergoing a protocol of intermittent theta burst stimulation delivered over the contralateral hemisphere will demonstrate enhanced behavioural recovery compared to rats receiving sham stimulation.

- **Hypothesis 3.1.1:** That rats receiving iTBS over the contralateral hemisphere will have a greater reduction, from post stroke impairment, in foot faults during grid walking as compared to rats receiving sham stimulation.
- **Hypothesis 3.1.2:** That rats receiving iTBS over the contralateral hemisphere will have a greater increase, from post stroke impairment, in paretic paw use during pasta handling as compared to rats receiving sham stimulation.

The concurrently running iSTART study returned results from its first two participants during this study. This is mentioned for completeness, however is awaiting publication (Lim et al.,

Unpublished work). The first participant, a 58-year-old male with a history of subdural stroke 16 months prior underwent electrode implantation over the contralateral motor cortex. Subsequently, he experienced greater improvements in Upper Extremity Fugl-Meyer score, Action Research Arm Test and grip strength during the period of iTBS coupled with rehabilitation, than with the preceding period of rehabilitation and sham stimulation. A second participant, data from whom has also not yet been published, demonstrated similar results. These examples support our prediction that iTBS will be associated with greater functional recovery than sham stimulation.

6.1.3 EFFECT OF INTERMITTENT THETA BURST STIMULATION ON INTERHEMISPHERIC INHIBITION

In Chapter 2, the evidence for an effect of low intensity iTBS on IHI was considered in detail. Previous work has demonstrated that iTBS has an effect on IHI when measured in an anaesthetised naïve animal using acute intracellular recordings (Barry, Boddington, Igelstrom, et al., 2014). There is also some evidence of its effect on modifying local inhibitory circuits in humans when delivered via TMS (Y. Z. Huang et al., 2005). Boddington et al. (2020) showed an interesting relationship between IHI and iTBS, which has been described earlier in this thesis. In brief, intermittent TBS treated animals did not show reduced IHI compared to sham, however there was a relationship between functional recovery (which was greater in iTBS treated animals) and ipsilesional excitability. Ipsilesional excitability, as measured by post synaptic potential amplitude in single neurons, was then in turn related to a reduction in IHI. Together these studies indicate that effect of TBS on IHI is likely indirect and is currently poorly understood.

In Chapter 5, IHI was measured before and in the early stages after stroke recovery. In the results in this chapter, IHI is again measured over time using EEG, in order to determine if there are further changes in IHI after iTBS or sham stimulation. The following hypothesis is given as a prediction for how IHI will change with delivery of low intensity iTBS.

- **Hypothesis 3.2** That rats receiving iTBS over the contralateral hemisphere will show a greater reduction, from post stroke inhibition, in interhemispheric inhibition as compared to rats receiving sham stimulation.

This addresses the following objective:

Objective 3.2 To determine if rats undergoing a protocol of intermittent theta burst stimulation delivered over the contralateral hemisphere will demonstrate changes in interhemispheric inhibition that differ from rats receiving sham stimulation.

6.2 GROUP STATISTICS FOLLOWING RANDOMISATION

Twenty-two animals were randomised to receive sham stimulation, and nineteen to receive iTBS. Complete data were available for 15 animals in each group. For this analysis, animals were excluded based on lack of functional impairment, as discussed previously in Chapter 5, leaving nine in the sham and five in the iTBS group with complete data sets.

6.3 MOTOR THRESHOLDS AND STIMULATION INTENSITIES

There is variation in the literature as to what constitutes a normal motor threshold in an awake rat. Two studies were found that used electrical stimulation to elicit a motor threshold response. In awake rats that had undergone photothrombotic stroke induction, with 1 Hz stimulation trains, Yang, Liu, Li, and Li (2017) found a baseline motor threshold of 1100 μA. In previous work in this laboratory, using a similar protocol to the one described in this thesis, the motor threshold was found at approximately 400 μA (Barry, Boddington, Igelstrom, et al., 2014).

In the present study, baseline motor thresholds were measured for those animals randomised to the iTBS group just after randomisation, so that stimulation intensity could be determined. There were 30 animals for which motor thresholds were measured, with an average threshold of 500 ± 20 μA. When 50% of motor threshold plus electrical threshold was calculated to determine stimulation intensity, an average stimulation intensity of 290 ± 10 μA was delivered. In comparison to previous work in this lab, the motor threshold measured in these experiments was slightly higher, and the electrical threshold slightly lower (as discussed in Chapter 5), which yielded an iTBS intensity that was similar to that used previously. In the present study, the average stimulation intensity was 60% of motor threshold, a level that altered IHI acutely in single cell recordings and produced functional benefit (Barry, Boddington, Igelstrom, et al., 2014; Boddington et al., 2020).

6.4 EFFECT OF INTERMITTENT THETA BURST STIMULATION ON GRID WALK PERFORMANCE

Grid walking results were available for 12 sham treated animals and 10 iTBS treated animals. Animals were excluded based on lack of impairment, according to criteria described in Chapter 5. They were also excluded if more than half of their data was missing. Group specific baseline and maximum stroke impairment results are given in Table 6.1 to demonstrate adequacy of randomisation.

	Sham (n=12)	**iTBS (n=10)**	**Unpaired t-test: p-value**
Baseline (%)	4.6 ± 0.5	4.1 ± 0.5	0.51 (not significant)
Maximum impairment - week one or two post stroke (%)	8.6 ± 0.5	10 ± 1.3	0.23 (not significant)

Table 6.1 Baseline grid walking results prior to randomisation (foot faults as a percentage of all steps taken)

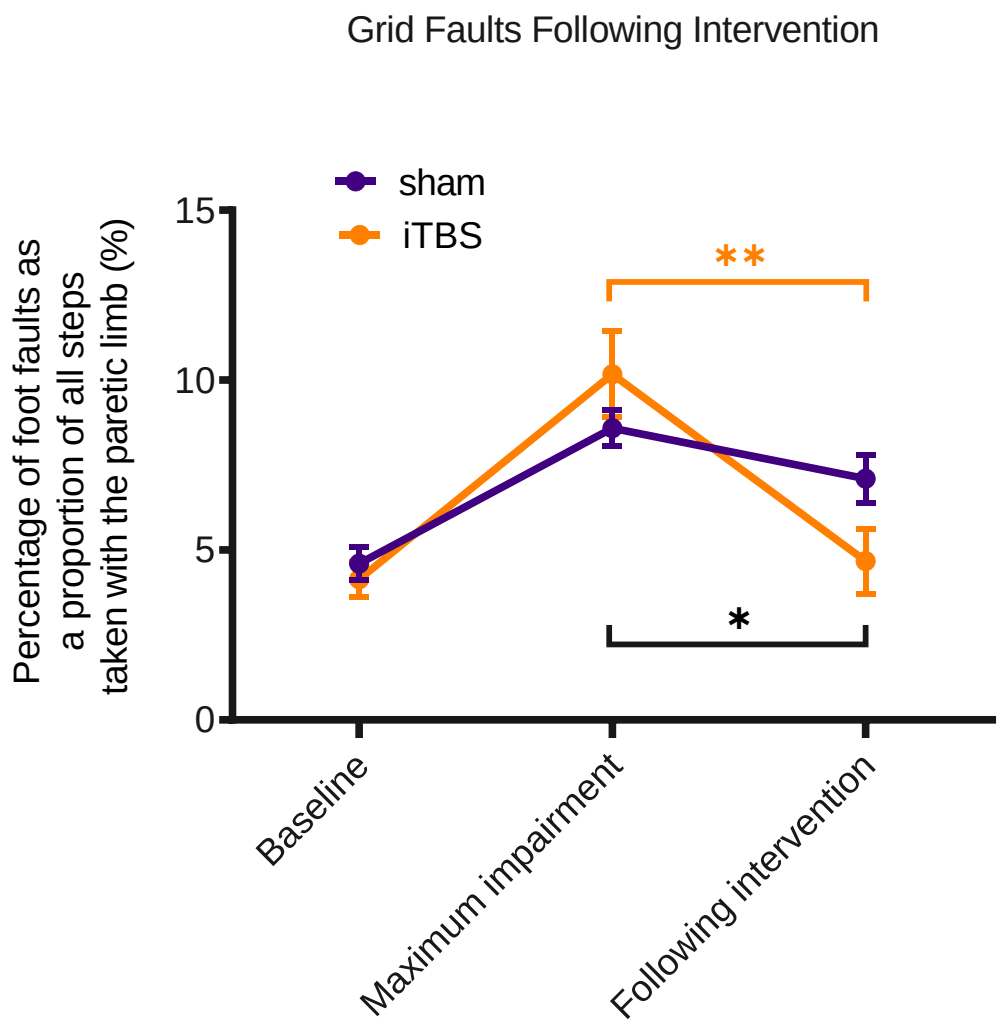

Figure 6.2 Grid walking results at baseline, at maximal impairment in the two weeks after stroke, and at the end of the intervention period. Results indicated are mixed effects analysis significant to p=0.011 (*) and within group comparison significant to 0.0021(**).

When comparing foot faults after stroke and following intervention, a mixed effects analysis of group x time revealed a statistically significant difference, $F(1, 19) = 8.0$, $p=0.011$, meaning that the iTBS group showed a greater change over time compared to the sham group. The nature of this difference was explored through multiple comparisons analysis, which showed a significant difference between the maximum level of foot fault impairment and the percentage of foot faults following intervention in the iTBS treated group, but not the sham group. The iTBS group showed a significant average decrease in foot faults of 5.5 per 100 steps ($p=0.0021$, 95% confidence interval of difference: 2.5 to 8.5) which equates to a functional improvement on average of 54% ± 8.7 following intervention. Contrast this with the sham group which showed an average non-significant decrease of 1.5 faults per 100 steps ($p=0.38$, 95% confidence interval of difference: -1.4 to 4.4), which equates to a functional improvement on average of 15% ± 11. Therefore, three weeks after stimulation had ceased, the group receiving iTBS showed significantly greater functional recovery than sham. Data for all timepoints is shown in Appendix 9 (page 285).

6.5 EFFECT OF INTERMITTENT THETA BURST STIMULATION ON PASTA HANDLING PERFORMANCE

Pasta handling results were available for 13 of the animals randomised to receive sham intervention, and 15 of the animals in the iTBS group. Other animals were excluded due to missing data (defined as having more than half of data missing or having data at baseline or the study end missing). Group specific baseline and maximum stroke impairment results are given in Table 6.2 to demonstrate adequacy of randomisation. All pasta handling results are reported as right (paretic) paw adjustments as a proportion of all adjustments made.

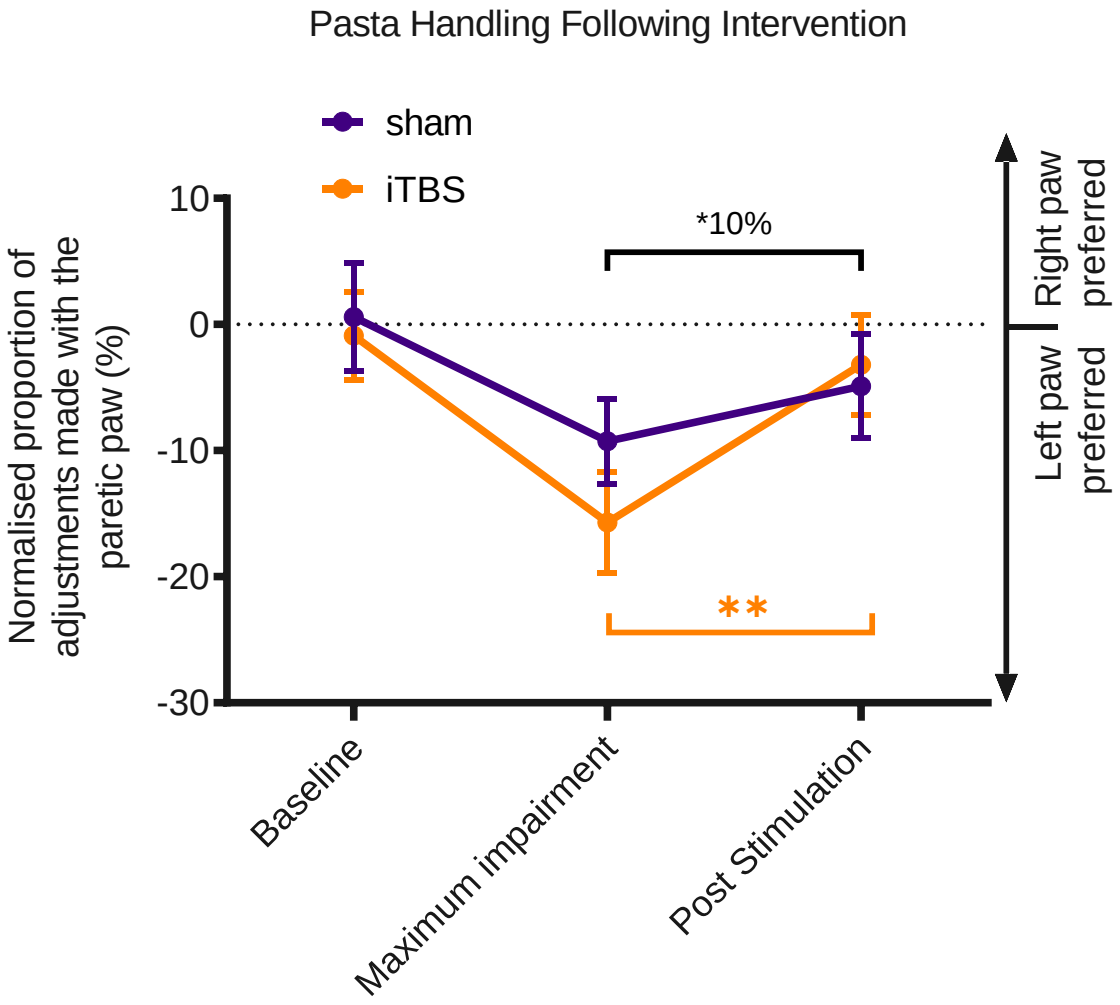

Figure 6.3 Pasta handling paw preference at baseline, at maximal impairment in the two weeks after stroke, and at the end of the intervention period. Positive values represent right (paretic) paw preference and negative values represent left paw preference. One-way ANOVA of group x time was significant at the 10% level (*), and difference indicated for iTBS group following randomisation was significant 0.0076 (**).

	Sham (n=13)	iTBS (n=15)	Unpaired t-test: p-value
Baseline (%)	0.6 ± 4.3	- 0.9 ± 3.5	0.78 (not significant)
Maximum impairment - week one or two post stroke (%)	-9.3 ± 3.4	- 15.7 ± 4.0	0.24 (not significant)

Table 6.2 Baseline pasta handling results prior to randomisation (positive result indicates right paw preference; negative result indicates left paw preference).

Results at relevant timepoints are shown in Figure 6.3. When comparing paretic paw impairment after stroke and following intervention, a two-way repeated measures ANOVA of group x time did not reveal a statistically significant difference, F (1, 26) =3.5, p=0.074. However, given the relationship was significant at the 10% level, a multiple comparison analysis was done. After within-group comparisons were made, it was found that there was a significant difference between the maximum level of paretic paw impairment and the degree of paretic paw use at the end of the study in the iTBS group (p=0.0076), while a similar difference was not seen in the sham group. Compared to following stroke, the iTBS group had an average increase in right paw use of 12.5 more adjustments with the paretic paw per 100 (95% confidence interval of difference: 3.4 to 21). Looking at this another way, paretic paw use increased by 56% ± 17 following intervention. The sham group had an average increase of 4.4 more adjustments per 100 (p=0.22, 95% confidence interval of difference: -2.2 to 11), or an increase in paretic paw use of 14% ± 7.4. Given that the data appears to 'cross' with the non-significantly more impaired iTBS treated animals showing more improvement than the relatively less impaired sham animals, an additional analysis was done to check for

confounding based on degree of initial impairment. There was at best a weak and non-significant relationship between degree of impairment and degree of improvement (p=0.0528, R^2=0.137), likely insufficient to explain the greater improvement in function in the iTBS group. See Appendix 9 (page 285) for data at all timepoints.

6.6 EFFECT OF INTERMITTENT THETA BURST STIMULATION ON INTERHEMISPHERIC INHIBITION

Interhemispheric inhibition measurements were available for nine animals in the sham stimulation group, and five in the iTBS group. The small animal numbers were a result of excluding those animals that were removed based on lack of functional impairment. Group specific baseline and maximum stroke impairment results are given in Table 6.3 to demonstrate adequacy of randomisation.

	Sham (n=9)	**iTBS (n=5)**	**Unpaired t-test: p-value**
Baseline (% inhibition)	30 ± 13	22 ± 6.8	0.69 (not significant)
Maximum IHI - week one or two post-stroke (% inhibition)	42 ± 9.9	44 ± 5.9	0.90 (not significant)

Table 6.3 Baseline interhemispheric inhibition prior to randomisation.

When comparing sham and iTBS interhemispheric inhibition results, data at all timepoints was included, rather than the abbreviated results shown for behavioural measures. This approach was taken due to the difficulty in finding changes at single timepoints, both in this thesis (Chapter 4) and in other work (Boddington et al., 2020). These data are shown in Figure 6.4.

