Uncovering the mechanisms of transcranial direct current stimulation-induced neuroplasticity after stroke

Research proposal for the Honours programme at the Radboud University’s Faculty of Science

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**Details**

**Details of proposal**

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Preface

This research proposal is the result of the interdisciplinary collaboration between second year bachelor students at the Radboud University during the academic year of 2015-2016. The proposal is part of the Radboud Honours programme, where ambitious students with common interests collaborate in groups under the supervision of a professor. The purpose of the programme is to investigate a scientific problem, in which each member of the group contributes from its own discipline. The programme spans 12 European credits. It contains presentations where each group presents its progress and receives feedback, as well as training for the development of academic skills. The programme includes a trip to a foreign university where the subject is discussed with expert researches. In the end, the students are expected to report a written research proposal. The final proposal is presented to the other groups and a jury.

This group was formed with a common interest in neuroplasticity and guided by Prof. P.H.E Tiesinga. Since all of us felt unfamiliar with the subject, initial parts of the research consisted of studying papers and chapters from the book Neuroscience by Dale Purves et al. The final proposal resulted primarily from the study of scientific papers and conversations with experts at the Radboud University and University College London.

Figure 1: The members from our group, left to right: Lisa Gerrits, Susan Waarlo, Yorick Busser, Kim Steenbakkers.
Abstract

Scientific abstract

On a yearly basis 46,000 people have a stroke in the Netherlands. Up until now, more than 175,000 people did not fully recover and live with the consequences of a stroke. The recovery from a stroke depends mostly on the level of neuroplasticity activated after the stroke. This means that other parts of the brain will compensate for the loss of function after a stroke. Right now physical therapy, which is used because activity will induce neuroplasticity, is the only used therapy. In order to increase activity and therefore neuroplasticity a non-invasive stimulatory technique can be used: transcranial direct current stimulation (tDCS). tDCS produces a direct current through a targeted brain area. However, the mechanisms of action are not fully understood. We hypothesise that tDCS will stimulate activity and will induce neuroplasticity after stroke. Previous research has demonstrated that tDCS can enhance the excitability of the targeted brain area. Furthermore, it has an effect on the NMDA (nitrosodimethylamine) receptor efficacy and on BDNF (brain-derived neurotrophic factor) which are both important factors in long term potentiation (LTP), which is a form of neuroplasticity. We propose an experiment that uses a rat model to understand these mechanisms. A stroke is induced in the motor cortex, tDCS is applied and the resulting activity will be measured by a $Ca^{2+}$ indicator, while the neuroplasticity will be measured by the formation of neurite outgrowths. We link these changes to the degree of functional recovery. This research hopefully results in a better understanding of the mechanisms of tDCS and will therefore be an important step in the improvement of stroke therapy.

Abstract for the broad scientific committee

Every year about 46,000 people suffer from a stroke in the Netherlands alone. There are 175,000 people in total that live with the consequences of a stroke every day. To regain function after a stroke the brain has to compensate for the lost area. This process is called neuroplasticity. The only used therapy is physical therapy, which is useful since neuroplasticity is stimulated by activity. It is however not very efficient. People take a very long time to recover if they recover at all. A way to improve the regain of function after stroke is transcranial direct current stimulation (tDCS), where a direct current is applied to a targeted brain area. The mechanisms of action are however poorly understood. We hypothesize that tDCS results in an increase of activity and therefore an increase of neuroplasticity. Previous research showed that tDCS increases the excitability of the brain, which would theoretically result in a faster recovery in stroke when applied with physical therapy. We propose an experiment that helps to understand these mechanisms. Our proposed experiment uses rats and tDCS is applied after an induced stroke. We measure the neuroplastic effects of tDCS and link these to functional recovery. This hopefully results in a better understanding of tDCS so it can be used in stroke therapy.
Abstract for general public in Dutch

In Nederland krijgen jaarlijks 46.000 mensen een beroerte. Veel mensen herstellen niet volledig en op dit moment leven 175.000 mensen met de consequenties van een beroerte. Om te kunnen herstellen van een beroerte moet het brein de functie van het beschadigde deel overnemen. Dit heet neuroplasticiteit. Op dit moment wordt een beroerte behandeld met fysiotherapie, omdat neuroplasticiteit wordt gestimuleerd door fysieke activiteit. Om ervoor te zorgen dat neuroplasticiteit beter wordt gestimuleerd kan de techniek tDCS gebruikt worden. Men weet echter nog niet hoe en waarom dit werkt. Om hier achter te komen zullen wij dit testen op ratten. We zullen eerst een kleine, gecontroleerde beroerte veroorzaken en ze daarna behandelen met tDCS. De effecten hiervan meten wij en kunnen gebruikt worden om de effictiviteit van tDCS als therapie voor een beroerte te verhogen.
Introduction

Ischemic stroke is the cause of 1 in 20 deaths in Western countries. Every year, more than 46,000 people suffer from a stroke in the Netherlands alone. Over 175,000 people in the Netherlands live with the consequences of a stroke. A stroke is therefore a serious issue that affects many people. As a result, many research groups focus their research on prevention of, or improving recovery from, this disease.

