THE UNIVERSITY OF MANCHESTER

Unravelling the genetic basis for cortical plasticity in the human swallowing motor system

Doctoral thesis

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Abstract

Swallowing is an important physiological function leading to nourishment of the organism, controlled by complicated interactions between the muscles, the cranial nerves and multiple brain structures. Swallowing impairments, also called dysphagia are a major health burden for patients with neurological diseases such as stroke, Parkinson's disease as well as community dwelling elderly individuals. It has been shown that activation of undamaged swallowing motor cortex compensates for the initial lost swallowing function in stroke patients. Non-invasive brain stimulation provides a tool to explore excitability within the areas of the motor cortex responsible for swallowing muscles. Repetitive transcranial magnetic stimulation (rTMS) is one such technique, with defined frequency parameters, however the underlying reasons for the heterogeneity is responses to low (1Hz) and high (5Hz) frequencies is unclear. These physiological interactions affecting the neurological control of swallowing may be influenced by multiple genes and proteins. Insights into the molecular basis of swallowing through genetic interactions could provide a source of information which can be further used in understanding and treating swallowing impairments. Existing evidence is limited in terms of candidate proteins, genes and pathways which might drive the neural control of swallowing.

The aim of my doctoral research was to explore genes which might be involved in swallowing neurophysiology and pathophysiology. My hypothesis is that swallowing due to its complicated physiology is most likely affected by multiple genes and interactions between genes and proteins.

To study this hypothesis I used two experimentally distinct study designs. Firstly I explored a number of single nucleotide polymorphisms (SNPs) and potential candidate genes presented in the existing literature. Then, I performed a SNP- and gene-based Genome-Wide Association Study (GWAS) of self-reported swallowing impairments compared with over 500,000 single nucleotide changes. For GWAS I used a group of 555 community dwelling individuals from the Dyne Steel Cohort from the areas of Manchester and Newcastle. Further research involved replication of selected genes and SNPs from literature screening and GWAS using two rTMS paradigms on the largest to date cohort of healthy young volunteers. Forty one volunteers (were assessed for corticobulbar excitability after single-pulse TMS. Repeated measurements of motor evoked potentials from the pharynx and the hand were recorded after the interventions of 1Hz and 5Hz rTMS. The subjects' individual responses were grouped according to multiple criteria and then associated with factors such as gender, ethnicity, time of day of the stimulation and individual genetic information.

GWAS analysis for association with swallowing impairment identified one SNP rs17601696 which achieved genome-wide significance (*P*-value=5×10(-8)) within a non-coding region of chromosome 10. Gene-based analysis did not result in any genome-wide significant association. In replication of these findings and following a priori selected genes from the literature (*BDNF, COMT, TRKB, APOE, DRD2, GRIN2B* and *GRIN1*) from neurophysiological studies applying TMS, two main conclusions were formed. Firstly, rTMS paradigms showed high variability in responses which made the phenotype more complicated. Secondly the result from GWAS could not be confirmed. By contrast, SNP rs6269 from the *COMT* gene was associated with responsiveness of the pharyngeal MEPs after delivering 1Hz paradigm and rs1800497 from the *DRD2* gene with responsiveness after 5Hz rTMS.

Lack of replication of the findings between two experiments might be caused by high variability in responsiveness with complex molecular networks of swallowing control where multiple genes with small genetic effects are involved. Although our findings support the hypothesis that molecular markers can be associated with swallowing, more studies are needed to understand the individual factors that determine responsiveness and effectiveness of treatment therapies of swallowing impairments.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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The Author

I was awarded Bachelor's Degree in Medical Biotechnology from the Medical Academy in Bydgoszcz (2009). I have continued my biotechnological education at the Adam Mickiewicz University in Poznan, Poland. To explore my interest in human genetics I have done my Master of Science dissertation project in the Polish Academy of Sciences in the Institute of Human Genetics, where I have worked for a year after graduation. In 2012 I was awarded a studentship form the Neuroscience Research Institute at the University of Manchester.

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My research would not be possible without very helpful group of volunteers who were kind enough to sacrifice their time to participate in the research.

Dedication

I would like to dedicate my PhD thesis to my mother Iwona, my husband Lukasz and my brother Jacek.

To my mother for spending last money for English private lessons over six years of primary school, for being the best example by gaining higher education as the first woman in my family and for being a working mother who did not and still do not accept ‘no’ for an answer when it comes to her children wellbeing.

I would like to dedicate this work to my husband, who has been my strongest motivator with the enormous patience over the last 10 years. This is the way I would like to thank for seeing and nurturing only the best of me and believing in me even when I did not believe in myself.

To Jacek, for showing me how to worry less, enjoy the life more, be good to people and in the end succeed.

List of abbreviations

APB	Abductor Pollicis Brevis
APOE	Apolipoprotein E
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
BDNF	Brain Derived Neurotrophic Factor,
CAMKII	Ca2+/calmodulin-dependent protein kinase II
CHARGE	A mnemonic for coloboma, heart defects, choanal atresia, retarded growth and development, genital abnormalities, and ear anomalies
CN	cranial nerve
CDT	conventional dysphagia therapy
COMT	Catechol-O-Methyltransferase
CPG	Central Pattern Generator
DNA	deoxyribonucleic acid
DRD2	Dopamine receptor D2
DZ	dizygotic twins
EAT-10	Eating assessment tool
EMG	Electromyography
FEES	Fibreoptic endoscopic evaluation of swallowing
FMRI	Functional Magnetic Resonance Imaging
GRIN1	Glutamate Receptor, Ionotropic, N-Methyl D-Aspartate 1
GRIN2B	Glutamate Receptor, Ionotropic, N-Methyl D-Aspartate 2B
GWAS	Genome-Wide Association Studies
ICMS	Intracortical microstimulation
LD	Linkage Disequilibrium
LTD	Long-term Depression
LTP	Long-term Potentiation
MEG	Magnetoencephelography
(P)MEPs	(Pharyngeal) motor evoked potentials
MZ	monozygotic twins
NMDA	N-methyl-D-aspartate receptor
NMES	neuromuscular electrical stimulation
NIHSS	National Institutes of Health stroke scale
NIRS	Near-infrared spectroscopy
NGT	Nasogastric feeding tube
PAs	Penetration-aspiration scale
PEG	Percutaneous Endoscopic Gastrostomy
PES	Pharyngeal Electrical Stimulation
PET	Positron emission tomography
PTLS	Potocki-Lupski syndrome
rMT	Resting Motor Threshold
rTMS	Repetitive Transcranial Magnetic Stimulation
SALTs	Speech and language therapists
SEM	Standard error of the mean
SRT	Swallowing Reaction Time
SSQ	Sydney Swallow Questionnaire
SWS	Stuve-Wiedemann syndrome
tDCS	Transcranial Direct Current Stimulation

TMS	Transcranial Magnetic Stimulation
TRKB (NTRK2)	Neurotrophic Tyrosine Kinase, Receptor, Type 2
NTKR2	Neurotrophic Tyrosine Kinase, Receptor, Type 2
UCHL1(3)	Ubiquitin Carboxyl-Terminal Esterase L1 (L3)
UOS	Upper oesophageal sphincter
VFS	Videofluoroscopy

Chapter 1

Introduction

Swallowing, also known as deglutition, is one of the rudimentary human physiological functions performed over 2000 times a day. The aim of deglutition is to cover the nutritional and hydration needs of the body. Swallowing has three different phases: oral, pharyngeal and oesophageal [1]. In the first oral stage, the bolus of food and liquid is mixed with saliva from three salivary glands (parotid, sublingual and submandibular) during the process of mastication in the oral cavity and is prepared for ingestion. From the oral cavity, the moisturised bolus travels through the pharynx into the oesophagus and eventually through the Lower Oesophageal Sphincter (LOS) into the stomach, where the swallowing process ends [2].

Understanding of the anatomy, physiology and neurophysiology of swallowing is the key step in explaining the origin and any swallowing impairments. Swallowing impairment (delay or misdirection) is called dysphagia. This PhD project focused on oropharyngeal dysphagia which is a symptom of many neurological diseases and a major health burden. Oropharyngeal dysphagia affects up to 12% of healthy elderly individuals [3], up to 55% of stroke patients [4] and up to 83% of patients with Parkinson's disease [5]. This symptom carries a serious risk for patients, and complications range from food and liquid aspiration, chocking, dehydration, malnutrition, increased of the risk of aspiration pneumonia and increased mortality rates.

Recovery from oropharyngeal dysphagia occurs and is observed in stroke patients [6], however physiological processes which are responsible for recovery are not well understood. One of the multifaceted mechanisms for recovery in stroke patients involves plasticity of the area of the motor cortex which controls swallowing musculature. Cortical plasticity can be defined as any morphological and/or functional change in cortical properties such as generation of new synapses between undamaged neurons and increasing efficacy of existing neuronal connections [7]. Neuroscientists have progressed the understanding of this phenomenon over the past three decades, because it is crucial not only in the recovery from neuronal injury, but also in human neurodevelopment, mechanisms of adaptation, learning and memory.

The process of cortical plasticity in the human motor cortex might be affected in part by genetic factors [8], thus studies at the molecular level might provide important insights in understanding these processes. Cortical reorganisation, as mentioned above, also affects recovery from swallowing impairments, however the literature about the genetic underpinnings of the neurophysiology of swallowing remains limited.

Here, I investigate a number of genetic changes which might influence human swallowing performance. Firstly, candidate genes with possible genetic associations in normal and impaired swallowing were identified from a detailed literature survey. Secondly, I conducted a Genome Wide Association Study (GWAS) of self-reported swallowing function in a cohort of older, healthy volunteers. These two sources supported the core experimental work using inhibitory and excitatory repetitive transcranial magnetic stimulation (rTMS) of human brain cortex regions responsible for deglutition in the cohort of healthy young volunteers, who have also agreed to provide DNA samples for genotyping of candidate markers.

In the following sections of this introduction, the background to the swallowing process, anatomy, physiology, pathophysiology are described along with methods of studying the neuronal network for swallowing and swallowing impairments, treatment and management of dysphagia and brief overview of genetic underpinnings underlying swallowing. I then review the genetic background of the neuronal control of swallowing from both human and animal studies, examine human studies on cortical plasticity following neurostimulation and assess genetic congenital syndromes with a high prevalence of dysphagia. The last part of this chapter described the principals of genetic association studies.

1.1. Anatomy and physiology of swallowing

The term swallowing describes the coordinated processes of swallowing which comprises a sophisticated series of manoeuvres between different craniofacial bones and muscles innervated by cranial nerves. Cranial nerves receive information from cortical and subcortical brain areas within an extensive neuronal network responsible for swallowing.

1.1.1. Anatomical structures involved in swallowing

Starting in the oropharynx, the first structure of importance in swallowing is the palatine, which consists of both the palate and the floor of the nasal passages [9]. Paired, multidimensional maxillae bones create a framework of mid-face, hard palate, oral cavity and nasal cavity. The hyoid bone plays a significant role during deglutition, creating a 'lever' for muscles and mouth components [9]. Other bones create a place for muscles attachment. The temporal bone forms the footing for the temporamandibular joint (TMJ). The mastication process is accomplished with the movement of the mandible and TMJ which allows three-dimensional movement of the jaw (elevation and depression, protraction, retraction and lateralization).

The swallowing process requires multiple facial and neck muscle movements. The facial muscles are responsible for lips, mouth and mandible movements, facial expressions and cheek flattening. All these muscles are innervated by the facial nerve (Cranial Nerve or CN VII). The muscles responsible for lip movements are the orbicularis oris, the zygomaticus minor (also known as the smiling muscle), the levator labii superiozygomaticus major, the depressor labii inferior and the mentalis. The muscles of lip angle movements comprise: the levator labii superior, the levator anguli oris, the depressor anguli oris and the risorius. The buccinator muscle is responsible for food holding in contact with teeth and retraction of mouth angles.

Mastication is the process of mandibular movements that involve muscles such as the temporalis, the masseter, the medial pterygoid and the lateral pterygoid. All muscles of mastication are innervated by the trigeminal nerve (CN V).

The palatal muscles are responsible for the soft palate, the uvula and the back tongue movements. Among muscles of the soft palate are the levator veli palatini (innervated by the vagus nerve-CN X and the accessory nerve- CN XI) and the tensor veli palatini (innervated by the CN V). A further three muscles linked to this action are innerved by the CN X and the CN XI: the palatoglossus (raising the back of the tongue), the palatopharyngeus (closes the nasopharynx) and the uvulae (raising and shortening the uvula).

The group of suprahyoid muscles play a role in the oral and pharyngeal stages of deglutition. The first muscle is the mylohyoid (CN V) which is responsible for tongue and floor of mouth elevation and jaw depression while the hyoid bone is in a fixed position. Next, the digastric muscle (CN V) raises the hyoid bone and depresses the jaw while the geniohyoid muscles draws the hyoid bone and depresses the mandible and the stylohyoid muscle elevates the hyoid and tongue base. Other muscles from this group are: the genioglossus (CN XII) muscle (responsible for protrusion and depression of the tongue and sucking), the styloglossus (CN IX) and the palatoglossus (CN X and CN XII) which raises the back of the tongue and lowers the soft palate.

The cricopharyngeus muscle (CN X) has a specific and important role in the swallowing process which is functionally and anatomically separating the pharynx and the oesophagus. This plays a role of the sphincter and relaxes the Upper Oesophageal Sphincter (UOS) when the bolus is entering the oesophagus [2].

The key cartilaginous structure during deglutition is the cricoid cartilage, which is located in inferior position to the thyroid cartilage in the neck, and is joined medially by the median cricothyroid ligament and postero-laterally by the cricothyroid joints.

1.1.2. Motor and physiological events of swallowing

Swallowing process consists of three main stages oral, pharyngeal and oesophageal [1]. Studies showed that all three phases are independent from each other [10], but can be inhibited and completed by each other. This means the pharyngeal stage can be performed without the oral stage and the oesophageal stage can be achieved without activating oral or pharyngeal stages [10].

Oral stage

The first, oral stage may be separated in two phases: the preparatory and the oral phase. The preparatory phase starts when food is transferred through the mouth and tongue in the oral cavity, followed by mechanical preparation and break-down of the food by mastication and fragmentation with the teeth. During the oral, voluntary [10] stage, the bolus is placed on the tongue from where it is propelled to the oropharynx [11, 12]. As the result, the soft palate is

raised, allowing the nasopharyngeal seal with the uvula closing the entrance to the nasal cavity (*Figure 1* a). The movement of the bolus is achieved through the integrated contraction of the tongue and hyoid muscles pulling the hyoid bone.

Pharyngeal stage

The pharyngeal phase is a continuation of the oral phase where several concurrent events take place. The epiglottis is pulled downwards to protect the airways, the vocal folds close, the larynx is elevated, while the bolus enters to the pharynx (*Figure 1.1.* b,c). These mechanisms causing apnoea, protect from the penetration and aspiration of food into the airway tract [13]. Simultaneously the pharynx widens and shortens which causes the elevation of the UOS. Posterior wall of the tongue pushes the pharynx, closing the nasopharynx. Simultaneously the laryngeal complex elevates upwards. Apart from gravitational forces, striated muscles of the pharynx generate the peristaltic wave which the progressive contraction is enabling the bolus to go through the UOS and the oesophagus. The velocity of this stage lasts 40cm/sec through the pharynx by the force of peristaltic movements of the pharyngeal muscles. When the bolus reaches the end of the pharynx, the muscles of the UOS relax as a result of complex series of actions such as laryngeal elevation and, peristaltic waves and gravitational pressures [14]. These actions enable entrance of the bolus into the oesophagus [12].

Oesophageal stage

Finally the UOS opens and the bolus passes to the last oesophageal stage of deglutition (*Figure 1* d). When the whole bolus is in the oesophagus, the epiglottis returns to the previous erected position and airways are reopened. In the smooth muscles of the oesophagus the next peristaltic wave occurs [15]. The wave of muscles contractions is immediately followed by inhibition of the contraction which is also called the latency gradient [16]. Peristaltic contraction waves push the bolus with the speed of 3-4 cm/sec [16]. When the tail of the bolus goes through the oesophagus, the UOS closes and once the bolus passes through (the UES closes within 1 sec) to the lower oesophageal sphincter (LOS), the swallowing phase ends.

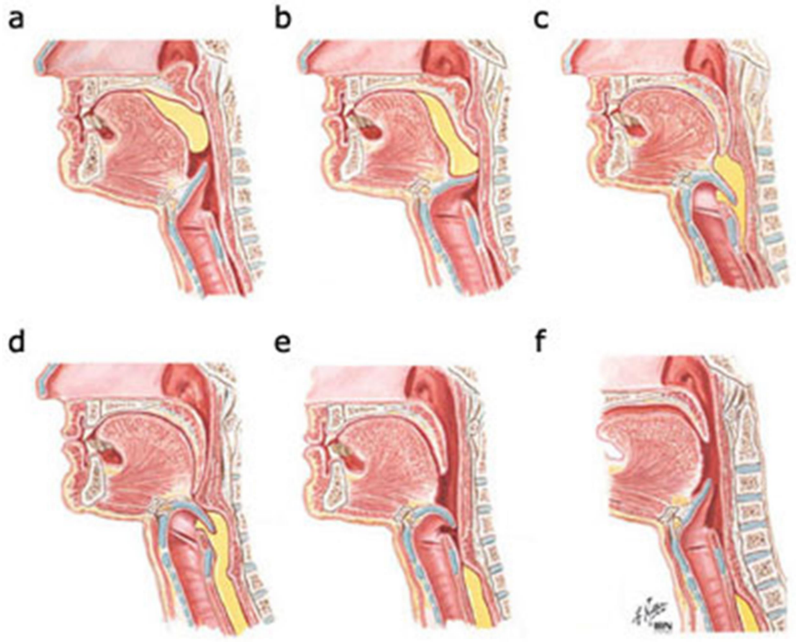

*Figure 1.1.***The motor events of swallowing the bolus** (yellow colour). Figures from a to f show the track from the oral cavity, the pharynx through the oesophagus.
Source: GI Motility online (May 2006) | doi:10.1038/gimo2

1.2. Neurophysiology of deglutition

The neural mechanisms of swallowing are highly complex and involve activation of multiple cranial nerves, the brainstem, the cerebral cortex and the cerebellum (*Figure 1.3.*).

1.2.1. Cranial nerves

Cranial nerves are responsible for afferent and efferent control of swallowing. There are five cranial nerves which take part in swallowing.

The trigeminal nerve (CN V) innervates the floor of mouth and the muscles responsible for mastication and sensation over the major surface of the tongue. The facial nerve (CN VII) which innervates the lip orifice and takes the input in taste sensation from the anterior two thirds of the tongue. The glossopharygeal (CN IX) nerve receives sensory inputs the sensation from the posterior part of tongue and innervates muscles important in the pharyngeal stage of swallowing. Along with CN X the glossopharyngeal nerve receives inputs from the pharynx, larynx and viscera. The vagus nerve (CN X) innervates not only the palate pharynx and the larynx, but also the lungs, the heart and muscles of other parts of gastrointestinal tract [17]. The CN X is the largest and the most important muscle which takes a part in swallowing process. The last nerve is the hypoglossal (CN XII) which controls tongue musculature during swallowing.

1.2.2. Brain stem

Within the medulla oblongata of the brain stem there is an area termed the central pattern generator (CPG) which plays a central role in the regulation of swallowing process [10, 18] (*Figure 1.3*). The CPG centre has two subnuclei: nucleus tractus solitarii (NTS) and nucleus ambiguus (NA). The NTS receives impulses from cranial nerves (CN V,VII, IX, X) and brainstem nuclei, whereas NA generates motor output through all cranial nerves involved in swallowing [17].

1.2.3. Cerebral cortex

Areas of the motor cortex are responsible for the volitional and non-volitional stages of deglutition such as recognition of the swallowed material and initiation. Moreover the human brain cortex has the ability to adapt to the changing environment, exhibiting neuronal plasticity (see Chapter 1.3.3).
Miller [19] in his review discussed the impact of cerebral cortex in swallowing regulation, from both human, and animal studies. Animal models remain the main contributor to our current understanding of cerebral involvement in swallowing function. The first animal studies revealing the evidence of cortical role were conducted at the beginning of 20th century [20]. The studies were conducted on rabbits and showed that stimulation of the cerebral cortex of both hemispheres affects mastication and swallowing. The authors also showed that swallowing is complex process most likely controlled by other brain regions.

Current evidence from animal studies (rodents) indicated involvement of the face primary motor area (M1) motor and sensory (S1) cortex (also termed sensorimotor cortex) in mastication and swallowing [21]. One of the functions of the facial M1 is the control of the semiautomatic functions such as swallowing (and mastication). Along with somatosensory facial S1 utilises inputs from the face and the mouth controlling motor events. Animal studies conducted on both mammals and subprimates showed neuronal plasticity within S1 and M1 followed by neurostimulation and tongue protrusion tasks [22, 23].

In human studies imaging techniques have enabled researchers to accurately define cortical areas activated during swallowing including: the primary M1S1, prefrontal cortex, anterior insula, premotor cortex, frontal operculum, the anterior cingulate, anterolateral and posterior parietal cortex, precuneus and superior-medial temporal cortex (*Figure 1.2*) [24, 25]. The cortical areas responsible for swallowing processes are located bilaterally, and show interhemispheric asymmetry, which does not depend of the handedness of the subject [26]. More studies examining cortical input in swallowing are listed in the Paragraph 1.4.5.

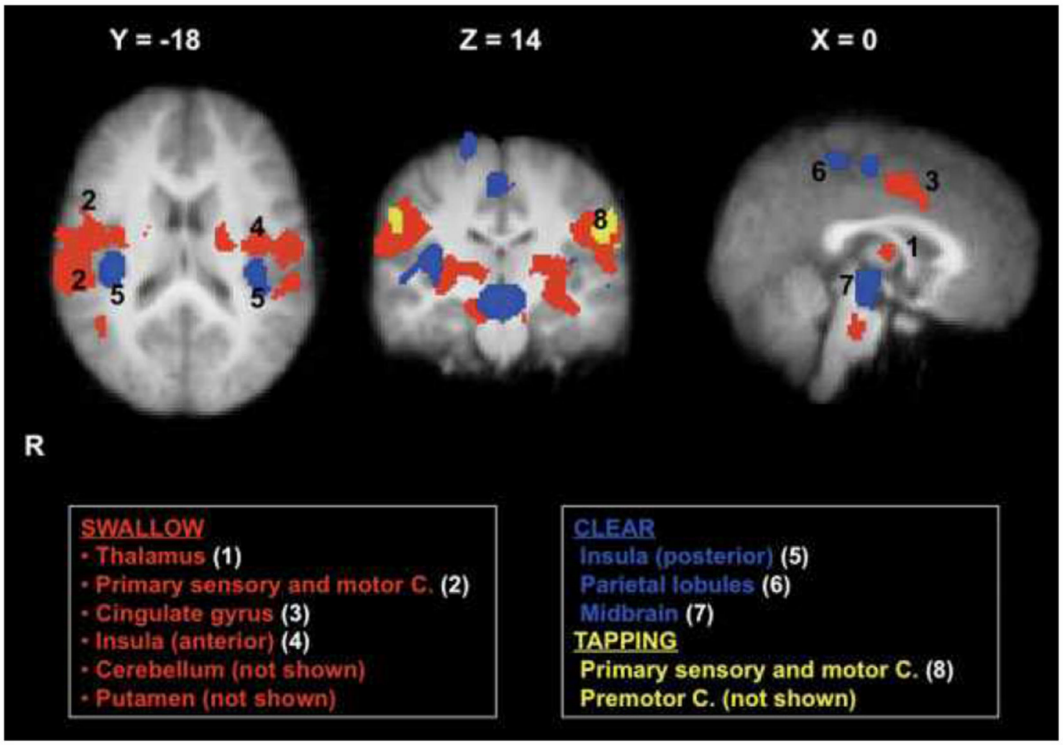

Figure 1.2. **The Figure shows areas of the strongest activation during swallowing.** (shown in red), during throat clearing (shown in blue), and during tongue tapping (shown in yellow). Boxes report the areas. Images are shown in radiological convention (the right hemisphere is shown on the left). (In: Malandraki *et al.* 2009, HBM, used with permission).

1.2.4. Cerebellum

Neuroimaging studies showed that during volitional swallowing, large areas of cerebellum are activated, for example for throat clearing task around the oral phases of swallowing [27, 28]. Neurostimulation techniques (see Paragraph 1.4.5.) were also used to study the role of cerebellum in swallowing [29]. Studies showed that magnetic stimulation of the cerebellum can evoke motor responses within the pharynx. Recent review [30], showed that the cerebellum monitors motor performance, may also play a role of intensifier neural responses and coordination of cortex execution and modulates information from different regions of the brain and passes information to M1 by functional connectivity.

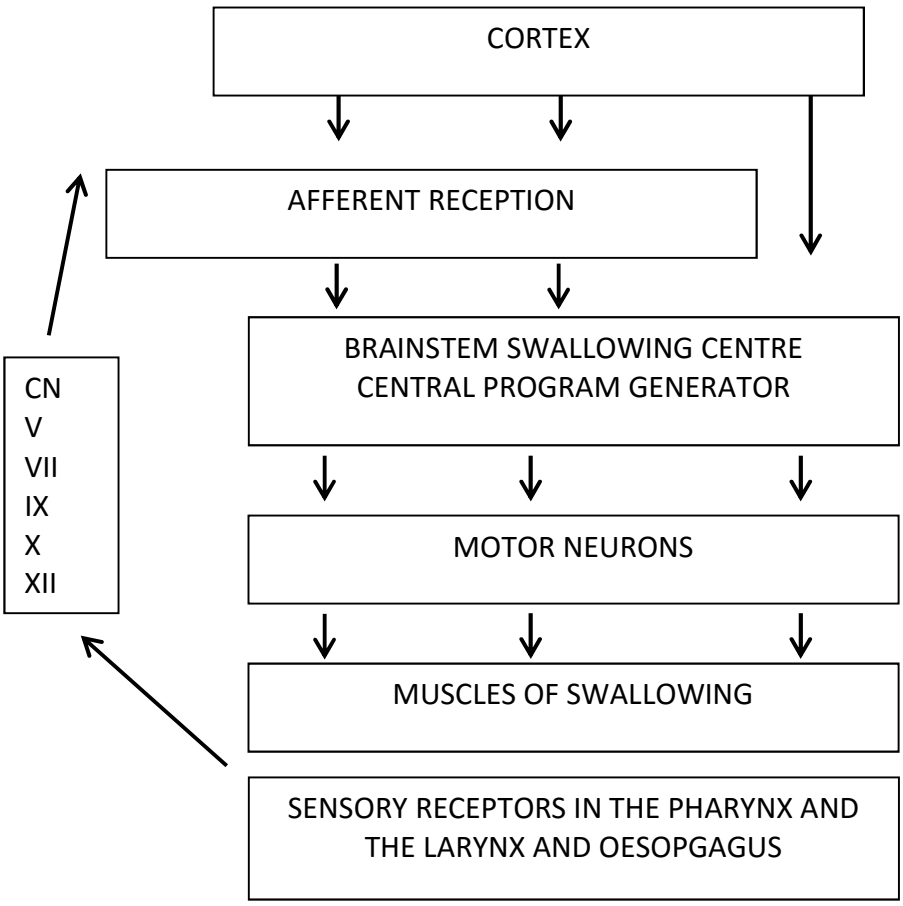

Figure 1.3. **Central regulation of sequential oral, pharyngeal and oesophageal contraction.** CN stands for Cranial Nerve in the figure. Adopted from Diamant N. [31]

Summary of the section 1.1.

Swallowing is a complicated physiological function involving integrated activity of central and peripheral nervous system with multiple facial, head and neck muscles involved. Other elements of swallowing anatomy are the submandibular joint and the hyoid bone. Swallowing process is coordinated by central pattern generator within the brain stem along with five cranial nerves (CN V, VII, IX, X, XII) which receive sensory inputs from the muscles of the oral cavity, the pharynx and the oesophagus. The areas of the cerebral cortex play important role in initiating swallowing as well as the autonomic control of deglutition.

1.3. Changes in swallowing in healthy ageing

The vast majority of studies investigating swallowing are conducted on healthy young volunteers. Replication of these findings in cohorts of elderly people requires the understanding of the changes in the swallowing performance caused by ageing. Alterations of swallowing do

not have to indicate pathological conditions, but should be considered in the study design and the interpretations of study results.

Elderly individuals develop mild, non-pathological swallowing problems without the presence of other diseases, the term is called presbyphagia [32]. Alterations of swallowing physiology, neurophysiology along with other alterations caused by non-pathological ageing affecting every stage of swallowing will be described below.

1.3.1. Anatomical and sensory changes

Dental loss and poor dentition cause problems with mastication and fragmentation of the bolus which affects other stages of swallowing [33]. Along with altered anatomy of the oral cavity other changes occur such as cartilages ossification, slight atrophy of the pharyngeal muscles and the vocal folds flaccidity and bowing [34].

Decreased sensation also affects swallowing by lowering the elicitating threshold of cough reflex which also increases the risk of aspirations [35]. Histological and morphological studies showed loss of myelinated fibres in the superior laryngeal nerve responsible for sensations for the aspired materials [36].

There are a number of motor changes affecting all phases swallowing performance in groups of elderly people compared to young healthy individuals. These include prolonged time to manipulate the bolus during the oral stage [37], which may be caused by poor dentition as well as by reduced lingual pressure [38]. Masticatory strength is significantly lower among individuals with advanced age [39].

In order to initiate the pharyngeal phase of swallowing, elderly individuals might require significantly larger portions of bolus comparing to young adults [40] and more residue of not digested bolus is observed in the pharynx [41], due to reduced cough reflex [35]. It has been described that elderly show decreased time of the cricopharyngeus muscles opening and overall delay of the pharyngeal swallow [42]. Lowered peak of the pharyngeal pressure during tongue hold swallows exercises have been observed [43].

1.3.2. Neurophysiological changes

Studies with functional Magnetic Resonance Imaging (fMRI see Section 1.4.5), revealed numerous differences between activation of the brain areas of healthy aged individuals compared to healthy young volunteers [25, 28, 41]. The studies differ in terms of brain activation areas depending on the behavioural or sensory tasks and the type of bolus changes which were examined. The first study examined the neural representations of voluntary saliva swallowing and water swallowing in older females [25]. Increased blood oxygenation was observed in multiple cortical regions such as the lateral pericentral, perisylvian, and anterior cingulate cortex during both saliva and water swallows. In the study by Humbert *et al.* [41], the effects of age,

bolus type (water saliva or barium liquid) were tested. Older subjects showed increased activity within the areas of the right and the pericentral gyri, the bilateral frontal lobe, the bilateral parietal regions and the right superior temporal gyri. The authors concluded that elderly individuals have to engage broader areas of the brain in order to initiate swallowing and increase their effort compared to the younger individuals to perform a swallow of the same bolus size and type [41]. However another fMRI study indicated that areas of the brain involved in sensorimotor control of swallowing, such as throat clearing, tapping the tongue or preparation of the bolus to swallow had decreased activation within the areas involved in sensory processing, sensorimotor integration and/or motor coordination in the elderly individuals compared to younger adults [44].

Summary of Section 1.3.

Swallowing performance is affected by numerous physiological changes caused by age. These changes in deglutition include decreased sensation, prolonged time of mastication, decreased tongue pressure, and altered sensation perception. Differences are also observed in the cortical representation of the swallowing process. More research is needed for conclusive evidence on the differences of the sensorimotor activation during swallow in older and younger groups of individuals. The studies with fMRI show differences in the activation within the sensor motor areas depending on the type of the study and bolus type. Even though described differences are non-pathological, they should be considered in the study design and analysis.

1.4. Pathology of swallowing process- dysphagia

Impairments in swallowing (dysphagia) pose a number of life-threatening complications following dysphagia include aspiration of the food and liquids to the airways, chocking, increased risk of aspiration pneumonia, malnutrition, dehydration as well as reduced quality-of-life due to increased risk of anxiety and depression. The following section will focus on oropharyngeal dysphagia caused by neurological impairment but not post-anatomical changes in the digestive track such as tumours of the oral cavity, the pharynx and the oesophagus.

1.4.1. Epidemiology of dysphagia

Dysphagia is a common symptom among elderly healthy and frail individuals. Increased number of cases with dysphagia is observed among residents of care homes (up to 52.5%) and hospital units compared with studies on individuals who live in their own homes (from 13%) [17, 45]. Another group of patients with up to 85% incidence of dysphagia are individuals with neurodegenerative disorders such as Parkinson's disease [5].

Perhaps the classic neurological condition associated with swallowing problems is stroke. The rate of dysphagia following stroke remains very high and with estimate of between 20% [46] and 63% [4, 47, 48] in different studies. The discrepancies between results published on dysphagia

after stroke may result from a number of factors including: patient demographic differences, ethnicity, age and gender; differences in stroke cause and type; method of identification and diagnosis of dysphagia and differences in study design such as interventional versus observational studies. Oropharyngeal dysphagia is often a symptom of other cerebrovascular diseases, multiple sclerosis, head injury, amyotrophic lateral sclerosis, myasthenia gravis [49].

The cost for oropharyngeal dysphagia is very high in the population of elderly adults. An estimated cost of annual economic of dysphagia is $547 million [50] in the USA. One-year cost of treatment stroke patient with oropharyngeal dysphagia in the United States was $4,510 higher than that for individuals without dysphagia post ischaemic stroke [51].

In cohorts of individuals with swallowing impairments, the risk of an adverse event is increased by a number of parameters [45, 47, 52]. Risk factors for worse outcome from dysphagia include:

a) age 75+ years [4, 53, 54]
b) aspiration [55]
c) dysarthia [55]
d) dementia [3]
e) baseline of the National Institutes of Health Stroke Scale (NIHSS) score ≥12 [55]
f) stroke features: lesion lateralization and loci of infract [4]
g) ethnicity – Japanese are more likely to suffer from stroke and poorer outcome, also from dysphagia [56, 57]
h) mental state of the patient- patients with low mood and depression are more likely to suffer for dysphagia [58].

1.4.2. Complications following dysphagia

Swallowing impairments lead to dangerous complications, where the commonest are aspiration of the material into the airways. Aspiration is the impaired transport of food and liquids below the vocal folds into the trachea and could cause pulmonary complications. The incidence of aspiration is high in the stroke patients and estimated between 22% and 52% [4]. Half of the cases of aspiration in the acute stroke patients populations are classified as ‘silent’ [59]. Silent aspiration does not cause any detectable symptoms such as coughing or changing voice which makes it more difficult to diagnose with the normal water swallowing test.

Overt or silent aspiration can give rise to the risk of pulmonary complications such as aspiration pneumonia. In her review Martino *et al.* [4] showed that patients with dysphagia have 3-fold increased risk and patients with aspiration 11-fold increased risk of developing aspiration pneumonia following stroke.

Other complications comprise malnutrition, dehydration of the patients which are again dangerous for fragile or elderly people [45].

Stroke patients with dysphagia show nearly 4 times higher likelihood to have a poorer outcome, prolonged length of stay in the hospital and increased mortality rates [60]. Apart from physical complications, patients with dysphagia have significantly lowered quality of life in the most severe cases leading to anxiety and depression causing poorer outcomes from the disease [58].

1.4.3. Swallowing impairment assessment

Detecting swallowing impairments in patients with neurological injuries or within the cohorts of elderly people remains problematic. Therefore diagnosis as well as estimating accurate prevalence of swallowing symptoms related to dysphagia within examined cohorts is challenging.

Healthcare professionals use a number of assessment tools for detecting dysphagia which comprise approaches such as videofluoroscopy (VFS), Fiberoptic endoscopic evaluation of swallowing (FEES), bedside screening tools and self-reported questionnaires. VFS and FEES are routinely used tools as 'gold standard' or first choice screening tools.

Videofluoroscopy (VFS)

VFS, modified barium swallow is a diagnostic tool which uses barium liquid or coated substances to evaluate all stages of swallowing. The procedure is performed in the radiology unit, where the patient is asked to swallow food or liquid with barium which enables tracking the bolus through the all stages of swallowing. The patient's swallowing performance is recorded in real time X-ray investigation using a video capture system. VFS test enables the study of the oropharynx, the pharynx, the larynx, and the upper oesophagus before, during and after the swallow. Assessment of these parts of swallowing is the main advantage of this screening tool comparing to other techniques. An important disadvantage is the need to expose the patient to radiation and it cannot be performed bedside.

Fiberoptic endoscopic evaluation of swallowing (FEES)

FEES uses flexible fiberoptic laryngoscope to evaluate the pharyngeal phase of swallowing. A small camera is integrated into the endocope which is then placed in the pharynx through the nose. Patients are asked to perform a number of swallows with different bolus types. The camera enables to detect aspirations, silent aspirations, residues etc. The main advantages of this method there there is no need for the radiation exposure and the device is portable so the study can be performed bedside. The main disadvantage is the inability to study other than pharyngeal stages of deglutition.

Bedside screening tools

Another popular group of screening tools are screening protocols, which are fulfilled by medical professionals (speech and language therapists, nurses, doctors) after a number of tasks given to patients. The commonest bedside screening tools for exploring swallowing impairments in acute neurological patients include: Massey Bedside Swallow Screen [61], Clinical Assessment of Swallowing [62], Toronto Bedside Swallowing Screening Test (TOR-BSST) [63]. The advantages of these are accessibility and low cost of the study. Some of the downsides are the inter-rater reliability and lowered accuracy with detecting silent aspirations.

Self-reported questionnaires

Self-reported swallowing questionnaires about swallowing symptoms related to dysphagia are especially useful in the early screening of both patients and healthy individuals in the early stages of swallowing problems. Here I describe five self-reported questionnaires most commonly used in the clinical practice and research.