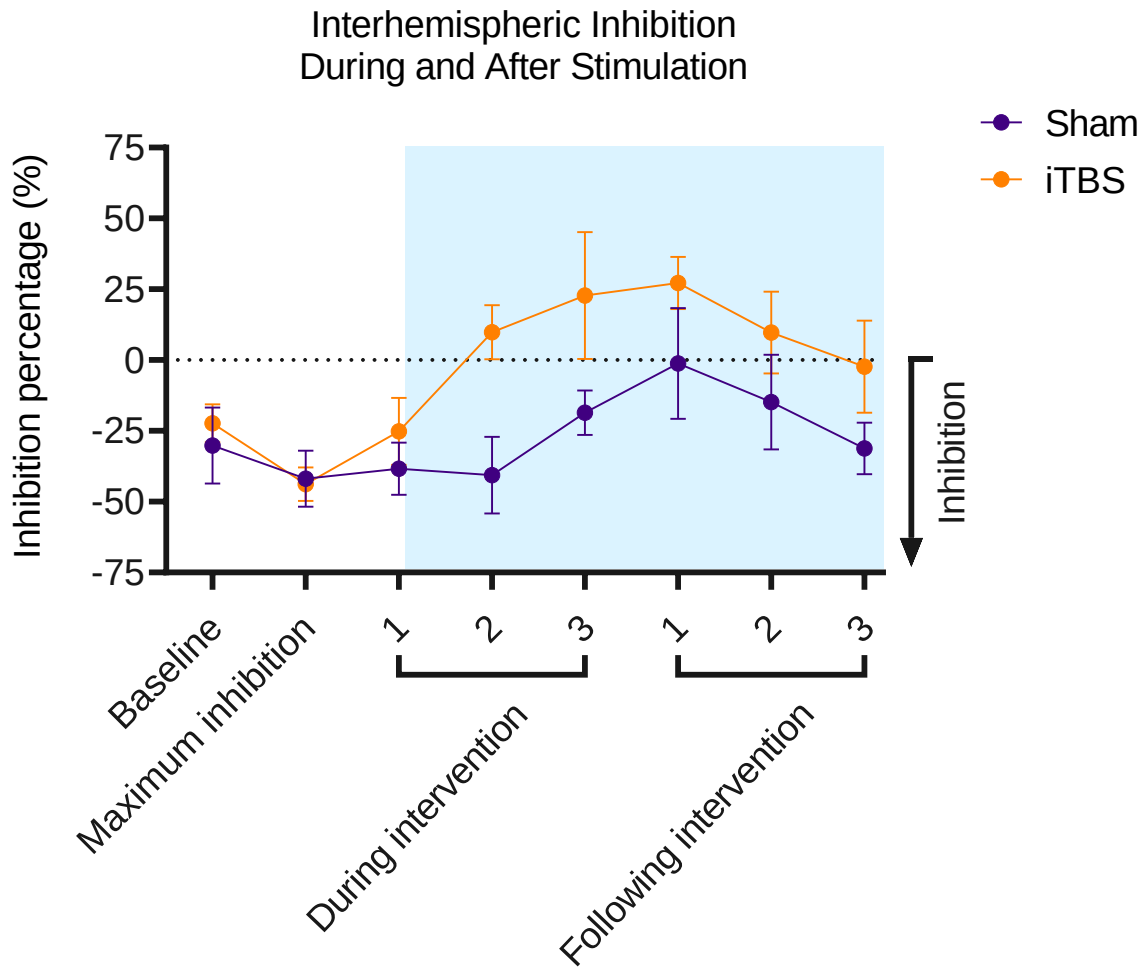

Figure 6.4 Interhemispheric inhibition at baseline, at the point of maximal inhibition in the two weeks after stroke, and both during and after intervention. The blue section highlights results following randomisation to sham or iTBS groups.

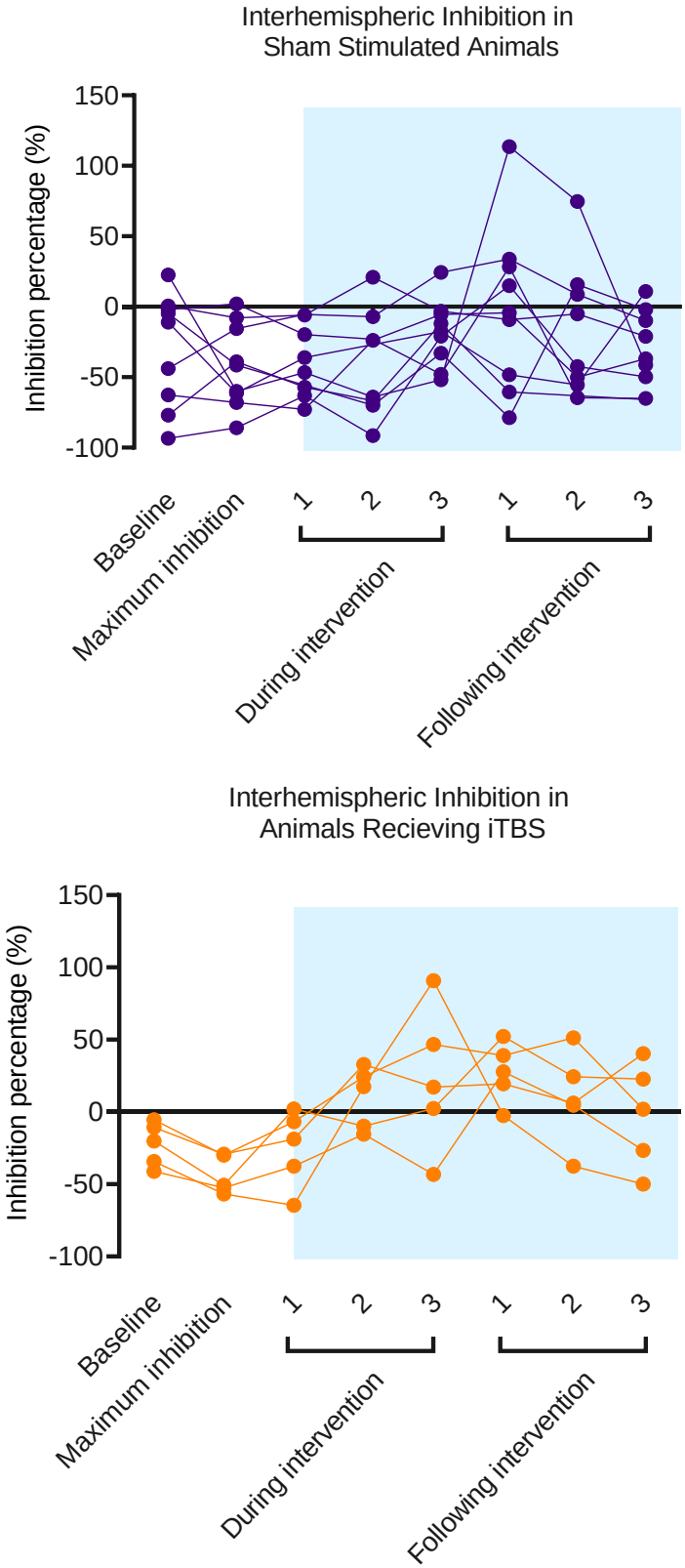

Figure 6.5 Interhemispheric inhibition data for individual animals separated by group for all time points, blue area corresponds to the stimulation and post-stimulation period for iTBS animals.

The data was analysed using a 'ROUT' (robust regression and outlier removal) analysis, which is designed to detect one or more outliers using regression analyses (Motulsky & Brown, 2006). The analysis works from a specified 'false detection rate' which for this study was set at 10%, representing a 10% chance that the removed outlier was incorrectly identified. One data point was identified in the sham group using this technique and was excluded from the analysis presented. A further two data points were visually identified as potential outliers, which contributed to the lower level of IHI at week one and two following intervention in the sham group, but these were not rejected by the ROUT analysis, and so were included in the final data set.

Once this analysis was complete, a mixed effects analysis of group against inhibition for all time points showed a significant effect of group on IHI, $F(1, 12) = 5.37$, $p = 0.039$. When only the data following randomisation was included, enhancing the effect of group on results, a significant result was also found, $F(1, 12) = 8.60$, $p = 0.013$. This is demonstrated clearly in Figure 6.5, where inhibition can be seen to increase following stroke, and then returns towards baseline and eventually becomes undetectable in the iTBS group.

In the sham group on the other hand, inhibition remains high (i.e. below zero) for at least two weeks following randomisation, and average inhibition never becomes undetectable. This finding is shown in greater detail in the individual animal plots in Figure 6.5, which shows the disappearance of IHI in the majority of iTBS treated animals following stimulation. In the sham treated animals, the level of inhibition is variable, without the consistent IHI reduction seen in the iTBS treated animals. This study was not designed to detect interhemispheric facilitation,

so the interpretation of 'positive IHI' is unclear. Rather than facilitation, positive values for IHI should instead be interpreted as in inability to detect IHI at all during testing.

6.7 ASSOCIATIONS BETWEEN BEHAVIOUR AND INTERHEMISPHERIC INHIBITION

When analyses of correlation were performed on grid walk and IHI data for time points following randomisation, the association shown in Chapter 5 was not maintained (change in IHI versus change in grid walk: $p=0.59$, $n=14$). There was also no association between IHI and pasta handling performance in the weeks following randomisation (change in IHI versus change in pasta handling: $p=0.15$, $n=11$). It may be that when the additional variable of stimulation was introduced, and animal numbers were fewer, any association was not strong enough to be detected.

6.8 DISCUSSION OF RESULTS WITHIN THIS SECTION

This chapter met the third objective of this thesis: "To determine whether, in an animal model of stroke, low intensity intermittent theta burst stimulation to the contralesional hemisphere can reduce interhemispheric inhibition and improve behavioural recovery." We showed both behavioural improvements and reduction in IHI after low intensity intermittent theta burst stimulation. Each section of results from this chapter will be discussed in detail, before moving on to a discussion of this body of work as a whole in Chapter 7.

6.8.1 BEHAVIOURAL RESULTS

- **Hypothesis 3.1.1:** That rats receiving iTBS over the contralateral hemisphere will have a greater reduction, from post stroke impairment, in foot faults during grid walking as compared to rats receiving sham stimulation.
- **Hypothesis 3.1.2:** That rats receiving iTBS over the contralateral hemisphere will have a greater increase, from post stroke impairment, in paretic paw use during pasta handling as compared to rats receiving sham stimulation.

We detected a significant increase in grid walk recovery in iTBS treated animals compared to sham. This likely represents an effect on gross motor functional recovery associated with iTBS, as has been previously described (Boddington et al., 2020). The degree of recovery detected was the same as has previously been shown for similar protocols, a 54% recovery of function was shown in both this study and by Boddington et al. (2020) with both studies showing significantly better recovery in iTBS treated compared to sham treated animals. In fact in the present study, the difference compared to sham was even greater, owing to an overall smaller improvement in the sham animals in this thesis (compare 15% spontaneous recovery here with 40% reported by Boddington). In addition to these findings for grid walking impairment, similar improvements were seen for pasta handling performance, representing a novel finding that contralateral iTBS following stroke can improve fine motor behaviour in rats. Both hypotheses 3.1.1 and 3.1.2 were accepted.

6.8.2 INTERHEMISPHERIC INHIBITION RESULTS

- **Hypothesis 3.2** That rats receiving iTBS over the contralateral hemisphere will show a greater reduction, from post stroke inhibition, in interhemispheric inhibition as compared to rats receiving sham stimulation.

The results given confirm the hypothesis that IHI from the contralesional cortex is reduced in animals receiving low intensity iTBS over this cortex. Rather than comparing IHI between sham and intervention animals at a single timepoint at the end of the study, as has been done previously, these experiments utilised repeated measures during the study. This enabled a difference to be detected where other studies have been unable to do so. A second strength of this approach is that the measurements could be made in freely moving animals, which has the potential to better capture the dynamic nature of IHI and eliminates the effect of anaesthesia on the recordings.

A next step for this study could be to pair IHI measurements with a single pellet reaching task, enabling measurements of IHI prior to movement initiation and during performance of the movement to be made, replicating the landmark experiments of Murase et al. (2004). Another approach to this question could be to pharmacologically block the effect of reduction of IHI following iTBS, using chemical inhibition of the predicted $GABA_A$ mechanism in the contralateral hemisphere as a target, e.g. by using a blocker of the cannabinoid receptor type 1 (CB1), AM251 (Barry, Boddington, Igelström, et al., 2014). Then, if IHI and behavioural changes with iTBS are not seen, it is more likely that a $GABA_A$ mediated interhemispheric pathway is involved. Without a better understanding of the mechanisms underlying IHI we cannot determine that the results in this chapter demonstrate a causal relationship between IHI

and behaviour. That said, this research does lend weight to the body of observational evidence from humans linking alterations of IHI with impairment following stroke.

As an additional consideration, recall that IHI was significantly increased in week one following stroke, with a non-significant increase in week two. Boddington found that longer delays between stroke induction and application of iTBS reduced the ability of iTBS to improve behavioural recovery (Figure 6.1). We have hypothesised that the behavioural benefit of iTBS is achieved through reduction of IHI. This would suggest that matching the delivery of iTBS to the time when IHI is greatest (i.e. week one following stroke in rats), would confer the greatest benefit. This was shown by Boddington, in their 'early-start' group, which showed the greatest behavioural improvements (Figure 6.1). Perhaps in the present study, even greater behavioural gains could have been achieved by starting iTBS one week earlier.

Tracking IHI changes in sham stimulated animals also gives an idea of what IHI changes might be expected in animals allowed to recover spontaneously. Figure 6.4 shows a relative increase in IHI from baseline for four weeks after stroke (non-significant effect). This four week period roughly corresponds to the 'sub-acute' recovery period in rats, which occurs approximately 1-3 months after stroke in humans (Vallone et al., 2016). Perhaps these first four weeks represent a window where iTBS intervention is more effective.

6.8.3 RELEVANCE OF HISTOLOGY

Histological results will not be discussed in this chapter. Data shown in Appendix 10 (page 290) show that there was no difference in lesion size between the two stimulation groups

(paired t-test, p=0.42). This excludes differences in lesion size as a potential explanation for any differences in behaviour or IHI. The model by which altering excessive contralateral IHI is predicted to improve behaviour is by allowing the area around the lesion to adopt the functions lost with the initial insult. This model does not rely on any change in lesion size or reforming of neural tissue. Any relevant differences in lesion size, had they been detected, would be unlikely to have a causal role in behavioural results. In addition, IHI was shown not to be associated with lesion volume in Chapter 5, excluding this as an explanation for the change in IHI that is shown.

6.9 SUMMARY AND CONCLUSIONS

This chapter concludes the description of results obtained during this study. These three results chapters have outlined the development of methods to measure interhemispheric inhibition in a minimally invasive fashion, and the application of this technique in a rat stroke model. In addition, they have described behavioural and electrophysiological changes associated with stroke induction, including an acute increase in interhemispheric inhibition following stroke. Finally, intermittent theta burst stimulation was shown to both improve functional recovery at a gross motor task, and to decrease interhemispheric inhibition. The final chapter of this thesis will discuss these findings.

7 DISCUSSION AND CONCLUSIONS

This discussion will consider conclusions made based on the experimental results reported herein and suggest future directions for researchers in this field. Each results chapter has contained individual discussion of the methods employed, and how each result might relate to the relevant literature, as well as any strengths and weaknesses in the approach taken, so this will not be repeated here. To demonstrate further the significance of our findings and highlight necessary areas for future work, three topics discussed within this thesis will be examined in more detail. The first is a closer evaluation of the literature relating to anatomy and physiology of the interhemispheric inhibition pathway, the second a discussion of the relationship between interhemispheric inhibition and post stroke functional impairment and thirdly a consideration of both the neuromodulation technique employed and the significance of this thesis in the field of neuromodulation for stroke.

7.1 STRUCTURAL AND ELECTROPHYSIOLOGICAL CORRELATES OF INTERHEMISPHERIC INHIBITION

One weakness of the experiments in this thesis was an uncertainty that the IHI measured here is the same as that identified in humans by Ferbert et al. (1992) and later measured in rats by Barry, Boddington, Igelström, et al. (2014). The experiments in Chapter 4 aimed to address this by showing characteristic similarities between our measured IHI and the literature. However, uncertainty remains, given that the underlying components of this pathway remain unknown. Building on the information in Chapter 2, the literature relating to this pathway is examined here in more detail to highlight areas that warrant future investigation.

Research from the early 90s examined closely the various receptor subtypes of the motor cortex, including those involved in transcallosal responses. In particular Kawaguchi (1992) studied the receptors involved in callosally evoked excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs). He found that early IPSPs in slices from the rat cortex occurred at a latency of approximately 30 ms and were mediated by $GABA_A$ receptors. Blockade of $GABA_A$ with picrotoxin or bicuculline caused the early IPSP to disappear and enhanced the later IPSP (which was found to be $GABA_B$ mediated). This effect was measured in layer V of the cortex.

The proposed pathway behind this inhibition was the activation of a callosal excitatory neuron (recorded as a very early EPSP) synapsing onto the dendrite of an inhibitory interneuron in the superficial cortical layers (seen as either early or late IPSPs) and is comparable to the model shown in Figure 2.5. This excitatory synapse was investigated with the use of N-methyl-D-aspartate (NMDA) and non-NMDA receptor antagonists. Early IPSPs were reduced by low concentrations of cyanquixaline, a non-NMDA receptor antagonist, and abolished at higher concentrations. EPSPs and later IPSPs (at a latency of 185 ms) were more sensitive to non-NMDA antagonists and showed some reduction in amplitude in the presence of NMDA antagonists. This suggests that different interneurons are involved in the production of early and late IPSPs. These experiments were later replicated in the mouse by Spalletti et al. (2017).

Palmer et al. (2012) specifically looked in the rat at the anatomic components of long latency interhemispheric inhibition (LIHI) occurring at a latency of 400 ms in response to sensory stimulation of the ipsilateral hind paw (i.e. the hind paw with inputs to the hemisphere opposite to the recording hemisphere). Like late IPSPs, this LIHI was shown to be $GABA_B$ and non-

NMDA receptor mediated and was inhibited by $GABA_B$ receptor antagonists and by cyanquixaline.

