Stimulation Genomics

Probing the effects of Genetic Variation on Human Cortical Plasticity and its Clinical Implications

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PhD Thesis

I, Binith Cheeran confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Make no little plans

Make no little plans. They have no magic to stir men's blood and probably themselves will not be realized. Make big plans; aim high in hope and work, remembering that a noble, logical diagram once recorded will never die, but long after we are gone will be a living thing, asserting itself with ever-growing insistency. Think big.

Daniel Burnham. (1846-1912)

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Abstract

The studies presented in this thesis employ neurophysiological outcome measures and the application of artificially induced cortical stimulation plasticity paradigms to study the effects of human genetic variation on human cortical neuroplasticity.

The introductory chapter includes a review of illustrative models of neuroplasticity. I also cover the principles, physiology and pharmacology of TMS and rTMS. With this background, I set out the scope and principles of such an approach applied to the study of human genetic variation, and define the field of Stimulation Genomics. I set out the case for such an approach, highlighting previous studies that have employed neurophysiological outcome measures and the application of artificially induced cortical stimulation plasticity paradigms to study the effects of disease-causing human genetic mutations. In the subsequent introductory chapters I have focused on the rationale of selecting the Brain Derived Neurotrophic Factor polymorphism Rs6265 (BDNF Val66 Met) as the candidate polymorphism for our studies, covering the molecular biology and physiological roles of this highly conserved protein, and with a particular focus on its diverse roles in neuroplasticity.

The 1st experiment presented here used established rTMS and TDCS paradigms to probe the effects of the BDNF Val66Met SNP on cortical plasticity and metaplasticity. The results generated from this study, and particularly the results suggesting an effect on metaplasticity, formed the basis for the studies in

patients. We investigated the influence of this SNP on the rate of onset of Levodopa-Induced Dyskinesia (LID) in patients with Parkinson's disease and on the penetrance of DYT1 Dystonia. The final experiment presented here was designed to confirm the effects of the BDNF Val66Met polymorphism on the iTBS paradigm, and quantify its effects alongside other variables thought to influence the response to rTMS paradigms. This study also provides some crucial insights into the iTBS paradigm itself.

Table of Contents

Index of Tables and Figures

1 Introduction

1.1 Neuroplasticity

The electro-chemical synapse is designed to serve the dual purpose of both an adaptable communication system and an information storage system. Numerous small coherent synaptic modifications produce the distributed memory that underlies learning. Perhaps no single discovery has quite captured the attention of neuroscientists as that of the synaptic modification phenomenon of Long Term Potentiation (LTP) in 1973(Bliss and Lomo, 1973). The discovery of LTP (and subsequently Long Term Depression (LTD)) in the hippocampus, an organ with the an accepted role in memory and earning, provided the catalyst for research into how the brain could adapt to experience at the level of the synapse - the broad based church of neuroplasticity. As evidence builds for both adaptive (like in learning, memory or recovery after head injury) or maladaptive (like in Dystonia or LID) neuroplasticity, interest in the field has spread from physiologists in labs to neurologists at the bedside.

1.1.1 Illustrative Concepts and Models of Neuroplasticity.

The Hebb Learning Rule

In 1949, Donald Hebb produced a theory of synaptic modification:
When axon in cell A is near enough to excite cell B and repeatedly and persistently takes part in firing it, some growth or metabolic process takes place in one or both cells such that A's efficacy in firing B, is increased.

The hebbian model of the synapse can be visualized mathematically.
If we imagine a simple synapse between neuron A (presynaptic) and neuron B (postsynaptic), and x as the strength of the presynaptic input, the postsynaptic output y is give by the equation:
$y=w.x$, where w is synaptic weight or efficacy.

Hebbs's theory for a synaptic basis for memory inspired the characterization of Long Term Potentiation (LTP) (Bliss and Lomo, 1973). Bliss and Lomo stimulated the perforant path and recorded extra cellular field potential before and after short volleys of high frequency stimuli. They found that the size of the field potentials increased, indicating an increase in synaptic efficacy.

The Hebb rule was borne out by initial studies producing LTP, but suffers from 2 main problems: 1) that repeated inputs force the neuron to the maximum values, predicting runaway excitability and 2) it did not provide for a synaptic basis for weakening connections when presynaptic input was poorly co-ordinated with post synaptic firing. Several attempts to overcome these key defect of the Hebbian model of the synapse followed, notably Gunther Stent's suggestion (Stent, 1973) that connections would weaken when a presynaptic neuron is active at the same time as the post synaptic neuron is inactive (a precursor to the Spike Timing Dependent Plasticity model) and Nas and Cooper's (Nass and Cooper, 1975) suggestion that the modification of synaptic strength would end when the response of the postsynaptic cell reached a maximum value. This can be visualized as a sigmoid function of the Hebbian

equation ($y=\sigma (w.x)$). However, the next major theoretical advance came in the shape of the BCM theory.

The BCM model.

The BCM model built on an earlier theory by Cooper et al. (Cooper et al., 1979), the CLO theory, which suggested the concept of a modification threshold for each synapse. If the postsynaptic cell response was greater than the modification threshold synaptic strength increased; if the postsynaptic cell response was lower than the modification threshold synaptic strength decreased. The major conceptual flaw with the CLO theory was the level at which the modification threshold needed to be set. Set too high, most patterns of input would produce decreased activity driving the cell response to zero. Set too low, most patterns of input would produce increased cell activity and the cell would lose specificity.

To circumvent this artificiality Bienenstock, Cooper and Munro (BCM) proposed in 1982 that the value of the modification threshold should vary as a nonlinear function of the average output of the postsynaptic neuron over a fixed time (Bienenstock et al., 1982). The equation can be visualized as:

$w(t)= \phi(y,\theta m)x$

Where $w(t)$ is the change in synaptic strength and x represents pre synaptic activity. ϕ is a function of the postsynaptic output (y) and θm, the modification threshold, which is itself a function of the history of activity of the cell.

The BCM theory inspired the search for experimental evidence of Long Term Depression (LTD), particularly by researchers looking to explain the effects of monocular deprivation in the visual cortex. Finally, in 1991 Mark Bear et al. showed that prolonged stimulation at low frequency (0.5-3 Hz) and low intensity (below that required to produce population spikes) produced a decrease in synaptic efficacy (Clothiaux et al., 1991).

The three key postulates of the BCM theory now have experimental backing. They are:

I. The change in synaptic weights, w(t), is proportional to the presynaptic activity. This postulates synapse specificity for LTP and LTD, shown by Dudek and Bear (Dudek and Bear, 1992).

II. w(t) is proportional to a function ϕ of the postsynaptic activity y. For low values of y, w(t) decreases (<0) and for large values of y w(t) increases (>0). In other words, the level of postsynaptic response relative to the modification threshold determines the polarity of modification. The modification threshold θm denotes the crossing over point between w(t)<0 and w(t)>0. Experimental evidence for postulate 2 has come from both hippocampal and cortical studies and from several independent groups (Dudek and Bear, 1992) (Brocher et al., 1992) (Mulkey and Malenka, 1992) (Kirkwood and Bear, 1994) (Mayford et al., 1995)).

III. The modification threshold θm is an increasing function of the history of postsynaptic activity. This was shown initially by Yang and Faber (Xian-Da Yang and Faber, 1990), that the recent history of synaptic activation determined the magnitude of synaptic modification. A more direct demonstration of the sliding threshold was provided in the elegant experiments of Kirkwood et al. (Kirkwood et al., 1996). By studying LTP and LTD in layer III of visual cortex slice preparations from dark reared and control rats, they demonstrated that θm was lower in light deprived rats compared to control rats in the visual cortex but not in the hippocampus (control site).

The sliding threshold also gives an elegant explanation for homeostatic plasticity and introduced the concept of the ease of inducing plastic change (the plasticity of plasticity or metaplasticity (Abraham and Bear, 1996)).

1.1.2 Studying LTP/LTD.

The first established evidence for the molecular basis of how a nervous system can display neuroplasticity and adapt its motor behavior was found in the invertebrate sea-slug, Aplysia californica, by Eric Kandel and his group in 1969 (Kandel et al., 1969) (Castellucci et al., 1970) (Kupfermann et al., 1970; Pinsker et al., 1973) and subsequently with the characterization of long-term potentiation (LTP) in the mammalian hippocampus 1973 by Bliss and Lømo,

providing a molecular mechanism for neuroplasticity that obeys Hebbian principles (Bliss and Lomo, 1973).

A long-lasting increase in synaptic efficacy long-term synaptic potentiation (LTP) can be produced in slices using either:

1) High-frequency stimulation (HFS) of presynaptic afferents (Bliss and Lomo, 1973).
2) By pairing presynaptic stimulation with postsynaptic depolarization (Markram et al., 1997).

A long-lasting decrease in the strength of synaptic transmission, long-term synaptic depression (LTD), is produced by low- frequency stimulation (LFS) of presynaptic afferents. The typical protocol for inducing LTD involves prolonged repetitive synaptic stimulation at 0.5–5 Hz. A robust change usually requires many stimuli (e.g., 900) (Dudek and Bear, 1992).

Prior to reviewing the putative molecular mediators of memory, it is important to consider certain lacunae of our current understanding of LTP/LTD.

1) The terms Long, Potentiation and Depression are rather non-committal - and have remained so for good reasons; several different flavors of LTP with distinct properties have been identified, and it increasingly accepted that LTP is not a unitary phenomena but a process that may vary in the details from one cell type to the next, one region of the brain to the next and with development. Even at a single synapse, LTP produced by different patterns of stimulation may not be the same (Kang et al., 1997).

2) The cellular locus of LTP/LTD is still under debate. Although this might seem like the first question that needed to be answered, there is a glut of evidence for each of the PreSynaptic, PostSynaptic and Both camps.

3) It has been difficult to separate the mediators necessary for these processes from the modulators that influence it.

4) LTP and LTP are experimental phenomena- experiential modification of synapses to encode memory are very likely to be mechanistically similar but not identical to these experimental phenomena.

Two mechanistically distinct forms of LTP have been reported:

-NMDAR dependent LTP: NMDAR- dependent form of LTP requires synaptic activation of NMDARs during postsynaptic depolarization, with influx of Ca2+ through the NMDAR channel and a rise in Ca2+ activating calcium/calmodulin-dependent protein kinase II (CaMKII) within the dendritic spine being the initial step. The subsequent intra-cellular cascade bridging LTP induction and expression is complex, and may have several distinct pathways. NMDAR dependent LTP expression however mostly requires increasing the number of AMPARs in the plasma membrane at synapses via activity-dependent changes in AMPAR trafficking (Bredt and Nicoll, 2003) (Malinow and Malenka, 2002) (Song and Huganir, 2002) and modification of the biophysical properties of AMPARs themselves via their direct phosphorylation (Benke et al., 1998) (Lee et al., 2003) (Malenka and Nicoll, 1999).

-Mossy Fiber LTP: This mechanistically distinct form of LTP coexists in the hippocampus at the synapses between the axons of dentate gyrus granule cells (i.e. mossy fibers) and the proximal apical dendrites of CA3 pyramidal cells (Nicoll and Malenka, 1995). Mechanistically similar forms of LTP have also been

observed at corticothalamic synapses (Castro-Alamancos and Calcagnotto, 1999) and cerebellar parallel fiber synapses (Salin et al., 1996). Mossy fiber LTP appears to involve a PKA-dependent, long-lasting modulation of the presynaptic release machinery leading to an increased probability of transmitter release. Like NMDAR-dependent LTP, it is likely mossy fiber LTP plays multiple functional roles and is known to be dendritic protein synthesis dependent. Unlike NMDAR-dependent LTP, the triggering of mossy fiber LTP does not require activation of NMDARs (Harris and Cotman, 1986).

Three mechanistically distinct forms of long-term depression (LTD) have been reported.

-NMDAR dependent LTD requires protein phosphatase activity. NMDA receptor subtypes (the NR2B subtype) and the rate and size of the calcium influx may mediate the induction of LTD over NMDAR dependent LTP, with subsequent dephosphorylation of postsynaptic PKC and PKA substrates serving as part of the postsynaptic cascade. Ultimately, internalization and dephosphorylation of AMPAR is required for the expression of NMDAR dependent LTD.

-mGluR dependent LTD : this form of LTD is best characterized in the cerebellar parallel fiber to Purkinje cell synapse, when co-stimulated with climbing fiber input. The evidence points to postsynaptic Group 1 mGluR activation leading to endocytosis of AMPARS through PKC mediated phosphorylation (Linden et al, 1991) (Wang and Linden, 2000).

eCB dependent LTD - this form of LTD is expressed widely- in the cortex (Sjostrom et al.,2003), striatum (Gerdeman et al., 2002) and hippocampus (Chevaleyre and Castillo, 2003). eCB dependent LTD requires postsynaptic

release of endo-cannabinoids acting on presynaptic CB1 receptors. The conditions for postsynaptic release of endo-cannabinoids appear to be different in the striatum (requiring post synaptic mGluR activation (Sung et al., 2001)) and in the cortex (requiring presynaptic NMDAR activation (Sjostrom et al., 2003)).

1.1.3 Human cortical plasticity

The study of neuroplasticity in awake human cortex has a shorter history, starting after the development of transcranial magnetic stimulation (Barker et al., 1985). Principles and protocols from the study of LTP and LTD in slices have informed and inspired the development of several TMS paradigms that enable us to probe neuroplasticity in the awake human cortex and this is discussed in the following chapter.

1.2 Transcranial Magnetic Stimulation

1.2.1 Principles

TMS (Barker et al., 1985) utilizes the principle of electromagnetic induction; by passing a rapidly varying electrical current through a coil of wound copper wire placed against the scalp, a rapidly varying magnetic field is produced, which can painlessly cross the scalp to induce a focal electrical current on the surface of the brain. Over the motor cortex, this focal electrical field elicits a Motor Evoked Potential (MEP) by depolarizing pyramidal neurons, and this MEP can be recorded using a EMG montage over a peripheral muscle.

Transcranial magnetic stimulation, when delivered as a single stimulus, produces a synchronous discharge of cortical interneurons and cortical pyramidal neurons. The result of this can only be easily detected if the stimulus is located in the primary motor cortex (M1) or the primary visual cortex (V1). When placed over the primary motor cortex, the discharges travel down the corticospinal tract and epidural electrodes in the cervical cord can detect several waves termed I-waves.

An interesting property of I-waves is that they occur at a fairly regular ~1.5-ms interval apart and it is unclear if the different I-waves (I1, I2, I3, and so forth) represent distinct populations of excitatory interneurons or the repetitive discharges of the same population of excitatory interneurons. A valuable

property of all these interneurons is that they have different thresholds of activation: inhibitory interneurons have the lowest activation threshold, followed by excitatory interneurons, then the cortical pyramidal neurons (Ilic et al., 2002). The activation threshold also changes depending on the activity of the underlying cortex; for example, excitatory interneurons have a lower threshold when the primary motor cortex is actively performing a task. This allows for experimental parameters to be manipulated so that a TMS pulse depolarizes only subsets of cortical neurons. The activation threshold (resting motor threshold, RMT) is increased by membrane-stabilizing drugs like lamotrigine but unaffected by GABA-ergic drugs (Ziemann et al., 1996a; Ziemann et al., 1996b), suggesting it represents resting membrane excitability of the pyramidal neurons.

Apart from the ubiquitous motor-evoked potential (MEP) measurement, paired-pulse TMS paradigms provide another avenue of measuring cortical physiology. In these paradigms, a conditioning stimulus modulates the amplitude of the MEP of a subsequent stimulus. By varying the interstimulus interval, the intensity of the conditioning stimulus, and even the type of the stimulus, various intracortical and corticocortical circuits can be studied. The most established measure is the short-interval intracortical inhibition (SICI) occurring when a subthreshold conditioning stimulus is delivered 1 to 5 ms before the test stimulus, resulting in inhibition of the MEP amplitude. SICI is mediated by GABA through GABAA receptors ((Ziemann et al., 1996b);(Di Lazzaro et al., 2006)) and appears to regulate plasticity (Teo et al., 2009). Other measures include intracortical facilitation (ICF), long-interval intracortical inhibition (LICI), short-interval afferent inhibition (SAI), long-interval afferent inhibition (LAI), and

interhemispheric inhibition (IHI) and appear to be modulated by different neurotransmitter systems and may perform different cortical functions.

1.2.2 Practice dependent plasticity

Normal human behavior is also associated with changes that mirror modifications produced by artificially induced plasticity: repeated motor practice increases MEP amplitude (Lotze et al., 2003) and alters motor representation in the primary motor cortex (Classen et al., 1998). This mirroring allows the study of the link between plasticity and motor learning with parallel experiments undertaking behavioral measurements and/ or physiological measurements (e.g., TMS measurements) before and after an intervention, which may be physiologically induced (e.g., practice).

1.2.3 Artificially Induced plasticity

When TMS is delivered repetitively (i.e., repetitive TMS or rTMS), an effect is produced on that outlasts the period of stimulation, and this can be harnessed to artificially induce cortical changes that are in many ways similar to those after motor practice.

The standard protocol for inducing depotentiation in animals uses long trains of low frequency stimulation (1 to 2 Hz for several minutes) whereas LTP is often induced using brief and repeated trains of high frequency stimulation (>50 Hz).

Such synaptic phenomena are probably reproducible in humans, because it is possible to deliver repetitive TMS (rTMS) of various frequencies. When TMS is delivered repetitively (i.e., rTMS), an effect is produced that outlasts the period of stimulation.

1.2.4 Paradigms for Artificially Induced plasticity

High frequency stimulation produces an increase in MEP amplitude, but low frequency stimulation produces a decrease in MEP amplitude, reflecting long-lasting changes in the excitability of the primary motor cortex (Pascual-Leone et al., 1998). rTMS protocols using patterned stimulation have been developed more recently: theta burst stimulation (continuous TBS, intermittent TBS) (Huang et al., 2005), I-wave interval rTMS (iTMS) (Cash et al., 2009), and quadripulse rTMS (QPS) (Hamada and Ugawa, 2010). These patterned forms of stimulation use principles from tissue models and Hebbian models of plasticity so that much lower intensities or shorter periods of stimulation are necessary to produce the change in MEP amplitude.

Theta Burst Stimulation (TBS)

In 1988, Hess and Donoghue showed that electrical stimulation patterns based on the pattern of theta burst firing seen in the hippocampus of rats during exploratory behavior, were very effective in producing LTP. This pattern of high frequency bursts of neuronal firing occurring at 4-6Hz (the theta frequency in

EEG terminology), is thought to represent the physiological pattern of synaptic plasticity.

Designed to mimic this physiological activity in hippocampal cells, Human Theta Burst Stimulation (Huang et al., 2005) consists of repeating bursts of stimuli. Each burst consists of 3 stimuli (at low intensity) repeating at 50 Hz; the bursts are repeated at 5 Hz. The nature of the after-effects differs according to the stimulation pattern. A continuous train of 100 bursts (300 stimuli), named cTBS, when given over the primary motor cortex (M1) produces a NMDAR dependent LTD-like effect, suppressing corticospinal excitability for several minutes afterward. An intermittent pattern (iTBS), delivering a 2 second train repeated every 10 seconds for 20 repetitions (600 stimuli), produces a NMDAR dependent LTP-like effect, enhancing corticospinal excitability for several minutes afterward (Huang et al., 2007). Continuous theta-burst produces this decrease in the excitability of cortical circuits at the level of the cortex, and may act preferentially on interneurons producing the I1 wave (Di Lazzaro et al., 2005).

