From micro to macro

From Micro to Macro: Unravelling the Underlying Mechanisms of Transcranial Magnetic Stimulation (TMS)

Alix Charlotte Thomson

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by

Alix Charlotte Thomson

Promotor FPN

Prof Dr. A.T. Sack

Maastricht University

Co-Promotors FPN

Dr. T.A. de Graaf

Maastricht University

Dr. T. Schuhmann

Maastricht University

Co-Promotor FHML

Dr. G. Kenis

Maastricht University

Assessment Committee

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Maastricht University

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Hasselt University, Hasselt, Belgium

Prof Dr. S.A. Kotz

Maastricht University

Prof Dr. J.H.H.J. Prickaerts

Maastricht University

Table of Contents

Chapter 1

General Introduction

The brain is a fascinating organ, made up of billions of neurons within complex structural and functional networks that contribute to human behavior, health and disease. Over time, advanced whole brain and cellular imaging techniques have been developed to study the human brain at different scales, from microscopes capable of visualizing individual neurons to magnetic resonance imaging (MRI) machines that are capable of imaging the whole brain in action. However, these microscopic to macroscopic techniques often remain the domain of distinct neuroscience disciplines, and communication between these fields can be limited. Bridging the gap between these different disciplines and promoting interdisciplinary neuroscience creates exciting opportunities to advance our understanding of human behavior and mental health.

One particularly interesting aspect of our brain is its capacity to undergo constant structural and functional reorganization, generally termed *neuroplasticity*. Proposed by Donald Hebb in 1949, the hypothesis that 'neurons which fire together, wire together' (1, 2), has led to most of our understanding on the mechanisms of activity-dependent plasticity (3-5). In other words, neural cells are capable of forming functional connections, which allow for the encoding of new information. *Neuroplasticity* can explain how certain neuronal connections can become strengthened, while unused ones can be lost. Neuroplastic mechanisms are therefore mediating complex, activity dependent cognitive processes, such as learning and memory, that ultimately impact human behavior. Many mental and neurological disorders are characterized by dysregulation of neuroplastic mechanisms, for example when part of the brain is damaged (such as in brain injury or stroke), degrades (such as in cognitive decline or dementia), or has aberrant connectivity to other parts of the brain (such as major depressive disorder (MDD) or schizophrenia).

Neuroplasticity can be studied at the molecular and cellular levels, but also at the whole-brain level. Dysregulated gene/protein expression can result in altered neuronal morphology and activity, leading to changes in neuronal firing that can be manifested at the whole-brain network level, and may result in altered human behaviour.

At the microscopic level, it is important to understand the molecular and cellular mechanisms underlying neuroplasticity. It is equally important to understand, at the macroscopic level, how these neuroplastic changes can lead to certain behavioural effects.

This thesis takes an interdisciplinary approach to investigating human neuroplastic mechanisms; from the microscopic to macroscopic level. This thesis focuses on how the non-invasive brain stimulation tool, transcranial magnetic stimulation (TMS), is able to induce neuroplastic effects in human neurons. At the microscopic level, human neurons are modelled using SH-SY5Y human neuroblastoma-derived cells.

These cells can be grown in a dish, and used to investigate molecular TMS-induced plasticity changes. At the macroscopic level, humans (both healthy and clinical participants) are stimulated with TMS, to indirectly measure plasticity changes in specific parts of the human brain. The overall aim of the series of studies presented in this thesis is to unravel the underlying mechanisms of neuroplastic changes induced by TMS, using both an *in vitro* model of human neurons, as well as healthy and clinical populations of human participants.

Transcranial Magnetic Stimulation (TMS)

TMS is a non-invasive form of brain stimulation, which uses electromagnetic pulses to induce an electric field in the underlying cortex, through stimulation which can pass through the intact skull (6). Electromagnetic pulses are delivered through a TMS coil, which is made of wound copper wires, the shape of which determines the depth and focality of the stimulation area (7, 8). In the most commonly used setup, electricity is sent through the TMS coil, generating a magnetic field of about 2 Tesla, which lasts about 100 µs (6, 7). Each TMS pulse reaches a depth of only a few centimetres, activating the outermost layer of the cortex directly below the stimulating coil (9). Computational modeling has shown that the TMS-induced electric field can directly activate axons in the stimulated area (10), causing an immediate, excitatory response in a focal area of proximal neurons (11).

In humans, a single TMS pulse delivered to the motor cortex can activate corticospinal circuits, resulting in a visible finger movement. Electrodes placed on the finger can quantify the amplitude of this movement as a measure of cortical excitability, called a motor-evoked potential (MEP) (12).

Interestingly, when multiple pulses are repeated in a certain pattern, as repetitive TMS (rTMS), lasting, neuroplastic effects on cortical excitability have been reported. The effects of different rTMS protocols on cortical excitability are often assessed indirectly, for example through MEPs. To do this, MEP amplitudes are compared before and after an rTMS protocol delivered to the motor cortex. rTMS protocols which decrease the amplitude of MEPs are considered to be inhibitory, and ones that increase the amplitude of MEPs, excitatory.

Low frequency rTMS refers to pulses delivered at 1 Hz or lower, which have been shown to have inhibitory aftereffects (13). Stimulation at frequencies higher than 5 Hz are considered high frequency protocols, and have been shown to be excitatory (14-16). These classical high and low frequency stimulation protocols require stimulation sessions of about 20-30 minutes, with aftereffects lasting about 30 minutes (17, 18). In 2005, a set of protocols called the theta burst stimulation (TBS) protocols were introduced, which cut stimulation time down to 3 minutes, with

effects lasting between 20 and 60 minutes (19). The TBS protocols were adapted from theta burst patterns used to induce plasticity in animal research (20, 21). These patterns consist of triplet pulses delivered at 50Hz between pulses, with triplets repeated at theta frequency (5Hz) (19). Two rTMS TBS protocols were introduced; the first, intermittent TBS (iTBS), requires only 3 minutes of stimulation time, and has shown excitatory effects lasting around 20 minutes (19). In contrast, continuous TBS (cTBS) requires only 40 seconds of stimulation, with effects lasting about 60 minutes (19). A visualization of the stimulation pulse parameters be seen in **Figure 1B**. Due to their short duration and opposite effects on cortical excitability in humans, the TBS protocols have been widely used both in research and in clinical applications over the past 15 years. All experiments in this thesis use the TBS protocols to induce neuroplasticity, **Chapters 3** and **4** use iTBS and cTBS, and **Chapters 6-8** use iTBS.

Recently however, several studies have been unsuccessful in attempts to replicate the expected effects of TBS protocols, namely that iTBS induces excitatory and cTBS inhibitory changes in excitability (22-25). This may be due to variability of MEPs as an indirect measure of cortical excitability. A large review of the range of variation in MEPs within healthy research subjects found that experimental factors such as coil placement, stimulation intensity, and pulse waveform, as well as biological factors such as skull thickness and brain volume, contribute to this variability (26).

Other factors such as the internal state of the brain at the time of stimulation can also influence MEP variability (27, 28). For example, the brain is in a dynamic state of neural network and excitability fluctuations, which occur spontaneously but also as a result of previous neural activity (such as a cognitive task, motor activity, or even prior brain stimulation) (28-30). One way to monitor and account for the impact of brain state in rTMS experiments is to record ongoing neuronal oscillations using electroencephalography (EEG) (29, 31-33).

Another benefit to recording simultaneous EEG in TMS experiments is to measure induced excitability changes in cortical areas other than the motor cortex. In addition to the intra and inter subject variability, another limitation of using MEPs as an outcome measure is that they are restricted to the motor cortex. Many research and clinical protocols apply rTMS to other cortical areas such as the dorsolateral prefrontal cortex (DLPFC), and assume that the effects are the same as in motor cortex. EEG-recorded responses to single TMS pulses are called TMS-evoked EEG potentials (TEPs), and show peaks at predictable latencies immediately following the TMS pulse (34-36). Early TEP components (within 100ms of the TMS pulse) are thought to represent neuroplastic processes (34, 37). In contrast to MEPs, TEPs have been shown to be highly reproducible (36, 38), but confounds exist with this outcome measure as well. For example, the loud ‘click’ of the TMS pulse activates auditory and somatosensory processes, which are also represented in both early and late TEP components (39).

Additionally, few studies have used TEPs to assess plasticity changes after rTMS, and more research is required to better understand the capacity for TEPs as an outcome measure for rTMS-induced plasticity.

In this thesis, **Chapter 6** describes the use of MEPs to assess motor cortex excitability following repeated iTBS in healthy human participants. **Chapter 7** combines MEPs and TEPs as outcome measures to assess neuroplasticity after iTBS. Finally, **Chapter 8** moves from the motor cortex to the DLPFC and examines the neuroplastic effects of iTBS in this cortical region.

Microscopic: Cellular Studies with TMS

In the first chapters of this thesis, exploratory experimental steps have been taken towards understanding the underlying molecular mechanisms of iTBS/cTBS induced plasticity in a human neuron model. The necessary background information, derived largely from research in animal and cellular model, is summarized in this section.

What is plasticity?

Neural plasticity refers to activity-dependent changes which can occur at the neuronal, neural network, and whole brain levels. At the molecular level, neuroplasticity is initiated by an influx of calcium, which can trigger signalling cascades, promote immediate and longer lasting gene expression changes, and result in protein expression and structural changes at the neuron/synapse (41, 42). Briefly, an activity-induced calcium influx activates a series of calcium dependent signalling cascades, triggering rapid changes in the expression of genes which can act as transcription factors to regulate the expression of important later-response plasticity genes (42, 43). These later-response genes can lead to alterations of neuron or synapse structure and excitability (43). Such structural alterations can include neurite outgrowth (axonal or dendritic), synapse formation or elimination, spine density, and changes to the excitatory/ inhibitory balance (44, 45).

The neuroplastic effects of rTMS are thought to work through long-term potentiation (LTP) or long-term depression (LTD) like mechanisms (17, 46, 47). LTP and LTD are among the most commonly studied forms of activity-dependent synaptic plasticity, and provide a mechanistic explanation to Hebb's theoretical postulate, which proposed that if a neuron repeatedly causes another neuron to fire, this connection will become strengthened (1, 3), i.e. "neurons which fire together, wire together" (2). For example, with LTP, a synapse that is repeatedly stimulated will be strengthened (4). With LTD, a synapse which is not stimulated frequently, will be weakened (48). The pattern (frequency) of stimulation determines whether LTP or LTD will be induced. Low frequency stimulation induces LTD and high frequency, LTP (48, 49). The

electrical stimulation protocol widely used to induce LTP *in vitro* is a 4-pulse burst at 100Hz, repeated at 5Hz for 10 bursts (21, 50).

Mechanisms such as intracellular signalling and synapse modifications are thought to underly neural plasticity effects. Such activity-dependent strengthening/weakening of specific synapses are thought to underlie the neural encoding of information, and may represent the fundamental mechanisms of learning and memory (51). Both LTP and LTD work through activation of N-methyl-D-aspartate (NMDA) receptors at the post synapse, which trigger calcium signalling and complex intracellular cascades. These changes result in post synaptic modifications, such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking which alter the synapse strength (48, 51-54).

Hypothesized rTMS-induced plasticity effects

Research in animal models and human pharmaceutical studies have provided evidence for rTMS induced neuroplasticity working through LTP/LTD like mechanisms. In humans, pharmaceutically blocking the NMDA receptor completely abolished TBS effects (59). Since the NMDA receptor is critical for LTP/LTD (59-61), this finding supports the hypothesis that TBS effects are related to LTP/LTD-like synaptic plasticity (59). The complex signalling pathways underlying rTMS induced neuroplastic effects have mainly been researched in animal models, or in brain tissue derived from rodents, either in organotypic slice cultures, or dissociated into neural cell cultures. Research has mainly been focused on two main neuron types: excitatory glutamatergic neurons, and inhibitory GABAergic neurons (46, 47, 62, 63).

About 80% of neurons in the neocortex are excitatory glutamatergic projection neurons which send axons to other, often remote, brain areas (64) and are known to be important in neuroplasticity (51). The remaining approximately 20% of the neocortex is comprised of interneurons, which, in contrast to excitatory projection neurons, have local connections and are mostly inhibitory, thereby modulating the activity of excitatory projection neurons (64). Interneurons are highly diverse, and can be classified based on several parameters such as the presence of different calcium binding proteins (CaBPs) (65). Neurons expressing calbindin D-28K (CB) are important in synaptic plasticity, those expressing calrectin (CR) exhibit control over other inhibitory interneurons, and neurons expressing paravalbumin (PV) are largely responsible for the output and synchronization of action potentials of large groups of excitatory projection neurons (65).

Studies in animal models such as rodents and non-human primates, mainly investigating effects on glutamatergic or GABAergic neurons (expressing different CaBPs), have uncovered a range of plasticity-related changes in calcium signalling, gene expression, and structural changes underlying rTMS effects.

Calcium Signalling

As briefly mentioned above, an initial rise in calcium levels and the resulting induction of calcium signalling is a critical first step in activity-dependent plasticity (66). During LTP/LTD, a transient increase in intracellular calcium can initiate calcium signalling, leading to synaptic modifications which can alter synaptic strength, thereby underlying activity dependent plasticity (48, 51-54). Measuring the change in amplitude of these immediate calcium transients can indicate that a particular intervention successfully induced plasticity changes, altering the responsiveness (excitability) of the neuron. An immediate increase in the amplitude of calcium transients can indicate LTP, while smaller amplitude changes can indicate LTD (67).

In animal organotypic slice cultures, single TMS pulses (68), as well as pulses delivered in a sequence as rTMS (69), were able to promote an immediate calcium release from intracellular stores of excitatory neurons (68, 69). Different patterned rTMS protocols (10Hz, cTBS) with different expected excitability effects (10Hz excitatory, cTBS inhibitory), both promoted an increase in intracellular calcium release (69). In this study, the intensity of stimulation was too low to induce an action potential, and yet stimulation induced a release of intracellular calcium (69). Intracellular calcium release independent of an action potential can indicate plasticity effects (70), providing support for the potential of rTMS to induce plasticity.

Few studies have investigated immediate calcium release in human neural cultures after iTBS or cTBS. Changes in intracellular calcium levels can be measured in living neurons through fluorescence probes, which bind to calcium and be visualized with fluorescence microscopy (41). In **Chapter 3**, the immediate effects of iTBS and cTBS on calcium activity in human neuron-like cells are measured. In this chapter, Fluo-4AM (F14201, ThermoFisher) fluorescence calcium indicator was used to visualize changes in calcium levels. This was done by quantifying fluorescence intensity immediately before and after iTBS, cTBS or sham stimulation, as well as after chemical depolarization with KCl. Fluorescence levels were measured before stimulation as an indicator of baseline fluorescence, after stimulation to visualize any spontaneous calcium activity indued by stimulation, and most importantly, after chemical depolarization, to measure the stimulation-induced change to neural responsiveness. An example of the increase in fluorescence which can be visualized following chemical depolarization in the human neuron-like model used can be seen in **Figure 1C**. These calcium effects detected immediately following stimulation are important first outcome measures in understanding rTMS-induced neuroplasticity, as an initial rise in calcium influx is a necessary first step in plasticity induction (66). In the next chapter (**Chapter 4**), plasticity effects such as gene expression and structural neuronal changes are investigated.

Gene Expression

LTP can be divided into two phases, the early phase (E-LTP) and the late phase (L-LTP). E-LTP occurs immediately following stimulation, and is induced by transient calcium influx and NMDA receptor activation (52). E-LTP is protein-synthesis independent, and works largely through the activation of enzymes and protein kinases such as Ca^{2+}/calmodulin -dependent protein kinase II (CaMKII) (56, 71). It is induced by brief stimulation and effects can last 1-3 hours. L-LTP can be induced with stronger or repeated stimulation, and the effects can last at least 24 hours (55, 57, 58). L-LTP requires protein synthesis, and can lead to longer lasting structural changes at the synapse (55-58). These changes rely on the activation of signalling cascades, and lead to the structural remodelling of synapses, which are thought to be the molecular events responsible for long term memory (45, 71). In contrast to live calcium imaging, changes in gene expression are measured from extracted tissue or cells, and the quantification of expression of single genes can be determined by quantitative PCR.

Immediately after an activity-induced calcium influx, for example in the induction of LTP, early gene expression effects are often measured in the levels of immediate early genes (), which are rapidly upregulated and do not rely on new protein synthesis (72-74). IEGs can act as transcription factors, which regulate the expression of other later response genes. These later response genes are often responsible for longer lasting plasticity effects (43). For example, LTP-inducing stimulation results in an immediate increase in the expression of the IEG early growth response 1 (*EGR1*) (76). *EGR1* expression is also critical in the switch from E-LTP to L-LTP (73), and initiates the expression of other plasticity genes (77).

In animal models, rTMS has been shown to alter the expression of genes important in cell firing activity (79-82), as well as genes involved in cell survival, cytoskeletal remodeling (69), and neuronal growth and regeneration (83). High frequency rTMS, thought to be excitatory (16), has been shown to increase the expression of the GLUR1 subunit of the AMPA receptor (84), which is known to be involved in synapse modifications during LTP/LTD (85). rTMS was also shown to increase the expression of brain-derived neurotrophic factor (BDNF) (82, 86, 87) important in LTP (88-90). rTMS has also been shown to increase the expression the neurotrophic tyrosine receptor kinase B (TrkB), the high-affinity receptor of BDNF, as well as several other genes involved in synapse formation (91).

In **Chapter 4,** changes in the expression of BDNF and TrkB, as well as downstream signalling targets in the BDNF-TrkB signalling pathway are investigated. When BDNF binds to TrkB, several intracellular signalling cascades are activated (71). A simplified diagram of important targets in this signalling pathway (adapted from Minichiello (2009) (71)) can be seen in **Figure 1D**. Gene expression changes in these three

pathways are responsible for effects such as plasticity behaviour, synaptic plasticity and growth and differentiation (71).

Structural Changes

Activity-induced calcium influx can regulate differential patterns of gene expression, which can lead to protein-synthesis dependent structural changes in neurons (45). Structural changes can include axon or dendritic growth, synapse strengthening or maturation, and dendritic spine density, all of which alter the strength and number of connections between neurons (44, 45). Structural changes in neurons are commonly measured in fixed cells or extracted tissue, using fluorescence microscopy to visualize the distribution of a particular protein of interest; for example, a protein known to be involved in plasticity effects. Important in this thesis are plasticity changes to the cytoskeleton, which consists of several proteins capable of re-organize in response to activity such as LTP-induction (93). Cytoskeletal remodelling, in particular of the protein F-actin, is thought to be important in LTP and memory consolidation, as well as involved in the conversion from E-LTP to L-LTP (94). These structural effects can be immediate (within 30 minutes of stimulation, but can also last for hours or days (45).

The effects of rTMS on neuron morphology have been studied in both animal and cellular models. High frequency rTMS has been shown to affect dendrites closest to the soma (cell body) of excitatory neurons (96). Using electrophysiological recordings, the synaptic responses were shown to be AMPA receptor-mediated, further supporting the hypothesis that rTMS effects are LTP/LTD plasticity-like. Additionally, high frequency rTMS has been shown to strengthen glutamatergic neurons through remodeling of small dendritic spines (97). This provides further support for LTP-like plasticity effects after high frequency rTMS, as the rTMS-induced changes involved the NMDA receptor, and resulted in an accumulation of AMPA receptors (97).

In addition to excitatory neurons, TMS can also alter the level of inhibition on excitatory neurons (63, 98-104). This has been shown following 10Hz stimulation, which is assumed to be excitatory (16), by measuring a reduction of inhibitory synaptic strength on excitatory neurons (105). iTBS has also been shown to affect the strength of inhibitory GABAergic interneurons (99).

The effects of iTBS on the expression on different inhibitory interneurons (PV, CB, or CR interneurons), was shown in rat studies to differ depending on the strain of rat (101). This is thought to be due to different basal expression patterns of CaBP's, which contribute to differences in cortical network properties. The maturity of PV interneurons was also shown to parallel iTBS effects, suggesting that iTBS may have different effects depending on when stimulation is applied during neurodevelopment (103, 106). iTBS has also been shown to specifically decrease the activity of PV-expressing interneurons, reducing their inhibitory tone on excitatory neurons (107).

Interestingly, these effects were abolished by co-application with an NMDAR antagonist (100), aligning with human pharmacological studies showing NDMAR-dependence of iTBS effects (59).

In summary, in animal models, iTBS has been shown to affect not only the structure of excitatory neurons but also the strength of inhibition of interneurons on excitatory neurons (98, 99, 101, 106, 108). TMS affects excitatory neurons at excitatory synapses closest to the cell body (96), and enlarges small excitatory dendritic spines (97). Additionally, iTBS specifically reduced PV-expressing interneurons, which are important for synchronizing activity of pyramidal cells (65). These effects were strongest after 2400 pulses (108), were rat-strain dependent (101), and thought to relate to neuronal growth and maturation, indicating an age-dependency of iTBS effects in rats (102, 103, 106).

In **Chapter 4** the effects of iTBS and cTBS on cytoskeletal re-organization in two important cytoskeletal proteins: βIII-Tubulin and microtubule associated protein 2 (MAP2) are investigated. βIII-Tubulin is the most dynamic of the six tubulin isotypes found in mammals, and is largely involved in axonal guidance and maturation (109, 110). βIII-Tubulin is predominantly expressed in axons, while MAP2 is expressed mainly in dendrites (111). In our human neuron-like model, βIII-Tubulin was very strongly expressed, in contrast to MAP2. Therefore, analyses on structural changes after iTBS/cTBS were done using βIII-Tubulin only. An example of the distribution of these two cytoskeletal proteins (βIII-Tubulin in green, MAP2 in red) can be seen in **Figure 1A**, showing the differentiation process, and **Figure 1E**, which shows the two proteins side by side and in a merged image.

Human Neuron-Like Model

While animal models have proven incredibly valuable in understanding the neuroplastic changes induced by TMS, there are limitations to the use of animals as models for the human brain. For example, there is often poor transferability of rodent models to specific human neurological disorders (113). In addition, human neuronal *in vitro* models may be able to more accurately represent human neuronal networks than rodent neurons (114). In order to fully understand the underlying mechanisms of rTMS, a reliable, human *in vitro* neuronal setup would be beneficial. A human neuronal model could both verify the evidence of neuroplasticity changes from studies on animals, and confirm that rTMS is capable of directly inducing neuroplasticity in human neurons. In light of the above-mentioned difficulty replicating the established effects of the TBS protocols (22-25), due largely to the variability of indirect outcome measures (MEPs) used to assess them, verification of the neuroplastic effects in a human neuronal setup is important.

However, only two studies have investigated TMS-induced neuroplasticity effects in a human neuronal cell culture model prior to **Chapters 3** and **4**. One used high frequency (5Hz) stimulation and reported an increase in the expression of cFOS and phosphorylated CREB, both of which are activity markers and indicate increased plasticity effects following stimulation (115). Another study looked specifically at the effects of different stimulation frequencies on catecholamine release, finding a decrease in dopamine, L-DOPA and norepinephrine after 3Hz stimulation and an increase in norepinephrine after 9Hz stimulation (116).

Both these studies were carried out on SH-SY5Y cells, a commonly used human neuronal cell line. These cells were originally derived from a neuroblastoma, and can be fully differentiated to a mature, neuronal-like state (92, 117-120). Once differentiated, SH-SY5Y cells express a catecholaminergic phenotype, and are commonly used to model Parkinson's disease (121). In addition, differentiated SH-SY5Y cells form functional synapses, and have therefore been extensively used to investigate human neuronal plasticity (92, 117, 120, 122-125). SH-SY5Y cells express many genes of interest for investigating TMS-induced neuroplastic effects such as those involved in synaptic plasticity and the BDNF-TrkB pathway (92, 120, 124, 126). They also express important morphological markers of plasticity, such as βIII-Tubulin and MAP2, which allow for the visualization of neuronal cytoskeletal structure (120). **Figure 1A** shows an example of SH-SY5Y cell morphology before and after differentiation. Functional effects can also be measured, with electrophysiology or live calcium imaging (120, 122, 127).

The main aim of the first three chapters of this thesis was thus to better understand the functional and molecular plasticity changes induced by rTMS in a human neuronal cell culture model, specifically at the level of calcium imaging, gene expression and morphology. The effects of rTMS on plasticity genes and morphological markers associated with plasticity were investigated, therefore it was important to first confirm that the cell growth and differentiation conditions would not interfere with (or potentially mask) our intended outcome measures. SH-SY5Y cells are derived from a tumour cell line, and consist of both neuron- and epithelial-like cells (128). To reduce the epithelial-like and promote the growth of neurons in the culture, SH-SY5Y cells are differentiated through addition of specific molecules such as retinoic acid (RA) and often by reducing the concentration of supplemented serum in the culture media, for example from 10% to 3% (117-119). Once differentiated, there is considerable variability in the pre-experimental handling of these cells, for example some completely remove all supplemented serum (the remaining 3%). This is done to prevent confounding effects of the molecular growth factors in the serum, as well as to ensure cells are all in the same phase of the growth cycle before experimentation (129, 130). However, since supplemented serum is removed to promote differentiation, we hypothesized that serum removal from differentiated cells may

result in molecular changes which could potentially interfere with the outcome measures we intended to measure following rTMS. In **Chapter 2,** the effects of removing supplementary serum from SH-SY5Y cell growth medium on gene expression and morphological markers of plasticity are described. We hypothesized that if serum supplementation has a strong effect on the gene expression and morphological outcome measures following TMS, this may confound the results and mask TMS-related plasticity effects. In **Chapter 3,** differentiated SH-SY5Y cells are used to visualize immediate effects of rTMS on calcium activity, and in **Chapter 4** to investigate the effects of rTMS on gene expression and morphological markers of neuroplasticity. A visualization of the progression of these first chapters can be seen in **Figure 1 A-E**.

Link to Human Studies

While the first half of this thesis focuses on the effects of rTMS in a living human neuron-like model, the second half investigates the neuroplastic effects of iTBS on living human subjects. To connect the cellular studies described in **Chapters 2-4** to the human studies described in **Chapters 6-8,** a mini review **(Chapter 5)** is included**.**

Chapter 5 reviews homeostatic metaplasticity and brain stimulation, providing a link between animal/cellular research and human brain stimulation studies. It is important to understand the underlying mechanisms induced by TMS, and the time scale of plasticity-promoting mechanisms. This chapter describes a way to harness metaplastic mechanisms to optimize research and clinical stimulation protocols.

Macroscopic: Human Studies with TMS

The range of cellular studies described so far are crucial for understanding and optimizing rTMS protocols for use in humans. In research, rTMS is used in humans to induce reversible neuroplastic changes non-invasively, for example to perturb and investigate the involvement of a brain area in a particular task or function, or to probe connectivity between different nodes in a neural network (7). rTMS also has many important clinical applications, for example in the treatment of depression (135-137). The neuroplastic effects of iTBS in healthy humans are investigated in **Chapters 6** and **8** and in a patient population with type II diabetes mellitus (T2DM) in **Chapter 7**.

Clinically, rTMS is a valuable treatment option for a range of psychiatric and neuropsychological disorders (137). It is most commonly used as a treatment option for major depressive disorder (MDD), where large, multi-centre clinical trials have shown beneficial results with a 37.5 minute, 10Hz stimulation protocol to the DLPFC (136, 138). Recently, iTBS has been shown to be equally as effective as the 10Hz protocol, indicating a time-effective alternative (139). This is beneficial to clinical treatment centres, as it increases the number of patients that can be treated per day

by a single TMS machine and TMS specialist. However, MDD treatment normally requires daily stimulation, with patients having to come to the clinic five days a week for four to six weeks. Such a treatment schedule is rather inconvenient for patients, certainly if they live far from treatment centres. To increase antidepressant response, and to decrease the number of days that patients have to come to the clinic, studies have begun testing protocols that condense multiple stimulation sessions into one treatment day. These protocols are called 'accelerated rTMS protocols', and have recently been shown to be safe and well tolerated by patients (140, 141). iTBS protocols are particularly interesting for accelerated protocols, due to their short stimulation duration.

Several studies have examined the efficacy of accelerated iTBS protocols for treating MDD, finding a decrease in the Hamilton Depression Rating Scale (HDRS-17) (142, 143), a score of depressive symptom severity that is often used to assess treatment responses in MDD. This clinical effect is intriguing, and prompts research in the neuroplasticity effects of accelerated iTBS protocols. As described earlier, these effects can be assessed indirectly in humans by measuring MEPs before and following a particular rTMS protocol. The results of such an experiment are described in **Chapter 6** of this thesis. Different stimulation conditions are compared: accelerated iTBS (consisting of five times the traditional iTBS protocol) with either i) 8, or ii) 15 minutes between each iTBS, iii) single iTBS, and iv) sham stimulation (**Figure 1F**). MEPs were assessed before and 90 minutes after the stimulation paradigms. This chapter provides valuable information on the neuroplastic effects of accelerated iTBS with varying (short) time intervals between iTBS sessions in the motor cortex.

While TMS is most commonly used in the treatment of MDD, it can also be used as a diagnostic tool in other clinical applications where neuroplasticity may be affected by disease progression. A rather unexpected example is the use of TMS in T2DM patients. T2DM is an increasingly prevalent disease worldwide, characterized by insulin resistance, and resulting in serious comorbidities such as cardiovascular disease (144), renal failure (145) and hypertension (146). In addition, damage to the central nervous system is present in the majority of T2DM patients, which is reflected, at least partly, in signs of altered neuroplasticity (147, 148).

Interestingly, T2DM patients are at a much higher risk of developing cognitive impairment and dementia (149-153), including Alzheimer's disease (154-158). This is likely a result of both altered glutamatergic neurotransmission and NMDA receptor-dependent LTP-like plasticity, which are affected at the earliest stages of insulin resistance (147). Further, T2DM patients have shown altered responses to iTBS and TMS-related methods of assessing cortical excitability (159). For example, the MEP amplitude following iTBS was decreased, suggesting altered NMDAR-dependent

plasticity, and cognitive decline was confirmed with lower verbal learning scores on cognitive tests (148).

However, T2DM patients with normal TMS-related measures and non-T2DM patients with impaired fasting glucose levels may be less likely to develop dementia (148). Therefore, TMS offers an excellent opportunity to diagnose patients with impaired neuroplasticity mechanisms at an early stage of insulin resistance. If these patients show abnormal reactivity to plasticity-inducing protocols (iTBS) or alterations in TMS-based outcome measures (i.e. MEPs or TEPs) before they show clinical manifestation of cognitive decline, this could serve as a potential 'biomarker' of T2DM-related cognitive impairment or even dementia. In **Chapter 7**, iTBS is used to assess neuroplasticity in patients with clinically diagnosed T2DM, as well as control group of participants with high BMI and no clinical diagnosis of T2DM. To characterize participants as having 'insulin resistance', blood samples were taken to calculate the degree of insulin resistance (using the homeostatic model assessment for insulin resistance (HOMA-IR) score). Participants are subjected to iTBS or sham stimulation over their motor cortex, followed by MEPs and TEPs to assess neuroplasticity effects across sessions. These measures are then compared across groups (insulin resistant versus matched control), and evaluated for an association between insulin resistance and plasticity measures.

This chapter adds several important dimensions to the overall main aim of assessing neuroplasticity induced by rTMS in an interdisciplinary approach in this thesis. First, it uses iTBS, the rTMS protocol used across all chapters in this thesis, both in humans and in cell culture. Secondly, it combines the outcome measure used in **Chapter 6** (MEPs) with simultaneous EEG to assess TEPs, and third, assesses neuroplasticity measures in a patient population known to have altered neuroplasticity mechanisms (147). The advantages of a multi-modal (combined TMS-EEG and fMRI) approach are further exploited in the last chapter of the thesis.

Multimodal TMS-EEG-fMRI

Finally, this thesis concludes with a chapter that brings several methods and techniques together to assess neuroplasticity in healthy human participants. As described in earlier sections, the neuroplasticity effects of rTMS can be examined at different human cellular to whole brain levels: for example, with calcium activity, gene expression, and neuronal morphology, as well as indirect measures of neural excitability such as with MEPs and TEPs. However, there are some limitations to both of these approaches. Cell culture *in vitro* modeling for example, lacks the complex structural organization of the human cortex. The use of MEPs, as described above, is hindered by substantial inter- and intra- subject variability (23-25). TEPs may be a more reliable alternative (36, 38), but are confounded by auditory and somatosensory

processes (39), and are not yet commonly used to assess neuroplastic changes after rTMS protocols.

Additionally, as was briefly mentioned earlier in the introduction, MEPs can only be assessed over the motor cortex. This is a major limitation when studying clinical applications, such as MDD, as rTMS is often delivered to the left DLPFC (135, 136). The choice for this area is based on imaging studies, which found that patients with MDD show altered functional connectivity within particular frontal areas and with deeper cortical networks (160-163). It is suggested that excitatory (10Hz or iTBS) rTMS to the left DLPFC has a facilitatory effect on frontal areas, and that antidepressant efficacy is related to functional connectivity to deeper cortical targets such as the subgenual anterior cingulate cortex (sgACC) (164, 165). It has been shown that single TMS pulses to the DLPFC can activate subcortical structures such as the (sg) ACC (166, 167), however, there is little evidence of any iTBS-induced modulation of such activation. In addition, the effect of ongoing brain state on how effective single pulses to DLPFC can propagate signal to ACC is relatively unknown.

In **Chapter 8**, TMS, EEG recordings, and fMRI are combined in a multimodal setup, aimed to activate and probe signal propagation through deeper cortical structures. Both rTMS and single TMS pulses are used. First iTBS is delivered to left DLPFC to induce neuroplastic changes, and then single TMS pulses are delivered to the left DLPFC, while in the MRI and simultaneously measuring the blood oxygenated level dependent (BOLD) signal. EEG adds another important dimension to the measurement, by assessing ongoing neural oscillatory firing patterns. Neurons firing at alpha frequency (~ 10Hz), have been shown to be related to response to TMS stimulation (32, 168), making this frequency particularly interesting for understanding whether brain state plays a role in TMS signal propagation.

Combining TMS-EEG-and fMRI in this final chapter makes it possible to answer several important questions regarding the neuroplastic potential of iTBS in the human brain. It allows for the investigation of whether iTBS stimulation is able to induce neuroplastic changes in network activity, and in deeper cortical structures, whether these neuroplastic iTBS effects can be probed by single TMS pulses to DLPFC, and finally whether TMS pulses delivered at high or low power of the ongoing alpha oscillation are capable of improving signal propagation. This final chapter adds an additional method for measuring human brain activity (fMRI), completing the thesis which consists of a range of research samples (cellular, human), techniques, and neuroplastic effects measured (summarized in the table below).

Outline of the Thesis

The interdisciplinary approach taken in this thesis has the overall aim of unravelling the underlying neuroplastic mechanisms of rTMS; progressing from human neuronal *in vitro* experiments to human *in vivo* experiments. Experimental chapters range from studying the molecular neuroplastic events induced by iTBS/cTBS in cell culture, to measuring the macroscopic effects of iTBS in human participants.

In **Chapter 2,** the human neuronal cell culture model, SH-SY5Y cells is introduced. This chapter is critical in describing the pre-experimental conditions of the human neuron-like model used in subsequent chapters.

In **Chapter 3,** the immediate effects of iTBS/cTBS are quantified using live calcium imaging. This chapter introduces the setup of stimulating living human neurons (SH-SY5Y cells) with rTMS, as well as uses an immediate outcome measure (calcium imaging), capable of detecting early neuroplastic-like changes in cell activity.

Chapter 4 moves past the immediate effects described in chapter 3 and quantifies the effects of iTBS, cTBS and sham stimulation on gene expression and morphological markers of plasticity, for up to 24 hours after stimulation. This is one of the first studies to investigate neuroplasticity-induced mechanisms after TBS in human neuron-like cells.

Chapter 5 links chapters 2-4 (the *in vitro* human neuron studies) to chapters 6-8 (the *in vivo* human studies) in a mini-review of metaplasticity in brain stimulation. It specifically discusses how evidence from animal and cell culture studies can be used to optimize rTMS protocols for use in research and clinics.

Chapter 6, is the first chapter involving healthy human participants, who were stimulated with accelerated (5x) iTBS over the motor cortex. Accelerated iTBS is a promising stimulation protocol for clinical applications, but the neuroplastic effects had yet to be established in healthy participants. This chapter is important to validate the neuroplastic effects of accelerated iTBS, and to optimize parameters for future clinical applications.