The proposed anatomical arrangement of this pathway, based on data from intracellular recording techniques, is shown in Figure 2.5, and consists of a transcallosal glutamatergic neuron, synapsing in layer I onto a $GABA_B$ inhibitory interneuron with a cell body in layer V. These findings seem to suggest that late IPSPs and LIHI could be mediated by the same pathways. Perhaps then, early IPSPs and short latency IHI (SIHI) are also mediated by the same pathway as one another. If this were the case, one would expect SIHI, as measured in this thesis, to be inhibited by $GABA_A$ antagonists, and by high concentrations of cyanquixaline. Future researchers interested in the mechanism underlying SIHI could conduct this experiment, perhaps in the context of *in vivo* or slice recordings from layer V neurons.

The SIHI pathway also has a characteristic low threshold for activation within the contralateral hemisphere. This has been shown by Barry, Boddington, Igelström, et al. (2014), and Boddington et al. (2020), and within this thesis. Only conditioning stimuli at low intensity were shown to produce inhibition of the ipsilateral response. Note that the threshold for activation of LIHI has not been reported previously, so cannot be compared. The most likely explanation for this is that low intensity stimulation of the contralateral cortex activates a population of low threshold neurons that synapse onto $GABA_A$ inhibitory interneurons, resulting in measurable inhibition of the normal response of the ipsilateral cortex. However, when stimulation intensities are higher, either these neurons are no longer activated, or they are activated in addition to a population of transcallosal neurons that have a downstream excitatory effect and counteract the initial inhibition. Again, these hypotheses could be scrutinised with recordings

from layer V neurons measuring the effects of high and low intensity transcallosal stimulation. The addition of pharmacological blockade of $GABA_A$ and glutamate receptors could help distinguish where in this putative pathway the different effects of high and low intensity stimulation are occurring.

When using TMS to measure IHI in humans, it is more challenging to create the precise conditions that allowed the difference between high and low intensity conditioning stimuli to be uncovered. It is likely that both excitatory and inhibitory circuits are activated by TMS pulses, rather than pure activation of the IHI pathway. Ipsilateral silent period measurements again represent activation of an independent pathway, which is more likely to be related to late IPSPs than to SIHI. This provides a potential explanation for the variation in conclusions reported in the human literature discussing changes in IHI following stroke. In addition, this demonstrates the value of developing a more precise tool for measuring IHI in humans, such as using electrical stimuli rather than the less targeted TMS pulses to conduct bilateral paired-pulse measurements (Appendix 11, page 291).

7.2 RELATIONSHIP BETWEEN INTERHEMISPHERIC INHIBITION AND IMPAIRMENT FOLLOWING STROKE

In the present study, greater IHI was shown to be weakly associated with functional impairment on the grid walk task. Another component explaining the variability in functional recovery was lesion volume, however, after accounting for this, over 50% of the variability in impairment remained unexplained. In human research, the link between increased IHI following stroke and functional impairment remains controversial. Some research suggests that the additional inhibition prevents those with less severe impairment from achieving full recovery, which was

the rationale behind the present study (Bertolucci et al., 2018; Du et al., 2018; Murase et al., 2004; Volz et al., 2017). Others suggest that it may be a compensation for or a consequence of incomplete recovery (Xu et al., 2019).

7.2.1 INCONSISTENCIES IN INTERHEMISPHERIC INHIBITION LITERATURE

The role of IHI in stroke recovery was called in to question in the recent paper by Xu et al. (2019). They measured IHI on to the stroke affected side immediately prior to movement initiation with the paretic limb, compared this to baseline IHI and expressed this as ΔIHI. They observed that pre-movement IHI increased over the course of recovery from stroke while behavioural impairment improved, implying that increased IHI was not driving behavioural impairment. They also found no correlation between behavioural measures and IHI in either the acute or chronic phase. The participants in the study were assessed for behavioural impairment using the Fugl-Meyer Motor Assessment (FMA) and were found to have a mean impairment score was 41/66, representing mild-moderate impairment. Individual data for the degree of recovery by 12 months was not given, but participants had a mean FMA of over 60/66 at 12 months. This minimal level of motor impairment overall may limit the conclusions that can be drawn from correlations of the two measures, especially during the chronic phase of the study.

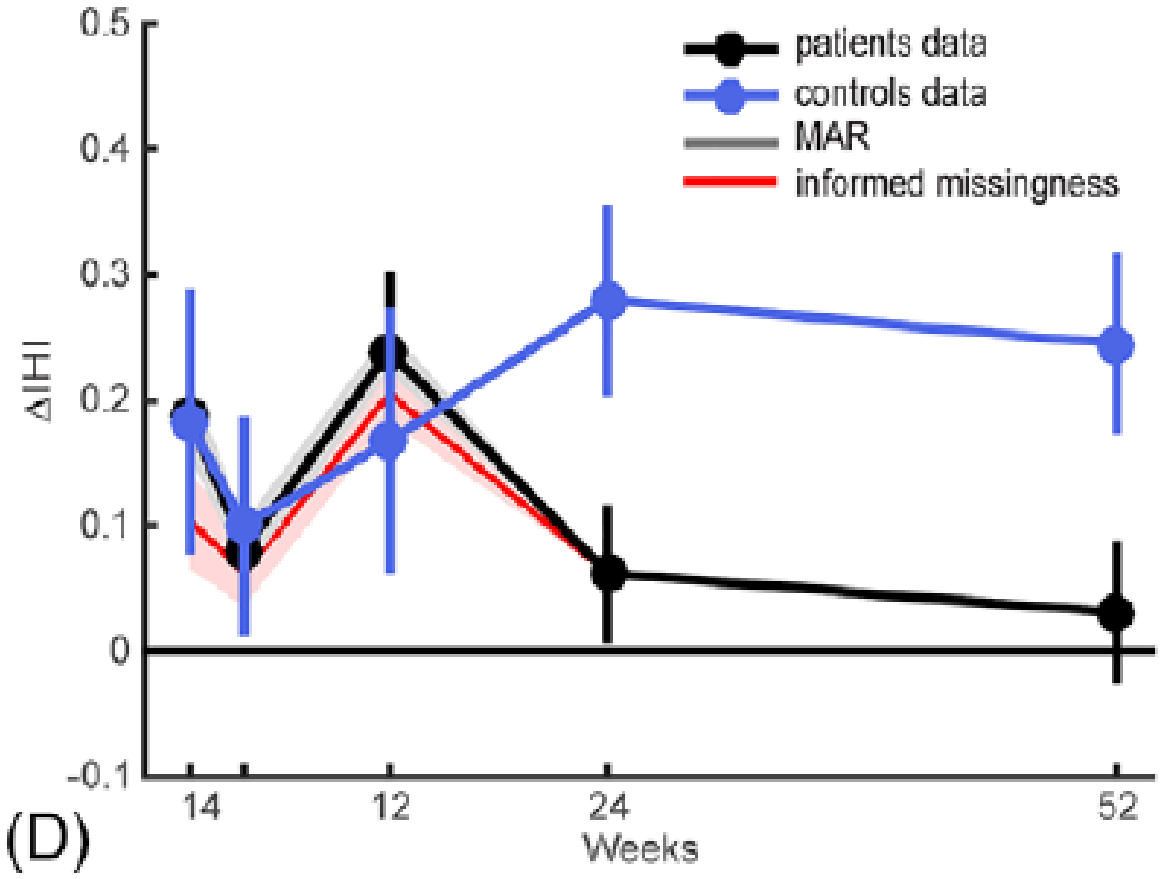

Figure 7.1 Evolution of ΔIHI for patients and controls over a 1-year period. Patients showed close to control level of ΔIHI in the acute/subacute periods ([week]1–12), but their ΔIHIs became abnormal at the chronic stage. Shaded plots in grey and red are sensitivity analyses. Copied without modification from Xu et al. (2019). (MAR: missing IHI values arose at random.)

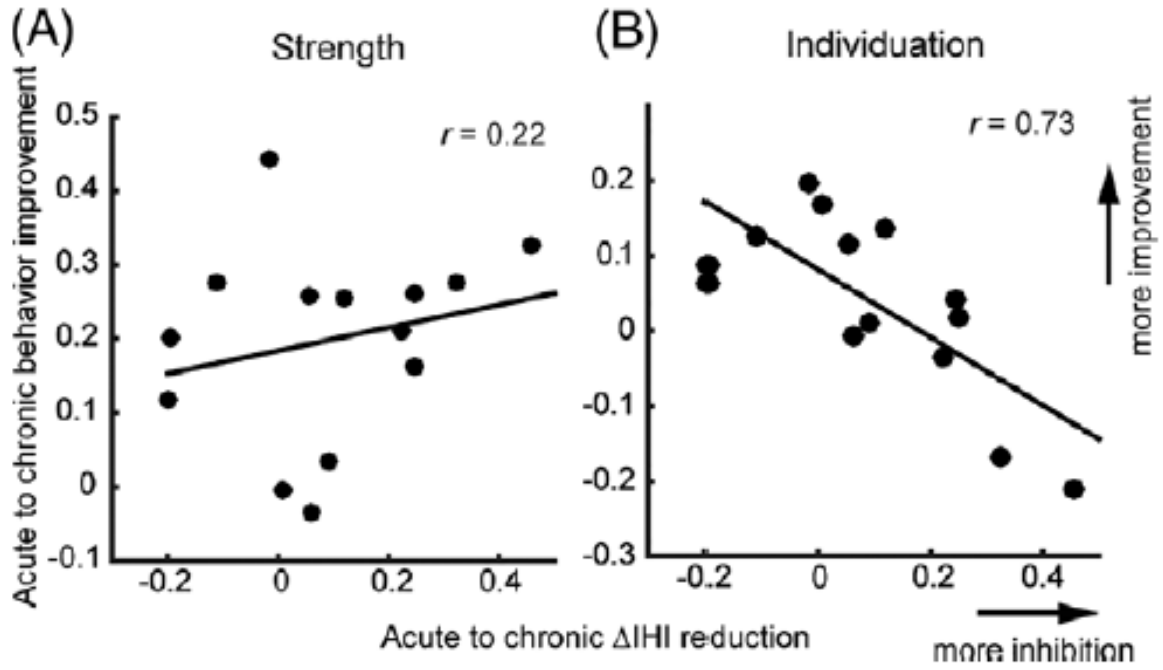

Figure 7.2 Correlations between the reduction of premovement IHI (ΔIHI) from acute/subacute to chronic stages and the amount of behavioural recovery: (A) Strength; (B) Individuation. x- and y-axes are the mean differences between chronic and acute/subacute behaviour measures and ΔIHI, respectively. Copied without modification from Xu et al. (2019), Figure 5(D)

Another aspect of the findings of Xu et al. (2019) that complicates interpretation of their results is the IHI changes seen in control participants. The variation in ΔIHI shown in the control group is nearly equal to that seen in the stroke affected group, without an explanation for this provided, nor its statistical significance commented on (Figure 7.1). The researchers draw the conclusion that, as pre movement IHI is increased in the chronic phase of stroke, at the point where behavioural impairment is less, that IHI does not play a role in behavioural recovery. They fail to account for the fact that control and patient pre movement IHI is also increased, to a similar degree to the later patient increase, during the first two weeks of the study, without listing any factors that could account for this.

This result may indicate problems with the measurement of IHI in this study accounting for the changing control participant readings. For example, in this study, TMS pulses were used to measure IHI, which, as we have suggested previously, may not be able to specifically target the low threshold inhibitory pathway responsible for SIHI. This may also indicate that there are two phases of increased IHI during recovery from stroke, accounting for the results seen by Xu in stroke patients. What is clear however is that development of IHI after stroke is complex, and results may vary widely depending on methodology.

One conclusion that the researchers did make in favour of the interhemispheric imbalance hypothesis was based on the finding that the development of more pre-movement IHI was associated with poorer recovery of individualised finger movements (Figure 7.2). This task scrutinises the control participants have over individual movements of their fingers. This correlation with a task that required coordination of fine movements is consistent with the hypothesis that IHI plays a role in motor coordination, rather than just strength (which did not

show the same correlation). The researchers conclude that this represents a common pathway affecting both IHI and fine motor recovery. Other potential factors in this pathway will now be discussed.

7.2.2 ADDITIONAL FACTORS FOR CONSIDERATION IN THE INTERHEMISPHERIC INHIBITION-RECOVERY RELATIONSHIP

In their rat studies, Boddington et al. (2020) showed a relationship between IHI and functional impairment, but only when excitability was included as an intermediary factor. This demonstrates a complex relationship between ipsilateral excitability (which was not measured in this thesis), contralateral IHI and stroke impairment. Here, the potential relationship between these factors will be discussed, as well as other elements that may play a role.

The measure of excitability used by Boddington et al. (2020) was post synaptic potential (PSP) amplitude. This provides a measure of the responsiveness of the cell to a monosynaptic input of fixed intensity and represents a measure of cellular excitability (intrinsic response to a synaptic input) not related to action potential generation. Cells with greater excitability are more likely to generate an action potential due to the increased membrane potential in response to stimuli. Therefore, upstream signals from other areas of the cortex, e.g. the pre-motor cortex, are more likely to generate outputs from an area of cortex that is more excitable. This is a crucial part of movement generation, and one explanation of the observed link between PSP amplitude and behavioural recovery seen by Boddington. In humans, Du et al. (2018) also showed a link between ipsilesional excitability measured using fMRI and hand function.

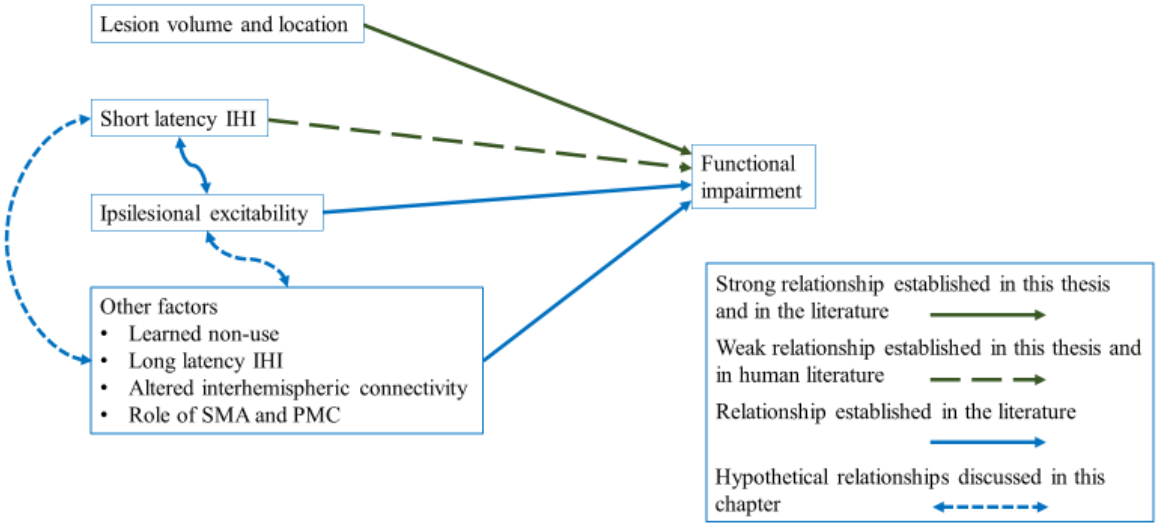

Figure 7.3 Simplification of established and proposed relationships between variables discussed in this chapter. Note that none of the relationships depicted, except for lesion volume, have been shown to be directly causal based on strong evidence.

Boddington also observed a link between PSP amplitude and IHI from the contralateral hemisphere. Inhibitory inputs, such as from interhemispheric inhibition, produce inhibitory post synaptic potentials which acutely alter the response of the stimulated area. However, changes in resting excitability require longer term changes in the neural tissue. The link between IHI and PSP amplitude indicates a process of change in the ipsilesional tissue that may be either as a result of, or the cause of, altered IHI. Several theories are proposed here and discussed to help unravel the potential nature of this relationship. As excitability has been shown to have a strong relationship with impairment, discussion of these theories also includes potential relationships between increased IHI and behavioural impairment. The relationships under discussion are listed below and represented in simplified format in Figure 7.3.

7.2.3 PROPOSED THEORIES RELATING TO RELATIONSHIPS BETWEEN INTERHEMISPHERIC INHIBITION, EXCITABILITY AND FUNCTIONAL RECOVERY

a) Interhemispheric imbalance model: stroke causes reduced ipsilateral excitability, which results in reduced inhibition from the ipsilateral to contralateral side, causing the contralateral inhibition to act unopposed. This could result in the strengthening of the IHI pathway from the contralateral side (see Figure 2.3). It is hypothesised that this increased inhibition impairs recovery of ipsilateral functions.
b) Additional factor(s) result in both increased SIHI and decreased ipsilesional excitability, as well as limiting behavioural recovery (Figure 7.3). Some potential factors that could be implicated are:
    a. Learned non-use of the paretic limb,
    b. Long latency IHI,

c. Altered neural networks, for example disrupted interhemispheric connectivity or altered coherence,
d. Stroke effects on the premotor cortex and the supplementary motor area.

7.2.4 WEAKNESSES OF THE INTERHEMISPHERIC IMBALANCE THEORY

Theory a) is a repetition of the interhemispheric imbalance theory discussed in the introductory Chapter 2. This relationship would explain the relationship between IHI and behavioural impairment, and the relationship between excitability and both impairment and IHI. In summary, as a result of anatomical or other damage following stroke, contralateral inhibition can act unopposed. This increase in inhibitory inputs from the contralateral side causes long term changes to the perilesional tissue which impede recovery (see Figure 2.3).