However, if subjects actively contracted during TBS, then effects of TBS were abolished (Huang et al., 2008). Contraction immediately after TBS enhanced the facilitatory effect of iTBS and reversed the inhibitory effect of cTBS into facilitation (Huang et al., 2008). Gentner et al. investigated these phenomena further, and showed that if the stimulation intensity was calculated using RMT rather than AMT, and thereby omitting the period of contraction (for experienced investigators 1-1.5 minutes) required for the assessment of AMT, the effect of cTBS on MEP amplitude was excitatory (Gentner et al., 2008b). The authors concluded that the inhibitory effects of cTBS shown by Huang et al. was a

metaplastic phenomenon, In many ways, these findings actually show that TBS induced changes in the brain are mediated at the synapse, by similar rules to that observed in experimental slices.

Paired- Associative Stimulation (PAS)

The pairing presynaptic stimulation with postsynaptic depolarization (Markram et al., 1997) to produce LTP in slices has inspired another type of artificially induced plasticity paradigm- paired- associative stimulation (PAS). PAS repeatedly pairs an afferent somatosensory input (an electrical stimulus in the periphery) with a TMS stimulus over the primary motor cortex at a specific time interval (Stefan et al., 2000). This induction protocol demonstrates Hebbian like spike-timing specificity with increases in MEP amplitude when the afferent pulse arrives in the primary motor cortex at the moment that the TMS pulse is delivered(Stefan et al., 2000). The peripheral stimulus is given approximately at the N20 latency of the somatosensory evoked potential (e.g. ISI of approximately 25 ms) to achieve this. MEP amplitude decreases when the afferent pulse arrives in the primary motor cortex before the TMS pulse is delivered (when shorter interstimulus intervals (e.g. ISI of approximately 10 ms) were used) (Wolters et al., 2003). Plasticity induced by paired stimulation evolved rapidly (within 30 min), was persistent (duration up to 60 min reported) and was topographically specific. *N*-methyl-d-aspartate (NMDA) receptor antagonist has been shown to block PAS-induced plastic changes (Stefan et al., 2002). This appears to be analogous to tissue models of spike-timing dependent plasticity.

However, the PAS protocol defined by Stefan et al., applied 90 paired stimuli, at 0.05 Hz over 30 minutes. This duration of significantly reduced the convenience of this protocol. Quartarone et al. (Quartarone et al., 2006a) showed that sub-motor threshold 5 Hz rENS of the right median nerve synchronized with sub-motor threshold 5 Hz rTMS of the left M1 at a constant interval for 2 min ('rapid rate PAS') could produce similar results. Most experimental protocols using PAS apply a rate of 0.25 Hz, which producing effects indistinguishable from the original paradigm. Motor learning, which alone results in an increase in MEP amplitude, prevents the subsequent induction of associative LTP- like plasticity with PAS (ISI N20) but enhanced LTD-like plasticity PAS (ISI<N20) at this rate (Ziemann et al., 2004). This implies that, like similar experiments in cortical slices, motor practice saturates synaptic connections and occludes subsequent effects of excitatory PAS at the synapse. The effects of PAS are reported to be affected by age of subject (Tecchio et al., 2008), attention to stimulation (Stefan et al., 2004) and sedentary lifestyle (Cirillo et al., 2009). Also, while PAS certainly involves long-term potentiation (LTP)-like mechanism in cortical synapses, changes in spinal excitability after PAS have been reported (Meunier et al., 2007), suggestive of parallel modifications in both cortical and spinal excitability.

Transcranial direct current stimulation (tDCS)

Transcranial direct current stimulation (tDCS) presents a different method to other rTMS paradigms (Nitsche and Paulus, 2000); TDCS involves continuous administration of weak currents of 1 mA through a pair of surface electrodes attached to the scalp. TDCS polarity refers to the electrode placed over the area

targeted for stimulation. For anodal TDCS, the anode was placed over the target area, whereas the cathode was over the target area during cathodal TDCS. The weak depolarizing current of anodal tDCS may shift the resting membrane potential of postsynaptic neurons such that postsynaptic neurons require less synaptic inputs to produce an action potential, thereby biasing the induction of LTP. The converse applies with the hyperpolarizing current of cathodal tDCS. Mechanistically this appears to be different from other artificial induction protocols. The weak currents simply bias the firing rates of neurons rather than directly inducing action potentials. However, the net result on cortical excitability appears superficially similar, with anodal tDCS produces enhancement of MEPs and cathodal tDCS producing inhibition of MEPs. Anodal stimulation of the primary motor cortex resulted in improved performance of a serial reaction time task (Nitsche et al., 2003b). Like other protocols, the after-effects may be NMDA receptor dependent (Nitsche et al., 2003a). However, the extensive spatial and temporal effects of tDCS need to be taken into account when tDCS is used to modify brain function (Lang et al., 2005).

In an elegant experiment, Siebner et al. interleaved repetitive transcranial magnetic stimulation (rTMS) with transcranial direct current stimulation (TDCS) to probe homeostatic plasticity in the motor cortex (Siebner et al., 2004). Excitatory preconditioning with anodal TDCS caused a subsequent period of 1 Hz rTMS (which on its own would produce inhibition of MEP amplitude) to reduce corticospinal excitability to below baseline levels. Conversely, inhibitory preconditioning with cathodal TDCS resulted in 1 Hz rTMS increasing corticospinal excitability.

1.2.5 Physiology and pharmacology of rTMS.

Changes in MEP amplitude may reflect either change in membrane excitability of either pyramidal or excitatory interneurons or change in the synaptic efficacy between neurons.

Any change in MEP is likely to reflect a process upstream from the pyramidal neurons, because the stimulation intensity of newer rTMS induction protocols is below the activation threshold of the cortical pyramidal neuron and rTMS pulses have only sufficient intensity to directly depolarize inhibitory interneurons and excitatory interneurons. This is supported by epidural recordings performed by Di Lazzaro and colleagues inasmuch as artificial induction with rTMS, I-wave rTMS, theta burst rTMS, tDCS, and PAS produce changes in the I-waves (Di Lazzaro et al., 2002) (Di Lazzaro et al., 2005) (Di Lazzaro et al., 2006b) (Di Lazzaro et al., 2006a) (Di Lazzaro et al., 2007) (Di Lazzaro et al., 2008) (Di Lazzaro et al., 2009) with some preferential effect on later I-waves (although continuous theta burst rTMS preferentially affected the early I-waves).

Some artificially induced plasticity paradigms also affect inhibitory interneurons that synapse onto I-wave interneurons (Peinemann et al., 2000), and the effects of some plasticity paradigms appear to be specific to certain populations of inhibitory interneurons (McAllister et al., 2009) (Russmann et al., 2009). The mechanism of this enhanced inhibitory activity remains unclear: it may be related to increased membrane excitability of these inhibitory interneurons or

changes in the GABA-ergic synaptic efficacy. Inhibitory interneurons also play a role in regulating the expression of artificially induced plasticity; enhancing the activity of GABA-ergic interneurons involved in SICI limit expression of artificially induced plasticity (Teo et al., 2009) , but decreasing the activity of GABA-ergic interneurons by peripheral de- afferentiation enhances the expression of artificially induced plasticity (Ziemann et al., 1998b).

Table 1-1: Comparison of Artificially Induced Plasticity with LTP/LTD

Similarities with LTP/ LTD		**Reference**
1)	Blocking NMDA receptors blocks changes in MEP amplitude (NMDA-dependent)	(Liebetanz and others 2002a; Stefan and others 2002a; Ziemann and others 2001)
1)	Repetitive transcranial magnetic stimulation increases or decreases MEP amplitude depending on frequency of stimulation (frequency-dependent)	(Pascual-Leone and others 1994; Chen and others 1997)
1)	Precise timing of stimuli can produce changes in MEP amplitude in paired-associative stimulation (spike-timing dependent)	(Wolters and others 2003)
1)	The changes in MEP amplitude have a degree of somatotopy in paired-associative stimulation (Hebbian plasticity)	(Stefan and others 2000)
1)	Consecutive sessions of PAS produces an effect similar to the BCM rule and metaplasticity	(Muller and others 2007)
1)	The effect of BDNF polymorphisms in human plasticity	(Cheeran and others 2008)
Differences with LTP/ LTD		
1)	The changes in MEP in some induction protocols do not always occur immediately after induction	
1)	High degree of inter-subject and intra-subject variability	
1)	Changes in the excitability of corticospinal neurons, rather than just the synaptic efficacy of excitatory interneurons synapsing onto corticospinal neurons, can also produce changes in MEP amplitude.	

The most convincing evidence that rTMS and all other artificial protocols involve long-term synaptic plasticity is that certain drugs block the rTMS effects. This

implies that rTMS artificially induced plasticity is dependent on specific neurotransmitters. For instance, the glutamatergic NMDA antagonist dextromethorphan (Ziemann et al., 1998a) and GABA-receptor agonists are both able to alter cortical plasticity induced by high frequency rTMS (Ziemann et al., 1996b). In addition, NMDA partial agonist D-cycloserine modulates the effects of theta burst rTMS and tDCS (Nitsche et al., 2004) (Teo et al., 2007). Thus although changes in membrane excitability may be a factor in artificially induced plasticity, the role of NMDA receptors suggests a localization of the effect to the synapse. Other characteristics point at LTP/LTD as being the mechanism by which artificially induced plasticity occurs (Table 1). The caveats should also be noted, however, because there is no direct way of confirming that the molecular biology so well described in animal models of synaptic plasticity hold true for human models of artificially induced neuroplasticity.

Table 1-2: Effects of Interventional Neurostimulation Paradigms on Neurotransmitter Systems

Table 2. Effects of Interventional Neurostimulation Paradigms on Neurotransmitter Systems

Neurotransmitter system	Drug	Plasticity in humans							
		rTMS	TBS		tDCS		PAS		Practice
			iTBS	cTBS	Cathodal	Anodal	Excitatory	Inhibitory	
Noradrenergic (NA) system	Combined noradrenergic stimulation (amphetamine or methylphenidate)	Unknown	Unknown	Unknown	Prolongs[1]	Strongly prolongs[1]	Unknown	Unknown	Enhanced[2]
	β-adrenoceptor antagonist (propanolol)	Unknown	Unknown	Unknown	Shortens duration[1]	Shortens duration[1]	Unknown	Unknown	Unknown
	A1-adrenoceptor antagonist (prazosin)	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Blocked[2]
Dopaminergic (DA) system	Dopamine precursor (levodopa)	Unknown	Unknown	Unknown	Prolongs inhibitory effect[3]	Changes excitation to inhibition[3]	Prolongs the effect[3]	Unknown	Unknown
	D1 and D2 agonist (pergolide or cabergoline)	Increases inhibition[4]	Unknown	Unknown	Inhibition from D2 antagonism not restored[5]	Inhibition from D2 antagonism not restored[5]	Unknown	Unknown	Enhanced and prolonged[2]
	D2 agonist (ropinirole)	Unknown	Unknown (results in press)	Unknown (results in press)	Inhibition at low or high doses[4]	Inhibition at low or high doses[4]	Inhibition at low or high doses[4]	No effect[6]	Unknown
	D2 receptor antagonists (sulpiride or haloperidol)	Unknown	Unknown	Unknown	Abolished inhibitory effect[5]	Abolished excitatory effect[5]	No effect	Abolished[7]	Blocked[2]
Cholinergic (Ach) system	Acetylcholinesterase inhibitor (rivastigmine or tacrine)	Unknown	Unknown	Unknown	No effect[8]	Abolished the excitatory effect[8]	Enhanced[8]	Prolonged[8]	Enhanced and prolonged[2]
	Muscarinic receptor antagonist (bupireden)	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Blocked[2]
Glucocorticoid system	Hydrocortisone (during cortisol circadian trough)	Unknown	Unknown	Unknown	Unknown	Unknown	Abolished[9]	Unknown	Unknown
Cannabinoid system	Unknown active agent	Unknown	No effect	Reverses inhibition[10]	Unknown	Unknown	Unknown	Unknown	Unknown
Glutamatergic system	NMDA receptor antagonist (dextromethorphan)	Blocked[11]	Blocked[12]	Blocked[12]	Abolished inhibitory effect[13,14]	Abolished excitatory effect[13,14]	Abolished[15]	Abolished[15]	Blocked[11]
	NMDA receptor partial agonist (d-cycloserine)	Unknown	Unknown	Excitation to inhibition[16]	No effect[17]	Prolonged the effect[17]	Unknown	Unknown	Unknown
GABA-ergic system	GABA-A receptor agonist (lorazepam or diazepam)	Blocked[18]	Unknown	Unknown	No effect[19]	Delayed excitation[19]	Unknown	Unknown	Blocked[18,20]
	GABA-B receptor agonist (baclofen)	Unknown	Unknown	Unknown	Unknown	Unknown	Abolished[21]	Unknown	Unknown

NMDA = N-methyl-D-aspartic acid; GABA = gamma-amino butyric acid; D1/2 = Dopamine receptor ½; tDCS = transcranial direct current stimulation; iTBS = intermittent theta burst stimulation; cTBS = continuous theta burst stimulation; PAS = paired associative stimulation.

Table references: 1, Nitsche and others 2004a; 2, Meintzschel and Ziemann 2006; 3, Kuo and others 2008; 4, Lang and others 2008; 5, Nitsche and others 2006; 6, Monte-Silva and others 2009; 7, Nitsche and others 2009; 8, Kuo and others 2007; 9, Sale and others 2008; 10, Koch and others 2009; 11, Ziemann and others 1998a; 12, Huang and others 2007; 13, Liebetanz and others 2002; 14, Nitsche and others 2003; 15, Stefan and others 2002; 16, Teo and others 2007; 17, Nitsche and others 2004b; 18, Ziemann and others 1998b; 19, Nitsche and others 2004c; 20, Teo and others 2009; 21, McDonnell and others 2007.

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Pharmacological modulation can be one such route, and painstaking pharmacological experiments have shown that artificially induced neuroplasticity protocols are differentially modulated by neuromodulator systems including dopaminergic, cholinergic, and nor-adrenergic systems just like in animal models of synaptic plasticity. This variance in their effect on different neurotransmitter systems (Table 1-2) probably reflects that the different induction protocols may be affecting different populations of interneurons and synapses, although the dependency of human neuroplasticity on the NMDA receptor and the dopaminergic system appears to be largely consistent across paradigms (Table 1-3) (Cheeran et al., 2010).

Table 1-3: Summary of Effects of Interventional Neurostimulation Paradigms on Neurotransmitter Systems from Pharmacological Studies

Type of plasticity		NMDA receptor	GABA-ergic system	Dopaminergic system	Noradrenergic system	Cholinergic system
Classical rTMS		NMDA-dependent	Modulation	Not established	Not established	Not established
Theta burst rTMS	***iTBS***	NMDA-dependent	Not established	Not established	Not established	Not established
	cTBS	NMDA-dependent	Not established	Not established	Not established	Not established
tDCS	***Cathodal***	NMDA-dependent	None	Dependent	Modulation	Modulation
	Anodal	NMDA-dependent	Modulation	Dependent	Modulation	Modulation
Paired associative stimulation	***Excitatory***	NMDA-dependent	Not established	Modulation	Not established	Modulation
	Inhibitory	NMDA-dependent	Not established	Dependent	Not established	Modulation
Practice-dependent plasticity		NMDA-dependent	Modulation	Modulation	Modulation	Modulation

Another route for understanding the molecular pathways is likely to come from magnetic resonance spectroscopy (MRS), a noninvasive imaging technique that allows accurate quantification of a number of neurochemicals. The results from MRS studies give a quantification of the total amount of that neurochemical within the selected volume of interest and are usually presented as a ratio of the amplitude of the peak of interest to the amplitude of a reference peak that is not expected to change during stimulation. Presenting the results as a ratio

prevents false positive results because of changes in cell volume within the voxel, for example because of edema.

A study using MRS to assess the physiological basis of TBS showed that inhibitory (continuous) TBS over the motor cortex leads to a significant increase in [GABA] within the stimulated area (Stagg et al., 2009b). It is not possible to definitively identify in which GABA pool this increase occurs, but previous studies have linked similar changes in [GABA] to changes in the rate of production of GABA within the presynaptic vesicles by GAD, the only synthetic enzyme for GABA in the human cortex (Floyer-Lea et al., 2006). GAD is the rate-limiting step for GABA metabolism within the presynaptic vesicles and its activity is dependent on neuronal activity (Patel et al., 2006).

That a change in glutamate was not seen after cTBS in conjunction with this GABA increase most likely reflects the differing sensitivities of MRS and TMS as techniques. MRS is not sensitive to changes in receptor density or strength, but only in neurotransmitter concentration. That a change in [GABA] was identified presumably reflects the fact that LTD-like plasticity within the GABA-ergic interneurons is primarily dependent on presynaptic neuronal changes (Tsumoto, 1990).

However, another inhibitory transcranial stimulation paradigm, cathodal tDCS, which exerts very similar effects on cortical excitability as cTBS, has very different effects on cortical neurotransmitters (Stagg et al., 2009a). This

suggests that different stimulation paradigms differentially stimulate different interneuronal pools, with very similar neurophysiological outcomes.

Although there are apparent contradictions in the results of MRS and TMS studies, these can be explained by the different sensitivities of the two techniques. As discussed above, MRS allows accurate quantification of the concentration of neurotransmitters primarily within presynaptic neurons, but gives no information concerning postsynaptic receptors. Conversely, TMS is sensitive to phasic changes at the synaptic receptors.

1.3 Stimulation genomics

The corticospinal motor system controls distal limb movements requiring a high degree of skill and flexibility. Genetic factors can impact on the structure and function of the corticospinal system in three different ways:

(i) Development: The influence of genes on the structure and function of corticospinal projections to the hand is strongest during development. Recent research has unraveled a machinery of regulatory genes that orchestrate the differentiation and connectivity of corticomotor projections (Dasen et al., 2005) (Kramer et al., 2006) (Dalla Torre di Sanguinetto et al., 2008). The regional expression of the relevant gene products critically depends on movement-related activity within the maturing corticospinal circuits. This explains why motor experience and genetic factors critically interact during the maturation of the corticospinal system and the differentiation of motor representations subserving skilled limb movements (Martin et al., 2007).

(ii) Neurodegeneration and aging: mutations in specific genes may trigger a progressive neurodegeneration of upper motor neurons in rare cases of familial amyotrophic lateral sclerosis (ALS) or in patients with hereditary spastic paraplegia (HSP). Genes that code for neurotrophic factors may also influence the potential of motor neurons to survive during normal aging (Sendtner et al., 1994).

(iii) Learning and skill acquisition: common variations in our genetic makeup may also influence function and structure of the corticospinal system beyond

maturation and aging. Even after its maturation, the corticospinal system has a substantial potential to undergo functional reorganization (Nudo, 2006) (Brown et al., 2007). This plasticity enables the acquisition and refinement of manual skills and mediates functional recovery in response to a focal or diffuse lesion. Neuronal substrates of reorganization include lasting changes in the efficacy of synaptic neurotransmission and formation of new synapses in cortical or spinal motor circuits, leading to a reconfiguration of cortical motor representations (Nudo et al., 1996).

