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Study of neurophysiological responses associated with the application of magnetic fields to the brain - repetitive Transcranial Magnetic Stimulation by Theta Burst

Nuno Filipe Cardoso Pinto

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Supervisor: Maria da Assunção Morais e Cunha Vaz Patto, MD, PhD
Co-supervisor: Jorge Manuel dos Reis Gama, PhD

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Jury:
President: Joaquim Mateus Paulo Serra, PhD
Arguer: Miguel de Sá e Sousa Castelo-Branco, MD, PhD
Arguer: Albino Jorge Carvalho de Sousa Oliveira-Maia, MD, PhD
Graça Maria Fernandes Baltazar, PhD
Maria da Assunção Morais e Cunha Vaz Patto, MD, PhD
Gisela Maria Henrique Maia, PhD

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Dedication

To my Family.

You are always in my heart.

"If I have seen further, it is by standing upon the shoulders of giants."

Sir Isaac Newton

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Thesis overview

This thesis is structured into eight chapters:

Chapter one: In this chapter – Introduction – we intended to survey and detail the essential information regarding transcranial magnetic stimulation, focused on the scope studied in the thesis. A guided literature approach was implemented, addressing elementary notions, historical background, basic principles, and technical details about transcranial magnetic stimulation and theta burst stimulation, in order to contextualize the topic and the purposes that will be explained and detailed in the following chapters. A full section is specifically dedicated to theta burst stimulation, the problems related to its use, doubts about its scope in some critical cortical regions. Possible methodologies to evaluate outcomes, such as the auditory P300, are contextualized.

Chapter two: Aims - In this chapter a detailed exposure of the research questions, hypotheses and objectives of the current thesis is presented.

Chapters three to seven: These chapters contain the essential research carried out attempting to fulfil the objectives defined in aims. Four scientific articles published in peer-reviewed journals are presented. The core of a registered patent using theta burst stimulation is also addressed.

Chapter eight: In this chapter, a critical integrative discussion is presented, focused on addressing the main research findings, and establishing connections between the studies. Future possibilities for innovative research that may complement the main findings are presented, and also possible applications for the achieved knowledge. Concluding notions regarding all the work of this thesis close this chapter.

Publications

Research included in this thesis lead to the publication of the following papers in indexed peer-reviewed journals:

Publication as First Author

- Pinto N, Duarte M, Gonçalves H, Silva R, Gama J, Pato MV. Bilateral theta burst magnetic stimulation influence on event-related brain potentials. PLoS One. 2018 Jan 5;13(1):e0190693. DOI:10.1371/journal.pone.0190693

- Pinto N, Duarte M, Gonçalves H, Silva R, Gama J, Pato MV. Theta burst stimulation is able to impact cognitive processing: A P300 and neuropsychological tests study. Neuropsychobiology. 2021; 1–11. doi:10.1159/000511605

- Pinto N, Gonçalves H, Silva R, Duarte M, Gama J, Vaz Pato M. Theta burst stimulation over the prefrontal cortex: effects on cerebral oximetry and cardiovascular measures in healthy humans. Neurosci Lett. 2021; 135792. doi:10.1016/j.neulet.2021.135792

- Pinto N, Oliveira I, Ferreira J, Gama J, Vaz Pato M. Can theta burst stimulation safely influence auditory hearing thresholds in healthy young adults? Clinical Neurophysiology. 2019 Oct;130(10):1900–7. DOI:10.1016/j.clinph.2019.07.019

Patent

- Patent register n. º 109800 - Method for repetitive transcranial magnetic stimulation on Creativity

Other TMS/TBS related publications during the Phd

Publications as Co-Author

- Roque C, Pinto N, Vaz Patto M, Baltazar G. Astrocytes contribute to the neuronal recovery promoted by high-frequency repetitive magnetic stimulation in in vitro models of ischemia. J Neurosci Res. 2021; jnr.24792. doi:10.1002/jnr.24792

- Tuna AR, Pinto N, Brardo FM, Fernandes A, Nunes AF, Pato MV. Transcranial Magnetic Stimulation in Adults With Amblyopia. J Neuro-Ophthalmology. 2020 Jun;40(2):185–92. DOI:10.1097/WNO.0000000000000828.

Resumo alargado

A estimulação magnética transcraniana (EMT) é uma técnica de diagnóstico e terapêutica não invasiva, que tem vindo a evoluir nos últimos 35 anos. A aplicação terapêutica da forma repetitiva da EMT (EMTr), tem vindo a demonstrar a sua utilidade científica e clínica, com aplicação em várias doenças neurológicas e psiquiátricas como a depressão major, a perturbação obsessivo-compulsiva, dor e reabilitação em doentes com acidentes vasculares cerebrais, ainda que as principais bases subjacentes à sua acção não sejam totalmente compreendidas.

A EMT baseia-se no princípio da indução magnética e na sua capacidade de induzir correntes elétricas no tecido cortical. Esses campos magnéticos (pulsos) originados por uma bobina adjacente ao couro cabeludo originam um fluxo iónico intracraniano que irá provocar a despolarização da membrana neuronal, desencadeando assim um potencial de ação. Embora a EMT exerça os seus efeitos predominantemente na área cortical adjacente à bobina, os potenciais de ação induzidos espalham-se trans-sinapticamente, originando a propagação da ativação para regiões corticais e subcorticais vizinhas pertencentes à rede neuronal em questão. Parece ocorrer ainda a aparente capacidade de influenciar a função do hemisfério contralateral à estimulação possivelmente por mediação calossal.

Os efeitos da EMTr ao nível da modulação da excitabilidade neuronal estão intrinsecamente dependentes das características da estimulação, nomeadamente ao nível da frequência e padronização dos estímulos. A aplicação de frequências inferiores ou iguais a 1 Hz (EMTr de baixa frequência) são associadas à indução de um efeito inibitório neuronal, enquanto que a aplicação de frequências acima de 1 Hz, normalmente acima dos 5 Hz (EMTr de alta frequência), podem induzir um efeito excitatório. Em 2005 surgiu uma forma padronizada de aplicação dos pulsos magnéticos, denominada Theta Burst Stimulation (TBS), na qual grupos de 3 pulsos com alta frequência (bursts de 50Hz) são enviados a cada 200 milissegundos (5 Hz – frequência teta), implicando normalmente a aplicação de 600 pulsos por cada sessão de estimulação. Este é um protocolo que assume particular importância pela sua rápida aplicação, levando menos de 3 minutos a executar, sendo significativamente mais célere do que os protocolos clássicos de EMTr (que podem exceder 30 minutos). Efeitos neuromodulatórios opostos podem ser igualmente induzidos com TBS, sendo que a aplicação ininterrupta da estimulação durante 40 segundos – TBS contínua (cTBS) –

parece originar uma diminuição na excitabilidade cortical com uma duração de até 50 minutos pós-estimulação, enquanto que a aplicação de apenas 2 segundos de TBS intervalada por 8 segundos de pausa – TBS intermitente (iTBS) – durante 190 segundos, terá a capacidade de induzir aumento na excitabilidade cortical até cerca de 60 minutos pós-estimulação.

Apesar do volume significativo de investigação acumulada na estimulação com EMTr e TBS, demonstrando a sua capacidade modulatória e a sua aplicabilidade na prática clínica, a investigação dos seus efeitos sobre algumas funções corticais superiores como a cognição ou os efeitos da aplicação em algumas regiões corticais menos estudadas como a região temporal tem sido mais limitada (principalmente com a TBS) e apresentado alguns resultados contraditórios.

O córtex pré-frontal assume particular importância associado à aplicação da EMTr/TBS dada a extensa rede de conexões com outras regiões corticais (como o córtex motor, o córtex sensitivo, a amígdala, o tálamo e o hipocampo), importantes em doenças como a depressão (desequilíbrio inter-hemisférico pré-frontal verificado por neuroimagem), e ainda pela sua aparente capacidade de influenciar funções autonómicas e cardiovasculares. Meta-análises como a de Lowe et al. 2018, avaliando os efeitos da TBS sobre o córtex pré-frontal, revelam que parece existir um efeito negativo no desempenho das tarefas de função executiva após estimulação com cTBS e um efeito positivo mas em menor grau após estimulação com iTBS. No entanto, o efeito mais definido da estimulação sobre as várias dimensões cognitivas permanece envolto em alguma dúvida, dado que por um lado têm surgido alguns resultados negativos e por outro lado a maioria dos estudos tem usado populações relativamente pequenas, com infrequente recurso a grupos sham. Um dos principais problemas na avaliação dos possíveis efeitos da estimulação magnética repetitiva prende-se com o uso de diversos métodos de avaliação, com diferentes sensibilidades para o estudo das várias dimensões cognitivas, ou ainda com técnicas com menor resolução temporal (como os estudos de imagem cerebral funcional) comparativamente a técnicas neurofisiológicas. Neste ponto, a utilização de estudos no âmbito da neurofisiologia, como os potenciais de longa latência, pode assumir particular importância.

O P300 auditivo, é um potencial evocado cognitivo, dependente da atenção e capacidade de discriminação do sujeito, traduzindo estadios mais superiores ou avançados de processamento associado a uma tarefa. As origens neuronais do P300 são múltiplas e bi-hemisféricas, associando-se a regiões como o hipocampo, o córtex pré-frontal ventrolateral e o córtex cingulado posterior. Até à data, são raros os estudos que

abordaram a associação entre o P300 auditivo e a EMTr e ainda mais raros combinando a estimulação com TBS e o P300. A avaliação dos resultados prévios sugere que a estimulação magnética pode ser capaz de influenciar o processamento cognitivo e que as alterações podem ser monitorizadas pelo P300, mas são encontrados alguns resultados contraditórios, existindo significativas discrepâncias na metodologia usada.

O uso da EMT em outros domínios cognitivos como a criatividade é ainda mais raro. Como parte das redes neuronais envolvidas no processo criativo ou pensamento divergente estão associadas a algumas das áreas-alvo geralmente usadas para ativação cognitiva, especificamente o córtex pré-frontal, pareceu-nos importante estudar se esta dimensão cognitiva poderia ser também influenciada pela TBS.

Ainda associado ao uso da estimulação magnética transcraniana sobre o córtex pré-frontal, salienta-se o ainda escasso conhecimento científico sobre a possível influência da EMT nas funções associadas ao sistema nervoso autónomo. Aparentemente, a activação de redes neuronais associadas à atividade simpática pós EMTr/TBS, principalmente se aplicada ao córtex pré-frontal dorsolateral, podem influenciar a oxigenação cerebral, o fluxo sanguíneo cerebral, a pressão arterial e até a frequência cardíaca, possivelmente por acção sobre as funções autonómicas cardiovasculares, embora continue a existir discordância significativa entre os raros estudos realizados.

Por outro lado, os efeitos da EMTr/TBS a nível sensorial são ainda pouco conhecidos e o alcance dos efeitos modulatórios na estimulação do córtex auditivo permanecem inconclusivos. Alguns estudos sugerem um possível efeito positivo na função auditiva de doentes com perda auditiva neurossensorial súbita ou com tinnitus, com melhoria dos limiares auditivos, mas também neste caso os resultados não são unânimes. Associada a este factor, é igualmente importante mencionar a segurança dos protocolos aplicados. Embora sendo considerada uma técnica segura, o ruído da EMTr e TBS em intensidades mais elevadas pode originar em alguns doentes o agravamento da hiperacusia e até hipersensibilidade sonora após as sessões, levando a que seja aconselhável proteção adicional como o uso de tampões de ouvido, principalmente se a bobina for posicionada próxima ao ouvido do doente.

Uma parte significativa do conhecimento científico sobre os efeitos da EMTr e especialmente sobre a TBS deriva de estudos em pacientes, com redes neuronais disfuncionais ou com áreas corticais hipo/hiperativas, o que adiciona desafios para a procura de evidências científicas em indivíduos saudáveis. Deve-se ainda salientar a problemática associada à dicotomia evidente entre a existência de inúmeros protocolos

de estimulação possíveis e as possíveis metodologias e ferramentas utilizadas para mensurar os resultados após a estimulação, tornando difícil a interpretação dos efeitos associados à estimulação cortical.

Perante as incertezas que subsistem relativamente ao alcance dos efeitos neuromodulatórios da estimulação magnética transcraniana repetitiva, especialmente na variante *Theta Burst*, o objectivo primordial desta tese centrou-se no aprofundar do conhecimento científico relacionado ao uso da estimulação TBS no cérebro saudável. Sumariamente, pretendeu-se estudar as respostas neurofisiológicas (como o P300 auditivo), funcionais (como os limiares auditivos) e fisiológicas (como a oximetria cerebral e a pressão arterial) associadas à aplicação de TBS nos córtices pré-frontais e temporais.

Todos os estudos realizados visaram uma população alvo com características similares, constituída por adultos saudáveis, com idades médias de aproximadamente 23 anos e idêntica escolaridade. O equipamento de estimulação magnética utilizado foi um *MagVenture MagPro*® G3 X100 5.0.1, com uma bobina tipo borboleta MCF-B70. O paradigma *Theta Burst* base utilizado foi o descrito por Huang et al. em 2005, com sessões de 600 pulsos, enviados em conjuntos de três pulsos de alta frequência (bursts de 50Hz), repetidos com uma frequência de 5 Hz, nas suas variantes contínua (40 segundos) ou intermitente (190 segundos). A metodologia base das intervenções baseou-se em protocolos duplamente cegos, controlados com grupos submetidos a estimulação sham e com distribuição aleatória pelos respectivos grupos. Globalmente, as análises estatísticas efectuadas basearam-se não só nas comparações pré-pós estimulação mas também na comparação entre os grupos intervencionados e os grupos sham.

O estudo abordado no capítulo III teve como objectivo principal estudar o efeito da TBS no córtex pré-frontal dorsolateral (CPFDL) de ambos os hemisférios cerebrais no processamento cognitivo. O objectivo foi avaliar se o P300 auditivo seria influenciado pela possível modulação cognitiva provocada pela estimulação. Os nossos resultados revelaram que a latência média do pico P300 após a TBS diminuiu apenas quando a iTBS foi realizada no hemisfério esquerdo, traduzindo uma maior rapidez nas respostas. A cTBS aplicada aos hemisférios direito e esquerdo originou um atraso significativo na latência do P300. A avaliação das amplitudes dos potenciais não revelou diferenças significativas. Assim, no nosso grupo de voluntários, a TBS pareceu influenciar efetivamente as redes neurais envolvida na formação do P300 auditivo, e os efeitos parecem distintos para os protocolos iTBS e cTBS (aumento da rapidez no primeiro e lentificação no segundo). Registou-se também um diferente comportamento hemisférico

no caso da iTBS, dado que apenas o lado esquerdo foi influenciado de forma significativa. Estes achados sugerem que o P300 auditivo pode ser uma técnica prática e eficaz para avaliar os efeitos cognitivos associados à estimulação com TBS, principalmente no caso da cTBS.

Os resultados abordados no capítulo IV visaram a aplicação da TBS sobre o CPFDL esquerdo, centrando-se na avaliação da possibilidade de os resultados do P300 auditivo pós-TBS terem alguma relação com os resultados de testes neuropsicológicos - *Trail Making Test* (TMT) e do Teste *Stroop* de Cores e Palavras, dada a documentada relação com o CPFDL. A avaliação pós-TBS revelou que o protocolo cTBS originou a lentificação do P300, influenciando também significativamente o Stroop Interferência e o desempenho esperado no *Stroop* C comparativamente com os grupos submetidos a iTBS e estimulação sham. Nenhum resultado significativo foi encontrado nos testes TMT associado ao uso da TBS, quer inibitória, quer excitatória. Estes resultados sugerem que o P300 e alguns parâmetros do Teste *Stroop* de Cores e Palavras podem ser influenciados de forma semelhante pelo mesmo protocolo de estimulação (cTBS), enfatizando que a avaliação dos efeitos da estimulação com TBS na cognição poderá ser monitorizada quer com recurso a testes neurofisiológicos quer com alguns testes neuropsicológicos.

No capítulo V, procurou-se avaliar os resultados da TBS sobre o córtex pré-frontal dorsolateral (CPFDL) de ambos os hemisférios cerebrais, avaliados agora com a oximetria cerebral medida de forma não invasiva (*Near Infra-Red Spectroscopy* - NIRS). Foram ainda estudados os eventuais efeitos (indirectos) sobre a pressão arterial e a frequência cardíaca. Nos nossos voluntários verificou-se uma redução significativa na oximetria na região frontal esquerda após aplicação de cTBS sobre o CPFDL esquerdo e uma redução próxima da significância estatística na região frontal direita. A inibição do hemisfério direito (cTBS sobre o CPFDL) foi associada a uma redução significativa de 8 mmHg na pressão arterial sistólica. Nenhuma modificação significativa foi observada na frequência cardíaca e na pressão arterial diastólica. Os achados sugerem que a modulação do córtex pré-frontal pode ter a capacidade de influenciar o sistema nervoso autónomo, podendo vir a ter um possível papel na avaliação de doenças cardiovasculares mediadas pelo sistema nervoso autónomo.

No artigo constante no capítulo VI, os objectivos principais centraram-se na avaliação dos efeitos associados à estimulação do córtex temporal esquerdo com TBS, estudando especificamente os limiares auditivos ipsilaterais à estimulação, no ouvido mais próximo da bobina. Tentou-se estudar não só o comportamento do córtex temporal mas perceber se com o uso de intensidades adequadas não existia o risco de interferir negativamente

com as funções auditivas básicas. A análise dos resultados revelou que nenhum dos grupos intervencionados apresentou efeitos colaterais relevantes, nem perda significativa do limiar auditivo após iTBS, cTBS ou sham. Foi ainda verificado que no grupo submetido a iTBS existiu uma tendência para a diminuição dos limiares auditivos após a estimulação, tendo sido encontradas diferenças significativas entre os grupos iTBS e sham para 500Hz e entre os grupos iTBS e cTBS para 4000Hz. Para além de adicionarem informação essencial para a utilização em segurança da estimulação com TBS, os resultados parecem apoiar a hipótese de que a iTBS pode exercer uma neuromodulação favorável no córtex auditivo, com o potencial de influenciar positivamente os limiares auditivos.

No capítulo VII é abordada uma patente com dados ainda não publicados, sobre a metodologia de estimulação e possível aplicação da TBS como método capaz de influenciar positivamente o processo criativo. Neste caso, o estudo decorreu em 24 voluntários divididos em dois grupos – um submetido a estimulação com iTBS sobre o córtex pré-frontal dorsolateral direito e outro a um protocolo sham. Ambos os grupos foram testados antes e após a estimulação usando uma seleção adaptada do Teste de Pensamento Criativo de Torrance. Os resultados revelaram que tanto a originalidade quanto a fluência de pensamento divergente melhoraram de forma significativa no grupo submetido a iTBS quando comparado com o grupo sham, sugerindo uma modulação funcional positiva no CPFDL direito devido a um possível efeito local excitatório da iTBS, aparentemente capaz de influenciar as redes neuronais envolvidas no processo criativo.

Uma análise crítica e integradora dos resultados provenientes de toda a investigação incluída nesta tese permite-nos pensar que os nossos objetivos principais foram alcançados, uma vez que parece verificar-se que a estimulação com TBS é capaz de influenciar as regiões corticais estimuladas e as suas respectivas redes cortico-subcorticais, com particular ênfase no córtex pré-frontal. Esta neuromodulação parece acontecer não só no córtex diretamente adjacente à bobina, mas também nas redes neuronais conectadas à região cortical alvo. Os achados suportam assim a noção científica da existência de um efeito trans-sináptico defendido especificamente para o método EMTr clássico, que após a publicação destes resultados poderá continuar a ser estendido com maior confiança para o protocolo TBS. Introduziu-se a noção de que é igualmente possível estudar os efeitos cognitivos induzidos pela estimulação recorrendo a métodos neurofisiológicos e outras técnicas complementares. Nesta matéria salientam-se os resultados que parecem sugerir que o P300 auditivo pode ser uma ferramenta eficaz para avaliar os efeitos relacionados com o uso da TBS, aparentemente com sensibilidade para detectar variações positivas e negativas no processamento neuronal.

Os resultados constantes nesta tese suportam também a teoria mais consensual sobre os efeitos modulatórios das duas principais formas de TBS - intermitente e contínua, especificamente no que concerne ao facto de que a iTBS ser capaz de induzir um efeito excitatório ou facilitador e que a cTBS é capaz de causar inibição cortico-subcortical, algo já documentado para o córtex motor. A capacidade inibitória da cTBS pode ser encontrada nos resultados pós estimulação do córtex pré-frontal, parecendo induzir uma lentificação do P300 (cap. III), limitando o efeito aprendizagem em alguns resultados do teste de Stroop (cap. IV) e também na diminuição da oximetria cerebral ipsilateral à estimulação (cap. V). Inversamente, os efeitos excitatórios da iTBS parecem originar latências mais rápidas do P300 após estimulação do hemisfério esquerdo (cap. III), uma neuromodulação aparentemente favorável ao estimular o córtex auditivo esquerdo - traduzida em limiares mais baixos após a estimulação (cap. VI), e finalmente no capítulo VII, onde o processo criativo parece ser positivamente modulado após o uso de iTBS no CPFDL direito.

Os resultados conjugados desta investigação parecem sugerir que a inibição induzida pela cTBS parece mais significativo, comparativamente com o efeito excitatório da iTBS: a iTBS apenas exerceu um efeito significativo no córtex auditivo (originando limiares auditivos mais baixos) e no CPFDL esquerdo (latências do P300 mais precoces). Esta não é uma noção consensual entre autores, principalmente em relação ao córtex motor. Pensamos que os nossos achados podem ser explicados por mais do que um factor: (i) o primeiro e possivelmente mais importante relaciona-se com a noção de que cada região cortical e suas respectivas redes neurais poderão responder de maneira distinta ao uso da iTBS e da cTBS, dado que estudamos regiões corticais diferentes não apenas funcionalmente, mas também na sua organização neuroanatómica; (ii) o segundo fator, também defendido por outros autores, relaciona-se com a possível resposta das regiões corticais estudadas nesta tese aos fenómenos de potenciação e depressão de longa duração (LTP e LTD), que poderão assumir magnitudes diferentes comparativamente ao que acontece no córtex motor primário.

Podemos também inferir que os efeitos da TBS parecem estar intrinsecamente correlacionados com a lateralização da estimulação e a razão poderá estar relacionada com as funções específicas de cada hemisfério e com as áreas corticais estimuladas. Os resultados dos capítulos III e V mostram que o hemisfério esquerdo parece mais susceptível à neuromodulação após a TBS ou que os efeitos pós-TBS à esquerda parecem ser mais intensos. Esta lateralização poderá por um lado traduzir uma maior dominância deste hemisfério para as funções estudadas ou, tal como reportado por outros autores, a

estimulação à esquerda poderá exercer um maior feito sobre neurotransmissores como a dopamina, algo menos comum com a estimulação magnética do hemisfério direito.

Depois dos estudos realizados com TBS em mais de uma região cortical, podemos inferir que esta é uma técnica segura, com efeitos colaterais raros e incipientes, desde que respeitadas as diretrizes de segurança preconizadas. Ainda sobre os resultados nesta temática, salientam-se os achados contantes no capítulo VI, nos quais a TBS não só não originou um compromisso dos limiares auditivos do ouvido próximo à bobina, como a iTBS parece ser capaz de originar limiares mais baixos após a estimulação. Estes dados encorajadores sugerem que a iTBS pode vir desempenhar um papel interessante na avaliação de possíveis intervenções terapêuticas em casos de perda auditiva neurossensorial.

A pesquisa detalhada ao longo desta tese permite sedimentar o conhecimento sobre a estimulação com TBS, mas abre também novas perspectivas relativamente a futuras implementações da técnica. A manutenção ou exacerbação dos resultados com a aplicação de múltiplas sessões, à semelhança do que se verifica com o uso da TBS na depressão, deverá ser estudada no futuro (em cérebros saudáveis ou com outras patologias), bem como a aplicação de protocolos com tempos de follow-up mais prolongados que permitam uma avaliação mais concreta da duração dos efeitos pós-TBS. Nestes pontos, destacamos particularmente o acompanhamento da possível melhoria cognitiva pós-iTBS sobre o córtex pré-frontal esquerdo, a diminuição dos limiares de audição pós-iTBS no córtex auditivo esquerdo e ainda o aparente favorecimento do processamento criativo pós-iTBS no córtex pré-frontal direito. De forma a tentar verificar uma possível generalização dos achados desta tese, será igualmente necessário alargar o espectro das idades estudadas, dado que o nosso estudo se centrou em jovens adultos. Acreditamos ainda que um passo necessário será aplicar o conhecimento derivado desta tese a estudos clínicos, nomeadamente tentando usar o P300 para avaliar os resultados da terapia com *Theta Burst* em doenças como a depressão ou em pacientes com perturbação cognitiva. Os resultados encorajadores após o uso da iTBS no córtex auditivo também deveriam ser replicados em pacientes, particularmente com perda auditiva neurossensorial leve, de forma a avaliar se este protocolo de estimulação pode ser uma técnica com capacidade terapêutica nestes casos. Salienta-se ainda que as técnicas utilizadas nesta tese de forma a estudar os efeitos associados ao uso da TBS podem ser muito úteis no futuro na tentativa de identificar a eficácia do uso terapêutico deste tipo de protocolos, possivelmente permitindo modificar e adaptar as intervenções idealizadas, orientando uma intervenção personalizada ao doente.

Finalmente, parece-nos muito importante ressalvar que a agregação dos resultados desta tese acrescenta informações valiosas para entender o espectro de efeitos da estimulação com o protocolo *Theta Burst*. Historicamente, os parâmetros éticos que habitualmente orientam o uso diagnóstico e terapêutico da estimulação magnética transcraniana estão predominantemente relacionados com a segurança do sujeito em ensaios clínicos, mas conhecer todos os possíveis efeitos positivos e negativos associados ao uso deste tipo de estimulação em humanos saudáveis é de primordial importância. Este é um ponto essencial no caminho para a conquista da credibilidade técnica e científica necessária para a TBS, procurando um uso clínico mais abrangente e confiável, numa altura em que cresce desreguladamente o seu uso como terapia off-label para inúmeras doenças neurológicas e psiquiátricas. Além de tentar explorar e compreender a abrangência do uso da estimulação magnética transcraniana repetitiva, esta investigação pretende promover as melhores práticas, tentando defender os melhores interesses do doente no uso futuro desta técnica.

Palavras-chave:

Estimulação magnética transcraniana; Estimulação *Theta Burst*; Córtex pré-frontal; Córtex auditivo; Potenciais evocados P300; Segurança; Testes neuropsicológicos; Oximetria; Pressão arterial; Criatividade

Abstract

Transcranial magnetic stimulation (TMS) is a non-invasive diagnostic and therapeutic technique used to stimulate the brain in several neurological and psychiatric diseases, even though the main bases underlying its action are not fully understood.

Theta Burst Stimulation (TBS), a patterned form of repetitive TMS, has been assuming particular importance due to its faster application. Research of TBS effects on some higher cortical functions such as cognition after stimulation of the prefrontal cortex (PFC), or its possible influence in some less studied cortical regions (as the temporal cortex) has been limited and revealed inconsistent results. One of the problems assessing the cognitive TBS after-effects relates to the use of multiple evaluation methods, with different sensitivities. In this matter, the use of neurophysiology studies such as the auditory P300, a cognitive evoked potential, may be of particular importance. To date, studies addressing the association between auditory P300 and TBS are scarce, and some contradictory results were found. The study of other higher cognitive domains such as creativity is even rarer, but it may be relevant given that part of the neural networks involved in creative processing are associated with the PFC. The effect of TMS over the PFC, studying the modulation of functions mediated by the autonomic nervous system has also been reported, but there is still a significant disagreement between the rare studies performed.

So far, the extent of the modulatory effects associated with TBS at the sensory level is still poorly known, and research with TBS over the auditory cortex, despite showing some positive results, remains inconclusive, with some reports of sound hypersensitivity after sessions with higher intensity stimulation. It should also be noted that a significant part of the knowledge about the effects of TBS derives from studies in patients, with dysfunctional neuronal networks or hemispheric lesions, which add challenges to the search for scientific evidence in healthy individuals.

Given the uncertainties that remain regarding the extent of the neuromodulatory effects of TBS, the primary objective of this thesis focused on increasing the scientific knowledge related to the use of TBS in the healthy brain. Therefore, we intended to study the neurophysiological responses (such as auditory P300), the functional responses (such as auditory thresholds), and the physiological responses (such as cerebral oximetry and blood pressure) associated with the application of TBS in the prefrontal and temporal cortices.

All studies used a target population of healthy young adults, with an average age of approximately 23 years, and similar education. TBS was performed accordingly to the 600-pulse paradigm described by Huang et al. (continuous and intermittent). Sham-controlled, double-blind intervention protocols were used, with random distribution by the respective groups.

The main objective of the study in chapter III was to evaluate the effect of TBS on the dorsolateral prefrontal cortex (DLPFC) of both cerebral hemispheres in cognitive processing. The objective was to assess if the auditory P300 would be influenced by the stimulation type. Results revealed that the mean P300 peak latency after TBS decreased only after leftward iTBS. A significant delay in P300 latency was originated from both right and left cTBS. Amplitude response did not change significantly.

The results covered in chapter IV derived from the use of TBS on the left DLPFC, studying the possibility of a relationship between the post-TBS auditory P300 and the post-TBS neuropsychological tests: Trail Making Test (TMT) and the Stroop Test of Words and Colours. Results revealed that cTBS led to a delay of the P300, also significantly influencing the expected performance on Stroop C and Stroop Interference when compared to the groups submitted to iTBS and sham stimulation. No significant results were found in the TMT tests for any type of TBS stimulation.

In Chapter V, we studied the cerebral oximetry using Near Infra-Red Spectroscopy, blood pressure, and heart rate, after applying TBS to the right and left DLPFC. We found a significant reduction in oximetry in the left frontal region after ipsilateral cTBS and a significant decrease in systolic blood pressure after cTBS to the right DLPFC.

Chapter VI covered the evaluation of the effects of TBS over the left temporal cortex, specifically studying the auditory thresholds in the ear closest to the coil. Results showed no major side effects after iTBS, cTBS, or sham stimulation. It was also found that iTBS led to lower hearing thresholds, especially when comparing the iTBS and sham groups at 500Hz and between the iTBS and cTBS groups at 4000Hz.

Chapter VII addresses a patent concerning the technique and possible use of iTBS as a method to influence creative processing. After iTBS over the right DLPFC, results of an adapted selection of the Torrance Tests of Creative Thinking suggest that divergent thinking, originality and fluency improved significantly compared to the sham group.

An integrative analysis of the results shows that TBS seems to effectively influence the underlying cortical neurons and cortico-subcortical networks. The findings thus support

the existence of a trans-synaptic effect advocated initially for the classic repetitive TMS, which after the publication of our research can continue to be extended with greater confidence to TBS protocols. Our results also support the most consensual theory about the modulatory effects of the two main forms of TBS – intermittent (excitatory) and continuous (inhibitory) – particularly on the prefrontal and temporal cortices.

The effects of TBS seem to be intrinsically correlated with the hemispheric lateralization and this may be related to the specific functions or dominance of each hemisphere and the specific stimulated cortical regions. The combined results of this investigation also seem to suggest that the inhibition induced by cTBS seems more effective when compared to the excitatory effect of iTBS, which seemed stronger in the left hemisphere.

After all our research with TBS in more than one cortical region, we can infer that this is a safe technique, with rare and incipient side effects.

The encouraging results after using iTBS in the auditory cortex opens new perspectives regarding future implementations of the technique and should be replicated in patients, particularly with mild sensorineural hearing loss, in order to assess whether this stimulation protocol can be a valid therapeutic technique in these cases. We also conclude that the techniques used to study TBS-related effects, as the P300 or the NIRS, can be very useful in the future, as an attempt to identify the effectiveness of the therapeutic use of TBS protocols, possibly allowing to adapt and modify the idealized interventions, leading to a personalized patient intervention.

Our findings provide relevant information, necessary to increase the technical and scientific credibility required for achieving a more comprehensive and reliable clinical use of TBS. This is crucial at a time when transcranial magnetic stimulation use as an off-label therapy for numerous neurological and psychiatric diseases grows unregulated, and the patient best interests must be defended.

Keywords

Transcranial magnetic stimulation: Theta burst stimulation; Prefrontal cortex; Auditory cortex; P300 evoked potentials; Safety; Neuropsychological tests; Oximetry; Blood pressure; Creativity

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List of acronyms and abbreviations

A	Ampere
AD	Alzheimer's disease
AMT	Active motor threshold
ANS	Autonomic nervous system
APB	Abductor pollicis brevis
ASHA	American Speech-Hearing-Language Association
BDNF	Brain-derived neurotrophic factor
BOLD	Blood-oxygen-level dependent contrast
C	Colours
cAPB	Contralateral abductor pollicis brevis
CBF	Cerebral blood flow
CE	Conformité Européene
CF	Cardiac frequency
cm	Centimetres
CMT	Cortical motor threshold
cTBS	Continuous theta burst stimulation
DAP	Diastolic arterial pressure
dB HL (dB)	Hearing level decibel
dB SPL	Sound pressure level decibel
DLPFC	Dorsolateral prefrontal cortex
ECT	Electroconvulsive therapy
EEG	Electroencephalogram
EMG	Electromyography
ERP	Event-related potentials
FDA	Food and Drug Administration
FHS	Faculty of Health Sciences
fMRI	Functional magnetic resonance imaging
g	Gram
GABA	Gamma-Aminobutyric acid
HbO2	Hyperbaric oxygen
Hz	Hertz
IBM	International Business Machines
iTBS	Intermittent theta burst stimulation
LSD	Least Significant Difference
LTD	Long-term depression
LTP	Long-term potentiation
MDO	Maximum device output
MEP	Motor evoked potential
min	Minutes

ml	Milliliter
mmHg	Millimeters of mercury
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
ms	Millisecond
MSO	Maximal stimulator output
NIRS	Near-infrared spectroscopy
NMDA	N-Methyl-D-aspartate
nparLD	Nonparametric Analysis of Longitudinal Data in Factorial Experiments
OCD	Obsessive-compulsive disorder
P-A	Posterior-anterior
PAC	Primary auditory cortex
PET	Positron emission tomography
PFC	Prefrontal cortex
PMC	Primary motor cortex
RCT	Randomized controlled trial
RMT	Resting motor threshold
rTMS	Repetitive transcranial magnetic stimulation
s	Seconds
SAP	Systolic arterial pressure
STWC	Stroop Test of Words and Colours
SD	Standard deviation
SPECT	Single-photon emission computerized tomography
SPSS	Statistical Package for the Social Sciences
T	Tesla
TBS	Theta burst stimulation
TES	Transcranial electrical stimulation
TMS	Transcranial magnetic stimulation
TMT	Trail Making Test
TTCT	Torrance tests for creative thinking
UBI	University of Beira Interior
V	Volt
W	Words
WC	Words/Colours
μV	Microvolt
Ω	Ohm

Chapter I

General Introduction

A primer to Transcranial Magnetic Stimulation

Historical background

Transcranial Magnetic Stimulation and Theta Burst Stimulation

Scientific research and clinical therapeutic possibilities of Transcranial Magnetic Stimulation over the cortex

Chapter I

General Introduction

1. A primer on Transcranial Magnetic Stimulation

Transcranial magnetic stimulation (TMS) is a non-invasive diagnostic and therapeutic brain stimulation technique that has been evolving over the last 35 years [1,2], since it was first introduced to the scientific community by Barker and colleagues in 1985 [3].

The first main use for the technique continues valid today, being an essential tool to evaluate the nervous motor function, as stimulation of the primary motor cortex activates the corticospinal tract, motor roots, and peripheral nerves [3,4]. The therapeutic use of TMS has achieved a place of reasoning and scientific solidification, finding its way into the mainstream for multiple neurological and psychiatric disorders, such as major depression, obsessive-compulsive disorder, stroke rehabilitation, pain disorders, and epilepsy [1,4], even if the main underlying bases of its action are not fully understood [5].

The neuromodulatory therapeutic potential of this technique is based mostly on the ability to trigger neuronal plasticity and potentiate synaptic transmission [2,4]. With TMS it is also possible to study the brain response and behaviour to online and offline interventions, both on healthy and compromised cortico-subcortical functions [6,7].

However, given the technical specificities, the number of methodological variants, and experimental options, it is of paramount importance to understand the TMS foundations and its historical origins.

2. Historical background

The principle of electromagnetic induction is the foundation of TMS, and dates back to the work of the English physicist Michael Faraday in 1831, that proved that electric energy could be converted in magnetic fields and these fields could also be converted into electric power [8,9]. He discovered this phenomenon by sending a pulse of electric

current passing through a wire coil, originating a secondary magnetic field in the proximity of the coil. Since then, many researchers have tried to create a safe and useful technique that could be used in the study of the nervous system. With a powerful magnetic coil in human subjects, D'Arsonval in 1896, was capable of inducing phosphenes, vertigo, and even syncope [9]. This was probably the most similar protocol to what is used nowadays. In 1903 in Austria, Adrian Pollacsek, and Berthold Beer patented what may have been the first idea about the application of TMS in neuropsychiatric diseases (Figure 1.2.1.). They described a process using an electromagnetic coil placed over the skull, in order to send vibrations to the brain and thus "treating depression and neuroses"[9].

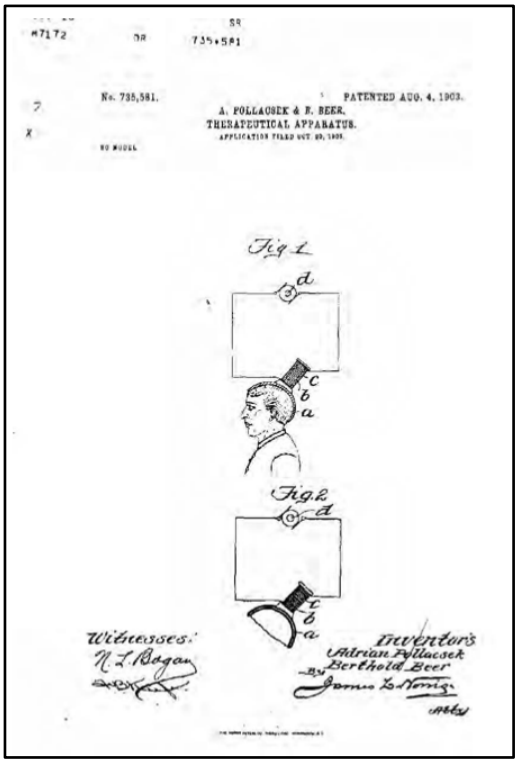

Figure 1.2.1. Patent from Adrian Pollacsek and Berthold Beer, from 1903, of an electromagnetic device used for treatment in neuropsychiatry.
Adapted from "Transcranial magnetic stimulation in clinical psychiatry" (p.6), by George MS and Belmaker RH, 2007, Washington, United States of America: American Psychiatric Publishing. Copyright © 2007 American Psychiatric Publishing, Inc.

Phosphene induction was a primary research area for TMS evaluation, as reported by Thompson in 1910 and Dunlap in 1911, but the rudimentary devices used were not capable of focal brain stimulation [9]. The first demonstration that magnetic fields can

stimulate muscle tissue (frog muscle preparation) dates back to Kolin and colleagues in 1959 [10].

Meanwhile, between 1937 and the late 1970s, parallel application and development of the transcranial electrical stimulation (TES) technique took place [7,11]. It occurred both in its therapeutic form - electroconvulsive therapy (ECT) in the field of psychiatry - and in its diagnostic form - evaluation of central motor conduction time and the study of motor evoked potentials, used in neurological diseases such as multiple sclerosis. This form of stimulation, although very effective, was significantly limited by the extreme discomfort caused in patients as it involved high-intensity electrical activation of the surrounding brain structures, contracting scalp muscles and activating local skin pain receptors [7,11].

The TMS golden age started in 1985 when the first modern TMS device was introduced (Figure 1.2.2). Barker and colleagues presented a reliable, relatively simple and small device, with a light small coil that could easily be placed near the subjects head and/or body, in a layout that still thrives today [3,7].

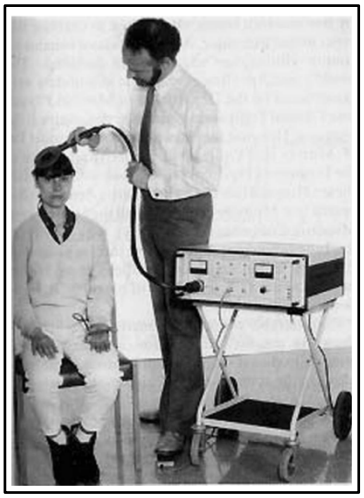

Figure 1.2.2. Barker with the transcranial magnetic stimulation machine (1985).
Adapted from "Transcranial magnetic stimulation in clinical psychiatry" (p.4), by George MS and Belmaker RH, 2007, Washington, United States of America: American Psychiatric Publishing. Copyright © 2007 American Psychiatric Publishing, Inc.

Even so, early devices were not capable of continuous usage since they were slow to recharge and coil overheating was frequent [9]. Initially, the device was first used in a single-pulse form in order to study the integrity of the corticospinal tract, gaining importance as a diagnostic tool. TMS was then seen as an adequate solution for the pain

stimulation-derived problem seen in TES, as it was capable of studying the central motor system through motor evoked potentials in a safer and less painful method [7]. Barker et al. demonstrated that a pulse over the primary motor cortex could evoke a peripheral motor response in the muscles linked to the stimulated cortical area. These responses could easily be recorded by electromyography (EMG) equipment and this lead to the establishment of the technique as a routine method to study the functional integrity of the motor pathways [3,12]. Although the concept was relatively simple, the demanding associated technical requirement has made the equipment expensive for many years, even after the main principles became well known [13].

Since then, it has been proven that TMS can have a relevant role as a therapeutic tool in neurological and psychiatric disease, as it can influence long-term excitability and connectivity of stimulated neural networks [2,14]. The breakthrough started in the 1990s, with the early studies that used a repetitive transcranial magnetic stimulation (rTMS) paradigm, as an attempt to originate a cortical effect that could be used in medical therapy [7,15,16]. In 1991 and 1994 Pascual-Leone and colleagues published results showing that rTMS could modify speech fluency and that the physiological cortical effect could last up to 4 minutes [15,16].

The first major attempt to use rTMS as a therapeutic tool occurred in depression [13]. In 1995 two very important papers were published. Kolbinger et al. used a low-frequency protocol over the vertex area of depressed patients, finding an improvement of the depressive symptoms [17]. George et al. followed a different direction, using high-frequency rTMS over the left prefrontal cortex [18]. These authors chose this area because it presented decreased metabolic activity in depressed patients in studies using positron emission tomography (PET). They argued that fast rTMS should induce a “tonic effect” by increasing cortical excitability [13,18].

Simultaneously, major concerns began to arise regarding the safety and ethical aspects of rTMS in humans, due to possible major side effects such as seizures or the ability to influence mood and cognition [7,19]. These concerns have been addressed over the years through guidelines and position papers, e.g. initially by Green et al. in 1997 (ethics)[20], later by Wassermann 1998 (ethics and safety – report from the International Workshop on the Safety of rTMS of 1996)[21], Rossi et al. in 2009 (by the International Federation of Clinical Neurophysiology - ethics and safety)[19], and more recently by Lefaucheur et al. in 2014 (general therapeutic use)[22] or Perera et al. in 2016 (depression)[23].

3. Transcranial Magnetic Stimulation

3.1 Basic principles and technical apparatus

Transcranial magnetic stimulation (TMS) originates a suprathreshold current in the cortex through electromagnetic induction [24]. TMS is supported by the principle of magnetic induction and its ability to induce electrical activity in the cortical tissue, following Faraday's law of electromagnetic induction [13,24]. These magnetic fields originated in the coil adjacent to the scalp suffer almost no attenuation through several resistive layers - scalp, skull bone, and meninges - being able to induce electrical currents in the brain [13,24]. This inducted electric field originates an intracranial ionic flow that modifies the electrical envelope around the neurons causing them to fire [13,19]. The ionic flow derived from TMS eliminates the need to "inject" charged particles into the scalp, as happens in TES [19]. The induction principle and the technical parameters involved are proposed both for cortical and peripheral nerve stimulation (as verified by studies in invasive stimulation during neurosurgery or epilepsy monitoring): the TMS pulse seems to be able to actively modify the charges on the inside and on the outside of cell membranes, originating hyperpolarization or depolarization of neurons, thereby resulting on the initiation of the action potentials, thus activating neuronal populations and related networks [19,24]. TMS is mostly capable of activating axons instead of cell bodies, as the axons present a lower excitability threshold, especially the larger diameter myelinated axons, which are easily stimulated with lower intensity stimulus [19,24]. This fact is even more pronounced when the axon ends (synapse), or bends sharply [19].

The basic equipment consists of a magnetic stimulator and a coil (Figure 1.3.1). The stimulator contains a source of energy that charges one or more capacitors, which are triggered depending on the type of stimulation and intensity required [13]. The apparatus consists of a peripheral device of copper wire wrapped circularly (coil), connected to large electrical capacitance terminals through a switch. The discharge device is a high-voltage (400 V– 3 kV), high-current (4 kA–20 kA) system [12]. The capacitance discharge happens when the switch is closed - this will originate the release of a large current of several thousands of amperes, transiently flowing through the coil, with a duration of less than 1 ms [24].

Figure 1.3.1. Transcranial Magnetic Stimulation apparatus (MagVenture MagPro® G3 X100 with a MCF-B70 coil) in the Faculty of Health Sciences, University of Beira Interior, Covilhã, Portugal.

The large current originating the TMS pulse can have two main configurations: monophasic or biphasic. The monophasic pulse has a strong initial current and a dampened return current. Only the initial current will induce a current flow in the stimulated cortical tissue, while the damped current induces no stimulation. The biphasic pulse is the most used one in therapeutic protocols, being a longer and more effective pulse configuration. Each phase induces two significant physiologically different current fluxes in brain tissue – with the same or opposite direction [24]. When the coil is placed tangentially on the scalp, each pulse originates a secondary electrical field in the intracranial tissue, with an opposite direction relative to the coil´s electrical current [12]. It is also important to realise that the induced currents and their routes in the cortico-subcortical tissue are not linear and are distorted by local differences in tissue conductivity since the human brain is not a homogeneous entity [12].

In order to evoke a cortical stimulation, the magnetic field has to be sufficiently strong to depolarize the neuron membrane, thus being able to trigger an action potential. This depolarization will mostly occur at the site where the induced electrical field is maximal [12]. Although the effects of TMS are predominantly dominant in the stimulated cortical area adjacent to the coil, its scope is not limited to that region. TMS induced action potentials in cortical axons spread trans-synaptically to other linked neurons, originating

a propagation of the neuronal activation to connected cortical and subcortical regions [12]. This networking action is an essential notion when addressing TMS neuromodulation capacity. As an example, the dorsolateral prefrontal region is known to have multiple connections, networking with areas like the prefrontal lateral cortex, the lateral orbitofrontal cortex, and with the mediodorsal nucleus of the thalamus [25], that in theory may be influenced by TMS. It also seems possible to modulate inter-hemispheric equilibrium, especially in motor areas, as TMS effects seem to spread not only in the stimulated hemisphere but also in the contralateral one [26–28]. This will be a quintessential notion when analysing the role of therapeutical TMS in stroke patients where this possible inter-hemispheric inhibition equilibrium, mediated by fibres of the corpus callosum, seems to be impaired. In this case, rTMS seems to have the capability to decrease the higher excitability of the contralesional hemisphere, which, after stroke, promotes an excessive inter-hemispheric inhibition of the damaged brain [26–28].

Magnetic field propagation is another important factor to address because the depth penetration of TMS is limited [12,24]. Pulses delivered using intensities below to 120% of motor threshold do not induce direct activation at depth of more than approximately 2 cm below the scalp [13,19]. Hence, the most classical coils – circular and figure-eight – are not able to reach deep neural structures, such as the thalamus, basal ganglia, or even the medial part of the temporal lobes. Even the stimulation of the primary cortical area of the leg is difficult when compared to the hand/arm areas, due to its interhemispheric fissure location, needing a higher intensity to be effective [12,24]. Increasing the distance to the coil there is an exponential decrease in the magnetic field originated by each TMS pulse. This means that the coil must be kept as close to the cortical target area as possible. Thus, the coil should be placed in direct contact with the scalp, with the horizontal plane of the coil parallel to the subject's head [12,24]. When designing interventions, it is important to take the target area into account. As an example, the average coil-cortex distance in the temporal lobe is usually shorter than the one in the primary motor cortex area [29] – therefore, in theory, the intensity should be adjusted accordingly to the specific cortical region.

3.2 Coil types and handling technique

Coils are an essential instrument in TMS, that may be presented in various sizes and shapes [12]. They can deliver a magnetic field with a strength of 1-2,5 Teslas, similar to a magnetic resonance imaging (MRI) equipment [12,24].

Initially, coils assumed large dimensions and a circular format (Figure 1.3.2), with a diameter close to 10 cm, allowing the unilateral or bilateral stimulation of the motor area related to the hand [13,24]. This type of circular wire coil induces an electrical current in a large volume of brain tissue but leads to a stimulation that is more effective in the outer rim under the coil and a minimal stimulation in the centre ring – resulting in a non-focal stimulation [12,24]. The tangentially placed circular coil can hypothetically cover a large area of the brain but unfortunately has a shallow penetration depth, as the induced current falls significantly with the distance to the coil; as an example, the induced current at a distance of 5 cm is around 1/3 of the peak value [24].

Figure 1.3.2. Circular coil (Magventure MCF-125).
Adapted from the Magventure website
https://www.magventure.com/media/k2/items/cache/be4e4fd1bcb87d92f342f6e3e3e1d9e2_XL.jpg

Technical advances allowed the use of two adjacent coils, using twice the number of windings, giving rise to the figure-eight or butterfly coil (Figure 1.3.3), a less powerful but very useful type of coil [12,24]. These two small overlapping round coils use oppositely directed passing currents [24]. The great advantage of this type of coil is that it allows a much more focal stimulation when the intersection zone of the coils is placed tangentially to the cortical target [13]. This way, the largest current induced is centred in the tissue under the intersection, with the major component of the electric field parallel-oriented to the centre plane of the coil [12].