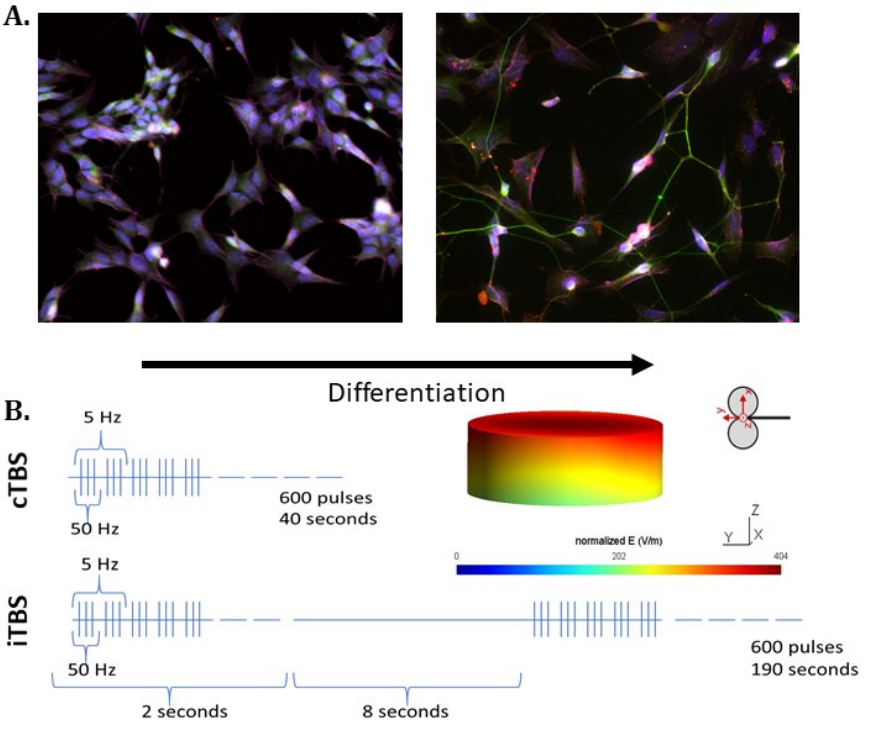
In **Chapter 7,** TMS is used to assess neuroplasticity in a clinical population (T2DM patients), known to have altered neuroplastic mechanisms due to severe insulin resistance (147). The main aim of this chapter was to use TMS-based measures of neuroplasticity and excitability to find a potential 'biomarker' of early cognitive decline in insulin-resistant patients.

Finally, in **Chapter 8,** a TMS-EEG-fMRI multimodal setup is used to measure the neuroplastic effects of iTBS stimulation on cortical and subcortical structures after stimulation of the left DLPFC. EEG signal is used to determine whether signal

propagation effects are dependent on brain state. This final chapter combines several techniques to assess neuroplasticity induced by iTBS in the human brain.

Finally, the general discussion summarizes the findings of the six experimental chapters and one mini-review chapter, and discusses these findings in the broader context of neuroplasticity induced by TMS. It considers both the strengths and limitations of the techniques and methods used, and proposes future studies, which can build on these findings to improve, optimize and even personalize the future of neuroplasticity inducing TMS-protocols.

Chapter	Purpose	Research Sample	Techniques Used	Neuroplastic Effect Measured
2	Set up a human neuronal model	SH-SY5Y Human Neuronal-like Cells	Cell Culture Gene Expression (qPCR) Morphology (Immuno-cytochemistry)	Gene expression and neuron morphology
3	Identify immediate effects of iTBS/cTBS/Sham	SH-SY5Y Human Neuronal-like Cells	Cell Culture Calcium Imaging	Functional calcium activity
4	Establish gene expression and morphological effects of iTBS/cTBS/Sham	SH-SY5Y Human Neuronal-like Cells	Cell Culture Gene Expression (qPCR) Morphology (Immuno-cytochemistry)	Gene expression and neuron morphology
5	Describe metaplasticity in rTMS	Mini-Literature Review	Review	Metaplasticity
6	Establish metaplastic effects of accelerated iTBS	Healthy Human Participants	MEPs TMS	Corticospinal and metaplasticity
7	Assess TMS-related neuroplastic effects in T2DM patients	Healthy, Early Insulin Resistant, and T2DM Patients	Data Analysis: MEPs EEG analysis (TEPs)	Corticospinal and TEP-related plasticity
8	Image effects of iTBS and single TMS pulses on DLPFC	Healthy Human Participants	TMS EEG fMRI	TMS-evoked BOLD signal State dependence (EEG)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600 pulses
40 seconds
normalized E (V/m)
0
202
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iTBS
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190 seconds
2 seconds
8 seconds
C.
D.
BDNF
TrkB
P
P
PLCY1
Ras
Calcium
AKT
PIK3G
ERK
Growth and Differentiation
Plasticity Behavior
CaMKII
Synaptic Plasticity
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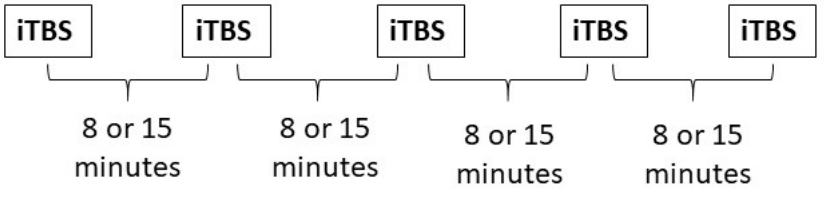
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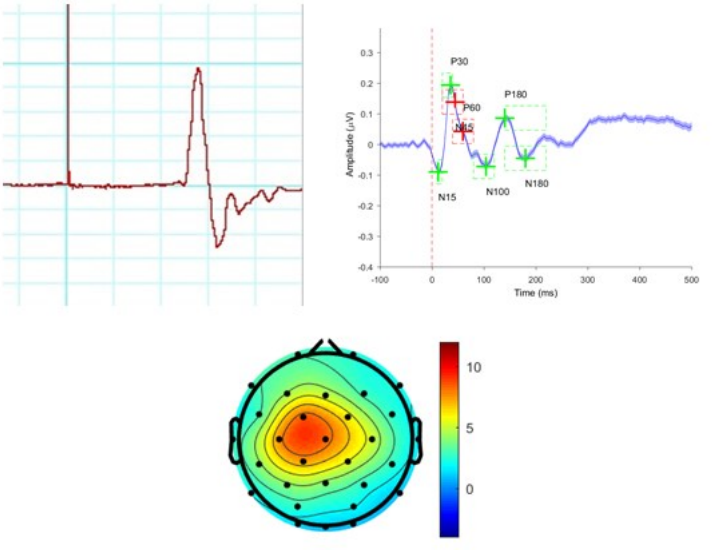
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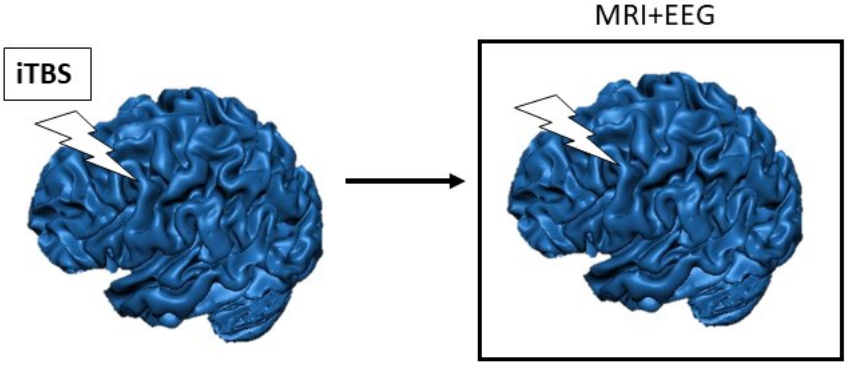
iTBS
MRI+EEG

Figure 1: Visual progression of thesis chapters. **A.** Differentiation of SH-SY5Y cells, from immature cells to a mature, neuron-like cell phenotype with long neurite extensions. Blue=DAPI (cell nucleus), Green=βIII-Tubulin, Red=MAP2. **B.** Theta burst stimulation protocols. Continuous theta burst stimulation (cTBS) is a 40 second protocol, assumed to be inhibitory. Intermittent theta burst stimulation (iTBS) is a 190 second protocol, assumed to be excitatory (19). The electric field induced within the cell culture dish, modelled using SimNIBS (169) using the cell culture dish parameters shared by (104). **C.** An example cell showing an increase in fluorescence-labelled calcium during the addition of 1M KCl. The background is turquoise, and pink indicates an increase in calcium (increase in cell activity). **D**. An adapted simplification of the BDNF-TrkB pathway (71) of interest in this thesis for gene expression in Chapter 4. **E.** An example neuron showing the proteins used for immunocytochemical analysis. Green=βIII-Tubulin, Red=MAP2, Yellow=Merge of both images. **F.** Stimulation setup for Chapter 6; accelerated iTBS. Stimulation with iTBS (5 times), with either 8 or 15 minutes between. For single iTBS, only the last iTBS was real, for sham, all stimulation was placebo. **G.** An example of an MEP, a TEP, and the distribution of the strength of the EEG signal (30ms after TMS pulse, in µV). **H.** Stimulation setup for chapter 8. iTBS was delivered offline (outside the MRI), followed by single TMS pulses every 15-17 seconds to left DLPFC while in the MRI with simultaneous EEG recording.

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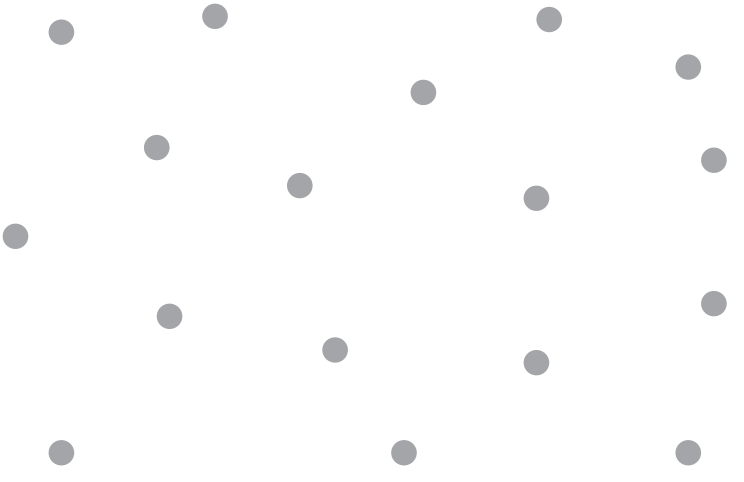
Chapter 2

The effects of serum removal on gene expression and morphological plasticity markers in differentiated SH-SY5Y cells



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Abstract:

Despite the widespread use of the SH-SY5Y human neuroblastoma cell line in modelling human neurons *in vitro*, protocols for growth, differentiation and experimentation differ considerably across the literature. Many studies fully differentiate SH-SY5Y cells before experimentation, to investigate plasticity measures in a mature, human neuronal-like cell model. Prior to experimentation, serum is often removed from cell culture media, to arrest the cell growth cycle and synchronize cells. However, the exact effect of this serum removal before experimentation on mature, differentiated SH-SY5Y cells has not yet been described. In studies using differentiated SH-SY5Y cells, any effect of serum removal on plasticity markers may influence results. The aim of the current study was to systematically characterize, in differentiated, neuronal-like SH-SY5Y cells, the potentially confounding effects of complete serum removal in terms of morphological and gene expression markers of plasticity. We measured changes in commonly used morphological markers and in genes related to neuroplasticity and synaptogenesis, particularly in the BDNF-TrkB signaling pathway. We found that complete serum removal from already differentiated SH-SY5Y cells increases neurite length, neurite branching, and the proportion of cells with a primary neurite, as well as proportion of βIII-Tubulin and MAP2 expressing cells. Gene expression results also indicate increased expression of *PSD95* and *NTRK2* expression 24 hours after serum removal. We conclude that serum deprivation in differentiated SH-SY5Y cells affects morphology and gene expression, and can potentially confound plasticity-related outcome measures, having significant implications for experimental design in studies using differentiated SH-SY5Y cells as a model of human neurons.

Keywords: SH-SY5Y Cells, Serum Deprivation, Plasticity, Human Neuron Model

Introduction

SH-SY5Y cells are a human neuroblastoma-derived cell line used to model human neurons *in vitro*. The original cells were derived from a bone marrow biopsy in 1970, and were cloned to produce the neuron-like SH-SY5Y cells that are used in a wide range of research applications today (1). These cells synthesize various neurotransmitters, express neural markers, and can be further differentiated *in vitro* to a mature human neuronal phenotype (2-5). Once differentiated, SH-SY5Y cells express a catecholaminergic phenotype, with the potential to synthesize both dopamine and noradrenaline (6). They can be used to study synapse modifications and functional cellular activity with live calcium imaging or electrophysiology (2, 7, 8). They are often used as a cell model for Parkinson's Disease (9), as well as Alzheimer's Disease (10), neuropathogenesis of viruses (11), screening for neurotropic properties of pharmaceuticals (12, 13), neurotoxicity (14, 15), and even as a multicellular 3D culture (16, 17).

With the widespread use of this cell line to study human neuron synapse activity and neuronal plasticity *in vitro*, it is important to understand the effects of cell handling, such as the removal of serum before experimental manipulation in fully differentiated cells.

SH-SY5Y cells are grown in a basic medium containing Dulbecco's Modified Eagle's-Medium (DMEM), glucose, antibiotics, and supplemented with 10-20% Fetal Bovine Serum (FBS) (9). The use of FBS in culture media to promote growth of cells and to maintain tissues *in vitro* was introduced in 1958 (18). This serum supplementation is vital for the growth and maintenance of cell lines, as it contains many crucial proteins, vitamins, hormones and growth factors important for cell survival and proliferation (19).

To induce differentiation of SH-SY5Y cells to a more mature neuronal phenotype, the serum concentration is commonly reduced to 1% or 3%, along with the addition of retinoic acid (3, 9). After 5-20 days, depending on the differentiation protocol, the cells reach their maximum differentiation state (2-4). Prior to experimental manipulation, e.g. exposure to potential pharmaceutical compounds, serum is often completely removed from the cultures. This is done to ensure all cells are in the same growth cycle phase before manipulation (20), and to prevent confounding effects of the myriad of proteins and other molecular factors present in serum, which differ by serum batch and therefore introduce phenotypic variations in cell cultures (21). Serum components may also mask certain intrinsic growth factor (e.g. brain-derived neurotrophic factor, BDNF) effects, therefore serum may be removed to assess the effects of BDNF in the absence of external growth factors (22).

Despite the common practice of serum removal before experimental manipulation in already differentiated SH-SY5Y cells, the effects of removing serum from culture media on plasticity-related gene expression and morphology markers have not yet been examined. Understanding the effects of serum removal is essential in standardizing pre-experimental protocols. If serum removal has strong effects on gene and morphological markers in already differentiated cells, any effect of experimentation may be confounded.

Here we aim to systematically characterize the effects of completely removing serum from differentiated SH-SY5Y culture media on gene expression markers of plasticity, specifically related to an important pathway in synaptic plasticity and long-term potentiation, the BDNF-TrkB signaling pathway (23-28). We also investigated the effects of serum removal on cytoskeletal markers of neuron morphology by visualizing changes in MAP2 and βIII-Tubulin.

Methods

Cell Culture

Cells were obtained from ATCC® (CRL2266™, RRID:CVCL_0019) and were maintained and expanded according to the provided protocol. For experiments, cells were not used above passage 26.

Undifferentiated cells were cultured in DMEM/ F12, GlutaMAX™ Supplement (GibcoTM, Thermo Scientific) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS, Merck), 1% penicillin-streptomycin (P/S) and 1% L-Glutamate at 37°C and 5% CO_2, and split at 80-90% confluency.

Differentiation

All cells were fully differentiated to a mature neuronal-like state before experimentation. For differentiation, cells were plated in 6-well culture plates (Greiner CELLSTAR®, Merck) at approximately $2.4x10^4$ cells per well. Serum concentration was decreased to 3% FBS three days prior to the addition of 10µM retinoic acid (RA; Sigma-Aldrich, R2625). A stock solution of RA was prepared in dimethylsulphoxide (DMSO; Sigma-Aldrich, 41640) at 10mM, and stored at -20°C until dilution in cell culture media to a final concentration of 10µM. Starting from the day RA was added, medium with 3% FBS supplementation was replaced every two days for a total of ten days.

Serum Deprivation

Differentiated SH-SY5Y cells were used for serum removal experiments. Medium containing 3% serum (FBS) was removed, and the cell surface was rinsed with PBS

(warmed to 37°C) to remove all remaining serum. Next, medium without supplemented serum (0% FBS), or medium with serum (3% FBS) was added for 1, 3, 6 or 24 hours. In total there were 8 different conditions; serum and no serum for each of the 4 time points (1, 3, 6, 24 hours).

Microscopy

Cells were grown on 12mm glass coverslips (VWR, 631-1577) coated with 100µg/mL Poly-L-Ornithine (Sigma, P4957) and 1µg/mL Laminin (Sigma, L2020) and differentiated as described above.

At the specified collection time points following complete serum removal, cells were washed in PBS (warmed to 37°C), and fixed for 10 minutes in cold 4% paraformaldehyde. To stop fixation, cells were washed 3 times 5 minutes in cold PBS and stored in PBS at 4°C for a maximum of two days before antibody incubation. Cells were then blocked in PBS-Tween 20, prepared with 0.2% Tween-20, and 10% donkey serum. Antibodies for visualizing neurite outgrowth (βIII-Tubulin; Cell Signaling, Cat #5568S, RRID:AB_10694505), and dendrites (MAP2; Sigma, Cat #M2320, AB_609904) were used. Both markers were chosen as they are often combined to capture all neuronal processes (29), and they have consistently been used as markers of differentiation in experiments with SH-SY5Y cells (2-4, 29, 30). Following primary antibody incubation, cells were washed in alternating PBS and PBS-Tween 20 and incubated with secondary antibodies donkey anti-rabbit Alexa 488 (Invitrogen, Cat #A-21206 RRID:AB_141708), donkey anti-mouse Alexa 594 (Invitrogen, Cat #A-21203, RRID:AB_141633), and cell nuclei were stained with DAPI (CarlRoth, Cat #6843.3). Following secondary antibody incubation, coverslips were washed in cold PBS and mounted on glass microscope slides. Fluorescence imaging was done with the Olympus BX51WI microscope and disc spinning unit. Pictures were taken using the 20X objective lens. Micro-Manager software (31)(RRID:SCR_016865) was used to collect images. Further details on the primary and secondary antibody dilutions as well as microscope exposure times can be found in the Supplementary Material (Table S1 and S2).

qRT-PCR

Cells in 6-well plates were first rinsed with PBS at 37°C and then kept on ice for the rest of the extraction. RNA was extracted with TRIzol (Invitrogen, 15596026) according to the manufacturer's protocol. RNA concentration was determined using the NanoDrop™ spectrophotometer, and cDNA was synthesized using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, K1632). RNA was stored at -80°C and cDNA at -20°C. Three cell culture replicates were collected per time point, per condition.

Primers for qPCR were designed using NCBI gene reference database and Primer-BLAST (National Library of Medicine). The following primers were analyzed: Activity Regulated Cytoskeleton associated protein (*ARC,*Gene ID: 23237), Early Response 1 (*EGR1*, Gene ID: 1958), cAMP Responsive Element Binding Protein 1 (*CREB1,* Gene ID: 1385), B-cell lymphoma 2 (*BCL2*, Gene ID: 596), BCL2- Associated X (*BAX,* Gene ID: 581), Brain Derived Neurotrophic Factor (*BDNF*, Gene ID: 627), Neurotrophic Receptor Tyrosine Kinase 2 (*NTRK2,* GeneID: 4915), Discs Large MAGUK scaffold protein 4 (*DLG4* also known as *PSD95,* GeneID:1742), Synaptophysin (*SYP,* GeneID:6855)*,* in conjunction with three House Keeping Genes (HKG's): Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH,* GeneID:2597), TATA-box Binding Protein (*TBP*,GeneID:6908), Peptidylprolyl Isomerase B (*PPiB*, GeneID:5479)*.* Primer sequences can be found in Supplementary Material Table S3. Primers at 600nM concentration were mixed with Fast Start Universal Sybr Green Master ROX (Roche,491385001). Samples were run in 384-well qPCR plates (Roche,4TI-0382), using the LightCycler 480 Real-Time PCR system (Roche Life Sciences). qPCR program details are described in Supplementary Material Table S4.

Analysis

Microscopy

Image processing and analysis was done in Fiji (ImageJ version 1.52i, RRID:SCR_002285) (30). A DAPI nucleus staining was used to count total cells in each image. Neurite length and branching was measured in the 488 (βIII-Tubulin) channel, using the segmented line tool at 20X magnification. Per condition 70-100 neurites were measured. Typically, in differentiated SH-SY5Y cultures, many cells have only one or no neurites. Therefore, from each cell, we measured the length of the primary neurite, defined as the single neurite, or the longest neurite for cells having more than one neurite. An example of tracings of primary neurites as well as cells without neurite extensions can be seen in Figure 2A. The NeuronJ plugin (33) was used to quantify neurite length and neurite branching. For each image, primary neurites and branches were semi-automatically traced, and manually labeled as either primary neurites, or branches. The number of branches were divided by the total neurons (counted with DAPI), to give the number of branches per neuron in each image. An example neuron with branching can be seen in Figure 2B. To identify whether the proportion of total cells with a primary extension changes due to serum deprivation, the number of primary neurites was also divided by total number of neurons (as counted with DAPI).

Total fluorescence of βIII-Tubulin staining was quantified using the 488 channel. First, the fluorescence threshold was set with the minimum intensity as the maximum

background intensity. The total fluorescence intensity in the image was then measured, and corrected for cell area by dividing by the total area of cells in the 488 channel.

βIII-Tubulin stained cells were counted manually by setting the brightness contrast settings to 834 (min) and 7474 (max). Cells with visible green neurites were counted. MAP2 was manually counted in the 594 channel, at brightness contrast settings of 596 (min) and 6007 (max). These cell counts were divided by the total cells to calculate the proportion of βIII Tubulin or MAP2 cells.

Gene Expression

A standard curve was used to calculate relative concentrations of gene expression per gene. An average of technical duplicates was made, and normalized to the average of 3 HKGs (TBP, PPiB, GAPDH). Analyses were performed with LightCycler 480 software version 1.5.1.62 (Roche Life Sciences) and Excel.

Statistics

Statistical analysis and graphs were made with Prism 5 (Graphpad Software, USA, RRID:SCR_002798) and IBM Statistics 24 (SPSS for windows version 24.0, Armonk, NY:IBM Corp). Data collected from 2 independent experiments were pooled for statistical analysis. This resulted in a total of 4-14 images per condition being included in statistical analysis. For analysis of neurite length, an average neurite length per image was calculated from 70-100 neurites, for a total of 8 images per condition included in the 2-way ANOVA. For the analysis of primary neurites per neuron and neurite branching per neuron, the number of primary neurites in an image (between 30-100 per image) were divided by the total neurons in the image (between 210-250 cells per image), with 4-6 images per condition included in the 2-way ANOVA. For analysis of βIII-Tubulin Immunoreactivity, a total of 10-14 images per condition were included in the 2-way ANOVA. A 2-way ANOVA with factors Serum (serum, no serum) and Time (1,3,6, 24 hours) was used for all comparisons of microscopy quantification and HKG normalized expression values. Bonferroni-corrected post-hoc tests were done in the case of significant interaction events. Reported results are mean ± standard error of the mean. Figures show bar graphs of the HKG normalized mean expression values; error bars are standard errors of the mean.

Results

Differentiation

Differentiation was verified as explained previously (34). Representative images comparing undifferentiated and differentiated cells can be seen in Figure 1.

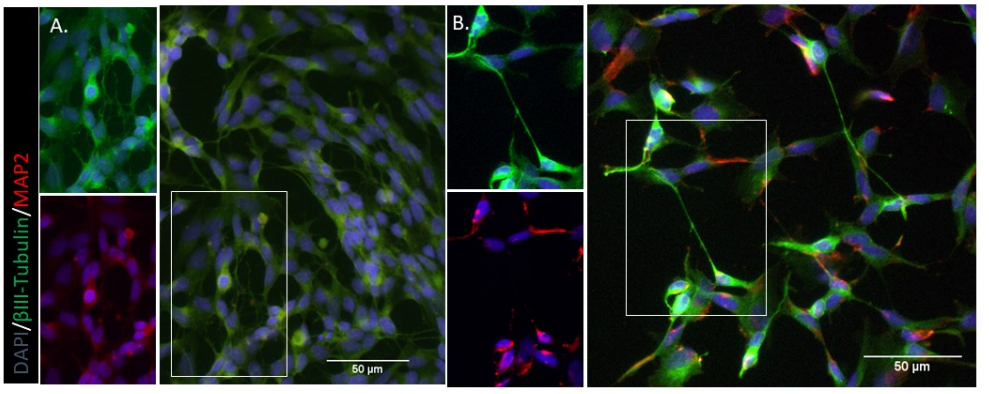

Figure 1. Visual representation SH-SY5Y cells **A.** Undifferentiated cells **B.** Neuron-like cells at 10 days differentiation.

Microscopy

Full statistical results of main effects (Time, Serum) and Time x Serum interaction effects for each parameter (Neurite Length, Neurite Branching, Primary Neurites, βIII-Tubulin immunoreactivity, and βIII-Tubulin and MAP2 positive cells) can be found in Supplementary Material Table S5. In case of significant main or interaction effects, the p-value of Bonferroni-corrected post hoc tests are reported below. An example of the parameters measured can be seen in Figure 2. An example of the morphology of cells immediately following serum removal can be seen in Supplementary Figure 2.

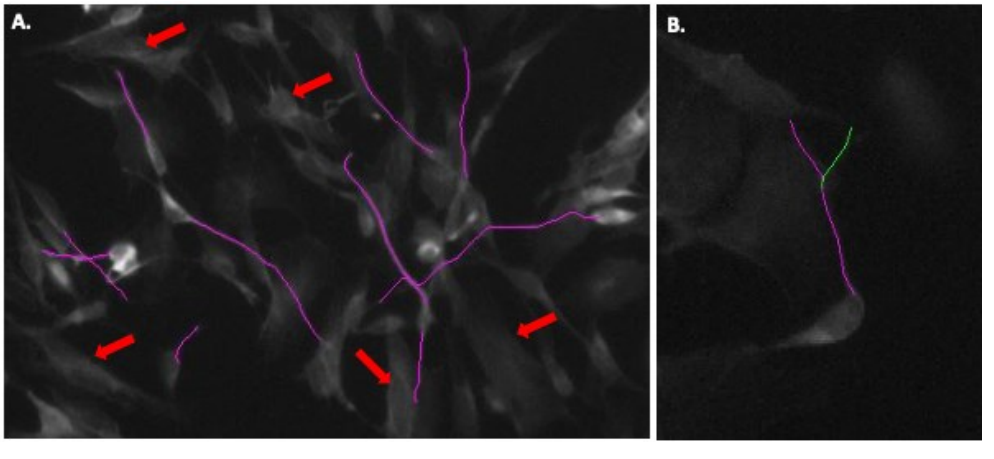

Figure 2. Morphological parameters analysed **A.** Tracing of primary neurite extension in purple. An example of cells showing no neurite extensions are indicated with red arrows **B.** An example neuron tracing with branch. Primary neurite is traced in purple, branch is traced in green.

Neurite Length

We found a significant effect of Serum (p<0.0001), but no significant effect of Time (p=0.382), or Time x Serum interaction (p=0.338) on neurite length. Serum deprived neurons had significantly longer outgrowths than neurons with serum at 3 hours (54.93±20.22 µm vs. 45.43±17.76 µm, p=0.03) and at 24 hours (65.53±27.74µm vs. 46.89±18.24 µm, p<0.0001) (Figure 3A).

Neurite Branching

There was a significant effect of Time (p=0.0105) and Serum (p<0.0001), but not a significant Time x Serum interaction (p=0.062) on neurite branching. Serum deprived neurons had significantly more branches per neuron at 3 hours (0.058±0.008 vs. 0.011±0.003, p<0.0001), 6 hours (0.041±0.007 vs. 0.015±0.003, p<0.028), and at 24 hours (0.033±0.008 vs. 0.0045±0.003, p<0.028) (Figure 3B).

Primary Neurites

Similarly, we found a significant effect of Time (p=0.0368), Serum (p=0.0006), but not a significant Time x Serum interaction (p=0.109). At 3 hours, serum deprived neurons showed a greater proportion with a primary neurite (0.45±0.034 vs 0.25±0.020, p=0.001) (Figure 3C).

βIII-Tubulin Immunoreactivity

A significant effect of Serum (p=0.0006) and a Time x Serum interaction (p=0.0035) was found, but no effect of Time (p=0.14) on βIII-Tubulin immunoreactivity. There was a significant increase in βIII-Tubulin immunoreactivity in the serum deprived cells at 3 hours (120.23±25.58 vs. 37.03±4.96, p<0.01) and 6 hours (110.49±28.55 vs. 22.94±3.03, p<0.01) (Figure 3D).

βIII-Tubulin and MAP2 Positive Cells

There was a significant effect of Time (p<0.0001), Serum (p<0.0001), and a Time x Serum interaction (p=0.0012), on cells expressing βIII-Tubulin after serum deprivation. Serum deprived neurons showed a significant increase in the percentage of βIII-Tubulin expressing cells at 3 hours (42.04±4.92% vs. 23.16± 3.78%, p=0.009) and 6 hours (30.80±2.77% vs. 17.40±2.21%, p=0.023), and 24 hours (26.69±2.41 vs. 8.90±2.35, p=0.00012) (Figure 3E).

There was also a significant effect of Time (p<0.0001), Serum (p<0.0001), and a Time x Serum interaction (p=0.0011), on percentage of cells expressing MAP2 after serum deprivation. Serum deprived cells also showed a significant increase in percentage MAP2 expressing cells at 3 hours (29.08±5.02% vs. 15.30±2.28%, p<0.01), 6 hours (23.65±2.12% vs. 12.51±1.87%, p<0.05), and 24 hours (19.85±2.50 vs. 7.27±2.21, p<0.01) (Figure 3F).

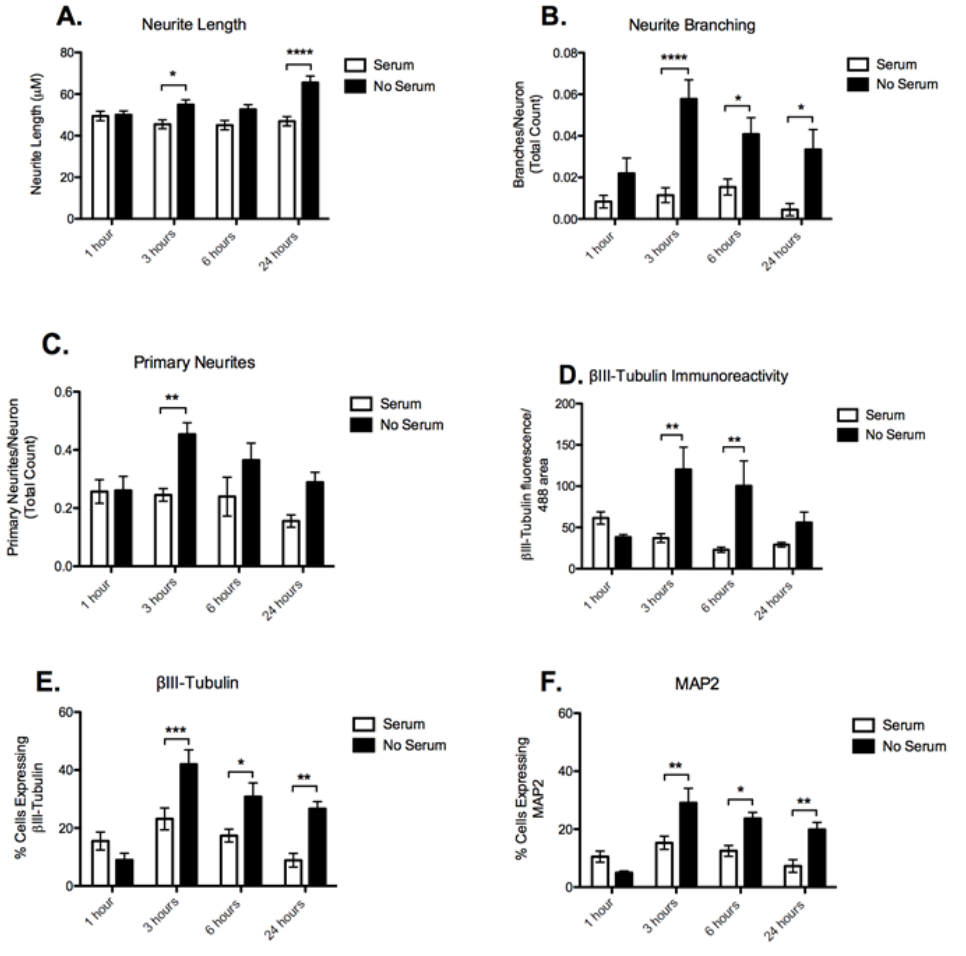

Figure 3. Morphological Parameters. **A.** Neurite Length **B.** Neurite Branching **C**. Primary Neurites **D.** βIII-Tubulin immunoreactivity **E.** Proportion βIII-Tubulin positive cells **F.** Proportion MAP2 positive cells. (Significant post hoc comparisons are indicated as **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001).

Gene Expression

We were most interested in gene expression changes following serum deprivation, specifically in genes related to IEG expression (*ARC, EGR1*), apoptosis (*BCL2*, *BAX*), plasticity (*BDNF, NTRK2*, *CREB1*) and synaptogenesis (*PSD95*, *SYP*). Full statistical results of main (Time, Serum) and Time x Serum interaction effects can be found in Supplementary Material Table S6. In case of significant main or interaction effects, p-values and Bonferroni-corrected post hoc tests are reported in the text. Graphs show

mean HKG normalized expression levels for each condition, error bars are standard error of the mean.

Immediate Early Gene Expression

We measured the expression of IEG's *ARC* and *EGR1*, finding high expression levels in both genes in the 1-hour serum condition only. Expression levels were low in all other samples (3,6 and 24 hours), many of which were too low to detect (Ct value ≥34). Gene expression results can be found in Supplementary Material and Supplementary Figure 1.

Apoptosis Markers

BCL2 Expression: We also found no significant main effects of Time (p=0. 362), Serum (p=0.618), or Time x Serum interaction (p=0.216) on BCL2 expression (Figure 4A).

BAX Expression: We found no significant main effect of Time (p=0.169), Serum (p=0.380), or Time x Serum interaction (p=0.228) on BAX expression (Figure 4B).

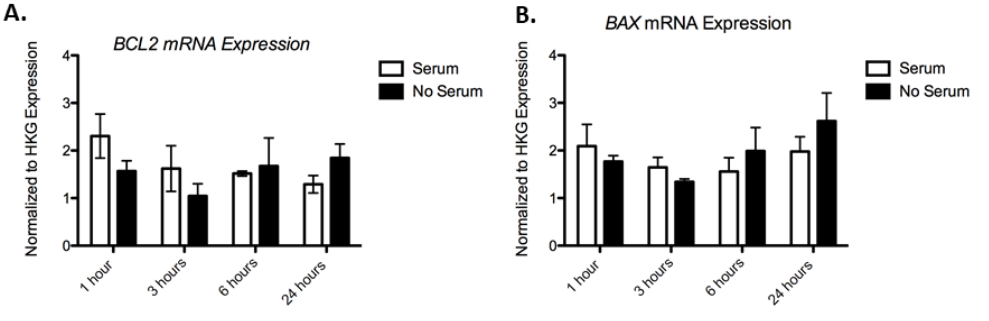

Figure 4. Results of gene expression analysis of **A.** *BCL2* mRNA and **B.** *BAX* mRNA. Expression values have been normalized to the average of 3 housekeeping genes (*TBP, PPiB, GAPDH*).

Expression of BDNF Signaling

BDNF Expression: We found a significant effect of Time (p<0.001), Serum (p<0.001) and Time x Serum interaction (p<0.001) on *BDNF* expression. There was a significant decrease in *BDNF* expression in the serum deprived cells at 1 hour (p<0.0001), 3 hours (p<0.0001), and 6 hours (p<0.0001). Compared to time-matched serum controls, serum deprived cells express 25.71±5.76% *BDNF* at 1 hour, 8.07±1.35% at 3 hours, 12.01±0.61% at 6 hours and 94.58±5.89% at 24 hours (Figure 5A).

