Electrophysiological transmission of motor signals during stroke recovery in rats

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Preface

The thesis 'Electrophysiological transmission of motor signals during stroke recovery in rats' is written for the submission for the master's degree of *Biology of Disease* at the Universiteit Utrecht. This thesis is the final result of a nine-month internship at the Biomedical MR Imaging and Spectroscopy group of the University Medical Centre Utrecht (UMCU). During my internship at the UMCU, I have been performing many animal experiments for my first time. This steep learning curve has brought me a lot of experience, knowledge, sometimes frustration but mostly even more curiosity to science. I want to express my gratitude to my daily supervisor Vera Wielenga for her guidance, brainstorm sessions and expertise during my project. But also the freedom and responsibilities that were essential for my personal development.

Secondly, I want to thank prof. dr. Rick Dijkhuizen for giving me the opportunity to work in his group and for his involvement in my project. Moreover, thanks to all the members of the Dijkhuizen lab, as everyone was always prepared to help me when I was lost. A special thanks to Julia Boonzaier, whose preliminary data I was allowed to use for my own analyses. I also want to thank dr. Bas Neggers for finding time to be my second reviewer.

Finally, I would like to mention the support of my boyfriend, family and friends through all the stages of my internship both emotionally and practically. Of course, it has been a labour of love and I hope you enjoy reading it as much as I enjoyed writing it.

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Abstract

Background **–** Stroke patients often experience motor function impairments as a result of damaged motor fibres such as the corticospinal tract (CST) and alternate motor fibres (aMFs). However, the relationship and contribution of these tracts in motor function recovery are still incompletely understood. Pre-clinical studies are needed to elucidate the role and relationship of aMFs in stroke recovery. Transcranial magnetic stimulation (TMS) is a non-invasive brain stimulation technique that can be used as 1) a diagnostic tool to study cortical excitability and integrity of motor tracts or 2) a therapeutic tool to alter cortical excitability. Currently, the effectiveness of repetitive TMS (rTMS) treatment in the clinic shows inconsistent findings on improved cortical activation and motor function. Therefore, further investigation is needed to test the effectiveness of inhibitory versus excitatory rTMS after stroke.

Methods **–** For the first part of this study, twelve adult female Long-Evans rats were given either a sham or a focal CST lesion to study the contribution of the aMFs on the electrophysiological transmission of motor signals. Action potentials were elicited in the motor cortex with TMS and recorded in the forepaw with needle electromyography to determine the lowest motor threshold (MT) that allowed signal transmission pre-stroke and four times post-stroke. In the second part of this study, 21 male Sprague-Dawley rats with a motor cortex lesion, were given nine rTMS sessions over nine days. They received one of three different rTMS interventions (inhibitory, excitatory and sham (n = 7/group)) to study their effect on motor signal transmission and motor function recovery post-stroke. MT was determined pre-treatment and 1 day and 8 days post-treatment.

Results **–** Part I: the animals with a CST lesion showed no increase in MT (60.83 ± 5.27) compared to the animals without lesion (60.00 ± 5.47) in the acute phase post-stroke. However, there was a significant increase of MT in the late sub-acute phase (65.33 ± 4.50). Part II: there was difference in MT and motor function between treatment groups.

Conclusions **–** This study shows that a focal lesion to the CST in the internal capsule does not affect the electrophysiological transmission of motor signals in the acute phase of stroke recovery, whereas it does decrease transmission in the late sub-acute phase. Secondly, this study shows that there is no effect of rTMS treatment on motor signal transmission and motor function in animals with a lesion in the motor cortex. Both these findings lay the groundwork for the improvement of diagnosis and prognosis for personalized therapies of stroke patients to improve their clinical outcome.

Layman summary

Stroke patients often experience long term movement impairments. This is the result of damage in brain regions that are responsible for movement. Even after excessive rehabilitation interventions, some patients never fully recover to their pre-stroke motor function. It is still unclear what group of patients and to what extent, will or will not benefit from rehabilitation. Due to the great variability of stroke patients and thus unstandardized research, animal studies are needed to elucidate the relationship of different brain regions responsible for movement. The corticospinal tract is the major structure in the brain through which motor signals are transmitted from the motor cortex in the brain to extremities in the body. But the brain also uses alternate routes to transmit those motor signals. In this study, we investigated the effect of a lesion in the corticospinal tract on motor signal transmission. Therefore, we damaged the corticospinal tract with a small infarct and left the alternate routes intact. With a non-invasive brain stimulation technique, called transcranial magnetic stimulation (TMS), we stimulated the motor cortex and with a recording technique, called electromyography (EMG), we recorded the motor signals in the muscle. The minimum strength of a TMS pulse to elicit movement in the forepaw muscle is called the motor threshold (MT). The MT gives information about the activity in the brain and the integrity of the tracts that transmit these signals. Secondly, TMS can also be used as a therapeutic tool to increase brain activity and promote the recovery of movement in stroke patients. However, as previously mentioned, not all patients benefit from this therapy, and it is not completely understood why. In this study, we applied two different types of repetitive TMS (rTMS) treatments to rats with a stroke in their motor cortex, to investigate their effect on motor signal transmission and motor function. We used TMS-EMG to evaluate signal transmission. Our results show that damaging the corticospinal tract with a small infarct had no effect on the signal transmission in early stages post-stroke. However, a loss of transmission was found in the late sub-acute phase. Secondly, the different rTMS treatments did not affect signal transmission and motor function. These findings help understand the role of motor tracts in stroke recovery to help diagnosis and prognosis in stroke patients. Additionally, it suggests that patients with a lesion in the motor cortex are less likely to benefit from a TMS intervention protocol as applied in this study.

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1. Introduction

Stroke is the second-leading cause of death globally and the third-leading cause of death and disability combined (1). A stroke is the result of a reduced blood flow and oxygen supply to brain tissue, caused by either a blocked or narrowed blood vessel (ischemic stroke) or the leakage or burst of a blood vessel (haemorrhagic stroke)(2). Many stroke patients experience loss of motor function, due to cell death leading to brain dysfunction in regions responsible for movement (3,4). Even though there are different rehabilitation interventions, many patients are left with incomplete recovery resulting in permanent functional disabilities. Additionally, patients with (partially) recovered motor function show compensation in adjacent neuronal regions rather than true neuronal network recovery, complicating the understanding of the reorganization of brain networks in poststroke recovery (5,6). Extensive research has shown that motor function impairment after stroke is related to the structural and functional integrity of motor fibres, such as the corticospinal tract (CST) (7,8), which is the main pathway that accounts for voluntary motor function and movement of distal extremities in healthy humans (9). Additionally, alternate motor fibres (aMFs) such as the cortico-rubro-spinal tract also contribute to the regulation of motor activity. Patients with a damaged CST, but an undamaged cortico-rubro-spinal tract score higher in motor assessment than patients whose both tracts have been damaged. The degree of aMF integrity might therefore explain an important part in motor function recovery after stroke (7,8,10,11). However, additional research is needed to establish the physiological significance of these aMFs in functional recovery.

Pre-clinical stroke studies are commonly carried out in rats (5) because of their cerebral vasculature and physiology that resembles a human, the opportunity to apply invasive recording techniques, but also because of standardization and reproducibility possibilities (5,12,13). Even though the role of the CST has developed during the evolution of vertebrates, phylogeny shows that the organization of the rat's locomotive system is still similar to humans (14), making rats an appropriate animal model to investigate changes in motor fibre organization after CST stroke. In this thesis, we aim to study the effects of a focal lesion to the CST in a rat stroke model on electrophysiological transmission of motor signals and motor recovery.

Transcranial Magnetic Stimulation

Electrophysiological transmission of motor signals can be studied with non-invasive brain stimulation (NIBS) techniques such as transcranial magnetic stimulation (TMS) (3,10,15–18). During TMS, a copper-wire coil is placed on the head, where an electric pulse can be discharged that generates a magnetic field perpendicular to the head. These magnetic pulses modulate cortical excitability and induce action potentials in the stimulated cortical neurons. Consequently, motor fibres conduct the elicited action potentials to the respective muscle to stimulate contraction. Needle electromyography (EMG) is a technique that records and analyses these electrical signals in the muscles (19). Combining TMS with EMG can give information about the electrophysiological transmission of motor evoked potentials (MEPs) generated in the motor cortex and thus the integrity of the motor tracts relaying these signals (18,20,21). In this thesis, TMS-EMG will be used as a diagnostic tool to evaluate the electrophysiological transmission of motor signals when the CST is damaged while the aMFs are still intact.

Moreover, repetitive TMS (rTMS) can been used as a therapeutic tool for neuropsychiatric disorders and is becoming more popular in the treatment of stroke patients as it promotes neuroplasticity and brain regeneration, improving functional recovery (13,15,17,18,22). In rTMS treatment protocols, a repetitive train of TMS pulses of the same intensity is applied to the motor cortex, altering cortical excitability for hours (21,23,24). Different rTMS protocols can have different effects on neural and synaptic activity (21,23–26). Low frequency rTMS (1 Hz) on the contralesional hemisphere reduces the increased interhemispheric inhibition after stroke and promotes the electrophysiological transmission of motor signals from the ipsilesional hemisphere. Contrarily, high frequency rTMS (> 5Hz) on the ipsilesional hemisphere directly promotes its cortical excitability and the electrophysiological transmission of motor signals, potentially improving motor function.