- Eating assessment tool (EAT-10) [64] is a brief questionnaire consisting of 10 questions. It is widely used to detect dysphagia of different ethnologies. The main disadvantage of EAT-10 is lack of information about the symptom frequency.
- MD Anderson Dysphagia Inventory (MDADI) [65] is a 20-item questionnaire focussed on dysphagia in head and neck cancer. Commonly used, multi-dimensional, but uses complex scoring.
- Mayo Dysphagia Questionnaire (MDQ) [66] consists of 27 items limited to the oesophageal stage of deglutition. Evaluates different bolus types, includes the information about validity, duration and frequency of symptoms. The main disadvantage is complex scoring and prolonged time of completion comparing to other questionnaires. Moreover, it is not able to evaluate oro-pharyngeal dysphagia.
- Swallowing Quality of life (SWAL-QOL) [67] questionnaire is a common, 44-item self-assessment tool used in different cultures. It focuses only on oropharyngeal dysphagia and remains rather cumbersome difficult to complete quality of life.
- Sydney Swallow Questionnaire (SSQ) [68] contains 17 questions with the visual analogue scale about swallowing symptoms related to dysphagia with different ethnology. The SSQ questionnaire is designed to evaluate mechanical swallow severity in oropharyngeal dysphagia, also widely used by clinicians to measure response following treatments. Short time of completion is one of the main advantages. Repeatability is the main disadvantage of SSQ due to using analogue scale. Each question from the SSQ is scored from 0 (no problem) to 100 (severe problem). The total score from SSQ is between 0-1700.

Self-reported questionnaires provide a very useful tool in population-based studies. Patients and healthy volunteers do not need to meet professionals so fulfilling the questionnaire is less

time consuming. Studies with self-reported questionnaires are less costly. Disadvantages of self-reported questionnaires comprise the lowered accuracy with detecting major swallowing problems comparing to VFS and FEES, lack of professional assessment of swallowing and inability to detect silent aspirations which may lower the percentage of individuals affected by dysphagia.

1.4.4. Recovery patterns in dysphagic stroke patients

Despite high prevalence of patients who show post-stroke self-recovery from swallowing symptoms [69], poorer stroke outcome compared to those in whom this is delayed/absent remains a matter of concern. The mechanisms involved in neuroplasticity are one of plausible factors involved in recovery from swallowing disorders after stroke [70]. In the recovery processes stronger activity of the unaffected by stroke swallowing motor cortex was observed in 3-monts recovery from stroke (*Figure 1.4.*). The process of neuronal plasticity involves the creation of new connections, synapses between intact neuronal cells. The cortical plasticity process is suggested to be the main mechanism influencing outcome from dysphagia following stoke [4, 71].

Synaptic plasticity processes within the cerebral cortex include modification of the neuronal properties can be caused by environmental and behavioural changes. These changes might be either pathological such as ageing, brain lesion, or non-pathological such as healthy development, memory and learning [7]. The main theoretical mechanism underlying the synaptic plasticity was hypothesised by Donald Hebb in 1949 [72] The original statement was as follows:

"Let us assume that the persistence or repetition of a reverberatory activity (or "trace") tends to induce lasting cellular changes that add to its stability.… When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased."

The theory was explained in more details in future experiments by the presence of short and long term potentiation (STP and LTP) of neuronal cells. Potentiation of a neuron is the ability to manipulate the strength of synapses action potentials. Action potentials within neurons may cause either excitation or depression depending of the concentration of neurotransmitters within or outside the synapses and impact of trans-cellular receptors. Potentiation and depression depends on time of stimuli presentation and can be divided in long and short lasting. Short-term potentiation tends to last milliseconds to few minutes whereas long-term potentiation last from minutes to hours.

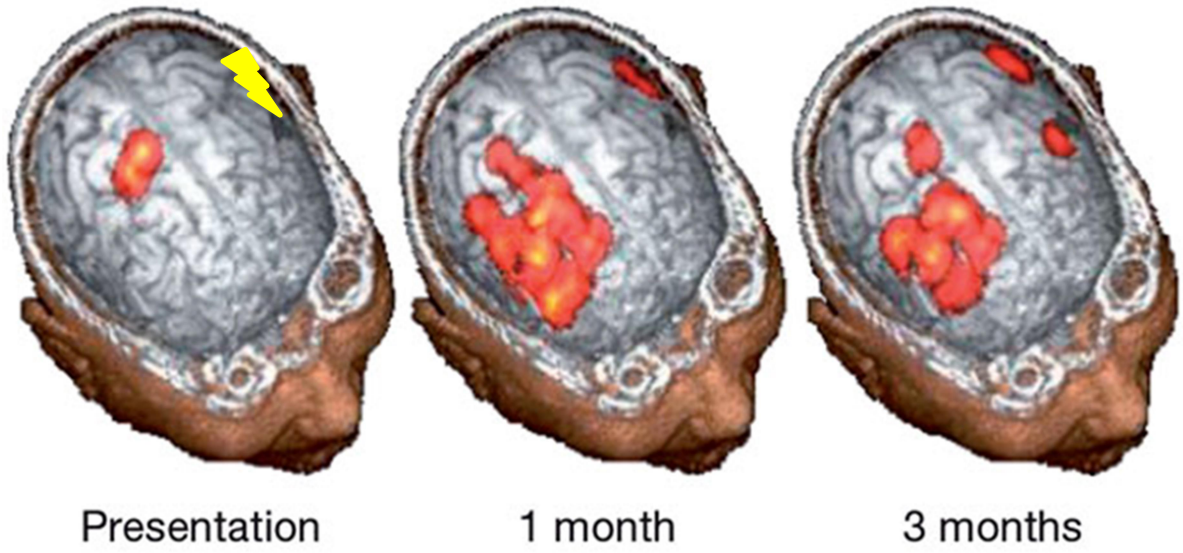

Figure 1.4. **The cortical maps show the size of pharyngeal cortical representation during recovery of a dysphagic patient from stroke.**

The affected side is marked with yellow arrow. The unaffected hemisphere showed increasing representation during 3 months recovery while the affected hemisphere showed small change. (*Source: GI Motility online* (May 2006) | doi:10.1038/gimo8)

1.4.4.1. Long term potentiation

LTP is one of the forms of synaptic activation and occurs when the presynaptic neuronal cell release neurotransmitters which activate receptors on the postsynaptic membrane increasing efficacy of the synapse. Depending of time of depolarization there are two types of LTP: early and late. Early LTP (E-LTP) tends to last from 1-3h and uses existing molecules in the postsynaptic density [73]. This type of LTP is induced by weak but high frequency tetanay. Late-LTP (L-LTP) requires de novo synthesis multiple proteins is required. This form of neuronal plasticity tends to last up to 10h and requires long high frequency stimulation. Most of the studies exploring synaptic plasticity were conducted on the hippocampus or the cerebellum, but both structures share common features which are likely to find a unifying pathway within the molecular mechanisms of cerebral cortex neuronal plasticity.

During LTP biochemical mechanisms, glutamate molecules are released to the intersynaptic space, which is followed by depolarization of the presynaptic cell. Glutamate molecules bond with the N-methyl-D-aspartate receptor (NMDA or NMDAR) which is physically blocked by Mg^{2+} ions. Simultaneously another receptor: The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (also known as AMPA receptor, AMPAR) allows for a small influx of Ca^{2+}. Both conditions have to be fulfilled: binding glutamate to NMDAR and AMPAR activity to unblock NMDARs from Mg^{2+} and allowing influx of Ca^{2+} and Na^{+} ions into the postsynaptic space *Figure 1.5*. The balance between both sides of the pre and postsynaptic membrane is crucial to retain physiological functioning of the brain.

After NMDAR opens the influx of Ca^{2+} ions, a cascade of biochemical reactions causing dephosporylation of Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) and other kinases is activated. Further biochemical changes lead to exocytosis of AMPARs to the postsynaptic membrane. Additional AMPARs inserted into the cellular membrane cause higher Na^{+} flow into the cell which increases depolarisation of the neuron.

Another stage of LTP-like plasticity is releasing energy from ATP by set of biochemical processes with different enzymes. Enzymes as all proteins are coded by specific genes and all changes within the DNA structure encoding these proteins can lead to alterations in the structure or functions of the protein affecting its efficacy. Prolonged high frequency stimulation causing L-LTP leads to increase in protein synthesis including F-actin which is used to build neuronal cells and synapses.

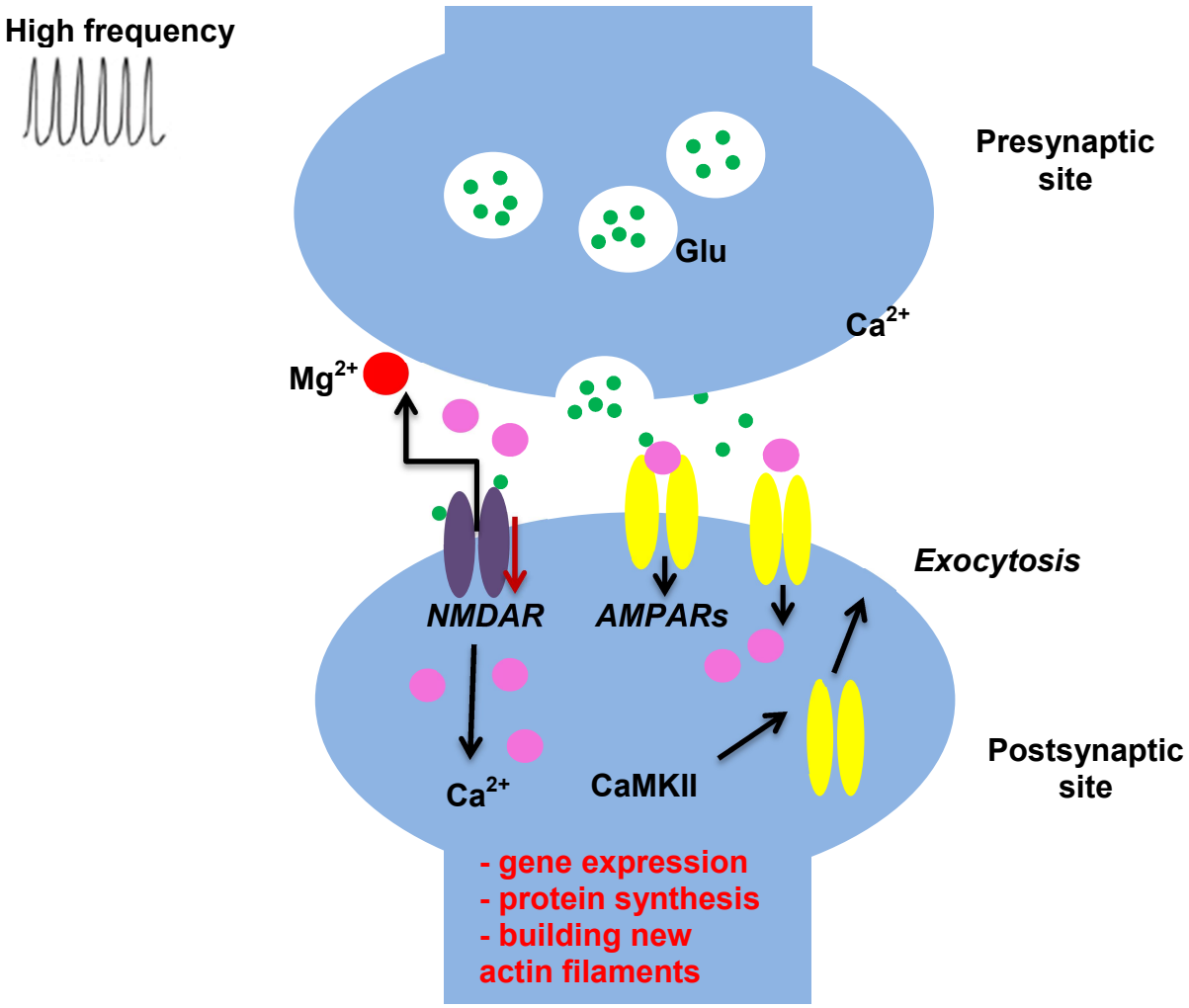

Figure 1.5. **Simple schematic representation of Long Term Potentiation induced by high frequency stimulation.** Activated presynapse releases glutamate (Glu●). Small amounts of Ca^{2+} ions go through AMPARs into the cell. Glu binds with NMDA receptors and Mg^{2+} which block NMDARs is released allowing influx of Ca^{2+}, which then dephosporylates CaMKII. Further biochemical changes lead to exzocytosis of AMPARs and increasing ion influx. Red arrow and fond indicate neuronal changes which occur during late phase LTP.

1.4.4.2. Long term depression

LTD is the mechanism opposite to LTP which causes lowering the efficacy of the synaptic junction between two neuronal cells (*Figure 1.6*). LTD can be induced by the minimal input and minimal depolarisation of the presynaptic cell. Another plausible mechanism of LTD is that long lasting polarisation of the cell, which reaches the threshold of a maximum efficacy leads to saturation and blocking the neuron of receiving a new information [74].

Two types of LTD were described: homosynaptic and heterosynaptic. Homosynaptic (input specific) occurs in the same synapse that receives induction and is activated by low or high

frequency stimulus where LTD is followed by LTP (depotentiation) or de novo LTD from the baseline conditions. Heterosynaptic LTD occurs at synapses located next to each other. Low frequencies (0.1-1 Hz) stimulations of the neuron lead to weaker depolarisation of the presynapse and as a result exzocytosis of the lower amount of glutamate. This will also cause Na^+ flow through the AMPARs but this activity does not cause removing Mg^{2+} molecules from the NMDARs. In the hippocampus this causes an activation of phosphatases: protein phosphatase 2B (PP2B) and protein phosphatase 1 (PP1) which dephosphorylate one of the AMPAR's subunits and as a result reduces AMPAR conductance and. LTD can be also divided on the basis of receptor which is triggered. First form uses NMDARs (NMDAR-LTD) which are built from 4 subunits two GluN1 and two GluN2. Two GluN2 subunits can be identical: 2A, 2B 2C and 2D- Diheteromer or different from each other and form a triheteromer along with 2 identical GluN1 subunits. Precursor of the brain derived neurothrophic factor (pro-BDNF) uses neurotropin receptor P75 ($p75^{NTR}$) which increases GluN2B expression and regulation [74].

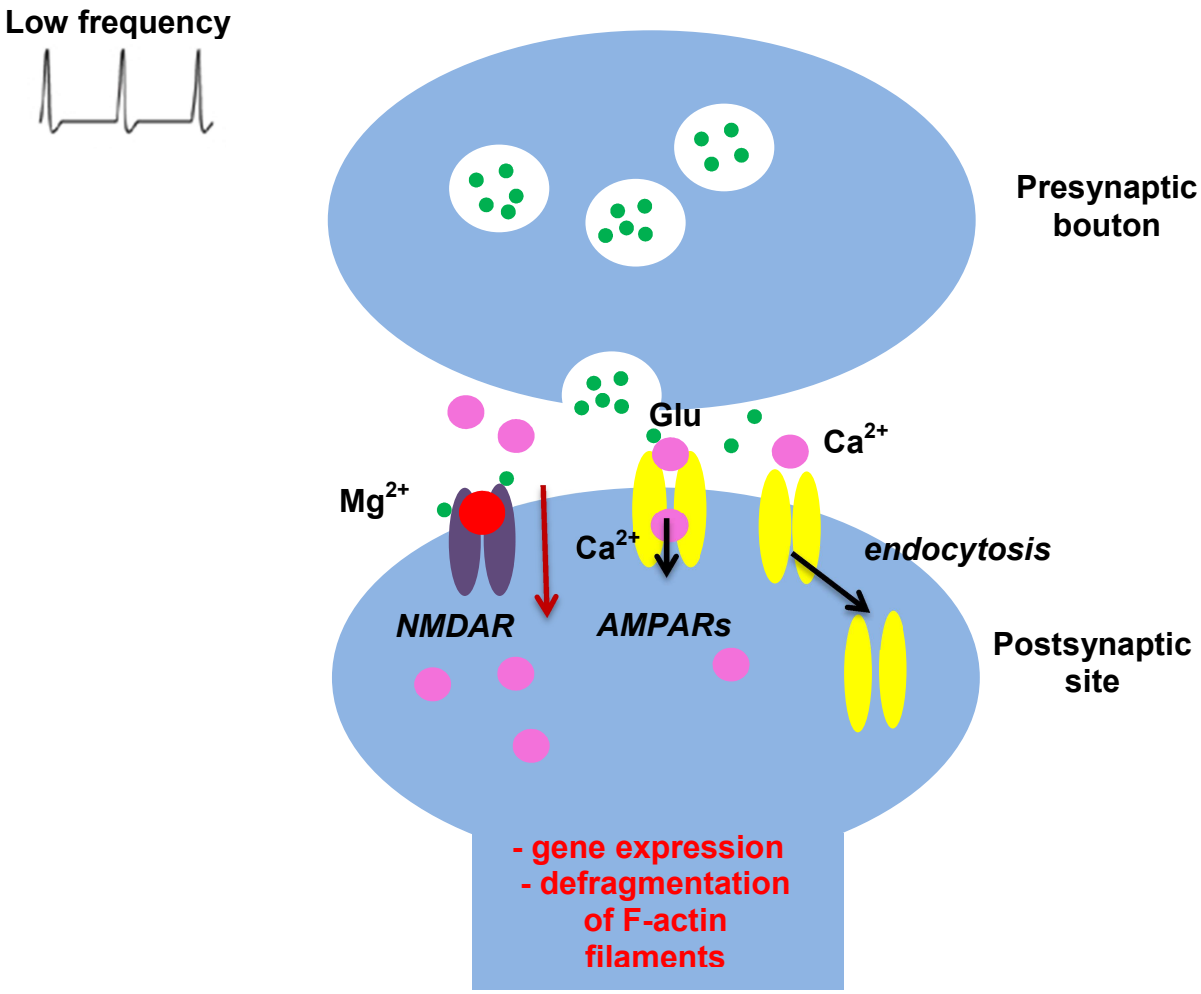

Figure 1.6. **Simple schematic representation of Long Term Depression induced by low frequency stimulation.**
Activated presynapse releases glutamate. Small amounts of Ca^{2+} ions go through AMPARs into the cell, but the amount of ions is not sufficient to cause excitation on the postsynaptic site. Further biochemical changes lead to endocytosis of AMPARs. Red arrow and fond indicate neuronal changes which occur during late phase LTD.

1.4.5. Methods of studying cortical plasticity involved in swallowing

Studies investigating functions of cortical structures in swallowing use different techniques of electrical or magnetic stimulation and neuroimagining to study the conscious brain [71].

These methods comprise:

- Transcranial Magnetic Stimulation (TMS) (see Paragraph 1.4.6.) uses an electromagnetic field to reveal neural activity just under the surface of the scull. This technique measures the motor evoked potential (MEP) amplitude of the pharyngeal response (muscle contraction associated in swallowing) by the electromyography (EMG) of the targeted muscle. TMS can be delivered

single (single-pulse TMS) or paired pulses (paired pulses TMS) to affect neuronal cortical excitability [75].

- Transcranial Direct Current Stimulation (tDCS) sends constant, low direct current through the electrodes placed in the brain area of interest. The current induces intracerebral current flow which either increases or decreases the neuronal excitability in the specific stimulated area [76].

- Functional magnetic resonance imaging (fMRI) - measures differences in cortical blood flow associated with hemodynamic activity within the brain areas. In neutrally active areas levels of oxyhaemoglobin (haemoglobin with oxygen atom) increase and deoxyhaemoglobin (haemoglobin without oxygen atom), which is used as a contrast decrease [28].

- Positron emission tomography (PET) - detects pairs of gamma rays emitted indirectly by a radiolabelled tracer, which is presented into the body on a biologically active molecule/ receptor [77].

- Magnetoencephalography (MEG) – measures magnetic fields produced by intracellular electrical currents in neuronal cells with very sensitive magnets [78].

- Near-infrared spectroscopy (NIRS) – optical neuroimaging technique measuring concentration changes of oxygenated and deoxygenated haemoglobin within the vessels of the cerebral cortex [79].

Depending on the study design, hypothesis and the resources researchers may choose one of the methods or combination. Studies conducted on exploring specific localization of the brain areas might find fMRI, PET, MEG or NIRS techniques more useful. The main advantage of these techniques is better localization the area of interest with a higher precision. The main disadvantage is high cost of the equipment and a single study. For less focal and more functional studies techniques such as TMS, tDCS should be more suitable. Unfortunately accuracy of localization is lowered compared to other techniques.

Additionally several techniques are used to study cortical facilitation within MI in animal models. These comprise:

- Intracortical microstimulation (ICMS) uses a microelectrode placed in the brain area of interest which delivers an electric stimuli. This creates a corticobulbar projection which passes the brain stem motorneurons and the information from the muscle is collected by the EMG method. Usually short 0.2ms pulses with high frequency are delivered every 35ms. In studies conducted on monkeys stimulation evoked both contra and ipsilateral elemental movements (such as tongue protrusion). Each region within the MI represents different groups of muscles [22, 80]

- Microelectrodes which record the activity of single or multiple neurons movement and EMG related activity [23]

Neither of genetic studies used these techniques to evaluate the cortical input in swallowing.

In the further sections of my doctoral thesis I will be focusing on the Trancranial Magentic Stimulation (TMS) technique (described below). I choose TMS method, because it is not invasive and gives robust approach to access changes in the muscle responses following focal brain stimulation.

1.4.6. Transcranial Magnetic Stimulation

Transcranial Magnetic Stimulation relies on the rule developed by Faraday in 1841: a current flow can be induced by placing (a wire) near the second conductor which generated a magnetic field. Nowadays TMS is used in clinical practice to induce an electromagnetic pulse in the coil by using the high current pulse generator to produce small electric currents in the human body. Magnetic field induces small electric currents within the pyramidal cells of the cerebral cortex which send the signal to the motor neurons activating the muscles.

Depending of the type of the coil TMS can induce more or less focal responses. Less focal stimulations are produced by the round coils (*Figure 1.7.*) and more focal by the double, eight-shaped. The pulse penetrates up to 3 cm under the scull. Placement of the coil is also crucial and the angle of the coil position may give different responses [81].

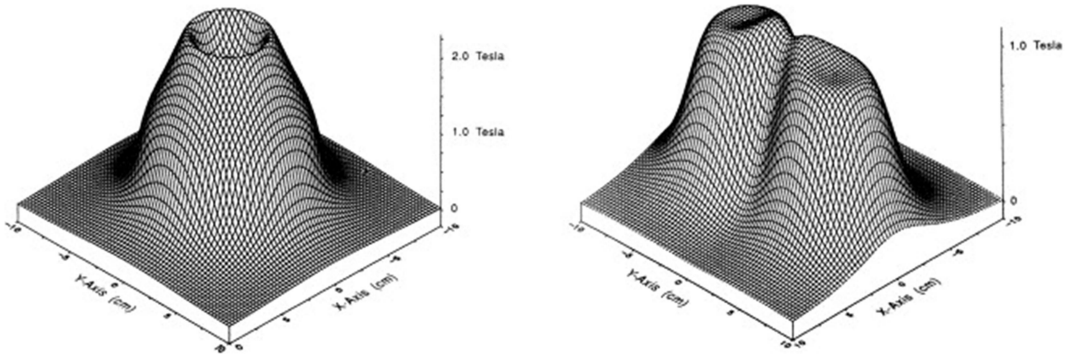

Figure 1.7. **Three dimensional representation of the peak magnetic flux produced on the surface by the circular coil (left) or double eight-shaped coil (right**). Double, coils (right) consist of two windings placed next to each other which produce a maximum electric field under the point where the two windings meet (used with permission).[82]

One of the first studies which used TMS to study swallowing neurophysiology was performed by Hamdy *et al.* in 1996 [26]. In the study topographical representation of the swallowing

musculature was identified. Swallowing muscles are represented on both hemispheres; there is an asymmetry and the presence of a dominant (revealing stronger responses) and non-dominant (revealing weaker responses) hemisphere. Swallowing performance controlled by the areas of the cerebral cortex is lateralized independently from handedness [83].

Recording motor responses of the swallowing musculature followed by TMS

Motor responses of the swallowing musculature are recorded with the electromyography (EMG) technique. EMG gives specific information about motor units which comprise the muscle fibres with a single motor neuron that reside in the brainstem or the spinal cord. At rest, a muscle fibre maintains a steady potential across the membrane. When an impulse travels along a nerve and arrives at the myoneural junction, acetylcholine is released from the motor end plate. This results in depolarization of the muscle fibres and muscle contraction. The depolarization generates the action potential which is called motor evoked potentials (MEP) (*Figure 1.8.*). MEPs are electromyographic responses at the level of peripheral musculature to centrally delivered stimuli (cortical level).

For analysis purposes two parameters of MEPs are measured: either the latency or amplitude. In simple terms, latency gives information of how long signal goes from the motor cortex to the muscle. For the pharyngeal muscles average latency of MEP is 8- 10ms and for the hand 18-22ms. Amplitude gives information about the magnitude of the response and is one of the parameters used to measure motor cortex excitability.

For TMS studies of swallowing a catheter with two electrodes at the end is usually placed in the pharynx or upper part of the oesophagus, depending on the study design (*Figure 1.10*). TMS pulses are given over the area of the motor cortex which gives the strongest responses of the swallowing muscle of interest. The coil position should be mapped to standard location maintaining an angle 45% (*Figure 1.9*). The catheter placement is slightly uncomfortable, but subjects habituate to the feeling after couple of minutes. In some cases the catheter can cause gag reflex, watery eyes, coughing or small irritation of the throat. Therefore the subject may choose whether the catheter should be applied through the mouth or the nose. TMS pulses reveal MEPs in the pharyngeal muscles which are subtle and not detectable by the subject. The catheter remains in the pharynx during the whole study (2.5-3h). There are small number of subjects who need to be excluded from the study, because inability to tolerate the catheter due to hypersensitivity of the pharynx.

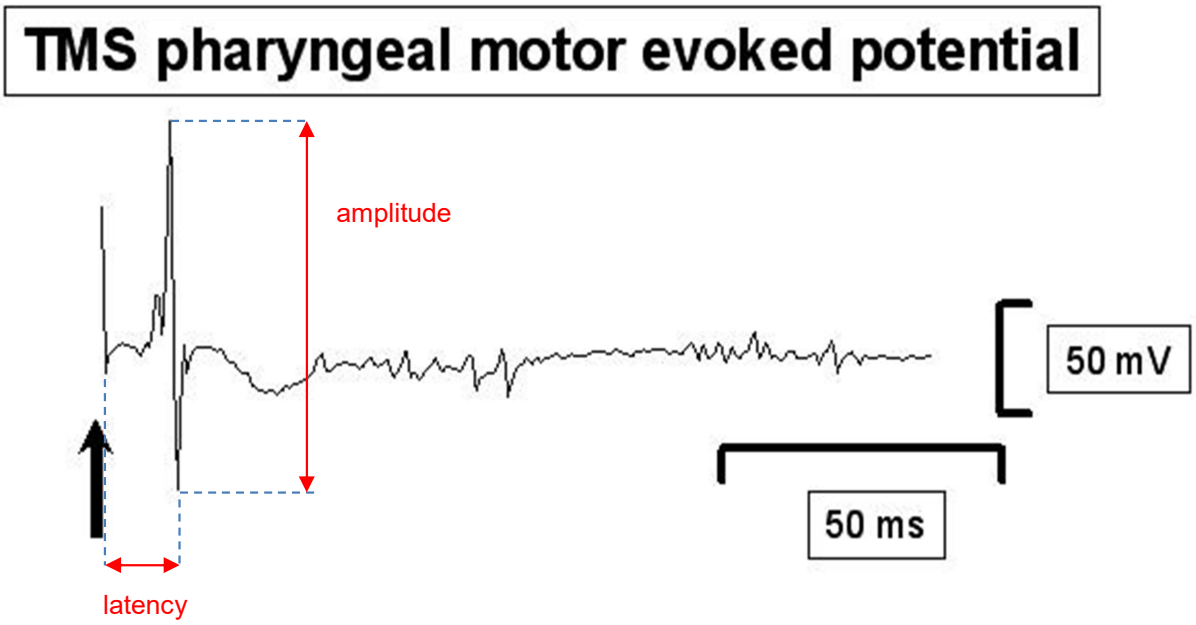

Figure 1.8. **Pharyngeal motor evoked potential followed single pulse TMS. Black arrow shows the stimuli artefact. Red arrows present amplitude and latency of the response.**

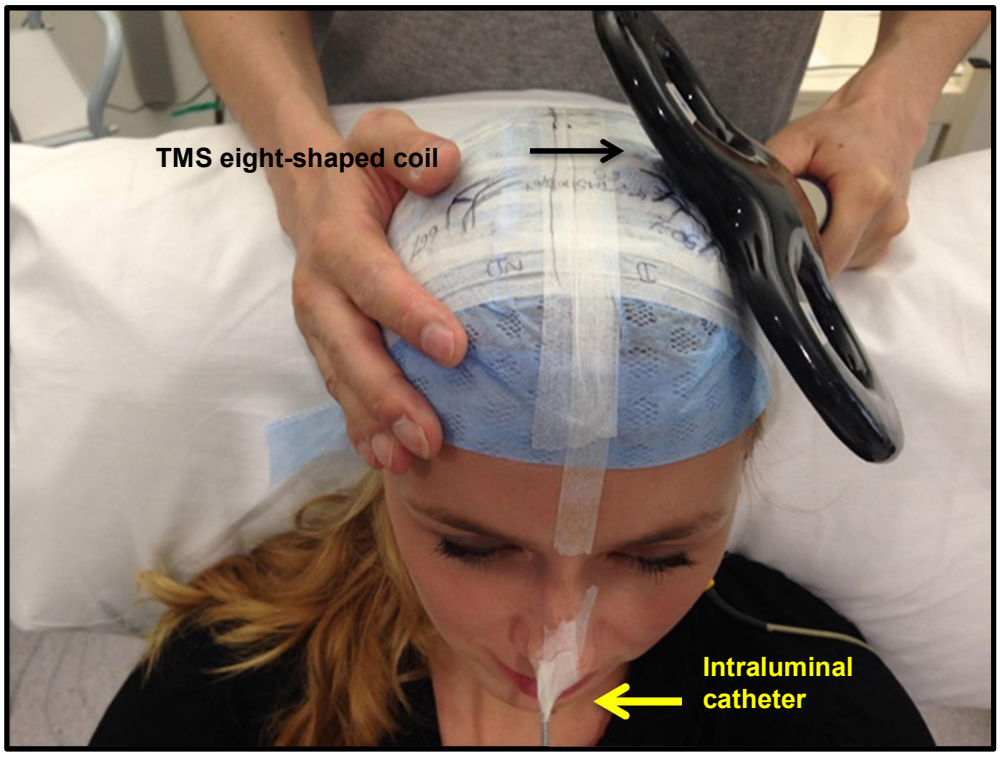

Figure 1.9. **Position of the coil located over the pharyngeal dominant motor cortex (45 degrees). The MEPs recorded with intraluminal catheter placed through the nose in the pharynx.**

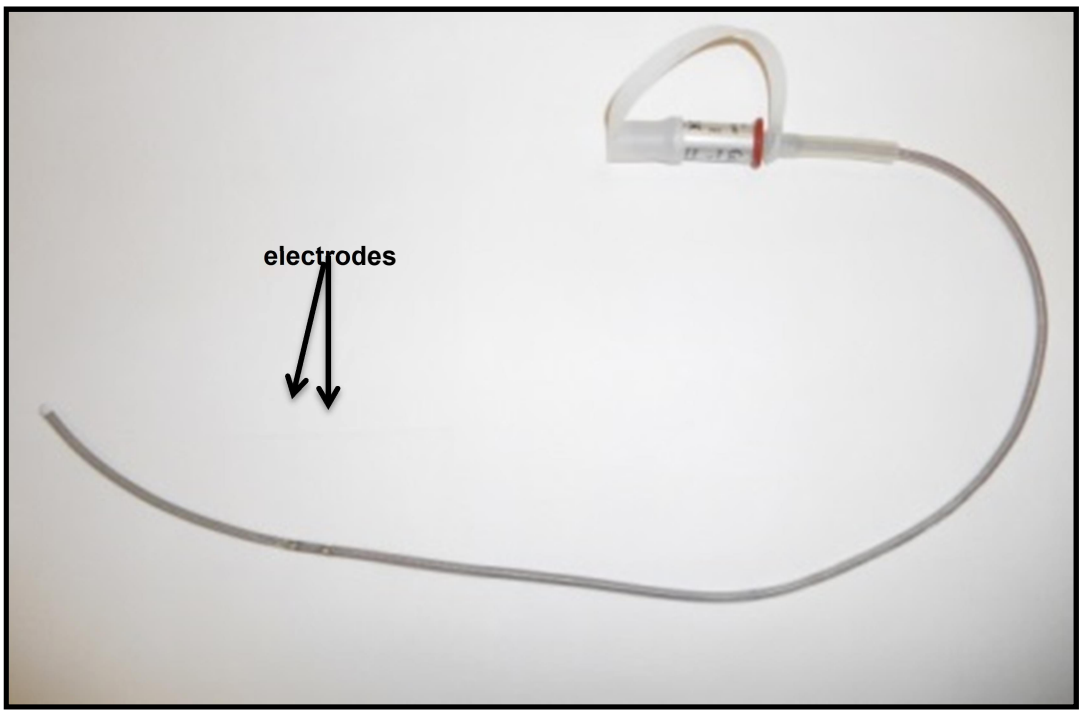

Figure 1.10. **The intraluminal Gaeltec catheter with marked by black arrows electrodes to measure pharyngeal MEPs.**

Other treatment therapies of dysphagia

Clinicians use a number of therapies which are aimed at improving swallowing recovery, these techniques comprise:

a) behavioural therapy; The commonest behavioural techniques use changing position of the whole body, tongue exercises, changing the strength of swallow;
b) changing diet and alteration of bolus size and texture;
c) pharmacological interventions are believed to help in preventing aspiration pneumonia (angiotensin I converting enzymes inhibitors treatment);
d) diversion by application of the feeding tubes placed in the stomach through the nose (the nasogastric tube (NGT)), the jejunum (naso-jejunal tube (NJT)) and surgically placed in the stomach (percutaneous endoscopic gastronomy tube-PEG). This supports the nutritional needs of patients whilst recovery of dysphagia is awaited;
e) neurostimulation techniques (neurorehabilitation) - described in more detail below.

Clinical studies about the effects of treatments for dysphagia (including swallowing therapy, nutritional and fluid supplementation) in stroke patients revealed that the majority of mentioned techniques did not have a significant effect on mortality caused by swallowing problems [84]. This poses the need for further evaluation of treatment approaches which would involve the

development of new methodology, combination of multiple techniques or discovery markers which might be used to stratification approaches in order to maximize the effectiveness of existing treatment therapies.

1.4.7. Neurorehabilitation

Neurostimulation techniques have been widely used in recent years as the potential treatment of dysphagia. Both peripheral and central nervous system are stimulated by electric and magnetic impulses (*Figure 1.11.*).

Central nervous system stimulations used in neurorehabilitation are most commonly repetitive TMS (rTMS) and tDCS. rTMS generates very short multiple stimuli used to induce changes in the excitability of neuronal cells which lasts longer than milliseconds as demonstrated by single pulse TMS.

The other non-invasive method used in neurorehabilitation is tDCS. Two types of tDCS are used in treatment therapies: anodal which aims to increase and cathodal tDCS to decrease cortical excitability. This simple technique uses two electrodes placed over the area of interest which send a constant current flow altering neurological performance of the subject. Evidence from pilot studies [85] and results from two clinical trials [86] suggest that tDCS intervention applied over the injured by stroke hemisphere combined with post-stroke rehabilitation significantly improves swallowing performance in stroke patients compared to sham stimulation also with rehabilitation. However all results from mentioned studies need to be replicated in bigger cohorts with randomised trial design.

Peripheral approaches include neuromuscular electrical stimulation (NMES) and pharyngeal electrical stimulation (PES). NMES uses electrodes to electrically stimulate targeted muscles to support muscle strength, increase muscle size and increase sensory awareness of volitional muscle control. In the PES technique electrodes are placed inside the pharynx within the intraluminal catheter to electrically stimulate the pharyngeal muscles. PES technique affects excitation within the pharyngeal motor cortex revealing long term plasticity by affecting sensory inputs within the pharynx [87]. Studies with PES technique on stroke patients showed increased excitation within the intact hemisphere followed by sensory stimulation [88, 89].

Paired associative stimulation (PAS) is a paradigm consisting of slow-rate repetitive low-frequency median nerve stimulation combined with TMS over the motor cortex [90].

One of the biggest issues in developing neurorehabilitation techniques is lack of consistency of patients' outcomes [91]. The discrepancy between the individual's outcomes in stroke patients followed by neurorehabilitation may be caused by targeting different muscles or area of the cerebral cortex, intensity and time of the stimulation and the severity and localization of the brain lesion. There is also the possibility that treatment efficacy may result from individual differences in neuronal function between patients.

In this project I will use one of the central stimulation technique - rTMS.

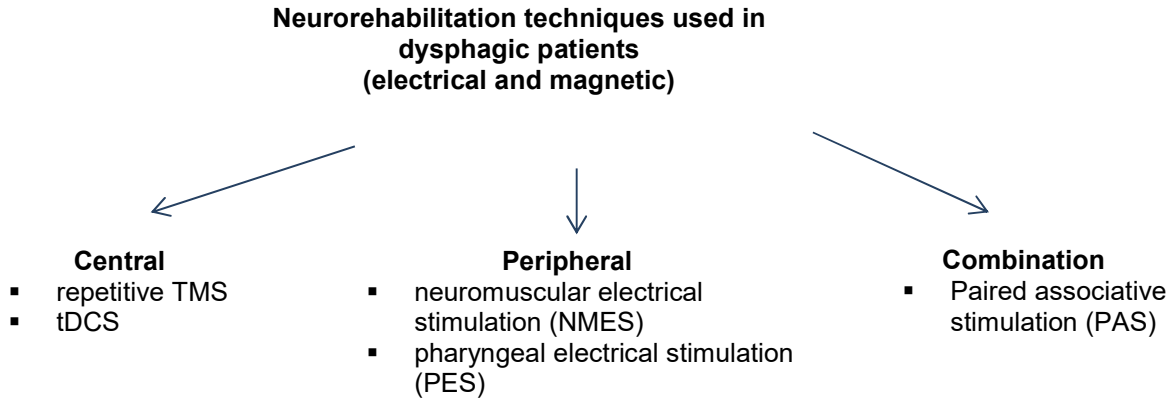

Figure 1.11. **Electrical and magnetic neurostimulation approaches used as the potential treatment therapies in stroke dysphagic patients.**

1.4.8. Repetitive Transcranial Magnetic Stimulation in the rehabilitation of stroke dysphagic patients

rTMS is a form of transcranial magnetic stimulation (described above), a current of magnitude produced by rTMS depreciates rapidly with the distance from the coil and stimulates the cerebral cortex, the cerebellum or the brainstem revealing long lasting effects in the pharyngeal musculature responses [75]. Low frequencies (1Hz) of TMS stimulation have an inhibitory effect whereas high frequencies (5Hz -10Hz) generate excitation [92].