NTRK2 Expression: There was also a significant effect of Time (p=0. 008), Serum (p=0.006), and Time x Serum interaction (p=0.021) on *NTRK2* expression. There was a significant increase in expression of *NTRK2* mRNA at 24 hours (p<0.01), with serum deprived cells expressing 242.11±33.00% of the *NTRK2* expressed in serum controls (Figure 5B).

CREB Expression: There was no significant main effect of Time (p=0. 393) or Serum (p=0.942), on CREB expression. However, there was a Time x Serum interaction effect (p=0.043). Initially, there is a decrease in *CREB* expression in serum deprived cells at 1 hour (to 65.25±1.26% serum controls), at 6 hours this is reversed (164.71±30.31% serum controls) (Figure 5C). None of these time points are significant in Bonferroni-corrected post hoc tests.

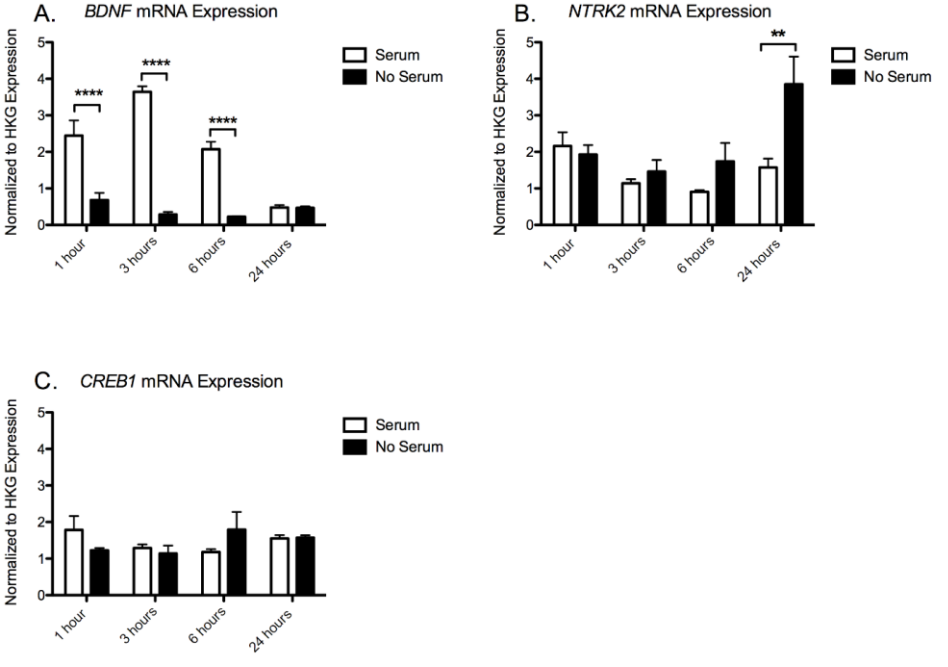

Figure 5. Results of gene expression analysis of **A.** *BDNF* mRNA and **B.** *NTRK2* mRNA, and **C.** *CREB* mRNA. Expression values have been normalized to the average of 3 housekeeping genes (*TBP, PPiB, GAPDH*). Significant post hoc comparisons are indicated as, **p<0.01, ****p<0.0001.

Synaptogenesis Genes

PSD95 Expression: There was a significant effect of Time (p=0.001), and a Time x Serum interaction (p=0.020), but no effect of Serum (p=0.153), on *PSD95* expression. There was a significant increase in expression in the serum deprived cells at 24 hours (p<0.01). Serum deprived cells express 192.85±17.78% of the serum controls at 24 hours (Figure 6A).

SYP Expression: Again, we found a significant effect of Time (p=0.017), but no effect of Serum (p=0.575). We found a trend towards a significant Time x Serum interaction effect (p=0.059). (Figure 6B).

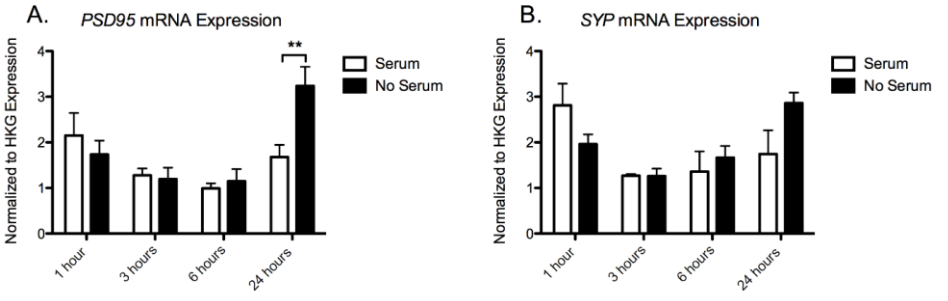

Figure 6. Gene expression analysis of A.*PSD95* mRNA and B. *SYP* mRNA. Expression values have been normalized to the average of 3 housekeeping genes (TBP, PPiB, GAPDH). Significant post hoc comparisons are indicated as, $^{**}p<0.01$.

Discussion

In this study we aimed to systematically characterize, in fully differentiated SH-SY5Y cells, the effects of complete serum removal on several morphological and gene expression markers of plasticity. We found that serum removal, over 24 hours, increased primary neurite length as well as neurite branching when compared to serum controls. Serum deprived neurons also showed higher levels of βIII-Tubulin immunoreactivity, and a greater proportion of βIII-Tubulin- and MAP2-positive neurons. MAP2 is mainly localized in mature dendrites (35), and βIII-Tubulin is a widely used neuronal maturity marker (36). These findings suggest that in fully differentiated cells, complete serum removal may promote additional plasticity-like effects. This can be seen as early as 3 hours following removal of serum, and lasts at least 24 hours.

We also found that complete serum removal has a specific effect on the expression of several genes involved in BDNF-TrkB signaling and synaptogenesis. Serum deprivation resulted in a significant increase in the expression of *NTRK2, PSD95* and *SYP* over time, with the strongest effect on the expression of *NTRK2* and *PSD95* mRNA at 24 hours following deprivation. *NTRK2*, the gene coding for the TrkB receptor, has been shown to be important in activity dependent plasticity leading to long term potentiation (LTP) (24, 37). PSD-95 is an important scaffolding protein, regulating the strength of excitatory synapses (38), and the *SYP* gene codes for synaptophysin, an important protein involved in neurotransmitter release (39, 40). An increase in the expression of *NTRK2, PSD95* and *SYP* mRNA over time in serum-deprived cells therefore aligns with our morphology results. Our results suggest that complete serum removal induces an increased expression of genes and morphological markers of plasticity and synaptic strength, potentially confounding experiments interested in these outcome measures.

While these observations are in line with the differentiation-inducing effect of serum deprivation (2-4, 41), these cells are already fully differentiated, therefore the additional changes in morphological markers that we present here may indicate additional, confounding plasticity effects. Indeed, systematic transcriptomic profiling SH-SY5Y cells has identified *NTRK2* as well as many genes involved in neurogenesis and cytoskeletal reorganization as upregulated in differentiated compared to undifferentiated cells (42). However, once cells have been differentiated, the expression of these genes is stable over time; in contrast to the serum removal effects we report here. This semi-acute increase in plasticity-related gene expression and morphological markers is problematic in studies using these genes or morphological plasticity markers as outcome measures.

Interestingly, we also report a strong effect of serum on the expression of *BDNF, ARC* and *EGR1*. *BDNF* expression increased in the in the serum control cells after 1, 3 and 6 hours, returning to low expression levels at 24 hours. The serum control cells underwent a regular medium change, including a PBS wash step. This increase in *BDNF* mRNA in the serum condition is surprising, but may be related to the addition of fresh medium and serum. In serum-deprived cells, this temporary increase is absent, likely due to the disruption of growth and protein production as a consequence of serum withdrawal (43-46). We also report an increase in expression of the immediate early genes *ARC* and *EGR1* in the serum condition at 1 hour after PBS wash and serum replacement. The removal and re-addition of serum could have induced an immediate but transient increase in the expression of *ARC* and *EGR1* mRNA, in line with the expected expression pattern of an immediate early gene (47, 48).

We did not find any effects of serum deprivation on the expression of genes linked to apoptosis, *BAX* and *BCL2*. Encinas et al. (2000) showed that SH-SY5Y cells show signs of apoptosis 6 and 24 hours after serum removal as measured by caspase activity and TUNEL assay (3). Encinas et al. (2000) used cells that were treated with RA for only five days and in medium containing 15% FBS. The shock of serum removal in the not-fully differentiated cells is likely much stronger compared to our protocol, and may explain the different finding. Based on the gene expression markers in our experiments, we cannot confirm that serum starvation influences apoptotic processes after 24h in in fully differentiated SH-SY5Y cells.

Conclusion

Despite being common practice to remove serum from the culture medium of already differentiated SH-SY5Y cultures before experimentation, the effects on morphology and gene expression had not been systematically characterized. Here, we show that complete serum deprivation has an effect on commonly used morphological and

gene expression markers of cellular and synaptic plasticity in differentiated SH-SY5Y cells, and may thus confound results when examining plasticity-related outcome measures. For future research involving differentiated SH-SY5Y cells as a model of human neural plasticity, our findings provide some key considerations for experimental design. Studies interested in measuring plasticity effects in differentiated SH-SY5Y cells should either refrain from complete serum deprivation 24 hours before experimentation, or include appropriate controls, e.g. cells which were not serum deprived, to confirm serum deprivation had no confounding effects on outcome measures.

Abbreviations

FBS	Fetal Bovine Serum
RA	Retinoic Acid
MAP2	Microtubule Associated Protein 2
ARC	Activity Regulated Cytoskeleton associated protein
EGR1	Early Growth Response 1
BCL2	B-cell Lymphoma 2
BAX	BCL2- Associated X
BDNF	Brain-Derived Neurotrophic Factor
NTRK2	Neurotrophic Receptor Tyrosine Kinase 2
CREB1	cAMP Responsive Element Binding Protein 1
PSD95	Discs Large MAGUK scaffold protein 4, *DLG4*
SYP	Synaptophysin
TBP	TATA-Box Binding Protein
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
PPiB	Peptidylprolyl Isomerase B

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Excitation/Emission	Marker of	Exposure Time (ms)	Color
358/461	DAPI (nuclei)	10	Blue
493/519	βIII-Tubulin	500	Green
591/614	MAP2	300	Red

Table S1. Exposure times for fluorescence microscopy. Olympus BX51WI microscope and DSU spinning unit used. Pictures were taken using the 20X objective lens.

Name	Company	Order number	Concentration used	Marker of
βIII Tubulin	Cell Signaling	5568S	1:300	Neurons; Axon guidance and maturation
MAP2	Sigma	M2320	1:300	Neurons; Dendrite formation
Donkey anti rabbit Alexa 488	Invitrogen	A21206	1:1000	βIII Tubulin
Donkey anti mouse Alexa 594	Invitrogen	A21203	1:1000	MAP2
DAPI	CarlRoth	6843.3	1:800	Nucleus

Table S2. Primary and secondary antibody dilutions used for immunocytochemistry.

GAPDH	FWD	CCAAATGCGTTGACTCCGA
GAPDH	REV	GCATCTTCTTTTGCGTCGC
TBP	FWD	TGCACAGGAGCCAAGAGTGAA
TBP	REV	CACATCACAGCTCCCCACCA
PPiB	FWD	GTTTGAAGTTCTCATCGGGG
PPiB	REV	AAAACAGCAAATTCCATCGTG
ARC	FWD	GGAGTACTGGCTGTCCCAGA
ARC	REV	ACTCCACCCAGTTCTTCACG
EGR1	FWD	CCCCGACTACCTGTTTCCAC
EGR1	REV	GACAGAGGGGTTAGCGAAGG
CREB1	FWD	CCCCAGCACTTCCTACACAG
CREB1	REV	CTCGAGCTGCTTCCCTGTTC
BCL2	FWD	ACATCGCCCTGTGGATGACT
BCL2	REV	CCGTACAGTTCCACAAAGGC
BAX	FWD	GGGGACGAACTGGACAGTAA
BAX	REV	CAGTTGAAGTTGCCGTCAGA
BDNF	FWD	ATAGAGTGTGGGAGTTTTGGGG
BDNF	REV	TGGTGGAACTTTTCAGTCACTACT
NTRK2	FWD	TGGATGCATATCGTGCTCCG
NTRK2	REV	GTGCTTGGTTCAGCTCTTGC
PSD95	FWD	CCCCAGGATATGTGAACGGG
PSD95	REV	CCGATGTGTGGGTTGTCAGT
SYP	FWD	CACTGATGACTTCCCAGAACTGT
SYP	REV	CTGGGCTTCACTGACCAGAC

Table S3. Primer Sequences, 5’ to 3’ orientation. All primers were designed using the NCBI gene reference database and Primer-BLAST (National Library of Medicine, https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Analysis Mode	**Program Name**	**cycles**	Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)
None	Pre-Incubation	1	95	None	0:10:00	4.8
Quantification	Amplification	45	95	None	0:00:10	4.8
			60	single	0:00:45	2.5
Melting Curves	Melting Curve	1	95	None	0:00:15	4.8
			60	None	0:00:30	2.5
			97	Continuous		0.11
None	Cooling	1	60	None	0:00:10	2.5

Table S4. qRT-PCR program. Run in 384 well qPCR plates (Roche,4TI-0382) using LightCycler® 480 Real-Time PCR (Roche LifeScience).

Full Statistical Results

Parameter	Time	Serum	Time x Serum Interaction
Neurite Length	$F(3,55)=1.041$ $p=0.382$	**$F(1,55)=22.33$ $p<0.0001$**	$F(3,55)=1.148$ $p=0.338$
Neurite Branching	**$F(3,29)=4.482$ $p=0.0105$**	**$F(1,29)=43.45$ $p<0.0001$**	$F(3,29)=2.727$ $p=0.0622$
Primary Neurites	**$F(3,29)=3.229$ $p=0.0368$**	**$F(1,29)=14.81$ $p=0.0006$**	$F(3,29)=2.203$ $p=0.109$
βIII-Tubulin Immunoreactivity	$F(3,73)=1.856$ $p=0.14$	**$F(1,73)= 13.04$ $p=0.0006$**	**$F(3,73)= 4.943$ $p=0.0035$**
βIII-Tubulin positive cells	**$F(3,56)=13.20$ $p<0.0001$**	**$F(1,56)=20.46$ $p<0.0001$**	**$F(3,56)=6.083$ $p=0.0012$**
MAP2 positive cells	**$F(3,56)=11.48$ $p<0.0001$**	**$F(1,56)=19.09$ $p<0.0001$**	**$F(3,56)=6.151$ $p=0.0011$**

Table S5. Morphological parameters main and interaction effects. Significant main effects in bold.

Gene	Time	Serum	Time x Serum Interaction
BCL2	*F*(3,16)=1.144 *p*=0.362	*F*(1,16)=0.258 *p*=0.618	*F*(3,16)= 1.659 *p*=0.216
BAX	*F*(3,16)=1.906 *p*=0.169	*F*(1,16)=0.816 *p*=0.380	*F*(3,16)=1.602 *p*=0.228
BDNF	***F*(3,15)=16.250** ***p*<0.001**	***F*(1,15)=128.71** ***p*<0.001**	***F*(3,15)=17.547** ***p*<0.001**
NTRK2	***F*(3,16)=5.650** ***p*=0.008**	***F*(1,16)=9.791** ***p*=0.006**	***F*(3,16)= 4.300** ***p*=0.021**
CREB1	*F*(3,16)=1.063 *p*=0. 393	*F*(1,16)=0.005 *p*=0.942	***F*(3,16)= 3.403** ***p*=0. 043**
PSD95	***F*(3,16)=8.338** ***p*=0.001**	*F*(1,16)=2.256 *p*=0.153	***F*(3,16)= 4.339** ***p*=0.020**
SYP	***F*(3,16)=4.550** ***p*=0.017**	*F*(1,16)=0.327 *p*=0.575	*F*(3,16)= 3.054 *p*=0.059

Table S6. Gene expression main and interaction effect results. Significant main effects in bold.

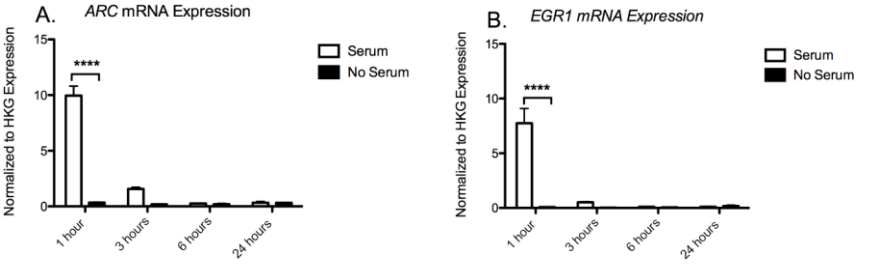
Immediate Early Gene Expression Results

Gene	Time	Serum	Time x Serum Interaction
ARC	***F*(3,16)=94.119** ***p*<0.0001**	***F*(1,16)=127.846** ***p*<0.0001**	***F*(3,16)=90.539** ***p*<0.0001**
EGR1	***F*(3,16)=21.815** ***p*<0.0001**	***F*(1,16)=25.455** ***p*<0.0001**	***F*(3,16)=22.007** ***p*<0.0001**

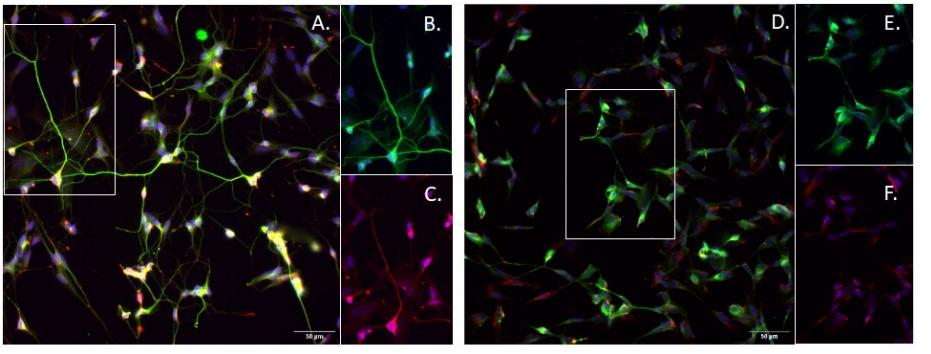
ARC Expression: We found a significant main effect of Time, Serum, and a Time x Serum interaction on *ARC* expression. Post-hoc analysis showed that cells that were serum deprived show a significant decrease in HKG normalized *ARC* expression compared to serum cells at 1 hour ($p<0.0001$). When normalized to the *ARC* expression in serum time-matched controls, serum deprived cells expressed 3.23±0.40% of the *ARC* mRNA at 1 hour, 11.76±1.60% at 3 hours, 76.74±26.40% at 6 hours, and 97.38±10.89% at 24 hours (Supplementary Figure 1A).

EGR1 Expression: Again, we found a significant main effect of Time, Serum, and a Time x Serum interaction on *EGR1* expression. Post-hoc analysis showed a significant

decrease in expression in the serum-deprived cells at 1 hour (p<0.001). Compared to the serum condition, *EGR1* expression in serum-deprived cells was 0.92±0.04% at 1hour, 5.18±0.60% at 3 hours, 54.10±10.03% at 6 hours, and 167.93±61.40% at 24 hours (Supplementary Figure 1B).



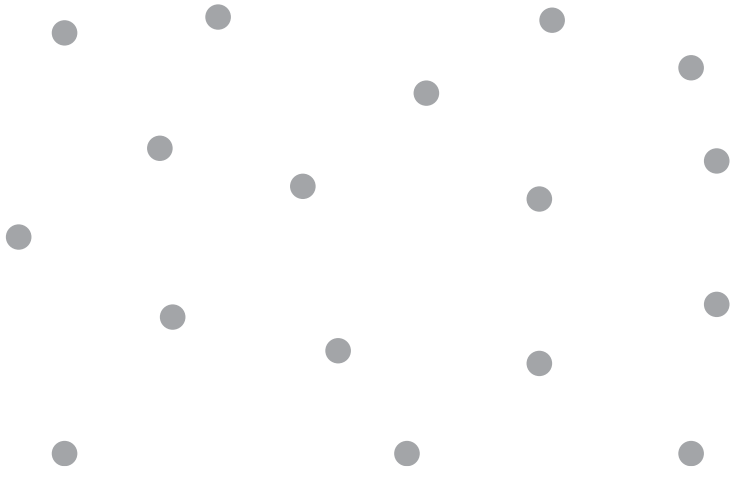
Supplementary Figure 1. Gene expression levels of **A.** *ARC* and **B.** *EGR1.* Expression levels are normalized to the average of 3 Housekeeping genes. Significant post hoc comparisons are indicated ****p <0.0001.



Supplementary Figure 2. A. Example morphology neurons immediately following serum removal (**A-C**) compared to neurons in serum-containing media(**D-F**). Nucleus (DAPI) in blue, βIII-Tubulin in green, MAP2 in red. **A.** Serum-deprived cells, merge of all channels, **B.** Serum-deprived cells, DAPI and βIII-Tubulin **C.** Serum-deprived cells, DAPI and MAP2 **D.** Cells in serum, merge of all channels **E.** Cells in serum, DAPI and βIII-Tubulin **F.** Cells in serum, DAPI and MAP2.

Chapter 3

Transcranial Magnetic stimulation (TMS) modulates functional activity of SH-SY5Y cells: An in vitro model provides support for assumed excitability changes



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* Equal contribution

Abstract

Repetitive transcranial magnetic stimulation (rTMS) is an established neuromodulation technique that, depending on stimulation parameters, can lead to lasting neural excitability changes. Intermittent and continuous theta burst stimulation (iTBS, cTBS), in particular, are increasingly popular rTMS protocols with widespread applications in both research and therapy. Most studies use in vivo neuroimaging measures such as fMRI, or neurophysiological recordings such as motor evoked potentials (MEPs) or simultaneous EEG signals to assess the lasting effects of rTMS/TBS on cortical excitability. However, these in vivo effects of TBS have shown substantial variability, arising from factors such as the complex structural organization of the human brain, the physiological-state dependence and/or the cortical network properties interacting with the external stimulation. Therefore, the assumed excitability effects of iTBS/cTBS have proven difficult to replicate on the single subject/patient level. We here describe a direct method of imaging rTMS effects in a human in vitro neuron model; differentiated SH-SY5Y cells. We use live calcium imaging to assess changes in neural activity following stimulation, through quantifying fluorescence response to chemical depolarization. We found that iTBS and cTBS have opposite effects on fluorescence response; with iTBS increasing and cTBS decreasing response to chemical depolarization. These results provide support for the assumed rTMS after-effects in an in vitro model of human neurons. Future work can build on this foundational evidence, for example using more complex human neuron models, to uncover where, between unorganized neuron-like cell cultures as here, and highly complex in vivo human cortical circuitry, the inter-subject variability of these rTMS protocols starts to affect their reliability, as a means to develop and evaluate subject/patient-specific brain stimulation protocols.

Keywords: Transcranial Magnetic Stimulation (TMS), Calcium Imaging, Intermittent Theta Burst Stimulation (iTBS), Continuous Theta Burst Stimulation (cTBS), Neuronal Excitability, In Vitro Human Neuron model, SH-SY5Y cells

Introduction

Transcranial brain stimulation describes all forms of neuromodulation in which neural activity is stimulated noninvasively by applying electric or electromagnetic pulses through the intact skull into the brain. In transcranial magnetic stimulation (TMS), magnetic pulses are applied transcranially to induce action potentials (1). When multiple TMS pulses are administered in a particular pattern/frequency (repetitive, rTMS) lasting effects on cortical excitability have been described (2, 3). Depending on parameters, a targeted cortical brain region can either show lasting increases or decreases of cortical excitability, having widespread implications both in research and the clinic. rTMS has proven effective as a treatment for various mental disorders; such as treatment resistant depressive disorder (4), offering a cost-efficient, painless alternative to pharmaceutical treatment with minimal side effects and risk (5, 6). However, to fully deliver on this potential, the modulatory effects of rTMS protocols must be understood, reliable, and ideally personalized.

Theta burst stimulation (TBS) is the prime example of a rapid rTMS protocol in which opposing neuroplastic effects can be induced depending on the chosen frequency/pattern of stimulation. “Theta burst” refers to triplet bursts (50 Hz) of magnetic stimulation administered at theta frequency (5 Hz). It has been shown that *intermittent* TBS (iTBS) increases, whereas *continuous* TBS (cTBS) decreases cortical excitability, for up to 1 hour following stimulation (2). However, recent reports have emphasized the difficulty of replicating the effects of these different TBS protocols (7-10), raising questions about their robustness and replicability, and subsequently about the generally assumed cellular basis of TBS-induced neuroplastic changes.

Currently in humans, cortical excitability and its modulation by rTMS are almost exclusively measured through motor-evoked potentials (MEPs), a contralateral muscle twitch which represents cortico-spinal excitability at the time of stimulation (11). However, this approach is hindered by large inter and intra subject variability (7, 9). Alternatively, rTMS-induced changes in cortical excitability can be assessed with EEG; for example with TMS-evoked potentials (TEPs) or resting state neuronal power/synchronization. However, substantial confounds exist with this method as well (12). This variability is difficult to control for, as rTMS effects are influenced by many biological and genetic factors (13-15), as well as brain state at the time of stimulation (16, 17). One alternative approach is to assess rTMS systematically, starting from a simple, unorganized in vitro human neuronal model, and building up to the complex circuitry of the human cortex.

Here, we took a first step in developing an in vitro model to assess the effects of rTMS/TBS. We perform functional imaging in SH-SY5Y human neuroblastoma cells, which we use as a model for neuronal activity and excitability following rTMS protocols. SH-SY5Y cells can be relatively quickly and consistently differentiated into a

mature neuron-like state, developing functional synapses and expressing many markers of mature human neurons (18-21). We therefore opted to first test our setup in these cells, with the aim of moving to a more complex human neuronal in vitro setup in the future.

We used calcium imaging to measure changes in neuron *activity* following cTBS and iTBS stimulation, and, crucially, to model effects on *excitability* through response to chemical depolarization. To measure calcium activity, we used Fluo-4 AM (F14201, Thermo Fisher), a fluorescent indicator which binds intracellular calcium, to quantify changes in calcium concentration in the 100nM-1mM range (22). In a neuron, the resting calcium levels range between 50-100nM, which can increase 100-fold during electrical activity (23). Therefore, we expected to measure low fluorescent signal at baseline, and an increase in signal intensity with cellular activity. To assess effects on *excitability*, or 'evoked' functional activity, we measured responses to 1M Potassium Chloride (KCl), which has been shown to immediately induce cellular activity during calcium imaging in differentiated SH-SY5Y cells (18).

Mature SH-SY5Y neurons were incubated with Fluo-4 AM dye, and imaged for baseline activity with fluorescence microscopy. We then stimulated our human neuron model with commonly used stimulation protocols: iTBS, cTBS, or sham, at an intensity of 100% Maximum Stimulator Output (MSO) and a distance of 1 cm from the dish surface. This has been done previously in *in vitro* and animal studies of rTMS effects (24, 25). The stimulation setup can be seen in Figure 1A. The distribution of the electric field (V/m) induced by rTMS within the cell culture dish from several viewpoints can be seen in Figure 1B-D, modelled with SimNIBS (26) using the cell culture dish mesh generously shared by the authors of (27). Post stimulation activity was the measured, followed by the addition of 1M KCl to induce cellular activity. A time-series montage showing an example of the increase in fluorescence in 1 cell as the KCl is added can be seen in in Figure 1E. We measured fluorescence intensity in 4 separate 2-minute blocks; 1. Baseline, 2. Post-stimulation, 3. During the addition of 1M KCl to induce depolarization, and 4. Post-KCl.

Methods

Experimental model

SH-SY5Y (ATCC® CRL2266™) neuroblastoma cell line, were used. Freezing and thawing of cell batches were performed according the provided protocols. Cells were not used above passage number 26. For all experiments, cells were grown in DMEM/Nut Mix F12 with Glut-L (Gibco™, Thermo Scientific, 31331-028) at 37°C and 5% CO^2. For maintenance and expansion of cell cultures, media was supplemented with 10% heat

inactivated Fetal Bovine Serum (FBS, MERCK), 1% penicillin-streptomycin (P/S) and 1% L-Glutamate.

For differentiation, cells were plated in round 35mm Poly-D-Lysine coated 10mm diameter glass bottom MatTek dishes (Matex Corp., P35GC-0-10-C) at approximately $2.4x10^4$ cells per well. FBS supplementation was decreased to 3% FBS 3 days prior to the addition of 10 µM retinoic acid (RA; Sigma-Aldrich, R2625). During differentiation, RA-supplemented media was replaced every 2 days for 10 days. This differentiation protocol has been shown to establish a mature neuron-like phenotype (28). While it has been reported that the addition of BDNF can promote differentiation and cell survival (28), we did not find this method to be superior to the addition of RA alone (data not shown).

Functional Imaging

For each condition, 6 separate cell dishes were measured. Cells were incubated in 5µM cell permeant calcium indicator Fluo-4 AM (F14201, Thermo Fisher) and 0.02% Pluronic Acid (ThermoFisher,P6867) made in RA-supplemented culture media for 20 minutes at 37°C and 5% CO_2. Cells were washed 3 times for 5 minutes in RA-supplemented culture media, the first wash containing 0.00002% Propidium Iodide (Molecular Probes, P3566) and 0.00001% Hoechst (Sigma, B2261). After washing, 1ml of Hank's Balanced Salt Solution (HBSS) was added to each dish for imaging.

Cells were imaged with the 10X magnification lens of an Olympus IX81 microscope (Olympus Nederland B.V), EXi Blue Fluorescence Camera (Q Imaging, Canada), and X-Cite 120 series fluorescent illuminator (EXFO Photonic Solutions Inc, Canada). A Pecon Tempcontrol 37-2 Digital 2-Channel heating plate (Meyer Instruments, Houston) was used to ensure cell dishes were kept at 37°C during imaging. Imaging was recorded using micromanager open-source software (29). To visualize the change in calcium response over time, 60 images with 2 seconds between each image were recorded per recording block: Baseline, Post Stimulation, KCl Addition and Post KCl. Following baseline recording, cells were removed from the microscope chamber and stimulated with rTMS as described below. Dishes were marked before removal in order to place them back in approximately the same position.

Magnetic Stimulation

Cells were placed 1 cm below the centre of a Cool-B65 figure 8 coil (Magventure, Denmark) and stimulated at 100% Maximum Stimulator Output (MSO) with a MagPro X100 with MagOption stimulator (Magventure, Denmark). Each stimulation session consisted of the Huang (2005) published protocol of 50Hz triplets repeated at 5 Hz. iTBS consisted of 2000ms trains with 8000ms inter-train intervals, while cTBS consisted of continuous triplets; both for 600 pulses (2). For the cTBS condition, cells were placed under the coil for an additional 150 seconds to ensure iTBS and cTBS cells

were out of the incubator for the same amount of time. For the sham condition, cells were placed under the coil for 190 seconds. The field induced within the dish was calculated using the SimNIBS toolbox (26). Electrical conductivities used in the simulation were the same as in Lenz et al., (2016), and the electric dish model used for modelling was generously shared by the authors (27). The rate of change of the coil current was 143 A/µs, corresponding to an intensity of 100% MSO. The resulting normalized electric field induced within the dish from several viewpoints can be seen in Figure 1B-D.

KCl addition

KCl was added drop by drop into the dish through a needle attached to a large 200ml plastic syringe, to reach a final KCl concentration in the dish of 1 M. This caused an approximately 200-fold increase in the extracellular potassium concentration. This high concentration of KCl required to visualize calcium activity in SH-SY5Y cells has been described previously in the literature (18). The 1 M KCl was left on the cell cultures, and a multi-channel image was then taken to quantify ROI's after KCl and to visualize cell morphological response to high KCl concentration, followed by another video to quantify stable KCl response for 2 minutes following addition. Fluorescence levels remained high in this measurement block, as has been shown previously in SH-SY5Y cells (18).

Quantification of Functional Imaging

Fiji (ImageJ, version 1.52i, RRID:SCR_002285) open source software (30) was used to analyze microscope-acquired images. Each condition (iTBS, cTBS, or sham), was measured from a different cell culture dish. The data is separated into 4 measurement blocks; 1. Baseline, 2. Post-stimulation, 3. During the addition of 1M KCl, and 4. Post-KCl.

First, 20 circular ROI's (width: 25, height: 25 pixels) of responding cells were randomly selected from the 4^{th} (Post KCl) block, and used to measure pixel intensity over time for every prior block of the measurement. Since the cell culture dish was removed between the baseline and post-stimulation blocks, these ROI's were shifted between the blocks and if possible, they were adjusted manually to quantify the same cell. Analysis was performed blinded to the condition. Measured intensity values were exported to excel and Prism 5 (Graphpad Software,USA, RRID:SCR_002798.) for statistics and graphing.

Intensity values were normalized to the average of the baseline intensity at each time point. All 20 ROI's in the baseline block were averaged, to calculate a baseline average at each time point ($BaseAv(T_x)$). Each intensity value for each ROI was then divided by the average baseline intensity at that time point ($Intensity\ (T_x)/BaseAv(T_x))*100$ to give a percent change from baseline.

This mean baseline normalized intensity of 20 ROI's for each time point, with error bars as standard error of the mean can be seen in Figure 1F. ROI outliers which were 2.5 standard deviations above the mean were removed.

Statistical Analysis

Analysis was done in Prism 5 (Graphpad Software,USA, RRID:SCR_002798) combining data from all 6 independent experiments. 2-way ANOVA with Bonferroni-corrected post-hoc comparisons were used to test the effect of CONDITION (iTBS, cTBS, sham) and TIME (60 time point measurements per block) on normalized fluorescence intensity in each of the measurement blocks; 1. Baseline 2. Post-stimulation, 3. Addition of KCl, 4. Post-KCl.

Results

We found that neurons which had been stimulated with iTBS, a protocol assumed to increase excitability, showed greater increase in fluorescence response to KCl than those which had been sham stimulated. In striking contrast, neurons which had been stimulated with cTBS, a protocol assumed to decrease excitability, showed the opposite response to KCl, i.e. a decreased response. A representative plot of the mean % change from baseline fluorescence during the entire experiment (error bars are standard error of the mean) is shown in Figure 1F.

We repeated this experiment in 6 independent cell cultures per stimulation condition (iTBS, cTBS, or sham). For statistical analysis, data from all experiments were combined. The mean normalized intensity of 20 cells per experiment was calculated for each time point, for each experiment, to give an n of 6, each with 60 time points per block. On the group level in block 2 (Post Stimulation), a two way ANOVA with factors condition (iTBS, cTBS, sham) and time reveals a significant effect of condition ($F(2,660)=83.53$, $p<0.0001$), and no effect of time ($F(59,660)=0.01716$, $p=1.00$) or interaction ($F(118,660)=0.01366$, $p=1.000$). Post-hoc Bonferroni corrected planned comparisons to normalized intensity after sham stimulation (101.4±0.23%) show a minor but significant decrease ($p<0.0001$) in baseline intensity after cTBS (99.27±0.08%), but no significant increase ($p=0.1287$) after iTBS (102.0±0.23%). Thus, cTBS/iTBS had very small, though significant, effects on 'baseline' neuronal activity as captured by pre-stimulation normalized fluorescence.