Even though rTMS treatment is already used in the clinic, the physiological mechanism and benefit of rTMS treatments are still incompletely understood. This is partly due to the non-standardized variety of clinical stroke patients and studies and limited pre-clinical models, resulting in variable effectiveness of rTMS treatment (13,17,18,26).

In the first part of this thesis, a rat TMS-EMG design is proposed to be applied as a validation and diagnostic tool after experimental focal stroke. This design can then be used to study the effect of a damaged CST on electrophysiological transmission of motor signals during stroke recovery. For the second part of this thesis, data of two different rTMS interventions will be analysed to evaluate their effect on electrophysiological motor signal transmission and motor function during stroke recovery in rats. The main research questions that will be discussed in this thesis are as follows:

Part I:

1. *Does a focal lesion in the corticospinal tract affect the threshold for electrophysiological transmission of motor signals?*

With the following sub-questions:

1.1. Is there recovery of the motor threshold overtime?

1.2. Does the recovery curve of the motor threshold correlate with the recovery curve of motor function?

Part II:

2. *Does a repetitive TMS intervention affect the threshold for electrophysiological transmission of motor signals?*

With the following sub-questions:

2.1. *Which intervention (inhibitory or excitatory) has the greatest effect on the motor threshold in the acute phase post-treatment?*

2.2. *Does repetitive TMS intervention affect motor function recovery?*

For the first part, we will optimize a previously developed TMS-EMG protocol (27) for measurements in the Long-Evans rat strain. After optimization, the protocol will be applied to evaluate electrophysiological transmission of motor signals in rats that have had a focal stroke in the CST to understand the role and relationship of aMFs in functional recovery.

For the second part, we will use preliminary data of *Boonzaier* where rats – who have had a phototrombotic stroke in the motor cortex – were given either rTMS inhibitory (1 Hz), excitatory (5 Hz) or sham treatment. The data will be used to investigate the effects

of different rTMS treatments on the gain or loss of electrophysiological transmission of motor signals and functional recovery.

Previous research has shown that patients with stroke-induced damage to the CST require a higher TMS intensity to evoke MEPs (21). It is therefore expected that rTMS treatment increases the excitability of the corticospinal system hereby lowering TMS intensity and improving functional recovery. If the data shows a beneficial effect for either one or both of the therapies, it could be translated into the clinic where patients can benefit from a more appropriate individual treatment and an improved clinical outcome.

2. Materials and Methods

2.1 Animals

For Part I, 12 adult (18 ± 1 week) female Long-Evans rats (Charles River Laboratories, Italy; 298 ± 28 g) were used for the experiments. Animals were only included when completed the study and when stroke or sham surgery was successful. A total of two rats was excluded from the study: one as a result of anaesthetic complications during TMS baseline measurements and one as a result of an injection with a too strong dilution of Gadolinium contrast agent (1:40 instead of 1:500), limiting the validation of a successful surgery during the day 0 MRI-scan. This resulted in the inclusion of ten animals (n = 10; sham: n = 4 and stroke: n = 6). For Part II, 23 adult (11 week) male Sprague Dawley rats (Charles River Laboratories, Sulzfield, Germany) were used (n = 23; inhibitory, excitatory and sham (n = 7/group)).

All animals were housed in a temperature-controlled (24 °C, 45 to 65% humidity) animal care facility with a 12-h light-dark cycle. All procedures were approved by and in accordance with the guidelines of the Animal Ethics Committee of the University Medical Center Utrecht, the Netherlands. And were conducted in agreement with Dutch laws ("Wet op de Dierproeven," 1996) and European regulations (Guideline 86/609/EEEC).

All animals were randomly allocated to I) stroke or sham surgery or II) inhibitory, excitatory or sham treatment. Both biotechnicians, who performed the surgical procedures, and the experimenter, who did MT determination and behavioural assessment, were blinded for the aforementioned randomized allocation of animals.

2.2 Stroke induction

2.2.1 Focal capsular stroke

Part I animals received a focal capsular stroke in the fore- and hindlimb regions of the CST (28–30). Animals were sedated with a mixture of medical air and oxygen (4:1) and 5% isoflurane, and 2.5% isoflurane for maintenance. During the entire procedure, body temperature was maintained at 37°C body temperature using a rectal probe and electrical feedback system. The animal was then placed in the stereotactic frame where the dorsal side of the skull was shaved, and lidocaine was administered subcutaneously. After an incision in the skin, a small craniotomy at coordinates -2.3 mm (AP), -4.3 mm (ML) was made. These coordinates are based on a previous study (29) and have been optimized in

our lab for ± 18-week-old female Long-Evans to target both forelimb and hindlimb fibres of the CST. A preloaded 27G needle, with a 4 μL N5-(1-iminoethyl)-I-ornithine (L-NIO) (0.8 μmol/μL) and gadolinium (2 μmol/mL) solution, was used to make an intracerebral injection in the posterior limb of the internal capsule (PLIC) and -7.4 mm (V; measured from the skull) in a 10 degrees angle. A microinjector automatically injected the solution at a constant rate of 1 μL/min. After 4 minutes, the infusion was terminated, and the needle was left in place to prevent the solution from running back. After 15 minutes, the needle was slowly retracted. The skin on the head was sutured and carprofen (5 mg/kg) was administered for analgesia. Sham animals received the same surgical procedures, but sterile saline (mixed with gadolinium (2μmol/mL)) was injected instead of L-NIO.

Directly after surgery, all animals were scanned in the MRI to validate a successful surgery. If surgery was unsuccessful or could not be validated, animals were excluded from the study.

2.2.2 Photothrombotic cortical stroke

Part II animals received a photothrombotic stroke in their motor cortex. Animals were sedated, maintained on body temperature, and prepared for incision as previously described. Additionally, the ipsilesional inside of the leg was shaved for the injection of Rose Bengal. After incision, the periost is scraped off the skull so the position of Bregma could be determined. From there the coordinates were calculated (-2.2 mm ML and -1.8 mm AP) for the placement of the centre of the green, fluorescent illumination over the motor cortex. A preloaded 27G needle with a 1 mL Rose Bengal solution (25 mg/mL) was inserted into the vena Femoralis. An infusion pump injected the solution at a constant rate of bodyweight of the rat in grams in μL per minute for 20 minutes. After injection, the needle was retracted immediately, and the green light source was directed over the motor cortex as closely as possible for another 20 minutes. Finally, the light source was switched off and the skull and leg were sutured. Sham animals received the same surgical procedures, but sterile saline was injected instead of Rose Bengal.

2.3 Anaesthesia and preparation during TMS

All animal groups were induced with a mixture of medical air and oxygen (4:1) and 5% isoflurane for anaesthesia. Under 2.5% isoflurane maintenance rate a lateral tail vein

catheter, preloaded with saline followed with a continuous propofol infusion (59 ± 4 mg/kg/h), was placed (see I in *figure 1*). As Long-Evans are pigmented rats, they required a higher dose than (albino) Sprague-Dawley rats (respectively 59 ± 4 mg/kg/h and 40 ± 2 mg/kg/h) to reach the required level of sedation (31). Research showed the use of propofol anaesthetic in a 4-hour period with no loss of MEP response (27,32). Isoflurane delivery was stopped after 5 minutes of propofol infusion and after a 20-minute washout period of isoflurane, MEP measurements could commence. Meanwhile, the rat was fixated in the stereotaxic frame where it continued to receive a mixture of medical air and oxygen (1:1) to counter oxygen deprivation caused by propofol (33). The top of its head was shaved to ensure optimal contact between skull and coil. The TMS coil was put on the starting position and needle electrodes were inserted. During the entire experiment, body temperature was maintained at 37°C using a rectal thermometer and an electrical heating pad system.

2.4 Electromyography

Monopolar uninsulated 28G stainless steel needle electrodes (REF. 74325-36/40, Ambu A/S, Denmark) were pre-soaked in saline (0.9% NaCl) and inserted in the belly of the biceps brachii; a muscle group that gets activated by CST activity upon motor cortex activation (10). The location of the brachialis muscle was determined by palpation of the extended forelimb (20,27). A reference electrode was positioned distally in the paw between two digits, see *Appendix 1* for visualisation of the needle placement. The rat was electrically grounded via a single 27G stainless steel disposable subdermal needle electrode (REF. TE/S43-438, Technomed Europe, The Netherlands) placed in the tail base (see II and III in *figure 1*). All electrodes were secured and held in place with adhesive tape. The EMG signal was band-pass filtered at 5-1000 Hz and amplified 164 times in the single output range of 60 mV (REF. NS006201.012, Neuro-MEP Amplifier Unit, Neurosoft, Russia; and Neuro-MEP-4 system, Neuro-MEP software, Version 4.2.5.6, Neurosoft, Russia). The EMG signal was digitized with 20 kHz sampling and stored for post-hoc analysis.