Figure 1.3.3. Figure-of-eight (butterfly) coil (Magventure MCF-B70).
Adapted from the Magventure website
https://www.magventure.com/media/k2/items/cache/1fc372946c0b98fb8d7f87d4c38ea83a_XL.jpg

Due to its higher focus, this type of coil is classically used in therapeutic applications of TMS, brain mapping, and research scenarios. Since the current induction occurring in the centre of the coil is two to three times greater than that occurring in the periphery of the coil, the focal stimulation obtained with the figure-eight coil can be achieved with low to moderate intensities (compared to circular coils) [12].

Figure-of-eight coil focus is directly dependent on the intensity applied. It can activate a target cortex patch (usually up to 2 cm^2) and the stronger the intensity, the larger the area of cortex that is activated (Figure 1.3.4) [30].

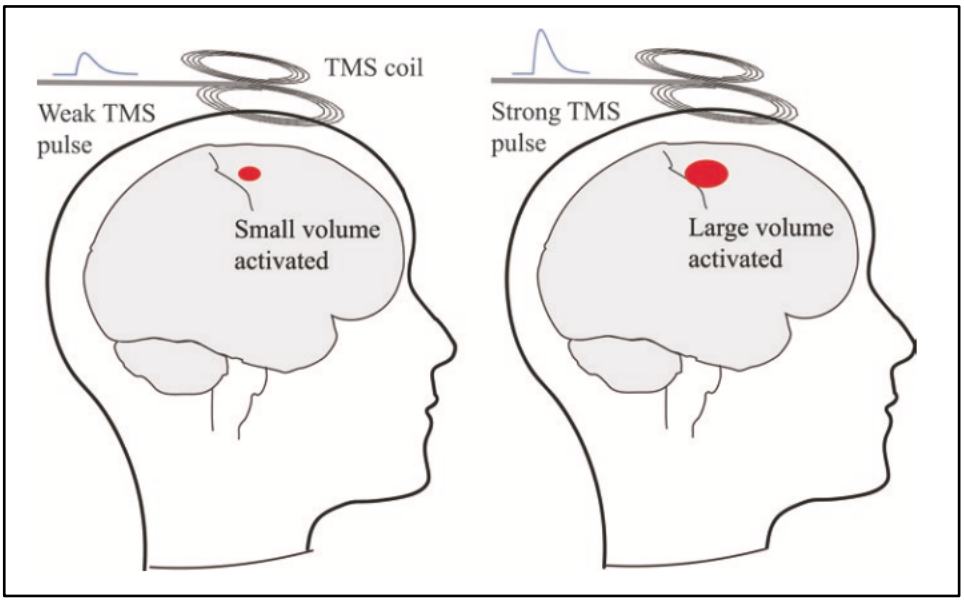

Figure 1.3.4. Pulse intensity and stimulated area with a figure-of-eight coil. Weaker pulses originate a smaller cortical activation area. With stronger pulses, the threshold of activation is exceeded in a larger cortical activation area.
Adapted from "Navigated transcranial magnetic stimulation in neurosurgery" (p.6), by Krieg SM., 2017, Cham, Switzerland: Springer Nature. Copyright © 2017 Springer International Publishing AG.

It should also be taken into account that the decrease in coil size originates a more focal stimulation but it also results in a more rapid coil heating when using rTMS or TBS [24]. Manufacturers use several methods to reduce coil heating, namely water, oil, or forced air [19].

Currently, the circular coil continues to be recommended for diagnostic use in the peripheral nervous system and over the primary motor cortex, mostly due to its ability to stimulate a larger cortical volume [12,14].

Coil design has specificities that should be considered and the induced current direction plays a relevant role in TMS [12]. In general, the position of the coil will guide the induced current direction, but theoretically, according to Ohm's law, the induction of the current inside the head can happen in a multiplicity of hypotheses, that is, in ways that will be guided by the conductance of the tissues [13]. This leads to the possibility that sometimes induction orientation may occur in another direction opposite to the one oriented by coil position [13]. The primary motor cortex, one of the most studied cortical areas, is best stimulated when a posterior-to-anterior direction is adopted [12]. It has been shown that stimulation with anterior-posterior, lateral-medial and posterior-anterior induced currents can originate different brain responses [14]. Each coil creates a current induction with different directions, always opposite to the direction of the electric current passing through the coil. Using a circular coil, the coil handle orientation is not

relevant because the spherical magnetic field induces a current that is also circular [12,14]. If an anticlockwise electric current passes through the coil, the induced current originated under the left part of the coil will be anteriorly oriented. The opposite effect occurs in the right side of the coil [14]. But if a figure-eight coil is used, the handle serves as a reference for the direction of the induced current. That is, if the handle is hold parallel to the midline (nasion-inion), with the coils anteriorly placed, the orientation of the induced current will be anteriorly oriented (Figure 1.3.5).

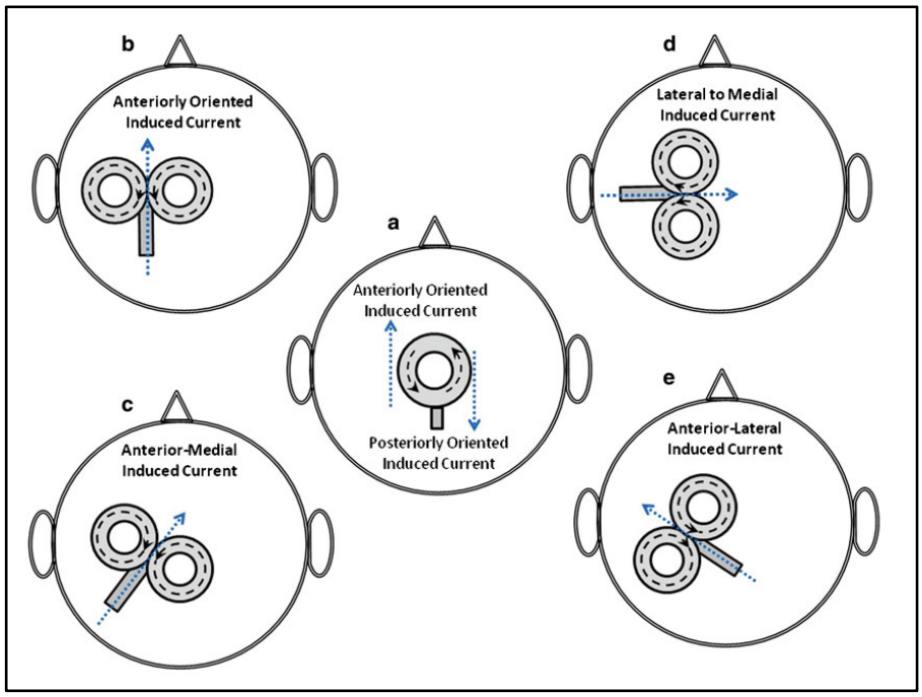

Figure 1.3.5. Representation of possible coil orientation in the head. (a) Round coil and (b–e) figure of eight (butterfly) coil. Different coil orientations and the respective direction of the induced currents (blue arrows).
Adapted from "Transcranial magnetic stimulation" (p.81), by Rotenberg AM, Horvath JC, and Pascual-Leone A., 2014, New York, United States of America: Humana Press. Copyright © 2014 Springer Science + Business Media.

Specific target location is also of the utmost importance since not all cortical areas respond similarly. For example, if the objective is to obtain the motor evoked potential of one of the hand muscles, one should use an approximate 45º angle relative to the midsagittal line, trying to induce a roughly perpendicular current concerning the central sulcus [12,14]. This oblique plane coil orientation was also used in the protocols approved by the Food and Drug Administration (FDA) in the United States for treatment in major depression, using the patient's nose as a reference [31,32]. But this approximate-positioning method may not be the most accurate one and so far the most efficient method to stimulate target cortical areas of varied lobes and structures remains in doubt

[33–35]. The cortex of each individual is unique and the factor that most influences the intracranial induced electrical currents is the position/orientation of the coil relative to each gyrus and sulci of the brain [33]. Even 1 cm or 10º variations may have a significant impact [35]. This was also found using computational models with realistic head models, where the optimal angle of stimulation could fluctuate over 90º at nearby cortical areas [33].

Using the motor cortex as a reference point to establish stimulation thresholds and also as an anatomical reference is the reference method for most studies so far [35,36]. As mentioned, the motor cortex is the cortical area that has an established optimal coil orientation, but for other cortical areas this knowledge is questionable. Recent studies evaluating the optimal coil orientation and position for TMS, trying to achieve a universal atlas, clearly state that it is preferable to use a personalised coil orientation in order to obtain the most effective stimulation [33].

Regular and specially designed coils can be used in sham/placebo stimulation protocols. One of the most used techniques, used in multiple studies over time, consists of tilting the coil away from the appropriate scalp placement, in a 90-degree angle, where the point of contact with the scalp becomes the edge of the coil and not the centre (Figure 1.3.6) [36–39]. Scalp-coil contact is similar to what is usually experienced in active stimulation but the magnetic field created does not reach neurons effectively or activate cutaneous receptors [36,38,40–43]. Sound is also present, adding to the roughly comparable actual stimulation experience [36]. New coils specifically for use in sham stimulation have been made, sounding and looking like the active coils, usually with a magnetic field passing to the cortex of less than 3%. The initial ones were not very successful, but more recent approaches, simulating the tapping sensation and/or delivering an electric stimulation during sham, apparently are more truthful to active stimulation [9]. Bae et al. compared the estimated placebo effect of different sham protocols in studies in epilepsy [44]. The comparison between modifying the coil position (orthogonal to the scalp), a spring-loaded sham coil and a double active sham coil, revealed that the placebo originated response was consistently low across all follow-up intervals, showing a relatively modest placebo response [44].

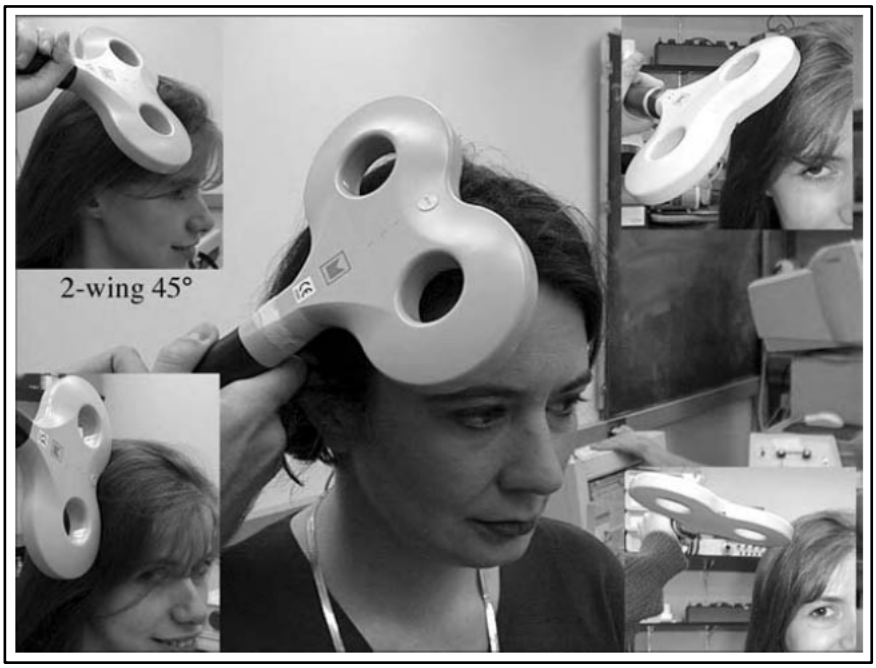

Figure 1.3.6. Coil positioning variants - active (centre) vs sham (different techniques). Active stimulation: figure-eight coil tangential to the scalp, with the mid-section of the coil in direct contact with the scalp. Sham stimulation: angling the coil off the head (one-wing or two-wing contact), with typical degree of 45 or 90 degrees.
Adapted from "Transcranial magnetic stimulation in clinical psychiatry" (p.128), by George MS and Belmaker RH, 2007, Washington, United States of America: American Psychiatric Publishing. Copyright © 2007 American Psychiatric Publishing, Inc.

3.3 Threshold intensity determination

Clinical TMS use implies the determination of the cortical motor threshold (CMT), either to be used specifically when rTMS occurs in the motor cortex, or to serve as a reference to other cortical areas (in the case of an initial evaluation through the motor cortex) [12,19]. CMT is an essential individual measure and must be specifically determined in every subject/patient. It also reflects a measure of the excitability of the corticomotor projection [12]. There are two forms of CMT - resting and active. Resting CMT of the primary motor cortex can be defined as the lowest stimulus intensity (intensity relative to the maximal stimulator output - MSO) which evokes a motor evoked potential of the target muscle in 50% of 10 trials. Each valid response should be larger than 50 μV [12,19]. Stimulation must be performed over the area in which pulses are able to evoke the largest response of the target muscle – "the hotspot" [12,22].

If the active CMT is used, the target muscle must be contracted at about 20% of the maximal voluntary strength during stimulation, usually using visual feedback. The accepted motor evoked potential (MEP) magnitude uses a cut-off value of 200 μV to avoid MEP misidentification [12]. This form of threshold determination was the one used

by Huang et al. and is the most commonly used for theta burst stimulation (TBS) [12,45,46].

The target muscles used for obtaining the motor evoked potentials vary, but the most used and recommended are the *abductor pollicis brevis*, the first *dorsal interosseous*, and the *abductor digiti minimi*, on the contralateral side to TBS [19,47].

It is also important to notice that physiological fluctuations impact the possible intra-subject and inter-subject variability in excitability measures such as the motor evoked potentials. These fluctuations can be found in the arousal state, attention level, and even time of day, possibly influencing both resting and active motor thresholds [35]. These factors should be controlled, especially when multiple sessions are implemented in the same subject.

3.4 Single-pulse stimulation

Recording the peripheral muscle response after stimulation of the corresponding cortical area in the contralateral primary motor cortex (PMC), was the initial use of TMS. This technique corresponds to the motor evoked potentials (MEP) [13,48]. MEPs using TMS, although simple in concept, allow not only the functional study of the motor pathway but also the analysis of the state of cortical excitability [13,48].

Single-pulse stimulation is the basis of magnetic stimulation. The study of this type of stimulation allowed researchers to realise that pulses preferentially stimulate axons following the plane of the stimulating current, that is, parallel to the coil plane [13]. Therefore, the most stimulated neurons are the tangentially oriented axons. However, it was found that there is also radial stimulation of pyramidal neurons, but that it happens indirectly [13,48]. Therefore, and knowing that TMS can only activate neurons very close to the cortical surface, it is assumed that the effects of TMS and rTMS on the deeper structures or on the radial fibres result predominantly from the synaptic transmission of the electrical induced stimulus [13]. Later on, the idea emerged of grouping two single-pulses, sent sequentially in a short time interval, using two TMS devices. This technique would allow the study of excitatory and inhibitory cortical function, and could be useful in neuropsychiatric disease diagnosis and therapy outcomes [13].

3.5 Repetitive stimulation (rTMS)

Since the early 1990s, studies using long short-interval trains of pulses started to be published. They aimed to test and possibly alter cortical activity and connectivity. It was the beginning of the use of repetitive TMS (rTMS) [13]. Pascual-Leone et al. published one of the early works studying the acute mechanisms of rTMS application, using motor evoked potential to study the physiological effects of rTMS trains over the motor cortex [15].

These early works exposed one of the phenomena associated with rTMS - the disruptive effect. By forcing a large group of neurons to fire simultaneously, causing a hypersynchrony of output cell firing, we also synchronize their respective refractory periods. This phenomenon gives rise to two different states: in the first one, any stimulus sent during the refractory period will not get a response or will be very small; in the second one, neurons will also be ready to fire at the same time, since they are artificially synchronised [13]. These phenomena have been described by Pascual-Leone et al. in 1994. By stimulating the motor cortex at 10 Hz, they obtained huge MEPs that alternated with no appreciable response. This alternation was due to the fact that each stimulus is sent at 100ms, which is half the time of the MEP recovery cycle (about 200 ms) [13,15]. This artificial hypersynchrony is the basis of the disruptive phenomena and apparently underlies the negative changes that occur in normal processing associated with a more acute effect of rTMS, as seen in online interventions.

There is an agreement regarding a direct association relating low and high frequency rTMS to different effects and degrees of risk. Frequencies equal to or below 1 Hz are identified as low-frequency stimulation or slow rTMS and all frequencies above the 1 Hz threshold are named high-frequency rTMS or fast simulation [19]. If rTMS delivery occurs in bursts of a small number of stimuli at high-frequencies, with short no-stimulation pauses between bursts, it is called patterned rTMS. Theta burst stimulation, in its different protocols, is the most common form of patterned stimulation [19].

3.5.1- Main probable mechanisms associated with the effects of rTMS

It is known that the magnetic field of each pulse will induce an intracranial electric current, thus activating the cortex, in a direction parallel to the plane of the coil. The magnetic field ends up functioning as an intermediary between the coil and the cortical electrical activity [49]. This phenomenon causes acute effects, such as the manifestation

of motor evoked potentials after stimulation of the primary motor cortex or the induction of phosphenes after stimulation of the visual cortex [49].

The application of multiple repeated pulses at fixed frequencies or forming patterns are able to have an effect that lasts beyond rTMS duration [49]. In general, we can separate these protocols into two different types of stimulation according to their effects: low-frequency stimulation (1 Hz or inferior), capable of reducing cortical excitability, and high-frequency stimulation (greater than 1 Hz - often above 5 Hz), capable of increasing cortical excitability [48,49]. The phenomenon of increased or decreased cortical excitability after rTMS is also found in the different forms of theta burst stimulation [48,49].

The successful therapeutic use of high and low-frequency rTMS, both in neurological and psychiatric disorders has been demonstrated in several randomized placebo-controlled studies, treating mood disorders, motor rehabilitation or pain, for example [49]. It is also known that these effects last over time, with durations that vary according to the specific pathology but that can reach 6 months [49], or in the case of depression up to 1 year in about half of the treated patients [50].

It is known that rTMS/TBS works, but what are the mechanisms underlying these effects?

Despite the increasing interest and large number of studies studying the effects of rTMS, these mechanisms remain involved in some doubt [1,49]. One of the running theories during the 90s was related to the increase or decrease of blood flow, depending on whether high-frequency or low-frequency rTMS were used, respectively [9]. But even then it was believed that these blood flow fluctuations would be related to the theories of long-term depression (LTD) and long-term potentiation (LTP), as described with more detail in sections 3.5.2, 3.5.3, and 3.5.4 [9]. Although it is accepted that rTMS can influence both blood flow and metabolism, this influence does not appear to be identical in the acute or chronic phase. As for rTMS acute effects (studied with fMRI and PET), discrepancies and contradictory results have been found [9]. More coherent results have been described concerning the long-term effects. In general, data are generally more consistent with an increase in blood flow if rTMS is delivered above 5Hz and a decrease in blood flow if rTMS uses frequencies below 1Hz [9,49]. These changes occur not only locally but are more widespread, in some cases [9]. This was also a very simplistic way to explain rTMS after-effects. Nowadays it is believed that the principles that originate the long term effects of rTMS are multiple, acting mostly at the neuronal level, in the neural-networks (focal and distal linked areas) and influencing synaptic function [49,51].

The effects of rTMS are due to specific combinations of stimulus frequency and intensity. These influence neuronal excitability, predominantly due to the shift in ionic balance relative to the studied neurons, and this is translated as synaptic plasticity [49,52]. One of the theories that supports that these changes remain over time is related to the phenomena of LTP and LTD [49,53]. The positive and negative reinforcement changes in synaptic strength seem to be significantly related to LTP and LTD, respectively [49,51].

LTP is produced when the activation of a presynaptic neuron is followed by the activation of a postsynaptic neuron (pre–post) within an interval of tens of milliseconds - this happens in high-frequency rTMS and theta burst stimulation. Low-frequency rTMS appears to evoke a reverse order stimulation, i.e. activating first the postsynaptic neuron and then the presynaptic neuron, which seems to originate LTD [49,51]. Activation of post synaptic membrane N-Methyl-D-aspartate (NMDA) receptors seems to be the origin of LTP. NMDA receptors have cationic channels, that are blocked by magnesium ions at rest, but these channel blocks can be eliminated by cell membrane depolarisation. This fact will allow the entry of calcium ions in the postsynaptic neuron, leading to larger excitatory post-synaptic potentials and the LTP phenomenon. This will only occur if a large and fast rate increase of postsynaptic calcium occurs, induced by high-frequency stimulation [47,49,51]. In contrast, low frequency rTMS will originate a slow flow of calcium ions, therefore inducing LTD. In vitro studies showed that LTD needs rTMS for long periods (600 pulses) but LTP can be induced by short train of high-frequency rTMS [49,51]. The reinforcement of synaptic strength that occurs in the phenomena of LTP and LTD takes place in an acute phase, lasting up to about 60 minutes, but a longer phase develops later, after changes in protein synthesis occur [51].

Theta burst stimulation mechanisms, although not fully known, are also linked to a possible LTP/LTD-like plasticity [6,45,54]. In the TBS protocols (continuous or intermittent), the NMDA receptors are also involved. So in TBS, the LTP and LTD-like phenomena are related to the activation of the calcium and NMDA channels at the post-synaptic membrane, both known to be key factors for the induction of synaptic plasticity [45,54]. But why continuous and intermittent TBS have inverse effects? It is assumed that the specific TBS protocol is able to induce a mixture of excitatory and inhibitory effects. The short 3 pulse burst at 50Hz originates a short-latency facilitation but also a longer-latency and weaker inhibition. The intermittent form, in which the TBS trains are short with pauses in between, maintain the excitatory effect dominant and thus induce an LTP-like effect. In contrast, if the TBS train is long and delivered continuously, the inhibitory effect will overcome the facilitatory effect, thereby producing an LTD-like effect [6,54].

One of the other theories associated with TBS effects relates to Glutamate Receptors, specifically with the modulation of their activity in neuronal circuitry. This modulation assumes a behavior identical to what happens in intermittent (iTBS)-LTP and continuous (cTBS)-LTD-like effects [6]. This is valid also for rTMS. In animal models, it is known that high-frequency rTMS (20 Hz) or similar techniques are capable of inducing lasting activation of the glutamate connections in structures such as the hippocampus and the nucleus accumbens, by stimulating the prefrontal cortex, suggesting a connection between fast frequency rTMS and possible therapeutic effects. Such an effect was not found when low-frequency rTMS was performed [55]. Yang et al. found that several sessions of rTMS high-frequency stimulation (10 Hz) over the left dorsolateral prefrontal cortex (DLPFC) in patients with major depressive disorder induced clinical improvement, and this was also associated with increased ipsilateral DLPFC glutamate levels measured by short echo proton magnetic resonance spectroscopy, suggesting a direct effect of glutamatergic influence in the possible therapeutic benefits of rTMS [56]. Increased glutamatergic ratios were also found by Croarkin et al. over the anterior cingulate cortex and the left dorsolateral prefrontal cortex after 10 Hz rTMS, not only after stimulation but also after 6-month follow-up [57].

Studies on the possible GABAergic role linked to rTMS have shown considerable variability and results have not been consistent, unlike what has happened with TBS. It appears that the TBS mechanisms associated with intracortical GABAergic activity seem to point to a possible difference between rTMS and TBS [6]. TBS effects may be related to GABA receptors [6,58], and these glutamatergic and GABAergic theoretical hypotheses are often mentioned together. Recent research points to a more important probable role of the GABAergic intracortical inhibition associated with TBS mechanisms [59,60]. TBS-related enhancement of synaptic plasticity appears to be related to presynaptic changes in GABA release [59]. LTP may be induced because the specific theta frequency is able to reduce postsynaptic inhibition and thus boost more postsynaptic depolarisation, possibly by consenting increased NMDA receptor responses [6,59]. This so-called disinhibitory behaviour is associated with a low GABA release. Thus, animal studies have shown that the blockade of the presynaptic GABA receptors (GABA-A or GABA- B) stops GABAergic inhibition, playing an essential role in LTP induction. As for LTD and the possible relation to GABAergic presynaptic activities, there seems to be some relationship but so far there are few conclusions on this topic [59].

LTP and LTD phenomena are also involved in neurotransmitter activity, and this is an essential part of the therapeutic effects of rTMS. Dopamine is one of the most studied neurotransmitters, with proven fluctuations associated with rTMS, in the treatment of

various neuropsychiatric diseases [49,51]. Changes in dopamine concentration have been documented in several TMS experimental studies, mainly in specific diseases such as Parkinson's disease. It is also important to note that these fluctuations occur cortico-subcortically and may occur in more distant locations from where the actual stimulation occurred [49]. Such cerebral stimulation can be more effective depending upon the activated hemisphere: some studies show that high-frequency rTMS administered to the left prefrontal cortex increased dopamine release [61]. Strafella et al. reported evidence that a single session of excitatory rTMS of the left prefrontal cortex increased dopamine significantly only over the left hemisphere, namely in the left caudate nucleus [62]. Ko et al. 2008 reported that the left dorsolateral prefrontal cortex (DLPFC) inhibition, using cTBS, resulted in impaired dopamine release compared to sham stimulation, whereas right brain inhibition showed no significant effect [63]. Cho et al. also found that the right and left hemispheres may also respond differently to rTMS. They applied high-frequency (10 Hz) rTMS to the left and right dorsolateral prefrontal cortex, finding an increase in ipsilateral dopamine release after left but not after right hemisphere stimulation [64].

There is also some evidence of neurotrophic effects relating rTMS to dendritic growth and neurotrophic factors. Studies like Ma et al., using hippocampal cell cultures, demonstrated that low vs high intensity (1.14T vs 1.55T) 1 Hz stimulation can have opposite effects - low intensity promotes growth and increases synaptic contact density, while higher intensities lead to a diminished number of synapses [49,65]. Other studies have focused on brain-derived neurotrophic factor (BDNF) function. This trophic factor is believed to be involved in several functions such as neuronal survival, dendrite growth and synapse formation. But so far data from BDNF studies using rTMS sessions have been controversial, with some rTMS studies showing an increase in BDNF serum levels and others showing no effect [49]. However, multiple sessions over several weeks are able to increase BDNF mRNA levels in the hippocampus as well as in the pyriform and parietal cortices. [49,66]. The positive effects of several rTMS sessions involving neurotrophic factors and their neuroprotective and neuroplastic effect have been shown by several studies [67–69], something that can happen in the stimulated area and in remote brain regions [49]. The effects of TMS on the central nervous system have been shown to vary, influencing neuronal morphology, neurogenesis, concentration of neuro-mediators, and neurotrophic factors, just to name a few. The probable combination of these factors may be linked to the positive therapeutic effects of transcranial magnetic stimulation [49].

3.5.2- High frequency rTMS and the Facilitatory effect

The results of scientific research leading to the use of rTMS as a therapeutic alternative showed that stimulating the cortex with approximately 5 Hz or more originates an increase in MEP responses, when MEPs are evoked with stimuli above threshold. These data suggested an increase in the excitability of the output neurons [13]. Underlying increase in excitability and MEP amplitude is thought to result from the modification of neuronal sensitivity to excitatory influences, perhaps associated with a change in the effectiveness of excitatory synapses. This phenomenon is associated with the theory of long-term synaptic potentiation (LTP) [13,70]. Support for this theory was provided by types of stimulation that resembled theta modulating frequencies, such as the theta burst pattern of stimulation. In these patterns, short bursts of high-frequency stimulation (50 Hz) are applied at a carrier frequency of 5 Hz [13,45]. This type of stimulation proved to be highly capable of not only inducing LTP in animal synapses but also significantly increasing human MEP amplitude, suggesting that there may exist a direct relationship between these two phenomena [13,45,71]. This excitatory effect and the possibility that this might influence behaviour was at the basis of the possible use of high-frequency rTMS in depression [18], for example.

3.5.3- Low frequency rTMS and the Inhibitory effect

The other main effect related to rTMS use over the motor cortex relates to MEP depression following the application of low-frequency stimulation trains, at 1 Hz or less [72]. Contrary to high-frequency stimulation, this low-frequency rTMS did not present relevant risks related to the induction of epileptic seizures, and it can be used for longer periods with more lasting effects [13]. The effect of low-frequency stimulation was compared with the phenomenon of synaptic long-term depression (LTD). This inhibitory phenomenon was also widely adopted by research in the area of cognition, with researchers using this technique as a safe form of temporarily impairing certain cortical areas, in order to study their relevance in behaviour, and relating their results to functional brain imaging studies [13].

3.5.4- Theta burst stimulation (TBS)

3.5.4.1- Theory and technique

With the technical development of magnetic stimulation devices, new forms of repetitive stimulation began to appear [54]. One of these new protocols was Theta Burst Stimulation (TBS) [45]. Despite being a method that initially appeared to be more powerful and with more reproducible effects than classical rTMS, premises that have not been consistently proven over the years, it is a protocol that assumes a particular importance because it is fast to implement [45]. It takes less than 3 minutes to apply, much faster than classic rTMS protocols, which may exceed 30 minutes (up to 37.5 minutes for the FDA-approved sessions for depression therapy) [54,73]. The theta burst technique derives from the theory related to hippocampal functioning, which is known to discharge in the theta band (4 to 7 Hz), frequencies which are also used in animal research in an attempt to induce brain plasticity [54]. Thus, the first stimulation protocol in 2005, promoted the continuous application of 20 seconds of a three pulse at 50 Hz burst, each delivered at 5 Hz, in the theta frequency (cTBS) (Figure 1.3.7). This type of stimulation with 300 pulses led to a decrease in amplitudes in motor evoked potentials (MEP) [45,54]. Next, researchers adapted the protocol, trying to mimic the types of stimulation that normally induced LTP in animal studies. In this case, the triple-pulse bursts were delivered only with a 2-second duration (sets of 10 bursts), repeated every 10 seconds, totalling 190 seconds (iTBS) [45]. This type of stimulation achieved an opposite effect to that previously seen, causing an increase in the amplitudes of MEPs, facilitating their formation and supporting the hypothesis of an LTP-like effect [45,54]. The most used TBS protocols currently apply 600 pulses, using iTBS for 190 seconds or cTBS for 40 seconds [47,73,74].

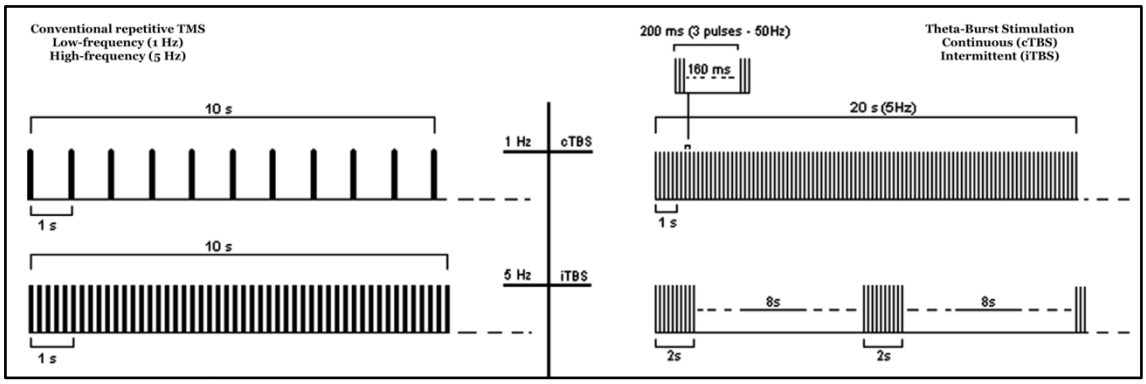

Figure 1.3.7. Representation of conventional repetitive transcranial magnetic stimulation (low and high-frequency rTMS) and theta burst stimulation (cTBS and iTBS).
Adapted from "Safety, ethical considerations, and application guidelines for the use of transcranial magnetic stimulation in clinical practice and research.", by Rossi S, Hallett M, Rossini PM, and Pascual-Leone A., 2009, Clin Neurophysiol, 120(12):2008–39.

Individual variability is an important factor to address when analysing TBS after-effects. Several relevant studies tried to identify predicting factors of the individual's response to TBS [54]. The most frequently suggested causes include genetic factors (such as BDNF Val66Met single nucleotide polymorphisms) or differences in intracortical networks between subjects [54].

TBS intensity is another important factor. Although the resting motor threshold (RMT) can be used, the most frequent in TBS studies is the use of the active motor threshold (AMT). Accordingly, the most common intensity used when applying iTBS and cTBS sessions is 80% of AMT [54].

3.5.4.2- Facilitatory and Inhibitory effects

It has been shown that in both forms of stimulation, iTBS and cTBS, after-effects are induced beyond the stimulation period, facilitating or inhibiting cortical functioning (Figure 1.3.8), for a period of 20 minutes in the case of iTBS and 60 minutes in the case of cTBS [54]. The theory behind these effects has been discussed in chapter 3.5.1. and these after-effects are found in a very solid manner in most studies. Wischnewski et al. in 2015 performed a systematic quantitative review on cortical excitability after iTBS and cTBS [47]. They found from the 64 studies that met their criteria, that change in cortical excitability duration at the primary motor cortex was different in iTBS vs cTBS. A session of iTBS for 190 seconds is able to increase cortical excitability for up to 60 minutes, originating a mean increase in excitability of approximately 36%. In contrast, 40 seconds

of cTBS induced a mean decrease in cortical excitability of -23%, with a duration of up to 50 minutes [47]. But a careful use of these protocols must be taken when trying to augment effect duration by increasing TBS duration. A study by Gamboa et al. has showed that inverse results may be found if double duration is used (i.e. iTBS becomes inhibitory and cTBS becomes facilitatory) [75]. This small sample study alerts to the fact that longer duration may reverse specific TBS induced effects. However, other studies using more than one block of 600 pulse iTBS did not find these inverse effects [76], again advising caution when performing and analysing TBS results.

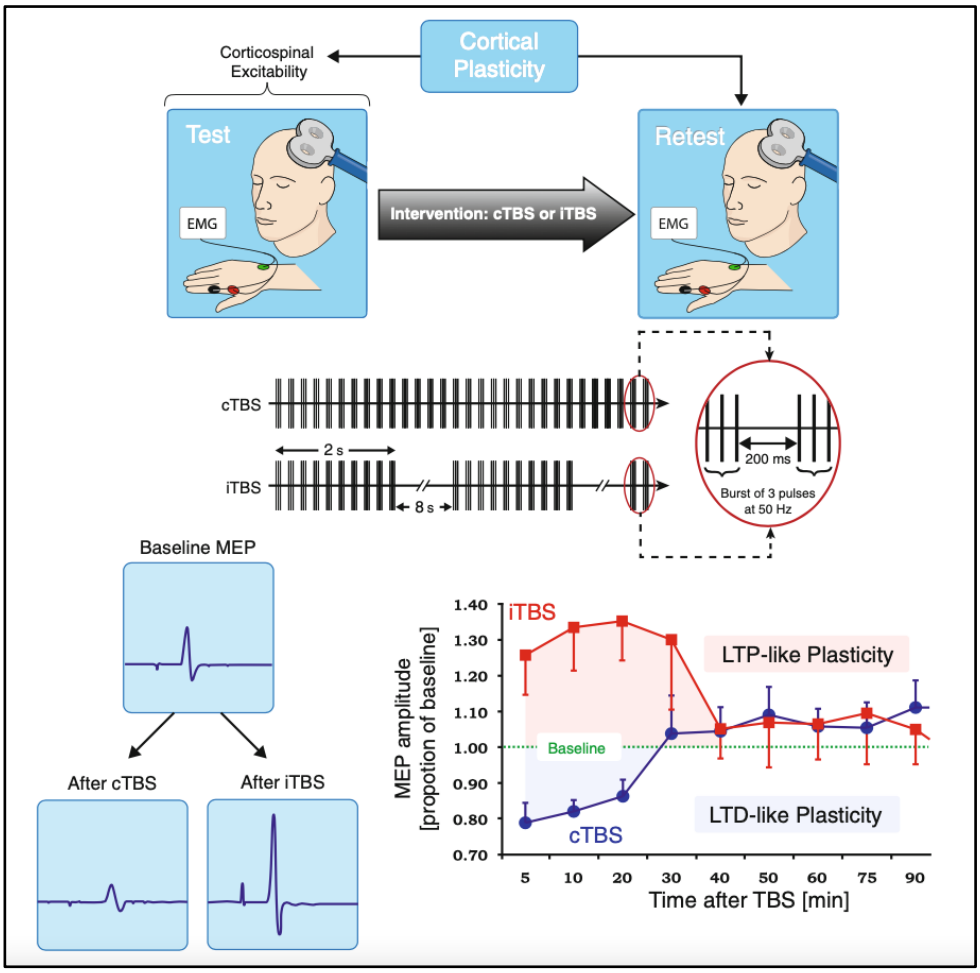

Figure 1.3.8. Schematic overview of the effects of theta burst stimulation (iTBS vs cTBS) over the motor cortex. After iTBS, single-pulse TMS-induced motor evoked potentials (MEP) feature increased amplitude. After cTBS, a MEP amplitude suppression is found.
Adapted from "Transcranial magnetic stimulation" (p.132), by Rotenberg AM, Horvath JC, and Pascual-Leone A., 2014, New York, United States of America: Humana Press. Copyright © 2014 Springer Science + Business Media.

3.5.4.3 TBS versus rTMS: an overview

Similarly to what happens with rTMS, TBS is also able to stimulate cortical neurons without inducing a relevant electrical current in the skin or subcutaneous tissue, so it is normally well tolerated by patients [12].

The use of TBS protocols helps to address one of the main problems associated with rTMS – classic repetitive stimulation is time-consuming. Thus, one of the main advantages of both iTBS and cTBS is their shorter duration [6,45]. As stated before, TBS protocols do not usually exceed 190 seconds. The shorter duration allows two main advantages in therapeutic use: to increase the number of patients that can be treated with a single rTMS equipment and to significantly reduce the cost associated with each treatment session [6,77]. A recent study by Mendlowitz et al. comparing the cost of iTBS vs 10 Hz rTMS treatment in depression, concluded that the shorter duration iTBS sessions allow increased treatment capacity and wider affordability [77]. Another important advantage relates to the lower stimulation intensity used. As mentioned before, the usual intensity suggested for TBS protocols is 80% of AMT [45]. This lower intensity allows a more comfortable stimulation experience, especially if the treatment procedure involves multiple sessions [6]. Finally another relevant advantage relates to safety, as TBS appears to originate fewer major side effects such as seizures when compared to rTMS protocols [19,46]. As an example, a 2017 literature review found no seizures or mania episodes reported as side-effects in the 26 controlled, open-label studies using TBS in the treatment of various psychiatric disorders [74].

General disadvantages, regardless of the cortical region to be treated, are similar to those associated with rTMS utilisation, relating to minor side effects such as mild headaches, tinnitus, mild discomfort involving cutaneous sensations, and muscle contraction [6,46]. Another mentionable disadvantage is that being a newer and less studied technique compared with rTMS, there is less information about the real modulatory effects of using iTBS and cTBS in cortical areas outside the motor area, which may limit its more comprehensive use [6,78,79].

3.6- Safety overview

TBS usage has been growing and with the increase in the number of stimulated patients as well as the increase in sessions per subject, more data regarding safety become available. Adverse reactions to TBS have been found, in part similarly to what is

described with rTMS [46,74], with symptoms such as transient headaches and neck pain comparable to or even less frequent than reported for high-frequency rTMS [74].

Reviews such as Oberman et al. (2011) and Rachid (2017), evaluating studies with healthy and ill subjects, concluded that the technique is safe [46,74]. Adverse events were considered mild, occurring in a very small percentage of the participants (around 5%). Controls have described discomfort or focal pain in the application zone, mild transitory headaches, cutaneous sensations, muscle contractions, and worsening tinnitus in patients [46,74]. Vagal responses have also been mentioned. Even though rare, transient impairment of working memory after TBS has also been found, alerting to the possible multiple layers of after-effects that can occur after TBS stimulation [19,46,74,80]. Perception of speech and auditory threshold appear not to be altered, at least if stimulation is kept below 84 dB SPL (64% maximum stimulator output with a figure-eight coil) [81], but specific TBS studies about these possible after effects are scarce [74].

So far, only one seizure was reported using TBS [46,74]. The event happened with a 33-year-old healthy man. No risk factor was identified but he had an altered sleep pattern due to a recent transatlantic flight. The protocol used was also slightly different, using 100% AMT instead of 80% AMT [46,74]. In TBS, a crude risk of seizure per subject is estimated at 0.1% [74]. Also, the crude risk of mild adverse events is 4.8% for healthy controls and almost 5% overall [74].

Studies in children using TBS are scant, so safety information about using TBS in children is limited. However, data so far suggest that adverse TBS effects in children are similar to those in adults [82]. In a systematic review of the literature about the safety of TMS/TBS use in children, Allen et al. found three TBS studies (90 healthy children and 40 children with disorders of the central nervous system) [82]. In these, some mild adverse events were reported, but no seizures occurred [82].

When testing new or significantly modified protocols, it is particularly advisable to adopt precautionary and monitoring procedures (e.g., blood pressure and heart rate), especially if TMS is associated with neuroimaging studies, to screen for after-effects and follow patient's safety [19]. One of the relevant areas to study relates to cardiovascular and blood flow monitoring and the relation between the autonomic system and rTMS [9,19]. Research using TBS on this topic is also scant. The first studies in the 1990s using rTMS showed that some of the known secondary effects were linked to autonomic responses, derived from nonspecific arousal and not resulting from direct cortical stimulation. This was attributed mainly to stimulus discomfort [9]. But later studies showed that the use of rTMS and TBS can actually influence cerebral oxygenation, blood

flow, blood pressure, and heart rate: however, results were not homogenous [19,83–85]. In a study in depressed patients, Udupa et al. evaluated heart rate variability after several sessions of rTMS, with over 18.000 stimulations. In these patients, a reduction in the sympathetic/ parasympathetic ratio was found, suggesting improvement in sympathovagal balance [86]. Tupak et al. used TBS to study if the stimulated prefrontal cortex could influence local oxygenation. After applying cTBS over either the left or right DLPFC, they concluded that a decrease of the prefrontal oxygenation occurred, reflecting an impairment in the prefrontal cortical areas after inhibitory TBS. In a more recent study, Poppa et al. studied the effects of both iTBS and cTBS over the right frontotemporal cortex on anxiety and cardiovascular responses [85]. In 24 volunteers, not all naïve to TMS, they used a cross-over design to evaluate heart rate variability and pulse transit time. Generalised increased anxiety was found. iTBS increased heart-rate variability wile cTBS increased pulse transit time latency, suggesting a reduction in systolic blood pressure. These results led to the conclusion that TBS is able to induce effects on visceromotor networks [85].

3.7- Contraindications

Unfortunately, not all subjects can undergo TBS / TMS. To guarantee the safety of patients and healthy subjects, some contraindications must be taken into account. The presence of any metallic elements close to the coil is the only absolute contraindication [19]. Intracranial foreign metal bodies or implanted devices, such as cochlear devices or deep stimulators, should not be present [19,87].

Some conditions are considered to increase the risk of seizure or have uncertain risk, such as conditions related with the technique, namely increasing frequencies or number of pulses beyond standard protocol limits [19], or conditions linked to the patient, specifically history of epilepsy or seizures, presence of lesions of the brain (vascular, tumoral, traumatic or metabolic), use of drugs that can lower seizure threshold such as amitriptyline, nortriptyline, clozapine, amphetamines, cocaine, ecstasy or alcohol) and sleep deprivation [19]. Caution is also advised when performing TBS in patients with severe or recent heart disease [19].

4. Scientific research and clinical use of Transcranial Magnetic Stimulation over the cortex

With the proliferation of scientific articles between the end of the 90s and the beginning of 2000, already with some guidelines in use, the use of rTMS as a therapeutic tool has been consolidated [88,89]. The initial use was based on an off-label implementation in several neurological and psychiatric diseases [7]. This fact drew increased attention to the implementation of the technique, as its use often took place in the context of a last resort for patients, posing serious challenges at ethical and scientific levels [7]. One of the most important steps in the attempt to regulate the use of TMS in research and clinical settings occurred at the consensus conference of 2008 and with the subsequent publication of the 2009 guidelines [7,19]. These guidelines addressed the TMS principles, technique, and rTMS application safety (frequencies, intensity, and train duration). They went even further, by analysing the risk of rTMS use in various neuropsychiatric illnesses, its general contraindications, and also the introduction of new stimulation paradigms [19].

The first therapeutic Food and Drug Administration (FDA) approved use for rTMS occurred in 2008, for application in the clinical treatment of a specific form of depression, namely medication-refractory depression (FDA K061053) [7,22]. This approval followed a 2007 study by O'Reardon et al., which used a multicentre, randomised clinical trial with nearly 300 major depression patients with at least one failed antidepressant treatment [32]. Patients were submitted to either active or sham rTMS over the left dorsolateral prefrontal cortex, and rTMS was proven effective in treating major depression and was associated with only minor side-effects [32]. The first approval was given to the NeuroStar device (Neuronetics Inc) and to the protocol using high-frequency stimulation (10 Hz rTMS), supra-threshold intensity, with daily sessions from 4 to 6 weeks [7].

Since then, both FDA and European CE Mark approvals were awarded to several devices, and one of the latest FDA approvals occurred in 2018 with the clearance of rTMS for the treatment of obsessive-compulsive disorder (OCD) [1,14,22]. Also in 2018, the FDA approved a short duration Theta Burst Stimulation (TBS) protocol (3 minutes with a MagVenture device), for treatment-resistant depression, following the positive therapeutic results of a randomised, multicentre clinical trial, comparing TBS with a "classic" rTMS protocol [90].

A guideline from the International Federation of Clinical Neurophysiology was subsequently published, based on scientific evidence published on the therapeutic use of rTMS or TBS [22]. Experts concluded that, in spite of frequent heterogeneity found across studies, the body of evidence was enough to assign level A - definite efficacy – to the antidepressant effect of high-frequency rTMS of the left dorsolateral prefrontal cortex. The same level A was assigned for the analgesic effect of high-frequency rTMS of the primary motor cortex contralateral to pain. Levels B and C (probable and possible efficacy, respectively) were assigned to therapeutic use in diseases like schizophrenia, stroke, and tinnitus. It was emphasised the necessity to optimise protocols in order to find the best way to apply the technique effectively in clinical practice [22]. Improvements of general study design and a decrease in clinical and methodological heterogeneity will contribute towards reducing efficacy uncertainties [4].

Next, we will evaluate some of the possible applications of TMS/TBS, focusing on the most relevant aspects, both historically and also regarding the scope of this thesis.

4.1 Stimulation of the Prefrontal cortex

4.1.1 Depression

The therapeutical use of rTMS and TBS in depression is an essential topic when addressing the stimulation of the prefrontal cortex.

Leading to FDA approval, more than 89 individual trials and 4 multicentre trials found significant effects of daily prefrontal rTMS, during 3–6 weeks compared with sham controls in patients with refractory major depression, previously treated with at least two antidepressant drugs [24]. The level A evidence relates to excitatory stimulation over the left DLPFC but inhibitory stimulation is also effective over the right DLPFC, with a level B classification [22].

Although there is a typical protocol accepted as effective, it remains to be seen which will be the most effective protocol. Parameters such as choosing the hemisphere to be stimulated, the best technique to select the area of the cortex to be stimulated, coil position (angle), the most appropriate distance away from the motor cortex hotspot (5 vs 6 vs 7 cm), stimulation intensity, ideal number of sessions, among others, may affect efficacy [24].

One of the recently asked questions is: what is the most effective therapeutic method for depression - rTMS or TBS? The main rTMS treatment protocol used consists of a 10 Hz rTMS session of 37.5 min, every day for 4-6 weeks, over the left DLPFC (5 cm anterior to the motor cortex). Intensity of 120% of the resting motor threshold is used, with pulses being delivered during 4 sec, with a 26-sec interval (off time) [32,91]. More recently, in a study that led to the FDA approval for use in refractory major depression (a randomised, multicentre clinical trial), Blumberger et al. found that iTBS to the left dorsolateral prefrontal cortex, administered daily for 4–6 weeks, was non-inferior to "classical" high-frequency rTMS for the treatment of depression [91]. Similar results were also found in the number of dropouts, side-effects, and tolerability profiles. A TBS treatment in these patients rather than rTMS will originate optimised laboratory management, allowing patients to save personal time and the laboratory to be able to treat more patients [91].

4.1.2 Cognitive domains related to the prefrontal cortex

TMS research in cognitive science is one of the most important non-invasive exploratory functional techniques, being used in several cortical regions, allowing the study of the human brain in vivo [24]. Its use has been dependent on different protocols, either online (where a direct intervention on a cortical area takes place, trying to influence its functioning at the same time it occurs, transiently impinging neuronal function), or as an offline intervention (where a lasting modulation of the cortical function is attempted, evaluating its effect beyond the exact time of stimulation) [19,24]. These methods allowed a better understanding of specific cortical areas in cognitive processes, by inhibiting, or increasing cortico-subcortical functioning. Studies in neurocognitive science using TMS can also be easily paired with other approaches such as traditional neuroimaging evaluations (fMRI or PET) [24].

However, the evaluation of outcomes and after-effects in cognitive studies is controversial and challenging [92]. Firstly, because some operational definitions are not universally accepted, and secondly, because there seems to exist a lack of consensus as to how the measurement of some cognitive executive functions should be performed [92]. Furthermore, the relatively low temporal resolution characteristic of the functional brain imaging studies (up to 6 seconds), limited by the haemodynamic response time, can be a real problem when evaluating more acute effects of TMS, possibly preventing the coupling of effects between stimulation and imaging [93]. In some study designs,

evaluating more acute results, it may be more effective to use electroencephalography or magnetoencephalography, which present a millisecond temporal resolution [93].