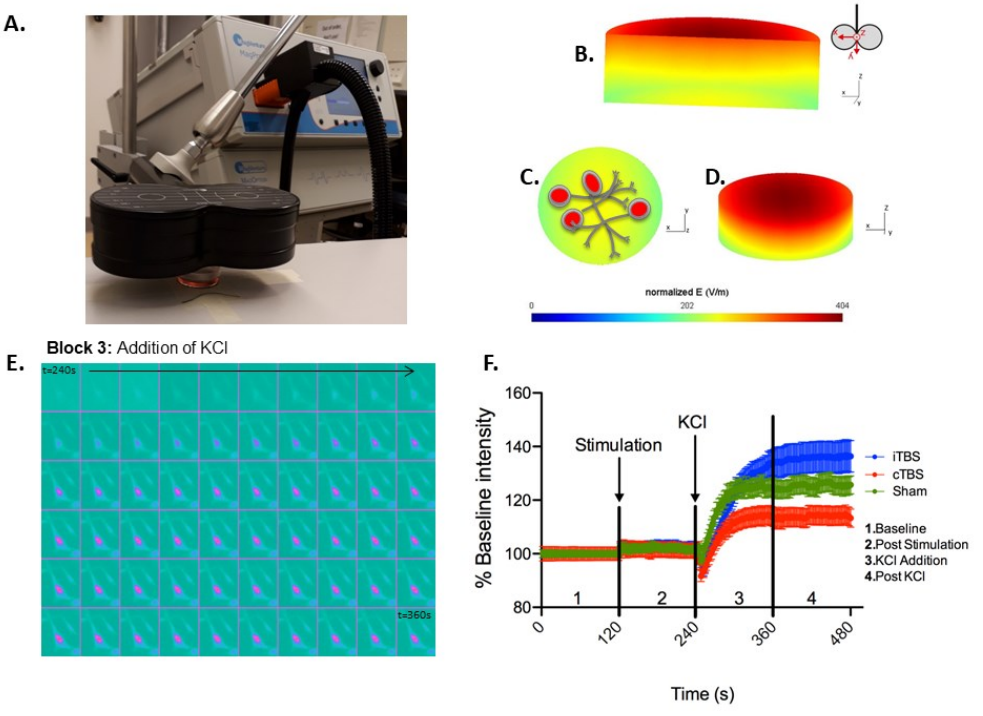

Figure 1. **A.** Position of the cell culture dish 1 cm below the center of the coil. **B-D.** Simulation of the induced electric field (V/m) within the cell culture dish. SimNIBS (26) was used to calculate the electric field induced within the cell culture dish, during TMS stimulation at 100% MSO. The simulation parameters (cell culture dish model and conductivity values) were generously shared by (27). **B.** A cross section of the cell culture dish, showing the gradient of induced electric field within the dish. The electric field is strongest (red) at the top of the dish, closest to the coil. Coil orientation shown beside. **C.** The bottom surface of the cell culture dish (furthest away from the coil, where the cells are plated) and **D.** Tilted view of the dish from the top surface. **E.** Example live cell fluorescence response to 1M KCl stimulation. Each of the 60 squares is a picture of the cell at 2 second intervals, for a period of 2 minutes as the KCl is added. Pink indicates an increase in fluorescence. **F.** Change in fluorescence intensity over time. Each line represents a different cell culture dish. Each data point is the mean percentage baseline intensity of 20 cells, error bars are standard error of the mean. Block 1 (0-120s): Baseline measurement. After this, cells were removed from the microscope and stimulated with iTBS, cTBS or sham, and placed back into the microscope. Block 2 (120-240s): Post Stimulation measurement. Block 3 (240s-360s): Addition of 1M KCl during recording. Block 4 (360-480s): Post KCl addition.

As hypothesized, TBS modulations were particularly apparent on 'evoked' neuronal activity. This functional activity was captured by the neuronal response to chemical depolarization (KCl), in block 4. We found a greater increase in fluorescence response to KCl in neurons stimulated with iTBS (125.4±0.44%) as compared to sham (122.2±0.42%). Neurons stimulated with cTBS instead showed a decreased

fluorescence response to KCl (114.8±0.65%). A two-way ANOVA of baseline-corrected intensity following KCl addition confirms a statistically significant, strong effect of condition ($F(2,840)=91.46$, $p<0.001$), but no effect of time ($F(59,840)=0.04$, $p=1.000$) and no interaction ($F(118,840)=0.035$, $p=1.000$). iTBS and cTBS comparisons to sham stimulated neurons showed statistically significant differences ($p<0.001$, Bonferroni-corrected). See Supplementary Material for 2-way ANOVA results of each block.

Discussion

Taken together, these findings suggest that rTMS was able to modulate human neuronal response to chemical depolarization with KCl; with iTBS facilitating, and cTBS inhibiting this response, compared to sham stimulation. This provides support for the hypothesized iTBS and cTBS effects on neuronal excitability on the human cell level.

These findings, which provide support for the positive and negative modulation of human neuronal calcium activity by iTBS and cTBS respectively, are reassuring. While they do not explain reported unreliability of TBS effects on other human excitability measures (e.g. MEP/TEP), they 1) validate at least on the cell level foundational assumptions about the mechanistic underpinnings of these globally implemented treatment/research protocols, and 2) demonstrate the value of *in vitro* human neuronal models for studying cellular effects of TMS (and potentially for other interventions and exposures linked to the onset or treatment of mental disorders). Therefore, this functional imaging setup of stimulated living human neurons in a dish provides a direct method for testing the immediate functional activity changes of human neurons following TMS.

Animal studies have previously provided support for the hypothesized opposing neuroplastic effects of iTBS and cTBS (31, 32), as well as an immediate effect of rTMS on intracellular calcium release (33, 34). Computational modelling of calcium-dependent plasticity effects following rTMS in a neural field model have shown that during iTBS/cTBS stimulation, the first burst of stimulation within a train causes an increase in calcium concentration, resulting in potentiation (35). In an iTBS protocol, 8 second breaks between trains of stimulation bursts allow for the maintenance of this potentiating increase in calcium concentration. In cTBS, the accumulation of calcium concentration results in an overall depressive effect (35). Previous studies have also discussed the importance of the intervals between stimulation trains in the iTBS protocols for its faciliatory effect (15). We also report an immediate depressive effect of cTBS and an immediate faciliatory effect of iTBS on calcium concentration, as measured by a response to KCl using fluorescence microscopy.

We modelled the induced electric field within the cell culture dish using SimNIBS as described in the methods. With stimulation at 100% MSO and 1 cm above the dish,

which is stronger than what is typically used for human cortex stimulation. In our cell culture dish, all neurons were affected by an electric field greater than 200V/m, while in models of the human cortex effected by stimulation, only a very superficial layer of cortex directly under the coil is affected by an electrical field of approximately 100V/m (36, 37). However, other rTMS animal and cell culture studies have reported using these stimulation parameters (24, 25), and in this first, exploratory setup we wanted to measure changes in response to the highest stimulation intensity possible.

One of the main limitations of this study, is the use of SH-SY5Y cells as a model for human neurons. SH-SY5Y cells are a human-derived neuroblastoma cell line, which can be differentiated to neural-like cells in a relatively short amount of time (28, 38, 39). They are well characterized, have been shown to develop functional synapses (18, 40), synthesize neurotransmitters (21), and are used as a model for various human neuropsychiatric and neurodegenerative disorders (41-43). However, they are not glutamatergic or GABAergic neurons, which are thought to be the primary cortical neurons affected by rTMS (44). SH-SY5Y cells have been shown to express many genes involved in axonal guidance and synaptic plasticity (18-20, 42), specifically in the BDNF-TrkB pathway, known to be involved in LTP (45). Therefore, while not the perfect human neuron model, we chose to first test our setup with SH-SY5Y cells as they are quick to culture, resulting in relatively reliable and stable phenotypes and they have been used as a model for human synaptic plasticity *in vitro*.

Additionally, we used a very high concentration of KCl to chemically induce depolarization in our differentiated SH-SY5Y cells. This has been done previously in calcium imaging of SH-SY5Y cells (18), however it is important to note that 1M KCl is a highly non-physiological concentration. This high concentration of KCl likely increases osmotic pressure, which may cause dehydration and shrinking of the cells as has been reported in SH-SY5Y cells previously (18). Therefore, an increase in calcium may also be explained by an increase in cytosolic calcium levels, due to an osmotically driven decrease in cytosolic volume. It is important to confirm our results using different methods, for example using potassium channel blockers such as 4-Aminopyridine (4AP) to depolarize the cells, or by lowering the KCl concentration.

Recently, brain-state has been suggested as a factor contributing to the variability of individual responses to rTMS protocols (17). rTMS is thought to lead to LTP/LTD-like synaptic plasticity effects, however, on a meta-plastic level, homeostatic mechanisms work to stabilize neural systems, keeping the threshold at which synaptic plasticity is induced within a physiologically relevant range (46-49). These metaplasticity mechanisms are thought to adjust this threshold for synaptic plasticity based on previous neural activity (50, 51), therefore brain-state is extremely influential in determining the excitability effects induced by rTMS. In our cell culture model, we did not test for the influence of metaplastic or homeostatic plasticity mechanisms, as we

were interested in 1.) establishing an *in vitro* setup capable of measuring excitability changes following rTMS in a human neuron model, and 2.) using this setup to quantify the *immediate* effects of iTBS and cTBS. Additionally, we have not yet proven that the functional activity effects we find are related to synaptic plasticity, which would be a necessary next step in establishing the presumed meta- or homeostatic plasticity effects of rTMS. Based on our work, future studies can be designed to specifically test the effects history of neural activity on TMS-induced plasticity changes in a human neural network model.

Looking ahead, this human neuron model has the potential to allow laboratory evaluation and optimization of rTMS protocols for individual patients, particularly when using patient-derived cells (e.g. skin-derived fibroblasts or peripheral blood mononuclear cells) and induced pluripotent stem cell (iPSC) reprogramming (52). For example, using iPSC techniques, individualized neuron models can be established by reprogramming skin cells taken from a treatment resistant patient into neurons which contain the genetic composition of that patient (52, 53). This can be taken one-step further with the cerebral organoid, as these patient specific neurons can organize into a 3D cell culture system to model whole cortical structures *in-vitro* (54). With this setup, patient-specific human neural models can be stimulated with rTMS, using calcium imaging to measure that patient's responsiveness to particular rTMS protocols. Further application and development of this model may enable assessment and exploration of planned patient-tailored rTMS treatments, thus optimising patient care.

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Supplementary Material Chapter 3

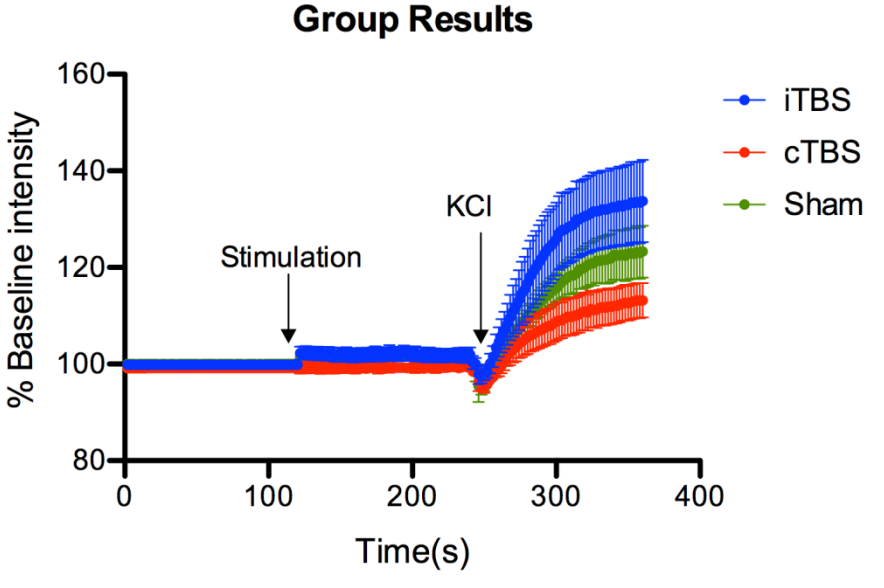

Figure S1. Group Results. Group results combining all independent experiments. Each data point is the mean of all percent baseline intensity values across the 6 experiments, error bars are standard error of the mean.

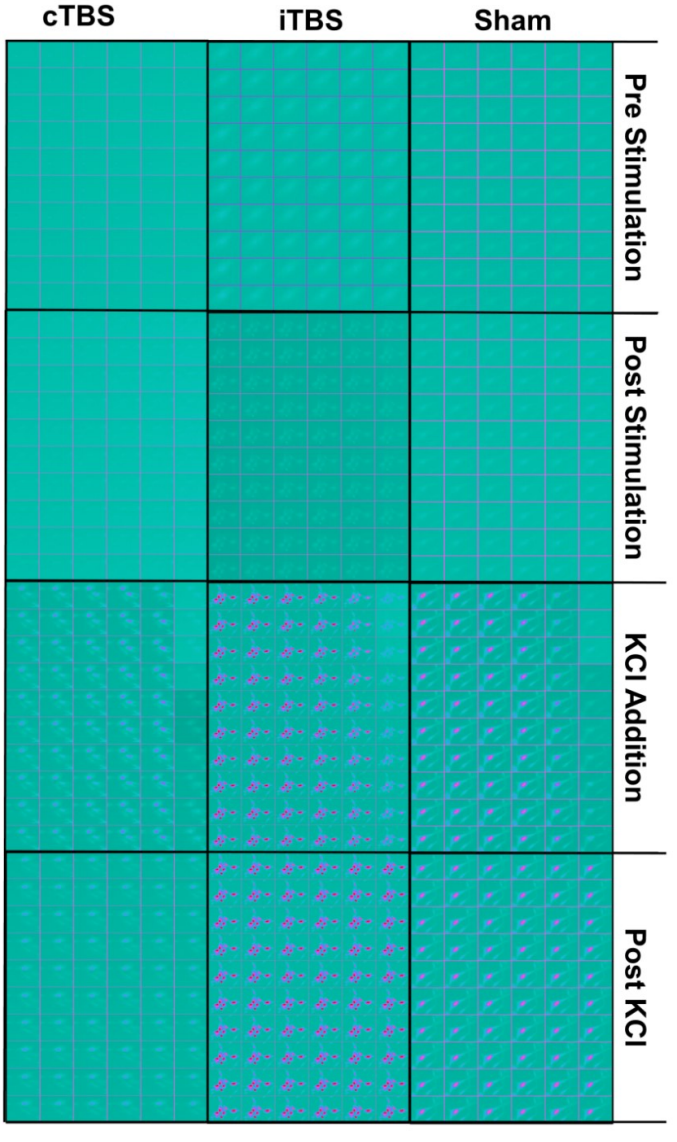

Figure S2. Change in Image Intensity over time in a single ROI. Image montages of change in fluorescence over time in an example cell selection for each stimulation condition (sham, iTBS, and cTBS). Each small purple-bordered box is one image in the time series, taken 2 seconds apart for a total of 60 images per measurement block. There are no increases in fluorescence in the pre stimulation block, as well as no large increases after stimulation. In the KCl Addition block; there is a visible increase in fluorescence (in purple) after about 20 seconds. This quickly reaches a maximum and is maintained throughout the Post KCl block. The mean intensity (a.u.) over the 60 images in the Post KCl block is stronger in the cells which had been stimulated with iTBS (3781.8±14.8), compared to sham stimulated (3449.9±9.74). The mean intensity is less in the previously-cTBS stimulated cells (3417.4±8.1).

2-way ANOVA results

1. Pre-Stimulation

Due to missing data, 6 independent experiments were included from the iTBS and sham conditions, and 5 from the cTBS condition. There was no effect of CONDITION $F(2,840)=0.260, p=0.7642$, TIME $F(59,840)=0.8191, p=0.8318$, or interaction $F(118,840)=0.9871, p=0.5233$ in the pre-stimulation block.

2. Post Stimulation

Data from 6 independent experiments for the iTBS and cTBS conditions were included, and 2 for the sham condition. There was a significant effect of CONDITION $F(2,660)=83.53, p<0.0001$, and no effect of TIME $F(59,660)=0.01716, p=1.00$ or interaction $F(118,660)=0.01366, p=1.000$. Post-Hoc Bonferroni corrected comparisons show a significant increase in percentage baseline intensity after iTBS (102.0±0.18%) compared to cTBS (99.27±0.08%) $t(660)=12.66, p<0.0001$ and a significant decrease after cTBS (99.27±0.08%)compared to sham (101.4±0.23%) $t(660)=6.922, p<0.0001$. There was no significant difference between iTBS and Sham ($p=0.1287$).

3. Addition of KCl

Data from 6 independent experiments for the iTBS and cTBS condition were included, and 3 independent experiments from the sham condition. As expected, since the measurement was during the addition of the KCl, there was a significant effect of TIME $F(59,720)=6.655, p<0.001$. There was also a significant effect of CONDITION $F(2,720)=124.2, p<0.0001$ but no interaction $(F118,720)=0.4828, p=1.000$. All Bonferroni-corrected post-hoc comparisons were significant ($p<0.0001$).

4. After KCl

Finally, in the last block, data from 6 independent experiments for the cTBS and sham conditions were included, and 5 from the iTBS condition. There was a significant effect of condition $F(2,840)=91.46$, $p<0.001$, but no effect of time $F(59,840)=0.04, p=1.000$ and no interaction $F(118,840)=0.003$, $p=1.000$. Bonferroni corrected Post Hoc Comparisons revealed all comparisons (Sham-cTBS, Sham-iTBS, iTBS-cTBS) to be significant, $p<0.001$.There was a significant increase in percentage baseline intensity in cells which had been stimulated with iTBS (125.4±0.44%,N=60) compared to sham (122.2±0.42%,N=60) $t(118)=3.925, p=0.0003$. There was also a significant increase in percentage baseline intensity in cells that had been stimulated with iTBS(125.4±0.44%,N=60) compared to cTBS (114.8±0.65%,N=60) $t(118)=12.99, p<0.0001$, and a decrease in cells which had been stimulated with cTBS (114.8±0.65%,N=60) compared to sham (122.2±0.42%,N=60) $t(118)=9.506$, $p<0.0001$.

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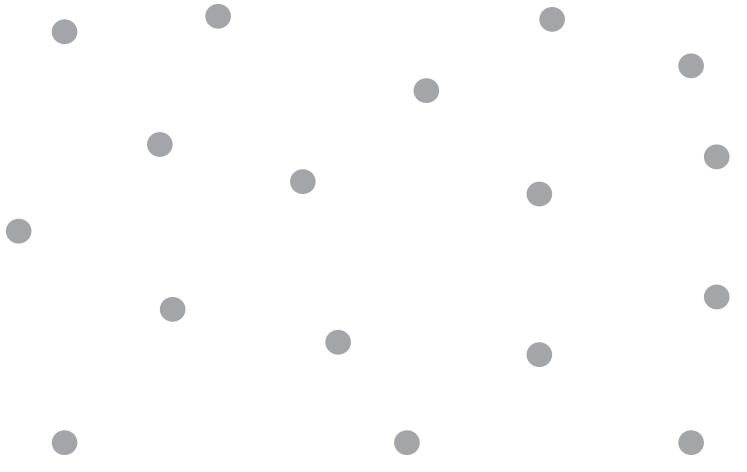
Key Resources Table

REAGENT or RRESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Fluo-4, AM, cell permeant	Invitrogen	Cat# F14201
Propidium Iodide stain	Molecular Probes	Cat# P3566
Bisbenzimide Hoechst 33342	Sigma-Aldrich	Cat# B2261
Pluronic™ F-127	ThermoFisher	Cat# P6867
Experimental Models: Cell Lines		
Homo sapiens; SH-SY5Y	ATCC	Cat# CRL-2266 RRID:CVCL_0019
Software and Algorithms		
Fiji (ImageJ)	Schindelin et al., 2012[1]	http://fiji.sc RRID:SCR_002285
Prism version 5.0	GraphPad Prism	www.graphpad.com RRID:SCR_002798
Micro-Manager software	Edelstein et al., 2014[2]	http://micro-manager.org/ RRID:SCR_016865
SimNIBS toolbox	Thielscher, Antunes, & Saturnino, 2015[3]	https://simnibs.github.io/simnibs/build/html/installation/simnibs_installer.html
JASP(Version 0.10.1)	JASP Team (2019)	https://jasp-stats.org RRID:SCR_015823

Chapter 4

Transcranial Magnetic Stimulation (TMS)-induced plasticity mechanisms: TMS-related gene expression and morphology changes in a human neuron-like cell model



Based on: Thomson A.C., Kenis, G., Tielens, S., de Graaf T.A., Schuhmann T., Sack A.T., Rutten, B.P.F., Sack A.T. (2020). Transcranial Magnetic Stimulation (TMS)-induced plasticity mechanisms: TMS-related gene expression and morphology changes in a neuron-like cell model. *Front. Mol. Neurosci.* 13:528396. doi:10.3389/fnmol.2020.528396.

Abstract

Transcranial magnetic stimulation (TMS) is a form of non-invasive brain stimulation, used to alter cortical excitability both in research and clinical applications. The *intermittent* and *continuous* theta burst stimulation (iTBS and cTBS) protocols have been shown to induce opposite after-effects on human cortex excitability. Animal studies have implicated synaptic plasticity mechanisms long-term potentiation (LTP, for iTBS) and depression (LTD, for cTBS). However, the neural basis of TMS effects has not yet been studied in human neuronal cells, in particular at the level of gene expression and synaptogenesis. To investigate responses to TBS in living *human* neurons, we differentiated human SH-SY5Y cells towards a mature neural phenotype, and stimulated them with iTBS, cTBS, or sham (placebo) TBS. Changes in a) mRNA expression of a set of target genes (previously associated with synaptic plasticity), and b) morphological parameters of neurite outgrowth following TBS were quantified. We found no general effects of stimulation condition or time on gene expression, though we did observe a significantly enhanced expression of plasticity genes *NTRK2* and *MAPK9* 24 hours after iTBS as compared to sham TBS. This specific effect provides unique support for the widely assumed plasticity mechanisms underlying iTBS effects on human cortex excitability. In addition to this protocol-specific increase in plasticity gene expression 24 hours after iTBS stimulation, we establish the feasibility of stimulating living human neuron with TBS, and the importance of moving to more complex human *in vitro* models to understand the underlying plasticity mechanisms of TBS stimulation.

Keywords: Theta Burst Stimulation (TBS), Brain Stimulation, Cortical Excitability, Long Term Potentiation (LTP), Gene Expression, SH-SY5Y

Introduction

Transcranial magnetic stimulation (TMS) is a widely used neuromodulation technique, where electromagnetic pulses can non-invasively stimulate cortical structures (1, 2). Multiple pulses administered in a certain frequency (repetitive TMS: rTMS), can have effects on cortical excitability lasting beyond the period of stimulation (3, 4). In humans, such effects are often revealed with physiological outcome measures, such as motor-evoked potentials (MEPs) (5). For example, the commonly used theta burst stimulation (TBS) protocols *intermittent* and *continuous* TBS (iTBS and cTBS) have been shown to increase or decrease MEPs for up to 1 hour following stimulation, respectively (4). Still, large inter and intra subject variability have been associated with the use of MEP's as an outcome measure (6). Several reports on the difficulty of replicating the assumed iTBS/cTBS effects have cast doubt on the efficacy of these protocols (7-10). A method to reliably verify rTMS effects, for example in an *in vivo* model, is urgently needed.

A widespread assumption is that such after-effects are attributable to neuronal plasticity mechanisms, such as long-term potentiation (LTP) and long-term depression (LTD) (11). Indeed, administering an N-methyl-D-aspartate receptor (NMDAR) antagonist to participants prior to iTBS/cTBS stimulation has been shown to completely abolish the after-effects on MEP amplitude, relating NMDAR-dependent LTP/LTD to TBS effects in humans (12).

LTP is a well-studied form of synaptic plasticity, often induced *ex vivo* through high frequency stimulation directly to individual neurons or groups of neurons (13). It can be divided into two phases, early-LTP, which is protein-synthesis independent, and occurs immediately after stimulation, and late-LTP, which requires protein synthesis and can lead to structural and functional changes lasting at least 24 hours *in vitro* (14-17). The phenomenon of late LTP depends heavily on brain-derived neurotrophic factor (BDNF) binding to its high affinity receptor, tyrosine kinase receptor B (TrkB), and initiating a series of signaling proteins leading to changes in expression of plasticity related genes (18-21).

In cultured mouse neurons, rTMS has been shown to activate this BDNF-TrkB signaling pathway (22-24), as well as to induce an immediate release of calcium from intracellular stores (25, 26), which is important in the induction of synaptic plasticity (27). In rats, high and low frequency rTMS stimulation showed differential activation of the immediate early genes *C-FOS* (a general marker for excitatory cell activity) and *EGR1* (a presumed marker of LTP or LTD induction) (28). In addition, iTBS and cTBS showed dose-dependent and protocol specific effects on the synthesis of these two proteins (29). iTBS and cTBS also differentially change the synthesis of calcium binding proteins in rats. The latter is related to modulation of inhibitory interneurons

(30-33), which has a functional impact on neuronal electrical activity, with iTBS, but not cTBS, enhancing spontaneous neuronal firing and EEG gamma band power (30).

In sum, several lines of cellular evidence from animal studies implicate different aspects of late LTP mechanisms in the after-effects of rTMS. But such plasticity effects of rTMS at the cellular and molecular level have mainly been examined in rodent-based models. Given the rapid development and increasingly widespread and accepted use of rTMS, particularly TBS, for both experimental and clinical applications in human volunteers and patients, it seems crucial to study and understand the cellular effects of TBS in human neurons. *In vitro* studies with human neurons could validate the animal results, and contribute to our understanding of the mechanisms of action underlying different TBS protocols that are already, and increasingly, (clinically) applied worldwide.

To our knowledge, only two previous studies have used human SH-SY5Y neuroblastoma cells to measure responses to rTMS *in vitro*, and both used classical high and low frequency protocols. One study reported protocol-specific effects of high (9Hz) and low (3Hz) frequency rTMS stimulation on catecholamine levels and neurotransmitter metabolism (34), and the other showed increased intracellular cAMP and CREB activation with high (5Hz) frequency rTMS (35). These studies provide the first evidence of the feasibility of using SH-SY5Y cells in this type of study. However, to date no study has used SH-SY5Y cells to investigate neural responses to TBS protocols.

Here, we developed an *in vitro* human neuron model to assess protocol-specific effects of iTBS/cTBS on plasticity markers of gene expression and neurite outgrowth. We chose to investigate changes in the BDNF-TrkB signaling cascade, given its importance in plasticity mechanisms, and because previous animal studies have shown an rTMS-induced effect on protein expression in this pathway [16-18]. We focused on hypothesis-driven gene expression targets in this pathway, to identify immediate effects to help tailor future protein or genome-wide screening analysis. We also wanted to quantify any structural changes to neurite morphology with commonly used cytoskeletal markers βIII-Tubulin and MAP2, which may indicate neuroplastic effects. We differentiated SH-SY5Y cells into a mature neuron-like phenotype, applied different TBS protocols, and collected cells immediately, 3 hours, 6 hours and 24 hours after stimulation. While in humans TBS effects have been shown to be strongest up to 30 minutes after stimulation (4), we chose these time points to capture the plasticity-dependent processes requiring longer periods of time (36-38).

We report a protocol-specific effect on expression of genes in the BDNF-TrkB pathway, with an increase in expression of *NTRK2* and *MAPK9* 24 hours after iTBS stimulation, but no change in cell count, neurite length, neurite branching, or levels

of neurite proteins. In a separate report, we showed that these TBS procedures, using the same *in vitro* model, did affect excitability as hypothesized. This suggests that the results reported here did originate from functionally active cell cultures, that responded to TBS as hypothesized (39). The current results, positive as well as negative, thus demonstrate the feasibility and value of *in vitro* human neuron studies to unravel plasticity mechanisms induced by TMS.

Methods

Cell culture

SH-SY5Y cells were obtained from ATCC® (Cat #CRL2266™, RRID:CVCL_0019) and were maintained and expanded according to ATCC® recommendations. For experiments, cells were not used above passage 26. Cells were grown in DMEM/Nut Mix F12 with Glut-L (Gibco™, Thermo Scientific) supplemented with 10% heat inactivated fetal bovine serum (FBS, MERCK), 1% penicillin-streptomycin (P/S) and 1% L-Glutamate at 37°C and 5% $C0_2$. Experiments were conducted on differentiated cells plated in round 35mm dishes at approximately $2.4x10^4$ cells per well. Differentiation was induced over a period of 13 days; FBS supplementation was decreased to 3% 3 days prior to the addition of 10 µM retinoic acid for 10 days (RA; Sigma-Aldrich, R2625). Medium was replaced every 2 days.

Magnetic Stimulation

Cells were placed 1 cm below the center of a Cool-B65 figure of 8 coil (Magventure, Denmark) and stimulated at 100% Maximum Stimulator Output (MSO) with a MagPro X100 with MagOption stimulator, realized output 143 A/µS (Magventure, Denmark). The setup is illustrated in Figure 1A. Each stimulation consisted of the Huang et al. (2005) published protocol of 50Hz triplets repeated in a 5 Hz rhythm. cTBS was a continuous train, while iTBS was a 2 second train of pulses, with an inter-train interval of 8 seconds, both for 600 pulses (4).

Cells stimulated with cTBS remained under the coil for an additional 150 seconds, and cells in the sham condition were placed under the coil for 190 seconds, to ensure that cells in all TBS conditions (cTBS, iTBS, sham) were out of the incubator for the same amount of time. The electrical field induced in the dish, with the stimulation conditions described above (100% MSO, dish placed 1cm below the coil), was simulated using the SimNIBS toolbox (40). The cell culture dish mesh was generously shared by the authors of (41). The distribution of the electric field (V/m) within the dish from several viewpoints can be seen in Figure 1B-D. The stimulation protocol is shown in Figure 1E.

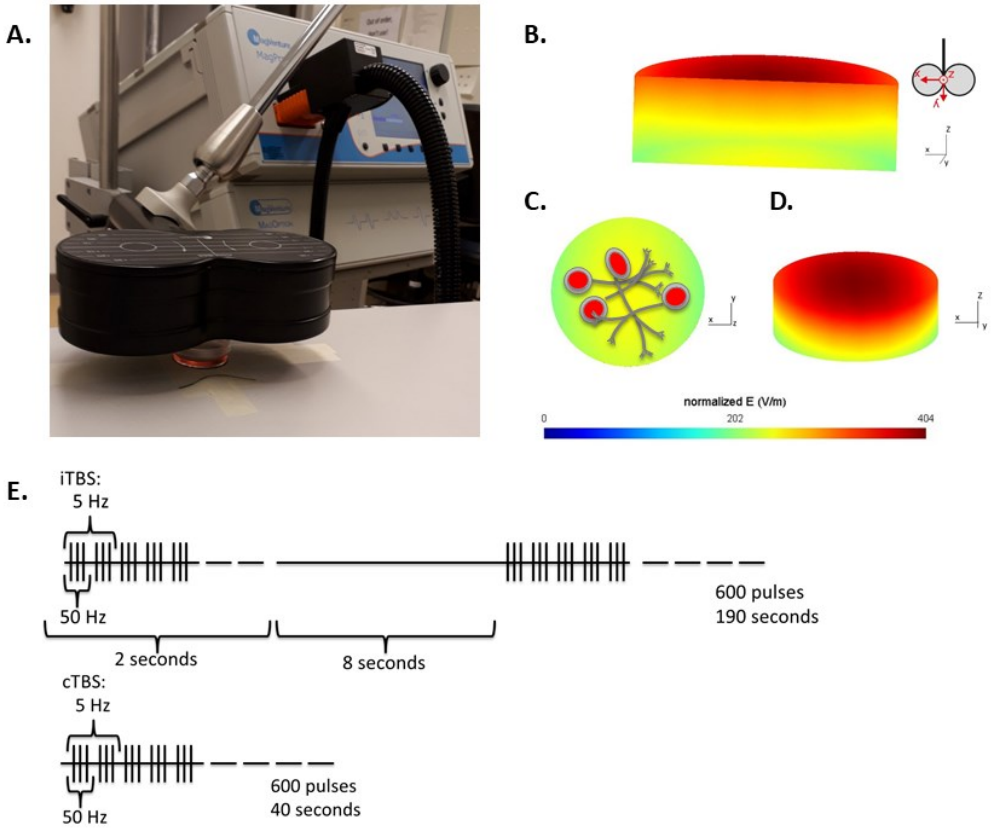


Figure 1. Experiment Setup**. A.** Position of the cell culture dish 1cm below the center of the coil.). **B-D.** Simulation of the induced electric field (V/m) within the cell culture dish. SimNIBS (40) was used to calculate the electric field induced within the cell culture dish, during TMS stimulation at 100% MSO. The simulation parameters (cell culture dish model and conductivity values) were generously shared by (41). **B.** A cross section of the cell culture dish, showing the gradient of induced electric field within the dish. The electric field is strongest at the top of the dish, closest to the coil. Coil orientation shown beside. **C.** Shows the bottom surface of the cell culture dish (furthest away from the cells are plated) and **D.** Is a tilted view of the dish from the top surface. **E.** Stimulation protocols used for stimulation, iTBS has been shown to increase cortical excitability (measured in motor evoked potentials), and cTBS to decrease it for up to 1 hour following stimulation (4).

qRT-PCR

Cells were collected immediately, 6 hours or 24 hours after stimulation. In humans, the maximal TBS effects are expected in the first 30 minutes after stimulation (4). However, we chose to measure at later time points because we were specifically interested in plasticity-dependent gene expression, which require hours or even days (36) While the rapid expression of immediate early genes could be effected by TBS

within the first 30 minutes (42, 43), most of the genes of interest in our study are expressed at later time points following plasticity-inducing protocols (37).RNA was extracted with TRIzol (Invitrogen,15596026) according to the manufacturer's instructions. Nanodrop was used to quantify the amount of RNA in each sample, and cDNA was synthesized using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, K1632). RNA was stored at -80°C, cDNA at -20°C. Eight biological replicates were collected per stimulation condition per time point, derived from at least two undifferentiated cell batches for differentiation. Due to quality of extracted RNA, some samples had to be discarded, leaving between four and eight biological replicates per condition. Each biological replicate was run in technical duplicates for qRT-PCR. A complete list of biological replicates and differentiation batches for each sample can be found in the Supplementary Material (Table S1).

Primers for qPCR were designed using the NCBI gene reference database and Primer-BLAST (National Library of Medicine). The following genes were analyzed (see Supplementary Material Table S2 for sequences): *NTRK2, BCL2, MAPK9*, *TUBB3*, *EGR1*, *CREB1*, and *GAPDH*, *PPiB*, and *TBP* were used as housekeeping genes (HKGs). Primers, 600 nM, were mixed with Fast Start Universal Sybr green ROX (Roche,491385001). Samples were run in 384 well qPCR plates (Roche,4TI-0382) using the LightCycler® 480 Real-Time PCR system (Roche LifeScience). qPCR program details are described in Supplementary Material (Table S3).

Microscopy

Cells were grown on 12mm round glass cover slips (VWR,631-1577), coated with 1µg/mL Laminin (Sigma,L2020) and 100 µg/mL Poly-L-Ornithine (Sigma,P4957), and cultured as described above.

Fluorescence microscopy was used to visualize morphological changes 3 hours, 6 hours, and 24 hours after stimulation. Again, this because these structural changes require longer time windows to visualize effects. Axonal reorganization has been shown to require several hours (2-6) to begin to show signs of microtubule movement (38). Cells were washed in PBS and fixed for 10 minutes at room temperature in 4% paraformaldehyde. Fixed and PBS-washed cells were blocked in PBS-T (PBS + 0.1% Tween-20) and 10% donkey serum followed by primary antibody incubation. Antibodies for marking neurite outgrowth (βIII-Tubulin (Cell Signaling, Cat #5568S, RRID:AB_10694505)) and axons (MAP2 (Sigma, Cat #M2320, AB_609904)) were used. Cells were washed in alternating PBS-T and PBS, and incubated with secondary antibodies donkey-anti-rabbit Alexa 488 (Invitrogen, Cat #A-21206 RRID:AB_141708), donkey-anti-mouse Alexa 594 (Invitrogen, Cat #A-21203, RRID:AB_141633), and with DAPI (CarlRoth, Cat #6843.3). The glass cover slips were then mounted on glass microscope slides and imaged with an Olympus BX51WI microscope and Disc Spinning Unit. Pictures were taken using the 20X objective lens.

Further details on primary and secondary antibodies and microscope settings are listed in the Supplementary Material (Tables S4 and S5). This experiment was repeated twice, with 4 images of each replicate analyzed. In total, 8 images per stimulation condition time point were included; with each image containing on average 163±73 cells.

Analysis

Gene expression

A standard curve was used to calculate relative concentrations of gene expression per gene. An average of technical duplicates was made, and normalized to the average of the 3 housekeeping genes (GAPDH, PPiB, TBP). Data were analyzed with LightCycler 480 software version 1.5.1.62 (Roche Life Sciences) and Microsoft Excel, and graphs were made in Prism 5 (GraphPad Software,USA, RRID: SCR_002798).

Microscopy

Images were processed and analyzed with Fiji (ImageJ version 1.52i, RRID:SCR_002285) (44). Cells in each picture were counted with the analyze particle tool, using the DAPI stain for the cell nuclei. Fluorescence intensity (immunoreactivity of βIII-Tubulin) was quantified by measuring the total 488 channel intensity in each image. This was then divided by the total fluorescence intensity in the 350 channel, to give a corrected fluorescence for the number of cells in the image. Neurite length and branching were quantified by tracing outgrowths in the 488 (βIII-Tubulin) channel. Neurite length was measured with the segmented line tool, at 20x magnification and quantifying 20 cells per image. From each cell only the primary neurite length was counted. The NeuronJ plugin (45) was used to quantify neurite branching. For each image, all neurites were semi-automatically traced, and manually labeled as either primary, secondary, or tertiary extensions. The number of branches (secondary or tertiary extensions) were divided by the total number of neurons (counted with DAPI), to give the number of branches per neuron in each image. Graphs were made with Prism 5 (GraphPad Software, USA, RRID:SCR_002798).