2.5 Transcranial magnetic stimulation

All animals were stimulated with a biphasic magnetic stimulator (REF. NS058201.005, Neuro-MS/D Main Unit, Neurosoft, Russia) and a small figure-of-eight TMS coil (REF. FEC-

03-25-C, 2x Ø25 mm Cooled Rat Coil, Neurosoft, Russia). During stimulation, the coil was fixed horizontally above bregma and secured to a stereotaxic frame (see V in *figure 1*). The stereotaxic frame and the aluminium foil underneath the animal were grounded to reduce noise signals (see IV in *figure 1*). See *figure 1* for a final setup of the experiment.

For the starting position, the posterior end of the coil was placed perpendicular to the interaural line of the rat so the centre of coil would be anterior respective to bregma. See *Appendix 2* for visualisation of the coil placement. The coordinates of bregma were calculated as follows:

$$bregma = interaural + (bodyweight * 0.0059) + 6.3968 \qquad (34).$$

The coil was moved posteriorly to place the centre of coil over bregma. Finally, the coil was relocated anterolaterally (+1 mm AP and ±2.5 mm ML) to place its centre over the ipsilesional motor cortex of the rat (35). See *Appendix 3* for the full TMS-EMG protocol.

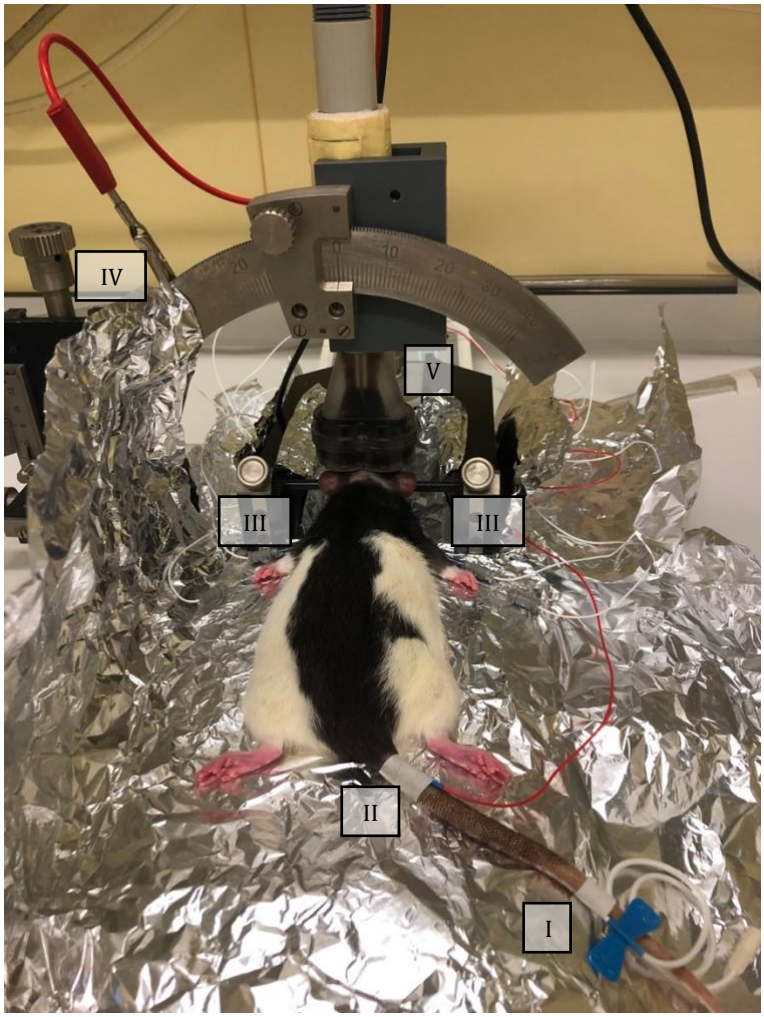

Figure 1 – Final setup of the experiment. The animal received a constant propofol infusion through the tail vein (I). One reference electrode was placed subdermal in the tail (II) and four electrodes were placed in the forelimbs (III). The stereotactic frame and aluminium foil underneath the animal and the electrodes are grounded to reduce noise signals (IV). The coil was placed perpendicular to the shaved head (V).

2.6 Motor threshold determination

To determine the location over the motor cortex where MEPs could be most reliably measured, the coil was moved mediolaterally over the affected hemisphere in steps of 1 mm (max. ±3 mm). At each of these locations, the motor threshold (MT) was obtained by starting stimulation at 50% of the maximum system machine output (MO) and increasing the intensity in steps of 5% or 2% until a positive MEP response was recorded. A positive MEP response is defined as a MEP with a peak-to-peak amplitude of at least 50 μV (36). The estimated MT was regarded as the minimum intensity at which minimally five out of ten consecutive trials resulted in positive MEPs. To exclude the possibility of low frequency rTMS-induced effects, a minimum of 10 seconds between stimulation pulses was maintained (37). From the mediolateral coordinate which showed the lowest MT value additional anteroposterior locations (max. ± 2 mm) were measured. From the entire measured grid, the lowest MT intensity is indicated to be the 'hotspot' location. In case of two locations where the intensity is the same, the location with the highest MEP peak-to-peak amplitude is selected. Finally, ten consecutive EMG traces were recorded at the central hotspot location with a 100% MT stimulation.

In the Part I of the study, MT was determined on five different days: pre-stroke (baseline), post-stroke day 3 (acute), week 1 (sub-acute), week 3 (late sub-acute) and week 10 (chronic). In Part II, MT was determined on three different days: pre-treatment day 3 (post-stroke), and post-treatment day 16 and day 23. See *figure 2* for an overview of the timeline for both studies.

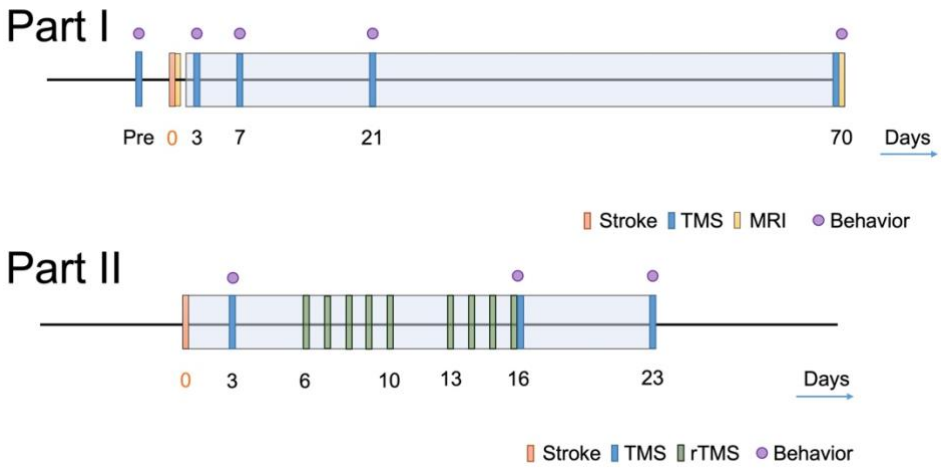

Figure 2 – Timeline for both studies. Part I: five different TMS and behaviour assessment days: pre-stroke and acute phase, sub-acute phase, late sub-acute and chronic phase post-stroke. MRI scans on day 0 and 70 were performed to validate successful surgery. Part II: three different TMS and behaviour assessment days post-stroke: pre-treatment, directly after the treatment sessions and one week after treatment sessions. The nine rTMS treatment sessions were given on nine consecutive dates, except for the weekend.

2.7 Repetitive transcranial magnetic stimulation

rTMS was only applied in Part II. rTMS treatment started on day 6 after stroke surgery and was composed of nine treatment sessions, one on each day, except for the weekend. Animals were prepared in the same manner as previously described to determine MT. Subsequently, animals would get either 1 Hz rTMS on the hotspot location of their unaffected hemisphere or 5 Hz rTMS on the hotspot location of their affected hemisphere. rTMS was given at 85% MT intensity and consisted of a continuous train of 1 pulse per second and a total of 1200 pulses (1 Hz inhibitory) or a 5-second train pulses with an interval of 55 seconds between trains and a total of 500 pulses (5 Hz excitatory). The sham-group experienced no active stimulation as the continuous train of 1 pulse per second (total of 1200 pulses) was given at 10% MT. This prevents the treatment from actively stimulating the brain.

2.8 Behavioural assessment

Behavioural assessment was performed with two different tests: 1) cylinder test to assess forelimb asymmetry (38) and 2) beam walk test to assess hindlimb motor coordination (39). In Part I both tests were performed pre-stroke (baseline) and four times post-stroke: on days 3 and 7 (acute and sub-acute) and week 3 and 10 (late sub-acute and chronic). In Part II, data from only the cylinder test is included, which was carried out on baseline (post-stroke, pre-treatment) and on days 3, 16 and 23 (post-treatment).