The use of rTMS or TBS to improve either cognitive impairment or even already installed dementia has become more frequent, with increasingly larger studies using more elaborate scientific designs and randomized controlled studies [94]. Recent reviews and meta-analyses have focused on the possible efficacy of these non-invasive techniques, and results are encouraging especially with regards to milder cognitive impairments [94–96]. Authors involved in this type of research have reinforced the notion that the technique has been shown to be safe and well-tolerated by patients with cognitive decline [94–96]. In their systematic review and meta-analysis about cognitive treatment of Alzheimer's disease (AD) using rTMS, Lin et al. included 12 studies (8 RCTs) with 231 pooled patients, using mainly high-frequency rTMS [94]. Unfortunately, multiple cortical target sites were used, again limiting generalisation of the results. Even so, the authors concluded that rTMS is able to significantly improve cognitive ability in mild to moderate AD [94].

Iriarte et al. analysed the use of TMS in elderly cognitive impairment, in applications with a Level I Evidence, still without FDA approval [97]. They suggested that a possible positive effect in these patients, although still unclear, maybe due to the increase in synaptic plasticity induced by rTMS, but alerted to the fact that, even though rTMS can be used to improve cognition, research limitations (small sample sizes and variability in protocols between studies), still limit data generalization [97]. These cognitive improvements were found in studies in patients with mild cognitive impairment (MCI) and Alzheimer’s disease, in memory and behavioural symptoms [97].

Rabey et al., conducted a study that led to a device approval and its clinical use in Europe, obtaining a CE Mark (certification of conformity with health, safety, and environmental protection standards for products sold within the European Economic Area), the Neuronix, Ltd. - neuroAD Therapy System [98]. In this study, the use of rTMS together with cognitive training, for 1 hour per day for 6 weeks, lead to an improvement in cognitive scores, in patients with probable mild to moderate AD [98]. These authors used an unusual and complex bi-hemispheric stimulation protocol, alternating hemispheres in each stimulation day. They also used a multi-site stimulation in each session, stimulating the DLPFC, Broca, and Wernicke regions, and even the parietal somatosensory association cortices [98].

The use of TBS in cognitive impairment remains scant, but results seem to be similar to those found for rTMS. A recent study on poststroke cognitive impairment compared the

effectiveness of 5 Hz rTMS and iTBS against sham stimulation, using small groups with a total of 41 patients [99]. All groups were subjected to 10 stimulation sessions over the left dorsolateral prefrontal cortex. After outcome evaluation, researchers concluded that both iTBS and 5 Hz rTMS were effective, improving global cognition, attention, and memory function [99].

Lowe et al., in a systematic review and meta-analysis studied the use of executive functions after TBS over several regions of the prefrontal cortex [92]. They included a total of 759 participants (a mean of approximately 20 subjects per study), in 29 cTBS and 8 iTBS studies, almost all young healthy adults (only one study with older adults). A negative effect on executive function task performance after cTBS was reliably found ($p<0.001$), and this effect was higher for left-sided stimulation. A lesser effect was also found after iTBS, being more task-specific, but clearly geared towards enhancing cognitive performance, particularly in working memory. These results support the theorised and expected modulatory directions for both cTBS and iTBS in these patients [92].

Continued investigation in this area remains essential. On one hand, because the number of studies and evaluated subjects is still low to support a robust scientific evidence. On the other hand, because not all studies have found positive results.

As an example, a recent study by Hill et al., studying cognitive impairment in Parkinson's disease, stimulated a group of 14 patients using iTBS, trying to induce excitatory plasticity [100]. Executive functions and working memory were evaluated with a double-blind sham-controlled crossover experimental design, and showed no significant improvement after stimulation, raising the doubt whether iTBS can be effective in all cognitive domains and/or diseases [100].

Studying and understanding cognitive control is an essential part of the potential use of TBS. Investigation in healthy subjects is one of the possible available methods. Gratton et al. tried to better understand cognitive control networks using a TBS intervention in 27 healthy right-handed subjects, with an outcome control through resting-state fMRI scans [101]. The author resorted to a left-sided multiple stimulation site protocol and evaluated the subsequent functional connectivity in the brain. They found that TBS induced increased connectivity between regions, especially in fronto-parietal network. They also reported that connectivity was increased between the DLPFC (right or left) and the frontal, parietal and cingulate cortex, confirming the theorised widespread change in brain functions linked to the stimulated networks [101].

The use of rTMS on other cognitive domains like creativity is even rarer, but since part of the neural networks involved in the creative process or even divergent thinking are connected to some of the target areas commonly used for cognitive activation in diseases with cognitive impairment, especially the prefrontal cortex, there is some theoretical reason for its study [102,103]. Kleinmintz et al. attempted to analyse the role of the left inferior frontal gyrus in the creative process formation [104]. These authors postulated that inhibition of this area could influence the increase in creativity, by "releasing" neural networks that may underlie such creativity. Using fMRI to evaluate the outcomes, they applied cTBS over the left inferior frontal gyrus, with a 900-pulse offline protocol in an attempt to induce an inhibitory effect. The rationale behind this approach was based on the authors' belief that this area plays an important role in idea evaluation, e.g. making sure that new ideas are feasible [104]. They found that cTBS on the left inferior frontal gyrus increases originality scores in a divergent thinking task compared with vertex stimulation [104]. However, the two sessions of the crossover protocol were only separated by 90 minutes, and it is not clear whether this can be a sufficient washout time for this cortical area. In a very recent study, Thakral et al. also used cTBS in order to study divergent thinking [105]. The main objective was to disrupt hippocampal brain networks, trying to impair divergent thinking. They compared stimulation on the vertex and at the left angular gyrus, guided by functional magnetic resonance imaging (fMRI), thus secondarily influencing hippocampal regions [105]. cTBS induced a reduction of the number of creative uses that are associated with each divergent thinking task, leading the authors to conclude that the hippocampal network plays a critical role in these cognitive functions [105]. These two studies, with different results, also suggest that the same stimulation type (cTBS) can influence neural networks differently, depending upon the primary stimulated area.

4.1.3. The P300 evoked potential and its use with Transcranial Magnetic Stimulation

Although there are known TMS after-effects associated with cognition, especially after stimulation of the prefrontal cortex, the full magnitude of these effects is still under research. Furthermore, investigation in this area is, so far, somewhat limited and has revealed some controversial results [106,107].

One of the techniques that can be very useful in identification, assessment of magnitude, and follow-up evaluation of after-effects is the use of event-related potentials (ERPs)

[36,106,107]. More specifically, the use of long-latency evoked potentials like the auditory P300, which is the most studied neurophysiologic diagnostic tool used to evaluate cognitive disorders. Although P300 in association with TMS has been scarcely used up until now, encouraging results have been found [106,107]. To date, less than 20 studies can be regarded as addressing the association between the auditory P300 and rTMS, and we found only two studies that used an intervention protocol combining TBS and P300 [106,108,109].

Cognitive-related responses can be studied by ERPs, as ERPs represent cerebral responses to specific stimuli associated with the cognitive process, thus conveying information from EEG neuronal dynamics [106,110,111]. They depend mainly on stimulus properties such as novelty, probability, and discriminatory difficulty [106].

4.1.3.1 The auditory P300

The auditory P300 (also known as P3 or P3b), is a positive cognitive evoked potential that typically peaks up to 300 ms, resulting from the discrimination of a rare, task-relevant stimulus (Figure 1.5.1) [106,111–115].

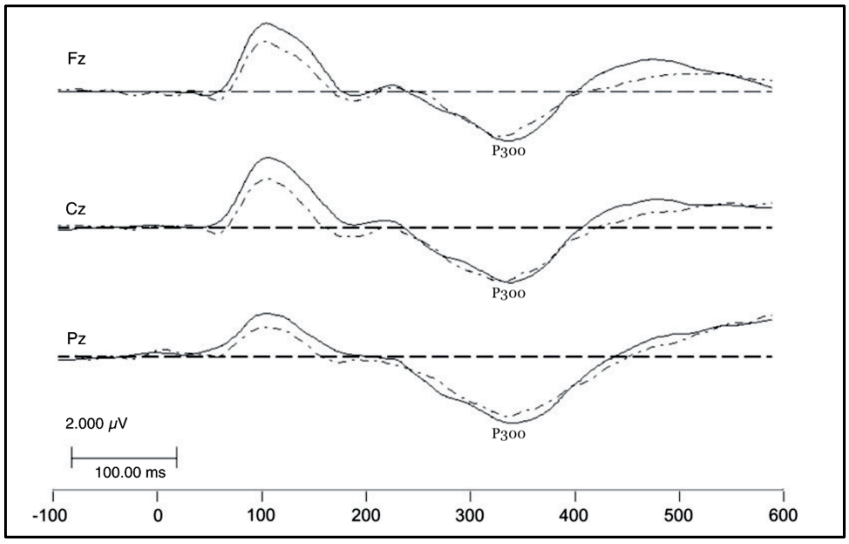

Figure 1.4.1. Representation of P300 event-related potential on different sites (Fz, Cz, and Pz).
Adapted from "P300 waveform and dopamine transporter availability: a controlled EEG and SPECT study in medication-naive patients with schizophrenia and a meta-analysis.", by Chen KC, Lee IH, Yang YK, Landau S, Chang WH, Chen PS, et al., 2014, Clin Neurophysiol, Psychol Med. 2014;44: 2151–2162.

P300 is an evoked potential known since 1965 when it was first reported by Sutton et al. [111], and is considered a cognitive potential related to an event, depending on subject's attention and discrimination, and which also reflects more advanced and purposeful stages of task processing [106,111–113]. Auditory ERPs can be elicited by a typical oddball task, which requires subjects to recognise infrequently presented target tones presented randomly between frequent stimuli, either by pressing a button or counting the stimuli. This paradigm seems to initially activate frontal activity and presents a regional centro-parietal scalp distribution, peaking over midline scalp [111,116]. Reflecting predominantly processing speed, P300 is an important tool in the study of cognitive processes and memory in normal subjects and in psychopathology, as its delay can be used as a marker in the identification of cognitive deterioration [113,116,117]. Amplitudes are usually directly associated with the amount of attentional resources assigned to the task [106], and seem to be more affected by the temporal-parietal junction integrity [116]. The source of P300 are still dubious, with multiple cortical and subcortical neural generators, such as the hippocampus, the superior temporal sulcus, the ventrolateral prefrontal cortex, the posterior cingulate cortex, and the intraparietal sulcus [111,116]. Inter-hemispheric connectivity may have also an important role since activity seems to propagate through *corpus callosum*, after the initial frontal activation, and larger callosal fibers are associated with better P300 performance (amplitude and latency) [116].

The N200 peak, the main negative component that precedes the P300, may also provide relevant information regarding cognitive processing, as it derives from the initial, subconscious processing of the stimulus involved in the oddball task. Even though it also has multiple cerebral origins, some authors found that its formation tends to significantly lateralise towards the left anterior region of the mid-cingulate cortex [118,119].

Auditory P300 performance can be affected by several biologic factors such as fatigue and old age (both with decreased amplitude and increased latency), and gender (amplitude: female>male, latency: female<male). The variability associated with these factors should be considered when performing group comparisons [111,116]. Addressing age as an example, P300 latencies are significantly lower in younger ages compared with older individuals, as can be seen in Figure 1.5.2, where latencies can be found just over 200 ms in the 20-30 year range of healthy subjects [114].

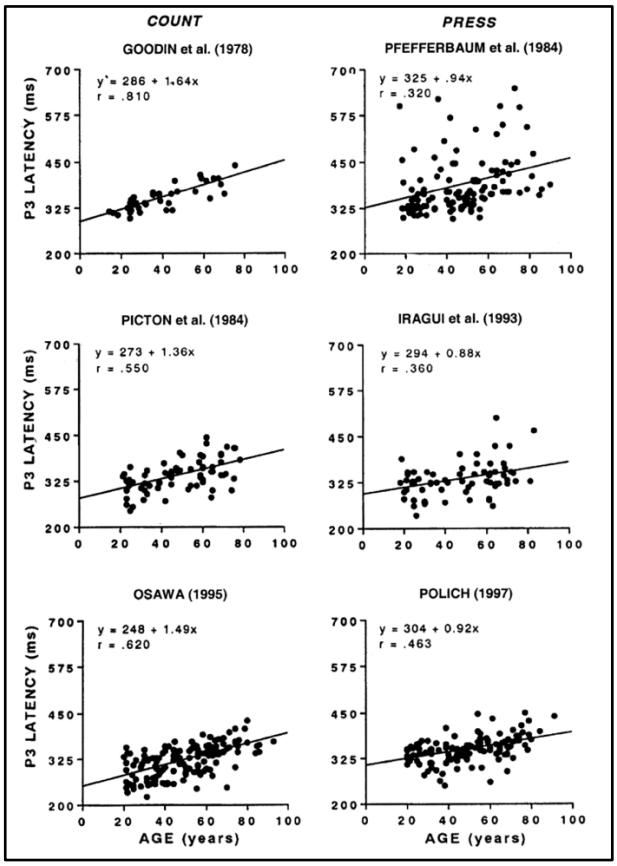

Figure 1.4.2. Representation of the scattergrams of P300 latency and age from representative normative aging studies that used auditory stimuli.
Adapted from "Clinical application of the P300 event-related brain potential.", by Polich J., 2004, Phys Med Rehabil Clin N Am., 15: 133–161.

Beyond its relevant role in cognition research, there are several clinical applications in which P300 is able to contribute to the diagnostic procedure, especially when cognitive dysfunction is present [111,114]. Impaired P300 can be found in some psychiatric and neurologic diseases such as dementia (mostly delayed latencies), schizophrenia (changes in latency and amplitude), mood disorders (variable changes), and multiple sclerosis (mostly prolonged latencies) [111,120].

4.1.3.2 P300 and rTMS/TBS

In most of the studies combining rTMS and ERPs, stimulation occurred over the dorsolateral prefrontal cortex (DLPFC), either left or right, rarely bilaterally, and few authors used sham/placebo stimulation [36,107]. The prefrontal cortex (PFC) has been associated to an important role in cognitive control. This may be related to the sensory projections from visual, auditory, and somatosensory cortices to the DLPFC, thus

integrating essential information for cognitive processing. It also has motor interconnections with the supplementary motor area, rostral cingulate, cerebellum, and dense interconnections to the basal ganglia. DLPFC directly interconnects with the other PFC areas, thus possibly influencing limbic connections with structures such as the hippocampus, neocortex, amygdala, and hypothalamus, which are essential structures for long-term memory and the processing of internal states, namely affect and motivation [121].

Rêgo et al. carried out a systematic review of the possible effects of TMS on the P300 potential, and found seven studies using the auditory P300 [106]. All studies used rTMS or repeated single pulses, mostly over the prefrontal cortex (one used the supramarginal gyrus). Six studies used healthy volunteers and only one used depressive individuals. However, Rêgo et al. found several methodology discrepancies and results were not uniform or were even contradictory. In five studies, authors reported changes in P300 parameters after stimulation of either left or the right hemisphere: significant changes occurred exclusively in latencies in two studies; amplitude changes occurred in other two studies (one of them being the one done in depressed patients); finally, rTMS was able to influence both latency and amplitude also in two studies. Rêgo et al. concluded that this review yielded evidence that rTMS is able to effectively affect cognition and that the related changes can be monitored by the P300 potential. Nevertheless, results highlight divergent data, as contradictory results were found in two articles (Jing et al. vs Hansenne et al.) [110,113]: both found increased P300 latencies after stimulation of the left DLPFC but used opposed rTMS frequencies (high-frequency 10Hz in the Jing et al. study and low-frequency 1 Hz for the Hansenne et al. study) [106,110,113]. The small number of subjects studied was also a highlighted factor since four studies had groups with fewer than eight subjects and one of these reported results from the stimulation of only one subject [106]. Since then, a few more studies arose, but the focus transitioned to the evaluation of the use of the P300 after rTMS in patients with dementia, schizophrenia, Parkinson disease, and even food craving [108,122,123]. Results again were not uniform. For example, Lin et al. reported increased P300 amplitude following high-frequency rTMS with no significant change in latency but Khedr et al. found decreased latencies after high-frequency rTMS with no change in amplitude [122,123]. Using cTBS, Lowe et al. found increased latencies but also increased amplitudes [108].

Although promising, the use of ERPs such as the auditory P300 to study and monitor the after-effects of rTMS/TBS sessions remains scarce, and the scientific evidence guiding its use and finding adequate protocol procedures is clearly needed.

4.2 Stimulation of the temporo-parietal cortex

Hearing impairment and conditions like tinnitus are also a developing area for rTMS use. As mentioned previously, given the number and characteristics of the studies published on tinnitus patients, a level C of possible efficacy was attributed to the use of rTMS over the left temporoparietal cortex in tinnitus and auditory hallucinations [22]. Transcranial magnetic stimulation in tinnitus attempts to modulate the hyperactivity of cortical and subcortical auditory and non-auditory areas usually present. Thus, usually, an inhibitory paradigm is used in tinnitus treatments. A 2016 systematic review and meta-analysis evaluating the therapeutic effect of rTMS/TBS on tinnitus found that the 15 randomised controlled trials were highly variable in study design (e.g. number of sessions) and also in the way they reported outcomes [124]. Nevertheless, a positive significant effect in follow-up was found after the use of the TMS sessions [124]. The most frequent protocol used the 1 Hz frequency. The only included study that used cTBS did not find significant results but used bi-hemipheric stimulation [124,125].

Recommendation classification for studies carried out in this field normally do not exceed Class III, and some published Class I studies did not show positive results, thus justifying the "possible efficacy" recommendation [22]. Despite being considered a safe technique, protection procedures such as the use of earplugs are advised. This relates to the noisiness of rTMS at higher intensities, with reports that some patients may complain of hyperacusis worsening and painful hypersensitivity to noises after the sessions [22].

As an example, TMS in tinnitus has shown promising results, although of partial and temporary nature. Due to these facts, some clinicians advise that the use of TMS to treat tinnitus is still not yet recommended [126]. Some studies have investigated in more detail the possible effects of rTMS/TBS on improving hearing function. Zhang et. al. studied rTMS as a treatment in patients with sudden sensorineural hearing loss, with partial recovery [127]. These authors aimed to reduce the perception of tinnitus and thus improve auditory processing. After 20 sessions of 1 Hz at the auditory cortex, they found a significant greater recovery of hearing function, seen in the pure tone audiograms, and improvement in tinnitus perception [127].

To date, the use of TMS/TBS in hearing and tinnitus is still not consensual and the extent of the effects of temporal cortex stimulation is still involved in some doubt. More studies on this topic are needed in order to achieve clearer results.

Chapter II

Aims and outline of the thesis

Chapter II

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Despite the amount of scientific knowledge in the area of repetitive transcranial magnetic stimulation (particularly theta burst stimulation), a significant part of this knowledge derives from studies in patients with dysfunctional neural networks or with hypo/hyperactive cortical areas [22,36,128–130]. As addressed previously, scientific evidence on healthy subjects exists, but with variable and some contradictory results [106,107,131]. This fact is even more evident in cortical areas less studied over the years, like the auditory cortex. It should be noted that, in addition to the existence of numerous possible stimulation variants, methodology and tools used to measure results after stimulation make it difficult to interpret the effects linked to cortical stimulation. Therefore, the main aim of this thesis was to contribute towards increasing scientific knowledge related to the use of theta burst stimulation in the healthy brain. Secondly, it also aimed to study the neurophysiological and concomitant physiological responses associated with TBS application in two specific cortical areas.

Descriptive aims:

1) To study the effect of theta burst stimulation over the prefrontal cortex on cognitive processing and to determine the importance of the stimulated hemisphere in the response.

1.1) To assess whether a neurophysiological technique of cognitive evaluation such as the auditory P300, is able to detect a possible cognitive modulation after TBS over the prefrontal cortex

1.2) To determine if the results of neurophysiological tests like the auditory P300 in post-TBS outcomes show similarity or overlap with the findings of other tests, namely neuropsychological tests (Trail Making Test and Stroop Test of Words and Colours) and non-invasive cerebral oximetry monitoring.

2) To explore the effects associated with the application of TBS to the temporal cortex and whether it can improve or impair hearing function (hearing thresholds), thus

limiting the use of neurophysiological methods such as the auditory P300 as a diagnostic or follow-up tool after TBS.

3) To assess whether TBS may have the ability to influence less studied neurological functions, such as creativity.

Main hypotheses:

a) TBS over the prefrontal cortex is capable of modulating, with just one session, cognitive processing associated with this cortical area, depending on the type of TBS applied.

b) The effects of TBS over the prefrontal cortex can be demonstrated with the use of neurophysiological techniques, such as the auditory P300, or indirectly such as the non-invasive cerebral oxygenation monitoring and/or neuropsychological tests.

c) Other findings may be associated with the use of TBS in less studied cortical regions, such as the auditory cortex.

General outline

According to the projected aims and main hypotheses, a structured outline was pursued, attempting to compartmentalise the core objectives.

The dorsolateral prefrontal cortex was used to study the effects of TBS stimulation on the auditory P300, brain oxygenation, and neuropsychological tests. We aimed to study the effects of inhibitory and excitatory TBS over the prefrontal cortex, using as reference: long-latency evoked potentials – the auditory P300 - for cognitive evaluation; the evaluation of cerebral oxygenation with non-invasive Near Infra-Red Spectroscopy; and neuropsychological tests - the Stroop test and the Trail Making Test.

The dorsolateral prefrontal cortex was also used to study the effect of TBS on higher functions, namely creativity, using an adaptation of the Torrance tests for creative thinking.

The temporal cortex was used to study the effects of TBS stimulation on basic hearing function. In this phase, emphasis was given to the study of the possible effects on hearing thresholds associated with the application of a single TBS stimulation session, evaluating if TBS could induce a threshold improvement or any possible unwanted side-effects. We also tried to increase the knowledge about hearing safety, given that it is a relevant issue associated with the use of TBS in this cortical area.

The resulting scientific production in the form of published and submitted papers is presented in the following chapters (III to VI). A patent derived from a specific method of this thesis is presented in Chapter VII.

Chapter III

Bilateral theta burst magnetic stimulation influence on event-related brain potentials

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Chapter III

Bilateral theta burst magnetic stimulation influence on event-related brain potentials.

Abstract

Theta burst stimulation (TBS) can be a non-invasive technique to modulate cognitive functions, with promising therapeutic potential, but with some contradictory results. Event related potentials are used as a marker of brain deterioration and can be used to evaluate TBS-related cognitive performance, but its use remains scant. This study aimed to study bilateral inhibitory and excitatory TBS effects upon neurocognitive performance of young healthy volunteers, using the auditory P300' results. Using a double-blind sham-controlled study, 51 healthy volunteers were randomly assigned to five different groups, two submitted to either excitatory (iTBS) or inhibitory (cTBS) stimulation over the left dorsolateral prefrontal cortex (DLPFC), two other actively stimulated the right DLPFC and finally a sham stimulation group. An oddball based auditory P300 was performed just before a single session of iTBS, cTBS or sham stimulation and repeated immediately after. P300 mean latency comparison between the pre- and post-TBS stimulation stages revealed significantly faster post stimulation latencies only when iTBS was performed on the left hemisphere ($p=0.003$). Right and left hemisphere cTBS significantly delayed P300 latency (right $p=0.026$; left $p=0.000$). Multiple comparisons for N200 showed slower latencies after iTBS over the right hemisphere. No significant difference was found in amplitude variation. TBS appears to effectively influence neural networking involved in P300 formation, but effects seem distinct for iTBS vs cTBS and for the right or the left hemisphere. P300 evoked potentials can be an effective and practical tool to evaluate transcranial magnetic stimulation related outcomes.

Keywords: Transcranial magnetic stimulation; Theta burst stimulation; P300; Event related potentials; Prefrontal cortex; Neuromodulation

Introduction

Transcranial magnetic stimulation (TMS) has become an essential tool for manipulation of cortical activity, thereby allowing the study of the functional organization of the human brain [132]. The continual development of techniques such as repetitive TMS (rTMS) and patterned rTMS, enhances their potential as a tool for clinical treatment of several psychiatric and neurological diseases [19,43,133–135]. TMS has been shown as a safe approach to non-invasive research of cognitive functions, both in healthy and pathologic brain. However, research focusing upon the cognitive therapeutic potential of rTMS over the last years has shown contradictory results, thereby perpetuating some doubts over its mechanisms [136,137].

It is known that stimulus characteristics such as frequency, intensity, train length or total number of pulses can induce lasting inhibitory or excitatory after-effects [19]. Theta burst stimulation (TBS) is a form of patterned rTMS which has some advantages including lower stimulation intensity, a short stimulation period and a more prolonged after-effect as compared to other rTMS protocols, both the excitatory (iTBS) and the inhibitory (cTBS) forms [45], and is additionally regarded by some authors to be safer than traditional rTMS [19,46].

Event related potentials (ERPs) are cerebral responses to external stimuli, which reflect the neurophysiology of cognition [110,138] and may be used to study the cognitive effects of TBS. The auditory P300, directly dependent upon subject's attention and discrimination, is the most extensively researched ERP component, resulting from the discrimination of rare, task-relevant stimuli, generally using an oddball paradigm. Predominantly reflecting processing speed, is an important tool in the study of cognitive processes and memory in normal subjects and in psychopathology, as its delay can be used as a marker of cognitive deterioration [113,139]. Playing a less prominent role in ERP studies, the N200 potential also yields important information regarding cognitive evaluation, as it represents the initial, subconscious processing of the stimulus involved in the oddball task, leaving the translation of more advanced and purposeful stages of task processing to P300.

Thus far, the use of ERPs remains scant [136,137], and there is still little research on auditory P300 and TBS. Therefore, in order to study TBS effects upon neurocognitive performance using a ERP evaluation tool, we delineated a study combining auditory P300 and TBS applied to young healthy volunteers. Our objectives were: a) to study the effects of a single TBS (iTBS or cTBS) session upon auditory P300 performance, b) to

analyse whether the stimulated side originates any lateralization on parietal P300 responses and c) to evaluate whether TBS protocol has any influence upon the volunteers' reaction time during P300 testing.

Materials and Methods

Subjects and study design

This was a double-blind sham-controlled study, involving healthy volunteers that were recruited after general advertisement with medical students enrolled at the Faculty of Health Sciences (FHS), University of Beira Interior (UBI), Covilhã, Portugal. Students were selected if they were between 18 and 30 years-old, and after answering a confidential screening questionnaire. Exclusion criteria included being left-handed or ambidexter; previous brain injury and/or severe head trauma; epilepsy or history of convulsions; presence of major medical illness (including neuropsychiatric diseases), intake of any medication during testing, pregnancy, implanted devices or foreign metal articles, sleep deprivation, alcoholism and history of drug intake [19]. All volunteers were instructed to avoid sleep deprivation, alcoholic beverages or other toxic/stimulant substances 24 hours prior to the application of the technique.

Volunteers were then randomly assigned to five different groups: two groups with active stimulation to the left dorsolateral prefrontal cortex (DLPFC) - Group A (iTBS) and Group B (cTBS), two other groups with active stimulation over the right DLPFC (Group D (iTBS) and Group E (cTBS) and finally, a placebo group - Group C (Sham).

After complete explanation of the procedures, all subjects signed a written informed consent. The study was approved by the Faculty of Health Sciences – University of Beira Interior Ethics Committee (no. CE-FCS-2011-001), in conformity with the Declaration of Helsinki.

Theta Burst Stimulation (TBS)

TBS was performed under medical supervision at FHS-UBI facilities, using a MCF-B70 figure-8 coil with a MagVenture MagPro® G3 X100 5.0.1 and recording EMG activity in a Dantec™Keypoint® - Keypoint.net v.2.03. Stimulation comprised a biphasic pulse

waveform and posterior-anterior (P-A) current direction in single pulse, iTBS and cTBS [19].

Stimulation intensity was defined using the active motor threshold (AMT), which consisted of the minimal stimulation intensity over the motor cortex that was necessary to produce a 150–200μV amplitude motor evoked potential (MEP) of the contralateral *abductor pollicis brevis* (APB), on more than five out of ten trials, while maintaining a voluntary mild contraction, using visual feedback. Active stimulation was performed over the right or left DLPFC area that can be defined as 5 cm rostral of the region from which the most prominent motor response of the contralateral APB muscle can be recorded [45,137,140].

The TBS protocol consisted of bursts of 3 pulses delivered at 50 Hz every 200 ms (i.e. at 5 Hz), at an intensity set to 80% AMT [45]. In the cTBS protocol the bursts were delivered without interruption, up to a total of 600 pulses. iTBS also comprised 600 pulses, but the bursts were delivered at 5 Hz during 2 seconds (groups of 10 bursts), repeated every 10 seconds [45].

Sham stimulation used the same coil, tilted away from the scalp at a 90 degree angle, but maintaining contact and sound (intensity reduced to 50% AMT), thereby giving the impression that the subject was being stimulated, although this stimulus does not reach cortical neurons [19,137]. During protocol application, subjects were seated in a comfortable declinable armchair and were told to relax and avoid any head movements.

P300

Auditory P300 recording was carried out in a quiet room, using an 8 channel Keypoint.net v.2.03. Active electrodes were placed in Cz, Pz, P3 and P4 of the 10/20 international system, with an anterior reference, trying to achieve a more accurate lateralization of the waves recorded in the right and left parietal electrodes. All recording sites were cleaned with alcohol and abraded to maintain a resistance below 5 kΩ [138,141,142]. A time constant of 1 second was used together with a high frequency filter of 50 Hz, with a time base of 1000 ms, using an automatic overload rejection mode. The auditory oddball paradigm consisted of 80% frequent stimuli presentation, 1000 Hz and 50 ms of duration, randomly mixed with a 20% target stimulus, 2000 Hz and 100 ms of duration. Both used a minimal intensity of 65 dB HL. Stimuli were presented binaurally,

with a random interval between 1 and 2 seconds. Each complete study recorded at least 400 stimuli (minimum of 100 target), divided into two series, and subjects were instructed to remain calm and relaxed, avoid blinking and to concentrate upon a focus point. Subjects were then asked to press a button for the rare stimuli as quickly as possible with the dominant hand in order to ensure attention and collaboration [138,143]. The chosen parameters were measured from the mean waveform of the two reproducible series and the epochs for the target and non-target tones were analysed separately. The largest negative peak, occurring between 160-260 ms, was considered as the N200. The P300 was defined as the largest positive peak arising after the N1, P2 and N2 components, increasing in amplitude at the posterior areas and occurring between 220-600 ms. Amplitude was measured in the N2-P3 complex, between the maximum negativity and positivity components [110,138,144,145].

Experimental design

The study design comprised three different timepoints for assessment, labelled as pre-TBS, TBS stimulation and post-TBS. Stimulation was always performed at the same time of day and randomly assigned to each volunteer according to the respective group. Each subject was submitted to a single TBS session on the DLPFC. The order of real and sham sessions was also randomized and counterbalanced across subjects. Only one member of the investigation team was aware of the type of stimulation applied. In pre-TBS stage, baseline P300 recording was performed. This step was followed by all the procedures regarding TBS protocol, performing either iTBS, cTBS or sham stimulation. Immediately after TBS or sham stimulation, the second auditory P300 recording was performed (post-TBS). Protocol available at: dx.doi.org/10.17504/protocols.io.kr3cv8n

Statistical analysis

Chi-square and Levene tests were used to study if there were any significant differences between groups. Normality was evaluated using Kolmogorov-Smirnov and Shapiro-Wilk tests. Due to the relative small number of group elements and data characteristics, we needed a robust nonparametric analysis test to evaluate pre-post stimulation mean result comparisons and multiple group comparison test, thus we used the R software package: Nonparametric Analysis of Longitudinal Data in Factorial Experiments

(nparLD) [146]. Analyses were performed using IBM SPSS Statistics 20® and R version 3.0.0., and the significance level was $p < 0.05$.

Results

Volunteers

This study involved 51 healthy volunteers (31 female and 20 male, aged 19–30 years, mean=22.84 +/- 1.98), and all study groups (Group A n=10; Group B n=10; Group D n=10; Group E n=11, and Group C n=10), were matched in terms of age and gender.

Pre-stimulation - N200 and P300

For all groups, N200 mean latency pre-stimulation ranged between 176.98 +/- 30.21 ms over Pz and 181.73+/- 23.05 ms over Cz. As for P300, the lowest mean latency was obtained over Cz – 255.65 +/- 45.07 ms – and the highest over P3 – 259.57+/-54.81 ms. Overall maximum latency recorded reached 256 ms and 483 ms, for N200 and P300 respectively. Amplitudes recorded regarding N2-P3 difference, showed mean results between 4.72 +/- 3.12 μV over Cz and 5.10 +/- 3.85 μV over Pz, with a maximum amplitude of 19.9 μV. Signalizing the rare stimuli by pressing the button on our oddball paradigm achieved an overall reaction time mean of 316,24 +/- 57,04 ms, ranging from 217 to 468 ms.

Pre- and post-stimulation latencies

Pre-stimulation and post-stimulation latencies, amplitudes and reaction times distributed per stimulation group are shown in Figure 3.1.

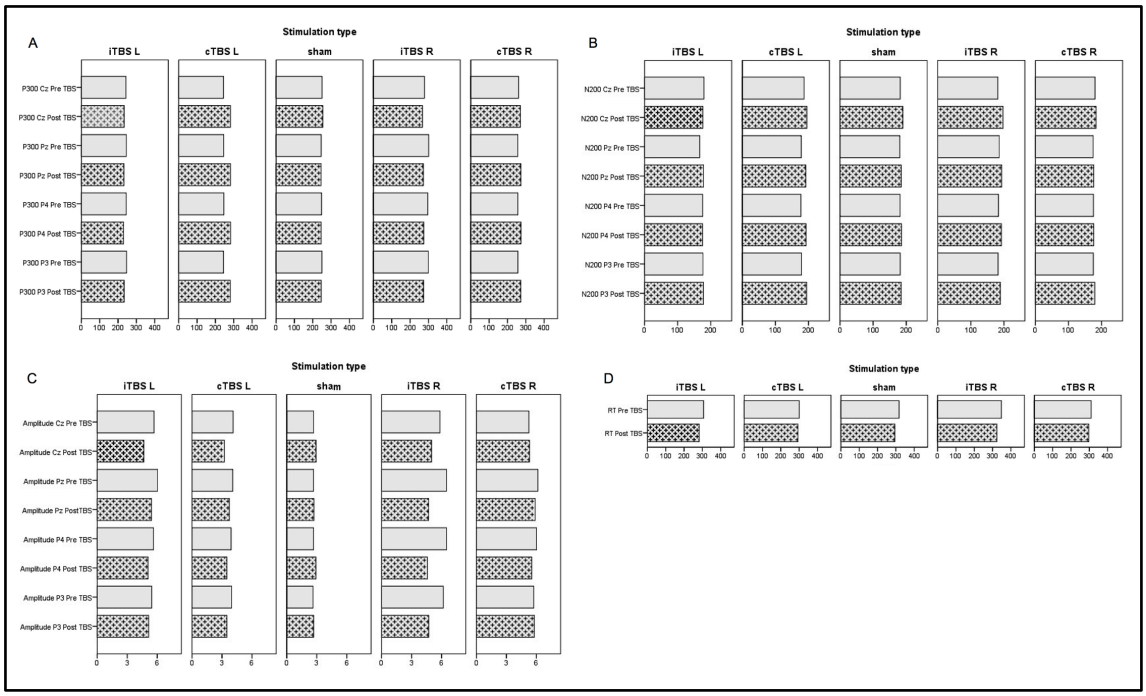

Figure 3.1 – ERP results per stimulation group. P300 latency (A), N200 latency (B), Amplitude (C) and Reaction Time (D).

Comparison of P300 latencies between the pre- and post-TBS stimulation stages are shown in Table 3.1.

Table 3.1
Group comparison - Pre vs Post stimulation - P300 and N200 latencies

	iTBS L		cTBS L		Sham		iTBS R		cTBS R	
	Mean Dif. (ms)	p-value[a]	Mean Dif. (ms)	p-value[a]	Mean Dif. (ms)	p-value[a]	Mean Dif. (ms)	p-value[a]	Mean Dif. (ms)	p-value[a]
P300 Cz Pre P300 Cz Post	-9,7	0.095	38,4	**0.009**	3,8	0.506	-10,8	0.604	9,91	*0.062*
P300 Pz pre P300 Pz Post	-12,9	**0,003**	36,8	**0,000**	-0,8	0,822	-28,4	0.084	16,64	**0.026**
P300 P4 Pre P300 P4 Post	-14,2	**0.006**	36,4	**0.000**	-2,4	0.829	-21,4	0.829	16,55	**0.009**
P300 P3 Pre P300 P3 Post	-13,3	**0.005**	37	**0.001**	-3,7	0.515	-26,2	0.345	15,18	**0.035**
N200 Cz Pre N200 Cz Post	-3,4	0,149	8,8	0,960	7,9	0.238	15,5	**0.006**	3,55	0.709
N200 Pz Pre N200 Pz Post	11,6	0.411	13,6	0.277	4,3	0.398	7,3	0.449	1,73	0.837
Reaction Time Pre Reaction Time Post	-24,2	**0,000**	-6,1	0,629	-22,4	**0,025**	-24,1	0,052	-13,45	0,176

[a]*nonparametric – nparLD package*

Differences were detected between groups, in terms of stimulation characteristics. iTBS groups showed a tendency towards decreasing P300 latencies after stimulation and cTBS groups showed a tendency towards a slower response time. In contrast, the sham group did not show a clear tendency.

Sham and right hemisphere iTBS groups showed no significant differences between the pre and post evaluations (nonparametric - nparLD package). iTBS over the left hemisphere showed significantly faster post stimulation latencies, mainly over the parietal recording sites (p=0.003, p=0.006 and p=0.005 for Pz, P4 and P3, respectively). cTBS over the left hemisphere significantly influenced P300 latency over all recording topographies, causing a delay in the P300 wave. In the right hemisphere, cTBS stimulation was associated with a significant parietal ERP delay (p=0.026, p=0.009 and p=0.035 for Pz, P4 and P3, respectively).

In terms of N200, latency showed a significant difference only when iTBS was performed on the right hemisphere. Contrasting with P300 behaviour to excitatory stimulation, N200 displayed longer latencies after stimulation. The remaining groups showed relatively small and inconstant changes in mean latencies.

Pre- and post-stimulation reaction times

Comparison of reaction times between the pre- and post-TBS stimulation stages are shown in Table 3.1.

All groups showed faster reaction times in the second ERP evaluation, after TBS and sham stimulation, but this was only significant in the sham group (mean difference= -22.4 ms; p=0.000) and the left iTBS group (mean difference= -24.2 ms; p=0.025). In contrast, right iTBS group only showed a trend towards reaction times being significantly faster (mean difference= -24.1 ms; p=0,052).

Pre- and post-stimulation amplitudes

Comparison of ERP amplitudes between the pre- and post-TBS stimulation stages are shown in Table 3.2.

Table 3.2
Group comparison - Pre vs Post stimulation - ERP amplitude

	iTBS L		cTBS L		Sham		iTBS R		cTBS R	
	Mean Dif. (μV)	p-value[a]	Mean Dif. (μV)	p-value[a]	Mean Dif. (μV)	p-value[a]	Mean Dif. (μV)	p-value[a]	Mean Dif. (μV)	p-value[a]
N2P3 Cz Pre N2P3 Cz Post	-1,01	0.189	-0,89	0.582	0,24	0.543	-0,84	0.295	0,06	0.876
N2P3 Pz Pre N2P3 Pz Post	-0,6	0.980	-0,33	0.850	0,04	0.963	-1,78	0.944	-0,28	0.454

[a]*nonparametric – nparLD package*

ERP amplitudes before and after stimulation in all groups, except for the sham group showed a trend towards a slight decrease after TBS, but no significant difference was found.

Group comparison – Stimulation vs Sham - P300

Comparison of Pz P300 results across all stimulation groups is shown in Table 3.3.

Table 3.3
Stimulation Group vs Sham Group multiple comparison test - P300 & N200 latencies

	P300 Lat. Pz	P300 Lat. Cz	N200 Lat. Pz	N200 Lat. Cz
	p-value[a]	p-value[a]	p-value[a]	p-value[a]
iTBS L vs Sham	**0.024**	0.805	0.250	0.764
cTBS L vs Sham	**0.001**	**0.016**	0.201	0.317
Sham vs iTBS R	0.167	0.837	0.262	**0.024**
Sham vs cTBS R	**0.042**	0.082	0.414	0.280

[a]*nonparametric ANOVA nparLD*

When we evaluate the outcomes through a multiple comparisons test, P300 latency over Pz results showed significant differences between the sham group and the left iTBS group (p=0.024), sham and left cTBS goups (p=0.001) and finally between sham and right cTBS groups (p=0.042).

Comparing groups using Cz P300 (Table 3), the only significant difference occurred between the sham and the left cTBS groups (p=0.016), with much slower latencies recorded after actual cTBS stimulation.

Group comparison – Stimulation vs Sham - N200

Multiple comparisons for N200 (Table 3.3) showed no significant differences over Pz recordings. N200 behaviour over Cz was significantly different between sham and right

iTBS groups, in this case because N200 was slower after excitatory TBS over the right hemisphere. ERP behaviour over P3 and P4 followed overall Pz results after pre- and post-stimulation, not showing any significant lateralization.

Discussion

The main goal of our work was to evaluate human cortical and subcortical network dynamics to TBS, via electrophysiological assessment using the auditory P300 ERP. Introducing a sham controlled design trial, we tried to verify if the effects were distinct for iTBS vs cTBS and for the right or the left hemisphere. To our knowledge, this is the first study that compared both excitatory and inhibitory TBS over the right and left DLPFC, evaluating its effects using neurophysiological tests like the auditory P300, with a placebo control group, in a young adult healthy population. Our sham-controlled results showed that ERPs responded differently to stimulation type and lateralization. Significantly slower P300 latencies were recorded over parietal locations after left and right inhibitory stimulation but faster P300 latencies were found only after excitatory stimulation over the left DLPFC. No apparent latency lateralization was found as P300 over P3 and P4 followed the same outcomes as the P300 recorded over Pz. Amplitudes showed no significant variation after cTBS or iTBS in either hemispheres. Reaction times behaved differently also with faster reaction times in the excitatory and sham groups, but with no significant changes in the inhibitory groups.

Using both inhibitory and excitatory TBS protocols, we found that the parietal P300 showed significantly slower latencies after cTBS stimulation bilaterally but the parietal P300 responses were significantly faster only after iTBS over the left cortex. These results suggest that the inhibitory protocol is capable of a more intense or more effective interference over the cerebral circuits that are implicated in P300 formation than excitatory TBS, as it seems to be able to modulate both hemispheres. Supporting these findings, Kaller et al. found interesting results when testing hemispheric relevance using bi-hemispheric cTBS and the Tower of London task. Their results showed that initial planning times could be influenced differently either by stimulating the right or the left hemisphere, with results directly dependent of hemisphere dominance - right hemisphere inhibition resulted in increased planning times and contralateral inhibition showed faster planning [147]. Such evidence is similarly defendable for ERPs global performance, since using a inhibitory stimulation over the frontal area originated decreases ERP amplitude in a modified P300 protocol [148].

Our results also propose an asymmetrical response to excitatory stimulation, since iTBS in our study seemed to be more effective over the left hemisphere, and P300 showed significantly slower latencies over Cz only after left cTBS. Leftward susceptibility to be more easily modulated was detected in other studies with excitatory stimulation, as shown by the faster latencies found after high frequency rTMS over the left hemisphere [149]. Overall, right hemisphere stimulation results tend to reveal fewer changes in ERP parameters, as showed when administering inhibitory rTMS over the right DLPFC [150,151], or excitatory rTMS over the right DLPFC [149]. Although asymmetries are reported, our overall recordings of P300 over the left and right parietal areas showed the same results as the P300 recorded over Pz. These findings suggest that lateralized cTBS and iTBS can influence the initial P300 neuronal generator behaviour but not the following bilateral wave formation and spreading. Our findings can be associated to TBS/rTMS modulation capacity to influence neurotransmitter production, as neurotransmitters trigger intracortical excitatory and inhibitory postsynaptic potentials that are the base for ERP formation. Magnetic stimulation capacity to modulate neurotransmitter dopaminergic and glutamatergic connection is known, especially if applied to the prefrontal cortex, and these neurotransmitter assume utmost importance in P300 formation [37,152]. Previous studies showed that high frequency magnetic stimulation increases anterior brain glutamate levels, in some cases with a left lateralization [38,56,57,153]. It is also known that dopamine modulation can influence both task performance testing and also event related potentials [154,155]. ERP latencies and amplitudes can be influenced by dopaminergic function, impacting cognitive speed processing and also neural resources magnitude allocation to a specific task. Magnetic stimulation can similarly impact dopaminergic function, with some studies showing that high frequency stimulation administered to left prefrontal cortex increases dopamine release [61,156]. Research also showed that in some studies this effect had also some degree of lateralization, as only the left hemisphere stimulation resulted in either dopamine increase after excitatory stimulation or impaired dopamine release after inhibitory stimulation [61,62,154–159]. These findings can strongly be correlated with our P300 latency results, since it is likely that cTBS over bilateral DLPFC can have a direct negative impact in either or both glutamate and dopamine production, essential in the electrogenesis of P300 potentials, resulting in ERP delay, even though it may be predominant over the ipsilateral hemisphere. We also found asymmetrical results, as it appears to exist a superior TBS influence over the left DLPFC, especially effective for iTBS and these findings can be related to the reported apparent iTBS superior capability to influence left hemisphere glutamatergic and dopaminergic release. Assuming that P300 test performance is related to mental processing speed affected by attentional

processing and cognitive operations, as shown in previous works [160], we can also assume that iTBS over the DLPFC worked has a facilitator of the cognitive and executive process.

As for N200 performance, reflecting the initial subconscious process of the ERP oddball task, our results showed small variations across the groups, except for the right iTBS group, revealing significantly slower N200 latencies, apparently divergent to P300 behaviour to excitatory stimulation. Previous experimental studies pointed to a left hemisphere N200 dominance, predominantly over the anterior mid-cingulate cortex, evaluated by magnetic resonance images, suggesting also a functional and neuroanatomical dissociation between N200 and P300 potentials [118]. We believe that this anatomical dissociation may explain the different P300 vs N200 response to TBS. In this case, the right inter-hemispheric inhibitory connectivity capabilities could have been potentiated by the right-sided iTBS [22,161,162], thus negatively influencing the N200 dominant left hemisphere, unbalancing right-left basal equilibrium, resulting in poorer N200 performance. Since N200 reflects the initial ERP phase, this result can also be related to right iTBS poorer P300 performance discussed earlier.

It is known that P300 amplitude is associated to the amount of attentional neuronal resources allocated throughout the P300 task, but amplitude evaluation is not straightforward, as it implies a relationship between attention and working memory that can originate higher amplitudes for easy targets and lower amplitude for more complex tasks, requiring more memory load [106,163]. In our groups, even though the task was not complex, probably our baseline psychological conditions were not ideal, as we were introducing a new, and somewhat unknown stimulation technic to our volunteers, that could have induced some anxiety. Our results did not reveal any significant change in ERP amplitude, neither in the stimulated groups or in the sham group. Our lack of significant changes in P300 amplitude, associated to a low baseline amplitude P300, could be related to a state of low excitability or a limited capacity to better allocate attentional neuronal resources, possibly related to the TMS protocol-disturbing physiological volunteer estate. It is also well established that P300 activity is influenced by individual internal physiologic state, ranging from circadian rhythms to fatigue and physical state [164]. Base line ERP results revealed latency and amplitude characteristics that can be explained by factors like our sample of young university students, capable of promoting a lower latency baseline ERP, and technical aspects as reference electrode position, as it is argued that anterior references are positioned within brain's electrical fields of the auditory ERP, being capable of voltage gradients which vary across subjects [141,160]. So, even though our primary aim was to reduce possible amplitude asymmetry

by electrode location and impedance discrepancies, this fact could have influenced amplitude and even latency baseline results [165].

When evaluating reaction time in ERP task we must remember that TMS has the capacity to induce local, trans-synaptic and system-level effects. We know as well that this ERP protocol involves a motor response and apparent significant involvement of the anterior cingulate cortex [164]. The fact that all groups, including sham group, tended to shorter reaction times suggests a mere habituation process. But careful analysis shows that stimulation type may influence this process because of right and left cTBS groups response speed wasn't significantly as fast as their counterparts. This result suggests that cTBS inhibitory capacity negatively influenced bilateral cerebral networking, preventing these groups to perform as fast as they normally would, supporting the notion that even though the DLPFC could be the most active region, it can activate cortical network relays, including deep subcortical relays, thus influencing motor response processes [61].

Using the TBS-P300 combination appears to be a useful approach to monitor stimulation effects, especially if applied when evaluating neurologic and psychiatric diseases, either in rehabilitation or diagnosis. This method may be also important to better understand neural network processing as it allows studying the direct and indirect influence of specific cortical and subcortical connectivity over cognitive performance. As mentioned, previous studies combining rTMS and event related potentials, magnetic stimulation tends to modulate brain responses accompanying the excitatory or inhibitory effects associated with high or low frequency stimulation, respectably, but most studies used only one stimulation type and one stimulation site, mostly without placebo control. Knowing that some previous results were even negative using bilateral inhibitory stimulation [166], a broader study using iTBS and cTBS was clearly necessary. Regardless the fact that there were already studies evaluating the effect of rTMS on the human cortex and the capacity to impact scalp ERPs, the significant variability in application technics and in some cases the incongruent results, enhance the scientific necessity to better understand this technic.

A limitation of our study was the sample size, translated into a small subject number per group, which did not allow us to have better statistical strength. Objective methodologies to evaluate volunteer stress and anxiety should also be used, but unfortunately these tests were not included in our initial study methodology as we did not expected that a TMS based stimulation could cause this level of apparent student solicitude towards the procedure. Nevertheless, we tried to provide ideal protocol application conditions,

previously by giving our volunteers all the information needed and during stimulation/recording procedures promoting a stress-free environment.