However, it is not known whether IHI increases first, causing both behavioural impairment and reduced excitability, or whether decreased excitability is the initiating factor causing both increased IHI and impairment. Confusingly, the relationships between excitability and IHI and that between excitability and impairment are strong, yet that between IHI and impairment is weak. Conversely, using iTBS over the contralateral hemisphere to target IHI consistently results in behavioural improvements and, as has been shown in this thesis and by Barry, Boddington, Igelström, et al. (2014), can drive a reduction in IHI. In addition, directly inhibiting the contralateral hemisphere with GABA antagonists has been shown to produce functional improvements in other rodent work (Clarkson et al., 2010; Spalletti et al., 2017). These results imply a more direct relationship between IHI and behaviour. Additional experiments are needed to probe the timeline of this relationship and the dependence of each

factor on the others. In the absence of answers to these questions, this discussion will also mention other factors that were identified in the literature that may complicate this relationship.

7.2.5 ROLE OF LEARNED NON-USE OF THE PARETIC LIMB

Other elements have a potential role in both the development of increased IHI and the establishment of functional impairment. One of these is learned non-use of the paretic limb, or alternately, learned overuse of the non-impaired limb. Each time the non-paretic limb is used, unopposed SIHI from the 'healthy' hemisphere would theoretically be triggered, acting on the lesioned hemisphere (Murase et al., 2004). In the healthy brain, this would be a helpful physiological reaction to prevent mirror movements (Beaulé et al., 2012; Koerte et al., 2010; Mayston et al., 1999). However, without the corresponding use of the impaired limb and by extension the lack of SIHI onto the healthy hemisphere, this could result in the development of imbalanced IHI.

The neglect of the impaired limb will also limit the ability for the perilesional tissue to obtain the lost functions of the lesioned area, resulting in both reduction in excitability and behavioural impairment, and this has been shown in both humans and animal models (Abdullahi, Truijen, & Saeys, 2020; Jones, 2017; Starkey et al., 2012; Takeuchi & Izumi, 2012). We know from the work in this thesis that rats reduce the use of the lesioned limb following stroke, as demonstrated by the pasta task. In humans, limiting the use of the healthy limb with constraint therapy has been shown in some studies to improve recovery of the impaired limb (Corbetta, Sirtori, Castellini, Moja, & Gatti, 2015; Langhorne et al., 2009; Stroke Foundation, 2017). In addition, a recent review showed links between constraint induced movement therapy and reduction of IHI, as well as links between this therapy and other

biomarkers of stroke recovery such as improved perfusion and metabolism and decreased level of GABA (Abdullahi et al., 2020). This indicates that avoiding development of unopposed contralesional IHI is one of the mechanism by which this constraint therapy is effective, as well as indicating a tangential link between IHI and other biomarkers of stroke recovery.

7.2.6 ROLE OF LONG LATENCY INTERHEMISPHERIC INHIBITION

A second possible mechanism by which increased contralateral IHI is related to reduced excitability requires us to consider again the different roles of LIHI and SIHI: the former a tonic inhibition acting to maintain hemispheric balance at rest, and the latter a movement related pathway that acts to control unimanual movements (R. Chen et al., 2003; Uehara et al., 2014). Stroke has been shown to cause increases in both LIHI and SIHI as measured by iSP and bilateral paired pulse techniques respectively (Duque et al., 2005; Murase et al., 2004; Takechi et al., 2014). Spalletti et al. (2017) showed reduced ipsilesional excitability and an increase in $GABA_B$ dependent contralateral inhibition (LIHI) following stroke. The reduction in amplitude of the PSP in those animals with stronger SIHI in Boddington's work could indicate an increase in tonic or $GABA_B$ dependent inhibition from the contralateral hemisphere that relates to both increased SIHI and decreased excitability.

A pathway for background inhibition from the contralateral hemisphere would be difficult to study, because by stimulating the suspected pathway, any effect recorded would no longer be a representation of the background state of the brain. However, it has been previously suggested that LIHI represents something close to background inhibition, in that it maintains the balance of inhibition between the hemispheres when one limb is tonically active (R. Chen et al., 2003; Uehara et al., 2014). Perhaps the effect of stroke is to increase both LIHI and SIHI from the

contralateral hemisphere, the former affecting baseline excitability, and the latter affecting active movement generation. This could explain the difficulty in interpreting the literature when iSP and bilateral paired pulse measurements are used somewhat interchangeably.

7.2.7 ROLE OF INTERHEMISPHERIC CONNECTIVITY

Functional connectivity is a concept described in the literature that relates to both stroke recovery and interhemispheric interactions. It is a description of the temporal correlation between the electrical or haemodynamic measures of brain activity from different brain areas (Westlake & Nagarajan, 2011). The assumption is that this correlation in activity represents a functional association between the areas, comprised of both anatomical connection and physiological cooperation. Descriptions of functional connectivity are simplified by modelling the brain as a collection of nodes, or functionally distinct brain regions, for example the primary motor cortex (M1) could be considered a node. The connections between multiple nodes are known as networks.

The various changes in functional connectivity following stroke have been studied extensively. A helpful review of this literature can be found in Westlake and Nagarajan (2011). Changes in functional connectivity after stroke are related not only to the area of brain that is lost due to ischaemia, but to a reorganisation of neural networks outside the stroke affected area. For example, research has shown a shift towards less efficient, more randomly organised neural networks following stroke, an element of recovery that could impair motor performance but not be captured by measures of IHI (L. Wang et al., 2010). Interestingly, primarily those changes in functional connectivity that related to interhemispheric connectivity, rather than changes confined to within one hemisphere, were shown to be directly associated with recovery

(Carter et al., 2010). These changes could represent a component of the relationship between functional impairment, excitability and changes in IHI that was not captured by the experiments in this thesis.

Disruption in connectivity between the hemispheres in the resting state, resulting in reduced connectivity between the two primary motor cortices has been shown to be associated with poor performance after stroke in human participants (Carter et al., 2010; Peng, Liu, Hua, Liang, & Yu, 2019; van Meer et al., 2012; van Meer et al., 2010). This was further characterised by Du et al. (2018) who showed with fMRI that inhibitory connectivity from the contralesional side was associated with functional impairment. This disruption perhaps reflects the imbalance in inhibition from the contralesional side at rest that was proposed earlier in discussions of the role of LIHI and the interhemispheric imbalance model.

Another aspect of functional connectivity is interhemispheric coherence, a measure of the covariance in phase and amplitude of electrical oscillations from different areas. Coherence correlates with behavioural recovery at certain frequencies, e.g. low gamma frequency, and is related to excitability (Bowyer, 2016; Schjetnan, Gidyk, Metz, & Luczak, 2019). Interhemispheric inhibition has been shown to increase when hemispheres are more coherent in the mu band, and when showing more excitable rhythms overall (Stefanou, Desideri, Belardinelli, Zrenner, & Ziemann, 2018). Interestingly, coherence can be modulated by bilateral electrical stimulation to produce lasting improvements in behaviour following stroke in the rat (Schjetnan et al., 2019). Coherence changes could therefore be considered as an alternative mechanism to explain the changes seen with stimulation in this study. However, the

unilateral nature of the stimulation in the experiments for the present study makes this less likely.

The experiments in this thesis were designed to test inhibition in freely moving animals, not at rest, so the role of resting state connectivity on both performance and interhemispheric inhibition cannot be evaluated. The transcallosal connection has a predominantly inhibitory role (Ferbert et al., 1992), so changes in connectivity and coherence across this pathway due to stroke will also result in changes to interhemispheric inhibition (Stefanou et al., 2018). One relationship to consider is whether stroke impairment is due to increased IHI resulting from changes in connectivity. Future researchers in this area should consider including measurements of resting state connectivity and coherence in their methodology to investigate the existence and nature of these relationships. In addition, coherence changes may represent a target for intervention using the techniques explored in this thesis.

7.2.8 ROLE OF THE PREMOTOR CORTEX AND SUPPLEMENTARY MOTOR AREAS

The literature relating to functional connectivity and coherence also emphasises the important roles of the contra and ipsilesional premotor cortices (PMCs), and supplementary motor areas (SMAs), see Figure 7.4. Again, these areas may have a role to play in the relationship between IHI, excitability and functional recovery. The PMC plays a role in coordinating more complex movements involving multiple muscle groups, such as a reaching action (Kandel et al., 2000). For example, immediately prior to movement onset, the PMC is seen to act in a coordinated manner with M1, with inhibition during movement preparation, releasing immediately prior to movement (Kroeger et al., 2010; Liuzzi et al., 2010). The SMA is involved with bilateral movement coordination and has some role in movement initiation (Kandel et al., 2000).

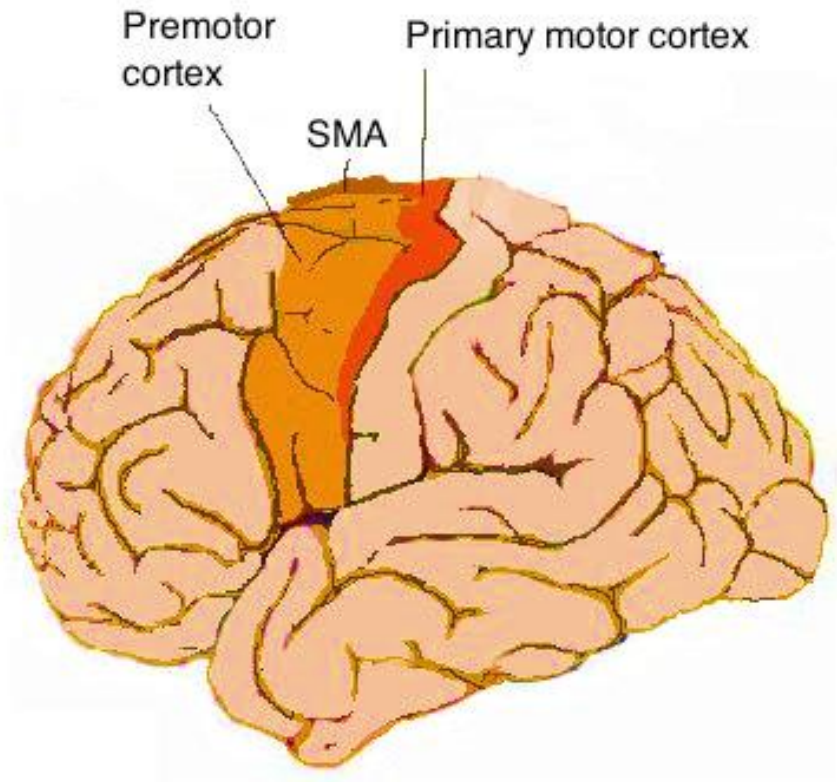

Figure 7.4 Location of the primary motor cortex (red), premotor cortex (orange) and SMA (brown) in the human brain. Adapted from image in the public domain: https://commons.wikimedia.org/wiki/File:Human_motor_cortex.jpg.

One simple way these additional motor areas could have relevance to this thesis is to consider whether one of the relevant functional 'nodes' was affected by the lesion itself. Given the heterogeneity of the lesions obtained with endothelin-1 in the present study, it is possible that in some animals the SMA or PMC was directly affected, causing greater functional impairment than would be expected for a lesion of that volume, as has been shown in humans (Alstott, Breakspear, Hagmann, Cammoun, & Sporns, 2009). The histological tools employed in this study were designed only to capture the extent of the lesion obtained and compare this to a generic model of the rat brain. This did not allow the variation in lesions to be aligned with the specific functional area affected beyond demonstrating a focus within M1, however this is unlikely to play a significant role, as most of the lesions were small and would not have affected multiple functional areas. Mapping of the functional areas affected by the lesion, for example with fMRI, could be utilised in future work to define the specific contributions of each area to functional recovery.

In addition to direct effects of stroke on the SMA or PMC, stroke can also have indirect effects on the function of these areas via network reorganisation. For example, those individuals who show greater task-related activity in the SMA following stroke show greater residual impairment, in one group studied (Quandt et al., 2019). This could perhaps represent maladaptive plasticity within the SMA contributing to residual impairment, or a reliance on areas outside M1 in less well recovered subjects. The connectivity between M1 and SMA is also shown to be altered following stroke, however this has not been shown to correlate with functional deficit (Gerloff et al., 2006; Grefkes et al., 2008; Serrien, Strens, Cassidy, Thompson, & Brown, 2004; Strens et al., 2004). However, changes in ipsilesional SMA and PMC connectivity with contralateral SMA, PMC and M1 do have a relationship with performance. Disruption of the connection between the left and right SMAs in the active and

resting state correlated with worsened motor performance in a similar way to the disruption between the bilateral M1s (Carter et al., 2010; Gerloff et al., 2006). In addition, increased information transfer from ipsilesional PMC to contralesional PMC correlated with better recovery, while the reverse was true of connectivity from contralesional to ipsilesional PMC (James et al., 2009).

The collaboration between different ipsilateral and contralateral areas was also important. For example, in bilateral movements, performance was worse when ipsilesional SMA failed to facilitate the activity of contralesional M1 (Grefkes et al., 2008). It is possible that the increased IHI measured in the present study is also capturing changes in these networks external to the M1-M1 connection, confounding the link between IHI and behavioural recovery. These networks may represent a pathway working in parallel with IHI to either impede or improve recovery. While complicating research in the field of IHI, techniques used in the present study to modulate IHI could potentially target these parallel pathways at the same time as addressing IHI and represent an interesting avenue for future investigation.

7.2.9 CONCLUSIONS ABOUT THE ROLE OF INTERHEMISPHERIC INHIBITION IN BEHAVIOURAL IMPAIRMENT

The above discussion has proposed that the interhemispheric imbalance hypothesis proposed in Chapter 2 to explain the role of IHI in behavioural impairment is likely to be too simplistic to capture the complexity of the relationship. Additional factors in this relationship, most importantly the role of ipsilesional excitability in the causal pathway, must be examined in order to fully understand the role of IHI in impairment. The brief description of other potential

components within this relationship also provide some ideas to explain the residual variation in impairment seen after lesion volume and IHI have been accounted for.

7.3 ANALYSIS OF THE CHOSEN INTERVENTION

7.3.1 COMPARISION OF NEUROMODULATION WITH MAGNETIC AND ELECTRICAL STIMULATION

Compared to transcranial magnetic and direct current stimulation, electrical stimulation has a distinct advantage which may explain its success where other techniques have struggled to show consistent results. Transcranial magnetic stimulation techniques have been shown to activate a large area of dendrites, while locally applied electrical stimulators have a more local effect on cell bodies (Day et al., 1989). It is quite possible that the mechanism of action of electrical stimulation in inducing plasticity is different to that of TMS, given this difference seen in recordings of single stimuli. One possible explanation for this could be that this more local effect allows the targeting of specific areas, such as the motor area homologous to the lesioned cortex that is suspected to be the source of increased interhemispheric inhibition, without distorting this with the activation of surrounding regions. In addition, the fact that the electrode can be implanted below the level of the skull allows a greater specificity of the intensity of stimulation delivered. Barry, Boddington, Igelström, et al. (2014) showed that this was crucial in producing the inhibition of IHI found in their work, with only low intensity iTBS resulting in abolition of IHI.

7.3.2 CURRENT UNDERSTANDING OF THE INTERACTION BETWEEN INTERMITTENT THETA BUST STIMULATION AND INTERHEMISPHERIC INHIBITION

One question that remains unanswered is exactly how the contralaterally applied iTBS, delivered at low intensity, is interacting with IHI to result in both reduced IHI and behavioural recovery. Intermittent TBS is not typically thought of as an inhibitory paradigm, in fact it is usually thought to have the opposite effect (Di Lazzaro et al., 2008; Gamboa et al., 2011; Y. Z. Huang et al., 2005). However, this thesis and previous related work has demonstrated an inhibition of inhibition by low intensity iTBS that lasts beyond the period of stimulation (Barry, Boddington, Igelström, et al., 2014; Boddington et al., 2020).

This was shown at the cellular level by Barry, Boddington, Igelström, et al. (2014), who further demonstrated that the depression of contralateral neurons by iTBS was cannabinoid receptor type 1 dependent. This cannabinoid receptor is involved pre-synaptically in the regulation of plasticity, and acts in a retrograde fashion to inhibit synaptic transmission (Harkany, Mackie, & Doherty, 2008; Hashimotodani, Ohno-Shosaku, & Kano, 2007). Thus, the potential mechanism of low intensity iTBS is to induce retrograde or self-suppression of the IHI pathway through induction of endocannabinoid-CB1 signalling pathways which inhibit the transcallosal pyramidal neuron (Harkany et al., 2008). Neurons affected by CB1 are either GABAergic or glutamatergic, which corresponds with the putative IHI pathway that has been described earlier (Figure 2.5).

The next step in this work is to investigate how low and high intensity iTBS affect this signalling pathway differently, perhaps by recording within a transcallosal glutamatergic neuron with single neuron recording techniques and recording the effect of high and low

intensity iTBS, and the effect of pharmacological activation and inactivation of CB1 receptors. Indeed, there may even be effects of iTBS on pathways that have not yet been considered, such as involving oligodendrocytes or other glia, alterations of learning patterns or alterations in neurotransmitter or receptor expression (Cullen et al., 2019; Deng, Lisanby, & Peterchev, 2013; Tang et al., 2018). This would add to the understanding of the physiology of both IHI and iTBS.

7.4 GENERALISABILITY OF THIS THESIS

As introduced in Chapter 1, the aim of research such as this is to investigate strategies for addressing the disease burden of stroke impairment in the human population. While the individual results chapters have described the internal validity of the results, it is also important to consider whether these results have significance for the human population. To this end, many of the methods in this study have been designed with translation to human work in mind.