Statistical Analysis

Statistical analysis was done using IBM SPSS 24 (SPSS for Windows version 24.0. Armonk, NY: IBM Corp). SH-SY5Y differentiation was verified through independent samples t-tests comparing undifferentiated and differentiated cells. Biological replicates were used for statistical analysis. For gene expression analysis, a 2-way ANOVA was used to first test housekeeping genes for an effect of stimulation condition or time on expression. None of the genes showed any significant effects (complete results in Supplementary Material). Since there was no significant effect of stimulation condition on expression of any genes at the immediate time point

(complete results in Supplementary Material) these levels were averaged across stimulation conditions, and used to calculate % immediate expression levels for the 6 and 24-hour time points.

Due to the small number of biological replicates and unequal variances across samples, non-parametric Kruskal Wallis testes were done for condition and time separately. However, using these non-parametric statistical tests did not allow for testing of interaction effects. We had expected gene expression effects to be strongest at one of the time points, therefore we performed hypothesis-driven analyses for the 6 hour and 24-hour time points separately, using the Kruskal-Wallis test for a significant difference in gene expression between stimulation conditions. Reported gene expression and microscopy results are presented as mean ± standard error of the mean. Bonferroni correction was used for post-hoc comparisons. Figures show bar graphs of the mean, error bars are standard error of the mean.

Results

SH-SY5Y differentiation

Differentiation status of SH-SY5Y cells was verified through visual inspection of increased neurite length, and confirmed by a significant increase in TrkB expression (*NTRK2)* at day 10 of differentiation ($t(7)=8.657$, $p<0.0001$), as reported previously (46, 47) (see Figure 2). Expression of all genes of interest was verified at day 10 of differentiation (Figure 2 G, H.). Complete results of t-tests are reported in Supplementary Material. Neurite outgrowth increased from day 0 to day 10 of differentiation (37.9 ± 2.8 µm and 112.3 ± 11.8 µm, respectively; $t(26)=6.163$, $p<0.0001$) (see Figure 2I).

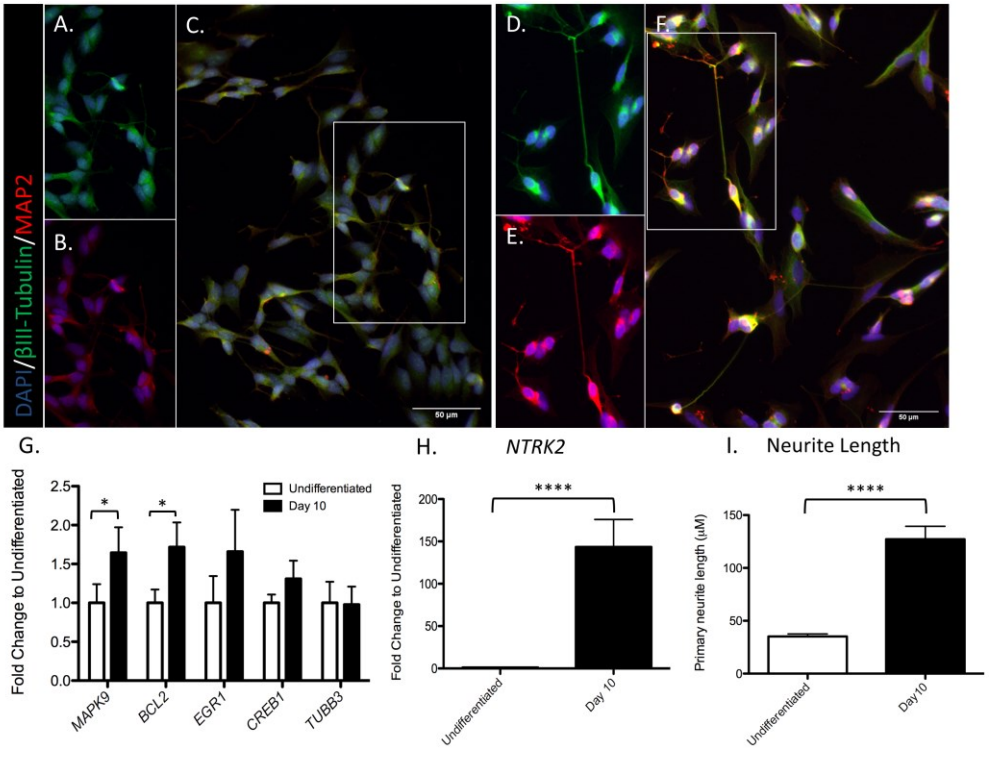


Figure 2. SH-SY5Y cell differentiation. Cells were marked for nucleus DAPI (blue), MAP2 (red) and βIII-Tubulin (green) in an undifferentiated state (A-C) or after 10 days of differentiation (D-F). A selection of neurons in the image were chosen to split by channel (βIII-Tubulin or MAP2), identified by the white box in C. and F. **A.** Selection of undifferentiated cells, βIII-Tubulin. **B.** Selection of undifferentiated cells, MAP2. **C.** Full image undifferentiated cells, merge of βIII-Tubulin and MAP2. **D.** Selection of 10 days differentiated cells, βIII-Tubulin. **E.** Selection of 10 days differentiated cells, MAP2. **F.** Full image of 10 days differentiated cells; merge of βIII-Tubulin and MAP2.**G.** RT-qPCR analysis was used to assess the expression of the indicated genes in differentiated and undifferentiated cells. **H.** Significant increase in *NTRK2* expression at day 10 of differentiation. **I.** Significant increase in primary neurite outgrowth at day 10 of differentiation. Values represent mean ± SEM (**p*<0.05, *****p* <0.001, Student's t-test).

Effects of stimulation condition on gene expression

We were interested in gene expression changes in the BDNF-TrkB signaling cascade, specifically in downstream targets related to plasticity. Therefore, we focused on the following genes involved in this pathway: Mitogen-Activated Protein Kinase 9 (*MAPK9*, GeneID: 5601), Neurotrophic Regulator Tyrosine Kinase 2 (*NTRK2,* GeneID: 4915), B-cell lymphoma 2 (*BCL2*, Gene ID: 596), Tubulin Beta Class III (*TUBB3,* Gene ID: 10381), cAMP Responsive Element Binding Protein 1 (*CREB1,* Gene ID: 1385). We also included Early Growth Response 1 (*EGR1*, Gene ID: 1958), which is considered an immediate early gene.

We found no significant effect of stimulation condition or time on several of the genes tested; *BCL2* (Condition: $H(2)=0.125$, $p=0.940$, Time: $H(1)=2.626$, $p=0.105$); *TUBB3* (Condition $H(2)=1.060$, $p=0.589$, Time $H(1)=1.298$, $p=0.255$); and *CREB1* (Condition $H(2)=0.651$, $p=0.722$, Time $H(1)=0.006$, $p=0.936$).

We found no significant effect of condition on *EGR1* expression ($H(2)=1.926$, $p=0.382$), but an effect of time ($H(1)=9.195$, $p=0.002$), with a decrease in the expression (% immediate) at 24 hours (54.5±5.6%) compared to 6 hours (77.4±5.0%) (Figure 3A). Similarly for *MAPK9* we find no significant effect of condition ($H(2)=1.043$, $p=0.594$) but an effect of time ($H(1)=4.152$, $p=0.042$), as well as for *NTRK2* expression (Condition $H(2)= 0.905$, $p=0.636$, Time $H(1)=4.022$, $p=0.045$).

***BCL2* expression:** There was no effect of stimulation condition at 6 hours ($H(2)=1.024$, $p=0.985$). However, at 24 hours, we observed a borderline statistically significant effect of stimulation condition on *BCL2* expression ($H(2)=5.981$, $p=0.050$); i.e. we observed an increase in *BCL2* expression in cells which had been stimulated with iTBS (119.8±18.7%) compared to cTBS (95.0±10.1%) and sham (77.2±3.2%), however none of the post-hoc comparisons were significant ($p>0.05$) (Figure 3B).

***NTRK2* expression:** We find no effect of stimulation condition at 6 hours ($H(2)=2.12$, $p=0.346$). At 24 hours we find a statistically significant effect of stimulation condition on *NTRK2* expression ($H(2)=8.010$, $p=0.018$). We observed an increase in expression in cells which have been iTBS stimulated compared to sham stimulated cells (139.7±30.85% and 83.8±5.2%, respectively; $p=0.036$) (Figure 3C).

***MAPK9* expression:** Again, we find no effect of stimulation condition at 6 hours ($H(2)=0.030$, $p=0.985$). When analyzing the 24 hour time point separately, we observed a statistically significant effect of stimulation condition ($H(2)=8.640$, $p=0.013$). *MAPK9* expression levels were significantly higher in iTBS stimulated cells, compared to sham stimulated cells (127.9±13.4% and 89.0±5.3%, respectively; $p=0.017$) (Figure 3D).

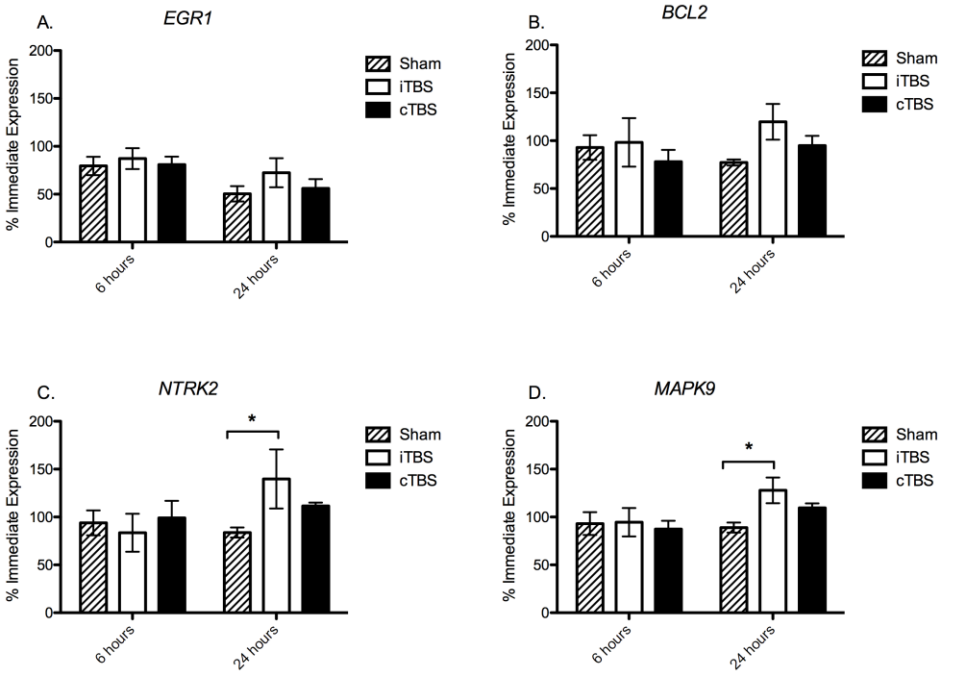


Figure 3. Gene Expression results 6- and 24-hours following stimulation. Values are normalized by the average of 3 Housekeeping Genes (*GAPDH,TBP,PPiB*) and divided by the average immediate expression. Bars shown are % immediate time point expression. **A.** Expression of *EGR1* **B.** Expression of *BCL2* **C.** Expression of *NTRK2* **D.** Expression of *MAPK9*.Significant Significant bonferroni-corrected post hoc tests are indicated with a * (p<0.05), n=4-8, see supplementary material for exact replicate numbers per condition.

Effect of Stimulation Condition on Neuron Morphology

Cell count: We found no significant effect of condition (H(2)=0.815, p=0.665) or time (H(2)=3.37, p=0.185) on cell count (Figure 4A).

βIII-Tubulin immunoreactivity: There was no significant effect of stimulation condition on total fluorescence intensity of βIII-Tubulin (H(2)=1.19, p=0.55). There was however a significant effect of time (H(2)=6.61, p=0.037), with an increase at 24 hours (1.23±0.05) compared to 6 hours (1.17±0.05) and 3 hours (1.09±0.007) (Figure 4B).

Neurite outgrowth: There was no significant effect of stimulation condition on primary neurite length (H(2)=0.336, p=0.85). There is a significant effect of time (H(2)=22.320, p<0.001). Neurites were longer at 3 hours (66.56±2.35µm) compared to 6 hours (49.82±2.00µm) and 24 hours (47.92±2.48µm) (Figure 4C).

Neurite Branching: There was no significant effect of stimulation condition on neurite branching ($H(2)=1.580$, p=0.45). There is a significant effect of time ($H(2)=13.901$, p=0.001). There were more branches per neuron at 3 hours (0.031±0.0044) compared to at 6 hours (0.018±0.0023), and 24 hours (0.012±0.0026) (Figure 4D).

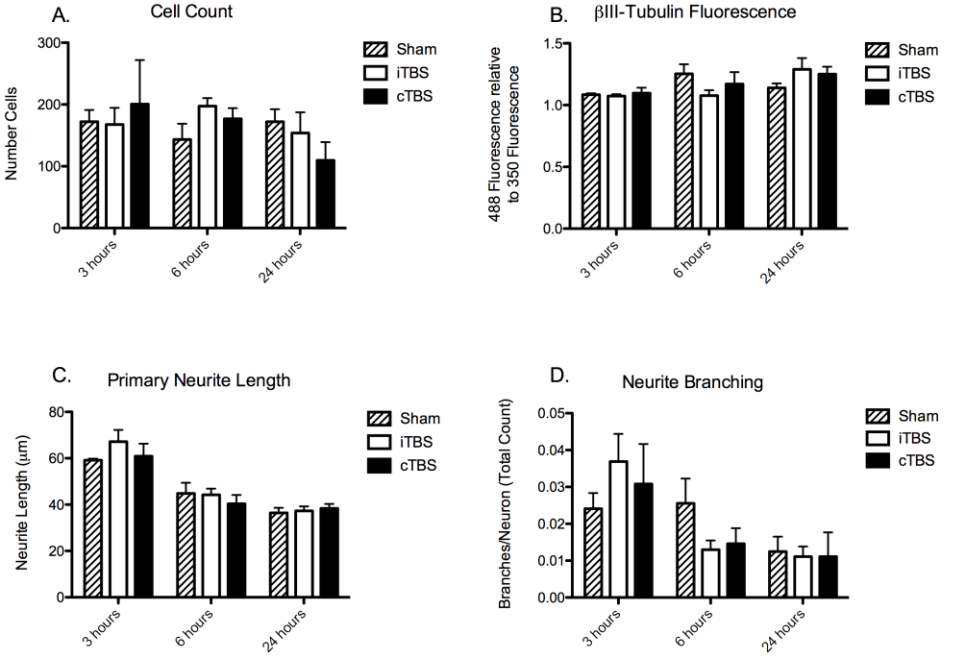


Figure 4. Morphological outcome parameters over time for each stimulation condition. **A.** Cell count **B.** Total fluorescence in the βIII-Tubulin channel (488), normalized to the DAPI channel (350). **C.** Primary neurite length **D.** Neurite branching. N=8 images per stimulation condition.

Discussion

To date, the molecular and cellular mechanisms underlying TMS have been mainly studied in living rodents or animal brain slices (11, 48). Here, we set out to investigate TMS induced plasticity mechanisms in an *in vitro* human neuron-like model, through stimulating differentiated SH-SY5Y neuroblastoma cells with either iTBS, cTBS, or sham stimulation. Previous animal and human TBS studies have suggested that iTBS/cTBS may rely on activity-dependent plasticity mechanisms of LTP/LTD (12, 29, 49-51). To investigate these changes in living human neurons, we focused on molecular (in particular genes related to BDNF/TrkB signaling) and morphological markers of plasticity. The induced electric field within the cell culture dish was

modelled using the SimNIBS toolbox (40), not with the aim of comparing stimulation strength to that usually achieved with TMS in a human brain, but only to confirm that stimulation was capable of depolarizing neurons. Our high-intensity stimulation parameters were based on other rTMS animal and cell culture studies (35, 52), with the primary goal of ensuring sufficient depolarization to induce excitations in our cells. Indeed, this was successful, as in another report (39) we could show that these procedures/parameters affected excitability in identically treated cell cultures as hypothesized: demonstrating after-effects in opposite directions for iTBS and cTBS as previously observed in animal and human *in vivo* studies.

We found that, compared to sham stimulation, iTBS increased the expression of *NTRK2* and *MAPK9* after 24 hours. *MAPK9*, also known as *JNK2*, has been shown in mice to be important in hippocampal synaptic plasticity (53, 54). MAPK9 knockout mice had impaired late but not early LTP, suggesting that *MAPK9* may be instrumental in the switch from early to late LTP (51). This switch is important, as late LTP is responsible for plasticity effects lasting at least 24 hours, requiring protein synthesis and structural changes (15, 16, 55). In humans, several studies have shown that repeating iTBS (9) or cTBS (56) at spaced intervals consolidates LTP/LTD-like effects, providing evidence for late-LTP or late-LTD mechanisms only after repeated iTBS/cTBS sessions. Our results showing an increase in *MAPK9* mRNA expression at 24 hours following iTBS could indicate this critical shift from early to late LTP mechanisms. Measuring *MAPK9* mRNA expression after repeated iTBS of SH-SY5Y cells could further support the evidence from human studies, that repeating iTBS sessions results in late-LTP mechanisms in humans.

Similarly, *NTRK2*, the gene that codes for the high affinity BDNF-receptor TrkB, is thought to be a critical regulator of hippocampal LTP (36). Mice lacking TrkB receptors showed reduced TBS-induced LTP, indicating the importance of this receptor in regulating synaptic plasticity (57). An increase in *NTRK2* mRNA expression indicates that the BDNF-TrkB signaling cascade may be upregulated 24 hours after iTBS. This supports the assumption that iTBS promotes LTP-like plasticity, specifically through up-regulation of the BDNF-TrkB pathway.

We also found a slight effect of condition at 24 hours following stimulation in expression of *BCL2*. This indicates an effect of TBS on the expression of this gene, but since no post-hoc comparisons were significant, we cannot conclude that this expression is protocol-specific. This expression of *BCL2* is similar to the expression of *MAPK9* and *NTRK2*, with iTBS stimulated cells showing increased expression compared to sham stimulated cells. *BCL2* is an integral outer mitochondrial membrane protein, and an important regulator of apoptosis (58). Its expression is strongly induced by BDNF-TrkB signaling, and has been shown to affect plasticity mechanisms (59). In other words, the increase in *BCL2* expression that we report may

be related to enhanced plasticity and neuroprotective mechanisms 24 hours after TBS. More specific apoptosis assays would be required to confirm this. Altogether, our gene expression findings support the hypothesis that the iTBS protocol enhances plasticity mechanisms induced by BDNF-TrkB signaling, confirming evidence from animal experiments. We also found a time effect for the expression of *EGR1*, an important neuronal immediate early gene, functioning as a transcriptional regulator for genes involved in differentiation and neuroplastic changes (60). This increase in *EGR1* expression is critical for the induction of LTP, as an initial increase in *EGR1* expression within 10 minutes to 2 hours after stimulation is required for protein-synthesis dependent late-LTP mechanisms (42, 43, 61). In other words, an increase in EGR1 expression immediately after stimulation supports TBS-induced plasticity mechanisms.

To examine possible effects of TBS on neuronal morphology, we used immunocytochemistry to visualize neurite outgrowths (βIII-Tubulin) and dendrites (MAP2). These are widely used as mature, neural cytoskeletal markers in studies of SH-SY5Y cells (46, 62-66). βIII-Tubulin is an important protein of the microtubule cytoskeleton, expressed primarily in neurons and is critical for axonal guidance and maintenance in mammals (67). We found, on average 30% (±10%) of neurons expressed βIII-Tubulin, 11.3% (±4.6%) MAP2 and 9.8% (±3.5%) expressed both markers. Representational images of neuron morphology after each stimulation condition, and each time point, can be seen in Supplementary Material (Supplementary Figure 2). Using qPCR as described above, we found no change in the mRNA expression of *TUBB3*, the gene coding for βIII-Tubulin protein, which aligns with our βIII-Tubulin immunoreactivity findings. We did observe a small decrease of axonal length and branches per neuron over time, but without an effect of stimulation condition. This might be related to manipulation of the cell cultures during the stimulation paradigm. Whether TBS induces structural plasticity changes should be further investigated over longer time periods.

In contrast to previous animal studies showing protocol specific changes in plasticity markers following TBS (29, 33, 68), we did not see any effects of cTBS on gene expression or neuron morphology. Additionally, the effects on gene expression that we did see were subtle, and in just two plasticity genes. Importantly, however, the protocol-specific effects reported in these animal studies were found in different cortical areas, therefore it is difficult to compare these results to cell culture which contain a single functional cell type in a single spatial organization. Animal models or slice cultures also contain a functionally relevant organization of different neuron types, such as a mix of inhibitory interneurons and excitatory pyramidal neurons. This neuronal organization might be important, as these studies suggest that iTBS/cTBS is related to differential effects on cortical inhibition (affecting the inhibitory

interneurons) (29, 30, 49). Indeed, as computational modelling has shown, the TMS-induced electric field depends critically on the complex microscopic and macroscopic anatomy of the human cortex (69-71). In light of this requirement for complex neuronal organization, our null results become more important, as they might begin to inform us on the minimal level of neuronal organization complexity required for TBS effects on expression of certain genes.

In addition, animal studies often use different stimulation parameters, for example repeating the established Huang 2005 TBS protocol up to five times (29, 33, 68). This greater number of stimulation pulses in these animal studies could also explain why we did not replicate any of the protocol specific changes described in the animal literature. On the other hand, as mentioned, in another set of experiments (39) we did successfully use calcium imaging to reveal the hypothesized TBS effects in our cell cultures, suggesting that our TBS protocols were at least sufficiently strong to induce excitability changes.

We opted to use SH-SY5Y neuroblastoma cells, a human-derived cell line widely used as an *in vitro* model of human neurons. These cells express a variety of neural markers, and can be further differentiated to a more mature neuronal phenotype, having longer neurite outgrowths, increased expression of mature neuron markers, and the formation of mature synapses (46, 62, 72). Differentiated SH-SY5Y cells have also been shown to produce action potentials (46, 73-75), and are therefore functionally active neural cells. We have also recently demonstrated TBS protocol-specific functional effects on SH-SY5Y cells using calcium imaging (39). They are a widely used model for a range of research applications such as Parkinson's disease (76), pathogenesis of viruses (63), drug efficacy and toxicity (64, 77, 78), and as a 3D cell culture (65, 66). These cells can also be used in the study of human neuron plasticity and synapse formation (46) for example in the context of examining treatment targets of depression (79, 80). They are also relatively easy to handle, making them a good candidate to investigate plasticity mechanisms following rTMS.

However, as these cells were derived from malignant tumors (72), cultures may contain two morphologically distant phenotypes, neuroblast-like and epithelial-like (81). While differentiation protocols aim to establish the most neuron-like phenotype among all cells (46, 62, 82), there are often inconsistencies among the proportion of phenotypes within each culture. Experimental conditions may also influence the consistency of differentiation or cellular phenotypes in our cultures. For example, removing the cells from the incubator for stimulation, and having a prolonged incubation for the 24-hour time point may have contributed to the time effects seen in the *EGR1* expression, primary neurite length, and βIII-Tubulin fluorescence intensity.

We chose to measure changes in *gene expression*, specifically in the BDNF-TrkB signaling cascade, shown to be important in LTP-dependent plasticity mechanisms (36, 57, 83). However, investigation of relevant changes at the protein level following stimulation are also important. For example, future studies could expand on our findings by focusing on protein phosphorylation in the BDNF-TrkB signaling cascade, or investigating whether these plasticity mechanisms are NMDA-receptor dependent. We have taken first steps towards investigating TBS-induced changes in human neurons *in vitro*, but more studies are needed to better understand the underlying mechanisms of TBS. Future studies in more advanced human neuron models such as (patient-specific) neuronal cultures derived from induced pluripotent stem cells (iPSC's) (84) or cerebral organoids (85), could help improve our understanding of individual differences in responsiveness to stimulation protocols.

Conclusion

The molecular mechanisms of rTMS remain largely undiscovered, and most of the evidence for plasticity effects following stimulation comes from animal models. In this study, we stimulated living *human* neurons (SH-SY5Y cells) with iTBS and cTBS protocols, and investigated changes in gene expression and morphology. We found evidence for a protocol specific increase in the expression of plasticity genes in the BDNF-TrkB pathway at 24 hours following iTBS, relative to sham. In this human neuron model, we show the feasibility of studying rTMS effects *in vitro,* and we identify several gene expression changes that support iTBS-induced plasticity. These findings pave the way to develop more complex *in vitro* models, such as neuronal cultures from patient-derived iPSCs, in order to better examine the molecular effects of TBS, which in turn is necessary to further optimize the stimulation parameters for human rTMS.

Data Availability Statement

The raw data supporting the conclusions of this manuscript is available through the following link: https://doi.org/10.34894/SP4AWN

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Supplementary Material Chapter 4

Immediate Time Point One-Way ANOVA

Each gene was analyzed separately, to test for an immediate effect of stimulation on gene expression. MAP2: $F(14)=1,438$, $p=0.276$, BCL2:$F(14)= 0.488$, $p=0.625$, EGR1: $F(17)= 0.174$, $p=0.842$, CREB1: $F(17)=0. 659$, $p=0. 532$, TUBB3: $F(17)= 0.074$, $p=0.926$, NTRK2: $F(14)= 0.857$, $p=0.449$.

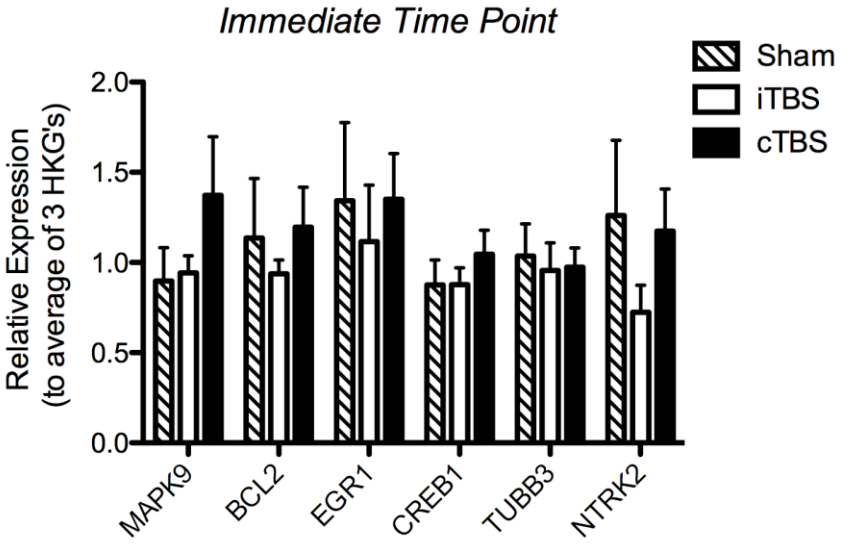
Differentiation analysis Gene expression

Independent samples student's t-tests (2-tailed) were performed for each gene separately, comparing expression levels between undifferentiated and 10 day differentiated cells. MAP2: $t(14)=-2.679$, $p=0.018$, BCL2:$t(14)=-2.769$, $p=0.017$, EGR1: $t(14)=-1.537$, $p=0.147$, CREB1: $t(14)=-1.389$, $p=0.186$, TUBB3: $t(14)=0.095$, $p=0.926$, NTRK2: $t(14)=-8.636$, $p<0.0001$.

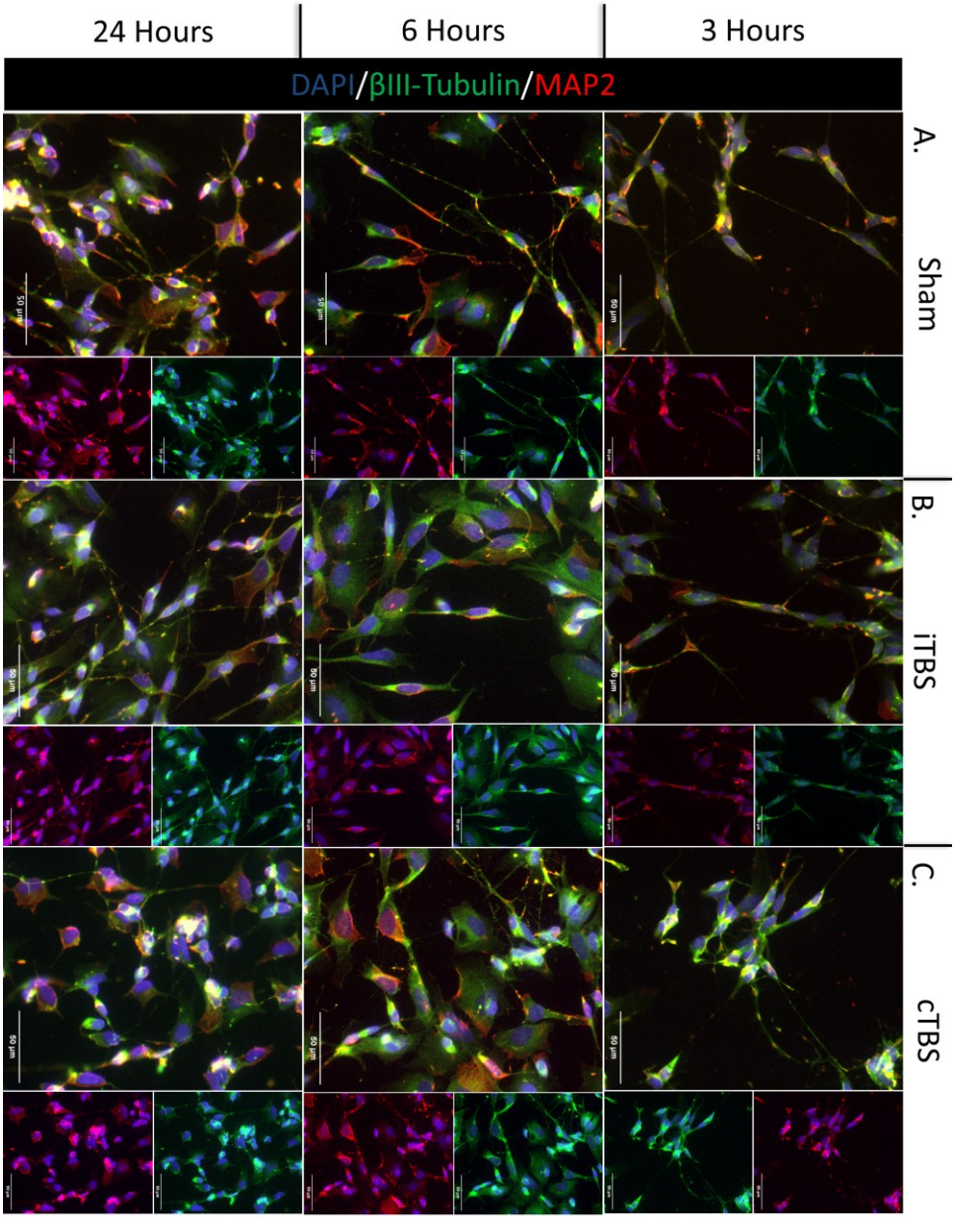
Effect of Stimulation on Housekeeping Genes

There was no significant effect of Time or Condition, or Interaction, on any of the housekeeping genes used to normalize gene expression values for further analysis. Housekeeping genes used: TBP Condition($F(2,30)=0.685$, $p=0.513$), Time($F(1,30)=0.001$, $p=0.977$), Interaction($F(2,30)=0.587$, $p=0.563$); GAPDH Condition($F(2,30)=0.091$, $p=0.913$), Time($F(1,30)=0.111$, $p=0.741$), Interaction($F(2,30)=0.058$, $p=0.944$); PPiB Condition($F(2,30)=0.269$, $p=0.766$), Time($F(1,30)=0.839$, $p=0.367$), Interaction($F(2,30)=0.009$, $p=0.991$). An average of these 3 genes was made, which was also not significant for Condition, Time or Interaction; Condition($F(2,30)=0.163$, $p=0.851$), Time($F(1,30)=0.146$, $p=0.705$), Interaction($F(2,30)=0.066$, $p=0.936$).

Supplementary Figures



Supplementary Figure 1. Gene Expression of all genes (*MAPK9, BCL2, EGR1, CREB1, TUBB3, NTRK2*) of interest in each of the conditions (cTBS, iTBS, sham) at the immediate time point (collected immediately after stimulation). None of the genes showed any significant effect of stimulation condition (p<0.05).



Supplementary Figure 2. Representative images of neuron morphology at each time point following each stimulation condition. DAPI (cell nucleus) in blue, βIII-Tubulin in green, and MAP2 in red. Each condition/time point image has 1 large merged image on the left (βIII-Tubulin and MAP2), and on the right 1 small βIII-Tubulin image, and 1 small MAP2 image. From Top to bottom, 3 hours, 6 hours, 24 hours. From left to right: **A.** Sham stimulation, **B.** iTBS stimulation, **C.** cTBS stimulation. Scale Bar is 50 μM.

Supplementary Tables

Condition	**Time**	**Biological Replicates**	**Differentiation batches**
cTBS	Immediate	6	4
iTBS	Immediate	7	4
Sham	Immediate	5	4
cTBS	6h	8	2
iTBS	6h	7	2
Sham	6h	6	2
cTBS	24h	5	2
iTBS	24h	4	2
Sham	24h	6	2

Table S1: qPCR sample collection

Name	Fwd/Rev	Sequence 5'→3'
NTRK2	Forward	TGGATGCATATCGTGCTCCG
NTRK2	Reverse	GTGCTTGGTTCAGCTCTTGC
BCL2	Forward	ACATCGCCCTGTGGATGACT
BCL2	Reverse	CCGTACAGTTCCACAAAGGC
MAPK9	Forward	TGGGCTACAAAGAGAACGTTGA
MAPK9	Reverse	GTGCCTTGGAATATCACACAACC
TUBB3	Forward	GGGGCCTTTGGACATCTCTTC
TUBB3	Reverse	GTGTAGTGACCCTTGGCCC
EGR1	Forward	CCCCGACTACCTGTTTCCAC
EGR1	Reverse	GACAGAGGGGTTAGCGAAGG
CREB1	Forward	CCCCAGCACTTCCTACACAG
CREB1	Reverse	CTCGAGCTGCTTCCCTGTTC
GAPDH	Forward	CCAAATGCGTTGACTCCGA
GAPDH	Reverse	GCATCTTCTTTTGCGTCGC
PPiB	Forward	GTTTGAAGTTCTCATCGGGG
PPiB	Reverse	AAAACAGCAAATTCCATCGTG
TBP	Forward	TGCACAGGAGCCAAGAGTGAA
TBP	Reverse	CACATCACAGCTCCCCACCA

Table S2: Primer Sequences, 5' to 3' orientation.

