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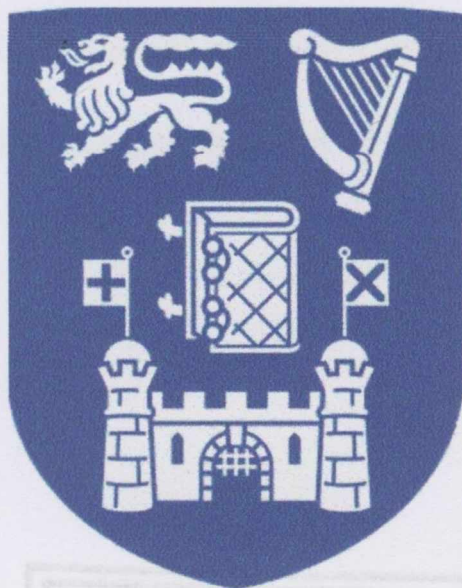
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Electrophysiological and functional responses to paired associative brain stimulation.



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A thesis submitted for the degree of Doctor of Philosophy at the

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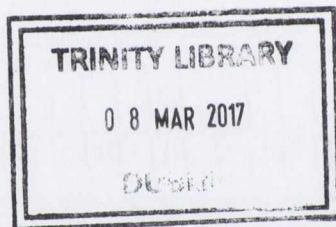
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Abstract

Non invasive brain stimulation (NIBS) methods have exploded in popularity over the last two decades. Despite exhibiting an exponential growth in the number of publications over that period, the field of NIBS research remains somewhat distant from either a sound mechanistic understanding of the effect that these techniques have, or from a clinically useful application of these approaches to therapeutic targets. Modern NIBS methods were developed from stimulation studies on animal models and reduced preparations. These origins have played a key role in shaping how the field has attempted to characterise the underlying mechanisms of action. This heritage has also influenced expectations of what may be practically achieved using NIBS.

The foci of this thesis are twofold. The first theme looks at whether the mechanisms observed in reduced preparation experiments can necessarily be extrapolated to the level of intact human systems that are more complex by many orders of magnitude. The second theme is an examination of whether the electrophysiological outcome measures used in stimulation studies in the human motor system are a useful predictor of behaviourally relevant change. The literature review in the first chapter explores each of these two main themes, providing the background and motivation for pursuing the experimental questions addressed by chapters two and three.

The first section of chapter one reviews the recent origins of some of the most common NIBS methods, their related *in vivo* human experimental interpretations, and whether it is reasonable to extrapolate the same mechanisms of action to these different levels of system. In particular, it focuses on one well-known NIBS technique, paired associative stimulation (PAS). The timing-dependent effects of PAS have been

described as an example of spike timing dependent-like plasticity in the intact human motor system. Evidence reviewed in chapter one suggests that a reappraisal of the mechanisms proposed to underlie PAS may be warranted, which leads to the experiments outlined in chapter two. The second part of chapter one examines the relationship between electrophysiological and functional changes that may be observed following the application of NIBS targeting the human primary motor cortex. Measures such as the motor evoked potential (MEP) signify a change in the excitability of the corticospinal tract. There exists an implicit assumption that modulation of the MEP indicates not only plastic change in the human motor system, but also denotes a greater *state* of plasticity and malleability. The second section of chapter one appraises evidence for the idea that functional (behavioural) measures such as motor task performance or learning should be expected to change as a result of altered electrophysiological responses (MEPs) subsequent to the application of NIBS.

Chapter two outlines empirical work that follows on from the reasoning outlined in the first section of chapter one. A series of seven experiments examine whether the discrete timing of the stimuli in PAS is indeed a necessary characteristic of the associative effects observed. As part of this examination a new method of NIBS was developed in which trains of peripheral nerve stimulation were repeatedly paired with short bursts of transcranial alternating current stimulation. Paired associative transcranial alternating current stimulation (PATACS) proved to be reliable and effective in bringing about change in corticospinal excitability (CSE) at a range of different alternating current intensities and frequencies, at different burst durations, and while utilising different peripheral stimulation modalities. Of note, clear evidence was provided that associative effects may be obtained in the absence of temporally discrete stimuli.

Chapter three outlines an empirical study designed to test the ideas expounded in the second part of chapter one. Two distinct methods were used to induce elevations in CSE prior to the practice of a task in which isometric wrist flexion contractions moved a cursor on a screen to hit each of a set of target gates in a repeated sequence. Speed and accuracy of gate acquisition were measured and combined using a speed accuracy trade-off function to provide an index of skill. In comparison to a sham control group in which levels of CSE were not elevated prior to the task, it was found that PATACS-induced changes in excitability conferred no advantage in motor learning. As an additional comparison, a third group participated in training with the opposite limb in place of receiving PATACS. Although elevations in CSE were also obtained for this ‘cross education’ group, the level of initial performance of these participants was superior that of the control group. The results demonstrated that increased CSE (in the context of this task) is an inconsistent predictor of improved motor performance or learning.

Chapter four includes a summary of the results from the experiments in chapter two and three. These outcomes are discussed in the context of the questions raised in chapter one. Several suggestions are made regarding novel avenues for future research related to these results.

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Abbreviations

AC - alternating current

AURC - area under the recruitment curve

CSE - corticospinal excitability

cTBS - continuous theta burst stimulation

ECR - extensor carpi radialis

EEG - electroencephalogram

EMG – electromyogram

EPSP - excitatory post-synaptic potential

FCR - flexor carpi radialis

FDI - first dorsal interosseous

GABA – gamma aminobutyric acid

ISI - interstimulus interval

iTBS - intermittent theta burst stimulation

LFP - local field potential

LTD - long term depression

LTP - long term potentiation

M1 - primary motor cortex

MEP - motor evoked potential

MUA - multi unit activity

mV.T - millivolt tesla

NIBS - non-invasive brain stimulation

NMDA - n-methyl d-aspartate

PAS - paired associative stimulation

PATACS - paired associative transcranial alternating current stimulation

PNS - peripheral nerve stimulation

PPS - paired pulse stimulation

QPS - quadro-pulse stimulation

RMT - resting motor threshold

rTMS - repetitive transcranial magnetic stimulation

SIFT – sequential isometric flexion task

STDP - spike timing dependent plasticity

tACS - transcranial alternating current stimulation

TBS - theta burst stimulation

tDCS - transcranial direct current stimulation

TMS - transcranial magnetic stimulation

VIBTACS - vibration paired transcranial alternating current stimulation

Chapter 1

Literature Review

1.1 Overview of the chapter

This introductory chapter has two main themes that are closely related to the two empirical strands of the thesis.

The first theme explores the *in vitro* and animal model experimental origins of the mechanistic theories for several broad groups of non invasive brain stimulation (NIBS) techniques, with an emphasis on the benefits and drawbacks of relying on extrapolation between microscopic and macroscopic levels of explanation. It will highlight how recent research is building upon simpler early models to bring us closer to satisfactory theories on the effects of different NIBS methods in humans. In particular, it examines one type of NIBS - paired associative stimulation (PAS). PAS has commonly been described as an *in vivo* example of spike timing dependent plasticity (STDP), a phenomenon that had previously only been observed *in vitro*. Many of the ostensive features of PAS mirror those of STDP however more recent theoretical and empirical developments warrant a reassessment of whether this is a valid description of the likely effect at the level of the human motor systems in associative brain stimulation. This discussion leads directly to the empirical work in chapter two which outlines a series of experiments that provide strong evidence in support of the idea that a STDP-like theory is unlikely to provide a full account of the mechanisms underlying PAS in humans.

The second theme examines modulation of corticospinal excitability (CSE) which is the outcome measure of interest in many non invasive stimulation protocols

and in particular whether it plays an important role in facilitating motor learning outcomes. There is evidence to suggest that many forms of NIBS may in some circumstances have a facilitatory influence on motor task performance and learning. Copious evidence also suggests that when the primary motor cortex is the target of stimulation corticospinal excitability may be modulated. There is an implicit assumption that increased CSE may be a precursor to enhanced functional plasticity or at least that the two phenomena may be in some way linked. In this section some of the empirical and theoretical contradictions to this line of reasoning are highlighted. This discussion is directly related to the second empirical strand of the thesis described in chapter three, which tested the *a priori* expectation (informed by the considerations discussed in the second theme of this chapter) that increased CSE brought about by NIBS may not prove to be an important factor in skill performance on a motor learning task.

1.2 Does non-invasive brain stimulation in humans engage the same mechanisms as its *in vitro* and animal model counterparts?

1.2.1 Introduction

The transcranial application of weak electrical currents or magnetic fields have over the past fifteen years become increasingly popular as a method of influencing cortical excitability and consequent behaviour (Ziemann *et al.*, 2008). These tools, broadly described as non-invasive brain stimulation (NIBS), have been applied to target a wide range of neural structures, to influence a broad spectrum of different behavioural and physiological outcomes, and to act in a therapeutic capacity in an improbable number of neuroclinical disorders ranging from addictions (Amiaz *et al.*, 2009) to chronic pain (Fregni *et al.*, 2007). As has become increasingly clear (Bestmann *et al.*,

2014), the enthusiasm with which NIBS techniques have been applied in an ever-growing range of circumstances has not been matched by advances in our mechanistic understanding of such methods.

Modern NIBS methods have been derived from classic stimulation experiments in small animals and reduced preparations (e.g. Bindman *et al.*, 1964; Bliss & Lomo, 1973). These origins have naturally shaped assumptions concerning the manner in which such techniques exert their effects in the intact human brain. Indeed, the appeal of these methods stems in part from the plausible expectation that we can study phenomena previously only accessible through reduced models (e.g. synaptic plasticity mechanisms) operating at a systems level in human. However, there is a growing recognition (de Berker *et al.*, 2013; Stagg, 2015) that the mechanisms apparent in reduced models do not necessarily provide a full account of the processes at work when adapted for application to the intact human nervous system.

This section first examines several simple, apparently parsimonious mechanistic explanations that have been provided for the action of several NIBS techniques with a view to assessing how likely it is that these reflect the true processes at work. These views are often widely accepted and offer a positive starting point in the search for explanations of the effects we observe when stimulating the intact human brain. Nevertheless, there are an encouraging number of more sophisticated models emerging through reconsideration of evidence generated in the rapidly building corpus of NIBS literature. The scope of this section is limited to four of the most frequently studied brain stimulation techniques; transcranial direct current (tDCS) and alternating current stimulation (tACS), repetitive transcranial magnetic stimulation (rTMS), and paired associative stimulation (PAS). It will focus upon studies in healthy human subjects whereby the primary motor cortex (M1) was the target of stimulation and where the

outcome of interest was corticospinal excitability (CSE) - rather than behavioural measures. This section places particular emphasis on the need to consider alternative explanatory models of one particular type of associative stimulation – PAS - ahead of the empirical study on this subject in chapter two.

1.2.2 Reduced model origins and putative mechanisms of tDCS, tACS and rTMS

1.2.2.1 Transcranial Direct Current Stimulation (tDCS)

Transcranial direct current stimulation in its contemporary form was first reported by Priori *et al.* (1998) and by Nitsche and Paulus (2000). These experiments were the precursor to an exponential growth in popularity of the technique in the years following (Liew *et al.*, 2014). TDCS has been used in a wide range of protocols with different electrode montages purportedly targeting many distinct neural structures and interacting with a wide range of electrophysiological (and ultimately behavioural) processes (Nitsche *et al.*, 2008). In its typical form, at least as applied in the context of motor control research, stimulation is directed to M1 by passing a weak (1-2mA) unidirectional current between a 25cm² conductive rubber electrode pad on the scalp over M1 and a second ‘reference’ electrode, most often placed over the contralateral supraorbital area, for a duration of 10-20 minutes. One of the most robust findings reported from this paradigm is that, when the anodal electrode is placed over M1, motor evoked potentials (MEPs) derived from subsequent single pulse TMS of the same area, are elevated in amplitude. When the cathode is placed over M1 the opposite effect is reported (Nitsche & Paulus, 2000). This is widely considered to be evidence that tDCS leads to a polarity-specific interaction with the excitability of the motor cortex. Pharmacological studies following on from these early experiments suggested that acute effects (during stimulation) resulted from membrane polarisation whereas aftereffects

outlasting the stimulation period represented changes in synaptic plasticity (Nitsche *et al.*, 2003; Nitsche *et al.*, 2004).

The origins of contemporary tDCS may be found in studies involving direct cortical stimulation in live animals. Bindman *et al.* (1964) found that delivering a constant current to the surface of the cortex in rat led to an increase in the amplitude of potentials (evoked through electrical stimulation of the paw) that outlasted the period of stimulation. Early studies in cat (summarised by Stagg and Nitsche, 2011) determined that the effects were likely due to sub-threshold membrane polarisation that depended upon the orientation of neurons relative to the electrical field. The duration of the current application was an important mediating factor; a low total charge delivered over a long duration current produced more durable after-effects than a higher total charge spread over a short period.

Early explanations of the effects of tDCS applied to the human motor cortex were to draw directly upon these rat and cat experiments. The opposing ‘anodal’ and ‘cathodal’ effects that increased and decreased MEP amplitude respectively led to descriptions in terms equivalent to those applied by the early investigators. Nitsche and Paulus, in their seminal paper referred to “anodal” and “cathodal” “polarisation” of neurons. While they provide a number of more conservative qualifying assessments in their discussion, such terms have nevertheless become common parlance in other descriptions of tDCS (e.g. Andrews *et al.*, 2011; Cohen Kadosh *et al.*, 2010). Even with stimulation electrodes placed on the scalp above other cortical areas such as dorsolateral prefrontal cortex (Fregni *et al.*, 2005) or cerebellum (Boehringer *et al.*, 2013), and where electrophysiological markers of altered excitability such as the MEP are not possible, researchers commonly refer to ‘inhibition’ and ‘excitation’ of the brain area under one or other electrode (Cohen Kadosh *et al.*, 2010). The early descriptions from

Bindmann *et al.* (1964), and others working with direct recordings from cat and rat cortex are well accepted mechanistic explanations of the effect in those circumstances. However, there is a growing awareness that simplistic extrapolation of such findings from largely uniform, direct cortical stimulation in experimental animals to the complex and dynamic human motor system are unlikely to be satisfactory (Bestmann *et al.*, 2014; de Berker *et al.*, 2013). Indeed, there are several factors relating to the transcranial delivery of such stimulation to the human brain that preclude simple interpretations.

The first and most obvious issue concerns the transmission of electrical charge to the target neocortical region. This question has attracted increasingly sophisticated examination in recent years via computational models of current flow. Early spherical models (Miranda *et al.*, 2006) based on techniques validated by Rush and Driscoll (1968) in experiments using the human skull have been superseded by elegant computational current flow models which acknowledge the non-uniformity of the human head in terms of its conductivity (Miranda *et al.*, 2013). More realistic approaches include individual anatomical features such as head fat (Truong *et al.*, 2012) and cortical folding (Salvador *et al.*, 2010). These models demonstrate clearly that current flow is not restricted to the area directly under the electrodes. Rather, it is widely dispersed and highly dependent upon individual morphology. The stimulation delivers charge to many distinct regions of the cortex, and at current densities sufficient to influence neural function at these distant (relative to the ‘stimulating’ electrode) structures (Neuling *et al.*, 2012b).

A second issue relates to the implicit assumption that a cogent region, e.g. M1, becomes uniformly polarised. The orientation of a neuron is critical in determining the ease with which it may be depolarised by induced electrical fields. Orientations vary widely according to position in the gyri and sulci of the cortex. It cannot be the case that

all neurons are similarly exposed. Some will receive a radial electrical field depolarising the soma and hyperpolarising the apical dendrites while others will be subject to current flow tangentially to the somatodendritic axis (Rahman *et al.*, 2013). Theories treating current delivery to a given region as purely ‘anodal’ or ‘cathodal’ are rendered less convincing when considered in the light of modelling studies (Neuling *et al.*, 2012b; Miranda *et al.*, 2013) showing that current is unlikely to flow through the brain in a linear path between cathode and anode.

The extrapolation from the initial ideas of hypo- and hyper-polarisation derived from groundbreaking early animal studies does not therefore provide a complete explanation of the action of tDCS on the intact human motor system, and cannot be easily reconciled with what has more recently become apparent through computational modelling. Recent animal studies (e.g. Kabakov *et al.*, 2012; Rahman *et al.*, 2013) are now proving more instructive, albeit by means of less straightforward explanations. The emerging field of *in silico* stimulation provides the promise of a more detailed understanding of the effects of tDCS on both neural (de Berker *et al.*, 2013), and non-neural elements (Ruohonen & Karhu, 2012) within the human brain.

1.2.2.2 Transcranial alternating current stimulation (tACS)

Transcranial alternating current stimulation was not developed directly from animal or reduced preparation research; rather it emerged as an extension of the work that had been done in the transcranial application of direct currents in humans (Antal *et al.*, 2008). TACS parameters have typically been similar to those of the familiar tDCS protocols, but instead of the current travelling in one direction between the anode and the cathode the current oscillates sinusoidally between positive and negative at a set frequency. Antal and colleagues (2008) conducted an exploratory study using tACS

(and ‘sinusoidal direct current’ stimulation which is tACS with a direct current offset) delivered using the typical tDCS M1 electrode montage. They measured electroencephalogram (EEG) spectral power, the amplitude of MEPs, and implicit motor learning in response to a range of different frequencies (1, 10, 15, 30, and 45 Hz) and durations (2 to 10 minutes) of alternating current. Stimulation had no impact upon EEG spectra or MEP amplitude, however one of the combinations (10Hz AC) led to improvements in implicit motor learning.

Given the number of different measurement time points, dependent variables and stimulation parameters examined in this pioneering exploratory experiment it could have been expected that additional results with the appearance of statistical significance might occur through chance alone. It is possible that the current intensities used would have been too low (250-400uA) to influence underlying cortical tissue (Francis *et al.*, 2003). Nevertheless, the study raised the prospect that tACS provides a means of interacting with underlying oscillatory neural activity and that this could act as an incisive tool in disentangling causality in the role of oscillatory activity in neural processes (Miniussi *et al.*, 2013). Further studies followed which appear to show that tACS influences a range of cognitive processes (Neuling *et al.*, 2012a) and physiological measures, (Kanai *et al.*, 2010) including corticospinal excitability (Moliadze *et al.*, 2010).

A central tenet motivating the application of tACS is that oscillatory rhythms in the human brain may be entrained through synchronising or desynchronising neural activity (Paulus, 2011). Although this inference is almost ubiquitous, unambiguous empirical evidence of entrainment has not been easy to demonstrate due to the technical difficulties associated with concurrent EEG and tACS. Recordings at the scalp are saturated by enormous artefacts from the application of tACS in proximity to these

electrodes (Herrmann *et al.*, 2013). Therefore the impact of stimulation upon perceptual processes that have long been theoretically linked to oscillatory activity at certain frequencies (Kanai *et al.*, 2008) has been used as indirect evidence that tACS led to entrainment or modulation.

Based on such studies, the view that tACS entrains oscillatory activity has been stated without direct evidence of concurrent oscillatory measures (e.g. Antal & Paulus, 2013). As with tDCS, it is unclear how much current is delivered through the scalp and where the current flows in brain, although, as with tDCS recent modelling developments are bridging this gap (Manoli *et al.*, 2012). As the polarisation mechanisms postulated in tDCS are unlikely to apply in a continually reversing current, the question of the influence that tACS has on neural structures remains open. The importance of these and other mechanistic questions have been recognised more recently (Herrmann *et al.*, 2013).

Animal and *in vitro* studies, rather than acting as an impetus to initiate the original tACS studies, have been carried out only following human tACS. As noted in the earlier section addressing tDCS, modelling work has demonstrated that the transcranial application of currents can result in electrical fields in neocortical tissue that are of sufficient magnitude to influence neural activity (Holdefer *et al.*, 2006; Neuling *et al.*, 2012b). Animal models have helped elucidate what influence these fields may have (Reato *et al.*, 2013). Alternating current is less effective in polarising cell membranes than direct current (Deans *et al.*, 2007). Nevertheless, at a network level, higher sensitivity may be exhibited. Fröhlich and colleagues (Fröhlich & McCormick, 2010; Ali *et al.*, 2013) showed in Ferret that *in vivo* recordings of spikes in multi unit activity (MUA) were synchronised to oscillatory local field potentials (LFPs) during intracranial stimulation. Furthermore, they demonstrated that cortical slices stimulated

in vitro displayed spiking activity that synchronised to the frequency of stimulating current. When stimulation is delivered transcranially rather than intracranially (Ozen *et al.*, 2010), alternating currents remain capable of synchronising firing rates in intracranial recordings.

The animal stimulation results, when considered in the light of current flow models, confirm that influencing neural oscillations in human cortex by means of tACS is plausible. Modelling studies have indicated that even weak polarisation can have an entrainment effect through magnification of dynamic network activity (Reato *et al.*, 2010). Previous studies using indirect measures (Kanai *et al.*, 2008) or EEG measured in the period following stimulation (Zaehle *et al.*, 2010) hinted at these prospects having been realised. Nonetheless, the field of tACS research awaits more conclusive evidence of stimulation-driven neural entrainment. Recent technological advances have indicated that it may be possible deal with problematic artefact issues. Simultaneous recording of EEG whilst delivering tACS was reported by Helfrich *et al.* (2014), while the same group (Neuling *et al.*, 2015) have published a report on the reconstruction of signals from MEG measured concurrently to stimulation. Such advances will doubtless move the field closer to an understanding of whether the tantalising prospect of entrainment is likely to be realised.

1.2.2.3 Repetitive transcranial magnetic stimulation (rTMS)

Following the first rTMS experiments conducted more than twenty years ago, it was reported that as well as being a method of quantifying change in the motor system, TMS delivered in repeated trains slightly below motor threshold could be used to modulate excitability. Pascual-Leone *et al.* (1994) found that rTMS delivered over M1 could bring about either inhibitory or excitatory changes measured at several upper limb

muscles, in a manner that depended upon the frequency and intensity of stimulation. Chen *et al.* (1997) reported that low frequency stimulation (0.9Hz) led to reliable depression of corticospinal excitability. They noted that the effective frequency of rTMS closely matched the frequency of tetanic stimuli used to bring about LTD-like changes *in vitro* in rat hippocampal (Dudek & Bear, 1992) and motor cortical (Hess & Donoghue, 1996) preparations. Much like tDCS, following this highly promising early work came the application of rTMS to what has been described as a “bewildering” breadth of different clinical goals (Ridding & Rothwell, 2007), occasionally with unclear theoretical rationale. In some non-motor areas this has proven a moderate success, e.g. in depression research (George *et al.*, 1995). Despite controversy over effectiveness (Mitchell & Loo, 2006) it has gained widespread medical usage, particularly in the USA. In motor control applications, much progress has been made in parameterising rTMS and understanding some of its mechanistic underpinnings (Hoogendam *et al.*, 2010).

Comprehensive reviews by Fitzgerald *et al.* (2006) and Hoogendam *et al.* (2010) summarise the general trends that have emerged through experiments on the many permutations of rTMS that can be derived through the use of different stimulation parameters. ‘Simple’ rTMS protocols involve using equally spaced TMS pulses. In a large number of studies rTMS delivered at 1Hz for 15 minutes has been demonstrated to reduce corticospinal excitability, whereas 5Hz stimulation (for various durations but commonly five minutes) will bring about increased excitability (Hoogendam *et al.*, 2010). In the absence of interceding voluntary motor output, these effects can last for up to an hour following the cessation of stimulation. ‘Patterned’ rTMS consists of variation in the inter stimulus intervals (ISIs). Paired pulse stimulation (PPS) (Thickbroom *et al.*, 2006) and quadro-pulse stimulation (QPS) (Hamada *et al.*, 2007) involve two or four

pulses at very short ISIs (1.5ms, to mirror the periodicity of I waves) repeated every five seconds. These generally induce increases in excitability, but excitability changes are reversed when the ISIs are lengthened (Hamada *et al.*, 2008). Theta burst stimulation (TBS) in particular is a patterned stimulation protocol that has attracted a large amount of attention. In TBS, three pulses at 50 Hz are delivered at five cycles per second (Di Lazzaro *et al.*, 2005; Huang *et al.*, 2005) mimicking the 5 Hz theta rhythm found in the hippocampus (Buzsáki, 2002). It was initially reported that when this pattern is delivered continuously (cTBS) for 40 seconds, MEP amplitudes are decreased, and that when delivered intermittently (iTBS) in a two second train every ten seconds for 190 seconds, corticospinal excitability is increased (Huang *et al.*, 2005).

Among the different NIBS method ‘families’, rTMS bears possibly the strongest resemblance to its related *in vitro* roots. The development of much of this work appears to have been undertaken with reasonably clear *a priori* hypotheses relating to the outcomes that might be expected (Huang *et al.*, 2005). At least superficially there are obvious shared characteristics between rTMS models of plasticity in M1 and fundamental *in vitro* research in tetanic stimulation and synaptic plasticity (Sanes & Donoghue, 2000). The burgeoning literature concerning rTMS in its myriad forms is drawn upon by Hoogendam *et al.* (2010) to summarise the extent of these similarities. In common with *in vitro* models, the timing and rhythm of pulses seems to be of critical importance in determining the outcomes of rTMS. Changes in corticospinal excitability following rTMS outlast the period of stimulation (Pascual-Leone *et al.*, 1994) and the duration of this period of modulated CSE depends on the number of pulses delivered (Peinemann *et al.*, 2004). Pharmacological studies have demonstrated that the effects may be NMDA receptor-dependent (Huang *et al.*, 2007). The history of activity in the

system has been reported to play an important metaplasticity-like role in the process (Iyer *et al.*, 2003).

The phenomenology of rTMS, at least as registered at a systems level, mimics the characteristics of neural plasticity as revealed at the cellular level. However, Hoogendam *et al.* (2010) stress that in the absence of invasive recordings in humans, these comparisons can only ever be indirect. The way that LTP and LTD are induced in slice preparations is vastly divergent from how rTMS is applied. Specific fibres are stimulated with higher frequency and intensity than rTMS. A single neuron is targeted at which the excitatory post-synaptic potential (EPSP) is measured. In TMS, magnetic fields (limited in intensity and frequency due to seizure risk; see Rossi *et al.*, 2009) are somewhat bluntly applied over a relatively large area of neocortex stimulating many neurons through both direct axonal depolarisation of pyramidal motor neurons and indirect, trans-synaptic potentials via a complex series of interneurons (Terao & Ugawa, 2002). Related to this point, while the *in vitro* EPSP is measured directly from one target cell, the results of rTMS over M1 are measured via responses evoked in the periphery, that are mediated by corticospinal neurons lying two or more synapses from the originally stimulated neurons. Finally, the neuronal target populations differ between *in vitro* experiments and rTMS studies; the former have primarily concentrated on CA1 and CA3 hippocampal pyramidal cells, whereas the latter target M1 motor neurons both directly and indirectly through horizontal interneurons. Horizontal pathways in M1 (which are likely to mediate plastic change) exhibit different response characteristics to tetanic stimulation protocols when compared with the more intensively studied hippocampal cells (Sanes & Donoghue, 2000).

As with the other forms of NIBS that impact upon plasticity processes in human M1, rTMS protocols have demonstrated an enormous amount of variability in effect

(Hamada *et al.*, 2012). While many of the features outlined above outwardly resemble LTP and LTD-like phenomena, there are still a number of issues to be resolved, including the reasons for the variability in response and the range of often contradictory findings (Fitzgerald *et al.*, 2007). Some of the outstanding issues that result from the indirect nature of the evidence gathered to date can only be resolved by invasive recordings. In rodents this is problematic as even with the use of specialist TMS coils, smaller brain size means that the relative area stimulated is even less focal than in humans. Recent advances in invasive recording in monkey (e.g. Mueller *et al.*, 2014) may go some way to resolving these questions. Through the development of more specialist stimulating coils and the use of microelectrode recordings in primates, it is hoped that future research may provide more conclusive evidence of whether changes in CSE brought about by rTMS are due to LTP and LTD-like processes in the human motor system.

1.2.3 Paired associative stimulation (PAS)

1.2.3.1 PAS and Spike Timing Dependent Plasticity

Since Stefan *et al.* (2000) reported the first experiments on the effects of paired associative stimulation (PAS) on corticospinal excitability, it has drawn widespread attention as another method by which LTP and LTD-like mechanisms might potentially be studied *in vivo* in human. While it has been adapted for use in non-motor systems (e.g. auditory - Schecklmann *et al.*, 2011), the majority of PAS research has remained focussed within the human sensorimotor system. In its prototypical form, an electrical peripheral nerve stimulus is applied to the median nerve of the forearm in advance of a single TMS pulse over contralateral M1. The repeated delivery of pairs of these stimuli leads to changes in the excitability of projections to peripheral muscles in the target

limb that appear dependent on the precise timing of the ISI (Stefan *et al.*, 2000). When the peripheral stimulus is timed so that the corollaries of the afferent volley are coincident with the TMS pulse delivered over M1 (typically at an ISI of 25ms), increased corticospinal excitability occurs. When a shorter ISI (typically 10ms) is used, such that an asynchronous arrival of the two stimuli is presumed, decreases in excitability are reported (Wolters *et al.*, 2003). While PAS, in common with all NIBS techniques, is subject to considerable variability in effect (Fratello *et al.*, 2006), these have proven robust findings across fifteen years of PAS research (Wischnewski & Schutter, 2015).

It has been suggested that the cardinal features of PAS not only mirror LTP and LTD-like plasticity effects observed *in vitro*, but more specifically that they could be accounted for by spike timing dependent plasticity-like (STDP) mechanisms (Müller-Dahlhaus *et al.*, 2010). In STDP *in vitro*, if the pre-synaptic cell spikes repeatedly just in advance of spiking in the post-synaptic cell, then potentiation results. The reverse occurs if the spiking is asynchronous or if the post-synaptic spike occurs in advance of the pre-synaptic spike (Markram *et al.*, 1997). The precise millisecond range of this relationship mirrors some of the first reported characteristics of PAS. There are other apparent LTP/LTD-like features of PAS; the effects outlast the period of stimulation (Stefan *et al.*, 2000), pharmacological studies reveal the importance of the NMDA receptor in mediating the effects (Stefan *et al.*, 2002a; Wolters *et al.*, 2003), the effects have been claimed to be effector (and by extension synapse)-specific (Nitsche *et al.*, 2007), the plasticity effects interact with motor learning related plasticity (Ziemann *et al.*, 2004) and it displays metaplasticity-like features (Müller *et al.*, 2007).