In general, during a stroke the blood flow disrupts, leading to oxygen and glucose deprivation in a certain brain area. Possible causes are blood clot, vessel disruption or temporary blockage of the blood flow, but all lead to similar consequences. As oxygen and glucose are essential for neurons to maintain their function, deprivation of these substances causes serious damage. Cell membranes are disrupted, after which cell death due to apoptosis (programmed cell death) or necrosis (pathological cell death) may follow. In addition, the blood brain barrier may be damaged and unfiltered blood enters the brain area, influencing regular cellular metabolism. As a result of these events, many neuronal connections are lost, leading to decreased function in the affected brain area.

Neuroplasticity is the brain’s ability to make changes in its structure and dynamical processes in order to adapt to changing circumstances throughout life. Post-injury neuroplasticity is strongly increased just after stroke. The primary area affected by stroke exists mainly of dead neurons and scar tissue. This area will therefore not benefit from plasticity changes and function regain. As regeneration of neurons is very limited in the human brain, other brain areas tend to partly take over the lost function. The brain area surrounding the traumatic area is called the peri-infarct zone. This area shows high neuronal activity shortly after stroke due to the release of factors, that are inducing and modulating plasticity. The time period during which this takes place is called the short-time window, which lasts for about three months in humans. This emphasizes the fact that the brain’s ability to heal itself by compensating the damage is only temporary. Thus, recovery is far from optimal. Therefore additional therapy is needed to stimulate recovery of stroke patients.

Current therapies for function improvement are generally not effective enough for full recovery. Physical therapy, the main therapy used in stroke recovery, has been shown helpful, but not sufficient. Stimulating factors, such as brain-derived neurotrophic factor (BDNF), are released during regular movement, stimulating brain areas and therefore contributing to recovery. However, many ischemic stroke patients remain physically and mentally impaired during their lives. Signs of function loss such as weakness in the hand, imbalance while walking and speaking difficulty often remain during and after the recovery process. Therefore, additional therapy options are subject to investigation. Stem cell therapy, where dead neurons are replaced by new ones, seems promising. There are many challenges still to be solved before this therapy would become a reality. Other research focuses enhancing neuroplasticity
induced after stroke to promote recovery. A way to do this is through non-invasive brain stimulation. One tool that gained attention as a possible method for recovery is transcranial direct current stimulation (tDCS). This technique generates an electrical current through brain tissue in order to influence neuroplasticity. Research shows this might help regain function in some cases under specific circumstance, meaning it could become a good addition to recent therapy. However, the mechanisms of this tool are mostly unknown, which makes it difficult to improve efficacy apart from trial and error. This represents a problem on ethical grounds. Hence, more fundamental research into mechanisms is necessary before this technique can be used in practice. Here we propose a comprehensive series of experiments to gain fundamental insight into how tDCS induces neural activity and plasticity.
Motivation

Our main goal is to further improve stroke recovery. tDCS is a promising form of therapy when applied to brain injuries or mental illness. For patients who suffer from a stroke, this form of therapy (in combination with physical therapy and/or medicine) could lead to very promising results.

It is needed to understand how and why this form of stimulation affects neuroplasticity for it to be applied and personalized when used as a therapy. The main step that is needed, before tDCS can be approved as a technique for stroke patients, involves evaluation of physical therapy when simultaneously applied with the correct tDCS treatment. If this combination proves to be more effective than physical therapy on its own, it can be used in practice. To determine whether it has the desired effect, activity in the brain needs to be measured.

For this main goal to be reliable and realistic, a good understanding of the tDCS effect at cellular level is necessary. As most of this is still unknown, the proposed experiment is focused on a better understanding of mechanisms underlying tDCS treatment after stroke. Imaging methods in humans are not precise enough to gain fundamental knowledge. We therefore propose and experiment in rats as an animal model to use better imaging techniques.

In the proposed experiment, we visualize changes on cellular level in a stroke affected brain area in rats, which receive tDCS treatment. In order to detect these changes, two-photon microscopy is used through a cranial window. We expect, based on previous literature, to see more activity in tDCS-treated rats, which we will link to functional improvement. These results can lead to a better understanding of how activation and/or neuroplasticity are induced during tDCS treatment and if combining this form of stimulation with currently used therapies could increase the chances or rate of recovery in stroke patients.
Scientific background

Neuroplasticity

The mammalian brain functions through constant changing of neuronal connections and reorganization of neural circuits. Cell proliferation is prominent during embryogenesis but very limited in the post-embryonic brain. Instead, the dynamics of neuronal connections give the mammal the opportunity to learn and memorize new tasks. The term that describes these processes of change in the brain is neuroplasticity. Plasticity mechanisms are distinguished in strength modifications at the synapse level and rewiring processes. Neuroplasticity therefore makes it possible for the brain to adapt during an entire lifespan.

Synaptic plasticity

Many synapses exhibit long-lasting forms of synaptic plasticity that are plausible substrates for more permanent changes in brain function. Because of their duration, these forms of synaptic plasticity may be the cellular process underlying learning and memory. Thus, significant effort has gone into understanding how they are generated.