Analysis Mode	**Program Name**	**cycles**	Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)
None	Pre Incubation	1	95	None	0:10:00	4.8
Quantification	Amplification	45	95	None	0:00:10	4.8
			60	single	0:00:45	2.5
Melting Curves	Melting Curve	1	95	None	0:00:15	4.8
			60	None	0:00:30	2.5
			97	Continuous		0.11
None	Cooling	1	60	None	0:00:10	2.5

Table S3: qPCR Program

Name	Company	Order number	Dilution	Marker of	Secondary Antibody
βIII-Tubulin	Cell Signaling	5568S	1:300	Neurons; Neurite outgrowth	donkey anti rabbit Alexa 488 (Invitrogen, A-21206)
MAP2	Sigma	M2320	1:300	Neurons; Dendrites	donkey anti mouse Alexa 594 (Invitrogen, A-21203)

Table S4: Secondary Antibody Information

Wavelength	Marker of	Exposure Time	Color
350	DAPI(nuclei)	10ms	Blue
488	βIII-Tubulin	500ms	Green
594	MAP2	300ms	Red

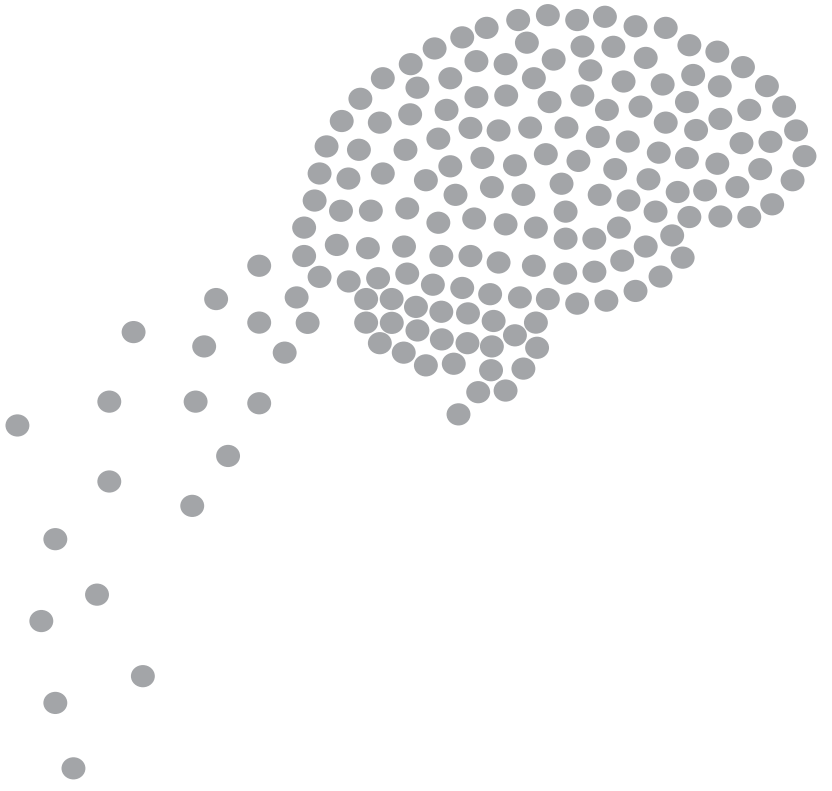
Table S5: Fluorescence Microscopy Detection. Olympus BX51WI microscope and DSU spinning unit, 20X objective.

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
βIII-Tubulin Rabbit mAb	Cell Signaling	Cat #5568S RRID:AB_10694505
Monoclonal Anti-MAP2 antibody produced in mouse	Sigma-Aldrich	Cat # M2320 RRID:AB_609904
Donkey Anti-Rabbit IgG Antibody	Molecular Probes	Cat #A-21206 RRID:AB_141708
Donkey Anti-Mouse IgG Antibody	Molecular Probes	Cat #A-21203 RRID:AB_141633
Experimental Models: Cell Lines		
Homo sapiens; SH-SY5Y	ATCC	Cat# CRL-2266 RRID:CVCL_0019
Software and Algorithms		
Fiji (ImageJ)	Schindelin et al., 2012	http://fiji.sc; RRID:SCR_002285
Prism version 5.0	GraphPad Prism	www.graphpad.com; RRID:SCR_002798
Micro-Manager software	Edelstein et al., 2014	http://micro-manager.org/; RRID:SCR_016865

Chapter 5

How to design optimal accelerated rTMS protocols capable of promoting therapeutically beneficial metaplasticity



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Abstract

Repetitive transcranial magnetic stimulation (rTMS) can induce longer lasting synaptic plasticity changes within the stimulated brain regions, similar to processes described by the theory of Hebbian plasticity. Hebbian synaptic plasticity works through post-synaptic modifications, such as NMDA receptor activation, calcium signaling, and AMPA receptor trafficking, leading to long-term potentiation (LTP) or long-term depression (LTD). Under Hebbian plasticity, a synapse which is repeatedly stimulated will undergo synaptic strengthening, i.e. LTP. This increases efficacy of signal transmission between specific synapses and is thought to underlie the molecular encoding of information, leading to learning and memory formation. However, if unregulated, this neural connection will be continuously strengthened, driving the synapse to an extreme maladaptive excitation level. Metaplasticity acts against this, regulating neuronal excitability by adjusting the threshold for LTP/LTD depending on previous neural activity. *Homeostatic* metaplasticity is a specific form of metaplasticity which acts to stabilize this LTP/LTD-threshold within a physiologically relevant range. These principles of (homeostatic) metaplasticity can be utilized to explain and optimize the effects of repetitive (rTMS) protocols on cortical excitability and synaptic plasticity. For example, reports have shown that the direction of the plasticity effects induced by a given rTMS protocol can be reversed by priming it with an identical stimulation intervention applied immediately before. This switch in direction of rTMS-induced plasticity provides indirect support for homeostatic metaplasticity, counteracting the (potentially maladaptive) additive metaplasticity effects of two stimulation protocols applied in quick succession. Recently, repeating stimulation protocols several times per treatment session (accelerated rTMS) has shown therapeutic potential, promoting stronger and longer lasting clinical outcomes. However, the time interval between repeated stimulation protocols is thus critical when developing optimal accelerated rTMS protocols, as homeostatic metaplasticity potentially works against the intended rTMS stimulation effects. The million-dollar question in this context refers to the optimal time interval between the repeated treatment sessions of accelerated TMS protocols for promoting additive while avoiding homeostatic plasticity. We here discuss animal and cellular models showing that longer time intervals may be needed between rTMS protocols in order to avoid stabilizing homeostatic metaplasticity mechanisms and to promote additive metaplastic effects. We argue that this may form the basis for developing optimal accelerated rTMS protocol intervals capable of promoting therapeutically beneficial metaplasticity.

Keywords: Metaplasticity, Homeostatic Plasticity, Hebbian Plasticity, Transcranial Magnetic Stimulation (TMS), Accelerated rTMS

Introduction

Our brain is comprised of billions of neurons, which can connect via synapses that rely on electrical signaling and the release of chemical messengers to communicate and propagate signals through neural networks. By forming such networks, neurons are capable of monitoring previous firing activity, and using this information to adapt subsequent firing rate. This so-called activity-dependent plasticity is critical for the encoding of new information, and the tuning of (low activity) connections (1-3). The physiological mechanisms of synaptic plasticity have largely been attributed to long-term potentiation (LTP) (4, 5), and long-term depression (LTD) (6-8), which result from molecular processes such as receptor trafficking or synaptic scaling (3). Both LTP and LTD are induced by postsynaptic NMDA receptor activation, which lead to an influx of calcium into the postsynaptic dendrites (8-10). This triggers a complex series of intracellular signaling cascades, resulting in synaptic modifications such as AMPA receptor trafficking (11, 12). The pattern of stimuli delivered to the post synapse determines whether LTP or LTD will occur; low frequency stimulation induces LTD, whereas high frequency stimulation induces LTP (8, 13). These processes underlie much of our knowledge on the molecular mechanisms of learning and memory.

However, if the principles of Hebbian synaptic plasticity (LTP, LTD) alone were to drive the strengthening and weakening of synaptic connections, activity would, over time, be driven towards destabilization. This is because continuously firing synapses could only become stronger (driven to saturation) and unused synapses quiescent (until completely lost) (14). Consider a synapse that is strengthened by LTP; meaning the presynaptic neuron becomes more effective at depolarizing the postsynaptic neuron. With each continued stimulation, the postsynaptic neuron will be more easily depolarized, in a positive feedback loop, resulting in a hyperexcitable postsynaptic neuron. Over time, not only will the original presynaptic connection be strengthened, but other unrelated presynaptic inputs could cause a depolarization of the hyperexcitable postsynaptic neuron, resulting in unregulated synaptic transmission (15). Therefore, other mechanisms must exist, which regulate synaptic plasticity on a global network level to maintain stability of synapses and maintain specificity of neural activity (16, 17).

Metaplasticity refers to any change in the direction or degree of synaptic plasticity (ex. LTP, LTD) based on prior neural activity (18). While both synaptic and metaplasticity are dependent on previous neural activity, metaplasticity does not *directly* alter the efficacy of synaptic transmission (as LTP/LTD), but it adjusts the neurons' ability to induce LTP/LTD with *subsequent* neural activity. Metaplasticity in some sense can be considered as the plasticity of synaptic plasticity, e.g. maintaining the dynamic nature of a neuron's firing threshold, when this neuron reaches a certain firing rate (16, 18, 19). Metaplasticity works through similar synaptic modifications as LTP/LTD, such as

NMDA receptor activation and modification (20), and changes in calcium signaling triggering complex signaling cascades (18). Metaplastic modifications, for example at NMDA receptors, can occur either at specific synapses or across the whole neuron, and on time scales from minutes to weeks (19). Depending on the temporal pattern and strength of previous neural activity, metaplastic mechanisms can be additive; for example promoting increased synaptic strengthening through repeated excitatory (LTP-inducing) stimulation. Metaplasticity can also be stabilizing; for example acting against subsequent synaptic strengthening when repeating excitatory (LTP-inducing) stimulation (19, 21). This stabilizing form of metaplasticity is often referred to as *homeostatic* metaplasticity, as it specifically regulates the dynamic threshold of synaptic plasticity to maintain equilibrium, or homeostasis (16, 17). We hypothesize, based on research from human and animal studies, that the timing between excitatory stimulations are what differentiate between promoting additive or homeostatic metaplasticity.

We focus on the role of metaplasticity in transcranial magnetic stimulation (TMS). We describe the recent use of accelerated (repeated) stimulation protocols, both in research and clinical applications, and the molecular mechanisms required to promote either homeostatic or additive metaplastic effects. Finally, we showcase the therapeutic potential of accelerated stimulation, and hypothesize that increasing the currently practiced stimulation intervals may be more efficacious in promoting additive metaplastic effects in various clinical applications of rTMS in rehabilitation, neurology, psychiatry, and cognitive decline.

Metaplasticity in TMS

TMS is a widespread and increasingly popular non-invasive brain stimulation technique, where electromagnetic pulses allow stimulation to pass non-invasively through the skull (22). When pulses are applied in a certain pattern, as repetitive TMS (rTMS), protocols can have lasting excitatory or inhibitory effects (23-25). Two commonly used stimulation protocols are *intermittent* theta burst stimulation (iTBS), requiring only 3 minutes of stimulation time, resulting in a lasting increase of cortical excitability, and *continuou*s theta burst stimulation (cTBS), requiring only 40 seconds of stimulation for a lasting decrease in cortical excitability (26). The after effects of these protocols have been shown for up to 1 hour following stimulation (26, 27).

While iTBS is normally an excitatory protocol, causing an increase in cortical excitability of the stimulated brain region, it has been shown that when applied twice in quick succession iTBS effects switch from excitatory to inhibitory (28). Conversely, when cTBS (an inhibitory protocol) is applied for double the normal duration, its effects switch from inhibitory to excitatory (28). Several studies have reported similar effects of repeating iTBS or cTBS stimulation protocols, with the timing between

protocols being an important factor in the magnitude and direction of aftereffects (19, 29, 30). For example, using a 'priming' iTBS protocol which does not induce plasticity, followed by a 'test' iTBS protocol has shown that short intervals of 5 minutes between priming and test resulted in homeostatic-like changes in excitability, i.e. an opposite effect. Interestingly, longer breaks of 15 minutes resulted in an increase in MEP amplitude after the test iTBS (30). However, 15 minutes between priming and test iTBS/cTBS has also been shown to induce in homeostatic-like metaplastic effects (29). While the timing between repeated TBS sessions is clearly important, the optimal interval is less clear. 15 minutes between iTBS sessions has shown to promote both homeostatic (29) and MEP enhancement after the second iTBS (30), while 10 minutes between priming and test iTBS has shown enhancement of MEP amplitude (31), but 5 and 20 minutes between iTBS sessions did not (32). Therefore, when 2 iTBS sessions are repeated with short (less than 30 minutes) between, conflicting effects on MEP amplitude have been reported.

'Accelerated' protocols, which consist of multiple stimulation sessions on a single day, have recently been introduced for the treatment of depression (33-37). Due to their short duration, the TBS protocols, in particular iTBS, have been promising candidates for accelerated protocols (38). Also, a large trial recently found that iTBS was not-inferior to the classical 10Hz rTMS protocol, confirming the clinical potential of this shorter stimulation protocol to treat depression (39). Indeed, several studies have shown additional benefits for accelerated iTBS protocols in the treatment of severe, treatment resistant depression (40, 41). In the clinic, an interval of 15 minutes is often used between iTBS sessions, with these sessions repeated up to 5 times on a single treatment day (40, 42).

We recently conducted a study investigating the effects of accelerated iTBS over motor cortex, consisting of 5 repeated iTBS sessions in a single day. iTBS with 8- or 15-minutes time interval between sessions were delivered to healthy participants in a fully within subject design; where participants received 4 different conditions (accelerated iTBS with 8-minute intervals, accelerated iTBS with 15-minute intervals, single iTBS and sham). (43). We compared change in motor evoked potential (MEP) amplitude up to 90 minutes following stimulation, across the stimulation conditions.

We found that there was no difference in the effects of accelerated iTBS on MEP amplitude, also when compared to sham stimulation, and thus no additive metaplasticity induced by five stimulation sessions applied successively in 8- or 15-minutes intervals. We argue that such intervals between iTBS protocols are likely too short to avoid processes of homeostatic plasticity. With only 8 or 15 minutes between sessions, homeostatic mechanisms may be working against additive metaplastic effects to maintain network stability and therefore result in a net effect of no change in excitability following these accelerated protocols (43).

In agreement with this notion, animal studies in rats and rat hippocampal slices have shown that a sufficiently long pause between excitatory stimulation sessions was necessary for additive (LTP) plasticity effects to occur (44-46). This may have to do with the time required for metaplasticity mechanisms, for example synapse strengthening with AMPA receptor trafficking (15).

It has been well established in animal studies, that a single round of TBS (a 4-pulse burst at 100Hz, repeated at 5Hz for 10 bursts) is effective at inducing LTP in CA1 hippocampal pyramidal neurons (47, 48). TBS has since then been used extensively to reliably induce LTP *in vitro* (49). Interestingly, repeating this single TBS protocol with a time interval of greater than 40 minutes, was capable of almost doubling the potentiation compared to the first TBS alone (44). This additional potentiation is thought to work through strengthening the smaller synapses which weren't strengthened by the first TBS protocol (44). This may have to do with the number of AMPA receptors; smaller synapses contain fewer AMPA receptors and therefore don't generate a response to trigger a depolarization following a single TBS (44). Several other studies have provided evidence for increased potentiation by spaced TBS, however the magnitude and duration of the effects depended on a series of factors such as rat strain, rat age, and the time interval. In adult Wistar rats, adult Long-Evans (LE) rats, and young LE rats, 4 hours was required between TBS to induce additional potentiation (45, 46). However, in young Sprague Dawley (SD) rats, a single TBS repeated at 1-hour intervals could induce further potentiation, following up to 3 repeated TBS stimulations (4 did not produce additional potentiation) (44, 46). These different studies used different stimulation intensities; Frey et al. (1995) (45) found that reducing stimulation intensity in the second stimulation was effective for promoting potentiation 4-hours later, while Cao & Harris (2014) (46) and Kramár et al. (2012) (44) kept stimulation intensities constant. However, these studies consistently show that additional potentiation following repeated TBS in animal slices is possible. Enhanced, additive LTP-like plasticity may be promoted when repeating TBS with 50-60 minutes between sessions (44, 46). After 3 TBS protocols, spaced 60 minutes apart, potentiation had been raised to 150% baseline, which is about three times higher than if just one protocol was given (44, 49). This suggests that 3 TBS protocols repeated at 60 minute-intervals may be effective at promoting maximal, additive metaplasticity effects (Figure 1A). If there is less time between TBS protocols, for example 10 minutes, homeostatic metaplasticity mechanisms may dominate, promoting a stabilizing rather than additive plasticity response (Figure 1B).

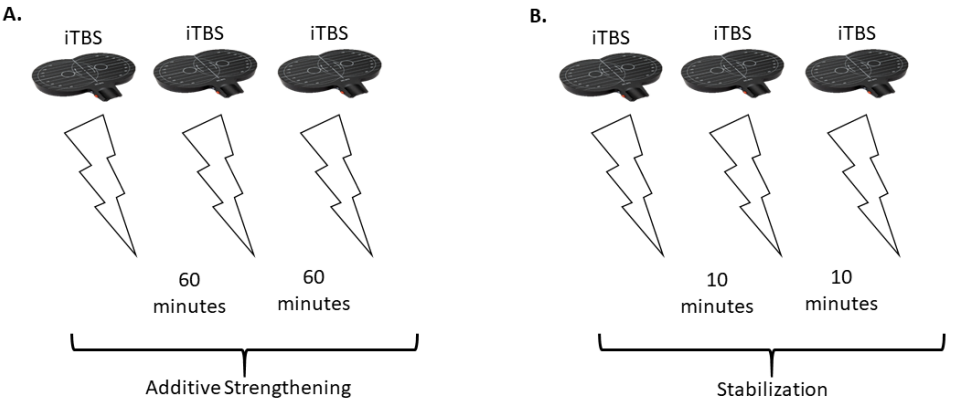

Figure 1: Theoretical stimulation setup and effects in response to different spacings between repeated stimulations. **A.** Repeating excitatory (iTBS) stimulation 3 times, with 60 minutes between sessions, promotes additive strengthening of stimulated synapses. Overall, the repeated stimulation increases potentiation (this has been shown in animals using a different TBS protocol (44, 46)). **B.** Repeating the same 3 iTBS stimulations, but with only 10 minutes between sessions results in stabilization (homeostatic metaplasticity) and no change in overall plasticity.

Discussion

Activity-dependent metaplasticity is considered to be *homeostatic* if the first stimulation protocol alters the threshold for subsequent LTP/LTD in the opposite direction, thereby stabilizing (network) brain activity (50). Interestingly, this reversal of aftereffects has been shown specifically when stimulation protocols were given with a short (0-5 minutes) interval (28, 30), providing support for homeostatic metaplasticity mechanisms in rTMS protocols (19). While homeostatic metaplasticity mechanisms are important for stabilizing network activity, they can be counteractive when promoting plasticity effects through rTMS. In fact, when applying rTMS protocols, the explicit goal is not stabilization but promotion of additive, increased plasticity effects.

Animal studies have shown that timing is important in the molecular mechanisms underlying metaplasticity. While there is overlap between the mechanisms of additive and homeostatic metaplasticity, there are temporal differences which may differentiate between both principles at the molecular level. Based on evidence form animal models, leaving 60 minutes between excitatory stimulation protocols may promote additive rather than homeostatic metaplastic effects in accelerated TMS treatment protocols.

Clinical Implications

If longer intervals between iTBS sessions are capable of promoting additive metaplasticity, as has been shown in animal studies (44) as well as improving clinical outcomes in the treatment of depression (51), longer spaced intervals between iTBS sessions will likely be beneficial for other therapeutic applications of iTBS. iTBS is increasingly being used as a treatment in a range of clinical applications such as rehabilitation, as well as neurological and psychiatric disorders. For example, to promote motor recovery after stroke (52), for managing spasticity associated with multiple sclerosis (MS) (53), and decreasing obsessive symptomatology associated with obsessive compulsive disorder (OCD) (54), just to name a few. These protocols all must adhere to the established safety guidelines (55), and recommendations for clinical TMS use (56, 57). These include total pulse number, interval between TBS session, intensity of stimulation, and cumulative weekly applications (55). Accelerated iTBS has been successfully and safely used in the treatment of depression (38, 40, 42), with patients receiving a total of 32400 pulses at 110% resting motor threshold, over 20 sessions (5 sessions per day, 15 minutes between sessions) in 4 days (41). Therefore, while following the established safety guidelines is the upmost priority, and local health authorities should always approve each stimulation protocol (55), delivering three iTBS sessions on a single day with 1 hour between sessions should theoretically be safe and tolerable for most patients.

rTMS is also used as a treatment for the cognitive decline associated with neurodegenerative disorders such as dementia, and Alzheimer's disease (AD) (58-62). However, there are ethical implications of using rTMS for cognitive enhancement, in particular in healthy participants (63). It is important to maintain the consensus ethical requirements that 1.) participants/patients provide informed consent, 2.) the benefit of the research outweigh the risks, and 3.) there is equal distribution of burdens and benefits across patients (this is violated if a particular group of patients with different economic, physical or social conditions (55).

Importantly, the here described principles of additive and homeostatic metaplasticity not only apply to the here discussed accelerated TMS treatments and the question of optimal time interval between its repeated stimulation sessions, but likewise can be used to explain and optimize other forms of plasticity-inducing TMS protocols such as paired associated-stimulation (PAS) or paired-coil TMS (pcTMS).

In humans, neural excitability and synaptic plasticity can be probed by TMS to peripheral nerves and motor cortex (64, 65). In such a transcortical loop, timings of afferent (muscle/nerve to brain), cortical, and efferent (brain to muscle) responses can be used to quantify central motor excitability (64). For example, delivering a conditioning TMS pulse to an afferent tract (ex. the wrist), followed (10-48ms) by stimulation of the efferent tract (motor cortex), will alter motor evoked potentials

(MEP's) measured from thumb flexor muscles (64). It has been shown that wrist stimulation 20-22 msec preceding motor cortex stimulation elicits a facilitated MEP, with a latency of about 1 ms, compared to MEPs given without the conditioning wrist stimulation (64). Repeating this afferent (wrist) efferent (motor cortex) stimulation, in paired associated stimulation (PAS), can induce lasting effects on motor cortex excitability (65, 66), providing evidence for synaptic plasticity. Interestingly, evidence of homeostatic and additive metaplastic responses has also been recorded using PAS stimulation (67, 68). When two LTP-inducing PAS protocols were separated by 30 minutes, a decrease in MEP amplitude was measured, indicating a homeostatic (stabilizing) metaplastic responses (67). Similarly, LTD-inducing PAS immediately preceding a motor-learning task facilitated motor-learning (68), again providing support for homeostatic plasticity mechanisms dominating at early time points following stimulation.

Additionally, the effects of brain stimulation are not only localized to the site of stimulation, but can also spread to different areas through complex cortical networks. Similarly to PAS, this has been shown using paired-coil TMS (pcTMS), where multiple coils are used to probe different cortical areas and assess connectivity (69, 70). For example, a single TMS pulse to motor cortex can cause a depression of the MEP measured following a subsequent (6-30ms) TMS pulse to contralateral motor cortex (71). Therefore, TMS can also be used to assess connectivity between brain areas (69). In other words, TMS stimulation can propagate to different cortical regions, having both local and remote effects on (meta) plasticity. This has valuable clinical implications, where inducing plasticity effects in a cortical network are important (70). In stroke patients for example, localized damage can disrupt connectivity and can have functional consequences (70), therefore stimulation effects should promote network plasticity, rather than localized plasticity. Similarly, in the treatment of depression, superficial stimulation uses cortical connectivity to influence deeper cortical structures, resulting in improvement of clinical symptoms (72, 73). Therefore, it is important to use TMS to strengthen connectivity, and to promote additive, metaplastic changes also on the network activity level.

With the increasing and widespread application of rTMS protocols in the clinic, it is important to optimize protocols to maximize their effects, while remaining within established safety and ethical guidelines for use in the clinic (55, 57). Single iTBS has proven promising, but accelerated iTBS at longer time intervals (60 minutes) between sessions could maximize clinical outcomes through additive metaplasticity, preventing homeostatic metaplasticity from stabilizing stimulation effects. Clinical efficacy of PAS and pcTMS protocols may be similarly increased by optimizing the timing between stimulations according to these principles of metaplasticity.

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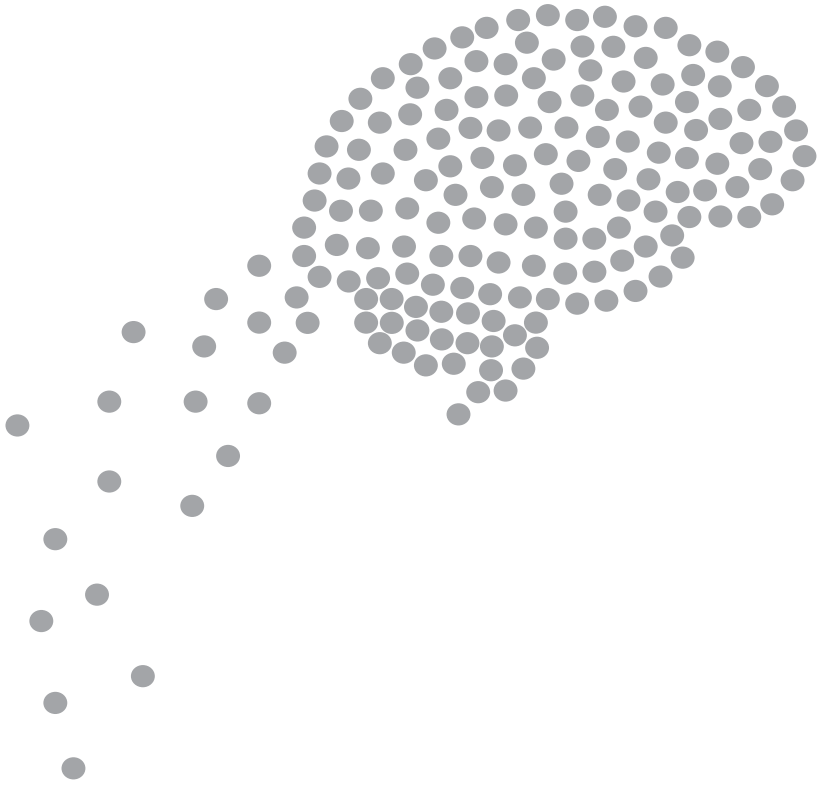
Chapter 6

No additive meta plasticity effects of accelerated iTBS with short inter-session intervals



Based on: Thomson, A. C., de Graaf, T. A., Kenis, G., Rutten, B. P. F., Schuhmann, T.*, & Sack, A. T.* (2019). No additive meta plasticity effects of accelerated iTBS with short inter-session intervals. *Brain Stimulation*. doi:https://doi.org/10.1016/j.brs.2019.05.012

* Equal contribution

Abstract

Intermittent theta burst transcranial magnetic stimulation (iTBS) can non-invasively increase cortical excitability beyond the duration of stimulation itself, having valuable research and clinical applications. However, recent reports suggest that such effects are not always reliable (1-3). Given its short duration, iTBS can be administered repeatedly ('accelerated iTBS'), which might stabilize and/or amplify neuroplastic effects. These accelerated iTBS protocols have been applied in depression treatment with positive results (4, 5). Yet, their efficacy has not been empirically demonstrated through objective, neurophysiological measures in healthy volunteers. We evaluated the effects of 1 (standard) or 5 (accelerated) iTBS, the latter with different short inter-protocol intervals, on primary motor cortex excitability as assessed with motor-evoked potentials (MEPs). 20 healthy participants were tested in a within-subject design of 4 pseudo-randomized conditions on different days; 1) standard iTBS, 2) placebo iTBS, accelerated iTBS with 3) 8-min or 4) 15-min inter-protocol intervals. Following each procedure, MEPs were elicited for 90 minutes. MEP amplitudes were grouped in time bins and analyzed with analyses of variance. In line with several previous reports, we did not find increased MEP amplitudes relative to placebo iTBS after standard iTBS. Importantly, accelerated iTBS did not amplify/stabilize neuroplastic effects, since neither the accelerated 8 nor 15-minute interval protocols had significant effects on MEPs. Standard iTBS did not yield reliable, consistent results, and short-interval accelerated iTBS did not appear to resolve the issue. We propose that longer intervals (45-60 minutes) may improve the effectiveness of accelerated iTBS protocols.

Keywords: Transcranial Magnetic Stimulation, Accelerated iTBS, Cortical Excitability, Primary Motor Cortex

Introduction

Transcranial magnetic stimulation (TMS) is a form of neuromodulation that uses electromagnetic pulses to non-invasively alter cortical excitability. When multiple pulses are applied in repetitive protocols (rTMS), lasting changes in neural firing can be induced (6). For example, classic rTMS protocols such as high frequency (10-20 Hz) stimulation have been shown to increase cortical excitability (7). However, repetitive protocols are notoriously unreliable; with large inter and intra subject variability hindering the reproducibility of these neuroplasticity effects (8, 9).

Many studies have aimed to optimize rTMS protocols, focussing on shortening protocol length, increasing plasticity effect size, and decreasing variability. For example, the theta burst stimulation (TBS) protocols are a widely used set of protocols which require only minutes of application duration. They reportedly induce increased plasticity effects which are less variable and longer lasting compared to classic rTMS protocols (10). Intermittent TBS (iTBS) is a three-minute protocol which has been shown to increase cortical excitability for up to 20 minutes post stimulation (10). However, several studies have reported difficulty in replicating these established iTBS effects (1-3). This is important, as iTBS is increasingly used in both research and clinical environments. If its effects on excitability, possibly extended to clinical treatment efficacy, are not very reliable, then research on protocol optimization must continue.

An additional benefit of iTBS compared to classical high frequency rTMS is its short duration, making it a more time and cost-effective alternative (11). Recently, clinicians using the FDA-approved rTMS protocol for depression therapy (12) have begun to take advantage of this short duration, by administering multiple iTBS sessions per day to treat their patients more cost- and time-effectively (5, 13, 14). The hope is that neuroplastic effects arise quicker, through condensing a classical week's worth of treatment into a single day. Such 'accelerated iTBS' has been successfully applied in the treatment of depression (4, 5), with short inter-session intervals (time between repeated iTBS administration).

Protocol developments in the clinic should go hand in hand with neurophysiological investigations of the underlying mechanisms of action. Theoretically, accelerated iTBS could not only shorten the treatment process, but also lead to enhanced and more stable (reliable) effects on cortical excitability. Initial iTBS sessions could prime, normalize, or amplify the neuroplastic effects of subsequent sessions (15). Yet, such effects may strongly depend on the time between sessions (16, 17).

In this study, we investigated the effects of standard iTBS (single-iTBS session) and accelerated iTBS (five repeated iTBS sessions) on motor cortical excitability, assessed with motor-evoked potentials (MEPs) in healthy volunteers. We included two short inter-session intervals, 15 minutes and 8 minutes, and evaluated effects on magnitude

and variability of MEPs. In this fully within-subject design, effects of standard and accelerated iTBS could be compared to placebo (sham) iTBS. We aimed to identify whether accelerated iTBS had stronger, more consistent aftereffects when compared to standard iTBS, if 8 minutes versus 15 minutes between iTBS sessions had different results, and if these effects were longer lasting. MEPs were measured for up to 90 minutes following each procedure. We were also interested in individual patterns of responsiveness to accelerated iTBS, compared to standard iTBS. We investigated whether cortical excitability response (increase or decrease in excitability) to accelerated iTBS could be predicted by response to standard iTBS, and if accelerated iTBS resulted in fewer opposite (clinically averse) responses as compared to standard iTBS. These analyses were therapeutically motivated; if patient response to accelerated iTBS can be predicted by response to a short standard iTBS protocol, this could benefit individualization of treatment options. Opposite response to iTBS, i.e. a decrease instead of an increase of excitability, could be harmful to patients, and therefore if accelerated iTBS has fewer opposite responders this would also be clinically relevant.

Methods

Participants

20 healthy, right-handed participants (7 male), with a mean age of 23.7 years (SD 3) were included in this study. All participants were screened for potential contraindications as in the established guidelines (18), and gave written informed consent before participating in the study. The experimental procedure was in accordance with the Declaration of Helsinki and approved by the local ethics committee at Maastricht University.

Experiment procedure

On the day of each experimental session, a pre-experimental check for safety was conducted, and caffeine intake and hours of sleep the night prior were recorded. None of these amounts of caffeine or hours of sleep were reported as being more or less than normal, or correlated with MEP amplitude.

Our within-subject design included 4 experimental conditions: accelerated iTBS with 8-minute breaks between sessions, accelerated iTBS with 15-minute breaks between sessions, single session iTBS, or placebo iTBS (Figure 1). The first 3 conditions were randomized across experiment days. The placebo condition was added after the first 11 participants had completed 3 experiment days, making placebo the condition of the 4th day. To placebo-counterbalance, placebo was the 1st experiment day for the second 9 participants. If possible, experiment days were planned 1 week apart, at the same time of day, and after 13:00 to reduce any potential variability caused by

circadian rhythms (19). On average, sessions 1, 2 and 3 (and 4 for the second 9 participants) were 9.13 days apart (SD 4.09). The average time between experiment days 3 and 4 for the first 11 participants was 93.72 days apart (SD 58.85).

TMS and EMG

Participants were seated in a comfortable chair with their left hand resting on a table. Disposable adhesive surface electrodes (Plaquette, Technomed Europe, Beek, and The Netherlands) were placed on the left index finger, belly of FDI muscle, and the wrist bone. EMG was recorded using a PowerLab 4/34 with a bio Amp system (ADInstruments, Oxford, UK), and LabChart Pro software (ADInstruments, Version 8) was used to monitor online muscle activity and for offline MEP analysis. Electrodes were adjusted to reach an online resting signal of below 0.02 mV peak to peak.

TMS was applied through a MagPro X100 stimulator (MagVenture, A/S, Farum, Denmark) and an MC-B70 figure-of-eight coil. The coil was hand held at a 45° angle to the midline, and biphasic pulses were given with the current flow in an anterior to posterior direction. Motor hotspot location was marked with Localite Neuronavigation system (Localite GmbH, Schloss Birlinghoven, Germany), to ensure that the same spot was targeted throughout the entire experimental session. Resting motor threshold (RMT) was determined as the maximum stimulator output (MSO) intensity where 5 out of 10 pulses gave peak-to-peak amplitude of below 0.05 mV. Active motor threshold (AMT) was the MSO where 5 out of 10 pulses elicited a response of below 0.20 mV while the participant activated their left hand. The RMT and AMT were re-assessed during each experimental day.

Single pulses were given in blocks of 30, at 120% of RMT. Pulses were delivered at intervals between 5 and 7 seconds. ITBS sessions were at 80% AMT. Each iTBS session consisted of the Huang et al. (2005) published protocol of 50Hz triplets repeated at 5 Hz, for 2000ms trains and 8000ms inter-train interval for 600 pulses (10). For the placebo sessions, the coil was held perpendicular to the skull. The single iTBS condition consisted of 4 placebo and 1 real iTBS, with 15-minute intervals between sessions. The placebo condition was with 8-minute intervals between the 5 placebo sessions. We took 3 baseline measurements, each 5 minutes apart. Following the stimulation protocol, lasting between 51.8-75.8 minutes, was an immediate MEP measurement block, and then one every 10 minutes for 90 minutes.

To establish a constant brain state between participants throughout the breaks, a relaxing movie clip was played on the computer screen approximately 35 cm in front of the participant. Movie clips were counterbalanced against the conditions, which were randomized across the 4 sessions.

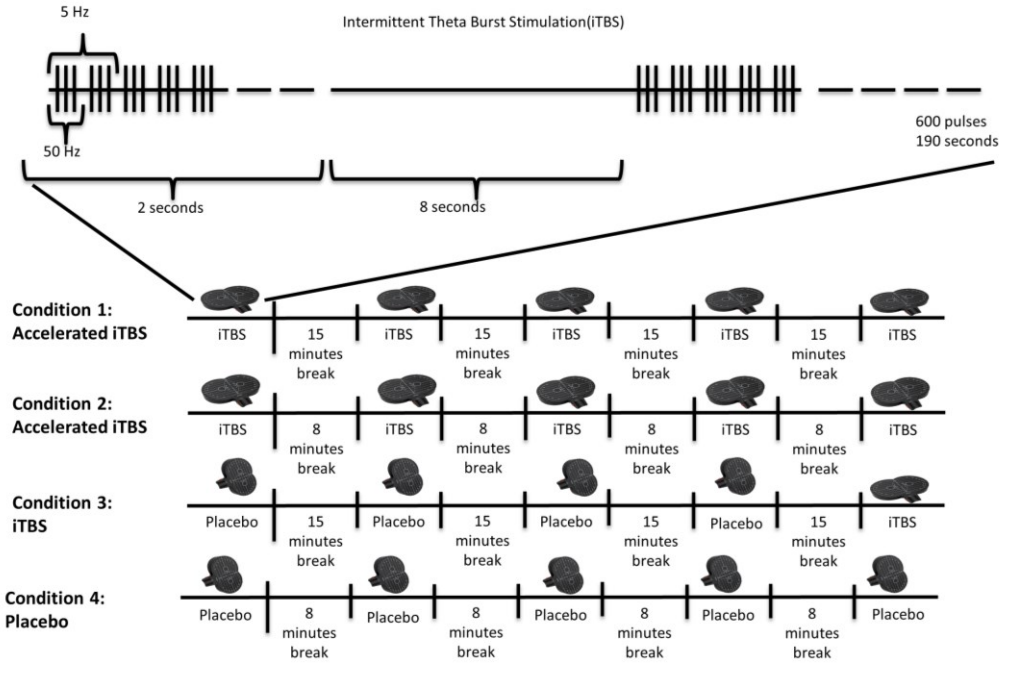


Figure 1. Experimental procedure. Each iTBS session consisted of the Huang et al. (2005) published iTBS protocol of 5Hz triplets repeated at 50Hz; repeated for 2 seconds, with 8 seconds in between. These were given with 8 or 15 minutes between. For real stimulation the coil was held tangential to the skull, and perpendicular to the skull for placebo.