Conclusions

Our results strongly support the hypothesis that TBS can effectively influence the cortical site of stimulation and also remote cerebral regions, directly or indirectly influencing neuronal excitatory/inhibitory networking, and that this influence is directly linked with stimulation characteristics and hemispheric lateralization. This significant capacity to modulate brain excitability should be further studied, either by neurophysiologic or behavioural testing in order to fully understand and dominate this noninvasive neuro-intervening tool. Further studies with larger subject number are required to confirm our findings and help understand whether these results have short duration, or if this neurocognitive influence is maintained for longer periods of time. We suggest also additional investigation studying and comparing these results using neuroimaging. It would be interesting to investigate the same protocol with repeated application of TBS in a daily scheme, with depression-like treatment sessions. Studies with a larger range of TBS intensities and different number of trains would also be important to evaluate in the future. We believe that P300 evoked potentials have the potential to be used as a useful tool to study and evaluate transcranial magnetic stimulation related outcomes.

Chapter IV

Theta burst stimulation is able to impact cognitive processing: A P300 and neuropsychological tests study.

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Chapter IV

Theta burst stimulation is able to impact cognitive processing: A P300 and neuropsychological tests study.

Abstract

Theta Burst Stimulation (TBS) is a safe non-invasive neurostimulation technique used to improve cognitive and neuropsychiatric impairments. Combined outcome evaluation using event-related potentials (ERPs) and neuropsychological tests may allow a more thorough assessment of TBS treatment efficacy, however some mixed results have been found and their use remains scarce. Our main objective was to evaluate whether a session of TBS to the left dorsolateral prefrontal cortex (DLPFC) can impact upon the performance of both neuropsychological and neurophysiological tests. This double-blind sham-controlled study involved 28 healthy adults, between 18 and 30 years of age. Volunteers were randomly allocated to receive excitatory (iTBS), inhibitory (cTBS) or sham stimulation on the left DLPFC. Subjects were evaluated using ERPs (auditory oddball paradigm P300) and neuropsychological tests (Trail Making Test (TMT) and Stroop Test of Words and Colours (STWC)), using a pre-post stimulation protocol. Inhibitory stimulation led to significantly delayed P300 peak latencies ($p<0.001$), with no consistent change in N2P3 amplitudes. cTBS also significantly influenced the expected group performance in Stroop C and Stroop Interference ($p=0.025$) compared to the iTBS and sham groups. No significant results were found in TMT tests after TBS. Our results suggest that P300 and specific STWC parameters can be similarly influenced by the same TBS protocol. This emphasizes the importance of mixed evaluation using neuropsychological and neurophysiological resources in research associated with the use of transcranial magnetic stimulation and cognition.

Keywords: Transcranial magnetic stimulation; theta burst stimulation; P300; Trail Making Test; Stroop Test

Introduction

Transcranial magnetic stimulation (TMS) is a safe noninvasive neurostimulation technique, with limited side effects, which has been widely used to study and treat several neuropsychiatric illnesses such as depression, stroke, epilepsy, Parkinson´s disease, and cognitive impairment [22,97]. Theta Burst Stimulation (TBS) is a specific form of TMS which has been shown to be as effective at modulating various brain functions, but using less time and lower intensity stimuli than those used by conventional TMS [45,90,167]. Specific TBS paradigms such as continuous TBS (cTBS) or intermittent TBS (iTBS) can have an inhibitory or an excitatory effect, respectively [45].

The frontal cortex is the main area involved in executive functions, having a fundamental role in behaviour regulation and cognitive functions [168–170]. Specifically, the dorsolateral prefrontal cortex (DLPFC) has an important role in attention networks. Through its connections with the dorsal striatum, DLPFC is related to higher-order processing tasks like working memory, conscious decision making and reasoning [42,171].

Cognitive processing and central nervous function can be studied by event related potentials (ERPs). One of the most used is the auditory P300, an ERP component with a major neural generator in the prefrontal cortex, and which is linked to decision making and attentional resource allocation [138,172–174].

Neuropsychological tests are essential tools for executive function assessment, evaluating aspects such as attention, working memory, cognitive flexibility or behaviour control [175]. Some of these functions can be evaluated using tests as the Stroop Test of Words and Colours (STWC) and the Trail Making Test (TMT). The STWC assesses executive functions such as selective attention, modulation, and inhibition, resistance to external interference and cognitive flexibility related to execution speed [176,177]. The TMT yields information about visual scanning, processing speed, mental flexibility, motor skills, and working memory, among other executive functions [178,179].

Accurate assessment of TMS neuromodulatory effects can be a challenge, especially when testing cognitive functions. The joint evaluation of event-related potentials with neuropsychological studies may allow a deeper patient assessment [180]. This may originate similar results when evaluating brain processes in some anatomically linked neurological and psychiatric diseases [180]. However, we know that this behavior is not stable and generalized, namely in the Stroop or in the Trail making tests, existing some

dissenting results, possibly dependent on the neural networks involved or activated by each test paradigm [181,182]. The use of TBS in the prefrontal area, together with P300 and neuropsychological tests may contribute towards understanding the neuronal basis of hemispheric laterality in brain functioning. The main aim of this study was to evaluate whether a single session of TBS to the left DLPFC can impact upon cognitive function and influence performance of neuropsychological and neurophysiological tests. We also wanted to evaluate whether the neuropsychological and neurophysiological results behave similarly throughout the process.

Materials and Methods

Participants

Twenty-eight healthy, right-handed volunteers, between 18 and 30 years old were recruited among students of the Faculty of Health Sciences of the University of Beira Interior, in Covilhã, Portugal. After answering a confidential screening questionnaire [183], students read and signed a written informed consent form and were asked to voluntarily take part in the study, receiving no monetary compensation. Screening included brief medical, substance use, and neuropsychiatric histories. Selected participants did not have any of the following exclusion criteria: left-handedness or ambidexterity; colour-blindness; neurological, psychiatric, cardiac, respiratory, infectious, tumoral or metabolic diseases; hearing loss; previous brain trauma/brain injury; epilepsy or personal history of one or more seizures; metallic prosthetics or other metallic elements located in the brain or skull; pregnancy; history of alcohol abuse; taking antidepressants, neuroleptics and other similar drugs that might induce seizures [7,19,46]. None of the volunteers had ever performed TMS in the past. Any other conditions the study team found problematic or doubtful also prevented the subject from being included in the study.

Experimental Design

All procedures were performed in accordance with the Declaration of Helsinki, were approved by the Faculty of Health Sciences UBI Ethics Committee (no. CE-FCS-2011-001) and were carried out under medical supervision. Study protocols were carried out

in the FHS-UBI TMS laboratory and volunteers were told to avoid sleep deprivation, alcoholic beverage intake or any other toxic/stimulant substances in the 24 hours prior to their participation.

The study was an experimental, double-blind sham-controlled study, of the effects of excitatory iTBS or inhibitory cTBS on the dorsolateral prefrontal cortex (DLPFC) on cognitive function, using both event-related potentials and neuropsychological tests. Double-blinding was ensured by keeping volunteers and team researchers who applied/evaluated the neuropsychological tests and the P300 results blinded to the assignment condition and without knowing whether active or sham stimulation was applied. Due to the technical study design, only the team researcher in charge of administering the TBS/Sham was aware of the stimulation characteristics.

Using simple randomisation, recruited volunteers were allocated to one of three groups, in order to receive either active or sham TBS in the left DLPFC:

Group A - submitted to iTBS; Group B – submitted to cTBS; Group C – submitted to sham TBS.

P300, TMT, and STWC were performed before (Steps 1 & 2) and after stimulation (Steps 4 & 5), as shown in Figure 4.1. A single TBS or Sham stimulation session was delivered to the left DLPFC of each volunteer.

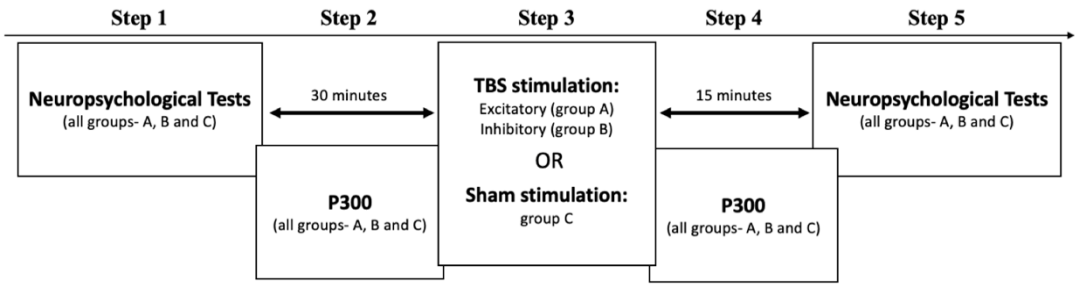

Figure 4.1. – Evaluation process

Transcranial magnetic stimulation (TMS)

Real and sham stimulations were conducted using a MagVenture MagPro1G3 X100 5.0.1, coupled with a Dantec™ Keypoint.net v.2.03 for motor threshold determination,

following the safety and ethics recommendations of the 2009 guidelines [19]. A parallel-handle positioned MCF-B70 butterfly coil was used. Primary stimulation was delivered to the left primary motor cortex, in order to identify the intensity to be used over the DLPFC. This intensity, expressed as a percentage of the maximum device output (MDO), was determined as 80% of the active motor threshold - the minimum intensity capable of inducing a motor response of at least 150μV in at least 5 of 10 stimuli, while the subject maintains a minimal contraction of abductor pollicis brevis (APB) muscle [184]. The left dorsolateral prefrontal cortex was found 5 cm anteriorly to the region that induced the most prominent motor response in the right APB, area where TBS was delivered [36,140]. Theta burst stimulation was applied consisting of a 3-pulse series at 50 Hz, applied repetitively with inter-series intervals of 200ms, according to Huang et al. [45]. The protocol of continuous TBS delivers all pulses continuously without interruption while intermittent TBS protocols deliver the bursts only during 2 s (groups of 10 bursts), repeated every 10 seconds. Both intermittent and continuous stimulation comprised a total of 600 pulses [45]. The same coil was used for sham stimulation placed in a perfectly vertical position (90-degree) on the subject's scalp, maintaining scalp contact [36].

Event related potential – P300

P300 recording was carried out before and immediately after real or sham stimulation (Fig. 1). An auditory oddball task was performed with an 8 channel Keypoint.net v.2.03., using a randomised 80%-20% presentation for the non-target and target stimuli, respectively. Stimulus characteristics consisted of 1000 Hz and 50 ms of duration for the non-target stimuli and 2000 Hz and 100 ms of duration for the target, using a binaural presentation with a minimum intensity of 65 dB HL at a random interval between 1 and 2 Hz. Each complete study recorded at least 100 target stimulus [138,143]. Volunteer attention and collaboration was ensured by signalling (pressing a button in the dominant hand) each time a target appeared [138,143]. P300 recording protocol focused the main topographic areas (central-parietal) for electrode placement, using the 10/20 international system, with anterior referencing [138,141,142,174]. Impedances were maintained below 5 kΩ. Subject brainwaves were analysed by one of the researchers blinded to stimulation type, in the mean waveform of the two reproducible series. P300 peak was found identifying the largest positive peak appearing after the N1, P2 and N2 components, with maximum posterior amplitude, occurring between 220-600 ms. N200

was considered the largest negative peak between 160-260 ms. For amplitude measurement, we used the N2-P3 complex [110,138,144,145,185].

Neuropsychological tests

All volunteers were submitted to TMT and STWC before they went through one of the three types of TBS: iTBS, cTBS or sham. Subjects completed neuropsychological tests about 30 minutes before TBS or Sham stimulation (step 1 - see Figure 1) and repeated them about 15 to 20 minutes after stimulation (Step 5). TMT consists of two parts: Part A, which requires a fast connection of numbers, sequentially and in ascending order; and Part B which requires a logical alphanumeric connection (1-A, 2-B), that is, the correspondence must be alternated between number (ascending order) and letter (alphabetical order). The results are related to the time needed to complete each part of the test, using a Portuguese version of the Stroop Test of Words and Colours [186].

The Stroop test is composed of three sheets in which the subjects must read or name the observed colours. The first sheet contains the words “green”, “red” and “blue”. The second sheet contains 100 similar elements - “XXXX” – printed in green, red and blue colours. On the third sheet, there are the words from the first sheet, printed in the colour of the second one, without correspondence between the colour of the ink and the meaning of the word. The result is directly related to the number of words/colours verbalized in 45 seconds [186].

Statistical analysis

Data were analysed using the IBM® SPSS Statistics® 25.0 package. Descriptive statistics such as means and standard deviations were calculated for each variable of the psychological tests. The effect of TBS stimulation on each of these variables in the two conditions (before and after TBS stimulation) was evaluated by a mixed repeated measures ANOVA. The assumptions of this ANOVA were investigated using the Shapiro-Wilk normality test and the Levene test, allowing the latter to evaluate the homogeneity of the variances. Due to the size of the sample and the fact that normality assumption was not always validated, analysis was also performed through a non-parametric version of Mixed Factorial ANOVA (Nonparametric Longitudinal Data in Factorial Experiments, using the "nparLD", version 2.1 package, for the statistical program R). However, since

the results obtained through the two analyses were compatible, we chose to present only the results obtained by the parametric version. Mean comparison between groups or conditions before/after TBS stimulation was performed with LSD (Least Significant Difference) test with Sidak's correction. All tests were two-sided and $p<0.05$ was considered statistically significant.

Results

Twenty-eight participants with a mean age of 22.6 years (SD=2.3 years), with an approximate 57% male - 43% female distribution, were included in this study and this ratio was maintained in the constitution of all groups. Mean age per group was 21.9 ± 1.9 years for the iTBS group, 23.7 ± 2.5 years for the cTBS group, and 22.0 ± 2.3 years for the sham group. None of the initial volunteers dropped out or reported any major side-effects. In Figure 4.2 we can observe a sample of the P300 wave recording from one of the volunteers.

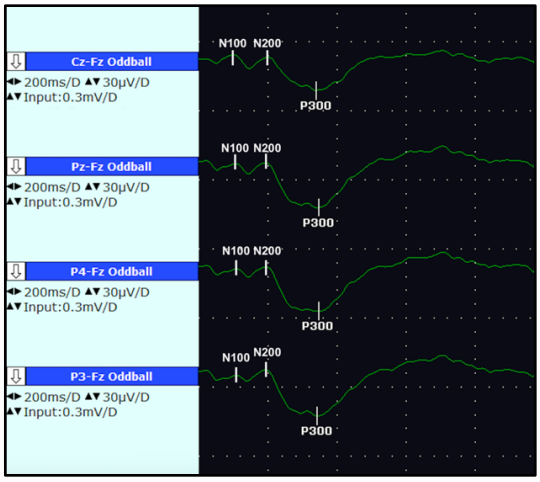

Figure 4.2. P300 sample. Pz latencies: N100=106ms; N200=192ms; P300=348ms. Pz Amplitude N2-P3=19.4µV.

Pre and post-stimulation assessment results (P300, TMT, and STWC) for all groups are shown in Table 4.1. For P300, results shown represent the main topographic representation (Pz).

Table 4.1
Descriptive analyses of response variables. Stimulation (pre and post) and stimulations types (groups)

Variables	Groups	Pre-stimulation Mean ± SD	Post-stimulation Mean ± SD
P300 Latency Pz (ms)	A - Excitatory (N=9)	248.7 ± 45.6	234.1 ± 31.6
	B - Inhibitory (N=10)	245.4 ± 39.1	282.2 ± 76.6
	C- Sham (N=9)	244.0 ± 31.7	243.7 ± 28.3
N2P3 Amplitude Pz (μV)	A - Excitatory (N=9)	5.9 ± 4.9	5.0 ± 2.8
	B - Inhibitory (N=10)	4.1 ± 2.8	3.7 ± 1.3
	C - Sham (N=9)	2.6 ± 1.1	2.6 ± 0.9
N200 Latency Pz (ms)	A - Excitatory (N=9)	167.0 ± 45.1	180.0 ± 26.0
	B - Inhibitory (N=10)	178.0 ± 31.0	191.6 ± 50.0
	C - Sham (N=9)	170.1 ± 22.2	183.1 ± 22.1
Stroop P	A - Excitatory (N=9)	97.2 ± 14.6	105.8 ± 16.8
	B - Inhibitory (N=10)	103.2 ± 10.3	107.9 ± 14.4
	C - Sham (N=9)	105.7 ± 9.9	112.1 ± 9.1
Stroop C	A - Excitatory (N=9)	78.0 ± 11.8	82.4 ± 11.4
	B - Inhibitory (N=10)	78.4 ± 11.3	81.9 ± 12.1
	C - Sham (N=9)	76.6 ± 11.2	85.0 ± 11.5
Stroop WC Estimated	A - Excitatory (N=9)	43.1 ± 6.1	46.1 ± 6.2
	B - Inhibitory (N=10)	44.4 ± 4.9	46.3 ± 6.3
	C - Sham (N=9)	44.2 ± 4.9	48.0 ± 4.4
Stroop Interference	A - Excitatory (N=9)	8.0 ± 3.6	6.4 ± 5.1
	B - Inhibitory (N=10)	2.6 ± 10.7	7.8 ± 11.3
	C - Sham (N=9)	5.0 ± 7.8	8.3 ± 7.4
TMT Part A (seg)	A - Excitatory (N=9)	18.0 ± 4.3	14.8 ± 2.9
	B - Inhibitory (N=10)	20.9 ± 4.9	16.2 ± 4.8
	C - Sham (N=9)	18.4 ± 4.2	15.1 ± 3.7
TMT Part B (seg)	A - Excitatory (N=9)	45.2 ± 16.5	31.4 ± 10.7
	B - Inhibitory (N=10)	45.1 ± 17.0	30.9 ± 7.7
	C - Sham (N=9)	39.4 ± 17.0	29.7 ± 10.1

Mean P300 latency pre-stimulation ranged between 244.0 ± 31.7 and 248.7 ± 45.6 ms, but post-stimulation results showed a greater difference between the fastest group (234.1 ± 31.6 ms for the iTBS group) and the longest P300 group (282.2 ± 76.6 ms for the cTBS

group). ERP (N2-P3) mean amplitude oscillated between 4.2 ± 3.5 μV before the stimulation session and 3.8 ± 2.0 μV in the recording made immediately after stimulation. As for N200 latencies, the shortest one was recorded in the iTBS group before stimulation (167.0 ± 45.1 ms) and the longest group was the cTBS group in the post-stimulation ERP (191.6 ± 50.0 ms). Only N200 latency results showed a global worsening after the real/sham stimulation session, with the P300 latency and N2P3 amplitude revealing mixed results.

Stroop test pre-stimulation results showed that the highest mean values were obtained by the cTBS group for the "C" and estimated WC variables, but the sham group scored highest for "W" variable. As for Stroop interference, iTBS group achieved the best results. We can also see in table 1 that in the post-stimulation evaluation, the sham group achieved the best results for all variables. It should be noted that all groups improved their results in the second evaluation (post-stimulation), except for iTBS group in the interference variable.

iTBS group obtained the best results (18.0 ± 4.3) in part A of the TMT test, before stimulation, whereas the sham group obtained the best result in part B (39.4 ± 17.0). In the post-stimulation evaluation, the best result in part A was obtained by the iTBS group (14.8 ± 2.9) and the best score in Part B was attained by sham group (29.7 ± 10.1). All groups improved their score in the post-stimulation step.

Table 4.2 shows global statistical analysis of the stimulation and group effects, as well as the interaction stimulation-group effect. In Table 4.3 we can see pair comparisons of the pre-post stimulation mean differences.

Table 4.2
Stimulation and type of stimulation (group) effects and interaction stimulation versus type of stimulation (group) - Mixed Factorial ANOVA

Variables	Mixed Factorial ANOVA		
	Stimulation pre-post p-value	**Between-Subjects Effects (Group Effect) p-value**	**Interaction Stimulation-Group p-value**
P300 Latency Pz	0.171	0.480	**0.001**
N2P3 Amplitude Pz	0.538	**0.026**	0.889
N200 Latency Pz	0.066	0.737	0.717
Stroop W	**<0.001**	0.458	0.195
Stroop C	**<0.001**	0.991	0.455
Stroop WC Estimated	**<0.001**	0.844	0.312
Stroop Interference	0.089	0.832	0.111
TMT Part A	**<0.001**	0.460	0.431
TMT Part B	**<0.001**	0.781	0.602

ERP result analysis showed a significant result (p=0.001) in the interaction stimulation-group variable and a significant amplitude group effect (p=0,026), as can be seen in table 2. Still, group comparisons in Table 4.3 showed a significant mean difference only in P300 latency for the cTBS group (p<0.001), with slower latency peaks emerging in the post-stimulation recordings. Group comparisons showed no significant results for N200 latency or ERP amplitude.

Table 4.2 also shows global pre-post significant results in all Stroop components in the stimulation variable, except for the interference. As can be seen in Table 4.3, all groups showed a significant difference between pre- and post-stimulation periods, regarding the W and WC estimated variables, with better results in the post-stimulation period. However, C variable results were not significant in the cTBS group (p=0.079), in contrast with what occurred with the iTBS and Sham groups (p=0.037 and p<0.001, respectively). Finally, the mean difference in the “interference” variable was only significant in the cTBS group (p=0.025). When analysing the results for the TMT test before and after stimulation, all groups showed significant differences, again with better results after the TBS session.

Table 4.3
Pre-Post evaluations – mean differences for each response variable and for each group – pair comparisons.

Variables	Group A iTBS		Group B cTBS		Group C Sham	
	Mean differences post-pre	p[1]	Mean differences post-pre	p[1]	Mean differences post-pre	p[1]
P300 Latency Pz (ms)	-14.6	0.124	36.8	**<0.001**	-0.3	0.971
N2P3 Amplitude Pz (μV)	-0.8	0.474	-0.3	0.764	0.1	0.962
N200 Latency Pz (ms)	12.9	0.179	13.6	0.137	4.0	0.672
Stroop W	8.6	**0.001**	4.7	**0.041**	6.4	**0.010**
Stroop C	4.4	**0.037**	3.5	0.079	8.4	**<0.001**
Stroop WC Estimated	3.0	**0.002**	1.9	**0.035**	3.8	**<0.001**
Stroop Interference	-1.6	0.499	5.2	**0.025**	3.3	0.163
TMT Part A (seg)	-3.2	**0.002**	-4.7	**<0.001**	-3.3	**0.001**
TMT Part B (seg)	-13.8	**<0.001**	-14.2	**<0.001**	-9.8	**0.009**

[1] LSD test

Mean differences between groups for pre and post-stimulation are shown in Table 4.4.

Table 4.4
Mean differences between groups for pre and post-stimulation (I – pre-stimulation; f – post-stimulation)

Variables	Mean differences A-B	p-value[1]	Mean differences A-C	p-value[1]	Mean differences B-C	p-value[1]
P300 Lat. Pz i (ms)	3.3	0.997	4.7	0.992	1.4	1.000
P300 Lat. Pz f (ms)	-48.1	0.155	-9.6	0.973	38.5	0.315
N2P3 Amp. Pz i (μV)	1.8	0.584	3.2	0.144	1.4	0.733
N2P3 Amp. Pz f (μV)	1.3	0.371	2.4	**0.028**	1.2	0.455
N200 Lat. Pz i (ms)	-11.0	0.865	-12.1	0.839	-1.1	1.000
N200 Lat. Pz f (ms)	-11.7	0.860	-3.2	0.997	8.5	0.940
Stroop W i	-6.0	0.625	-8.4	0.364	-2.5	0.958
Stroop W f	-2.1	0.983	-6.3	0.713	-4.2	0.885
Stroop C i	-0.4	1.000	1.4	0.991	1.8	0.980
Stroop C f	0.5	0.999	-2.6	0.956	-3.1	0.920
Stroop WC Est. i	-1.3	0.937	-1.1	0.964	0.2	1.000
Stroop WC Est. f	-0.2	1.000	-1.9	0.872	-1.7	0.892
Stroop Int. i	5.4	0.398	3.0	0.824	-2.4	0.887
Stroop Int. f	-1.4	0.979	-1.9	0.952	-0.5	0.999
TMT A i (seg)	-2.9	0.432	-0.4	0.996	2.5	0.569
TMT A f (seg)	-1.4	0.824	-0.3	0.997	1.1	0.911
TMT B i (seg)	0.1	1.000	5.8	0.854	5.7	0.852
TMT B f (seg)	0.5	0.999	1.8	0.971	1.2	0.989

[1] LSD test with Sidak's correction

The only significant result when mean results are compared among groups was found in the post-stimulation comparison between the iTBS and sham groups (p=0,028), with shorter mean difference between groups.

Discussion

In our group of TMS naïve young volunteers, we found that a single session of cTBS over the left prefrontal cortex can influence both P300 and Stroop test, by slowing P300 peak latencies and significantly changing the expected cTBS group performance in Stroop C and Stroop Interference compared to the iTBS and sham groups. In contrast, no changes associated with any of the TBS sessions occurred in TMT performance. Assessment of the neuropsychological test results showed that all study groups had a significant tendency towards improving their performance. During testing, minor side effects were reported and no dropouts occurred. In this context, our study brings novel data suggesting that neurophysiological and some neuropsychological performances can be similarly influenced by the same TBS protocol in normal volunteers.

In our study, both TMT and STWC tended to improve significantly in the second test in all groups, including the sham group. This test-retest behaviour is known and has been reported in several published studies as "the learning effect". This effect apparently develops when a neuropsychological test is repeated within a short period of time [187–189]. It is important to emphasise that the groups showed no significant baseline differences in their characteristics or performance. As expected, the evaluation of our sham group showed an improvement in performance between testing and re-testing. Results found in iTBS and cTBS groups evaluated with TMT A and B, Stroop W and Stroop WC also confirm a significant improvement after the second test, in agreement with the learning effect hypothesis. In contrast, cTBS group response in Stroop C did not follow the significant improvement of the results recorded in the Sham and iTBS groups. The cTBS group also behaved differently in Stroop interference, with a stronger improvement compared to the sham group and with opposite performance compared to the iTBS group.

The Stroop test is based upon two sets of data: verbal fluency (W and C variables) and lability (WC variable), with lability being the capacity to answer independently when comparing with previous answers [177,190]. The "C" variable in the cTBS group did not achieve a significant improvement, in contrast with the iTBS and sham groups. This task, related to reading and verbalization of colours, can probably be more affected by left hemisphere inhibition, thereby accounting for the worse result of the cTBS group compared to the other groups. MacLeod et al. (2000) state that in the Stroop test there is an asymmetry of the Stroop effect – the interference effect - since words interfere in colour naming, but not the reverse, concluding that reading words is more automatic

than naming colours [176]. We found the highest interference result in the cTBS group and it is possible that the impaired left hemisphere performance may have led to a left-right hemisphere imbalance, by enhancing right hemisphere competences. This result may contradict the trend defending that there is a left hemisphere dominance related to interference in naming the colour of a word printed in an incorrect word (e.g, the word "blue" printed in green) [191,192], but these assumption may not be so linear. In 1993, Bench et al. already stated that the interference task was associated with right frontal activation [193], so hemisphere dominance in Stroop testing is not a consensus topic in literature.

In a recent literature review, Banich claimed that the Stroop effect results from a cascade-like process, in which different anatomical areas are activated in sequence [194]. A left hemisphere language area activation is mostly seen when confronting the congruent and incongruent trials using word and colours [194,195], and a right hemisphere activation is also seen, depending upon task demands. Studies have shown that network hubs with right regional activation (inferior frontal sulcus and anterior insula) can be found in higher demand Stroop protocols [194,196]. The idea that lateralization was linked to the task was already reported suggesting that the right prefrontal cortex assumes a more preeminent role when attentional control is need in order to reduce conflict [197]. However using rTMS and measuring reaction time, contradicting results were found with stroop tests: Kern et al. found no left hemisphere dominance for the cognitive control implementation but Vanderhasselt et al., using a protocol that also involved rTMS, showed that the left hemisphere activation could improve Stroop task performance [197–199]. It is important to emphasize that these findings were related to reaction time only. Using high frequency rTMS, stimulating either the right DLPFC and the left DPFC, the same team only found results in the reaction time of the volunteers with no significant result in the Stroop interference effect [198,200].

Using other techniques, such as functional near-infrared spectroscopy, bi-hemispheric activation was also found for the Stroop test with a congruent-incongruent word-colour task [201] and for a Stroop test task based on spatial trails [202]. When comparing results from different techniques we have to remain cautious: for instance, functional imaging is able to show changes with some delay compared to more immediate functional assessment techniques such as functional near-infrared spectroscopy or even evoked potentials.

We believe that the results found in our study were most likely originated by a real left hemisphere inhibitory effect through cTBS, affecting volunteer cognitive functions, thus

counteracting the expected learning effect that we found in the remaining groups. This left hemisphere impairment effect induced by TBS affects neuropsychological tests like Stroop, TMT, and even the P300 differently. Our results in the Stroop test may also be explained by the notion that the right hemisphere not only processes the whole stimulus [191] but is linked to a more complex protocol or even that it may be more involved in the decision making procedure [191,194,196]. It is also important to mention that our protocol is a Portuguese Stroop test adaptation, with emphasis on the number of hits and not on reaction time [186]. Likewise, a direct comparison with other studies should be carried out with caution because our protocol only used incongruent trials without congruent trial presentation and without congruent-incongruent trial comparison.

It is known that DLPFC is an area of tremendous relevance in the formation and regulation of brain function associated with P300, Stroop and also TMT tests [92,163,203]. The fact that we found different test results after stimulating the same left DLPFC with excitatory or inhibitory stimulation suggests that this region has a different weight on each specific test. The left DLPFC seems to have a more direct influence on neural networks allocated to P300 and Stroop C, being mainly influenced by inhibitory stimulation. The P300 protocol used on these volunteers revealed a significant increase in P300 latency only after cTBS, with no significant change after iTBS. This left hemisphere lateralization may also be linked to a greater capacity of the left hemisphere to influence dopamine release, either by lowering or promoting its release depending upon stimulation characteristics (inhibitory vs excitatory) [63,156].

Dopamine is known to influence both event related potentials and task performance testing [154,155]. Lower P3 latencies and faster reaction times were also found by Evers et al. after excitatory stimulation of the left PFC (only), again suggesting a leftward susceptibility to being more easily influenced by TMS [149]. Lowe et al., in a 2018 systematic review evaluating TBS targeting the prefrontal cortex of healthy subjects, found a significant effect in modulating executive functioning associated with stimulation, suggesting that cTBS decreased performance [92]. They also found that these effects were larger if the left PFC was used. Our results also support the notion that left frontal cTBS may originate changes in both neurophysiological and neuropsychological testing results. Which factors are involved in this biased response are still unknown, thus emphasizing the need for more research to determine the factors that may lead to such behaviour.

Our results also show that a single iTBS session on the left hemisphere appears to have little ability to modulate or influence cognitive functions assessed by P300, N200, TMT

and Stroop tests. This result supports the hypothesis that the inhibitory capacity of cTBS appears to be superior to the excitatory ability of iTBS, as suggested in previous studies with various forms of assessment [6,45,204].

These results highlight the importance of mixed evaluation using neuropsychological and neurophysiological tools in the evaluation of research findings and clinical results related to the use of transcranial magnetic stimulation in several diseases that may impair cognitive processing. Isolated evaluations such as response time, although objective, do not allow to assess how long it takes for a stimulus to be encoded in the brain. The partial similarity of P300 behaviour and Stroop test found in our results supports the notion of a common cognitive pathway between the two tests.

It is also important to note that no major side effects were reported with our stimulation protocol, following findings described in the literature for TBS, as we had no dropouts and only a few volunteers mentioned short-term headaches and negligible focal pain during stimulation.

One of our study limitations is the relatively small number of volunteers for each group, which may have limited the statistical strength of the tests used. Another possible limitation is related to the fact that the duration of the stimulation effects has not been evaluated, a process that was difficult to implement given our study design. Replication of this study should be performed with a larger number of subjects, in order to try to achieve a more robust result. The duration of the TBS effect on these tests should also be evaluated in the future, especially if a multiple session protocol is used. It would be also interesting to monitor volunteers in these types of studies with an anxiety scale in order to control the possible influence of this parameter in naïve subjects. We may assume that agitation or concentration difficulties originated by study procedures could have interfered with the subjects due to the novelty of the experiment. Finally, identification of the DLPFC could have benefited from the use of a neuronavigation tool, not available in our laboratory.

In summary, in spite of a small number of volunteers and a learning effect due to test repetition, our study showed that when an inhibitory stimulation is applied on the left hemisphere, an impairment of this hemisphere's functions is observed, but these effects do not seem to affect or influence long-latency evoked potentials and neuropsychological tests similarly. Our results suggest that when trying to evaluate magnetic stimulation success as a therapeutic tool, researchers should always opt for a battery of multiple tests, sensible enough to detect the expected clinical improvement.

Chapter V

Theta burst stimulation over the prefrontal cortex: effects on cerebral oximetry and cardiovascular measures in healthy humans

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Chapter V

Theta burst stimulation over the prefrontal cortex: effects on cerebral oximetry and cardiovascular measures in healthy humans

Abstract

Theta Burst Stimulation (TBS) is a non-invasive neurophysiological technique, able to induce changes in synaptic activity. Research suggests that TBS may induce changes in cerebral oxygenation, cerebral blood flow, blood pressure and heart rate but there are conflicting results across studies. Thus, the objective of our sham-controlled study is to evaluate if TBS applied to the dorsolateral prefrontal cortex (DLPFC) of healthy volunteers produces changes in cerebral oximetry, heart rate and blood pressure. Forty-nine volunteers of both sexes were randomly allocated to one of five stimulation groups. Before and after real TBS or sham stimulation, blood pressure, heart rate, and cerebral oxygenation of the volunteers were measured. Cerebral oxygenation values were obtained with a near infra-red spectroscopy system. We found a significant reduction in left cortex oximetry after continuous TBS (cTBS) over the left DLPFC ($p=0.039$) and a non-significant reduction in right cortex oximetry ($p=0.052$). Right hemisphere inhibition (using cTBS) seemed to originate a significant reduction of 8 mmHg in systolic arterial pressure. No other changes were seen in oximetry, cardiac frequency and diastolic arterial pressure. In our group of normal subjects, cTBS applied to the left DLPFC was able to reduce oxygenation in the left cortex. Right hemisphere inhibition was associated with a significant reduction in systolic pressure.

Keywords: Transcranial Magnetic Stimulation; Theta burst stimulation; Near infrared spectroscopy; Oximetry; Blood pressure

Introduction

The therapeutical use of repetitive transcranial magnetic stimulation (rTMS) is based on the premise that the application of recurring magnetic pulses can modify neuronal excitability [205–207], being able to alter the plasticity of neurons and synapses. A specific rTMS protocol called Theta Burst Stimulation (TBS) was proposed by Huang et al. [45,208], inducing lasting electrophysiological changes (up to 60 min), not only in the primary motor cortex but also in other cortical regions [45,209]. Research distinguishes two types of TBS: intermittent TBS (iTBS), which appears to increase transmission efficiency between synaptic connections of stimulated neurons (excitatory effect); and continuous TBS (cTBS), which decreases neuron transmission capacity (inhibitory effect). Because of its abilities and minor side-effects, rTMS and TBS have been used for therapeutically use in individuals with neuropsychiatric conditions, such as depression [210–214] and stroke [215,216].

Cerebral oxygenation is dependent on cerebral blood flow (CBF), cerebral metabolism, and neuronal activity. Neurons are unable to produce energy through anaerobic mechanisms, and therefore depend on a constant supply of O2. Thus, we can promote an increase in CBF by vasodilatory mechanisms, thereby increasing the percentage of oxygen delivered to the cells per minute and also influence CBF by changing cerebral vascular resistance and perfusion pressure [217–219].

Several techniques are used to measure cerebral blood flow, but transcutaneous oximetry allows a more economical, portable, faster, and non-invasive way to obtain localized, reliable results without increasing risks to the individual being studied and with a time frame closer to the actual neurophysiologic process [220,221]. Frontal cerebral oxygen saturation can be measured using near-infrared spectroscopy (NIRS) which measures cerebral oxygenation through the balance between oxygenated and deoxygenated haemoglobin [222–224], thus performing an indirect measurement of the concentration of O2 in the underlying tissues. TMS effects can be studied using NIRS since changes in neuron firing rates after stimulation seem directly related to changes in local hemodynamic behaviour [225,226].

Evidence from animal and human research (level A of evidence) showed that interneuronal activation following rTMS/TBS may activate networks associated with sympathetic activity in humans [227]. Some authors suggest that stimulation can induce changes in cerebral oxygenation, CBF, blood pressure, and heart rate [228–232], but there is disagreement between the studies, evidencing the need for further work. One of

the main cortical regions involved is the dorsolateral prefrontal cortex (DLPFC), possibly by influencing cardiovascular autonomic functions [227,228,231]. Cardiovascular diseases and hypertension can be influenced by impaired cardiovascular neuromodulation [233,234], and the importance of indirect modulation of the autonomic nervous system through cortical stimulation may prove to be a promising method for controlling these diseases. In addition, as far as we can ascertain, no studies have been published on iTBS/cTBS applied to the DLPFC and their relationship with brain oxygenation, as well as their relationship with blood pressure and heart rate in normal volunteers.

Given that DLPFC is a region with a large neural network and has been found to play a relevant role influencing sympathetic activity and autonomic functions, we hypothesized that the use of TBS over the left and right DLPFC of normal volunteers may be capable of modifying physiologic parameters like oximetry, heart rate, and blood pressure. Therefore, we expected to (i) find significant changes after TBS in the studied parameters, (ii) observe different parameter behaviour after the iTBS session (excitatory effect) compared with the cTBS session (inhibitory effect), and (iii) clarify whether a hemispheric dominance might be present.

Material and methods

Subjects

Our study focused on a sample of forty-nine right-handed, 18-30 year-old healthy volunteers, students from the Faculty of Health Sciences (FHS) - University of Beira Interior, Covilhã, Portugal. Exclusion criteria included: metallic elements in the skull; epilepsy or seizures; diagnosis of vascular, tumour, infectious or uncontrolled metabolic brain disease; pacemakers, intracardiac lines or severe heart disease; increased intracranial pressure; drug intake; pregnant women or suspected pregnancy; and chronic alcoholism. All volunteers were naïve to rTMS/TBS. In order to ensure volunteers' safety, we followed the safety considerations of Rossi et al. (2009) [19]. The study was approved by the Ethics committee of FHS/UBI (No. CE-FCS-2011-001), and all volunteers signed a written informed consent form, following the principles of the Declaration of Helsinki. All volunteers were evaluated in the FHS/UBI Neurophysiology laboratory and were contacted within 24 h after stimulation to see if there were any adverse effects to report.

Experimental paradigm

Volunteers were randomly assigned to one of five stimulation groups (using a closed envelope method). The final sample was composed of the following groups:

- Left iTBS group - 9 volunteers exposed to left dlPFC excitatory stimulation; Left cTBS group - 10 volunteers exposed to left dlPFC inhibitory stimulation; Sham Group - 10 volunteers exposed to either left or right dlPFC sham stimulation; Right iTBS group - 10 volunteers exposed to right dlPFC excitatory stimulation; Right cTBS group - 10 volunteers exposed to right dlPFC inhibitory stimulation.

All groups had an approximate 60% female vs 40% male distribution.

This was a double-blind study since only the researcher performing the technique knew which TBS stimulation type was given to each volunteer and both volunteers and the researchers evaluating the results were not aware of which stimulation type had been given.

Figure 5.1 shows a schematic overview of the experimental methodology.

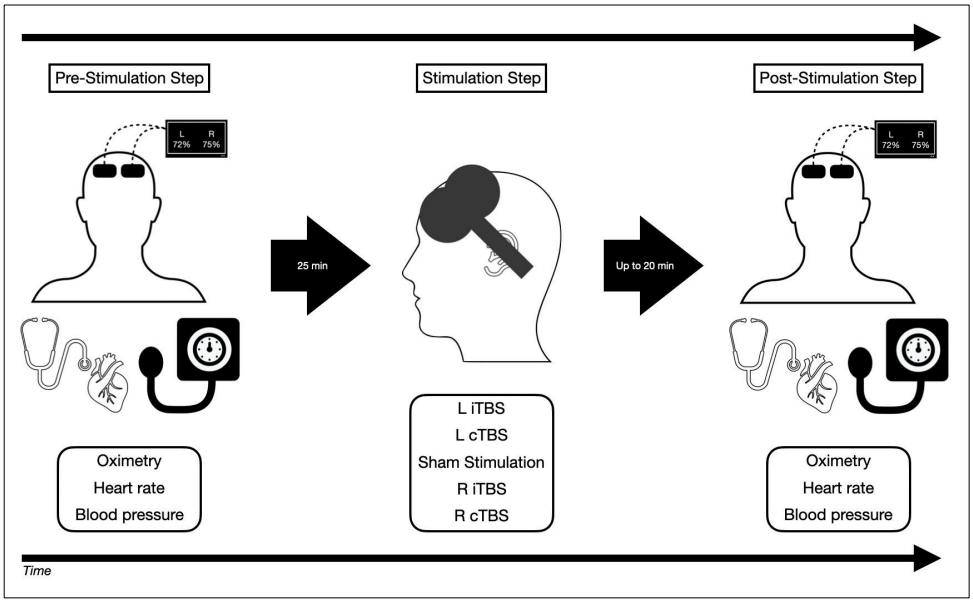

Figure 5.1. Evaluation process.

All participants were evaluated once before the active or sham stimulation session – the pre-stimulation step – and then after the stimulation – the post-stimulation step (occurring up to 20 min afterwards). In the pre-stimulation step, blood pressure, heart rate, and oximetry were measured. After this step, each volunteer was submitted to a single TBS or Sham stimulation session, accordingly to the volunteer´s assigned group. Measurements of blood pressure, heart rate, and oximetry were re-evaluated in the post-stimulation step.

Blood pressure and heart rate

Considering that all subjects who participated in the study were right-handed and had no documented disease, all measurements were made on the left upper limb. Therefore, two to three blood pressure and heart rate measurements were performed, each separated by 1 to 2 minutes. At the end of the evaluation, mean values were calculated and registered, for heart rate and both systolic arterial pressure (SAP) and diastolic arterial pressure (DAP). An automatic MEDEL - Model Check 3 - meter was used to perform the measurements.

Oximetry

Cerebral oxygenation measurements were obtained with the aid of the EQUANOX ™, Model 7600 Regional Oximeter System with EQUANOX Classic ™ Sensor, Model 8000CA. Oximetry was measured using two sensors placed bilaterally in the frontal region, approximately 5 cm above the eyebrow line, one corresponding to the left cortex and another to the right. In each of the pre and post-stimulation steps, four measurements separated by 30 s (0, 30, 60, and 90 s) were performed for each hemisphere, and the final result was a mean of the four values. This mean was assumed to be the final oxygenation value of the elapsed period. This short-timed multiple-evaluation method results in a mean value, thus less susceptible to variation.

Theta burst stimulation

For the TBS sessions a MagVenture MagPro® G3 X100 5.0.1 magnetic stimulator was used, with a butterfly-type coil. Recording of motor responses necessary for the location of the stimulation point was obtained in the contralateral abductor pollicis brevis (cAPB), through a Dantec ™ Keypoint® electromyograph - Keypoint.net v.2.03. TBS stimulation was performed on the left dlPFC and on the right dlPFC for each specific group, in a zone 5 cm anterior to the previously defined left/right primary motor area. The intensity used was 80% of the active motor threshold value (AMT), defined as the minimum intensity of the stimulation for which there is a motor response in 5 or more of 10 stimuli, while the subject is in a minimum contraction of the cAPB [137].

TBS stimulation followed the method described by Huang et al. [45] and consisted of bursts of three pulses at 50 Hz, applied repetitively in intervals of 200 ms (5 Hz). In the iTBS groups, stimulation lasted 2 s, with intervals of 8 s without stimulation, totalling a stimulation period of 600 pulses. In relation to the cTBS groups, bursts appeared continuously at 5 Hz until they reached 600 pulses. In the placebo group, the same coil and the same protocols were used, but the coil was placed at a perpendicular angle (90° angle) to the skull of the volunteer in order to avoid inducing cerebral stimulation and thereby maintaining the previously described characteristics associated with the stimulus.

Figure 5.2 shows the main equipment used in the experimental process.

Figure 5.2. Transcranial magnetic stimulation (TMS) apparatus, oximeter system, and blood pressure meter.

Statistical analysis

Data were analysed using IBM SPSS (v22), and a mixed-measure ANOVA was applied. For univariate tests (ANOVA), sphericity was assumed. With the Levene test, it was also verified that the assumption of the homogeneity of the variances of each of the variables in the five stimulation groups was not violated. Assumption of normality was violated in some cases. For the cases of interest, multiple comparisons were made using LSD test (Least Significance Difference) with Sidak´s correction when necessary, based on the estimated marginal means, since this allows in particular to make such comparisons without there being any significant effect of any of the factors or interaction between them. For comparison of ages between stimulation groups or sexes, Kruskal-Wallis or Mann-Whitney tests were used, respectively. For pairwise comparisons of stimulation groups, Dunn's test with Bonferroni correction (Dunn-Bonferroni) was used. Results with a test value (p-value) less than or equal to 0.05 were considered statistically significant. Confidence intervals were considered at 95%.

Results

Subjects

Our group of 49 volunteers consisted of 19 men and 30 women, with a mean age of 22.8 years. Left iTBS group mean age was 21.67±1.58 years, left cTBS group mean age was 23.70±2.54 years, right iTBS group mean age was 23.90±0.88 years and right cTBS group mean age was 23.90±0.88 years. Sham group mean age was 23.00±1.16 years. There was a significant difference in age distribution (Kruskall Wallis; p=0.012) but not in sex distribution between the various stimulation groups. Mean age differences (max 2,23 years) were considered to be irrelevant to the physiologic parameters in evaluation.

Oximetry

Mean oximetry values were obtained before the stimulation session on the right and the left frontal areas and were compared with the results obtained after stimulation. Table 5.1 and Figure 5.3 briefly present the data observed for the variables under study.

Table 5.1

Oximetry values observed in sham and TBS stimulation groups

Side	Time	Stimulus	N	Sample mean	SD	CI 95% For mean
Left cortex	Before	iTBS Left	9	73.03	4.24	69.77 – 76.29
		cTBS Left	10	73.08	1.81	71.78 – 74.37
		Sham	10	71.93	3.89	69.15 – 74.71
		iTBS Right	10	69.95	3.68	67.32 – 72.58
		cTBS Right	10	71.93	6.02	67.62 – 76.23
	After	iTBS Left	9	74.33	3.65	71.52 – 77.14
		cTBS Left	10	69.95	4.34	66.84 – 73.06
		Sham	10	72.95	4.73	69.57 – 76.33
		iTBS Right	10	68.68	3.48	66.19 – 71.16
		cTBS Right	10	70.95	6.09	66.60 – 75.30
Right cortex	Before	iTBS Left	9	74.28	3.55	71.55 – 77.01
		cTBS Left	10	73.10	4.36	69.98 – 76.22
		Sham	10	71.93	3.89	69.15 – 74.71
		iTBS Right	10	72.00	4.56	68.74 – 75.26
		cTBS Right	10	72.03	5.64	67.99 – 76.06
	After	iTBS Left	9	74.00	3.47	71.34 – 76.67
		cTBS Left	10	70.50	3.66	67.88 – 73.12
		Sham	10	72.95	4.73	69.57 – 76.33
		iTBS Right	10	69.95	6.00	65.66 – 74.24
		cTBS Right	10	70.05	5.78	65.92 – 74.19

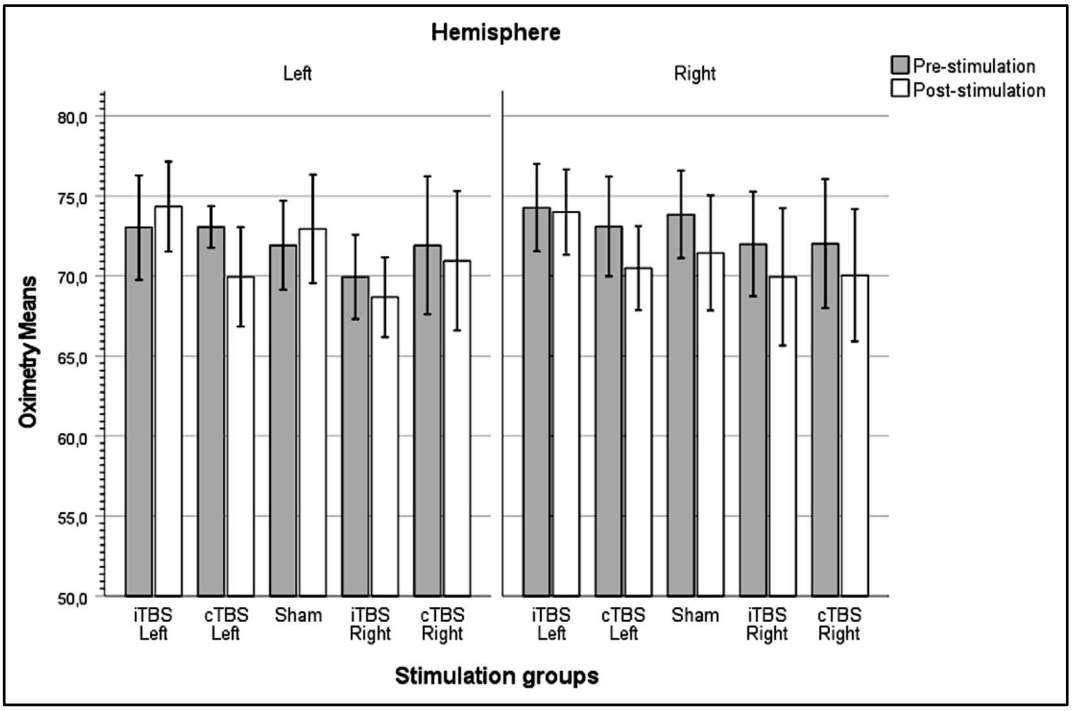

Figure 5.3. Oximetry values – graphic presentation.

A mixed repeated measures ANOVA was performed to assess whether the factors under study had a significant effect over left and /or right oximetry. This analysis did not find any significant effect of the Stimulation Moment (Time) on the multivariate compost (oximetry values from left or right Side) (p=0.153) nor a significant effect of the Stimulation Groups (p=0.275; p=0.099). Interaction between the stimulation process and stimulation type was not significant (p=0.447; p=0.155).