Changes in neuronal connections are caused by processes on synaptic level. When an action potential reaches the presynaptic terminal, calcium influx is increased, leading to neurotransmitter release. The neurotransmitter travels to the postsynaptic side, where it binds to receptors after which the post-synaptic membrane potential either depolarizes or hyperpolarizes. This way, neurons communicate. The synapse can be strengthened or weakened through changes of the receptor density at the post-synapse. When multiple action potentials reach the presynaptic terminal in a short time period, calcium influx increases to high levels, allowing more neurotransmitter to be released. The boost of neurotransmitter induces an increase in post-synaptic receptors and therefore strengthens the connection, as the communication is more efficient.

Neuroplasticity can be distinguished in short term and long term plasticity. The main difference is the duration of the change in synaptic strength. Short term plasticity only lasts for about a few minutes or less. It mostly affects the amount of neurotransmitter released from pre-synaptic terminals in response to an action potential reaching the terminal. Long term plasticity last for more than a 30 minutes and is caused by continuous stimulation of the synapse.

In mammals, there are two forms of long term plasticity: long term depression and long term potentiation. Long term potentiation (LTP) results in a long-lasting increase in synaptic strength. In LTP, the neurotransmitter glutamate has a major role, together with two receptors present on the postsynapse, the AMPA and NMDA receptor. Glutamate binds the receptors, which undergo conformational changes that allow influx of calcium. However,
the NMDA channel is blocked by Mg2+. During high-frequency stimulation, many action potentials follow in such a short time period that there is summation of the excitatory postsynaptic potential (EPSP). As a result, there is a prolonged depolarization, leading to the expel of Mg2+ from the NMDA receptor. The resulting increase in Ca2+ concentration in the dendritic spines of the postsynaptic cell induces LTP. As soon as calcium is not able to enter the post synapse, no LTP processes are observed.

Calcium induces several signal transduction pathways in the post-synapse, including Ca2+/calmodulin-dependent protein kinase (CaMKII). This protein can stimulate changes in gene expression and drive the insertion of additional AMPA receptors in the synapse for more efficient communication (figure 2). When there is a defect in this signaling protein, LTP is also negatively affected. So, glutamate needs to be present along with the depolarization and calcium to release Mg2+ from the NDMA pore and increase CaMKII concentration.

LTD is the result of lower frequency activity and weakens synapses. EPSPs are repressed for several hours, leading to a slower influx of calcium through the NMDA receptors. So, both LTP and LTD are stimulated by activity and calcium influx. They can compensate each other, regulating the synapse plasticity.

**Rewiring processes**

Besides neuroplasticity between individual neurons, plasticity causing reorganization of entire neuronal pathways in the brain is also observed. This is also referred to as rewiring within brain tissue. Rewiring is also induced by LTP and reduced or repressed by LTD (figure 2). A few main processes can be observed regarding circuitry, such as dendritic outgrowth and axonal sprouting. In addition, an increase in dendritic spines, where axonal input from other neurons is received, can be seen. The communicatory parts of nerve cells therefore enable a neuron to gain or reduce efficiency of its communication sites. So, these processes show the creation of new connections, which is also a major part of neuroplasticity.

Several substances either stimulate or inhibit the rewiring processes. The main ones are: growth factors, brain derived neurotrophic factor (BDNF) and reticulon-4, also referred to as NOGO. Growth factors and BDNF are positive factors which stimulate dendritic outgrowth and increase in number of synapses, while NOGO, being a negative factor, inhibits these processes. Especially BDNF has important functions regarding the stimulation of plasticity. BDNF encourages differentiation, neuron survival and is important for long-term memory. Negative factors exist to limit plasticity to maintain acceptable structural changes, ensuring that the formation of connections is limited. This way, rewiring is regulated.
The reorganisation of brain connections is generally seen as beneficial for the individual. However, it can be detrimental as well. When improvement of the individual’s behavior is seen after plasticity changes, the neural plasticity is seen as adaptive: neural plasticity helps the individual adapt to changing circumstances. When plasticity causes behavioral decline, it is referred to as maladaptive.22

Figure 2: Left: Mechanisms of long-term potentiation. When calcium influx increases through the NMDA receptor, CamKII (green) is activated, after which a signal transduction pathway is induced. This stimulates insertion of AMPA receptors, which leads to more efficient communication. From Paoletti et al.23 Right: A two-photon microscopy image of axonal sprouting after LTP. From Purves.24
Neuroplasticity after stroke

Neuroplasticity and its effects also play a major role after stroke. A stroke, often occurring in sub-cortical areas and also affecting cortical areas, can be caused by a blood clot blocking blood flow. As a result, many neurons die or undergo damage due to ionic imbalance in the membrane and oxygen deprivation. Along with loss of neurons goes the loss of neuronal circuits, as connections get lost. This leads to a loss of function, the extent of which depending on the stroke location.