Preprocessing/data analysis of the MEP's

EMG data recorded in LabChart consisted of epochs beginning 50ms before the TMS pulse and ending 100ms after the TMS pulse. Peak-to-peak MEP amplitudes were calculated by LabChart software. To account for any muscle tension before the MEP, single MEPs where the EMG recording before the TMS pulse was above 0.1 mV were removed. Matlab (R2016a, Mathworks Inc.) was used for further analysis. In first outlier analysis, MEP's that were 2.5 standard deviations above the mean per participant, per condition were removed. At the group level, MEP time point values for individual participants that had a Z score of 3 standard deviations above the mean for that condition were removed. Statistical analysis was completed in IBM SPSS 24 (SPSS for Windows version 24.0. Armonk, NY: IBM Corp) and JASP (JASP Team 2018 version 0.9).

Statistical analysis

For each participant, an average of the 3 baseline MEP blocks was taken. Each of the 10 post-TMS blocks was then normalized to this baseline average: expressed in percentage baseline. In the group level analysis, mean normalized MEPs over factors

TIME and CONDITION were tested in a repeated-measures analysis of variance (RM-ANOVA). For further analysis, specific time bin grand averages were calculated. In case of violation of the sphericity assumption (Mauchly's test), we report Greenhouse-Geisser corrected statistics.

For the placebo-subtracted analysis, the normalized values of placebo were subtracted from each of the normalized MEP time block values. MEP time bins were collapsed into 30-minute blocks, and analyzed in a 3x3 RM-ANOVA with factors TIME and CONDITION. The placebo-subtracted responses were characterized into responders or non-responders, for each stimulation condition. For both groups, a 3x2 RM-ANOVA was run with TIME and CONDITION (the 2 conditions not underlying the response classification).

Results

Baseline measures and stimulation intensities

All participants completed the 4 experimental sessions, and none reported any negative side effects. There were no differences in MEP Baseline ($F(3, 74)=0.911$, $p=0.440$), RMT ($F(3, 76))=0.532$, $p=0.662$), or AMT ($F(3, 76))=0.050$, $p=0.985$) across conditions.

Group level analysis

To evaluate the changes in cortical excitability over time, a RM ANOVA of CONDITION (1. Accelerated iTBS with 8 minute breaks, 2. Accelerated iTBS with 15 minute breaks, 3. iTBS, and 4. Placebo) vs. TIME (0, 10, 20, 30, 40, 50, 60, 70, 80, and 90 minutes) was conducted. There was no main effect of CONDITION (F (3, 19) =0.769, p=0.516), TIME (F (9, 19) =1.695, p=0.093), and no interaction (F (27, 19) =0.988, p=0.483). Results, with baseline values included as reference, are plotted in Figure 2.

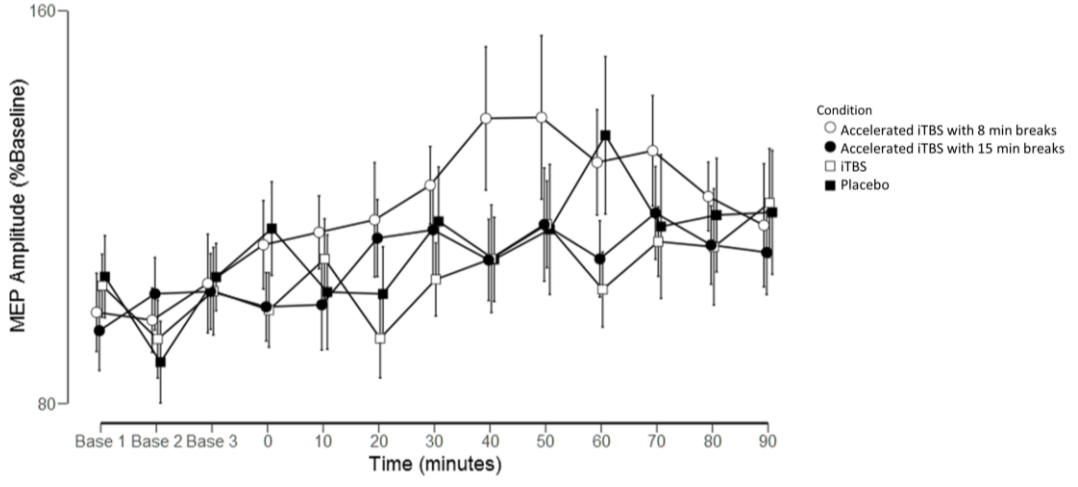


Figure 2. Baseline-normalized MEP amplitude changes over time. Three baseline measurements were taken (Base 1-3) before stimulation, then measurements every 10 minutes from 0-90 minutes following stimulation. Presented error bars are standard error of the mean.

Placebo subtracted

After subtraction of placebo, we found no significant main effect of CONDITION (accelerated iTBS with 8 minute breaks, accelerated iTBS with 15 minute breaks, iTBS; $F(1.509, 19)=1.830$, $p=0.185$) or TIME (0-30, 30-60, 60-90 minutes; $F(1.833, 19)=0.056$, $p=0.934$), and no interaction ($F(2.954, 19)=1.829$, $p=0.153$). Results, including baseline as a reference for starting values, are shown in Figure 3.

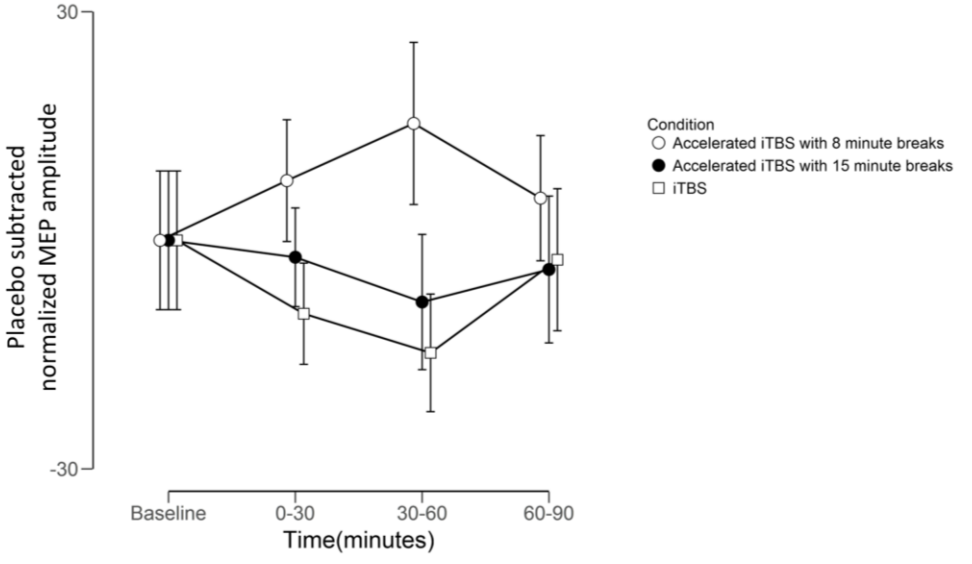


Figure 3. Baseline-normalized, placebo subtracted MEP amplitude changes over time. Three baseline measurements were averaged, and 30-minute time bins were calculated for the post-rTMS measurements. Presented error bars are standard error of the mean.

Total Response 0-60 minutes after stimulation

Overall, we found no significant effect of accelerated iTBS or iTBS motor cortex stimulation on MEP amplitude, both when baseline-normalized and when subtracted from placebo. In post-hoc analysis, we aimed to better understand the pattern of responses; why they were so variable, and if we could predict responsiveness to one stimulation protocol based on responsiveness to another stimulation protocol. In our iTBS protocol, 40% of participants responded with a decrease in MEP amplitude, i.e. the 'opposite' response. We hypothesized that certain protocols, for example; accelerated iTBS with 8-minute breaks, produced fewer opposite responders.

Based on the literature, we should expect the greatest potential modulation of MEP amplitudes in the 0-60-minute time window (10). To investigate our responses in this time interval, we took a grand average of percentage baseline MEP amplitude from 0-60 minutes post stimulation. Per condition, this allowed classification into facilitated response (greater than 110% of the baseline MEP amplitude), inhibited response (less than 90%), or 'Unchanged' (between 90% and 110%). Results are shown in Figure 4. For none of the iTBS conditions was the distribution of responses altered relative to placebo (Stuart Maxwell test for marginal homogeneity, p's>0.05).

Opposite Responders

If we consider only the inhibited responders compared to the other responders (facilitated or unchanged), using a χ^2 McNemar's exact test we found that neither

accelerated iTBS with 8 minute breaks; χ^2 (1, N=20) =0.208, p=0.289; Accelerated iTBS with 15 minute breaks; χ^2 (1, N=20) =0.469, p=0.344; nor iTBS χ^2 (1, N=20) =0.035, p=1.000 show a significantly different number of opposite responders than placebo.

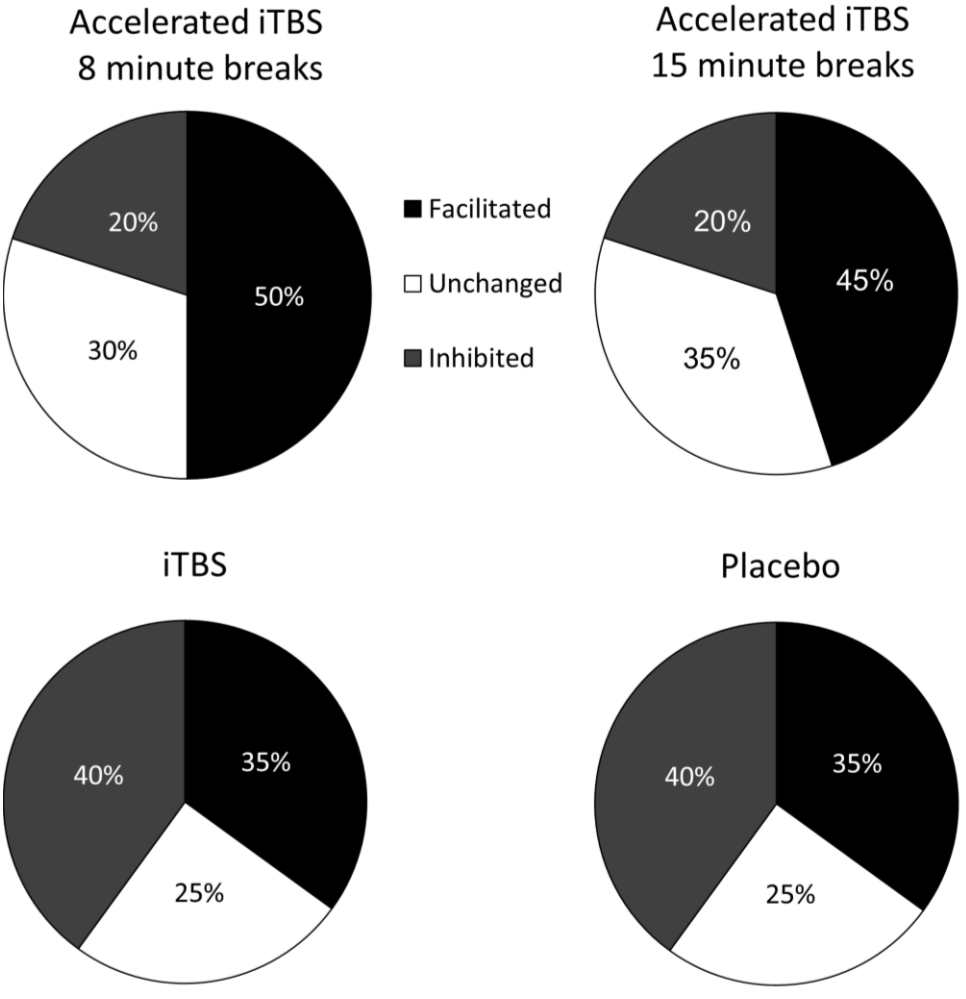


Figure 4. Response distribution of participants following stimulation protocol. Responses from 0-60 minutes following stimulation were averaged. Greater than 110% baseline MEP amplitude was counted as a facilitated response, less than 90% was counted as an inhibited response, and between 90% and 110% was counted as an unchanged response.

Responders vs. Non-Responders

Above, we performed RM-ANOVAs to assess the effects of stimulation condition and time on normalized MEP amplitudes, finding no effects. Here, we wanted to evaluate whether these analyses yield different outcomes if performed separately for

responders and non-responders. Since partitioning the participant sample into 3 response categories (facilitated, unchanged, inhibited) results in small group sizes, for these analyses we split participants into 2 groups; responders or non-responders. To do this, we first took a grand average of percentage baseline MEP amplitude from 0-60 minutes post stimulation, and then subtracted the grand average of the placebo stimulation. A response of greater than 0 was classified as a responder, smaller than 0 as a non-responder. A RM-ANOVA was run with TIME (0-30mins, 30-60 mins, and 60-90 mins) and CONDITION (the 2 conditions that the underlying response classifications were not based on). Graphs are shown in Figure 5.

Single iTBS: 11 participants classified as iTBS responders. In this subsample, there was no effect of CONDITION (F(1, 10)=0.901, p=0.365), TIME (F(1.778, 10)=0.457, p=0.618), and no interaction (F(1.097, 10)=0.308, p=0.610). Therefore, those who respond to iTBS are likely to respond to accelerated iTBS with 8- and 15-minute breaks as well. 9 participants classified as iTBS non-responders. There was no effect of CONDITION F(1, 8)=4.609, p=0.064, TIME F(1.446, 8)=0.432, p=0.597, and no interaction F(1.323, 8)=1.931, p=0.195. Those who do not respond to iTBS are unlikely to respond to accelerated iTBS with 8- or 15-minute breaks.

Accelerated iTBS (15-minute breaks): 12 participants classified as responders to accelerated iTBS with 15-minute breaks. In this subsample there was no effect of CONDITION F(1, 11)=0.248, p=0.628 and no interaction F(1.848, 11)=0.581, p=0.555. There was, however, a significant effect of TIME F(1.884, 11)=4.711, p=0.022. Bonferroni-corrected post-hoc comparisons revealed only a significant effect between 30-60 minutes and 60-90 minutes (t (11) =-3.107, p=0.030). The responders to accelerated iTBS with 15-minute breaks are also likely to respond to accelerated iTBS with 8-minute breaks, and to iTBS, with the strongest response at 30-60 minutes post stimulation. 8 participants classified as non-responders. There was no effect of TIME F(1.590, 7)=0.880, p=0.436, and no interaction F(1.647, 11) =2.638, p=0.120. There was, however, an effect of CONDITION; F(1, 7) =9.216, p=0. 019. This suggests that for those who do not respond to accelerated iTBS with 15-minute breaks, accelerated iTBS with 8-minute breaks may be a good alternative, not producing an opposite response. This is important as opposite responses are detrimental during clinical treatment.

Accelerated iTBS (8-minute breaks) There were 14 participants who classified as responders to accelerated iTBS with 8-minute breaks. In this subsample there was no effect of CONDITION F(1, 13)=0.113, p=0.743, TIME F(1.471, 13)=0.863, p=0.406, and no interaction F(1.473, 13)=1.073, p=0.342. Thus, participants who respond to accelerated iTBS with 8-minute breaks are likely to respond to all stimulation conditions. The other 6 participants classified as non-responders. There was no effect of CONDITION F(1, 5)=0.141, p=0.723, TIME F(1.047, 5)=0.253, p=0.646, or an

interaction effect $F(1.841, 5)=0.318$, $p=0.719$. Those who do not respond to accelerated iTBS with 8-minute breaks are likely to not respond to the other 3 conditions.

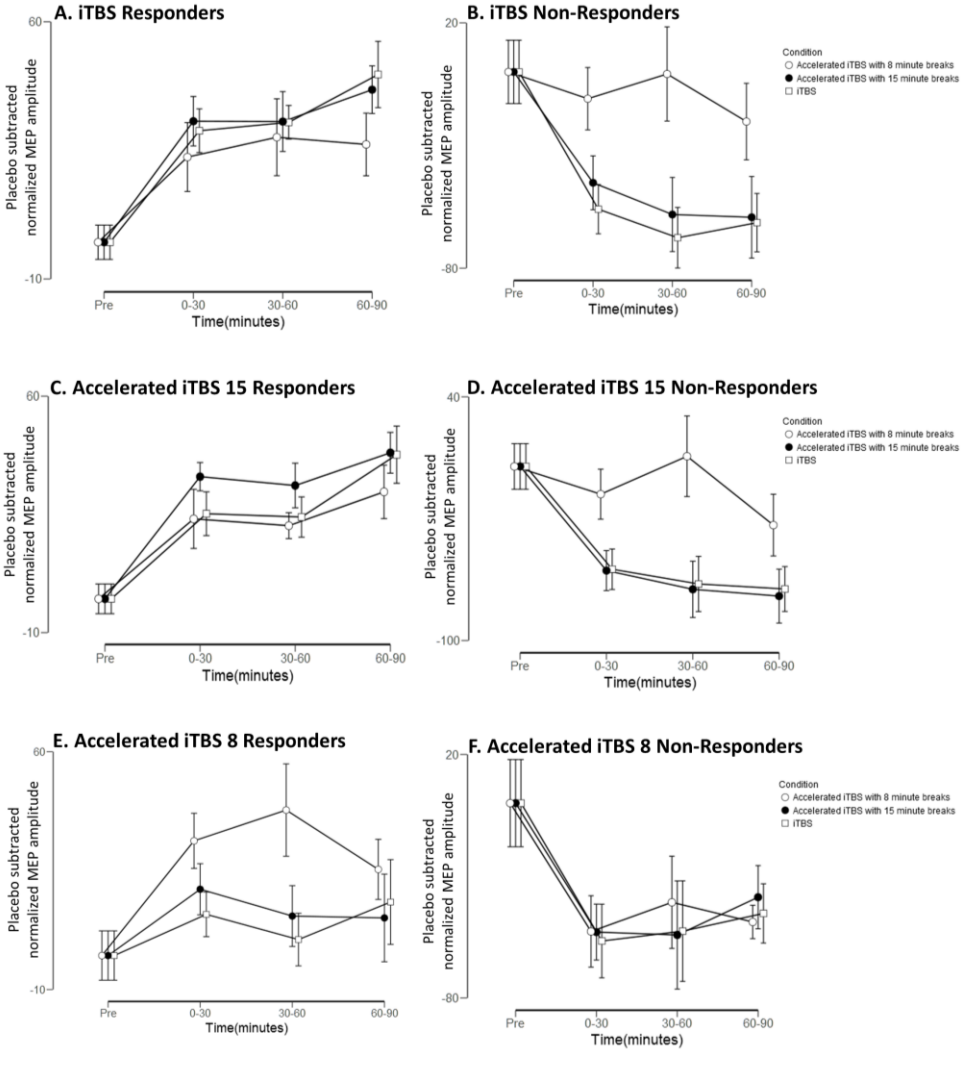


Figure 5. Responders and non-responders for each different stimulation protocol. Grand average of percentage baseline MEP amplitudes from 0-60 minutes following stimulation was calculated. Placebo grand average from the same time window was then subtracted. Responders showed a response greater than 0. Non-responders showed a response less than 0. **A.)** Responders to iTBS. **B.)** Non-responders to iTBS. **C.)** Responders to Accelerated iTBS with 15-minute breaks. **D.)** Non-responders to accelerated iTBS with 15-minute breaks. **E.)** Responders to Accelerated iTBS with 8minute breaks. **F.)** Non-responders to Accelerated iTBS with 8-minute breaks. Presented error bars are standard error of the mean.

Variability as a covariate

To account for participants' individual variability, we calculated total individual variance by taking the variance of the 90 baseline MEP's, across all sessions. When this between-subject variability was added as a covariate, we found a significant interaction between TIME and CONDITION (F(4.069, 1)=2.808, p=0.031). However, there was also a significant interaction between TIME and CONDITION and the covariate (total variance) (F(4.069, 1)=4.248, p=0.001). Therefore, we centred the covariate around either 1 standard deviation below (0.010) or 1 standard deviation above (0.574) the mean total variance. When centring the covariate on the high variance value, we see a significant effect of TIME and CONDITION at 30-60 minutes (F(3,16)=3.914, p=0.028), with pairwise comparisons indicating a significant difference (Sidak-adjusted for multiple comparisons) at 30-60 minutes post stimulation between accelerated iTBS with 8 min breaks and iTBS (p=0.027). This suggests that if the participant has high baseline MEP variance, accelerated iTBS with 8-minute breaks is significantly better than iTBS at increasing MEP amplitude at 30-60 minutes post stimulation, however further research is needed to confirm this finding.

Discussion

In 2005, Huang and colleagues presented the theta burst stimulation (TBS) protocols, a set of novel patterned rTMS protocols which were able to alter cortical excitability for 60 minutes following stimulation (10). Since then, an increasing number of studies have used these protocols, not only in motor cortex but also in other brain areas, and as a treatment for various movement, psychiatric and neurological disorders (For review, see: (20)). However, TBS protocols have also shown large inter-subject variability and reproducing the initially published effects has proven difficult (1, 2, 9, 21, 22).

Recently, optimization of the TBS protocols has included administering multiple TBS sessions in a single day (23). Therapeutically, accelerated iTBS protocols condense five iTBS sessions into one day (5, 24). Accelerated iTBS protocols have been successful in depression treatment, where recent studies have found the treatment to be safe and tolerated (4, 5), with the additional benefit of condensing a treatment schedule which would normally consist of daily sessions for four to six weeks, into a few days (13).

We decided to investigate the potential additive plasticity effects of these accelerated iTBS protocols over the motor cortex in healthy volunteers. We were also interested if the time interval between iTBS protocols had an effect on cortical excitability changes. Specifically, we were interested in if we could find stronger and less variable iTBS effects if we reduced the time interval between iTBS sessions.

In this protocol testing study, we found there to be no effect of iTBS on MEP amplitude over time. Interestingly, we saw an increase in MEP amplitude across all protocols, including placebo. There was, however, no difference between a single iTBS, accelerated iTBS with 8-minute breaks, accelerated iTBS with 15-minute breaks, and placebo.

Homeostatic Plasticity

We hypothesized that accelerated iTBS exerts its effects through different plasticity mechanisms than a single session of iTBS, which would be represented through MEP amplitude. This is because there is evidence that neural networks have the ability to monitor previous activity, and to modify subsequent firing rate (25). This activity-dependent plasticity, is critical for the encoding of new information, and the tuning of low activity connections. However, to avoid destabilization, other mechanisms must exist to maintain a homeostatic range of neural activity (26). These theories are relevant to brain stimulation research, where neural activity can be non-invasively altered, and resulting neural activity can be measured (for review see (15, 27, 28)). For example, taking homeostatic plasticity into account, we could hypothesize that one iTBS session would raise the threshold of neuron firing, so that the next session; if given in an appropriate time following the previous session, would result in decreased MEP amplitude.

This has been shown in several studies, for example priming with an identical TBS session (iTBS-iTBS) or (cTBS-cTBS) caused a reversal of expected effect directionality (28). Similarly, sessions that last twice as long compared to standard TBS showed a reversal of effects (16). A recent study repeated iTBS sessions at either 5 or 15-minute intervals, and found a reversal of effects (a decrease in MEP amplitude) when iTBS sessions were administered 5 minutes apart, and an enhancement (increase in MEP amplitude) when two iTBS sessions were administered with 15 minutes between (3). Similar to our results, they reported no significant effect of a single iTBS session, with only 33% of participants showing a facilitated response, 27% no change, and 40% an inhibited response. As a comparison, in our study we found 35% showed a facilitated response, 25% no change, and 40% an inhibited response. On the group level, only 53% of their participants showed a facilitation of MEP amplitude following their repeated iTBS sessions with 15 minutes in between (3). This is similar to our findings, where 45% showed a facilitation of MEP amplitude following accelerated iTBS with 15-minute breaks.

Inter-Session Interval

We found no differences in MEP modulation by single vs accelerated iTBS, which is seemingly in contrast to reports of clinical efficacy of accelerated iTBS (4, 5, 14). One explanation for this discrepancy might be that our measurements were constrained to a single day, whereas clinical effects often emerge after two weeks or more (13, 14).

Additionally, in the clinic patients received 20 iTBS sessions over four days (5, 14), while in our study healthy participants received only five iTBS sessions. Yet, if iTBS or accelerated iTBS do indeed have clinical effects, they must be through plastic mechanisms affecting cortical excitability, which should be measurable in motor cortex. One potential reason why we see no such effects of accelerated iTBS might lie in the short intervals between repeated iTBS protocols. Animal studies in rat hippocampal slices have shown that a delay of about one hour between iTBS sessions was necessary for additive LTP effects to occur (29). Specifically, the longer inter-protocol intervals were required for recruitment of synapses that were not affected by the first iTBS stimulation. The first iTBS session is thought to induce LTP in low-threshold synapses, and to lower the threshold for higher-threshold synapses. Shorter breaks of 10-30 minutes are not long enough for the high threshold synapses to be lowered (29). There is evidence that 40-50 minutes is required for the initiation and protein synthesis of the synaptic machinery necessary for refractory LTP effects in synapses with different plasticity thresholds (29, 30).

Recently, increasing the inter-protocol interval to 50 minutes has been successful in the treatment of severe depression (31). This could explain our results have not found an effect on MEP amplitude of repeating iTBS sessions with short (up to 15 minute) time between sessions.

MEP variability

Finally, substantial inter-subject variability has been reported among studies relying on MEP's to assess cortical excitability (1, 2, 9, 21, 22). We also found large inter-subject variability between our participants (Figure 6). In addition to this, intra-subject variability can be seen between different experiment days, as found by a recent study in our group comparing iTBS effects in the same subjects over their first and second visits (8).

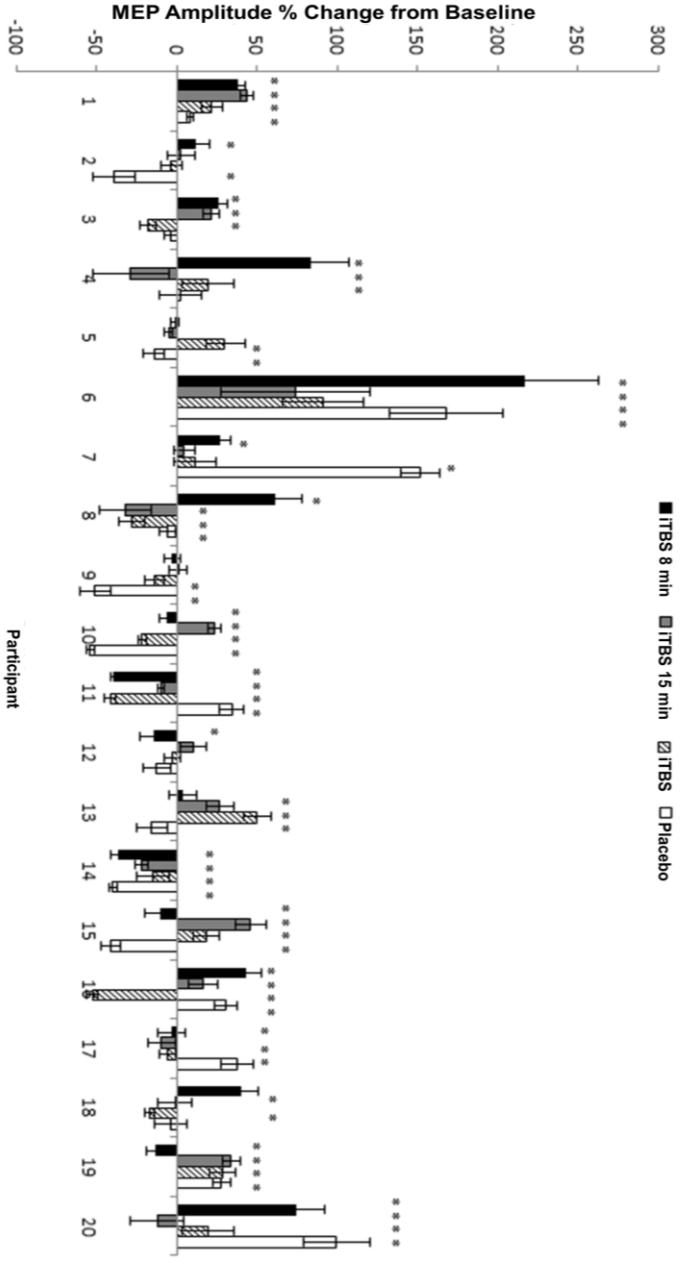


Figure 6. Variability of MEP responses. Individual baseline-corrected MEP grand averages from 0-60 minutes post stimulation are plotted, and 2-sided t-tests against 0 were performed for each condition, for each subject. Significant t-tests are marked with a *.

Conclusion

In this accelerated iTBS protocol testing study, we found no effect of iTBS on MEP amplitude on the group level. We found that a single iTBS session did not increase MEP amplitude, which has been shown previously (1, 2, 9, 21, 22). We also found no evidence for accelerated (5 times) iTBS with short breaks between iTBS sessions to be a more effective alternative, with no difference between 8 or 15-minute breaks. Our results could be explained by evidence from animal research, where at least 45-minute breaks are required for additive efficacy of iTBS sessions (29, 30). Accelerated iTBS with longer intervals between sessions may also be effective as a depression treatment (31). As further, exploratory analysis, we computed post-hoc analysis of response patterns. We were able to characterize an 'opposite responder', i.e. a response in the other direction than expected. This is important in clinical applications, where an opposite response is detrimental to recovery. Future studies should consider the high variability and patterns of response to brain stimulation protocols. Established protocols such as iTBS cannot be assumed to increase cortical excitability at the individual or even group level, which has implications for studies using brain stimulation on other cortical areas for their involvement in cognitive tasks. Moreover, we found no evidence of potential additive plasticity effects through accelerated iTBS protocols, in which several sessions are repeated within one day with short inter-session intervals of up to 15 minutes.

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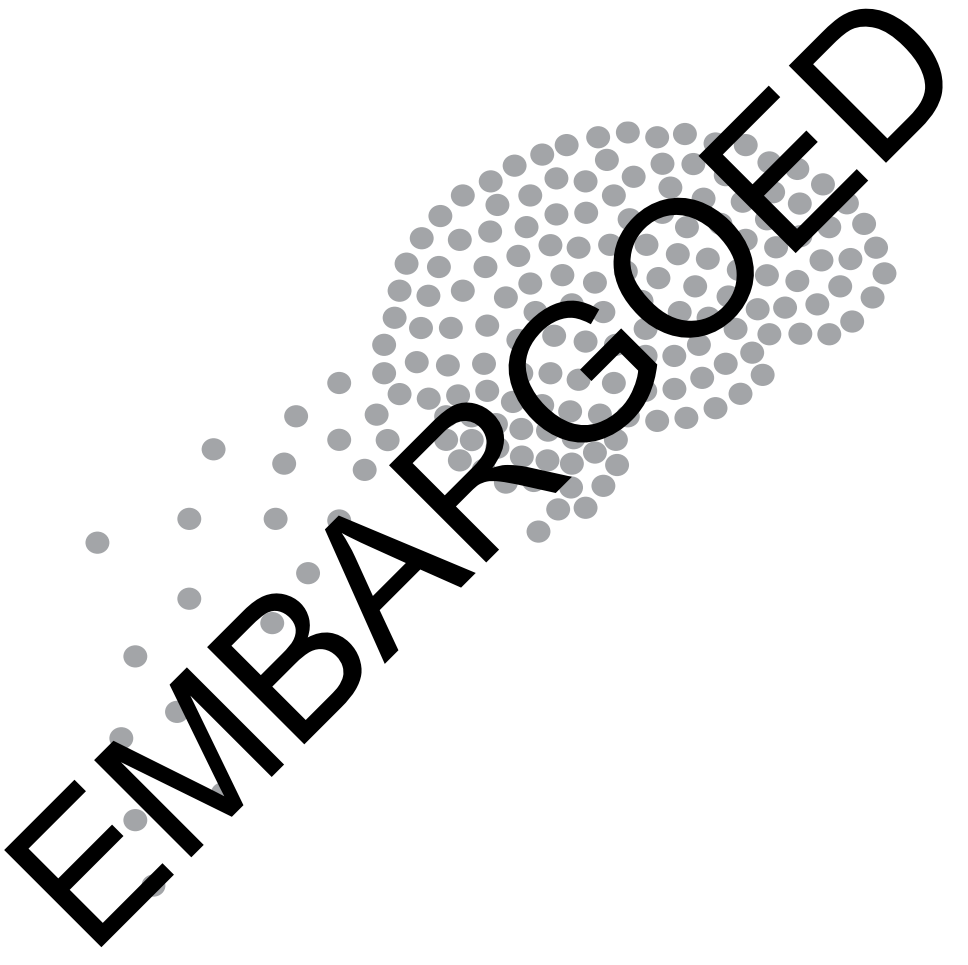
Chapter 7

iTBS does not induce neuroplasticity in insulin resistant patients or matched controls



Based on: Thomson, A.C.*, Schilberg, L.*, Schuhmann, T., de Graaf, T.A., ten Oever, S., Sack, A.T. iTBS does not induce neuroplasticity in insulin resistant patients or matched controls. *In preparation*

* Equal contribution

Chapter 8

Simultaneous TMS-EEG-fMRI to visualize TMS-induced plasticity effects within the depression network

Based on: Thomson, A.C.*, de Graaf, T. A.*, Janssens, S.J.W., Henauer, S., Duecker, F., Schuhmann, T., Sack, A.T. Simultaneous TMS-EEG-fMRI to visualize TMS-induced plasticity effects within the depression network. *In preparation*

*Equal contribution

Chapter 9

General Discussion

The overall aim of this thesis was to investigate the underlying neuroplastic mechanisms induced by transcranial magnetic stimulation (TMS) in humans, through an interdisciplinary series of studies using a variety of techniques. The experimental chapters of this thesis describe six different studies, beginning with three in an *in vitro* human neuronal cell culture model, and moving to three in human participants. In human neurons, the aim was to investigate the plasticity-related mechanisms induced by both excitatory and an inhibitory repetitive TMS (rTMS) protocols, specifically through measuring neural activity (calcium imaging), gene expression, and morphological and structural changes. We found evidence for immediate changes to cell activity following stimulation, but few changes in gene expression and morphology. In humans, the aim was to use indirect measures of assessing plasticity after the same excitatory protocol used in the first three chapters. We found in healthy participants that repeating multiple stimulation sessions in a single day did not promote additive plasticity effects. We also did not find evidence that TMS could be used to assess plasticity in participants with altered neuroplasticity (insulin resistance). Finally, we show using concurrent EEG-TMS and fMRI that excitatory stimulation to left dorsolateral prefrontal cortex (LDLPFC) was able to promote activation in several important cortical and subcortical structures. Overall, from stimulation of living human cell cultures to human participants rTMS has been used in this thesis to induce and investigate neuroplastic changes with a range of microscopic to macroscopic outcome measures.

Summary of findings

This thesis begins with molecular studies *in vitro*, and moves towards TMS stimulation of human participants. For the molecular, *in vitro* studies, the human SH-SY5Y cell line was introduced. SH-SY5Y cells are derived from a human neuroblastoma, and they can be differentiated *in vitro* to a mature, neuronal-like state (1-3). They can be used as a model of human neuronal plasticity, as they express many plasticity-related genes as well as morphological and functional characteristics of mature neurons (3-5).

SH-SY5Y cells are grown in a cell culture medium that is commonly supplemented with 3-10% fetal bovine serum (FBS, serum). To differentiate SH-SY5Y cells to a mature state, retinoic acid (RA) is often added to the cell culture medium, and the concentration of supplemented serum is reduced, for example from 10% to 3% (1, 6, 7). Once fully differentiated, the remaining supplemented serum is commonly removed from the culture medium before experimentation. This is to synchronize the cells to the same phase of the growth cycle (8) as well as to remove all growth factors and proteins which may have confounding effects on the experimental intervention (9, 10). However, the acute effects of such a complete serum removal from

differentiated SH-SY5Y cells had not previously been examined, in particular the effects on plasticity gene expression and structural outcome measures.