The main strength of this research in comparison to previous studies was the use of serial measurements of short latency IHI (SIHI) before and after stroke onset, and during and after delivery of an intervention designed to modulate IHI. This study design is unique to the best of our knowledge. Crucially we were able to measure the effect of stroke on IHI, which is not possible in human patients where there are no measurements of IHI made pre stroke. This allowed us to identify an acute rise in IHI following stroke, which was weakly associated with behavioural impairment. Human studies in this field rely on comparison with healthy controls, and have made a variety of contradictory conclusions about the role of IHI in functional recovery (Bütefisch et al., 2008; Du et al., 2018; Duque et al., 2005; Murase et al., 2004; Stinear et al., 2015; Takechi et al., 2014; Xu et al., 2019). In our study of anaesthetised rats, we showed

comparisons with healthy controls to be unreliable, and only detected an acute increase in IHI following stroke when comparisons with pre-stroke IHI were made within animals.

Spalletti et al. (2017) used optogenetic stimulation in mice during stroke recovery to examine the transcallosal response and found with field potential measurements, and CSD analysis that inhibition from the intact to lesioned hemisphere was increased at day 30 after stroke. The measured inhibition was long latency and $GABA_B$ dependent. The present study adds to this work, by using bilateral paired pulse recordings to demonstrate that short latency inhibition measured from the intact to lesioned hemisphere was also increased one week following stroke.

With the exception of Spalletti et al. (2017), who used pharmacological blockade to demonstrate reduction of transcallosal inhibition and a return to pre-stroke behaviour, this study is also unique in making a series of bilateral paired pulse IHI measurements following intervention in an animal model. In most other animal studies aiming to target IHI, measurements are typically made at the conclusion of the intervention period, giving a static measure of IHI at one time point, in an anaesthetised animal (Barry, Boddington, Igelström, et al., 2014; Boddington et al., 2020). The methodology in the present study eliminates the effects of anaesthesia (which was shown in Chapter 4 to have a significant effect on raw measures of IHI) and allows changes over time to be detected. This increased the sensitivity of the present study, allowing the detection of changes which might have been overlooked if IHI was measured at a single time point.

Other components which also increase the translatability of the results within this thesis have been discussed in Chapter 2, including the use of the endothelin-1 model of stroke, the inclusion

of two behavioural tasks (one gross motor and one fine motor), and the use of EEG recordings rather than more invasive intracellular recordings. Chapter 2 also explained the strengths and weaknesses of using a more targeted electrical stimulation approach for both measurement and modulation of IHI, as compared to traditional magnetic stimulation and transcranial direct current stimulation techniques.

The primary weakness of this study relating to translatability of the results are the significant differences between a rat model of stroke and human stroke patients. Rats experience a greater and faster degree of spontaneous recovery without rehabilitation compared to humans. This means that any alterations in functional recovery, especially from a small volume stroke, are superimposed on a large amount of spontaneous functional recovery (Karthikeyan et al., 2018). The effect of any intervention to modulate this recovery can therefore be difficult to detect. In saying this, the results presented here did demonstrate greater functional recovery in those rats treated with iTBS, especially in the gross motor grid task.

Furthermore, it is difficult to determine the equivalent dose, intensity and timing of stimulation that could produce equivalent benefit in humans. This was touched on in Chapter 2, where we mentioned that no studies have been done that compare the degree of functional impairment in animals models and human patients (Jickling & Sharp, 2015; Turner et al., 2011). Previous work has been done in this area by Boddington et al. (2020), and in the iSTART trial. Measurement of the efficacy of intervention is also not equivalent in rat and human samples, and the grid walking and pasta handling tasks employed in the present study are only a proxy for the equivalent gross and fine motor activities that are impaired in human patients. An additional limitation related to this is the added difficulty of combining a behavioural task with

bilateral paired pulse recordings in rats, for example to measure IHI at the moment of movement initiation. An experiment to do just this was considered in the planning of this thesis, but ultimately fell outside the scope of our objectives.

A final consideration is the fact that in these experiments we used an electrode which pierced the rat dura, not one secured to the dural surface as is used in human applications of this technique. This is further complicated by the fact that in human applications the intensity of current required to pierce the dura is greater (Fonoff et al., 2009). There may be a danger of activating multiple populations of neurons with the epidural electrodes, losing the specificity achieved by using the electrical technique. What is clear is that careful investigation of the stimulation intensities used in humans will be necessary to match the results seen in rats. These difficulties demonstrate the importance of designing experiments such as these with translation in mind so that human research building from the findings can be more easily planned and results more accurately predicted.

7.4.1 POTENTIAL FOR TRANSLATION OF THIS WORK

Firstly, the techniques outlined in Chapter 3 and analysed in Chapter 4 for measuring IHI in an awake animal using EEG are able to be used by researchers in future IHI work, especially if further work is done to correlate the electrophysiological measures used here with the underlying anatomical components of the transcallosal pathway. These techniques were designed for rats, and could be used in future for this purpose, however they were also designed with human applications in mind. An EEG measure of IHI has not been previously reported, nor has the bilateral paired-pulse technique been used with electrical pulses in humans. Appendix 11 (page 291) describes an as yet untested protocol for how these techniques could

be incorporated into human experiments and validating the accuracy of these techniques could be a useful tool for further research.

It is also important that the findings from this work are utilised to direct human neuromodulation research. The iSTART study, which is ongoing, was designed to test the safety and feasibility of using contralateral iTBS in conjunction with rehabilitation. The next step would be to test whether use of this technique in humans can produce the same enhanced behavioural recovery and reduction in IHI as was shown in this work and by Boddington et al. (2020). With the body of work in rat literature that now exists to support this technique, translation to a larger scale human trial should become more feasible. The work within this thesis has also shown the importance of measuring interhemispheric inhibition alongside behavioural improvements during the intervention period, as a secondary measure of whether the protocol is having the intended effect.

7.4.2 OVERCOMING THE LIMITATIONS OF INVASIVE NEUROMODULATION

One major hurdle that exists with translating this work for humans is the necessarily invasive nature of the implantable pulse generator. Undergoing neurosurgery in the months after having a stroke is likely to intimidate patients and dissuade them from participating in research and exclude others altogether. While this may be mitigated by good recruitment strategies and ethical research design (Ferreira et al., 2019; Hendriks et al., 2019), work still needs to be done to improve the acceptability of these types of interventions among the general public.

This will be particularly important for patients of New Zealand Māori ancestry, who consider the head to be tapu (sacred) and so may be more reluctant to consider interventions of an invasive nature in the brain (Sachdev, 1989). This is an important consideration in the context of this research which takes place under the legislation of Te Tiriti o Waitangi, which demands that the considerations of Māori be considered, and that equity for Māori be upheld (Health Promotion Forum of New Zealand - Runanga Whakapiki ake i te Hauora o Aotearoa, 2002; Stroke Foundation of New Zealand & New Zealand Guidelines Group, 2010). Māori people, and Māori young people especially, bear a disproportionate burden of disability from stroke in New Zealand (Krishnamurthi et al., 2018). The weakness of an epidural electrical stimulation approach in this context is that it risks further widening this disparity, because the intervention itself is less culturally appropriate. On the other hand, successful development of this technique in partnership with Māori to ensure it is delivered in a culturally sensitive manner, could go some way towards addressing the inequitable burden of disability from stroke faced by this population.

The success from the work in this thesis and initial success with the iSTART trial derives from focussing on targeted, low-intensity, contralateral stimulation and coupling this with rehabilitation and assessment of both behaviour and IHI. These principles could be applied to less invasive strategies such as transcranial burst stimulation, enabling this work to reach those for whom neurosurgery interventions are not possible or culturally appropriate.

7.5 CONCLUSION

In this thesis we aimed to build on prior work which demonstrated behavioural recovery during application of iTBS to the contralateral hemisphere following stroke. Specifically, we wished

to investigate the link between interhemispheric inhibition and behavioural recovery during application of intermittent theta burst stimulation. We successfully demonstrated an increase in IHI in the week following stroke induction, and a weak link between IHI and behavioural impairment during the two weeks following stroke induction. In addition, we demonstrated that IHI was reduced in animals receiving iTBS both during and after the application of this intervention, and that iTBS treated animals had improved behavioural recovery compared to sham stimulated rats on both a gross and a fine motor task.

As was described earlier, we had an additional aim of designing this experiment with human applications in mind. We successfully adapted methods of both IHI measurement and stroke induction to reflect this aim. In saying this, there remains work to be done, primarily in developing our understanding of the IHI pathway and the components of this that are affected by stroke, and in the translation of this research for human trials. Overall, we believe that the work in this thesis provides a stepping-stone for development of this and other neuromodulation techniques, ultimately providing benefit to stroke patients worldwide who experience difficulties with recovery of function.

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APPENDICES

APPENDIX 1 MEASUREMENTS OF INTERHEMISPHERIC INHIBITION USING THE PRODIGY IMPLANTABLE PULSE GENERATOR

An additional aim relates to the previously discussed need to develop these experiments in such a way that they can be translated to human applications of this work. As part of this, the Prodigy IPG was used as an electrical stimulator to deliver one of the bilateral paired pulses during IHI measurement in anaesthetised animals. The 'conditioning' pulse was delivered with the IPG and the MC_Stimulus system was arranged to deliver the 'test' pulse.

- **Hypothesis A1** That IHI measured using the Prodigy IPG will be the same as that measured using other techniques.

APPENDIX 1.1 METHOD

The MC_Stimulus test pulse needed to be timed to the delivery of the conditioning IPG pulse, the signal was diverted from the AxoClamp 900A and was high pass filtered at approximately 5 kHz using a NeuroLog 125/126 filter module (Digitimer, UK), to remove low frequency EEG waves. Stimulation artefacts from the IPG were detected using the NeuroLog 201 spike trigger module (Digitimer, UK), which allowed a test pulse to be triggered at a set time interval after the IPG conditioning pulse. A function generator (Tektronix AFG3102C, Beaverton, Oregon, USA), using input from the spike trigger module, triggered an ipsilateral test pulse at a set time interval after every second IPG pulse. This allowed the IPG to be used for this novel purpose.

The recording of IHI using the Prodigy IPG as the conditioning stimulus was limited by the capabilities of the IPG itself. The IPG, programmed remotely through a custom St Jude Patient

Controller application (St Jude, USA) for iPad (Apple, USA) was able to deliver a square monophasic pulse with a width of 500 μs, at a limited range of amplitudes and frequencies. The iPad could be used to start the programme, and the stop feature used to terminate the protocol immediately once the required number of trials had been completed. The amplitudes available were multiples of 100 μA, and so conditioning intensities were rounded to the nearest 100 μA. Single pulses could be delivered at a minimum frequency of 2Hz. The function generator could be programmed to respond and deliver a test pulse after the IPG conditioning pulse every 2 seconds, giving an inter-trial interval of 2 seconds. However, the IPG continued to fire pulses to the conditioning electrode every 500 ms between the test pulses, owing to the minimum allowed frequency. Blocks of 4 trials under each testing condition were recorded in a randomly assigned order. Unconditioned ipsilateral trials could take place in the previously described manner, however, to control for the reduced inter-trial interval precluded by the IPG, the inter-trial interval was reduced to 2 seconds.

APPENDIX 1.2 INHIBITION INDUCED WITH THE PRODIGY IMPLANTABLE PULSE GENERATOR

In a group of eight rats, the conditioning pulse was delivered using the Prodigy IPG. In these animals, comparable results are seen to those observed when using the MC_stimulus system. Thresholds used in the eight animals tested using the IPG to deliver conditioning stimuli are given in Appendix 6 (page 281), and were similar to those for the MC_stimulus system, although were constrained by the limitations of the device. On average, the threshold found was 200 ± 33 μA, n=8. At threshold conditioning intensity, at the point of maximum inhibition for each animal, the maximum slope of the second peak of the test response was inhibited by 8.6 ± 11.2%, n=8, and once normalised to linear regression: -11.1%. This is not statistically

different from the percentage inhibition reported earlier in this chapter (9.2 ± 4.4%, n=16), or from levels of IHI reported previously in the literature (Barry, Boddington, Igelström, et al., 2014; Boddington, 2016). At suprathreshold conditioning intensity (at mean 167% of threshold), facilitation of ipsilateral response was 4.7 ± 12.7%, n=8 with inhibition of -3.0% once normalised. The difference between inhibition with threshold and suprathreshold conditioning was not significant for the normalised results. These results are shown in Figure A.1, with a complete set of results in Table A.1.

Owing to the necessity of pairing each conditioning pulse individually to its test pulse, there was a greater degree of variation in interstimulus intervals tested, and intervals were not an exact number of milliseconds. When aligned to the maximum inhibition of the second peak maximum slope, the average ISI used was 7.9 ± 0.6 ms, n=8, comparable to that reported in Chapter 4 and in other literature.

When peak-to-peak amplitudes were studied, effects were consistent with those previously described. When raw results were analysed, there was significantly greater inhibition shown with threshold conditioning stimuli (paired t-test, mean difference 14.6 ± 5.9, p=0.044), which disappeared when results were normalised, see Figure A.2 and Table A.1.

	Subthreshold	Threshold	Suprathreshold	High intensity
Change in the maximum slope - raw result (%)	Inhibition: 2.5 ± 16.1, n=6	Inhibition: 8.6 ± 11.2, n=8	Facilitation: 4.7 ± 12.7, n=8	Facilitation: 18.7 ± 12.8, n=7
Change in the maximum slope - normalised result (%)	Inhibition: 1.1 ± 16.1, n=6	Inhibition: 11.1 ± 11.2, n=8	Facilitation: 0.21 ± 12.7, n=8	Facilitation: 4.6 ± 12.8, n=7
Change in the peak to peak amplitude - raw result (%)	Inhibition: 10.5 ± 6.4, n=6	Inhibition: 15.0 ± 4.9, n=8*	Inhibition: 0.40 ± 7.9, n=8*	Facilitation: 8.5 ± 5.4, n=7
Change in peak to peak amplitude - normalised result (%)	Inhibition: 5.5 ± 6.4, n=6	Inhibition: 14.6 ± 4.9, n=8	Inhibition: 4.9 ± 7.9, n=8	Facilitation: 4.0 ± 5.4, n=7

*Table A.1 Results obtained from bilateral paired pulse investigations of interhemispheric inhibition using the Prodigy IPG as conditioning stimulation. Shaded boxes indicate that the test response was inhibited. *Indicates significant difference (p=0.044).*

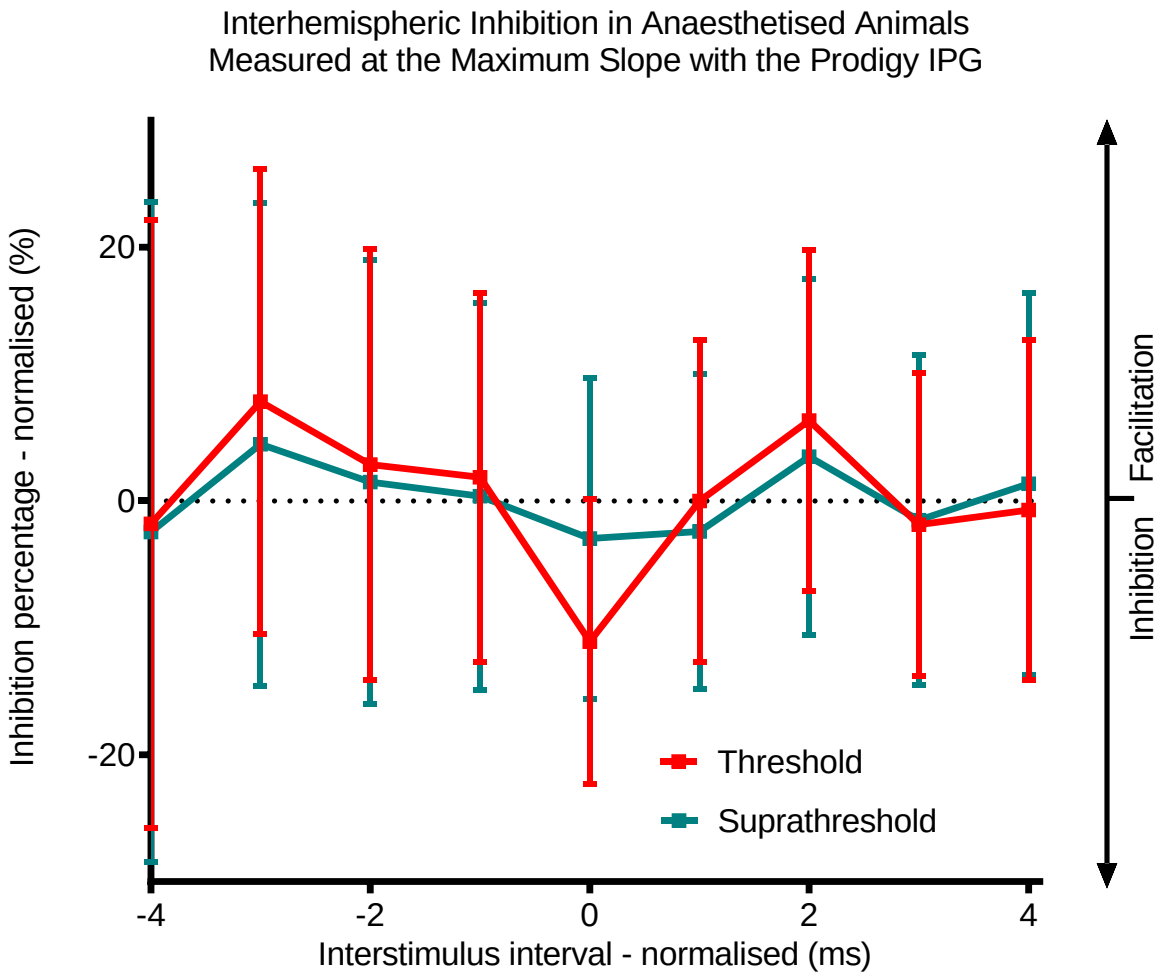

Figure A.1 Inhibition of the maximum slope of the second peak of the local field potential where he conditioning pulse was delivered using the Prodigy IPG, at threshold (low) and suprathreshold (high) conditioning intensities, expressed as a percentage of the mean non-conditioned response. There is inhibition of the test response with threshold stimulation, but this is not statistically different from the effect of suprathreshold stimulation. Data are aligned at x=0 to the interstimulus interval (ISI) corresponding to the maximum inhibitory effect for each animal and are normalised to a linear regression of the central data points. Vertical bars display standard error of the mean.