1.3.1 Genes and plasticity

Mechanisms of synaptic plasticity have been studied extensively in the hippocampus, and have provided a good deal of insight into the complexity of the processes involved in long term potentiation and depression (LTP/LTD) of synaptic transmission. These involve changes in pre- synaptic release of transmitter, expression of postsynaptic receptors and even synaptic growth that occur over a variety of different time ranges from seconds to months or years. Sanes and Lichtman (Sanes and Lichtman, 1999) pointed out that 121 molecules had been implicated in LTP and LTD. Their general areas of action are summarized in Table 1. Clearly, there is ample scope in principle for natural variations in the genetics of these processes to influence not only learning in health but also in recovery from damage. A number of novel approaches have produced results that are consistent with this idea and allow us for the first time

to open a window into the role of functional variations in genes coding for the molecular building blocks of neuroplasticity on motor function in humans.

Table 1-4: Molecular mediators of motor plasticity

Intercellular factors
Neurotransmitters, Neuromodulators and their receptors (glutamate, NMDAR, mGluR, dopamine and D1 receptor, GABA and GABA-B receptor, 5-HT and 5HT3 receptor).
Neurotrophins and their receptors (NGF, FGF, BDNF, GDNF, NT3, TrK A-C, p75NTR)
Nitric oxide
Intracellular factors
Transcription factors (CREB)
Ca regulators (calmodulin, calretinin, CaMK)
Structural proteins
Synaptic vesicle associated proteins
Synapse associated proteins and adhesion molecules
Kinases and proteases
ERK, MAPK, CaMK, tPA, plasmin

1.3.2 Application TMS techniques to study the effects of genetic mutations.

Patients with specific genetic mutations and different diseases may present with abnormal patterns of cortical excitability when tested with artificially induced plasticity protocols.

Fedi and others (Fedi et al., 2008) have utilized paired-pulse paradigms to study the effects of a missense mutation in the GABAA receptor, linked to inherited human generalized epilepsy, on intracortical excitability. Subjects affected by the GABARG2(R43Q) mutation showed reduced short-interval intracortical

inhibition (SICI) compared to controls, but there was no effect of the mutation on RMT. These findings are as would be predicted from the neurochemical basis of these probes, SICI being GABAA mediated whereas RMT is mediated largely by voltage- gated Na+ and Ca2+ channels. The study is able to provide a physiological basis for the increased epileptogenic potential in carriers of this mutation.

Similarly, Turner and others (Turner et al., 2005) utilized the ability of paired-pulse techniques to assess GABAergic function in patients with the D90A SOD1 mutation. Patients with this genetic mutation develop motor neuron disease (MND), but have a prolonged survival compared to patients with sporadic MND. Patients with sporadic MND have an increased cortical excitability compared to patients with the D90A SOD1 mutation. It is unclear if the increased cortical excitability may contribute to the excitotoxicity or the reduced survival in sporadic MND.

1.3.3 Application of TMS techniques to study the effects of genetic mutations on neuroplasticity.

A useful disease model to study neuroplasticity is dystonia; dystonia is a neurological movement disorder characterized by sustained involuntary muscle contraction causing twisting and repetitive movements or abnormal postures (Marsden and Sheehy, 1990). Neuroplasticity may be an underlying mechanism for this disorder inasmuch as adult-onset dystonias are often associated with excessive practice of particular movements in humans (task-specific dystonias)

and that dystonia can occur or worsen following trauma to the affected body part (e.g., (Jankovic, 2001)); this leads to the emerging consensus that excessive ability to undergo plastic change could drive the development of dystonia (Quartarone et al., 2006b).

DYT1 dystonia, caused by a mutation (a single GAG deletion) in the DYT1 gene on chromosome 9q34, is the commonest cause of young-onset primary generalized dystonia. Only 30% of carriers of the DYT1 mutation develop dystonia; 70% never develop symptoms. Edwards and colleagues demonstrated that the responsiveness to the artificially induced neuroplasticity seems to be associated with whether or not patients with the DYT1 gene mutation develop clinical symptoms (Edwards et al., 2006).
DYT1 gene carriers who have symptoms have a significantly longer response to artificially induced plasticity compared to normal subjects with MEPs still maximally suppressed at 35 min after conditioning. However, DYT1 gene carriers who have not developed the disease showed no significant change in MEP size at all at any time point after conditioning, significantly different in this respect from both normal and dystonic subjects.

This study showed two key points: 1) patients with DYT1 gene and who expressed the disease were excessively responsive to artificially induced plasticity; and 2) asymptomatic DYT1 gene carriers appeared resistant to plastic changes. The authors of this study speculated that this resistance protects them against the development of clinical symptoms. Thus, susceptibility to artificially induced plasticity reflects an “endophenotype” of dystonia. The molecular

mechanisms of this resistance is unknown, although in light of the emerging knowledge of the neurochemical consequences of cTBS, one would need to question whether the changes observed are part of the pathology or were in fact the result of adaptive compensatory changes.

1.3.4 Stimulation genomics

We defined the field as the study of the effects of human genetic variation on cortical plasticity, by employing neurophysiological outcome measures and the application of artificially induced plasticity paradigms.

Stimulation genomics focuses on the effects of common genetic variations on cortical plasticity in healthy human volunteers, providing clues as to their potential influence on subsequent brain disease.

1.3.5 Candidate gene selection

This approach involves assessing the association between a particular allele (or set of alleles) of a gene that may be involved in the disease or outcome (i.e., a candidate gene) and the disease or outcome itself. Candidate gene studies are better suited for detecting genes underlying common and more complex diseases where the risk associated with any given candidate gene is relatively small (Risch and Merikangas, 1996).

This approach requires:

- Plausible role for candidate gene - the candidate gene selected must have a predefined role in the process being investigated; i.e. the physiological or pathophysiological role of the candidate gene must be known and the candidate gene must be influenced by the selected intervention(s).

- A suitable SNP - the candidate gene must have identified genetic variants, ideally with previously known functional role, and where the minor allele is common enough to be found in sufficient numbers - a rare allele may require impractically large cohorts.

Polymorphisms affecting the dopaminergic system (in DAT, COMT and Dopamine Receptors) would perhaps be the obvious targets for a pilot study, as repetitive transcranial magnetic stimulation (rTMS) of the human prefrontal cortex leads to striatal dopamine release. However, our attention was drawn to Brain Derived Neurotrophic Factor (BDNF), stored and released (like Dopamine) from Large Dense Core Vesicles. Chronic rTMS (like chronic ECT) (Bocchio-Chiavetto et al., 2006) has been reported to increase serum BDNF levels in patients with depression (Zanardini et al., 2006), and animal studies of chronic rTMS show an up-regulation of BDNF mRNA (Muller et al., 2000).

1.4 Brain Derived Neurotrophic Factor

1.4.1 Gene

Maisonpierre et al. localized the BDNF gene to 11p13 (Maisonpierre et al., 1991). Translation of BDNF is governed by complex regulatory mechanisms, to accommodate its varied and at times contrarian roles. Transcription of BDNF initiates from multiple promoters in response to distinct stimulation cues, with evidence of epigenetic influences on transcription (hippocampal epigenetic modification at the brain-derived neurotrophic factor gene induced by an enriched environment). The human BDNF gene, extending over 70 kb, contains 11 exons and nine functional promoters, leading to a number of transcript variants. Particular transcript variants may predominate in particular brain areas. Human BDNF contains two more exons than rodent BDNF. Another variation in the regulation of BDNF in rodents compared to humans is that noncoding antisense RNAs are transcribed from the human BDNF gene locus (Pruunsild et al., 2007). These antiBDNF transcripts could have an important role in the regulation of BDNF expression in human.

1.4.2 Molecular biology.

During development, BDNF protein expression is more abundant in the nervous system compared to other tissues and its levels are dramatically increased in the brain during postnatal development (Katoh-Semba et al., 1997). In the adult

nervous system, BDNF displays a widespread distribution pattern, with the highest levels of mRNA and protein in the hippocampus, amygdala, cerebral cortex, and hypothalamus (Ernfors et al., 1990).

BDNF was initially thought to be responsible for neuronal proliferation, differentiation, and survival (Ip et al., 1993) through its uptake at nerve terminals and retrograde transport to the cell body. A more diverse role for BDNF emerged progressively from observations showing that it is also transported anterograde, is released upon activity dependent neuron depolarization, and triggers rapid intracellular signals in central neurons (Lohof et al., 1993) (Kang and Schuman, 1995) (Stoop and Poo, 1996).

While most neurotrophins are secreted constitutively, BDNF secretion at central synapses is activity dependent (Castren et al., 1992) (Lu, 2003), possibly by virtue of a 'sorting motif' in the 'pro' region of its precursor molecule ProBDNF. This 'sorting motif' allows binding with sortilin, a protein that packages ProBDNF into Large Dense Core Vesicles for activity dependent secretion. In response to neuronal depolarisation, BDNF is released, predominantly as ProBDNF into the synaptic cleft. Cleavage of the ProBDNF into BDNF relies on extracellular proteases like Tissue Plasminogen Activator/ Plasmin and metalloproteases. The co-release of proteases and proBDNF is critical to the effects on synaptic connectivity. The emerging molecular biology of BDNF suggests that the intra-synaptic ratio of BDNF to its secreted precursor form ProBDNF may influence the relative ease of producing increases or decreases in synaptic efficacy (Lu et al., 2005). Transcript variants can cause variations in the precursor to ProBDNF

(PreProBDNF), but it is not known if this impacts the functions of the ProBDNF 'sorting motif'.

Mature BDNF binds to Trk (neurotrophin) receptors (Lohof et al., 1993), single transmembrane catalytic receptors with intracellular tyrosine kinase activity, on the postsynaptic membrane. There are four members of the Trk family; TrkA, TrkB and TrkC and a related p75NTR receptor. Each family member binds different neurotrophins with varying affinities. TrkB has highest affinity for BDNF and is inherently involved in the actions of BDNF neuronal plasticity, longterm potentiation and apoptosis of CNS neurons. p75NTR binds neurotrophin precursors like ProBDNF with high affinity and retains low affinity to the mature cleaved forms. ProBDNF binding to p75NTR may promote LTD (Woo et al., 2005) or apoptosis. Trk receptors are coupled to the Ras, Cdc42/ Rac/ RhoG, MAPK, PI 3-K and PLC gamma signalling pathways. p75NTR lacks tyrosine kinase activity and signals via NF-kappaB activation.

1.4.3 Roles in synaptic plasticity.

During development:

Maturation of the visual cortex is influenced by visual experience during an early postnatal period. Huang et al. (Huang et al., 1999) examined the maturation and plasticity of the visual cortex in transgenic mice in which the postnatal rise of BDNF was accelerated. In these mice, the maturation of GABAergic innervation

and inhibition was accelerated and age-dependent decline of cortical long-term potentiation induced by white matter stimulation occurred earlier. The transgenic mice also showed an earlier termination of the critical period for ocular dominance plasticity. The authors proposed that BDNF promotes the maturation of cortical inhibition during early postnatal life, thereby regulating the critical period for visual cortical plasticity. Guillin et al. (Guillin et al., 2001) used lesions and gene-targeted mice lacking BDNF to show that BDNF from dopamine neurons is responsible for inducing normal expression of the dopamine D3 receptor in nucleus accumbens both during development and in adulthood.

Modulating synaptic signaling:

Brain-derived neurotrophic factor (BDNF) is known to play a role in long-term potentiation (LTP), in the hippocampus (Patterson et al., 1992) (Kang and Schuman, 1995). Also, LTP in the hippocampus is reported to be impaired in BDNF knockout mice (Korte et al., 1995). Akaneya et al. (Akaneya et al., 1997) studied the effects of BDNF application on LTP induced by tetanic burst stimulation of layer IV in visual cortical slices prepared from young rats and confirmed potentiated field potentials and EPSCs that outlasted the BDNF application. Moreover, the actions of BDNF were blocked by preincubation of slices with TrkB-IgG fusion protein, a BDNF scavenger, or co-application of K252a, an inhibitor for receptor tyrosine kinases. TrkB-IgG or K252a itself completely blocked LTP, suggesting that endogenous BDNF or another TrkB ligand plays a role in LTP in the developing visual cortex.

BDNF appears to have a crucial role in the homeostatic regulation of cortical excitability, by selectively modifying excitation and inhibition within cortical networks (Desai et al., 1999) (Rutherford et al., 1998). In cell cultures of rat sympathetic neurons innervating cardiac myocytes, Yang et al. (Yang et al., 2002) showed that BDNF rapidly (within 15 minutes) shifted the neurotransmitter release properties of the neurons from excitatory to inhibitory cholinergic transmission in response to neural stimulation via the presynaptic p75 neurotrophin receptor.

1.4.4 Physiology of the BDNF Val66Met variation

In humans the 5-prime 'pro' region of BDNF, critical for intracellular trafficking and activity dependent secretion, shows a single nucleotide polymorphism (SNP) - BDNF Val66Met (rs6265).

Egan et al. (2003) (Egan et al., 2003) examined the effects of this SNP in humans. The met 66 allele was associated with poorer episodic memory, abnormal hippocampal activation assayed with functional magnetic resonance imaging (fMRI), and lower hippocampal N-acetyl aspartate, (a putative marker of neuronal integrity and synaptic abundance) assayed with MRI spectroscopy. Neurons transfected with met66-BDNF-GFP showed lower depolarization-induced secretion, while constitutive secretion was unchanged. met 66-BDNF - GFP did not localize to secretory granules or synapses. These results

demonstrated a role for BDNF and its V66M polymorphism in human memory and hippocampal function and suggested that V66M exerts these effects by impacting intracellular trafficking and activity-dependent secretion of BDNF.

Several other studies have reported an association between BDNF genotype and episodic memory, hippocampal activation during fMRI and hippocampal volume (Hariri et al., 2003) (Hashimoto et al., 2008) (Pezawas et al., 2004). This effect was independent of age, IQ, number of voxels, hippocampal volume or gray matter content in the voxels of interest.

1.4.5 Effects of the BDNF Val66Met SNP on practice dependent plasticity.

In a pioneering study, Kleim et al. (Kleim et al., 2006) asked whether the val-to-methionine substitution at codon 66 (val66met) of the BDNF gene would also influence the process of motor learning. They used TMS to probe learning-induced increases in excitability of the hand area of M1. Participants performed a 30 min training protocol that focused on ballistic movements of the index finger. Training consisted of blocks of a finger tapping and a pinch grip task. Both tasks were repeated twice in an alternating order. Each training block lasted 5 min. In the finger tapping task, subjects were asked to press the 1 and 3 key on a keyboard with the right index finger as fast as possible. In the pinch grip task, participants had to press the pad of a pinch grip gauge with the right

index finger every 5 s in order to reach a force of at least 5 kg. Before and after the training period, Kleim et al. used neuro-navigated single-pulse TMS to map the cortical area from which MEPs could be elicited in the first dorsal interosseous muscle of the practicing hand. Single-pulse TMS over the motor hot spot also was employed to characterize the input–output relationship between the intensity of stimulation and the size of the MEP (i.e. the stimulus–response curve) before and after training.

There were no significant differences in the motor performance of each group before and after the training. However, TMS data suggested that despite this, there was a difference in the effect of training on the function of the corticospinal system. Individuals with the val66val genotype showed the expected larger volume of the cortical motor map and a homogeneous increase in MEP amplitude across all intensities used to test the input–output curve. There was also a substantial shift in the center of gravity (COG) of the cortical map with a mean shift of approximately 2 cm. Conversely, individuals carrying one or two met alleles did not show a consistent change in map volume and tended to show a relative decrease in MEP amplitude after training, especially at low stimulus intensities. The shift in the COG was also less prominent (<1.0 cm) compared to the val66val carriers.

Although these results are highly suggestive of a functional role of BDNF polymorphisms in mechanisms of motor learning, there are a number of caveats that should be addressed in future studies. First, it should be noted that the post-training shift of the COG in the val66val group (mean shift of approximately 2 cm) was much larger than found in previous TMS studies and therefore may

not be repeatable in a study with larger numbers of subjects. For example, in stroke patients, Liepert et al. (Liepert et al., 2000) have also found shifts in the COG but the magnitude was well below 1 cm. Second, the tasks used are motor practice tasks (pinch grip) with a low skill acquisition component, thus limiting the inferences to effects on motor adaptation rather than more complex tasks such as sequence learning. Finally, it is unclear to what extent the reported excitability changes are caused by changes in the efficacy of synaptic connections in the cortex or to changes in neuronal excitability at the spinal level.

Despite these caveats, the pioneering study by Kleim et al. (2006) provided preliminary evidence that the val66met SNP affects activity driven changes in corticospinal circuits. This was only demonstrated with TMS and would have been missed by merely relying on behavioral measurements because the genotype dependent difference in training-induced corticospinal excitability changes did not translate into altered performance in simple motor tasks.

2 Methods

2.1 BDNF genotyping technique

Genotyping was carried out twice with known positive controls. In GenBank sequences and the public SNP database (http://www.ncbi.nlm.nih.gov/), we identified a common coding variant in the BDNF gene, a G→A polymorphism responsible for a Val66Met change. Whole blood was taken into EDTA tubes and DNA was extracted using a standard phenol–chloroform method and checked for quality and concentration using a spectrophotometer. Part of exon 2 of the BDNF gene was amplified using the polymerase chain reaction (PCR) and primers (SBDNF1-AAA GAA GCA AAC ATC CGA GGA CAA G; SBDNF2-ATT CCT CCA GCA GAA AGA GAA GAG G) resulting in a 274base pair (bp) PCR product. A Perkin Elmer 9700 thermal cycler was used for DNA amplification. Amplification reactions were performed in a total volume of 25µl, containing approximately 50 ng of genomic template, 1 µm of each primer, 200 µm deoxyribonucleotide triphosphate (dNTP), 10× buffer inclusive of 2.5 mM magnesium chloride and 1 U of Taq polymerase. The PCR cycling conditions consisted of an initial denaturation for 10min at 94○C, followed by touchdown program of 25 cycles of 94○C for 30 s, 60○C for 30 s and 72○C for 45 s. After each cycle the annealing temperature was reduced by 0.4○ C down to 50○ C.

There were then 12 cycles of 94○C for 30s, 50○C for 30s and 72○C for 45 s and a final extension at 72○C for 10 min. The PCR was checked for success on a 2% agarose gel. The PCR product was then digested with the restriction enzyme Hsp92II. The reaction consisted of 10 µl of PCR product, 2 µl buffer, 1 µl Hsp92II, 0.2 µl bovine serum albumin and 6.8 µl of water. In the presence of

the G allele, Hsp92II digestion produced two products, 57 and 217 bp, whereas the A allele produced three products, 57, 77 and 140 bp. The presence of a second Hsp92II site served as a restriction digest control, identifying incomplete digests for repeat analysis. Polymerase chain reaction products were electrophoresed on a 2% agarose gel and visualized using a transilluminator and ethidium bromide staining. All participants were successfully genotyped.

2.2 Transcranial magnetic stimulation methods

We employed a variety of rTMS methods to test synaptic plasticity in healthy human subjects.

The primary motor cortex of the dominant hemisphere was stimulated in all experiments.