Shortly after stroke, many activity patterns are visible in the brain. The core of the stroke, where neural death is common, does not show enhanced activity. However the area surrounding the stroke is subject both excitatory and inhibitory activity. This area is called the peri-infarct zone, in which activity stimulates plasticity changes as an attempt to compensate for the lost neuronal connections. Homeostatic and Hebbian plasticity are major processes after stroke. In Homeostatic plasticity, synaptic activity is upregulated to restore activity to a set point. Hebbian plasticity results in strengthening of existing connections. This way, the peri-infarct zone takes over some of the lost function. So, during a short time window of about 4 weeks the brain shows spontaneous recovery, leading to partial regain of function. Patients that have experienced a stroke show continued functional recovery for years after damage.

The reorganization of circuits and neural structure depends on LTP and LTD balance. Multiple experiments have tried to determine reorganisation after stroke and how this affect functioning of the brain tissue as a whole. It was found that after a stroke, axonal remodeling is induced. Neurofilaments, which are part of the cytoskeleton in neurons and influence the structure and organization, are phosphorylated after stroke is initiated. As a result, axonal growth is stimulated to form new connections with neighboring cells. In addition, several factors are released as a reaction of the peri-infarct area on the stroke. These include positive and negative factors. Positive factors stimulate stroke-affected circuits to show axon sprouting and increase the amount of dendrites and spines. Also, brain derived neurotrophic factor (BDNF) is increased significantly after stroke, stimulating axonal growth, pathfinding of synapses and the modulation of dendritic growth and morphology. As a reaction to the increase of positive factors, negative factors such as NOGO and chondroitin sulphite proteoglycans reach higher levels later during the spontaneous recovery. Also inhibiting neurotransmitters, such as gamma-aminobutric acid (GABA), are increased post-stroke. The amount of GABA is usually regulated by astrocytes which have the ability to take up this neurotransmitter. In the area surrounding the damage, however, this ability is repressed, resulting in a higher expression of the negative factors.
Research is done on the prevention and the optimization of the recovery from a stroke. As neural genesis is not a common process after damage in the adult mammalian brain, true recovery is a challenge. Research based on the idea that true recovery involves replacement of damaged and dead neurons, is focused on stem cell therapy as a potential tool for recovery. However, this approach can only succeed when a number of challenging problems are solved, such as stem cell transport, implantation and correct differentiation. Therefore, recovery of patients who suffered from stroke is primarily based on the plasticity of the remaining neurons in and around the damaged area. By upregulating neuroplasticity through therapy, stimulation of the compensation process is studied.

A main idea in neuroscience following the previous experimental results is that the stimulation of positive factors and inhibition of negative factors would lead to better recovery of stroke patients. More induced plasticity will provide active rewiring. Therefore, LTP induction is desired shortly after stroke. To accomplish optimal recovery, we would want to stimulate long range rewiring processes, followed by re-balancing the local circuit, where both LTP and LTD regulate plasticity.

Figure 3: Expression of factors influencing plasticity during the critical recovery period. Factors stimulating plasticity are expressed shortly after stroke (red line). Inhibitory factors are gradually expressed a few weeks later (green line). Some promoting and inhibiting factors are transiently expressed during the critical period (dashed lines). From Murphy and Corbett.

Non-invasive stimulation methods

One potential tool for stroke recovery, which has gained popularity over the last years, is non-invasive stimulation of the brain. Non-invasive stimulation has been shown to affect behavior such as learning and memory. Now, it is also thought it has an effect on brain plasticity, making this form of stimulation a potential tool for treatment of neurodegenerative disease.
The three most common non-invasive stimulation techniques are: transcranial magnetic stimulation (TMS), transcranial direct current stimulation (tDCS) and transcranial alternating current stimulation (tACS). TMS induces a magnetic field which easily passes the skull and generates a current in the brain. tDCS uses a constant, low current delivered to the brain by electrodes placed on the skull, achieving neural polarity changes. Finally, tACS delivers a low current as well, yet alternating.

In this proposal, we choose to focus on stimulation using tDCS. It is together with TMS the most promising stimulatory non-invasive technique used for clinical application and brain research. The magnetic field created by TMS is however less specific when targeting brain tissue and this form of stimulation needs more equipment. In addition, any form of placebo effect cannot be ruled out when using TMS. When the placebo effect is tested, the subject does not know whether the treatment is mimicked or not. With TMS, mimicking the effects is difficult. Both tDCS and TMS were shown to have few side-effects. To conclude, current stimulation is thought to be more promising.

A reason to use tACS instead of tDCS would be that an alternating current is applied instead of a direct current. tACS can manipulate cortical oscillations in the brain with the alternating current. However, previous studies suggest that tACS effects may be weaker than tDCS effects. In addition, tDCS parameters are better identified and more is known about this form of stimulation. Thus, we see potential for tDCS as a recovery tool and base our proposal on this stimulation method.