The main principal of rTMS in neurorehabilitation of dysphagia is to target the cerebral cortex areas in order to increase (excite) or decrease (inhibit) neuronal activity in the brain swallowing areas and as a result affects the muscles involved in certain stage of swallowing. rTMS intervention does not cause reflexive swallowing, but only simple swallowing muscles responses called motor evoked potentials (MEPs). MEPs are stronger or weaker when compare to the baseline. Muscle responses depend on multiple factors such as the intensity and time of stimulation, different equipment for rTMS and cortical representation of the muscles.

Four cortical swallowing areas have been targeted in different studies: oropharyngeal (mylohyoid) [93, 94], pharyngeal [75, 91, 95], oesophageal [96] and tongue [97].

Two types of paradigms (high and low frequency) have been used to study mechanisms underlying swallowing cortex plasticity and recovery in healthy subjects and patients. Clinical trials conducted on stroke dysphagic patients' explored the potential of rTMS as a treatment

therapy for dysphagia. Each study used a different design including the choice of different hemisphere by stimulation, frequency, duration of the stimulation etc. Results were inconsistent which might be due to both differences in patient level factors or experimental design. Surprisingly all studies showed positive results. Summary of the studies is presented in the *Table 1.1 and Figure 1.12* and short description below. Presented studies do not include 3Hz paradigms delivered over the swallowing motor cortex [96, 98].

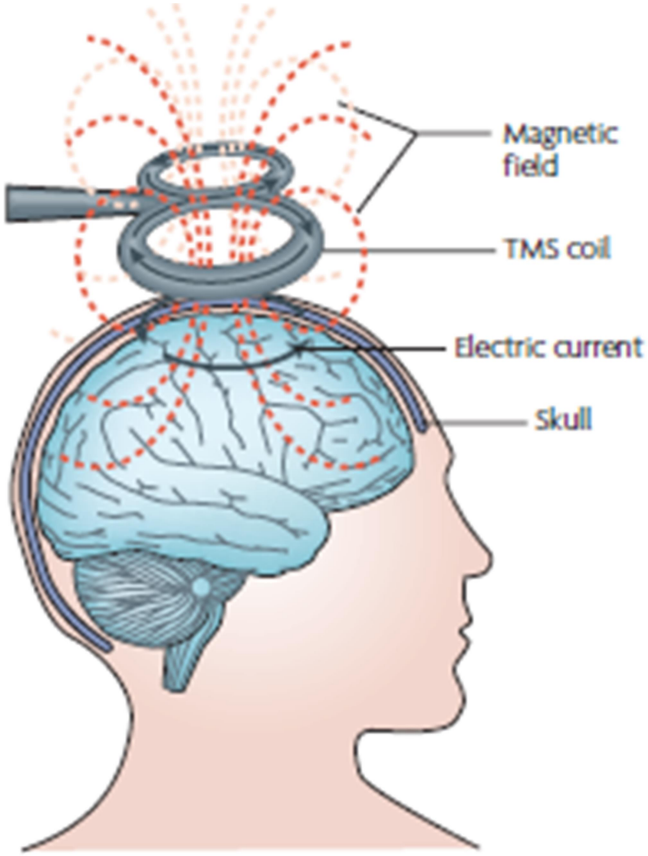

Figure 1.12. **Schematic representation of the generation of the electric current within the brain induced by TMS.**

1.4.8.1. Safety of TMS

TMS have been proven to be both: safe and non-invasive technique used worldwide. However as every technique also TMS has side effects described in the updated safety guidelines for the application of TMS which comprise: headache, local pain, transient hearing changes [99]. There have been also few seizures reported in the literature caused by low-frequency TMS.

Key advantages of the rTMS over other techniques are, that it is non-invasive, can be performed in healthy volunteers and patients, stimulation is relatively focal and depending on the intensity impulses can reveal inhibition or excitation

Presented studies on rTMS interventions in healthy individuals and stroke dysphagic patients provide an evidence of diagnostic and therapeutic potential of rTMS, which can be used as a tool to explore mechanisms underlying cortical plasticity and recovery from brain injuries. Before clinical research trials were conducted all paradigms were explored in the cohorts of healthy volunteers. Therefore in my research I will use a cohort of young, healthy volunteers to study the molecular basis of swallowing physiology followed by rTMS. Young healthy volunteers should not be affected by genetic changes caused by age or coexisting diseases potentially confounding study findings.

1.4.8.2. Low frequency rTMS

rTMS with low frequency has been used to examine mechanisms of excitability by down regulation MEPs. The following paragraph shortly describes studies on healthy young volunteers followed by studies on stroke patients.

The first study with 1Hz rTMS paradigm was performed by Gow *et al.* in 2004 [75] on a group of healthy volunteers. This study showed that rTMS interventions lead to long lasting changes in excitability measured with MEPs amplitudes. The next was performed by Mistry *et al.* [83] parameters for the most consistent inhibition in PMEPs with 1Hz stimulation were obtained- 250 pulses at 120% of pharyngeal resting motor threshold. Jefferson *et al.* [100] used the 1Hz rTMS intervention to study effects of inhibition in MEPs, also called 'virtual lesion', and reported that this type of 'lesion' can be reversed by an applying excitatory paradigm to the opposite (unlesioned) hemisphere afterwards. Similar study design with using 1Hz rTMS intervention to cause 'virtual lesion' was used in Michou *et al.* studies [101] and Jayasekeran *et al.* [89]. In all mention studies 1Hz rTMS intervention over the hemisphere giving stronger PMEPs caused inhibition in pharyngeal MEPs in healthy volunteers. Another study by Verin *et al.* [94] discovered that 1 Hz rTMS applied over the oropharyngeal cortex (hemisphere giving stronger responses - 'dominant') in healthy individuals cause short decrease in MEPs. Stimulation delivered over the non-dominant hemisphere did not result in any changes in the process of swallowing.

In the pilot study, Verin *et al.* [93] used 7 stroke patients with dysphagia to study the effects of low-frequency rTMS on swallowing recovery. The protocol consisted of 1Hz, 20 minutes TMS sessions over the intact hemisphere for 5 days a week on 120% hand resting motor threshold (RMT). Dysphagia severity was assessed by VFS examination before TMS sessions and with standardized 8-point penetration-aspiration scale. Improvement of swallowing performance was observed in all patients.

Kim *et al.* [95] conducted a randomised controlled trials (RCT) on 30 patients with brain injury and dysphagia comparing frequencies rTMS applied to the affected hemisphere (n=10), low-frequency applied to the intact hemisphere (n=10) and sham rTMS the intact hemishphere (n=10). All interventions were delivered for 20 minutes five days in a row for 2 weeks. Only 1Hz

rTMS, delivered over the intact hemisphere enhanced recovery from swallowing impairment. Swallowing was not affected by either sham or high-frequency rTMS.

Another study used dysphagic patients with subacute, unilateral hemispheric stroke [102] to study effects of 1Hz rTMS and NMES intervention. Patients were randomly assigned to the conventional dysphagia therapy (CDT), rTMS, or NMES groups. Results of the following study indicated that both low-frequency rTMS and NMES could induce recovery from dysphagia.

These results suggest that decreasing the excitability within the intact hemisphere may improve swallowing recovery from dysphagia following experimentally induced or pathological brain lesions. However the evidence remains somewhat controversial.

1.4.8.3. High frequency rTMS

In contrast to the above described studies, another hypothesis was explored with paradigms using high frequency rTMS either to the intact or stroke affected hemisphere to improve recovery from swallowing difficulties.

Gow *et al.* showed the potential application of 5 Hz rTMS applied over the pharyngeal motor cortex in a group of healthy individuals [75]. The stimulation revealed long-term excitability of pharyngeal MEPs. The analysis included comparison between excitability in the pharynx and the hand followed by 5Hz neurostimulation. The results demonstrated that rTMS is not only frequency but also muscle specific. RTMS intervention revealed different mechanisms depending on the muscle which was stimulated. Further studies by Jefferson *et al.* validated parameters used for the intervention and showed that giving 250 rTMS pulses at 5Hz might maintain the excitatory responses up to 2 hours post intervention [100]. In the recent study Vasant *et al.* used cerebellar rTMS stimulation to reveal increase in the PMEPs caused by facilitation of the pharyngeal motor cortex [103].

Park *et al.* performed double-blinded randomized controlled clinical trials with 5Hz rTMS applied over the intact hemisphere (n=18) in stroke dysphagic patients [104]. Stimulation was applied for 2 weeks for 10 minutes every weekday. VFS was conducted on each participant before the studies (baseline) and after 2 weeks of therapy. Significant improvement of pharyngeal phase of swallowing was observed within the group of patients who received real stimulation. Targeting the pharyngeal motor cortex may explain that improvement of the swallowing performance was observed only in the pharyngeal stage of swallowing.

Another clinical research trial was performed to examine responses to three different types of stimulation [91]: rTMS, PAS, PES applied to the intact hemisphere of chronic stroke, dysphagic patients. Patients were subjected to ether of 3 stimulation and sham as a control. Swallowing was assessed by VFS. All types of stimulations showed functionally significant changes. However the smallest improvement was observed in patients within the group of rTMS

stimulation. This may be caused by longer time of stimulation and using PAS and PES for (10 minutes) comparing to standard 5Hz stimulation of 250 pulses in 5 blocs (2-3 min).

Lee *et al*. in his studies on subacute stroke patients used high frequency 10Hz [105] delivered over the cortical representation of the suprahyoid muscle or the abductor pollicis brevis muscle. rTMS was performed at 110% of MEP threshold, dysphagia status was measured by the Functional Dysphagia Scale (FDS), the Penetration-Aspiration Scale (PAS), and the Dysphagia Outcome and Severity Scale (DOSS) measured before, immediately, and 4 weeks after rTMS. Patients who received the stimulation over the suprahyoid muscle motor cortex showed an improvement in swallowing performance immediately and 4 weeks after rTMS.

A recent pilot study used different study approach and targeted the tongue motor cortex [97]. The authors used 4 stroke patients with dysphagia assigned to two groups of 5Hz rTMS and sham stimulation. The swallowing performance was improved in the group receiving real stimulation and effects lasted up to 4 weeks. Main disadvantages of this study were small sample and targeting only the tongue muscles which do not affect the pharyngeal stage of swallowing.

Presented studies showed contribution of rTMS in neurorehabilitation of dysphagic stroke patients, precipitated by pilot studies conducted on healthy volunteers. Reassuringly both paradigms: excitatory applied for both intact and affected hemisphere and inhibitory applied for the intact hemisphere, showed significant improvements of swallowing performance in stroke patients.

Despite these promising observations limitations of both rTMS paradigms exist. These include lack of access to functional anatomy (except Vasant *et al.* [103] studies) and measurement time differences during application and possible adverse effects (sporadic epileptic seizures and headache).

Paradigm	subjects	n	Hz	Intensity of the stimulation (RMT)	Duration of the intervention (min x days)	hemisphere	Swallowing cortical area assessed	Swallowing assessment (technique x follow up)	Outcome x author
Inhibitory	Healthy volunteers	12	1	80% thenar motor threshold	100 pulses	Contra pharynx and ipsilateral hand	pharyngeal	MEPs with single pulse TMS	Corticobulbar and corticospinal responses may differ in both paradigms in hand and swallowing motor cortex. Gow *et al.* [75]
		9	1	120% pharyngeal motor threshold	250 pulses	'dominant' pharyngeal hemisphere	pharyngeal	MEPs with single pulse TMS, swallowing reaction times	The most consistent inhibition in PMEPs was observed I after applying 1Hz intervention at 120%. Mistry *et al.* [83]
		23	1	120% pharyngeal motor threshold	Once 600 pulses	'dominant' pharyngeal hemisphere	pharyngeal	MEPs with single pulse TMS, swallowing reaction times	1 Hz intervention caused decrease in the PMEPs and increased swallowing reaction time. Jefferson *et al.* [100]
		13	1	120% pharyngeal motor threshold	250 pulses	'dominant' pharyngeal hemisphere	pharyngeal	MEPs with single pulse TMS, swallowing reaction times	Decreased PMEPs and increased swallowing reaction times. Jayasekeran *et al.* [89]
		9	1	120% mylohyoid motor threshold	20 minutes	'dominant', non-dominant and Sham over the dominant	oropharyngeal (mylohyoid)	VFS x 5min, 30 min, 60 min post	In dominant hemisphere increased Oral Transit Time and increased and Pharyngeal Response Time, no signs on non-dominant and sham. Verin *et al.* [94]
		12	1	120% pharyngeal motor threshold	250 pulses	'dominant' pharyngeal hemisphere	pharyngeal	MEPs with single pulse TMS, swallowing reaction times	1 Hz intervention caused decrease in the PMEPs and increased swallowing reaction time. Michou

									et al. [101]
	Stroke, dysphagic patients	7	1	120%	20 minutes x 5 days	Intact hemisphere	oropharyngeal (mylohyoid)	Dysphagia handicap index and VFS before, 1 and 3 weeks after	Improvement of specific dysphagia symptoms up to 3 week post intervention Verin *et al.* [93]
		n=10, n=10 sham (n=1 traumatic brain injury)	1	100%	5sec for 20 min, 10 days (2x5working days)	Intact hemisphere	pharyngeal	VFS , before and after rTMS sessions	Low- frequency improved recovery from dysphagia. Kim *et al.* [95]
		n=14 in rTMS group. N=15 traditional dysphagia therapy, n= 18 MNES	1	100%	20 minutes per session (5 days per week for 2 weeks)	Intact hemisphere	pharyngeal	Functional dysphagia scale (FDS), pharyngeal transit time (PTT), the penetration-aspiration scale (PAS), and the American Speech-Language Hearing Association National Outcomes Measurement System (ASHA NOMS)	All patients showed improved swallowing performance after 1Hz rTMS intervention. Lim *et al.* [102]
Excitatory	Healthy volunteers	12	5	80% hand motor threshold	100 pulses	Contra pharynx and ipsilateral hand	pharyngeal	MEPs with single pulse TMS	Corticobulbar and corticospinal responses may differ in both paradigms in hand and swallowing motor cortex. Gow *et al.* [75]
		23	5	90% hand motor threshold	250	'dominant' pharyngeal hemisphere	pharyngeal	MEPs with single pulse TMS, swallowing reaction times	Increase in PMEPs and swallowing reaction times was. Jefferson *et al. [100]*

		17	10	90% hand motor threshold	250	Stronger cerebral	pharyngeal	MEPs with single pulse TMS	Increase in PMEPs. Vasant *et al.* [103]
	Stroke, dysphagic patients	18 (RCT) 9 active, n=9 sham	5	90% hand motor threshold	10 min x 2 weeks	Intact hemisphere	pharyngeal	VFS and penetration-aspiration scale (PAS) before, just after and 2 weeks	Improvement of pharyngeal phase of swallowing results maintained up to 2 weeks. Park *et al.* [104]
		18, n=6 PAS, n=6 PES, n=6 rTMS	5	90% hand motor threshold	250 pulses of rTMS	Intact hemisphere	pharyngeal	VFS and PAS before, just after, 30 min and 60 min	Improvement is swallowing observed after 3 types oof stimulations. Michou *et al.* [91]
		N=10, n=10 sham	5	100% hand motor threshold	5sec for 20 min, 10 days (2x5working days)	Affected hemisphere	pharyngeal	BI before, FDS and VFS , before and after rTMS sessions	High frequency did not have an effect on swallowing performance. Kim *et al.* [95]
		24 (n=12 rTMS over the swallowing motor cortex)	10	110% hand motor threshold	10 seconds, and then repeated every minute for 10 minutes	Affected hemisphere	the suprahyoid muscle	Functional Dysphagia Scale (FDS), the Penetration-Aspiration Scale (PAS), and the Dysphagia Outcome and Severity Scale (DOSS) using the results of a videofluoroscopic swallowing study	rTMS over the suprahyoid muscle motor cortex causes more improvement in swallowing function when compared to that over the interconnected site. Lee *et al.* [105]
		N=2 active rTMS n=2 sham	5	90% hand motor threshold	3000 pulses rTMS per day for 10 days	Affected hemisphere	tongue region	Tongue pressure assessment; swallowing-related quality of life questionnaire; and videofluoroscopic swallowing study.	Participants who received real rTMS stimulation showed improvement in swallowing performance. Cheng *et al.* [97]

Table 1.1. **Summary of studies with different rTMS paradigms conducted on healthy subjects and patients with brain lesions.**

Summary of the Section 1.4.

Swallowing difficulties, called dysphagia affects substantial number of individuals with neurological diseases as well as healthy older people. This work focuses on neurogenic oro-pharyngeal dysphagia which might be caused by stroke, Parkinson's disease and ageing. Swallowing impairment leads to serious life threatening complications such as aspiration, pneumonia and death. Accurate diagnosis and selection of the appropriate treatment therapy is a key step in improving the recovery process. A number of swallowing assessment tools are widely used in clinical practices, such as 'gold standard' VFS and FEES, bedside screening questionnaires and self-reported questionnaires. Recovery from swallowing impairments is observed in stroke patients and is most likely caused by changes in neuronal excitability within the swallowing motor cortex. Cortical neuronal excitability associated with swallowing performance remains an area of interest of multiple studies with diverse tools chosen on the basis of a study design and costs. The areas can be broadly divided into neuroimaging techniques to delineate neuroanatomy, comprising fMRI, PE, MEG or NIRS and therapeutic tools to influence function including TMS, PAS, tDCS. In this project I will focus on the non-invasive and safe technique of TMS, which is suitable for exploring pharyngeal muscle responses after the stimulation.rTMS with low and high frequency paradigms were used on healthy individuals and few clinical trials as a potential therapy of post-stroke dysphagia. All studies conducted on patients showed positive results which confirm diagnostic and therapeutic potential of rTMS interventions.

Using rTMS paradigms to treat neurogenic dysphagia is very challenging. One of the reasons is that some of patients show inhibition or excitation, while others do not respond to rTMS paradigms. Combining results from rTMS studies and individuals genetic information may give an insight in molecular mechanisms which underlie cortical excitability following neurostimulation. Individual genetic information might be potentially used to stratify treatment approaches in order to increase their effectiveness.

1.5. Genes and swallowing

One of the factors associated with the observed variation between individuals' outcome from dysphagia symptoms could be driven by individual's genetic 'make-up'. Knowledge about differences in an individual's genotype is also a potential direction for developing novel treatment strategies with stratified medicine. Stratified medicine (also called personalised or precision medicine) is an approach which subdivides patients into groups based on their risk of developing specific diseases/symptoms or their response to particular treatment therapies.

All processes in living organisms are controlled by sophisticated mechanisms on the molecular level. Each cell has a unique individual code - deoxyribonucleic acid (DNA). Even though 99.9% of the human genetic information (genome) is the same, there are still multiple differences

which make a single person unique. 0.1% of the DNA which differs between individual is one focus of investigation and is believed to cause genetically driven phenotypic differences as well as individual predispositions for certain outcomes from diseases. Some studies focus on small single nucleotide changes, other explore whole genes (protein coding sequences) or chromosomal regions, products of genes transcription, post transcription and posttranslational (epigenetic) alterations. This research will explore single nucleotide changes called single nucleotide polymorphisms (SNPs) within the genes of interest.

1.5.1. Single nucleotide polymorphisms

Each SNP represents a difference in a single 'brick' (nucleotide) which builds the DNA strands. Change (substitution, deletion or insertion) of one nucleotide may lead to changes in the amino acid codon and structural change in the protein. SNPs have a frequency of ≥1% in the population and should not be confused with mutations. Depending on the localization we can divide SNPs into main 5 categories [106]:

- within the coding sequence, but non-synonymous, non-conservative- do not cause change of the codon for specific amino acid, localized in the region on conservative regions (least common);
- within the coding sequence, non-synonymous, conservative- without causing the change, but located in the conservative regions which are not changing during the evolution processes;
- within coding sequence, synonymous- cause change of the codon and change of the amino acid;
- non-coding SNPs within the 5' untranslated region and 3' untranslated region - SNPs located there may lead to change the length of the protein or may affect regulatory mechanism;
- other non-coding – these are the commonest, usually with unknown function.

An important parameter for describing SNPs is Minor Allele Frequency (MAF) which refers to the frequency at which the least common allele occurs in a given population.

In this doctoral research I will use a number of approaches exploring SNPs and their possible association with the swallowing processes. SNP-focused studies have number of advantages over the other techniques: SNPs are very frequent and allow to capture significant proportions of the human genome, SNPs from coding regions causing non-synonymous amino acid changes may be used as markers for specific diseases, SNPs are more conserved (did not change frequently during the process of evolution), are useful in population-based studies [107].

The following sections will present a current state of knowledge on the genetic background of swallowing processes from both human and animal studies which focus on genes, SNPs or chromosomal regions.

1.5.2. Heritability and dysphagia

Heritability in the SNP-based studies is defined as the degree to which individual genetic variation affects phenotypic variation seen in a population. Complex disease/symptom is classified as heritable usually after conducting family or twin studies. Twin studies explore the differences between monozygotic (MZ- genetically identical) and dizygotic (DZ- share only around 50% of the genetic information) couples of twins. The most common twin study design compares the similarity of MZ and DZ twins. If MZ twins show significantly more similarities than DZ twins (which is found for most traits), this might indicate a potential role of genetic factors in analysed traits. By comparing many hundreds of families of twins, researchers explore the roles of genetic effects on certain outcomes.

Unfortunately, no twin studies on swallowing processes have been done to date. However as it was mentioned in paragraph 1.4.4., cortical plasticity remains a significant driver in the recovery of dysphagia following stroke. Twin studies showed that cortical excitability caused by rTMS intervention delivered over the motor cortex responsible for hand movements might be in part a heritable process. The heritability estimate for brain motor excitability was 0.68, which means 68% of the variance can be explained by genetics [8]. Therefore, we can hypothesise that the genetic contribution towards neurological control of swallowing may also be in part driven by genes. These results should, however, be interpreted with caution, because they were conducted only on female twins and examined only one SNP rs6265 from the Brain Delivered Neurotropic Factor (*BDNF*) (See further sections of the Introduction).

1.5.3. Genetics of swallowing in humans

The literature remains very limited in terms of studies about the genetic background of swallowing impairments with a neurogenic aetiology.

The most relevant study exploring the genetic basis of neurological control of swallowing was conducted by Jayasekeran *et al.* [108]. The study focused on a single nucleotide polymorphism from the *BDNF* gene. The main aim of the study was to find an association between Val66Met (rs6265) SNP and its impact on the pharyngeal muscle responses followed by inhibitory and excitatory rTMS paradigms and PES.

The *BDNF* gene is located on the chromosome 11, locus 11p13 and is a member of the nerve growth factor family. *BDNF* is expressed by cortical neurons, and is necessary for survival of striatal neurons in the brain. Multiple studies showed that rs6265 from the *BDNF* gene affects cortical plasticity (See paragraph 1.5.4). Polymorphism rs6265 located in the coding region of the *BDNF* causes substitution of valine (Val) to methionine (Met) in the codon 66.

Jayasekeran *et al.* [108] showed the link between provoked neuronal plasticity of the pharyngeal area and the impact of the polymorphism rs6265. Twelve healthy individuals underwent excitatory (5Hz) and inhibitory (1Hz) rTMS stimulations over the dominant pharyngeal cortex. As an outcome, MEP from the pharyngeal muscles were collected with intra-luminal catheter placed in the individual's throat. The individuals were divided in two groups according to their genotype from the codon 66 of *BDNF* into: Val/Val and non-Val/Val (carrying Val/Met of Met/Met) groups. Statistical analysis showed significant differences between the pharyngeal MEPs in homozygous participants with Val/Val comparing to participants carrying at least one *BDNF* Met allele after 5Hz rTMS (*P*-value = 0.04). This study suggests the plausible hypothesis of a genetic factor on pharyngeal cortical plasticity. Jaysekeran's *et al.* study was the first to use a human model of this nature to study swallowing neurophysiology and genetics. Animal studies, however informative, may reveal results limited by species. The main disadvantage of this research was the examination of the single gene polymorphism, while the majority of common diseases are most likely multi-factorial and polygenic (complex), that may include gene-gene or gene-environmental interactions [106].

Another study by Vasant *et al.* [109] used electrical stimulation of the oesophagus of healthy subjects to measure sensitivity and its association with rs6265. Study explored the relationship between oesophageal sensitivity and *BDNF r*s6265 genotype and found that the Met allele was likely to lower levels of sensory tolerance to oesophageal electrical stimulation.

Mentz *et al.* [110] performed the first association analysis between self-reported swallowing symptoms from the cohort of heathy elderly volunteers and the *APOE* gene (OMIM 107741). The *APOE* gene, encodes apolipoprotein essential for normal catabolism of triglyceride-rich lipoprotein constituents. It has been discovered that isoforms of *APOE* are related to neurological conditions and cognitive decline [111-113]. This study used a more global approach assessing 634 volunteers. Volunteers completed self-reported SSQ (See section 1.4.2.) questionnaire about the swallowing problems. The score was classified as clinically significant if was ≥120. The study showed that there is an association between *APOE* E4 homozygosity and higher score from the SSQ questionnaire (*P*-value= 0.033).

The main advantage of the study was the number of individuals included, which gives a better statistical power of the result. Self-reported questionnaires, despite lowered accuracy, remain a useful tool for swallowing symptoms diagnosis. However there are disadvantages of this tool such as: recall biases, silent aspirations, undetectable by individuals; response biases (although response rates in this work were >80%). Which may suggest that people reporting swallowing problems had a real swallowing problems, however the control, 'healthy group' may have hidden swallowing symptoms.

A major limitation in these studies is using a candidate genetic analysis experimental approach. This limits association to the choice of the genetic marker. As we have limited understanding in

the mechanisms involved in neurogenic dysphagia the choice of a genetic marker is made on extrapolating information from associated phenotypes.

1.5.4. Genes and cortical excitability induced by non-invasive brain stimulations

The *BDNF* gene is the most commonly explored gene in terms of its role of regulating motor cortical plasticity [114, 115]. *BDNF* has been identified as a gene with a strong pleiotropic effect in various neurological diseases such as schizophrenia and depression [116] or stroke [117]. Studies focused on the neuroplasticity of the other motor functions such as hand movements followed by rTMS interventions explored SNP from *BDNF*, however without consistency in the results.

Two of the studies where the high-frequency rTMS interventions suggested that individual's genotype for rs6265 may predict a response followed by TMS [115] [114]. On the contrary two other studies showed that individual's genotype for rs6265 does not predict responses after rTMS stimulation [118, 119]. In the study by Hwang *et al.* [120] individuals with Val/Val and Val/Met had higher hand MEPs comparing to individuals with Met/Met genotype (*P*–value = 0.025).

Cheeran *et al.* [115] used three stimulation techniques: continuous and intermittent theta burst TMS; median nerve paired associative stimulation; and homeostatic plasticity to cathodal tDCS to study excitability and plasticity of neuronal circuits in human motor cortex in healthy volunteers. Subjects were divided into two groups- homozygous for Val allele and hetero- or homozygous for the Met allele (Met allele carriers). Carriers of the Met allele had different results comparing to non-carriers (higher excitation after iTBS, lower after cTBS and higher after tDCS). These results suggest a link might exist between *BDNF* polymorphism and cortical excitability after various non- invasive brain stimulation protocols.

Glutamate receptors play an important role in plasticity mechanisms (See Paragraph 1.4.4.2) so therefore become another area of interest in studies exploiting cortical plasticity mechanisms. Mori *et al.* [121] used paired-pulse TMS to study intracortical inhibition (ICI) and facilitation (ICF) in 77 young volunteers. Two SNPs were genotyped rs4880213 and rs6293 from the *GRIN1* gene and three rs3764028, rs7301328 and rs1805247 from the *GRIN2B* gene. Homozygotes for rs4880213 minor allele TT had less intracortical inhibition comparing to hetero- (CT) and homozygotes (CC). Increased intracortical facilitation was observed in the individuals carrying the G allele of rs1805247 *GRIN2B* following iTBS. Both genes code NR1 and NR2B subunits of NMDA receptors similarly to *BDNF* gene which also regulate NMDARs. These studies also suggest that examined genes might influence individual cortical excitability.

All studies described above chose genes for further analysis on the basis of homology with other physiological process, without selecting candidate genes to study from other potential

sources. These studies indicate the importance of replication of the results and considering effects of interactions between genes or proteins.

1.5.5. Genetics of swallowing- evidence from the animal studies

Methodological issues around recruitment and detailed investigation and variability within the outcomes within the human studies make animal studies, in spite of their limitations, an informative source of the genetic data associated with swallowing.

BDNF gene was examined in animal models, with the linkage to *TRKB* gene (other name *NTRK2*) [122, 123]. *TRKB* (OMIM 600456) gene encodes a member of the neurotrophic tyrosine receptor kinase (NTRK) family. This kinase is a membrane-bound receptor that, upon neurotrophin binding, phosphorylates itself and members of the MAPK pathway.

Bariohay *et al*. [122] showed that *BDNF* inhibits the swallowing reflex in rats. Injection of *BDNF* in dorsal vagal complex resulted in inhibition of regular swallowing induced by electrostimulation. Moreover the inhibition is probably stimulated by interaction of *BDNF* and GABAegric interneurons and is associated with *TRKB* activation (*P*-value>0.05, n=14). Bariohay's studies overlook the impact of cortical areas while focusing only on dorsal vagal complex (DVC) and its effect on swallowing. Other limitations include methodological problems in clearly showing dysphagia in the rat is homologous to humans. The author's conclusions were based on the presence of masticated, but not digested food in rats' cages.

Comparatively, Schaser *et al.* [123] in a rodent model used 48 rats divided into three age groups: 16 young (9–10 months), 16 middle-aged (24–25 months) and 16 old (32–33 months). Immunocytochemistry tests showed that immunoreactivity of *TRKB* in the sensorimotor system decreases with age (*P*-value = 0.03). Additionally *BDNF* expression increased after tongue pressure exercises, but only in the young rats (*P*-value = 0.0003). Among the group of old and middle aged rats there were no significant decrease of immunochemistry of this protein. Moreover there were no significant increases in *TRKB* and *BDNF* expression after tongue muscles exercises in old and middle aged animals. These studies were only preliminary and further, more detailed investigation is needed.

Kurihara *et al.* [124] examined the influence of two hydrolases encoded by genes *UCHL1* and *UCHL3* on dysphagia in mice. The authors reported that Uch-L1gad and Uch-L3Delta3-7 double homozygote mice had a 45% weight reduction compared to the wild type (*P*-value< 10^{-6}) which they used as a proxy for a direct measurement of dysphagia. They also used the method of identification of un-digested, but masticated food in the animals' cages. As mentioned previously, the loss of weight could have different causes. Further limitation is that the authors examined only the pathological changes in the nucleus tractus solitarious (NTS), not examining

the cerebral cortex. Presence of protein aggregation in the mouse's brains might be evidence of neurological causes of swallowing impairments in these animals.

Another protein which has been reported to possibly affect swallowing control is leptin encoded by *OB* gene. Leptin plays a role in the regulation of feeding behaviour. Felix *et al.* [125] showed the inhibitory effect of the *OB* gene on swallowing in rats. The results showed effects of leptin on the swallowing central pattern generator (SwCPG) as well as the motor neurons activity (motor outputs). Dysphagia in rats was diagnosed in the same way as in previous studies- presence/absence of masticated, undigested food. There is no confirmation of these studies since 2006. The authors were examining swallowing in general, not specifically dysphagia and the effects on appetite cannot be excluded.

Swallowing difficulties were also studied in terms of orofacial pain which often occurs with dysphagia. Tsujimura *et al.* [126] investigated the effects of orofacial stimulation on the swallowing reflex, phosphorylated extracellular signal-regulated kinase (pERK) within the area of the NTS. Anaesthetized rats had stainless steel wire electrodes placed in the mylohyoid muscle to record EMG activity. Changes in swallowing performance were assessed by laryngeal movement and by the mylohyoid EMG activity. The findings provided evidence that facial pathways between skin and NTS as well as lingual muscle and the NTS might modulate swallowing reflex by facial and lingual pain, respectively. This study was not focused on genetics of swallowing, but might provide some evidence for involvement of the gene encoding pERK protein. Studies examined only the involvement of the brainstem and no cortical areas in control of swallowing. The main advantage was the more reliable and detailed method of swallowing assessment.

The last potentially relevant study explored the recovery processes not from swallowing, but other motor function (paw movement) showed the influence of *BDNF* polymorphism rs6265. Mice received sham or transient middle cerebral artery occlusion (animal model of stroke). Motor functions were assessed regularly for 6 months after stroke and then anatomical analysis was performed. Mice with genotype Met/Met showed increased neuronal plasticity in the intact parts of the brain, especially in the contralateral striatum. Authors suggested that rs6265 may play an adaptive function in the recovery from stroke. In this case, the presence of this polymorphism could imply a significant role in maintaining the balance between inhibitory and excitatory circuits within the brain [127].

Animal studies from the field of neuroscience, apart from multiple advantages, carry different kinds of other disadvantages, thus analysis of the results should be considered with caution. One of the potential causes may be differences in brain structures even among the same species [128]. One of the advantages of using rats as the animal model is that they have a short life span (36 months) which allows studying physiological changing with ageing, responsiveness to different kinds of interventions.

Despite the limitations, significant proportion of results from animal models can go on to replication in human studies; therefore replication of the genetic loci from this work is warranted in my experimental work.

1.5.6. Genetic syndromes, where one of the features is dysphagia

Dysphagia is a common symptom observed in congenital genetic syndromes. Studies conducted on patients with these genetic syndromes, where the detailed genetic background is examined, may provide another source of valuable information of swallowing genetics. The literature describing these complex genetic diseases could provide evidence about chromosomal localization of genes which may play a role in swallowing difficulties.

The following sections exclude syndromes where swallowing difficulties are caused by: severe cleft palate (frequently observed in Pierre Robin Syndrome), inappropriate mastication and eating quickly which can cause choking (e.g. Prader-Willi syndrome).

1.5.6.1. Potocki-Lupski syndrome- Ch 17 (dup(17)(p11.2p11.2)

Potocki-Lupski syndrome (PTLS) is caused by micro duplication of chromosome 17p11.2 [dup(17)(p11.2p11.2)]. The phenotype is characterised by a number of dysmorphic features, hypotonia, sleeping problems, cardiovascular diseases and gaining insufficient weight. Moreover patients suffer from neurological and cognitive features including intellectual impairment and autism. However not every patient presents all of these features. Genetically patients have duplicated region of the short arm of chromosome 17.

Soler-Alfonso *et al.* [129] published studies about the association of oropharyngeal dysphagia and failure to thrive in PTLS. A limitation of this study was the number of patients (18 with available swallowing function study analysis), which is understandable, with an extremely rare disease. Another limitation with the author's approach was the method of dysphagia identification by radiographic views of chewing and swallowing.

1.5.6.2. Stuve-Wiedemann syndrome- locus 5p13.1

Stuve-Wiedemann syndrome (SWS) is a rare, genetic autosomal recessive disease with main features associated with bone dysplasias, respiratory distress and physical disability and early mortality. Most of the patients suffer from swallowing difficulties followed by aspiration pneumonias which are a key contributor to cause of death among these children [130].

Dagoneau *et al*. [131] investigated 19 families of SWS patients. Using a linkage analysis the authors screened 24 patients with SWS and 19 families and revealed that chromosomal region 5p13.1 may be associated in the pathogenesis of this syndrome. Moreover they analysed in more detail one of the genes from chromosome 5q13.1 – *LIFR* and analysed the mRNA transcripts. Most of the children from analysed families had swallowing problems with diagnosis of dysphagia. Another study on a two-year old female with SWS and severe dysphagia, confirmed the mutation in *LIFR* gene [130]. *LIFR* is probably not associated in swallowing

difficulties, because it main function is bone formation. Thus during further analysis other genes from 5p13.1 chromosomal region should be considered and investigated.

1.5.6.3. CHARGE syndrome- locus 8q12.

CHARGE syndrome is a mnemonic for coloboma of the eye, heart defects, atresia of the choanae, retarded growth and development, genital and/or urinary abnormalities, and ear anomalies. CHARGE Syndrome is most likely caused by mutations within the chromosomal region 8q12. Main features of the CHARGE syndrome comprise of coloboma (abnormality of the eye caused by the missing tissue of the iris or the retina-choroid), one or two-sided choanal atresia (blocking of the nasal passage), cranial nerve dysfunction causing hearing and swallowing impairment, orofacial clefts, developmental delays and cardiovascular problems.

One of the studies indicated that swallowing problems affect 79% of children with CHARGE syndrome [132]. The swallowing impairment was assessed by parents reporting. Proportion of the cases with swallowing impairment may be caused by the clef palate which occurs in 20% of children affected by the syndrome. Nevertheless swallowing difficulties lead to more severe feeding difficulties which remain the leading cause of neonates death with CHARGE syndrome. The main gene related to the CHARGE syndrome is the CHD7 gene from the chromosome 8q12 which encodes Chromodomain Helicase DNA Binding Protein. The exact mechanisms of the pathways with CHD7 gene remain unknown.

1.5.6.4. DiGeorge syndrome- locus 22q11

DiGeorge syndrome is caused by a small deletion of the chromosome 22q11. Clinical features are difficult to describe and vary between all individuals with Di George Syndrome, even within the families. Main features include: heart defects and orofacial abnormalities. Patients with DiGeorge syndrome develop autoimmune disorders such as rheumatoid arthritis, breathing and hearing impairments, seizures caused by low level of calcium, gastrointestinal problems such as dysphagia.