In Chapter 2**,** the effects of serum removal on gene expression and morphological markers of plasticity were assessed. We found that serum removal from differentiated SH-SY5Y cells does cause acute changes in plasticity markers, in particular in the gene expression and morphological outcome measures, which we were interested in investigating after iTBS and cTBS. Therefore, in all subsequent chapters we did not deprive differentiated SH-SY5Y cells of serum before stimulation. This chapter also provides important information for future studies using differentiated SH-SY5Y cells as a model for plasticity effects on gene expression and morphology. We show that the removal of serum causes acute changes in the expression of genes related to plasticity and in neuron morphology, which may mask any plasticity effects of the particular intervention. It is therefore important to consider the impact of serum removal before experimentation, and perhaps refrain from or thoroughly verify that such acute removal would not affect specific target genes/markers of interest before applying the intervention.

Chapter 3 describes the first in-vitro study using the theta burst stimulation (TBS) protocols on human neuron-like cells in culture. We were first interested in whether stimulation with intermittent TBS (iTBS), a three-minute protocol assumed to have excitatory effects, and continuous TBS (cTBS), a 40-second protocol assumed to have inhibitory effects (11) have opposite effects on immediate neuronal excitability. To do this, we used differentiated SH-SY5Y cells to investigate functional changes in neuronal activity, as measured through live calcium imaging. Cells were incubated with a fluorescence calcium indicator (Fluo-4AM, Thermo Fisher) which binds calcium at concentrations in the 100nM-1mM range (12). Resting calcium levels in neurons are between 50-100nM (13), therefore there is almost no fluorescence signal detected at baseline. Cells were then stimulated with iTBS, cTBS, or sham stimulation. Immediately after stimulation, there was a slight increase in fluoresce levels, however, when a depolarization was chemically induced with 1M KCl, a large increase in fluorescence levels was recorded. Importantly, cells that had been stimulated with the excitatory protocol (iTBS) showed a stronger increase in fluorescence compared to sham stimulated cells. Cells stimulated with the inhibitory protocol (cTBS) showed a lower fluorescence response to chemical depolarization compared to sham stimulated cells. These results provide support for the expected opposite effects of iTBS/cTBS on neuronal excitability, namely that iTBS can increase and cTBS can decrease neural responsiveness to subsequent depolarization. Further research in more complex human neuron models is needed, however these results provide preliminary support for the generally assumed effects of these two commonly used protocols, as well as provides a potential outcome measure for assessing the responsiveness of neurons to different rTMS protocols.

To investigate the longer-lasting effects of iTBS and cTBS, we chose to measure changes in gene expression and neuron morphology. This is done in Chapter 4, using the same human neuronal model (differentiated SH-SY5Y cells) as in Chapter 3. Gene expression changes specifically related to plasticity were measured, as well as morphological changes in the organization of proteins βIII-Tubulin and MAP2, which have also been related to plasticity (14-17). We found that stimulation did not lead to dramatic morphological or gene expression changes in any of the plasticity markers measured. There was however a slight increase in two genes we measured, *NTRK2* and *MAPK9,* 24 hours after stimulation. iTBS has been shown to increase excitability, as shown in studies in human motor cortex (11), as well as in SHSY5Y cells in Chapter 3 of this thesis. Therefore, an increase in the expression of these genes may indicate plasticity processes induced by iTBS.

Thus, in Chapters 2-4 the human neuronal cell model (differentiated SH-SY5Y cells) was introduced and established, and the effects of rTMS stimulation on immediate neuronal activity, gene expression, and morphology in this model were described. Evidence for increased neuronal excitability was shown, as well as some support for iTBS-induced plasticity effects on gene expression.

There are benefits to using a human *in vitro* neuronal model to measure the plasticity-inducing mechanisms of rTMS, which are further discussed below. However, it is important to complement human *in vitro* studies with *in vivo* ones. There are many large differences between the human brain and human neurons grown in a dish, and to fully understand and optimize rTMS for use in research and clinically, its effects need to be examined across all levels. Chapter 5 offers an example of how cellular and animal studies can be used to inform and design rTMS protocols for use in the clinic. This chapter reviews the concept of metaplasticity, and the importance of fundamental research to inform the necessary timing between subsequent rTMS sessions to maximize stimulation effects. This review is also important in the interpretation of the results of Chapter 6**,** where iTBS sessions are repeated five times in a single day in order to maximize excitatory effects of stimulation.

In the first chapter involving healthy human participants (Chapter 6), we were interested in investigating the effects of 'accelerated iTBS', a protocol consisting of repeated iTBS sessions in a single day, which has shown efficacy in the treatment of depression (18, 19). Despite its success in the clinic, the efficacy of accelerated iTBS over motor cortex using motor evoked potentials (MEPS) as an outcome measure, had yet to be shown. In this chapter, the effects of accelerated iTBS on corticospinal excitability (using MEPs) for up to 90 minutes following the stimulation were assessed. Effects on MEP amplitude were compared to that after a single iTBS session, and to sham. In a fully within-subject design, we found that there was no effect of accelerated iTBS on motor cortex excitability compared to sham. As discussed in

Chapter 5, evidence from animal studies showed that longer breaks (60 minutes) may be required to maximize excitability effects in repeated stimulation protocols. This could explain why, in Chapter 6, we were unable to measure stronger excitability effects, as the breaks between stimulation sessions were maximally 15 minutes and thus may have been not long enough to promote measurable neuroplastic effects.

However, in Chapter 6, the effects of iTBS on neuroplasticity were assessed only through motor evoked potentials (MEPs), which can be influenced by many sources of variability (biological, experimental, etc. (20)). Therefore, in Chapter 7**,** MEPs were combined with TMS-evoked potentials (TEPs) to assess excitability after iTBS. Additionally, this chapter investigated iTBS-induced neuroplasticity in type II diabetes (T2DM) patients, known to have altered neuroplasticity mechanisms. T2DM patients (and high-BMI matched control participants) were classified using blood samples as having insulin resistance (IR) or as being matched controls. The aim of this study was to investigate whether the degree of insulin resistance correlated with TMS-based measures of excitability (MEPs, TEPs). We found no difference in TMS-based neuroplastic responses between the IR and matched control groups, and no correlation between IR and TMS-based measures of excitability. However, we did not find evidence for iTBS-inducing neuroplastic mechanisms in our control group, indicating that future studies using a more effective plasticity inducing protocol, such as accelerated iTBS, are needed to draw any conclusions from this clinical population.

In Chapters 6 and 7, iTBS protocols were applied to either a healthy or clinical population, and TMS-based measures of assessing neuroplasticity (MEP/TEP's) were used. In both these studies, we found no effect of iTBS stimulation on promoting neuroplasticity when stimulating the motor cortex. This adds to recent reports from other groups showing difficulties in replicating the assumed excitatory effects of iTBS (21-23).

In the clinic, iTBS has been shown to be an effective option for the treatment of depression (24), where stimulation is delivered to frontal cortical areas such as the DLPFC (24-26), as opposed to the motor cortex. Therefore, it would be beneficial to assess neuroplastic effects of iTBS directly in the DLPFC rather than the motor cortex.

The study described in Chapter 8 uses a multimodal approach combining TMS, EEG and fMRI, pioneered several years ago at Maastricht University (27, 28), to examine the neuroplastic effects of iTBS in the DLPFC. This chapter presents preliminary results of a within-subject design on eight healthy participants. Offline iTBS stimulation (or sham) was delivered to the left DLPFC, followed by concurrent single TMS-pulses in the 3T MRI, while simultaneously recording the EEG signal. The project is ongoing, but preliminary results suggest that TMS pulses to the DLPFC are able to activate deeper cortico-limbic structures such as the anterior cingulate cortex (ACC) and insula, and

that alpha power can modulate the signal elicited by high intensity TMS pulses at subcortical structures.

Throughout the six experimental chapters in this thesis, the neuroplasticity mechanisms induced by TMS have been investigated using several different techniques and experimental setups. The effects of commonly used rTMS protocols iTBS and cTBS have been assessed in a human neuronal cell culture model (neural activity, gene expression, morphology), a healthy human population (MEPs), a clinical human population (TEPs, MEPs), and finally in a multimodal approach combining TMS-EEG and fMRI. Our human neuronal cell model was used to establish strong functional effects of iTBS and cTBS, as recorded using live calcium imaging (Chapter 3), while effects on plasticity-related gene expression and neuron morphology showed fewer clear differences between iTBS and cTBS over several later time points (Chapter 4). In two of the chapters describing human studies (Chapters 6 and 7), we were unable to replicate the established effects of iTBS on MEP amplitude. This difficulty replicating the expected iTBS effects has been reported by several other studies (21, 22, 29). Limitations of the findings *in vitro* and of the indirect human neuroplasticity outcome measures *in vivo* are described in the section below.

A human neuron-like model for assessing TMS effects

SH-SY5Y human neuroblastoma cells were chosen as a human neuronal model for the studies described in Chapters 2-4 of this thesis, as they are relatively easy to culture, can be fully differentiated to a mature neuronal-like state (1, 2, 7), and express mature neural markers and functional synapses which have been well documented in the literature (3, 30-33). This makes them a good model for exploratory, pilot studies such as those described in this thesis.

Why move to human *in vitro* neural models?

Animal models have been critical in advancing our understanding of the underlying mechanisms of TMS (for reviews; see (34-36)). Animal models have provided evidence for the hypothesized opposing neuroplastic effects of iTBS and cTBS (37-39), and showed that TMS is capable of inducing an immediate release of intracellular calcium following stimulation (40, 41). However, there are several key aspects which limit the use of animal models when modelling the complexity of the human brain (42). Several animal studies have been carried out in cat (43) and non-human primates (44-46), though most studies have used rodents or cell cultures derived from rodents in TMS studies (37-41, 47-52). Despite the obvious difference in size and organizational complexity between the human and rat brain, human neurons also show different gene regulation and expression patterns (42, 53), and different baseline neuronal excitability (54-56). Thus, as TMS is thought to work through altering neuronal excitability (57), it is important to use human neurons to verify findings from animal models. Before the experiments described in this thesis, only two studies had used a

differentiated SH-SY5Y cells to investigate the effects of rTMS (33, 58), and none had investigated the effects of iTBS/cTBS in a human neuron or neuron-like model.

Chapters 3 and 4 are the first to describe the effects of iTBS/cTBS on calcium activity, gene expression, and morphological markers of plasticity in a human neuron-like model. In contrast to animal studies (38, 39, 59), in Chapter 4 we did not show strong, opposite effects of iTBS/cTBS on plasticity markers. However, this may be due to the different stimulation parameters used, and the lack of cortical organization and inhibitory interneurons in our human neuronal setup. We stimulated at 100% maximum stimulator output, in order to ensure that TMS was able to induce activity in our cells. This is verified by our findings from Chapter 3, which convincingly show that iTBS and cTBS are able to immediately induce changes in response to chemical depolarization using calcium imaging. However, animal studies often repeat iTBS/cTBS protocols several (up to five) times (38, 39, 59), while we stimulated our cells with a single iTBS/cTBS protocol. Therefore, the many more stimulation pulses could explain our lack of strong findings in SH-SY5Y cells on the gene expression and morphological levels.

Additionally, our findings in Chapter 4 describing the gene expression and morphology effects after stimulation can also be explained by several limitations of our SH-SY5Y human neuron-like cell model. First, they are derived from human neuroblastoma cells; and while differentiation protocols aim to establish mature, neuron-like phenotypes in the majority of cells (1, 7), there is still dish-to-dish variation between cultures (6). For instance, there are different ratios of mature, neuron-like cells and undifferentiated, epithelial-like cells, which likely respond differently to TMS.

Second, SH-SY5Y cells develop a catecholaminergic-like phenotype, with the potential to synthesize dopamine and noradrenaline (30). They do not represent the mix of excitatory (glutamatergic) and inhibitory (GABAergic) neurons thought to be most involved in rTMS response (34), or the complex spatial organization of cortical columns within the human brain. Animal studies often investigate TMS effects over the whole cortex, or in slice cultures, which contain various neural cell types. Animal studies have found TMS effects to be specific to the cortical region (59), and largely working through inhibitory interneurons (37, 39, 48, 60-62) both of which are not represented in our SH-SY5Y setup.

In humans, many biological and experimental factors contribute to the high degree of variability associated with indirect assessment of TMS-induced neuroplasticity using MEPs (20, 21, 36). By performing experiments in a human neuron-like model, we show the value of systematically assessing the neuroplastic effects induced by iTBS/cTBS from the most basic level up. In Chapter 3 we show that iTBS can increase the excitability of neurons as expected. Future studies can build on this finding, in subsequently more complex human neural models (from iPSC-derived neurons, to

cerebral organoids, to human cortex), to better understand which layers contribute to variability of TMS responsiveness. Through *in vitro* human studies, these factors can be measured and controlled, and can contribute to a better understanding of how TMS is able to induce neuroplasticity in human neurons.

Future Directions of human *in vitro* modelling

Future studies using the SH-SY5Y cells could be informative by applying different TBS protocols (such as accelerated protocols, or repeating sessions up to five times as in some rodent studies (38, 39, 59)), different gene expression or morphological assessment techniques, or following up on protein/phosphorylation changes in these cells. However, based on the limitations of SH-SY5Y cells as a model for human neurons following TMS, it would also be beneficial to consider more complex, advanced human neuronal setups. The studies described in this thesis were important in piloting the setup in SH-SY5Y cells, and serve as a basis for future studies in more complex human *in vitro* neural models to better understand TMS neuroplasticity effects.

For example, it is now possible to take fibroblasts from a skin biopsy and transform them through treatment with a series of reprogramming factors to become induced pluripotent stem cells (iPSCs) capable of differentiating to any cell fate, including neurons (63). Since this ground-breaking discovery in 2006, protocols for direct differentiation from fibroblast to neural progenitor cells have become available (64-66). After differentiation to functional neural progenitors, neurons can then be further differentiated into specific neural phenotypes (glutamatergic, GABAergic, dopaminergic, serotonergic, motor neurons, etc.) (67). iPSCs can also organize into a 3D structure, or cerebral organoid, which can be used to model the complexity of neurodevelopment of the human brain (68, 69).

The development of these advanced, *in vitro* human neural model systems has allowed for progress in disease modelling, but also in personalized medicine. For example, TMS is widely used as a clinical treatment for a range of psychiatric and neuropsychological disorders (70). It is most widely used as a treatment for depression (25, 26, 71), however many patients are nonresponsive to treatment, with about a third of patients completing stimulation treatment in remission (72). iPSC-derived neurons offer the possibility to pre-screen patient-derived neurons for responsiveness to particular stimulation protocols, *before* undergoing TMS treatment. In this way, the parameters of stimulation protocols could first be optimized *in vitro*, which would likely improve the remission rate in the clinic. Building on the results presented in Chapter 3, one relatively quick way to assess whether neurons respond to specific TMS protocols could be to test their responsiveness to rTMS protocols with calcium imaging. This method could potentially verify whether patient-specific neurons respond to a particular stimulation protocol within a relatively limited time

window. This method would furthermore benefit from validation in iPSC-derived neural setups, while forming a proof-of-principle study of how *in vitro* human neural models and interdisciplinary research can be extremely valuable for the future of TMS applications.

Indirectly assessing neuroplasticity in humans

In the first half of the thesis, techniques such as calcium imaging, qPCR and immunocytochemistry were used to directly measure the molecular neuroplasticity changes induced by iTBS and cTBS in SH-SY5Y cells. In the second half, such a direct assessment of molecular changes following stimulation was not possible. The studies done in humans relied on indirect measures to assess neuroplasticity, such as corticospinal (MEP) or cortical (TEP) excitability assessment measures, as well as neuroimaging (fMRI). In Chapters 6 and 7, we report difficulty in replicating the established, excitatory effects of iTBS. In Chapter 6 and 7, we found no difference between MEP amplitude induced by sham, iTBS or accelerated iTBS. Essentially, we were unable to validate the assumption that iTBS increases cortical excitability in humans, as measured by corticospinal assessment (MEP amplitude).

This difficulty in replicating an iTBS-induced increase in corticospinal excitability as assessed through MEP amplitude has also been reported by several other groups (21, 23, 29). Our null findings do not necessarily indicate that iTBS does not work as previously hypothesized, but highlight the limitations of using MEP amplitude as an indirect assessment outcome measure following stimulation.

For example, the use of MEPs can be confounded by substantial variability related to both experimental and biological factors (20). Several uncontrollable neurobiological dynamics such as cortical network activity, developmental factors and neurotransmitter availability are thought to influence the variability of responses (36). Substantial intra-subject variability has also been reported, for example with subjects showing highly variable responses to iTBS stimulation on different experimentation days (22). To control for as many factors as we could, in Chapters 6 and 8 we planned all experiments (if possible) at the same time of day, 1 week apart, and told participants to maintain a normal routine and drink the same amount of caffeine as normal. While we aimed to control for as many confounding factors as possible, MEPs remained a variable outcome measure in these two studies.

Additionally, brain-state has been suggested as a factor contributing to variability of individual responses to rTMS protocols (73). For example, in a setup such as that in Chapter 6, where iTBS sessions were repeated multiple times within a single day, the effects of brain state can strongly influence results. Even the effect of the metal visualization of activity can prime the motor cortex and affect MEP amplitude (74-76). In Chapter 6, we aimed to control for this impact of mental visualization differences

between participants during the breaks between iTBS sessions. We did this by playing the same video clips to all participants, to hopefully maintain a relatively controlled group brain state.

An alternative to MEPs as an assessment of corticospinal excitability is to use simultaneous TMS-EEG, and to record TMS-EEG evoked potentials (TEPs). TEPs are recorded in the ongoing EEG signal, where positive (P) and negative (N) fluctuations at predictable latencies milliseconds after the TMS pulse (N15, P30) can indicate neural excitability (77-79). TEPs are not yet commonly used to assess neuroplasticity, however they have been shown to be highly reproducible (80, 81), in contrast to MEPs (22). In Chapter 7, we found no effect of iTBS on any TEP component measured (N15, P30, N15-P30). As this study was carried out in an elderly population of participants with high BMI or clinical T2DM diagnosis, it is difficult to draw conclusions on the use of TEPs to assess neuroplasticity, as iTBS may have been unable to induce the hypothesized neuroplastic effects in this clinical population. Since we also found no effect of iTBS on MEP amplitude, it is feasible that a single iTBS protocol is not sufficient to induce neuroplastic changes in this participant group. However, the accelerated protocols, such as those used in Chapter 6 may be a promising alternative. The mini-review in Chapter 5 also hypothesizes that accelerated protocols with longer breaks between stimulation sessions may be more effective at promoting stronger neuroplasticity effects in the clinic.

In this thesis, both Chapters 6 and 7 describe a lack of iTBS effects on indirect outcome measures; MEPs and TEPs. Additionally, in Chapter 4, gene expression and morphological markers of plasticity show little to no modulation by iTBS at the cellular level. Therefore, three chapters in this thesis do not provide support for the assumed excitatory-effects of iTBS. Importantly, we do show evidence for an immediate modulation of neural calcium activity induced by iTBS at the cellular level (Chapter 3). Cells that were stimulated with iTBS showed a stronger fluorescence response to chemical depolarization thank sham and cTBS stimulated cells. This fundamental finding in a simple, monolayer human neural setup is important to contrast with the null in vivo and *in vitro* findings described in Chapters 4, 6 and 7.

Homeostatic Plasticity

The underlying neuroplastic effects of rTMS are thought to work through synaptic plasticity, as well as by altering molecular mechanisms, which maintain a dynamic threshold for subsequent plasticity (82-85). Therefore, if the first stimulation primes the neuron for a particular direction of plasticity (for example LTP), homeostatic plasticity would act against this to promote plasticity in the opposite direction (LTD, in this example) following the subsequent stimulation. A full review on metaplasticity and its relation to brain stimulation studies can be found in Chapter 5.

Accelerated iTBS (Chapter 6) is also important to interpret in light of the mini review of metaplasticity in Chapter 5. With accelerated iTBS in particular, homeostatic metaplasticity mechanisms may act against intended stimulation effects. Accelerated protocols have also been shown to be effective when given with 50 minutes between stimulation (86), which aligns with animal research describing that 40-60 minutes between stimulation sessions is required to ensure additive LTP effects (87, 88). Accelerated iTBS protocols offer a promising alternative as an optimized treatment protocol, but it is important to consider homeostatic plasticity effects from *in vitro* studies, to best determine optimal spacing between stimulations.

Interdisciplinary Research

The research in this thesis aims to provide some insight into the neuroplasticity mechanisms induced by iTBS and cTBS. More generally, the interdisciplinary aspect (studies in both *in vitro* and *in vivo,* and using a range of techniques) of this research can highlight the value of these combined approaches. For example, as described in the mini review in Chapter 5, fundamental research on *in vitro* models is critical for determining the optimal parameters for clinical or research stimulation protocols in humans. Additionally, starting from a simple, unorganized neuronal model such as SH-SY5Y cells and building up to more complex *in vitro* models containing relevant functional organization can provide valuable information on processes influencing the large variability of rTMS responses in humans. Combining both fundamental, cellular studies where such stimulation protocols can be directly tested for effectiveness, and clinical studies where these protocols can be tested in patients are important. In the future, this presents the possibility for personalized medicine, by deriving patient or person-specific neural cells (iPSC-derived) and testing the effects of various stimulation protocols, before stimulation of the patient/person in the clinic or lab. This could greatly benefit the effectiveness of brain stimulation protocols.

However, there have been some additional challenges in the dissemination of some of the studies described in this thesis, which may be partially related to their interdisciplinary nature. For example, we have received many critiques of our studies, such as not fitting the scope of the journal, and not having a strong, realistic human neuronal model. While there are certainly limitations to the use of SH-SY5Y cells (discussed in detail in the discussion of Chapters 2,3 and 4 as well as above), the novel and interdisciplinary aspect of the studies was often overlooked. Therefore, in addition to the high-risk nature and variety of techniques in these studies, this difficulty with dissemination is an additional barrier that we have encountered in this interdisciplinary research.

Different perspectives, skill sets, and communication methods are critical to start unravelling the complexities of the human brain. For the studies included in this thesis in particular, if we are better able to understand the tools we use to study the brain,

such as TMS, we can move towards optimizing them, personalizing treatment options, and predicting the most successful outcome measures. The exploratory studies presented in this thesis provide a basis from which future research can build on, to hopefully better understand, develop and optimize rTMS protocols for patient/research use.

Concluding remarks

The main aim of this thesis was to investigate the neuroplasticity mechanisms induced by TMS (iTBS/cTBS) in humans. Starting from the neuronal level and working up to studies in human participants, the experiments described in this thesis begin to unravel the neuroplastic mechanisms induced by TMS, and pave the way for future understanding, optimization and maybe even personalization of TMS protocols.

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Appendix

-Impact paragraph

-Curriculum Vitae

-List of publications

-Acknowledgements

Impact Paragraph

"If all you have is a hammer, everything looks like a nail."
-Abraham Maslow, "The Psychology of Science", 1966

This thesis describes a series of experiments which advance our understanding of a widely used neuroscientific and therapeutic tool, Transcranial Magnetic Stimulation (TMS). In addition to the scientific and societal impact of the results described, this thesis is an example of approaching a research question from different perspectives and combining different disciplines, expertise, and research techniques to approach scientific bottlenecks. It is the first funded project in a newly established collaboration between the Faculties of Psychology and Neuroscience (FPN) and Health Medicine and Life Sciences (FHML) called the "Centre for Integrative Neuroscience (CIN)". This initiative has allowed for the bridging of research, expertise, techniques and communication between two different neuroscience disciplines at Maastricht University.

The research and main findings described in this thesis are important for advancing our understanding of how TMS exerts its effects. TMS is a form of non-invasive brain stimulation, which is widely used in neuroscience research around the world. It uses electromagnetic pulses to briefly and painlessly send electricity into the brain (1). For example, when a pulse is given over the motor cortex, it can directly activate the neurons beneath, carrying an electrical signal along a specific neural circuit to a finger muscle, and causing a visible finger twitch (2). When many of these pulses are repeated in a specific pattern (as repetitive, rTMS), longer-lasting stimulation effects can be induced, including increased or decreased activity of a particular brain region (3). In other words, giving a short round of rTMS over a particular brain region can deactivate or increase activity in that specific brain region for a short time after the stimulation is over. This is very useful in understanding what role a particular brain region plays in certain processes.

Perhaps the most important use of rTMS is in the clinic, where it is a treatment option for several psychiatric and neurological disorders. It is most commonly used as a treatment for depression (4-6), but obsessive-compulsive disorder (OCD), pain and stroke are some other examples of disorders being treated with rTMS (7). Despite the widespread use of rTMS both in research and therapeutically, the underlying mechanisms are relatively unknown. Understanding the very basic, molecular machinery which underly rTMS effects would allow us to design stimulation patterns to be the most safe and effective for research and clinical treatment. With advances in neuroscientific methods, it may even be possible personalize treatment protocols, but only with a strong background of research identifying reliable molecular targets of rTMS effects.

This thesis takes a unique, inter- and cross disciplinary approach to understanding the underlying molecular mechanisms of TMS. It is interdisciplinary for combining studies on a molecular level using human neurons grown in the lab (**Chapters 2-4**), with rTMS studies in human participants (**Chapters 6-8**). Several important findings are reported in these chapters, and they contribute to a better understanding of how rTMS is able to create lasting effects in humans. We show in **Chapter 3** that human neurons stimulated with different rTMS protocols respond in the expected way. Neurons stimulated with an rTMS protocol thought to *increase* the activity of a particular brain region were more strongly activated than neurons stimulated with an rTMS protocol thought to *decrease* it. Similarly, in **Chapter 4** we show evidence for increased expression of a few important genes after stimulation with the excitatory protocol, but more research is needed on the specific molecular pathways activated by the different stimulation protocols. In humans, we show that it can be difficult to replicate the expected effects of stimulation due to many sources of variability, which again supports that future research in human cellular models such as in Chapters 3 and 4 is important. We hypothesize that combining multiple stimulation sessions could enhance stimulation effects, and in the final chapter (**Chapter 8**) provide preliminary evidence that stimulation at one area of the brain could lead to activation of different, remote brain areas. Overall, the findings of this thesis both in human neuronal cell culture and in human participants add to our understanding of the underlying mechanisms of TMS, and offer suggestions for future research in this area.

One of the larger, societal impacts of these findings lies in the therapeutic potential of TMS. We are currently in the middle of a serious global pandemic, where many are forced to self-isolate, work from home and are burdened with financial and health worries. This understandably has a large impact on the mental health of millions worldwide, with likely consequences such as an increase in the global burden of depressive disorders for years to come (8).

Depression is one of the most severe mental health disorders, ranked by the WHO as the single largest contributor to global disability (9), and with several large studies consistently placing it within the highest for disease burden and disability adjusted life years (DALYS) (10-12). Depression therefore has a huge impact on the quality of lives of millions of people worldwide, having substantial social and economic consequences. In addition to severely reducing the quality of lives of people suffering from depression, the economic costs of depression are huge. Global estimates of costs due to lost productivity are in the billions (US dollars) (13), not to mention the burden on the healthcare system. This indicates the critical need for an effective and quick treatment option. The findings of this thesis highlight the potential of a short-duration rTMS protocol: intermittent Theta Burst Stimulation (iTBS), which requires only 3 minutes to apply, and has also been shown to be effective as a treatment for depression (14). iTBS is additionally promising as it is quick, and therefore could be

used to treat many people per day. This thesis provides support for iTBS as a treatment protocol, in particular when repeated several times in a single day (as accelerated iTBS). A review on this idea is also included as a chapter in this thesis.

There are several steps required for the findings described in this thesis to have a clinical impact, not only in the treatment of depression but also in the treatment of other mental and neurological disorders. First, more research needs to be done on the efficacy of accelerated iTBS with longer intervals (50-60 minutes). For instance, research with healthy participants in a similar setup as described in **Chapter 6**, but as proposed in **Chapter 5**, with three iTBS sessions separated by 50-60 minutes. If this is proven effective in healthy participants, large clinical trials could test whether it is also as effective as a treatment. Some potential disorders where this could be effective could be in cognitive decline and dementia, where patients could do cognitive enhancement tasks (memory games, reading or drawing tasks) during the long breaks between iTBS sessions. Similarly, as a treatment for depression, Cognitive Behavioral Therapy (CBT) could be done in the breaks between iTBS sessions, as this combination has proven to improve treatment efficacy (15).

At the molecular level, further research following up on the findings of this thesis using TMS and human neuronal cells is needed. Identifying specific, molecular mechanisms of rTMS effects could help us to understand why some people respond well to rTMS treatment, but others do not. This thesis provides evidence for calcium imaging being a potential indicator of iTBS efficacy (**Chapter 3**), but further research would be needed to confirm this. Studies could use patient-derived neurons to indicate whether a particular patient would be responsive to a particular stimulation protocol. For example, if a particular cellular response (such as calcium imaging) can indicate responsiveness to a certain rTMS protocol, then patients could have their neurons tested for responsiveness before undergoing rTMS treatment. Much more research is needed for this to be a realistic option in the future; however, this thesis provides a step in this direction towards identifying molecular targets to indicate rTMS efficacy, and to use these targets as predictors for patient responsiveness to treatment.

Future studies can use more advanced human neuron models to investigate different molecular targets, and can work with other TMS users from different disciplines to better understand the limitations and main research questions in their research areas. Combining input from experts in computational modelling, engineers and physicists who can work to develop optimal TMS coil designs, and researchers/clinicians who use TMS on human participants or can establish a neuronal cell model are all important in advancing our understanding of how TMS works. In conclusion, this thesis provides an example of the benefits of interdisciplinary research, and describes several important findings which have a larger societal and clinical impact. Notably in

the treatment of depression, but also for other applications, where support for accelerated treatment protocols as well as molecular targets for assessing stimulation responsiveness are reported. Future interdisciplinary research into these ideas will lead us to gain deeper insights into the underlying mechanisms of rTMS, and optimize its use in research and the clinic.

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Appendix

-Impact paragraph

-Curriculum Vitae

-List of publications

-Acknowledgements

Alix Charlotte Thomson was born on April 14th, 1991 in Vancouver, British Columbia, Canada. She grew up in Victoria, and moved back to Vancouver to begin her bachelors of science degree at the University of British Columbia. She was always interested in interdisciplinary science, and pursued a unique interdisciplinary sciences programme, combining courses in microbiology, immunology, and neuroscience. She also ran varsity cross country and track and field (marathon) and represented UBC at many (inter)national races in Canada and the USA. During her degree, she also went on exchange to the University of Glasgow, to study the molecular mechanisms of disease and stem cells. She was also accepted into the science co-op program, and spent an extra year gaining paid work experience in two different research labs across Canada. She spent eight months at a biochemistry lab at the Jack Bell Research Centre in Vancouver, and four months in a neuroscience lab the Université Laval in Quebec City.

Following her bachelors degree, she moved across Canada to begin a Masters of Science in Global Health at McMaster University across the country in Hamilton, Ontario, Canada. During this one-year masters program, she went on exchange to study healthcare innovation and transferability at Maastricht University, as well as a one month research stay at Manipal University in India.

After her masters degree, she moved back to Maastricht, and began working as a research assistant at Maastricht University in the Faculty of Psychology and Neuroscience (FPN) department of Cognitive Neuroscience in the group of Prof Dr. Alexander Sack. She first worked with Dr. Franziska Emmerling, as well as Dr.Tom de Graaf. In September 2016 she began her PhD in the group of Prof Dr. Alexander Sack, and in September 2017 began as the first PhD project funded by the newly established Centre for Integrative Neuroscience (CIN) in a collaboration between Prof Dr. Alexander Sack (FPN) and Prof Dr. Bart Rutten (Faculty of Health, Medicine, and Life sciences, FHML), and under the supervision of Dr. Tom de Graaf, Dr. Teresa Schuhmann and Dr. Gunter Kenis. She was the PhD student representative, both of the department of Cognitive Neuroscience at the FPN graduate school, but she also represented FPN at the Central PhD Candidates Platform (CPCP) of Maastricht University. In her last year of her PhD, she was awarded NENS best student's posted award at the FENS conference 2020. Upon completion of her PhD, she will continue to spread her passion for interdisciplinary science.

Appendix

-Impact paragraph

-Curriculum Vitae

-List of publications

-Acknowledgements

Journal Publications

Thomson, A. C., Schuhmann, T.G., de Graaf, T. A., Sack, A. T., Rutten, B. P. F., & Kenis, G. (2021). "The effects of serum removal on gene expression and morphological plasticity markers in differentiated SH-SY5Y cells" *Cell Mol Neurobiol*. 10.1007/s10571-021-01062-x.

Thomson, A. C., Sack, A.T. (2020). "How to design optimal accelerated rTMS protocols capable of promoting therapeutically beneficial metaplasticity" *Front. Neurol.* 11:599918. 10.3389/fneur.2020.599918

Thomson, A. C., Kenis, G., Tielens, S., de Graaf, T. A., Schuhmann, T., Rutten, B. P. F. & Sack, A. T. (2020). "Transcranial Magnetic Stimulation (TMS)-induced plasticity mechanisms: TMS-related gene expression and morphology changes in a human neuronal-like cell model " *Front. Mol. Neurosci.* 10.3389/fnmol.2020.528396

de Graaf, T.A., **Thomson, A.**, Janssens, S.E.W., van Bree, S., Sack, A.T. (2020). "Does alpha phase modulate visual target detection? Three experiments with tACS-phase-based stimulus presentation" *Eur J Neurosci.* 51:2299-2313. doi.org/10.1111/ejn.14677

Thomson, A.C., de Graaf, T. A., Kenis, G., Rutten, B. P. F., Schuhmann, T.*, & Sack, A. T.* (2019). " No additive meta plasticity effects of accelerated iTBS with short inter-session intervals" *Brain Stimulation.*12(5):1301-3. doi.org/10.1016/j.brs.2019.05.012. *Equal contribution

Emmerling, F., Martijn, C., Alberts, H. J., **Thomson, A. C.**, David, B., Kessler, D., Schuhmann, T., & Sack, A. T. (2017). "The (non-)replicability of regulatory resource depletion: A field report employing non-invasive brain stimulation" PloS one, 12(3), e0174331. https://doi.org/10.1371/journal.pone.0174331

Preprint Articles

Thomson, A. C., de Graaf, T. A., Schuhmann, T., Kenis, G.*, Sack, A. T.*, & Rutten, B. P. F.*, (2020). "Transcranial Magnetic stimulation (TMS) modulates excitability of SH-SY5Y cells: An in vitro model provides support for assumed excitability changes" *Preprint:* bioRxiv doi: https://doi.org/10.1101/2020.08.19.257295. * Equal contribution

Book Chapters

de Graaf, T.A., **Thomson, A.**, Duecker F., & Sack, A.T (2021). CNS Medical Devices: The Various Forms of Noninvasive Brain Stimulation and Their Clinical Relevance. In R. Schreiber (ed.)., Modern CNS Drug Discovery. Springer Nature. https://doi.org/10.1007/978-3-030-62351-7_7

Zwamborn, R. A. J., Snijders, C., Ning, A., **Thomson, A.**, Rutten, B. P. F., & de Nijs, L. (2018). Wnt signaling in the hippocampus in relation to neurogenesis, neuroplasticity, stress and epigenetics. In BPF. Rutten (Ed.), Neuroepigenetics and Mental Illness (Vol. 158, pp. 129-157). Elsevier Science. Progress in Molecular Biology and Translational Science.

Conference Posters

Thomson, A.C., Kenis, G., Schuhmann,T., deGraaf, T.A., Rutten,B., Sack, A.T. (2020). *A human in vitro neuron model of Transcranial Magnetic Stimulation (TMS)-induced plasticity mechanisms.* FENS 2020 Virtual Forum July 11-15 2020 * Best Poster Award

Thomson, A.C., Tielens,S., Schuhmann,T., deGraaf, T.A., Kenis, G., Rutten,B., Sack, A.T. (2019). *The effect of transcranial magnetic stimulation on living human neurons.*doi.org/10.1016/j.brs.2018.12.724. 3rd International Brain Stimulation Conference, Vancouver, Canada. February 24-27, 2019

In Preparation

Schilberg, L.*, **Thomson, A.C.***, Schuhmann, T., de Graaf, T.A., ten Oever, S., Sack, A.T. iTBS does not induce neuroplasticity in insulin resistant patients or matched controls. *In preparation.* * Equal contribution

Thomson, A.C.*, de Graaf, T. A.*, Janssens, S.J.W., Henauer, S., Duecker, F., Schuhmann, T., Sack, A.T. Simultaneous TMS-EEG-fMRI to visualize TMS-induced plasticity effects within the depression network. *In preparation.* *Equal contribution

Appendix

-Impact paragraph

-Curriculum Vitae

-List of publications

-Acknowledgements

Promotion Team

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FPN

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FHML

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