Transcranial direct current stimulation

Transcranial direct current stimulation is a neuro-stimulation technique that delivers constant intracortical low current (usually in a range of 1 mA) to the area of interest through the scalp for about 9 to 15 minutes. It uses surface electrodes soaked in isotonic sodium chloride solution. Two electrodes are placed on the skull: the anodal, positively charged electrode and cathodal, negatively charged electrode. Between the two, a current flow which passes the cortical brain area is generated. The current can either go from the cathodal to anodal or vice versa (Figure 4). The actual current within the brain is caused by ion flow. Positively charged ions flow towards the cathode, while negatively charged ions flow towards the anode.

Where anodal stimulation is positive and enhances neural excitability, cathodal stimulation is negative and decreases excitability. Thus, it is important to use anodal stimulation if we want to generate more activity in the targeted brain area. In anodal stimulation, the reference electrode is placed in a supraorbital region (region above the eye sockets) and the anodal electrode is placed over the brain part of interest.

The effect of tDCS application depends on several parameters. Two are important for the
optimization targeting: The current flow through the skin, skull and brain, also defined as the current direction, and the field strength applied. There are conflicting results on what gives the best result. The used tDCS intensity usually lies within a range of 0.05 mA/cm². The optimal field strength was defined using realistic head models in 2014. The electric field generated by tDCS was modeled as a function of the electrode placement. When treating human individuals, the optimal placement for the electrode pair should be placed around the targeted area, since that will result in an optimal field strength. As the current flows with the least resistance, a significant amount tends to get lost through the skin instead of entering the skull with higher resistance. This should be taken into consideration when applying tDCS.

Next, direction of the current should be considered. It is difficult to predict current flow in a human brain, as the human brain is convoluted, meaning that the brain surface consists of many folds to increase the total area. However, this problem is non-existent within rats and mice, since the cerebrum of these mammals does not have gyri (ridge on cerebral cortex) and sulci (groove in cerebral cortex).

![Figure 4: The difference in direction with anodal and cathodal stimulation. Either anodal or cathodal tDCS can be applied. In anodal stimulation, the current flows from the active electrode to the reference electrode. In cathodal stimulation, the current flows from the reference electrode to the active electrode. From Schlaug et al.](image)

**Known mechanisms of action on plasticity**

Direct current stimulation is shown to change brain excitability and plasticity. In addition, behavioural changes such as decreased depression and enhanced learning were previously observed after tDCS application. The mechanisms of tDCS however are poorly understood.
There are a few things known about the mechanism of tDCS. First, anodal tDCS application seems to enhance excitability of the targeted brain area. It is hypothesized that tDCS provides a sub-threshold stimulation which alters the chance of neurons to fire. Neurons closer to threshold are more likely to generate action potentials, resulting in more activity in the targeted brain area. Not only the targeted brain area is affected, activity changes can be seen in other areas as well. However, the maximal activation is seen in the area of interest.

Although mechanisms on molecular basis when applying tDCS are not well identified, previous studies suggest the importance of receptor composition. The long-term effects of tDCS may be caused by changing of the NMDA receptor density, due to the altering of intracellular Ca2+ concentration. The changing of the concentration might be due to the depolarization of the tissue after the subthreshold stimulation from tDCS. As already explained earlier the NMDA receptors play an important part in LTP, this supports our hypothesis that tDCS would stimulate neuroplasticity. In addition, previous work suggests that tDCS stimulates rewiring processes. Furthermore, it is shown that tDCS induced LTP only occurs in presence of activity dependent BDNF-secretion. The absence of BDNF would cause an impairment of the motor skill learning. So, induction of LTP by tDCS is thought to be activity-dependent (figure 5). It has already been proven that tDCS results in an increase of BDNF concentration (figure 6).

![Figure 5: Activity induces a signal transduction pathway involving calcium, CaMKII and BDNF, which leads to LTP. Without BDNF, tDCS is not able to induce LTP. We therefore hypothesize that tDCS induces activity leading to BDNF expression.](image)

In order to understand the mechanisms of tDCS, animal models have been used, including rodents. Y.Takano et all (2011) studied the effectiveness of tDCS using functional magnetic resonance imaging (fMRI). They concluded that anodal tDCS over the frontal cortex induces neuronal activation in the frontal cortex and in its connected brain region. In addition, recent research showed chancing plasticity during tDCS treatment. Thus, rodents are well suited for studies on tDCS effects as they well represent plastic brain changes in tDCS studies.
Figure 6: tDCS treatment influences BDNF levels in the targeted brain area. BDNF levels were increased 24h and 1 week after tDCS. The assay was performed in duplicate. Data retrieved from Podda et al.47.

**tDCS in stroke recovery**

tDCS is already used in studies in order to enhance stroke recovery. Proof-of principle studies have shown that long lasting benefits can be replicated. More activation is seen when tDCS is applied48. This could make tDCS an important tool in stroke recovery. tDCS also has several practical benefits in stroke recovery: it is easy to use, safe and non-invasive. Furthermore, it can be combined with other recovery therapies, like physical therapy, in order to improve stroke recovery due to relatively simple placement of electrodes. It is hypothesized that tDCS could help to restore the imbalance of interhemispheric inhibition after a stroke39. The non-affected hemisphere could then possibly inhibit the lesional hemisphere in an unopposed or imbalanced fashion, which would result in an interference in the recovery process. tDCS could either increase the excitability in the damaged hemisphere or inhibit activation in the healthy hemisphere37.
Research question and hypothesis

tDCS is a promising technique to improve functional recovery after stroke\textsuperscript{59}. The current induces activity in neural tissue. Since many plasticity processes are activity dependent, tDCS could help to enhance these processes. However, the mechanisms of action of tDCS on neuroplasticity are not well understood. In the proposed work, we try to quantitatively investigate the effect of tDCS after stroke on a cellular level.