1.2.3.2 Phenomena of PAS in humans that may require reinterpretation

There are clear similarities in this set of comparisons and those made between rTMS and cellular level plasticity experiments. Like in rTMS, several pertinent questions arise when these features are examined more closely, particularly when extrapolating from stimulation and recording in single cells or small numbers of hippocampal neurons to the delivery of relatively large and diffuse stimuli targeting the enormously complex human sensorimotor system. Carson and Kennedy (2013) provide a comprehensive review of the mechanistic underpinnings of PAS. They underline the fact that, in order to account for findings that have arisen since the first examples of PAS were reported, novel explanatory models are necessary. Several phenomena are highlighted in the review, which upon critical examination prove difficult to conciliate with STDP.

In order to adhere to the characteristics of the STDP theory, PAS in humans should reflect several key characteristics. Timing dependency is an integral feature; the coincident arrival of two stimuli in STDP described *in vitro* should be within a tightly restricted window, with the pre-synaptic spike occurring just before or coincident to the post synaptic spike. On balance, the evidence in human examples of PAS indicates that excitatory effects can occur over a span of at least 50ms; this may be larger than range normally characteristic of STDP (Carson and Kennedy, 2013). Additionally there is an unexpected variability between the effective time windows for different people; Huber *et al.* (2008) report that some participants demonstrated potentiating effects at an ISI of 10ms and an inhibitory effect at 25ms which is the opposite pattern to that predicted by theory. Another key characteristic of STDP is synapse specificity - the changes in excitability should be restricted to the synapse targeted. The *in vivo* homologue of this feature is muscle specificity. One of the widely assumed and reported features of PAS is

that adaptations, i.e. altered excitability, occurred only (or to a greater extent) at the muscles innervated by same nerve that was the target of the peripheral nerve stimulation. There are many instances from across the literature that suggest that this is 'more the exception than the rule' (Carson and Kennedy, 2013). It has been demonstrated that hand muscles neighbouring the target muscle but innervated by a different nerve may display greater modulations in CSE than the target itself (Cheeran *et al.*, 2008).

The unfolding events that occur in response to both the peripheral and cortical stimuli are, upon deeper reflection, problematic to characterise as spatially discrete. While TMS at intensities which exceed motor threshold (described in more detail elsewhere in this chapter) leads to a periodic high frequency bombardment of the corticospinal tract pyramidal neurons under the focal point of the stimulating coil, EEG reveals that TMS also brings about a propagating pattern of activation lasting around 300ms and can be registered as positive and negative deflections over ipsilateral motor, premotor and parietal regions (Ferreri *et al.*, 2012; Carson & Kennedy, 2013). It is equally elusive to pinpoint the arrival of the afferent volley generated by the peripheral nerve stimulus to one small, well-defined locality. The EEG N20 somatosensory related potential shows that the sensory afferent volleys, which result from peripheral stimulation, first arrive at primary somatosensory cortex (S1). However, in order to register such a response, many thousands of neurons must be activated. The peak of such SEPs represent only a part of the overall deflection, and should not necessarily be considered the most important or informative part of the evoked potential (Luck, 2005). Furthermore, since peripheral nerve stimulation often occurs at levels sufficient to generate muscle responses, the afferent volley will also consist of responses from the muscle spindles. The N20 response is largely cutaneous in origin (Kunesch *et al.*, 1995)

and has a maximal impact upon Brodmann area 3b, whereas muscle spindle afferent volleys are registered maximally in area 3a. Area 3a has the most extensive connections with M1 while projections from 3b are sparse (Burton & Fabri, 1995). Therefore the many sequelae of the PNS arrive at different areas of the cortex dependent on their sources (cutaneous or muscle spindle) and are unlikely to consist of one discrete time-locked event. The timing of the N20 (of cutaneous origin and arriving at area 3b) has been used extensively in justifying the ISI between the peripheral and cortical stimuli. However the anatomical connections between area 3a (in receipt of the muscle spindle afferents) is likely to be more impactful upon M1, the eventual putative site of associative adaptations in PAS.

The evidence thus suggests that the timing of the peripheral and cortical stimuli may be effective outside of the window necessary for STDP *in vitro*, and it appears that the muscle-specificity of the effect initially reported is actually the exception more than the rule. Most pertinently, the interaction between the constituent elements of PAS after their arrival via several distinct pathways at M1 cannot be thought of as a single time-locked event at one discrete locality. Consequently, the known features of the impact of these two stimuli upon circuits in M1 appears to lack similarity to the concept of the temporally and spatially restricted presynaptic and postsynaptic ‘spike’ which would constitute the conditions necessary for *in vitro* STDP.

These observations form the motivation for the first empirical strand of this thesis. If it could be clearly demonstrated that associative plasticity effects may be brought about in a paired associative protocol using stimuli that were by design non-discrete, then it would further underline the point that the STDP mechanism postulated for the effects of PAS is likely to be insufficient to fully explain the effect.

1.2.4 Conclusions

While reduced preparation and animal models are a sensible place to start in the search for mechanisms that may account for the effects of NIBS in humans, it is readily apparent that simplistic extrapolation is not likely to provide a full explanation. Granted, the overt features shared by NIBS techniques and their *in vitro* counterparts are at first glance suggestive of common mechanisms. Without more direct evidence however, there remains the possibility that these are in some cases merely superficial similarities. Some common mechanistic basis notwithstanding, (at least as presently articulated) *in vitro* models do not provide a complete account of extant empirical data concerning the action of NIBS *in vivo*. With respect to each family of NIBS methodologies, there have been recent theoretical developments that seek to address these shortcomings (Herrmann *et al.*, 2013; Carson & Kennedy, 2013; Bestmann *et al.*, 2014) and empirical studies that have tested the associated underlying assumptions (McNickle & Carson, 2015). These initiatives are complemented by modelling and *in silico* simulations of NIBS (de Berker *et al.*, 2013).

In acknowledging the shortcomings of current explanatory frameworks, and seeking to develop radical experimental designs, there is the conviction that a deeper understanding of the mediating processes may be achieved. By extension, more effective therapeutic applications of these techniques may ultimately be derived. As noted above, chapter two outlines the first strand of empirical work related to this motive; a series of studies that examine the assumptions around mechanisms of associative stimulation.

1.3 Changes in corticospinal excitability following NIBS can be thought of as an indication of plastic change but not as an indication of greater ‘plasticity’.

1.3.1 Introduction

The sections that follow relate directly to the second empirical strand of the thesis in chapter three, and focus on one of the main outcome variables from NIBS targeting the human motor system, the motor evoked potential (MEP). MEPs have proven a useful marker of physiological changes in the corticospinal tract following the application of non-invasive brain stimulation. Many different forms of brain stimulation can attenuate or facilitate MEP amplitude (e.g., Nitsche & Paulus, 2000; Stefan *et al.*, 2000). The change in MEP amplitude is inferred to reveal something about the influence that the interventional NIBS has had on the subsequent state of the brain.

The changes in the amplitude of MEPs brought about by various NIBS protocols have frequently been described not just in terms of corticospinal ‘excitability’ (CSE) but also as manifestations of ‘plasticity’ (e.g. Stefan *et al.*, 2000; Thickbroom *et al.*, 2006). There are many indications that the modulation of MEPs following NIBS does indeed reflect plastic change in the motor system (Ziemann *et al.*, 2008; Stefan *et al.*, 2002; Nitsche, 2003). The application of the term ‘plasticity’ is appropriate when used to mean precisely this. However, there may exist in certain contexts a degree of ambiguity regarding whether plastic change has occurred or whether a greater *state* of plasticity has been induced. A state of enhanced plasticity can be thought of as a period of greater malleability during which more effective functional plasticity can be induced through, e.g. motor training. This concept is central to the point made in the following sections;

there may be doubt whether changes in MEP amplitude reflect greater ‘plasticity’ as opposed to evidence of ‘plastic change’ having occurred.

Frequently, increased malleability is the explicit goal of NIBS; the stimulation is applied with the intention of increasing the state of plasticity in order to prime the motor system for improved learning or performance (Reis *et al.*, 2009). The use of NIBS to bring about changes in CSE, while subject to considerable variability (Wiethoff *et al.*, 2014; López-Alonso *et al.*, 2014), is a robust finding (Bastani & Jaberzadeh, 2012; Wischniewski & Schutter, 2015). The effect of NIBS in improving performance and learning on motor tasks may be even more variable, but it does appear to be effective in some circumstances. (Nitsche *et al.*, 2003; Orban de Xivry & Shadmehr, 2014) The two phenomena are, however, infrequently studied together in the same experimental context, and the degree to which they may be associated is seldom an object of enquiry in studies involving neurologically healthy participants. In the absence of brain stimulation, motor learning itself is strongly associated with increases in CSE (Ljubisavljevic, 2006). Nonetheless, it is not generally possible to determine whether accompanying changes in CSE are causal of, or a corollary to, the neural adaptations that mediate motor learning. The sections that follow describe the physiological effects of TMS in generating the MEP and the limits of this measure in predicting behavioural outcomes. This literature is reviewed in order to set the context for the second empirical strand of the thesis, chapter three, which is an experiment designed to test the hypothesis that a change in CSE does not necessarily predict a modulated behavioural response to a motor training task.

1.3.2 The motor evoked potential

TMS, when applied over the primary motor cortex (M1) can elicit motor evoked potentials detectable using surface electromyography (EMG) over contralateral skeletal muscles (Terao & Ugawa, 2002). An electrical current passed through a copper coil held over the scalp creates a transient magnetic field ($\sim 200\mu\text{s}$) which in turn induces an electrical current in the underlying cortex. When the magnetic field is of sufficient strength this current can depolarise neurons and create action potentials. The result of the activation caused by magnetic stimulation over M1 is a corticospinal volley in the descending pathways from M1, which can cause contraction of the muscle if the stimulation is of sufficient intensity.

The MEP is an output measure reflecting excitability of the corticospinal tract (Rothwell, 1997). It is a compound signal resulting from several distinct descending waves as detectable using spinal recordings (Di Lazzaro *et al.*, 2004). The first component, the D (direct) wave, results from direct axonal depolarisation of the pyramidal motor neurons. At intervals of 1.5ms following the D wave come a series of I (indirect) waves which are due to trans-synaptic activation of the pyramidal motor neurons by a series of interneurons which lie at respectively further synaptic distances from the motor neuron (Terao & Ugawa, 2002). The MEP is therefore not only a 'read-out' of the excitability of cortical motor neurons, but also an indication of the state of excitability of intracortical and transcortical networks of interneurons that synapse with neurons of the corticospinal tract. Measured at the peripheral muscle, it must also be considered that the MEP is influenced by the state of the spinal segment of the pathway (Taylor, 2006) and by extension also influenced by direct corticospinal projections from non-M1 areas such as the supplementary motor area (SMA) (Dum & Strick, 2002), and by all other factors that alter the post-synaptic excitability of spinal motor neurons.

The MEP has been intensively studied over the past thirty years and many of the contributions to the MEP have been well characterised (Di Lazzaro & Ziemann, 2013). Paired pulse stimulation (Kujirai *et al.*, 1993) has been used to examine the contribution of certain I waves in the MEP. GABAergic interneurons, which project on to the pyramidal motor neuron, respond to lower stimulation intensity. They can be preferentially targeted by weaker pulses, thus allowing one to probe the influence of these circuits. Changing the direction of current flow by manipulating coil position can also activate different I waves and change the latency of the D wave (Di Lazzaro & Ziemann, 2013). These types of approaches permit examination of some of the different elements that contribute to the MEP. Despite such developments, the MEP and the factors that determine its makeup (and how it is modified) are incompletely understood. Bestmann & Krakauer (2015) review the use of the MEP with a view to outlining what conclusions may or may not be inferred from its alteration in experimental or clinical contexts. One of the cautionary points that they make is that MEPs do not result from the stimulation of a uniform set of neural elements; they are a compound measure with multiple known (and probably several unknown) influencing factors. In most experimental paradigms it is not a trivial matter to clearly delineate the influences of these factors on the amplitude of the MEP.

1.3.3 The semantics of ‘plasticity’

In vitro experimental investigations of synaptic plasticity have had an impact upon how we describe the effects of NIBS in the intact human motor system. This extends beyond mechanistic theories to the linguistic terms within which we couch observed effects. In classical Hebbian plasticity, when cell A repeatedly takes part in firing cell B, its efficiency in firing cell B is increased (Hebb, 1949). The long term

potentiation (LTP) of the connection between the two cells is referred to as neural plasticity. *In vivo* experimental approaches using non-invasive stimulation to induce change in corticospinal excitability have retained this descriptive terminology. When NIBS was demonstrated in several different forms to change the amplitude of the MEP for a period outlasting the stimulation, the authors of formative studies concerning tDCS (Nitsche & Paulus, 2000), rTMS (Chen *et al.*, 1997) and PAS (Stefan *et al.*, 2000) labelled the change that they observed “neural plasticity”. While, as noted previously, precisely that which causes modulation in MEP amplitude in each of these cases remains unclear, several characteristics of the observed effects permit description of the change as an expression of neural plasticity.

A key objective of the present discourse is to emphasise however, that the use of a term such as ‘PAS-induced plasticity’ rather than the alternative ‘PAS-induced plastic change’, has the potential to lead to erroneous interpretation of the meaning of the observations made in these studies. This is not merely a matter of semantics. That which is being described in each of these cases is evidence of plastic change having occurred. That which is sometimes inferred however, is that a greater *state* of plasticity has been induced (Bolognini *et al.*, 2009). In literature describing the use of NIBS as a possible adjunct to rehabilitation this is particularly evident (Schabrun & Chipchase, 2012). The point to be made here is that changes in CSE that outlast neurostimulation are evidence of plastic change having occurred. They are not necessarily reflective of on-going ‘plasticity’ in the ‘malleability’ sense of the word. There is a substantial literature which suggests that NIBS may have a positive effect on motor learning (Reis & Fritsch, 2011; Orban de Xivry & Shadmehr, 2014). On an *a priori* basis at least, it thus appears that exposing the processes through which this occurs necessitates a line of enquiry

conceptually distinct from that which has been followed in assessing the means through which exogenous stimulation gives rise to changes in CSE.

1.3.4 The MEP during and after motor learning

TMS has been used in a variety of different ways with a view to elucidating the mechanisms underlying motor learning. CSE increases during voluntary contraction of muscles (Ridding *et al.*, 1995). CSE is also increased by the learning of a motor task (Pascual-Leone *et al.*, 1995; Abbruzzese *et al.*, 1996) in a manner that appears independent of the changes brought about by the training movements themselves. This is a widely reported finding that appears to apply to many different types of motor learning task (Ljubisavljevic, 2006). Muellbacher *et al.* (2001) demonstrated that learning a ballistic pinch task led to increased CSE during the initial period of training but while the performance improvements remained evident upon follow-up some weeks later, the increased CSE was no longer apparent. The authors suggested that this provided evidence of reorganizational processes in M1 due to motor learning during the initial session. In addition, it has been reported that a period of motor learning can influence the 'plasticity-inducing' effects of some forms of NIBS. Ziemann *et al.* (2004) demonstrated that learning a fast-as-possible thumb abduction task immediately prior to administration of PAS(N20), occluded the LTP-like effect of stimulation. Moreover, the LTD-like effects of PAS(N20-5) were enhanced by the prior learning of the ballistic abduction task. The conclusions drawn by the authors were that LTP and LTD-like mechanisms can be measured via changes in CSE, and that these mechanisms were involved in the learning that was manifest in response to task practice.

On the basis of this evidence one may conclude that motor learning and CSE are intricately linked (Ljubisavljevic, 2006). There are however, a number of additional

issues that are pertinent to the assumption that an increase in CSE is in some direct way causal to, or a necessary pre-requisite for, motor learning. The first is that there are examples where the posited relationship is not clearly evident. Lissek and colleagues (Lissek *et al.*, 2013; Lissek, 2014) demonstrate that certain motor learning protocols may actually lead to a decrease in CSE. There are several instances where motor learning has been found to occur without any clear effect on CSE (Ziemann *et al.*, 2001; Smyth *et al.*, 2010). A second issue is that of causality. Even if increased MEP amplitude was without exception displayed in all cases of motor learning, it is difficult to determine causality in the process. The increased excitability could be an epiphenomenon; TMS might more effectively recruit or activate neural populations after a period of learning, but these neural elements may in fact have little causal relationship to the synaptic plasticity processes presumed to take place as a result of task practice (Bestmann & Krakauer, 2015). A third question relates to points made earlier on the nature of the constituent elements of the MEP. There remains a degree of ambiguity as to what precisely contributes to a change in MEP amplitude. Any one of several neural elements (or any combination thereof) that are known to contribute to the MEP may have changed in relation to learning. If it were established clearly that there was a meaningful link between enhanced MEP amplitude and motor learning-related plasticity, it is difficult to disentangle what the physiological significance of the link means without probing deeper into the elements that make up the MEP (Bestmann & Krakauer, 2015).

1.3.5 Does increased CSE facilitate learning?

There is a considerable body of evidence which supports the assertion that NIBS can bring about changes in CSE (Hoogendam *et al.*, 2010; Bastani & Jaberzadeh, 2012;

Wischnewski & Schutter, 2015). There is also evidence that NIBS can positively modulate motor performance and learning (Orban de Xivry & Shadmehr, 2014). There is enormous variability in effect of NIBS upon CSE both within and between subjects (e.g. Wiethoff *et al.*, 2014; López-Alonso *et al.*, 2014; 2015). When motor performance and learning are the outcome measures in NIBS studies the variability is likely to be larger again, and the results reported are highly task specific. Saucedo-Marquez *et al.* (2013) found that ‘anodal’ tDCS applied during a sequential finger-tapping task led to immediate benefits in performance, whereas application of stimulation during a visual pinch task led to gains only evident at retention. With an emphasis on novel approaches in the field rather than on straightforward replication of previous results, it is difficult to derive meaningful effect sizes for NIBS in motor learning (although see Jacobson *et al.*, 2012). Nevertheless, sufficient evidence exists to suggest that in some cases, there may be an advantage conferred to motor learning by prior or concurrent delivery of NIBS (Stagg *et al.*, 2011; Rajji *et al.*, 2011; Kang *et al.*, 2015) .

Karok and Witney (2013) report an increase in CSE and in measures of motor performance at a group level following tDCS, however there are in general few incidences of increases in both phenomena reported together in the same experiment. In order to make a stronger case for the relationship between increased CSE and enhanced motor learning, it would be instructive to assess examples where a change in one has been correlated with a change in the other. This evidence is conspicuous by its absence from the literature. Hummel *et al.* (2005) provide evidence of a correlation between changes in CSE and motor learning, however this was in the context of stroke rehabilitation and specifically within the framework of seeking to redress hemispheric excitatory imbalance following stroke (Hummel & Cohen, 2006) .

Convincing evidence demonstrating that increased corticospinal excitability may bring about more effective motor learning is therefore missing. There is also a lack of any compelling theoretical model that would account for such a phenomenon. Schabrun and Chipchase (2012) highlight the rehabilitation opportunities presented by several neuromodulatory techniques including tDCS, peripheral nerve stimulation and rTMS. They note that these methods appear capable of promoting cortical neuroplasticity, and that their excitability-enhancing properties may be used to prime the brain ahead of physiotherapy. However, there is little evidence, and no clear theoretical model that would lead one to predict that increased CSE via NIBS may be beneficial in motor training.

Learning related plastic change in M1 likely occurs through synaptic strengthening in neural circuits related to the effectors involved in learning (Rioult-Pedotti *et al.*, 2000). Evidence explored in the earlier part of this chapter demonstrates that contemporary forms of NIBS are unlikely to selectively stimulate specific targets, but rather lead to a more global impact over a relatively large area (e.g. Carson & Kennedy, 2013; Neuling *et al.*, 2012*b*). During learning, there is little reason to believe that these forms of intervention would have an impact restricted to the circuits in M1 correspondent to the task-relevant effectors. The stimulation could conceivably influence neural targets that are responsive to subsequent stimulation by TMS but have no direct relationship to learning.

Why should the addition of NIBS-driven elevations in CSE, even within circuits engaged in motor learning, lead to more effective learning? Stochastic resonance models (Kitajo *et al.*, 2003) have been used to make a case in NIBS that weak sensory signals may be boosted above detection threshold by the addition of ‘noise’ to the system (Miniussi *et al.*, 2013). While in this framework the addition of noise is

conceived of as a ‘pedestal’ that may serve to boost a weak signal, it may also act as an impediment if the signal is already of sufficient strength to be detected. The addition of NIBS-derived increases in CSE during normal motor learning might be thought of in the same way; an additional source of activation is being introduced to the system via an extraneous stimulus that is not systematically related to the adaptations relevant to the task being learned. Thus, NIBS-derived increases in CSE might conceivably act as an impediment to motor learning in some circumstances.

A second theory might also predict that there could in some circumstances be detrimental rather than beneficial effects caused by extra CSE from stimulation in addition to the changes caused by natural learning. Homeostatic metaplasticity refers to the process whereby synaptic plasticity is determined by the historical activity of the synapse (Bienenstock *et al.*, 1982). If the post-synaptic cell exhibits high excitability prior to a normally potentiation-inducing stimulus from a pre-synaptic cell, LTD will result rather than LTP. If low excitability is exhibited in the prior state of the post-synaptic neuron then the opposite effect is observed. This is thought to be a mechanism through which neural networks are kept in check to prevent a scenario of ‘runaway potentiation’ (Abraham *et al.*, 2001). In the context of this conceptualisation, CSE increases driven by NIBS may bring the system to a state of excitability which acts counter to the facilitation of plastic change via the CSE changes that occur naturally as a motor task is being learned (Siebner *et al.*, 2004; Stagg *et al.*, 2011).

There are however relatively few reports of increased CSE leading to a detriment in motor learning, with the exception of experiments deliberately designed to manipulate homeostatic metaplasticity (e.g. Stagg *et al.*, 2011). A more likely conclusion that can be drawn from this then, is that CSE increases due to motor learning

and those driven by NIBS may not interact with each other in any meaningful way either to the benefit or detriment of motor learning.

1.3.6 Conclusions regarding MEPs and functional plasticity

The MEP is a compound measure of excitability influenced by multiple neural elements. The precise implications of changes in MEP amplitude for voluntary motor behaviour are far from clear (Bestmann & Krakauer, 2015). Despite the development of sophisticated methods that aid the analysis of separate contributions to the MEP, it remains the case that a change in MEP amplitude allows us only to infer that something has changed, not precisely what has changed. Increases in CSE are associated with motor learning, but it is not possible to establish causality in this relationship and there are instances where motor learning can occur in the absence of increased corticospinal excitability. CSE is also modulated in response to NIBS, however there is a lack of any clear relationship between these changes and impact upon the efficacy of motor learning following stimulation.

NIBS-driven modulation of CSE might reasonably be described as plastic change. It is more problematic to describe these techniques as bringing about an increase in ‘neural plasticity’. The implication, which may be conveyed unintentionally by this terminology, is that a state of greater malleability has been induced in the motor system. While this distinction is abundantly clear to those who are steeped in NIBS and motor plasticity research, it is likely to be much less obvious to physiotherapists or other practitioners seeking novel methods of optimising rehabilitation outcomes (Shabrun & Chipchase, 2012; Carr & Shepherd, 2011). It is therefore an opportune time to categorically separate expectations of malleability from measures of excitability in NIBS protocols. The experiment outlined in chapter three tests this line of reasoning.

Increases in CSE may be sustained for a time following the cessation of stimulation. In this time period a benefit to performance or learning of a motor task might be expected if CSE is an important precursor to motor learning.

1.4 Chapter summary

The two themes of this introduction chapter introduce the two empirical strands in chapters two and three. The motivation for the series of experiments in chapter two arose from the observation that many of the current theories for the mechanisms underlying brain stimulation, which arose through extrapolation from reduced models, may not provide complete explanations of the effects observed at the level of the intact human motor system. One particular type of NIBS technique, PAS, is examined in the light of more recent theory and experimental evidence. Studies conducted *in vitro* originally prompted comparisons of PAS outcomes to a STDP-like effect, however reconsideration of recent findings makes this explanation appear less likely. Chapter two outlines experiments that test this theoretical link between PAS and STDP.

The second part of the chapter addresses the links between changes in MEP amplitude and functional plasticity outcomes following NIBS. An implicit assumption (in studies where M1 is the target of stimulation) is that increased CSE resulting from such interventions may be a useful primer ahead of physical rehabilitation. This section reviews the evidence and theoretical rationale that supports this assertion, with the conclusion that while various NIBS techniques may promote functional plasticity and may enhance corticospinal excitability, the two phenomena are not necessarily causally related. Chapter three reports an experiment that tests the link between changes in CSE and changes in performance on a motor learning task.

Chapter 2

Paired associative transcranial alternating current stimulation increases the excitability of corticospinal projections in humans.

2.1 Abstract

Many types of non-invasive brain stimulation alter corticospinal excitability (CSE). Paired associative stimulation (PAS) attracts particular attention as its effects ostensibly adhere to Hebbian principles of neural plasticity. In prototypical form, a single electrical stimulus is directed to a peripheral nerve in close temporal contiguity with transcranial magnetic stimulation (TMS) delivered to the contralateral primary motor cortex (M1). Repeated pairing of the two discrete stimulus events (i.e. association) over an extended period either increases or decreases the excitability of corticospinal projections from M1, contingent on the interstimulus interval (ISI). We studied a novel form of associative stimulation, consisting of brief trains of peripheral afferent stimulation paired with short bursts of high frequency (≥ 80 Hz) transcranial alternating current stimulation (tACS) over contralateral M1.

Elevations in the excitability of corticospinal projections to the forearm were observed for a range of tACS frequency (80Hz, 140Hz and 250Hz), current (1mA, 2mA and 3mA) and duration (500ms, and 1000ms) parameters. The effects were at least as reliable as those brought about by PAS or transcranial direct current stimulation (tDCS). When paired with tACS, muscle tendon vibration also induced elevations of CSE. No such changes were brought about by the tACS or peripheral afferent stimulation alone. In demonstrating that associative effects are expressed when the timing of the peripheral and cortical events is not precisely circumscribed, these findings suggest that multiple

cellular pathways may contribute to a LTP-type response. Their relative contributions will differ depending on the nature of the induction protocol that is used.

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2.2 Introduction

2.2.1 Background

Among the various forms of non-invasive brain stimulation that have been explored experimentally during the past three decades, paired associative stimulation (PAS) has attracted particular attention as a means by which to investigate the expression of neural plasticity at a systems level in humans (e.g. Müller-Dahlhaus *et al.*, 2010). In the prototypical variant (Stefan *et al.*, 2000), a single electrical stimulus is applied over a peripheral nerve in advance of transcranial magnetic stimulation (TMS) delivered to the contralateral primary motor cortex (M1). Repeated pairing of the stimuli (i.e. association) over an extended period (e.g. 13 to 30 min) tends to increase or decrease the excitability of corticospinal projections from M1 in manner that depends on the interstimulus interval (ISI). If the ISI is set such that the first component of the ascending afferent volley (initiated by the shock to the nerve) reaches the cortex marginally in advance of the magnetic stimulus, “long term potentiation (LTP) – like” increases in corticospinal excitability (CSE) are observed (Stefan *et al.*, 2000). Conversely, if the ISI is adjusted to ensure that the first corollary of the afferent volley registered at M1 arrives following the TMS, “long term depression (LTD) - like” decreases in corticospinal excitability may be obtained (Wolters *et al.*, 2003).

In light of these observations, it has been noted that the effects of PAS are in accordance with Hebbian principles (Stefan *et al.*, 2000; Quartarone *et al.*, 2003; Stefan *et al.*, 2004). More specifically, since the polarity of the induced changes in CSE appears contingent upon the order of the stimulus-generated cortical events, and the effective inter-stimulus intervals lie within a restricted (milliseconds) range, it has been proposed that the resemblance is to spike-timing dependent plasticity (STDP) (Müller-Dahlhaus *et al.*, 2010).

Although it is widely assumed that TMS over M1 exerts an effect on chains of interneurons with fixed temporal characteristics that produce a periodic bombardment of corticospinal neurons (Amassian *et al.*, 1987), it is also apparent that the magnetic pulse produces complex spreading patterns of cortical activity that are not localised in either space or time. Similarly, the administration of a single shock to a peripheral nerve gives rise to an unfolding series of neural events that can be registered in many parts of the brain. There also exist multiple routes through which the sequelae of TMS applied to M1, and peripheral nerve stimulation, may converge and interact (Carson & Kennedy, 2013). As such, an *a priori* assumption that there is discrete temporal convergence of activity generated by the two associated sources of stimulation is not necessarily warranted. We reasoned that if the generation of a temporally discrete cortical event is not a critical determinant of the effects induced by PAS, it should be possible to replace the TMS element of the induction protocol with another cortical stimulation modality that is - by design, extended in time.

Transcranial alternating current stimulation (tACS) is one of many other non-invasive methods that can alter brain activity. Moliadze and colleagues (2010) applied high frequency - 140Hz tACS over M1 for 10 minutes, and observed consequent increases in the amplitude of motor-evoked potentials (MEPs) elicited by TMS. This effect (which was not obtained for 80Hz or 250Hz stimulation) was attributed to an influence of the tACS on endogenous 'ripple range' activity. Sharp wave ripple complexes occur in short (200-700ms duration) bursts (Ego-Stengel & Wilson, 2009; O'Keefe and Nadel, 1978; Buzsaki *et al.*, 1983), and have been associated with the consolidation of some forms of memory (Buzsaki, 2006; Logothetis *et al.*, 2012).

In the present study, we used brief bursts of high frequency (≥ 80 Hz) tACS in place of TMS in the context of an associative stimulation protocol. We hypothesised

that pairing peripheral afferent stimulation with the application of high frequency alternating current over M1 would lead to changes in corticospinal excitability comparable to those obtained using conventional PAS.