Figure-of-eight coils with outer diameter of 70 mm (Magstim Co., Whitland, Dyfeld, UK) were used for the experiments. According to the guidelines of the International Federation of Clinical Neurophysiology, we defined the resting motor threshold (RMT) as the minimum stimulation intensity over the motor hot-spot, which can elicit a MEP of no less than 50 μV in 5 out of 10 trials. AMT was calculated with the biphasic stimulator prior to the TBS session. AMT was defined as the minimum single pulse intensity that produced an MEP of at least 0.2mV on more than 5 out of 10 trials, while the subjects were maintaining a

background contraction of 15% of their maximum power. The motor hot-spot was defined as the location where TMS consistently produced the largest MEP size at 120% RMT in the target muscle. The coils were held at an angle of 45 deg away from the mid-sagittal line with the handle pointing backwards.

EMGs were recorded via Ag–AgCl electrodes placed over the target muscle(s) of the dominant hand using a belly tendon montage.

The change in corticospinal excitability produced by each intervention was assessed by measuring the amplitude of the MEP response to a standard test pulse that remained constant throughout the experiment. In each subject the intensity of this pulse was individually adjusted at the start of the experiment to produce a stable MEP (of 0.5–1 mV) with the subject at rest. In all cases, the effects are quantified by examining changes in the amplitude of EMG responses evoked by standard single TMS pulses (Motor Evoked Potential (MEP)) before and at various time-points after rTMS.

Signals were filtered (30 Hz to 2 kHz) and amplified (Digitimer 360, Digitimer Ltd, Welwyn Garden City, Herts, UK) and then stored on computer via a Power 1401 data acquisition interface (Cambridge Electronic Design Ltd,Cambridge, UK). Analysis was carried out using Signal Software (Cambridge Electronic Design).

2.2.1 Theta burst stimulation (TBS).

The established conditioning intensity for TBS is 80% of the active motor threshold (AMT) (Huang et al., 2005). AMT was calculated with the biphasic stimulator prior to the TBS session. TBS was applied over the motor cortex hot-spot as described by Huang et al. (2005). Each burst consisted of three stimuli (80% AMT) given at 50 Hz. Continuous TBS (cTBS), which usually suppresses corticospinal excitability, was delivered as a sequence of 100 bursts (300 stimuli) given at a rate of 5 Hz (total duration of 20 s); intermittent TBS (iTBS) involved giving a 2s train repeated every 10s for 20 repetitions (600 stimuli).

2.2.2 Transcranial Direct Current Stimulation (TDCS) preconditioned 1 Hz Stimulation

TCDS Stimulation (Nitsche and Paulus, 2000) was delivered using a battery-driven DC stimulator (Schneider Electronic, Germany) via two conductive – rubber electrodes, placed in saline soaked sponges (5x7 cm), positioned over the primary motor cortex (the TMS hotspot for FDI was used) and above the contralateral eyebrow. A constant current flow of 1mA was applied for 10 min. The current is always ramped up/ down slowly in the first and last 10 seconds of stimulation. The after effects depend on the polarity used; we used the cathodal polarity (cathode over the FDI TMS hot-spot), which produces an inhibitory effect if applied for 15 minutes.

1 Hz rTMS delivered at intensities at or above RMT suppresses corticospinal excitability. The duration of the after-effects depends on the total number of

pulses given. In this study, 1Hz rTMS was delivered for 15 minutes (900 pulses) at subthreshold intensity (85% RMT) 10 minutes after the end of TDCS. RMT was assessed with the biphasic stimulation using the same criteria as above.

2.2.3 Paired Associative Stimulation

PAS involves pairing electrical stimulation of the median nerve at the wrist with single pulse TMS of the hand area of motor cortex. Our protocol applied 200 electrical stimuli to the median nerve of the dominant hand at the wrist paired with a single TMS pulse over the hot spot of the APB muscle hand area of the contralateral hemisphere at a rate of 0.25 Hz. TMS was delivered through a Magstim 200 magnetic stimulator (Magstim Company, UK). The TMS pulses were applied at the same stimulus intensity as the test stimulus (stimulator intensity adjusted to achieve 1mV MEP in the APB). Electrical stimulation was applied through a bipolar electrode (cathode proximal), using square wave pulses (0.2 ms duration). Intensity was adjusted to three times the perceptual threshold. The threshold for visible motor twitch was also recorded. The interstimulus interval between peripheral and TMS stimulus was 25 ms. Subjects were instructed to look at their stimulated hand and count the peripheral electrical stimuli they perceived to control for attention. After 200 pairings applied at a rate of 0.25 Hz, where the electrical stimuli precedes the TMS pulses by 25ms, MEPs in median nerve innervated hand muscles increase in size consistent with an LTP-like mechanism (Quartarone et al., 2006a). Spread of excitability to ulnar nerve innervated hand muscles occurs to a limited extent in normal subjects and is reported to occur more prominently in patients

with focal hand dystonia (Quartarone et al., 2003). Several studies have demonstrated that MEP facilitation induced by PAS is greater when subjects were tested at 8o'clock in the evening than at 8 o'clock in the morning, possibly due to diurnal variations in the levels of neuromodulators like cortisol (Sale et al., 2008). To minimize daytime-dependent changes, in the present experiments PAS was always delivered between 11:00 and 15:00 h.

3 Effects of the BDNF Val66Met SNP on artificially induced plasticity

3.1 Introduction

In this pilot study (Cheeran et al., 2008a) we employed a number of non-invasive TMS techniques that directly test the excitability and plasticity of neuronal circuits in human motor cortex. Even in healthy subjects, the response to these protocols is highly variable between different individuals. A number of factors have already been described that contribute to this variation such as the prior history of brain activation (Gentner et al., 2008a) (Huang et al., 2008), the subject's age (Muller-Dahlhaus et al., 2008), the time of day (Sale et al., 2008), and the menstrual cycle (Inghilleri et al., 2004). Here we ask whether genetic factors like the BDNF Val66Met SNP might also influence these measures.

3.2 Ethical approval

The UCL/UCLH Regional Ethics Committee approved all experimental procedures.

Sixty-one volunteers were recruited after informed consent was obtained. Subjects recruited did not have any chronic illnesses requiring treatment. Epilepsy and chronic or recent use of prescription medication (like antidepressants, analgesics, etc.) other than the oral contraceptive pill were specifically excluded.

3.3 Recruitment of subjects

Subjects were genotyped after informed consent was obtained, using the previously described method and primers.

All subjects carrying a 'Met' allele were invited for all experiments and recruited into the non-Val/Val group. Only two Met allele homozygotes were identified and only one volunteered for experiments 1a, 2 and 3. Subjects homozygous for the 'Val' allele, matched for age, sex and ethnicity (see Table 3-1), were then recruited into the 'Val/Val' group. Female volunteers were not matched for phase of menstrual cycle, but since the timing of the experiments was random, this would be unlikely to bias the results in any significant way. In total, 18 subjects (9 in each group) took part in experiments 1a, 1b and 3. Sixteen subjects (8 in each group) took part in experiment 2. Investigators blinded to the subject's genotype collected electrophysiological measures. Experiments were conducted at least 1 week apart.

3.4 Transcranial magnetic stimulation methods

One subject in each group in experiments 1a, 2 and 3 was left handed and therefore the right hemisphere was stimulated in these two subjects in these experiments. A monophasic Magstim 200 was used to define the motor hot-spot and to assess MEP size. A second coil was connected to a biphasic

stimulator, a Super Rapid Magstim package (Magstim Co., UK), and was used to deliver rTMS. (see Table 3-2B for mean baseline MEP amplitudes for each experiment).

For experiments 1 and 2, EMGs were recorded via Ag–AgCl electrodes placed over the first dorsal interosseous (FDI) of the dominant hand using a belly tendon montage. For experiment 3, EMGs were recorded via Ag–AgCl electrodes placed over the abductor pollicis brevis (APB) and the abductor digiti minimi (ADM) of the dominant hand using a belly tendon montage.

For the TBS experiments twenty baseline MEPs were collected and averaged at baseline. Then, after cTBS (experiment 1a) and iTBS (experiment 1b) over the same hot-spot, 20 MEPs were recorded at 1–5, 6–8, 9–11, 12–15 and 16–24 min after TBS and averaged.

In a second set of experiments, we examined the role of the polymorphism in the control of synaptic plasticity. To study homeostatic plasticity, 10 min of cathodal transcranial direct current stimulation (TDCS) was given initially to reduce motor cortical excitability; it was then followed by a short period of sub-threshold 1 Hz rTMS. For this TDCS experiment (experiment 2), 20 MEPs were collected and averaged at baseline as for experiment 1(time-point T0). Subjects then received 10 min of priming with cathodal TDCS, followed by 15 min of sub-threshold 1 Hz rTMS (both to the hand area of the motor cortex).

MEPs were recorded immediately after TDCS (time-point T1), immediately after rTMS (time-point T2) and at 10 min after rTMS (time-point T3), and then averaged. Sub-threshold 1 Hz rTMS alone is insufficient to induce any after-effects, but when pre-conditioned by cathodal TDCS it generates facilitation of the motor cortex, producing a homeostatic-like effect (Siebner et al. 2004) that has been shown to be impaired in patients with focal dystonia (Quartarone et al. 2005).

PAS (Stefan et al. 2000; Quartarone et al. 2006) was delivered using pairs of median nerve electrical and single pulse TMS over the abductor pollicis brevis (APB) hot-spot at an inter-stimulus interval of 25ms. MEPs were recorded from the median-innervated APB and the ulnar-innervated abductor digiti minimi (ADM) muscles at baseline (T0) and at 1 min (T1), 15 min (T2), 30 min (T3), 45 min (T4) and 60 min (T5) after PAS, and then averaged. Note that the test stimulus was optimized for APB.

3.5 Data analysis

Data were analyzed using SPSS for Windows version 11.0 on log transformed peak–peak amplitudes of the mean MEPs of each subject. Note that graphs show untransformed data. Repeated measures ANOVA with within subject factor of TIME (before/after intervention) and between subjects factor of GENOTYPE (Val/Val/non-Val/Val) was used to compare variables before and

after each experimental intervention. Dose effect analysis (i.e. Val/Val versus Val/Met versus Met/Met) was not done as only a single Met allele homozygote participated in the study (and in 3 of 4 experiments only). Post hoc paired t tests were applied when necessary. In all figures, error bars refer to the standard error.

3.6 Results

Table 3-1: Demographics of volunteers included in experiments

	Exp 1a: cTBS		Exp 1b: iTBS		Exp 2: TDCS preconditioning		Exp 3: PAS	
Genotype	Val/Val	Non-Val/Val	Val/Val	Non-Val/Val	Val/Val	Non-Val/Val	Val/Val	Non-Val/Val
	($n = 9$)	($n = 9$)	($n = 9$)	($n = 9$)	($n = 8$)	($n = 8$)	($n = 9$)	($n = 9$)
Mean age (± s.d.) (years)	26.45 (±5)	26.45 (±5)	29.3 (±3)	28.7 (±3)	25.8 (±5)	26.5 (±5)	27.1 (±4)	28 (±5)
Female	5	5	3	3	3	3	4	4
Caucasian	5	6	6	7	5	5	6	6
Asian	4	3	3	2	3	3	3	3

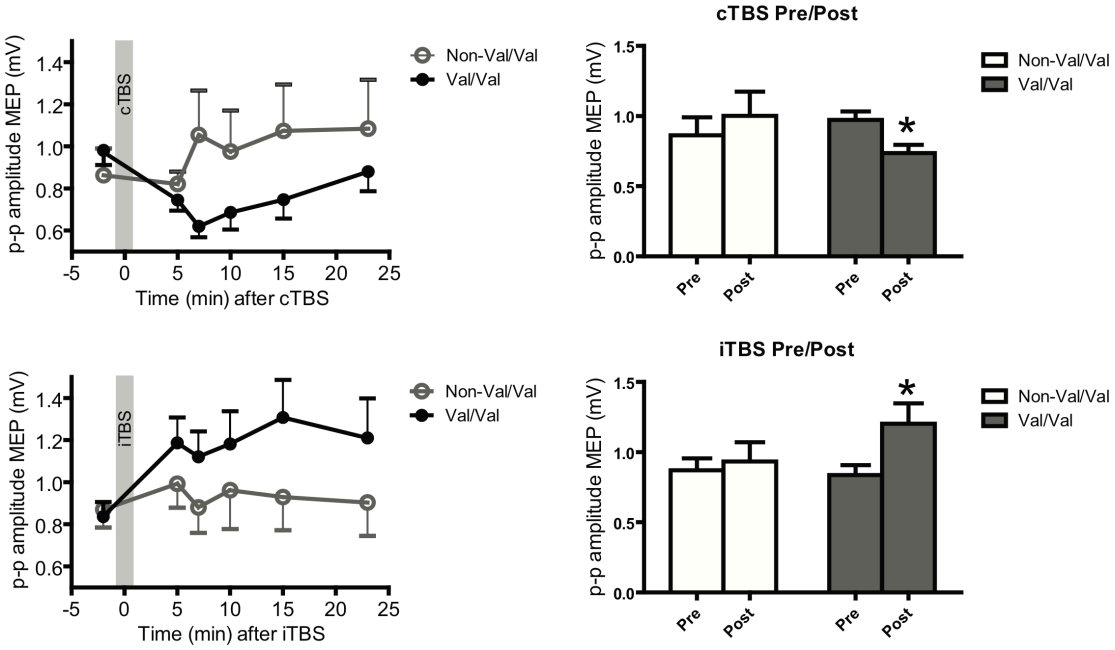
Table 3-2: Stimulation intensity (expressed as percentage of maximum stimulator output) and baseline MEP amplitudes (mV)

A % Stimulation intensity	Val/Val	Non-Val/Val	*t* test
cTBS (AMT)	37	40	0.47
iTBS (AMT)	37	39	0.48
TDCS (RMT)	40	42	0.37
B **MEP (Baseline)**	**Val/Val**	**Non-Val/Val**	***t* test**
cTBS	0.97	0.86	0.44
iTBS	0.87	0.81	0.66
TDCS	1.1	1	0.39
PAS (ADM)	0.55	0.57	0.88
PAS (APB)	0.82	0.97	0.38

No significant differences were noted between groups.

3.6.1 Induction of LTP/LTD-like change

Figure 3-1: Effect of BDNF Val66Met polymorphism on cortical excitability in response to cTBS (top row) and iTBS (bottom row)

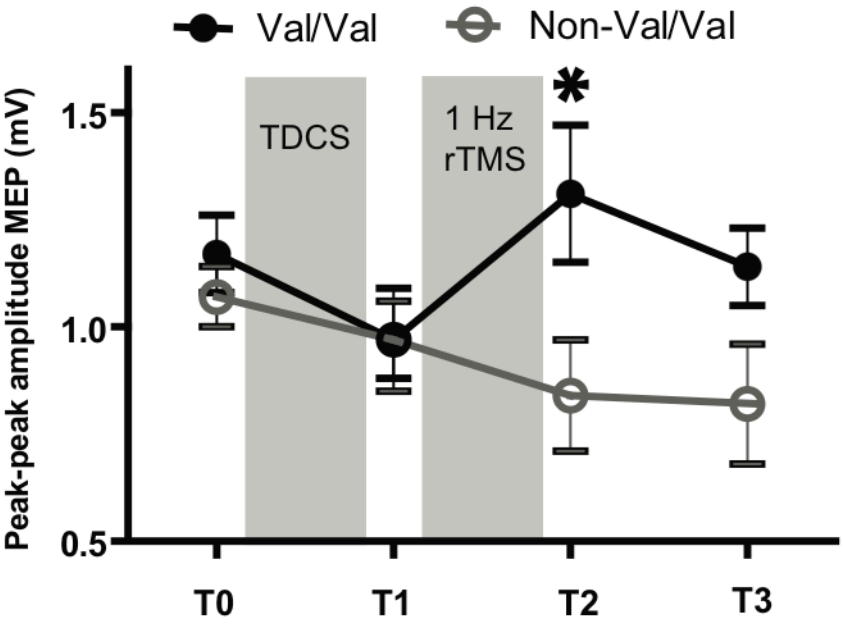


In the first set of experiments we tested whether the Val/Met polymorphism in the BDNF gene would affect the response to human theta burst stimulation. The data from all individual time points are plotted in Fig.3-1A and B for the inhibitory cTBS and excitatory iTBS interventions, respectively. Since there was no difference in the post-TBS values at any time point in either group, these were averaged and the mean pre/post data are shown in the corresponding Fig.3-1C and D. Two-way ANOVA of the log transformed data revealed a significant TIME∗GENOTYPE interaction for both cTBS ($F_{1,16} = 16.08$; $P = 0.001$) and iTBS ($F_{1,16} = 8.59$; $P=0.01$). This was due to the fact that there was a significant decrease in MEPs after cTBS in the Val/Val individuals ($P=0.0002$; paired ttest) but not in the non-Val/Val group. Similarly,

there was a significant increase in MEPs after iTBS in the Val/Val individuals (P=0.003; paired ttest) but not in the non-Val/Val group.

3.6.2 Control of homeostatic plasticity

Figure 3-2: Effect of BDNF Val66Met polymorphism on cortical excitability in response to cathodal TDCS preconditioning followed by sub-threshold 1 Hz rTMS

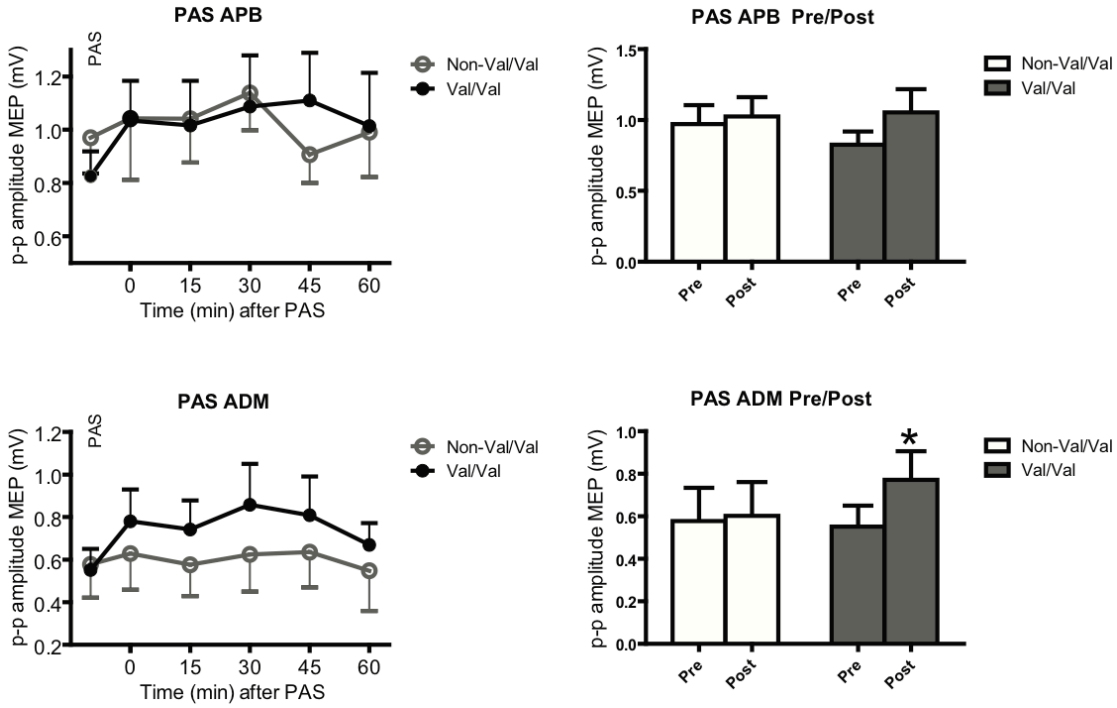


In a second set of experiments, we examined the role of the polymorphism in the control of synaptic plasticity. Analysis was performed using a mixed ANOVA design on the log transformed data with GENOTYPE as between-subjects factor (levels Val/Val and non-Val/Val) and TIME as within-subject factor; levels for factor TIME were baseline (T0), T1, T2 and T3. Figure 3-2 shows that subjects in the Val/Val group showed the expected pattern of effects: cathodal TDCS initially suppressed corticospinal excitability and this

was followed by facilitation after 1Hz rTMS. Subjects in the non-Val/Val group showed the same suppression after TDCS but no further effect after 1Hz rTMS. ANOVA showed a significant GENOTYPE×TIME interaction ($F_{3,42} = 4.44$, $P = 0.009$). Pair-wise comparisons revealed significantly higher MEP amplitudes after 1 Hz (T3) (t test: $P = 0.025$) in the Val/Val group compared to the non-Val/Val group.