The main objective of this research is to answer:

\textit{What are the effects of tDCS on the recovery of the peri-infarct region after a stroke in a rodent model?}

We expect an overall increase in neuroplastic recovery processes in the peri-infarct region after tDCS treatment, because earlier results suggest that neuroplasticity can be stimulated by tDCS. For the experiment to yield quantitative results, we measure neuroplasticity indicators. A few examples of these indicators are: rewiring processes, overall neuronal activity and synapse strength, which can give a good indication of the occurring plasticity.

To answer the main question, we formulate four sub questions:

1. \textit{What are the effects of tDCS on cellular calcium concentrations in the peri-infarct region after stroke?}

Monitoring intracellular calcium concentration yields information for overall activity in distinct neurons. Intracellular Ca\textsuperscript{2+}-concentration has a crucial role in LTP and LTD since a chelation of intracellular Ca\textsuperscript{2+}-ions prevented the LTP and LTD processes\textsuperscript{18,49}. Therefore, calcium concentration is often used as an indication for plasticity (see for example Monai et al.\textsuperscript{50}). As we expect tDCS treatment to induce plasticity, we hypothesize that calcium concentrations are elevated.

2. \textit{What are the effects of tDCS on rewiring processes in the peri-infarct region after stroke?}

The rewiring processes we want to visualize are axonal sprouting and dendritic outgrowths. In addition to overall neuronal activity, formation of new synapses compensates for circuits lost in the stroke. Axonal sprouting\textsuperscript{49,51,52,53,60} and dendritic spine production after stroke\textsuperscript{7,54} are processes that can help to return post-stroke synaptic activity to target levels. Synaptic outgrowths can be observed within hours after stimuli that induce LTP. We expect to see axonal sprouting and dendritic outgrowths after stroke, due to earlier implications that this plasticity is induced by tDCS\textsuperscript{44}.
3. What are the effects of tDCS on CaMKII-protein concentration in the peri-infarct zone after stroke?

It was shown that there is an increase of CaMKII concentration at the postsynaptic side directly after LTP induction\textsuperscript{55}. This indicator will be measured post mortem. CaMKII is involved in the signalling cascade leading to LTP. Thus, we hypothesize that the concentration of this protein increases as a result of tDCS treatment.

4. How does the plasticity link to functional recovery?

We link plasticity changes to functional recovery. We hypothesise that, if the above plasticity processes intensify, more functional recovery occurred. Motor cortex damage can be linked easily to functional recovery. Therefore, we choose to apply a stroke in the motor cortex region. What the motor cortex is responsible for, is not exactly known and often discussed. However, previous work showed that the motor cortex in rodents is essential for motor skill. Therefore, we test their recovery in the motor cortex area by defining their ability to perform a motor skill task. We expect the rats which receive tDCS treatment to learn new tasks faster than the untreated rodents.

As described, we monitor plasticity using three plasticity indicators: calcium, CaMKII and rewiring. This way, we can provide a good overview of the effect of tDCS on plasticity processes. To fully characterise the plasticity, the above 3 indicators must be monitored during the critical period of recovery of stroke. The quantitative characterisation of the plasticity indicators yields an overview of the cellular recovery processes that are induced by the tDCS treatment. Overall we would expect that all of the indicators would give a stronger signal in the rats that received tDCS treatment, than in the control group.
Methodology

To visualize the plasticity in the rat’s brain, we must have access to the brain, which requires a cranial window. The cranial window is also used to induce stroke through the exposure of light at the targeted area. After stroke induction, we treat the peri-infarct region with tDCS. To monitor possible neural changes we use fluorescent imaging techniques and two-photon imaging. We will also monitor functional recovery and link this to the plasticity indicators.

Rat as animal model

Monitoring the plasticity indicators requires experiments that need access to the brain. For this reason we use an animal model. Rats are the most appropriate as they are widely used in research and can provide insight into the mechanisms of tDCS on stroke recovery. We found that extensive literature is available for the use of rats specifically as animal models. This includes: realistic stroke models, imaging methods for the plasticity indicators and methods to assess functional recovery. We find that photothrombosis can induce small and localized strokes and is a feasible technique. Additionally, rats share a similar layering of motor cortex structure as humans. BDNF in animal models was shown to have a major role in homeostatic plasticity and altered motor function, which supports a link between human plasticity and that of rats. Lastly, mice have smaller brains but allow for a wider range of genetic manipulations, whereas rats of larger brains and are easier to train on motor cortex related tasks, but have more limited possibilities for genetic manipulation. Since the experiment links plasticity to recovery, which requires training, we use rats.