2.8.1 Cylinder Test

A 30 cm high Perspex cylinder with a 20 cm diameter was placed in a box with two mirrors behind the cylinder to ensure a complete view of the animal. The animal was placed in the cylinder and was only retrieved when the total number of forepaw touches on the cylinder wall added to at least twenty in the final score. Touches were counted for affected, unaffected or both paws (simultaneous contact) (40). A touch started when at least one of the forelimbs touched the cylinder in its entirety and ended when the last forelimb stopped making full contact. A simultaneous contact was counted when the second forelimb touched the cylinder before the first forelimb was replaced or retracted. If the first forelimb touch replaced or retracted without the use of the second forelimb, the touch was counted as an individual affected or unaffected touch. Touches are counted

towards a total paw placement score for 'affected', 'unaffected' or 'both', from which the forelimb asymmetry could be scored:

$$forelimb\ asymmetry\ = \frac{affected\ forelimb}{affected\ forelimb + both + unaffected\ forelimb} - \frac{unaffected\ forelimb}{affected\ forelimb + both + unaffected\ forelimb} * 100\%$$

2.8.2 Beam Walk Test

A 175 cm long tapered beam, varying 6 to 1.5 cm width from start to end, was used. An underhanging ledge (2 cm below the beam and 2 cm width) worked as a crutch for the animal. As the beam narrowed to the end, the difficulty increased, and more foot faults were made. The first 30 cm and last 15 cm of the beam were not counted when scoring foot faults. A dark 'safe box' was placed at the end of the beam in which the animal jumped after completing a trial on the beam and where it was left alone for a minute. The beam was equipped with mirrors on the wall to help visualize all limb side movements.

On three separate days before the baseline measurement, rats were trained by completing three consecutive trials on the beam without the ledge. On each test day, the ledge was re-attached, and five consecutive trials were performed and recorded, from which the first one is always discarded (41).

The beam walk test consisted of two scoring items: 1) total steps to traverse and 2) foot faults. The former is defined by the total steps the individual rat limb took to traverse from the wide to the narrow side of the beam. Counting started when the respective limb crosses the 30 cm mark and stopped when the same limb crosses the 15 cm mark. The foot faults were scored based on 'half-slips' (0.5 pt) and 'full slips' (1 pt). A full slip is defined by the full placement of the paw on the ledge, whereas a half-slip is defined as slipping of the beam and slightly touching the side of the beam, but not placing the paw on the ledge (42). All full- and half-slips were added up and the percentage of foot faults per stap (FpS) was calculated per hindlimb, from which the hindlimb-asymmetry could be scored:

$$foot\ faults\ per\ step\ (FpS) = \frac{(half\ slips * 0.5) + full\ slips}{all\ steps} * 100\%$$

$$hindlimb\ asymmetry = FpS_{unaffected} - FpS_{affected}$$

2.9 Data analysis

All data were firstly examined on outliers and normality in Windows SPSS (IBM SPSS Statistics, Version 28.0.1.1, IBM Corp., USA). Outliers were determined by *z-scores*, in which *z-scores* of < -2.68 or > 2.68 was regarded as an outlier. Normality was measured using the Shapiro-Wilk test ($p < 0.05$). Finally, RStudio (RStudio, Version 2021.09.01, RStudio, PBC, USA) was used for the statistical analysis of the MT-values and motor assessment scores.

To analyse whether a lesion in the CST has any effect on MT or whether rTMS treatment has any effect on MT or motor function, all three datasets were separately statistically analysed using the linear mixed models and Anova functions in RStudio, with a Bonferroni correction for multiple comparisons ($p < 0.05$). Post-hoc testing could follow on the significant data, using the F-test and the one-sided independent t-test. Respectively used to evaluate on what timepoint(s) the MT values differ between surgery or treatment groups. For both tests, the Bonferroni correction for multiple comparisons was applied.

To follow recovery, the paired samples t-test was used in RStudio ($p < 0.0125$) with the baseline measurement as the control group against which other timepoints were tested. The *p-value* is based on the Bonferroni correction for multiple comparisons ($0.05/4 = 0.0125$).

Finally, to study the correlation between MT and either forepaw or hindlimb asymmetry, their graphs were graphically visualized and inspected for similarity.

For the visualisation and possible qualitative analysis of the Neurosoft software MEP signals, MATLAB (MATLAB R2018b, The MathWorks. Inc., USA) was used. To analyse possible changes in the MEP amplitude the difference between minimum and maximum MEP signal, between 10 and 25 ms after stimulus, was calculated for the ten recorded 'hotspot' traces on 80% MO for all animals. However, due to the great variable morphology of the traces and their amplitude within the same measurement, the qualitative analysis was not included in this thesis.

3. Results

All different datasets have been checked on outliers and normal distribution. Visually there were some outliers in the some of the datasets, however, not statistically significant (*z-scores* between -2.68 and 2.68). Secondly, all data was normally distributed ($p > 0.05$).

3.1 Part I

3.1.1 Focal capsular stroke leads to elevated motor threshold in the late sub-acute phase

To evaluate whether a focal CST lesion has any effect on the electrophysiological transmission of motor signals on the ipsilesional hemisphere, we analyse the MT-values of the stroke group (n = 6) and sham group (n = 4) over all five different timepoints. The results are shown in *figure 3*.

The MT-values of both groups are marginally significantly different from another ($p = 0.070$). Time has the greatest significant effect on the MT-value ($p = 0.008$).

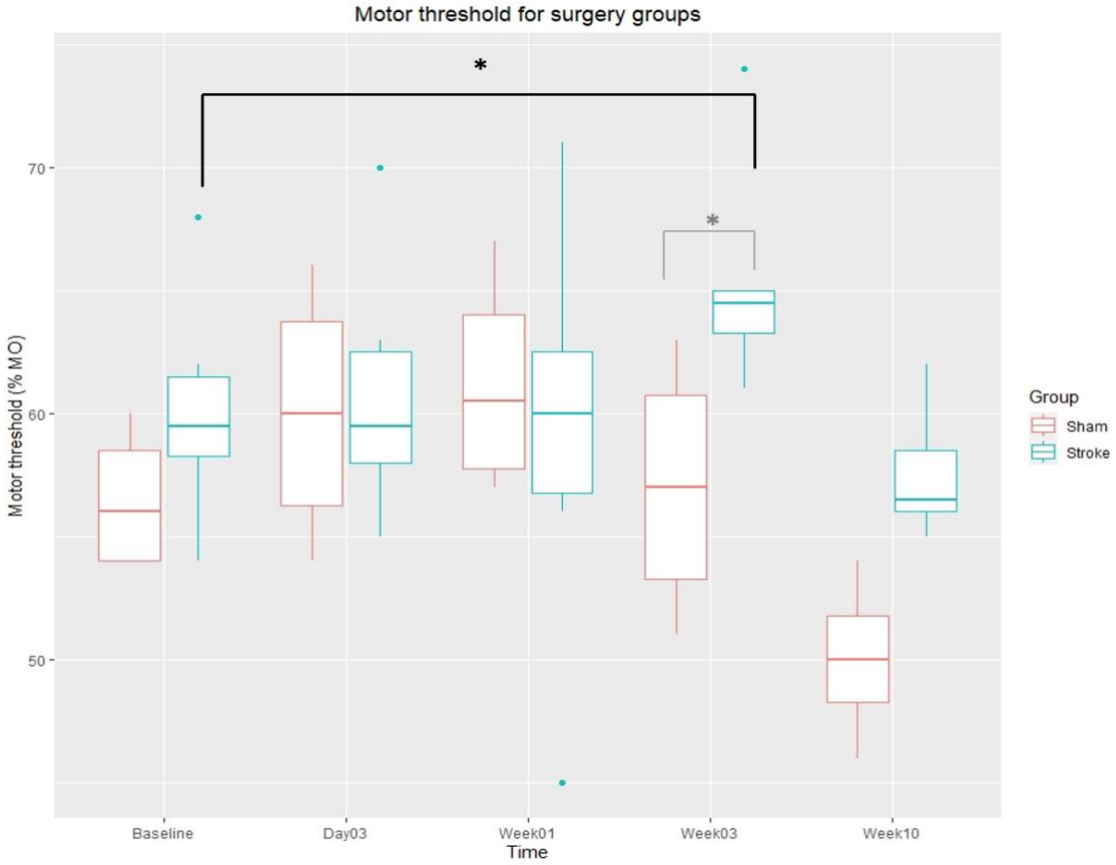

*Figure 3 – Motor threshold for surgery groups given as a percentage of the machine output. MT of the ipsilesional hemisphere was measured on five different occasions for both sham (n = 4) and stroke (n = 6) groups. All values are expressed as mean ± SD. The mean scores for the sham group on the different timepoints are: 56.50 ± 3.00 (baseline), 60.00 ± 5.47 (day 3), 61.25 ± 4.65 (week 1), 57.00 ± 5.48 (week 3) and 50.00 ± 3.37 (week 10). The mean scores for the stroke group on the different timepoints are: 60.17 ± 4.67 (baseline), 60.83 ± 5.27 (day 3), 59.17 ± 8.59 (week 1), 65.33 ± 4.50 (week 3) and 57.50 ± 2.59 (week 10). A marginal significant difference between stroke and sham group is found on week 3 (*p = 0.085*). The MT for the stroke group is only significantly increased on week 3 (*p = 0.012*). On other timepoints and in the sham group, there is no significant difference in MT between pre-stroke and post-stroke.*

Post-hoc analysis was performed to evaluate on which timepoints the MT of the stroke group differs significantly from the sham group. This timepoint is found on week 3 ($p = 0.085$) where the MT of the stroke group (65.33 ± 4.50) is marginally higher than that of the sham group (57.00 ± 5.48), see *figure 3*.