A Univariate Anova was performed for values of oximetry for each hemisphere. Table 5.2 present the results of the univariate ANOVA for each side (all groups together).

Table 5.2

Repeated measures ANOVA (Univariate)

	Side	Stats	p	η2p	Potency	Mean diference (after-before)
Time (before-after)	Left	F(1.44)=0.838	0.365	0.019	0.146	-0.609
Interaction Time- rTMS		F(4.44)=1.487	0.223	0.119	0.422	-
Time (before-after)	Right	F(1.44)=3.994	*0.052*	0.083	0.498	-1.176
Interaction Time- rTMS		F(4.44)=1.329	0.274	0.108	0.380	-

Overview of the results in Table 2 show no statistically significant findings both in Time and Interaction, but near-marginal in the comparison before-after for Time over the right (p=0,052).

From the multiple comparisons with Student´s *t*-test and Sidak correction, when necessary, we found results presented in Table 5.3. Mean Oximetry results showed different behaviours between right and left cortex, with a tendency to a reduction in oximetry on the right when right and left hemisphere were stimulated. Left cortex showed a non-homogeneous picture.

Table 5.3

Comparisons between oximetry mean pairs regarding stimulation moments, with side and stimulation groups fixed

Side	TBS	Mean differences (Before - after)	p[1]
Left	iTBS Left	1.31	0.404
	cTBS Left	-3.13	**0.039**
	Sham	1.03	0.490
	iTBS Right	-1.28	0.391
	cTBS Right	-0.98	0.511
Right	iTBS Left	-0.28	0.840
	cTBS Left	-2.60	*0.052*
	Sham	1.03	0.435
	iTBS Right	-2.05	0.122
	cTBS Right	-1.98	0.136

[1] LSD test

When comparing oximetry mean values of the three stimulation groups (cTBS, iTBS and Sham), we did not find any significant differences, before or after stimulation, on the left or right side of the cortex or when comparing left with right side of the cortex.

When we compared oximetry mean values between stimulation moments (Table 5.3), we found a significant reduction in the left cortex oximetry after cTBS on the left (p=0.039) and also a reduction, but not significant on the right cortex oximetry after cTBS on the left (marginal p=0.052). No other differences were found.

Blood pressure and heart rate

Tables 5.4 and 5.5 presents cardiac frequency, systolic (SAP) and diastolic pressure (DAP) results, according to the type of stimulation. There was a significant interaction between “Time” and cardiac frequency, and the behaviour of diastolic pressure showed a significant change after stimulation (p=0.008). Interaction between “Time“ and “Stimulation” was significant only for SAP.

Table 5.4

Repeated measures ANOVA and mean and SD of physiological parameters.

	Time	Stimulation type	N	Mean±SD	Time effect p-value	Interaction Time*TBS p-value	TBS effect
SAP	Before	iTBS Left.	9	121.56±14.41	0.537	**0.050**	0.299
		cTBS Left	10	128.10±15.32			
		Sham	10	110.01±13,55			
		iTBS Right	10	119.60±12.48			
		cTBS Right	10	120.10±13.00			
	After	iTBS Left.	9	123.33±14.58			
		cTBS Left	10	120.18±14.42			
		Sham	10	125.10±15.98			
		iTBS Right	10	123.00±16.48			
		cTBS Right	10	111.80±12.92			
DAP	Before	iTBS Left.	9	76.56±10.91	0.895	0.771	**0.008**
		cTBS Left	10	81.10±8.12			
		Sham	10	81.70±8.59			
		iTBS Right	10	70.90±6.97			
		cTBS Right	10	72.90±6.94			
	After	iTBS Left.	9	74.78±7.40			
		cTBS Left	10	82.20±7.22			
		Sham	10	81.10±9.92			
		iTBS Right	10	73.60±8.29			
		cTBS Right	10	72.25±8.37			
Cardiac frequency	Before	iTBS Left.	9	75.67±9.43	0.006	0.930	0.375
		cTBS Left	10	76.80±7.54			
		Sham	10	78.20±13.83			
		iTBS Right	10	70.70±8.96			
		cTBS Right	10	73.20±10.64			
	After	iTBS Left.	9	74.11±9.64			
		cTBS Left	10	74.70±10.06			
		Sham	10	75.50±15.69			
		iTBS Right	10	66.80±8.97			
		cTBS Right	10	69.50±9.96			

Table 5.5

Comparisons between mean pairs for each physiological parameters related to the stimulation, with fixed side and stimulation groups.

Side	rTMS Group	Mean Difference (After - Before)	p[1]
SAP	iTBS Left	1.78	0.614
	cTBS Left	2.40	0.474
	Sham	5.40	0.111
	iTBS Right	3.40	0.312
	cTBS Right	-8.30	**0.016**
DAP	iTBS Left	-1.78	5.15
	cTBS Left	1.10	0.671
	Sham	-0.60	0.817
	iTBS Right	2.70	0.299
	cTBS Right	-0.65	0.802
CF	iTBS Left	-1.56	0.495
	cTBS Left	-2.10	0.333
	Sham	-2.70	0.215
	iTBS Right	-3.90	0.076
	cTBS Right	-3.70	0.092

[1] LSD Test

Mean pairs comparisons in Table 5 showed a significant reduction of 8.3 mmHg (p=0.016) in the systolic pressure, after right hemisphere inhibition (cTBS). It should be further noted that this group was the only one where a decrease in systolic blood pressure was recorded, with all others showing an increase. Cardiac frequency (CF) showed a global tendency to decrease after stimulation (Table 4), especially after right hemisphere stimulation, but without any significant results in any single group.

No significant changes were found in diastolic pressure measurements.

When comparing the averages of SAP, DAP and CF between the three stimulation groups (cTBS, iTBS and Sham), before or after stimulation on the left or right sides, no significant difference was found. The biggest difference before stimulation was related to DAP between the Sham and cTBS groups on the right side (difference Sham-right cTBS=10.8 mmHg, p=0.059).

After stimulation, the biggest difference was found in SAP between the groups cTBS, inhibited on the left side, and cTBS, inhibited on the right side (difference left cTBS-right cTBS=18.7 mmHg, p=0.073).

Discussion

In our group of subjects submitted to bilateral TBS over the dorsolateral prefrontal cortex, we found a significant reduction in left frontal cortex oximetry after ipsilateral cTBS stimulation. No other significant differences in oximetry were found. Right DLPFC cTBS was associated with a significant reduction of systolic mean pressure. Our results seem to suggest that inhibition of the left DLPFC can originate a nearly identical reduction in oximetry in both hemispheres, possibly associated with a bilaterally decreased cerebral blood flow, even when subjects are at rest. Inibitory stimulation over the right DLPFC seems to influence autonomic dependent responses, leading to a decrease in SAP.

During brain stimulation there is an immediate short increase in deoxygenated haemoglobin concentration at stimulation, believed to be due to an immediate rise in the rate of oxygen utilization by the brain [235]. However, this change is almost immediately followed by a fast auto-regulation phenomenon leading to a consistent decline in the concentration of deoxygenated haemoglobin, due to the blood flow response following the shift of the cerebral metabolic rate of O2 consumption [235]. Increased brain metabolism due to brain stimulation results in greater demand for oxygen that is linked to increased perfusion. It is known that magnetic stimulation not only activates cerebral neurons and synapses but also has an effect on CBF: for instance, CBF velocity is decreased in the middle cerebral artery when applying low-frequency rTMS to the DLPFC [236,237], while high-frequency rTMS induced a localized and transitory increase in CBF [238]. However, studies with apparently dissonant results have been found. Park et al. reported a lasting increase in the concentration of oxyhemoglobin in the hemisphere contralateral to one session of low-frequency rTMS, but these authors studied the primary and premotor cortices with a 10 subject cross-over protocol [207].

In our group of volunteers, we found a bi-hemispheric reduction in frontal cortex oximetry after left cTBS, which was statistically significant ipsilaterally, and only marginal in the right hemisphere. Effects of cTBS on CBF/oximetry in the ipsilateral cortex and in other areas of the brain were reported previously: Cho et al. using cTBS and PET, found reduced CBF both ipsilaterally and in other pre-frontal areas after stimulating the right DLPFC [239]. Orosz et al. [229] used cTBS in the right motor cortex during a motor task with the left hand and fMRI and found an increase in CBF in the primary motor cortex, probably resulting simultaneously from the cTBS effect and the motor task performed by the volunteers. Kozel et al. and Tian et al. reported a decrease

in oxygenated haemoglobin in both ipsilateral and contralateral sides of the motor cortices when stimulating at 1 Hz for a short period of time (10 s) [240,241]. This simultaneous response of oximetric values to stimulation in both hemispheres suggests a connection between both hemispheres, probably similar to inhibitory connections detected in other experiments, since the DLPFC is bilaterally active in multiple high cortical functions that need a network to be performed [240,241]. Similar connections have been already reported: using rTMS and NIRS, Mochizuki et al. [232] demonstrated an inhibitory interaction between primary motor cortices reflected in CBF responses; Vernieri also found a bilateral effect on CBF when low-frequency rTMS was applied to the left motor area and suggested either a commissural effect or an effect on the autonomic nervous system via the sympathetic nervous system to explain it [242]. Tupak et al. evaluated the relationship between oximetry and TBS (cTBS) applied to DLPFC [231] :they analysed surface oximetry with functional NIRS, and concluded that cTBS, when applied to the left hemisphere, was able to decrease prefrontal cortex oxygenation bilaterally, a finding corroborated by our study. However, their subjects' oxygenation was evaluated during an emotional Stroop task while our subjects were evaluated in a resting state, therefore not subjected to other stimuli and with a mean response of 4 measurements in order to obtain the best response. Thus, our data suggest that the observed decrease in oximetry in our volunteers is a direct effect of cTBS, in subjects at rest, without interference by any performed task.

Our findings also suggest that the inhibitory stimulation of the left DLPFC may also influence the right frontal cortex oximetry, consistent with the theoretical hypothesis of a previously mentioned callosal mediation. However, other mechanisms may be present because we could not find any significant changes with the excitatory stimulation on the left hemisphere and no significant results with any stimulation of the right hemisphere. Some hemispheric dominance or even the small number of subjects evaluated in our study could account for these results.

The right hemisphere stimulation, independently of type, does not evoke significant changes in cerebral regional vascularization/oximetry. Mochizuki et al. [232] only observed inhibitory-like responses in the contralateral hemisphere when stimulating the left DLPFC and they attributed this asymmetry to the dominance of the left hemisphere. Cardenas Morales et al. [243] also examined the left motor cortex with iTBS and found a decrease in BOLD signal in motor areas on the left hemisphere and other remote areas with no changes observed for CBF at rest in the right hemisphere. The variation of oximetry values in our results can also be explained by volunteers who had left hemisphere dominance (since it was one of the criteria for selection). We suggest that

connectivity for CBF present in both hemispheres is probably dominated by the left hemisphere in right-handed subjects at rest, with no apparent significant influence of the right dorsolateral prefrontal cortex on CBF, independently of the type of stimulation.

Although we did not study the association between the effects of cTBS on oximetry (reduction) and a functional inhibition, we could assume that they occur simultaneously. In vitro and in vivo studies able to make this distinction would be valuable, mainly in the context of the use of TMS in stroke patients. The neuro-vascular mechanism activated by magnetic stimulation is a complex one, with neural components as well as myogenic and metabolic ones [242,244]. It is suggested that TMS exerts its therapeutic effects by changing several neurosynaptic events but also cerebral blood flow in a frequency-dependent manner, but these effects are yet to be fully understood.

All types of TBS (excitatory, inhibitory, and placebo) on the right and left hemispheres were associated with a significant effect on heart rate in our volunteers ($p=0.006$), and we observed an overall mean reduction of approximately 3 heartbeats per minute after the stimulation. Due to its presence even in the placebo group, no clinical significance was attributed to it. Since volunteers were naïve in terms of TBS, this could be an effect of post-performance.

Right hemisphere stimulation, however, was associated with significant result in cardiovascular physical parameters: in our subjects, the interaction between the stimulation moment and systolic blood pressure showed a significant difference only when there was an inhibition of the right DLPFC ($p=0.016$), with a reduction of approximately 8 mmHg. Although it is a controversial subject, different authors support the hypothesis of a right hemisphere dominance over the sympathetic nervous system, after studying parameters such as heart frequency and blood pressure. Studies such as those of Hilz et al., Yoon et al., and Oppenheimer et al. [245–247] in which the impairment of the right hemisphere was promoted, resulted in a significant decrease in blood pressure. This supports our result that only the inhibitory TBS on the right hemisphere seems to induce a blood pressure reduction.

Sibon et al. did not find significant changes in heart rate and blood pressure after the application of several blocks of excitatory rTMS (10 Hz) on the DLPFC [248]. Other authors also argued that there is an influence of rTMS over blood pressure through effects on the autonomic nervous system. Yoshida et al. concluded that inhibitory rTMS (0.2 Hz) applied to the vertex region causes a transient stimulation of the sympathetic nervous system, and Vernieri et al. concluded that excitatory stimulation (17 Hz) applied

to the primary motor cortex of the dominant hemisphere causes changes in brain hemodynamics due to alteration in the autonomic nervous system [242,249]. More recently, Li et al. [250] and Zhang et al. [251] reported an effect of rTMS on blood pressure, when applied to the carotid sinus of humans and rabbits: systolic and diastolic blood pressure was significantly reduced after low rate magnetic stimulation of the carotid sinus in 15 subjects. We found a similar effect with our results, with a reduction of systolic blood pressure when cTBS is applied in the right DLPFC. In the opposite direction and studying stroke patients, Yozbatiran et al. found that high-frequency rTMS applied ipsilaterally to the stroke lesion, promoted a systolic blood pressure increase of 7 mm/Hg [252]. Our study could not find similar results after iTBS, possibly because we performed only a single session of stimulation and excitatory effects may need more stimulation sessions. Our results showed a change in SAP measures, which supports the theory that DLPFC appears to be involved in the control of the autonomic nervous system, together with the posterior insular cortex, by forming a central network, which integrates and processes information from higher cognitive centres and transmits it to autonomous centres of the reticular formation, triggering the most appropriate autonomous response to the stimulus received [253–255].

A possible limitation in the interpretation of the results of our study may be related to the fact that the emotional state of our volunteers has not been controlled. In a recent study, Poppa et al. found that there is an increased anxiety state affecting the subjects submitted to TMS [233]. This can be a problem when evaluating autonomous nervous system responses, cardiovascular studies, and neuropsychological performance associated with TMS. In the future, study protocols directed to autonomic studies and rTMS should address and measure anxiety levels in volunteers, in order to try to eliminate this possible confounding factor. Although the volunteers had strict rules to follow prior to the study, trying to control eventual confounding factors, we must also admit that other factors like tiredness or the number of hours of sleep, could also have influenced our final results.

Since studies involving both oximetry and blood pressure in the field of transcranial magnetic stimulation have revealed some contradictory results and reviews have shown difficulty in highlighting the role that TMS may play in the study of cardiovascular diseases mediated by the autonomic nervous system and possible intervention in these patients [227,256], we think that our bi-hemispheric sham-controlled study brings new information that will help to understand TBS-related brain dynamics.

Conclusions

Our results suggest that inhibition of the left dorsolateral prefrontal cortex can originate a reduction in oximetry in both hemispheres, especially in the left, linked to a possible bilateral decreased cerebral blood flow, when subjects are at rest. We can also observe that right hemisphere inhibition appears associated with a modulation of some autonomic responses. TBS effects on oximetry and possibly on blood pressure may offer interesting clinical perspectives in patients, particularly with cerebrovascular diseases.

Chapter VI

Can theta burst stimulation safely influence auditory hearing thresholds in healthy young adults?

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Chapter VI

Can theta burst stimulation safely influence auditory hearing thresholds in healthy young adults?

Abstract

This TBS sham-controlled study aimed to evaluate the effects of intermittent TBS (iTBS) and continuous TBS (cTBS) over the ipsilateral hearing thresholds after stimulation over the left auditory cortex.

Sixty healthy adults, aged between 19 and 32 years (median of 23 years), were randomly distributed to three groups and underwent iTBS, cTBS or sham stimulation. Each double-blind experimental session comprised two pure tone audiometric evaluations per subject, before and after stimulation. To assess volunteer safety, a follow-up of at least 48 hours was implemented.

The iTBS group mean thresholds displayed a tendency to decrease after stimulation, predominantly in the 500Hz-6000Hz interval and group comparisons revealed significant differences between the iTBS and sham groups for 500Hz ($p=0.041$) and between the iTBS and cTBS groups for 4000Hz ($p=0.038$). No relevant side effects nor any significant hearing threshold impairment after active or sham stimulation were found.

A single stimulation session led to a favourable neuromodulation of the auditory cortex, translated in lower thresholds when using iTBS.

These encouraging results with this safe noninvasive tool suggest that iTBS may have the potential to positively influence hearing thresholds.

Keywords: Transcranial magnetic stimulation; theta burst stimulation; auditory threshold, audiometry; safety; auditory cortex

Introduction

Repetitive transcranial magnetic stimulation (rTMS) is a neuromodulation tool, capable of influencing neural networks through the application of repetitive and patterned stimuli [13,22]. It can be used in several clinical applications, and is a promising technique in the treatment of auditory related disorders such as tinnitus, auditory hallucinations, and hearing loss [19,81,257]. However, the noise levels achieved with the coils at higher intensities have the theoretical ability to impair hearing if long stimulation procedures are used [19,81,258]. Accordingly, the exposure to excessive noise during stimulation, with sound levels that can exceed the 120dB barrier, poses a health risk concerning possible sensorineural hearing loss, underlining the importance of using hearing protection [81]. So far, studies following safety guidelines suggest that this technique is relatively safe and well-tolerated [19]. Even in short duration sessions discomfort, minor hearing losses and hypersensitivity to noise have been described, but which rapidly disappear in most cases [19,22,81,127,259].

Theta burst stimulation (TBS) is an optimized rTMS paradigm, using significantly shorter duration sessions and lower stimulation intensities [6,45]. TBS paradigms may be capable of inducing more pronounced and enduring effects in cortical excitatory and inhibitory phenomena when compared to rTMS [6,260]. TBS benefits from shorter duration protocols (typically 40-190 seconds for TBS vs around 30 minutes for rTMS), achieving similar therapeutic effectiveness (namely in depression) [91], allowing for better time management in laboratory application [71,261,262]. These effects are attributed to changes in synaptic strength associated with long-term potentiation and long-term depression phenomena induced by a single TBS session [263,264]. However, the exact neural mechanism that underlies the auditory cortical modulation and the possible degree of cortical reorganisation remains unknown [127,265].

Hearing related disorders have been studied with rTMS/TBS and treatment protocols have been developed, especially in tinnitus, both in human and animal studies [22,127,266]. Interventions are based on the premise that the primary and secondary auditory cortices can be modulated and that the stimulation has the ability to promote cortical plasticity [22,127,266]. Auditory cortical stimulation must comply with this area´s anatomical specificities: the human ear is able to discern a spectrum of frequencies between 20Hz and 20000Hz and these are spread according to a tonotopic distribution, which in the primary auditory cortex (PAC) manifests identically in both hemispheres in the Heschl's gyrus, with bilateral ear representation, thus a unilateral

intervention may be able to modulate this frequency range, with results being dependent on correct PAC targeting [267–269]. Although promising, scientific evidence in this area requires a greater number of studies in rTMS and especially in TBS in order to ensure patient hearing safety, particularly relevant in the ear closer to the coil, and to identify the most effective protocols to effectively intervene.

With this TBS sham-controlled study in a group of healthy young adults, we aimed to assess ipsilateral hearing safety after TBS exposure over the left PAC and also to evaluate the effects of both iTBS and cTBS over the ipsilateral hearing thresholds.

Methods

Subjects and study design

Sixty healthy adults agreed to participate in this prospective double-blind sham-controlled study, recruited among students enrolled at the Faculty of Health Sciences, University of Beira Interior (FHS-UBI), Covilhã, Portugal. After answering a confidential screening questionnaire, students were included in the study if they met the inclusion and exclusion criteria, that were as follows - inclusion criteria: age between 18 and 35 years and had no hearing complaints; exclusion criteria: altered initial pure tone audiometry, previous ear diseases, tinnitus or other hearing related complains, brain injury or suspected diagnosis of organic brain damage, severe head trauma, epilepsy or convulsions, presence of major medical illness (including neuropsychiatric diseases), recent intake of any drugs or medication, pregnancy, implanted devices or foreign metal articles in the head or chest areas, sleep deprivation, alcoholism and history of drug abuse [19]. Participants were instructed to rest as usual, avoid being exposed to excessive noise and avoid taking alcoholic beverages or other toxic/stimulant substances 24 hours prior to the application of the technique.

Volunteers were randomly allocated to three equally sized separate groups according to stimulation type: the intermittent TBS group (iTBS group), the continuous TBS group (cTBS group), and sham group (placebo stimulation), with 20 volunteers per group. A sealed envelope randomization protocol was used.

Fully informed about all procedures, subjects signed a written informed consent and anonymity was ensured. Study protocols were approved by the Faculty of Health

Sciences-UBI Ethics Committee (CE-FCS-2011-001), in conformity with the Declaration of Helsinki.

Theta Burst Stimulation (TBS)

TMS protocols were performed in accordance with the safety and ethics recommendations of the 2009 guidelines [19], in the FHS-UBI TMS laboratory, using a MagVenture MagPro1G3 X100 5.0.1, with a Dantec™ Keypoint.net v.2.03 for motor threshold determination. All procedures were carried out under medical supervision. Using a butterfly coil MCF-B70, stimuli were applied accordingly to the classic TBS protocols, using biphasic pulses, with a total of 600 pulses sent in 3 pulse bursts, repeated at 5Hz, either in iTBS or cTBS. In continuous mode, bursts occurred without interruption for 40 seconds, and in the intermittent mode bursts were delivered only for 2 seconds (sets of 10 bursts), repeated every 10 seconds for a total of 190 seconds [45]. Coil handle was positioned parallel to the midline [270].

Stimulation target site was the left primary auditory cortex and it was found for each individual: in order to set the stimulation coil over the left PAC, a procedure based on the 10/20 international system for electrode placement it was used to find that specific site. Starting from the T3 position, we measured 2,5 cm towards Cz (following the coronal plane) and then measured 1,5 cm posteriorly, perpendicular to the plane T3-Cz [271–274]. The primary motor cortex (PMC) was used as a marker for stimulus intensity and was identified by the single pulse vs visible thumb-twitch relation. Active motor threshold (AMT) was defined as the lowest stimulation intensity over the left PMC capable of a consistent contralateral abductor pollicis brevis (APB) motor evoked potential (150–200μV), while maintaining minimal voluntary contraction, on more than half of the pulses applied [36,45,184]. For real stimulation over the left PAC, intensity was defined as 80% AMT. For sham stimulation, the same coil was used, maintaining scalp contact, using a 90-degree tilted position (magnetic field pointing downwards), also emitting sound of randomly cTBS or iTBS, simulating actual stimulation, even though this technique is not capable of effective neuronal activation [19,36,184]. Subjects were instructed to use disposable earplugs (Ohropax® Germany - noise reduction rating of 22-27 dB; 125-8000Hz) during active or sham stimulation. All volunteers were relaxingly seating in a comfortable reclining armchair during active or sham stimulation and stimuli application was always performed by the same technician.

Threshold Audiometry

Audiological measurements were implemented in a noise isolated room. Standard pure tone audiometry was executed using a calibrated clinical screening audiometer - MAICO Audiometer GmbH®, ST 20 model (steps of 10dB) – evaluating the following frequencies: 250Hz, 500Hz, 1000Hz, 2000Hz, 3000Hz, 4000Hz, 6000Hz and 8000Hz. Audiometry protocol followed the Guidelines for Manual Pure-Tone Threshold Audiometry from the American Speech-Hearing-Language Association (ASHA) [275].

Experimental design

Preceding every volunteer participation, procedure explanation was presented, and informed consent was obtained, followed by the audiological evaluations and TMS stimulation. Therefore, each experimental session comprised two audiometric evaluations of the left ear per subject (stimulation ipsilateral ear), before and after real or sham stimulation: a pre-TBS audiometry and a post-TBS audiometry. TBS or sham protocols were applied immediately after the first audiometry, following the protocol mentioned earlier (AMT determination and PAC stimulation). In order to standardise procedures and minimise changes in immediate TBS sound impact, the second audiometry always occurred 5 minutes after the end of the TBS/Sham stimulation. All experimental sessions took place at the same time of the day and each volunteer was subjected to only one session of real or sham TBS over the left PAC, according to his/her previous randomised group allocation. Both volunteer and team member that performed the audiometry were blind to the stimulation type used (cTBS, iTBS or Sham). Audiometries and the stimulation sessions were always performed in two completely separate rooms, and only the team member in charge of performing the TBS/Sham session was aware of the actual stimulation type (sham, iTBS or cTBS) applied to each volunteer. The researcher responsible for the audiometry had no information about what type of stimulation was performed. None of the volunteers had been previously submitted to rTMS/TBS and was not aware of the stimulation type performed, thus contributing to the blinding method success.

In order to assess volunteer safety and control eventual side effects, a follow-up of at least 48 hours was implemented (focusing on self-reported unwanted effects).

Statistical analysis

Statistical analysis was performed with IBM® SPSS Statistics® 25.0, using a mixed-design repeated measures ANOVA. ANOVA assumptions were verified using the Shapiro-Wilk normality test and the Levene test, allowing the latter to evaluate homogeneity variance. Due to sample size and the fact that the normality assumption was not validated, analysis was also performed with a non-parametric version of repeated measures ANOVA (Nonparametric Longitudinal Data in Factorial Experiments, with the "nparLD" package, version 2.1, for the statistical program R). However, since the results obtained through the two analyses were similar and compatible, we opted to present only the results obtained by the parametric version which can be interpreted more easily. Only one repeated measures ANOVA with a single repetition (post) and one factor (group) was used. There is no random effect other than that of the volunteers. For the comparisons between the intensity average pairs of the iTBS, cTBS and Sham groups or for the pre and post-TBS, the Sidak test (Student's t-test for independent samples or paired – Least Significant Difference-Sidak's correction) was used for each group. Hypotheses tests were considered significant when test value (p-value, p) did not exceed the significance level of 5% ($p<0.05$).

Results

Among the 60 volunteers who agreed to participate in this study, with a median age of 23 years, 44 (73.3%) were females aged 19 to 28 years and males between 21 and 32 years. Sex age distribution showed no significant differences (Mann-Whitney U, $p=0.773$), with a median age of 23 years in both sexes and similar averages of 23.10 years (SD=1.96 years) and 23.87 years (SD=2.85 years) for the female and male sex, respectively.

The iTBS group consisted of 14 females and 6 males; in the other two groups, distribution consisted of 15 females and 5 males. Age distribution of the three groups were not significantly different (Kruskal-Wallis, $p=0.273$), the age medians in the three groups were 23 years and averages were approximately 23.30 years (SD=0.73 years), 23.85 years (SD=3.03 years) and 22.65 years (SD=2.23 years) in the iTBS, cTBS and Sham groups, respectively.

Due to the reduced number of male volunteers in each group, it was decided not to consider the influence of sex on the variables related to the audiometry.

During the stimulation procedure, no incidents occurred and in the 48 hour follow-up, only two volunteers reported mild focal discomfort related to the cTBS stimulation site and one volunteer submitted to iTBS mentioned a mild headache. No other major adverse events were reported and none of the volunteers dropped out.

Pre-TBS audiometry and post-TBS audiometry mean threshold intensities per stimulation group are shown in Figure 6.1.

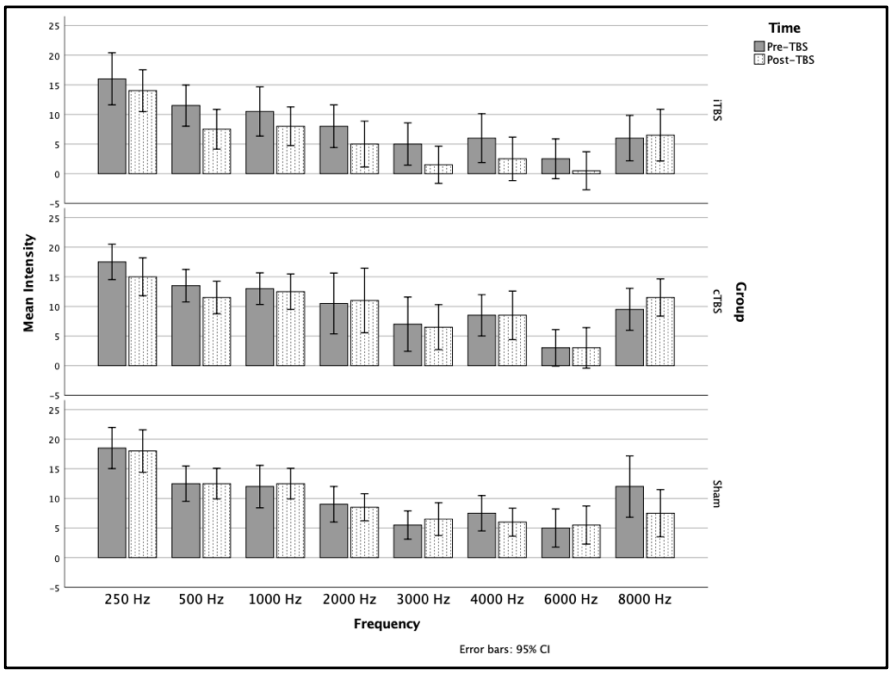

Figure 6.1 – Audiometry mean threshold intensities (dB HL) per frequency (Hz) and stimulation group.

Regardless of the group, the mean auditory thresholds of all the volunteers evaluated for the range of frequencies tested revealed that the highest threshold was found at 250Hz (17,33+/-7,78dB) and the best (lowest) thresholds were found between 2000Hz (9,17+/-9,49dB) and 6000Hz (3,50+/-6,84dB), as previously described in other studies [276]. Fig. 1 also seems to display that group behaviour was not similar, with the iTBS group results displaying a tendency for threshold decrease after stimulation, mainly for the 500Hz-6000Hz interval (mean difference between -2 and -4dB), with an exception for the 8000Hz, in which there was a slight increase (+0.5dB). The cTBS group showed

mixed results, with slight threshold increases in the 2000Hz and 8000Hz (mean difference between +0.5 and +2dB), slight decreases after stimulation in the 250Hz, 500Hz, 1000Hz and 3000Hz (mean difference between -0.5 and -2.5dB), and unaltered thresholds in the 4000Hz and 6000Hz. We can also observe that the sham group did not show a clear tendency, with variations between 0.5 and 1.5dB, except for the 8000Hz, in which there was a decrease (-4.5dB). Moreover, it should be noted that when an increased threshold occurred after active TBS or sham, these variations were of small degree.

Table 6.1 presents stimulation effect and interaction versus group type (repeated measures ANOVA) and Table 6.2 the pre-TBS audiometry and post-TBS audiometry mean difference for each group (iTBS, cTBS, and sham).

Table 6.1
Stimulation effect and interaction versus group type - repeated measures ANOVA.

Frequency (Hz)	Pre vs Post TBS *p-value*	Between Groups effect *p-value*	Interaction Stimulation-Group *p-value*
250	**0,025**	0.349	0,506
500	**0,001**	0.197	**0,028**
1000	0,192	0.193	0,152
2000	0,098	0.280	0,055
3000	0,140	0.248	**0,026**
4000	**0,015**	0.169	0,107
6000	0,470	0.187	0,298
8000	0,489	0.197	**0,019**

Table 6.2

Audiometry results: pre-TBS vs post-TBS mean difference for each group.

	iTBS Group		cTBS Group		Sham Group	
Frequency (Hz)	Intensity: mean diff. pre-post (dB HL)	p-value[1]	Intensity: mean diff. pre-post (dB HL)	p-value[1]	Intensity: mean diff. pre-post (dB HL)	p-value[1]
250	2.0	0.116	2.5	0.051	0.5	0.691
500	4.0	**<0.001**	2.0	0.056	0.0	1.000
1000	2.5	**0.026**	0.5	0.649	-0.5	0.649
2000	3.0	**0.005**	-0.5	0.629	0.5	0.629
3000	3.5	**0.004**	0.5	0.668	-1.0	0.392
4000	3.5	**0.004**	0.0	1.000	1.5	0.198
6000	2.0	0.098	0.0	1.000	-0.5	0.676
8000	-0.5	0.763	-2.0	0.231	4.5	**0.009**

[1] *LSD test with Sidak's correction*

Pre-post iTBS group threshold mean difference showed statistically significant differences in frequencies between 500Hz and 4000Hz (500Hz $p<0.001$; 1000Hz $p=0.026$; 2000Hz $p=0.005$; 3000Hz $p=0.004$; 4000Hz $p=0.004$), with lower thresholds after stimulation. No significant differences were found in cTBS group threshold mean differences. Sham group results showed also no statistically significant differences between 250Hz and 6000Hz. However, results in the 8000Hz of the sham group revealed a significant difference ($p=0.009$).

Group comparisons between the pre-TBS audiometry and post-TBS audiometry mean differences are shown in Table 6.3.

Table 6.3
Group comparisons: mean differences between the pre-TBS audiometry and post-TBS audiometries

Frequency (Hz)	Intensity: mean diff. pre-post **cTBS-iTBS**	p-value[1]	Intensity: mean diff. pre-post **cTBS-Sham**	p-value[1]	Intensity: mean diff. pre-post **iTBS-Sham**	p-value[1]
250 [i]	1.5	0.908	-1.0	0.970	-2.5	0.683
250 [f]	1.0	0.964	-3.0	0.495	-4.0	0.251
500 [i]	2.0	0.715	1.0	0.951	-1.0	0.951
500 [f]	4.0	0.134	-1.0	0.942	-5.0	**0.041**
1000 [i]	2.5	0.655	1.0	0.966	-1.5	0.897
1000 [f]	4.5	0.082	0.0	1.000	-4.5	0.082
2000 [i]	2.5	0.739	1.5	0.927	-1.0	0.977
2000 [f]	6.0	0.098	2.5	0.748	-3.5	0.506
3000 [i]	2.0	0.802	1.5	0.904	-0.5	0.996
3000 [f]	5.0	0.079	0.0	1.000	-5.0	0.079
4000 [i]	2.5	0.663	1.0	0.967	-1.5	0.900
4000 [f]	6.0	**0.038**	2.5	0.641	-3.5	0.363
6000 [i]	0.5	0.994	-2.0	0.740	-2.5	0.587
6000 [f]	2.5	0.603	-2.5	0.603	-5.0	0.082
8000 [i]	3.5	0.539	-2.5	0.770	-6.0	0.118
8000 [f]	5.0	0.171	4.0	0.344	-1.0	0.974

[i] *pre-stimulation;* [f] *post-stimulation*
[1] *LSD test with Sidak's correction*

Baseline audiometry records showed no statistically significant differences between the iTBS, cTBS and sham groups, in any of the frequencies evaluated, thereby revealing no inconsistencies between groups at baseline.

On the other hand, post-stimulation results revealed statistically significant mean differences between the iTBS and sham groups for 500Hz (p=0.041) and also between the cTBS and iTBS groups for 4000Hz (p=0.038).

As can be seen in Table 6.3, reinforced by Figure 1, none of the stimulated groups had a significant mean threshold worsening, after active or sham stimulation, supporting that the technique is safe to use, as long as you use adequate protection.

Discussion

Our study, using a sham-controlled protocol, revealed no relevant side effects nor any significant hearing threshold impairment of the ipsilateral ear after iTBS, cTBS or sham stimulation over the PAC, contributing to better understand possible safety limitations in these protocols. Further analysis revealed that iTBS seems to have a greater capacity to influence hearing thresholds when compared to cTBS and sham stimulation, resulting in lower thresholds after stimulation between 500Hz and 4000Hz. Direct group comparison showed significantly lower thresholds at 500Hz after iTBS compared to Sham and at 4000Hz also after iTBS compared to cTBS stimulation. Our data suggest that this specific TBS method can be a safe approach to influence hearing sensitivity through non-invasive neurostimulation.

One of our main objectives was to assess hearing safety of the ipsilateral ear after exposure to one session of TBS over the left primary auditory cortex. Even though rTMS and TBS stimulation may involve some health side effects, they are considered safe techniques, and major risks when applying these techniques following accepted safety protocols are negligible, both in children and adults [19,46,277]. Special attention should be taken when undergoing PAC stimulation because secondary effects can occur both from the neural stimulation and from the noise related to rTMS at higher intensities [19]. Whereas higher stimulation can achieve the 120dB SPL threshold, thereby risking exposure to excessive noise and possible sensorineural hearing loss, usually the sound levels do not go beyond 60-70dB SPL [81]. While some studies found hearing impairment related to cochlear effects [278], some tinnitus patients reported worsening of hyperacusis after rTMS [19], or headaches, tinnitus worsening and increased sensitivity to noise after TBS [125], other studies did not report any hearing decline or significant complaint after 20 sessions of TBS stimulation [81]. The temporal cortex is not a frequent location to apply TBS, but mixed results concerning secondary effects after few stimulation sessions can be found. Poreiz et al. used a 3 session TBS (iTBS+cTBS+imTBS) protocol in tinnitus patients and reported complaints of discomfort, headaches and 3 patients suffered a worsening in tinnitus-related complaints [279]. On the other hand, De Ridder et al. applied one session of modified cTBS in 46 tinnitus patients and reported no significant secondary effects [128]. Our results revealed no significant global threshold increase after either iTBS, cTBS or sham stimulation for all the frequencies tested, nor other relevant side effects (such as tinnitus or perceived hearing loss). These results are particularly relevant since our objective was to test the ipsilateral ear immediately after stimulation, in order to evaluate the ear

closest to the stimulation coil and more likely to reveal any changes linked to excessive noise from the coil. The fact that there was no threshold worsening, none of the volunteers mentioned any hearing related complaints and none of the volunteers dropped out, suggests that TBS stimulation over PAC can be a safe procedure if the safety guidelines are followed. Furthermore, we observed an encouraging tendency to a threshold reduction in some frequencies with iTBS. In terms of side effects, they can be considered negligible [19], because our volunteers described only two cases of mild focal discomfort and one case of mild headache related to active stimulation. Our data clearly support the scarce information to date that TBS can be a safe technique when applied to PAC, suggesting good auditory tolerance.

The other main objective focused upon the study of the auditory effects of both iTBS and cTBS in ipsilateral hearing thresholds using a placebo-controlled protocol, after stimulating the left PAC. Regarding auditory information processing, evaluated by positron emission tomography or functional magnetic resonance imaging, it is important to mention the apparent existence of a left hemisphere dominance, either at rest [280] or after auditory stimulation of only one (pure-tone or speech) [281] or both ears [282]. Thus, we opted to primarily stimulate the left cortex because it seemed to be the hemisphere in which we would probably have the higher chance of influencing hearing capabilities, either in terms of improving the hearing thresholds, or in terms of inducing a negative change related to the stimulation procedure. TMS modulatory capacity has been tested and proven in auditory related research. Inhibitory properties using the temporal or temporoparietal cortices were found on studies using schizophrenic patients with auditory hallucinations (reduction) and especially studying patients with tinnitus stimulating the hypermetabolic areas (PAC stimulation for tinnitus reduction) [271]. TBS experiments, though scant and mostly using cTBS protocols, have been used to treat or improve tinnitus symptoms, namely hearing thresholds, but the results have been controversial. Positive results using one stimulation session over the auditory cortex [128] and 20 sessions over the auditory temporoparietal cortex [129] revealed improved tinnitus symptoms. In contrast, Poreisz et al. and Plewnia et al. showed no significant positive results stimulating the temporoparietal cortex, but concluded that the protocols used were safe [125,279]. Disparities between stimulation protocols and study designs can explain these results as some studies did not use placebo/sham-controlled designs and used diverse protocols – different session numbers, different stimulation locations and slight differences in the used intensities (De Ridder et al. 2007; Poreisz et al. 2009; Soekadar et al. 2009; Plewnia et al. 2012). Evaluation of our results revealed a statistically significant reduction in mean hearing thresholds between the 500Hz and

4000Hz interval for the iTBS group after stimulation. However, for the 250Hz, 6000Hz and 8000Hz frequencies, there was no significant change in the thresholds. Since we are working with groups with a relatively small number of volunteers per group and our audiometer only operates in steps of 10dB, these results should be evaluated against direct comparison with the cTBS and Sham groups. Group comparison using the Sidak test revealed significantly lower thresholds only at 500Hz ($p=0.041$) comparing iTBS vs Sham groups (mean threshold in the Sham group remained stable and the iTBS threshold significantly decreased) and at 4000Hz ($p=0.038$) when comparing iTBS vs cTBS groups (mean threshold in the cTBS group remained unaltered and iTBS threshold significantly diminished). In addition to accentuating the safety of the technique, these results suggest that the iTBS may be able to influence auditory capability, specifically decreasing auditory thresholds in some frequencies. Cortical modulation process and respective synaptic hearing circuits are still to be clarified in their totality; however, our results can be understood based on the probable neuron modulation in distinct zones of the tonotopic map of the primary auditory cortex. The observed significant changes, since they are located at different frequencies but mostly between 500Hz and 6000Hz, are also supported by the hypothesis of stimulated neurons integrating different sound frequencies among themselves. In the PAC, tonotopic organisation manifests itself equally in both hemispheres, in the form of two gradients in Heschl's gyrus, which make up a pattern of high, low and high frequencies again [268,269]. Even if the stimulation coil has a slight target area deviation, it is believed that this can be within an acceptable margin of error because it is known that figure 8 coils can produce a magnetic field directly over an area extending around 3cm of length and 2cm of width [271]. Thus, we can consider that even if there are some deviations, stimulation will still be performed in PAC but maybe more focused on low to mid frequency areas, thereby justifying more effective results in some frequencies to the detriment of others. Perhaps results would be more homogeneous if a neuronavigation system was be used to identify the target zone, thus promoting a more accurate stimulation of the PAC. Other limitation when analysing these results is that it is still unclear what are the underlying mechanisms mediating potential iTBS benefits, as they can be explained by more than one hypothesis. A possible hypothesis for the results is the cortical plasticity aptitude: functional magnetic resonance imaging (fMRI) studies have demonstrated that the auditory cortex has the capacity to reorganise and change its expression of excitatory and inhibitory neurotransmitters [127]. Neurotransmitter modulation by transcranial magnetic stimulation is an known outcome, namely in the upregulation (increased levels) of the excitatory neurotransmitter glutamate related to excitatory stimulation [38,56,57], or in the left hemisphere down-regulation in glutamate, directly linked to a reduced loudness

level of tinnitus awareness, found by Cacace et al. using a 5 day inhibitory protocol over the left auditory cortex [264]. Even though we cannot confirm these theories, we believe they can explain some of our findings. Our findings also support some previous studies reporting that excitatory stimulation can influence hearing, such as the work of Andoh et al. in which 10Hz rTMS applied to the Heschl's gyrus originated improved auditory performance, but only in females [258].

Results for the cTBS group show that for all frequencies evaluated, no significant statistical change ($p>0.05$) was found in hearing thresholds after stimulation. This lack of significant results continues when we compare the mean differences of the cTBS group with the iTBS and Sham groups, highlighting the frequent variations in the thresholds, either decreasing or either rising. These results suggest that a single session of the cTBS protocol used in this investigation has no ability to significantly modulate neuronal auditory activity, emphasizing that effects and possible efficacy of iTBS and cTBS techniques may be distinct, at least when applied over the auditory cortex. The result for 8000Hz obtained from the group undergoing sham stimulation, fails to reach statistical significance when compared to the results of the excitatory and inhibitory groups - thus, its significance may therefore be negligible. The reasons that led to a threshold decrease for 8000Hz in the sham group may only be speculated, but a placebo effect is admitted as a possibility, mainly because it is a very high frequency in the auditory spectrum, therefore more difficult to assess. Several studies [283–285] support the multifactorial nature inherent in the mechanisms underlying the placebo effect; however, in this specific case, it is possible that the main mechanism can be related to an intrinsic expectation that the volunteers had in relation to this study in order that TBS could improve their hearing capabilities.

Future work should focus upon the study of not only the ipsilateral thresholds but also the contralateral ones. Ipsilateral vs contralateral dominance and which side can be the most effective in the auditory cortex stimulation can be a controversial issue because there are several contradictory studies. For instance, some studies in tinnitus gave stimulation primacy to the left cortex independently of the complaints being lateralized to the right, left or bilateral, others reported better results stimulating the contralateral cortex related to the existing complaints [127,270,286]. Another limitation to our rationale is that we don't know for how long the iTBS effects last. It would still be interesting to study whether two daily sessions, separated by at least 15 minutes [263], could have an enhanced or more prolonged effect. It should also be mentioned that despite some studies are increasing stimulus intensity, our objective was to comply with

the standard safety guidelines, even more so that stimulation outside the primary motor area may incur some inaccuracies related to distance-adjusted intensities [29].

As for study design, despite all participants officially declared that they did not take any drugs, we were not able to include in our protocols a screening test to evaluate their presence, thus limiting our control over this experiment. Even though our design was simple and all our subjects were completely naïve regarding stimulus characteristics and effects, we should also have included a blinding assessment in order to increase result reliability.

In this area, very few studies used TBS, and most studies used the technique mainly in patients, neglecting its effects in normal healthy volunteers. No study focused on the use of both iTBS and cTBS, comparing the results with sham stimulation. Our study protocol is therefore unique and to the authors' knowledge this is the first approach to a healthy volunteer placebo-controlled research using both cTBS and iTBS over the left PAC, showing diverse effects between these two stimulation modalities, thus contributing to a better understanding of this type of noninvasive neurostimulation over the auditory cortex. Our results using only a single session point to a favourable neuromodulation of the PAC, translated in lower thresholds when using iTBS. It can be assumed that several sessions could be more effective, as most of the protocols that formed the basis of the previous studies mentioned applied several sessions (between 3 to 20 sessions) [81,125,127,264,286–289], similarly as is currently used in depression therapy. It is also noteworthy that threshold improvement occurred manly around the human speech/voice frequency range (500-2000Hz) [290,291]. This possible hearing improvement in the low to mid frequency range can be particularly important if similar stimulation protocols can be used in sensorineural hearing loss, often attributed to hereditary factors and congenital conditions [292], specifically if trying to enhance patient speech perception. These interventions may aim to improve life quality in patients, however, this possible use for TBS should be approached carefully, after reproduction of this method in further studies with a large number of healthy subjects and, finally, after patient investigation.

Conclusions

These are encouraging results about the use of this safe noninvasive neuromodulation tool, suggesting that iTBS has the potential to positively influence hearing thresholds in healthy young adults. We believe that the same iTBS protocols could be reproduced in older adults with minor sensorineural hearing loss, presbycusis or other hearing loss cases, trying to improve patients hearing capacity by modulating PAC to become more sensitive to the auditory stimuli and thus helping patients to improve their auditory assessment of the world and to increase patients' life quality.

Chapter VII

Method for repetitive transcranial magnetic stimulation on Creativity Patent n. º 109800

The patent discussed in this chapter, made official in May 2020, concerns a method to improve the creative process in normal subjects using non-invasive brain stimulation, specifically with theta burst stimulation.

Chapter VII

Method for repetitive transcranial magnetic stimulation on Creativity

Patent n. º 109800

Rationale

Defining creativity is a complex process as it concerns all domains of brain activity. It is also challenging due to the difficulty of materializing a single definition of creativity [293,294]. One definition can be expressed as the appearance or presentation of anything innovative and new, positive for society, which goes beyond the familiar and socially accepted. Most authors consider essential in the creative process characteristics such as originality, functionality, and the surprise element [293–295].

Knowledge about the neurologic mechanisms underlying human creativity is scant [296]. Neuroimaging studies have not yet been able to clarify the exact brain network processing in creativity. So far, it is believed that it does not depend on a specific brain location, being apparently dependent on a process of bi-hemispheric interaction, resultant from the action of the corpus callosum and its bridging effect between the right and left hemispheres [293,297]. Although some authors propose that human creativity arises from the different combinations of previously established neural patterns forming new and useful ones [298], a more mainstream line of thought defends that a more creative brain is one in that the right hemisphere is not very inhibited by the left counterpart [299]. Macroscopic approaches to creativity have focused on specific hemispheric locations and hemispheric asymmetry. In this context, Bogen and Bogen (1969) suggested that one of the main obstacles to a high level of creativity would be the left hemisphere's inhibition of the functions of the right hemisphere [296]. This theory can be supported by several studies, e.g. Martindale (1999) claims that creative people present higher activity in the right than in the left hemisphere (parieto-temporal EEG), contrary to low creative people on a creative task [300]. As an example, Einstein's enhanced spatial imagination (right hemisphere dependent) could be explained by the

documented abnormal development of his left hemisphere (lower neuron ratio compared to supporting glial cells) [299]. Also, other brain changes associated with predominant lesions in the left hemisphere, such as dysfunction of the frontotemporal area, seem to increase figurative creativity [301–303], suggesting the existence of a competition effect between both hemispheres. Finally, this concept is also supported by imaging studies carried out in normal volunteers [304].

The specific location of the brain areas involved in the creative process has thus been the object of study by several groups over the years. The prefrontal cortex can be an essential area, as it is known that creativity involves multiple cognitive abilities (e.g. working memory, attention, and cognitive flexibility) associated with the prefrontal cortex functions [296]. Evidence from cognitive neuroscience, using functional imaging studies and electroencephalography, support the notion that the prefrontal cortex is a key area in the cognitive functions essential in creative thinking [102,297]. It is also known that focal compromise of these areas originates impairments in creativity tasks [102,297], as reported by de Souza et al., finding impaired results in all dimensions of the Torrance tests for creative thinking (TTCT) in patients with a frontal variant of a frontotemporal lobar degenerative disease [305], or by Shamay-Tsoory et al. that described diminished levels of originality, using not only the TTCT but also the Alternate uses task (AUT), in patients with lesions in the prefrontal cortex [302].