VFS study performed on 75 children with DiGeorge Syndrome [133] identified problems with coordinating the suck/swallow/breath pattern leading to gagging or regurgitation. Karpinski *et al.* [134] recently developed an animal model of DiGeorge syndrome, 22q11 knockout mice were compared with mice with normal genotype. 21 genes were selected to the analysis. Apart from features such as altered jaw morphology mice had swallowing impairments and chest infections caused by aspirations. Swallowing problems and aspirations were assessed post mortal by the presence of milk in the nose and the sinuses of mice infants. This may be a limitation of the study, because swallowing impairment assessment in mice and rats is problematic (see section 1.5.4.1.). Mice pups had disrupted development of cranial nerves crucial for feeding and swallowing (CN X, CN IX, CNX). Different expression with knockout mice and wild type was

observed in the *COMT* gene, as may play a role in the cortical plasticity in humans (see paragraph 1.5.3.). Thus there is a role for testing this genetic locus in my experimental studies in this work

The studies presented above have major genetic contribution to the clinical problems. There are also well specified regions of the genome implicated as causal in the problems patients experience including swallowing. However considering the aims of this doctoral research, one of the disadvantages is the fact that swallowing problems within the cohorts of patients with congenital syndromes might be due to the brainstem problems with no evidence of the cerebral cortex involvement. Nevertheless the genetic loci implicated in this work will be considered in the experimental work conducted.

Summary of Section 1.5.

Unravelling the genetic basis of dysphagia is a key step in understanding the physiology and pathophysiology of swallowing processes. Molecular studies focus on diverse areas, but this research project will focus on single nucleotide polymorphisms within genes or non-coding regions. Genetic studies on human swallowing remain very limited, with lack of twin studies confirming heritable features of swallowing. Existing literature highlight a number of single genes or SNPs which might take a part in swallowing neurophysiology. The most commonly studied gene is *BDNF*, however depending of the study design might or might not predict motor responses followed by neurostimulation paradigms. Another gene highlighted in population studies is *APOE* which probably affects swallowing. *COMT* gene might take a role in motor cortical plasticity and is located in the area of chromosome 22q11 (deletion of this area cause DiGeorge syndrome with severe swallowing impairments) and should be evaluated in the further research. Animal studies provide an evidence of the impact of genes *BDNF, TRKB, UCHL1, UCHL3* and protein encoded by *ERK* gene. Main disadvantage of animal studies is lack of accurate assessment of swallowing impairment in rats and mice. The last area of the literature which may provide the important insight of genetic basis of dysphagia comprise of exploring genetic syndromes where one of the features is dysphagia. These studies provide information on chromosomal localization of genes which may take a part in the process of impaired swallowing development. Swallowing impairments are common in Potocki- Lupski syndrome (chr17p11.2), Stuve-Wiedemann syndrome (chr5q13.1), CHARGE syndrome (chr8q12) and DiGeorge Syndrome (chr22q11).

Swallowing due to its complicated physiology is most likely controlled by numerous genes and pathways between these genes. Presented studies show the need of more comparative integrative research protocols, consistency of methodological approach and replication of existing findings in order to find numerous genetic candidates which may control neurophysiology of swallowing.

1.6. Searching for candidate genes underlying complex genetic traits such as swallowing impairment.

There is very limited evidence of molecular pathways involved in swallowing physiology and pathophysiology. Existing literature provide an evidence on the need to use more global approach before conducting another experimental studies. One of the first steps in exploring genetic traits of complex disorders is through performing Genome-Wide Association Studies [135].

1.6.1. Principals of Genome-Wide Association Studies

National Institutes of Health (NIH) defines Genome-Wide Association Studies (GWAS) as a tool used to identify common genetic variants across the entire human genome that influence health and disease (www.nih.gov). Direct or indirect genetic associations (*Figure 1.13*) are confirmed between any two characteristics (gene/SNP vs disease) when are present more often than it would be expected by chance [106].

The GWA Studies were successfully used to identify the genetic determinants of common diseases the most famous example the Welcome Trust Case Control Consortium with approximately 14000 cases of conditions where researchers were examining type 1 and 2 diabetes, coronary heart disease, rheumatoid arthritis, Crohn's disease, bipolar disorder, and hypertension [107].

The main hypothesis behind GWAS studies states that common disorders are likely influenced by genetic variation that is common in the population. Common SNPs which are the most successfully identified with GWAS design have small genetic effects (penetrance) *Figure 1.14*. Additionally if common allele has small penetrance, but common disorder shows heritability in families (twin studies) this means that most likely multiple common alleles influence disease susceptibility. This causes experimental challenges with a large number to amount of possible genetic factors that might play a role in the disease, even in successful large population-based studies [136].

There are several criteria for the population-based studies such as GWAS studies which have to be fullfield. First one is determining the location and density of common SNPs. The genetic markers from the array should capture majority of genetic variants with sufficient power of the study in order to produce true, unbiased results. Then, population specific differences have to be determined (population stratification). The International HapMap Project has been designed to identify variation for specific populations with European descent, the Yoruba population of African origin, Han Chinese individuals from Beijing, and Japanese individuals from Tokyo [137]. Another criterion is that SNPs should have determined is Linkage Disequilibrium between them in order to reject redundant information. Linkage disequilibrium (LD) is a property of SNPs

that describes the degree to which an allele of one SNP is inherited or correlated with an allele of another SNP within a population[136].

Association studies carry a high risk of generating false positives results, therefore before the statistical analysis the sufficient power of at least 80% should be obtained and a number of quality control procedures on the genotypic and phenotypic data should be performed.

Considering all these points, Genome Wide Associations Study analysis used in the following project was performed in order to find associations between genetic locus and swallowing impairment among the population of elderly, healthy individuals to inform the experimental work to follow.

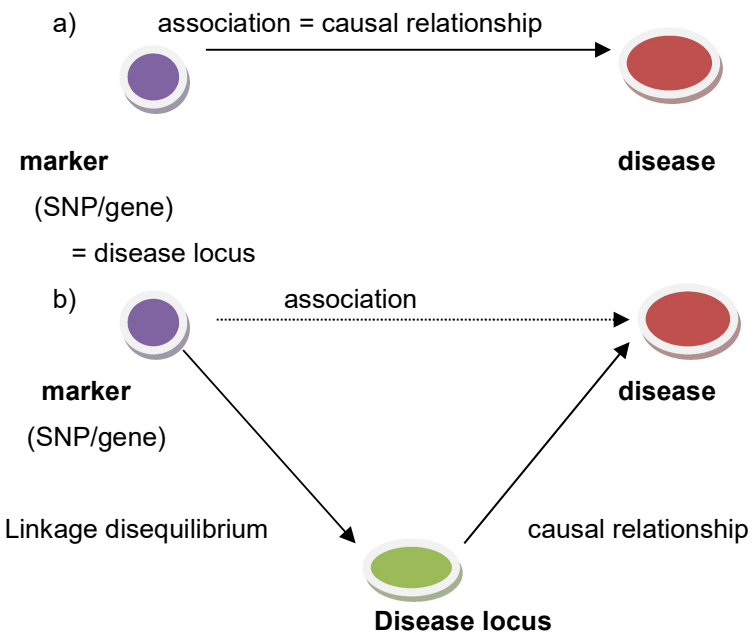

Figure 1.13. **Two types of associations: direct association between marker and the disease (a) and indirect association between marker and locus of the disease (b).**

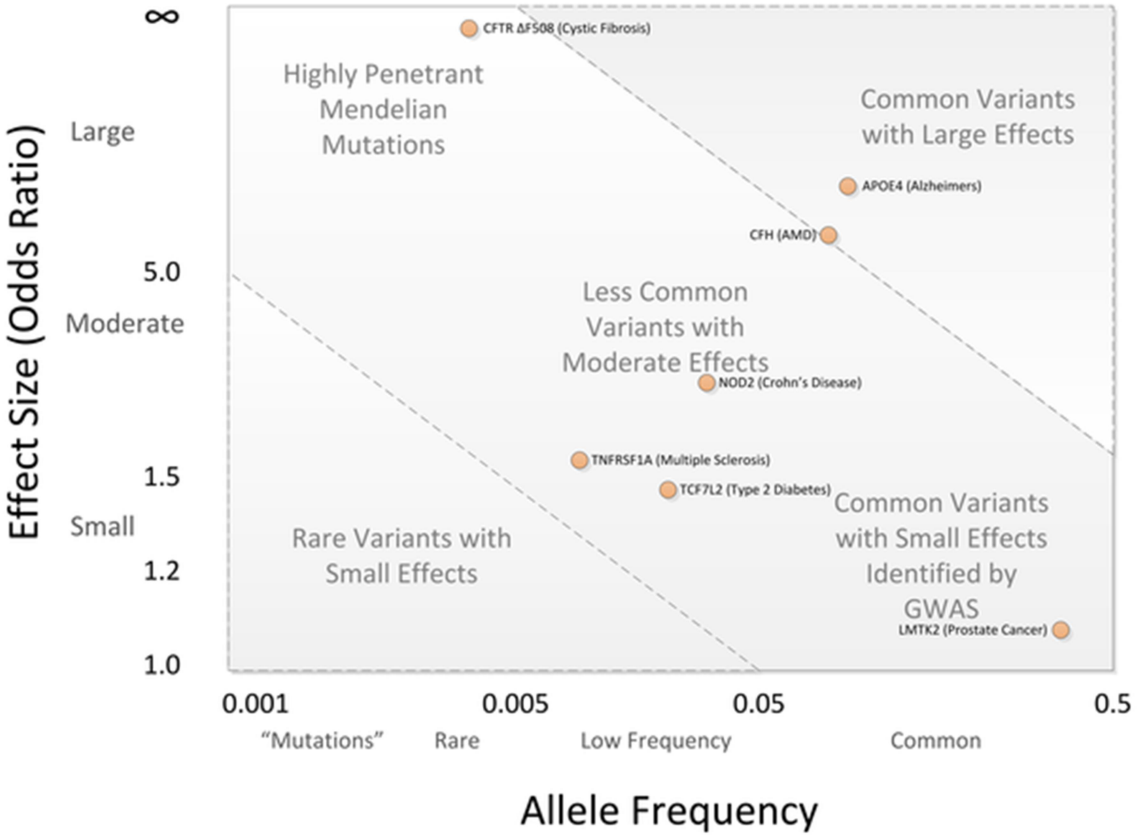

Figure 1.14. **Spectrum of Disease Allele Effects.** Disease associations are usually described by allele frequency and effect size. Alleles for Mendelian disorders (inherited according to follows the laws proposed by Gregor Johann Mendelin 1865) are extremely rare with large effect sizes (upper left). Most GWAS findings are associations of common SNPs with small effect sizes (lower right).
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Following this introductory chapter, I will present a series of experiments exploring genetic underpinnings of swallowing impairment and swallowing motor system. Schematic representation of steps which has been undertaken is presented in *Figure 1.15.* This will be followed by an over-arching concluding chapter synthesising the work presented, considering the findings interpretion, considering all the limitations and positioned within the existing literature. This is followed by discussion how this work will develop in the future to understand genetic components of swallowing and dysphagia further.

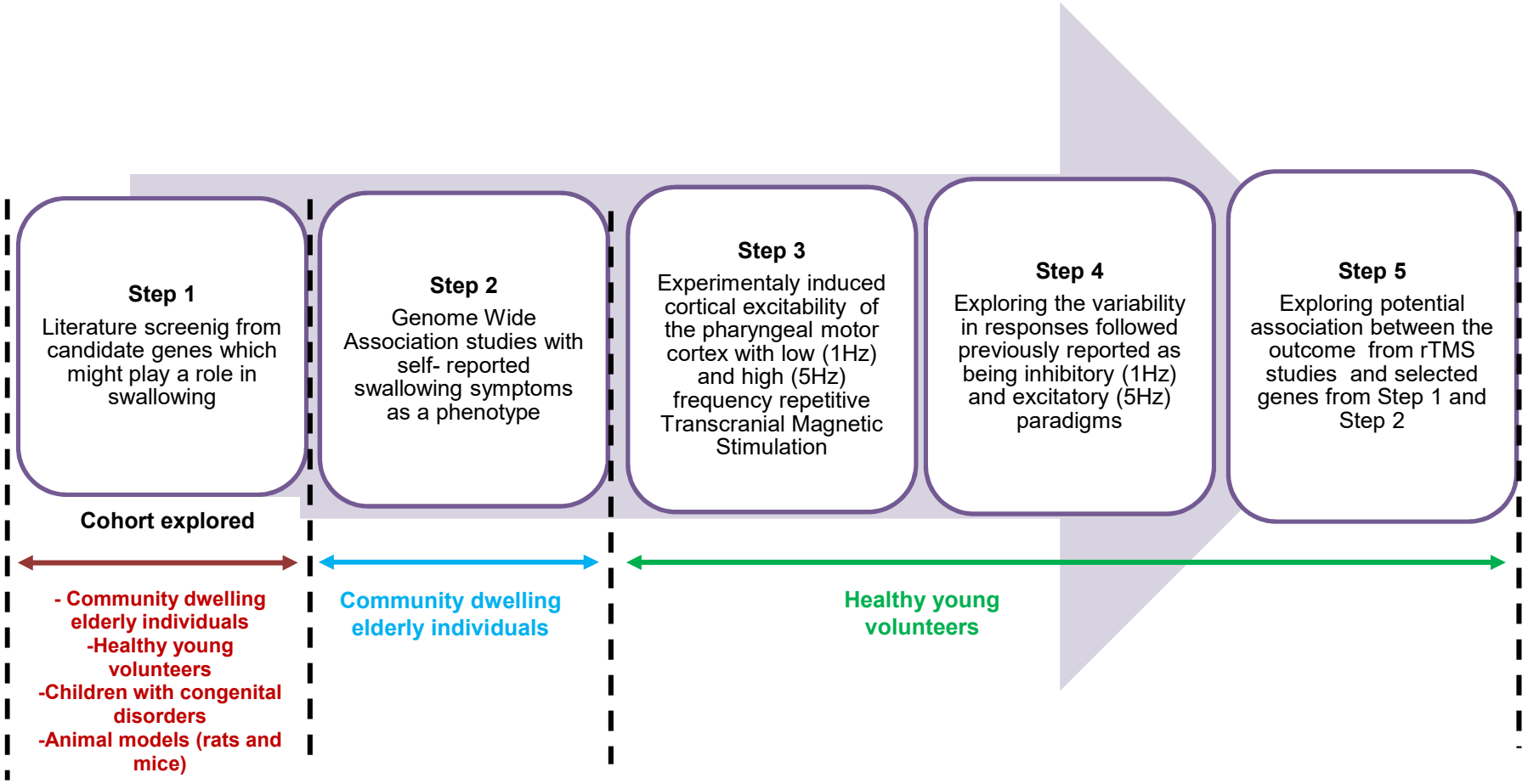


Figure 1.15. **Schematic representation of steps of presented doctoral research.**

Chapter 2

Genetic determinants of swallowing impairments among community dwelling older population

Abstract

Background: Swallowing difficulties (dysphagia) affect a significant proportion of community dwelling older individuals, being more prevalent in age-associated neurological conditions such as stroke and Parkinson's disease. The genetic determinants of dysphagia are still being explored and have largely been studied through candidate gene analysis approaches. The aim of the study was to perform a genome-wide association study (GWAS) of common genetic single nucleotide polymorphisms (SNP) and self-reported swallowing impairments in a longitudinal cohort of community dwelling older adults.

Materials and methods: We performed a case-control genome-wide association study of self-reported swallowing symptoms using the Sydney Swallow Questionnaire. The analysis included 555 community dwelling, unrelated, older adults (mean years of age=81.4; SD=5.349) with known phenotype and genetic information consisting of 512,806 single nucleotide polymorphisms. Gene-based association analysis of these traits was also conducted.

Results: Analysis of the cohort confirmed European ancestry with no major population stratification. Further analysis for association with swallowing impairment identified one SNP rs17601696 which achieved genome-wide significance (*P*-value=5×10(-8)) within a non-coding region of chromosome 10. Gene-based analysis did not result in any genome-wide significant association.

Conclusion: SNP rs17601696 may have an impact on swallowing impairment among elderly individuals. The results require replication in an independent cohort with appropriate phenotype/genotype data.

2.1. Introduction

The swallowing process is controlled by a coordinated neuromuscular system regulated by areas of the brain stem and the cerebral cortex. Difficulty in the ability to swallow solid or liquid materials is termed dysphagia. Approximately 15% of healthy, ageing population may be affected by swallowing difficulties [138]. The presence of stroke, Parkinson's disease and other neurological conditions increases rates of dysphagia [4, 5]. Swallowing impairments are associated with higher risk of pneumonia, dehydration, malnutrition and lowered quality of life due to increased risk of anxiety and depression [139]. Patients with dysphagia reveal different recovery patterns [48], which often impacts on the effectiveness of existing therapies.

Recent evidence suggests that the swallowing process may in part be affected by genetic variations. Previous studies conducted by Jayasekeran *et al.* [108] reported an association between a Brain–Derived Neurotrophic Factor *(BDNF)* (OMIM 113505) gene polymorphism rs6265 and the response to neurostimulation in the area of the brain responsible for swallowing. *BDNF* is a member of the nerve growth factor family, expressed in cortical neurons and is necessary for survival of striatal neurons in the brain. *BDNF* was previously described as being a gene with pleiotropic effect, playing a role in neurological and psychiatric diseases which include depression and schizophrenia [116]. Mentz *et al.* [110] performed the first association analysis between self-reported swallowing symptoms among older individuals and two single nucleotide polymorphisms (SNPs) within the *APOE* gene (OMIM 107741). The *APOE* gene which encodes apoliporotein is essential for normal catabolism of triglyceride-rich lipoprotein constituents and has been reported as a risk factor in dementia and cognitive decline in an elderly cohort [112, 113].

A potential limitation to current candidate gene based analysis models for swallowing symptoms and dysphagia are that they depend on a-priori assumptions about biological processes in a complex system. A genome-wide association study (GWAS) offers an effective method to identify novel genetic variation which confer susceptibility to complex genetic disorders [140]. As yet there have been no GWAS investigations of dysphagia.

The aim of this chapter was to examine the contribution of SNPs to swallowing impairment in older people using a genome-wide screening approach. We present the results from the first case-control GWAS of self-reported swallowing symptoms related to dysphagia derived from a cohort of non-hospitalised, community dwelling older adults.

2.2. Methods

2.2.1. Study cohort

A subset of individuals from the Dyne-Steel DNA archive for cognitive genetics of older adults was used where data on swallowing had been collected. The Dyne-Steel archive is an on-going study established by the "University of Manchester Longitudinal Studies of Cognition in Normal Healthy Old Age" initiated in 1981 [12]. This cohort of 6542 healthy older adults aged between 42-92 years comprised community dwelling older adults from Manchester and Newcastle in the United Kingdom with contemporary cognitive, lifestyle and health information. Between 1999 and 2001, approximately 2000 volunteers consented to donating blood samples for genetic studies of cognitive ageing. Only 800 continued the study in 2004 when the Sydney Swallowing Questionnaire was send. The numbers decreased due to death of participants or withdrawing from the study. The swallowing questionnaire was send again to all participants in 2008 from whom 634 completed forms giving response rate of 79%. From 634, 555 had full genetic and phenotypic information used in the following studies.

This study relates to a sample of 555 volunteers from this cohort which had appropriate genetic and clinical data (*Table 2.1*).

2.2.2. Swallowing phenotype

Swallowing phenotype is constructed from participants' answers to the Sydney Swallow Questionnaire (SSQ) [68]. The SSQ contains 17 questions, scored from 0 to 100, about the difficulty of swallowing (maximum score from the questionnaire is 1700). For each question a score of 0 means no problem at all whereas 100 indicate severe difficulty. Swallowing impairment was judged to be present, when the total score from the SSQ for each individual was equal or above 180 (based on previous findings presented by Wallace *et al.* [68]). Volunteers were classified as being cases when the total score from the SSQ was ≥180 and controls when total score from the SSQ was <180.

2.2.3. Neurological and depression phenotype

Information about participants' demographics such as age and gender were available from data collected. Advanced age, presence of Parkinson's disease or history of stroke and clinical depression have been described as major risk factors for swallowing symptoms related to dysphagia. Self-reported presence of stroke or Parkinson's disease have been assessed using the Cornel Medical Index (CMI) Health Questionnaire [141]. To measure emotional health in these individuals, responses to the Geriatric Depression Scale (GDS) 15 item version was used [142]. This information was included in the analysis as confounders.

	Total	Mean	Percentage	Range
Subjects: Male Female	 125 430	 82±5.3 (years) 81±5.4 (years)	 22.5% 77.5%	 69-98 (years) 69-98 (years)
Score ≥180 from SSQ for swallowing impairment	71		12.8%	
Presence of neurological disorder Parkinson's Disease and/or stroke	52		9.4%	
GDS score >5	43	1.46	7.45%	0-12

Table 2.1. **Study cohort characteristic.** GDS stands for geriatric depression score

2.2.4. Phenotype for 'sensitivity analysis'

For additional, sensitivity analysis was performed in which subjects with stroke (n=48) and Parkinson's disease (n=4) or both (n=1) were excluded and GDS 15 score was not included as a covariate. In this analysis only age and sex were included as confounders.

2.2.5. Genotyping and quality control

All DNA samples underwent genome-wide genotyping using the Illumina Human 610-Quad v1.0 Genotyping BeadChip (approximately 620000 markers). This was performed at the Edinburgh University Wellcome Trust Clinical Research Facility, Edinburgh, UK.

Genotyping and quality control for the Dyne-Steel cohort which included participants used in the current study have been described elsewhere [143]. Briefly, corrections of sex differences errors, chromosomal abnormalities, relatedness between individuals and population substructure were corrected with additional analysis using PLINK software (http://pngu.mgh.harvard.edu/purcell/plink) [144]. Individuals were excluded from this study based on unresolved gender discrepancy, relatedness, call rate (≤ 0.95), and evidence of non-Caucasian descent. SNPs were included in the analyses if they met the following conditions: call rate ≥ 0.98, and Hardy-Weinberg equilibrium test with *P*-value ≥ 0.001, minor allele frequency (MAF) ≥ 0.05. The levels of MAF were increased from MAF ≥ 0.01 in the current GWAS considering the sample size and power for this association analysis.

A total number of 512,806 of the 549,692 SNPs passed quality control procedures and were available for two approaches of the analysis.

2.2.6. Statistical analysis

To calculate the statistical power of the study the G*Power programme was used. SNP-based GWAS was performed using the PLINK toolset for genotype-phenotype analysis using logistic regression (http://pngu.mgh.harvard.edu/~purcel/plink) [144]. Four covariates: age, sex, GDS depression scores and presence/absence of key neurological disorders (stroke and Parkinson's disease) were included in subsequent analyses. A second 'sensitivity analysis' model was performed with only age and sex as covariates on sub-sample of the cohort (see 2.4). Tests for association were performed by PLINK v.107 separately for each SNP in the additive disease model. A quantile-quantile (Q-Q) plot was used to characterize the extent to which the observed distribution of the test statistic follows the expected (null) distribution. Results for both SNP-based genome-wide analysis (Manhattan plot) and Q-Q plot were visualised using RStudio v.0.96.331 (http://www.rstudio.com) [145]. Gene-based tests for association were carried out using results from the SNP-based analysis using the VEGAS programme (http://gump.qimr.edu.au/VEGAS/) [146]. To plot regional association results from SNP- and Gene- based GWAS Locuszoom was used [147].

Statistical significance following multiple testing was assessed using Bonferroni correction (62), for the total number of SNPs (n=512,806, *P*-value ≤ $1.0x10^{-7}$) and genes (n=17,676; *P*-value ≤ $2.8x10^{-6}$) analysed in the study.

Multidimensional scaling analysis (MDS) was carried out in PLINK and visualized in SPSS software (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0 Armonk, NY) for study participants from and 210 unrelated samples from the International HapMap Project, using a subset of 390,142 SNPs (Supplementary material 2.1.).

Previous studies showed the involvement of *BDNF* and *APOE* genes in the swallowing performance in humans [108, 110]. Therefore result for rs6265 (*BDNF*) has been extracted from SNP- based GWAS analysis and *APOE* from additional data was committed on same subjects using Sequenom (Sequenom Inc, San Diego, USA) using the iPLEX method. This method has been described previously by Ghebranious *et al.* [148]. *APOE* genotype requires additional analysis with 3 different alleles (APOE- ε2 , APOE- ε3 and APOE- ε4) defined by two SNPs rs429358(C) + rs7412(T). APOE- ε4 allele is associated with multiple neurological conditions such as dementia [112], therefore we divided our cohorts into 2 groups of carrying one or two APOE- ε4 alleles and those who do not carry the APOE- ε4 allele. 550 individuals from the initial cohort had phenotypic and genetic information for *APOE*. From whom 130 individuals had at least one APOE- ε4 allele. We have performed simple chi square test between APOE- ε4 carriers and non-carriers and swallowing phenotype.

Imputation to 1000 Genomes catalogueswas performed at the Arthritis Research UK Centre for Genetics and Genomics, Institute of Inflammation and Repair, University of Manchester. Briefly, IMPUTE2 software with human genome issue HG 19 aligned to 1000 Genomes reference panel from 16th June 2014. We used European populations panel with MAF >0.01 and PLINK v1.90b3b 64-bit programme used to convert to PLINK format. Pre analysis filtering included: excluding SNPs with and IMPUTE2 INFO score of 0.8; in the conversion to PLINK format, SNPs were hard-called using a posterior probability cut-off of 0.9. In the analysis this resulted in inclusion of 4,196,861 SNPs.

2.3. Results

Characteristics of the participants' age, gender and GDS scores used in the following analysis are shown in *Table 2.1.* The analysed cohort consists of elderly individuals, largely female. Participants with self-reported scores from 0-180 were classified as controls. The presence/absence of Parkinson's disease, stroke, GDS scores were used as covariates in the analysis. Less than 10% of participants had a history of stroke, 4 individuals had Parkinson's disease and 43 individuals had GDS scores >5, a significant threshold widely used to indicate mood disorders or poor emotional health.

In the second sensitivity analysis approach only individuals without stroke and Parkinson's disease were included without adjusting for GDS score. This resulted in total sample of 503 volunteers (392 females, mean years of age=81; SD=5.4) and 59 (11.3%) with total Sydney Swallow Questionnaire score >180.

2.3.1. Statistical analysis

Statistical power for the study was calculated for 555 individuals with G*Power programme [149]. 71 volunteers of this sample being defined within the case category and a case-control ratio of 1:9. A log-additive model was used, with an allele frequency between 0.1 and 0.4 and statistical significance set at *P*-value = $5x10^{-7}$. This indicates that for allele frequencies 0.1-0.2, there is 83% power and for 0.3-0.4, 96% power to detect effect size with odds ratios above 1.6.
Analysis for biases due to population stratification was performed using MDS components and the data visualised using a plot of first two eigenvectors. The output was visualised for individuals lying further than approximately one tenth the distance to non CEU HapMap sample and this did not show any evidence of substructures within the analysed population (*Figure 2.1*). This matches findings described in a previous analysis of the cohort [150].

In the SNP-based GWAS analysis (*Figure 2.2*), one SNP rs17601696 (chromosome 10q26.13) achieved genome-wide significance (OR=4.75; 95%CI 2.72-8.32; *P*-value=1.7×10^{-8}). The rs17601696 T allele was associated with higher scores on SSQ and therefore an increased risk of dysphagia symptoms. The rs17601696 polymorphism is located within the intergenic region, ~117 kb downstream of the fibroblast growth factor receptor 2 (*FGFR2*) gene.
Further genome wide analysis identified 11 SNPs with *P*-value ≤ 1.7×10^{-6} and 56 SNPs showing association at *P*-value ≤ 10^{-5}. Additionally, the top 25 SNPs below the multiple testing correction threshold are summarized in the *Table 2.2*.

Gene-based analysis was performed to provide genome-wide evidence for specific genes potentially associated with self-reported symptoms related to dysphagia. This did not reveal genes which retained significance following correction for multiple testing. *Supplementary material 2.1.* summarises 16 genes with the highest significance (*P*-value ≤ 10^{-4}). The most

significant genes, *CCNJL* (encoding Cyclin J-Like) and *C1QTNF2* (encoding C1q and tumour necrosis factor related protein 2) are localized within the region of chromosome 5q33.3, however SNP-based genome-wide analysis did not support the evidence of high significance of this chromosomal region.

SNP rs17601696 with the highest significance from SNP-based analysis, *CCNJL* and *KIAA0513* genes chromosomal locations and 400kb regions flanking were plotted in LocusZoom (Supplementary material 2.3.).

The Q-Q plot (*Figure 2.3*) shows slight deviation of observed versus expected - log(*p*) values at the higher ends of significance but within an acceptable range. The Genomic inflation factor, also known as lambda gc (λgc) was 1.020209 for the analysis indicating that there was no appreciable inflation of the test statistics.

Nine of the top 25 SNPs, were located within intronic regions of *KIAA0513* (rs17789174), *TTC23L* (rs163233), *BAZ2B* (rs4665083, rs1269553), *KCNIP4* (rs4911134), *CRMP1* (rs11945849), *GPR109A* (rs109516642), *CTB43E* (rs4868308), *RWDD1* (rs11755235).

Considering their previous association with swallowing phenotype, we performed two additional analyses with *BDNF* rs6265 and *APOE* genotype (presence or absence of APOE- ε4 allele) with chi square nominal significance threshold (*P*-value < 0.05). There was no association between *BDNF* (*P*-value=0.15) and *APOE* (*P*-value = 0.63) and swallowing phenotype.

Further sensitivity analyses using the cohort of older adults with no history of neurological disorders (Parkinson's disease or stroke) or adjustment for GDS 15 score was performed using subgroup of 503 individuals. In the SNP-based GWAS analysis (*Figure 2.4.*), SNP rs17601696 (chromosome 10q26.13) again achieved highest significance (OR=4.75; 95%CI 2.72-8.32; *P*-value = 1.7×10^{-8}*).*

No additional significant SNPs were identified with the analysis based on the 1000 Genomes imputed data compared to the genotyped data. 100 SNPs with the highest significance level are listed in the *Supplementary material 2.4*.

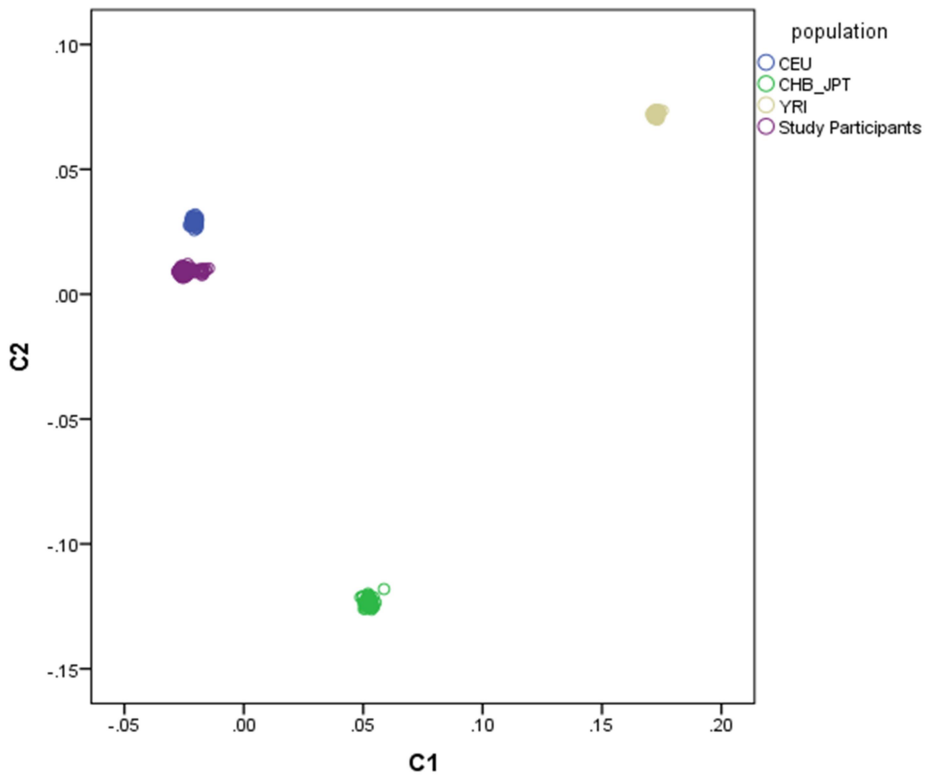


Figure 2.1. **Multidimensional scaling plot of study participants compared to HapMap data.** The blue points represent individuals with European ancestry (CEU), yellow points represent individuals from Yoruban population (YRI), green points represent individuals from Chinese and Japanese population (CHB_JPT).

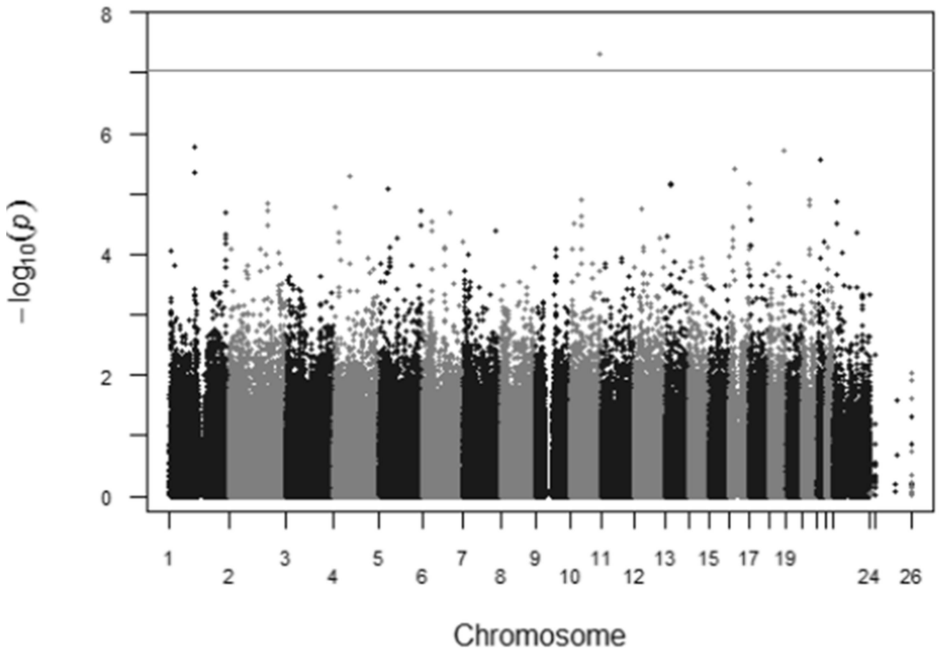


Figure 2.2. **Manhattan Plot for genome- wide association results for the analysed cohort.**
The Figure shows the results for 555 participants from the Dyne Steel Cohort. The $-\log_{10}$ *P*-values (y axis) of 512,806 SNPs are presented based on their chromosomal localization (x axis). The grey line indicates the genome-wide significant threshold for multiple testing.

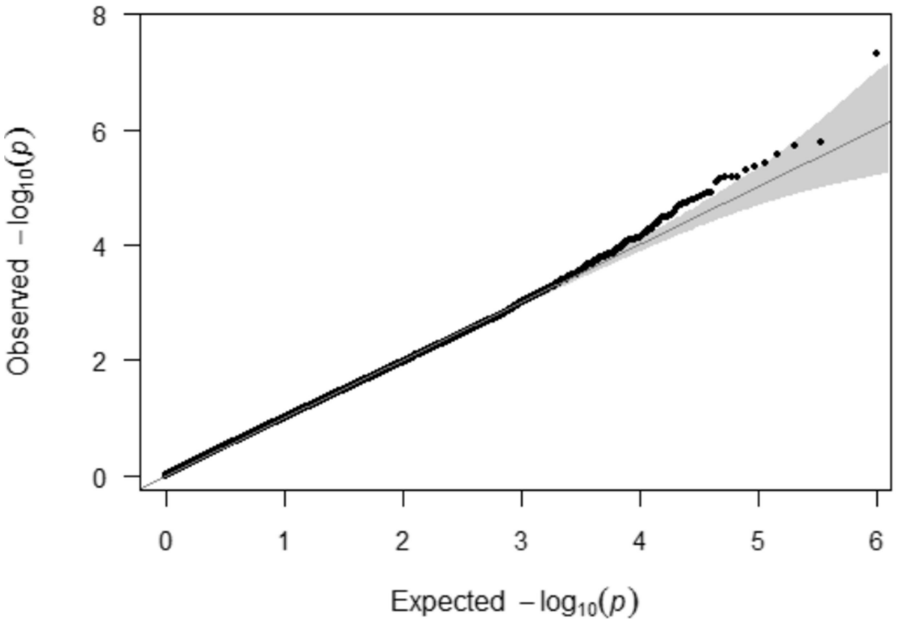


Figure 2.3. **Quantile-quantile plot of P-values for genome- wide association results for the 555 participants from the Dyne Steel Cohort.**

The black points represent the observed results, the dark grey line represents expected results under the null hypothesis of no association and 95% confidence interval in the shaded area.