3.1.2 Motor threshold recovers in the chronic phase after stroke induction

Since time has a significant effect on the MT. Post-hoc analysis was performed to evaluate on what timepoints this effect is significant. The two groups were analysed individually to follow their respective change in MT over time. For the sham group no mean value on different time points is significantly different from the baseline (p = 0.322, p = 0.045, p = 0.713, p = 0.039 for the respective days following baseline). Suggesting that there is no significant increase or decrease and recovery of MT in the sham group.

In the stroke group, MT is significantly higher on week 3 (65.33 ± 4.50) than on baseline (60.17 ± 4.67, $p = 0.012$), see *figure 3*. But again, on week 10 (57.50 ± 2.59) the MT is not significantly different from baseline ($p = 0.315$). Suggesting that the animals in the stroke group experienced an increase in MT on week 3 that recovered on week 10.

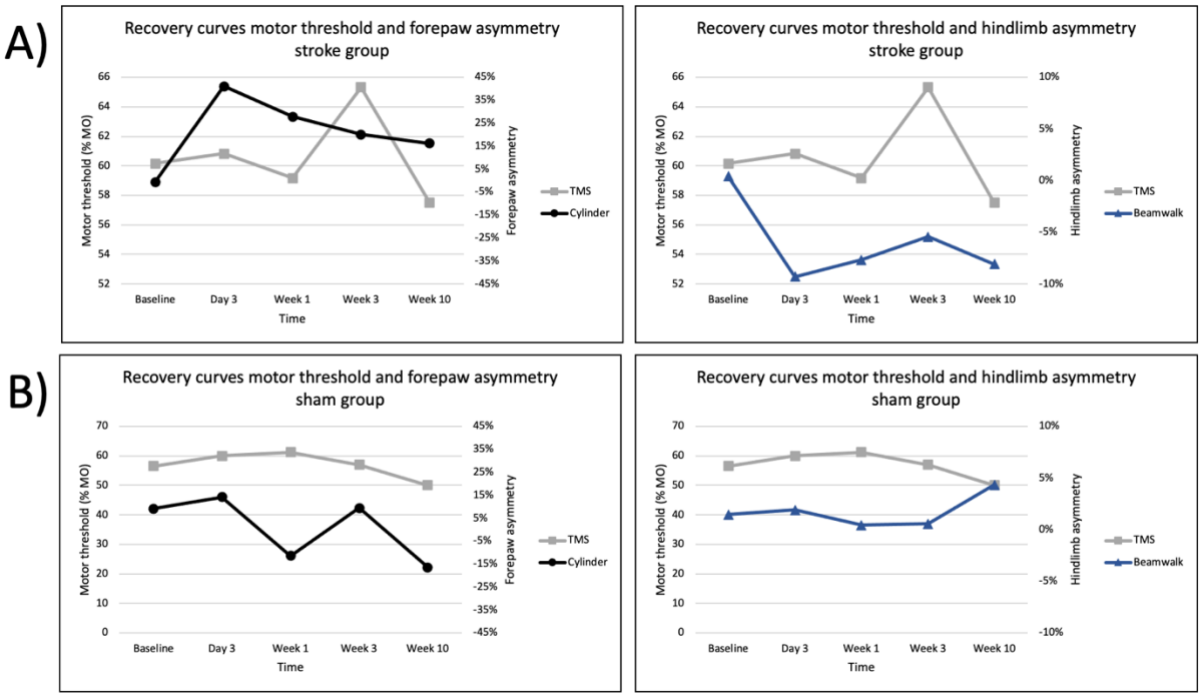

Figure 4 – Relation between recovery of MT and motor function. A) Stroke group: left the correlation between cylinder forepaw asymmetry and MT. Right the correlation between beamwalk hindlimb asymmetry and MT. No similarity was observed as the behaviour curves show deterioration in the early stages while the MT shows deterioration in the late sub-acute stage. B) Sham group: left correlation between cylinder forepaw asymmetry and MT. Right the correlation between beamwalk hindlimb asymmetry and MT. There is more similarity between MT and fore- and hindlimb asymmetry for sham animals compared to stroke animals.

3.1.3 Motor threshold and motor function do not correlate

To assess whether MT can give additional information of motor function or vice versa, we visually examined the elapse of both curves overtime to evaluate similarity. The curve of MT in the stroke group is visually not similar to the motor function curves of forepaw and hindlimb asymmetry as seen in *figure 4a*. As both behaviour curves have greater asymmetry in the early stages, associated with deteriorated motor function. While the MT curve is elevated in the late sub-acute phase, associated with reduced transmission of motor signals.

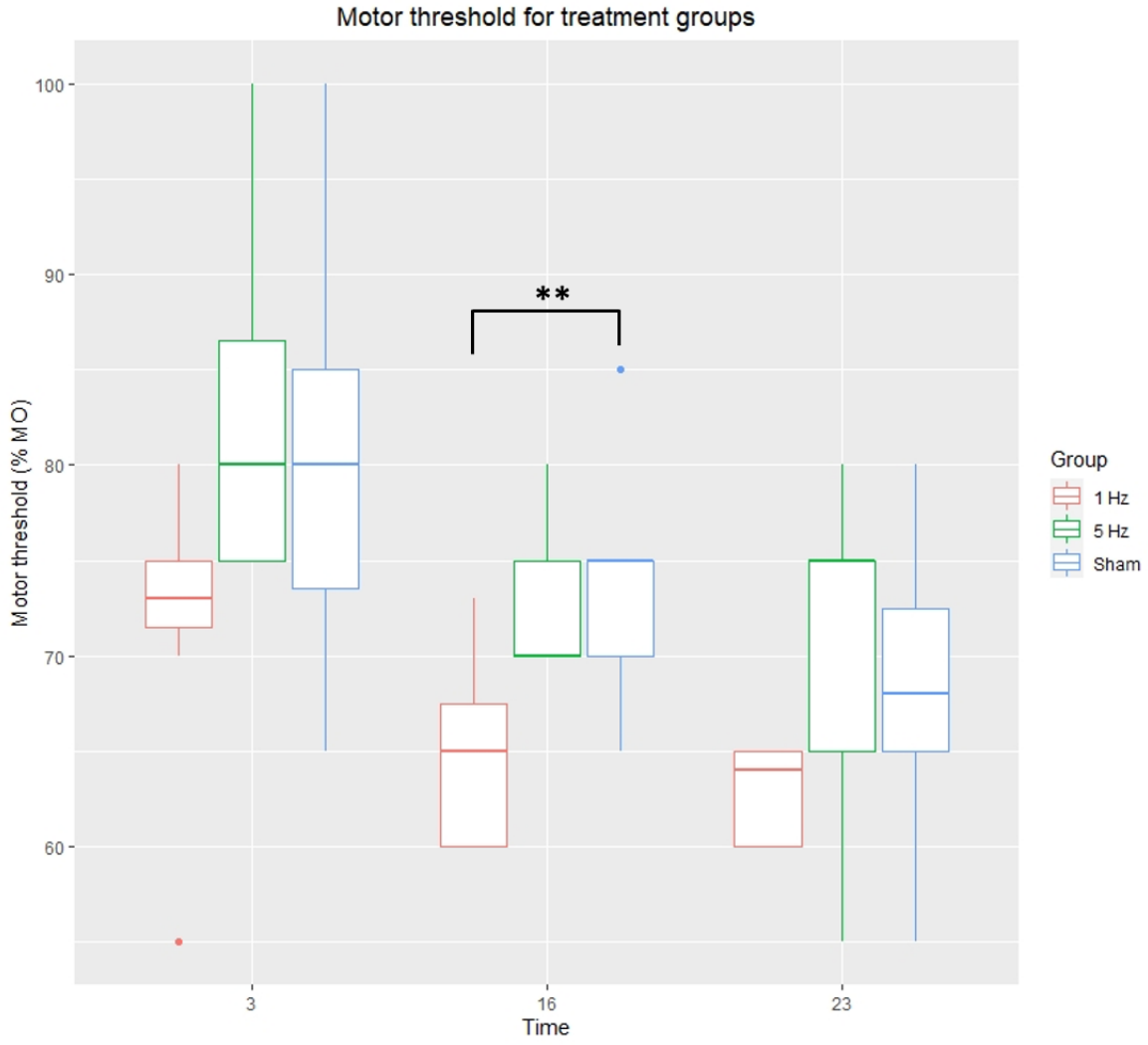

Figure 5 – Motor threshold for treatment groups. MT of the ipsilesional hemisphere was measured on three different occasions for all groups. The mean scores for the 1 Hz treatment group (n = 7) on the different timepoints were: 71.57 ± 7.33 (day 3), 64.71 ± 4.83 (day 16) and 62.80 ± 2.32 (day 23). The mean scores for the sham treatment group (n = 7) on the different timepoints were: 80.29 ± 10.44 (day 3), 73.57 ± 5.80 (day 16) and 68.29 ± 7.40 (day 23). The mean scores for the 5 Hz treatment group (n = 7) on the different timepoints were: 82.57 ± 8.76 (day3), 72.86 ± 3.64 (day 16) and 70.00 ± 8.02 (day 23). Inhibitory 1 Hz treatment decreased MT significantly compared to sham treatment (p = 0.007). On other timepoints there was no significant difference in MT between treatment groups.