Signs of higher creativity existent in patients with frontal lobe dysfunction, as seen in frontal lobe dementia [306], suggest a significant role of the frontal lobe in the creative process. The concepts underlying divergent thinking, which forms the basis of the creative process, also appear to be located in the frontal lobe [293] although the parietal lobes and other cortical and subcortical brain regions may also play a role in creativity [296]. It is important to note that some authors suggest that the creative impulse is controlled through interactions between the frontal and temporal lobes and the limbic system [296]. In particular, the creative impulse also increases when there are dysfunctions of the temporal lobe and when the dopaminergic tone increases, while the creative block increases with a reduction in the dopaminergic tone. These aspects suggest also a clear functional role for neurotransmitters [293].

One of the most important neurotransmitters is dopamine. Dopamine is capable of inducing a low degree of latent inhibition (capacity to screen and ignore irrelevant stimuli) [296,307], and low latent inhibition together with higher intelligence has been shown to be a characteristic of creative individuals [296]. Additionally, it can also be important in the creative discovery, through its effects on the ability to search for

novelties, increasing the creative impulse [296]. Studies by Lhommée et al. and Inzelberg et al. showed that enhanced creative thinking may be found in Parkinson's disease patients who were submitted to a dopamine agonist treatment [308,309].

As mentioned in the previous chapters of this thesis, TMS has the ability to influence and modify neural networks, with both acute and chronic capacities, also assuming the ability to influence dopaminergic networks. Thus, TMS can undertake a pivotal role in the study of the processes associated with creativity, both in the functional assessment of the cortical areas that are most associated with the creative process, as well as in the potential for the facilitation of the creative process. To the author's knowledge, no relevant publications studying theta burst stimulation and creativity existed by the time we conducted our investigation.

If the origin and definition of creativity are complex, its assessment is also not simple, although there are already validated instruments that allow the study and evaluation of the process. The Torrance tests for creative thinking (TTCT) can be used to test creativity [310]. These tests can be used to test healthy subjects but are also able to detect creative deterioration in patients with prefrontal cortex degeneration, revealing a decrease in the TTCT creativity index scores [311]. TTCT are the most used tests in this field and are validated for the Portuguese population. TTCT administration is simple to apply and they do not require much time to administrate [306,310,312]. The system for assessing the creativity of Torrance tests depends on [306,310,313]:

a) Fluency: the number of relevant answers to questions that test the ability to produce and consider many alternatives

b) Flexibility: the total number of categories covered by the responses is compared with a table, in order to assess the ability to produce responses with a broader perspective

c) Originality: number of less frequent ideas (statistically speaking) showing the ability to produce ideas that differ from others, with an evaluation grid that classifies the most common ideas as 0 points, and the others with 1 point according to a list originality prepared by the evaluator

d) Elaboration: ability to produce ideas in detail

For the study we carried out under this patent, only fluency was assessed, and the other criteria will not be approached.

The TTCT are made up of Verbal and Figurative versions [312]. The verbal component consists of six different activities in which the person presents possible scenarios in the face of a design, reinvents existing products, or predicts the consequences of an unlikely situation. The Figurative component has three activities that aim to transform geometric figures into products or situations through their use and combination. After applying the tests, the creativity index is calculated, which is based on five quantified variables [312]. The methodology used in our work resorted to a simplified adaptation of the TTCT, in order to carry out a simpler but also faster assessment, since we did not know the duration of the TBS after-effects. Thus, we maintained the binomial Verbal and Figurative relation in the pre-post stimulation assessment. The protocol understood the application of a verbal test, where the volunteer was confronted with an improbable situation, being asked to formulate the maximum possible creative outcomes for the situation in question.

The figurative tests included: a) the construction of novel pictures and forms from a pattern diamond shape; b) a sequence of tasks - Use, Create and Complete: 1) use a circle shape as the base of a new drawing, 2) create a novelle idea incorporating a selection of shapes, and finally 3) complete an incomplete drawing (incomplete square and a small circle). Full evaluation totalized 9 minutes.

The importance of creativity in enhancing human activities and wellbeing is widely recognized, allowing for social, artistic, and economic development [314,315]. With these premises in mind, the patented technique aimed to stimulate creativity by exciting neurons in the left prefrontal cortex and associated neural networks by the use of a specific repetitive transcranial magnetic stimulation (rTMS) – the intermittent Theta Burst Stimulation (iTBS). We aimed to demonstrate that with the specific parameters, methodology, and cortical area used, it is possible to improve a particular capacity of the human complex cognitive abilities – creativity or improve/influence the creative process.

Patent description

The process of neuronal modulation in order to promote creative thinking is based on the use of theta burst stimulation (TBS). Methodology implies the use of a butterfly coil, using biphasic pulses, oriented in order to induce postero-anterior activation. In our case, a MagVenture MCF-B70 coil was used, without the need for cooling, connected to

a MagVenture MagPro® G3 X100 magnetic stimulator, capable of performing both "classical" repetitive magnetic stimulation and Theta Burst Stimulation (TBS).

The definition of the specific stimulation site over the prefrontal cortex was preceded by the definition of the intensity to be used, through the stimulation of the primary motor area (PMC). Pulses applied over PMC, induce an action potential that propagates through the corticospinal tract reaching a target muscle, causing the activation of the muscle fibres – the motor evoked potential. To detect the hotspot (cortical area that corresponds to the maximal muscle response when stimulated), the integrity of the short abductor muscle of the left thumb is initially analysed, followed by the placement of the coil over the right PMC, using for initial anatomical reference the C4 electrode location of the 10/20 EEG international system. Then, pulses are applied at lower levels of intensity until the active motor threshold (AMT) is reached - minimum intensity with which it is possible to obtain a minimum muscle response in 50% of the pulses sent, with the muscle in tonic contraction. After determining the threshold intensity, the coil is moved 5 centimetres in the anterior direction of the head, placing the centre of the coil on the right dorsolateral prefrontal cortex. With the coil fixed in this position, an intensity of 80% of the AMT will be used.

The stimulation technique used is the Theta Burst Stimulation, in its intermittent form (iTBS), in which grouped series of three pulses at 50Hz, are applied repeatedly in 200ms (5Hz) intervals, in 2-second bursts, alternating with intervals 8 seconds without effective stimulation, totalling 600 pulses.

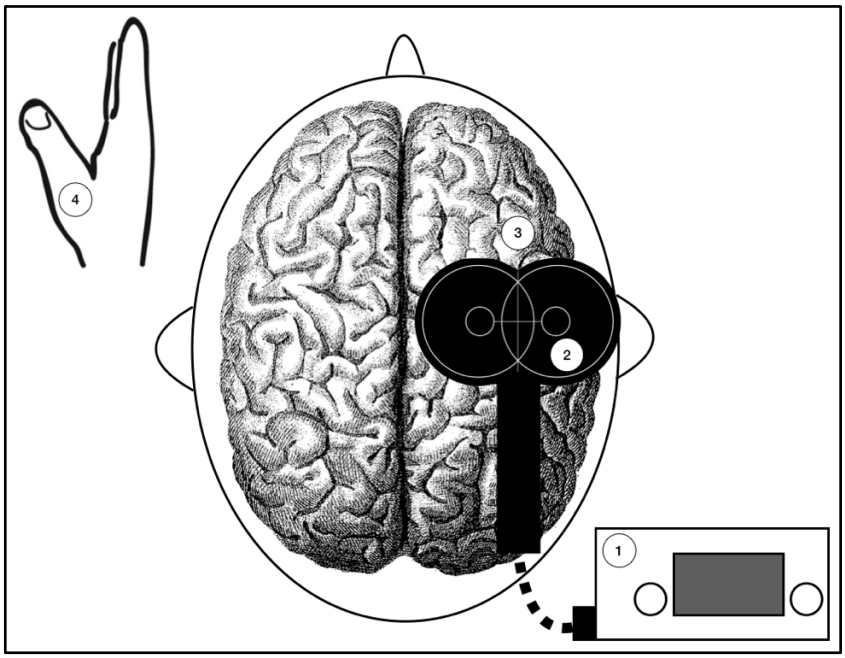

Figure 7.1 – Schematic representation of the primary phase of the process described in the patent, where the magnetic stimulation equipment (1), the butterfly coil on the C4 position and / or hotspot of the right primary motor area (2) is shown, located anatomically posterior to the right dorsolateral prefrontal cortex (3), whose stimulation will allow the contraction of the left thumb by activating the short abductor of the thumb (4).

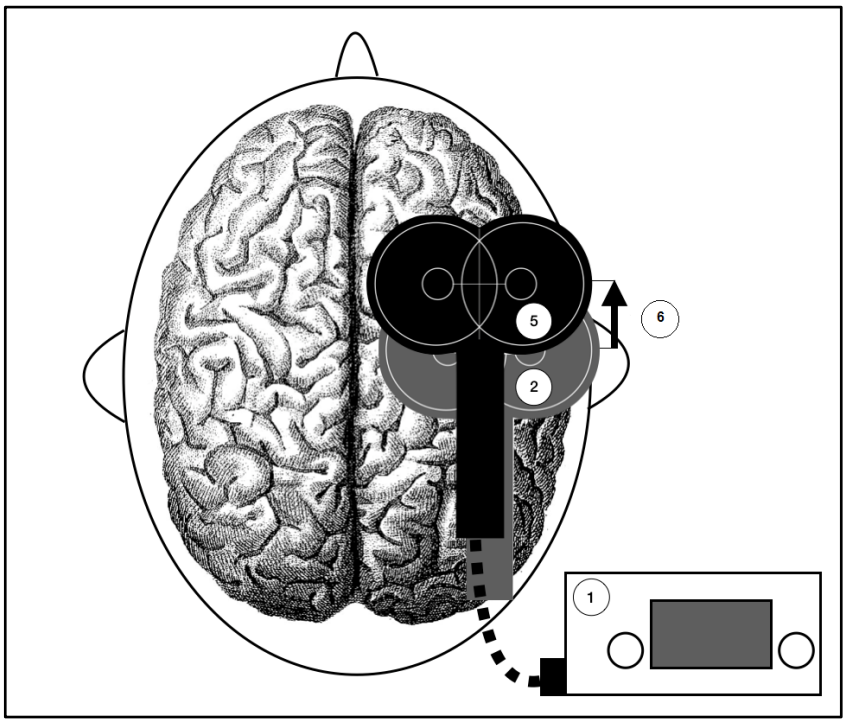

Figure 7.2 – Schematic representation of the secondary phase of the process described in the patent, where the repositioning of the coil is presented from its initial position (2) to its final position (5), located 5 centimetres anterior to the hotspot (6), where the 600 stimuli of the iTBS protocol will be applied.

The methodology patented comprehends the following steps:

a) stimulation is performed using the intermittent theta burst (iTBS) technique [45] using a butterfly-shaped coil connected to a MagVenture MagPro® G3 X100 5.0.1 system.

(b) detection of the hotspot in the primary motor area of the right hemisphere;

(c) identification of the active motor threshold by activating the left thumb;

(d) iTBS stimulation is applied 5 cm anteriorly to the motor hotspot, over the right prefrontal dorsolateral cortex, using 80% of the active motor threshold, with the coil handle parallel to the midline.

(e) apply iTBS through bursts of 3 pulses at 50Hz, repeatedly applied at 200 ms intervals, for 2 seconds, followed by 8 seconds without stimulation, reaching a total of 600 pulses [45].

The present method was developed and tested using 24 volunteers (medical students over 18 years old), divided into two smaller groups: the stimulated group and the Sham group, stimulated with iTBS or with sham stimulation respectively. The iTBS group was composed of 12 volunteers (six men and six women). The placebo group consisted of 12 volunteers (five men and seven women). Both groups were tested before and after stimulation using selected adapted parts of the Torrance Test of Creative Thinking [310,313] in a double-blind trial. Results revealed that both originality and divergent thinking fluency significantly improved in the stimulated group (iTBS) when compared to the placebo group, suggesting improved functional changes in the right DLPFC due to a possibly excitatory local effect form the iTBS, capable of influencing neuronal networks involved in the creative process [45,296].

The current patented methodology also aims to protect the method of stimulation on the left hemisphere using inhibitory stimulation through the use of a continuous form of TBS (cTBS). We also argue that, so that the effect can be more effective, the method may also require the daily repetition of the stimulation process, between 10 to 20 sessions.

This patent was submitted using preliminary data from our early work in this field, dating to December of 2016. According to the rules instituted for patent registry, the results of the studies should not be published in advance, hence the non-presentation of more concrete results. Over the time since the initial submission, the extension of our

initial study took place, with the stimulation of the contralateral hemisphere and with other variations in stimulation. We are currently starting a new phase of this study and we expect to have more data at the end of this year, then moving on to the full publication of the results.

Chapter VIII

Summarising discussion & conclusions

Summary of the findings in this thesis

Future perspectives

Concluding remarks

Chapter VIII

Summarising discussion & conclusions

Summary of the findings in this thesis

In this chapter, the results from each of the pursued research will be addressed in a summarised and integrative manner, trying to create bridges that highlight the findings in order for them to be used for a broader implementation of the theta burst stimulation technique. The focus will remain on the main findings from the published papers within the scope of this thesis and also from the registered patent. This approach also refers to the discussions on the previous chapters and does not exempt consulting the main articles.

The overall content of this thesis focused on the study of neurophysiological responses and other physiological measures, associated with the application of magnetic fields to the brain, resorting to evaluation techniques that may be sensitive to its effects.

As discussed in chapter I (general introduction), despite TBS continuous consolidation and arising preponderance in clinical practice, predominantly in the treatment of major depression but also in obsessive-compulsive and pain disorders [4,22,130], there are still doubts associated with the degree of modulation and its main effects over the central nervous system. Doubts also extend to which may be the potential array of side effects, and even in what cortical (and subcortical) areas of implementation it may be more beneficial. Bearing in mind that this has been a rapidly expanding scientific discipline, these premises were even more pressing at the time that this thesis was projected and began to be developed, guiding all the research that surrounded the main objectives.

Critical analysis of the results derived from the research included in this thesis allows us to think that our main objective was achieved since it was possible to verify with our data that theta burst stimulation seems capable of influencing the healthy prefrontal and temporal cortices, and their respective cortico-subcortical networks. It was also possible to register the induced after-effects through neurophysiological methods and other complementary techniques.

After stimulating more than one hundred and thirty healthy individuals with TBS in more than one cortical region, we can infer that this is a safe technique, with rare and

minor side effects, as long as the safety guidelines recommended in its application are complied with [12,19,22,46]. In our research, a few subjects reported focal discomfort after TBS and mild transient headaches, independent of the stimulated region. These side-effects occurred up to 48 hours after stimulation of the prefrontal cortex (4 subjects with headaches), and temporal cortex (1 subject with headache and 2 with focal discomfort). None of the stimulation sessions had to be interrupted due to major side-effects, again reinforcing the favourable acceptability of this technique. Regarding TBS safety, crucial findings were also addressed in chapter VI (stimulation of the auditory cortex), specifically regarding hearing safety. Our sham-controlled study allowed to assess hearing safety of the left ear (the one closest to the coil and more likely to be impaired) after exposure to one session of TBS over the ipsilateral primary auditory cortex, and no major side effects nor any significant hearing threshold impairment occurred after iTBS, cTBS or sham stimulation.

An overview of this thesis findings supports the theory that theta burst stimulation is able to stimulate the cortex directly below the coil but is also able to exert an excitatory or inhibitory effect on neuronal networks interconnected to the targeted region. These activated or impaired neural networks may be functionally simpler, such as the auditory cortex and the auditory pathways (as studied in chapter VI), or more complex neural networks, involved in cognitive processes or creativity (as studied in chapter III, IV, and VII). We believe that these are very relevant results and an asset for the current knowledge about TBS. Our data support scientific notions of a trans-synaptic effect defended specifically for the classic rTMS method [316–318], which can thus be extended with greater confidence to the TBS technique. Neural networks that control more distal physiological processes contoured by the central nervous system also appear to be influenced by TBS, as is the case of the decrease in systolic arterial pressure after right hemisphere cTBS (as studied in chapter V), a divergent result compared with some studies evaluating arterial pressure. In this case, the scientific evidence is scarcer, contradictory, and used classical rTMS: e.g. Sibon et al. did not find significant changes in blood pressure after high-frequency rTMS and Yozbatiran et al. reported that high-frequency rTMS applied ipsilaterally to stroke lesions increased systolic blood pressure [248,319]. Different methodology (rTMS vs TBS and number of sessions) may explain these differences. Nevertheless, decreases in blood pressure in line with our results were found by research also using inhibitory transcranial magnetic stimulation, in the form of low-frequency rTMS. Both Li et al. and Zhang et al. reported a decrease in blood pressure after inhibitory stimulation, but stimulation was applied over the carotid sinus of humans and rabbits, respectively [250,251].

Our results support the most accepted knowledge about the modulatory after-effects of the two main forms of TBS – intermittent and continuous, specifically regarding that iTBS is able to induce an excitatory or facilitating effect and that cTBS is capable of causing cortico-subcortical inhibition [209,320], with different duration of after-effects, as recently described [209]. The inhibitory effects were described in chapter III, where cTBS was able to significantly delay the auditory P300 latency, after stimulation of either right or left hemisphere; in chapter IV where cTBS over the left hemisphere impaired the expected learning effect performance in Stroop C and Stroop Interference compared with the excitatory and sham groups; and finally, in chapter V, where cTBS originated a significant reduction in left cortex oximetry after stimulation of the left pre-frontal cortex and a significant reduction in systolic arterial pressure after right hemisphere stimulation. In contrast, the excitatory iTBS effects were reasserted in chapter III - faster P300 latencies after stimulation of the left hemisphere; also in chapter VI, with an apparently favourable neuromodulation reflected in lower thresholds when using iTBS over the left auditory cortex; and finally in chapter VII, where the creative process seems to be significantly improved after iTBS on the right DLPFC. When all of these findings are evaluated comprehensively, we can also observe that the inhibitory phenomenon induced by cTBS appears to have a superior capacity to exert its effects, compared with the excitatory effect of iTBS. In almost all of our comparisons between cTBS and iTBS (and versus sham stimulation) in the same hemisphere, cTBS seems to be more effective, being able to: (i) bilaterally inhibit P300 evoked potentials (iTBS only succeeded in evoking an effect on the left hemisphere), (ii) impair the results of the neuropsychological tests after stimulation of the left hemisphere (no significant effect after iTBS) or even (iii) induce a decrease in oximetry and systolic blood pressure (again with no significant effect after iTBS). Only in the auditory threshold study (chapter VI) after stimulation of the primary auditory cortex did we observe a predominance in the iTBS after-effects over the possible cTBS modulation. As discussed in the previous chapters, results for each cortical region are not unanimous. However, when comparing with the results presented by Wischnewski et al. in their systematic review on cortical excitability of the motor cortex [209], our results do not support the notion that iTBS can have a greater excitatory effect than the inhibitory effect evoked by cTBS. In our opinion, more than one factor may explain these findings. The first and possibly most important factor is that each cortical region and its respective neural networks may be modulated differently, responding in a different manner to iTBS and cTBS, given that we are addressing different areas (motor vs non-motor cortices), not only functionally but also in their neuroanatomical organisation: (i) the prefrontal cortex is highly developed and processes higher-order functions (such as planning and executive decision), having no

direct association with discrete peripheral nerves but benefiting from complex afferent and efferent connections to other cortical, subcortical, and brainstem regions (e.g. sensory and motor cortices, amygdala, thalamus, and hippocampus); (ii) the motor cortex presents a less developed (rudimentary) layer IV and is centred around creating and planning the impulses that will originate muscle contraction, through all the direct nervous connections that come out of the region (both cranial and spinal nerves) [321–324]. The second factor, also defended by Wischnewski et al. [209], is related to the possible response of the prefrontal and auditory cortical regions to the effects of long-term potentiation and long-term depression, which are probably different in magnitude from what happens in the primary motor cortex.

We can also infer that the effects of TBS appear to be intrinsically interconnected with the lateralisation of stimulation. Strictly speaking, the effects of TBS (either iTBS or cTBS) of the right hemisphere appear to be different from those resulting from stimulation of the left hemisphere. But this variance is, in turn, linked to the specific functions of each hemisphere and directly linked to the stimulated cortical area. Results from chapters III and V show that the left hemisphere seems more likely to undergo neuromodulation following TBS or that the after-effects associated with leftward stimulation seem to be more intense. As an example, asymmetric results were also found studying neurotransmitter functioning: Ko et al., using PET scans, found that dopamine release seems to be impaired in both hemispheres after the use of cTBS over the left DLPFC in healthy adult volunteers. These results were not found after right DLPFC stimulation [325]. The other theory, also discussed in the aforementioned chapters (III and V), is that the left hemisphere is dominant for the functions studied in the chapters in question. Cognitive processing is highly correlated with linguistic functions and, as such, an attempt to modulate these processes is more likely to be successful in stimulating the left hemisphere – the dominant hemisphere [326–328]. This left dominance appears to be the reason behind the results in some studies evaluating ERPs and neurovascular measures [149–151,232,243] but other results after supposedly excitatory rTMS over the left frontal hemisphere are not unanimous, as demonstrated by Jing et al., reporting a delay in the auditory P300 latency after 10Hz stimulation over this area [328]. Our results support the notion of a left hemisphere dominance, as we found an asymmetrical response to excitatory stimulation, with iTBS over the left DLPFC leading to significant faster auditory P300 latencies, a result not found after right-sided iTBS (chapter III). We also found a leftward asymmetry after cTBS stimulation over the left DLPFC, but in this case, originating a significant reduction in left cortex oximetry (chapter V). Therefore, these results reinforce the theory that the left hemisphere may

have some dominance over the right [149–151,232,243], at least in these studied functions and with this type of evaluation.

As mentioned, and discussed in chapter I, the prefrontal cortex and specifically the dorsolateral prefrontal cortex is a key area when addressing cognitive investigation using transcranial magnetic stimulation. The analysis of the results presented in chapters III, IV, and V allow a better understanding of the effects of excitatory and inhibitory TBS on the prefrontal cortex, further proving that we can study the effects of cognitive neuromodulation using long-latency evoked potentials - auditory P300 - that are usually only used in clinical practice to assess patients with cognitive impairment and dementia.

The sham-controlled protocol using a one session hemispheric cTBS/iTBS approach seems to support the concept that TBS may effectively influence neural networking involved in P300 formation, with different effects seen for iTBS and cTBS: (i) slower evoked potentials being found after cTBS in both hemispheres, and (ii) faster evoked-potentials arising after iTBS on the left DLPFC. These results appear to corroborate that the auditory P300 may be an effective tool to evaluate transcranial magnetic stimulation related outcomes, having the sensitivity to detect both positive and negative neuronal processing variations. Furthermore, the association of the auditory P300 with the neuropsychological testing evaluating stimulation of the left hemisphere, showed only a partial overlapping capacity between these types of tests, evidencing differences in the ability to detect the neuromodulatory effects of TBS. While the possible excitatory effects of iTBS seem to escape the sensitivity of the used tests, cTBS significantly slowed P300 responses but only partially altered Stroop testing, changing the expected cTBS group performance only in Stroop C and Stroop Interference. Trail Making Test results did not suffer any significant changes either with iTBS or cTBS. These results can convey different possible meanings. On one hand, the auditory P300 may be more sensitive for detecting changes in the neural networks affected by the cTBS neuromodulatory effects, when compared with the Stroop and TMT tests. Another perspective is that assuming that a global impairment in cognitive processing occurs after cTBS, the magnitude of the impairment may be higher in the neural networks that are more likely to influence the auditory P300, especially its processing speed, since the main parameter altered is P300 latency.

With the bi-hemispheric sham-controlled evaluation performed in chapter V, using a simple non-invasive test - Near Infra-Red Spectroscopy, it was also possible to realise that cTBS appears to influence the cerebral oxygenation of these subjects, resulting in decreased cerebral blood flow, bilaterally but only statistically significant on the left

hemisphere. By simultaneously taking into account the results of chapters III and V, we can also argue that the P300 appears again more able to detect the bilateral cTBS induced changes when compared with the used non-invasive oximetry evaluation technique and arterial pressure monitoring, suggesting a possible higher sensitivity of this P300 technique when studying DLPFC-TBS induced outcomes.

Beyond improving scientific knowledge regarding TBS safety, chapter VI findings allowed to better understand the after-effects of iTBS and cTBS over the auditory cortex, stimulating near the subjects' ear, and two major inferences can be drawn: the first is that a single session of TBS (iTBS or cTBS), with the coil in the proximity of a protected ear (earplug usage), was not capable of significantly impairing ipsilateral hearing, thus adding to the notion that TBS noise cannot negatively influence hearing-related outcomes; secondly, an interesting result was found after iTBS, as this type of stimulation was able to influence hearing thresholds, resulting in lower thresholds after stimulation. These encouraging results suggest that iTBS may have the potential to positively influence hearing thresholds in healthy young adults. Furthermore, they also suggest that iTBS may play an interesting role in studying a possible therapeutic intervention in sensorineural hearing loss cases. Further studies in this specific domain are being prepared by our research team.

It is also important to note that the inhibitory and excitatory effect of cTBS and iTBS, respectively, do not always translate into an impairment with negative after-effects or activation with positive effects. The negative or positive outcome after TBS seems to be directly dependent upon the cortical area and function to be modulated. TMS and TBS are used therapeutically in rehabilitation in several illnesses, namely depression, stroke, and tinnitus, in order to generate interhemispheric rebalancing [22,91,329]. The most accepted theory suggests that in the healthy brain, hemisphere equilibrium is controlled by an inter-hemispheric inhibition, mediated by fibres of the corpus callosum [26–28]. This inter-hemispheric competition model may be impaired in diseases like stroke, in which a lesion of one hemisphere leads to an ipsilateral hypofunction and a reduction of the inhibition to the healthy hemisphere. This will inadvertently originate an increase in the activity and excitability of the non-lesional hemisphere, leading to an ever-greater secondary inter-hemispheric inhibition of the damaged brain [26–28]. In general, the objective is to use cTBS as a method to inhibit a specific brain area and its respective neural networks, which are abnormally hyperactive (e.g. tinnitus) [22,124] or which, despite being normal, are overactive when compared with the homologous hemisphere with hypofunctional abnormal activity (e.g. stroke) [22].

Specific study limitations of the research developed during this thesis were addressed in the previous chapters, but a few should be mentioned here given their transversality. The first relates to the relatively small subject number per group, even though the total number of subjects per article was relevant for the type of research. This may limit the statistical strength of the tests used, restraining the possibility to assume a wider generalization of the results. The second relates to the volunteers itself: after analysing the results, we think that these types of protocols may benefit from the use of objective techniques to evaluate volunteer stress and anxiety, in order to control a possible influence of these parameters. We cannot exclude that this fact may have possibly influenced cognitive and autonomic related responses. Finally, we must mention that our laboratory does not have a neuronavigation tool to be used in conjunction with TMS. Although all the techniques used in our investigation to determine the DLPFC and the auditory cortex are well documented and have been proven before, the use of a neuronavigation tool could have increased the reliability of our studies.

Future perspectives

Despite the limitations that have been addressed, it is relevant to highlight some points that may be important to consider in future research.

To further improve TBS assessment and to complement the research addressed in this thesis, it would be important to study and evaluate the hemisphere homologous to those studied in chapters IV and VI. In this sense, the evaluation of the right hemisphere after TBS becomes relevant, also comparing the auditory P300 with neuropsychological tests. It is expected that, with the leftward dominance of some functions of the prefrontal cortex and a left hemisphere that may somehow be more sensitive to stimulation, the after-effects after rightwards TBS may not assume similar characteristics. A minor dominance is also documented for the left auditory cortex, so it would be relevant also to perform TBS on the right auditory cortex and evaluate if the thresholds are similarly influenced by iTBS.

All of our research focused on studying the effects of TBS after a single session, occurring within a short time window, where we expected to find a meaningful change after the TBS session in the proposed parameters. Therefore, none of the protocols considered

subjects follow-up. Future research in these areas would benefit from follow-up periods, designed to identify after-effects duration. These extended study protocols would allow to better understand the potential influence of a single session of iTBS over the following hours or even days. In this regard, we particularly stress the follow-up of the possible cognitive improvement after iTBS over the left prefrontal cortex, the favourable decrease in hearing thresholds after iTBS on the left auditory cortex, and even the apparent increment of the creative processing following iTBS of the right prefrontal cortex. Future protocol advances should also ponder testing using multiple stimulation sessions, with a daily depression-like treatment scheme, evaluating whether enhanced or more prolonged effects could be found. However, it is also important to note that, following what is suggested in the safety guidelines, it will be necessary to ensure the maintenance and even the increase of the safety procedures during and after the stimulation sessions, given that the risks associated with stimulation may fluctuate with the increased number of sessions.

Additional research studying the scientific fields addressed in this thesis (cognition, hearing, creativity, and cerebral oxygenation/blood flow) would benefit tremendously if associated with other neurophysiologic techniques such as multichannel electroencephalography, with caps over 64 channels (preferably over 128), linking our findings to possible and more immediate changes in regional frequencies and/or amplitudes, also studying synchronisation/desynchronisation dynamics. We also suggest that these studies could benefit from an association with neuroimaging techniques, such as functional magnetic resonance imaging.

As for the methodology implemented, future research in these scientific domains would probably benefit from the use of a neuronavigation system for the coil target placement. Although the methodology used in our studies is widely used with good results, a more precise target definition technique using an image-based neuronavigator to place the coil may be helpful in some cortical non-motor areas, in which adequate target identification may be more difficult.

We also find it important to note that all of our studies were developed in healthy young subjects. Thus, it will be important in the future to replicate the methods and techniques applied in this research in the brain and nervous systems of subjects of older ages, thus trying to understand if the behaviour found in the previously presented chapters remains the same in different ages.

Finally, we believe that a paramount step should be taken to study and possibly apply the knowledge derivative from this thesis to clinical studies, preferably multicentre based. Larger studies with patients should be undertaken attempting to use the auditory P300 technique to evaluate theta burst stimulation related outcomes in diseases like depression, obsessive-compulsive disorder, or even stroke. The use of the auditory P300 may be even more relevant in patients with cognitive impairment. Given that the use of the auditory P300 is already an important part of the diagnostic aid, especially in the early diagnosis, the comparative evaluation pre-post treatment with TBS can be very relevant, allowing to assist in the more rigorous follow-up of these patients. Following the same reasoning, the use of non-invasive cerebral oxygenation measurements, a simple and fast evaluation method, could also be a valid form of supporting assessment, especially in vascular-related diseases. The encouraging results following iTBS use on the auditory cortex, apparently able to positively influence hearing thresholds, should also be replicated in patients, particularly with mild sensorineural hearing loss or other types of hearing loss, first in adults and possibly also in children. If results prove to be as positive, this stimulation protocol may be a useful neuromodulatory technique able to improve patients' hearing.

Concluding remarks

Our findings suggest that this non-invasive neuromodulation technique may, in fact, be a relevant asset in the quest to understand brain functioning and also be able to increase the range of possible therapeutic applications. A single session of this fast application technique seems capable of modifying brain functioning in the studied regions, with a magnitude that allows the identification of the functional changes with specific sensitive techniques. It was also clear that iTBS and cTBS are capable of inducing opposing effects, emphasising the implicit need to properly adapt interventions with TBS according to the advocated intention - to excite or inhibit the cortico-subcortical functions to be studied or treated.

Our results also highlight the potential importance of neurophysiological assessment of the cognitive outcomes associated with TBS interventions, using the auditory P300, isolated, or in association with other assessment methods such as neuropsychological tests. The use of the auditory P300 may be a useful approach to study and monitor stimulation effects in the healthy brain, with the potential to evaluate neurologic and psychiatric diseases. Our findings involving the after-effects on both oximetry and blood

pressure may bring new insights regarding the role that TMS may eventually play in the study of cardiovascular diseases mediated by the autonomic nervous system, and even guiding a possible intervention in these patients.

Overall results from our research emphasise that TBS over the auditory cortex proved to be not only safe but also to have the potential to be used as a mean to improve patient's hearing capacity. Finally, it was possible to use TBS to influence and modulate less studied higher cognitive functions such as the creative process, attempting to uncover a new process of neuronal modulation in order to promote creative thinking.

We believe that these thesis findings may add valuable information to understand the real spectrum range of rTMS, specifically TBS. Historically the ethical parameters that guide the diagnostic and therapeutic use of TMS are mainly related to the safety of the subject in clinical trials [7], but it is of equal importance to know all the possible positive and negative effects associated with the use of rTMS/TBS in healthy humans. In this case, our objectives were achieved, since we were able to evaluate the use of theta burst stimulation in more than one cortical region, understanding more about the real neuromodulatory possibilities of the technique, and acknowledging which aspects of the technique do not work as well or may even give rise to less desirable side effects. These points in unison are of the utmost importance on the path towards gaining the necessary technical credibility for TBS, trying to achieve a more comprehensive and trustworthy clinical use. In view of the myriad of possibilities and variants in TMS and its clinical applications, it is essential to understand the scope of each specific variant in rTMS - this was a basic objective that we carried out in this thesis by studying the theta burst stimulation. It became clearer that TBS seems capable to neuromodulate cognitive processes and that these can be evaluated with neurophysiological tests, which are usually used in the clinical/diagnostic practice. It seems also clearer that TBS may be able to modify functional regions associated with the auditory and creative processes. We also highlight that all the methods used were unquestionably safe, with the absence of any major negative side-effects, once more clarifying the potential impact of the TBS risk-benefit profile.

It is also important to note that the techniques used in this thesis in order to study the effects associated with TBS may be very useful in the future when trying to identify the effectiveness of therapies instituted with TBS, possibly allowing to modify and adapt the idealised interventions, thus guiding a personalised, tailor-made intervention.

Finally, it is important to substantiate the struggle opposing the inappropriate use of this technique. Although the on-label use is very well defined, we have been witnessing the exponential worldwide growth of several off-label therapies for numerous neurological and psychiatric diseases, especially in patients who have exhausted other forms of classic treatment [7]. This raises several ethical problems and that can lead to the appearance of serious adverse consequences due to the misuse of rTMS/TBS. In fact, the unfounded use of this technique can lead to the emergence of criticism and the discrediting of its potential, something that can be tragic given the potential capacity of rTMS/TBS to assist in the treatment of various diseases with proven results, thus helping a vast number of patients. In addition to trying to explore and understand the scope of the use of TMS, research like ours tries to promote the best practices associated with TMS, attempting to defend the patient's best interests.

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Appendices

Appendix A

PLOS ONE

RESEARCH ARTICLE

Bilateral theta-burst magnetic stimulation influence on event-related brain potentials

Nuno Pinto[1,2☯]*, Marta Duarte[1☯], Helena Gonçalves[1☯], Ricardo Silva[1☯], Jorge Gama[1,3☯], Maria Vaz Pato[1,4☯]

1 CICS-Health Sciences Research Centre, Faculty of Health Sciences, University of Beira Interior, Covilhã, Portugal, **2** Dr. Lopes Dias School of Health - Polytechnic Institute of Castelo Branco, Castelo Branco, Portugal, **3** Department of Mathematics - University of Beira Interior, Covilhã, Portugal, **4** Sousa Martins Hospital, Guarda Local Health Unit, Guarda, Portugal

☯ These authors contributed equally to this work.
* nfcpinto@gmail.com

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Abstract

Theta-burst stimulation (TBS) can be a non-invasive technique to modulate cognitive functions, with promising therapeutic potential, but with some contradictory results. Event related potentials are used as a marker of brain deterioration and can be used to evaluate TBS-related cognitive performance, but its use remains scant. This study aimed to study bilateral inhibitory and excitatory TBS effects upon neurocognitive performance of young healthy volunteers, using the auditory P300' results. Using a double-blind sham-controlled study, 51 healthy volunteers were randomly assigned to five different groups, two submitted to either excitatory (iTBS) or inhibitory (cTBS) stimulation over the left dorsolateral pre-frontal cortex (DLPFC), two other actively stimulated the right DLPFC and finally a sham stimulation group. An oddball based auditory P300 was performed just before a single session of iTBS, cTBS or sham stimulation and repeated immediately after. P300 mean latency comparison between the pre- and post-TBS stimulation stages revealed significantly faster post stimulation latencies only when iTBS was performed on the left hemisphere ($p = 0.003$). Right and left hemisphere cTBS significantly delayed P300 latency (right $p = 0.026$; left $p = 0.000$). Multiple comparisons for N200 showed slower latencies after iTBS over the right hemisphere. No significant difference was found in amplitude variation. TBS appears to effectively influence neural networking involved in P300 formation, but effects seem distinct for iTBS vs cTBS and for the right or the left hemisphere. P300 evoked potentials can be an effective and practical tool to evaluate transcranial magnetic stimulation related outcomes.

Introduction

Transcranial magnetic stimulation (TMS) has become an essential tool for manipulation of cortical activity, thereby allowing the study of the functional organization of the human brain [1]. The continual development of techniques such as repetitive TMS (rTMS) and patterned rTMS, enhances their potential as a tool for clinical treatment of several psychiatric and neurological diseases [2–6]. TMS has been shown as a safe approach to non-invasive research of

cognitive functions, both in healthy and pathologic brain. However, research focusing upon the cognitive therapeutic potential of rTMS over the last years has shown contradictory results, thereby perpetuating some doubts over its mechanisms [7, 8].

It is known that stimulus characteristics such as frequency, intensity, train length or total number of pulses can induce lasting inhibitory or excitatory after-effects [4]. Theta-burst stimulation (TBS) is a form of patterned rTMS which has some advantages including lower stimulation intensity, a short stimulation period and a more prolonged after-effect as compared to other rTMS protocols, both the excitatory (iTBS) and the inhibitory (cTBS) forms [9], and is additionally regarded by some authors to be safer than traditional rTMS [4, 10].

Event related potentials (ERPs) are cerebral responses to external stimuli, which reflect the neurophysiology of cognition [11, 12] and may be used to study the cognitive effects of TBS. The auditory P300, directly dependent upon subject's attention and discrimination, is the most extensively researched ERP component, resulting from the discrimination of rare, task-relevant stimuli, generally using an oddball paradigm. Predominantly reflecting processing speed, is an important tool in the study of cognitive processes and memory in normal subjects and in psychopathology, as its delay can be used as a marker of cognitive deterioration [13, 14]. Playing a less prominent role in ERP studies, the N200 potential also yields important information regarding cognitive evaluation, as it represents the initial, subconscious processing of the stimulus involved in the oddball task, leaving the translation of more advanced and purposeful stages of task processing to P300.

Thus far, the use of ERPs remains scant [7, 8], and there is still little research on auditory P300 and TBS. Therefore, in order to study TBS effects upon neurocognitive performance using a ERP evaluation tool, we delineated a study combining auditory P300 and TBS applied to young healthy volunteers. Our objectives were: a) to study the effects of a single TBS (iTBS or cTBS) session upon auditory P300 performance, b) to analyse whether the stimulated side originates any lateralization on parietal P300 responses and c) to evaluate whether TBS protocol has any influence upon the volunteers' reaction time during P300 testing.

Materials and methods

Subjects and study design

This was a double-blind sham-controlled study, involving healthy volunteers that were recruited after general advertisement with medical students enrolled at the Faculty of Health Sciences, University of Beira Interior, Covilhã, Portugal. Students were selected if they were between 18 and 30 years-old, and after answering a confidential screening questionnaire. Exclusion criteria included being left-handed or ambidexter; previous brain injury and/or severe head trauma; epilepsy or history of convulsions; presence of major medical illness (including neuropsychiatric diseases), intake of any medication during testing, pregnancy, implanted devices or foreign metal articles, sleep deprivation, alcoholism and history of drug intake [4]. All volunteers were instructed to avoid sleep deprivation, alcoholic beverages or other toxic/stimulant substances 24 hours prior to the application of the technique.

Volunteers were then randomly assigned to five different groups: two groups with active stimulation to the left dorsolateral pre-frontal cortex (DLPFC)—Group A (iTBS) and Group B (cTBS), two other groups with active stimulation over the right DLPFC (Group D (iTBS) and Group E (cTBS) and finally, a placebo group—Group C (Sham).

After complete explanation of the procedures, all subjects signed a written informed consent. The study was approved by the Faculty of Health Sciences UBI Ethics Committee (no. CE-FCS-2011-001), in conformity with the Declaration of Helsinki.

Theta burst stimulation (TBS)

TBS was performed under medical supervision at FCS-UBI facilities, using a 70 mm figure-8 coil with a MagVenture MagPro® G3 X100 5.0.1 and recording EMG activity in a Dantec™K-eypoint®—Keypoint.net v.2.03. Stimulation comprised a biphasic pulse waveform and antero-posterior (A-P) current direction in single pulse, iTBS and cTBS [4].

Stimulation intensity was defined using the active motor threshold (AMT), which consisted of the minimal stimulation intensity over the motor cortex that was necessary to produce a 150–200μV amplitude motor evoked potential (MEP) of the contralateral *abductor pollicis brevis* (APB), on more than five out of ten trials, while maintaining a voluntary mild contraction, using visual feedback. Active stimulation was performed over the right or left DLPFC area that can be defined as 5 cm rostral of the region from which the most prominent motor response of the contralateral APB muscle can be recorded [8, 9, 15].

The TBS protocol consisted of bursts of 3 pulses delivered at 50 Hz every 200 ms (i.e. at 5Hz), at an intensity set to 80% AMT [11]. In the cTBS protocol the bursts were delivered without interruption, up to a total of 600 pulses. iTBS also comprised 600 pulses, but the bursts were delivered at 5 Hz during 2 s (groups of 10 bursts), repeated every 10 seconds [9].

Sham stimulation used the same coil, tilted away from the scalp at a 90 degree angle, but maintaining contact and sound (intensity reduced to 50% AMT), thereby giving the impression that the subject was being stimulated, although this stimulus does not reach cortical neurons [4, 8]. During protocol application, subjects were seated in a comfortable declinable armchair and were told to relax and avoid any head movements.

P300

Auditory P300 recording was carried out in a quiet room, using an 8 channel Keypoint.net v.2.03. Active electrodes were placed in Cz, Pz, P3 and P4 of the 10/20 international system, with an anterior reference, trying to achieve a more accurate lateralization of the waves recorded in the right and left parietal electrodes. All recording sites were cleaned with alcohol and abraded to maintain a resistance below 5 kΩ. [11, 16, 17]. A time constant of 1 second was used together with a high frequency filter of 50 Hz, with a time base of 1000 ms, using an automatic overload rejection mode. The auditory oddball paradigm consisted of 80% frequent stimuli presentation, 1000 Hz and 50 ms of duration, randomly mixed with a 20% target stimulus, 2000 Hz and 100 ms of duration. Both used a minimal intensity of 65 dB HL. Stimuli were presented binaurally, with a random interval between 1 and 2 seconds. Each complete study recorded at least 400 stimuli (minimum of 100 target), divided into two series, and subjects were instructed to remain calm and relaxed, avoid blinking and to concentrate upon a focus point. Subjects were then asked to press a button for the rare stimuli as quickly as possible with the dominant hand in order to ensure attention and collaboration [11, 18]. The chosen parameters were measured from the mean waveform of the two reproducible series and the epochs for the target and non-target tones were analysed separately. The largest negative peak, occurring between 160–260 ms, was considered as the N200. The P300 was defined as the largest positive peak arising after the N1, P2 and N2 components, increasing in amplitude at the posterior areas and occurring between 220–600 ms. Amplitude was measured in the N2-P3 complex, between the maximum negativity and positivity components [11, 12, 19, 20].

Experimental design

The study design comprised three different timepoints for assessment, labelled as pre-TBS, TBS stimulation and post-TBS. Stimulation was always performed at the same time of day and randomly assigned to each volunteer according to the respective group. Each subject was

submitted to a single TBS session on the DLPFC. The order of real and sham sessions was also randomized and counterbalanced across subjects. Only one member of the investigation team was aware of the type of stimulation applied. In pre-TBS stage, baseline P300 recording was performed. This step was followed by all the procedures regarding TBS protocol, performing either iTBS, cTBS or sham stimulation. Immediately after TBS or sham stimulation, the second auditory P300 recording was performed (post-TBS). Protocol available at: dx.doi.org/10.17504/protocols.io.kr3cv8n

Statistical analysis

Chi-square and Levene tests were used to study if there were any significant differences between groups. Normality was evaluated using Kolmogorov-Smirnov and Shapiro-Wilk tests. Due to the relative small number of group elements and data characteristics, we needed a robust nonparametric analysis test to evaluate pre-post stimulation mean result comparisons and multiple group comparison test, thus we used the R software package: Nonparametric Analysis of Longitudinal Data in Factorial Experiments (nparLD) [21]. Analyses were performed using IBM SPSS Statistics 20® and R version 3.0.0., and the significance level was $p < 0.05$.

Results

Volunteers

This study involved 51 healthy volunteers (31 female and 20 male, aged 19–30 years, mean = 22.84 +/- 1.98), and all study groups (Group A n = 10; Group B n = 10; Group D n = 10; Group E n = 11, and Group C n = 10), were matched in terms of age and gender.

Pre-stimulation—N200 and P300

For all groups, N200 mean latency pre-stimulation ranged between 176.98 +/- 30.21 ms over Pz and 181.73+/- 23.05 ms over Cz. As for P300, the lowest mean latency was obtained over Cz– 255.65 +/- 45.07 ms—and the highest over P3—259.57+/-54.81 ms. Overall maximum latency recorded reached 256 ms and 483 ms, for N200 and P300 respectively. Amplitudes recorded regarding N2-P3 difference, showed mean results between 4.72 +/- 3.12 μV over Cz and 5.10 +/- 3.85 μV over Pz, with a maximum amplitude of 19.9 μV. Signalizing the rare stimuli by pressing the button on our oddball paradigm achieved an overall reaction time mean of 316,24 +/- 57,04 ms, ranging from 217 to 468 ms.

Pre- and post-stimulation latencies

Pre-stimulation and post-stimulation latencies, amplitudes and reaction times distributed per stimulation group are shown in Fig 1.

Comparison of P300 latencies between the pre- and post-TBS stimulation stages are shown in Table 1.

Differences were detected between groups, in terms of stimulation characteristics. iTBS groups showed a tendency towards decreasing P300 latencies after stimulation and cTBS groups showed a tendency towards a slower response time. In contrast, the sham group did not show a clear tendency.

Sham and right hemisphere iTBS groups showed no significant differences between the pre and post evaluations (nonparametric—nparLD package). iTBS over the left hemisphere showed significantly faster post stimulation latencies, mainly over the parietal recording sites ($p = 0.003$, $p = 0.006$ and $p = 0.005$ for Pz, P4 and P3, respectively). cTBS over the left

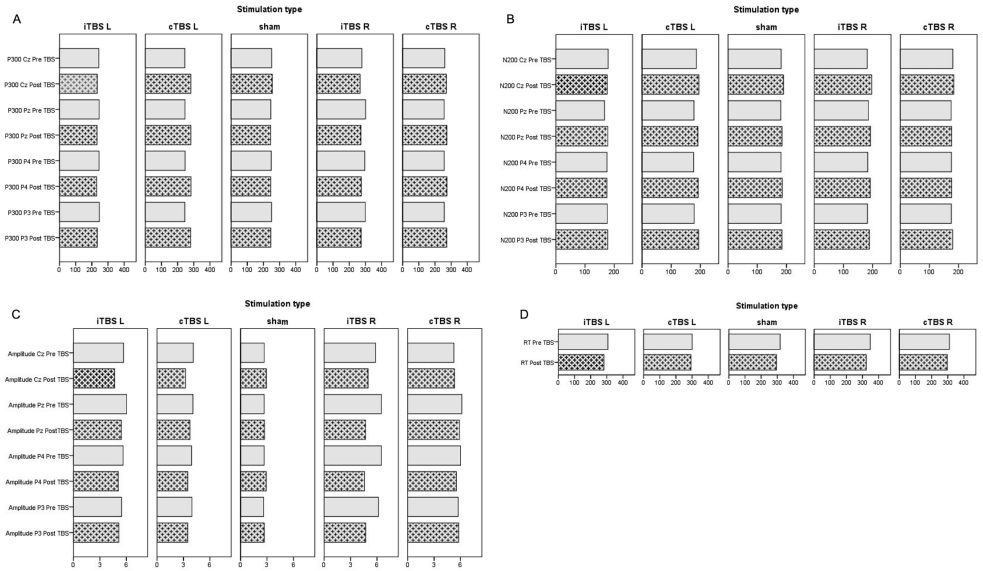

Fig 1. ERP results per stimulation group. P300 latency (A), N200 latency (B), Amplitude (C) and Reaction Time (D).

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Table 1. Group comparison—Pre vs Post stimulation—P300 and N200 latencies.

	iTBS L		cTBS L		Sham		iTBS R		cTBS R	
	Mean Dif. (ms)	p-value[a]	Mean Dif. (ms)	p-value[a]	Mean Dif. (ms)	p-value[a]	Mean Dif. (ms)	p-value[a]	Mean Dif. (ms)	p-value[a]
P300 Cz Pre	-9,7	0.095	38,4	**0.009**	3,8	0.506	-10,8	0.604	9,91	*0.062*
P300 Cz Post										
P300 Pz pre	-12,9	**0,003**	36,8	**0,000**	-0,8	0,822	-28,4	0.084	16,64	**0.026**
P300 Pz Post										
P300 P4 Pre	-14,2	**0.006**	36,4	**0.000**	-2,4	0.829	-21,4	0.829	16,55	**0.009**
P300 P4 Post										
P300 P3 Pre	-13,3	**0.005**	37	**0.001**	-3,7	0.515	-26,2	0.345	15,18	**0.035**
P300 P3 Post										
N200 Cz Pre	-3,4	0,149	8,8	0,960	7,9	0.238	15,5	**0.006**	3,55	0.709
N200 Cz Post										
N200 Pz Pre	11,6	0.411	13,6	0.277	4,3	0.398	7,3	0.449	1,73	0.837
N200 Pz Post										
Reaction Time Pre	-24,2	**0,000**	-6,1	0,629	-22,4	**0,025**	-24,1	0,052	-13,45	0,176
Reaction Time Post										

[a]nonparametric—nparLD pakage

https://doi.org/10.1371/journal.pone.0190693.t001

hemisphere significantly influenced P300 latency over all recording topographies, causing a delay in the P300 wave. In the right hemisphere, cTBS stimulation was associated with a significant parietal ERP delay (p = 0.026, p = 0.009 and p = 0.035 for Pz, P4 and P3, respectively).