3.6.3 Spread of LTP-like excitability

Figure 3-3: Effect of BDNF Val66Met polymorphism on cortical excitability in response to paired associative stimulation in the target (homotypic) abductor pollicis brevis (top row) and (heterotopic) ulnar-innervated abductor digiti minimi (bottom row)



In the final experiment, we explored the spread of LTP-like excitability using the paired associative stimulation protocol (experiment 3). Figure 3A and B

plots the mean data at each individual time point for the APB and ADM muscles, respectively. Since there was no difference in any of the post-PAS values these were averaged together to form the summary pre/post comparisons in Fig. 3-3C and D. Three-way ANOVA on the log transformed data with within subject factors of MUSCLE (APB, ADM) and TIME (Pre, Post), and between subject factor of GENOTYPE revealed a significant TIME$*$ GENOTYPE interaction ($F_{1,16} = 4.41$; $P = 0.05$) as well as a significant main effect of MUSCLE ($F_{1,16} = 8.39$; $P = 0.011$). Post hoc paired t tests showed that interaction was due to the fact that in Val/Val subjects, PAS produced a significant increase of the MEPs in ADM ($P = 0.01$) and a borderline significant increase in APB ($P = 0.07$). There were no significant effects in non-Val/Val individuals. The effect of MUSCLE was due to the fact that the MEPs were larger overall in the APB than the ADM.

3.7 Discussion

The present data show that the response of healthy subjects to three different plasticity-inducing protocols in motor cortex is associated with the polymorphism of the BDNF gene that they carry. The implication is that genetic variation in the normal population can produce significant differences in the after-effects of rTMS protocols. If this conclusion is valid in more physiological conditions, then the same variations may influence behavioural learning as well as recovery from brain damage.

Experiment 1 examined the after-effect of an inhibitory (cTBS) and an excitatory (iTBS) rTMS protocol on corticospinal excitability. In non-genotyped healthy controls, cTBS suppresses MEPs for 30 min or so whereas iTBS facilitates them. The present results show that the after-effects of both iTBS and cTBS are reduced or absent in subjects carrying the 'Met' allele of the BDNF gene. This was not related to any initial differences in thresholds or MEP size in the two groups, and presumably indicates that 'Met' carriers are less susceptible to the effects of TBS than the Val66Val individuals.

There are several possible reasons for the difference in response between the groups. The most likely is that it is more difficult to induce plasticity of neural circuits in the non-Val/Val individuals. However, the present experiments do not address the question of whether this difference arises because non-Val/Val individuals lack any response to TBS, or because they have a different input–output relationship between the intensity of stimulation and the duration/depth of the after-effects. Further experiments with a range of TBS intensities would be needed to address this. A rather different possibility is raised by the report of Gentner et al. (Gentner et al., 2008a) in which they pointed out that the after-effects of cTBS are extremely sensitive to the past history of motor cortex activation ('rapid metaplasticity'). Although there was no difference in the amount of voluntary movement prior to stimulation in the two groups, it is possible that subjects differ in their sensitivity to prior activation and this could account for the apparently different response to TBS

protocols. Despite the exact mechanism, we conclude that the after-effects of TBS protocols are affected by genetic variation in the normal population.

In experiment 2 we selected a 'metaplastic' conditioning protocol that employed 10 min cathodal TDCS to prime the response to presentation of 900 sub-threshold TMS pulses at 1 Hz. In a non-genotyped population of healthy subjects, MEPs are suppressed by the TDCS. This then transforms a subsequent period of 1 Hz rTMS, which on its own has no effect on corticospinal excitability, into facilitation. In the present experiments, cathodal TDCS produced the same amount of LTD-like suppression of corticospinal activity in all subjects, although there was a tendency for a smaller effect in the 'Met' carriers. More impressive, however, was a lack of the expected homeostatic effect of this stimulation on subsequent 1 Hz rTMS in the same subjects: the Val/Val subjects showed the expected reversal of corticospinal excitability towards facilitation, whereas MEPs remained suppressed in the non-Val/Val individuals.

Given the rather small number of subjects studied we cannot say with certainty that subsequent work will never reveal a difference in the response to TDCS. Nevertheless if the conclusion holds it would be consistent with the idea that TDCS and rTMS act on different neural circuits, which are differentially responsive to the BDNF polymorphism (Lang et al., 2005). The lack of any pre-conditioning effect on the response to a subsequent period of

1 Hz rTMS in the non-Val/Val group could be due to a number of reasons. For example, the duration of the 'metaplastic window' following TDCS could be shorter in 'Met' carriers, so that if we could have applied TMS more quickly after stopping TDCS, or if we had prolonged the duration of TDCS to increase the duration of the 'metaplastic window', we may have seen a smaller difference between the groups. Another possibility is that 'Met' carriers have an increased sensitivity to 1 Hz rTMS compared with Val/Val subjects. This could make it more difficult to reverse into facilitation than in Val/Val group. However, this seems unlikely in view of the generally reduced level of plastic changes we observe in the non-Val/Val individuals.

If the group differences reflect a true reduction in metaplastic interactions, then the results may relate to those in experiment 1. As noted above, one possible explanation of the lack of response to TBS protocols in non-Val/Val subjects is a lack of 'rapid metaplasticity' in motor cortex, where the prior level of activation preceding the TBS protocols determines the duration and direction of the after-effects on MEP amplitude.

Experiment 3 probed the effects of paired associative stimulation of median nerve and motor cortex on MEPs on the median nerve innervated APB muscle and the ulnar innervated ADM muscle. Using an interstimulus interval of 25 ms in non-genotyped healthy controls, this leads to a variable 0 to >100% facilitation of MEPs in the APB lasting 30–60 min after the end of PAS.

Effects in the ADM are also variable: Stefan et al. (Stefan et al., 2000) originally reported that there was no significant difference in the facilitation of APB and ADM, but others have suggested that effects in ADM are generally smaller than in APB, consistent with a topographic specificity of PAS. In the present experiments, non-Val/Val subjects had no significant response to PAS in either muscle, whereas Val/Val individuals responded with an increase that was significant in ADM, and borderline in APB. Again, this suggests that individuals carrying the Met allele have a reduced response to LTP-like plasticity induction by rTMS protocols.

At first sight it may seem odd that the amounts of PAS-induced facilitation in APB were less than those in ADM. However, if we had mixed the data from all subjects in the present experiments, as would have been the case in previous reports, we would have found a 20–30% mean increase in both muscles, which is within the range of values reported by others. It should also be noted that we carried out all the PAS examinations between 11:00 and 15:00 h in order to avoid daytime-related changes in levels of PAS that have been reported in early evening vs early morning comparisons (Sale et al., 2008).

Since we examined only one homozygous Met/Met carrier, we were not able to make any analyses of ‘dose’ effect. However, in some other studies ((Egan et al., 2003)) Met/Met carriers had more pronounced differences compared with Val/Val or even Val/Met individuals, and we presume that the same may well be true of the measures we examined here. Kleim et al. (Kleim et al.,

2006), however, did not demonstrate such an allele dose effect on motor map expansion after FDI exercise tasks.

Many previous studies have pointed out the variability of individual responses to the newly developed TMS and TDCS protocols that probe synaptic plasticity in motor cortex. The present data suggest that genotype is one factor that can influence these effects, and it may therefore be useful to include this as a potential co-variate in analysis of the data, particularly in studies utilizing these protocols as a therapeutic intervention (for example in stroke rehabilitation or depression). In smaller studies utilizing rTMS as an experimental intervention, our results highlight the importance of ethnicity matching, as the prevalence of SNPs like BDNF Val66Met varies widely among different populations.

Several neurological conditions such as dystonia (Edwards et al., 2006) (Quartarone et al., 2003) and phantom limb pain (Karl et al., 2001) have been proposed to involve abnormal plasticity at central synapses. Similarly, disorders of metaplasticity have been postulated to underlie susceptibility to L-DOPA-induced dyskinesia in Parkinson's disease (Picconi et al., 2003) (Linazasoro, 2005). The recovery of function after brain injury (e.g. stroke), is also thought to be modulated by the ability of synapses to undergo plastic change. The fact that this common polymorphism of the BDNF gene influences experimental protocols that are thought to induce synaptic plasticity

in the adult human brain suggests that this polymorphism could be a factor in the development of or recovery from certain neurological disorders.

4 Clinical Application: DYT1 Dystonia

4.1 Introduction

The observations that Dystonia can occur after excessive practice of particular movements in humans (task specific dystonias) and that Dystonia can occur or worsen following trauma to the affected body part (e.g. (Jankovic, 2001)) has lead to the emerging consensus that excessive ability to undergo plastic change could drive the development of dystonia.

DYT1 dystonia, caused by a mutation in the DYT1 gene on chromosome 9q34 and the commonest cause of young-onset primary generalized dystonia (Bressman, 2004), is an excellent model within which to test this hypothesis. Only 30% of carriers of the DYT1 mutation develop dystonia; 70% never develop symptoms, and these individuals appear to be protected by a reduced potential for synaptic plasticity. Studies have recently demonstrated how the sensitivity of the neuroplasticity system, tested with TBS, seems to directly determine whether patients with the DYT1 gene mutation develop clinical symptoms or not (Edwards et al., 2006). As discussed earlier, we have found that a common polymorphism (BDNF Val66Met) in the gene coding for Brain Derived Neurotrophic Factor (BDNF) determines sensitivity to these very plasticity probes. The presence of a Met allele significantly reduces the responsiveness of the brain to these protocols.

We hypothesized that:

1. That genetic polymorphisms that are functionally important in determining the sensitivity of the neuroplasticity system like BDNF Val66Met will be distributed differently in DYT1 mutation carriers with and without symptoms.

2. That DYT1 carriers with symptoms will more often carry the alleles that confer increased sensitivity to undergo plastic changes (like the BDNF Val allele), with the opposite pattern seen in DYT1 carriers without symptoms.

4.2 Methods

Study participants and mode of recruitment: DYT1 mutation carriers were identified from an existing database of patients and relatives who have expressed a desire to participate in future studies. They were initially approached and recruited from the movement disorders clinics supervised by Prof K Bhatia at the National Hospital for Neurology. Inclusion criteria for patients (manifestors): (i) genetic analysis positive for the typical DYT1 gene mutation; (ii) onset of limb dystonia prior to the age of 25 years with or without subsequent progression. Inclusion criteria for carriers (non-manifestors): (i) genetic analysis positive for the typical DYT1 gene mutation; (ii) clinical absence of dystonia, (iii) Age over 30 (the age by which symptoms manifest in almost all patients).

We contacted 61 patients and carriers with the DYT1 mutation (45 manifestors, 16 non manifestors) for informed consent. We obtained informed consent from 9 carriers (non-manifestors) and 28 patients (manifestors).

Genotyping for BDNF val66met genotypes was performed by a 5' exonuclease allelic discrimination Taqman assay on stored DNA. Pearson's Chi square was used to compare the frequency of the polymorphisms between DYT1 mutation carriers with and without symptoms.

4.3 Results

The Pearsons Chi-Square test (2-sided, df=1) suggests a significant effect (p=0.044) of BDNF genotype on DYT1 penetrance. Polymorphisms that reduce the sensitivity of the neuroplasticity system, like the Met allele in BDNF Val66Met, may confer a protective effect on expression of dystonia.

Table 4-1: DYT1 mutation carriers by genotype.

Genotype	Manifestor	Non-Manifestor	Total
Val/Met	7	11	18
Val/Val	2	17	19
Total	9	28	37

4.4 Discussion

Despite the small sample size in this study, the results summarized in table 2, taken together with the study of Edwards et al. suggest an intriguing explanation for reduced penetrance in DYT1. The results will need to be confirmed in a future study with more subjects. If these results were reproduced, it would provide the first genetic risk factor for the penetrance of DYT1. Moreover, other genetic variations that reduce the response to 'plasticity probes', may explain more of the risk for penetrance.

Although this study was analyzed as a case control study, several (but not all) controls (non-manifestors) are related to cases (manifestors). However, a transmission disequilibrium test, which would add credence despite the small sample size, was not possible; relationships were not recorded in several cases and ethical constraints limited direct contact with non-manifestors.

Other studies have subsequently explored this issue in cranio-cervical dystonia. Cramer et al. (Cramer et al., 2010) studied the prevalence of the BDNF genotype in 34 subjects with cervical dystonia, 54 age-matched healthy controls, and 53 subjects with Parkinson's disease (as a control group with another movement disorder). In this study subjects with cervical dystonia, the val66met polymorphism was approximately twice as prevalent when compared to either control group. Martino et al. (Martino et al., 2009) also explored the influence of the Val66Met SNP of the *BDNF* gene on the risk of cranial and cervical dystonia in a cohort of 156 Italian patients and 170 age- and gender-matched healthy control subjects drawn from the same

population, but found that the presence of the Met allele was not significantly associated with risk of developing cranio-cervical dystonia. This study was adequately powered to detect a 50% change in the risk of developing cranial-cervical dystonia; but this also means that a risk less 50% (which is more likely) could be missed.

5 Clinical Application: Levodopa-Induced Dyskinesia

5.1 Introduction

Chronic dopaminergic treatment in Parkinson's disease is complicated by the development of levodopa-induced dyskinesia (LID) in around 40% of patients after 4-6 years of levodopa therapy (Ahlskog and Muenter, 2001). However, some patients remain untroubled by LID even after 6 years of therapy with Levodopa. The reason for such variable susceptibility to this complication is unclear. Several risk factors have been established for the earlier onset of dyskinesias, which include younger age, greater disease severity at baseline and higher daily levodopa dose, but these factors do not explain all the variability observed in time to dyskinesia onset.

Studies in rat models of PD have shown that plasticity of cortico-striatal synapses, measured as the ability to undergo long term potentiation or depression (LTP, LTD) is reduced or abolished by dopaminergic denervation following chemical lesions of the nigrostriatal tract. LTP is restored by chronic l-dopa therapy, but in some animals synaptic depotentiation ('forgetting') is not restored and these then go on to develop dyskinesia (Picconi et al., 2003). Indirect evidence from human studies also is consistent with a role of synaptic plasticity in development of dyskinesias in susceptible individuals. Thus LTP-like plasticity in motor cortex evaluated by transcranial magnetic stimulation is deficient in PD patients off medication and is restored by levodopa in non-dyskinetic but not in dyskinetic patients (Morgante et al., 2006). One

explanation for the variable susceptibility to LID is that exposure to l-dopa could have a much greater effect on someone with PD who carries a genetic vulnerability (based on a genetic influence on synaptic plasticity) to develop dyskinesias (Linazasoro, 2005). Thus both animal and human work is compatible with the idea that a genetically determined difference in the regulation of synaptic plasticity determines the susceptibility to develop LID.

The neuromodulatory role of has been demonstrated in several settings, especially in the dopaminergic system and PD. BDNF synthesized by dopamine neuron is responsible for the appearance of the dopamine D3 receptor during development and maintains its level of expression in adults (Guillin et al., 2001). New findings supporting this demonstrate that levodopa induces BDNF release from cortico-striatal fibers, which in turn enhances the expression of D3 receptors; and that this effect is associated with motor dyskinesias (see review by (Kostrzewa et al., 2005). Monkeys that are rendered parkinsonian with 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine and develop levodopa-induced dyskinesia show an overexpression of the D3 receptor. Treatment with partial agonists to the D3 receptor can reduce dyskinesias in the MPTP monkey model. Again in unilaterally 6-OHDA-lesioned rats, it appears that behavioural sensitization to levodopa is dependent on D3 receptor expression (Bordet et al., 1997). Thus there is experimental evidence to link 1) levodopa and BDNF release 2) BDNF and expression of the D3 receptor and 2) the D3 receptor and levodopa induced dyskinesia.

Our results (Cheeran et al., 2008b) show that homeostatic plasticity is reduced by the Met allele. Results from Picconi et al. (Picconi et al., 2003)) in rats and LTP induction in patients by PAS from Morgante et al., (Morgante et al., 2006)), as discussed earlier, implicated impaired homeostatic plasticity in levodopa induced dyskinesia. It was tempting to hypothesise that BDNF genotype could influence corticostriatal synaptic plasticity in PD patients, with carriers of val alleles able to potentiate and depotentiate corticostriatal synapses more effectively than met allele carriers, who thus develop a pathological storage of information that would normally be erased leading to the earlier development of abnormal motor patterns i.e dyskinesias.

Taking into account its critical role in activity dependant modulation of synaptic plasticity and our results showing that the val66met polymorphism functionally influences this role, we hypothesized that this polymorphism could influence the time to develop dyskinesia in a large prospectively followed cohort of PD patients (Foltynie et al., 2009).

5.2 Methods

We obtained access through collaboration to the clinical and DNA database of patients seen and assessed at the Cambridge Centre for Brain Repair in Cambridge, UK as part of an ongoing prospective study of Parkinson's disease. Only patients meeting UKPDS Brain bank criteria for the diagnosis of PD were included in this study. All patients had provided written consent for genetic analysis of their DNA, extracted using standard techniques from their peripheral blood samples. Genotyping for BDNF val66met genotypes was performed by a 5' exonuclease allelic discrimination Taqman assay. All patients were assessed with the Unified Parkinson's Disease Rating Scale (which incorporates dyskinesia assessment). Patients' medications were not adjusted as part of attendance at this clinic. To maximise the accuracy of recording the presence/ absence and date of onset of dyskinesias, only patients who were free of dyskinesias at the time of their first assessment were included in the study. Doses of dopaminergic medication were noted at the first patient assessment and converted to equivalent levodopa doses with a previously used formula. Equivalent levodopa dose= (levodopa (x1.2 if COMT inhibitor) (x1.2 if 10mg selegiline OR x1.1 if 5mg selegiline)) + (pramipexole x400) + (ropinirole x40) + (cabergoline x160) + (pergolide x 200) + (bromocriptine x 10) + (lisuride x 160), all doses in mg. This allows a comparison between patients on different dopaminergic regimes and takes account of the risk of dyskinesia that exists from both levodopa and to a lesser extent from dopamine agonist use. All patients were UK Caucasians

apart from one Afro-Caribbean individual, 2 Asian-Indian individuals and one individual who was half Caucasian and half Asian-Indian.