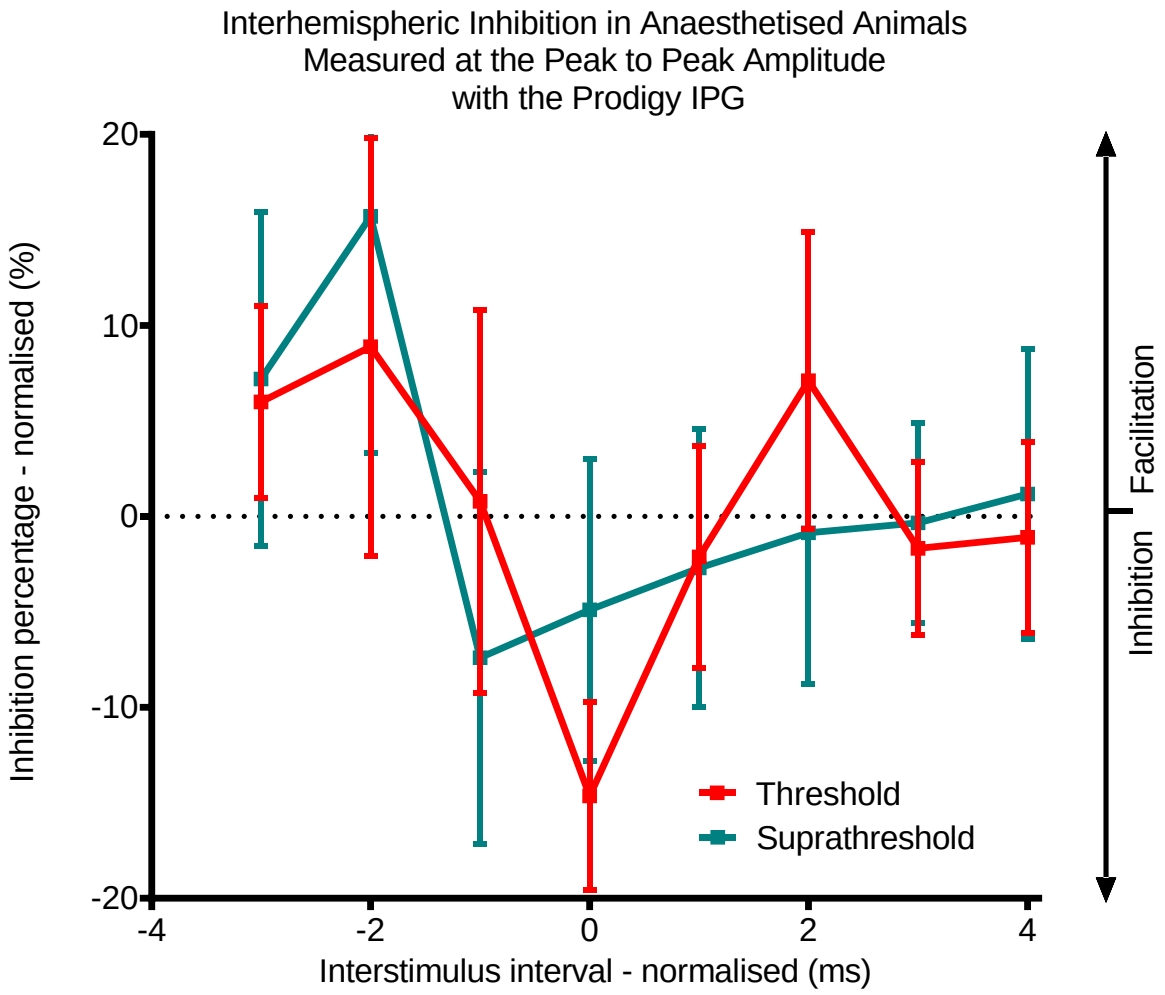

Figure A.2 Inhibition of the maximum slope of the second peak of the local field potential where he conditioning pulse was delivered using the Prodigy IPG, expressed as a percentage of the mean non-conditioned response. There is inhibition of the test response with threshold stimulation, not statistically different from the effect of suprathreshold stimulation. Data are aligned at x=0 to the interstimulus interval (ISI) corresponding to the maximum inhibitory effect for each animal and are normalised to a linear regression of the central data points. Error bars display standard error of the mean.

APPENDIX 1.3 EFFECTS OF ALTERING CONDITIONING AMPLITUDE ON INHIBITION OF THE MAXIMUM SLOPE USING THE PRODIGY IMPLANTABLE PULSE GENERATOR

The results using the IPG to deliver conditioning stimulation showed the same trend towards inhibition using threshold and subthreshold stimulation at comparable magnitude and ISI to that produced using the MC_stimulus system. Notably, even in this smaller sample the results are of similar reproducibility and precision. These trends were present without normalisation to a linear regression. Results for higher intensity stimuli were similar to the reported suprathreshold stimulation results in that inhibition was not seen. Subthreshold stimulation also produced inhibition, although to a lesser degree than the threshold conditioning. A full table of these results is given; Table A.1.

The hypothesis A1 was accepted; with a small group of animals (n=8), a trend towards inhibition of the ipsilateral response with low intensity contralateral conditioning was seen. This has implications outside of this thesis, as this technique was developed with the idea of translation into human studies of this work in mind. Appendix 11 (page 291) contains a description of the methodology that was developed, using this preliminary work in rats, to measure IHI in human participants implanted with the Prodigy IPG. This methodology has yet to be trialled in a human participant.

APPENDIX 2 INTERHEMISPHERI INHIBITION MEASUREMENTS WITH TRANSCRANIAL MAGNETIC STIMULATION

The use of small ‘rat’ TMS coils as a method for delivering test stimuli in rat experiments was also piloted, but ultimately abandoned as a cortical response was not able to be recorded. Six animals were used as part of these trials. Two coils were available for use, a 25 mm figure-of-eight coil for use with the MagStim (MagStim Rapid2, Wales, UK) and an 8 mm custom ‘mini’ coil (supplied by A. Tang, University of Western Australia). Responses were measured using an implanted silver wire, as previously described. While using the larger MagStim coil a blanking circuit was connected to turn off recording at the moment of stimulation, to ‘blank’ the oversized artefact which obscured the signal. This was unsuccessful, and no response could be discriminated in the extradural recording either with or without blanking activated. The smaller 8 mm coil was also trialled. This failed to produce any visible response in the EEG recording other than a stimulation artefact.

APPENDIX 3 MED-PC PROGRAMMES

To produce ipsilateral or contralateral stimulation in isolation:

```
\  MEDSTATE NOTATION program for the PHM-152
\  Note that the Stim Node is set in the 'STIMULATE' set up command
\  and the port number (1 or 2 for each stimulator node) is set with the STIMON command.

\Set Aliases

Var_Alias Con Pulse Amplitude (uA)       =L \Default 200uA
Var_Alias Test Pulse Amplitude (uA)      =E \Default 400uA
Var_Alias Number of trials               =X \Default 10

\K pulses used

\K1 - GO contralateral
\K2 - E + 10  \Ipsilateral stim amplitude
\K3 - E - 10
\K5 - L + 10  \Contralateral stim amplitude
\K6 - L - 10
\K10 - GO ipsilateral
\K11 - contra stim
\K12 - ipsi stim


S.S.1,  \***** Show Parameter Values *****\
S1,
  1": SHOW 1,ipsiWIDTH,D, 2,ipsiAMP,E, 3,contraWIDTH,K, 4,contraAMP,L, 5, ISI, C ---> S1

\Contralateral stimulation\

\H = Pulse 1 Width (us)
\I = Pulse 1 Amplitude (uA)
\J = Delay between Pulse 1 and 2 (us)
\K = Pulse 2 Width (us)
\L = Pulse 2 Amplitude (uA)
\M = Frequency (Hz)
\N = Duration (ms)
\NODE = Node of stimulator module - it has two ports 1 and 2 that can be turned on and off independently
but
\ if activated together will output the same waveform (intensity included)


\set node from BOX variable to '5' which is the number for Stim Unit#5 (contra)


S.S.2,  \***** Main Program *****\

S1,
  \Set the default values
  .01": SET H = 100, I = 1, J = 100, K = 500, L = 200, M = 1, N = 1000 ---> S2
```

```
S2,
  #START: ---> S3

S3,
  .01": ~STIMULATE(MG, BOX+4, H, I, J, K, L, M, N);~ ---> S4

S4,
  #K11: ~STIMON(MG, BOX+4, 1);~ ---> S5

S5,
  2": ---> S3


S.S.3,  \***** Change amplitude *****\
S1,
  #K5: SET L = L + 10; ---> SX
  #K6: SET L = L - 10; ---> SX

\Ipsilateral stimulation\

\A = Pulse 1 Width (us)
\B = Pulse 1 Amplitude (uA)
\C = Delay between Pulse 1 and 2 (us)
\D = Pulse 2 Width (us)
\E = Pulse 2 Amplitude (uA)
\F = Frequency (Hz)
\G = Duration (ms)
\NODE = Node of stimulator module - it has two ports 1 and 2 that can be turned on and off independently
but
\ if activated together will output the same waveform (intensity included)


\set node from BOX variable to '6' which is the number for Stim Unit#6 (ipsi)


S.S.4,  \***** Main Program *****\


S1,
  \Set the default values
  .01": SET A = 100, B = 1, C = 100, D = 500, E = 400, F = 1, G = 1000 ---> S2

S2,
  #START: ---> S3

S3,
  .01": ~STIMULATE(MG, BOX+5, A, B, C, D, E, F, G);~ ---> S4

S4,
  #K12: ~STIMON(MG, BOX+5, 1);~---> S5

S5,
  0.1": ---> S3


S.S.5,  \***** Change amplitude *****\
```

```
S1,
  #K2: SET E = E + 10; ---> SX
  #K3: SET E = E - 10; ---> SX


S.S.7, \Repeat contralateral stim

S1,

#K1 ---> S2

S2,

5":K11 ---> S3

S3,

0.1": ---> S2

S.S.8, \Repeat ipsilateral stim

S1,

#K10 ---> S2

S2,

5":K12 ---> S3

S3,

0.1": ---> S2

S.S.9,

S1, \Set trial number

.01": SET X = 10;
SHOW 6, Trial number, T ---> S2

S2,

#K12:ADD T ---> S1
#K11:ADD T ---> S1


S.S.10,\Kill after X trials

S1,

1": IF T>= X [@TrueEnd, @FalseContinue]
            @End:K10 ---> S2
            @Cont: ---> SX

S2,
 1" ---> STOPKILL
```

For bilateral paired pulse stimulation for recording of IHI:

```
\  MEDSTATE NOTATION program for the PHM-152
\  Note that the Stim Node  (1 to 8 with our operant chambers at time of writing) is set in the 'STIMULATE' set up command
\  and the port number (1 or 2 for each stimulator node) is set with the STIMON command.
\for box with nodes 5,6

\Set Aliases

Var_Alias Con Pulse Amplitude (uA)       =L \Default 200uA
Var_Alias Test Pulse Amplitude (uA)      =E \Default 400uA
Var_Alias ISI (+100 us)                  =C \Default 7900us ISI=interstimulus interval
Var_Alias Number of trials               =X \Default 10

\K pulses used

\K1 - GO
\K2 - E + 10  \Ipsilateral stim amplitude
\K3 - E - 10
\K5 - L + 10  \Contralateral stim amplitude
\K6 - L - 10
\K7 - C + 100 \ISI
\K8 - C - 100


S.S.1,  \***** Show Parameter Values *****\
S1,
  1": SHOW 1,ipsiWIDTH,D, 2,ipsiAMP,E, 3,contraWIDTH,K, 4,contraAMP,L, 5, ISI, C ---> S1

\Contralateral stimulation\

\H = Pulse 1 Width (us)
\I = Pulse 1 Amplitude (uA)
\J = Delay between Pulse 1 and 2 (us)
\K = Pulse 2 Width (us)
\L = Pulse 2 Amplitude (uA)
\M = Frequency (Hz)
\N = Duration (ms)
\NODE = Node of stimulator module - it has two ports 1 and 2 that can be turned on and off independently but
\ if activated together will output the same waveform (intensity included)


\set node from BOX variable to '5' which is the number for Stim Unit#5 (contra)


S.S.2,  \***** Main Program *****\

S1,
  \Set the default values
```

```
  .01": SET H = 100, I = 1, J = 100, K = 500, L = 200, M = 1, N = 1000 ---> S2

S2,
  #START: ---> S3

S3,
  .01": ~STIMULATE(MG, BOX+4, H, I, J, K, L, M, N);~ ---> S4

S4,
  #K1: ~STIMON(MG, BOX+4, 1);~ ---> S5

S5,
  2": ---> S3


S.S.3,  \***** Change amplitude *****\
S1,
  #K5: SET L = L + 10; ---> SX
  #K6: SET L = L - 10; ---> SX

\Ipsilateral stimulation\

\A = Pulse 1 Width (us)
\B = Pulse 1 Amplitude (uA)
\C = Delay between Pulse 1 and 2 (us)
\D = Pulse 2 Width (us)
\E = Pulse 2 Amplitude (uA)
\F = Frequency (Hz)
\G = Duration (ms)
\NODE = Node of stimulator module - it has two ports 1 and 2 that can be turned on and off independently
but
\ if activated together will output the same waveform (intensity included)


\set node from BOX variable to '6' which is the number for Stim Unit#6 (ipsi)


S.S.4,  \***** Main Program *****\


S1,
  \Set the default values
  .01": SET A = 100, B = 1, C = 7900, D = 500, E = 400, F = 1, G = 1000 ---> S2

S2,
  #START: ---> S3

S3,
  .01": ~STIMULATE(MG, BOX+5, A, B, C, D, E, F, G);~ ---> S4

S4,
  #K1: ~STIMON(MG, BOX+5, 1);~---> S5

S5,
  0.1": ---> S3
```

```
S.S.5,  \***** Change amplitude *****\
S1,
  #K2: SET E = E + 10; ---> SX
  #K3: SET E = E - 10; ---> SX


S.S.6,  \***** Change ISI *****\
S1,
  #K7: SET C = C + 100; ---> SX
  #K8: SET C = C - 100; ---> SX

S.S.7, \Repeat contralateral stim

S1,

#START ---> S2

S2,

5":K1 ---> S3

S3,

0.1": ---> S2


S.S.8, \Kill after X trials

S1, \Set trial number

.01": SET X = 10;
SHOW 6, Trial number, T ---> S2

S2,

#K1:ADD T ---> S1

S.S.9,

S1,

1": IF T>= X [@TrueEnd, @FalseContinue]
            @End:K10 ---> S2
            @Cont: ---> SX

S2,
 1" ---> STOPKILL
```

APPENDIX 4 FILTERING EXAMPLE

The example in Figure A.3 and Table A.2 is used to show that whether or not a 30 Hz high pass filter was applied, the level of IHI recorded remained the same. The high pass filter was used in on-line recordings where stimulation artefacts were large (although of low frequency) and made it difficult to determine whether an EEG potential was being recorded successfully. The high pass filter reduced the size of the artefact, and enabled recordings to be checked for quality on-line.

Recording	**No filter**	**High pass filter (30 Hz)**
Maximum slope (mV/ms)		
Example trace	-0.34	-0.37
Average trace	-0.35	-0.38
Maximum IHI	35%	38%
Peak to peak amplitude (mV)		
Example trace	1.5	1.6
Average trace	1.4	1.7
Maximum IHI	38%	41%

Table A.2 Response characteristics with and without 30 Hz filter, results are given for a single response, for an average of 10 responses from a single animal, and for maximum IHI measurement from that animal. Note that the responses are changed by 0.03 mV or less with filtering in each case from this example animal.