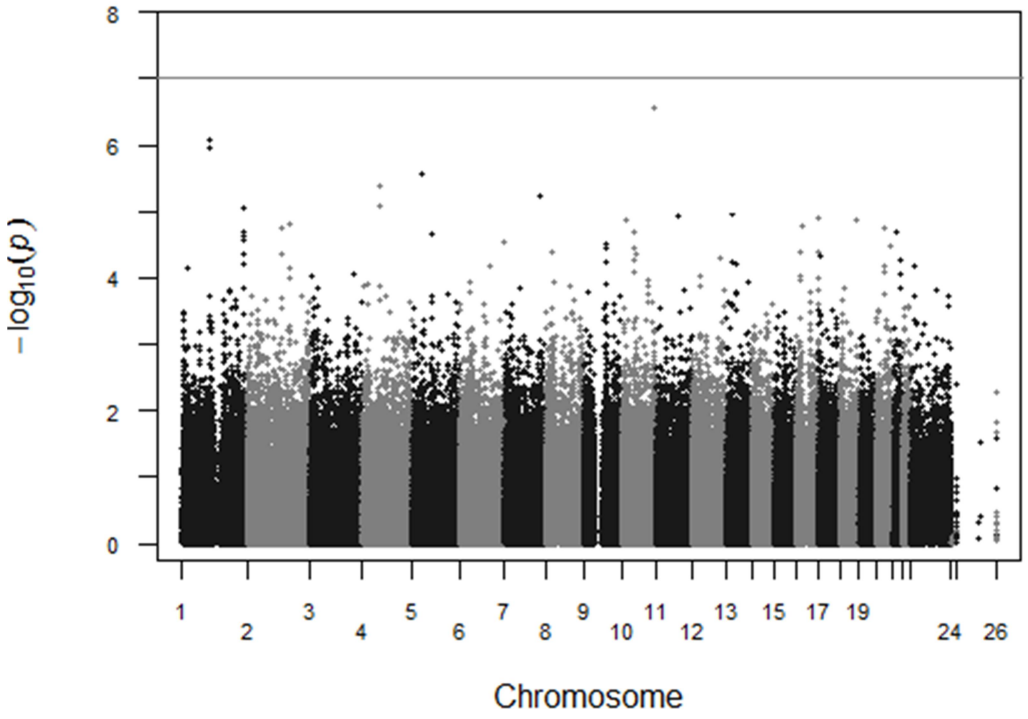


Figure 2.4. **Manhattan Plot for genome- wide association results from the sensitivity analysis.**

The Figure shows the results for 503 participants from the Dyne Steel Cohort without the history of stroke and Parkinson's disease. The $-\log_{10}$ *P*-values (y axis) of 512,806 SNPs are presented based on their chromosomal localization (x axis). The grey line indicates the genome-wide significant threshold for multiple testing.

2.4. Discussion

In this study we reported results from the first genome-wide screen of self-reported symptoms related to dysphagia. This identified one SNP (rs17601696) on chromosome 10, whose function remains unknown, which was significantly ($<10^{-8}$) associated with the dysphagia phenotype. This SNP and others which neared genome wide significance have not previously been implicated with swallowing physiology or pathophysiology and therefore require replication using an independent cohort. One reason for the lack of overt association could be the polygenic nature of the symptom as seen in other common complex conditions.

The strongest associated SNP (rs17601696) is 117 kb downstream of the *FGFR2* (OMIM 176943) gene. *FGFR* (fibroblast growth factor receptor 2) is a member of fibroblast growth factor receptor (FGFR) family, which acts as cell-surface receptors for fibroblast growth factors. Members of the FGFR family play a key role in the regulation of cell proliferation, differentiation, and in the regulation of embryonic development. Previous studies have reported the relationship between *FGFR2* gene and the regulation of *ERK* gene [151, 152]. Evidence from animal studies demonstrated that *ERK* gene may have a regulatory function to swallowing reflex in rats [126]. This suggests that a weak link might exist between regulation of *FGFR2* gene and the indirect relationship with swallowing process.

None of the genes from the gene-based analysis retained significance following correction, however genes with the highest significance may be worthy of further analysis as novel loci which may contribute swallowing impairment. Neuroplasticity of neuronal cells within the cerebral cortex responsible for volitional swallowing is believed to be a key driver in the recovery from swallowing impairment in stroke patients [153]. Evidence from twin studies indicates that such neuronal plasticity within the human cerebral cortex is a heritable process [8]. Genes associated with the cortical plasticity may therefore play a role in swallowing physiology. Despite the lack of statistically significant results, both SNP-based and gene-based analysis highlighted the *KIAA0513* gene (OMIM 611675; chromosome 16) in both SNP-based and gene-based analysis. *KIAA0513* encodes a protein, which has been shown to play a role in neuroplasticity [154]. SNP-based analysis, but not gene-based analysis also highlighted the SNP rs4911134 (located on the 20 chromosome) within the *KCNIP4* gene. *KCNIP4* belongs to a family of genes encoding K channel-interacting proteins, which modulate the activity of Kv4 A-type potassium channels thus playing a significant role in the firing of action potentials within the neurons and the shape of the action potential in the heart [155].

'Sensitivity analyses' with the cohort of individuals without stroke and Parkinson's disease patients showed similar results with lower significance. This is likely to be explained by attenuation of the result caused by reduced sample size which challenges the statistical power of the study.

The findings of the current study do not support previous research. Animal studies have identified two genes which may play a role in swallowing: *BDNF* gene and its receptor encoded by *TRKB* gene [122, 123]. The *TRKB* (OMIM 600456) gene encodes a member of the neurotrophic tyrosine receptor kinase (NTRK) family. An animal study performed by Kurihara *et al.,* investigated the influence of two hydrolases encoded by genes *UCHL1* and *UCHL3* on dysphagia in mice [124]. Both hydrolases showed effects on maintenance of neurons on nucleus tractus solitarius, which receives signals from cranial nerves that carry sensory information from the tongue, the palate and the pharynx required for swallowing.

One of the possible explanations of the lack of association between the *APOE* genotype and swallowing impairments in our study is that Mentz *et al.* [110]have found the significance only in APOE- ε4 homozygotes. We have decided to divide our cohort into combined homo- and heterozygotes of APOE- ε4 and those without the allele as our sample had a very low number of homozygotes for APOE- ε4 (n = 7). Our finding confirms the results of Mentz *et al.* who also failed to show an association between APOE- ε4 heterozygotes and the swallowing outcome.

Lack of significance of rs6265 from the *BDNF* gene and the swallowing impairment in our analysed cohort might be expected if one considers the differences between the two experimental models. In Jayasekeran's *et al.* studies highly specific, artificially induced subtle changes in neurophysiology of swallowing were examined in a younger healthy cohort. In contrast self-reported questionnaire used in the following study provide clinical phenotype of swallowing which could be affected by multiple factors, molecular pathways and genes involved.

Despite these novel findings of potential genetic loci influencing the neurophysiological processes found in swallowing impairment, there are important limitations we recognise to the study. We appreciate that sample size is the main limitation of this study and caution must be applied until further replication is conducted. Regardless of the high prevalence of dysphagia, questionnaires used to collect data relating to swallowing impairments are not routinely included in the design of large cohort observational studies. As yet we have not been able to identify investigators with appropriate healthy or patient adult samples to undertake a replication study. Another limitation of this study is the possibility that we have overlooked other associations by applying too strict criteria for multiple testing. We have reduced potential known biases to the analytical models used such as important covariates including mood, but there is still the possibility that unmeasured confounders or population strata not detected could affect our results. We also accept the swallowing phenotype used is a surrogate of more specific invasive experimental models. However, these experimental paradigms would pose problems in generating sample sizes sufficient for GWAS.

In support of our approach, there are strengths in this research that need to be considered. We have used a validated self-report questionnaire and previously published threshold to construct our dysphagia phenotype. The genotyping data has been through an extensive QC process on which we have based the findings [150]. We have adjusted for biases in our analysis both for

genetic architecture and known confounders for dysphagia such as low mood. The sample used comprises over 500 older community dwelling adults with self-reported swallowing symptoms and is unique as demonstrated by the difficulty in finding any similar resource for our planned replication.

Despite such potential limitations, our study shows a significant genome-wide association between the SNP rs17601696 and self-reported swallowing impairment in older adults. This with several other SNPs on the suggestive significance level could offer candidates for further investigation of the process of swallowing and dysphagia. Further confirmatory analysis of these loci need replication in an appropriate cohorts or experimental studies to investigate the underlying genetic determinants of neural mediated dysphagia.

2.5.Supplementary material

Supplementary material 2.1. **Top 16 genes from the VEGAS gene- based analysis of the study cohort based on *P*-values ≤ 10^{-4}.**

Chromosome	Gene	number of SNPs	*P*-value
5	*CCNJL*	22	0.000077
5	*C1QTNF2*	23	0.000176
16	*KIAA0513*	41	0.000207
6	*RPS10*	9	0.000233
13	*POSTN*	42	0.000309
16	*ZDHHC7*	34	0.000347
7	*RAC1*	17	0.000432
7	*RAC1*	17	0.000433
12	*C12orf35*	44	0.000489
7	*MGC12966*	13	0.000492
20	*CHMP4B*	27	0.000542
5	*SLU7*	26	0.000675
10	*BLNK*	34	0.000752
5	*CARD6*	19	0.000828
3	*NEK4*	14	0.000879
5	*LOC63920*	24	0.000917

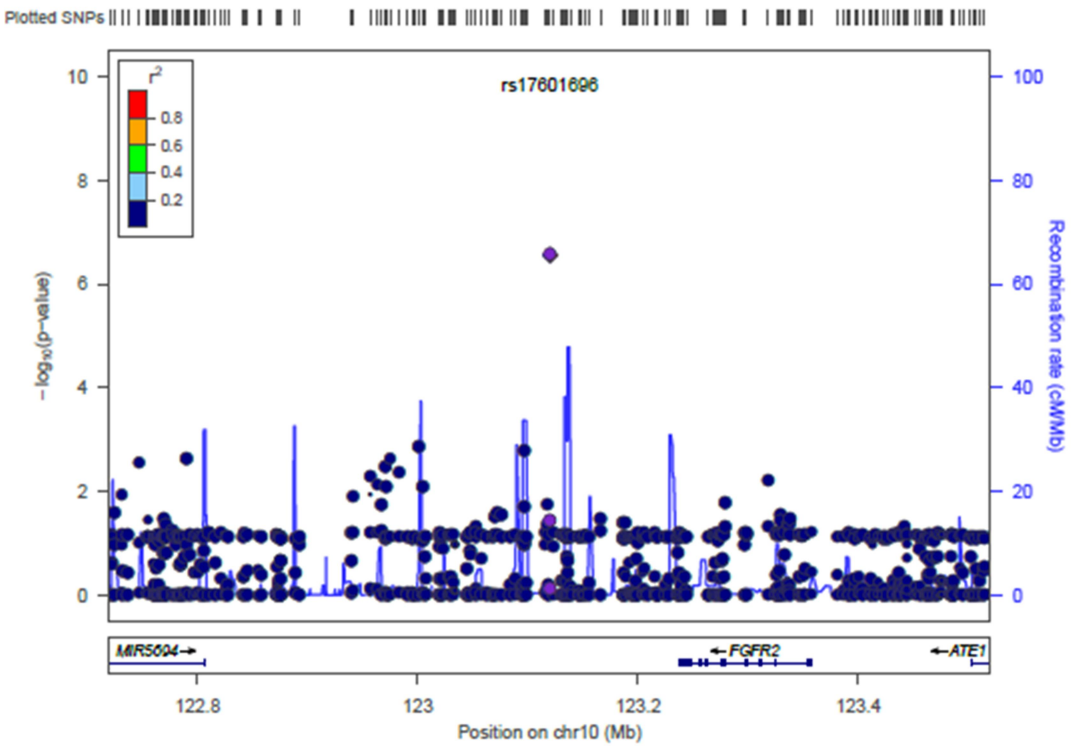
Supplementary material 2.2 **Top 25 SNPs from the genome- wide analysis of the examined cohort ordered by *P*-values.** The information about localization, alleles and Gene/Region was based on annotation from Ensembl built 54 (inter-genic polymorphisms fall >20 kb from an annotated gene). MAF indicates minor allele frequency within the analysed cohort, OR= odds ratio, 95%CI= 95% confidence intervals show the 95% confidence interval boundaries.

Chr	SNP	Physical location	Major allele	Minor allele	*P*-value	MAF	Gene/Region	OR (95% CI)
10	rs17601696	123110026	C	T	4.80E-08	0.08163	intergenic	4.75 (2.72-8.32)
1	rs17440619	107232118	T	C	0.000001683	0.1552	intergenic	2.79 (1.83-4.25)
18	rs8087995	63873981	T	C	0.000001962	0.1309	intergenic	3.4 (2.05-5.62)
21	rs2226441	22063716	T	C	0.000002762	0.2899	intergenic	2.45 (1.68-3.56)
16	rs17761993	24415800	T	G	0.000003864	0.2389	intergenic	2.49 (1.69-3.66)
1	rs12137571	107232002	C	T	0.000004485	0.1596	intergenic	2.66 (1.75-4.05)
4	rs7654157	66770084	G	A	0.000005197	0.3439	intergenic	2.35(1.63-3.38)
16	rs2352930	84728781	A	G	0.000006541	0.239	intergenic	2.5 (1.68-3.73)
13	rs9594221	37016531	C	T	0.000006713	0.1332	intergenic	2.98 (1.85-4.79)
16	rs17789174	83661386	C	T	0.000006758	0.2217	*KIAA0513*	2.57 (1.71-3.89)
13	rs9547919	36989131	C	T	0.000007441	0.1991	intergenic	2.65 (1.73-4.05)
5	rs163233	34894982	G	A	0.000008304	0.3135	*TTC23L*	0.32 (0.2-0.53)
20	rs7261505	31918074	G	A	0.00001285	0.05321	intergenic	4.28 (2.23-8.22)
10	rs812545	48246485	C	T	0.0000129	0.1619	intergenic	2.59 (1.69-3.97)
23	rs5979748	12757584	G	A	0.00001373	0.3572	intergenic	0.33 (0.2-0.55)
2	rs4665083	160138373	A	G	0.00001411	0.3776	*BAZ2B*	2.38 (1.61-3.51)
20	rs4911134	31915876	C	T	0.00001513	0.1012	*KCNIP4*	3.01 (1.83-4.95)
4	rs11945849	5901244	A	G	0.00001636	0.1705	*CRMP1*	2.66 (1.71-4.15)
16	rs9936836	83613657	A	G	0.00001653	0.1832	intergenic	2.48 (1.64-3.76)
12	rs11051642	31916867	T	C	0.00001719	0.2252	*GPR109A*	2.46 (1.63-3.71)
2	rs126925	160099094	T	C	0.00001852	0.2567	*BAZ2B*	2.39 (1.6-3.55)

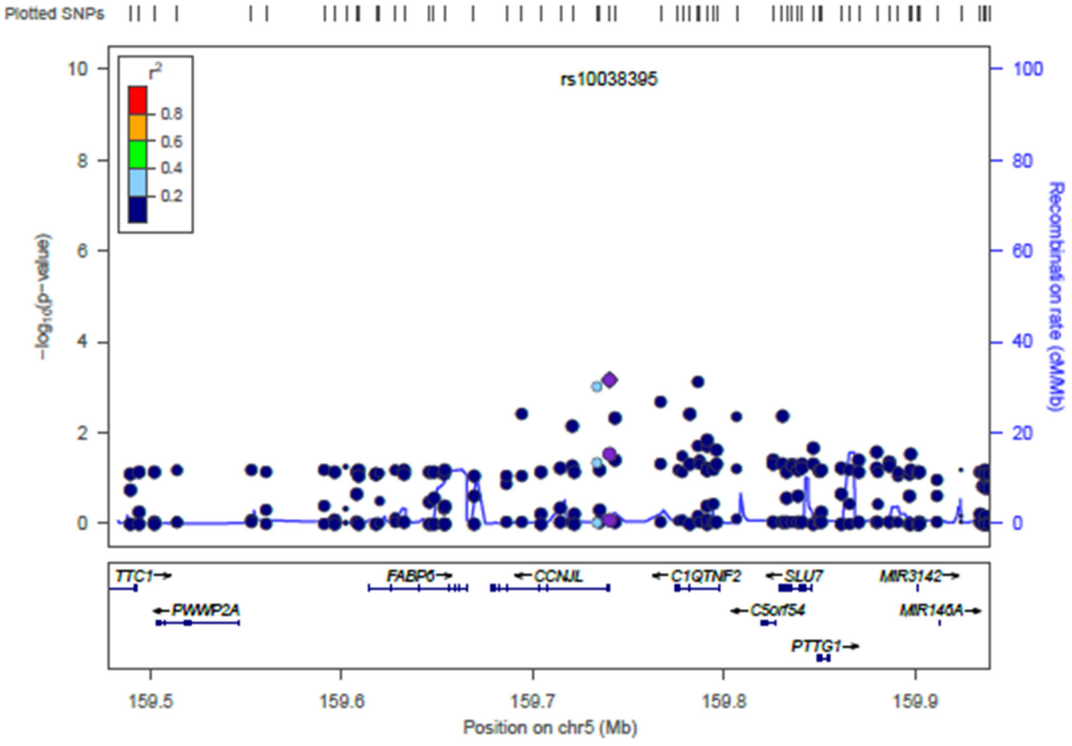
	53							
5	rs4868308	173094991	A	G	0.00001924	0.347	*CTB43E*	2.29 (1.57-3.34)
6	rs11755235	117005695	C	T	0.00002038	0.05182	*RWDD1*	4.02 (2.12-7.62)
1	rs11803645	236791997	T	C	0.00002057	0.388	intergenic	2.36 (1.59-3.5)
10	rs786795	48244366	G	A	0.00002339	0.163	intergenic	2.51 (1.64-3.85)

Supplementary material 2.3. **LocusZoom plots for regions of interest.** (A) statistically significant SNP rs17601696 located near *FGFR2* gene from SNP-based analysis (B) statistically suggestive gene *CCNJL* from gene-based analysis (C) suggestive locus near gene *KIAA0513.*

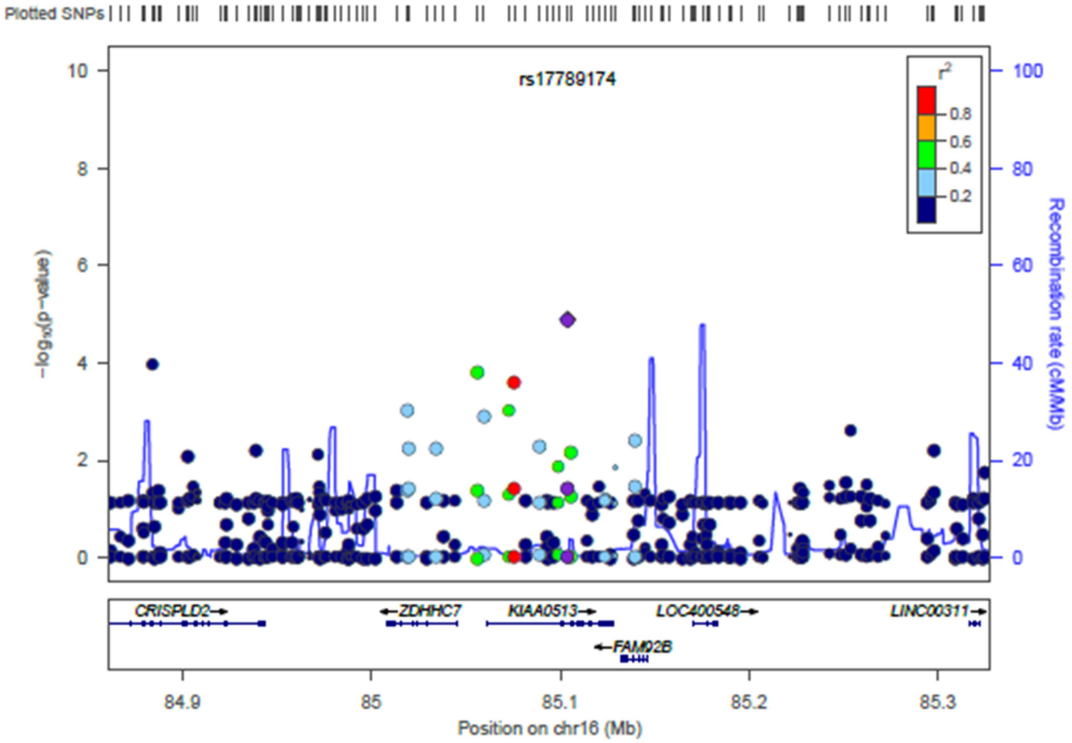
A



B



C)


Plotted SNPs
rs17789174
r²
0.8
0.6
0.4
0.2
−log10(p−value)
Recombination rate (cM/Mb)
CRISPLD2
ZDHHC7
KIAA0513
LOC400548
LINC00311
FAM92B
84.9
85
85.1
85.2
85.3
Position on chr16 (Mb)

Supplementary material 2.4. **Top 100 SNPs from the genome- wide analysis imputed in 1000 Genome Project.** The examined cohort ordered by *P*-values with the information about localization, chromosome and odds ratio (OR).

CHR	SNP	physical location	*P*- value	OR
10	rs17601696	123120036	4.63E-08	4.761
21	rs2827025	23144602	1.64E-06	2.521
1	rs17440619	107430595	1.68E-06	2.789
18	rs12964323	65718956	1.74E-06	3.419
1	rs17017852	107431001	1.79E-06	2.781
18	rs34932443	65724331	1.96E-06	3.398
18	rs9965705	65724551	1.96E-06	3.398
18	rs8087995	65723001	1.96E-06	3.398
18	rs9953488	65724543	1.96E-06	3.398
21	rs2226441	23141845	2.99E-06	2.444
4	rs7675861	5849821	3.16E-06	2.885
4	rs11946892	5850377	3.16E-06	2.885
20	rs2747539	32418368	3.84E-06	3.389
16	rs17761993	24508299	3.86E-06	2.487
16	rs4597304	85055741	4.10E-06	2.733
1	rs12137571	107430479	4.49E-06	2.663
16	rs4782686	85057207	4.50E-06	2.722
2	rs6751744	160407485	4.72E-06	2.584
1	rs12085171	107429673	4.76E-06	2.656
1	rs11184998	107430137	4.76E-06	2.656
1	rs1410258	238722872	4.82E-06	2.572
4	rs7654157	67087489	5.20E-06	2.345
5	rs295688	73716118	5.51E-06	2.523
13	rs9547929	38116392	5.82E-06	3.001
13	rs9594221	38118531	6.44E-06	2.985
13	rs9547932	38118068	6.44E-06	2.985
13	rs9547935	38118613	6.44E-06	2.985
16	rs2352930	86171280	6.54E-06	2.504
16	rs17789174	85103885	6.76E-06	2.574
13	rs7330705	38098719	7.11E-06	2.661
13	rs9547919	38091131	7.44E-06	2.648
13	rs973497	38092457	7.44E-06	2.648
13	rs1812957	38092059	7.44E-06	2.648
5	rs163233	34859225	7.98E-06	0.3232
20	rs6087532	32422597	8.17E-06	3.177
20	rs68074572	32421026	8.17E-06	3.177
20	rs4911368	32421742	8.17E-06	3.177
16	rs8061532	86169716	8.39E-06	2.478
2	rs6432541	160450827	9.86E-06	2.414
2	rs6738215	160453305	9.86E-06	2.414
2	rs6432540	160450703	9.86E-06	2.414
2	rs12692554	160451288	9.86E-06	2.414

2	rs7563368	160457075	9.86E-06	2.414
20	rs4911369	32452176	0.00001029	3.087
20	rs61208627	32452627	0.00001039	4.37
2	rs10204535	160463692	0.00001055	2.434
1	rs1519877	107431656	0.00001056	2.577
18	rs12966040	65729820	0.00001122	4.301
2	rs4665100	160505790	0.00001139	2.39
2	rs7595639	160533044	0.00001157	2.383
2	rs13032135	160532099	0.00001163	2.384
2	rs4665104	160532358	0.00001163	2.384
2	rs12386214	160531523	0.00001163	2.384
2	rs73967899	160537569	0.00001163	2.384
2	rs12692558	160519196	0.00001166	2.384
2	rs10176436	160515394	0.00001172	2.385
2	rs2357526	160524596	0.00001172	2.385
2	rs4664296	160523572	0.00001172	2.385
2	rs7559127	160515935	0.00001172	2.385
2	rs7604482	160513826	0.00001172	2.385
6	rs117165579	117012261	0.00001182	4.214
2	rs11676412	160508071	0.00001185	2.387
2	rs10184034	160505427	0.00001185	2.387
2	rs10193402	160507782	0.00001185	2.387
2	rs4665098	160484974	0.00001185	2.387
2	rs4664293	160505752	0.00001185	2.387
2	rs6432542	160480536	0.00001185	2.387
20	rs59357366	32454379	0.00001285	4.279
20	rs7261505	32454413	0.00001285	4.279
20	rs59519100	32456567	0.00001285	4.279
20	rs61643008	32453919	0.00001285	4.279
1	rs56151262	238717153	0.00001314	2.409
20	rs8122909	32454448	0.00001326	4.271
10	rs812545	48626479	0.00001391	2.58
2	rs13391919	160560160	0.00001452	2.355
10	rs796814	48615419	0.0000147	2.574
20	rs4911134	32452215	0.00001513	3.008
2	rs6432548	160545537	0.00001557	2.344
2	rs7590607	160545635	0.00001557	2.344
2	rs7597488	160547096	0.00001557	2.344
2	rs4380179	160541575	0.00001557	2.344
2	rs10803758	160548219	0.00001557	2.344
2	rs7597482	160547091	0.00001557	2.344
12	rs9888381	109843666	0.00001586	3.163
10	rs812005	48624479	0.00001598	2.564
4	rs11945849	5850343	0.00001636	2.661
16	rs9936836	85056156	0.00001653	2.483
5	rs246959	132810935	0.00001676	2.852

5	rs34105391	34856326	0.00001744	0.309
2	rs13386318	160398357	0.00001791	2.389
5	rs4868308	173162385	0.00001828	2.292
2	rs75811077	160559438	0.00001909	2.317
2	rs12692553	160390848	0.0000191	2.383
2	rs12611922	160462749	0.00001933	2.329
2	rs6745766	160454795	0.00001933	2.329
2	rs1963848	160458090	0.00001933	2.329
16	rs62048450	85069539	0.00001944	2.454
16	rs12597107	85070381	0.00001944	2.454
2	rs4665083	160430127	0.0000195	2.33

Chapter 3

The effects of 1Hz and 5Hz repetitive Transcranial Magnetic Stimulation on the pharyngeal motor cortex in the cohort of young healthy individuals.

Abstract

Introduction: Cortical excitability studies following non-invasive brain stimulation paradigms over the pharyngeal motor cortex have been previously published with clear parameter, specific directional effects and therapeutic potential. Repetitive transcranial magnetic stimulation (rTMS) is one such technique, where defined frequency parameters have been established, however the variation in responsiveness to low and high frequencies is unclear. I therefore examined responses following both reported inhibitory (1Hz) and excitatory (5Hz) rTMS paradigms in a large cohort of young individuals.

Materials and methods: Healthy volunteers (n=41, 25.4 ± 4.6 years old) were assessed for corticobulbar excitability after single-pulse TMS. Electromyographic responses were measured from the pharyngeal muscles termed pharyngeal motor evoked potentials (PMEPs) with single pulse TMS. Repeated measurements of PMEPs were recorded before and for up to one hour after the interventions of 1Hz and 5Hz rTMS. The data were analysed with repeated-measures ANOVA. Both interventions were applied on two separate days at least a week apart.

Results: Initial observations showed large variability in the responses to 1Hz (n=39) and 5Hz (n=40) rTMS. Overall, group responses from both the 5Hz and 1Hz paradigms showed no directional specific change in response in the analysed cohorts ($F(3.692, 129.211) = 0.782$, $P = 0.564$); ($F(4.079, 146.850) = 1.375$, $P = 0.245$) respectively).

Conclusions: Understanding the variability in outcomes following 1Hz and 5Hz rTMS requires different approaches in future analysis. Further investigations on individual factors will thus delineate the underlying mechanisms for the responsiveness and will increase our knowledge for the stratified application of these therapeutic paradigms on dysphagic patients.

3.1. Introduction

Swallowing is controlled by an extensive neural network. One important brain locus involved control of swallowing muscles is located in the sensorimotor motor cortex of both hemispheres [26]. Additionally one of the hemispheres shows stronger activity and gives stronger responses to non-invasive brain stimulations and therefore is sometimes called the “stronger/dominant’’ hemisphere for swallowing.

Lesions within the cortical areas involved in swallowing may lead to impairments (dysphagia). Swallowing impairments are commonly observed in patients with stroke (up to 55%) [5, 50, 54], leading to complications such as dehydration, malnutrition and increased risk of aspiration causing prolonged hospitalization and increased mortality rates [4]. The most frequently observed gastrointestinal impairment in stroke patients is oropharyngeal dysphagia which affects the upper digestive tract [4].

Recovery from oropharyngeal dysphagia is observed in stroke patients; however the exact mechanism remain unknown. Neuroimaging techniques have showed increased activity and functional reorganisation in the unaffected hemisphere, which is most likely due to neuronal plasticity mechanisms [6]. Recently developed treatment therapies for dysphagia in stroke patients have proposed the use non-invasive brain stimulations (NIBS) to enhance cortical excitability within the swallowing motor cortex [156].

Repetitive Transcranial Magnetic Stimulation (rTMS) is one of NIBS in which magnetic pulse is delivered over the motor cortex of the swallowing musculature [26, 81]. The stimuli reveals corticobulbar excitability measured as pharyngeal motor evoked potentials (PMEPs) and the after effects of this stimulation remain up until 1h post intervention [75].

Studies on healthy volunteers showed consistent pathways of responses followed by rTMS stimulation delivered over the dominant pharyngeal motor cortex [83, 100, 108]. For low (1Hz) frequency rTMS paradigms, subjects showed consistent inhibition [83, 108] and high frequency (5Hz) rTMS, consistent excitation [100, 108].

Different rTMS paradigms delivered over the swallowing motor cortex were used as treatment therapies in patients with acute brain lesions such as stroke. Surprisingly both types of stimulations with high (5Hz) [91, 95, 104] as well as low frequency (1Hz) [94, 102] caused improvements in swallowing performance.

One of the biggest limitations of existing studies both on healthy individuals and patients is using small, diverse cohorts. Also not all healthy subjects and patients respond to rTMS interventions in the same manner.

The following study will examine the effects of 1Hz and 5Hz rTMS paradigms delivered over the dominant pharyngeal motor cortex on the largest to date cohort of younger volunteers. This homogeneous group will be used to replicate existing studies of the effects of 1Hz rTMS paradigm previously described as being inhibitory stimulation and 5Hz rTMS paradigm previously described as being excitatory.

3.2. Material and methods:

3.2.1. Participants

Forty-one young, healthy volunteers (mean age= 25.6 ± 4.6 years old, age range 18-35, 43.9 % female) were recruited for the study. Participants were excluded from the study if they have had a history of epilepsy, metal in their head, throat, have had a brain surgery, a cardiac pacemaker, were pregnant or were already involved in other research. Prior to the study, informed consent was obtained from all the volunteers.

3.2.2. The procedure for measurements

Pharyngeal electromyographic (EMG) measurements

Subjects were asked to swallow a 3.2-mm diameter inraluminal catheter (Gealtec Ltd, Dunvegan, Isle of Skye, Scotland) either transorally or transnasally depending of participant's preference. The catheters house a pair of bipolar platinum ring electrodes positioned in the pharynx to record electromyographic (EMG) traces. The catheter is connected via a preamplifier and interface to a personal computer which records the traces through the Signal Application Program (Cambridge Electronic Design Ltd, Cambridge). An earth was connected to a skin electrode placed on one of the sternocleidomastoid muscles on the neck. The catheter was connected via preamplifier (CED Cambridge Electronic Design Ltd, Cambridge), amplifier (CED 1902, Cambridge Electronic Design Ltd, Cambridge) and interface (CED 1401, Cambridge Electronic Design Ltd, Cambridge) to a personal computer enabling real time visualization and recording of the traces using Signal Application Program v.4.11 (Cambridge Electronic Design Ltd, Cambridge). This has filters set at 200 Hz to 2 kHz and allows a sampling rate of 4-8 kHz. Analysis of the amplitudes was also performed with the Signal program.

Single pulse Transcranial Magnetic Stimulation

Single- pulse TMS was applied to both hemispheres by a figure-of-eight coil with an outer diameter of 70 mm, with produces a maximum output of 2.2 Tesla (Magstim 200; The Magstim Company, Whitland, Wales, England). The cranial vertex was marked on a surgical cap placed over the scalp and the magnetic stimulator was discharged over both hemispheres to identify the site evoking the greatest pharyngeal response (dominant hemisphere), which will then be marked on the head. At this site the motor threshold will be identified using single pulses of stimulation to achieve motor evoked potentials (MEP) of at least 20μv on 50% of occasions. Pharyngeal MEP (PMEP) amplitude was assessed by applying TMS at 120% of pharyngeal motor threshold, with 10 stimuli being given and repeated over both hemispheres. Additionally MEP for the control thenar response will be collected by delivering 10 pulses of TMS at 120% of the threshold at each time point over the hemisphere giving stronger PMEPS. PMEPs have been collected before each intervention and at 5 time points after the intervention (immediately and 15, 30, 45, 60 minutes after the intervention) (*Figure 3.1.*).

3.2.3. Stimulation Techniques

Repetitive Transcranial Magnetic Stimulation – 5Hz paradigm

5Hz rTMS was used to excite pharyngeal motor cortex. Magstim Super Rapid stimulator (The Magstim Company) was used to deliver pulses through the figure-of-eight shaped coil with a maximum output of 1.8 Tesla and run through 'Magstim Rapid Session' (Magstim Company) computer software. The optimal excitatory parameters to excite the pharyngeal motor cortex have been shown to be a frequency of 5Hz, at intensity of 90% of resting thenar motor threshold. A train consisting of 250 pulses in 5 blocks of 50 pulses with a 10 second pause between the block was delivered over the dominant pharyngeal motor cortex.

Repetitive Transcranial Magnetic Stimulation – 1Hz paradigm

To study inhibiting cortico-pharyngeal circuits (focal suppression) 1Hz rTMS has been given at up to 110% of pharyngeal resting motor threshold for 10 minutes. Two sets of 300 pulses with 30 seconds pause between sessions have been delivered over the dominant pharyngeal motor cortex.

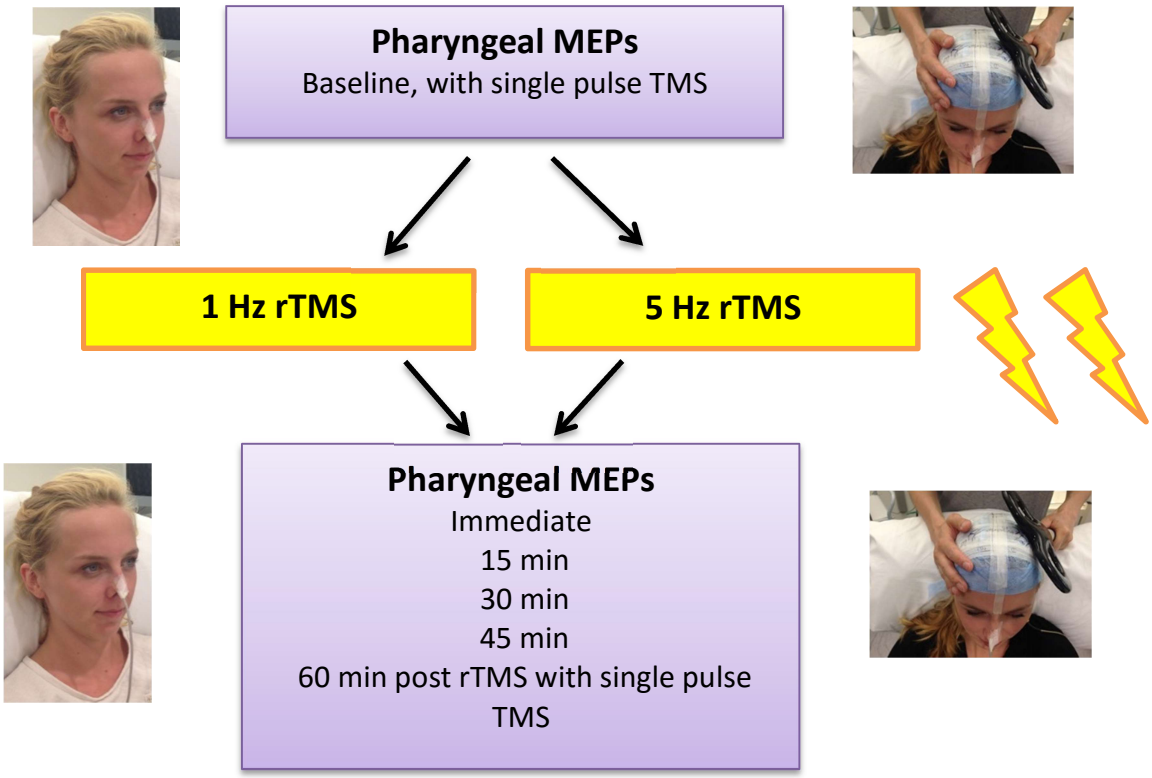


Figure 3.1. **Schematic representation of the experimental protocol.**

3.2.4. Statistical analysis

The peak-to-peak PMEPs amplitude was used as a measure of cortical excitability of the dominant (evoking the stronger responses) hemisphere. These data were tested for normal distribution with Kolmogorov-Smirnoff test for each time point. Baseline PMEPs were compared with Wilcoxon Mann-Whitney test. Baseline measurements data are reported as mean value ± standard error of the mean (SEM) (*Table 3.1.*). Analysis of repeated measurements ANOVA (rmANOVA) with general linear model were conducted with log transformed PMEPs values for 6 time points. In the analysis Kolmogoro-Smirnof transformation was used. Data were analysed using SPSS-software (SPSS ver.20.0 for Windows; SPSS Inc.).

3.3. Results:

3.3.1. Participants

Thirty eight subjects underwent both stimulation paradigms. Two subjects could not tolerate the catheter during the second study and one subjects' results for single studies did not have sufficient quality for further analysis and were therefore withdrawn after completing only one study (*Table 3.1.*). Thirty nine subjects underwent 1Hz and 40 subjects 5Hz paradigms. No one developed any adverse effects followed by rTMS intervention. All subjects were <35 (18-35 years old) and healthy. There was no statistical significance between baselines expressed as raw data between 1Hz and 5Hz rTMS paradigms.

	1Hz rTMS	**5Hz rTMS**
No. of subject	39	40
Age (*y*), mean ± SEM	25.95	25.65
Male/ Female n	23 / 16	22 / 18
% of the stimulator output used for the rTMS intervention mean ± SEM	96.42 ± 1.17	56.72 ± 0.94
baseline PMEP (μV) mean ± SEM	98.4 ± 8.5	106.9 ± 9.5

Table 3.1. **Descriptive statistics of the cohort and baseline parameters for both paradigms.**

3.3.2. Effects of 1Hz rTMS paradigm on the pharyngeal motor cortex excitability

1Hz rTMS revealed a range of different responses in PMEPs in the dominant for swallowing motor cortex for each individual (*Figure 3.2.* and *Table 3.2.*). Kolmogorov-Smirnov tests for normality showed lack of normal distribution for 3 from 6 time points (*Supplementary Table 3.1.* and *Supplementry Figure 3.1*). Therefore log transformation was performed which converted the data set for normal distribution (*Supplementary Table 3.2.* and *Supplementary Figure 3.2.*).