In terms of N200, latency showed a significant difference only when iTBS was performed on the right hemisphere. Contrasting with P300 behaviour to excitatory stimulation, N200 displayed longer latencies after stimulation. The remaining groups showed relatively small and inconstant changes in mean latencies.

Pre- and post-stimulation reaction times

Comparison of reaction times between the pre- and post-TBS stimulation stages are shown in Table 1.

All groups showed faster reaction times in the second ERP evaluation, after TBS and sham stimulation, but this was only significant in the sham group (mean difference = -22.4 ms; p = 0.000) and the left iTBS group (mean difference = -24.2 ms; p = 0.025). In contrast, right iTBS group only showed a trend towards reaction times being significantly faster (mean difference = -24.1 ms; p = 0,052).

Pre- and post-stimulation amplitudes

Comparison of ERP amplitudes between the pre- and post-TBS stimulation stages are shown in Table 2.

ERP amplitudes before and after stimulation in all groups, except for the sham group showed a trend towards a slight decrease after TBS, but no significant difference was found.

Group comparison—Stimulation vs Sham—P300

Comparison of Pz P300 results across all stimulation groups is shown in Table 3.

Table 2. Group comparison—Pre vs Post stimulation—ERP amplitude.

	iTBS L		cTBS L		Sham		iTBS R		cTBS R	
	Mean Dif. (μV)	p-value[a]	Mean Dif. (μV)	p-value[a]	Mean Dif. (μV)	p-value[a]	Mean Dif. (μV)	p-value[a]	Mean Dif. (μV)	p-value[a]
N2P3 Cz Pre	-1,01	0.189	-0,89	0.582	0,24	0.543	-0,84	0.295	0,06	0.876
N2P3 Cz Post										
N2P3 Pz Pre	-0,6	0.980	-0,33	0.850	0,04	0.963	-1,78	0.944	-0,28	0.454
N2P3 Pz Post										

[a]nonparametric—nparLD pakage

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Table 3. Stimulation group vs Sham group multiple comparison test—P300 & N200 latencies.

	P300 Lat. Pz	P300 Lat. Cz	N200 Lat. Pz	N200 Lat. Cz
	p-value[a]	p-value[a]	p-value[a]	p-value[a]
iTBS L vs Sham	**0.024**	0.805	0.250	0.764
cTBS L vs Sham	**0.001**	**0.016**	0.201	0.317
Sham vs iTBS R	0.167	0.837	0.262	**0.024**
Sham vs cTBS R	**0.042**	0.082	0.414	0.280

[a]nonparametric ANOVA nparLD

https://doi.org/10.1371/journal.pone.0190693.t003

When we evaluate the outcomes through a multiple comparisons test, P300 latency over Pz results showed significant differences between the sham group and the left iTBS group ($p = 0.024$), sham and left cTBS goups ($p = 0.001$) and finally between sham and right cTBS groups ($p = 0.042$).

Comparing groups using Cz P300 (Table 3), the only significant difference occurred between the sham and the left cTBS groups ($p = 0.016$), with much slower latencies recorded after actual cTBS stimulation.

Group comparison—Stimulation vs Sham—N200

Multiple comparisons for N200 (Table 3) showed no significant differences over Pz recordings. N200 behaviour over Cz was significantly different between sham and right iTBS groups, in this case because N200 was slower after excitatory TBS over the right hemisphere. ERP behaviour over P3 and P4 followed overall Pz results after pre- and post-stimulation, not showing any significant lateralization.

Discussion

The main goal of our work was to evaluate human cortical and subcortical network dynamics to TBS, via electrophysiological assessment using the auditory P300 ERP. Introducing a sham controlled design trial, we tried to verify if the effects were distinct for iTBS vs cTBS and for the right or the left hemisphere. To our knowledge, this is the first study that compared both excitatory and inhibitory TBS over the right and left DLPFC, evaluating its effects using neurophysiological tests like the auditory P300, with a placebo control group, in a young adult healthy population. Our sham-controlled results showed that ERPs responded differently to stimulation type and lateralization. Significantly slower P300 latencies were recorded over parietal locations after left and right inhibitory stimulation but faster P300 latencies were found only after excitatory stimulation over the left DLPFC. No apparent latency lateralization was found as P300 over P3 and P4 followed the same outcomes as the P300 recorded over Pz. Amplitudes showed no significant variation after cTBS or iTBS in either hemispheres. Reaction times behaved differently also with faster reaction times in the excitatory and sham groups, but with no significant changes in the inhibitory groups.

Using both inhibitory and excitatory TBS protocols, we found that the parietal P300 showed significantly slower latencies after cTBS stimulation bilaterally but the parietal P300 responses were significantly faster only after iTBS over the left cortex. These results suggest that the inhibitory protocol is capable of a more intense or more effective interference over the cerebral circuits that are implicated in P300 formation than excitatory TBS, as it seems to be able to modulate both hemispheres. Supporting these findings, Kaller et al. found interesting results when testing hemispheric relevance using bi-hemispheric cTBS and the Tower of London task. Their results showed that initial planning times could be influenced differently either by stimulating the right or the left hemisphere, with results directly dependent of hemisphere dominance—right hemisphere inhibition resulted in increased planning times and contralateral inhibition showed faster planning [22]. Such evidence is similarly defendable for ERPs global performance, since using a inhibitory stimulation over the frontal area originated decreases ERP amplitude in a modified P300 protocol [23].

Our results also propose an asymmetrical response to excitatory stimulation, since iTBS in our study seemed to be more effective over the left hemisphere, and P300 showed significantly slower latencies over Cz only after left cTBS. Leftward susceptibility to be more easily modulated was detected in other studies with excitatory stimulation, as shown by the faster latencies found after high frequency rTMS over the left hemisphere [24]. Overall, right hemisphere

stimulation results tend to reveal fewer changes in ERP parameters, as showed when administering inhibitory rTMS over the right DLPFC [25, 26], or excitatory rTMS over the right DLPFC [24]. Although asymmetries are reported, our overall recordings of P300 over the left and right parietal areas showed the same results as the P300 recorded over Pz. These findings suggest that lateralized cTBS and iTBS can influence the initial P300 neuronal generator behaviour but not the following bilateral wave formation and spreading. Our findings can be associated to TBS/rTMS modulation capacity to influence neurotransmitter production, as neurotransmitters trigger intracortical excitatory and inhibitory postsynaptic potentials that are the base for ERP formation. Magnetic stimulation capacity to modulate neurotransmitter dopaminergic and glutamatergic connection is known, especially if applied to the prefrontal cortex, and these neurotransmitter assume utmost importance in P300 formation [27, 28]. Previous studies showed that high frequency magnetic stimulation increases anterior brain glutamate levels, in some cases with a left lateralization [29–32]. It is also known that dopamine modulation can influence both task performance testing and also event related potentials [33, 34]. ERP latencies and amplitudes can be influenced by dopaminergic function, impacting cognitive speed processing and also neural resources magnitude allocation to a specific task. Magnetic stimulation can similarly impact dopaminergic function, with some studies showing that high frequency stimulation administered to left prefrontal cortex increases dopamine release [35, 36]. Research also showed that in some studies this effect had also some degree of lateralization, as only the left hemisphere stimulation resulted in either dopamine increase after excitatory stimulation or impaired dopamine release after inhibitory stimulation [33–40]. These findings can strongly be correlated with our P300 latency results, since it is likely that cTBS over bilateral DLPFC can have a direct negative impact in either or both glutamate and dopamine production, essential in the electrogenesis of P300 potentials, resulting in ERP delay, even though it may be predominant over the ipsilateral hemisphere. We also found asymmetrical results, as it appears to exist a superior TBS influence over the left DLPFC, especially effective for iTBS and these findings can be related to the reported apparent iTBS superior capability to influence left hemisphere glutamatergic and dopaminergic release. Assuming that P300 test performance is related to mental processing speed affected by attentional processing and cognitive operations, as shown in previous works [41], we can also assume that iTBS over the DLPFC worked has a facilitator of the cognitive and executive process.

As for N200 performance, reflecting the initial subconscious process of the ERP oddball task, our results showed small variations across the groups, except for the right iTBS group, revealing significantly slower N200 latencies, apparently divergent to P300 behaviour to excitatory stimulation. Previous experimental studies pointed to a left hemisphere N200 dominance, predominantly over the anterior mid-cingulate cortex, evaluated by magnetic resonance images, suggesting also a functional and neuroanatomical dissociation between N200 and P300 potentials [42]. We believe that this anatomical dissociation may explain the different P300 vs N200 response to TBS. In this case, the right inter-hemispheric inhibitory connectivity capabilities could have been potentiated by the right-sided iTBS [43–45], thus negatively influencing the N200 dominant left hemisphere, unbalancing right-left basal equilibrium, resulting in poorer N200 performance. Since N200 reflects the initial ERP phase, this result can also be related to right iTBS poorer P300 performance discussed earlier.

It is known that P300 amplitude is associated to the amount of attentional neuronal resources allocated throughout the P300 task, but amplitude evaluation is not straightforward, as it implies a relationship between attention and working memory that can originate higher amplitudes for easy targets and lower amplitude for more complex tasks, requiring more memory load [46, 47]. In our groups, even though the task was not complex, probably our baseline psychological conditions were not ideal, as we were introducing a new, and

somewhat unknown stimulation technic to our volunteers, that could have induced some anxiety. Our results did not reveal any significant change in ERP amplitude, neither in the stimulated groups or in the sham group. Our lack of significant changes in P300 amplitude, associated to a low baseline amplitude P300, could be related to a state of low excitability or a limited capacity to better allocate attentional neuronal resources, possibly related to the TMS protocol-disturbing physiological volunteer estate. It is also well established that P300 activity is influenced by individual internal physiologic state, ranging from circadiam rhythms to fatigue and physical state [48]. Base line ERP results revealed latency and amplitude characteristics that can be explained by factors like our sample of young university students, capable of promoting a lower latency baseline ERP, and technical aspects as reference electrode position, as it is argued that anterior references are positioned within brain's electrical fields of the auditory ERP, being capable of voltage gradients which vary across subjects [16, 41]. So, even though our primary aim was to reduce possible amplitude asymmetry by electrode location and impedance discrepancies, this fact could have influenced amplitude and even latency baseline results [49].

When evaluating reaction time in ERP task we must remember that TMS has the capacity to induce local, trans-synaptic and system-level effects. We know as well that this ERP protocol involves a motor response and apparent significant involvement of the anterior cingulate cortex [48]. The fact that all groups, including sham group, tended to shorter reaction times suggests a mere habituation process. But careful analysis shows that stimulation type may influence this process because of right and left cTBS groups response speed wasn't significantly as fast as their counterparts. This result suggests that cTBS inhibitory capacity negatively influenced bilateral cerebral networking, preventing these groups to perform as fast as they normally would, supporting the notion that even though the DLPFC could be the most active region, it can activate cortical network relays, including deep subcortical relays, thus influencing motor response processes [35].

Using the TBS-P300 combination appears to be a useful approach to monitor stimulation effects, especially if applied when evaluating neurologic and psychiatric diseases, either in rehabilitation or diagnosis. This method may be also important to better understand neural network processing as it allows studying the direct and indirect influence of specific cortical and subcortical connectivity over cognitive performance. As mentioned, previous studies combining rTMS and event related potentials, magnetic stimulation tends to modulate brain responses accompanying the excitatory or inhibitory effects associated with high or low frequency stimulation, respectably, but most studies used only one stimulation type and one stimulation site, mostly without placebo control. Knowing that some previous results were even negative using bilateral inhibitory stimulation [50], a broader study using iTBS and cTBS was clearly necessary. Regardless the fact that there were already studies evaluating the effect of rTMS on the human cortex and the capacity to impact scalp ERPs, the significant variability in application technics and in some cases the incongruent results, enhance the scientific necessity to better understand this technic.

A limitation of our study was the sample size, translated into a small subject number per group, which did not allow us to have better statistical strength. Objective methodologies to evaluate volunteer stress and anxiety should also be used, but unfortunately these tests were not included in our initial study methodology as we did not expected that a TMS based stimulation could cause this level of apparent student solicitude towards the procedure. Nevertheless, we tried to provide ideal protocol application conditions, previously by giving our volunteers all the information needed and during stimulation/recording procedures promoting a stress-free environment.

Conclusions

Our results strongly support the hypothesis that TBS can effectively influence the cortical site of stimulation and also remote cerebral regions, directly or indirectly influencing neuronal excitatory/inhibitory networking, and that this influence is directly linked with stimulation characteristics and hemispheric lateralization. This significant capacity to modulate brain excitability should be further studied, either by neurophysiologic or behavioral testing in order to fully understand and dominate this noninvasive neuro-intervening tool. Further studies with larger subject number are required to confirm our findings and help understand whether these results have short duration, or if this neurocognitive influence is maintained for longer periods of time. We suggest also additional investigation studying and comparing these results using neuroimaging. It would be interesting to investigate the same protocol with repeated application of TBS in a daily scheme, with depression-like treatment sessions. Studies with a larger range of TBS intensities and different number of trains would also be important to evaluate in the future. We believe that P300 evoked potentials have the potential to be used as a useful tool to study and evaluate transcranial magnetic stimulation related outcomes.

Supporting information

S1 File. Data TBS_P300_PLOSONE. Study SPSS data.
(SAV)

S2 File. Ethics Committee.
(PDF)

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Author Contributions

Conceptualization: Nuno Pinto, Maria Vaz Pato.

Data curation: Nuno Pinto, Jorge Gama.

Formal analysis: Nuno Pinto.

Investigation: Nuno Pinto, Marta Duarte, Helena Gonçalves, Ricardo Silva, Maria Vaz Pato.

Methodology: Nuno Pinto, Jorge Gama, Maria Vaz Pato.

Supervision: Maria Vaz Pato.

Validation: Maria Vaz Pato.

Writing – original draft: Nuno Pinto.

Writing – review & editing: Nuno Pinto, Maria Vaz Pato.

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Appendix B

Neuropsychobiology

Research Article

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Theta-Burst Stimulation Is Able to Impact Cognitive Processing: A P300 and Neuropsychological Test Study

Nuno Filipe Cardoso Pinto[a, b, c] Marta Duarte[a] Helena Gonçalves[a]
Ricardo Silva[a] Jorge Gama[a, d] Maria Vaz Pato[a, c, d]

[a]Faculty of Health Sciences, University of Beira Interior, Covilhã, Portugal; [b]Dr. Lopes Dias School of Health, Polytechnic Institute of Castelo Branco, Castelo Branco, Portugal; [c]CICS-Health Sciences Research Centre, University of Beira Interior, Covilhã, Portugal; [d]Department of Mathematics, University of Beira Interior, Covilhã, Portugal

Keywords
Transcranial magnetic stimulation · Theta-burst stimulation · P300 · Trail making test · Stroop test

Abstract
Introduction: Theta-burst stimulation (TBS) is a safe non-invasive neurostimulation technique used to improve cognitive and neuropsychiatric impairments. Combined outcome evaluation using event-related potentials (ERPs) and neuropsychological tests may allow a more thorough assessment of TBS treatment efficacy; however, some mixed results have been found, and their use remains scarce. Our main objective was to evaluate whether a session of TBS to the left dorsolateral prefrontal cortex (DLPFC) can impact upon the performance of both neuropsychological and neurophysiological tests. ***Methods:*** This double-blind sham-controlled study involved 28 healthy adults, between 18 and 30 years. Volunteers were randomly allocated to receive excitatory (intermittent [iTBS]), inhibitory (continuous TBS [cTBS]) or sham stimulation on the left DLPFC. Subjects were evaluated using ERPs (auditory oddball paradigm P300) and neuropsychological tests (Trail making test [TMT] and Stroop test of words and colours [STWC]), using a pre-post stimulation protocol. ***Results:*** Inhibitory stimulation led to significantly delayed P300 peak latencies ($p < 0.001$), with no consistent change in N2P3 amplitudes. cTBS also significantly influenced the expected group performance in Stroop C and Stroop interference ($p = 0.025$) compared to the iTBS and sham groups. No significant results were found in TMT tests after TBS. ***Conclusion:*** Our results suggest that P300 and specific Stroop colour and words test parameters can be similarly influenced by the same TBS protocol. This emphasizes the importance of mixed evaluation using neuropsychological and neurophysiological resources in research associated with the use of transcranial magnetic stimulation and cognition.

Introduction

Transcranial magnetic stimulation (TMS) is a safe non-invasive neurostimulation technique, with limited side effects, which has been widely used to study and treat several neuropsychiatric illnesses such as depression, stroke, epilepsy, Parkinson's disease, and cognitive impairment [1, 2]. Theta-burst stimulation (TBS) is a specific form of TMS which has been shown to be as effective

karger@karger.com
www.karger.com/nps

Nuno Filipe Cardoso Pinto
CICS-Health Sciences Research Centre, Faculty of Health Sciences
University of Beira Interior
Avenida Infante D. Henrique, PT–6200-506 Covilhã (Portugal)
nfcpinto@gmail.com

at modulating various brain functions, but using less time and lower intensity stimuli than those used by conventional TMS [3–5]. Specific TBS paradigms such as continuous TBS (cTBS) or intermittent TBS (iTBS) can have an inhibitory or an excitatory effect, respectively [3].

The frontal cortex is the main area involved in executive functions, having a fundamental role in behaviour regulation and cognitive functions [6–8]. Specifically, the dorsolateral prefrontal cortex (DLPFC) has an important role in attention networks. Through its connections with the dorsal striatum, DLPFC is related to higher order processing tasks like working memory, conscious decision-making, and reasoning [9, 10].

Cognitive processing and central nervous function can be studied by event-related potentials (ERPs). One of the most used is the auditory P300, an ERP component with a major neural generator in the prefrontal cortex, which is linked to decision-making and attentional resource allocation [11–14].

Neuropsychological tests are essential tools for executive function assessment, evaluating aspects such as attention, working memory, cognitive flexibility, or behaviour control [15]. Some of these functions can be evaluated using tests as the Stroop test of words and colours (STWC) and the trail making test (TMT). The STWC assesses executive functions such as selective attention, modulation, and inhibition, resistance to external interference, and cognitive flexibility related to execution speed [16, 17]. The TMT yields information about visual scanning, processing speed, mental flexibility, motor skills, and working memory, among other executive functions [18, 19].

Accurate assessment of TMS neuromodulatory effects can be a challenge, especially when testing cognitive functions. The joint evaluation of event-related potentials with neuropsychological studies may allow a deeper patient assessment [20]. This may originate similar results when evaluating brain processes in some anatomically linked neurological and psychiatric diseases [20]. However, we know that this behaviour is not stable and generalized, namely in the Stroop or in the Trail making tests, existing some dissenting results, possibly dependent on the neural networks involved or activated by each test paradigm [21, 22]. The use of TBS in the prefrontal area, together with P300 and neuropsychological tests, may contribute towards understanding the neuronal basis of hemispheric laterality in brain functioning. The main aim of this study was to evaluate whether a single session of TBS to the left DLPFC can impact upon cognitive function and influence performance of neuropsychological and neurophysiological tests. We also wanted to evaluate whether the neuropsychological and neurophysiological results behave similarly throughout the process.

Materials and Methods

Participants

Twenty-eight healthy, right-handed volunteers, between 18 and 30 years old were recruited among students of the Faculty of Health Sciences of the University of Beira Interior, in Covilhã, Portugal. After answering a confidential screening questionnaire [23], students read and signed a written informed consent form and were asked to voluntarily take part in the study, receiving no monetary compensation. Screening included brief medical, substance use, and neuropsychiatric histories. Selected participants did not have any of the following exclusion criteria: left-handedness or ambidexterity; colour blindness; neurological, psychiatric, cardiac, respiratory, infectious, tumoral, or metabolic diseases; hearing loss; previous brain trauma/brain injury; epilepsy or personal history of one or more seizures; metallic prosthetics or other metallic elements located in the brain or skull; pregnancy; history of alcohol abuse; taking antidepressants, neuroleptics and other similar drugs that might induce seizures [24–26]. None of the volunteers had ever performed TMS in the past. Any other conditions the study team found problematic or doubtful also prevented the subject from being included in this study.

Experimental Design

All procedures were performed in accordance with the Declaration of Helsinki, were approved by the Faculty of Health Sciences UBI Ethics Committee (No. CE-FCS-2011-001), and were carried out under medical supervision. Study protocols were carried out in the FHS-UBI TMS laboratory, and volunteers were told to avoid sleep deprivation, alcoholic beverage intake, or any other toxic/stimulant substances in the 24 h prior to their participation.

This study was an experimental, double-blind sham-controlled study, of the effects of excitatory iTBS or inhibitory cTBS on the DLPFC on cognitive function, using both event-related potentials and neuropsychological tests. Double-blinding was ensured by keeping volunteers and team researchers who applied/evaluated the neuropsychological tests and the P300 results blinded to the assignment condition and without knowing whether active or sham stimulation was applied. Due to the technical study design, only the team researcher in charge of administering the TBS/sham was aware of the stimulation characteristics. Using simple randomization, recruited volunteers were allocated to 1 of 3 groups, in order to receive either active or sham TBS in the left DLPFC:

- Group A – submitted to iTBS.
- Group B – submitted to cTBS.
- Group C – submitted to sham TBS.

P300, TMT, and Stroop colour and words test (SCWT) were performed before (Steps 1 and 2) and after stimulation (Steps 4 and 5), as shown in Figure 1. A single TBS or sham stimulation session was delivered to the left DLPFC of each volunteer.

Transcranial Magnetic Stimulation

Real and sham stimulations were conducted using a MagVenture MagPro1G3 X100 5.0.1, coupled with a Dantec™ Keypoint.

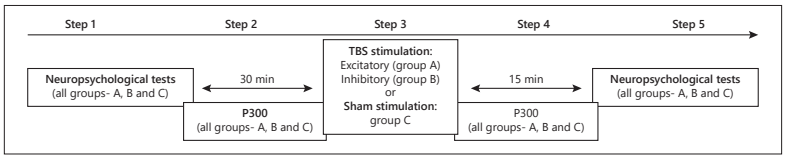

Fig. 1. Evaluation process.

net v.2.03 for motor threshold determination, following the safety and ethics recommendations of the 2009 guidelines [24]. A parallel-handle positioned MCF-B70 butterfly coil was used. Primary stimulation was delivered to the left primary motor cortex, in order to identify the intensity to be used over the DLPFC. This intensity, expressed as a percentage of the maximum device output, was determined as 80% of the active motor threshold – the minimum intensity capable of inducing a motor response of at least 150 μV in at least 5 of 10 stimuli, while the subject maintains a minimal contraction of abductor pollicis brevis muscle [27]. The left DLPFC was found 5 cm anteriorly to the region that induced the most prominent motor response in the right abductor pollicis brevis, area where TBS was delivered [28, 29]. Theta-burst stimulation was applied consisting of a 3-pulse series at 50 Hz, applied repetitively with inter-series intervals of 200 ms, according to Huang et al. [3]. The protocol of cTBS delivers all pulses continuously without interruption while intermittent TBS protocols deliver the bursts only during 2 s (groups of 10 bursts), repeated every 10 s. Both intermittent and continuous stimulation comprised a total of 600 pulses [3]. The same coil was used for sham stimulation placed in a perfectly vertical position (90°) on the subject's scalp, maintaining scalp contact [28].

Event-Related Potential – P300

P300 recording was carried out before and immediately after real or sham stimulation (Fig. 1). An auditory oddball task was performed with an 8 channel Keypoint.net v.2.03., using a randomized 80–20% presentation for the non-target and target stimuli, respectively. Stimulus characteristics consisted of 1,000 Hz and 50 ms of duration for the non-target stimuli and 2,000 Hz and 100 ms of duration for the target, using a binaural presentation with a minimum intensity of 65 dB HL at a random interval between 1 and 2 Hz. Each complete study recorded at least 100 target stimulus [14, 30]. Volunteer attention and collaboration was ensured by signalling (pressing a button in the dominant hand) each time a target appeared [14, 30]. P300 recording protocol focused the main topographic areas (central-parietal) for electrode placement, using the 10/20 international system, with anterior referencing [13, 14, 31, 32]. Impedances were maintained below 5 kΩ. Subject brainwaves were analysed by one of the researchers blinded to stimulation type, in the mean waveform of the 2 reproducible series. P300 peak was found identifying the largest positive peak appearing after the N1, P2, and N2 components, with maximum posterior amplitude, occurring between 220 and 600 ms. N200 was considered the largest negative peak between 160 and 260 ms. For amplitude measurement, we used the N2P3 complex [14, 33–36].

Neuropsychological Tests

All volunteers were submitted to TMT and SCWT before they went through one of the 3 types of TBS: iTBS, cTBS, or sham. Subjects completed neuropsychological tests about 30 min before TBS or sham stimulation (step 1 – see Fig. 1) and repeated them about 15–20 min after stimulation (Step 5). TMT consists of 2 parts: part A, which requires a fast connection of numbers, sequentially and in ascending order; and part B, which requires a logical alphanumeric connection (1-A, 2-B), that is, the correspondence must be alternated between number (ascending order) and letter (alphabetical order). The results are related to the time needed to complete each part of the test, using a Portuguese version of the Stroop colour and words test (SCWT) [37].

The Stroop test is composed of 3 sheets in which the subjects must read or name the observed colours. The first sheet contains the words "green," "red," and "blue." The second sheet contains 100 similar elements – "XXXX" – printed in green, red, and blue colours. On the third sheet, there are the words from the first sheet, printed in the colour of the second 1, without correspondence between the colour of the ink and the meaning of the word. The result is directly related to the number of words/colours verbalized in 45 s [37].

Statistical Analysis

Data were analysed using the IBM® SPSS Statistics® 25.0 package. Descriptive statistics such as means and standard deviations were calculated for each variable of the psychological tests. The effect of TBS stimulation on each of these variables in the 2 conditions (before and after TBS stimulation) was evaluated by a mixed repeated measure ANOVA. The assumptions of this ANOVA were investigated using the Shapiro-Wilk normality test and the Levene test, allowing the latter to evaluate the homogeneity of the variances. Due to the size of the sample and the fact that normality assumption was not always validated, analysis was also performed through a non-parametric version of Mixed Factorial ANOVA (Non-parametric Longitudinal Data in Factorial Experiments, using the "nparLD," version 2.1 package, for the statistical program R). However, since the results obtained through the 2 analyses were compatible, we chose to present only the results obtained by the parametric version. Mean comparison between groups or conditions before/after TBS stimulation was performed with least significant difference test with Sidak's correction. All tests were two-sided, and $p < 0.05$ was considered statistically significant.

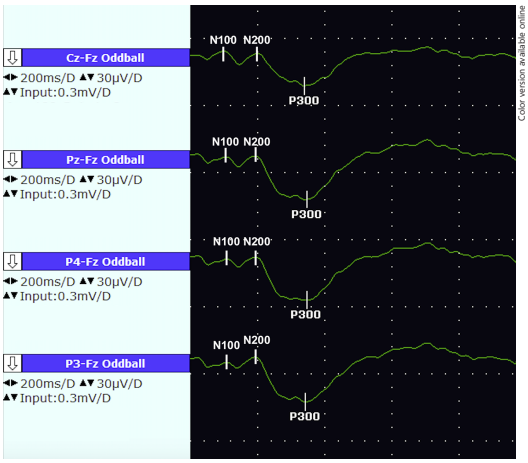

Fig. 2. P300 sample. Pz latencies: N100 = 106 ms; N200 = 192 ms; P300 = 348 ms. Pz amplitude N2P3 = 19.4 µV.

Results

Twenty-eight participants with a mean age of 22.6 years (SD = 2.3 years), with an approximate 57% male – 43% female distribution, were included in this study, and this ratio was maintained in the constitution of all groups. Mean age per group was 21.9 ± 1.9 years for the iTBS group, 23.7 ± 2.5 years for the cTBS group, and 22.0 ± 2.3 years for the sham group. None of the initial volunteers dropped out or reported any major side effects. In Figure 2, we can observe a sample of the P300 wave recording from one of the volunteers.

Pre- and post-stimulation assessment results (P300, TMT, and SCWT) for all groups are shown in Table 1. For P300, results shown represent the main topographic representation (Pz).

Mean P300 latency pre-stimulation ranged between 244.0 ± 31.7 and 248.7 ± 45.6 ms, but post-stimulation results showed a greater difference between the fastest group (234.1 ± 31.6 ms for the iTBS group) and the longest P300 group (282.2 ± 76.6 ms for the cTBS group). ERP (N2P3) mean amplitude oscillated between 4.2 ± 3.5 µV before the stimulation session and 3.8 ± 2.0 µV in the recording made immediately after stimulation. As for N200 latencies, the shortest one was recorded in the iTBS group before stimulation (167.0 ± 45.1 ms), and the longest group was the cTBS group in the post-stimulation ERP (191.6 ± 50.0 ms). Only N200 latency results showed a global worsening after the real/sham stimulation session, with the P300 latency and N2P3 amplitude revealing mixed results.

Stroop test pre-stimulation results showed that the highest mean values were obtained by the cTBS group for the "C" and estimated WC variables, but the sham group scored highest for "W" variable. As for Stroop interference, iTBS group achieved the best results. We can also see in Table 1 that in the post-stimulation evaluation, the sham group achieved the best results for all variables. It should be noted that all groups improved their results in the second evaluation (post-stimulation), except for iTBS group in the interference variable.

iTBS group obtained the best results (18.0 ± 4.3 s) in part A of the TMT test, before stimulation, whereas the sham group obtained the best result in part B (39.4 ± 17.0 s). In the post-stimulation evaluation, the best result in part A was obtained by the iTBS group (14.8 ± 2.9 s) and

Table 1. Descriptive analyses of response variables. Stimulation (pre and post) and stimulations types (groups)

Variables	Groups	Pre-stimulation mean ± SD	Post-stimulation mean ± SD
P300 latency Pz, ms	A – excitatory, *N* = 9	248.7±45.6	234.1±31.6
	B – inhibitory, *N* = 10	245.4±39.1	282.2±76.6
	C – sham, *N* = 9	244.0±31.7	243.7±28.3
N2P3 amplitude Pz, μV	A – excitatory, *N* = 9	5.9±4.9	5.0±2.8
	B – inhibitory, *N* = 10	4.1±2.8	3.7±1.3
	C – sham, *N* = 9	2.6±1.1	2.6±0.9
N200 latency Pz, ms	A – excitatory, *N* = 9	167.0±45.1	180.0±26.0
	B – inhibitory, *N* = 10	178.0±31.0	191.6±50.0
	C – sham, *N* = 9	170.1±22.2	183.1±22.1
Stroop P, seg	A – excitatory, *N* = 9	97.2±14.6	105.8±16.8
	B – inhibitory, *N* = 10	103.2±10.3	107.9±14.4
	C – sham, *N* = 9	105.7±9.9	112.1±9.1
Stroop C, seg	A – excitatory, *N* = 9	78.0±11.8	82.4±11.4
	B – inhibitory, *N* = 10	78.4±11.3	81.9±12.1
	C – sham, *N* = 9	76.6±11.2	85.0±11.5
Stroop PC estimated, seg	A – excitatory, *N* = 9	43.1±6.1	46.1±6.2
	B – inhibitory, *N* = 10	44.4±4.9	46.3±6.3
	C – sham, *N* = 9	44.2±4.9	48.0±4.4
Stroop interference, seg	A – excitatory, *N* = 9	8.0±3.6	6.4±5.1
	B – inhibitory, *N* = 10	2.6±10.7	7.8±11.3
	C – sham, *N* = 9	5.0±7.8	8.3±7.4
TMT part A, seg	A – excitatory, *N* = 9	18.0±4.3	14.8±2.9
	B – inhibitory, *N* = 10	20.9±4.9	16.2±4.8
	C – sham, *N* = 9	18.4±4.2	15.1±3.7
TMT part B, seg	A – excitatory, *N* = 9	45.2±16.5	31.4±10.7
	B – inhibitory, *N* = 10	45.1±17.0	30.9±7.7
	C – sham, *N* = 9	39.4±17.0	29.7±10.1

TMT, trail making test.

the best score in part B was attained by sham group (29.7 ± 10.1 s). All groups improved their score in the post-stimulation step.

Table 2 shows global statistical analysis of the stimulation and group effects, as well as the interaction stimulation-group effect. In Table 3, we can see pair comparisons of the pre-post stimulation mean differences.

ERP result analysis showed a significant result (p = 0.001) in the interaction stimulation-group variable and a significant amplitude group effect (p = 0.026), as can be seen in Table 2. Still, group comparisons in Table 3 showed a significant mean difference only in P300 latency for the cTBS group (p < 0.001), with slower latency peaks emerging in the post-stimulation recordings. Group comparisons showed no significant results for N200 latency or ERP amplitude.

Table 2 also shows global pre-post significant results in all Stroop components in the stimulation variable, except for the interference. As can be seen in Table 3, all groups showed a significant difference between pre- and post-stimulation periods, regarding the W and WC estimated variables, with better results in the post-stimulation period. However, *C* variable results were not significant in the cTBS group (p = 0.079), in contrast with what occurred with the iTBS and sham groups (p = 0.037 and p < 0.001, respectively). Finally, the mean difference in the "interference" variable was only significant in the cTBS group (p = 0.025). When analysing the results for the

Table 2. Stimulation and type of stimulation (group) effects and interaction stimulation versus type of stimulation (group) – mixed factorial ANOVA

Variables	Mixed factorial ANOVA		
	stimulation pre-post *p* value	between-subjects effects (group effect) *p* value	interaction stimulation-group *p* value
P300 latency Pz	0.171	0.480	**0.001**
N2P3 amplitude Pz	0.538	**0.026**	0.889
N200 latency Pz	0.066	0.737	0.717
Stroop W	<**0.001**	0.458	0.195
Stroop C	<**0.001**	0.991	0.455
Stroop WC estimated	<**0.001**	0.844	0.312
Stroop interference	0.089	0.832	0.111
TMT part A	<**0.001**	0.460	0.431
TMT part B	<**0.001**	0.781	0.602

TMT, trail making test. Figures in bold indicate significance ($p < 0.05$).

TMT test before and after stimulation, all groups showed significant differences, again with better results after the TBS session.

Mean differences between groups for pre- and post-stimulation are shown in Table 4. The only significant result when mean results are compared among groups was found in the post-stimulation comparison between the iTBS and sham groups ($p = 0.028$), with shorter mean difference between groups.

Discussion

In our group of TMS naïve young volunteers, we found that a single session of cTBS over the left prefrontal cortex can influence both P300 and Stroop test, by slowing P300 peak latencies and significantly changing the expected cTBS group performance in Stroop C and Stroop interference compared to the iTBS and sham groups. In contrast, no changes associated with any of the TBS sessions occurred in TMT performance. Assessment of the neuropsychological test results showed that all study groups had a significant tendency towards improving their performance. During testing, minor side effects were reported and no dropouts occurred. In this context, our study brings novel data suggesting that neurophysiological and some neuropsychological performances can be similarly influenced by the same TBS protocol in normal volunteers.

In our study, both TMT and STWC tended to improve significantly in the second test in all groups, including the sham group. This test-retest behaviour is known and has been reported in several published studies as "the learning effect." This effect apparently develops when a neuropsychological test is repeated within a short period of time [38–40]. It is important to emphasize that the groups showed no significant baseline differences in their characteristics or performance. As expected, the evaluation of our sham group showed an improvement in performance between testing and retesting. Results found in iTBS and cTBS groups evaluated with TMT A and B, Stroop W and Stroop WC also confirm a significant improvement after the second test, in agreement with the learning effect hypothesis. In contrast, cTBS group response in Stroop C did not follow the significant improvement of the results recorded in the sham and iTBS groups. The cTBS group also behaved differently in Stroop interference, with a stronger improvement compared to the sham group and with opposite performance compared to the iTBS group.

The Stroop test is based upon 2 sets of data: verbal fluency (W and C variables) and lability (WC variable), with lability being the capacity to answer independently when comparing with previous answers [17, 41]. The "C" variable in the cTBS group did not achieve a significant improvement, in contrast with the iTBS and sham groups. This task, related to reading and verbalization of colours, can probably be more affected by left hemisphere inhibition, thereby accounting for the worse result of the cTBS group compared to the other groups. MacLeod and MacDonald [16] state that in the Stroop test there is an asymmetry of the Stroop effect – the interference effect – since words interfere in colour naming, but not the reverse, concluding that reading words is more automatic than naming colours [16]. We found the highest interference result in the cTBS group, and it is possible that the impaired left hemisphere performance may have led to a left-right hemisphere imbalance, by enhancing right hemisphere competences. This result may contradict the trend defending that there is a left hemisphere dominance related to interference in naming the colour of a word printed in an incorrect word (e.g., the word "blue" printed in green) [42, 43], but these assumptions may not be so linear. In 1993, Bench et al. [44] already stated that the interference task was associated with right frontal activation, so hemisphere dominance in Stroop testing is not a consensus topic in literature.

In a recent literature review, Banich [45] claimed that the Stroop effect results from a cascade-like process, in which different anatomical areas are activated in se-

Table 3. Pre-post evaluations – mean differences for each response variable and for each group – pair comparisons

Variables	Group A iTBS		Group B cTBS		Group C sham	
	mean differences post-pre	*p* value[1]	mean differences post-pre	*p* value[1]	mean differences post-pre	*p* value[1]
P300 latency Pz, ms	−14.6	0.124	36.8	**<0.001**	−0.3	0.971
N2P3 amplitude Pz, μV	−0.8	0.474	−0.3	0.764	0.1	0.962
N200 latency Pz, ms	12.9	0.179	13.6	0.137	4.0	0.672
Stroop W, seg	8.6	**0.001**	4.7	**0.041**	6.4	**0.010**
Stroop C, seg	4.4	**0.037**	3.5	0.079	8.4	**<0.001**
Stroop WC estimated, seg	3.0	**0.002**	1.9	**0.035**	3.8	**<0.001**
Stroop interference, seg	−1.6	0.499	5.2	**0.025**	3.3	0.163
TMT part A, seg	−3.2	**0.002**	−4.7	**<0.001**	−3.3	**0.001**
TMT part B, seg	−13.8	**<0.001**	−14.2	**<0.001**	−9.8	**0.009**

LSD, least significant difference; TBS, theta-burst stimulation; cTBS, continuous TBS; iTBS, intermittent TBS. [1] LSD test.

Table 4. Mean differences between groups for pre and post-stimulation

Variables	Mean differences A–B	*p* value[1]	Mean differences A–C	*p* value[1]	Mean differences B–C	*p* value[1]
P300 lat. Pz i, ms	3.3	0.997	4.7	0.992	1.4	1.000
P300 lat. Pz f, ms	−48.1	0.155	−9.6	0.973	38.5	0.315
N2P3 amp. Pz i, μV	1.8	0.584	3.2	0.144	1.4	0.733
N2P3 amp. Pz f, μV	1.3	0.371	2.4	**0.028**	1.2	0.455
N200 lat. Pz i, ms	−11.0	0.865	−12.1	0.839	−1.1	1.000
N200 lat. Pz f, ms	−11.7	0.860	−3.2	0.997	8.5	0.940
Stroop W i, seg	−6.0	0.625	−8.4	0.364	−2.5	0.958
Stroop W f, seg	−2.1	0.983	−6.3	0.713	−4.2	0.885
Stroop C i, seg	−0.4	1.000	1.4	0.991	1.8	0.980
Stroop C f, seg	0.5	0.999	−2.6	0.956	−3.1	0.920
Stroop WC Est. i, seg	−1.3	0.937	−1.1	0.964	0.2	1.000
Stroop WC Est. f, seg	−0.2	1.000	−1.9	0.872	−1.7	0.892
Stroop int. i, seg	5.4	0.398	3.0	0.824	−2.4	0.887
Stroop int. f, seg	−1.4	0.979	−1.9	0.952	−0.5	0.999
TMT A i, seg	−2.9	0.432	−0.4	0.996	2.5	0.569
TMT A f, seg	−1.4	0.824	−0.3	0.997	1.1	0.911
TMT B i, seg	0.1	1.000	5.8	0.854	5.7	0.852
TMT B f, seg	0.5	0.999	1.8	0.971	1.2	0.989

TMT, trail making test; i, pre-stimulation; f, post-stimulation. [1] LSD test with Sidak's correction.

quence. A left-hemisphere language area activation is mostly seen when confronting the congruent and incongruent trials using word and colours [45, 46], and a right hemisphere activation is also seen, depending upon task demands. Studies have shown that network hubs with right regional activation (inferior frontal sulcus and anterior insula) can be found in higher demand Stroop protocols [45, 47]. The idea that lateralization was linked to the task was already reported suggesting that the right prefrontal cortex assumes a more preeminent role when attentional control is need in order to reduce conflict [48]. However, using rTMS and measuring reaction time, contradicting results were found with Stroop tests: Kerns et al. [49] found no left hemisphere dominance for the cognitive control implementation but Vanderhasselt et al. [48, 50], using a protocol that also involved rTMS, showed that the left hemisphere activation could improve Stroop task performance. It is important to emphasize that these

findings were related to reaction time only. Using high-frequency rTMS, stimulating either the right DLPFC or the left DPFC, the same team only found results in the reaction time of the volunteers with no significant result in the Stroop interference effect [50, 51].

Using other techniques, such as functional near-infrared spectroscopy, bi-hemispheric activation was also found for the Stroop test with a congruent-incongruent word-colour task [52] and for a Stroop-test task based on spatial trails [53]. When comparing results from different techniques, we have to remain cautious: for instance, functional imaging is able to show changes with some delay compared to more immediate functional assessment techniques such as functional near-infrared spectroscopy or even evoked potentials.

We believe that the results found in our study were most likely originated by a real left hemisphere inhibitory effect through cTBS, affecting volunteer cognitive functions, thus counteracting the expected learning effect that we found in the remaining groups. This left hemisphere impairment effect induced by TBS affects neuropsychological tests like Stroop, TMT, and even the P300 differently. Our results in the Stroop test may also be explained by the notion that the right hemisphere not only processes the whole stimulus [42] but is linked to a more complex protocol or even that it may be more involved in the decision-making procedure [42, 45, 47]. It is also important to mention that our protocol is a Portuguese Stroop test adaptation, with emphasis on the number of hits and not on reaction time [37]. Likewise, a direct comparison with other studies should be carried out with caution because our protocol only used incongruent trials without congruent trial presentation and without congruent-incongruent trial comparison.

It is known that DLPFC is an area of tremendous relevance in the formation and regulation of brain function associated with P300, Stroop, and also TMT tests [54–56]. The fact that we found different test results after stimulating the same left DLPFC with excitatory or inhibitory stimulation suggests that this region has a different weight on each specific test. The left DLPFC seems to have a more direct influence on neural networks allocated to P300 and Stroop C, being mainly influenced by inhibitory stimulation. The P300 protocol used on these volunteers revealed a significant increase in P300 latency only after cTBS, with no significant change after iTBS. This left hemisphere lateralization may also be linked to a greater capacity of the left hemisphere to influence dopamine release, either by lowering or promoting its release depending upon stimulation characteristics (inhibitory vs. excitatory) [57, 58].

Dopamine is known to influence both event-related potentials and task performance testing [59, 60]. Lower P3 latencies and faster reaction times were also found by Evers et al. [61] after excitatory stimulation of the left PFC (only), again suggesting a leftward susceptibility to being more easily influenced by TMS. Lowe et al. [56], in a 2018 systematic review evaluating TBS targeting the prefrontal cortex of healthy subjects, found a significant effect in modulating executive functioning associated with stimulation, suggesting that cTBS decreased performance. They also found that these effects were larger if the left PFC was used. Our results also support the notion that left frontal cTBS may originate changes in both neurophysiological and neuropsychological testing results. Which factors are involved in this biased response is still unknown, thus emphasizing the need for more research to determine the factors that may lead to such behaviour.

Our results also show that a single iTBS session on the left hemisphere appears to have little ability to modulate or influence cognitive functions assessed by P300, N200, TMT, and Stroop tests. This result supports the hypothesis that the inhibitory capacity of cTBS appears to be superior to the excitatory ability of iTBS, as suggested in the previous studies with various forms of assessment [3, 62, 63].

These results highlight the importance of mixed evaluation using neuropsychological and neurophysiological tools in the evaluation of research findings and clinical results related to the use of transcranial magnetic stimulation in several diseases that may impair cognitive processing. Isolated evaluations such as response time, although objective, do not allow to assess how long it takes for a stimulus to be encoded in the brain. The partial similarity of P300 behaviour and Stroop test found in our results supports the notion of a common cognitive pathway between the two tests. It is also important to note that no major side effects were reported with our stimulation protocol, following findings described in the literature for TBS, as we had no dropouts and only a few volunteers mentioned short-term headaches and negligible focal pain during stimulation.

One of our study limitations is the relatively small number of volunteers for each group, which may have limited the statistical strength of the tests used. Another possible limitation is related to the fact that the duration of the stimulation effects has not been evaluated, a process that was difficult to implement given our study design. Replication of this study should be performed with a larger number of subjects, in order to try to achieve a more robust result. The duration of the TBS effect on

these tests should also be evaluated in the future, especially if a multiple session protocol is used. It would be also interesting to monitor volunteers in these types of studies with an anxiety scale in order to control the possible influence of this parameter in naïve subjects. We may assume that agitation or concentration difficulties originated by study procedures could have interfered with the subjects due to the novelty of the experiment. Finally, identification of the DLPFC could have benefited from the use of a neuronavigation tool, not available in our laboratory.

In summary, in spite of a small number of volunteers and a learning effect due to test repetition, our study showed that when an inhibitory stimulation is applied on the left hemisphere, an impairment of this hemisphere's functions is observed, but these effects do not seem to affect or influence long-latency evoked potentials and neuropsychological tests similarly. Our results suggest that when trying to evaluate magnetic stimulation success as a therapeutic tool, researchers should always opt for a battery of multiple tests, sensible enough to detect the expected clinical improvement.

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Statement of Ethics

This study complies with the guidelines for human studies and was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. All procedures were approved by the Faculty of Health Sciences UBI Ethics Committee (No. CE-FCS-2011-001).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Author Contributions

N.P. and M.V.P conceived and supervised all work. N.P., M.V.P., and M.D. wrote the main manuscript text. N.P., H.G., R.S., and M.D. conducted the experiment(s). Statistical analysis conducted by J.G. All authors analysed the results and reviewed the manuscript.

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Appendix C

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Theta burst stimulation over the prefrontal cortex: effects on cerebral oximetry and cardiovascular measures in healthy humans

Nuno Pinto [a, b], Helena Gonçalves [a], Ricardo Silva [a], Marta Duarte [a], Jorge Gama [a, c], Maria Vaz Pato [a, b]

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Highlights

- Left hemisphere cTBS originated a significant reduction in frontal cortex oximetry;
- Right hemisphere cTBS originated a significant reduction of systolic mean pressure;
- TBS seems to influence both oximetric measures and autonomic dependent responses;

Abstract

Theta Burst Stimulation (TBS) is a non-invasive neurophysiological technique, able to induce changes in synaptic activity. Research suggests that TBS may induce changes in cerebral oxygenation, cerebral blood flow, blood pressure and heart rate but there are conflicting results across studies. Thus, the objective of our sham-controlled study is to evaluate if TBS applied to the dorsolateral prefrontal cortex (DLPFC) of healthy volunteers produces changes in cerebral oximetry, heart rate and blood pressure. Forty-nine volunteers of both sexes were randomly allocated to one of five stimulation groups. Before and after real TBS or sham stimulation, blood pressure, heart rate, and cerebral oxygenation of the volunteers were measured. Cerebral oxygenation values were obtained with a near infra-red spectroscopy system. We found a significant reduction in left cortex oximetry after continuous TBS (cTBS) over the left DLPFC ($p = 0.039$) and a non-significant reduction in right cortex oximetry ($p = 0.052$). Right hemisphere inhibition (using cTBS) seemed to originate a significant reduction of 8 mmHg in systolic arterial pressure. No other changes were seen in oximetry, cardiac frequency and diastolic arterial pressure. In our group of normal subjects, cTBS applied to the left DLPFC was able to reduce oxygenation in the left cortex. Right hemisphere inhibition was associated with a significant reduction in systolic pressure.

Keywords

Transcranial Magnetic Stimulation; Theta Burst Stimulation; Near infrared spectroscopy; Oximetry; Blood pressure

Appendix D

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Can theta burst stimulation safely influence auditory hearing thresholds in healthy young adults?

Nuno Pinto [a,b,*,1], Iris Oliveira [a,1], Joana Ferreira [a,1], Jorge Gama [a,c,2], Maria Vaz Pato [a,d,1]

[a] CICS-Health Sciences Research Centre, University of Beira Interior, Covilhã 6200-506, Portugal
[b] Dr. Lopes Dias School of Health – Polytechnic Institute of Castelo Branco, 6000-767, Portugal
[c] University of Beira Interior – Department of Mathematics, Covilhã 6200-506, Portugal
[d] Sousa Martins Hospital, Guarda Local Health Unit, Guarda 6300-858, Portugal

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HIGHLIGHTS

- Theta-burst stimulation (TBS) of auditory cortex was not associated with hearing impairment of other side effects.
- Intermittent TBS (iTBS) of auditory cortex resulted in lowering of hearing threshold.
- This effect occurred most prominently at 500 Hz and 4000 Hz.

ABSTRACT

Objective: This TBS sham-controlled study aimed to evaluate the effects of intermittent TBS (iTBS) and continuous TBS (cTBS) upon ipsilateral hearing thresholds after stimulation on the left auditory cortex.
Methods: Sixty healthy adults, aged between 19 and 32 years (median of 23 years), were randomly distributed into three groups and underwent iTBS, cTBS or sham stimulation. Each double-blind experimental session comprised two pure tone audiometric evaluations per subject, before and after stimulation. To assess volunteer safety, a follow-up of at least 48 hours was implemented.
Results: The iTBS group mean thresholds displayed a tendency to decrease after stimulation, predominantly in the 500 Hz–6000 Hz interval and group comparisons revealed significant differences between the iTBS and sham groups for 500 Hz ($p = 0.041$) and between the iTBS and cTBS groups for 4000 Hz ($p = 0.038$). Neither relevant side effects nor any significant hearing threshold impairment after active or sham stimulation were found.
Conclusions: A single stimulation session led to an effective neuromodulation of the auditory cortex, reflected in lower thresholds when using iTBS.
Significance: These encouraging results with this safe noninvasive tool suggest that iTBS may have the potential to positively influence hearing thresholds.