2.2.2 Rationale for experimental parameters

In the course of these experiments, the parameters of both the peripheral and cortical elements of stimulation were systematically altered in order to address a sequence of questions on how paired stimulation may modulate CSE. The principle associative excitability effect explored here constituted a novel finding. Therefore in several of the experiments we also sought to explore and establish the boundaries of the range of effective stimulation parameters.

2.2.2.1 Frequency and intensity of tACS

As discussed in chapter one, a prime motive behind the application of tACS in humans is that it may modulate oscillatory brain activity. Alternating currents have previously been used most commonly within the lower range of ‘traditional’ EEG frequency bandwidths, aiming to interact with, e.g., 8 – 12 Hz alpha or 12 – 20 Hz beta activity (Antal *et al.*, 2008). Moliadze *et al.* (2010) demonstrated that high frequency tACS delivered over a sustained period of ten minutes could elevate the amplitude of MEPs in the period following stimulation. Previous studies have shown that following excitatory PAS protocols there are elevations in excitability that may last for over thirty minutes following stimulation (Carson *et al.*, 2013). Moliadze and colleagues found effects of comparable duration with 140 Hz tACS.

As noted, sharp wave ripple complexes are short bursts of high frequency activity in the range around 140 Hz that have been associated with memory

consolidation in mammals (Logothetis *et al.*, 2012). Should this prove to be a uniquely influential frequency in paired associative effects, it would have possible implications for the mechanisms of the effect. As with the Moliadze (2010) study, 80 Hz and 250 Hz frequencies were chosen as comparator conditions for the purpose of parameter exploration.

The intensity of stimulation used in previous tACS experiments has closely mirrored tDCS experiments, generally between 1mA and 2mA. We chose 2mA on the basis that previous modelling and animal studies (Reato *et al.*, 2013) have suggested that this should be sufficient to ensure enough current is delivered to cortex to modulate neural function. 1mA and 3mA were tested to explore whether there was a ‘floor’ or ‘ceiling’ effect evident with intensity or indeed whether there might be any evidence of a dose dependent effect.

2.2.2.2 Stimulation duration, cycle duration and number of pairings

A key consideration for this series of experiments was to address the question of whether associative effects could be derived from stimulation protocols that utilised ‘non-discrete’ stimuli. Therefore, a PAS protocol was selected which had been used in previous studies that has demonstrated excitability changes; thirty minutes of total duration, with paired stimuli occurring every ten seconds plus or minus a five second jitter (e.g. Carson *et al.*, 2013). To ensure maximum comparability to this protocol, the PATACS sessions and all other control sessions mirrored this stimulation and cycle duration.

The duration of each paired stimulation event was set at 500ms. This was in order to preserve comparability to previous PAS studies that have utilised trains of five peripheral nerve stimuli at 10 Hz. This duration also ensured that, temporally, the two

overlapping stimuli were confirmedly ‘non-discrete’ in nature. Again, due to the novelty of the protocol, it was of interest to explore the upper and lower boundaries of effectiveness for the duration of stimuli. Hence 250ms and 1000ms duration bursts were included as comparators conditions.

2.2.2.3 Muscle vibration

As noted in chapter one, anatomical evidence suggests that following PAS at levels exceeding motor threshold, activation of muscle spindle fibres is likely to have greater influence on circuits converging upon afferent input to M1 than cutaneous afferents (Carson & Kennedy, 2013). Therefore we sought to explore the effect of an afferent stimulation modality that activated muscle spindles to a greater and cutaneous afferents to a lesser extent than electrical peripheral nerve stimulation. The kinaesthetic illusory threshold (for intensity) and the frequency of muscle tendon vibration were selected to match previous studies that had found reliable effects of vibration on corticospinal excitability (Forner-Cordero *et al.*, 2008).

2.3 Methods

2.3.1 Participants

Ninety healthy volunteers each participated in one of seven experiments. Their characteristics (with respect to age and sex) are provided below in the description of each experiment. In no instance was there a statistically reliable imbalance in terms of the age or sex of the participants. No individual was involved in more than one experiment. All were right handed according to the Edinburgh handedness inventory (Oldfield, 1971), and gave informed consent to procedures approved by the Trinity College Dublin Ethics Committee, which were conducted in accordance with the Declaration of Helsinki. For any given experiment, the order of allocation to conditions was pseudo-randomised and counterbalanced across participants. In line with current recommendations (Nitsche *et al.*, 2008), successive testing sessions were separated by at least 7 days.

2.3.2 Recording Procedures

The participants were seated with the upper limbs supported and stabilized by vacuum cushions, the forearms in mid-pronation and the elbows semi-flexed (100-120°). Electromyographic (EMG) activity was recorded from the right flexor carpi radialis (FCR) and extensor carpi radialis brevis (ECR) muscles, using pairs of silver chloride (AgCl) electrodes. EMG signals were amplified (gain = 1000), bandpass filtered (20 or 30 – 1000 Hz), and digitized at a sampling rate of 4 kHz.

Magnetic stimuli were delivered to the left primary motor cortex (M1) by a Magstim 200 stimulator using a figure of eight coil (internal wing diameter 70mm), located at the optimal position (“hot spot”) to obtain a motor evoked potential (MEP) in

the FCR muscle of the contralateral (right) arm. The coil was placed so that the axis of intersection between the two loops was oriented at approximately 45 degrees to the sagittal plane, to induce posterior to anterior current flow across the motor strip. Once the hot spot was established, the lowest stimulation intensity at which MEPs with peak-to-peak amplitude of approximately 50 μ V were evoked in at least 5 of 10 consecutive trials was taken as resting motor threshold (RMT).

Prior to each intervention (Pre), a MEP recruitment curve was obtained by delivering TMS at 10% increments of intensity between 90% and 160% of the RMT. Six stimuli were delivered at each level of intensity. A further twelve stimuli were delivered at 120% RMT. The order of delivery was randomised. The interval between successive stimuli varied between 4 and 6 seconds. The total duration of the sequence was approximately five minutes. The average MEP amplitudes obtained at 90% and 100% RMT was calculated to ensure that the threshold had been correctly determined. In cases where the averaged MEPs for these intensities did not correspond to the expected values (i.e. <50 μ V at 90%RMT, 50-100 μ V at 100%RMT), the threshold intensity was adjusted accordingly and another recruitment curve was obtained. Equivalent sets of stimuli (without adjustments of threshold) were delivered immediately following the intervention (Post0) and at 10 (Post10), 20 (Post20) and 30 (Post30) minutes thereafter. The first of these sets (i.e. Post0) always commenced within 30s following completion of the intervention. There was a break of five minutes after the delivery of each such set of stimuli prior to commencement of the subsequent set.

2.3.3 Methods Used in the Interventions

2.3.3.1 *Peripheral nerve stimulation (PNS)*

A constant-current stimulator (Grass S88 Dual Output Square Pulse Stimulator; Grass technologies, Rhode Island, USA) was used to locate the motor point of FCR by moving a bipolar surface electrode over the muscle belly. The principle identification criterion was a reliable, visible displacement of the FCR tendon at the wrist. Two AgCl electrodes were affixed in line with to the orientation of the muscle fibres – one on either side of the location thus defined. These were used for both stimulation and EMG recording. The intensity of stimulation delivered during an intervention was the minimum at which visible displacement of the FCR tendon was observed. Depending on experimental condition, 10 Hz trains of 3, 5 or 10 pulses (each of 1 ms duration) were employed.

2.3.3.2 *Transcranial alternating current stimulation (tACS)*

Flexible electrode paddles were placed within two saline-soaked 5cm x 5cm sponges and fixed securely on the scalp using non-conducting elastic straps. One electrode was placed over left M1 at the FCR “hot spot” determined previously by TMS. The other electrode was placed over the contralateral supraorbital area. A battery-driven stimulator (AM Systems Model 2200) controlled by Signal software (Cambridge Electronic Design, Cambridge, UK.) was used to deliver short (≤ 1000 ms) bursts of bipolar sinusoidal alternating current at a fixed frequency and amplitude (values dependent on experimental condition). The current density was 0.04 mA/cm^2 at 1 mA, 0.08 mA/cm^2 at 2mA, and 0.12 mA/cm^2 at 3mA. Electrode impedance was monitored and maintained below $5\text{k}\Omega$. In all tACS conditions, 180 bursts were delivered at

approximately ten seconds intervals, corresponding to a stimulation period of 30 minutes.

2.3.3.3 Transcranial Direct Current Stimulation (tDCS) and Sham stimulation

The anode was placed over left M1 at the FCR “hot spot” determined previously by TMS. The cathode was placed over the contralateral supraorbital area. In the tDCS condition, the stimulator was driven by Spike software (Cambridge Electronic Design) to deliver current at 1mA for 30 minutes, including an 10-second period at the beginning and at the end when the current was ramped up/down. For the sham stimulation condition, in order to provide a skin tingling sensation, the current was ramped up to 1mA over the first ten seconds and ramped back down to zero over the subsequent ten seconds.

2.3.3.4 Transcranial Magnetic Stimulation (PAS condition)

We utilised a variant of PAS described by Castel-Lacanal *et al.* (2007) (see also Carson *et al.*, 2013). The peripheral nerve stimulation consisted of a train of 5 pulses delivered at 10Hz. Single pulse TMS at 120% RMT (FCR) was delivered 25ms after the final pulse of the train. The protocol was in other respects equivalent to that described for tACS.

2.3.3.5 Muscle Vibration

Vibration was applied to the distal tendon of the right FCR, 3cm proximal to the radiocarpal joint, by means of an exciter (Type 4810 mini-shaker, Bruel & Kjaer, Sydney) driven by Signal software (Cambridge Electronic Design) via a power

amplifier (Ling Dynamic Systems UK model PA 25). A notched plastic probe attached to the exciter was applied perpendicular to the tendon with a comfortable but firm load, which remained constant throughout the experiment through stable fixation of both the arm and the exciter. Sinusoidal vibration at 80Hz was delivered at the threshold level necessary to evoke the kinaesthetic illusory sensation (Naito *et al.*, 1999). To define this threshold participants were asked to look away from the hand while the amplitude of the vibration was increased gradually over approximately 20 seconds. The participants were asked to report the first moment at which they perceived the wrist to flex towards the body midline. The threshold was defined as the mean amplitude of vibration necessary to evoke the illusion in three such trials.

2.3.4 General Procedures

The procedures for the seven experiments differed only in the parameters of stimulation applied during the intervention. Figure 1 illustrates the time course of the experiments. In all experiments, regardless of intervention condition, PNS was used to establish the motor point and define threshold stimulation intensity.

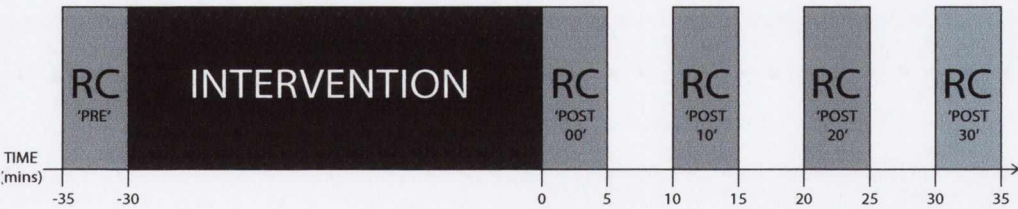


Figure 2.1. Time course of each experimental session. A recruitment curve ('Pre') was measured at the beginning of testing followed by a 30 minutes intervention that differed according to the experimental condition. Further recruitment curves were measured immediately following ('Post00') and at 10 ('Post10'), 20 ('Post20') and 30 ('Post30') minutes following the completion of the intervention.

The first MEP recruitment curve was recorded prior to commencement of the intervention. Thereafter, the tACS electrodes were placed on the scalp, and the bipolar surface electrodes on the FCR were switched from EMG measurement to PNS delivery - regardless of whether the forthcoming condition required peripheral stimulation. Following the (30 minutes duration) intervention, further recruitment curves were obtained at fixed intervals (~0, 10, 20, and 30 minutes).

In five of the seven experiments the participants were asked to attend testing on three occasions. These were separated by at least one week. Testing was conducted at the same time of day for each participant to control for potential effects of circadian cortisol fluctuations (Sale *et al.*, 2007). In examining the effects of sham stimulation (Experiment 4) and peripheral nerve stimulation alone (Experiment 7) – see below, there were one and two sessions respectively. Testing was carried out double-blinded in experiments 1, 2, 3 and 7, and in all other experiments the participants remained naïve to the specific parameters of stimulation. In all cases, a jitter (± 5000 ms maximum) was introduced in relation to the timing of successive stimulation events (mean separation 10 seconds) in order to ensure that, for conditions in which at least one modality was perceptible (tACS and tDCS were in general not perceived), the participants could not anticipate their onset.

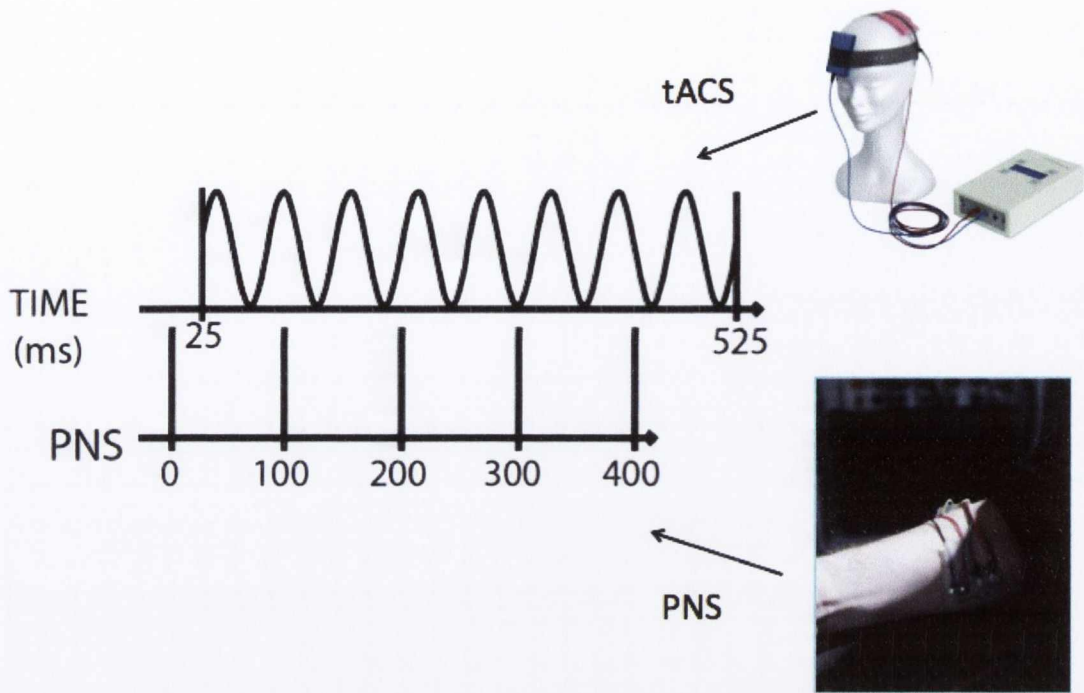
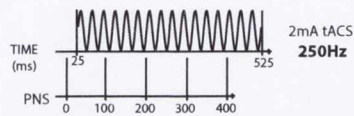
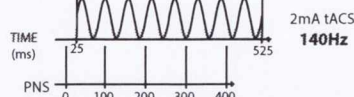
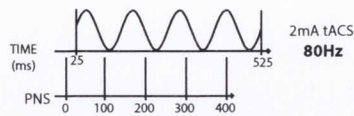
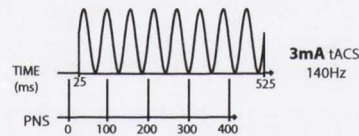
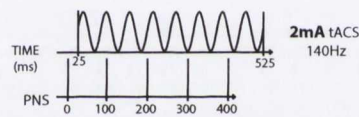
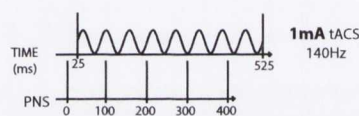


Figure 2.2 Electrode montage for elements of stimulation. One tACS electrode was placed over the left M1 and the second electrode was placed over the right supraorbital area. The peripheral nerve stimuli (PNS) were delivered via surface electrodes placed either side of the motor point of the FCR muscle.

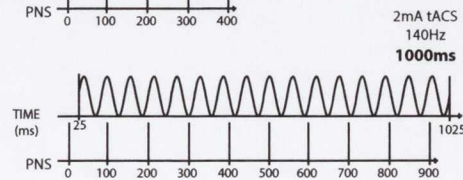
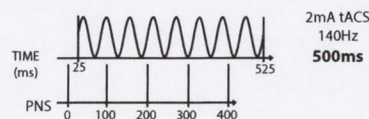
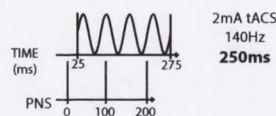
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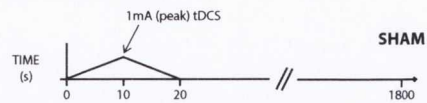
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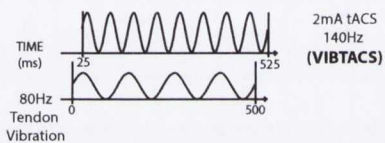
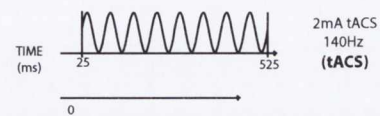
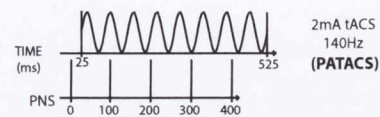
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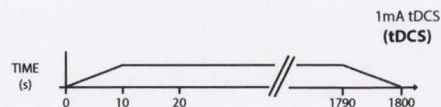
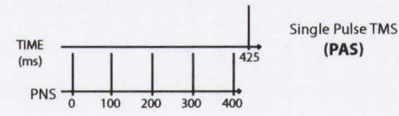
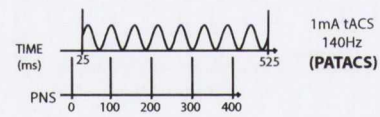
D (EXPERIMENT 4)



E (EXPERIMENT 5)



F (EXPERIMENT 6)



G (EXPERIMENT 7)

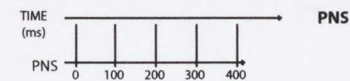
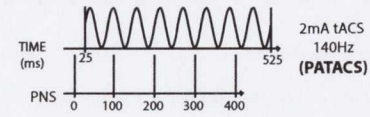


Figure 2.3. Stimuli presented during the interventions applied in each experiment. In its prototypical form PATACS comprised a train of electrical peripheral nerve stimulation (PNS - five pulses at 10 Hz) commencing 25 ms prior to the onset of a 500 ms duration burst of tACS with frequency 140Hz and current 2 mA. There were 180 such paired stimuli, one every ~10 s over a period of 30 minutes. **A)** In Experiment 1 the frequency of tACS (80Hz, 140Hz or 250Hz) was varied across conditions. **B)** In Experiment 2 the current intensity (1 mA, 2 mA and 3 mA) was varied. **C)** In Experiment 3 the duration of stimulation was varied - either a train of 3 PNS pulses was paired with 250 ms of tACS, 5 PNS pulses were paired with 500 ms of tACS, or 10 PNS pulses were paired with 1000 ms tACS. **D)** In Experiment 4 transcranial unipolar direct current was ramped up from 0mA to 1mA over ten seconds, then ramped back down to 0 mA during the ten seconds following. **E)** In Experiment 5 the PATACS condition consisted of the prototypical form of stimulation. The tACS condition involved AC stimulation unaccompanied by PNS. In the VIBTACS condition 80Hz vibration of the muscle tendon was applied for 500ms, commencing 25ms prior to the onset of 500 ms tACS. **F)** In Experiment 6, PATACS was delivered in its prototypical form, with the exception that the tACS current was 1mA. In the PAS condition, single pulse TMS at 120% RMT (FCR) was delivered 25ms after the final pulse of the PNS train. In the tDCS condition, 1mA ‘anodal’ direct current was applied for 30 minutes. **G)** In Experiment 7, PATACS was delivered in its prototypical form. The PNS condition involved electrical peripheral afferent stimulation unaccompanied by tACS.

2.3.5 Interventions

2.3.5.1 Experiment 1 – paired associative transcranial alternating current stimulation (PATACS) with variations of tACS frequency

Each of the 180 paired stimulation events (separated by ≈ 10 s) comprised a train of PNS (five pulses at 10 Hz). The train commenced 25ms prior to the onset of a 500 ms duration burst of 2mA tACS. In the three separate conditions, the frequency of tACS was 80Hz, 140Hz or 250Hz (Figure 2A). The twelve participants (6 male) were aged 18–28 (mean, 21.8 ± 4.3 years).

2.3.5.2 Experiment 2 –PATACS with variations of tACS current

Each of the 180 paired stimulation events comprised a train of PNS (five pulses at 10 Hz). The train commenced 25ms prior to the onset of a 500 ms duration burst of 140Hz tACS. In three separate conditions, the tACS current was either 1mA, 2mA or 3mA (Figure 2B). The twelve participants (3 male) were aged 20–29 (mean, 24.0 ± 3.7 years).

2.3.5.3 Experiment 3 – PATACS with variations of tACS and PNS duration

Each of the 180 paired stimulation events comprised a train of PNS at 10Hz which commenced 25ms prior to the onset of a burst of 140Hz 2 mA tACS. In the first condition PNS (train of 3 pulses) was paired with tACS lasting 250ms. In the second, PNS (5 pulses) was paired with tACS of duration 500ms. In the third condition, PNS (10 pulses) was paired with tACS of duration 1000ms (Figure 2C). The twelve participants (3 male) were aged 18–40 (mean, 24.7 ± 6.2 years).

2.3.5.3 Experiment 4 - Sham stimulation

Transcranial unipolar direct current was ramped up from 0mA to 1mA over ten seconds, then ramped back down to 0 mA during the ten seconds following. During the 30 minutes intervention period peripheral afferent stimulation was not applied (Figure 2D). The twelve participants (4 male) were aged 20–30 (mean, 22.7 ± 3.3 years).

2.3.5.4 Experiment 5 – PATACS with variation of peripheral afferent stimulation modality

In the VIBTACS condition, 80Hz vibration of the FCR muscle tendon was applied for 500ms at the kinaesthetic illusory threshold. This commenced 25ms prior to the onset of a 500 ms burst of 140Hz 2 mA tACS. This pairing was repeated approximately every ten seconds for 30 minutes. In the PATACS condition, each of the 180 paired stimulation events comprised a train of PNS (5 pulses at 10Hz), which commenced 25ms prior to the onset of a burst of 140Hz 2 mA tACS. In a further control condition, 500ms bursts of 140Hz 2 mA tACS were delivered at approximately ten seconds intervals for 30 minutes. In this condition there was no peripheral stimulation (Figure 2E). The twelve participants (5 male) were aged 18–24 (mean, 20.2 ± 2.0 years).

2.3.5.5 Experiment 6 – Comparison of PATACS with PAS and tDCS

In the PATACS condition, each of the 180 paired stimulation events comprised a train of PNS (5 pulses at 10Hz), which commenced 25ms prior to the onset of a burst of 140Hz 1 mA tACS. In the PAS condition, single pulse TMS at 120% RMT (FCR) was delivered 25ms after the final pulse of the PNS train (Castel-Lacanal *et al.*, 2007; Carson *et al.*, 2013). In the tDCS condition, unipolar direct current was ramped up from 0mA to 1mA over a period of ten seconds. Stimulation continued at a constant current of 1mA for 30 minutes, before being ramped back down to 0mA over ten seconds (Figure 2F). The twelve participants (9 male) were aged 20–31 (mean, 24.6 ± 4.6 years).

2.3.5.6 Experiment 7 – Comparison of PATACS with PNS alone

In the PATACS condition, each of the 180 paired stimulation events comprised a train of PNS (5 pulses at 10Hz), which commenced 25ms prior to the onset of a burst of

140Hz 2 mA tACS. In the PNS alone condition, 180 PNS events were delivered over the course of the 30 minutes intervention period in accordance with the PATACS schedule. These were preceded by three paired PNS-tACS events. Eighteen participants were enrolled in this experiment. One person withdrew on account of a dislike of the peripheral nerve stimulation. With respect to the remaining seventeen participants (10 male), they were aged 18–32 (mean, 24.1 ± 3.8 years).

2.3.6 Data analysis

The root mean square (rms) of the background EMG recorded in FCR and ECR was calculated for a window 93ms to 3ms before TMS onset. If the value was greater than $5\mu\text{V}$ for either muscle, the corresponding MEP was disregarded. As a further means of eliminating instances in which elevated excitability of the spinal motoneuron pool may have influenced the MEP amplitude, we first calculated for each participant (separately for FCR and ECR) the quartiles for all background rms EMG values retained following the screening procedure described above. In the event that an individual rms value was above the upper whisker of the distribution (in this instance set to the third quartile plus 1.5 times the interquartile range) the corresponding MEP was disregarded. Overall, 88.4% of the responses were retained.

For the retained recordings, the mean (peak-to-peak) amplitude of the MEPs elicited at the eight respective stimulation intensities was calculated. For each time of measurement (Pre, Post0, Post10, Post20 and Post30), the summated area under the recruitment curve (AURC) - bounded by magnetic stimulation intensity and MEP amplitude (in units of mV.T), was obtained using the trapezoidal rule. It has been demonstrated elsewhere (Carson *et al.*, 2013) that the AURC is an extremely reliable

measure of the state of corticospinal projections to hand and forearm muscles, which has construct, face, and concurrent validity.

It is widely recognised (e.g. Abelson, 1995) that in contrast to between-groups analyses, inferential tests in repeated measures designs, including ANOVA and mixed effects models, are highly vulnerable to violation of underlying assumptions, including normality of the sample distribution. In order to address this issue, the normality of the distribution of AURC values obtained in each analysis cell (i.e. separately for each experiment) was assessed using the Shapiro-Wilks test. On the basis of these analyses it was established that for 42% (38/90) of the cells, the AURC values were not normally distributed ($p < 0.05$). To increase the symmetry of the sample distribution for the purposes of inferential analyses, the AURC values were therefore subject to a log transformation. Following application of the transformation, there were no cells for which the outcome of the Shapiro-Wilks test indicated that the values were not normally distributed.

Mixed effects models in which participant was a random effect, and time was a fixed effect (levels = Pre, Post0, Post10, Post20, Post30), were conducted separately for each experiment (using the lmerTest package in R). The fitting of the models employed restricted maximum likelihood (REML) estimation and an unstructured covariance matrix. On the basis of these models, planned contrasts were conducted between the (log) AURC value obtained prior to the intervention (Pre), and the (log) AURC calculated for each time point following the intervention (Post0, Post10, Post20, Post30). The exact probabilities associated with each comparison are reported in Tables 1-7. The relevant degrees of freedom were obtained using Kenward-Roger's approximation that - in the case of the balanced designs employed in the present study, yields values equivalent to those of a repeated measures ANOVA design.

A series of supplementary analyses (using mixed effects models) were performed that contrasted at each post-intervention time point, the (log) AURC values obtained in the various conditions employed in each study. In all such instances, the AURC value obtained prior to the intervention in each condition was used as a covariate.

In order to further assist in the interpretation of the tests of significance, in particular with a view to comparing the respective conditions included in each experiment, the unbiased effect size index for ANOVA (f) (Cohen, 1988) was calculated for each planned contrast following Nakagawa and Cuthill (2007). This is a dimensionless index, which describes the degree of departure from no effect, in other words, the degree to which the phenomenon is manifested. A small effect size is considered by convention to be indicated by an f of 0.1, a medium effect size by an f of 0.25, and a large effect size by an f of 0.4.

2.4 Results

2.4.1 Experiment 1 - PATACS with variations of tACS frequency

In the 80Hz condition, indices of corticospinal excitability (AURC) were elevated reliably (relative to initial values) at all time points following the intervention. (Figure 3). The largest increase was observed 30 minutes following the cessation of stimulation. A statistically reliable increase was observed in the 140 Hz condition at 30 minutes post-intervention. In the 250 Hz condition, AURC values increased monotonically following the cessation of stimulation. They were elevated reliably relative to initial values at 30 minutes post-intervention (Table 1). The AURC values obtained in the three conditions were not however distinguished reliably from each other ($F(1, 65.4)$, $p = 0.05 - 0.93$, $f = 0.01 - 0.23$).

Table 2.1 – Experiment 1: F ratios, p values and effect sizes for comparisons between the AURC obtained prior to the intervention (Pre), and the values obtained at each of 4 time points following the intervention.