A repeated measures ANOVA with a Greenhouse-Geisser correction determined that mean PMEPs did not differ statistically between time points ($F(3.692, 129.211) = 0.782$, P-value < 0.564). Post hoc tests using the Bonferroni correction confirmed lack of differences between each time points. Therefore, overall 1Hz rTMS stimulation had no simple representative effect type (excitation or inhibition) for this cohort.

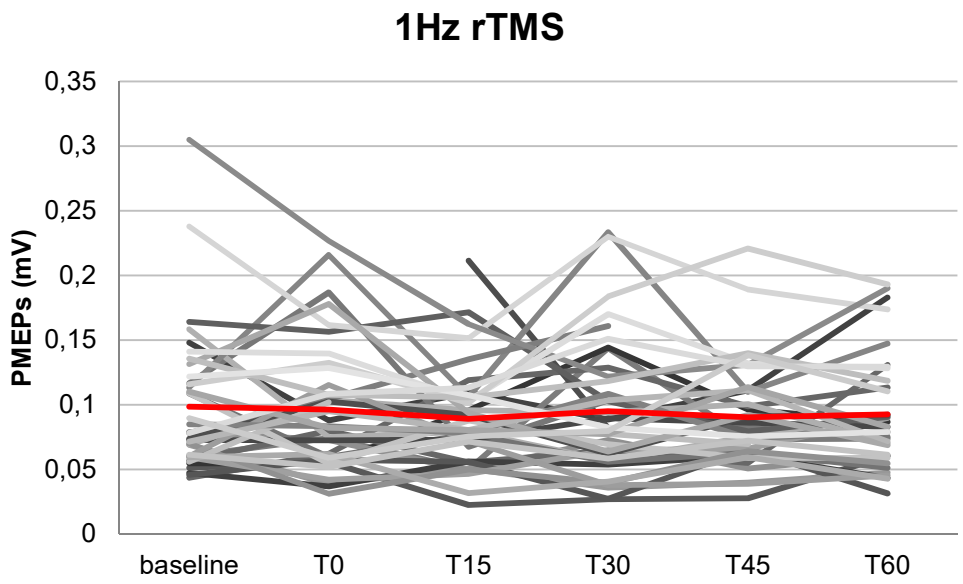


Figure 3.3. **Plot of the raw PMEP data from all subjects for 1Hz rTMS stimulation.** There was a large variation in response between all individuals. The red line indicates the average PMEPs

		baseline 1Hz rTMS	T0 1Hz rTMS	T15 1Hz rTMS	T30 1Hz rTMS	T45 1Hz rTMS	T60 1Hz rTMS
N	Valid	39	38	38	39	37	38
	Missing	2	3	3	2	4	3
Mean		0.098	0.096	0.089	0.095	0.090	0.093
S.E.M.		0.009	0.008	0.006	0.008	0.007	0.007
Median		0.078	0.083	0.080	0.082	0.079	0.083
Std. Deviation		0.054	0.049	0.039	0.051	0.040	0.042
Range		0.261	0.196	0.189	0.207	0.193	0.162

Table 3.2. **Descriptive statistics of the raw data for each time point of measurements for 1Hz paradigms.**

3.3.3. Effects of 5Hz rTMS paradigm on the pharyngeal motor cortex excitability

Similar results were observed in the studies with 5Hz rTMS, where a range of different individuals traits shown in the *Figure 3.3.* occurred (descriptive statistics for each time point shown in *Table 3*). Kolmogorov-Smirnov tests for normality showed lack of normal distribution for 3 from 6 time points (*Supplementary Table 3.2.* and *Supplementary Figure 3.3.*). Therefore log transformation was performed which converted the data for normal distribution (*Supplementary Table 3.1.* and *Supplementary Figure 3.4.*).

A repeated measures ANOVA with a Greenhouse-Geisser correction demonstrated that mean PMEPs of all time points were not statistically different ($F(4.079, 146.850) = 1.375$, P-value<

0.245). Post hoc tests using the Bonferroni correction confirmed lack of differences between each time points. Therefore, overall 5Hz rTMS stimulation had no effect in the group responses for this cohort.

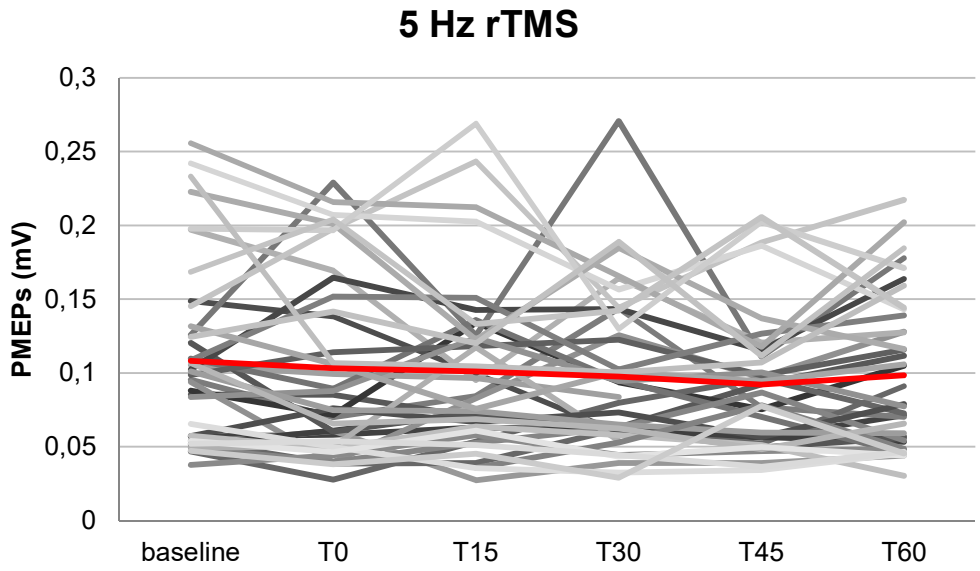


Figure 3.3. **Plot illustrating the raw PMEPs data from all subjects for 5Hz rTMS stimulation.**

Variation in response between all individuals is observed. The red line indicates the average PMEPs.

		baseline 5Hz rTMS	T0 5Hz rTMS	T15 5Hz rTMS	T30 5Hz rTMS	T45 5Hz rTMS	T60 5Hz rTMS
N	Valid	40	39	40	40	38	38
	Missing	0	1	0	0	2	2
Mean		0.100	0.097	0.091	0.098	0.091	0.098
S.E.M.		0.009	0.008	0.007	0.008	0.007	0.008
Median		0.083	0.082	0.082	0.078	0.082	0.078
Std. Deviation		0.056	0.053	0.046	0.052	0.046	0.052
Range		0.242	0.242	0.171	0.187	0.171	0.187

Table 3.3. **Descriptive statistics of the raw data for each time point of measurements for 1Hz paradigm.**

3.4. Discussion

My study has now shown the group effects of two rTMS paradigms which in previous studies revealed inhibition (1Hz) and excitation (5Hz) in the PMEPs. Unexpectedly, no constant differences were found in ranges between baseline and five consecutive follow up measurements for both 1Hz and 5Hz rTMS stimulations.

The results differ from previously described studies by Mistry *et al.* [83], Jefferson *et al.* [100] and Jayasekeran *et al.* [108] where common trends in PMEPs responses as well as swallowing behaviours after both stimulation paradigms were observed. Neurophysiological findings from these studies with the same parameters demonstrated that 5Hz reveals excitation and 1Hz inhibition in the pharyngeal motor cortex.

A possible explanation for these discrepancies between the results is the use of smaller cohorts, where detecting variability is less quantifiable. Mistry *et al.* [83] in his studies used 9 subjects in the group who was delivered 1Hz rTMS stimulation and Jefferson *et al.* [100] 12 subjects in the group with 5Hz rTMS intervention. Jayasekeran *et al. [108]* use a cohort of 21 subjects to study the effects of 1Hz paradigm and 22 for 5Hz paradigm from different age groups.

Another possible explanation for variability in the responses followed by rTMS is the influence of multiple covariates which can affect cortical plasticity. RTMS is a non-invasive technique where activity of the motor cortex might be affected by multiple intra- and inter-individual factors such as time of the day when the study is performed [18], caffeine levels, stress, physical activity, body mass index or recently studied genetic predispositions [108]. Further analysis should be done to analyse intra-subject and intra-intervention analysis in order to know the mechanisms of neuronal plasticity within the pharyngeal motor cortex.

One of the limitations was subjects' tolerability of the catheter which limited collecting the data from 2 studies which do not give the full insight in responses profile. Another limitation is lack of sham stimulation, although for the purposes of the later analyses of these data, no sham arm was deemed necessary.

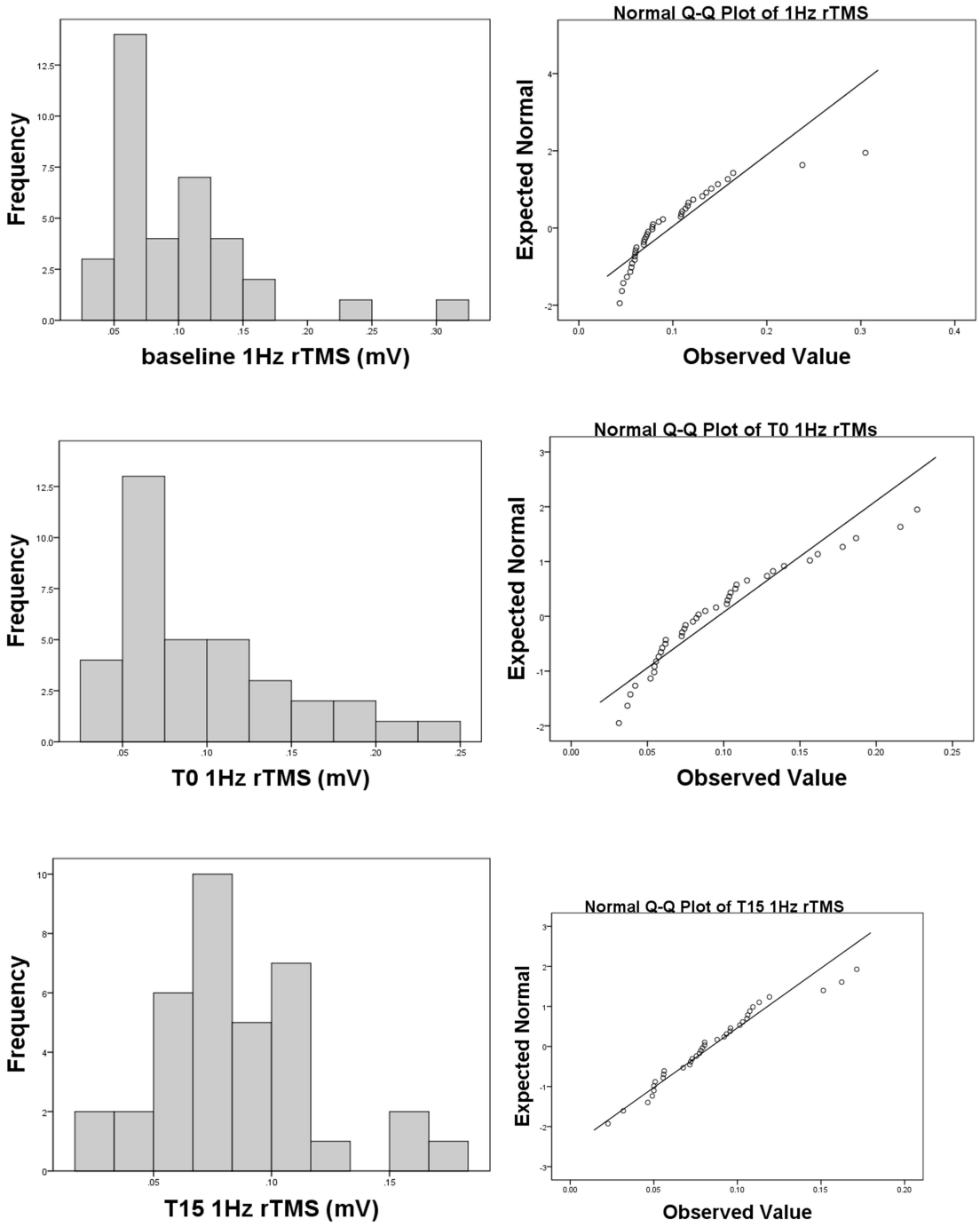
Despite the absence of the common trends in the overall responses of the cohort, individual subject's PMEP responses showed both excitation and inhibition in the pharyngeal motor cortex. Current statistical analysis with ANOVA is not able to detect a constant response pattern by stimulus applied (1Hz or 5Hz rTMS). Therefore using other approaches (such as grouping individuals according to their excitatory or inhibitory outcomes) to analyse physiological data prove to be a useful tool for further analysis of variability.

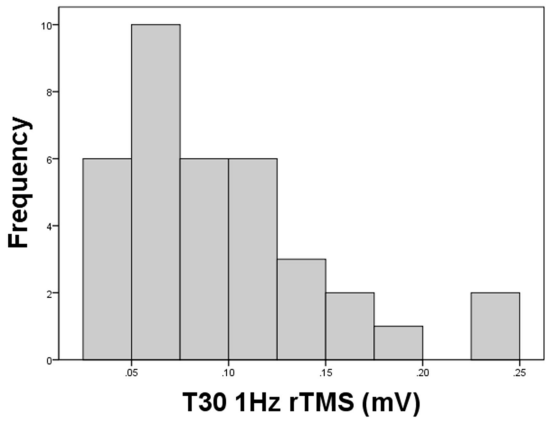
3.5. Conclusions

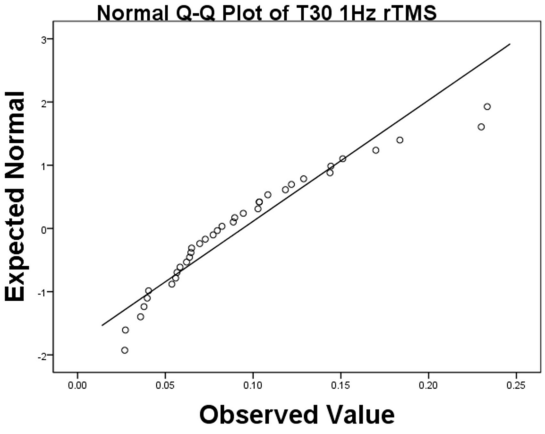
Low (1Hz) and High (5Hz) frequency rTMS paradigms reveal a range of different outcomes in a large, homogeneous cohort of young, healthy individuals. This suggests that responses to standardized protocol of single rTMS intervention may produce heterogeneous group responses. Studying the factors determining this variation may lead to deeper understanding cortical control of swallowing and application for future treatments of dysphagia.

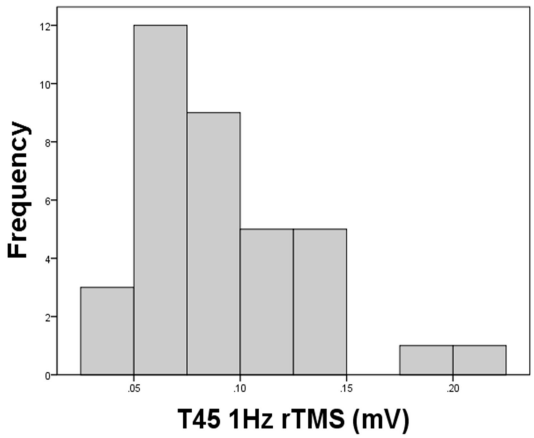
3.6.Supplementary material

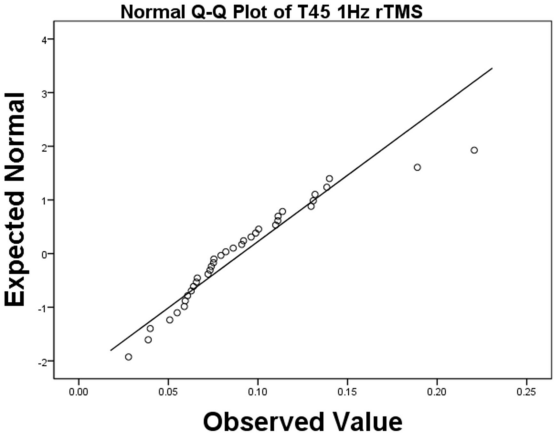
Supplementary Figure 3.1. **Histograms of the frequencies and normality Q-Q plots of the raw 5Hz rTMS** paradigm data. Only T45 follow up measurement after 45 minutes post stimulation showed normal distribution.

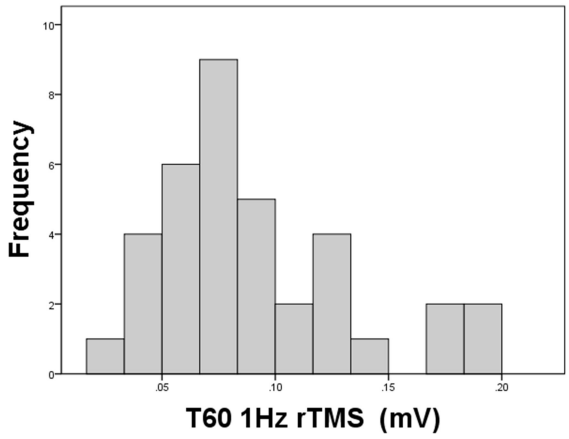


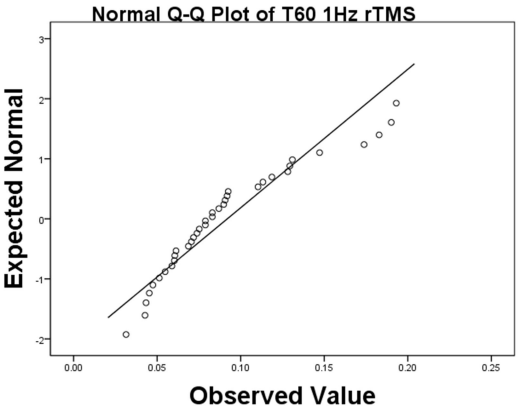

Frequency
T30 1Hz rTMS (mV)


Normal Q-Q Plot of T30 1Hz rTMS
Expected Normal
Observed Value

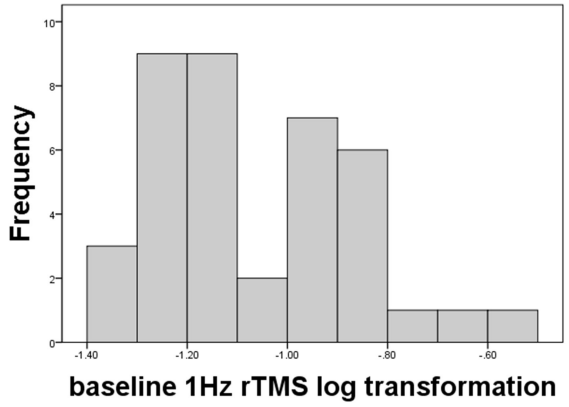

Frequency
T45 1Hz rTMS (mV)

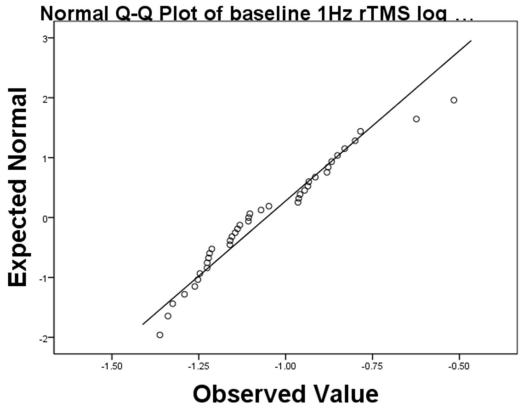

Normal Q-Q Plot of T45 1Hz rTMS
Expected Normal
Observed Value

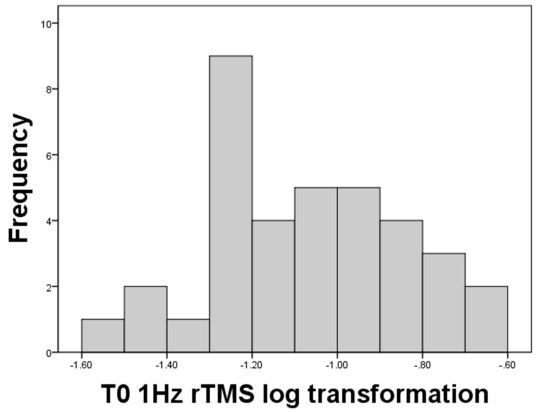

Frequency
T60 1Hz rTMS (mV)

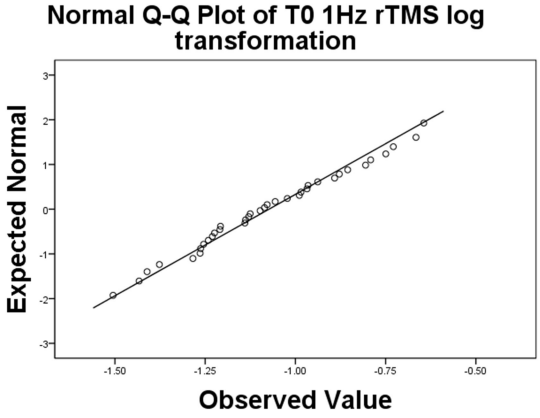

Normal Q-Q Plot of T60 1Hz rTMS
Expected Normal
Observed Value

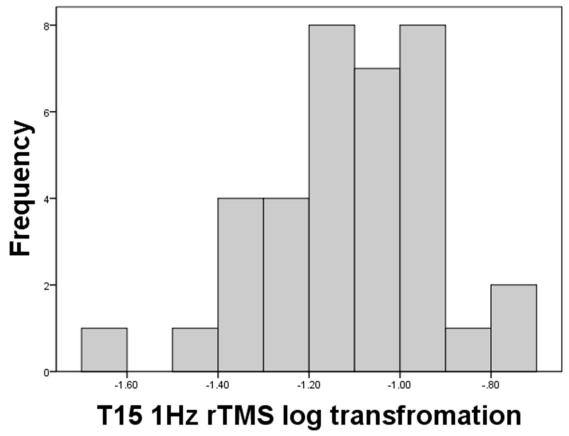
Supplementary Figure 3.2. **Histograms of the frequencies and Q-Q plots of the raw 1Hz rTMS paradigm** data. Only baseline measurement did not show normal distribution.

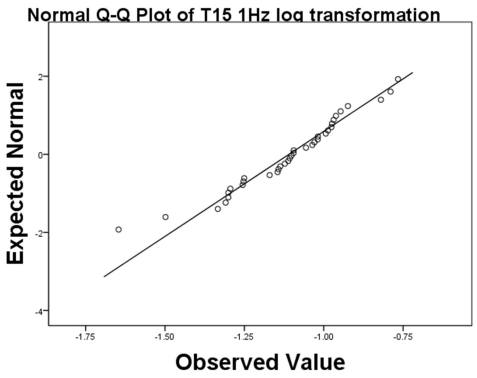


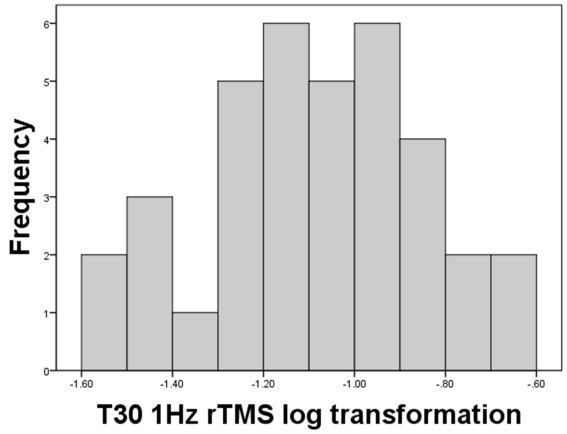


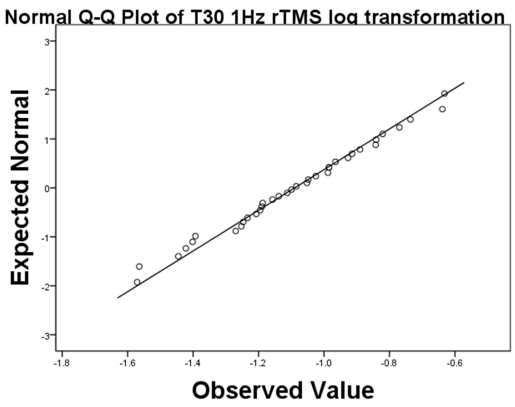


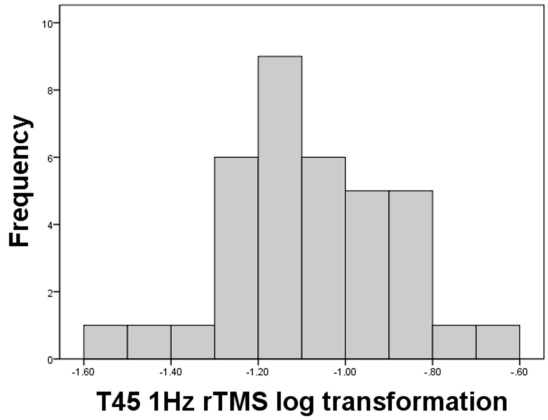


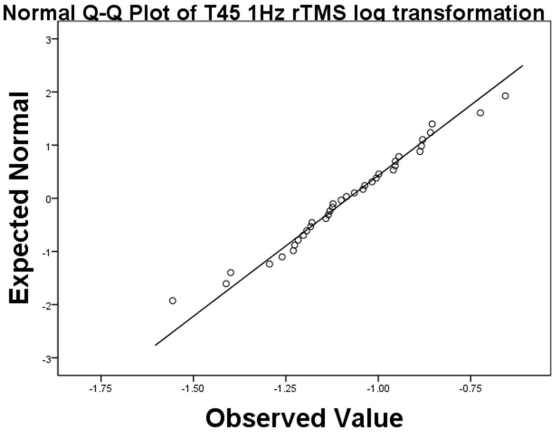


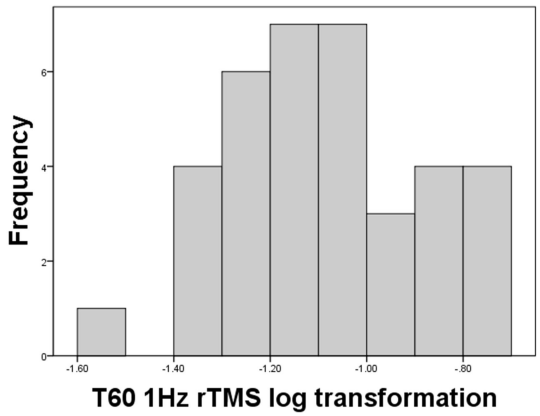


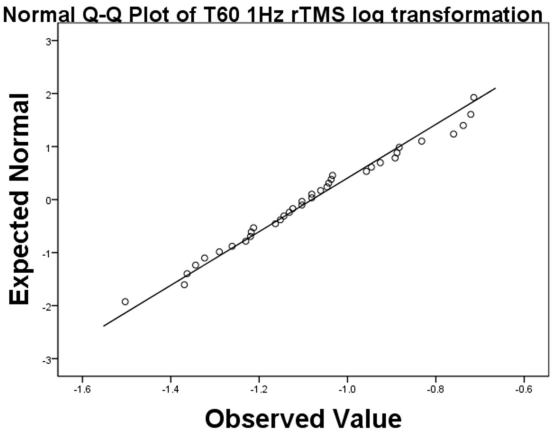

Frequency
T30 1Hz rTMS log transformation


Normal Q-Q Plot of T30 1Hz rTMS log transformation
Expected Normal
Observed Value


Frequency
T45 1Hz rTMS log transformation


Normal Q-Q Plot of T45 1Hz rTMS log transformation
Expected Normal
Observed Value

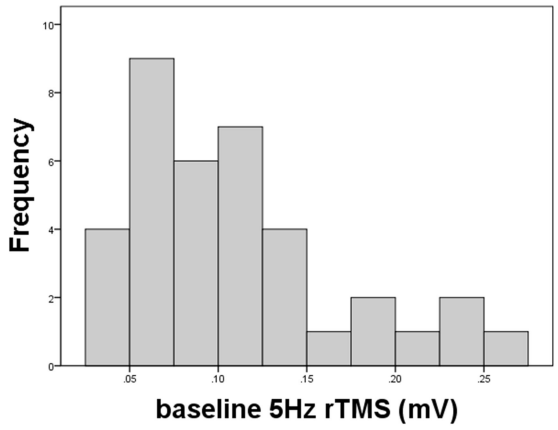

Frequency
T60 1Hz rTMS log transformation

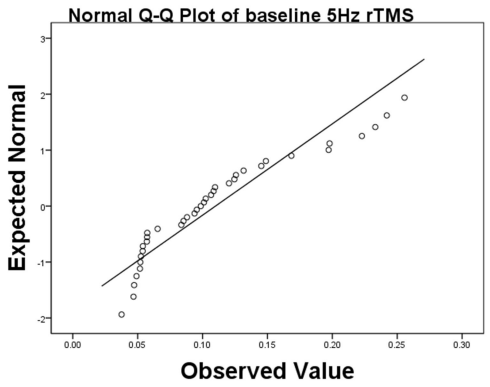

Normal Q-Q Plot of T60 1Hz rTMS log transformation
Expected Normal
Observed Value

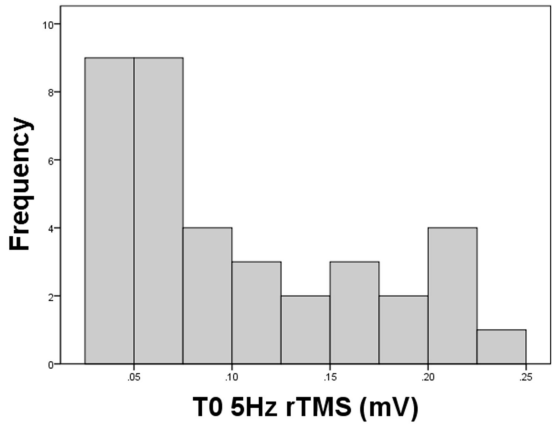
Raw data	Kolmogorov-Smirnov significance	Log transformed data	Kolmogorov-Smirnov significance
baseline 5Hz rTMS	0.033	baseline 5Hz rTMS	0.033
T0 5Hz rTMS	0.003	T0 5Hz rTMS	0.200*
T15 5Hz rTMS	0.018	T15 5Hz rTMS	0.200*
T30 5Hz rTMS	0.008	T30 5Hz rTMS	0.096
T45 5Hz rTMS	0.089	T45 5Hz rTMS	0.194
T60 5Hz rTMS	0.012	T60 5Hz rTMS	0.200*

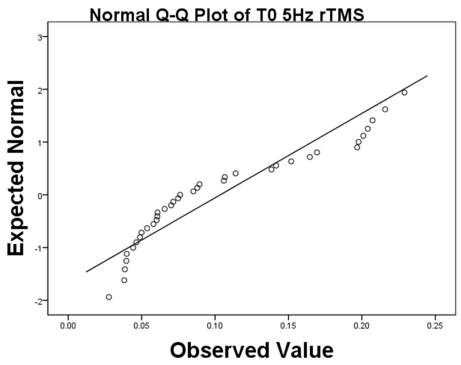
*. This is a lower bound of the true significance.

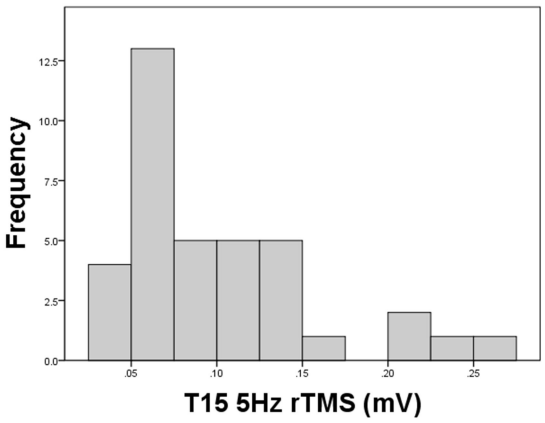
Supplementary Table 3.2. **Normality test results for raw and log transformed data from 5Hz stimulation measurements for the baseline and 5 consecutive time points after the stimulation.** *P*-value >0.05 indicate normal data distribution.

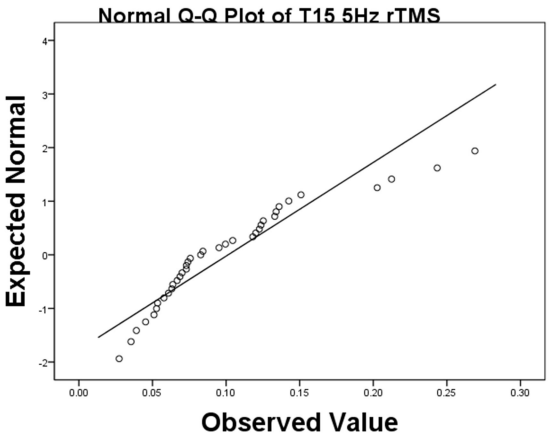


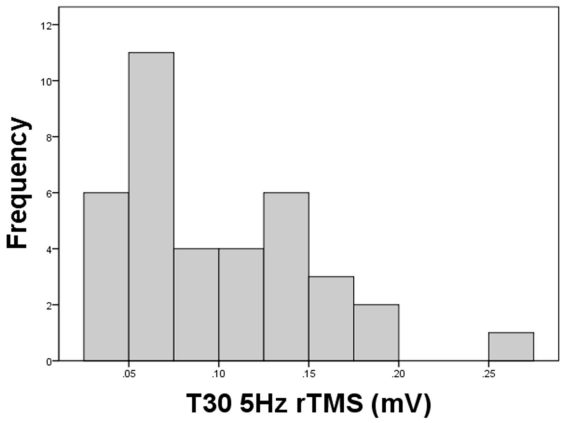


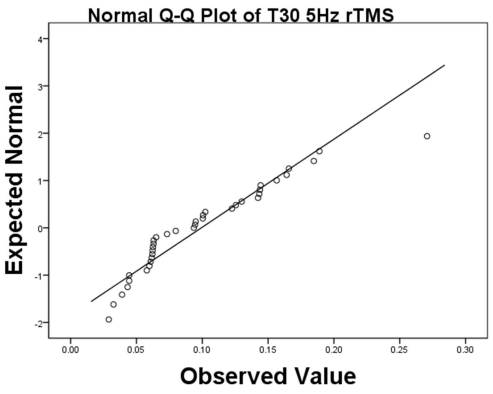


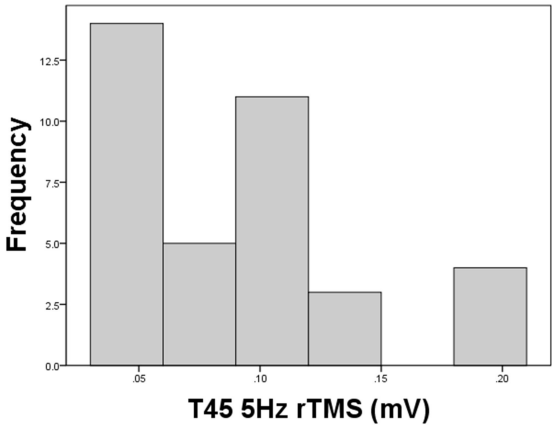


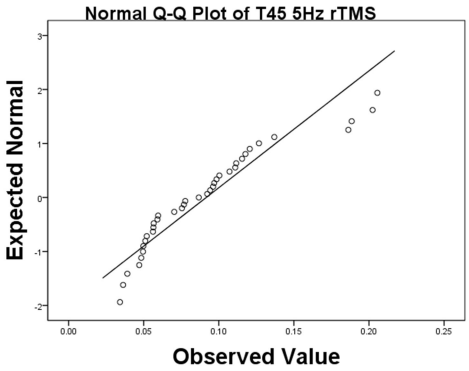

Frequency
T15 5Hz rTMS (mV)

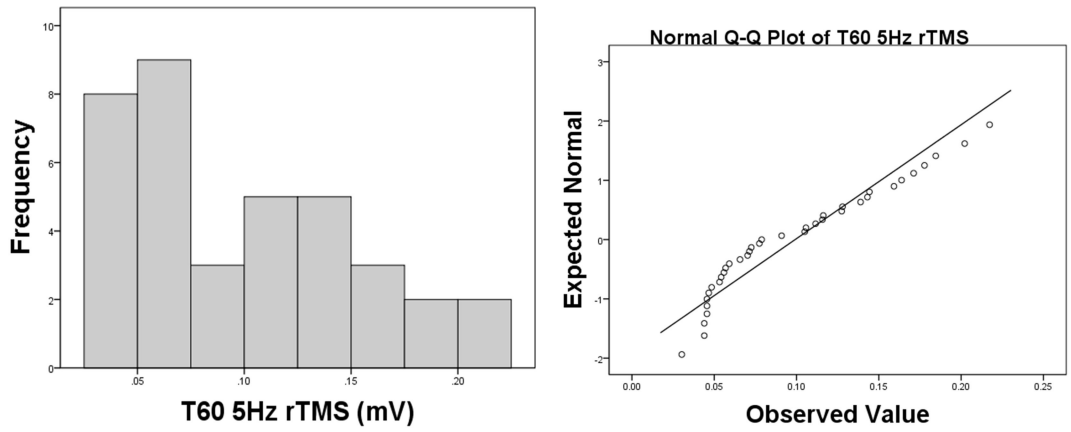

Normal Q-Q Plot of T15 5Hz rTMS
Expected Normal
Observed Value


Frequency
T30 5Hz rTMS (mV)