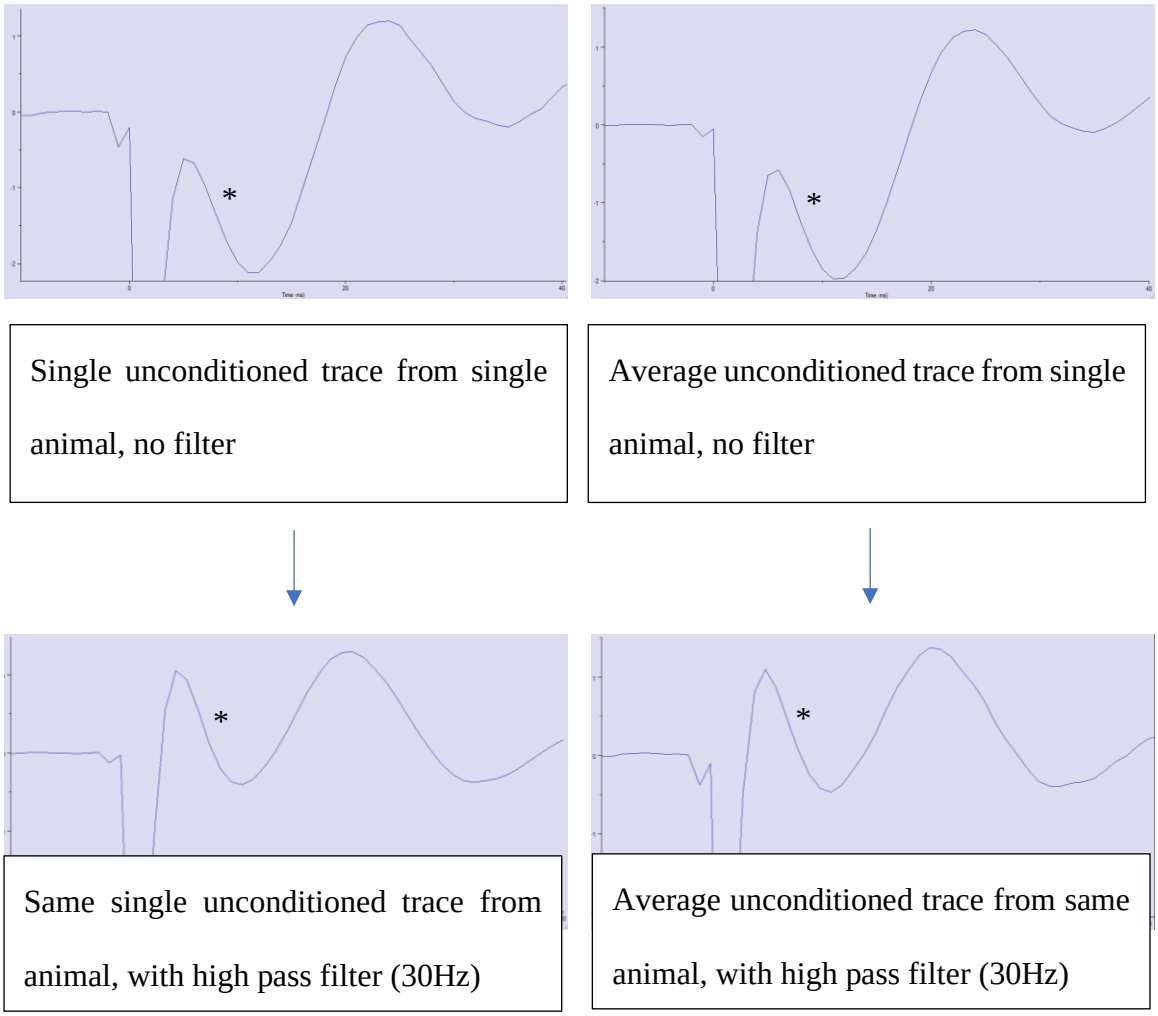

*Figure A.3 Example traces showing filtered and unfiltered responses. On the left is a single response, and on the right is shown an average of 10 responses from the same animal. Note that in both examples the morphology of the response is unchanged by the addition of the filter. The region of interest is denoted with *.*

APPENDIX 5 HISTOLOGY PROTOCOL

1. Mix Cresyl Violet solution: 90 mL 0.1 M acid solution (0.57 mL glacial acetic acid made up to 100 mL with milliQ), 10 mL 0.1 M base solution (.41 g anhydrous sodium acetate in 50 mL milliQ) and 5 mL Cresyl Violet stock solution (2% aqueous solution dissolved overnight and filtered through fast Whatman paper).
2. Delipidise:
    a. 2 minutes in 70% ethanol
    b. 2 minutes in 95% ethanol
    c. 2 minutes in 95% ethanol
    d. 2 minutes in 100% ethanol
    e. 2 minutes in 100% ethanol
    f. Into xylene and place in fume hood for 5 minutes
    g. Repeat f in fresh xylene
3. Rehydrate
    a. 2 minutes in 100% ethanol
    b. 2 minutes in 100% ethanol
    c. 2 minutes in 95% ethanol
    d. 2 minutes in 95% ethanol
    e. 2 minutes in 70% ethanol
    f. 2 minutes in 50% ethanol
    g. 30 seconds in deionized H_2O
4. Stain
    a. 60 minutes in Cresyl Violet solution
5. Wash
    a. Dip into deionized H_2O
    b. 30 seconds in deionized H_2O
6. Dehydration
    a. 30 seconds in 50% ethanol
    b. 30 seconds in 70% ethanol
    c. 30 seconds in 95% ethanol
    d. 30 seconds in 95% ethanol
    e. 30 seconds in 100% ethanol
    f. 30 seconds in 100% ethanol
    g. Into xylene and place in fume hood for 5 minutes
    h. Into fresh xylene and place in fume hood for 10 minutes
7. Coverslip using DPX mountant (Sigma-Aldrich, Germany)

APPENDIX 6 TEST AND CONDITIONING INTENSITIES USED FOR ANASETHETISED ANIMALS

Intensities used as test and conditioning stimuli											
	Contralateral - conditioning								Ipsilateral - test		
	Subthreshold		Threshold		Suprathreshold		High-suprathreshold				
	Absolute (µA)	% threshold	Absolute (µA)	% threshold	Absolute (µA)	% threshold	Absolute (µA)	% threshold	Intensity where max. response seen (µA)	stimulus intensity (µA)	%
Animals where MC_Stimulus was used to deliver conditioning stimulus											
A	100	50	200						300	200	66.7
B	50	50	100	100	150	150	300	300	1100	700	63.6
C	50	50	100	100	150	150	300	300	900	600	66.7
D	40	80	50	100	100	200	300	600	1100	800	72.7
E	100	67	150	100	200	133	400	267	900	600	66.7
F	30	60	50	100	100	200	150	300	1100	800	72.7
G	125	50	250	100	375	150	750	300	900	600	66.7
H	25	50	50	100	75	150	150	300	900	600	66.7
I	50	50	100	100	150	150	300	300	900	600	66.7
J	75	50	150	100	225	150	450	300	1100	800	72.7
K	100	50	200	100	300	150	600	300	900	600	66.7
L	50	50	100	100	150	150	300	300	900	600	66.7
M	150	50	300	100	450	150	900	300	700	500	71.4
N	50	50	100	100	150	150	300	300	900	600	66.7
O	25	50	50	100	75	150	150	300	1100	800	72.7
P	125	50	250	100	375	150	700	280	1100	800	72.7
Q	50	50	100	100	150	150	300	300	1100	800	72.7
R	75	50	150	100	225	150	450	300	1100	800	72.7
Average	69	53	132	100	200	155	400	315	982	682	69.0
Median	50	50	100	100	150	150	300	300	900	600	66.7
Animals where IPG was used as conditioning stimulus											
S	100	50	200	100	300	150	600	300	1100	800	72.7
T	100	50	200	100	300	150	600	300	900	600	66.7
U			100	100	200	200	300	300	1100	800	72.7
V	200	50	400	100	600	150			900	600	66.7
W	100	50	200	100	300	150	600	300	800	600	75.0
X	100	50	200	100	300	150	600	300	800	600	75.0
Y	100	50	200	100	300	150	600	300	800	600	75.0
Z			100	100	200	200	300	300	1100	800	72.7
Average	117	50	200	100	313	163	514	300	938	675	72.0
Median	100	50	200	100	300	150	600	300	1100	600	72.7

Table A.3 Intensities used as test and conditioning stimuli.

APPENDIX 7 MEASURING PEAK TO PEAK AMPLITUDE TO DETERMINE INHIBITION

The other measure which was measured to determine IHI was the difference between the maximum and minimum peaks of the response, which is referred to as peak to peak amplitude, see Figure 4.4. When peak-to-peak responses were analysed, the overall trends remained the same, especially when observing the comparison between threshold and suprathreshold stimulation. These data are shown in Figure A.4 and Table A.4. Using this measure, the inhibitory effect was smaller, and the difference between threshold and suprathreshold stimulation was non-significant. At threshold conditioning intensity, at the point of maximum inhibition for each animal, the peak to peak amplitude of the test response was inhibited by 2.2 ± 3.0%, n=17 using the raw data, and when normalised inhibition increased to 8.4%. At suprathreshold conditioning intensity (at mean 155% of threshold), there was facilitation of 2.3 ± 4.8%, n=17 using the raw data, and inhibition of 2.4% once normalised. The effects of high intensity and subthreshold stimulation were less consistent for this measure, and did not follow suprathreshold and threshold responses (respectively) as closely as for the peak second slope measure, see Table A.4.

	Subthreshold	**Threshold**	**Suprathreshold**	**High intensity**
Change in the peak to peak amplitude - raw result (%)	Facilitation: 0.5 ± 5.0, n=18	Inhibition: 2.2 ± 3.0, n=17	Facilitation: 2.3 ± 4.8, n=17	Facilitation: 1.0 ± 5.3, n=17
Change in peak to peak amplitude - normalised result (%)	Inhibition: 4.4 ± 5.0, n=18	Inhibition: 8.4 ± 3.0, n=17	Inhibition: 2.4 ± 4.8, n=17	Inhibition: 3.9 ± 5.3, n=17

Table A.4 IHI measured with bilateral paired pulse investigations using the MC_Stimulus system. Shaded boxes indicate that the test response was inhibited. The peak to peak amplitude was used to determine inhibition.

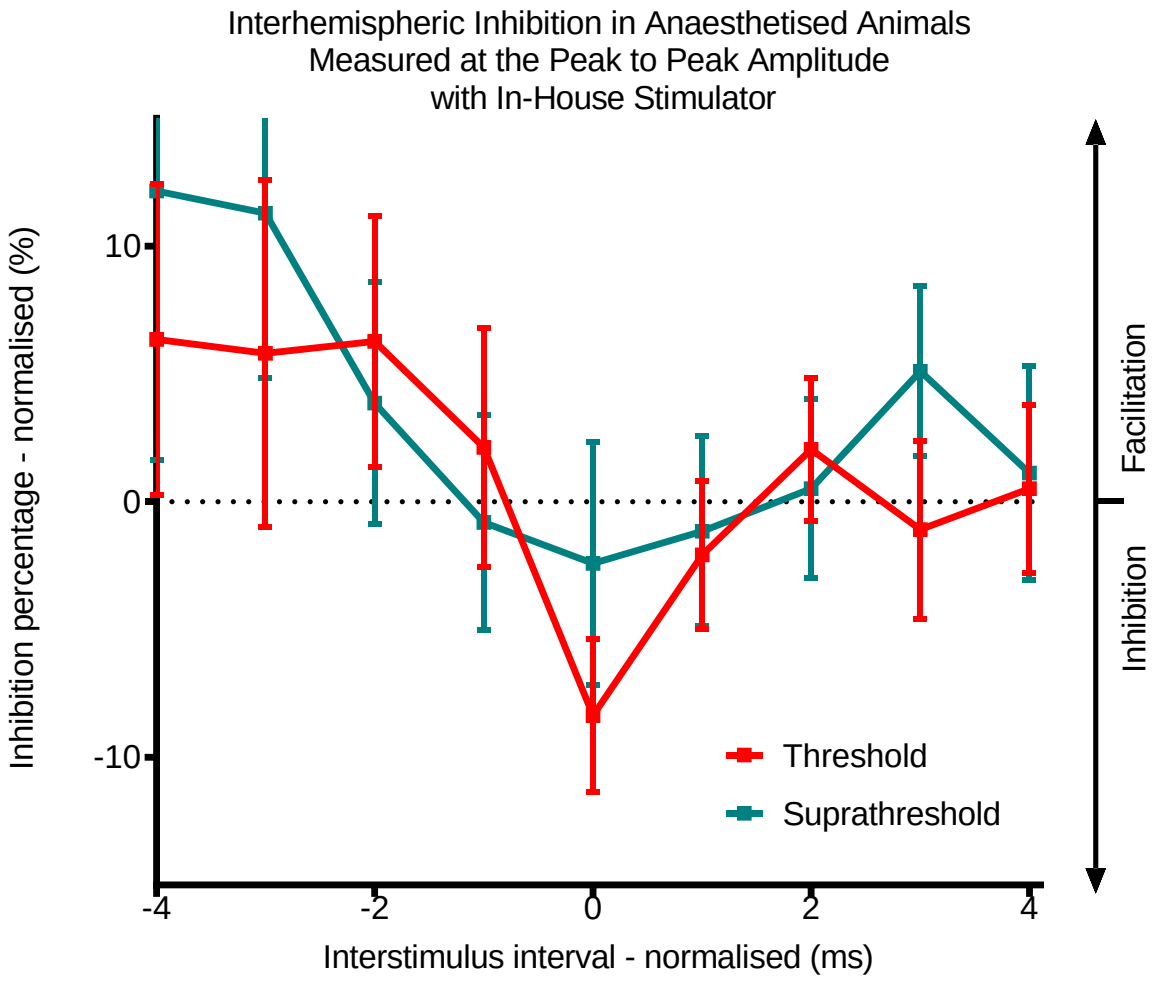

Figure A.4 Inhibition of the peak to peak amplitude of the local field potential where the conditioning pulse was delivered using MC_Stimulus, at threshold and suprathreshold conditioning intensities, expressed as a percentage of the mean non-conditioned response. There is inhibition of the test response with threshold stimulation, but this is not statistically different from the effect of suprathreshold stimulation. Data are aligned at x=0 to the interstimulus interval (ISI) corresponding to the maximum inhibitory effect for each animal and are normalised to a linear regression of the central data points. Error bars display SEM.

APPENDIX 8 EFFECTS OF ADDITIONAL CONDITIONING AMPLITUDES ON INHIBITION

In addition to the threshold and suprathreshold conditioning stimuli used with bilateral paired pulse measurements, two additional conditioning amplitudes were also used. These were equivalent to 50% of threshold (subthreshold) and 300% of threshold (high supra-threshold).

There were no differences between the effects of threshold and subthreshold stimulation; both produced an inhibitory effect of similar magnitude, see Figure A.5. In addition, high intensity stimulation, which was delivered at 300% of threshold intensity, did not produce a different effect to suprathreshold stimulation, which was 150% of threshold, see Figure A.5 and Table A.5.

	Subthreshold	**Threshold**	**Suprathreshold**	**High intensity**
Change in the maximum slope - raw result (%)	Inhibition: 1.2 ± 2.9, n=18	Inhibition: 3.1 ± 2.6, n=17	Facilitation: 9.2 ± 4.4, n=16	Facilitation: 8.2 ± 4.8, n=16
Change in the maximum slope - normalised result (%)	Inhibition: 5.5 ± 2.9, n=18	Inhibition: 9.2 ± 2.6, n=17	Facilitation: 0.5 ± 4.4, n=16	Inhibition: 2.4 ± 4.8, n=16

Table A.5 Interhemispheric inhibition measured with paired pulse investigations using the MC_Stimulus system at four conditioning pulse amplitudes. Shaded boxes indicate that the test response was inhibited.

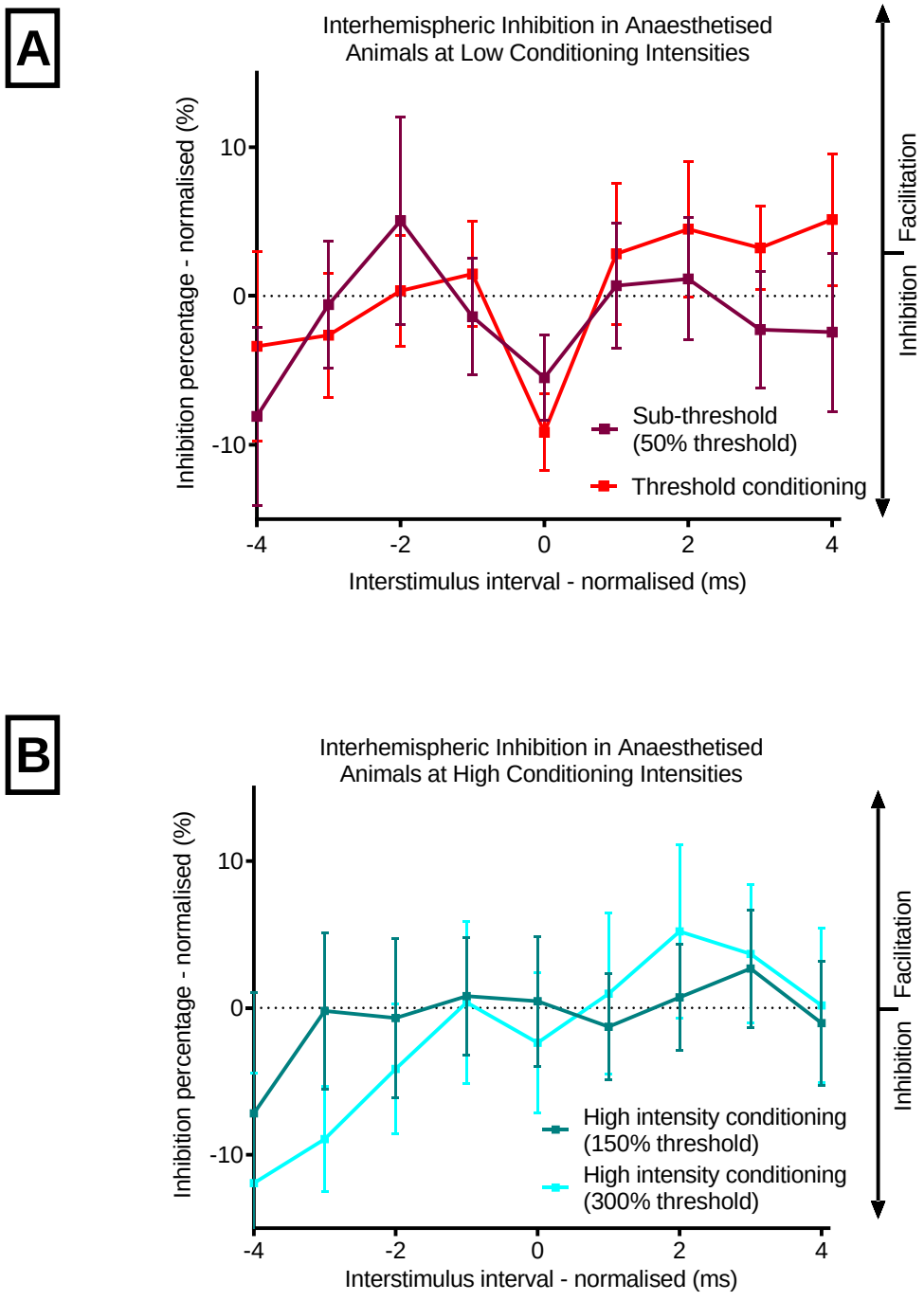

Figure A.5 Inhibition of the maximum slope of the second peak of the local field potential. A: Subthreshold and threshold conditioning stimulation with inhibition of the test response. B: Suprathreshold and high intensity conditioning stimulation, with no inhibition of the test response. Inhibition is expressed as a percentage of the mean non-conditioned response. Data are aligned at x=0 to the interstimulus interval corresponding to the maximum inhibitory effect for each animal and are normalised to a linear regression of the central data points. The conditioning pulse was delivered using MC_Stimulus. Error bars display SEM.