The curve of MT in the sham group is similar to both the curves of forepaw and hindlimb asymmetry as seen in *figure 4b*. Even though the forepaw asymmetry curve slightly oscillates between different timepoints, both behavioural tests show no effect of sham surgery. The hindlimb asymmetry curve only increases slightly on week 10. Similarly, the MT-values show no effect of sham surgery.

3.2 Part II

3.2. Inhibitory treatment has a lower motor threshold in the acute phase post-treatment

In part II of this thesis, we studied the effect of three different rTMS treatments on electrophysiological transmission of motor signals in the ipsilesional hemisphere. All animals received similar surgeries but were treated with one of three different rTMS interventions: inhibitory 1 Hz (n = 7), excitatory 5 Hz (n = 7) or sham stimulation (n = 7). The outcome parameter MT was measured on three different occasions for all treatment groups. The results are shown in *figure 5*. To evaluate any effect of treatment on MT, the MT-values of all three groups were analysed over all timepoints. The MT-values of the three groups are statistically significantly different from another ($p = 0.011$).

The following post-hoc analysis shows no statistical difference in MT-values pre-treatment ($p = 0.107$) or chronically post-treatment ($p = 0.258$). But it shows statistical significance directly post-treatment on day 16 ($p = 0.009$).

Since we found a statistically significant difference in MT on day 16. A second post-hoc analysis was performed to evaluate which treatment group has a significantly lower MT than the sham group on day 16. The two treatment groups were individually compared to the sham group to find out which treatment is more beneficial in decreasing MT. The MT of the 1 Hz treatment group (64.71 ± 4.83, $p = 0.007$) is significantly lower than the MT of the sham treatment (73.57 ± 5.80) as seen in *figure 5*. Whereas the MT of the 5 Hz treatment group (72.86 ± 3.64, $p = 0.402$) is not significantly lower than sham treatment. This indicates that only the 1 Hz treatment has a significant effect on the MT.

3.2.2 Relative motor threshold values show no effect of treatment

Even though the inhibitory treatment group experiences a significant lower MT on day 16, it is evident from group means at day 3 that the inhibitory treatment group starts with an already lower MT – although not significantly lower. Therefore, we analysed the relative MT values of the treatment groups to evaluate any significant decrease in MT on the post-treatment timepoints compared to pre-treatment. See *figure 6* for the relative MT post-treatment. Analysis shows no significant effect ($p = 0.771$) of treatment of the relative MT values on both post-treatment timepoints for all treatment groups.

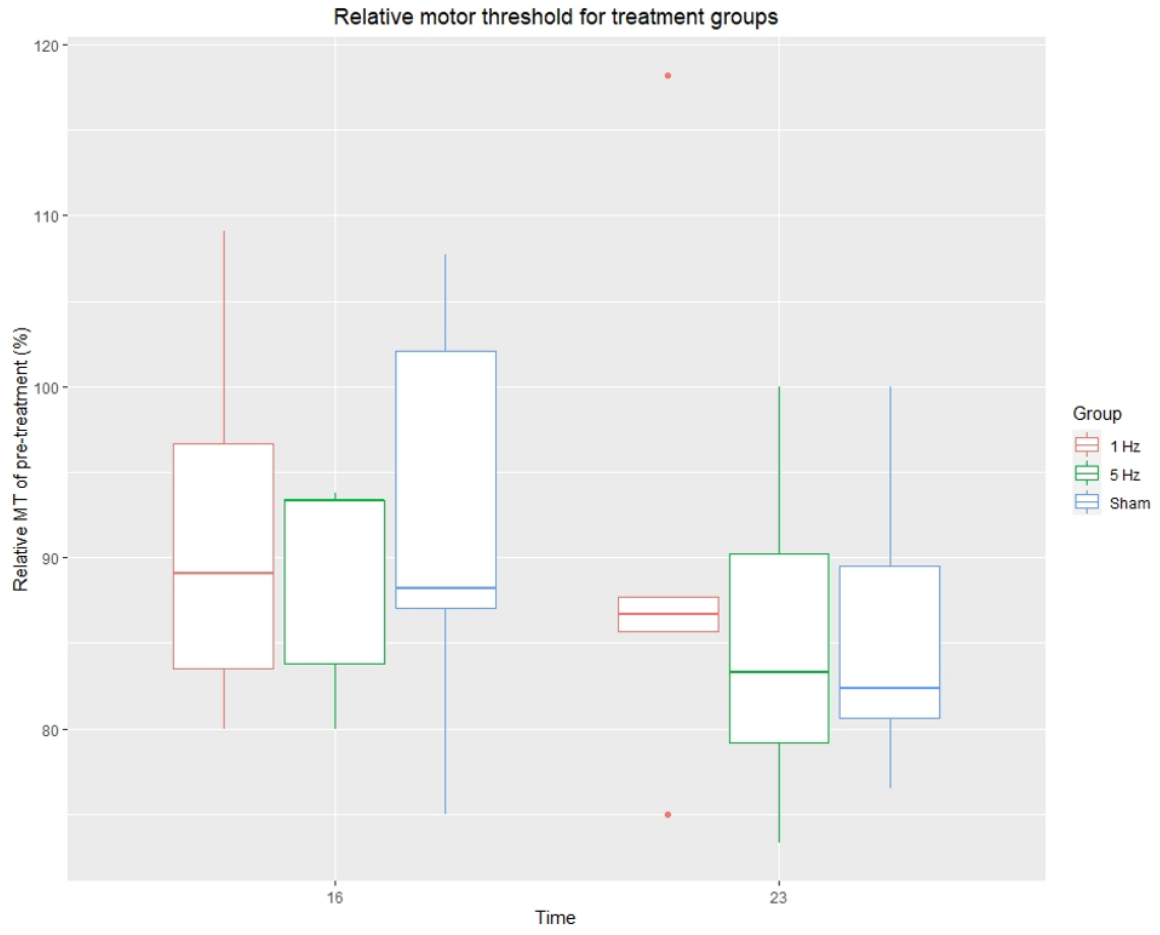

Figure 6 – Relative MT values of post-treatment days as a percentage of pre-treatment MT. The relative mean scores for the 1 Hz treatment group (n = 7) on the post-treatment timepoints were: 90% ± 8% (day 16) and 88% ± 3% (day 23). The mean scores for the sham treatment group (n = 7) on the different timepoints were: 93% ± 8% (day 16) and 85% ± 10% (day 23). The mean scores for the 5 Hz treatment group (n = 7) on the different timepoints were: 88% ± 5% (day 16) and 84% ± 11% (day 23). No significance in relative MT value was found between treatment and sham groups.

3.2.3 rTMS intervention does not affect motor function

To evaluate if rTMS intervention has an effect on motor function, the cylinder test was performed on three different days for all treatment groups. The results are shown in *figure 7*. The forepaw asymmetry scores of all three groups were analysed over all timepoints. The asymmetry scores are not statistically different between the treatment groups ($p = 0.319$).