5.3 Results

A survival analysis was performed with date of diagnosis used as baseline and censoring occurring at 1) onset of dyskinesia, or 2) latest date of follow up assessment if free from dyskinesia.

Four hundred and twenty one patients were free from dyskinesia at their first assessment and had reliable data regarding date of PD onset. 358/421 were on dopaminergic treatment at the time of their first visit. Table 5-1 shows a description of these patients at the time of their first assessment divided according to their BDNF genotype (confirmed to be in Hardy-Weinberg equilibrium). Analysis of variance found no significant differences between BDNF genotypes in patients, with respect to age at diagnosis, duration of disease at first clinic attendance, UPDRS motor scores, whether or nor they were on dopaminergic treatment, mean equivalent levodopa dose, or duration of follow up in clinic.

At the time of analysis, 52 patients had developed new onset of dyskinesia having been dyskinesia free when first assessed in the clinic, 25/260 val/val patients, 22/146 val/met patients, and 5/15 met/met patients. Figure 5-1 shows Kaplan-Meier curves for the development of dyskinesia for each BDNF genotype from PD onset. Calculating scaled residuals confirms that the

proportional hazards assumption is met, and univariate Cox regression analysis produces a hazard ratio of developing dyskinesia of 1.68 (95%CI 1.08-2.61) for each additional met allele (p=0.02). Multivariate Cox regression analysis with adjustment for possible confounding variables -equivalent L-dopa dose at baseline, age at diagnosis, gender, total UPDRS score at baseline, and duration of clinical follow up increases the hazard ratio for developing dyskinesia to 1.74 (95%CI 1.09-2.78)(p=0.021) for each additional met allele. Repeating the analysis with exclusion of patients who had not yet received L-dopa throughout their period of follow up (n=44 still on no dopaminergic treatment, n=49 still on dopamine agonists only), made no difference to the magnitude or significance of the univariate or multivariate hazard ratios observed.

5.4 Discussion

This study suggests that PD patients with BDNF met alleles are at risk of developing LID earlier in the course of their disease than val/val homozygotes. Further research is needed to confirm these results in additional populations of patients with PD.

However, if the development of disabling dyskinesias is shown to be related to the presence of one or two met alleles, then this could have implications for the treatment of PD patients.

It would be a considerable advance in the treatment of PD if it were possible to predict who was likely to develop dyskinesias before starting levodopa. This would:

1) Enable the earlier use of levodopa in patients with a low risk of developing dyskinesias, increasing their chance of good symptom control.

2) Enable selection of patients at high-risk of developing dyskinesias for alternative treatments to oral levodopa. Several such treatments are currently restricted until late in the disease process according to current NICE guidelines.

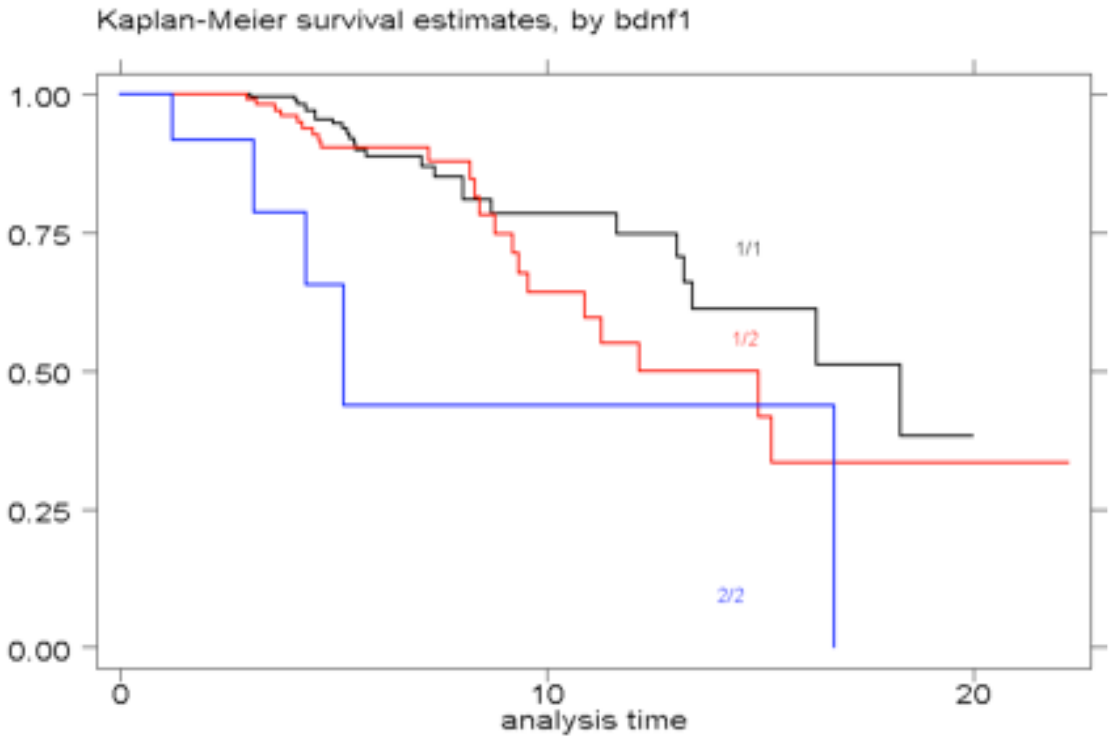


Figure 5-1 Kaplan Meier curves to show duration from PD diagnosis (years) until first record of dyskinesia among BDNF val/val patient (1/1), val/met (1/2), and met/met patients (2/2).

Table 5-1: Demographics and statistical comparison of patients at time of their first assessment by genotype- all patients were free from dyskinesia at baseline.

BDNF genotype	N	Age at diagnosis Median (Range)	On Dopa meds	Equivalent Levodopa dose-mg Mean (SD)	Duration disease at first visit - years Median (Range)	UPDRS motor score at first visit Mean (SD)	Duration of clinic follow up-years Median (Range)
Val/Val	260 (153 male)	65 (31-89)	217 (83%)	407 (433)	1.1 (0-16.9)	24.9 (14.1)	2.7 (0-7.3)
Val/Met	146 (85 male)	64 (41-91)	127 (87%)	410 (422)	1.3 (0-19)	23.3 (13.6)	2.8 (0-7.7)
Met/Met	15 (8 male)	60 (44-80)	14 (93%)	355 (354)	0.5 (0-15)	22.7 (14.4)	2.95 (0-7.2)
χ^2 / Anova	χ^2 =0.18 p=0.91	F=0.14, p=0.87	χ^2 =1.8 p=0.42	F=0.11 p=0.88	F=0.49 p=0.62	F=0.69 p=0.50	F=0.72 p=0.49

6 Studying the role of BDNF Val 66 Met in the variability in response to rTMS

6.1 Introduction

The aim of the present study was to provide some information in a sample of 115 young volunteers of the variation in response to a commonly used rTMS protocol, intermittent Theta Burst Stimulation (iTBS) (Huang et al., 2005). The analysis presented here was designed to address two questions of practical importance when interpreting single session studies of an rTMS protocol. These were: what proportion of individuals will have the expected response to the protocol; and is this proportion affected by time of testing (morning or afternoon between 9AM and 6PM) (Sale et al., 2008), sex (Inghilleri et al., 2004), or genotype (the val88met polymorphism of the BDNF gene)? In addition, we took advantage of the relatively large numbers of individuals to perform a cluster analysis on the time course of the responses to iTBS to test whether it is possible to classify particular patterns of response that have similar time courses.

6.2 Methods

Research ethics committees in London and A Coruna approved the study.

Subjects were recruited in 3 cohorts at a single site, each with a target of 50 subjects. In total, 139 subjects were recruited. Each subject was allocated a 4 digit alpha-numeric study ID to enable effective blinding.

Blood samples for DNA were obtained from 139 subjects. Genotyping for the BDNF Val66Met SNP (rs6265) was performed using commercially available primers and standard techniques described previously. 119 subjects attended for the TMS experiments, with each cohort being studied over a 6-week period. The study was completed over a 10-month period.

115 subjects are included in the analysis presented here; 2 subjects with missing data points and 2 subjects with the BDNF Met66Met genotype (AA genotype) have been excluded. We excluded the first 2 subjects because missing data points adversely affect the categorical outcome variables (sustained response and futility) as well as affecting the clustering algorithm. The subjects with the BDNF AA genotype were excluded, as with just 2 subjects, there are insufficient numbers in this group for meaningful analysis.

All TMS experimental data was collected by 3 experienced investigators, and each experiment was done by a pair of investigators to minimize inter-observer variation in technique (e.g. assessment of Active Motor Threshold (AMT, optimal site of stimulation, etc)). Within each pair, experimenters took it in turns to collect data or to observe data collection. Every recorded TMS variable (RMT, AMT, 1 mV stimulator intensity, etc.) required the agreement of experimenter and observer. The observer had the additional role of monitoring EMG traces online to delete traces with muscle activation,

attention, subject movement, etc. Subjects and experimenters were blinded to genotype.

Experiments are done between 9 AM and 6 PM; as a result, fewer subjects were examined in the morning. The primary motor cortex of the dominant hemisphere was stimulated in all experiments. Two figure-of-eight coils with outer diameter of 70 mm (Magstim Co., Whitland, Dyfeld, UK) were used for the experiments. A monophasic Magstim BiStim was used to define the motor hot-spot and to assess MEP size. A second coil was connected to a biphasic stimulator, a Super Rapid Magstim package (Magstim Co., UK), and was used to deliver rTMS. Active motor thresholds were obtained with both the BiStim and Super Rapid Magstim packages.

As in the experiments presented previously, the effect of iTBS on corticospinal excitability was quantified by measuring the amplitude of MEPs evoked in the FDI by a constant-intensity TMS pulse given over the contralateral motor cortex. At the start of the experiment the intensity of this pulse was adjusted so that it evoked an MEP of about 1 mV peak-to-peak amplitude in each individual. Twelve such MEPs were collected and averaged at baseline. Theta burst stimulation (TBS) was applied over the motor cortex hot-spot as described by Huang et al. (Huang et al., 2005). 12 MEPs were recorded at fixed 3-minute intervals (minutes 3, 6, 9... 21).

Here we analyze data describing the variability in response to rTMS (MEP amplitudes pre/post iTBS) and three previously described factors influencing the response to rTMS (BDNF genotype, Sex, Time of Day). Phase 2 of this study, which is ongoing, will attempt to predict the variability in response within an individual studied on two separate occasions.

6.3 Analysis

Data pre-processing and analysis was carried out using Signal Software (Cambridge Electronic Design) - the experimenter involved is blinded to demographic and genotype information during this process as data is coded by the study ID. Statistical analyses and cluster analyses were done using SAS JMP 8 ®.

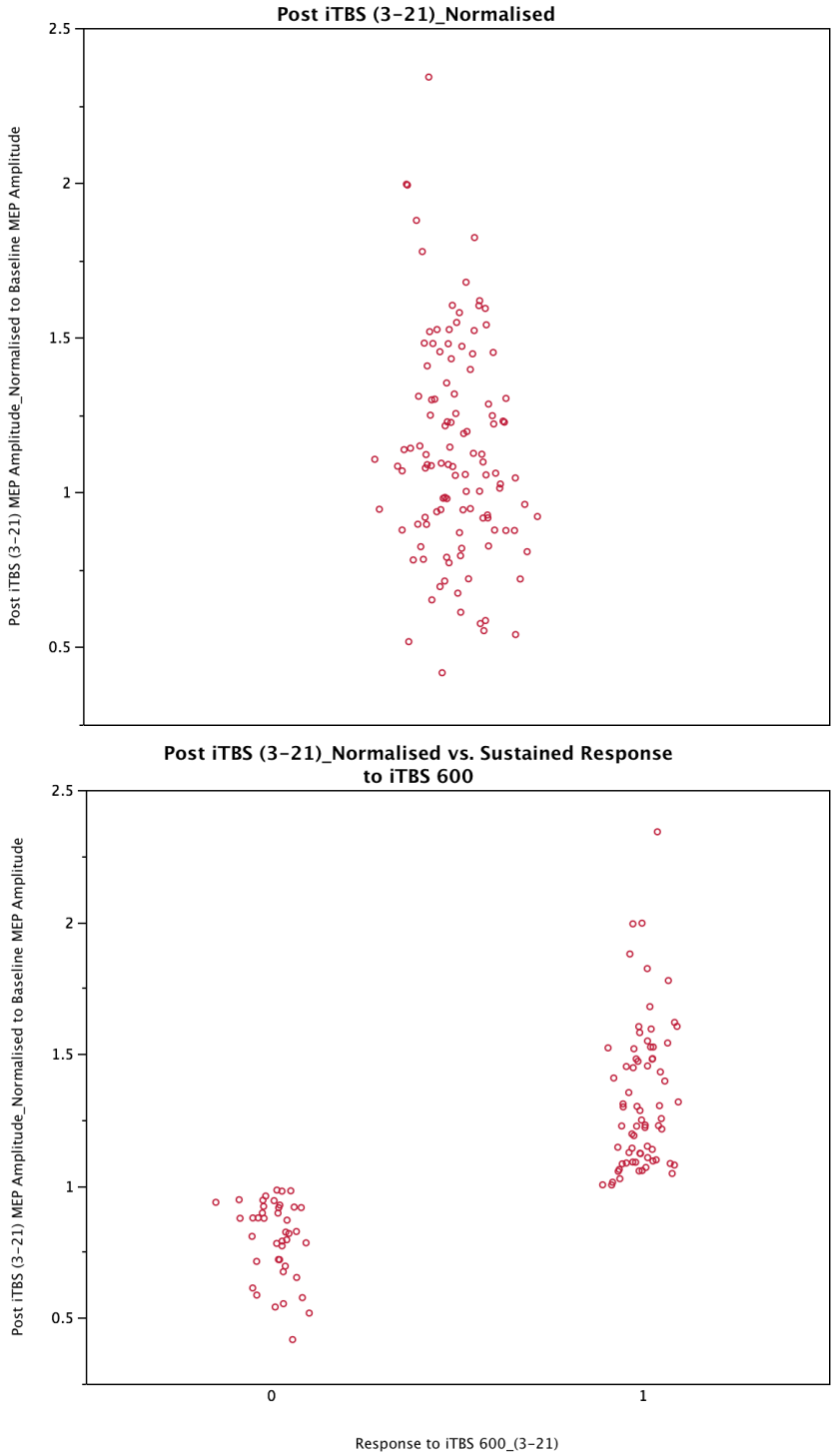
The primary outcome measure was the MEP amplitude response to iTBS post-stimulation, determined by averaging the MEP amplitudes obtained at all time-points over between minutes 3 and 21(Mean (Post- iTBS Min 3-21) MEP Amplitude) compared to the MEP amplitude at baseline, pre-iTBS (Mean (Baseline) MEP Amplitude). Three previously reported between subjects factors (BDNF Genotype (Val/Val (GG) Vs. Val/Met (GA)), Sex, Time of Day (experiment done AM Vs. PM).

Two additional categorical variables were also constructed from the MEP amplitude measurements to answer specific questions likely to be useful for future studies employing TMS paradigms:

6.3.1 Sustained Response

Subjects were assigned a value of 1 if the mean MEP ratio to baseline from 3-21 min iTBS was greater than 1 (i.e. they had a positive overall response). If the MEP ratio was ≤1, then they were assigned a value of 0. Effectively the proportion of subjects with a value of 1 is a measure of the expected number of responders in the population. We averaged over 3-21 min since this is a typical assessment duration or time during which the effects of iTBS might be combined with a second behavioral intervention.

Figure 6-1: Understanding the Sustained Response categorical variable.

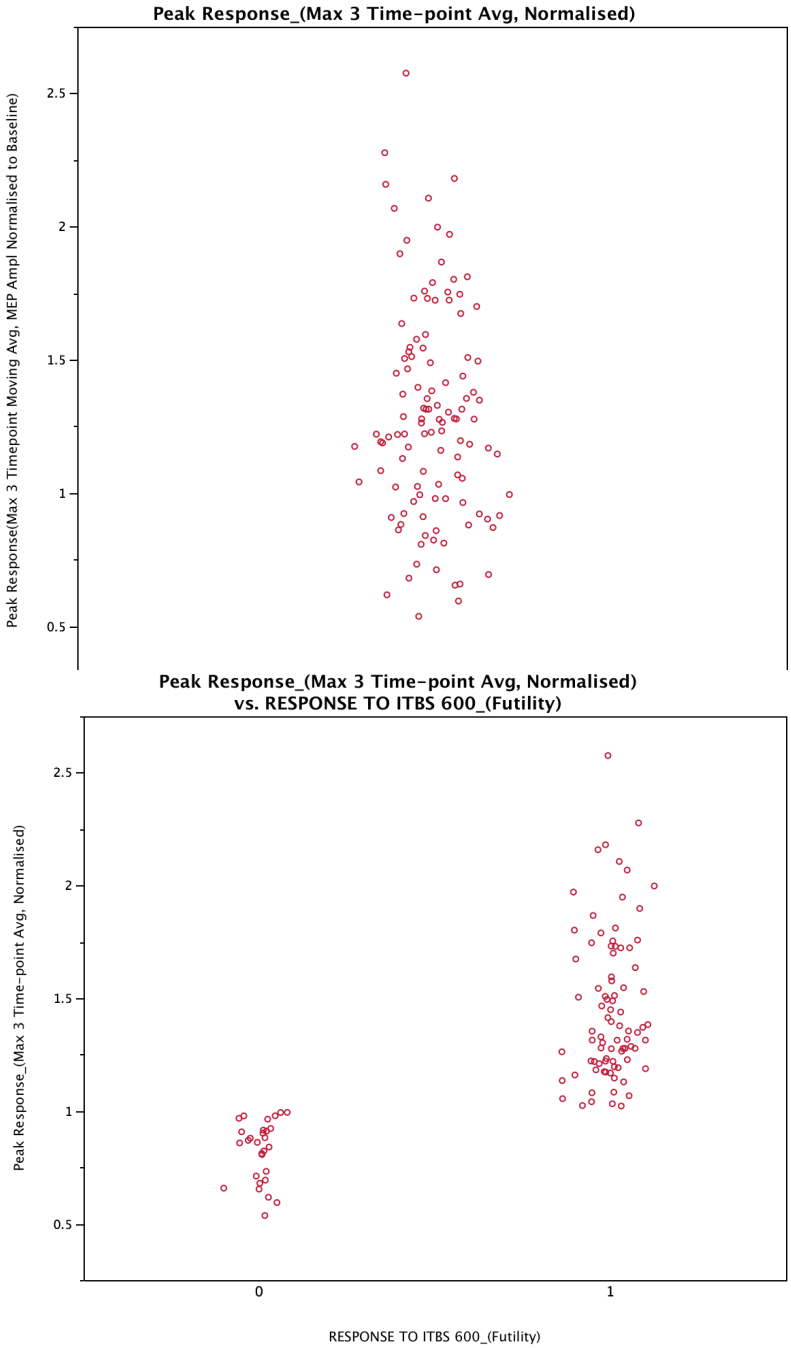


This variable is designed to capture if the average response over 21 minutes post iTBS is the expected increase in MEP amplitude (signifying an effective increase in corticospinal excitability over 21 min post-iTBS). The sustained response categorical variable is calculated by normalizing the average of MEP amplitudes obtained at all time-points over between minutes 3 and 21(Mean (Post- iTBS Min 3-21) MEP Amplitude) to the pre-iTBS baseline MEP amplitude (Mean (Baseline) MEP Amplitude) (plotted in Figure 6A. A value of ≤1 signifies that there was no sustained increase in corticospinal excitability produced by iTBS-600, and is coded by State 0 in Fig 6B. A value >1 signifies a sustained increase in corticospinal excitability over 21 minutes following iTB 600, independent of the degree of that increase, and is coded for by State 1 in Fig 6B.