Cranial window

We require access to the motor cortex of the brain in order to observe plasticity and induce stroke. This is done by surgically preparing a cranial window.

During the preparation of the cranial window, rats are anesthetized with urethane. This is a standard anesthesia method on rats. The cranial window, a 6 mm diameter craniotomy, is made at the site of the motor cortex. The dura mater needs to be surgically removed. After dyeing the target cells (this will be discussed in the section "Observing plasticity"), the craniotomy is covered with an agarose gel. The gel must be dissolved as 1.5% agarose in artificial cerebrospinal fluid. The agarose gel must be 37 degrees Celsius when it is applied to the brain surface. The gel cover is used to prevent tissue damage due to exposure to the environment. It is sealed with a thin glass coverslip (6 x 6 mm) of 0.12 mm thickness. Fixation is done with dental cement. Figure 7 shows the application of the cranial window.
Figure 7: Application process of the cranial window. 

**a.** A bone flap is removed from the brain tissue.

**b.** A cotton-tipped applicator is used to apply the window.

**c.** Removal of the agarose gel. From Goldey et al. 59

### Photothrombosis to induce stroke

Photothrombosis uses a light-sensitive dye that is injected systemically. The open neural tissue exposed by the cranial window, is incident to a green laser. The light-sensitive dye reacts to the laser by forming singlet oxygen species that lead to platelet activation and microvascular occlusion 62. Since the laser is locally precise and the exposure time of the dye to the laser is controlled, photothrombosis yields an accurate stroke model.

The experiment aims to characterise synaptic changes and rewiring processes induced by tDCS. We therefore suggest a stroke model such that these processes are activated and yield optimal recovery. If the damaging effect of stroke spares some circuitry in the peri-infarct region, such that it is still able to execute minimal motor function, Hebbian-like activity dependent learning can partially recover these circuits 9. Additionally, rewiring processes in the peri-infarct region compete with adjacent healthy tissue, particularly in animal stroke models that affect 5-15 percent of a hemisphere. These strokes compare in size to survivable human strokes 7,63. For these reasons it is important that the stroke is quite small.

To link plasticity processes to functional recovery, we induce a stroke in the sensorimotor forelimb area (sFL) of the cortex that controls the rat’s forelimb. Additionally, this area is surrounded by tissue that has a similar function and therefore damage promotes the rewiring processes. The procedure to induce the stroke is done immediately after the preparation of the cranial window so that the rats do not have to undergo two surgical procedures. A laser beam of approximately 0.5 mm in diameter and a wavelength of 560 nm is incident
on the sFL area. We are only interested in the peri-infarct region of the stroke. Therefore, we expose at the center of the cranial window so that it assures a large peri-infarct region is to be inside the cranial window. To guarantee a small stroke, we illuminate the sFL area for approximately 15 minutes. During the first 2 minutes of illumination, 0.133 mL/kg body weight of the light-sensitive dye (Bengal Rose) is injected systemically near the sFL area as suggested by Schmidt et al. Figure 8 schematically shows the location of the stroke we tend to achieve.

Figure 8: Left: Mapping of major cortical areas of a rat’s brain. The primary motor cortex (number 3 in yellow) is the area where we apply a small, controlled stroke. From Mohajerani et al. Right: Coronal section of the primary motor cortex. The double arrow indicates the relocating of function. The sFL area labeled in green. From Murphy and Corbett.

tDCS treatment

After the initiation of stroke, rats are treated daily with tDCS. A daily treatment consists of a period of 30 exposure to direct currents.

The placement of the anode and cathode must be in such a way, that the current flows through the peri-infarct region. As the infarct is in the motor cortex, the anode is placed anterior to the infarct. The cathode is placed on the neck skin Monai et al. In previous experiments tDCS treatment in rats used current densities between 0.04 mA/mm$^2$ and 2mA/mm$^2$. The exposure time varied between 10 and 60 minutes. The overall safety limit in rats is 0.142 mA/mm$^2$ for exposure times of 10 to 90 minutes. To determine the current density and the applied current, we measure the effect on neural activity of the current densities in the range of 0.04 to 0.5mA in rats with an induced stroke for a duration of 30 minutes. The size of the electrodes should be approximately 3.5mm$^2$ since the safety limit will be broken otherwise. We start measuring at 0.04mA and increase the current with steps of 0.1mA. The current density that induces the most effect on neural activity is used for the experiment.
**Observe plasticity**

We observe the plasticity indicators through the cranial window (figure 9A) in the peri-infarct zone (figure 9B) with two photon imaging (figure 9C). The principle of two-photon imaging is based on the occurrence of two low-energy photons exciting the same electron at the same time. If the electron falls back to its original state, it emits a photon of twice the energy as the incident photons.

We monitor calcium concentrations with two photon imaging (for example, see figure 10). The calcium ions in the sFL area are marked by injecting the membrane permeable dye “Oregon Green 488BAPTA-1 AM (OGB-AM)”. This method was proved successful earlier by Ohki et al.\[69\]. Synthesis of the dye and the in vivo injection of tissue is performed according to their protocol. After the cells are loaded with the dye, we use two-photon fluorescent imaging on a cellular level to obtain fluorescent images of calcium concentrations in a small brain area during short anesthetization.