Condition	Pre vs.	F (1,88)	p value	Effect Size (f)
80Hz	Post00	5.59	0.020	0.24
	Post10	6.36	0.010	0.26
	Post20	8.67	0.004	0.30
	Post30	17.78	< 0.001	0.44
140Hz	Post00	0.14	0.706	0.04
	Post10	3.10	0.081	0.18
	Post20	2.28	0.135	0.16

250Hz	Post30	24.40	< 0.001	0.51
	Post00	0.97	0.330	0.10
	Post10	1.37	0.250	0.12
	Post20	3.77	0.056	0.20
	Post30	12.75	< 0.001	0.37

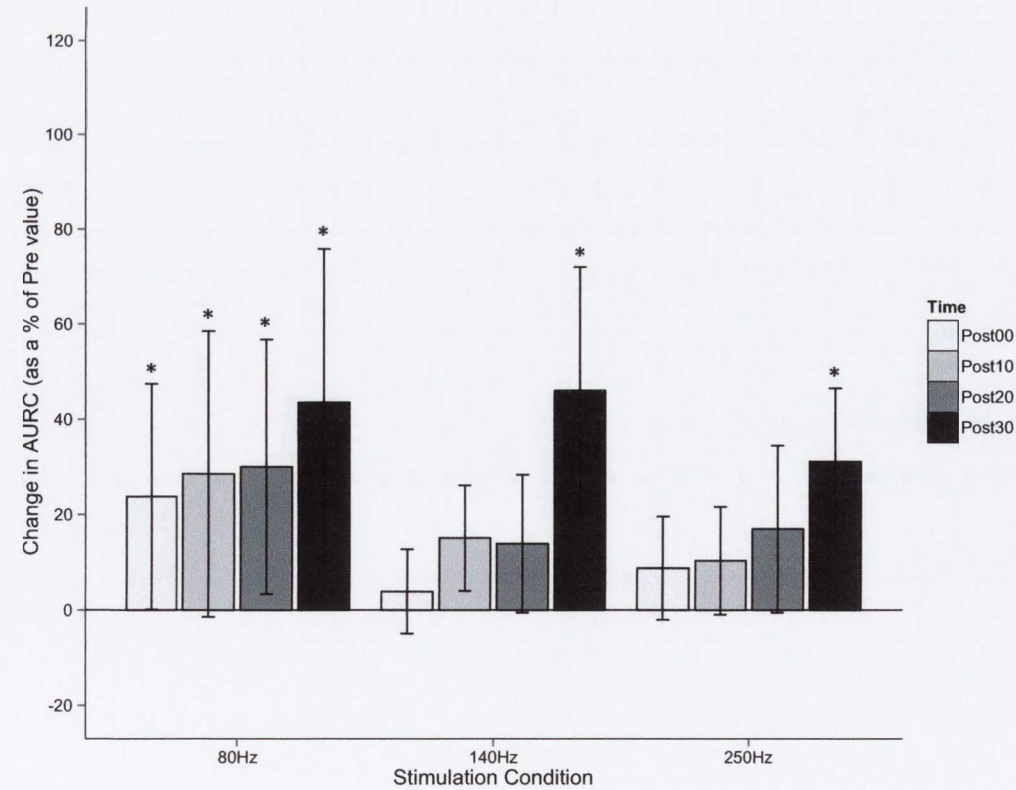


Figure 2.4. For Experiment 1 – in which the frequency of tACS was varied, the AURC for each post-intervention time point is expressed as the (percentage) change relative to the value obtained prior to the

intervention. All values are the mean of twelve participants. The error bars are the corresponding 95% confidence intervals calculated across participants. Values recorded following the intervention that differed reliably ($p < 0.05$) from those obtained prior to the intervention are represented by an asterisk symbol above the error bar.

2.4.2 Experiment 2 –PATACS with variations of tACS current

When 1mA tACS current was employed, elevations in CSE following the interventions were observed ten minutes following the cessation of paired stimulation. These increased in magnitude thereafter (Figure 4). In the 2mA condition, a similar trend expressed at Post20 (Table 2) was expressed reliably thirty minutes following the cessation of paired stimulation. In the 3mA condition, elevations of CSE were evident 20 and 30 minutes following paired stimulation. The AURC values obtained in the three conditions were not however distinguished reliably from each other ($F(1, 64.6)$, $p = 0.42 - 0.83$, $f = 0.01 - 0.09$).

Table 2.2 – Experiment 2: F ratios, p values and effect sizes for comparisons between the AURC obtained prior to the intervention (Pre), and the values obtained at each of 4 time points following the intervention.

Condition	Pre vs.	F (1,88)	p value	Effect Size (f)
1mA	Post00	1.41	0.230	0.12
	Post10	4.96	0.029	0.23
	Post20	4.77	0.032	0.23
	Post30	14.59	< 0.001	0.40

2mA	Post00	0.18	0.670	0.04
	Post10	1.36	0.250	0.12
	Post20	4.81	0.031	0.23
	Post30	5.60	0.020	0.25
3mA	Post00	0.97	0.330	0.10
	Post10	2.22	0.140	0.15
	Post20	8.19	0.005	0.30
	Post30	11.74	< 0.001	0.36

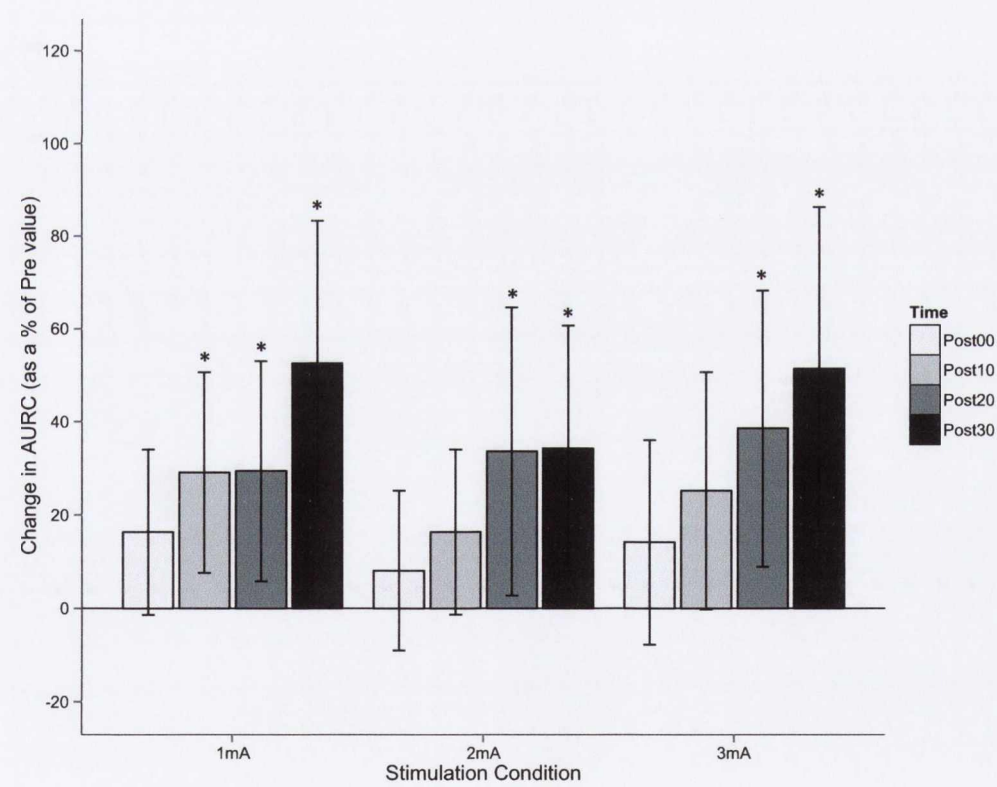


Figure 2.5. For Experiment 2 – in which the tACS current was varied, the AUCR for each post-intervention time point is expressed as the (percentage) change relative to the value obtained prior to the intervention. All values are the mean of twelve participants. The error bars are the corresponding 95%

confidence intervals calculated across participants. Values recorded following the intervention that differed reliably ($p < 0.05$) from those obtained prior to the intervention are represented by an asterisk symbol above the error bar.

2.4.3 Experiment 3 – PATACS with variations of tACS and PNS duration

Although the mean AURC values obtained following paired stimulation in the 250ms and 500ms conditions were larger than those recorded prior to the intervention, these changes were not statistically reliable (Table 3). When the duration of the stimulation events was 1000ms, CSE was elevated reliably at 20 and 30 minutes post-paired stimulation (Figure 5). The AURC values obtained in the three conditions were not however distinguished reliably from each other ($F(1, 64.7)$, $p = 0.19 - 0.98$, $f = 0.003 - 0.16$).

Table 2.3 – Experiment 3: F ratios, p values and effect sizes for comparisons between the AURC obtained prior to the intervention (Pre), and the values obtained at each of 4 time points following the intervention.

Condition	Pre vs.	F (1,88)	p value	Effect Size (f)
250ms	Post00	1.14	0.290	0.11
	Post10	1.14	0.290	0.11
	Post20	0.68	0.410	0.08
	Post30	2.34	0.130	0.16
500ms	Post00	0.83	0.363	0.09
	Post10	1.31	0.255	0.12
	Post20	0.41	0.524	0.07

1000ms	Post30	1.56	0.214	0.13
	Post00	0.16	0.694	0.04
	Post10	1.69	0.198	0.13
	Post20	5.96	0.017	0.25
	Post30	4.34	0.040	0.22

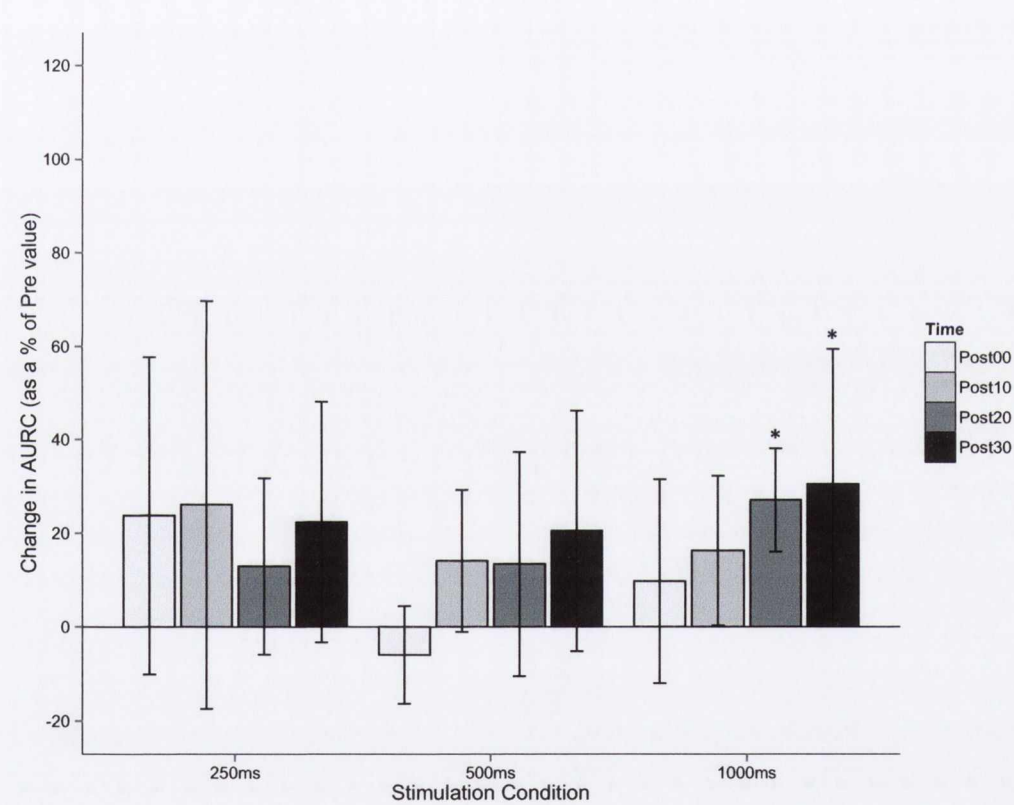


Figure 2.6. For Experiment 3 – in which the duration of the paired stimulation events was varied, the AURC for each post-intervention time point is expressed as the (percentage) change relative to the value obtained prior to the intervention. All values are the mean of twelve participants. The error bars are the corresponding 95% confidence intervals calculated across participants. Values recorded following the intervention that differed reliably ($p < 0.05$) from those obtained prior to the intervention are represented

by an asterisk symbol above the error bar.

2.4.4 Experiment 4 - Sham stimulation

Sham stimulation failed to produce reliable changes in corticospinal excitability (Figure 6 and Table 4).

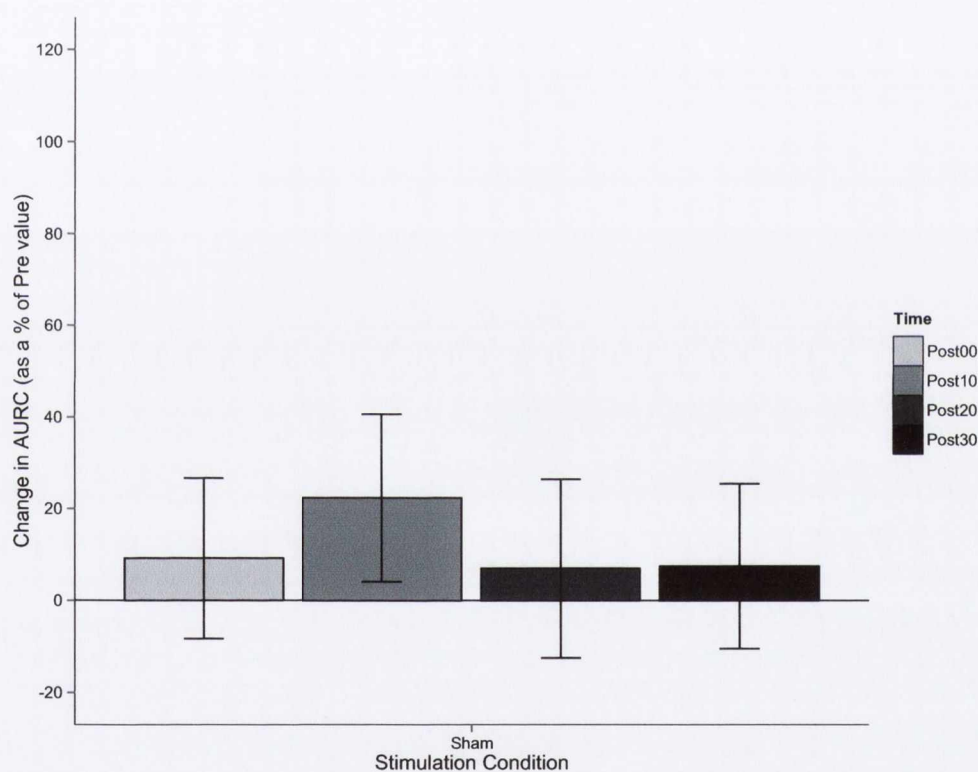


Figure 2.7. For Experiment 4 – in which sham stimulation was delivered, the AURC for each post-intervention time point is expressed as the (percentage) change relative to the value obtained prior to the intervention. All values are the mean of twelve participants. The error bars are the corresponding 95% confidence intervals calculated across participants. There were no instances in which values recorded following the intervention differed reliably from those obtained prior to the intervention.

Table 2.4 – Experiment 4: F ratios, p values and effect sizes for comparisons between the AURC obtained prior to the intervention (Pre), and the values obtained at each of 4 time points following the intervention.

Condition	Pre vs.	F (1,44)	p value	Effect Size (f)
Sham	Post00	0.36	0.554	0.09
	Post10	3.28	0.077	0.26
	Post20	0.03	0.861	0.03
	Post30	0.09	0.763	0.04

2.4.5 Experiment 5 - PATACS with variation of peripheral afferent stimulation modality

In the PATACS condition, reliable elevations in corticospinal excitability were obtained 30 minutes following the cessation of paired stimulation (Figure 7). In the VIBTACS condition - when measured at all time points following the cessation of paired (tACS and tendon vibration) stimulation, the AURC was markedly larger than pre-intervention values (Table 5). No reliable increases in corticospinal excitability were observed at any time point following the administration of tACS alone (i.e. without peripheral stimulation). Additional analyses revealed that in the PATACS condition, corticospinal excitability was greater than that observed in the tACS only condition, when contrasted (df = 1, 61.8) at 30 minutes following the end of the intervention (p = 0.02, f = 0.28). Upon the end of the intervention (Post00), the AURC values obtained in the VIBTACS condition were larger than those in the tACS only condition (p = 0.02, f = 0.30), with a similar pattern being expressed 10 (p = 0.07, f =

0.23) and 30 ($p = 0.07$, $f = 0.23$) minutes thereafter. The AURC values obtained in the PATACS and VIBTACS conditions were not distinguished reliably from each other ($F(1, 61.8)$, $p = 0.10 - 0.69$, $f = 0.05 - 0.21$).

Table 2.5 – Experiment 5: F ratios, p values and effect sizes for comparisons between the AURC obtained prior to the intervention (Pre), and the values obtained at each of 4 time points following the intervention.

Condition	Pre vs.	F (1,88)	p value	Effect Size (f)
PATACS	Post00	1.52	0.221	0.13
	Post10	1.83	0.180	0.14
	Post20	2.11	0.150	0.15
	Post30	11.20	0.001	0.36
TACS	Post00	0.09	0.767	0.03
	Post10	1.03	0.312	0.11
	Post20	0.43	0.514	0.07
	Post30	0.01	0.921	0.01
VIBTACS	Post00	15.43	< 0.001	0.41
	Post10	20.75	< 0.001	0.48
	Post20	12.28	< 0.001	0.37
	Post30	12.10	< 0.001	0.37

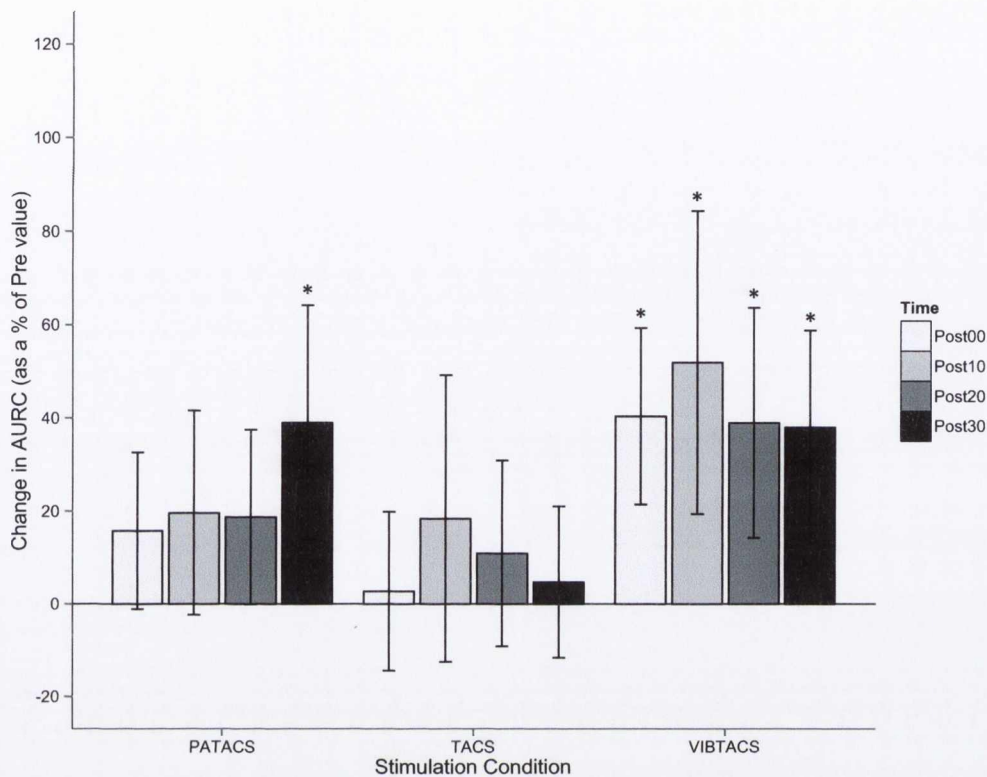


Figure 2.8. For Experiment 5 – in which the nature of the afferent stimulation was varied, the AURC for each post-intervention time point is expressed as the (percentage) change relative to the value obtained prior to the intervention. All values are the mean of twelve participants. The error bars are the corresponding 95% confidence intervals calculated across participants. Values recorded following the intervention that differed reliably ($p < 0.05$) from those obtained prior to the intervention are represented by an asterisk symbol above the error bar.

2.4.6 Experiment 6 - Comparison of PATACS with PAS and tDCS

In the PATACS condition, reliable elevations in corticospinal excitability were obtained 30 minutes following the cessation of paired stimulation (Figure 7). In the tDCS and PAS conditions, no changes in corticospinal excitability met conventional

criteria for statistical significance (Table 6). The AURC values obtained in the three conditions were not distinguished reliably from each other ($F(1, 65.2)$, $p = 0.24 - 0.99$, $f = 0.002 - 0.14$).

Table 2.6 – Experiment 6: F ratios, p values and effect sizes for comparisons between the AURC obtained prior to the intervention (Pre), and the values obtained at each of 4 time points following the intervention.

Condition	Pre vs.	F (1,88)	p value	Effect Size (f)
PATACS	Post00	1.25	0.266	0.12
	Post10	2.01	0.160	0.15
	Post20	1.37	0.245	0.12
	Post30	4.16	0.044	0.21
PAS	Post00	1.63	0.204	0.13
	Post10	2.50	0.117	0.16
	Post20	1.25	0.267	0.12
	Post30	2.59	0.111	0.17
TDCS	Post00	0.21	0.650	0.05
	Post10	2.94	0.090	0.18
	Post20	1.80	0.184	0.14
	Post30	2.44	0.122	0.16

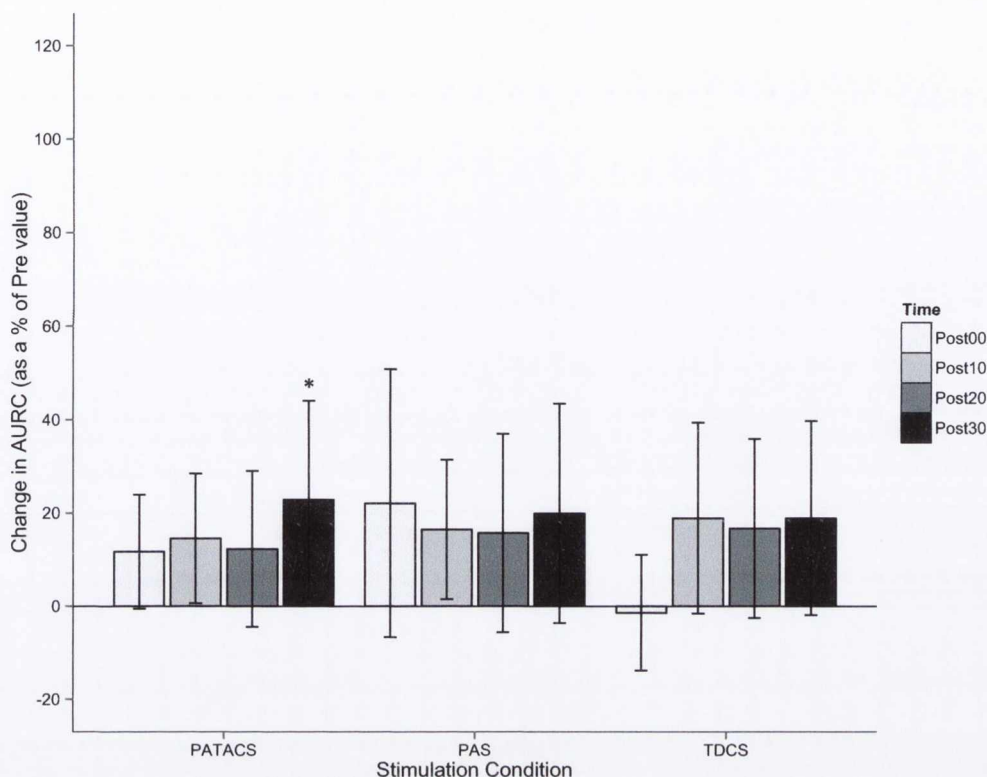


Figure 2.9. For Experiment 6 – in which the effects of PATACS were compared with PAS and tDCS, the AURC for each post-intervention time point is expressed as the (percentage) change relative to the value obtained prior to the intervention. All values are the mean of twelve participants. The error bars are the corresponding 95% confidence intervals calculated across participants. Values recorded following the intervention that differed reliably ($p < 0.05$) from those obtained prior to the intervention are represented by an asterisk symbol above the error bar.

2.4.7 Experiment 7 – Comparison of PATACS with PNS alone

In the PATACS condition, reliable elevations in corticospinal excitability were obtained 10, 20 and 30 minutes following the cessation of paired stimulation (Figure 9). No reliable increases in corticospinal excitability were observed at any time point following the administration of peripheral nerve stimulation alone (i.e. without tACS) (Table 7). Additional analyses revealed that in the PATACS condition, corticospinal excitability was greater than that observed in the PNS only condition, when contrasted

(df = 1, 47.7) at 0 (p = 0.01, f = 0.38), 10 (p = 0.05, f = 0.28) and 30 (p = 0.04, f = 0.31) minutes following the end of the intervention.

Table 2.7 – Experiment 7: F ratios, p values and effect sizes for comparisons between the AURC obtained prior to the intervention (Pre), and the values obtained at each of 4 time points following the intervention.

Condition	Pre vs.	F (1,64)	p value	Effect Size (f)
PATACS	Post00	3.16	0.074	0.22
	Post10	5.22	0.022	0.29
	Post20	5.12	0.024	0.28
	Post30	3.93	0.046	0.25
PNS	Post00	1.17	0.273	0.14
	Post10	0.06	0.799	0.03
	Post20	0.47	0.484	0.09
	Post30	0.06	0.810	0.03

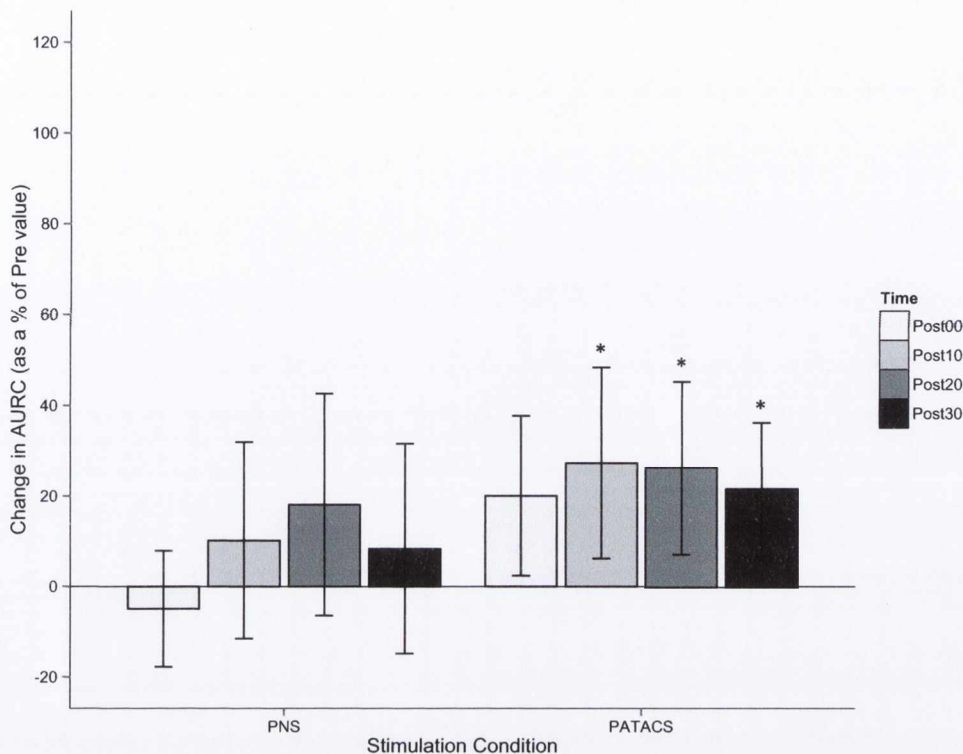


Figure 2.10. For Experiment 7 – in which the effects of PATACS were compared with PNS alone, the AURC for each post-intervention time point is expressed as the (percentage) change relative to the value obtained prior to the intervention. All values are the mean of seventeen participants. The error bars are the corresponding 95% confidence intervals calculated across participants. Values recorded following the intervention that differed reliably ($p < 0.05$) from those obtained prior to the intervention are represented by an asterisk symbol above the error bar.

2.4.8 Pooled Data –PATACS comprising 140 Hz, 2 mA tACS of 500 ms duration

Sixty-four participants (drawn from five experiments) were exposed to the same PATACS protocol (i.e. PNS paired with 140 Hz, 2 mA tACS of 500 ms duration). In analysing these pooled data, the relatively large sample size permitted the calculation of confidence intervals (95%) for the effect size estimates (Smithson, 2001). When assessed thirty minutes following the cessation of stimulation, the observed changes in corticospinal excitability constituted a medium to large effect (95% C.I. (Cohen's f

unbiased) 0.27 – 0.48; $F(1, 251) = 36.5$, $p < 0.0001$). There was a small to medium effect when assessed ten (95% C.I. (Cohen's f unbiased) 0.13 – 0.34; $F(1, 251) = 14.2$, $p = 0.0002$) and twenty 95% C.I. (Cohen's f unbiased) 0.14 – 0.35; $F(1, 251) = 15.5$, $p = 0.0001$) minutes post-stimulation. There was no basis upon which to conclude that corticospinal excitability was elevated immediately following the intervention (95% C.I. (Cohen's f unbiased) 0 – 0.20; $F(1, 251) = 2.27$, $p = 0.13$).

2.4.9 Additional observations

In Experiment 1, eleven of the twelve participants reported experiencing phosphenes in the 80Hz condition. No such percepts were reported in any of the other conditions or experiments.

2.5 Discussion

We have demonstrated that a novel form of associative stimulation, in which bursts of tACS are paired with trains of peripheral afferent stimulation, increases the excitability of corticospinal projections to the forearm. In the context of a series of seven experiments (engaging distinct groups of participants), in which various stimulation parameters were manipulated, it was evident that the defining effect is highly replicable. Most notably, it is not contingent upon temporally discrete cortical or peripheral stimulation events. Rather it was obtained using extended (500 and 1000 ms) periods of excitation.

Among the many means by which LTP and LTD can be induced in reduced preparations, it has been suggested (e.g. Wolters *et al.*, 2005) that STDP occupies a unique position in so much as the polarity of the induced change in synaptic efficacy is determined by the sequence of pre- and postsynaptic neuronal activity (for reviews see Dan & Poo, 2004; Markram *et al.*, 2011). In the classical model of STDP (e.g. Song *et al.*, 2000), strengthening (potentiation) arises if the presynaptic neuron fires no more than 50 ms in advance of the postsynaptic neuron (Feldman, 2000), whereas weakening (depression) occurs if postsynaptic spikes precede presynaptic action potentials – (or transpire without activity in the presynaptic neuron) (Levy & Steward, 1983; Bi & Poo, 1998; Cooke & Bliss, 2006).