6.3.2 Futility

This categorical variable was designed to capture if there was any experimentally or therapeutically useful window of increased corticospinal excitability produced by iTBS 600. We arbitrarily determined the 'useful window' to be any 3 consecutive time-points. As the MEP amplitude measurements were taken 3 minutes apart following iTBS, this window is book-ended by measurements 6 minutes apart but signifies a 9-minute window of response. This variable is calculated by determining the maximum average MEP amplitude over any three consecutive time-points after iTBS for each subject (maximum value obtained in a 3 time-point moving average), and then normalizing it to baseline MEP amplitude for each subject. A value for this outcome measure of >1, signifies that there was some net positive modulation of corticospinal excitability over 3 consecutive time-points, at some point between 3-21 minutes after iTBS 600, independent of the degree of response and when that response occurred, coded for by State 1. A value of ≤1 is coded by State 0 and signifies that iTBS 600 did not increase corticospinal excitability for even a 6-9 minute window.

Figure 6-2: Understanding the Futility categorical variable



This categorical variable was designed to capture if there was any experimentally or therapeutically useful window of increased corticospinal excitability produced by iTBS 600. We arbitrarily determined the 'useful window' to be any 3 consecutive time-points. As the MEP amplitude measurements were taken 3 minutes apart following iTBS, this window is book-ended by measurements 6 minutes apart but signifies a 9 minute window of response. This variable is calculated by determining the maximum average MEP amplitude over any three consecutive time-points after iTBS for each subject (maximum value obtained in a 3 time-point moving average), and then normalizing it to baseline MEP amplitude for each subject. The values obtained are shown in Fig 7A. A value for this outcome measure of >1, signifies that there was

some net positive modulation of corticospinal excitability when averaged over 3 consecutive time-points, at some point between 3-21 minutes after iTBS 600, and is coded for by State 1 in Figure 7B. It is independent of the degree of that response and when the response occurred. A value of ≤1 is coded by State 0 and signifies that iTBS 600 did not increase corticospinal excitability for even a 6-9 minute window.

6.3.3 Hierarchical clustering

Finally, we used Hierarchical clustering, an unsupervised multivariate technique of grouping rows that share similar values together, to determine if there are underlying patterns of response to iTBS. Effectively this tells us whether we should expect individuals to have a particular time course(s) of response to iTBS. MEP Amplitudes, normalized to baseline MEP amplitude, at minute 3,6,9,12,15,18 and 21 (8 levels) across 115 subjects was used. Hierarchical clustering is a process that starts with each point in its own cluster. At each step, the two clusters that are closest together are combined into a single cluster until there is only one cluster containing all the points. In Ward's minimum variance hierarchical clustering method, the distance between two clusters is the ANOVA sum of squares between the two clusters added up over all the variables. To determine the number of clusters in the data, a scree plot, that plots the distance that was bridged to join the clusters at each step, was done, showing a natural break ('elbow') around 3-5 clusters where the distance jumps up suddenly.

6.4 Results

Demographics of the study sample are shown in Fig 6-3. The majority of the participants were young, between 18-21 years and approximately one quarter were female. 90% had not participated in any previous rTMS study. As expected from a typical European population in northern Spain, almost one third carried the less common val66met polymorphism of BDNF. Most of the evaluations were conducted in the afternoon.

Figure 6-3: Cohort Demographics.
Distribution of subjects by Age, BDNF Val66Met Genotype, Sex and Time of Day.

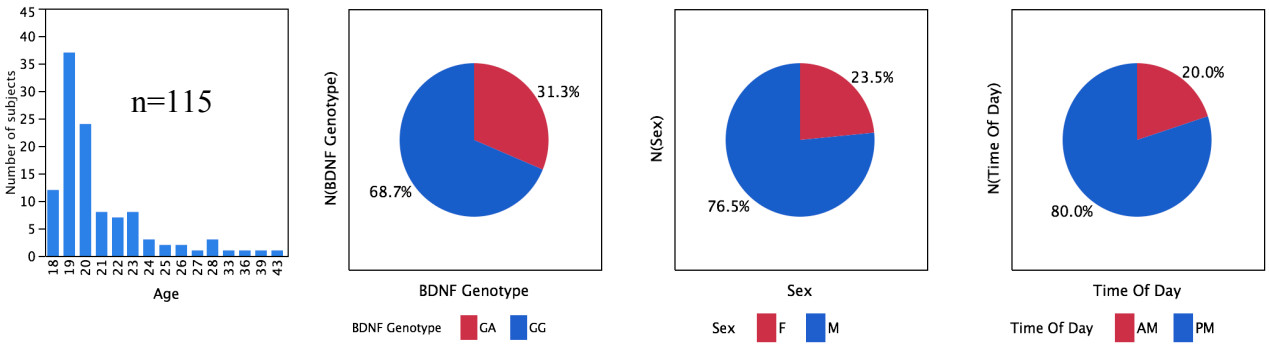
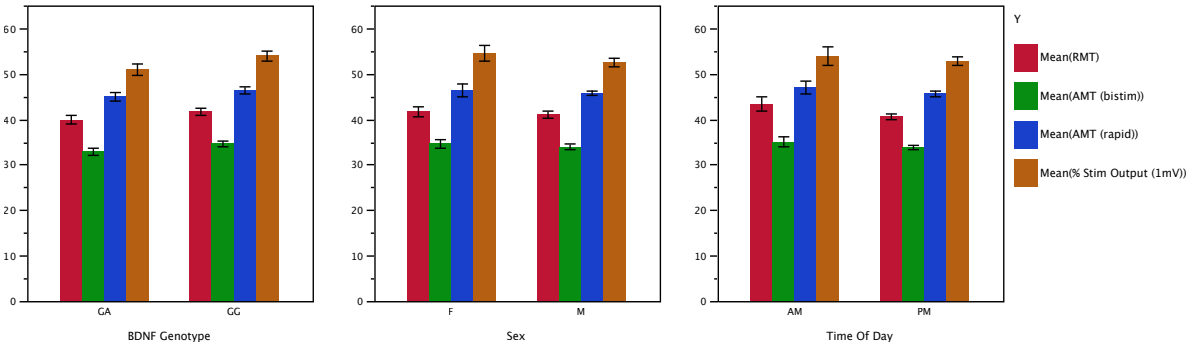


Figure 6-4: Mean values for RMT, AMT (bistim and rapid Magstim packages), Stimulator output for 1 mV baseline MEP

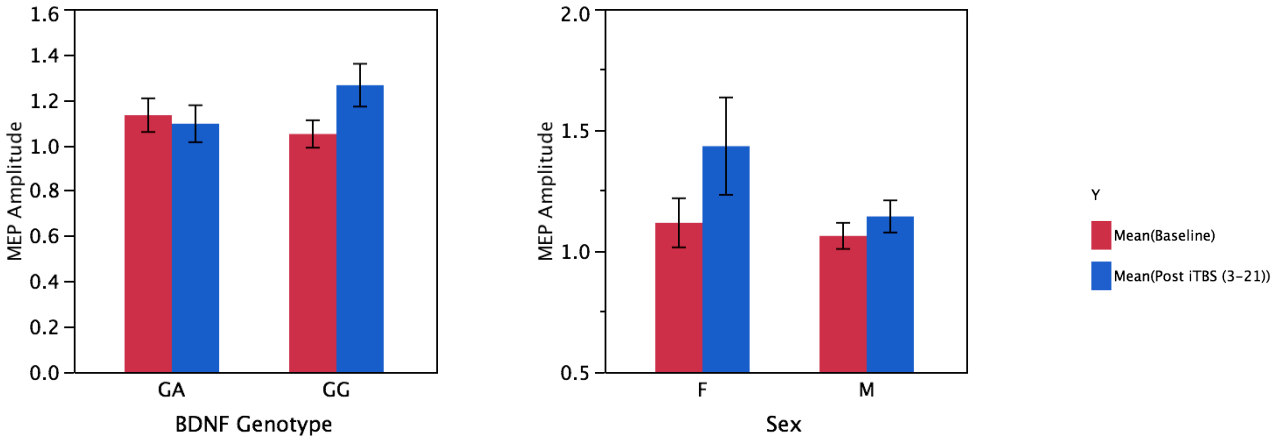


A scatter plot of all the data is shown in Fig 6-1: values greater than 1 indicate that a participant had mean increase in MEP over the 3-21 min post-iTBS. The majority of individuals had a positive response, indicating that iTBS increased MEPs over the period of testing. The mean effect was an increase in MEP amplitude to 113% baseline size.

Table 6-1: Effect of iTBS 600

Variable	Mean	Std Dev	Number
Baseline	1.075162	0.509124	115
Post iTBS (3-21)	1.211133	0.748648	

Figure 6-5: Testing factors previously known to affect the response to rTMS.



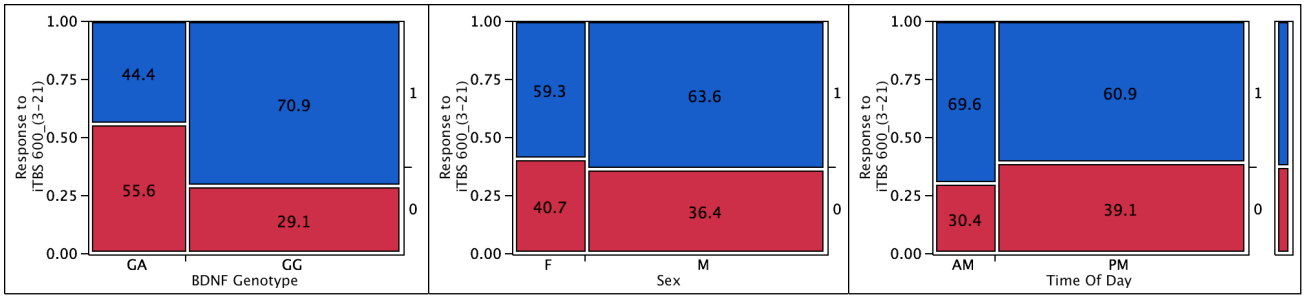
Three Separate 2 way repeated measures ANOVAs, combining the main factor of TIME (pre-iTBS versus the mean post-iTBS average from 3-21min) with one of the three independent factors of BDNF genotype (val66val or val66met), sex (M/F) and time of day (AM or PM) were performed. These revealed a significant TIME x Genotype ($F(1,111)=8.6$; $p= 0.004$) and Time x Sex interaction ($F(1,111)=5.7$; $p=0.02$). There were no significant effects of time of day. Post-hoc paired t-tests showed that there was a significant increase in MEP amplitude following iTBS600 for the group with the BDNF Val/Val (GG) genotype but no response for the Val/Met (GC) group. There was a slightly greater effect of iTBS in females, although there was a significant increase in MEP amplitude following iTBS600 for both male ($p=0.01$) and female ($p=0.02$) subjects (Fig 6-5).

6.4.1 Sustained response to iTBS

In the categorical analysis, individuals with a sustained effect of ≥ 1 were assigned a value of 1; the remainder were assigned a value of 0. The results grouped according to genotype, sex and time of day are shown in Fig 6-6. The combined analysis over all individuals is shown in the rightmost column. It indicates that just fewer than 40% of participants had no sustained response to iTBS. The rate of sustained response to iTBS by BDNF Genotype, Sex, and Time of Day was tested with 2 tailed Fisher's Exact tests; there was a significant effect of BDNF genotype ($p=0.012$) indicating that individuals with

the val66met polymorphism were less likely to have a sustained response than those with the more common val66val polymorphism. No other effects were significant, indicating that presence of a sustained response was independent of time of day or of sex.

Figure 6-6: Sustained Response to iTBS 600 Mosaic Plot

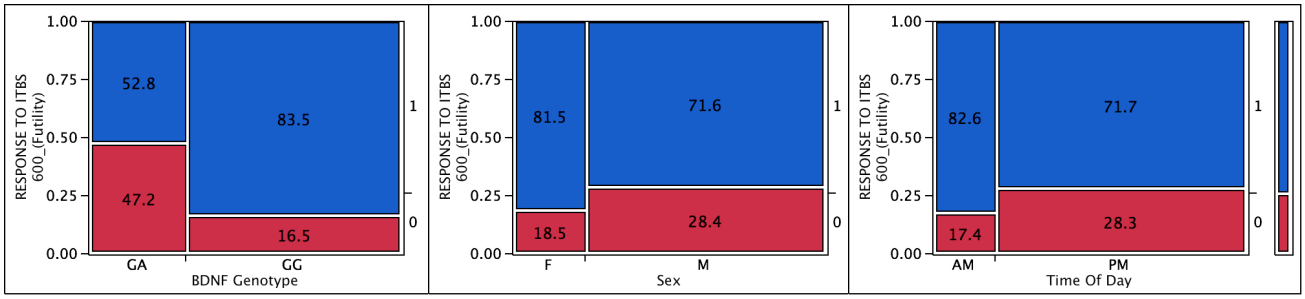


6.4.2 "Futility" of response to iTBS

The term "futility" refers to the inability of an intervention to achieve its objectives. For example, if iTBS is being employed to enhance the effect of physical therapy after stroke then we might consider that we need at least 10min sustained positive interaction if we are to achieve any measureable effect. Thus we would consider that it would be futile to use iTBS if it failed to increase corticospinal excitability for 10min. In this analysis we assigned a categorical value of 1 to all individuals who had a mean post-iTBS increase in MEP $\geq$1 when averaged over 3 consecutive time points (i.e. for at least 9 min). Individuals who never achieved a mean increase for this duration were

assigned a value of 0. Fig 6-7 plots the data separated by genotype, sex and time of day. The combined analysis over all individuals is shown in the rightmost column. It indicates that approximately 25% of participants had no persisting response to iTBS. Rate of Futility of iTBS by BDNF Genotype, Sex, and Time of Day was tested with 2 tailed Fisher's Exact tests; there was a significant effect of BDNF genotype (p=0.001) indicating that individuals with the val66met polymorphism were less likely to have a response than those with the more common val66val polymorphism.

Figure 6-7: Futility Measure Mosaic Plot



6.4.3 Time Course Analysis: Clustering

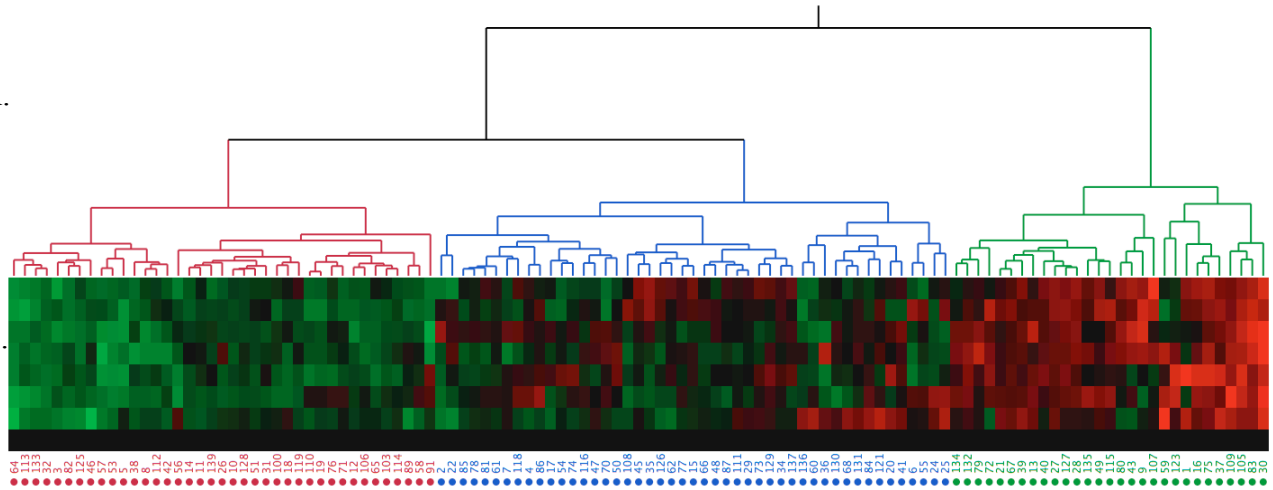
This analysis tests whether the time course of the response to iTBS follows particular patterns in different subjects. The results are plotted in Fig 6-8. Fig 5B colour codes the time course of response for all 115 individuals. The

numerical identifier of each person is shown at the bottom of the colour plots (Fig 6-8B). The column above each number consists of 8 rectangular rectangles that are coloured according to the normalized amplitude of the MEP at each time point studied. The bottom rectangle is black in all cases (=1). Green rectangles indicate an MEP ≤1; red rectangles a value of >1; intensity of colour indicates increasing or decreasing values. Visual inspection suggests that there are 3 main groupings. There is a mainly red group on the right and a mainly green group on the left, with a mixed group in-between. This was borne out using Ward's hierarchical clustering and is indicated by the dendrogram of Fig 6-8A above. The mean time courses from the 3 main groups are plotted in Fig 6-8C. They consist of a group with a sustained large response, an intermediate group with a much smaller but still positive response characterized by a late increase in MEP amplitude similar to that originally reported (Huang et al., 2005), and a group with a negative response to iTBS. The categorical analysis of these groups according to genotype, sex and time of day is plotted in Fig 6-8D. The mean data (given in the narrow column on the extreme right) show that approximately one third of the population has a negative time course, whereas the remainder has a positive effect. Of these, the majority have the intermediate facilitation with a smaller proportion showing a much larger sustained effect. The Val/Met group contains an excess of the negative response type (chi sq= 12.2; p=0.0024) while females tended to have more of the sustained large response than males (chi Sq = 6.5, p=0.04). There was no effect of time of day.

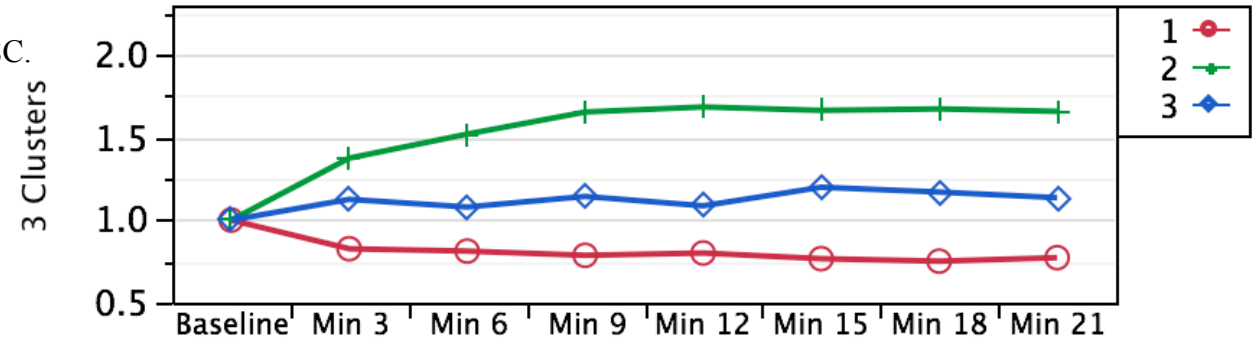
Figure 6-8: Fig 6-8A - Dendrogram with 3 clusters, Fig 6-8B - Colour map, Fig 6-8C - Time-series plot of 3 clustered MEP response patterns to iTBS 600, normalized to baseline pre-iTBS MEP amplitude, Fig 6-8D - Mosaic plot of BDNF Genotype, Sex and Time of Day by Cluster.