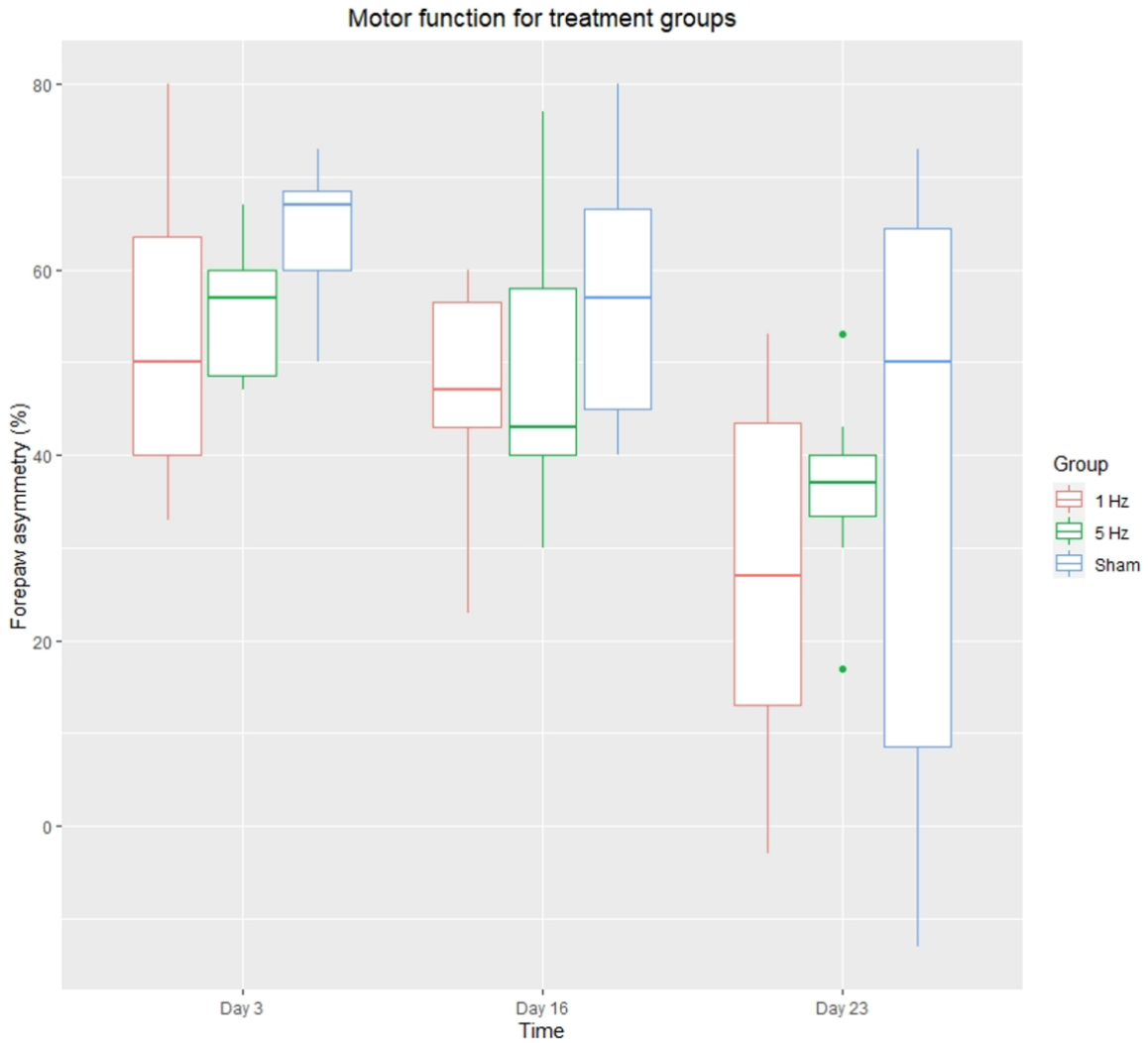

Figure 7 – Motor function for treatment groups. Forepaw asymmetry was measured on three different occasions for all groups. The mean scores for the 1 Hz-group on the different timepoints are: 53% ± 16% (day 3), 47% ± 12% (day 16), 27% ± 19% (day 23). The mean scores for the sham-group on the different timepoints are: 64% ± 7% (day 3), 57% ± 14% (day 16), 37% ± 34% (day 23). The mean scores for the 5 Hz-group on the different timepoints are: 55% ± 7% (day 3), 50% ± 15% (day 16), 36 ± 10% (day 23). There is no significant difference in motor function between the different treatment groups on different timepoints.

4. Discussion

4.1 Part I

The aim of the first part of the thesis was to evaluate electrophysiological transmission of motor signals in rats that have had a focal capsular stroke in the CST to help understand the role and relationship of aMFs in functional recovery.

Part I of the study demonstrated that there was no effect of a focal capsular lesion to the CST on the MT in the acute phases after stroke. This was evident from the MT-values of the surgery and sham groups that did not show any differences on day 3 and week 1. This was unexpected as we hypothesized that the MT would increase in the more acute phases after surgery. As previous research has shown that reduced density of motor fibres results in inefficient or loss of signal conduction (21,43), which should thus lead to an increase of MT. In the acute phase post-stroke, neurons in the ischemic core region die while neurons in the penumbra survive but lose their dendritic spines. As a result, there is reduced sensory specificity and activity in this region (43). However, since we did not find an increase in MT in the acute phase, we hypothesize that aMFs compensate for the loss of conduction by the CST, as they do in stroke animals and patients (10). However, it is unlikely that this compensation is already established in the early phases post-stroke (43,44). Therefore, the observation of an unaffected MT in the acute phase might be caused by one of the limitations of our model, as we will discuss more extensively later. The magnetic field of the coil is likely to penetrate deeper and broader into the brain (27), stimulating parts of the motor tract that were not damaged during surgery. Therefore, we did not find any effect of surgery on the electrophysiological transmission of motor signals.

Secondly, there was a significant increase in MT of the stroke group in the late sub-acute phase. This result also went against our expectations based on previous research. In the late sub-acute stages, sustained homeostatic processes of growth-promoting factors increase dendritic spine turnover and synaptogenesis of both ipsi- and contralesional regions, restoring the neuronal connectivity (43,45). During this process, the neurons become hyperexcitable as they lack sensory specificity to engage in the neuroplastic processes. This means that the MT should decrease instead of increase in the late sub-acute phase. The results did show a recovery of MT of the stroke group in the chronic phase, as was expected from previous research (43). In the chronic phase, there is refinement of synaptic connections and adjacent neurons have rewired to compensate

for the loss of electrophysiological transmission previously by the CST (43,45). The recovery of MT in the chronic phase is therefore a process of compensation rather than true recovery.

The deteriorated behaviour assessment scores of the stroke group in the early stages showed that a focal CST lesion does influence motor function. However, the results demonstrated that there is no similarity between the curves of MT and motor function during stroke recovery. This is explained by the fact that the motor function is mostly affected in the acute phase of stroke (5,7,11) whereas MT was not.

The beam walk hindlimb test shows an unexpected increase in asymmetry on week 10 in the sham group and a great variability in the stroke group. We assume that this is the effect of repetitive exercise and training rather than a reflection of real motor function.

4.2 Part II

The aim of the second part of the thesis was to evaluate the effects of different rTMS treatments on the electrophysiological transmission of motor signals and functional improvement in stroke rats to improve the clinical use of rTMS.

From the second part of the results, it is evident that the treatment did not have a significant effect on MT-value and motor function. Even though the MT of the 1 Hz treatment group was lower directly post-treatment, the relative post- and pre-treatment MT of the 1 Hz group were not statistically different, indicating that applying these interventions does not lower MT. This is not in line with our hypothesis that rTMS intervention would increase the excitability of the motor cortex and therefore lower MT. Some studies suggest that rTMS treatment – irrespective of the type of rTMS – is not sufficient to have any beneficial effect on MT and motor function when used as the sole intervention strategy (17,24,26,46). A second behavioural therapy, such as physiotherapy or speech therapy in patients or enriched environment in animals, next to rTMS treatment can have a more permanent and vigorous effect on neuronal plasticity (47). This is because neuronal plasticity is dependent of motor learning and task-specific practice (48). Motor training can strengthen the newly formed neuronal circuits of rTMS by emphasizing the desired interaction between presynaptic stimuli and postsynaptic action potentials, making novel durable synaptic connections (43,48). Contrarily, undesired connections are weakened. This process is referred to as Hebbian plasticity and might explain the need for combination rTMS interventions.

Additionally, the 9 rTMS sessions during the acute to sub-acute phases of stroke recovery might not have been enough to stimulate cortical excitability and functional improvement (49). Studies showed that rTMS might only be effective when applied within hours of stroke onset in rats (44) or when at least 20 rTMS sessions are given. Therefore, when applied sooner and more generously, rTMS could possibly counter the deterioration of cortical activity in early stages and improve functional recovery.

Finally, recent studies have researched the effect of lesion location and effectiveness of rTMS treatment (50–52). They show that patients with a damaged cortical region have less benefit from rTMS than patients with only subcortical damage. This might be explained by the lack of signal transmission of the increased ipsilesional excitability by rTMS to different parts of the motor network, due structural or functional disconnection and an overall lower brain activity. Our results support this hypothesis, since all the animals in Part II of this study experienced cortical damage and did not react to rTMS treatment. These findings are relevant for clinical interventions as it suggests that patients with cortical involvement are less likely to benefit from rTMS treatment.

4.3 Limitations

As previously mentioned, there were several limitations when conducting this research. First, the small sample sizes for all groups, the variability of the MT data and the spread within groups, may have contributed to the fact that we have not found any statistical significance. Similarly, caution has to be taken when interpreting the results where a significant effect was found. It would therefore be interesting to see what effects are seen when the datasets are expanded. And even though we attempted to maintain a standardized animal model, there still was variability within the data. A contributing factor could be that in Part I we have not taken the preference paw into account when giving the lesion. This means that some animals were more likely to use their impaired limb, accentuating the Hebbian plasticity, resulting in a lower MT. Or vice versa, where MT was more elevated because the lack of use of the impaired limb.

Secondly, the anaesthetic regime might also have contributed to variability in the data. As the regime used in both parts alters cortical physiology and excitability, hereby affecting consistent TMS measurements (18,32). Even though we aimed to maintain all animals in the equally sedative states consistently over time by checking their pain response after a pinching its toe, it is difficult to compare the depth of sedation between

animals and between different timepoints. Differences in depth of sedation may therefore have affected cortical excitability and MT-value for each animal. However, current rat TMS protocols require the use of anaesthetics (20), making it hard to circumvent this limitation.

Lastly, even though TMS has been applied to rats to study neurological disorders, the technique still faces some limitations (17,27,32,53). This is partly related to the small-sized rat brain and the broad volume of electrical current induced by a TMS coil, even a rodent-specific TMS coil. Since the broad volume of electrical current may lead to the simultaneous stimulation of both hemispheres and/or the stimulation of distal components of descending motor pathways (17,27,32,53). However, there are currently no coils on the market with more focal stimulation as properly cooling them is a great challenge (54).