In foundational descriptions of PAS (e.g. Wolters *et al.*, 2003) it was highlighted that increases in corticospinal excitability are achieved if PNS is timed such that the initial phase of input to M1 arising as its corollary occurs synchronously with the delivery of a magnetic pulse over that region of cortex. If the relative timing is adjusted such that TMS is applied prior to the time at which a corollary of the (single pulse) PNS is likely to reach M1, repeated pairings may lead to a subsequent reduction in the

excitability of corticospinal projections. Since these initial reports indicated not only that the order of the stimulus-generated cortical events is critical, but also that the effective inter-stimulus intervals lie within a very restricted range, it was concluded that PAS induced adaptation represents a form of associative LTP and LTD which exhibits the defining features of STDP (Müller-Dahlhaus *et al.*, 2010).

Increases in corticospinal excitability have been observed previously following the application of associative protocols that comprised extended trains of afferent stimulation. Ridding and Taylor (2001) administered TMS 25ms after the *onset* of 500 ms trains applied over the motor point of first dorsal interosseus (FDI). Following a 30 min intervention, increases in the amplitude of MEPs elicited in FDI were observed (see also McKay *et al.*, 2002). When the TMS is administered 25 ms following the *last* shock of the train, effects of a similar nature are obtained when either the ECR (Castell-Lacanal *et al.*, 2007) or the FCR (Carson *et al.*, 2013) motor point are in receipt of stimulation. While at first glance these results suggest that the range of effective ISIs is larger than is supposed in STDP-oriented accounts, in all cases the delay of the magnetic stimulus with respect to the preceding peripheral shock was 25 ms. It might be argued therefore that in these protocols also, the *effective* ISI was within the range of timing intervals associated with STDP.

In the present study however, the application of a range of tACS frequencies (80, 140 and 250 Hz), in conjunction with the use of both discrete (i.e. trains of electrical pulses) and continuous (80 Hz tendon vibration) methods of generating peripheral afference, precludes the possibility that the phenomenon was attributable to either a specific order or timing of the stimulus-generated cortical events. In thus revealing that associative effects are expressed when the timing (or order) of the contributory elements

is not precisely circumscribed, these findings suggest that multiple cellular pathways may mediate the LTP-type response typically ascribed to PAS.

With regard to the assumption that the associative nature of the stimulation protocol was instrumental in promoting the observed increases in corticospinal excitability, it has been reported on many previous occasions that peripheral nerve stimulation of the type used here (1 ms duration shocks applied at 10 Hz), induces elevations of CSE only when it is applied for extended (≥ 2 hours) periods, using high duty cycles (e.g. 50% - 500 ms on; 500 ms off) (Ridding *et al.*, 2000a; 2001; Charlton, 2003) Similarly, Forner-Cordero *et al.* (2008) and Steyvers *et al.* (2003) have shown that continuous 80Hz vibration of the FCR muscle tendon applied for 60 minutes is insufficient to induce either acute or chronic changes in the excitability of corticospinal projections to FCR. In the present study we also demonstrated that administration of the peripheral nerve stimulation alone was insufficient to bring about changes in corticospinal excitability. Similarly, bursts of tACS (2mA; 140 Hz; 500 ms duration) – when applied in isolation over a period of 30 minutes, failed to alter CSE. Sham stimulation was likewise ineffective. It seems reasonable to conclude therefore that the critical factor was the repeated association of tACS with peripheral afferent stimulation.

Recent developments in modelling current density suggest that tACS at the intensities utilised here is capable of altering the membrane potentials of neurons in M1. Neuling and colleagues (2012b) demonstrated that superficial areas of grey matter that protrude into the cerebrospinal fluid (a characteristic typical of the M1 representations of upper limb muscles) are likely to be subject to the highest current densities. When 1mA current is applied, these can reach 0.1A/m^2 , and generate electrical fields of up to $417\mu\text{V/mm}$. In the majority of conditions employed in the present study, currents of at least 2mA were used. These are likely to have been sufficient to provide current

densities well in excess of the minimum ($140\mu\text{V}/\text{mm}$) thought necessary to alter the polarisation of neural membranes (Francis *et al.*, 2003). Nonetheless, since such levels of tACS (or indeed tDCS) are not known to generate action potentials in descending corticospinal neurons, it might be supposed that the associative effects were mediated at the level of the cerebral cortex (i.e. rather than at the level of the spinal cord).

It has been hypothesised that direct current stimulation (tDCS) has the potential to bias (i.e. depolarise or hyperpolarise) the membrane potential of cortical neurons (Nitsche & Paulus, 2000). Due to the oscillating polarity of the applied current, it is unlikely that the effects of tACS can be accommodated in these terms. It is currently believed possible however, that at least in relation to the endogenous frequencies registered traditionally by EEG, tACS is capable of entraining oscillatory activity in the cortex (Antal *et al.*, 2008; Antal & Paulus, 2013; Herrmann *et al.*, 2013; Helfrich *et al.*, 2014). The literature concerning the use of high frequency (i.e. ≥ 80 Hz) tACS to influence intrinsic cortical rhythms is less extensive, however ripple range stimulation (Moliadze *et al.*, 2010) and high frequency random noise stimulation (Terney *et al.*, 2008) have both been used to increase corticospinal excitability. Moliadze and colleagues (2010) have speculated that the effect of 140Hz stimulation in particular, could be due to an interaction with sharp wave ripple complexes - short bursts high frequency oscillatory activity thought to be important in memory consolidation (Logothetis *et al.*, 2012). The conjecture that ripple range tACS is uniquely influential was not supported by the present results.

It might also be noted in this context that the impact of extended (i.e. 10 min) 140 Hz tACS (potentiating vs. inhibiting) is reportedly sensitive to the intensity of stimulation that is applied (Moliadze *et al.*, 2012). Specifically, a reduction in corticospinal excitability was demonstrated for 0.4 mA tACS, whereas (as per Moliadze

et al., 2010) an increase was obtained if 1 mA current was used. Due to variations in the size of electrode placed over the target M1 (25 cm² in the present study; 16 cm² in the Moliadze *et al.* studies), the current densities resulting from 1mA stimulation are not equivalent (0.04 mA/cm² vs. 0.06 mA/cm²). Nonetheless, at least within the range examined, variations in the level of applied tACS current – and thus in the current density (0.04 mA/cm² at 1 mA, 0.08 mA/cm² at 2mA, and 0.12 mA/cm² at 3mA) did not reliably influence the magnitude of the effects observed in the present study.

In experiment 1, 80 Hz, 140 Hz, and 250 Hz tACS – when paired with PNS, all brought about elevated levels of CSE. There remains the possibility that each of these frequencies were responsible for entrainment of a lower endogenous oscillatory frequency through sub-harmonic resonance. Bursts of high frequency (60-90 Hz) gamma oscillations have been ascribed a facilitating role in movement initiation (Cheyne, 2013). Half harmonic resonance from 140Hz stimulation, quarter harmonic resonance from 250Hz, and first harmonic resonance at 80Hz could match this high frequency gamma range. In principle, the timing of the peripheral nerve shocks relative to the peaks and troughs of the AC stimulation cycle may also have a determining influence on the effects that are induced.

Whether achieved by high intensity of individual shocks, and/or by elevated frequencies or periods of delivery, increases in the excitability of corticospinal projections from the primary motor cortex can in some circumstances be induced by afferent stimulation alone (e.g. Chipchase *et al.*, 2011; Khaslavskaja *et al.*, 2002; Knash *et al.*, 2003; McKay *et al.*, 2002; Ridding *et al.*, 2000b; Schabrun *et al.*, 2012, see also Luft *et al.*, 2002). The related point that may be made concerning PAS is that, in addition to the relative timing of its delivery in relation to TMS, the intensity of afferent stimulation may play an instrumental role in determining the magnitude of the effects

that are induced. Furthermore, these considerations serve to highlight the possibility that the impact of the cortical stimulation delivered over M1 is to augment the weak effects that arise from the peripheral stimulation.

As a corollary of this line of reasoning, dose-dependency might be anticipated. In this regard, we observed that epochs of paired stimulation (onsets staggered by 25 ms) spanning 175 ms were ineffective, whereas 375 ms and 875 ms periods of simultaneous PNS and tACS gave rise to reliable increases in CSE. When these three conditions were compared directly however, no statistically reliable differences were apparent. As such, there is presently no evidence that the magnitude of the PATACS induced effect is dose dependent.

An alternative proposition is that the efficacy, rather than simply the dose of afferent stimulation is an important determining influence in relation to the effects of associative stimulation. There are differences in the manner in which the corollaries of vibratory and electrical afferent stimuli exert an influence upon circuits within M1. The N20 component of the somatosensory evoked potential, measured by EEG in response to electrical nerve stimulation, is dominated by cutaneous input (Kunesch *et al.*, 1995) . The origin of the associated N20 response is thought to be a deep tangential generator in area 3b (e.g. Desmedt & Ozaki, 1991; McLaughlin & Kelly, 1993) , which has sparse if any connections with M1 (Burton & Fabri, 1995). In contrast, the source generator for cortical potentials invoked by muscle spindle afference (e.g. in response to tendon vibration) is principally area 3a (McKinnon *et al.*, 2000) . As this area has extensive direct projections onto pyramidal and multipolar neurons in deep (V and VI) layers of M1 (Porter *et al.*, 1990), it has been highlighted previously that, in the context of associative stimulation protocols, muscle spindle afference - such as that generated by tendon vibration, may represent the most efficacious source of peripheral input (Carson

& Kennedy, 2013). Although it was the case that when paired with tACS, muscle tendon vibration induced more reliable elevations of CSE than electrical nerve stimulation, when contrasted directly the PATACS and VIBTACS interventions were not differentiated. The present study therefore failed to provide a basis upon which to conclude that the magnitude of the associative effect is instrumentally related to the modality of afferent stimulation.

Miniussi *et al.* (2013) have proposed that some forms of non-invasive brain stimulation (NIBS) exert their effects through stochastic resonance. In a psychophysical context, it is believed that stochastic noise acts as a pedestal sufficient to raise the effective level of the relevant stimulus above the detection threshold. When delivered in isolation, the forms of afferent stimulation applied in the present study are not known to induce sustained changes in corticospinal excitability. In circumstances in which high frequency tACS is applied concurrently, it is possible that the activity in M1 circuits that receive inputs from sensory areas may be rendered sufficient to alter the state of corticospinal neurons engaged (subsequently) by TMS. In relation to this conjecture, a number of caveats necessarily apply. The stochastic resonance model of NIBS was developed i) to explain effects reported during ‘online’ stimulation, as opposed to the ‘offline’ aftereffects obtained here; ii) in relation to signal detection rather than motor systems plasticity; iii) to account for the addition of ‘noise’, rather than a regularly oscillating stimulus. There is no indication in any of the *in vitro* or *in vivo* research reported to date (Reato *et al.*, 2013), that tACS applied over the scalp at a fixed frequency translates into random (i.e. broad spectrum) noise in the cortex.

The effects of all forms of non-invasive brain stimulation vary markedly across individuals (Cheeran *et al.*, 2008; Ridding & Ziemann, 2010; Hamada *et al.*, 2012). In our experiment six, tDCS and PAS failed to yield reliable increases in corticospinal

excitability, despite the use of protocols that are ostensibly effective (for reviews see Nitsche *et al.*, 2008; Carson & Kennedy, 2013). In the same group of individuals PATACS generated an elevation in CSE. Nonetheless, when compared directly the three conditions were undifferentiated. On the other hand, in Experiment three increases in CSE occurred after 1000ms (2 mA, 140 Hz) PATACS but not after 500ms (2 mA, 140 Hz) PATACS - a variant that increased corticospinal excitability in four other groups of participants. In this context, the value of large samples is clearly revealed by the outcomes of the pooled analysis – which comprised the sixty-four participants (drawn from five experiments) who were exposed to the same PATACS protocol (i.e. PNS paired with 140 Hz, 2 mA tACS of 500 ms duration). Beyond the increased statistical power that is accrued, the use of larger samples permits the derivation of confidence intervals for the associated effect size estimates. These impose reasonable bounds on the interpretations that may be derived (Smithson, 2001). It follows that in the present instance, given same-sized samples under identical conditions, we should expect that in 95 of 100 repetitions, the population value of the effect size for the pre vs. post30 comparison (following this form of PATACS) will range between medium ($f = 0.27$) and large ($f = 0.48$).

It might be instructive to further manipulate parameters of the PATACS intervention. The three stimulation frequencies examined herein elevated CSE. Thus it is as yet unclear whether high frequency entrainment is taking place, or whether stimulation using i) frequencies of lower than 80Hz; ii) higher than 250Hz; or iii) high frequency random noise stimulation (tRNS,) (Terney *et al.*, 2008) would be equally effective. Similarly, with respect to the currents that were applied, we did not encounter either a floor or a ceiling effect. It is possible that intensities lower than 1mA may remain effective (although there are doubts about the efficacy of tACS below this level -

(Antal *et al.*, 2008; Moliadze *et al.*, 2012). In consideration of safety and comfort it is unlikely that current levels will be routinely extended beyond 3mA. Periods of paired stimulation much shorter than 500ms may not be effective. There is also a possibility that periods extending beyond one second may further accentuate the induced effects. We observed a largely monotonic increase in corticospinal excitability over the 35 minutes interval post-stimulation. While the origin of this pattern of response is unclear, it was reliable, being replicated in each PATACS condition. The extent to which it is sustained thereafter awaits further exploration.

The principal significance of the present study lies in the demonstration that associative effects are expressed when the timing of the peripheral and cortical events is not precisely circumscribed. One interpretation of these findings is that an account of the mechanisms underlying associative plasticity of the human motor system that emphasises only STDP is unlikely to be complete. The results suggest instead that multiple cellular pathways contribute to the LTP-type response that is engendered by associative stimulation protocols.

Chapter 3

Stimulation-induced elevations in corticospinal excitability confer no advantage in learning or performance of a sequential force task.

3.1 Abstract

Non-invasive brain stimulation (NIBS) techniques may be used to bring about changes in corticospinal excitability (CSE). Changes in CSE may also occur in the context of tasks that through practice promote motor learning. The degree to which these phenomena reflect the expression of common mediating mechanisms remains a matter of debate.

In order to examine this question, we used two distinct methods to induce elevations in CSE prior to the practice of a task in which isometric wrist flexion contractions moved a cursor on a screen to hit each of a set of target gates in a repeated sequence. Speed and accuracy of gate acquisition were measured and combined using a speed accuracy trade-off function to provide an index of 'skill'. Before training, one group of participants received paired associative transcranial alternating current stimulation (PATACS), a stimulation protocol demonstrated in chapter two to induce increases in CSE. A second group received no brain stimulation in the intervention period but instead undertook training with the opposite limb (cross education). The third group were a sham control. In all cases CSE was monitored throughout the observation period, using TMS evoked motor potentials.

The PATACS group exhibited increases in CSE relative to baseline and to the control group however, the subsequent initial performance of this group on the motor task was inferior to that of controls. Although elevations in CSE were also obtained for

the cross education group, the level of initial performance of these participants was superior that of the control group. The results show that increased CSE (in the context of this task) is an inconsistent predictor of improved motor performance or learning.

3.2 Introduction

3.2.1 Background

A plethora of non-invasive brain stimulation techniques have been demonstrated to be effective in modulating corticospinal excitability (CSE) (e.g. Stefan *et al.*, 2000; Nitsche & Paulus, 2000; Huang *et al.*, 2005). In a commonly used paradigm, CSE is measured using transcranial magnetic stimulation (TMS). When applied over the region of the primary motor cortex (M1) from which corticospinal projections to muscles of the upper limb originate, the magnetic pulse leads to a descending volley, the outcome of which - the motor evoked potential (MEP) - is detectable by surface electromyography (EMG) at the peripheral target (Rothwell, 1997). The amplitude of the MEP may be modulated when the population of neurons which lie under the focal point of the TMS coil become more or less readily recruited by magnetic stimulus dependent upon the prior or concurrent application of one or other of the NIBS protocols.

Modulation of CSE following or during NIBS is often referred to as a change in 'neural plasticity' (Ziemann *et al.*, 2008). This terminology may be an artefact of the roots that modern NIBS techniques have in reduced preparation and animal model research (Baranyi *et al.*, 1991). Tetanic stimulation of cell preparations (Bliss & Lomo, 1973) or direct current polarisation of animal cortex (Bindman *et al.*, 1964) may lead to a plastic change in the Hebbian sense of the word; there is a strengthening of the efficiency with which one cell or population of cells communicates with another or a lasting change in the amplitude of evoked potentials, closely akin to a long term potentiation-like mechanism. The ostensibly common characteristics in the observed effects in some forms of NIBS at the level of the human motor system (Chen *et al.*,

1997; Stefan *et al.*, 2000; Nitsche & Paulus, 2000) may have led naturally to the adoption of the same descriptive language.

Following stimulation, changes in CSE can be reasonably assumed to indicate that some plastic change has occurred, hence the common usage of the term ‘plasticity’. There is however, little evidence that a greater *state* of plasticity has been induced. While rarely stated explicitly, it is implicit in much NIBS research on the human motor system that an increased MEP amplitude is evidence of the system being primed for more effective learning or rehabilitation within the window of time when increased excitability is exhibited (Bolognini *et al.*, 2009; Schabrun & Chipchase, 2012). In such instances the word ‘plasticity’ may be taken to mean ‘malleability’.

Despite notoriously fickle results, at least partially due to inter and intra-individual variability in its effects on CSE (Hamada *et al.*, 2012; Wiethoff *et al.*, 2014; Horvath *et al.*, 2014) and on behavioural outcomes (Reis *et al.*, 2008), sufficient evidence has accumulated over the past two decades to suggest that in the right circumstances NIBS may produce measurable effects. There are however, few instances in which positive changes in both behaviour and CSE have been reported in the same study, and there is as yet little convincing evidence that they are related in consistent and reliable manner. This is a consideration of vital importance, given that NIBS has regularly been proffered as a basis upon which to expedite physical and cognitive rehabilitation in wide variety of clinical contexts, including the amelioration of functional impairment following stroke (Schabrun & Chipchase, 2012) and Parkinson’s disease (Boggio *et al.*, 2006).

In light of these considerations, it is therefore opportune to examine the veracity of the assumption that prior induction of increased CSE may be a positive modulator of motor skill performance and learning. In the present study we used paired associative

transcranial alternating current stimulation (PATACS) (McNickle & Carson, 2015) to induce changes in CSE before training in a sequential isometric flexion task. Changes in motor skill performance and learning were compared between the brain stimulation group, a sham control group and a cross education group, who in place of stimulation received training in the task with the opposite limb. Cross education is the phenomenon whereby training of one limb may give rise to improvements in performance of the opposite untrained limb (see Ruddy & Carson, 2013 for a review). This is a reliable method of inducing increases in contralateral M1 excitability as well as increasing opposite limb performance. In comparison with the PATACS stimulation group, which was predicted to increase excitability but not influence subsequent performance or learning, the aim was to examine whether increased excitability was in itself was a positive primer ahead of motor skill performance and learning.

3.2.2 Rationale for experimental conditions

3.2.2.1 Selection of the motor learning task.

Selection of the most appropriate motor task for this experiment was influenced by several studies that used the same sequential pinch force task combined with several other techniques relevant to the present study, including interventional brain stimulation (Reis *et al.*, 2009), cross education (Camus *et al.*, 2009) and TMS measures of corticospinal excitability (Cantarero *et al.*, 2015; López-Alonso *et al.*, 2015).

Participants demonstrated continual learning on the task over time without reaching asymptote, which was an important characteristic for the present purposes. Reis *et al.* (2009) independently validated a speed accuracy trade-off model associated with the task. Camus *et al.* (2009) reported a significant cross education transfer effect

over time, which indicated that an adaptation of the sequential pinch force task was an appropriate design for the present purposes.

The series of experiments in chapter two that developed and parameterised the PATACS stimulation technique targeted the wrist flexor muscle. Peripheral nerve stimulation was delivered to the motor point of the flexor carpi radialis (FCR) muscle. TMS was used to locate the FCR hot spot over the primary motor cortex, which was then used in the tACS electrode montage. MEPs were measured from surface electrodes placed over the FCR. Therefore, to remain consistent with these experiments, it was important that the wrist flexor remained the target of the motor learning task. The sequential pinch grip force task used by Reis and others was adapted so that forces were generated from wrist torque to ensure that the FCR was the principal effector engaged in the task.

3.2.2.2 Analysis of skill and learning on the SIFT

Most motor skill tasks have separate speed and accuracy elements. An increase in one element comes at a cost to the other (Reis *et al.* 2009). During motor training paradigms this can lead to difficulty in ascertaining whether improvements in either speed or accuracy reflect a genuine improvement in skill or a shift in emphasis from one element to the other. Reis and colleagues validated a speed accuracy trade off (SAT) model, which is described in further detail in the method section. They first dictated speed on the task by asking participants to move to the rhythm of a metronome, and plotted error rate against the different pre-set movement times. The resultant curve was used to derive a mathematical function yielding a 'skill' score. Post training, Reis and colleagues showed that the area under this curve reduced and the skill parameter of their model changed, representing learning of the skill.

Changes in skill in the present adaptation of the sequential force task were calculated using the same model following confirmatory steps (outlined in the method section) to ensure that learning on our adaptation of the task displayed the same features as the model used by Reis and colleagues. The speed and accuracy scores were also analysed and reported separately. Performance scores in speed, accuracy and skill were compared between different time points across the experiment. Participants completed the task for one block with the right hand initially, followed by an intervention which involved either sitting passively while receiving real or sham brain stimulation, or training on the task with the opposite limb. They then performed eight further right hand blocks. One week later they returned and performed four more blocks of the task with the right hand (figure 3.4). Performance change within the training session is considered separately from 'learning' which is defined as either a change in performance that is retained following an extended time period, or which is evident in transfer from one effector or domain to another (Magill, 2004). Comparisons of interest were therefore defined as follows. Changes from block one prior to the intervention to block two immediately following the intervention were defined as performance improvements due to the intervention (and initial experience of performing the task). Changes from block two to block nine (the end of the main sequence of training) were considered due to the effect of training. Changes from block one to block ten (the first block upon return one week later) were considered the 'learning' effect – improvements in performance relative to the very first trials were evidence that skill had been learned and retained. The final comparison between blocks 10 and 13 represented the rate of performance improvement during the second training session.

3.2.2.3 Cross education as a comparison condition

Cross education is the process whereby training of one limb gives rise to performance improvements in the contralateral limb. The transfer of learning from the trained to the untrained effector is often associated with increased CSE measured at the opposite limb (Carson *et al.* 2004; Hortobagyi, 2003), possibly through interactions between primary motor cortices. It has been postulated that bilateral activity during unilateral movements may drive adaptations in the untrained hemisphere (Hellebrandt, 1951). While many studies have reported increased excitability in the opposite limb, this does not constitute evidence that the change in CSE directly contributes to the skill transfer. Several studies have tested the correlation between degree of skill transfer and level of CSE increase (Carroll *et al.*, 2008; Hinder *et al.*, 2011; Dickins *et al.*, 2015; Reissig *et al.*, 2015), reporting a null relationship in each instance.

Therefore, cross education acts as an ideal comparison condition in this experiment. Although increases in CSE of the untrained limb are highly task dependent, it might be expected that changes would occur. This would provide a counter point to the brain stimulation condition. In both cases it might be expected that increases in CSE would occur. In the cross education condition these changes would likely be accompanied by an increase in skill learning or performance. In the PATACS condition it might be expected that increased CSE would occur in the absence of improved skill learning. Thus a dissociation between electrophysiological and functional plasticity may be demonstrable.

3.3 Methods

3.3.1 Participants

Forty-five healthy volunteers (14 male) aged 18–34 years (mean 22.7 ± 3.4 years) participated in the experiment. All were right handed according to the Edinburgh handedness inventory (Oldfield, 1971), and gave informed consent to procedures approved by the Trinity College Dublin Ethics Committee, and conducted in accordance with the Declaration of Helsinki. The allocation of participants to the three experimental groups was pseudo-randomised. The investigators were blind as to the allocation of participants to groups. There were no statistically significant differences in the age or sex of the participants in the three experimental groups

3.3.2 EMG recording procedures and transcranial magnetic stimulation

Participants were seated with the upper limbs supported and stabilized by vacuum cushions, the forearms in mid-pronation and the elbows semi-flexed ($100\text{--}120^\circ$). The hands were secured in manipulanda that were mounted on torque transducers located coaxially with the (flexion-extension) axes of rotation of the wrists. Electromyographic (EMG) activity was recorded from the right flexor carpi radialis (FCR) and extensor carpi radialis brevis (ECR) muscles, using pairs of silver chloride (AgCl) electrodes. EMG signals were amplified (gain = 1000), bandpass filtered (20 – 1000 Hz), and digitized at a sampling rate of 4 kHz.

Magnetic stimuli were delivered to the left primary motor cortex (M1) by a Magstim 200 stimulator using a figure of eight coil (internal wing diameter 70mm), located at the optimal position (“hot spot”) to obtain a motor evoked potential (MEP) in the FCR muscle of the contralateral (right) arm. The coil was placed so that the axis of

intersection between the two loops was oriented at approximately 45 degrees to the sagittal plane, to induce posterior to anterior current flow across the motor strip. Once the hot spot was established, the lowest stimulation intensity at which MEPs with peak-to-peak amplitude of approximately 50 μ V were evoked in at least 5 of 10 consecutive trials was taken as resting motor threshold (RMT).

Prior to each intervention (Pre), a MEP recruitment curve was obtained by delivering TMS at 10% increments of intensity between 90% and 160% of the RMT. Six stimuli were delivered at each level of intensity. A further twelve stimuli were delivered at 120% RMT. The order of delivery was randomised. The interval between successive stimuli varied between 4 and 6 seconds. The total duration of the sequence was approximately five minutes. The average MEP amplitudes obtained at 90% and 100% RMT was calculated to ensure that the threshold had been correctly determined. In cases where the averaged MEPs for these intensities did not correspond to the expected values (i.e. <50 μ V at 90%RMT, 50-100 μ V at 100%RMT), the threshold intensity was adjusted accordingly and another recruitment curve was obtained. Equivalent sets of stimuli (without adjustments of threshold) were delivered immediately following the intervention (Post0) and at 10 (Post10), 20 (Post20) and 30 (Post30) minutes thereafter. The first of these sets (i.e. Post0) always commenced within 30s following completion of the intervention. There was a break of five minutes after the delivery of each such set of stimuli prior to commencement of the subsequent set. Mid-way through the right hand training sequence a final set of stimuli were delivered (Post55).

3.3.3 Experimental group conditions

3.3.3.1 Paired associative transcranial alternating current stimulation (PATACS)

Procedures for stimulation were identical to those described in chapter two and by McNickle and Carson (2015). PATACS involved the repeated pairing of trains of afferent peripheral nerve stimulation (PNS) with bursts of transcranial alternating current stimulation (tACS) (figure 1).

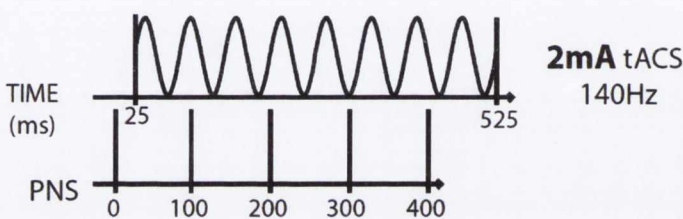


Figure 3.1. Schematic of one paired stimuli event from PATACS. Five peripheral nerve stimuli were delivered overlapping with 500ms of tACS. The first PNS pulse was delivered 25ms in advance of the commencement of the tACS. (Frequency of tACS oscillations not to scale).

For the PNS a constant-current stimulator (model DS7A, Digitimer, Hertfordshire UK) was used to locate the motor point of FCR by moving a bipolar surface electrode over the muscle belly. The principle identification criterion was a reliable, visible displacement of the FCR tendon at the wrist. Two AgCl electrodes were affixed in line with to the orientation of the muscle fibres – one on either side of the location thus defined. These were used for both stimulation and EMG recording. The intensity of stimulation delivered during an intervention was the minimum at which visible displacement of the FCR tendon was observed. This motor point stimulation was delivered in trains of 5 pulses (each of 1 ms duration).

For the tACS flexible electrode paddles were placed within two saline-soaked 5cm x 5cm sponges and fixed securely on the scalp using non-conducting elastic straps.

One electrode was placed over left M1 at the FCR “hot spot” determined previously by TMS. The second electrode was placed over the contralateral supraorbital area. A battery-driven stimulator (AM Systems Model 2200) controlled by Signal software (Cambridge Electronic Design, Cambridge, UK.) was used to deliver short (500 ms) bursts of bipolar sinusoidal alternating current at a fixed frequency of 140 Hz and amplitude of 2mA. Electrode impedance was monitored and maintained below 5k Ω . In all tACS conditions, 180 bursts were delivered at approximately ten seconds intervals, corresponding to a stimulation epoch of 30 minutes. Therefore the total duration of all tACS stimulation applied within this period was 90 seconds.

Each of the 180 paired stimulation events (separated by ≈ 10 s) comprised a train of PNS (five pulses at 10 Hz). The train commenced 25ms prior to the onset of a 500 ms duration burst of 2mA tACS. A random jitter (± 5000 ms maximum) was introduced in relation to the timing of successive stimulation events (mean separation 10 seconds) in order to ensure that the participants could not anticipate the onset of stimuli.

3.3.3.2 Sham stimulation

The equipment and electrode montage were identical to those described for PATACS. The transcranial stimulator was driven by Spike software (Cambridge Electronic Design). In order to provide a skin tingling sensation, the current was ramped up to 1mA over the first ten seconds and ramped back down to zero over the subsequent ten seconds. In the sham group the motor point was found using PNS in the manner employed for the PATACS group. Before commencement of the sham protocol, three trains of PNS were delivered to verify the afferent stimulation threshold, thus ensuring consistency with the PATACS condition.

3.3.3.3 Cross education

During the 30 minutes intervention period the stimulating electrodes for tACS and PNS were placed on the scalp and arm respectively regardless of the fact that stimulation was not delivered. In the cross education group the motor point was found using PNS exactly in the manner employed for the PATACS group. Before commencement of the sham protocol, three trains of PNS were delivered to verify the afferent stimulation threshold, thus ensuring consistency with the PATACS condition.