APPENDIX 9 BEHAVIOURAL RESULTS FOLLOWING INTERVENTION AT ADDITIONAL TIMEPOINTS

Chapter 6 discussed grid walking results for the most clinically relevant timepoint following delivery of sham or iTBS, that is at the conclusion of the study period. For completeness, grid walk results for other timepoints following randomisation are given in Figure A.6. Data are normalised to the level of post stroke impairment, so improvement can be seen more easily, and animals without grid walk impairment are excluded, as discussed in Chapter 5. Animals receiving iTBS showed immediate improvement compared to sham animals. This difference disappeared in the third to fifth week of the study, however at the conclusion of the experiment a residual benefit was seen compared to sham. Relevant statistics are discussed in Chapter 6. A mixed effects model did not show a significant difference by group when all time points were included, $F(8, 150) = 1.6$, $p = 0.13$.

Chapter 6 also showed results for paw preference at the conclusion of the study. The results for individual timepoints relating to pasta handling, specifically paw preference is given in Figure A.7. Figure A.7 shows that in sham stimulated animals, post stroke paw preference remained (represented by the dotted line in the graph), but that iTBS treated animals began using their paretic paw more frequently over time. These observations did not reach the level of statistical significance in a mixed effects model including all timepoints, $F(1, 26) = 0.14$, $p = 0.71$.

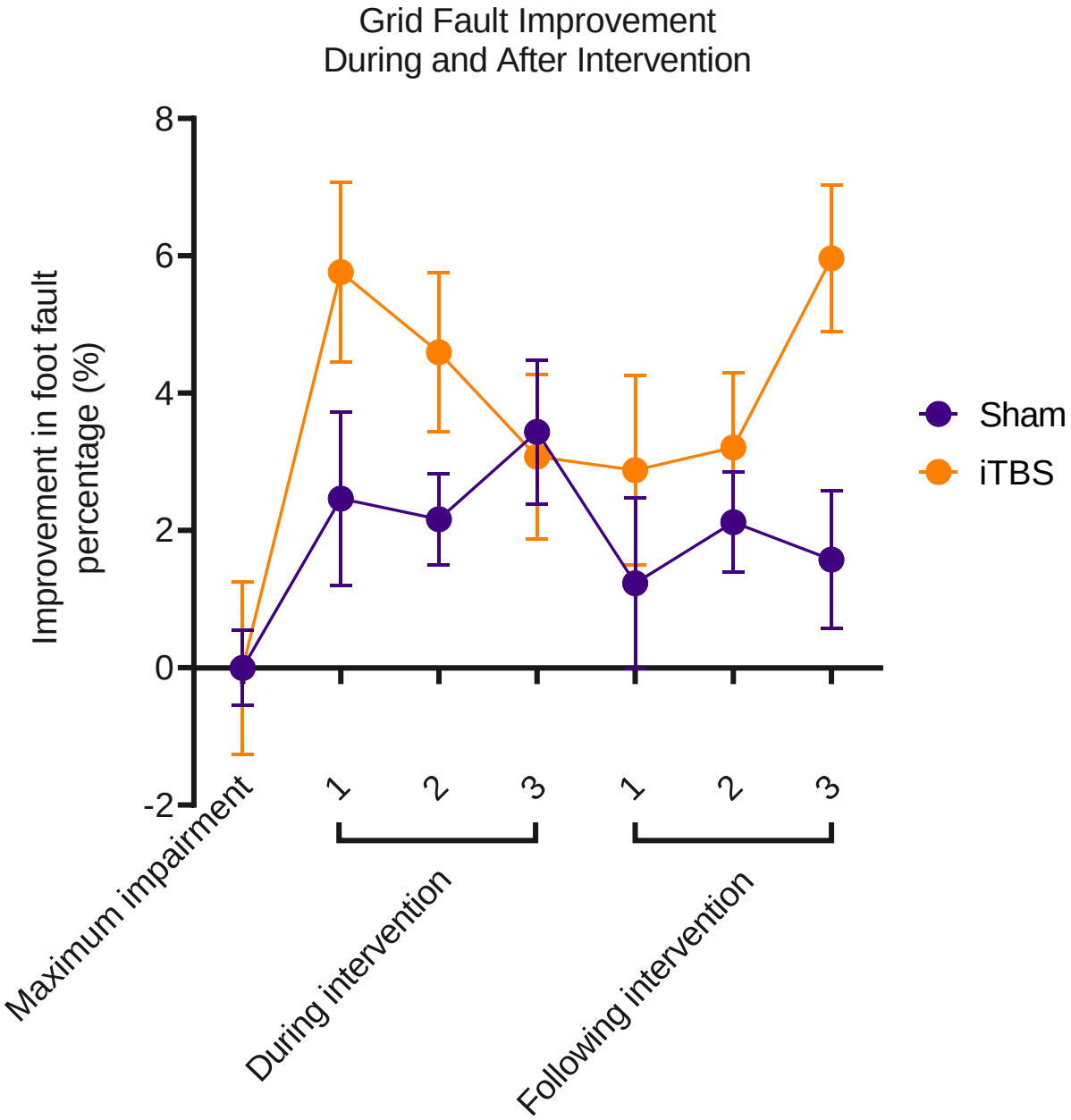

Figure A.6 Improvement in grid walk performance compared to post stroke impairment for individual timepoints, where zero is the maximum post stroke fault level. Data are mean ± SEM.

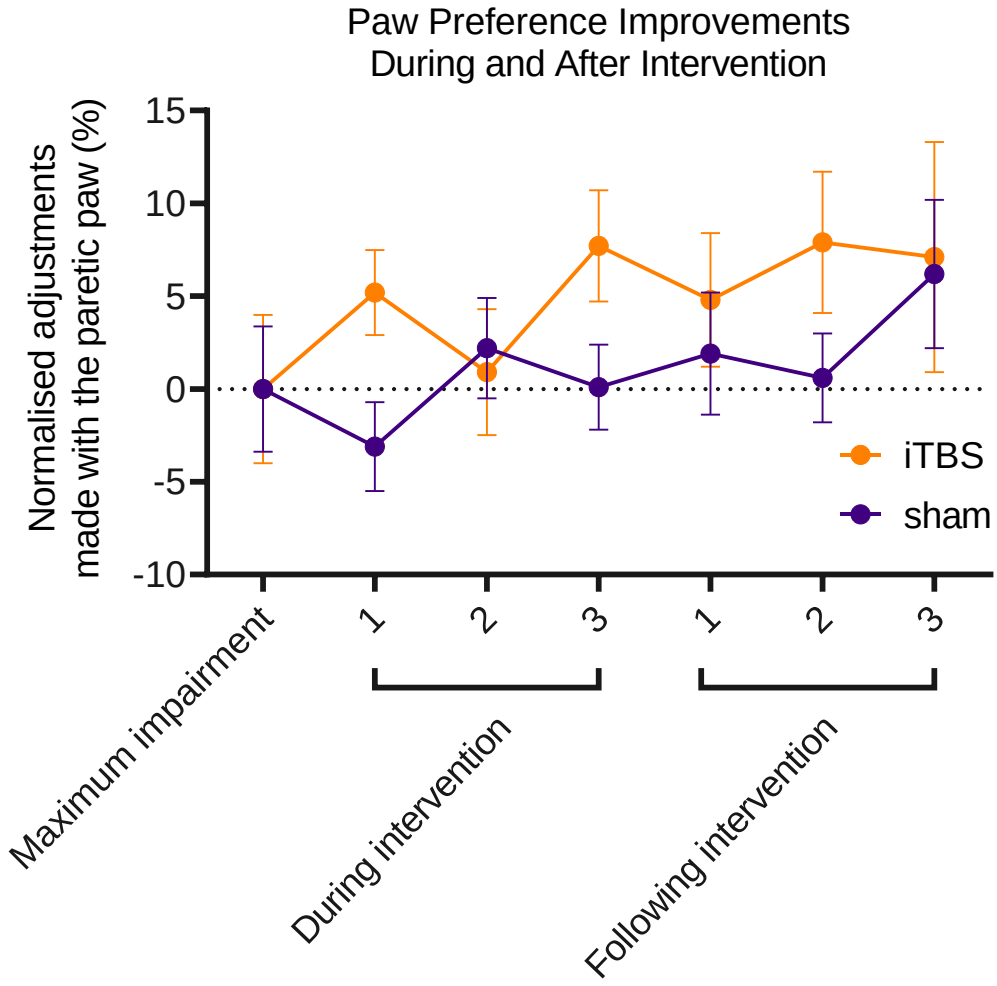

Figure A.7 Increase in paretic paw use compared with post stroke paw use (represented by the dotted line). Increases in paretic paw use are demonstrated by data above the dotted line and decreases by that below it. Data are mean ± SEM.

APPENDIX 10 LESION VOLUME RESULTS BY GROUP

Sham stimulated animals had an average lesion volume of 7.8 ± 1.5 mm^3, and iTBS animals an average volume of 9.7 ± 1.7 mm^3. An unpaired, two tailed t-test showed that these groups were not significantly different (p=0.42), excluding lesion volume as the cause of differences in behaviour and IHI between groups.

APPENDIX 11 PROTOCOL FOR MEASUREMENT OF INTERHEMISPHERIC INHIBITION IN IMPLANTED PATIENT

1. Steps 2 and 3 are used to find the appropriate intensity for the IPG conditioning pulse
2. Find threshold for IPG to induce a cortical response
    a. The patient will have a single lead EEG (positive) attached over the area directly opposite the IPG implant, i.e. over the lesioned hemisphere, with a reference electrode over the forehead (negative), and a ground electrode on the earlobe.
    b. A continuous recording of EEG activity will be taken, while the intensity of IPG stimulation at 2Hz is increased in steps of 0.1mA. The EEG recording will be monitored for signs of a cortical response following the stimulation artefact. The intensity at which this occurs will be known as 'cortical threshold'.
    c. Stimulation intensities used to find cortical threshold will not exceed the manufacturer's recommended maximum (nominally 6mA) to avoid tissue damage.
    d. If cortical threshold is not found within the specified range, the IPG shall be set to 80% of active motor threshold (if found), or to 0.5mA (intensity of iTBS).
3. Find motor threshold for IPG
    a. The patient will have 2 electrodes positioned over the first dorsal interosseous muscle (and/or extensor carpi radialis brevis) contralateral to the IPG, with a ground electrode on the lateral epicondyle of the elbow. I.e. if IPG on the left – the electrodes will be on the right. Electrodes for MEP studies on the opposite limb should also be positioned at this time for convenience.
    b. The patient will be asked to lightly pinch their index finger and thumb together to increase the likelihood of motor neuron activation following a cortical stimulus.
    c. Epochs of electromyography activity will be recorded, each epoch being triggered by a single IPG artefact recorded in the EEG signal, while the intensity of IPG stimulation at 2Hz is increased in steps of 0.1mA. The electromyography recording will be monitored for signs of a MEP response following the stimulation artefact. The intensity at which this occurs will be known as 'active motor threshold'.
    d. If a MEP is seen, the intensity of the IPG should not be increased further.

e. Stimulation intensities used to find motor threshold will not exceed manufacturer's recommended maximum so as to avoid tissue damage.
    i. If both cortical and active motor thresholds are found, then the IPG shall be set to 50% of (cortical + active motor threshold).
    ii. If cortical threshold is found but active motor threshold is not found within the specified range, the IPG shall be set to 50% of (cortical threshold + 1 mA).
    iii. If neither cortical nor active motor thresholds are found, IPG intensity will be set to 0.5mA (intensity of iTBS).

4. EEG responses at conditioning intensity will be recorded (360 – 3 minutes).
5. Find test stimulus location and intensity, using TMS on the opposite side to IPG implantation (i.e. over the lesioned hemisphere)
    a. Active and reference EEG electrodes for recording over the lesioned hemisphere will be removed at this point.
    b. The patient will have two electromyography electrodes positioned over the first dorsal interosseous muscle ipsilateral to the IPG, with a ground electrode at the elbow. (I.e. if TMS on the left – the electrodes will be on the right).
    c. The patient will be given a cap to wear over their head and EEG electrodes to allow for accurate positioning of the coil.
    d. The likely location for stimulation will be marked (5 cm lateral and 2 cm anterior of the vertex) and this area explored with TMS pulses ~50% of maximum TMS output while the patient lightly pinches their index finger and thumb. Until a MEP is seen the intensity will continue to be increased and the likely area explored.
    e. Determine test TMS intensity. Once the optimal stimulation site is located, the TMS intensity should be increased until MEPs are observed in 4/8 of trials and then further increased until the size of the MEP plateaus.
        i. If motor threshold and plateau response are found, then test intensity will be 50% of motor threshold + intensity for plateau response.
        ii. If only motor threshold found, then test intensity will be 50% of (motor threshold + 100% machine output).
        iii. If neither motor threshold nor plateau intensity found, then experiment will be terminated.

f. During this time, the size of the TMS artefact in the EEG should be monitored to give an estimate of how much voltage is being delivered to the IPG. (Initial testing has shown that a pulse of 15V from a TMS coil did not damage the IPG nor disrupt its normal function.)
g. Baseline MEPs at test intensity should be recorded (3 x 20).

6. EEG response to TMS and sham
    a. The EEG response to TMS should be recorded (2 x 20)
    b. The EEG response to sham TMS should be recorded (2 x 20)
        i. These can then be compared to remove the effect of the auditory stimulus from the response to real TMS.
7. Bilateral paired pulse protocol (see Figure A.8)
    a. The IPG should be started at the conditioning intensity, using 2 seconds on, 8 seconds off cycle.
    b. The EEG signal will be filtered of low and high frequency signal components (bandpass filtered at 5-50 kHz), so that the IPG artefact can be detected
    c. The IPG artefact will be used to trigger a TMS pulse at 10ms after the IPG artefact
    d. 2 x 20 responses of this type should be recorded, followed by 2 x 20 with TMS alone
    e. Step d should be repeated as necessary
    f. If wanted, the conditioning intensity and ISI can be altered to explore IHI in more detail, and to increase the likelihood of finding the crossed inhibitory response.

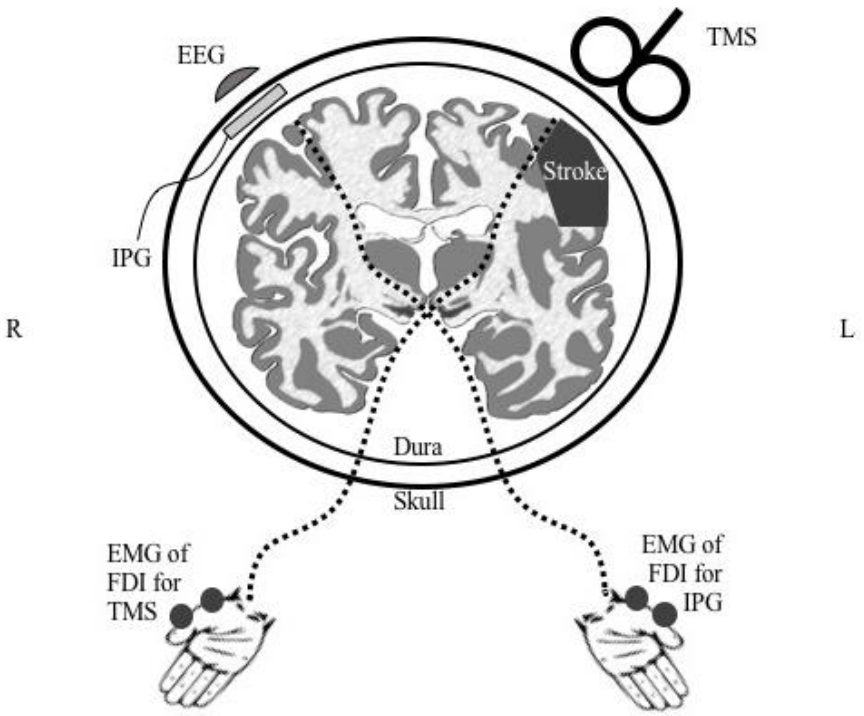

Figure A.8 Diagram of proposed interhemispheric inhibition measurement setup in a human participant implanted with an epidural stimulator.