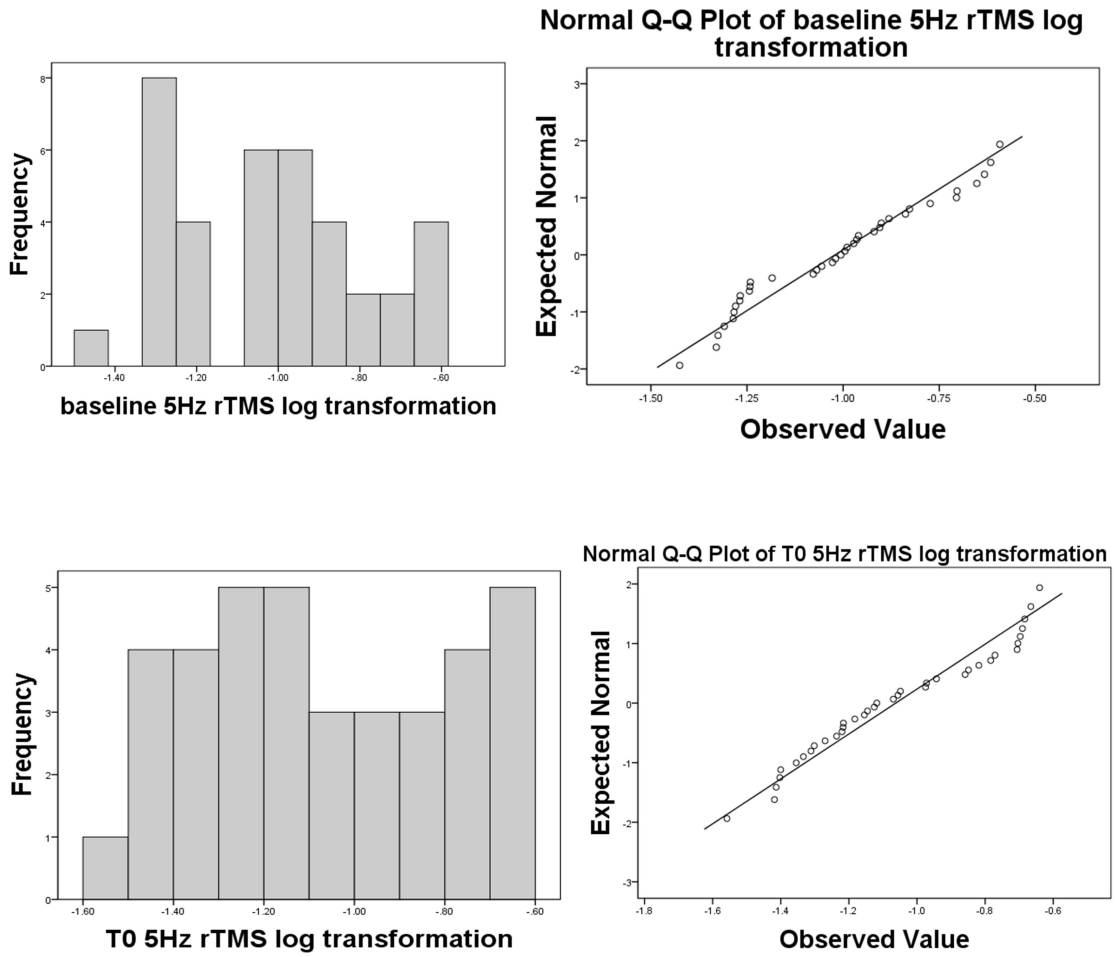

Normal Q-Q Plot of T30 5Hz rTMS
Expected Normal
Observed Value

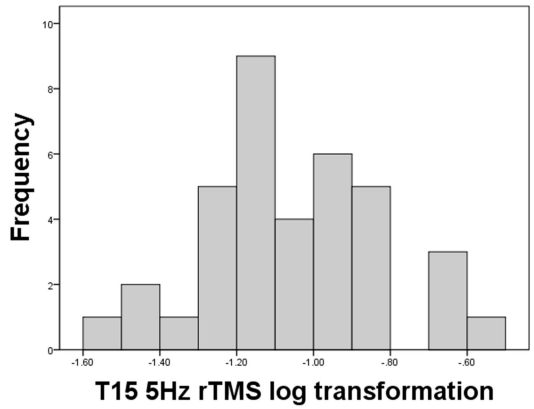

Frequency
T45 5Hz rTMS (mV)

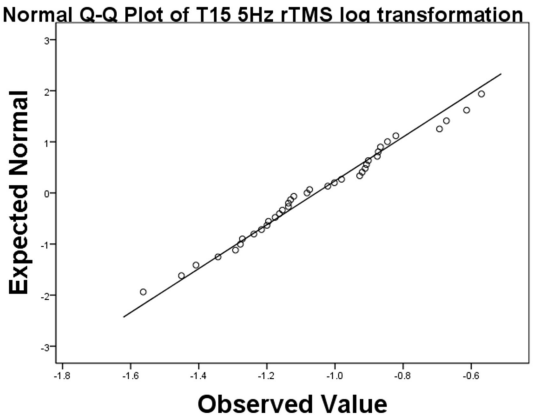

Normal Q-Q Plot of T45 5Hz rTMS
Expected Normal
Observed Value

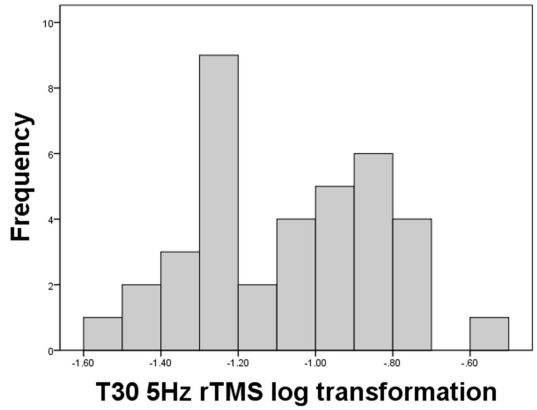


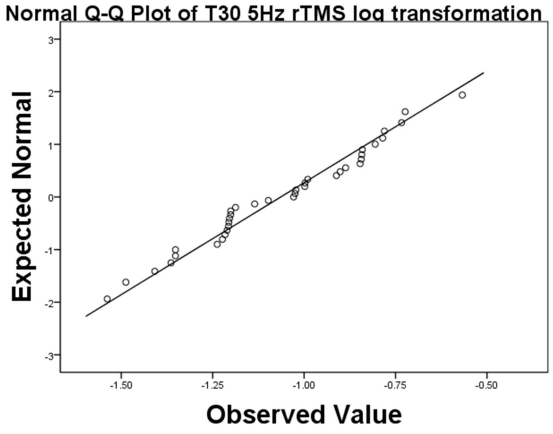
Supplementary Figure 3.3. **Histograms of the frequencies Q-Q plots of the raw 5Hz rTMS paradigm data.** Only T45 follow up measurement after 45 minutes post stimulation showed normal distribution.

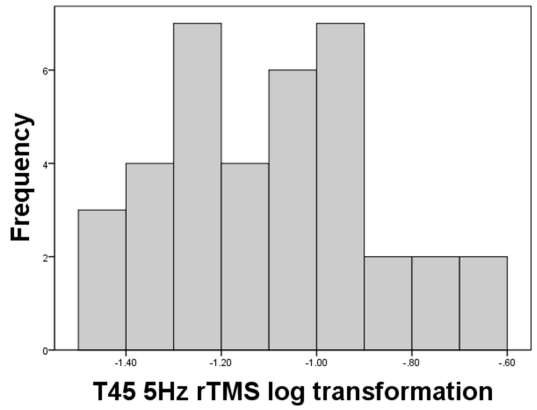


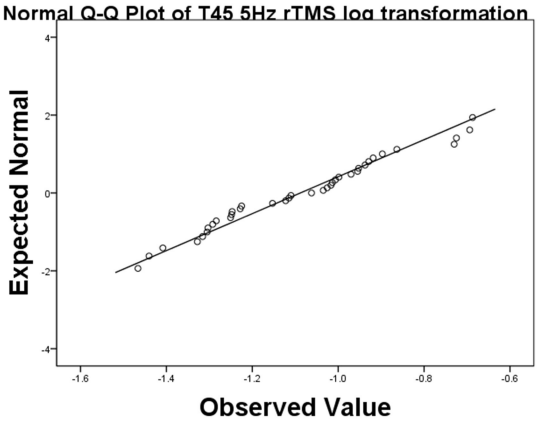

Frequency
T15 5Hz rTMS log transformation

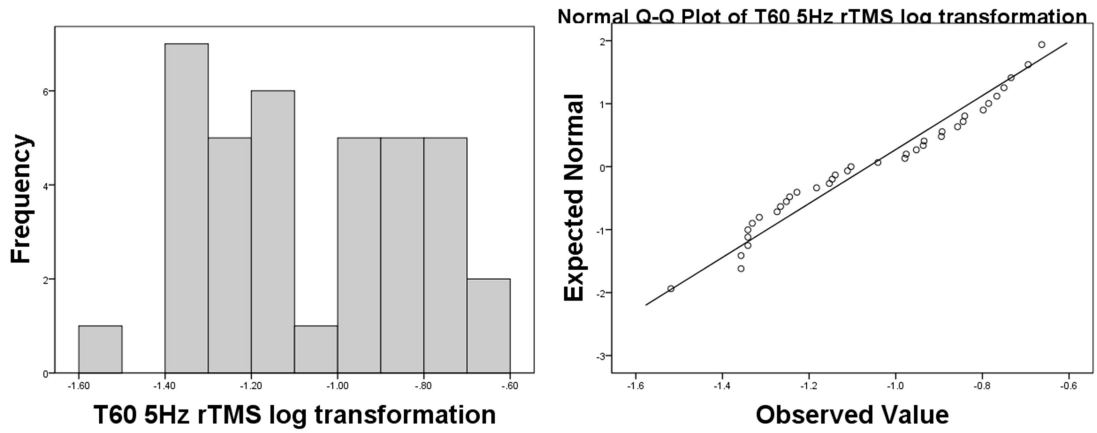

Normal Q-Q Plot of T15 5Hz rTMS log transformation
Expected Normal
Observed Value


Frequency
T30 5Hz rTMS log transformation


Normal Q-Q Plot of T30 5Hz rTMS log transformation
Expected Normal
Observed Value


Frequency
T45 5Hz rTMS log transformation


Normal Q-Q Plot of T45 5Hz rTMS log transformation
Expected Normal
Observed Value



Supplementary Figure 3. 4. **Histograms of the frequencies Q-Q plots of the raw 5Hz rTMS paradigm data.** Only baseline measurement did not show normal distribution.

Chapter 4

Title: Exploring variability in responses of the pharyngeal motor cortex following 1Hz and 5Hz rTMS paradigms.

Abstract

Background: High and low frequency rTMS paradigms have been used as therapeutic tools for neurogenic swallowing impairments. The effectiveness of these therapies seems to vary between individuals. Exploring factors which can influence intra-subject diversity in responsiveness on healthy, young group of people might provide an insight into mechanisms or factors affecting pharyngeal cortical facilitation

Aim: To explore the variability in responsiveness after 1Hz and 5Hz rTMS previously identified as inhibitory and excitatory respectively.

Materials and methods: Healthy volunteers (n=41, 25.4 ± 4.6 years old) were assessed for corticobulbar excitability after single-pulse TMS. Repeated measurements of motor evoked potentials from the pharynx and the hand were recorded before and for up to one hour after the interventions of 1Hz and 5Hz rTMS. The subjects' individual responses were grouped according to multiple criteria and then associated with factors such as gender, ethnicity and time of day of the stimulation.

Results: 1Hz rTMS decreased pharyngeal cortical excitability in almost half of the subjects. Unexpected increase or lack of change was observed in 33% and 23% of subjects respectively. Only 33% of subjects showed expected increase in excitability following 5Hz rTMS while the majority of subjects revealed unexpected decrease and lack of responses (40% and 23% respectively). Just 14% of individuals showed expected outcomes (inhibition after 1Hz and excitation after 5Hz) for both stimulation paradigms. There were no significant effects of gender, ethnicity and time of the stimulation on cortical excitability.

Conclusion: The large variability in response to 1Hz and 5Hz paradigms is in line with similar studies using other forms of non-invasive brain stimulation on somatic motor responses. The results highlight the need to understand the individual factors that determine responsiveness and effectiveness of treatment therapies of swallowing impairments with rTMS.

4.1. Introduction

Non-invasive brain stimulations (NIBS) have been recently used as therapeutic tools to help in the recovery of aerodigestive functions in patients with neurogenic swallowing impairments [97, 102, 156].

One of the concerns in applying NIBS as a therapy for swallowing impairments is variability of patients' responses. There are a number of factors which influence somatic motor responses (e.g. hand movements) followed by NIBS even in the neurologically intact population such as: physical activity, age, attention, gender, pharmacological substances, time of day of the intervention [157] and stress [158].

In the previous Chapter 3 of my thesis I have shown that two paradigms delivered over the pharyngeal motor cortex of healthy, young volunteers show a wide range of responsiveness. Raw data collected as pharyngeal motor evoked potentials (PMEPs) did not show a clear pattern of responses. These data therefore need to be analysed more systematically to identify changes between individuals. Single individual responses identified excitatory, inhibitory and mixed responses followed by 1Hz and 5Hz rTMS stimulation paradigms previously described as being primarily inhibitory and excitatory, respectively. One of the ways of analysing the data I will propose is grouping individuals into clusters of similar patterns of cortical facilitation responses or expected outcomes. In this chapter, therefore, the effects of gender, time of day of the intervention and ethnicity of participants on these cluster patterns will be also examined.
There are no studies exploring the variability in responses followed by rTMS interventions over the swallowing motor cortex. However inter- and intra-subject variability analyses were previously reported in studies examining effects of other NIBS such as transcranial Direct Current Stimulation (tDCS) [159, 160] and Theta Burst Stimulation (TBS) [158, 161, 162] over the hand motor cortex in the cohorts of healthy young volunteers. Previous studies showed that the hand muscles respond independently from the pharyngeal muscles [83]. Therefore the hand motor evoked potentials (MEPs) will be used as control for the pharyngeal muscles outcome.
The aim of this study is to explore variability in the responses to 1Hz and 5Hz rTMS paradigms over the pharyngeal motor cortex with defined parameters by using different clusters of common individual responses.

4.2. Materials and methods

Methods are identical to Chapter 3 and only briefly summarized below.

4.2.1. Participants

Forty-one young, healthy volunteers (mean age= 25.6 ± 4.6 years old, age range 18-35, 43.9 % female) were recruited for the study. Participants were excluded from the study if they have had a history of epilepsy, metal in their head, throat, have had a brain surgery, a cardiac pacemaker, were pregnant or already involved in other research. Prior to the study, informed consent was obtained from all the volunteers.

4.2.2. The procedure for measurements

Pharyngeal electromyographic (EMG) measurements and thenar electromyographic (EMG) measurements were collected. As a control hand motor responses from the abductor pollicis brevis (APB) muscles were measured. APB electrode was placed contralateral to the dominant hemisphere.

4.2.3. Single pulse Transcranial Magnetic Stimulation

Additionally MEP for the control thenar response will be collected by delivering 10 pulses of TMS at 120% of the threshold at each time point over the hemisphere giving stronger PMEPS before each intervention and at 5 time points after the intervention (immediately and 15, 30, 45, 60 minutes after the intervention) (*Figure 4.1*).

4.2.4. Stimulation Techniques

Two Repetitive Transcranial Magnetic Stimulation paradigms (1Hz and 5Hz) were delivered on separate days over the pharyngeal motor cortex in the hemisphere giving larger responses.

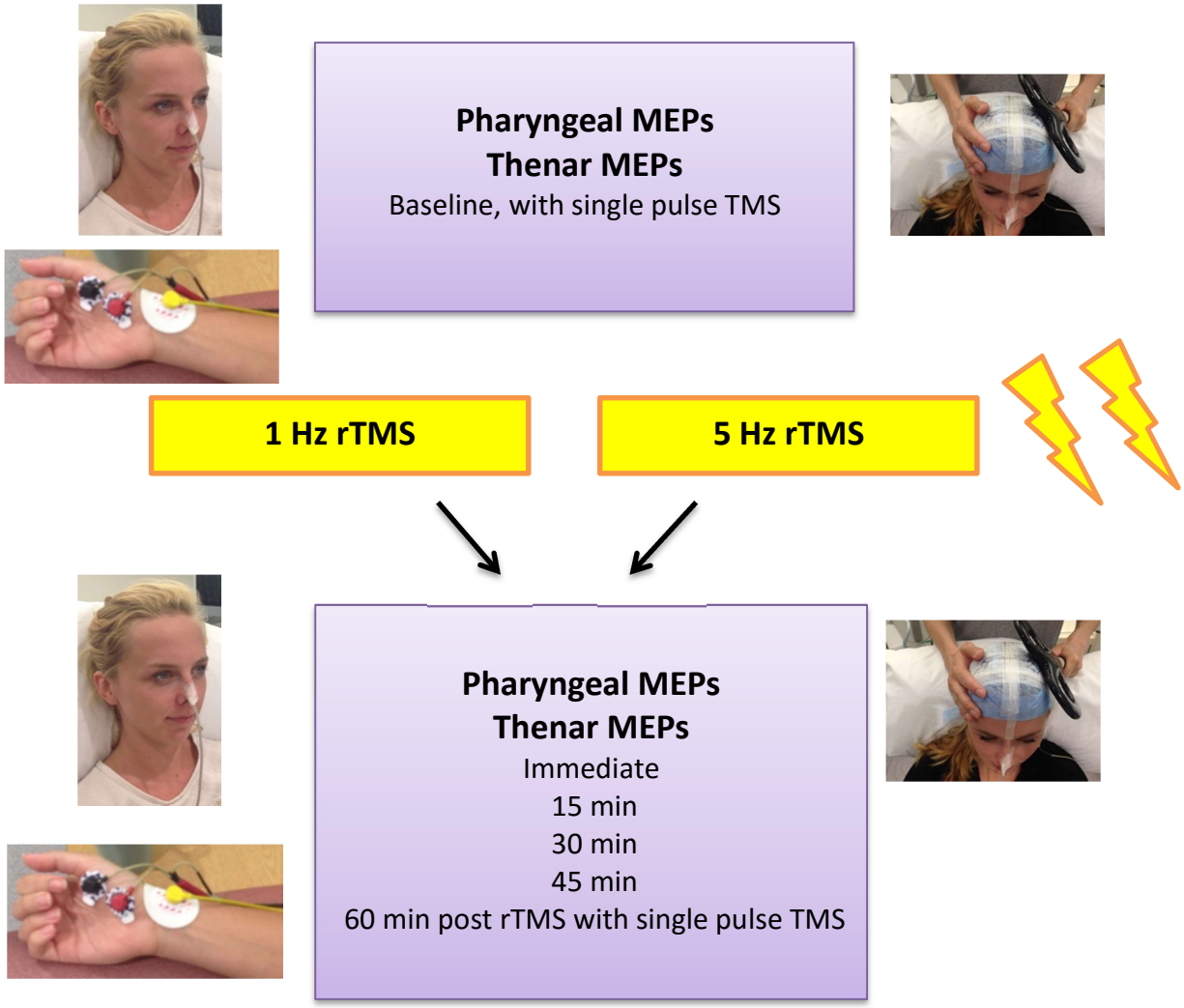


Figure 4.1. **Schematic representation of the experimental protocol.**

4.2.5. Analysis

Subjects were clustered into groups according to their individual PMEPs patterns after 1Hz and 5Hz rTMS interventions (*Figure 4.2.*). For this analysis, normalized to baseline data of PMEPs amplitudes have been used, which are expressed as % of change from the baseline, which eliminates the effects of time factor within the study. For each individual the grand average (GA) of the percentage of change for 5 consecutive follow up time points was calculated and used for grouping. Time of day of the stimulation was called 'a.m.' when the intervention was delivered before 12:30 p.m. and 'p.m.' after 12:30. Subjects were clustered into two groups according to their ethnicity into Caucasians and non-Caucasians to test the effects of gender, ethnicity and time of the day (morning or afternoon) with the chi square test has been used.

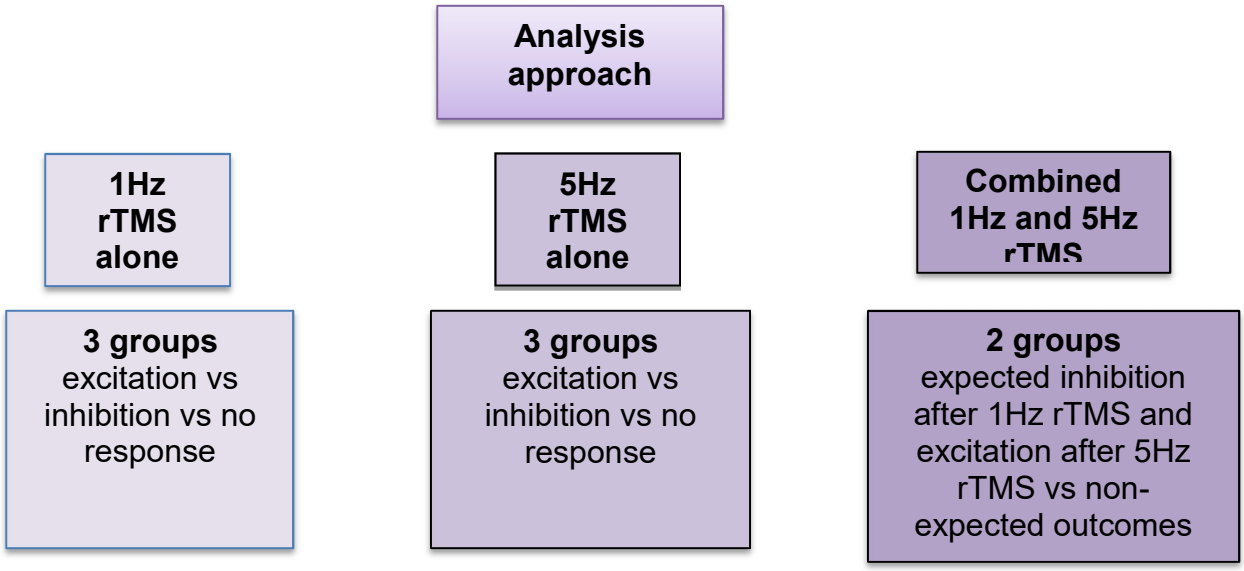


Figure 4.2. **Schematic representation of approach used to grouping of the study cohort.**

4.2.5.1. Classification according to the grand average (GA) results

Subjects were clustered into groups according to their individual GA for the five time points after 1Hz and 5Hz rTMS intervention (excluding baseline). Subjects with GA >10% were classified into facilitation group; <-10% inhibition group and values between 10% and -10% as non-responders. The 10% threshold has been previously used in studies with theta burst stimulation rTMS interventions over the hand motor cortex as a potentially clinically significant threshold [163].

4.2.5.2. Classification for combined 1Hz and 5Hz paradigms into two groups of expected and unexpected outcomes.

Another grouping has been made according to responses for both stimulations and so the group called 'expected outcome' included individuals who showed inhibition in responses followed by 1Hz rTMS and excitatory pattern for 5Hz rTMS, all the other responses were classified as unexpected outcome.

4.2.5.3. Comparison between the pharyngeal motor cortex excitability and the hand motor cortex excitability

Hand MEPs were used as controls to compare the effects of both 1Hz and 5Hz rTMS paradigms on the hand and the pharyngeal muscles. To test normality in the raw hand MEPs Kologorov-Smirnov test was performed on the raw data of hand MEPs. Log transformation was used to transform the data to normal distribution.

To compare effects of log transformed data of PMEPs and hand MEPs, two-way repeated measures ANOVA was performed with two factors: muscle (the pharynx or the hand) and time (for 6 time points). The normalised approach with z-scores was used to remove dimension issue with amplitude of response difference in the hand and the pharynx.

4.3. Results

4.3.1. Variability in the responses followed by 1Hz rTMS paradigm- classification into 3 groups of excitatory, inhibitory and non- responders

Figure 4.3. and *Table 4.1.* show basic descriptive data for three groups of individuals separated into those with excitatory, inhibitory and non-responders group according to individual GA. 44% of subjects were clustered into the group of expected inhibitory responses, a third into excitatory and 23% were classified as non-responders with GA under 10% and over -10%.

There were no statistically significant differences between 3 groups of outcomes according to individual's GA from 5Hz rTMS intervention and gender, ethnicity and time of the day of the stimulation (χ= 0.416, *P-value* = 0.812; χ= 1.086, *P-value* = 0.581; χ= 1.582, *P-value* = 0.453 respectively) *Table 4.2*.

Group	n	mean GA of % of change ± S.E.M.
excitatory	13	48.92 ± 8.93
inhibitory	17	-28.36 ± 2.8
non-responders	9	2.24 ± 1.03

Table 4.1. **Frequencies and mean of individual GA values of normalized to baseline PMEPs amplitudes expressed as % of change for 5 time points ± S.E.M. for each group after 1Hz rTMS.**

Feature	χ^2	*P-value*
Gender	0.416	0.812
Ethnicity	1.086	0.581
Time of the day	1.582	0.453

Table 4.2. **Chi square (χ^2) and P-values for the relation between gender, ethnicity and time of the day and GA of the responses after 1Hz rTMS.**

Chi (χ^2) and *P-value***s** for the relation between gender, ethnicity and time of the day and GA of the responses after 1Hz rTMS.

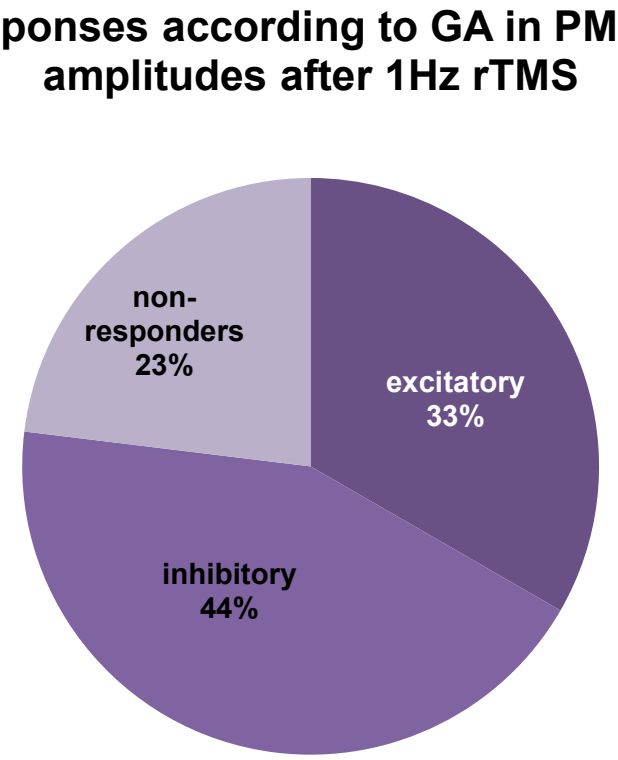


Figure 4.3. **Percentage of subjects classified according to their GA of normalized to baseline PMEPs after 1Hz rTMS paradigm**

into subjects who respond with no change (non-responders), excitation and inhibition.

4.3.2. Variability in the responses followed by 5Hz rTMS paradigm - classification into 3 groups of excitatory, inhibitory and non-responders

Classification according to the GA intro 3 groups after 5Hz paradigm. *Figure 4.4.* and *Table 4.3.* show basic statistics for individuals separated into 3 groups: excitatory, inhibitory and non-responders group. 40% of subjects were clustered into the group of inhibitory responses, a third into excitatory and 28% were classified as non-responders with GA under 10% and over -10%.

There were also no statistically significant differences between 3 groups of responses after 5Hz rTMS intervention and gender, ethnicity and time of the day of the stimulation (χ= 0.776, *P-value* = 0.679; χ= 2.469, *P-value* = 0.291; χ= 2.731, *P-value* = 0.604 respectively) shown in *Table 4.4.*

Group	n	mean GA of % of change
excitatory	13	29,1943 ± 3,36
inhibitory	16	-19,7511 ± 6,3
non-responders	11	0,0419 ± 1,72

Table 4.3. **Frequencies and mean of individual GA values**
of normalized to baseline PMEPs amplitudes expressed as % of change for 5 time points ± S.E.M. for each group after 5Hz rTMS.

Feature	χ^2	*P-value*
Gender	0.776	0.679
Ethnicity	2.469	0.291
Time of the day	2.731	0.604

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Table 4.4. **Chi square (χ^2) and P-values for the relation between gender, ethnicity and time of the day and GA of the responses after 1Hz rTMS.**

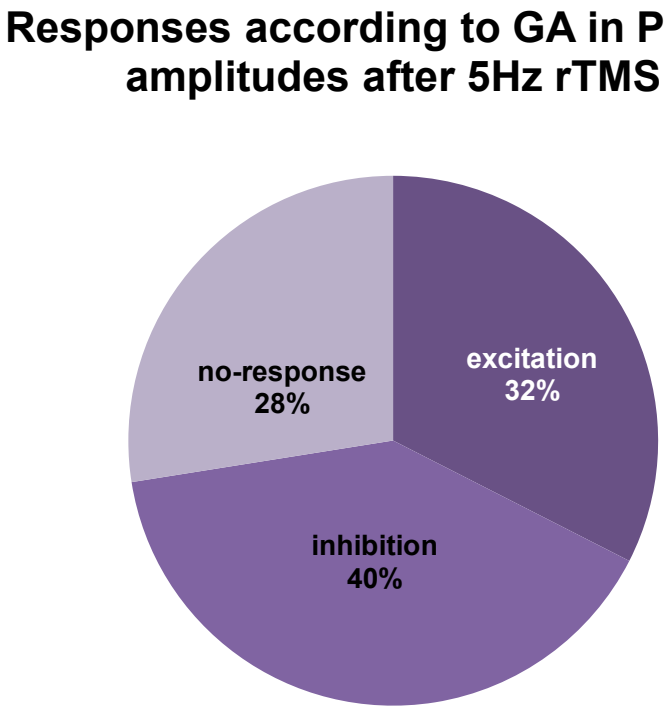

Figure 4.4. **Percentage of subjects classified according to their GA of normalized to baseline PMEPs.**
after 5Hz rTMS paradigm into subjects who respond with no change (non-responders), excitation and inhibition.

4.3.3. Grouping individuals into two groups of expected and unexpected outcomes followed by combined 1Hz and 5Hz rTMS paradigms

Another way of classification individuals into clusters takes into consideration two rTMS interventions at the same time and grouping subjects according to both post-intervention outcomes.

Only 13% of individuals presented expected results followed both stimulations: inhibition after 1Hz rTMS paradigm and excitation after 5Hz rTMS paradigm. Of the 38 participants who received both rTMS stimulations, 33 showed unexpected outcomes for one or two paradigms (*Figure 4.5*).

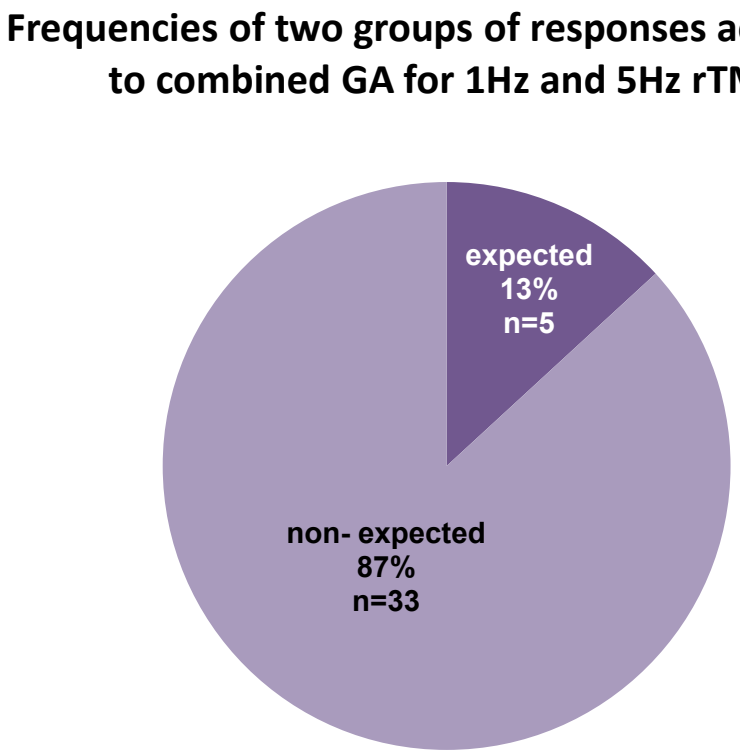

Figure 4.5. **The figure shows frequencies of two groups according to GA classification for both stimulations.**

into expected (inhibition after 1Hz and excitation after 5Hz) and unexpected outcomes.

4.3.4. Comparison between the hand MEPs and the pharyngeal MEPs responses followed by 1Hz rTMS

As with the pharyngeal MEPs, raw data for hand MEPs amplitudes did not have a normal distribution (*Figure 4.6.*), and log transformation was performed. Normality was tested with Kolmogorov-Smirnov tests (*Supplementary table 4.1.*). Log transformed data were standardized for both the hand and the pharyngeal log-transformed amplitudes of MEPs and expressed as z-scores for further analyses.

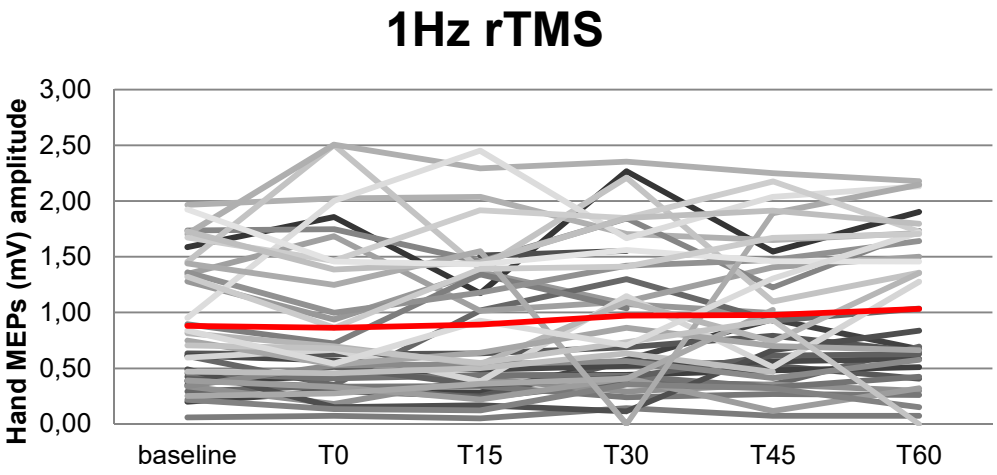

Figure 4.6. **Plot of the raw hand MEP data from all subjects for 1Hz stimulation.** Large variation in response between all individuals was observed. The red line indicates the average hand MEPs.

There was no significant effect of muscle ($F(1, 31) = 0.257$, P-value = 0.616) and time ($F(3, 107) = 0.225$, P-value = 0.902). There was also no statistically significant difference between muscles and change over time ($F(4, 125) = 0.125$, P-value = 0.974).

4.3.5. Comparison between the hand MEPs and the pharyngeal MEPs responses followed by 5Hz rTMS

Again hand MEPs raw amplitudes had a range of different responses (*Figure 11*), so data did not have a normal distribution and log transformation was performed. Normality was tested with Kolmogorov-Smirnov tests (*Supplementary Table 2*). Log transformed data were standardized with z-scores for both the hand and the pharyngeal log-transformed amplitudes in MEPs for further analyses.

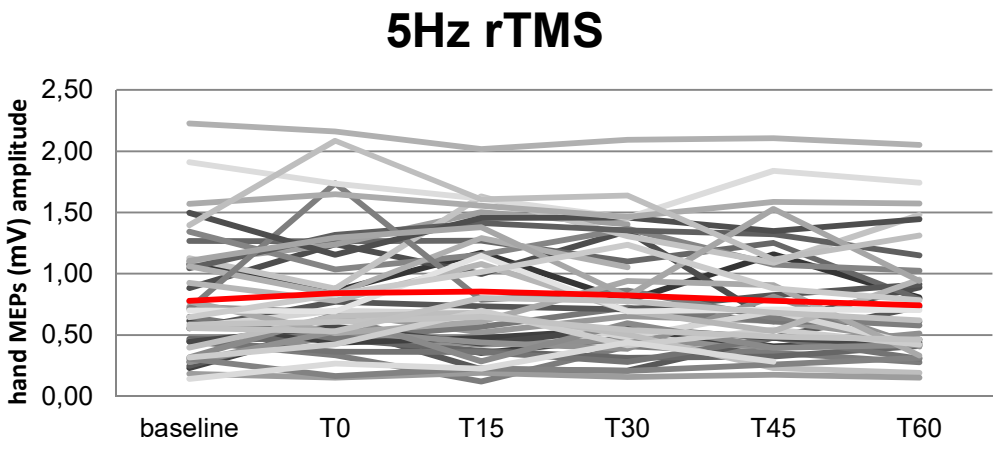

Figure 4.7. **Plot of the raw hand MEP data from all subjects for 5Hz stimulation.**

Large variation in response between all individuals was observed. The red line indicates the average hand MEPs.

There was a no significant effect of muscle ($F(1, 35) = 0.009$, P-value = 0.926) and mean time ($F(5, 175) = 0.033$, P-value = 0.999) followed by 5Hz rTMS paradigm. There was also no significant difference between muscles and change over time ($F(5, 175) = 0.159$, P-value = 0.977).

4.4. Discussion

In this chapter effects of 1Hz and 5Hz rTMS paradigms over the pharyngeal motor cortex area have been explored by clustering participants into groups selected by different criteria.
1Hz rTMS paradigm revealed a variety of individual responses firstly grouped in 3 main categories: excitation, inhibition or no response according to GA for each individual with 10% above and below baseline threshold. Almost half of individuals showed inhibitory pathways. One interesting finding was the observation that a proportion of individuals showed the reverse trend to inhibition, i.e. unexpected excitation. Similarly 5Hz stimulation showed large variability in subjects' responsiveness, from which only 32% were classified in the group of expected excitation.

These unexpected, limited or "reverse" responses might explain why mean ratio values of raw PMEPs showed in the previous Chapter 3 are close to 0.1 indicating lack of responses over time. Findings of the current study seem to differ from previous research. When considering both stimulations, just over 15% of subjects revealed expected inhibition after 1Hz paradigm and excitation after 5Hz rTMS. In previous studies with the same parameters clear overall inhibition [83, 108] and excitation [100, 108] in the corticobulbar projection was observed. One possible explanation for these inconsistencies in the reports may be the lack of adequate power of previous studies or more diversity within the cohort in this study.