Participants completed the sequential isometric flexion task (SIFT) used their *left* hand for eight blocks of trials, as in the manner of the right hand motor training described below. In this case the target force levels were defined in relation to the maximum voluntary contraction (MVC) values obtained for the left limb (see below).

3.3.4 Development of the sequential isometric flexion task (SIFT)

The SIFT was derived from the sequential isometric visual pinch task (SIVPT) used by Reis *et al.* (2009). In this specific instantiation, isometric generation of wrist flexion torque was used in place of a pinch grip. The on-screen task presentation was developed through incremental pilot studies and coded specifically for this experiment using the Spike2 programming language (Cambridge Electronic Design, Cambridge, UK). An experimental apparatus was built which used a manipulandum attached to a force transducer. Force applied by a hand within the manipulandum moved a cursor between a series of targets presented on-screen. The same Spike2 program recorded the voltage output from the force transducer. Following data collection, a bespoke offline Matlab (Mathworks Inc., USA) program was used to capture the position of the cursor in relation to the onscreen targets and to provide the speed and accuracy data for each trial.

A series of target gates were presented on a wall-mounted 27-inch monitor approximately 60cm from the participant (figure 2). Participants moved a cursor horizontally from left to right across the screen by applying force against a manipulandum (i.e. by generating isometric wrist flexion torques). When no force was applied the cursor returned to the 'home' gate. The level of force required to move the cursor to each of the gates was scaled relative to each participant's maximum voluntary contraction (MVC). The furthestmost gate required a force equivalent to 40% of the participant's MVC.



Figure 3.2. Gate sequence display for the SIFT. The home gate was red at the beginning of each trial and changed to green to signal 'go'. The cursor (in this diagram illustrated at the 'home' gate) moved from left to right in response to wrist flexion.

The MVC for each limb was obtained prior to the first exposure to the task. Participants were asked to flex against the handle with maximal effort. The mean of the peak torque generated on each of three such attempts was taken as the reference value.



Figure 3.3. The manipulandum used to measure force of wrist flexion during the SIFT. The wrist was secured using vacuum packs and stabilising metal rods. The static handle was attached to a torque transducer.

At the commencement of each trial the participant was asked to wait until the colour of the ‘home’ gate turned from red to green on screen before commencing movement. The target gates were numbered 4-1-3-5-2. They were required to move the cursor between the home gate and each of the five target gates in numerical order, returning to the home gate after each was acquired, and instructed to avoid ‘overshooting’ the right margin or ‘undershooting’ the left margin of each gate. Upon reaching gate five, the participant was to hold the cursor in place for 400ms. Satisfaction of this requirement triggered the end of one trial, and the start of the next. If the participant did not reach gate five within 20 seconds of the start of the trial, the trial ended automatically, and was counted as “missed”. Each participant was told to move as quickly and as accurately as possible.

3.3.5 General Procedures

The procedures for the three experimental groups differed only in the nature of the intervention. Figure 3 illustrates the time course of the experiments. The first MEP recruitment curve was recorded prior to commencement of block one (B1) of the SIFT (in all cases performed by the right hand). The intervention immediately followed B1. The tACS electrodes were placed on the scalp, and the bipolar surface electrodes on the FCR were switched from EMG measurement to PNS delivery - regardless of whether the forthcoming condition required stimulation. Following the (30 minutes duration) intervention, further recruitment curves were obtained at fixed intervals (~0, 10, 20, and 30 minutes). At the completion of the fifth (post30) recruitment curve participants commenced a 30 minute period of training on the SIFT using the right hand. This involved eight blocks each of ten trials (B2 – B9), with breaks lasting around one minute between each block. Mid way through the main SIFT learning sequence (between block five and block six) another TMS recruitment curve (post55) was obtained.

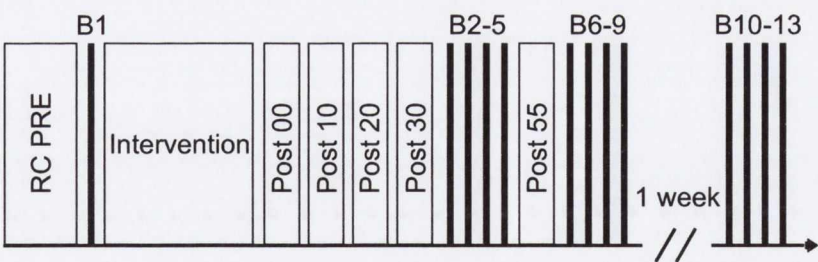


Figure 3.4. Time course of the experiment for all groups. The ‘pre’ recruitment curve (RC) was measured prior to completion of the first SIFT block (B1). Following the intervention were four RCs (Post0-30), four SIFT blocks (B2-5), a final RC (Post55) and four further SIFT blocks (B6-9). One week later participants returned for a retention test of four SIFT blocks (B10-13)

Participants returned one week after the main testing session to complete a retention test. They completed four blocks of the SIFT (B10 – B13) following the same procedure as in the main testing session.

3.3.6 Analysis of motor evoked potentials

The root mean square (rms) of the background EMG recorded in FCR and ECR was calculated for a window 93ms to 3ms before TMS onset. If the value was greater than $3\mu\text{V}$ for either muscle, the corresponding MEP was disregarded. As a further means of eliminating instances in which elevated excitability of the spinal motoneuron pool may have influenced the MEP amplitude, we first calculated for each participant (separately for FCR and ECR) the quartiles for all background rms EMG values retained following the screening procedure described above. In the event that an individual rms value was above the upper whisker of the distribution (in this instance set to the third quartile plus 1.5 times the interquartile range) the corresponding MEP was disregarded.

For the remaining recordings, the mean (peak-to-peak) amplitude of the MEPs elicited at the eight respective stimulation intensities was calculated. For each time of measurement (Pre, Post0, Post10, Post20, Post30 and Post55), the summated area under the recruitment curve (AURC) - bounded by magnetic stimulation intensity and MEP amplitude (in units of mV.T), was obtained using the trapezoidal rule. It has been demonstrated elsewhere (Carson *et al.*, 2013) that the AURC is a reliable measure of the state of corticospinal projections to hand and forearm muscles. In general, samples of AURC values are not normally distributed. To increase the symmetry of the sample distribution for the purposes of inferential analyses, the AURC values were therefore subject to a log transformation.

Mixed effects models in which participant was a random effect, and time was a fixed effect (levels = Pre, Post0, Post10, Post20, Post30), were conducted using the lmerTest package in R. Measurements derived at the Post55 time point were analysed using a separate model. In the context of these models, planned contrasts were conducted between the (log) AURC value obtained prior to the intervention (Pre), and the (log) AURC calculated for each time point following the intervention (Post0, Post10, Post20, Post30). The relevant degrees of freedom were obtained using Kenward-Roger's approximation that in the case of the balanced design employed in the present study, yields values equivalent to those of a repeated measures ANOVA design.

In order to further assist in the interpretation of the tests of significance eta-squared and the effect size index for ANOVA (f) (Cohen, 1988) were calculated for each planned contrast. Linear mixed effects model analysis was used to conduct comparisons of changes in AURC at various time points between the three groups, with the Pre AURC used as a covariate. Between groups planned comparisons were made between PATACS and sham and between cross education and sham after 0, 10, 20, and 30 minutes post-intervention.

All inferential tests were carried out two-tailed with the exception of those including the PATACS group. In these cases, based on prior evidence (McNickle & Carson, 2015), there was a clear expectation that AURC values would be elevated both with respect to baseline (Pre) values obtained for the PATACS group, and in relation to the group of participants that received sham stimulation.

3.3.7 Speed Accuracy Trade-off Function (SAF) and skill parameter

Two measures were derived from performance of the SIFT; the mean trial duration per block (time taken from the start of the trial until the cursor was held within

gate 5 for 400ms) and the error rate (proportion of targets missed over the entire block). Individuals may place varying emphasis on one of either speed or accuracy in performance of a given task, leading to difficulties in ascertaining whether changes in one measure or the other reflect skill improvement or merely a shift along the speed accuracy trade-off continuum. Therefore a speed accuracy trade-off function (SAF) was used to generate a skill score. The following mathematical model with two free parameters was developed and validated empirically for a pinch force version of the same task by Reis and colleagues (2009) and others (Cantarero *et al.*, 2015; López-Alonso *et al.*, 2015).

$$a = \frac{1 - \text{error rate}}{\text{error rate}(\ln(\text{duration})^b)},$$

Reis *et al.* (2009) found that training on the sequential force task brought about a change in the *a* parameter while the *b* parameter remained constant; they therefore proposed that the ‘*a*’ parameter equates to skill while the ‘*b*’ parameter remains a fixed constant value. In order to ascertain that the same *b* constant was applicable to this adaptation of the sequential force task, participants’ individual values for *a* were calculated for a range (0.1 – 100 in steps of 1) of iterations of *b* using the error and duration values for each block. A learning curve for each of these sets of *a* values was plotted, and a curve fitting program in Matlab (using the Nelder-Mead simplex method of algorithm fitting) was used to determine goodness of fit to a power curve.

The power law of practice is one of the most robust findings in psychological research (see Newell & Rosenbloom, 1993 for a review). This was therefore the premise for the reasonable assumption that goodness of fit (and consequently the

appropriateness of any given b value) should be determined by the derivation of a set of a values that most closely fitted a power function. It was found that on an individual basis, varying the value of b did not have any discernible impact upon the closeness of the curve of a values to a power curve (see appendix x). Therefore, the b value was set at the same constant value (5.424) used in the previous research using this task (Reis *et al.*, 2009; Cantarero *et al.*, 2015).

Reis and colleagues performed a log transformation as part of the calculation of the a skill parameter due to the finding that change in a over blocks was multiplicative. The same step was taken with these data.

3.3.8 Statistical analysis of changes in SIFT performance

A number of key comparisons were made for both within- and between-groups analyses in the SIFT data (table 1).

Table 3.1. Statistical comparisons of interest in the SIFT

B1 – B2	Change in performance from Block 1 to Block 2; initial changes following the intervention.
B1 - B10	Change in performance from Block 1 to Block 10; ‘learning’ as demonstrated by retention following return after one week.
B2 - B9 (-B1)	Change in performance from Block 2 to Block 9; effect of main training sequence on task performance (with B1 as covariate)
B10 – B13 (-B1)	Change in performance from Block 10 to Block 13; effect of second retention training sequence on task performance (with B1 as covariate)

For within-groups comparisons in skill, duration and error rate, two linear mixed effects models were conducted using the lme package in R. In both, participant was a random effect, and time (Block) was a fixed effect.

The first model had three levels of the fixed effect (B1, B2; B10). In the context of this model planned comparisons were carried out between B1 and B2, and between B1 and B10.

The second model had four levels of the fixed effect. (B2, B9, B10, B13) Each of these was expressed as change from B1, i.e. minus the participant's B1 value. Therefore the fixed effects levels were B2-B1, B9-B1, B10-B1, B13-B1. In the context of this model these are in future referred to as B2, B9, B10, B13. Planned comparisons were carried out between B2 and B9, and between B10 and B13.

Between-groups comparisons were carried out on skill, duration and error rate using the linear model function in the R base package. For differences between intervention groups in the degree of change from B1 to B2, and from B1 to B10, one way anovas were carried out followed by planned comparisons between PATACS vs. sham and between cross education vs. sham. For between-groups differences in the degree of change from B2 to B9 and from B10 to B13, ancovas were carried out using participants' performance at B1 as a covariate. These were followed by planned comparisons between PATACS vs. sham and cross education vs. sham.

3.4 Results

3.4.1 Effect of intervention group on levels of corticospinal excitability

Indices of corticospinal excitability (AURC) taken after the intervention were higher than measurements taken at baseline ('pre') for the PATACS group. These increases became statistically reliable after 10 minutes (table 2 and figure 4) and remained so for measurements taken 20 and 30 minutes following the cessation of stimulation. In the cross education group increases in excitability were evident in measurements taken at 10, 20 and 30 minutes following the completion of the intervention. At no time point was the AURC reliably distinguished from baseline measurements in the sham group.

Table 3.2 – F ratios, p values and effect sizes for comparisons between the AURC obtained prior to the intervention (Pre), and the values obtained at each of 4 time points following the intervention. P values are expressed two-tailed for Sham and Cross-Ed, one tailed for PATACS.

Condition	Pre vs.	F (1,163)	p value	Effect Size (f)
PATACS	Post00	0.01	0.45	0.01
	Post10	2.91	0.05	0.13
	Post20	2.70	0.05	0.13
	Post30	3.20	0.04	0.14
SHAM	Post00	0.62	0.43	0.06
	Post10	0.72	0.39	0.07
	Post20	0.08	0.78	0.02
	Post30	0.11	0.74	0.03
CROSS-ED	Post00	2.91	0.09	0.13
	Post10	7.78	<0.01	0.21
	Post20	8.16	<0.01	0.22

Post30	7.75	<0.01	0.21
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Between-groups analysis revealed that in the PATACS group, AURC reliably exceeded measurements taken in the sham group after 10 minutes ($F(1, 84) = 4.30, p = 0.02, f = 0.23$) and after 30 minutes ($F(1, 81) = 3.59, p = 0.03, f = 0.21$). When compared with the sham group at the same time points, AURC was higher in the cross education group after 10 minutes ($F(1, 80) = 8.79, p <0.01, f = 0.33$), 20 minutes ($F(1, 80) = 5.16, p = 0.03, f = 0.25$) and 30 minutes ($F(1, 80) = 7.16, p <0.01, f = 0.30$).

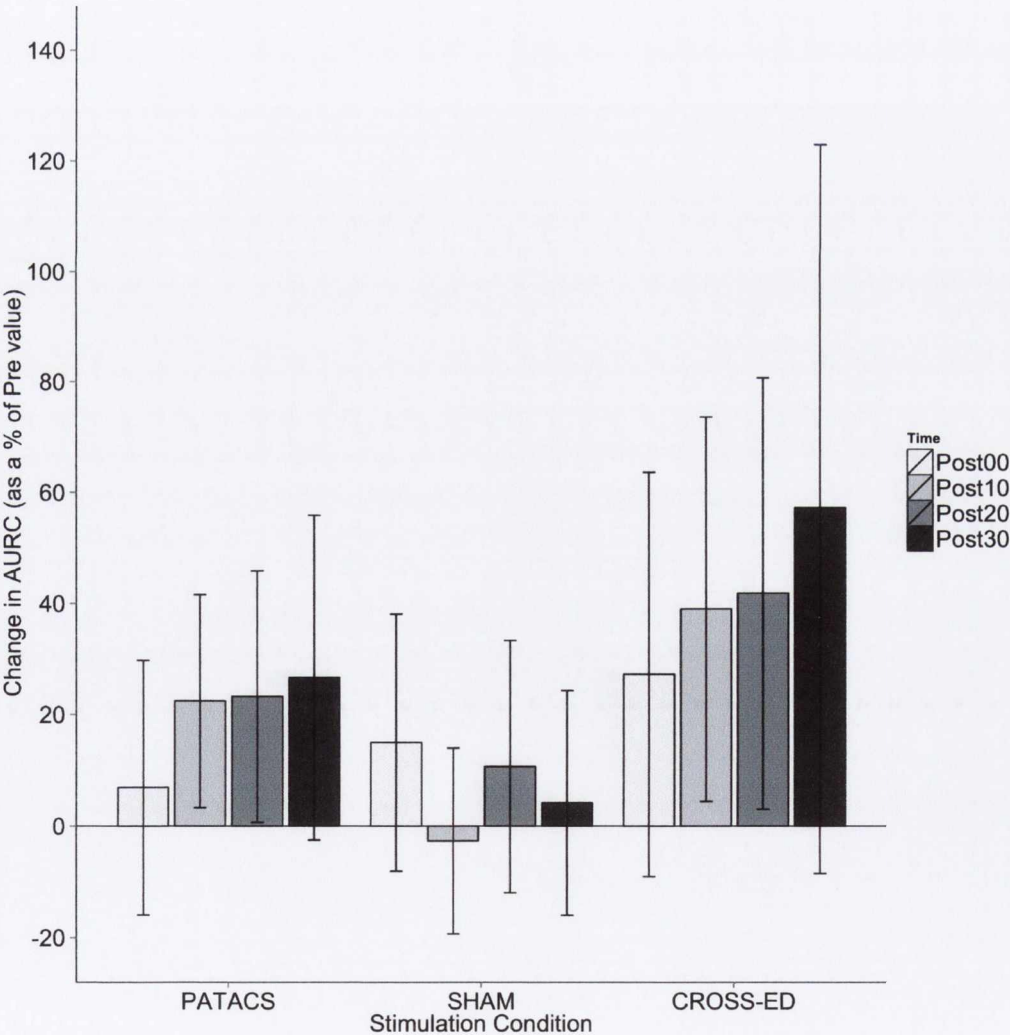


Figure 3.5. Effect that each intervention had on indices of CSE. The AURC for each post-intervention time point is expressed as the (percentage) change relative to the value obtained prior to the intervention. All values are the mean of 15 participants in each group. The error bars are the corresponding 95% confidence intervals calculated across participants. Values recorded following the intervention that differed reliably ($p < 0.05$) from those obtained prior to the intervention are represented by an asterisk symbol above the error bar.

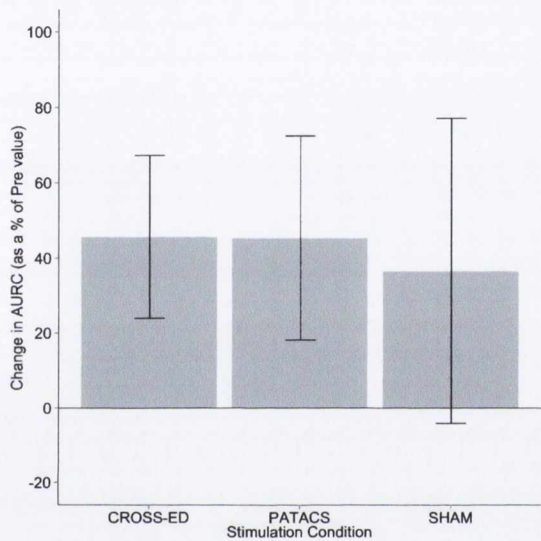


Figure 3.6. Levels of CSE in each of the three groups measured after block five of the motor learning task (at ‘Post55’). All values are the mean of 15 participants in each group. The error bars are the corresponding 95% confidence intervals calculated across participants.

3.4.2 Sequential isometric force task – within group changes over time

3.4.2.1 Block 1 to Block 2

In the sequential isometric force task (SIFT), the cross education group demonstrated a higher mean skill score at block two (the first block following the intervention) when compared to block one (table 3 and figure 6). Increases in skill for the PATACS and sham groups were not statistically reliable at block two. The cross education group also demonstrated a significantly lower mean trial duration from block

one to block two, while there were no reliable differences in duration for the PATACS and sham groups (table 3 and figure 7). Decreases in error rate (table 3 and figure 8) between block one and two were not significant in any of the three groups.

Table 3.3. Mean change within each group from block one to block two. Skill scores are the ‘a’ parameter, duration is mean time in seconds, and error rate is % of total gate targets missed per block. T values and probabilities are from planned comparisons following lme repeated measures analyses.

B1 vs. B2	Group	Mean change	t	p
Skill (a)	CE	0.89	-3.180	0.004
	PATACS	0.37	0.272	0.787
	SHAM	0.66	-0.411	0.684
Duration (s)	CE	-1.94	2.658	0.013
	PATACS	-1.44	0.898	0.377
	SHAM	-0.92	-0.485	0.632
Error rate (%)	CE	-10.1%	1.702	0.100
	PATACS	-2.0%	-0.529	0.601
	SHAM	-10.1%	0.643	0.525

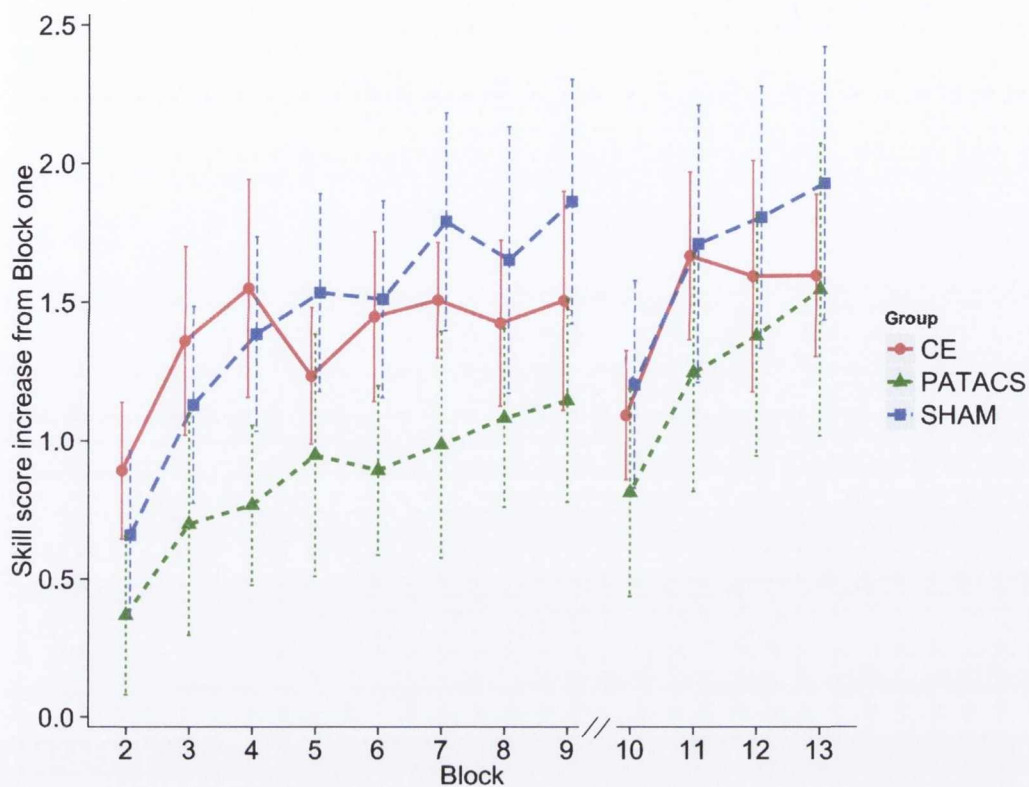


Figure 3.7. Skill score expressed in as change from block one (i.e. B_i minus B_1) for each of the three groups. Error bars are 95% confidence intervals. The space represents the interval (one week) between the main testing session and the retention test. “CE” = cross education group.

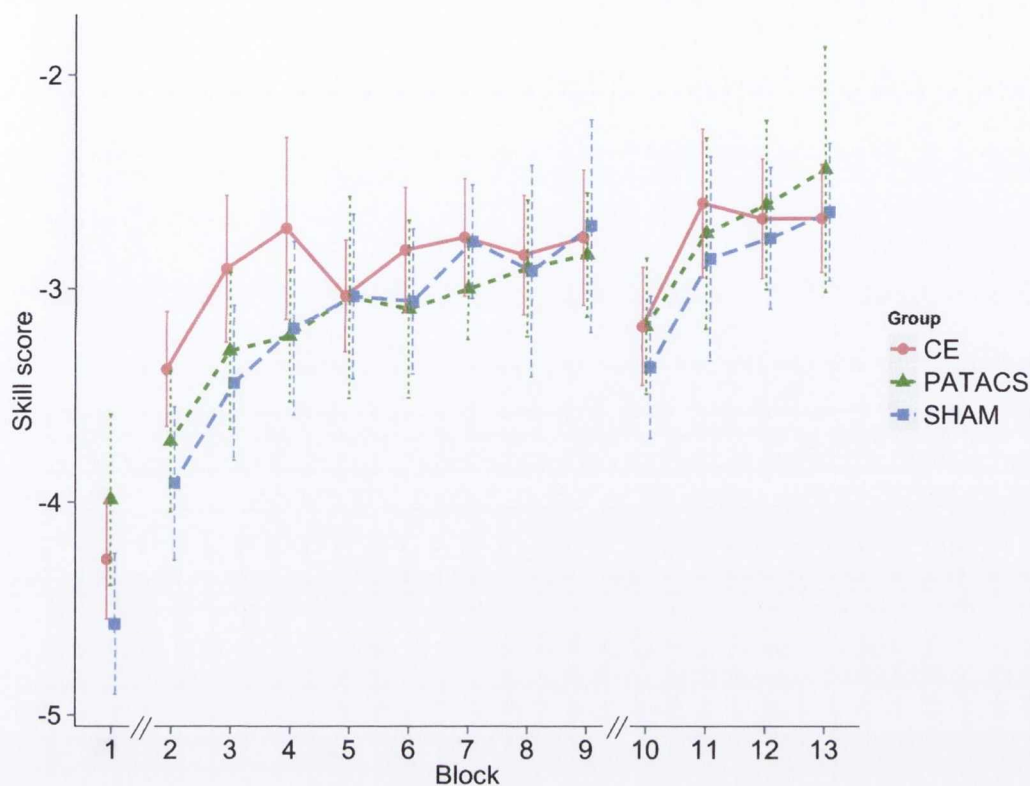


Figure 3.8. Skill score across blocks (non-normalised) for each of the three groups. Error bars are 95% confidence intervals. The space between block 1 and 2 represents the intervention and between block 9 and 10 represents the interval (one week) between the main testing session and the retention test. “CE” = cross education group.

3.4.2.2 Block 1 to Block 10

Compared to block one, there were reliable increases in skill at block ten (the first block upon return for the retention session) for all three groups (table 4 and figure 6). There were reliable decreases in mean trial duration (table 4 and figure 7) and error rate (table 4 and figure 8) for all three groups.

Table 3.4. Mean change within each group from block one to block ten. Skill scores are the ‘a’ parameter, duration is mean time in seconds, and error rate is % of total gate targets missed per block. T values and probabilities are from planned comparisons following lme repeated measures analyses.

B1 vs. B10	Group	Mean change	t	p
Skill (a)	CE	1.09	-5.942	<0.001
	PATACS	0.81	-4.549	<0.001
	SHAM	1.20	-6.231	<0.001
Duration (s)	CE	-2.47	5.708	<0.001
	PATACS	-2.37	5.839	<0.001
	SHAM	-2.17	4.883	<0.001
Error rate (%)	CE	-34.6%	2.551	0.017
	PATACS	-6.8%	2.205	0.036
	SHAM	-37.6%	3.486	0.002

3.4.2.3 Block 2 to Block 9

Within-group differences between block two and block nine (spanning the main training sequence) were assessed using each participant’s performance at block one as a covariate (table 5). There were reliable increases in skill (figure 6) and reliable decreases in mean duration (figure 7) and error rate (figure 8) for all three groups between block two and block nine.

Table 3.5. Mean change within each group from block two to block nine, with block one as a covariate. Skill scores are the ‘a’ parameter, duration is mean time in seconds, and error rate is % of total gate targets missed per block. T values and probabilities are from planned comparisons following lme repeated measures analyses.

B2 vs. B9	Group	Mean change	t	p
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Skill (<i>a</i>)	CE	0.61	-4.142	<0.001
	PATACS	0.78	-4.217	<0.001
	SHAM	1.20	-6.410	<0.001
Duration (s)	CE	-0.83	4.762	<0.001
	PATACS	-1.43	5.673	<0.001
	SHAM	-1.65	5.741	<0.001
Error rate (%)	CE	-6.5%	2.550	0.015
	PATACS	-8.3%	3.234	0.002
	SHAM	-12.2%	3.948	<0.001

3.4.2.4 Block 10 to Block 13

Within-group differences between block ten and block thirteen (the four blocks of the retention session) were assessed using each participant’s block one performance as a covariate (table 6). Reliable increases in skill were demonstrated by all three groups over this time period (figure 6). Reliable decreases in duration (figure 7) were demonstrated by cross education and sham groups, but not the PATACS group. Reliable decreases in error rate (figure 8) were evident in the cross education and PATACS groups, but not the sham group.

Table 3.6. Mean change within each group from block ten to block thirteen, with block one as a covariate. Skill scores are the ‘a’ parameter, duration is mean time in seconds, and error rate is % of total gate targets missed per block. T values and probabilities are from planned comparisons following lme repeated measures analyses.

B10 vs. B13	Group	Mean change	t	p
Skill (<i>a</i>)	CE	0.50	-3.403	0.002

	PATACS	0.73	-3.980	<0.001
	SHAM	0.73	-3.864	<0.001
Duration (s)	CE	-0.46	2.636	0.012
	PATACS	-0.37	1.486	0.145
	SHAM	-0.70	2.435	0.019
Error rate (%)	CE	-6.2%	2.408	0.021
	PATACS	-6.9%	2.702	0.010
	SHAM	-7.6%	2.457	0.099

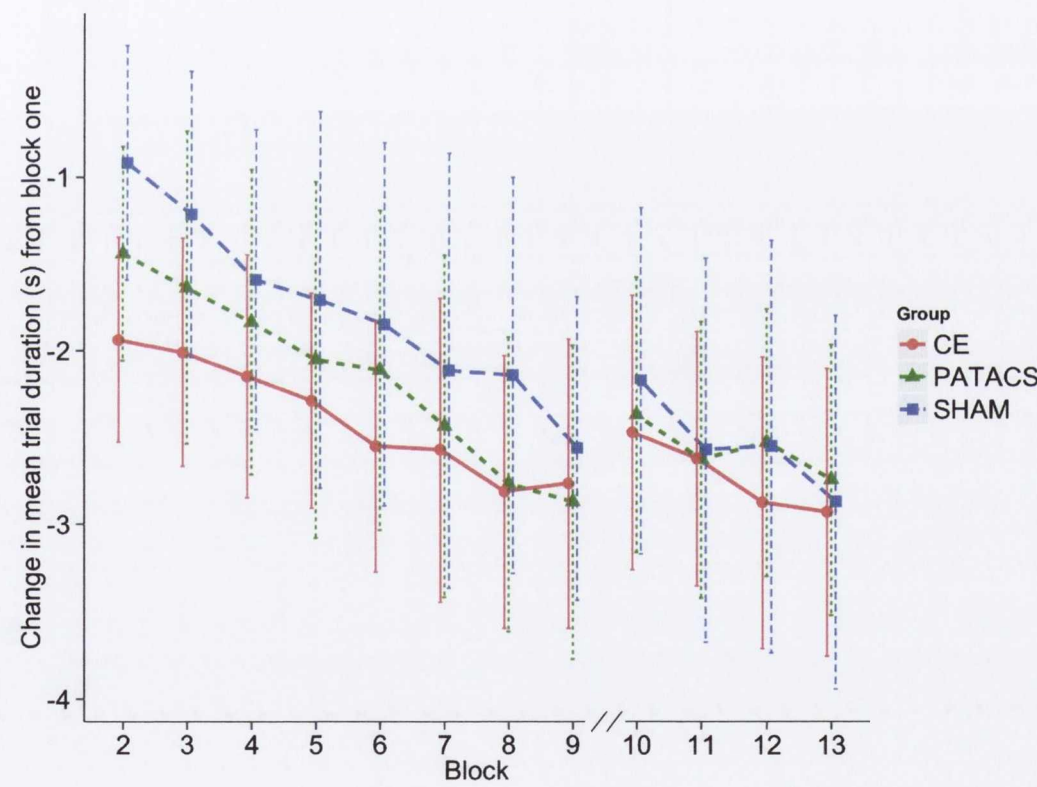


Figure 3.9. Mean trial duration (in seconds) expressed as change from block one (i.e. Bx minus B1) for each of the three groups. Error bars are 95% confidence intervals. The space represents the interval (one week) between the main testing session and the retention test. “CE” = cross education group.