Synaptic sprouting and dendritic outgrowths are also be monitored with two-photon imaging. Calcein is used as a fluorescent dye. We use a local superfusion technique as described by Florian Engert Tobias Bonhoeffer. It is challenging to monitor these outgrowths since only outgrowths that lie exactly in the plane we are observing are visible. These fields of view contain layers 2 and 3 of the cortex\[50\]. We observe in the same region of the sFL area and take images of the outgrowths and count them.

To determine if tDCS induced the expression of CaMKII we visualise the autophosphorylated CaMKII-complex using primary and secondary antibodies, as described by a protocol used previously\[70\]. This is only possible post mortem. It was shown that CaMKII-concentrations increased after TMS treatment of 4 weeks in non-stroke rats\[55\]. This post mortem observation is done in the control and experimental group.

We monitor the calcium concentrations and synaptic outgrowths during the monthly treatment period with tDCS. Since the first week after the induction of a stroke yields most plasticity, we observe more often in this period. As the measurement of overall activity and neuritic sprouting both use the same fluorescent marking, we observe these indicators in distinct rats to ensure no interference of images.

Approximately 50 rats are used for the experiment. The rats are divided in 2 groups of 25 rats each. One group undergoes the calcium imaging and one group undergoes measurement of neuritic outgrowths.

During the experiment, there are 5 post mortem measurements of CaMKII concentrations. For each measurement, 5 rats from the two different groups are killed. We think by mon-
itoring these indicators a comparison of the effects of tDCS on the plasticity can be made between rats that underwent tDCS treatment and rats that did not. Figure 11 shows the time-line for each of the two groups during the course of the experiment.

The experiment is repeated in a control group of 50 rats which do not undergo tDCS treatment, which means a 100 rats are used in total.

Figure 9: A schematic representation of the experimental setup. a: A cranial window to visualize the rat’s brain. b: Placement of the anode and cathode for tDCS on the rat’s head. The anode is placed anterior to the infarct. The cathode is placed posterior to the infarct. The red marked area is the location of the motor cortex, and the site of the infarct. The cranial window is located above the infarct region. c: Placement of the two photon microscope and tDCS elektrodes for observation of plasticity. From Takebe et al.11

Figure 10: An example image of two-photon images acquired in vivo of OBG-1 labelling of calcium concentration after stroke. In green, calcium is labeled. This way, activity is visualised on a cellular level.
Link functional recovery to plasticity

In addition to a characterisation of the occurring plasticity as a result of tDCS, we link these changes to functional recovery. We suspect better a functional recovery in rats that showed more plasticity.

We perform a skilled reaching test on the rats to quantise their function. We use the Montoya staircase test\(^\text{[71]}\), in which rats that are slightly deprived from food are placed on a platform and must obtain pallets from a set of stairs on either side of the platform. Forepaw dexterity can be measured by comparing the number of pallets eaten to the number of pallets dropped or left behind. This quantifies a forelimb reaching score\(^\text{[9]}\).

Ethics on in vivo models

As mentioned before, stroke affects many people every day. We therefore feel that it is necessary to investigate the effectiveness of a tool such as tDCS. The societal relevance of this work is extant. We believe we can justify sacrificing this amount of test animals.

The protocols we address for our proposed work were approved by international institutes, including RIKEN Institutional Animal Care and Use Committee and Science and Technology Agency Ethical Board for Experiments on Animals. As our work involves similar animal procedures, we expect our protocol to be approved as well.

Backup plan

If no results are acquired within the proposed range of tDCS range, the current intensity is adjusted while activity is monitored by imaging calcium. are used as a direct feedback for the effectiveness. This should cover any potential problems with the current strength of the tDCS application. For two photon imaging, we need to make sure enough images are made per experiment to compensate for any problems with focus images or blurriness. This observation method has been used widely before\(^\text{[50]}\) and we therefore do not expect any hurdles with the application on this experiment.

If it is not possible to link functional recovery to the observed neuroplasticity, we propose a different method for measuring the functional recovery. It has been shown, that the motor cortex of rats may not be as involved in motor function as we presumed\(^\text{[72]}\). If this feature of the motor cortex limits us in measuring functional recovery in paw movement, we can try to measure functional recovery by learning the rats a new task. If the same task is mastered by rats with tDCS treatment more quickly than the control group, it can be seen as faster functional recovery due to the treatment if this is supported by observations of plasticity in the brain.
Figure 11: Timeline of the events a rat undergoes during the experiment. Surgery starts at day 0 and includes the preparation of the cranial window. On day 1, tDCS treatment and measurement of the plasticity indicators start. The rats are divided in two groups: half of the rats undergo the calcium concentration measurement, the other half undergoes measurement of neuritic outgrowths, as both imaging methods interfere. All rats will be tested for motor skill improvement on the days indicated. In addition, after each motor skill test, 5 rats from each group are sacrificed for post mortem measurement of CaMKII expression.
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