5. Conclusion

In conclusion, this thesis suggests that a focal capsular CST lesion does not change the electrophysiological transmission of motor signals in the acute phases post-stroke. Unexpectedly, the electrophysiological transmission of motor signals of the stroke group decreased in the late sub-acute phase and recovered in the chronic phase. However, due to some limitations, caution must be taken with these interpretations.

It might be possible that aMFs are capable of compensating for the loss of transmission, but future research is needed to confirm this. It would therefore be interested to study the effects of different lesion combinations, such as: a lesion in the CST and/or the cortico-rubro-spinal tract and/or the cortico-reticulo-spinal tract etc. When studying these combinations and their respective effect on electrophysiological transmission of motor signals, a full understanding of the contribution and relationship of all the motor tracts in the brain during stroke recovery could be gained. Consequently, stroke patients can benefit from a more personalized diagnosis and prognosis.

Furthermore, no beneficial treatment effect has been measured for either the excitatory or inhibitory rTMS intervention. Suggesting that both rTMS approaches may not be suited as a therapeutic strategy to increase a patient's cortical excitability and improve motor function recovery for patients with a cortical lesion. Rather, TMS therapy may be more effective when used in even more acute stages post-stroke or in combination with another form of intervention. Therefore, prospective studies should focus on combination rTMS therapies in stroke recovery, such as rTMS and physiotherapy or pharmacological treatment. Laying the groundwork for the improvement personalized therapies of stroke patients to improve clinical outcome.

Together these directions open up clinical possibilities to improve diagnosis and prognosis for personalized therapies in stroke patients to reduce chronic disability and improve quality of life.

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Appendices

Appendix 1: Coil placement

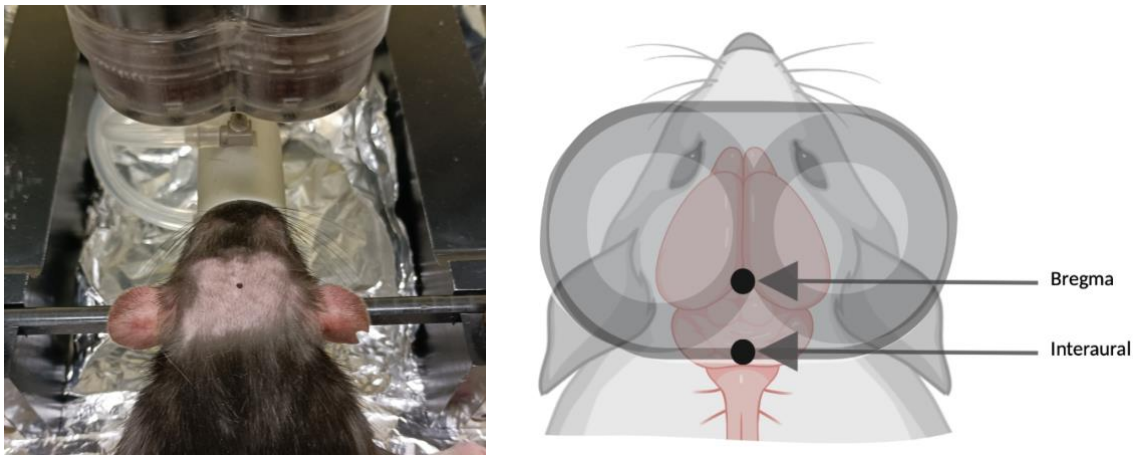

Figure 8 – Coil placement in the final setup of the experiment. Visually, the interaural line is marked on the shaved head of the rat. The marked posterior end of the coil is positioned right on top of the mark on the head. The centre of the coil is now anterior of Bregma.

Appendix 2: Needle electrode placement

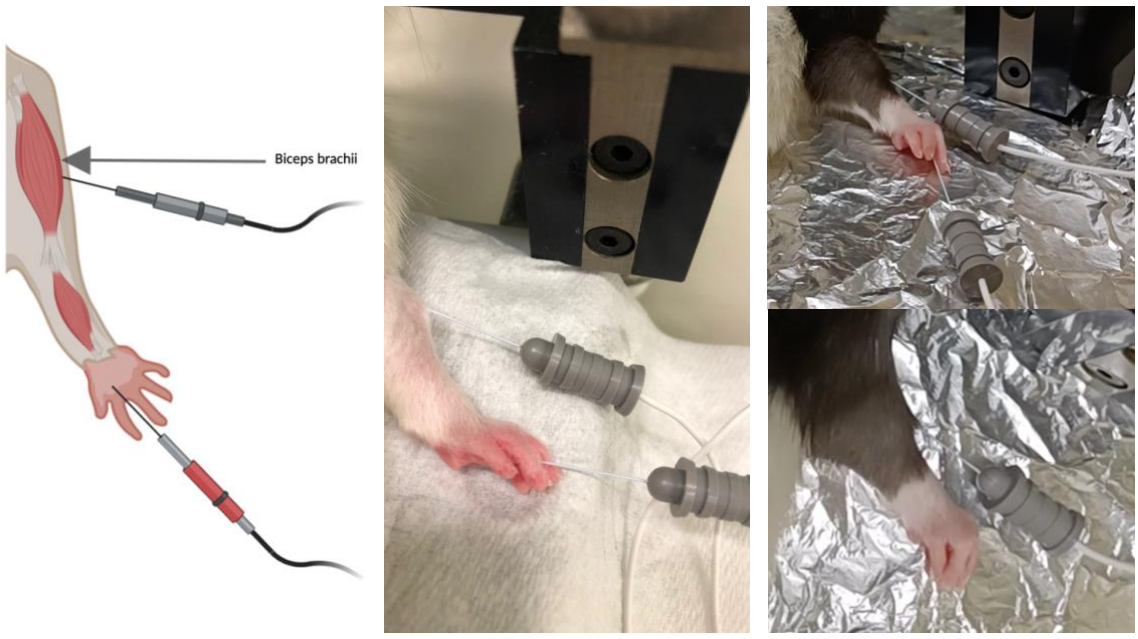

Figure 9 – Needle electrode placement. The 27G monopolar electrode only records at the tip of the needle. With palpation, we locate the bicep brachii and the needle is inserted. Secondly, a needle is placed in-between the digits of the paw, without touching any bones or tendons.

Appendix 3: TMS-EMG protocol

Materials:

- 4x 28G monopolar stainless steel needle electrode (Neuroline, Ambu A/S, Ballerup, Denmark) (lichtgrijs; 25 x 0.36 mm; 1" x 28G)
- 1x Disposable subdermal needle electrode (Technomed Europe, The Netherlands) (blauw)
- Stereotactic with place for attachment
- EMG NeuroMEP apparatus
- Lenovo lab laptop with Neuro-MEP.NET software version 4.2.5.6 Neurosoft
- Isoflurane
- Saline
- Shaver
- Electric heat mat
- Aluminium foil
- Wires to ground
- Propofol (10 mg/mL)
- Butterfly needle 23 G
- Infusion pump
- Plastic line: 0.38 x 1.09 mm

Preparations:

Place the heat mat underneath the tooth bar and cover with a big sheet of aluminium foil. Place aluminium foil underneath the location of the electrode wires. Connect one wire to the stereotactic and one wire to the aluminium and connect them to the ground of the electrical outlet.

Measurements:

<u>Anaesthesia</u>

1. Induction with isoflurane (5% induction, 2.5% maintenance)
2. Apply tail vail catheter
3. Maintain anaesthesia with continuous propofol infusion (59 ± 4 mg/kg/h)
4. Turn down isoflurane after 5 minutes of start propofol infusion

5. Wait 20 minutes before starting with MEP measurements for washout of isoflurane. Start putting the rat in the stereotactic.

Stereotactic/coil positioning

6. Place the rat on top of the heating mat and fixate on the ears
7. Shave the dorsal side of the skull
8. Mark the middle of the intraural line on the head
9. Place the posterior end of the coil central over the mark
10. Calculate location of Bregma and the M1 of the desired hemisphere
11. Place the coil over the respective M1

Electrode placement

12. Presoak the needle electrodes in saline to minimalize resistance
13. Pull the forelimb slightly backwards and find the brachii muscle with palpation
14. Insert the negative (black) 27G electrode in the belly of the muscle
15. Insert the positive (red) 27G electrode in the footbed of the same forepaw, the needle should be directed into the body
16. Use adhesive tape to ensure placement
17. Repeat steps 14 - 16 for the opposite forepaw
18. Insert the 28G ground electrode in the tail. Ensure with tape

Motor threshold determination

19. Start stimulation from the calculated start position over M1 on 50% MO
20. Increase with steps of 5% or 2% until a reliable MEP is found, make sure there is a minimum of 10 second in-between stimulations to limit the effects of rTMS
21. Move over a grid of maximum 3 mm lateral, 2 mm medial and ± 2 mm anteroposterior from the M1 and repeat steps 19 & 20 for each new location
22. Pick the location on the grid with a lowest MT as the hotspot location
23. Measure 10 additional traces on this location on 100% MT

End experiment

24. Remove the coil from the head
25. Remove the electrodes from the forelimbs and tail
26. Stop propofol infusion and extract the needle
27. Take the rat out the stereotactic