1. Introduction

Repetitive transcranial magnetic stimulation (rTMS) is a neuromodulation tool, capable of influencing neural networks through the application of repetitive and patterned stimuli (Wassermann and Zimmermann, 2012; Lefaucheur et al., 2014). It can be used in several clinical applications, and is a promising technique for the treatment of auditory related disorders such as tinnitus, auditory hallucinations, and hearing loss (Rossi et al., 2009; Mennemeier et al., 2013; Schraven et al., 2013). However, noise levels achieved with the coils at higher intensities have the theoretical ability to impair hearing if long stimulation procedures

* Corresponding author at: University of Beira Interior – Faculty of Health Sciences, Avenida Infante D. Henrique, 6200-506 Covilhã, Portugal.
E-mail addresses: nfcpinto@gmail.com (N. Pinto), a30345@fcsaude.ubi.pt (I. Oliveira), a21912@fcsaude.ubi.pt (J. Ferreira), jgama@ubi.pt (J. Gama), mariavazpato@gmail.com (M. Vaz Pato).
[1] University of Beira Interior – Faculty of Health Sciences, Avenida Infante D. Henrique, 6200-506 Covilhã, Portugal.
[2] University of Beira Interior – Department of Mathematics, Rua Marquês D'Ávila e Bolama, 6201-001 Covilhã, Portugal.

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are used (Rossi et al., 2009; Andoh and Zatorre, 2011; Schraven et al., 2013). Accordingly, exposure to excessive noise during stimulation, with sound levels that can exceed the 120 dB barrier, poses a health risk concerning possible sensorineural hearing loss, underlining the importance of using hearing protection (Schraven et al., 2013). So far, studies following safety guidelines suggest that rTMS is relatively safe and well-tolerated (Rossi et al., 2009). Even in short duration sessions discomfort, minor hearing losses and hypersensitivity to noise have been described, but rapidly disappear in most cases (Rossi et al., 2009; Schönfeldt-Lecuona et al., 2012; Schraven et al., 2013; Lefaucheur et al., 2014; Zhang and Ma, 2015).

Theta burst stimulation (TBS) is an optimized rTMS paradigm, using significantly shorter duration sessions and lower stimulation intensities (Huang et al., 2005; Cárdenas-Morales et al., 2010). TBS paradigms may be capable of inducing more pronounced and enduring effects in cortical excitatory and inhibitory phenomena when compared with rTMS (Cárdenas-Morales et al., 2010; Clavagnier et al., 2013). TBS benefits from shorter duration protocols (typically 40–190 seconds for TBS vs around 30 minutes for rTMS), achieving similar therapeutic efficacy (namely in depression) (Blumberger et al., 2018), allowing better time management in laboratory application (Huang et al., 2007, 2009; Bakker et al., 2015). These effects are attributed to changes in synaptic strength associated with long-term potentiation and long-term depression phenomena induced by a single TBS session (Cacace et al., 2018; Tse et al., 2018). However, the exact neural mechanism that underlies the auditory cortical modulation and the possible degree of cortical reorganisation remains unknown (Jäncke et al., 2002; Zhang and Ma, 2015).

Hearing related disorders have been studied with rTMS/TBS and treatment protocols have been developed, especially in tinnitus, both in human and animal studies (Lefaucheur et al., 2014; Zhang and Ma, 2015; Mulders et al., 2016). Interventions are based on the premise that primary and secondary auditory cortices can be modulated and that the stimulation has the ability to promote cortical plasticity (Lefaucheur et al., 2014; Zhang and Ma, 2015; Mulders et al., 2016). Auditory cortical stimulation must comply with the anatomical specificities of this area. Human ear is able to discern a spectrum of frequencies between 20 Hz and 20000 Hz and these are spread according to a tonotopic distribution which, in the primary auditory cortex (PAC), occurs identically in both hemispheres in Heschl's gyrus, with bilateral ear representation. Thus a unilateral intervention may modulate this frequency range, with results being dependent on correct PAC targeting (Pérez-González and Malmierca, 2014; Gardumi et al., 2017; Yuan et al., 2018). Although promising, scientific evidence in this area requires a greater number of studies in rTMS and especially in TBS in order to ensure patient hearing safety, particularly relevant in the ear closer to the coil, and to identify the most effective protocols to effectively intervene.

With this TBS sham-controlled study in a group of healthy young adults, we aimed to assess ipsilateral hearing safety after TBS exposure over the left PAC and also to evaluate the effects of both iTBS and cTBS over the ipsilateral hearing thresholds.

2. Methods

2.1. Subjects and study design

Sixty healthy adults agreed to participate in this prospective double-blind sham-controlled study, recruited among students enrolled at the Faculty of Health Sciences, University of Beira Interior (FHS-UBI), Covilhã, Portugal. After answering a confidential screening questionnaire, students were included in the study if they met the following inclusion criteria: age between 18 and 35 years with no hearing complaints. Exclusion criteria were as follows: altered initial pure tone audiometry, previous ear diseases, tinnitus or other hearing related complains, brain injury or suspected diagnosis of organic brain damage, previous severe head trauma, epilepsy or convulsions, presence of major medical illness (including neuropsychiatric diseases), recent intake of any drugs or medication, pregnancy, implanted devices or foreign metal articles in the head or chest areas, sleep deprivation, alcoholism and history of drug abuse (Rossi et al., 2009). Participants were instructed to rest as usual, avoid being exposed to excessive noise and avoid taking alcoholic beverages or other toxic/stimulant substances 24 hours prior to the application of the technique.

Volunteers were randomly allocated to three equally sized separate groups according to stimulation type: intermittent TBS group (iTBS group), continuous TBS group (cTBS group), and sham group (placebo stimulation), with 20 volunteers per group. A sealed envelope randomisation protocol was used.

After being fully informed about all procedures, subjects signed a written informed consent and anonymity was ensured. Study protocols were approved by the Faculty of Health Sciences-UBI Ethics Committee (CE-FCS-2011-001), in conformity with the Declaration of Helsinki.

2.2. Theta burst stimulation (TBS)

TMS protocols were performed in accordance with safety and ethics recommendations of the 2009 guidelines (Rossi et al., 2009), in the FHS-UBI TMS laboratory, using a MagVenture MagPro1G3 X100 5.0.1, with a Dantec™ Keypoint.net v.2.03 for motor threshold determination. All procedures were carried out under medical supervision. Using a butterfly coil MCF-B70, stimuli were applied according to classic TBS protocols, using biphasic pulses, with a total of 600 pulses sent in 3 pulse bursts, repeated at 5 Hz, either in iTBS or cTBS. In continuous mode, bursts occurred without interruption for 40 seconds, and in the intermittent mode bursts were delivered only for 2 seconds (sets of 10 bursts), repeated every 10 seconds for a total of 190 seconds (Huang et al., 2005). Coil handle was positioned parallel to the midline (Lefaucheur et al., 2012).

Stimulation target site was the left primary auditory cortex and it was found for each individual: in order to set the stimulation coil over the left PAC, a procedure based on the 10/20 international system for electrode placement was used to find that specific site. Starting from the T3 position, we measured 2.5 cm towards Cz (following the coronal plane) and then measured 1.5 cm posteriorly, perpendicular to the plane T3-Cz (Langguth et al., 2006; Lorenz et al., 2010; Minami et al., 2011; Schecklmann et al., 2011). The primary motor cortex (PMC) was used as a marker for stimulus intensity and was identified by the single pulse vs visible thumb-twitch relation. Active motor threshold (AMT) was defined as the lowest stimulation intensity over the left PMC capable of inducing a consistent contralateral *abductor pollicis brevis* (APB) motor evoked potential (150–200 μV), while maintaining minimal voluntary contraction, on more than half of the pulses applied (Huang et al., 2005; Di Lazzaro et al., 2008; Sandrini et al., 2011). For real stimulation over the left PAC, intensity was defined as 80% AMT. For sham stimulation, the same coil was used, maintaining scalp contact, using a 90-degree tilted position (magnetic field pointing downwards), also emitting sound of randomly cTBS or iTBS, simulating actual stimulation, even though this technique is not capable of effective neural activation (Di Lazzaro et al., 2008; Rossi et al., 2009; Sandrini et al., 2011). Subjects were instructed to use disposable earplugs (Ohropax® Germany - noise reduction rating of 22–27 dB; 125–8000 Hz) during active or sham stimulation. All volunteers were relaxed, seating in a comfortable reclining armchair

during active or sham stimulation and stimuli application was always performed by the same technician.

2.3. Threshold audiometry

Audiological measurements were implemented in a noise isolated room. Standard pure tone audiometry was performed using a calibrated clinical screening audiometer – MAICO Audiometer GmbH®, ST 20 model (steps of 10 dB) – evaluating the following frequencies: 250 Hz, 500 Hz, 1000 Hz, 2000 Hz, 3000 Hz, 4000 Hz, 6000 Hz and 8000 Hz. The audiometry protocol followed the Guidelines for Manual Pure-Tone Threshold Audiometry from the American Speech-Hearing-Language Association (ASHA) (Campbell et al., 2005).

2.4. Experimental design

Preceding every volunteer participation, procedure explanation was presented and informed consent was obtained, followed by audiological evaluations and TMS stimulation. Therefore, each experimental session comprised two audiometric evaluations of the left ear per subject (stimulation ipsilateral ear), before and after real or sham stimulation: a pre-TBS audiometry and a post-TBS audiometry. TBS or sham protocols were applied immediately after the first audiometry, following the protocol mentioned earlier (AMT determination and PAC stimulation). In order to standardise procedures and minimise changes in immediate TBS sound impact, the second audiometry always occurred 5 minutes after the end of the TBS/Sham stimulation. All experimental sessions took place at the same time of the day and each volunteer was subjected to only one session of real or sham TBS over the left PAC, according to his/her previous randomised group allocation. Both volunteer and team member that performed the audiometry were blind to the stimulation type used (cTBS, iTBS or Sham). Audiometries and stimulation sessions were always performed in two completely separate rooms, and only the team member in charge of performing the TBS/Sham session was aware of the actual stimulation type (sham, iTBS or cTBS) applied to each volunteer. The researcher responsible for the audiometry had no information about what type of stimulation was performed. None of the volunteers had been previously submitted to rTMS/TBS and was not aware of the stimulation type performed, thus contributing to the blinding method success.

In order to assess volunteer safety and control eventual side effects, a follow-up of at least 48 hours was implemented (focusing on self-reported unwanted effects).

2.5. Statistical analysis

Statistical analysis was performed with IBM® SPSS Statistics® 25.0, using a mixed-design repeated measures ANOVA. ANOVA assumptions were verified using the Shapiro-Wilk normality test and the Levene test, allowing the latter to evaluate homogeneity variance. Due to sample size and the fact that the normality assumption was not validated, analysis was also performed with a non-parametric version of repeated measures ANOVA (Nonparametric Longitudinal Data in Factorial Experiments, with the "nparLD" package, version 2.1, for the statistical program R). However, since results obtained with both analyses were similar and compatible, we opted to present only the results obtained by the parametric version, which can be more easily interpreted. Only one repeated measures ANOVA with a single repetition (post) and one factor (group) was used. There is no random effect other than that of the volunteers. For comparisons between the intensity average pairs of the iTBS, cTBS and Sham groups or for the pre and post TBS, the Sidak test (Student's t-test for independent samples or paired – Least Significant Difference-Sidak's correction) was used for each group. Hypotheses tests were considered significant when test value (p-value, p) did not exceed the significance level of 5% ($p < 0.05$).

3. Results

Among the 60 volunteers (median age of 23 years) who agreed to participate in this study, 44 (73.3%) were females aged 19–28 years and males between 21 and 32 years. Sex and age distributions showed no significant differences (Mann-Whitney U, $p = 0.773$), with a median age of 23 years in both sexes and similar averages of 23.10 years (SD = 1.96 years) and 23.87 years (SD = 2.85 years) for the female and male sex, respectively.

The iTBS group consisted of 14 females and 6 males; in the other two groups, distribution consisted of 15 females and 5 males. Age distribution of the three groups was not significantly different (Kruskal-Wallis, $p = 0.273$), the age medians in the three groups were 23 years and averages were approximately 23.30 years (SD = 0.73 years), 23.85 years (SD = 3.03 years) and 22.65 years (SD = 2.23 years) in the iTBS, cTBS and Sham groups, respectively.

Due to the reduced number of male volunteers in each group, it was decided not to consider the influence of sex on the variables related to the audiometry.

During the stimulation procedure, no incidents occurred and in the 48 h hour follow-up, only two volunteers reported mild focal discomfort related to the cTBS stimulation site and one volunteer submitted to iTBS mentioned a mild headache. No other major adverse events were reported and none of the volunteers dropped out.

Pre-TBS audiometry and post-TBS audiometry mean threshold intensities per stimulation group are shown in Fig. 1.

Regardless of the group, mean auditory thresholds of all the volunteers evaluated for the range of frequencies tested showed that the highest threshold was found at 250 Hz (17.33 ± 7.78 dB) and the best (lowest) thresholds were found between 2000 Hz (9.17 ± 9.49 dB) and 6000 Hz (3.50 ± 6.84 dB), as previously described in other studies (Johnson, 2012). Fig. 1 also seems to show that group behaviour was not similar, with the iTBS group results displaying a trend towards a threshold decrease after stimulation, mainly for the 500 Hz–6000 Hz interval (mean difference between −2 and −4dB), except for 8000 Hz, in which there was a slight increase (+0.5 dB). The cTBS group showed mixed results, with slight threshold increases in 2000 Hz and 8000 Hz (mean difference between +0.5 and +2 dB), slight decreases after stimulation in the 250 Hz, 500 Hz, 1000 Hz and 3000 Hz (mean difference between −0.5 and −2.5 dB), and unaltered thresholds in the 4000 Hz and 6000 Hz. We can also observe that the sham group did not show a clear trend, with variations between 0.5 and 1.5 dB, except for the 8000 Hz, in which there was a decrease (−4.5 dB). Moreover, it should be noted that when an increased threshold occurred after active TBS or sham, these variations were of small degree.

Table 1 presents the stimulation effect and interaction regarding group type (repeated measures ANOVA) and Table 2 shows the pre-TBS audiometry and post-TBS audiometry mean difference for each group (iTBS, cTBS, and sham).

Pre-post iTBS group threshold mean difference showed statistically significant differences in frequencies between 500 Hz and 4000 Hz (500 Hz $p < 0.001$; 1000 Hz $p = 0.026$; 2000 Hz $p = 0.005$; 3000 Hz $p = 0.004$; 4000 Hz $p = 0.004$), with lower thresholds after stimulation. No significant differences were found in cTBS group threshold mean differences. Sham group results showed no statistically significant differences between 250 Hz and 6000 Hz. How-

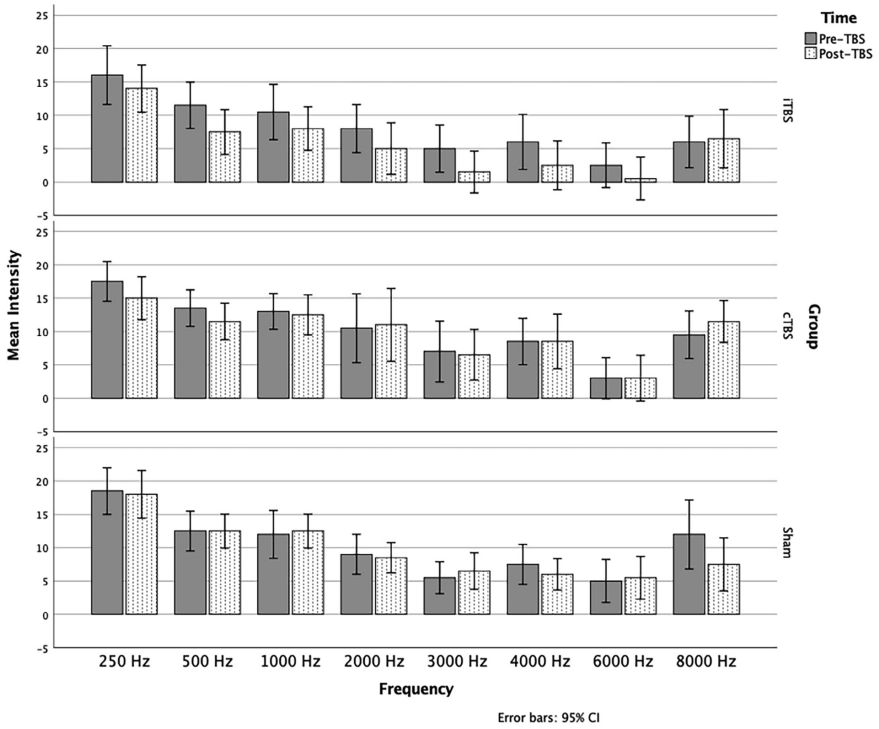

Fig. 1. Audiometry mean threshold intensities (dB HL) per frequency (Hz) and stimulation group.

Table 1
Stimulation effect and interaction versus group type – univariate repeated measures ANOVA.

Frequency (Hz)	Pre vs post TBS *p-value*	Between groups effect *p-value*	Interaction stimulation-group *p-value*
250	**0,025**	0.349	0,506
500	**0,001**	0.197	**0,028**
1000	0,192	0.193	0,152
2000	0,098	0.280	0,055
3000	0,140	0.248	**0,026**
4000	**0,015**	0.169	0,107
6000	0,470	0.187	0,298
8000	0,489	0.197	**0,019**

$p < 0.05$.

ever, results in the 8000 Hz of the sham group revealed a significant difference (p = 0.009).

Group comparisons between the pre-TBS audiometry and post-TBS audiometry mean differences are shown in Table 3.

Baseline audiometry records showed no statistically significant differences between the iTBS, cTBS and sham groups, at any of the evaluated frequencies, thereby revealing no inconsistencies between groups at baseline.

On the other hand, post-stimulation results showed statistically significant mean differences between the iTBS and sham groups for 500 Hz (p = 0.041) and also between the cTBS and iTBS groups for 4000 Hz (p = 0.038).

As can be seen in Table 3, and also in Fig. 1, none of the stimulated groups had a significant worsening of the mean threshold, after active or sham stimulation, supporting that the technique is safe to use, as long as you use adequate protection.

4. Discussion

Our study, using a sham-controlled protocol, revealed neither relevant side effects nor any significant hearing threshold impairment of the ipsilateral ear after iTBS, cTBS or sham stimulation over the PAC, thereby contributing to better understanding the possible safety limitations in these protocols. Further analysis showed that iTBS seems to have a greater capacity to influence hearing thresholds when compared with cTBS and sham stimulation, resulting in lower thresholds after stimulation between 500 Hz and 4000 Hz. Direct group comparison showed significantly lower thresholds at 500 Hz after iTBS compared to Sham and at 4000 Hz also after iTBS compared to cTBS stimulation. Our data suggest that this specific TBS method can be a safe approach to influence hearing sensitivity through non-invasive neurostimulation.

One of our main objectives was to assess hearing safety of the ipsilateral ear after exposure to one session of TBS over the left primary auditory cortex. Even though rTMS and TBS stimulation may involve some health side effects, they are considered safe tech-

Table 2
Audiometry results: pre-TBS vs post-TBS mean difference for each group.

Frequency (Hz)	iTBS group		cTBS group		Sham group	
	Intensity: mean diff. pre-post (dB HL)	p-value[1]	Intensity: mean diff. pre-post (dB HL)	p-value[1]	Intensity: mean diff. pre-post (dB HL)	p-value[1]
250	2.0	0.116	2.5	0.051	0.5	0.691
500	4.0	**<0.001**	2.0	0.056	0.0	1.000
1000	2.5	**0.026**	0.5	0.649	−0.5	0.649
2000	3.0	**0.005**	−0.5	0.629	0.5	0.629
3000	3.5	**0.004**	0.5	0.668	−1.0	0.392
4000	3.5	**0.004**	0.0	1.000	1.5	0.198
6000	2.0	0.098	0.0	1.000	−0.5	0.676
8000	−0.5	0.763	−2.0	0.231	4.5	**0.009**

$p < 0.05$.
[1] LSD test with Sidak's correction.

Table 3
Group comparisons: mean differences between the pre-TBS audiometry and post-TBS audiometries.

Frequency (Hz)	Intensity: mean diff. cTBS-iTBS	p-value[1]	Intensity: mean diff. cTBS-Sham	p-value[1]	Intensity: mean diff. iTBS-Sham	p-value[1]
250[i]	1.5	0.908	−1.0	0.970	−2.5	0.683
250[f]	1.0	0.964	−3.0	0.495	−4.0	0.251
500[i]	2.0	0.715	1.0	0.951	−1.0	0.951
500[f]	4.0	0.134	−1.0	0.942	−5.0	**0.041**
1000[i]	2.5	0.655	1.0	0.966	−1.5	0.897
1000[f]	4.5	0.082	0.0	1.000	−4.5	0.082
2000[i]	2.5	0.739	1.5	0.927	−1.0	0.977
2000[f]	6.0	0.098	2.5	0.748	−3.5	0.506
3000[i]	2.0	0.802	1.5	0.904	−0.5	0.996
3000[f]	5.0	0.079	0.0	1.000	−5.0	0.079
4000[i]	2.5	0.663	1.0	0.967	−1.5	0.900
4000[f]	6.0	**0.038**	2.5	0.641	−3.5	0.363
6000[i]	0.5	0.994	−2.0	0.740	−2.5	0.587
6000[f]	2.5	0.603	−2.5	0.603	−5.0	0.082
8000[i]	3.5	0.539	−2.5	0.770	−6.0	0.118
8000[f]	5.0	0.171	4.0	0.344	−1.0	0.974

$p < 0.05$.
[i] Pre-stimulation.
[f] Post-stimulation.
[1] LSD test with Sidak's correction.

niques, and major risks when applying these techniques following accepted safety protocols are negligible, both in children and adults (Rossi et al., 2009; Oberman et al., 2011; Kukke et al., 2017). Special attention should be taken when undergoing PAC stimulation because secondary effects can occur both from neural stimulation and from noise related to rTMS at higher intensities (Rossi et al., 2009). Although higher stimulation can achieve the 120 dB SPL threshold, thereby risking exposure to excessive noise and possible sensorineural hearing loss, sound levels usually do not go above 60–70 dB SPL (Schraven et al., 2013). While some studies found hearing impairment related to cochlear effects (Tringali et al., 2012), some tinnitus patients reported worsening of hyperacusis after rTMS (Rossi et al., 2009), or headaches, tinnitus worsening and increased sensitivity to noise after TBS (Plewnia et al., 2012), other studies did not report any hearing decline or significant complaint after 20 sessions of TBS stimulation (Schraven et al., 2013). The temporal cortex is not a frequent location where to apply TBS, but mixed results concerning secondary effects after few stimulation sessions in that location can be found. Poreiz et al., used a 3 session TBS (iTBS + cTBS + imTBS) protocol in tinnitus patients and reported complaints of discomfort, headaches and three patients suffered a worsening in tinnitus-related complaints (Poreisz et al., 2009). On the other hand, De Ridder et al., applied one session of modified cTBS in 46 tinnitus patients and reported no significant side effects (De Ridder et al., 2007). Our results showed neither significant global threshold increase after either iTBS, cTBS or sham stimulation at all tested frequencies, nor other relevant side effects (such as tinnitus or perceived hearing loss). These results are particularly relevant since our objective was to test the ipsilateral ear immediately after stimulation, in order to evaluate the ear closest to the stimulation coil and more likely to reveal any changes linked to excessive noise from the coil. The fact that there was no threshold worsening, none of the volunteers mentioned any hearing related complaints and none of the volunteers dropped out, suggests that TBS stimulation over PAC can be a safe procedure if safety guidelines are followed. Furthermore, we observed an encouraging trend towards threshold reduction in some frequencies with iTBS. In terms of side effects, these can be considered negligible (Rossi et al., 2009), because our volunteers described only two cases of mild focal discomfort and one case of mild headache related to active stimulation. Our data clearly support the scarce information to date that TBS can be a safe technique when applied to PAC, suggesting good auditory tolerance.

The other main objective focused upon the study of the auditory effects of both iTBS and cTBS in ipsilateral hearing thresholds using a placebo-controlled protocol, after stimulating the left PAC. Regarding auditory information processing, evaluated by positron emission tomography or functional magnetic resonance imaging, it is important to mention the apparent existence of a left hemisphere dominance, either at rest (Geven et al., 2014) or after auditory stimulation of only one (pure-tone or speech) (Millen et al., 1995) or both ears (Bernal et al., 2004). Thus, we opted to primarily

stimulate the left cortex because it seemed to be the hemisphere in which we would probably have the higher chance of influencing hearing capabilities, either in terms of improving the hearing thresholds, or in terms of inducing a negative change related to the stimulation procedure. TMS modulatory capacity has been tested and proven in auditory related research. Inhibitory properties using the temporal or temporoparietal cortices were found in studies using schizophrenic patients with auditory hallucinations (reduction) and especially studying patients with tinnitus stimulating the hypermetabolic areas (PAC stimulation for tinnitus reduction) (Langguth et al., 2006). TBS experiments, though scant and mostly using cTBS protocols, have been used to treat or improve tinnitus symptoms, namely hearing thresholds, but results have been controversial. Positive results using one stimulation session over the auditory cortex (De Ridder et al., 2007) and 20 sessions over the auditory temporoparietal cortex (Soekadar et al., 2009) showed improved tinnitus symptoms. In contrast, Poreisz et al., and Plewnia et al., showed no significant positive results when stimulating the temporoparietal cortex, but concluded that the protocols used were safe (Poreisz et al., 2009; Plewnia et al., 2012). Disparities between stimulation protocols and study designs can explain these results as some studies did not use placebo/sham-controlled designs and used diverse protocols – different session numbers, different stimulation locations and slight differences in used intensities (De Ridder et al., 2007; Poreisz et al., 2009; Soekadar et al., 2009; Plewnia et al., 2012). Evaluation of our results revealed a statistically significant reduction in mean hearing thresholds between the 500 Hz and 4000 Hz interval for the iTBS group after stimulation. However, for the 250 Hz, 6000 Hz and 8000 Hz frequencies, there was no significant change in the thresholds. Since we are working with groups with a relatively small number of volunteers per group and our audiometer only operates in 10 dB steps, these results should be evaluated in direct comparison with the cTBS and Sham groups. Group comparison using Sidak test showed significantly lower thresholds only at 500 Hz ($p = 0.041$) comparing iTBS vs Sham groups (mean threshold in the Sham group remained stable and the iTBS threshold significantly decreased) and at 4000 Hz ($p = 0.038$) when comparing iTBS vs cTBS groups (mean threshold in the cTBS group remained unaltered and iTBS threshold significantly diminished). In addition to highlighting the safety of the technique, these results suggest that iTBS may influence auditory capability, by specifically decreasing auditory thresholds at some frequencies. Cortical modulation processes and respective synaptic hearing circuits are still to be clarified in their totality; however, our results can be understood on the basis of probable neuron modulation in distinct zones of the tonotopic map of the primary auditory cortex. Since the observed significant changes are located at different frequencies but mostly between 500 Hz and 6000 Hz, they are also supported by the hypothesis of stimulated neurons integrating different sound frequencies among themselves. In the PAC, tonotopic organisation manifests itself equally in both hemispheres, in the form of two gradients in Heschl's gyrus, which make up a pattern of high, low and high frequencies again (Gardumi et al., 2017; Yuan et al., 2018). Even if the stimulation coil has a slight target area deviation, it is believed that this can be within an acceptable margin of error because it is known that figure-8 coils can produce a magnetic field directly over an area extending around 3 cm of length and 2 cm of width (Langguth et al., 2006). Thus, we can consider that even if there are some deviations, stimulation will still be performed in PAC although probably more focused on low to mid frequency areas. This would account for more effective results seen with some frequencies but not with all. Perhaps results would be more homogeneous if a neuronavigation system was be used to identify the target zone, thus promoting a more accurate stimulation of the PAC. Another limitation when analysing these results is that it is still unclear what the underlying mechanisms mediating potential iTBS benefits are, since they can be explained by more than one hypothesis. A possible hypothesis for the results is that of the cortical plasticity aptitude: functional magnetic resonance imaging (fMRI) studies have demonstrated that the auditory cortex has the capacity to reorganise and change its expression of excitatory and inhibitory neurotransmitters (Zhang and Ma, 2015). Neurotransmitter modulation by transcranial magnetic stimulation is a known outcome, namely in the upregulation (increased levels) of the excitatory neurotransmitter glutamate related to excitatory stimulation (Yang et al., 2014; Croarkin et al., 2016; Dlabac-de Lange et al., 2017), or in the down-regulation in glutamate in the left hemisphere, directly linked to a reduced loudness level of tinnitus awareness, found by Cacace et al., These authors used a 5 day inhibitory protocol over the left auditory cortex (Cacace et al., 2018). Even though we cannot confirm these theories, we believe they may explain some of our data. Our findings also support some previous studies reporting that excitatory stimulation can influence hearing, namely the work by Andoh et al., in which 10 Hz rTMS applied to the Heschl's gyrus originated improved auditory performance, but only in females (Andoh and Zatorre, 2011).

Results for the cTBS group showed that, for all frequencies evaluated, no significant statistical change ($p > 0.05$) was found in hearing thresholds after stimulation. This lack of significant results is still observed when we compare the mean differences of the cTBS group with the iTBS and Sham groups, highlighting the frequent variations in the thresholds, either decreasing or increasing. These results suggest that a single session of the cTBS protocol used in this investigation does not significantly modulate neuronal auditory activity. This indicates that effects and possible efficacy of iTBS and cTBS techniques may be distinct, at least when applied over the auditory cortex. The result for 8000 Hz obtained in the group undergoing sham stimulation, failed to reach statistical significance when compared with the results of the excitatory and inhibitory groups - thus, its significance may be negligible. The reasons that led to a threshold decrease at 8000 Hz in the sham group may only be speculated, but a placebo effect is a possibility, mainly because it is a very high frequency in the auditory spectrum, which is therefore more difficult to assess. Several studies (Chan, 2014; Požgain et al., 2014; Morral et al., 2017) support the multifactorial nature inherent to the mechanisms underlying the placebo effect; however, in this specific case, it is possible that the main mechanism may be related to an intrinsic expectation that the volunteers had that TBS could improve their hearing capabilities.

Future work should focus upon the study not only of the ipsilateral thresholds but also of the contralateral ones. Ipsilateral vs contralateral dominance and which side can be the most effective for auditory cortex stimulation can be a controversial issue because there are several contradictory studies. For instance, some studies in tinnitus gave stimulation primacy to the left cortex independently of the complaints being lateralized to the right side, to the left side or bilateral. In contrast, other studies reported better results when stimulating the contralateral cortex related to the existing complaints (Khedr et al., 2010; Lefaucheur et al., 2012; Zhang and Ma, 2015). Another limitation to our rationale is that we do not know how long iTBS effects last. It would still be interesting to study whether two daily sessions, separated by at least 15 minutes (Tse et al., 2018), could have an enhanced or more prolonged effect. It should also be mentioned that although some studies use increasing stimulus intensity, our objective was to comply with standard safety guidelines, even more so because stimulation outside the primary motor area may yield some inaccuracies related to distance-adjusted intensities (Stokes et al., 2005).

As for study design, despite all participants officially declaring that they did not take any drugs, we were not able to include in

our protocols a screening test to evaluate their presence, thus limiting our control over this experiment. Even though our design was simple and all our subjects were completely naïve regarding stimulus characteristics and effects, we should also have included a blinding assessment in order to increase result reliability.

In this area, very few studies used TBS, and most studies used the technique mainly in patients, neglecting its effects in normal healthy volunteers. No study focused on the use of both iTBS and cTBS, comparing the results with sham stimulation. Our study protocol is therefore unique and to the authors' knowledge this is the first approach to a healthy volunteer placebo-controlled research using both cTBS and iTBS over the left PAC, showing diverse effects between these two stimulation modalities, thus contributing to a better understanding of this type of noninvasive neurostimulation over the auditory cortex. Our results using only a single session point to an effective neuromodulation of the PAC, reflected in lower thresholds when using iTBS. It can be assumed that several sessions could be more effective, as most of the protocols that formed the basis of various previous studies applied several sessions (between 3 to 20 sessions) (Loo et al., 2001; Khedr et al., 2008, 2010; Plewnia et al., 2012; Barwood et al., 2013; Schraven et al., 2013; Zhang and Ma, 2015; Cacace et al., 2018), as is currently used in depression therapy. It is also noteworthy that threshold improvement occurred manly around the human speech/voice frequency range (500–2000 Hz) (Williams et al., 2005; Anjos et al., 2014). This possible hearing improvement in the low to mid frequency range can be particularly important if similar stimulation protocols can be used in sensorineural hearing loss, which is often attributed to hereditary factors and congenital conditions (Shah et al., 2005), specifically if trying to enhance patient speech perception. These interventions may aim to improve life quality in patients; however, this possible use for TBS should be approached carefully, after replication of this method in further studies with a larger number of healthy subjects and, finally, after patient investigation.

5. Conclusions

iTBS, a safe, non-invasive neuromodulation tool has the potential to positively influence hearing thresholds in healthy young adults. The same iTBS protocols amy be reproduced in older adults with minor sensorineural hearing loss, presbycusis or other hearing loss cases. This would allow improvement of patients' hearing capacity by modulating PAC to become more sensitive to the auditory stimuli, thereby helping patients to improve their auditory assessment of the world and increasing patients' quality of life.

Author contributionsss

N.P. and M.V.P conceived, supervised all work and wrote the main manuscript text. N.P., I.O., J.F., conducted the experiment(s). Statistical analysis conducted by J.G. All authors analysed the results and reviewed the manuscript.

Data availability

Data sets analysed during the current study are available on request.

Declaration of Competing Interest

There are no conflicts of interest and the authors have not received any specific grant.

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Appendix E

Direção de Marcas e Patentes
Departamento de Patentes e Modelos de Utilidade

RELATÓRIO DE EXAME DO PEDIDO DE PATENTE DE INVENÇÃO NACIONAL Nº 109800

Pedido nº 109800	**Data de prioridade:** 2016.12.22

Requerente:
UNIVERSIDADE DA BEIRA INTERIOR

Data dos documentos examinados:

Reivindicações: **2020.02.26**
Descrição: **2020.02.26**
Desenhos: **2020.02.26**

Data da pesquisa: 2017.10.20, 2020.01.03
Bases de Dados Pesquisadas: INTERNET, EPODOC, WPI, $LOGDB, PATENW, PATDEW, PATFRW

Exame
Na sequência do exame ao presente pedido de patente, sou de parecer que:

- [x] Não há objeções à concessão do pedido.
- [ ] Há objeções à concessão do pedido por:
  - [ ] carecer de novidade.
  - [ ] carecer de atividade inventiva.
  - [ ] carecer de aplicação industrial.
  - [ ] não preencher os requisitos estabelecidos nos artigos 52.º, 53.º,61.º, 62.º, 63.º, 118.º, 119.º, 124.º ou 125.º do CPI.
  - [ ] falta de unidade da invenção prevista nos artigos 71.º ou 135.º do CPI
  - [ ] outros motivos previstos nos artigos 73.º, 137.º ou 161.º do CPI.

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DIREÇÃO DE MARCAS E PATENTES
DEPARTAMENTO DE PATENTES E MODELOS DE UTILIDADE

FOLHA DE DESPACHO

Patente de invenção n.º 109800

Pedido

Requerente(s): UNIVERSIDADE DA BEIRA INTERIOR

Data: 2016.12.22

Publicado no Boletim da Propriedade Industrial n.º 2018/06/22

Oposição ❑ Sim ☒ Não

❑ Reclamação ___ / ___ / _____

❑ Contestação ___ / ___ / _____

❑ Exposições ___ / ___ / _____

Concessão ☒	**Recusa** ❑
❑ Total (nos termos do n.º 4 do artigo 70.º)	❑ Nos termos da alínea a) do nº 1 do artigo 23º
☒ Total (nos termos do n.º 7 do artigo 70.º)	❑ Nos termos do nº 7 do artigo 62º
❑ Parcial (nos termos do artigo 71.º)	❑ Nos termos do nº 5 do artigo 67º
	❑ Nos termos do nº 8 do artigo 70º
	❑ Nos termos do nº 9 do artigo 70º
Relatório de Exame em anexo ☒	❑ Nos termos do nº 1 do artigo 71º
Justificação em anexo ❑	❑ Nos termos do nº 4 do artigo 72º
	❑ Nos termos da alínea a) do nº 1 do artigo 75º
	❑ Nos termos da alínea b) do nº 1 do artigo 75º
	❑ Nos termos da alínea c) do nº 1 do artigo 75º
	❑ Nos termos da alínea d) do nº 1 do artigo 75º
	❑ Nos termos da alínea e) do nº 1 do artigo 75º
	❑ Nos termos da alínea f) do nº 1 do artigo 75º
	❑ Nos termos da alínea g) do nº 1 do artigo 75º
	❑ Nos termos do nº 3 do artigo 75º
	Relatório de Exame em anexo ❑
	Justificação em anexo ❑

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Instituto Nacional da Propriedade Industrial, 2020.05.18

O Técnico Superior,
João Marcelino

Concordo,	Concordo e defiro. Por subdelegação de competências do Conselho Diretivo
A Chefe de Departamento, Inês Cristovão da Silva	O Diretor, André Robalo

Appendix F

P300 (extended theory)

Event related potentials (ERPs), specific neurophysiological tests that allow the study of cognitive processes, are manly cerebral responses to external stimuli [1,2]. The auditory P300, also known as P3 or P3b, is the most extensively researched ERP component. It spurred the use of these neurophysiological tests to study cognition. It is known since 1965 when it was first reported by Sutton et al. [2]. Directly dependent to subject's attention and discrimination, it is considered a cognitive potential related to an event [2–4]. It has been suggested that it indexes memory storage, serving as a link between stimulus characteristics and attention [5].

It is a large, broad, positive evoked potential that typically peaks around 300 ms, resulting from the discrimination of rare, task-relevant stimulus [2]. The P300 (P3b) has a centro-parietal scalp distribution that is maximal over midline scalp sites, while the more anterior P3a as an earlier latency and a midline fronto-central maximum [2]. P300 origins are still dubious, with multiple cortical and subcortical neural generators, such as hippocampus, superior temporal sulcus, the ventrolateral prefrontal cortex, posterior cingulate cortex and the intraparietal sulcus [2,6]. Inter-hemispheric connectivity may have also an important role since activity seems to propagate through corpus callosum, after the initial frontal activation, and larger callosal fibers are associated with better P300 performance (amplitude and latency) [6].

The neurotransmitters and related processes involved in P300 formation are not yet fully understood [7]. Nevertheless, research has shown that the frontal activity is mostly mediated by dopamine, and that the posterior P300 (more related to the temporal-parietal activity) is associated to a denser norepinephrine input [7].

Auditory ERPs can be elicited by a typical oddball task, which requires subjects to recognize infrequently presented target tones that are presented randomly between frequent stimuli, either by pressing a button or counting the stimuli. This paradigm seems to initially activate frontal activity and presents a regional centro-parietal scalp distribution, peaking over midline scalp [2,6]. This type of protocol requires the activation of both attention (by selecting the deviant stimulus from the other irrelevant

stimuli) and working memory (supporting the selection by retaining the characteristics of the standard stimulus for comparison) [8].

Reflecting predominantly neural processing speed, P300 is an important tool in the study of cognitive processes and memory in normal subjects and in psychopathology, as its delay can be used as a marker in the identification of cognitive deterioration [4,6,9]. Amplitudes are usually directly associated with the amount of attentional resources assigned to the task, and seem to be more affected by the temporal-parietal junction integrity [6,10]. Amplitudes vary significantly, depending upon paradigm characteristics, but usually range between 5 and 20 μV [2,5].

Playing a less prominent role in ERP studies, the N200 also give important information in cognitive evaluation [11,12]. This negative peak arises after the presentation of a specific auditory stimulus [5]. It represents the initial, subconscious processing of the stimulus involved in the oddball task, leaving for P300 the translation of more advanced and purposeful stages of task processing. Even though it has also multiple cerebral origins, some authors found a significant lateralization towards the left anterior region of the mid-cingulate cortex [11,12].

Auditory P300 performance can be affected by several biologic factors such as fatigue and old age (both with decreased amplitude and increased latency), and gender (amplitude: female>male, latency: female<male). The variability associated with these factors should be considered when performing group comparisons [2,6]. Addressing age as an example, P300 latencies are significantly lower in younger ages compared with older individuals, where latencies can be found just over 200 ms in the 20-30 year range of healthy subjects [13]. As another example, the post aerobic exercise period after physical activity is associated with increased P3 amplitude [5]. Despite assuming some intra-individual variability, the P300 is considered a reliable evoked potential. and testing reliability is an important parameter to consider. The common oddball paradigm has revealed good test-re-test correlation coefficients for both amplitude and latency in repeated testing [14].

Beyond its relevant role in cognition research, there are several clinical applications in which P300 is able to contribute to the diagnostic procedure, especially when cognitive dysfunction is present [2,13].

Its clinical use in neurological and mental illnesses has become popular due to some relevant characteristics such as its reliability (it is considered reliable and can be

elicited with relatively simple paradigms) and its sensitivity (its latency might even be more sensitive than reaction-time events to minor changes in cognitive processing) [8].

Impaired P300 can be found in some psychiatric and neurologic diseases [2]. A recent review has also stated that patient evaluation with P300 seems a sensitive way to detect cognitive changes associated with therapies in clinical trials [15]. In dementia we may find predominantly delayed latencies [2,12]. Using the P300 latency can also help to distinguish dementia from depression-associated pseudodementia, as the latter patients present only minor delays in latency, usually associated with normal aging [14]. The use of the P300 in patients with Alzheimer's disease and cognitive impairment, especially if used in conjunction with neuropsychological tests, can be particularly useful in the assessment of population with low socio-cultural background, which may pose challenges to patient assessment [16]. In schizophrenia, we may find changes in latency but also in amplitude. This may be associated with the described fronto-temporal atrophy and impaired attention seen in patients with schizophrenia. It has also been suggested that P300 abnormalities in these patients may reflect mnemonic defects [5,14]. In patients with traumatic brain injury, the most frequently found P300 impairment relates to amplitude: auditory P300 amplitudes tend to be significantly reduced in this situation, and researchers believe that there is a relation to auditory processing deficits [14].

Diseases affecting the glycemic status also affect the P300, with slower responses found in hypoglycemia [5]. The evaluation of patients with addiction related disorders also have shown attenuated P3b amplitudes in individuals considered at high-risk for alcoholism and at-risk individuals [5].

Neuropsychological tests (extended theory)

Neuropsychological tests are essential tools for executive function assessment, evaluating aspects such as attention, working memory, cognitive flexibility or behaviour control [17]. Some of these functions can be evaluated using tests as the Stroop Test of Words and Colours (STWC) and the Trail Making Test (TMT).

Stroop Test of Words and Colours (STWC)

The STWC is a neuropsychological test that has been used for both experimental and clinical purposes, originally proposed by John Ridley Stroop in 1935 [18]. The STWC assesses executive functions such as selective attention, modulation, and inhibition, resistance to external interference and cognitive flexibility related to execution speed [18,19]. Thus, STWC can be used to measure various cognitive functions [18].

The most common SCWT implies that subjects read three different sheets as quickly as possible, reading or naming the observed colours [18]. The first two sheets imply a "congruent condition": one contains the words "green", "red" and "blue", and participants are required to read the color names printed in black ink; the second sheet contains similar elements - "XXXX" – printed in green, red and blue colours. On the third sheet, there are the words from the first sheet, printed in the colour of the second one, without correspondence between the colour of the ink and the meaning of the word [18,20].

The results are directly related to the number of words/colours verbalized in 45 seconds. Acquiring results with a fixed time of 45 seconds can be advantageous, contributing to the reduction of fatigue and/or refusal to finish the test. [18,20].

The Stroop test is based upon two sets of data: verbal fluency (word and colour variables) and lability (word-colour variable), with lability being the capacity to answer independently when comparing with previous answers [18,21]. In the incongruous condition (third sheet), participants are required to name the ink color rather than read the word, thus being asked to perform a less automated task (ie, naming the ink color) while inhibiting interference from a more automated task (ie read the word) [18]. This added difficulty in inhibiting the more automatic process is called the Stroop effect [18].

Regarding SCWT, factors such as age and gender have provided inconclusive results. There seems to be a trend that suggests a female superiority in most of the variables studied and that younger volunteers may be more subject to influence by interference [20]. Usually, middle aged volunteers commit fewer errors than younger or older participants [22].

Despite the Stroop test being widely used to assess cognitive impairment, in the case of milder impairments results may not be as evident. The study of mild cognitive impairment (MCI) typically reveals poorer behavioral execution in amnestic MCI than in controls, but sometimes no significant differences are observed [22].

Trail Making Test (TMT)

The Trail Making Test is one of the best-known tests in the field of neuropsychological assessment, being frequently included in neurocognitive test batteries [23–25]. Originally, this test was introduced by Partington to assess the ability of divided attention and later was incorporated the Halstead-Reitan Battery [26]. Although trail making tests are simple, it yields information about visual scanning, processing speed, mental flexibility, motor skills, and working memory, among other executive functions [23,24]. Despite its known popularity, there is little normative data about it [23].

The standard version of the Trail Making Test consists of two different tasks – TMT A and TMT B. Part A delivers useful information concerning attention, visual scanning and speed of eye–hand coordination [26,27]. In TMT A, the participant has to draw lines connecting the numbers in a sequence (1–2–3…). Part B evaluates with more accuracy the ability to alternate between two cognitive sets of stimuli. In this case, the participant must connect numbers and letters in an orderly and alternating numerical and alphabetical sequence (1-A-2-B…) [26,27]. In both TMT A and TMT B, the participant is instructed to finish both tasks as quickly and accurately as possible, without lifting the pen from the paper [27]. The total time each subject takes to complete each test reflects its performance.

TMT A provides a baseline measure of psychomotor speed, visuospatial search and target-directed motor tracking [27]. TMT-B performance seems to reflect higher order processes. This idea is based on studies that validated TMT-B outcome measures against commonly used tests of executive functions [27]. Performance on TMT, especially part B, decreases with lower levels of education and subjects with older age

also generally score poorer in performance, even in the absence of cognitive impairment, but this should not be taken into account in the scoring [25,26].

Although TMS is used to test executive functions, it seems less capable of detecting improvements in the effects of physical exercise on cognition compared to tests such as the Digit span forward test, Digit span backward test and Stroop Tests [28].

Factors likely to influence the results of both the SCWT and the TMT, like the learning effect, cannot be ignored. The learning effect occurs when a neuropsychological test is re-administered in a short period of time, the expected results may improve from the previous application, even if the subject reveals a cognitive deficit. This results from when the same item or test being presented to the same individual on repeated occasions or as a result of gaining experience in solving certain problems in the same way [29,30].

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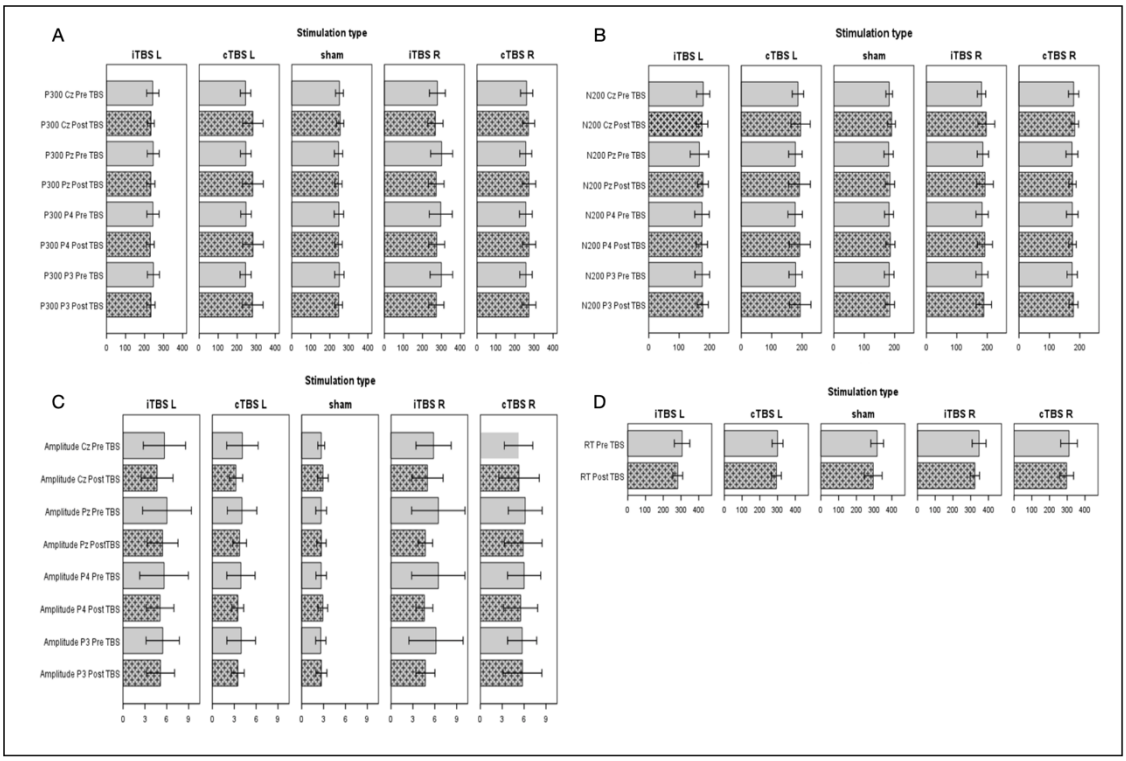

Figure 3.1 – ERP results per stimulation group. P300 latency (A), N200 latency (B), Amplitude (C) and Reaction Time (D), with confidence intervals. (Chapter III)