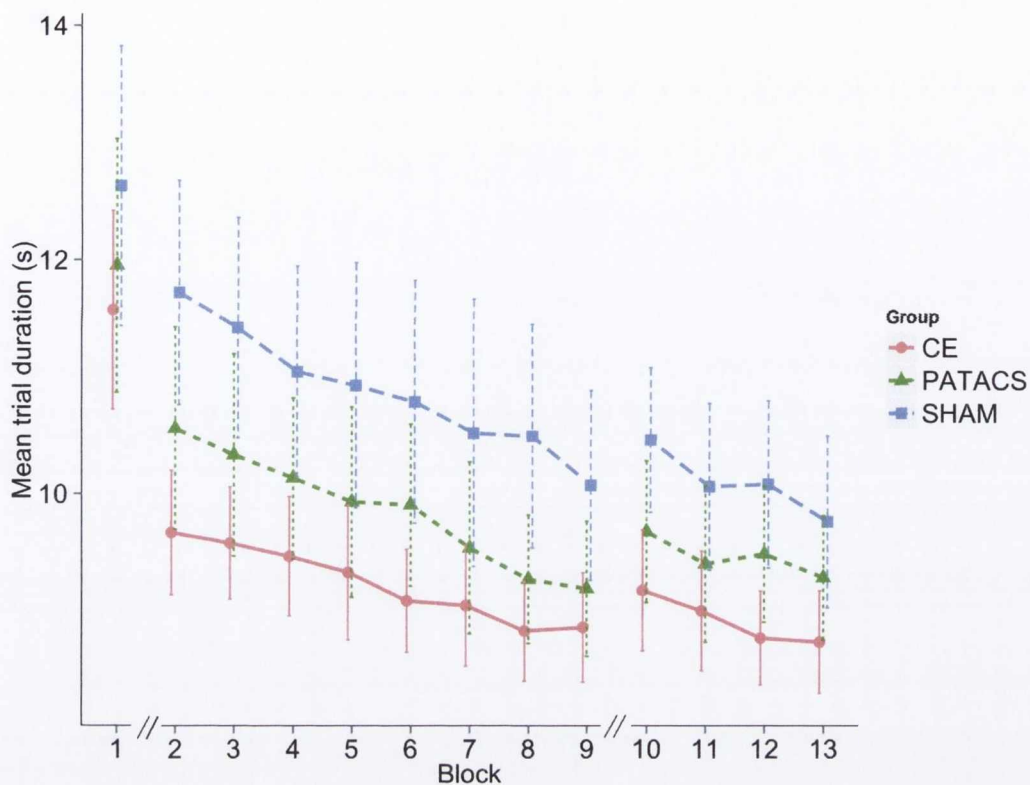


Figure 3.10. Mean trial duration (in seconds) across blocks (non-normalised) for each of the three groups. Error bars are 95% confidence intervals. The spaces between blocks 1 and 2 represents the intervention and between blocks 9 and 10 represents the interval (one week) between the main testing session and the retention test. “CE” = cross education group.

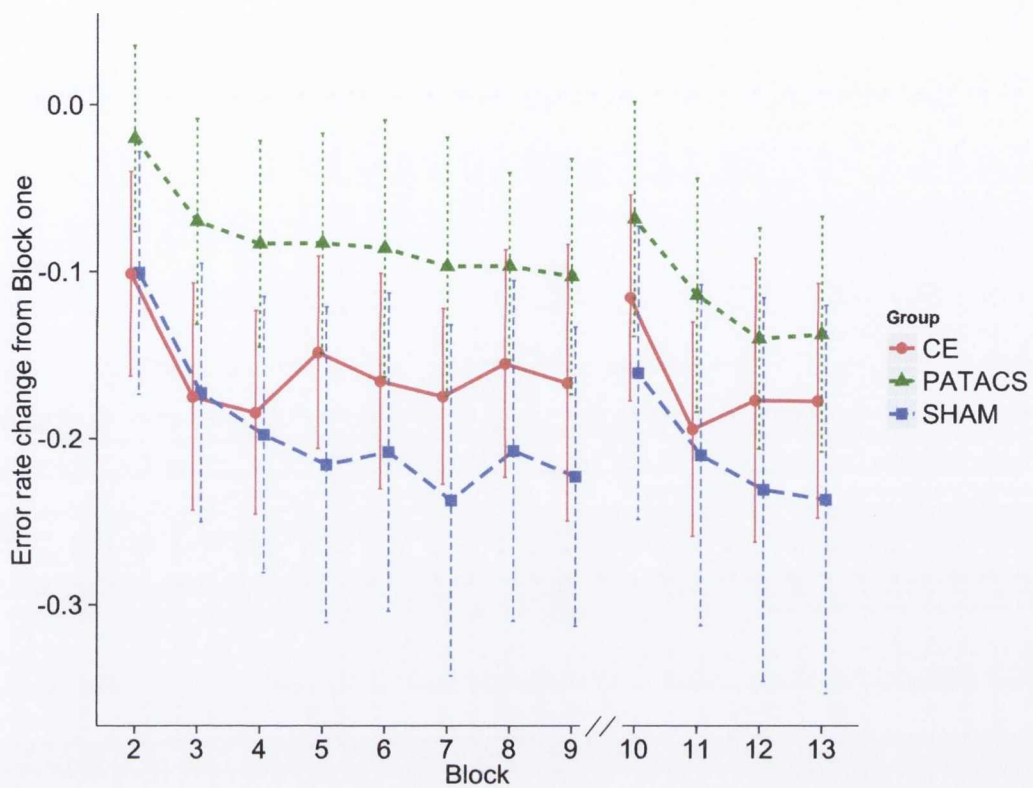


Figure 3.11. Error rate (proportion of missed gates per block) expressed as change from block one (i.e. B_x minus B_1) for each of the three groups. Error bars are 95% confidence intervals. The space represents the interval (one week) between the main testing session and the retention test. "CE" = cross education group.

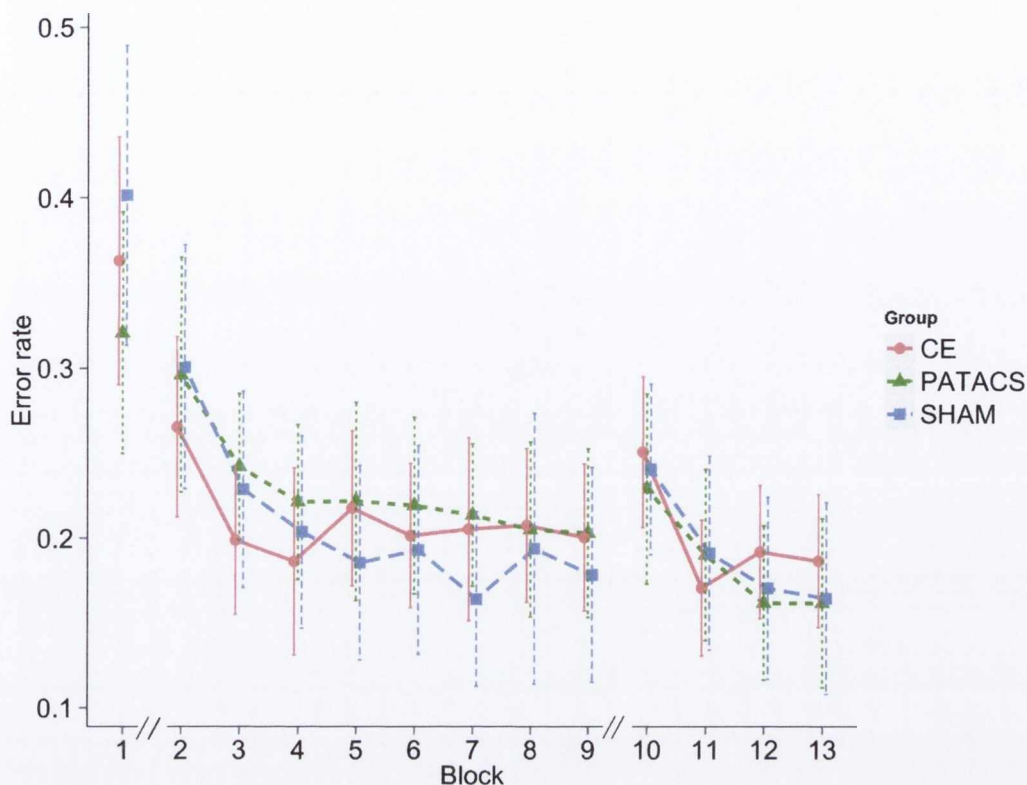


Figure 3.12. Error rate (proportion of missed trials) across blocks (non-normalised) for each of the three groups. Error bars are 95% confidence intervals. The spaces between blocks 1 and 2 represents the intervention and between blocks 9 and 10 represents the interval (one week) between the main testing session and the retention test. “CE” = cross education group.

3.4.3 Sequential isometric force task – between group differences

3.4.3.1 Block 1 to Block 2

There were reliable differences in the degree to which the three groups’ skill scores increased from block one to block two (table 7 and figure 9). The cross education group increased to a greater degree than the sham group, whereas skill increase in the PATACS group was lower than in the sham group. Error rate in the PATACS group did not decrease as much as decreases displayed in the sham group (figure 10). There were

no reliable differences in error rate between cross education and sham. There were no reliable differences in the degree of change in mean trial duration between any of the three groups (table 7 and figure 10).

Table 3.7. Difference in degree of change from block one to block two for the cross education group compared to the sham group and for the PATACS group compared to sham group. The differences are displayed for the skill score, the mean trial duration in seconds, and the error rate. T values and probabilities are for planned comparisons following one way anova.

B1 vs. B2	Comparison with	Mean difference	t	p
Skill (<i>a</i>)	Sham			
	CE	0.23	2.156	0.037
Duration (s)	PATACS	-0.29	-2.320	0.025
	CE	-1.02	-1.935	0.060
Error rate (%)	PATACS	-0.52	-0.032	0.975
	CE	-0.1%	-1.028	0.310
	PATACS	8.1%	2.034	0.048

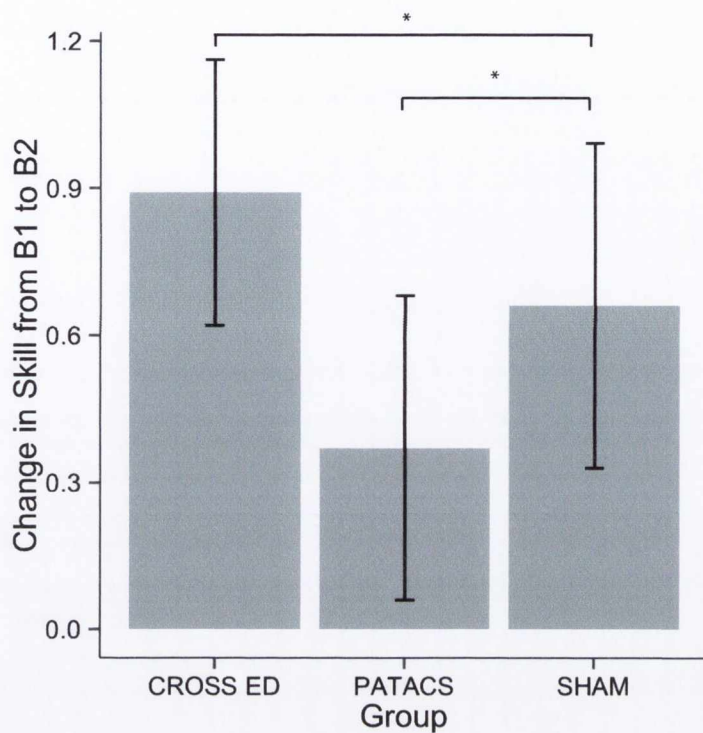


Figure 3.13. Change in skill score from block one to block two for each of the three groups. Error bars are 95% confidence intervals. Asterisks represent a statistically reliable group difference.

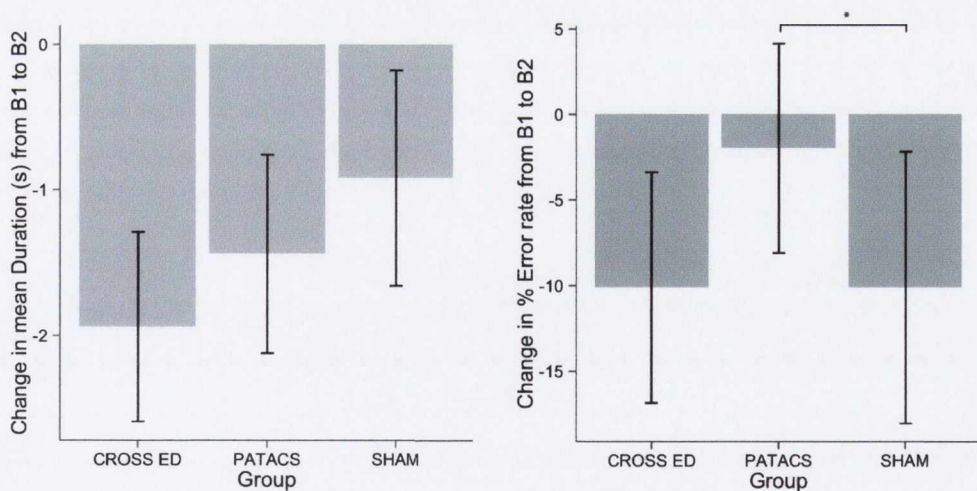


Figure 3.14. Difference in degree of change from block one to block two for both mean trial duration (left) and error rate (right) for all three groups. Error bars are 95% confidence intervals. Asterisks represent a statistically reliable group difference.

3.4.3.2 Block 1 to Block 10

There were no reliable differences in the degree of change (in skill, mean duration or error rate) between block one and block ten when either the PATACS or the cross education groups were compared with the sham group (table 8, figures 11 and 12).

Table 3.8. Difference in degree of change from block one to block ten for the cross education group compared to the sham group and for the PATACS group compared to sham group. The differences are displayed for the skill score, the mean trial duration in seconds, and the error rate. T values and probabilities are for planned comparisons following one way anova.

B1 vs. B10	Comparison with	Mean difference	t	p
	Sham			
Skill (a)	CE	-0.11	0.403	0.689
	PATACS	-0.39	-1.601	0.117
Duration (s)	CE	-0.30	-0.373	0.711
	PATACS	-0.20	-0.088	0.930
Error rate (%)	CE	4.5%	-0.025	0.980
	PATACS	9.2%	1.509	0.139

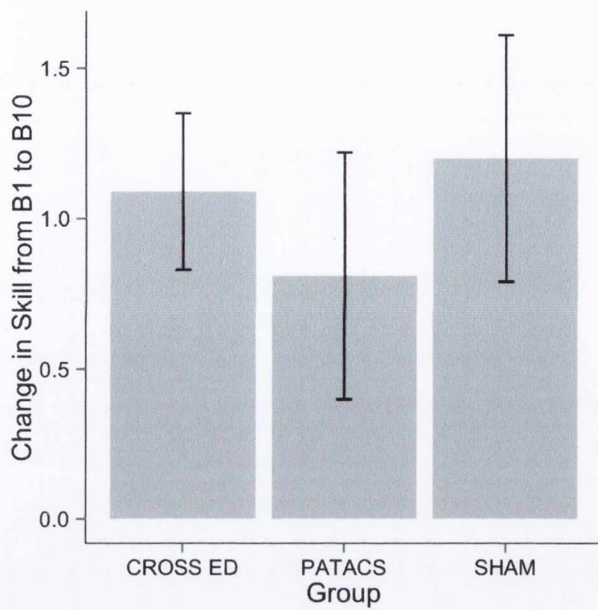


Figure 3.15. Change in skill score from block one to block ten for each of the three groups. Error bars are 95% confidence intervals.

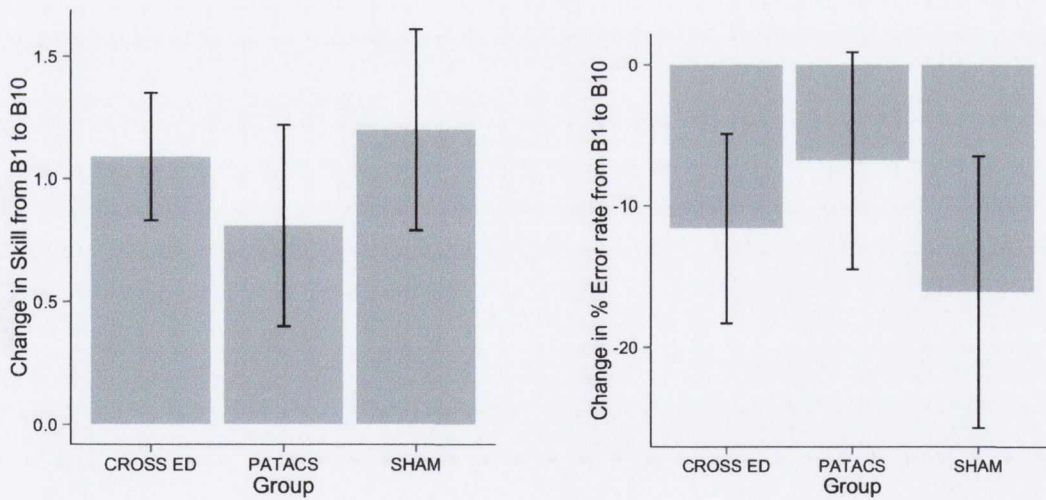


Figure 3.16. Difference in degree of change from block one to block ten for both mean trial duration (left) and error rate (right) for all three groups. Error bars are 95% confidence intervals.

3.4.3.3 Block 2 to Block 9

Between block two and block nine, there were no reliable differences in the degree of change in skill or error rate when either the PATACS or the cross education groups were compared with the sham group (table 9, figures 13 and 14). The cross education group demonstrated a smaller decrease in mean trial duration than the sham group, while there was no reliable difference in change in duration between the PATACS group and the sham group over this period.

Table 3.9. Difference in degree of change from block two to block nine (with block one as covariate) for the cross education group compared to the sham group and for the PATACS group compared to sham group. The differences are displayed for the skill score, the mean trial duration in seconds, and the error rate. T values and probabilities are for planned comparisons following one way ancova.

B2 vs. B9	Comparison with	Mean difference	t	p
	Sham			
Skill (a)	CE	-0.59	-1.878	0.067
	PATACS	-0.43	-0.254	0.801
Duration (s)	CE	0.82	2.293	0.027
	PATACS	0.21	-0.955	0.345
Error rate (%)	CE	5.7%	1.392	0.171
	PATACS	4.0%	-0.465	0.644

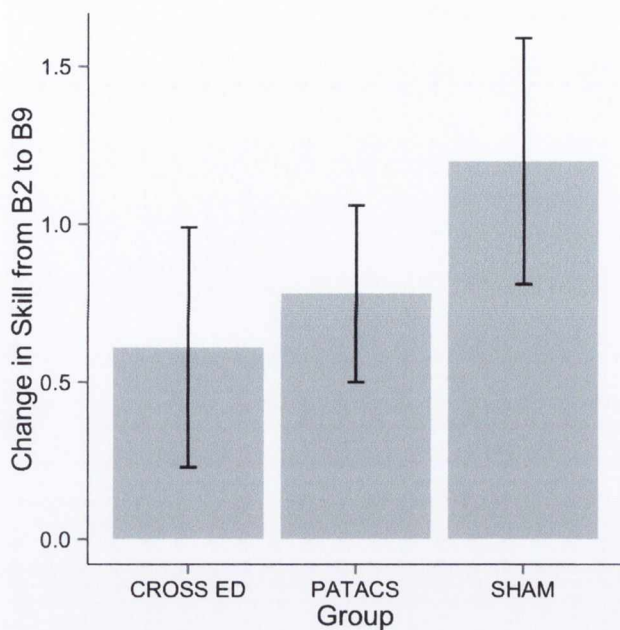


Figure 3.17. Change in skill score from block two to block nine for each of the three groups. Error bars are 95% confidence intervals.

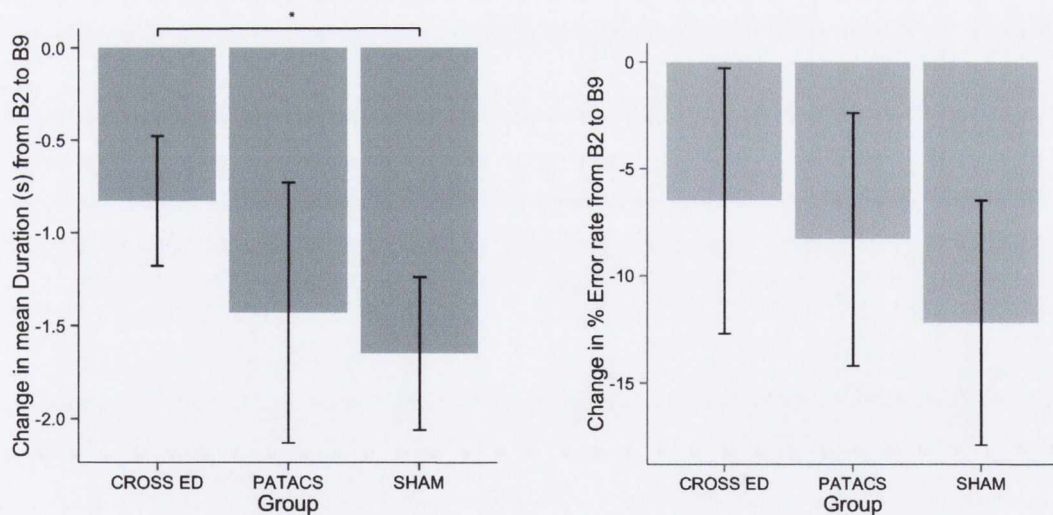


Figure 3.18. Differences in degree of change from block two to block nine for both mean trial duration (left) and error rate (right) for all three groups. Error bars are 95% confidence intervals. Asterisks represent a statistically reliable group difference.

3.4.3.4 Block 10 to Block 13

There were no reliable differences in the degree of change (in skill, mean duration or error rate) between block ten and block thirteen when either the PATACS or the cross education groups were compared with the sham group (table 10, figures 15 and 16).

Table 3.10. Differences in degree of change from block ten to block thirteen (with block one as covariate) for the cross education group compared to the sham group and for the PATACS group compared to sham group. The differences are displayed for the skill score, the mean trial duration in seconds, and the error rate. T values and probabilities are for planned comparisons following ancova.

B10 vs. B13	Comparison with	Mean difference	t	p
	Sham			
Skill (a)	CE	-0.22	-1.036	0.306
	PATACS	0.01	0.683	0.498
Duration (s)	CE	0.24	0.224	0.824
	PATACS	0.32	0.767	0.447
Error rate (%)	CE	10.2%	0.421	0.676
	PATACS	10.0%	-0.467	0.643

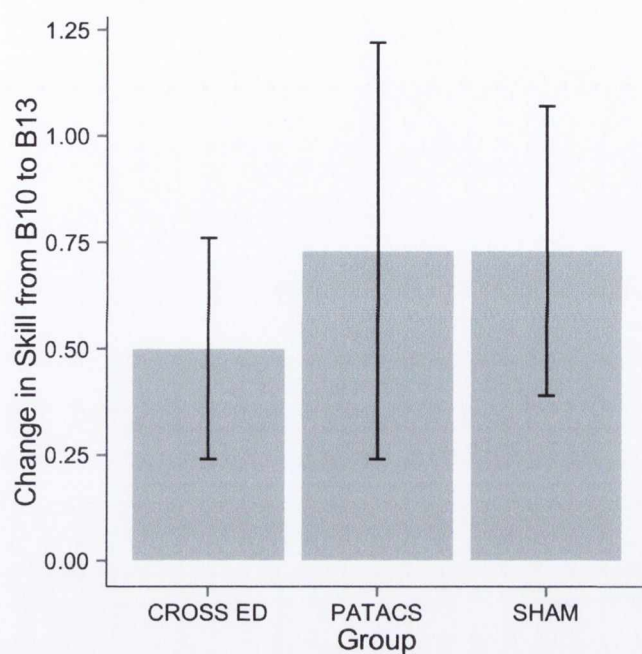


Figure 3.19 Change in skill score from block ten to block thirteen for each of the three groups. Error bars are 95% confidence intervals.

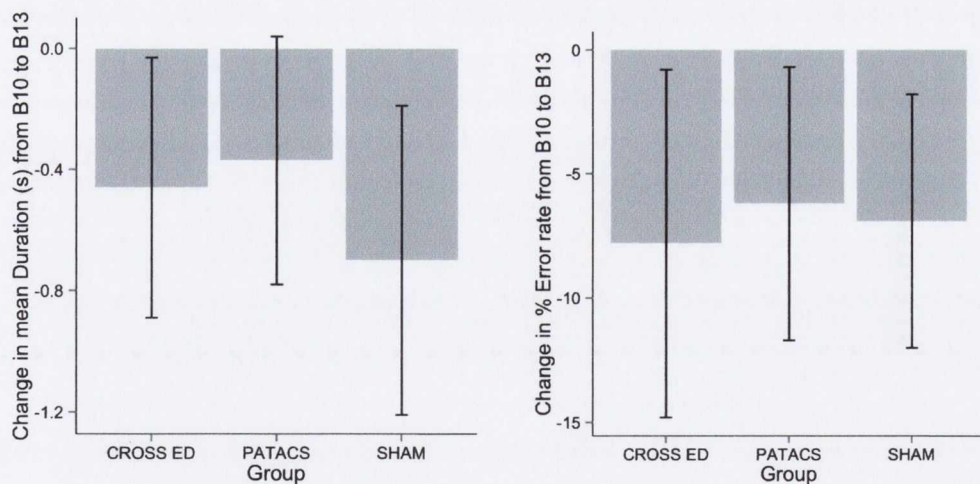


Figure 3.20. Differences in degree of change from block ten to block thirteen for both mean trial duration (left) and error rate (right) for all three groups. Error bars are 95% confidence intervals.

3.5 Discussion

The principle aim of the study was to examine whether increases in corticospinal excitability induced by a non-invasive brain stimulation protocol (PATACS) would lead to benefits in the subsequent performance or learning of a motor task. In order to investigate this, PATACS was administered in a group of participants prior to a period of motor training. Subsequent task performance and learning in this group were compared with that of a sham stimulation group and with a second comparison group; the cross education group. This second comparison group trained on the task with the opposite limb rather than receiving brain stimulation. It was expected that this group might also demonstrate increased in CSE (measured using TMS over the left 'untrained' M1), and may exhibit contrasting effects in performance or learning characteristics when compared with the PATACS group.

Increased CSE was evident in the PATACS group both relative to baseline and to the sham group after thirty minutes post-stimulation. However, this did not lead to any beneficial effects in either performance improvements during training of the task (block two to block nine), or learning of the task when measured at the retention test the following week (block one to block ten). Indeed, the PATACS group (relative to the sham group) actually displayed a small but reliable negative effect on gains in motor skill performance immediately following the intervention. All groups improved between block one and block two, but skill gains in the PATACS group over this time were significantly lower than those of the sham group. This seems to have been mediated by a detrimental effect on the proportion of errors made relative to the sham group.

The cross education group also demonstrated an increase in CSE relative to baseline and to the sham group. Immediately following the intervention they exhibited a SIFT performance benefit relative to the sham group; their skill score improved to a

relatively greater degree than their sham control counterparts. However, they did not demonstrate any greater gains in performance across the main training sequence (blocks two to nine) than the sham group, nor did they exhibit any advantage relative to sham when learning (retention) was examined upon return a week later (block ten compared with block one).

It could be questioned whether the sequential isometric flexion task is sensitive enough to detect subtle between-group differences. The task was carefully developed and validated by Reis *et al.* (2009) and has been used frequently in studies of motor learning (e.g. Cantarero *et al.*, 2015; Camus *et al.*, 2009). Certainly within-group learning occurs as one might expect, and continues throughout the training sequence without reaching asymptote. On an individual basis, participants' highest scoring performances tended to be in the last block (see frequency plot in appendix y). There are clear between-group differences in degree of change in the skill parameter between block one and block two.

A greater degree of transfer benefit may have been expected from the cross education condition in performance improvement across the main training sequence and in learning as measured at the retention test. The initial advantage appears to have been effectively 'washed out' by subsequent gains from training with the right limb. Camus *et al.* (2009) used a pinch force version of this task in a cross education paradigm and reported significant transfer effects. However, the high degree of task dependency in the outcomes of motor tasks is again highlighted; Camus and colleagues reported that there were no increases in CSE measured via recruitment curves at the untrained M1, which is contrary to the findings of the wrist flexion task used presently.