8A.

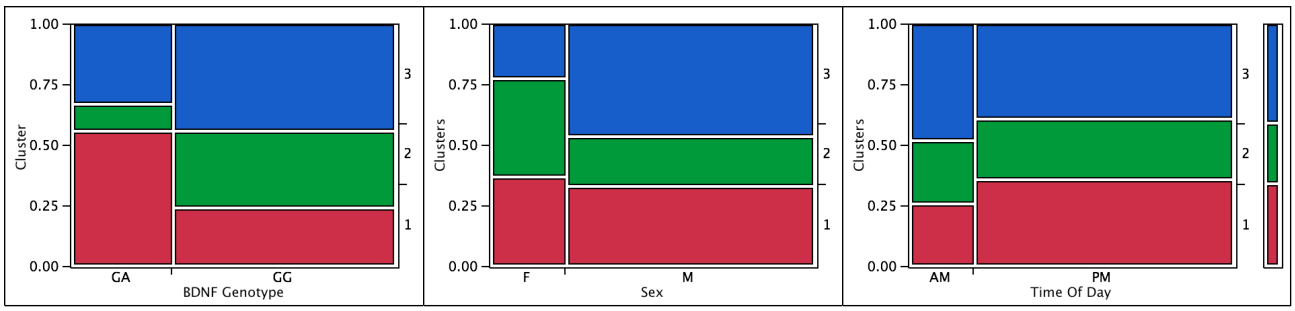
8B.



8C.



8D.



At the bottom of figure 8B, the row of numbers lists every participant by the numeric portion of the alphanumeric study ID. The color map above this row plots the value of the data on a graduated color scale for each subject to provide a visual indicator on how the clustering process arrives at its conclusions. Each column in the color map codes an individual subject's response to iTBS 600 across individual time-points from pre-iTBS Baseline MEP amplitude at the bottom to Minute 23 post iTBS at the top. A MEP amplitude equal to Baseline is coded black, an increasing MEP amplitude is coded by brighter shades of red and an inhibition of MEP's is coded by brighter shades of green. The colour map does not follow the color scheme of the Fig 8A and Fig 8C. The clustering sequence itself is visualized with the help of the dendrogram, a tree diagram that lists each individual subject's response to iTBS, shows which cluster the response pattern is in and when it entered the cluster (Fig 8A). The dendrogram is drawn to scale, showing the actual joining distance (based on the F-statistic) between each join point, and is the same scale used on the scree plot. Fig 8C is a time-series plot showing the mean MEP Amplitude for each cluster. Figures 8A, 8C and 8D share the same color scheme.

6.5 Discussion

This is the largest study to date of the variation between individuals in response to a standard “plasticity enhancing” TMS paradigm, iTBS. The results show that there is a wide variation in the effect on corticospinal excitability as measured by the MEP evoked by a standard single TMS pulse: in some individuals, MEPs double in size whereas in others, MEPs are reduced by half. On average, responses increase to 113% baseline, which is in line with other values published in the literature following the initial report of Huang et al. (Huang et al., 2005) (Talelli et al., 2007).

6.5.1 Clinical implications of the results

Since iTBS, like many other rTMS/TDCS protocols is being used in a number of therapeutic trials we analysed the data in terms of the proportion of individuals who would be expected to have a positive response in a typical trial by assuming that a positive therapeutic effect can be directly predicted by a positive response to iTBS. If therapeutic benefit requires a sustained 20 min response to iTBS, then 37% of individuals would fail to respond. This outcome measure is relevant to studies employing rTMS as a ‘virtual lesion’ in behavioral and connectivity studies, as they require a consistent response (either facilitation or inhibition of corticospinal excitability) over the post-rTMS period. Without a sustained excitatory response, the experimental blocks done at e.g. minute 5 may vary from that at minute 15. As shown in the color

map (fig 6-8B), the timing of the excitatory response can be very early or very late.

However, there may be instances when a useful therapeutic response requires only that iTBS is effective for only 6-9 min. In this case a smaller proportion of approximately 25% would fail to respond. This outcome measure might be relevant for studies that employ rTMS as a therapeutic adjunct. Very often, iTBS or other protocols are added to standard physiotherapy, or robotic training, in stroke rehabilitation. In such circumstances it is reasonable to suppose that patients might benefit from a boost in corticospinal excitability and enhancement of therapy, even if it is only for 6-9 minutes.

6.5.2 Implications of clustering analysis

Hierarchical clustering analysis has not been used on rTMS data previously. Here it revealed 3 main clusters that define the typical time courses of response within the population (Fig 6-8C). Approximately one third of individuals that tended to group around the inhibitory response pattern (clustered response pattern 1), about 20% were grouped around an extreme facilitatory response (clustered response pattern 2) while the majority were similar to a more modest response pattern with an early and a late peak (clustered response pattern 3). The latter is similar in time course, but lower in magnitude than the mean data in the original report of Huang et al (2005). Suppression of MEPs after iTBS has not been noted in the literature very

frequently, although it is evident in the responses of some individual subjects in the data of Talelli et al (Talelli et al., 2007).

Fig 6-8D plots proportion of subjects in each cluster pattern by BDNF genotype, Sex and Time of Day. Fig 6-8D suggests that the results obtained in the 2 way repeated measures anova for BDNF genotype may be explained by the higher proportion of the inhibitory response pattern 1, and that the effect of sex in that analysis may explained by the proportion of response patterns 2 and 3. It is reassuring to note that there is little change in proportion of subjects falling into different response patterns with variation in the timing of the experiment.

If the hierarchical clustering is done for 4 and 5 clusters (see figures in supplemental data), response pattern 2 decomposes into a progressively rising and early sustained response (see Fig 6-9) and followed by a split in response pattern 3 into an early (first peak at minute 3) and late (first peak at minute 9) bimodal response (see Fig 6-10), as reported by Huang et al. in the original paper on human Theta Burst Stimulation) (see supplemental data, Fig 9). The clustered response patterns remain significantly different (main effect and cluster-time interaction) even at 5 clusters when tested with 2-way repeated measures Anova. The study is adequately powered to detect up to 7 clusters.

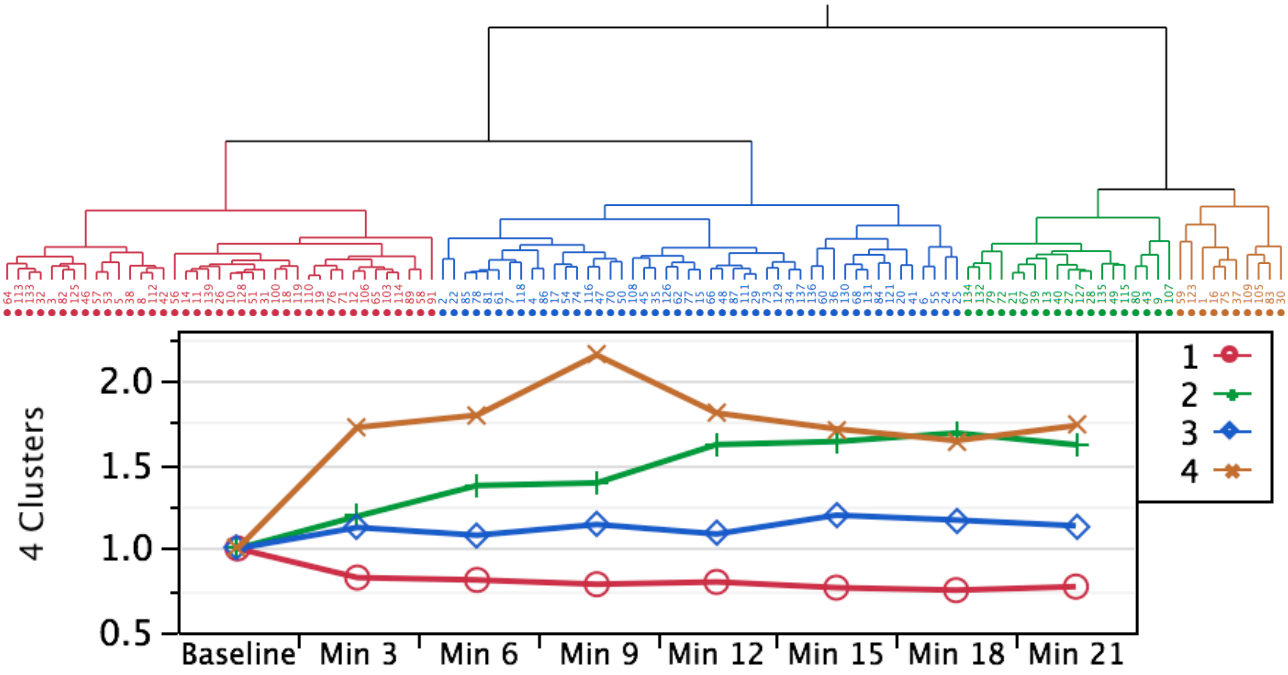


Figure 6-10:
Fig 6-9A. Dendrogram with 4 clusters.
Fig 6-9B. Time-series plot of 4 clustered MEP response patterns to iTBS 600, normalized to baseline pre-iTBS MEP amplitude.

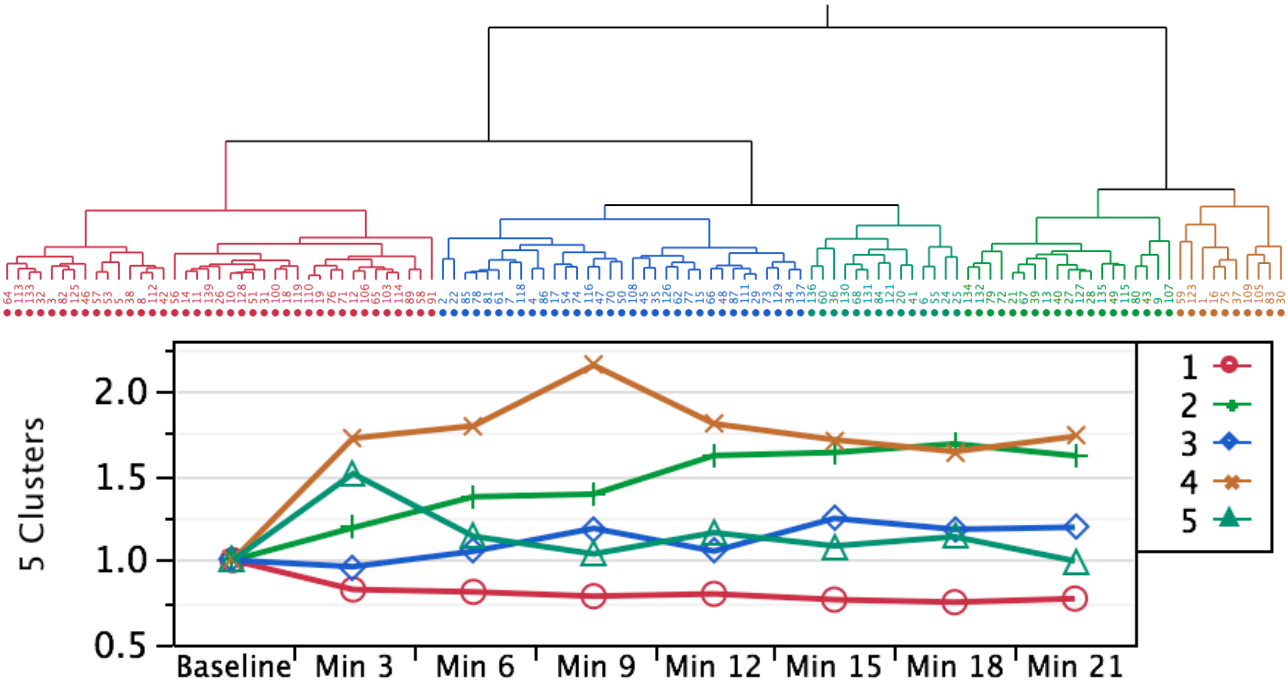


Figure 6-9:
Fig 6-10A. Dendrogram with 5 clusters.
Fig 6-10B. Time-series plot of 4 clustered MEP response patterns to iTBS 600, normalized to baseline pre-iTBS MEP amplitude.

This study does not shed light on why these factors (BDNF genotype and Sex) influence the response to iTBS. With over 90% of subjects in this study never having participated in rTMS experiments before, it is necessary to explore the effects of novelty and anxiety on the response to iTBS, particularly with the reported effects of the BDNF Val66Met SNP on anxiety and depression. An extension of this study is designed to provide those insights. Another caveat is that, by design, this study does not address age as a variable that is likely to influence the variability in response. A much larger sample size is necessary to study the effects of age fully alongside other factors, and this was not feasible due to resource restrictions. One suggestion that has been raised is that iTBS may be affecting other parameters, other than corticospinal excitability as measured by MEP amplitude. However, as the paradigm (as are most rTMS paradigms), was defined by the effects on MEP amplitude, we do not feel this is a valid explanation.

In summary, of the previously recognized variables affecting the response to rTMS paradigms, BDNF genotype and sex of subject show a significant interaction with time. The BDNF Val66Met polymorphism (rs6265) (GA genotype) alone shows higher rates of futility and lower rates of sustained facilitation of MEPs following iTBS 600. The response to rTMS paradigms like iTBS maybe more complex and variable than previously recognized, and it may account for the inconsistent results across rTMS studies.

7 Conclusions

Following our initial study into the effects of the BDNF Val66Met SNP, Antal et al. retrospectively analyzed experimental data collected and identified subjects with the Val66Met allele. For iTBS (15 subjects, 5 heterozygotes), plasticity could be only induced in the Val66Val allele carriers. However, for facilitatory tDCS (24 subjects, 10 heterozygotes), as well as for inhibitory tDCS, (19 subjects, 8 heterozygotes), carriers of the Val66Met allele displayed enhanced plasticity.

The conclusions of this study were not dissimilar to our own pilot study - 'met' allele carriers failed to respond to iTBS 600. However the final study presented here suggests that the effect of the met allele on the response to rTMS paradigms like iTBS maybe more complex than previously recognized. One explaination for the spectrum of responses to the same iTBS protocol would be consistent with the idea that TBS produces a mixture of inhibitory and excitatory effects. According to the model of Huang et al (Huang et al., 2010), the proportion of each can be modulated by the pattern of TBS, being primarily facilitatory with iTBS and inhibitory with cTBS. Variation between individuals in the proportion of inhibition/facilitation for a given pattern of TBS could be one explanation of the variation in responses we observed here. Indeed, the high response group that was revealed in the hierarchical cluster analysis could represent individuals with a bias towards facilitatory effects of TBS whereas the negative responders in the same analysis would have a bias towards inhibitory effects. However another insight into why this common

SNP in a highly conserved polymath gene would influence the response to TMS paradigms may come from the work of Gentner et al (Gentner et al., 2008a).

The BCM equation states as its third postulate that the recent history of synaptic activity determines the crossover point (the modification threshold - θ_m) between weakening and strengthening of synaptic weight. When cTBS is preceded by a period of muscle contraction, the post synaptic activity would shift θ_m to the right, and subsequent presynaptic input is more likely to be a weakening of synaptic weight resulting in a decrease in corticospinal excitability. Without the recent history of postsynaptic activity, the same presynaptic input is more likely to produce an excitatory response.

The results obtained here can be explained if the met allele shifts θ_m to the right. The response probability for a fixed presynaptic input to result in weakening of synaptic strength (and subsequent reduction in corticospinal excitability assessed by MEP's) would be higher, resulting in a greater proportion of met allele carriers having an inhibitory response to iTBS 600.

Huber et al. investigated the effects of BDNF on long-term potentiation (LTP) and long-term depression (LTD) in visual cortex slices in rats (Huber et al., 1998). The slices treated with BDNF showed no difference from control slices when a 'strong' tetanus was used (theta-burst stimulation) to elicit a maximal level of LTP but displayed significantly greater synaptic potentiation in

response to a 'weak' (20 Hz) tetanus. The BDNF-treated slices also showed significantly less LTD in response to a1 Hz tetanus. They concluded that BDNF alters the relationship between stimulation frequency and synaptic plasticity in the visual cortex, shifting the modification threshold to the left. The cellular phenomena corresponds best with shifts in modification threshold is synaptic scaling (Turrigiano, 2008) and BDNF was the first molecule implicated in synaptic scaling (Rutherford et al., 1998). Abidin et al. showed that chronic reduction in the expression of BDNF attenuates the efficiency of presynaptic glutamate release in response to repetitive stimulation and subequently impairs presynaptically evoked LTP in the visual cortex in BDNF heterozygous knockout BDNF(+/-) mice (Abidin et al., 2006). As the met allele reduces activity dependent synaptic release of BDNF, the suggestion that a rightward shift in the modification threshold in heterozygotes compared to val/val homozygotes has basis in studies of LTP/LTD induction in slices.

Other factors, both intrinsic immutable factors like other genetic polymorphisms, sex of subject or variable factors like estrogen levels/ fatigue/ attention (Inghilleri et al., 2004)(Stefan et al., 2004) (Tecchio et al., 2008) may similarly affect the response to fixed presynaptic input by affecting θ_m or postsynaptic depolarization. The net effects on θ_m would determine the individual difference in response to an identically conducted rTMS experiment, resulting in the spectrum of responses reported here. Immutable factors would account for a significant portion of inter-individual variation in the

response to rTMS. Mutable factors would determine the test-retest intra-individual variability.

Recognition of the variables that influence response to rTMS will enable us to better predict an individual's response to rTMS paradigms, and this in turn is necessary for the success, safety and replicability of studies employing rTMS paradigms. Missitzi et al. (Missitzi et al., 2010) probed the heritability of corticospinal excitability changes induced by the paired-associative stimulation paradigm 32 healthy female twins (9 monozygotic and 7 dizygotic pairs). Intra-pair differences in the changes in MEP amplitudes measured at 25-30 min post intervention were almost double for dizygotic twins (1.25) in comparison to monozygotic twins (0.64). The heritability estimate for brain plasticity was found to be 0.68. Recognizing and quantifying the role of immutable factors like genotype alone therefore will be a significant advance in our ability to predict the response to rTMS paradigms. The effects of mutable factors will need to be controlled for by adopting larger study sample sizes than has been the norm, and by establishing normative values for the commonly used TMS outcome measures. In conclusion, we propose that the experiments presented here make a strong case for the study of the effects of human genetic variation on cortical plasticity, employing neurophysiological outcome measures and the application of artificially induced plasticity paradigms.

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