There is a possibility that the relatively small gains made by the cross education group when compared with sham may have been due to the counterintuitive mapping

relationship between cursor movement and the direction of wrist flexion. In order to maintain consistency in the spatial displays between the right and left hand training, the order of the gates and the motion of the cursor were designed as identical in the left and right hand versions of the task. This meant that in the left hand version of the task, the cursor moved in the same direction as the applied wrist force but in the right hand version, the rightward flexion still led to leftward cursor motion. It is possible that switching the display arrangement might lead to more rather than less cross education transfer effect, as directional compatibility between display and control can have an important influence on performance (Worringham & Beringer, 1989).

With regards to the PATACS group, two observations may be made. The first point is related to the induced changes in CSE. These were of a smaller magnitude than those reported in previous demonstrations of the effect (McNickle & Carson, 2015), underlining once again the large degree of variability common to all NIBS methods (e.g. López-Alonso *et al.*, 2014; 2015). The changes in CSE were subsequently overwhelmed by the excitability-inducing influence of simply moving the right limb during training. The final TMS recruitment curve was measured after block five of the flexion task. The levels of CSE were virtually identical in all three groups at this time point, meaning that any increases in excitability due to stimulation were no longer detectable among the increases in CSE brought about by right hand training on the flexion task. This has obvious implications for any hypothesis regarding the usefulness of increased CSE ahead of training or physical rehabilitation; simply engaging in movement influences CSE (Ridding *et al.*, 1995) and could do so to a greater degree than the NIBS protocol, thus negating any hypothetical advantage of artificially inducing extra excitability prior to training.

The second point, as noted already, is that if any difference was apparent at all it was that PATACS was associated with a slight detrimental effect in the initial task performance following the intervention. There are many indications in the recent literature that there is no straightforward relationship between CSE and measures of motor learning. Initial evidence that NIBS may be used to enhance motor learning through an interaction with plasticity processes (Nitsche *et al.*, 2003; Hummel *et al.*, 2005) has been complicated by more recent examples showing that the interactions between excitability and functional plasticity are complex and highly dependent on task (Saucedo Marquez *et al.*, 2013) and timing (Stagg *et al.*, 2011) among myriad other factors. Stagg and colleagues (2011) found that training on a motor learning task immediately following anodal transcranial direct current stimulation (tDCS) actually acted as an impairment to learning. The authors described this phenomenon as possibly being due to regulatory metaplasticity mechanisms (Bienenstock *et al.*, 1982). Later experiments by the same group revealed (through the use of short interval intracortical inhibition) that this metaplastic effect may be underpinned by an increase in GABA_A activity (Amadi *et al.*, 2015). Motor training may itself act as a positive modulator of excitability in M1 and training-induced excitability can interact with subsequent attempts to manipulate excitability through NIBS (Ziemann *et al.*, 2004; Rosenkranz *et al.*, 2007). Metaplasticity mechanisms act to prevent the process of continual positive feedback and ever-building ‘runaway potentiation’ (Abraham *et al.*, 2001) in neural networks. As such, they may represent a natural barrier to the prospective gains that might be achieved through adding NIBS-derived excitability to the motor system, at least in healthy populations with normal levels of cortical excitability.

Changes in the excitability of the ‘untrained’ M1 due to training of the left (ipsilateral) limb are largely task dependent (for a review see Ruddy & Carson, 2013).

In the present study cross education gave rise to reliable increases in CSE. These were at least as large as the increases brought about in the PATACS group, yet clearly they did not serve as a barrier to subsequent improvements in performance. These observations somewhat undermine the possibility that the (relatively) diminished gains in performance exhibited by the PATACS group between blocks one and two can be accounted for in terms of regulatory metaplasticity mechanisms.

There is convincing evidence to suggest that NIBS can change excitability (Bastani & Jaberzadeh, 2012) and that it can in some circumstances impact upon various measures of motor behaviour and motor learning (Reis *et al.*, 2008; 2009). Nevertheless, there are fewer convincing instances where excitability and motor learning have occurred together in the same experiment (however, see Karok & Witney, 2013). Bestmann and Krakauer (2015) comment upon the difficulties inherent in making inferences about volitional motor behaviour based upon responses to TMS. A change in CSE as measured by MEP amplitude may be a useful indication that *something* has changed in inhibitory and excitatory circuits that contribute, via the corticospinal tract, to potentials detectable at a peripheral muscle. However the transcortical, intercortical and corticospinal factors that contribute to systematic changes in its amplitude in different circumstances remain incompletely understood. The use of MEP amplitude as an assay of motor learning is complex and highly dependent on task (Bagce *et al.*, 2013), and the relevance of change in MEP amplitude to voluntary motor output in general is at best unclear. This study further underlines the fact that while facilitation of motor performance and enhanced CSE are both commonly reported outcomes of NIBS, there are few theoretical or empirical reasons to expect increased MEP amplitude to represent more optimal conditions for subsequent motor training.

Chapter 4

General Conclusions

4.1 General discussion

Mechanistic theories on the effects of non invasive brain stimulation in the human motor system human have been influenced by their *in vitro* and animal model origins. The first aim of this thesis was to examine how close these proposed mechanisms are likely to be to the processes which have been characterised in their reduced model counterparts. The reduced model heritage of stimulation techniques is also likely to have influenced expectations of what may be practically achieved using NIBS in humans. The second aim of the thesis was to examine whether changes in electrophysiological measures derived following NIBS are likely to indicate an influence upon behaviourally relevant (i.e. functional plasticity) outcomes.

4.2 Summary of findings

4.2.1 Chapter one

Chapter one had two themes. The first involved an overview of the literature concerning mechanistic theories proposed for four families of non-invasive brain stimulation techniques; tDCS, tACS, rTMS and PAS. The focus of this review was to highlight where explanatory models for the effects of these stimulation methods in humans had been extrapolated from their *in vitro* and animal model heritage. The conclusions drawn from this review were that while reduced models were an important starting point, the over-extrapolation of somewhat simplistic mechanistic theories to the

much more complex level of the intact human motor system may not always yield a complete explanation. It was also noted that novel modelling approaches to investigating the effects of NIBS have recently been explored. Furthermore, this section expanded on the idea of over-extrapolation of *in vitro* to *in vivo* mechanisms by exploring in greater detail the purported mechanisms for PAS. This technique has commonly been described as an example of spike timing dependent plasticity (STDP), a phenomenon that had previously only been observed *in vitro*. Many of the ostensive features of PAS mirror those of STDP however more recent theoretical and empirical developments warrant a reassessment of whether this is a valid description of the likely effect at the level of the human motor systems in associative brain stimulation. This frames the topic of the experimental work outlined in chapter two.

The second part of chapter one examined how changes in CSE following NIBS might relate to behaviourally relevant outcomes. The amplitude of the motor evoked potential is the yardstick of efficacy in several NIBS paradigms. While this can be said to reflect plastic change in the human motor system, there is little evidence that increased CSE is causal to modulations in performance or learning of motor tasks during (or subsequent to) the application of NIBS. This review explores the origins of the often implicitly held assumption that modulated electrophysiological plasticity responses (MEPs) should lead to more effective functional plasticity outcomes in physical motor learning, which frames the primary topic of the experiment in chapter three.

4.2.2 Chapter two

Chapter two outlines a series of seven experiments which examined whether the discrete timing of the stimuli in PAS is indeed a necessary characteristic of the

associative effects observed, thus testing the postulate that it is a clear example of STDP in the intact human motor system. As part of this examination a new method of NIBS was developed in which trains of peripheral nerve stimulation were repeatedly paired with short bursts of transcranial alternating current stimulation. Paired associative transcranial alternating current stimulation (PATACS) proved to be reliable and effective in bringing about changes in corticospinal excitability (CSE) at a range of different alternating current intensities and frequencies, at different burst durations, and while utilising different peripheral stimulation modalities. Several further experiments were conducted in order to replicate the effect, to gain an appreciation of the effect size, and to gauge the effects of changing the parameters of the stimuli. Importantly, clear evidence was provided that associative effects may be obtained in the absence of temporally discrete stimuli. This was interpreted as an indication that alternative mechanistic models may be necessary to describe some of the effects observed in PAS.

4.2.3 Chapter three

Chapter three outlines an experiment which was designed to examine the question raised in the second theme of chapter one: should NIBS-driven elevations in CSE be expected to act as a positive 'primer' and predictor of more effective training on a motor task? The PATACS stimulation technique developed in chapter two was utilised to induce increases in CSE in a group of participants ahead of training on a sequential isometric wrist flexion task. The increased CSE demonstrated in this group conveyed no advantage in the task; rather, upon initial task performance following stimulation, this group exhibited slightly lower skill scores than the sham control group (who had not exhibited elevated levels of CSE). Elevated CSE was also exhibited in a third group, who in place of PATACS stimulation underwent training on the sequential

flexion task using the opposite limb (cross education). In contrast to the PATACS group however, the cross education group initially performed better than the sham control group. These results served to illustrate that there is dissociation between augmentation in measures of CSE and subsequent task performance.

Initial between-group differences in performance demonstrated immediately following stimulation were no longer evident at the end of the main sequence of right hand training. All three groups demonstrated equivalent learning of the task as defined by measurements of retention one week later; there were no between-group differences at this point. Thus, the impact of intervention group led to differences only in the initial performance of the task following the intervention, but did not impact upon training performance or retention of skill.

4.3 Limitations of findings

4.3.1 STDP-like mechanisms

The development of the PATACS stimulation method in chapter two served to illustrate that associative effects may be brought about when the timing of the stimuli is not precisely circumscribed. This does not absolutely preclude the possibility that a STDP-like mechanism may account for some of the features of PAS. However, as noted by Stagg (2015), the fact that effects can also be derived when ‘non-discrete’ stimuli are utilised does make this interpretation less likely.

Evidence such as that presented by Wolters *et al.* (2003) provides the suggestion that a STDP-like mechanism is responsible for PAS effects. ISIs of 0ms, 5ms and 10ms tended to provide inhibition (although only 10ms was reliable). These results reflect the effective window for LTD-like effects in line with STDP. Excitability inducing PAS25

timings can be viewed as consistent with the PATACS results; stimuli that to an extent overlap in time are sufficient to bring about increases in CSE. However, these inhibitory ISIs and other demonstrations of the LTD-like PAS₁₀ effect (which it should be noted are less often reported than facilitatory PAS₂₅ effects) remain to an extent unexplained in light of the PATACS findings.

4.3.2 Variability of the PATACS effect on CSE

In common with all NIBS methods, PATACS exhibits large variability in effect. In the first set of experiments, pooled data from five replications of the prototypical paradigm enabled the calculation of a confidence interval around the effect size. However, when PATACS was applied in a new group of participants in chapter three, levels of variability in the effect were such that the effect size derived fell below the lower bracket of the previously calculated confidence interval. This is a frustrating if familiar theme in NIBS research. It is for this reason that approaches which reveal the true underlying mechanisms of action are of vital importance. Only through a more comprehensive understanding of the effects that NIBS has will there be more effective development of methods that seek to ameliorate this variability.

4.3.3 Degree of transfer on the SIFT

As noted in the discussion of chapter three, it might have been expected that training using the left hand would have led to greater learning and performance advantages in subsequent training with the right hand in the sequential isometric flexion task. While an initial advantage was evident immediately following the intervention, this was quickly ‘washed out’ by continuous training with the right hand. Use of an alternative display for the motor learning task may have led to a greater cross education

transfer effect not only in initial performance but also to rate of learning, and degree of retention when tested one week later. The task involved ‘counterintuitive mapping’ between the display and the force direction (as discussed earlier). The right and left hand versions of the task both moved the cursor across the screen from left to right, with the rationale that the two displays would be identical for comparison. This meant that the left hand pushed the cursor in the same direction as the force it was generating, but the right hand (also pushing towards the body’s midline) pushed the cursor in the opposite direction to the force it generated. Had the right hand version of the task moved the cursor from right to the left of the screen instead, degree of transfer may have been influenced, however without further experimental work this remains unclear. One potential solution which would enable an identical screen display in both conditions and avoid a discrepancy in counterintuitive and intuitive mapping would be to design a display that moved the cursor upwards to reach vertically placed targets in both conditions.

4.4 Future directions

4.4.1 Effective parameters of PATACS

PATACS represents yet another novel method of non-invasively inducing changes in corticospinal excitability. Numerous experimental adaptations of the method were investigated, however the technique has not yet been fully parameterised. It may be instructive to explore whether further manipulations of the stimulation frequency or duration might affect outcomes. Frequencies higher than 250 Hz or lower than 80 Hz are yet to be tested. Additionally, it may be possible obtain similar results using pulses of random noise stimulation (frequencies randomly distributed in a broad bandwidth) in

place of the tACS of uniform oscillatory frequency. Paired stimuli of a duration greater than one second have not been applied. Furthermore, it is as yet unknown how long following the intervention the increases in CSE remain.

4.4.2 Timing of peripheral stimuli in PATACS

It may be of interest to examine whether the timing of the peripheral stimuli relative to the peaks and troughs of the oscillatory tACS waveform may impact upon changes in CSE. The corollaries of peripheral stimuli delivered around 20ms prior to the peak of the tACS wave might theoretically coincide with the maximal current travelling in one direction through the primary motor cortex, while 20ms prior to the trough of the wave may coincide with the opposite current direction. Should these two timings lead to divergent MEP characteristics recorded subsequent to stimulation, it may provide the impetus to generate new avenues of investigation into the mechanisms of associative stimulation. The caveats in relation to this line of reasoning, however, mirror those outlined in chapter one; the distribution and timing of transcranially delivered currents and the arrival of the PNS volley at M1 are far from certain. This makes planning the ISIs for such designs problematic.

4.4.3 Mechanistic investigations of the effect of PATACS

As outlined in the second section of chapter one, there are numerous paired pulse TMS techniques that may be used to preferentially engage GABAergic circuits in M1. Stimulation techniques such as short latency intracortical inhibition (SICI) could be applied following PATACS to investigate whether such circuits are involved in the effect. Through the use of such techniques, the involvement of different circuits has

been investigated in tDCS (Hummel *et al.*, 2005), rTMS (Huang *et al.*, 2005), tACS (Moliadze *et al.*, 2010), and PAS (Carson & Kennedy, 2013).

Pharmacological interventions have also been utilised frequently in NIBS research to highlight some of the possible molecular mechanisms of the effects observed, e.g. Nitsche *et al.*, (2003). Among the compounds that have been administered to human participants previously, several candidates could be used to examine PATACS. Dextromethorphan, an NMDA antagonist, blocks both the excitability enhancing effect of PAS₂₅ and the excitability reducing effect of PAS₁₀, implicating the NMDA receptor in these plastic effects (Carson & Kennedy, 2013). It may be revealing to demonstrate, using dextromethorphan, whether the effects of PATACS are mediated by the same receptor. In a similar vein, magnetic resonance spectroscopy has been applied to examine whether changes in GABA concentrations may be detected following tDCS (Stagg *et al.*, 2011). These lines of investigation could possibly delineate some of the molecular mechanisms of PATACS.

4.4.4 The effects of NIBS on motor learning

Advances in the understanding of the underlying mechanisms of NIBS may ultimately aid their application in practical settings. The results of chapter three help to demonstrate the argument made in chapter one; the influence that NIBS may have on motor learning is likely to be due to other characteristics than simply the change in CSE.

It must be acknowledged that any effects observed in motor training experiments will commonly be task-specific. The evidence in chapter three points to the fact that prior application of PATACS seemed to be of no benefit in performance or learning of the sequential isometric flexion task. However, the task-dependency of effects in other

investigations of NIBS and motor learning highlighted in chapter one suggest that there could well exist circumstances where prior application of PATACS may have a clearer modulatory effect. It may therefore be instructive to test a similar experimental paradigm to that outlined in chapter three using a number of motor learning tasks which aim to target substantially different types of motor learning, e.g. tasks which emphasise sequence learning, grip strength tasks, tasks which require simultaneous coordination of multiple effectors, and ballistic motor tasks which involve 'fast-as-possible' movements.

4.5 Implications and concluding remarks

This thesis explores the electrophysiological and functional impact of a novel associative brain stimulation technique. Ultimately, it provides evidence that more sophisticated models may be necessary to explain the effects observed following non-invasive brain stimulation, specifically paired associative stimulation. It also demonstrates that modulation of the amplitude of motor evoked potentials (the outcome of focus in many NIBS paradigms) may demonstrate that something has changed, but that this may not be related to changes in voluntary behaviour. It is hoped that in future, through cautious interpretation of experimental results, more informative explanatory models will emerge. This process of refinement can ultimately lead to a clearer understanding of non-invasive stimulation effects, and eventually to effective practical applications of these techniques.

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Appendices



COLÁISTE NA TRÍONÓIDE, BAILE ÁTHA CLIATH | TRINITY COLLEGE DUBLIN
Ollscoil Átha Cliath | The University of Dublin

F.A.O. Emmet McNickle

School of Psychology Research Ethics Committee

2 March 2015

Dear Emmet,

The School of Psychology Research Ethics Committee has reviewed your application entitled "Associative Stimulation to facilitate motor learning" and I am pleased to inform you that it was approved.

Please note that you will be required to submit a completed **Project Annual Report Form** on each anniversary of this approval, until such time as an **End of Project Report Form** is submitted upon completion of the research. Copies of both forms are available for download from the Ethics section of the School website.

Adverse events associated with the conduct of this research must be reported immediately to the Chair of the Ethics Committee.

Yours sincerely,

Richard Carson
Chair,
School of Psychology Research Ethics Committee

Histogram of Bestblock\$Block

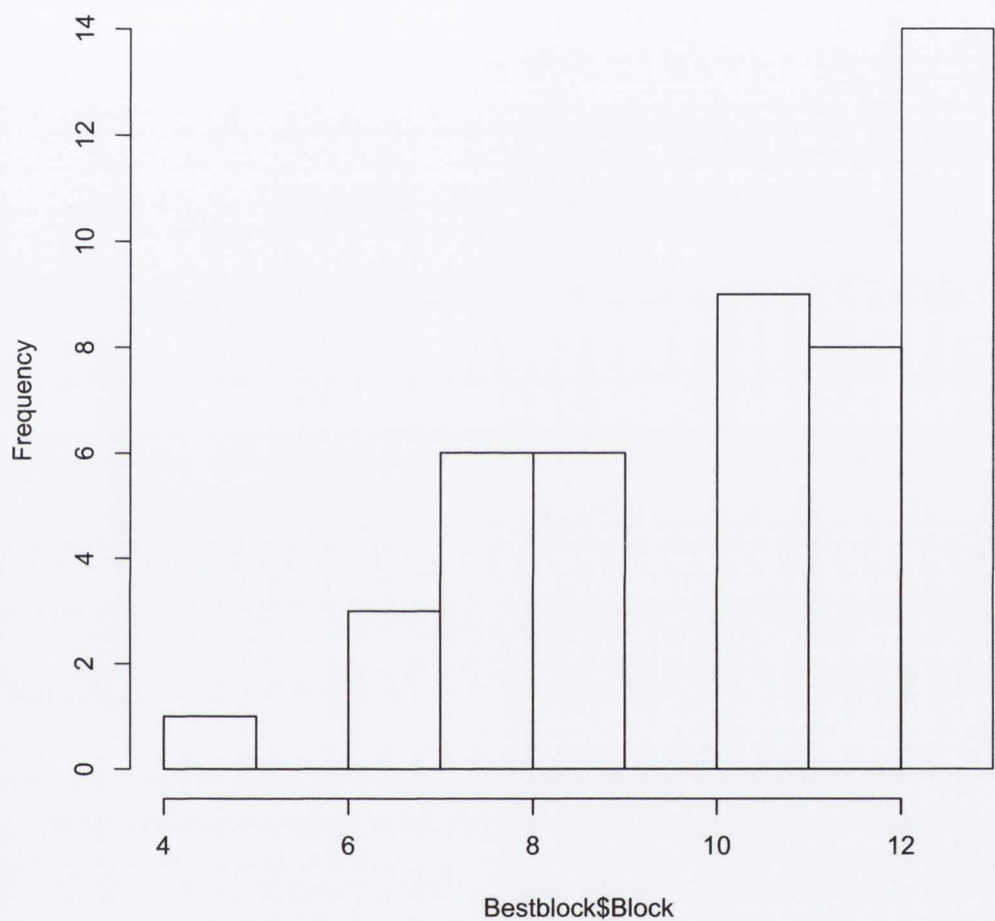


Figure y – Frequency plot illustrating count of which block participants achieved their highest score.

Table x – Curve fitting results which demonstrate that for a large range of ‘b’ values, the impact upon the goodness of fit of ‘a’ values across blocks to a power curve.

B_Value	Iterations	count	a	b	R-squared	KS-test value	KS-test prob	Theil U
0.1	40	8	2.360743	0.150681	0.614914	0.375	0.51894	0.013346
1.1	21	8	-0.040778	1.045546	0.125186	0.5	0.18768	0.014054
2.1	33	8	-0.931578	-0.809192	0.307841	0.5	0.18768	0.011074
3.1	40	8	-1.171167	-0.662867	0.621618	0.5	0.18768	0.061678
4.1	39	8	-1.438058	-0.463501	0.627061	0.5	0.18768	0.043612
5.1	38	8	-1.648496	-0.375041	0.628052	0.375	0.51894	0.035265
6.1	40	8	-1.822449	-0.324203	0.628247	0.375	0.51894	0.030422
7.1	38	8	-1.970607	-0.290733	0.628185	0.375	0.51894	0.02723
8.1	38	8	-2.099621	-0.266802	0.628039	0.375	0.51894	0.024949
9.1	38	8	-2.213896	-0.248712	0.627932	0.375	0.51894	0.023224
10.1	38	8	-2.316423	-0.234463	0.627742	0.375	0.51894	0.02187
11.1	41	8	-2.409401	-0.222899	0.627629	0.375	0.51894	0.02077
12.1	39	8	-2.494417	-0.213281	0.62754	0.375	0.51894	0.019854
13.1	39	8	-2.572799	-0.205135	0.627408	0.375	0.51894	0.019082
14.1	41	8	-2.645451	-0.198117	0.627303	0.375	0.51894	0.018417
15.1	38	8	-2.713176	-0.19201	0.627212	0.375	0.51894	0.017839
16.1	40	8	-2.776583	-0.186614	0.627124	0.375	0.51894	0.017328
17.1	39	8	-2.83621	-0.181815	0.627064	0.375	0.51894	0.016873
18.1	37	8	-2.892447	-0.177505	0.626956	0.375	0.51894	0.016467
19.1	40	8	-2.945712	-0.173617	0.626898	0.375	0.51894	0.016099
20.1	36	8	-2.996265	-0.170085	0.626839	0.375	0.51894	0.015766
21.1	41	8	-3.044377	-0.166845	0.626773	0.375	0.51894	0.01546
22.1	39	8	-3.090278	-0.163872	0.626721	0.375	0.51894	0.01518
23.1	41	8	-3.134147	-0.161129	0.626653	0.375	0.51894	0.014922
24.1	42	8	-3.17618	-0.158588	0.62663	0.375	0.51894	0.014681
25.1	38	8	-3.216515	-0.156222	0.626582	0.375	0.51894	0.014459
26.1	40	8	-3.25527	-0.154013	0.626524	0.375	0.51894	0.014251

27.1	36	8	-3.292569	-0.151945	0.626487	0.375	0.51894	0.014056
28.1	38	8	-3.328538	-0.150005	0.626436	0.375	0.51894	0.013874
29.1	37	8	-3.363252	-0.14818	0.626403	0.375	0.51894	0.013702
30.1	38	8	-3.39681	-0.14646	0.626379	0.375	0.51894	0.01354
31.1	40	8	-3.429254	-0.14483	0.626345	0.375	0.51894	0.013387
32.1	38	8	-3.460677	-0.143286	0.626295	0.375	0.51894	0.013242
33.1	38	8	-3.491158	-0.141822	0.62627	0.375	0.51894	0.013105
34.1	40	8	-3.520714	-0.140429	0.626237	0.375	0.51894	0.012974
35.1	38	8	-3.549444	-0.139104	0.626207	0.375	0.51894	0.01285
36.1	39	8	-3.577341	-0.137837	0.626193	0.375	0.51894	0.01273
37.1	37	8	-3.604492	-0.136628	0.626166	0.375	0.51894	0.012617
38.1	38	8	-3.630945	-0.135474	0.626142	0.375	0.51894	0.012509
39.1	41	8	-3.65668	-0.134364	0.626115	0.375	0.51894	0.012405
40.1	40	8	-3.681771	-0.133299	0.626088	0.375	0.51894	0.012305
41.1	38	8	-3.706284	-0.132282	0.626064	0.375	0.51894	0.012209
42.1	37	8	-3.730158	-0.131298	0.62603	0.375	0.51894	0.012118
43.1	40	8	-3.753518	-0.130357	0.626014	0.375	0.51894	0.012029
44.1	37	8	-3.776323	-0.129447	0.625996	0.375	0.51894	0.011944
45.1	38	8	-3.798607	-0.128568	0.625981	0.375	0.51894	0.011861
46.1	41	8	-3.820404	-0.12772	0.625941	0.375	0.51894	0.011782
47.1	38	8	-3.841772	-0.126906	0.625939	0.375	0.51894	0.011706
48.1	37	8	-3.862665	-0.126117	0.625929	0.375	0.51894	0.011632
49.1	38	8	-3.883119	-0.12535	0.625901	0.375	0.51894	0.01156
50.1	41	8	-3.903181	-0.124611	0.625893	0.375	0.51894	0.011491
51.1	38	8	-3.922854	-0.123897	0.625881	0.375	0.51894	0.011424
52.1	38	8	-3.942101	-0.123195	0.625845	0.375	0.51894	0.011359
53.1	39	8	-3.961021	-0.122522	0.625822	0.375	0.51894	0.011296
54.1	39	8	-3.979606	-0.121871	0.625835	0.375	0.51894	0.011234
55.1	37	8	-3.99782	-0.121234	0.625806	0.375	0.51894	0.011175
56.1	40	8	-4.015721	-0.120617	0.625791	0.375	0.51894	0.011117
57.1	38	8	-4.033296	-0.120014	0.625755	0.375	0.51894	0.011061

58.1	38	8	-4.050559	-0.119429	0.625763	0.375	0.51894	0.011006
59.1	39	8	-4.067538	-0.118857	0.62573	0.375	0.51894	0.010953
60.1	41	8	-4.084245	-0.118305	0.62574	0.375	0.51894	0.010901
61.1	39	8	-4.100671	-0.117765	0.625721	0.375	0.51894	0.010851
62.1	39	8	-4.116836	-0.117239	0.625699	0.375	0.51894	0.010802
63.1	40	8	-4.132709	-0.116722	0.625691	0.375	0.51894	0.010753
64.1	41	8	-4.148408	-0.116228	0.625716	0.375	0.51894	0.010706
65.1	39	8	-4.163814	-0.115737	0.62569	0.375	0.51894	0.010661
66.1	37	8	-4.178961	-0.115255	0.625664	0.375	0.51894	0.010616
67.1	38	8	-4.193902	-0.114788	0.625641	0.375	0.51894	0.010573
68.1	41	8	-4.208621	-0.11433	0.62563	0.375	0.51894	0.01053
69.1	39	8	-4.223135	-0.113885	0.625618	0.375	0.51894	0.010488
70.1	39	8	-4.237439	-0.113448	0.625595	0.375	0.51894	0.010448
71.1	38	8	-4.251537	-0.113021	0.625601	0.375	0.51894	0.010407
72.1	37	8	-4.265448	-0.112602	0.625587	0.375	0.51894	0.010368
73.1	37	8	-4.279149	-0.112193	0.625566	0.375	0.51894	0.01033
74.1	38	8	-4.292714	-0.111797	0.625583	0.375	0.51894	0.010293
75.1	37	8	-4.306054	-0.111404	0.625579	0.375	0.51894	0.010256
76.1	38	8	-4.319229	-0.111019	0.625537	0.375	0.51894	0.010221
77.1	41	8	-4.332222	-0.110642	0.625555	0.375	0.51894	0.010185
78.1	39	8	-4.345067	-0.110275	0.625539	0.375	0.51894	0.010151
79.1	38	8	-4.357737	-0.109912	0.625547	0.375	0.51894	0.010117
80.1	38	8	-4.370227	-0.109551	0.625506	0.375	0.51894	0.010084
81.1	39	8	-4.382588	-0.109204	0.625495	0.375	0.51894	0.010051
82.1	38	8	-4.394777	-0.10886	0.625491	0.375	0.51894	0.010019
83.1	40	8	-4.406859	-0.108527	0.625473	0.375	0.51894	0.009988
84.1	38	8	-4.418772	-0.108198	0.625514	0.375	0.51894	0.009957
85.1	40	8	-4.430502	-0.107867	0.625447	0.375	0.51894	0.009927
86.1	38	8	-4.442174	-0.107556	0.625485	0.375	0.51894	0.009897
87.1	37	8	-4.453638	-0.107237	0.62545	0.375	0.51894	0.009868
88.1	39	8	-4.465052	-0.106934	0.62548	0.375	0.51894	0.009839

89.1	40	8	-4.47627	-0.106627	0.625439	0.375	0.51894	0.009811
90.1	37	8	-4.487398	-0.106333	0.625439	0.375	0.51894	0.009783
91.1	36	8	-4.498405	-0.106041	0.625433	0.375	0.51894	0.009756
92.1	38	8	-4.509261	-0.105752	0.625405	0.375	0.51894	0.009729
93.1	38	8	-4.520026	-0.105469	0.625427	0.375	0.51894	0.009702
94.1	40	8	-4.530659	-0.105189	0.625387	0.375	0.51894	0.009677
95.1	39	8	-4.541198	-0.104918	0.625413	0.375	0.51894	0.009651
96.1	37	8	-4.551627	-0.104648	0.625406	0.375	0.51894	0.009626
97.1	39	8	-4.561915	-0.104378	0.625376	0.375	0.51894	0.009601
98.1	39	8	-4.572108	-0.104115	0.625381	0.375	0.51894	0.009576
99.1	40	8	-4.582205	-0.103858	0.625369	0.375	0.51894	0.009553