In our cohort ethnicity, gender and time of day had no statistically significant effects on responses no matter what criteria were used. Therefore the variability responses of within this cohort cannot be explained by these factors. However another explanation might be associated with possible effects of other factors which were not examined in these analyses such as stress, attention or genetic factors (see later chapters). All these were highlighted in the previous studies on NIBS over the M1 of the motor cortex of the hand area to have influence on subject's cortical responsiveness [157, 158].

As a control, the PMEPs for both 1Hz and 5Hz rTMS were compared with the MEPs of the hand abductor pollicis brevis (APB). No differences were found regardless which criteria were applied (transformed data or grouping). Similar variability of responses was observed in raw MEPs outcomes. Possible explanation of this similarity would be that the hand motor cortex is located close to the pharyngeal sensorimotor cortex area, where the signal for rTMS stimuli can spread. In this study I did not apply rTMS directly to the hand area of the motor cortex however intensity was high enough to cause small visible twitches in contralateral thenar muscles.

Although results of this chapter used the same parameters from published studies of the pharyngeal motor cortex excitability, they are consistent with those examining the variability in responsiveness of the hand muscles followed by other NIBS delivered over the M1 of the hand area [158-162]. Researchers used either tDCS [159, 160] or TBS [158, 161, 162] to affect motor cortex. Volunteers were classified as responders and non-responders or those with excitatory,

inhibitory or lack of responses. In all the aforementioned research, the GA of individual responses was used for classification. The only limitation of this approach is that using the GA of the results overlooks bidirectional responders. High excitation then low inhibition or the other way around responses were commonly observed in my study and might not be considered as lack of response. There is also inconsistency in defining threshold from which excitation or inhibition is ascertained. We have used 10% above and below the GA for PMEPs, however results should be considered with caution, because this was arbitrary approach.

One of the limitation of this research studies were lack of sham stimulation and participants were used as their own controls. Another reason of not showing an effect could also reflect differences in how the parameters used were applied (such and type or orientation of the coil).
One of the biggest advantages is that these findings might help us to understand inter- and intra-individual differences in the mechanisms for neuronal plasticity within the pharyngeal motor cortex. All the subjects in the study showed unique pathways of responses which might explain why both 1Hz [93-95, 102] and 5Hz [75, 95, 104] rTMS stimulations over the swallowing motor cortex show positive results in studies conducted on both healthy individuals and stroke dysphagic patients.

To develop a full picture of intra- and inter-individual variability additional studies will be needed. These findings should be confirmed across multi test sessions and pathological population. Another research question that could be asked is whether similar variability is present in subcortical areas involved in swallowing such as the cerebellum [30].

The results of present study raise the need to create more stratified approaches in the therapy of swallowing impairment with rTMS. Tailored treatment therapies might increase their effectiveness and decrease recovery time from neurogenic swallowing impairment.

4.5. Conclusions

This project was undertaken to evaluate variability after 5Hz and 1Hz rTMS paradigms. The results of this investigation show that while there were a subset of subject who showed expected (from previous research) outcomes, a number of subjects showed unexpected (reverse or lack) responses. Understanding the individual capacity of rTMS to induce long lasting changes in plasticity in the human pharyngeal motor cortex is critical for their application as therapeutic tools in neurogenic oropharyngeal swallowing impairments.

4.6. Supplementary material

Supplementary Table 4.1. **Normality test results for raw and log transformed data from 1Hz stimulation measurements for the hand**: baseline and 5 consecutive time points after the stimulation. Significance value >0.05 indicates normal data distribution.

Raw data	Kolmogorov-Smirnov significance	Log transformed data	Kolmogorov-Smirnov significance
baseline 1Hz rTMS	0.012	baseline 1Hz rTMS	0.149
T0 1Hz rTMS	0.001	T0 1Hz rTMS	0.200*
T15 1Hz rTMS	0.009	T15 1Hz rTMS	0.200*
T30 1Hz rTMS	0.020	T30 1Hz rTMS	0.200*
T45 1Hz rTMS	0.196	T45 1Hz rTMS	0.200*
T60 1Hz rTMS	0.012	T60 1Hz rTMS	0.037

*. This is a lower bound of the true significance.

Supplementary Table 4.2. **Normality test results for raw and log transformed data from 5Hz stimulation measurements for the hand**: baseline and 5 consecutive time points after the stimulation. Significance value >0.05 indicates normal data distribution.

Raw data	Kolmogorov-Smirnov significance	Log transformed data	Kolmogorov-Smirnov significance
baseline 5Hz rTMS	0.017	baseline 5Hz rTMS	0.200*
T0 5Hz rTMS	0.002	T0 5Hz rTMS	0.200*
T15 5Hz rTMS	0.200*	T15 5Hz rTMS	0.200*
T30 5Hz rTMS	0.035	T30 5Hz rTMS	0.200*
T45 5Hz rTMS	0.028	T45 5Hz rTMS	0.200*
T60 5Hz rTMS	0.027	T60 5Hz rTMS	0.200*

*. This is a lower bound of the true significance.

Chapter 5

Exploring the association between genetic polymorphisms and the swallowing motor cortex excitability induced by repetitive transcranial magnetic stimulation

Abstract

Background: Non-invasive brain stimulation such as repetitive Transcranial Magnetic Stimulation (rTMS) are used to affect excitability in the swallowing motor cortex. Molecular mechanisms controlling the excitability remain unknown. Swallowing neurophysiology and impairments might be in part driven by genes. The aim of this Chapter is to determine whether the variability in excitability after 1Hz and 5Hz rTMS within the pharyngeal motor cortex might be affected by selected single nucleotide polymorphisms (SNPs).

Materials and methods: 11 SNPs from 7 genes (*BDNF, COMT, TRKB, APOE, DRD2, GRIN2B* and *GRIN1*) were selected to explore possible link between neurophysiological outcomes after rTMS intervention with high (5Hz) and low (1Hz) frequencies. 41 healthy young volunteers were used for the study. Different statistical approaches were used.

Results: Only analysis with grouped phenotype according to grand average of percentage of change in pharyngeal motor evoked potentials showed statistically significant results. Non-carriers of the minor G allele from SNP rs6269 from *COMT* gene (*P*-value = 0.026) are more likely to be non-responders, while those carrying G allele are more likely to have inhibitory and excitatory outcomes after delivering 1Hz rTMs. Cross-tabulation analysis with chi square indicated there was a significant difference between 5Hz rTMS outcome and one SNP - *DRD2* rs1800497. Carriers of minor allele A from rs1800497 (*DRD2* gene) showed inhibition while non-carriers were non-responders (*P*-value = 0.03).

Discussion and conclusion: Presented studies showed a possible evidence of genetic association with the neuromuscular control of swallowing affected by rTMS paradigms. Two SNPs from *COMT* and *DRD2* genes might play a role in pharyngeal cortex excitability depending on the stimulation applied. Further research is needed to establish more detailed information which might be used in developing more stratified approaches in the field of dysphagia therapy with non-invasive brain stimulation.

5.1. Introduction

Non-invasive brain stimulation (NIBS) techniques have been recently used as promising therapies in neurogenic swallowing difficulties in patients with acute brain injuries such as stroke [97, 102, 156]. Despite their therapeutic potential and improvement in swallowing impairments in some patients, there is a group of patients who remain resistant to treatments with NIBS [164].

In the previous Chapters (3 and 4) I showed that variability in responsiveness following two rTMS paradigms over healthy pharyngeal motor cortex could not be explained by factors such as gender, time of the day of stimulation, ethnicity and age.

Other factors which can influence responsiveness in pharyngeal cortex excitability might be associated with individual genetic predisposition. Recent studies [108, 110] and my findings from Chapter 2, suggest that individual genetic make-up might play a role in swallowing performance in elderly individuals [110, 165] as well as the responsiveness of the pharyngeal motor cortex and pharyngeal hypersensitivity after for the brain stimulation [108, 109]. Existing literature exploring genetics of swallowing remains limited without evidence of a strong candidate gene. One of the biggest disadvantages of existing studies is the fact that single genetic polymorphism or genes do not provide sufficient power for the studies to show causality. Moreover, none of the existing findings have currently been replicated.

A possible link between overall motor cortex excitability and genetic factors has been studied in a different branch of neurogenetic research, which measured motor evoked potentials in the hand after non-invasive brain stimulation. Twin studies with TMS provided some evidence for the potential genetic components with hand motor cortical excitability [8]. In these studies only the *BDNF* gene was studied which is believed to play an important role in cortical plasticity. Studies conducted on healthy volunteers have highlighted a number of genetic polymorphisms from various genes which might affect responsiveness of the hand motor cortex followed by non-invasive brain stimulations [114, 118, 121, 166-169].

The hypothesis is that the neurological control of swallowing might be controlled by multiple genes with small effect size. Therefore one of the biggest challenges in exploring the genetic underpinnings of neurological conditions is gene selection.

Cortical plasticity mechanisms of Long Term Potentiation (LTP) and Long Term Depression (LTD) (described in Chapter 1) are controlled by a highly sophisticated network of multiple genes and proteins [73, 74]. Studies highlight a number of genes which could play a potential role in cortical plasticity. The first group of importance are genes encoding subunits of receptors on the postsynaptic membranes (NMDA receptors) - glutamate receptor ionotropic, NMDA 1 (*GRIN1*) and Glutamate receptor ionotropic, NMDA 2B (*GRIN2B*). The *BDNF* gene encoding brain delivered neurotropic factor is the most commonly studied gene in cortical plasticity studies, also associated with the pharyngeal motor cortex [108]. Another gene, *APOE,* apart

from playing a potential role in neuronal connectivity following non-invasive brain stimulation in cohorts of non-demented elderly [170], was also related to self-reported swallowing impairments also in a cohort of healthy elderly individuals [110]. Both *APOE* and *BDNF* genes are involved in multiple genetic pathways and diseases such as depression, stroke and neurodegenerative diseases [116, 117, 127, 171].

Another candidate gene which could be further evaluated is *TRKB.* The evidence from swallowing reflex experiments in rats suggests that *TRKB* gene along with *BDNF* might affect neurological control of swallowing [122]. Recently published epidemiological studies highlighted the importance of SNPs from *COMT* gene and self-reported swallowing impairments [165]. *COMT* gene also plays a role in cortical plasticity after non-invasive brain stimulation [166, 168].

In this chapter I will explore possible associations between outcomes from rTMS (1Hz and 5Hz) paradigms delivered over the pharyngeal motor cortex (Chapter 3 and 4) and a number of selected SNPs from Chapter 2 (rs17601696 and rs17789174 from the *KIAA0513* gene) as well as other a priori selected genes which might influence swallowing impairments: *APOE*, *BDNF* and *COMT*. Additionally SNPs from 3 genes from studies with rTMS delivered over the hand motor cortical area will be genotyped: *GRIN1, GRIN2B* and *DRD2*.

5.2. Materials and methods

5.2.1. RTMS experiments summary

NIBS methods are identical to Chapter 3 and are only briefly summarized below.

5.2.2. Participants

Forty-one young, healthy volunteers (mean age= 25.6 ± 4.6 years old, age range 18-35, 43.9 % female) were recruited for the study. Participants were excluded from the study if they have had a history of epilepsy, metal in their head, throat, have had a brain surgery, a cardiac pacemaker, were pregnant or already involved in other research. Prior to the study, informed consent was obtained from all the volunteers.

5.2.3. The procedure for measurements

Pharyngeal electromyographic (EMG) measurements measurements were collected.

5.2.4. Single pulse Transcranial Magnetic Stimulation

PMEP responses were collected by delivering 10 pulses of TMS at 100% and 120% of the threshold at each time point over the hemisphere giving stronger PMEPS before each intervention and at 5 time points after the intervention (immediately and 15, 30, 45, 60 minutes after the intervention) (*Figure 5.1.*), see earlier chapters.

5.2.5. Stimulation Techniques

Two Repetitive Transcranial Magnetic Stimulation paradigms (1Hz and 5Hz) were delivered on separate days over the pharyngeal motor cortex in the hemisphere giving stronger (larger) responses.

5.2.6. DNA collection

Saliva samples were collected with the ORANGENE DNA (OG-500) after the first study from all subjects for DNA analysis.

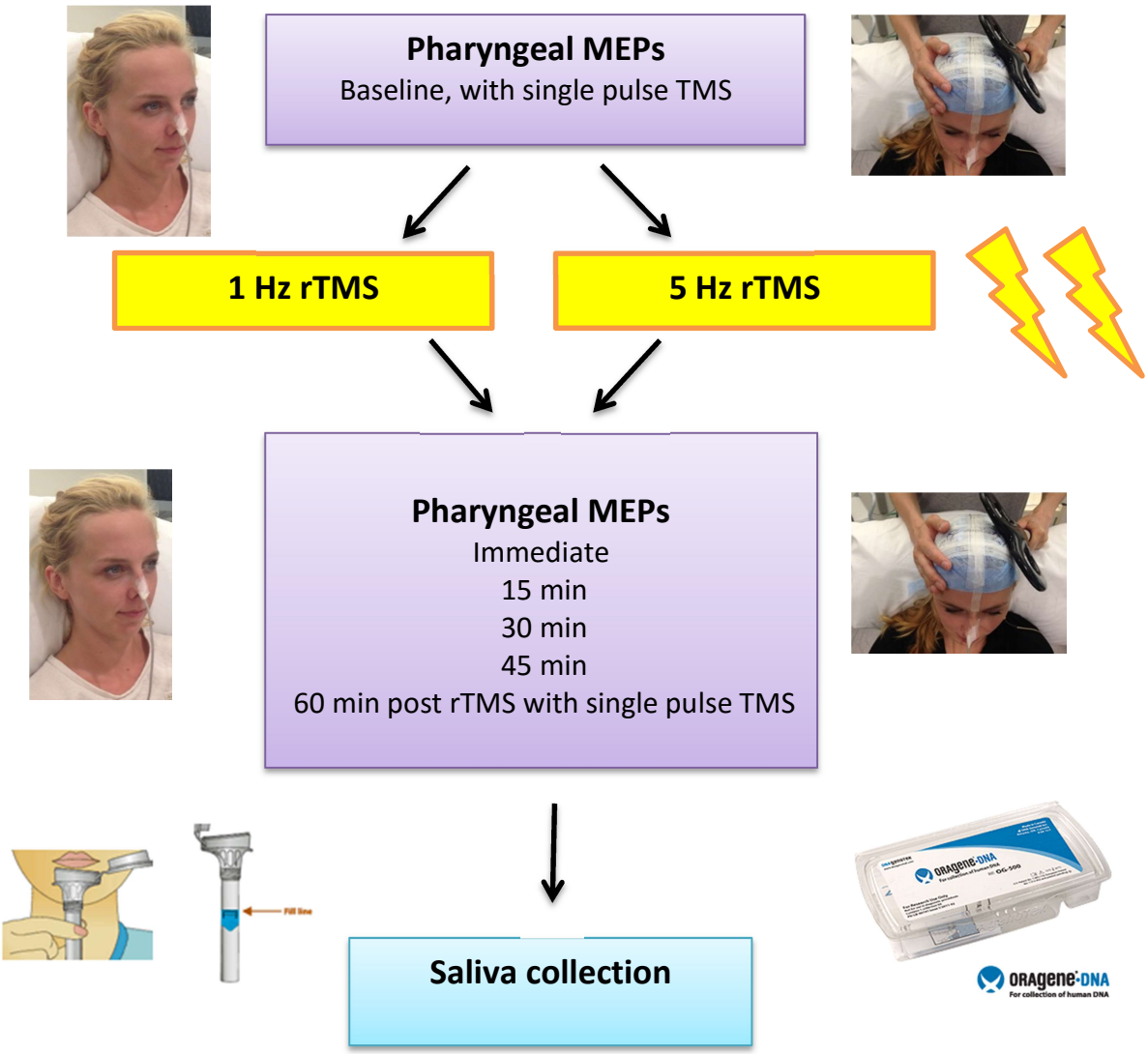

Figure 5.1. **Schematic representation of the experimental protocol.**

5.2.7. Genotyping

All processes were carried out at the Centre for Integrated Genomic Medical Research, University of Manchester. Saliva sample collection for extraction of DNA was obtained using Oragene-250 self-contained DNA collection kits (DNA Genotek Inc, Ontario, Canada). For saliva DNA, standard operating procedures were used to extract and purify the salivary DNA. SNPs were genotyped using Applied Biosystems Assays-by-Demand kit (Applied Biosystems, Warrington, Cheshire, England, UK). Genotyping was carried out using a reaction mixture containing 2.5 µL of DNA Probe Master (Roche Diagnostics, West Sussex, UK), 15 ng genomic DNA in a 5- µL reaction volume, comprising 2 µL DNA, 0.125 µL Assay Mix (40×) and 0.375 µL nuclease-free water. The PCR conditions were 95°C for 10 min then 50 cycles of 95°C for 10 s and 60°C for 30 s. The reaction was allowed to run and the PCR products were

electrophoresed. The sample reporter fluorescence was measured using a Roche Lightcycler 480 genotyping real-time PCR platform (Roche Diagnostics, West Sussex, England, UK).

5.2.8. SNP selection

15 SNPs were selected for genotyping on the basis of previous work and literature searches (*Table 5.1.* and *Figure 5.2.*). SNPs were grouped in 3 categories:

a) SNPs from GWAS studies on swallowing (See Chapter 2):

- rs17601696 SNP- cytogenetic location 10q25 which has obtained the highest significance in SNP-based GWAS analysis

- *KIAA0513*, rs1778917 (OMIM 611675; cytogenetic location 16q24.1) - Gene highly expressed in several brain regions such as the cerebellum, the cerebral cortex, the hippocampus, the pons, the putamen, and the amygdala. Lauriat *et al.* [154] suggested that *KIAA0513* is involved in synaptic and apoptotic signalling.

b) SNPs from genes associated with swallowing impairments selected in other studies as significant:

- *BDNF* gene, rs6265 (OMIM 113505*;* cytogenetic location: 11p14.1) - The most commonly studied SNP from the *BDNF* gene involved in cortical plasticity. During development *BDNF* promotes the survival and differentiation of selected neuronal populations, participates in axonal growth, pathfinding and in the modulation of dendritic growth and morphology. BDNF protein is a regulator of synaptic transmission and plasticity at adult synapses in many regions of the central nervous system. It contributes to a range of adaptive neuronal responses such as LTP, LTD, certain forms of short-term synaptic plasticity, as well as homeostatic regulation of intrinsic neuronal excitability [172].

- *COMT* gene rs6269 (OMIM 116790; cytogenetic location: 22q11.21) - Protein product of this gene catalyses the inactivation, of catecholamine neurotransmitters and catechol hormones. COMT protein shortens the biological half-lives of certain neuroactive drugs such as L-dopa, alpha-methyl DOPA and isoproterenol [172].

- *APOE* gene 2 SNPs from s429358, rs7412 (OMIM 107741, cytogenetic location: 19q13.32) - APOE protein mediates the binding, internalization, and catabolism of lipoprotein particles. APOE plays an important role in various molecular pathways in the central nervous system including neuronal plasticity [173]. Individuals

who are carrier of one of *APOE* isoforms *APOE Ɛ-4* are more likely to suffer from self-reported swallowing impairments [110].

c) Other SNPs associated with cortical plasticity of motor cortex studied with rTMS procedures delivered over the other

- *TRKB* (other name *NTRK2*) 3 SNPs: rs10868223, rs1659412, rs11140778-(OMIM 600456, cytogenetic location: 9q21.33) - *TRKB* is a *BDNF* receptor and plays a role in learning and memory by regulating both short and long-term potentiation and in communication between neurons and glia [172]. Animal studies conducted on rats and brain controlled swallowing impairment showed that interacting *BDNF* and *TRKB* influence swallowing frequency (See Chapter 1- Introduction) [122, 123]. Human studies with 5Hz rTMS found an association between *TRKB* gene and cortical excitability in of the hand motor cortex [169].

- *GRIN1* 2 SNPs rs4880213, rs6293 (OMIM 138249, cytogenetic location: 9q34.3) and *GRIN2B* 3 SNPs: rs3764028, rs7301328, rs1805247 (OMIM 138252, cytogenetic location: 12p13.1) - The proteins encoded by these genes builds one of the subunits of NMDA receptor subtype of glutamate-gated ion channels. NMDA receptors are heterotetramers composed of 2 NMDA receptor-1 (NR1, or GRIN1) subunits and 2 NR2 subunits, *GRIN2B*. These channels have high calcium permeability and voltage-dependent sensitivity to magnesium. *GRIN1* is believed to regulate synaptic plasticity, synaptogenesis and excitotoxicity, memory acquisition and learning. Human studies with non-invasive brain stimulations (paired pulse TMS and intermittent Theta Burst Stimulation) highlighted the impact of SNPs rs4880212 from *GRIN1* and rs4805247 on intracortical reactivity [121].

- *DRD2* rs1800497 (OMIM 608774, cytogenetic location: 11q23.2) - The D2 dopamine receptor is a G protein-coupled receptor located on postsynaptic dopaminergic neurons involved in corticolimbic pathways [174]. Dopamine is of the major neurotransmitter which is involved in modulating dopamine-mediated control of motor activity [167].

A list of all the SNPs from chosen genes for further exploration is shown in *Table 5.1.*:

	SNP	GENE	Chromosome
1	rs17601696	non- coding sequence	10
2	rs17789174	*KIAA0513*	16
3	rs6265	*BDNF*	11
4	rs6269	*COMT*	22
5	rs10868223	*TRKB*	9
6	rs1659412	*TRKB*	9
7	rs11140778	*TRKB*	9
8	rs429358	*APOE*	19
9	rs7412	*APOE*	19
10	rs4880213	*GRIN1*	9
11	rs6293	*GRIN1*	9
12	rs3764028	*GRIN2B*	12
13	rs7301328	*GRIN2B*	12
14	rs1805247	*GRIN2B*	12
15	rs1800497	*DRD2*	11

Table 4. **Selected SNPs for further analysis.**

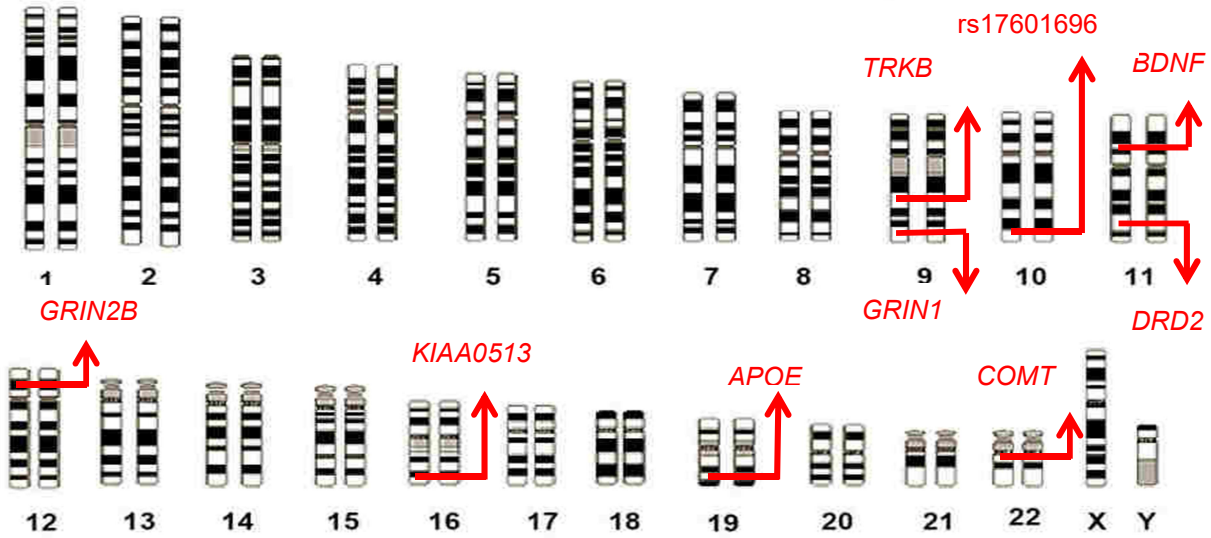

Figure 16. **Chromosomal localization of selected SNPs**

(marked with red arrows). Numbers 1-22 represent autosomes and X and Y sex chromosomes.

a. SNP quality control procedures

All SNPs went for quality check procedures such as genotyping control. The SNPs showed a call rate higher than 95%, with no significant departure from the Hardy-Weinberg Equilibrium.

b. Power calculation

To calculate the power and sample size for the TMS experimental work and possible genetic effects I used Power calculator G*Power 3.1.92 [149].

c. Statistical analysis

SNPs which passed quality control procedures underwent a number of statistical analyses in order to find with specific outcomes from rTMS experiment. Firstly individual genotypes were clustered into two main groups of carriers of at least one minor allele and non-homozygotes for major allele.

This grouping has been used in two types of analysis with neurophysiological data (See Chapter 4 and *Figure 5.3.*)

a) Raw log transformed data from neurophysiological studies was used to performed repeated measures ANOVA with within subject factor TIME and between subject GENOTYPE were used;
b) Grouped neurophysiological data was used according to individual grand average (See Chapter 4) (inhibitory, excitatory pattern and non-responders) examined with two groups and using Chi Square test;
c) Grouped for both paradigms into two groups of expected (inhibition for 1Hz and excitation after 5Hz paradigms) and non-expected vs two groups of genotypes with Chi Square test.
d) Additionally I performed the analysis between selected genes the continuous outcome with grand average value both paradigms alone with *t*-test. The results are presented in the Supplementary section (*Supplementary Table 5.1., Supplementary material 5.1.)*

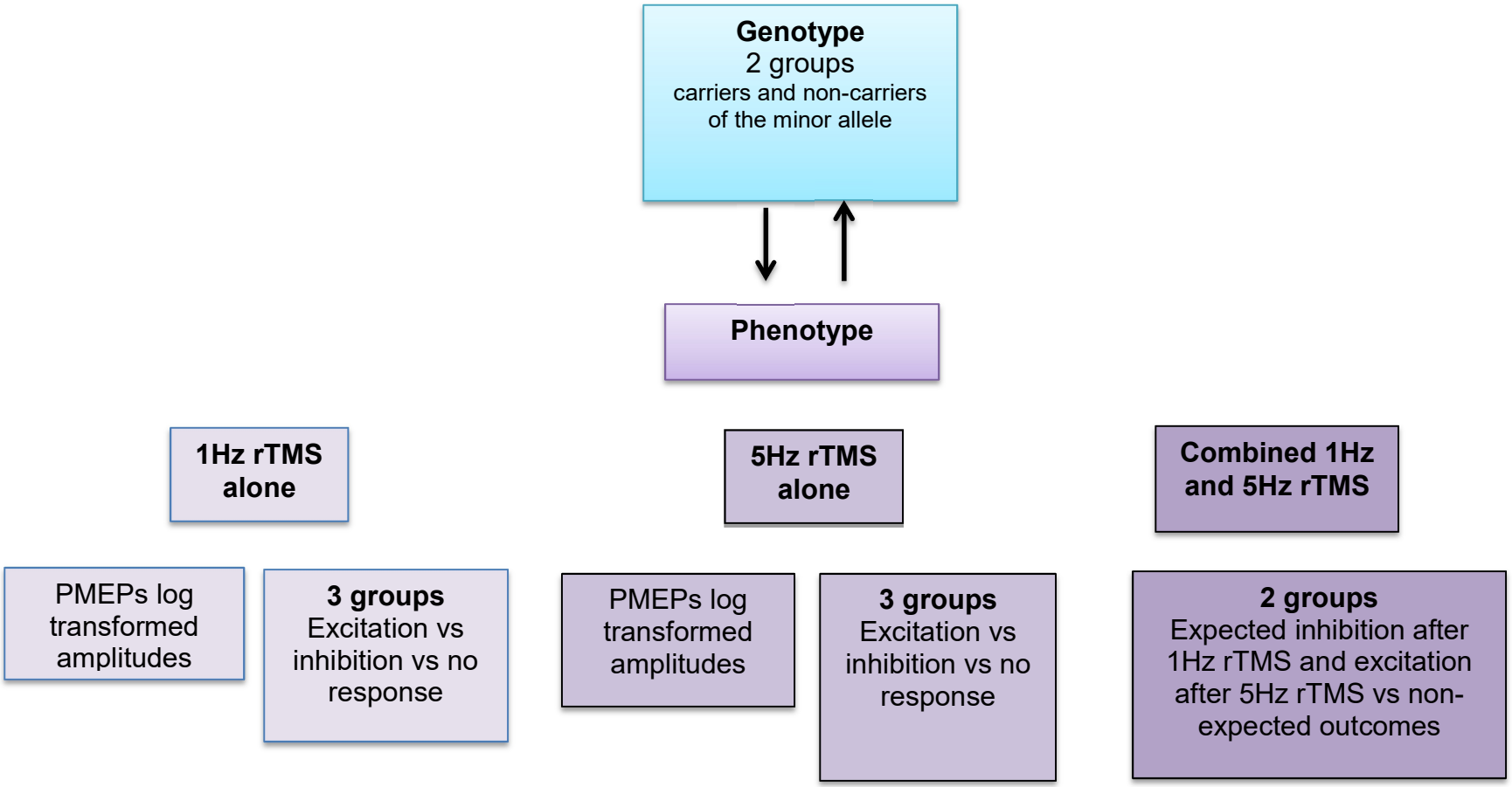

Figure 17. **Schematic representation of approach used to grouping of the study cohort.**

5.3. Results

5.3.1. Quality control

Initial 15 SNPs from 8 genes and one single genetic polymorphism underwent quality check of call rate, Hardy- Weinberg Equilibrium (HWE), 12 SNPs were selected for the analysis. However to obtain a full information about the *APOE* gene, 2 SNPs had to be genotyped. Rs7412 from the *APOE* gene failed the genotyping procedures therefore the second SNP- rs429358 had to be excluded from the analysis due to insufficient genetic information (*Figure 5.4*.). The final list of SNPs for further statistical analysis is presented in *Table 5.3.*

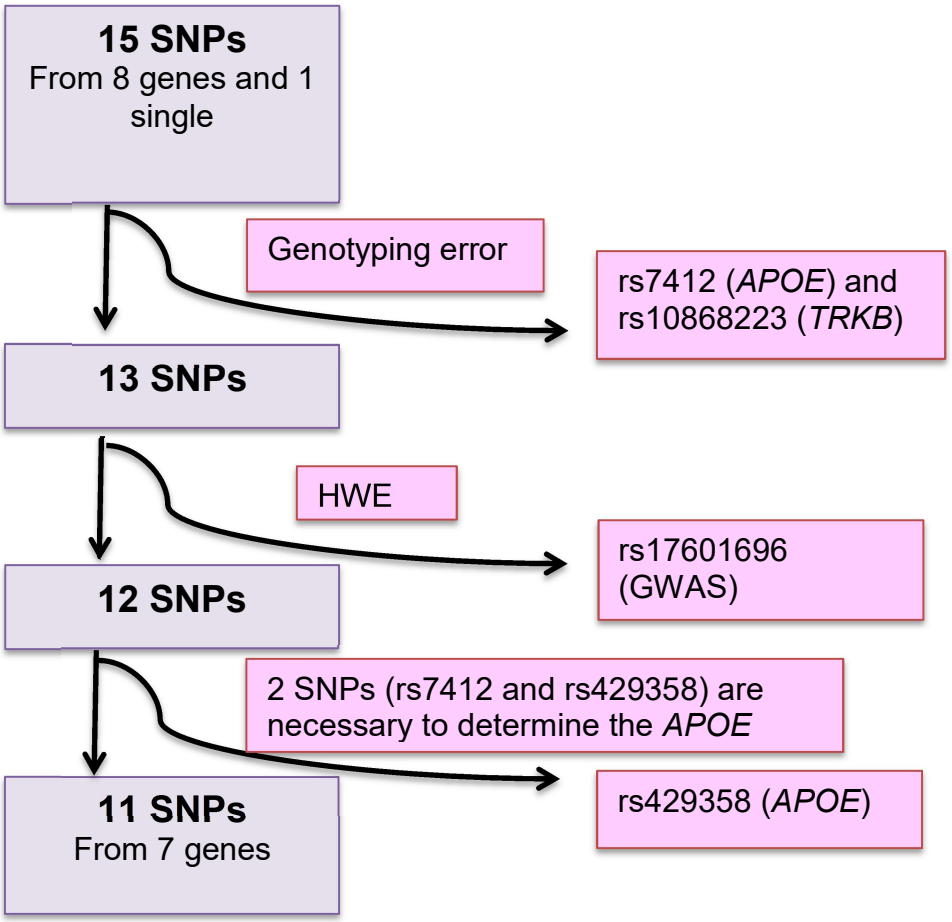

Figure 18. **Table shows quality control procedures of the genotyping data for 15 SNPs selected for genotyping.**

Two SNP failed genotyping, one did not meet Hardy- Weinberg Equilibrium (HWE) and rs429358 was withdrawn from further analysis due to lack of information on rs7412 which is obligatory to determine the *APOE* isoform status.

rs number	gene name	minor allele	Minor allele frequency (Ensembl)
rs17789174	*KIAA0513*	T	0.13
rs6265	*BDNF*	T	0.20
rs6269	*COMT*	G	0.36
rs1659412	*TRKB*	G	0.08
rs11140778	*TRKB*	T	0.14
rs4880213	*GRIN1*	T	0.50
rs6293	*GRIN1*	G	0.14
rs3764028	*GRIN2B*	T	0.20
rs7301328	*GRIN2B*	C	0.44
rs1805247	*GRIN2B*	G	0.22
rs1800497	*DRD2*	A	0.33

Table 5.3. **Final list of SNPs with minor allele after quality control procedures.**

5.3.2. Power calculation

I used repeated measures ANOVA with between factors model with α =0.05 and power of 80%. Caution small effect size (f=0.2) for calculation was used. Concluding from the G*Power output summarized in the *Table 5.4* below and *Figure 5.5.,* a sample of 42 individuals would be sufficient for our project to detect genetic changes. In the analysis only individuals with full genetic and phenotypic information were used and these numbers vary between 28 to 38 which would detect small genetic effect with the power between 68% and 78%.

G-Power output:

F tests -	ANOVA: Repeated measures, between factors		
Analysis:	A priori: Compute required sample size		
Input:	Effect size f	=	0.2
	α err prob	=	0.05
	Power (1-β err prob)	=	0.8
	Number of groups	=	2
	Number of measurements	=	5
	Corr among rep measures	=	
Output:	Noncentrality parameter λ	=	8.4000000
	Critical F	=	4.0847457
	Numerator df	=	1.0000000
	Denominator df	=	40.0000000
	Total sample size	=	42
	Actual power	=	0.8073289

Table 5.4. **Summary of the G*Power output parameters used for power calculation.**

According to these calculations 42 individuals are necessary for 80% power to detect small/moderate genetic effect .

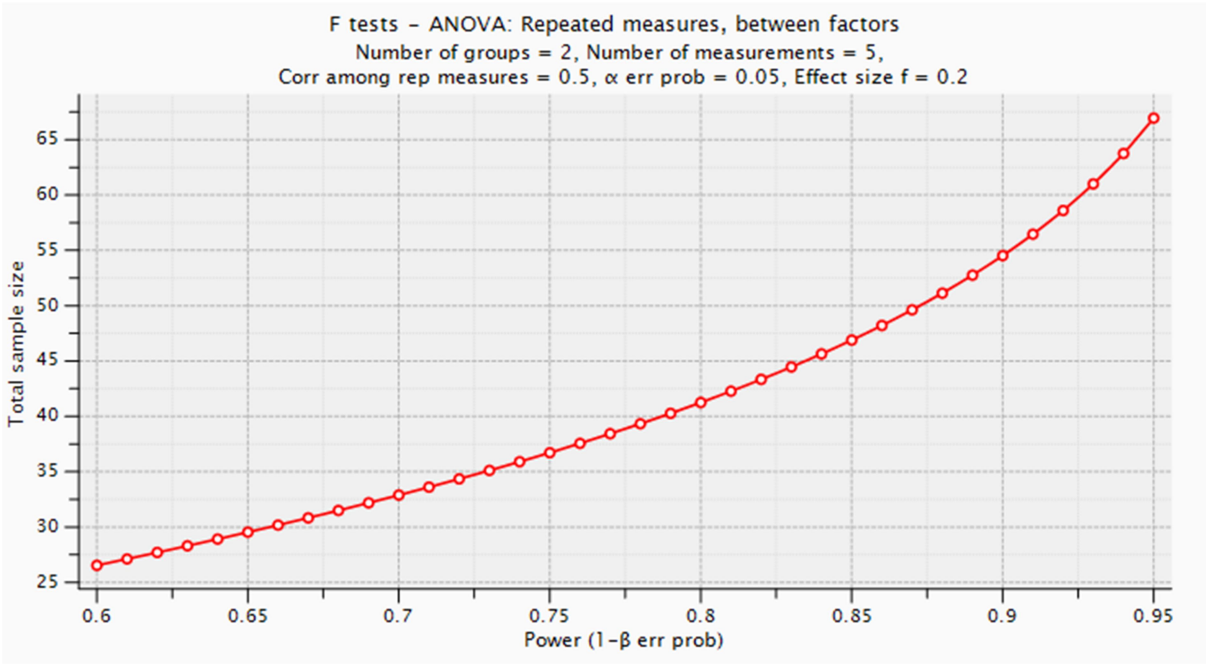

Figure 19. **Plot of sample size versus power for the model described above.**

5.3.3. Statistical analysis results

In this section results from different types of statistical analysis were presented. The analysis was divided into two parts for two stimulations. *Figure 5.2* shows all statistical approaches of dealing with neurophysiological phenotype used in this chapter.

5.3.3.1. 1Hz rTMS results

5.3.3.1.1. Analysis with raw data

Repeated measures one way ANOVA with between-subject factor GENOTYPE and within subject TIME did not show any statistical significance for any of 12 analysed SNPs after applying 1Hz rTMS paradigm (*Table 5.4.*).

SNP	Carriers of the minor allele	Non-carriers of the minor allele	time		time*genotype	
			F	sig	F	sig
rs17789174(*KIAA0513*)	19	13	0.524	0.758	1.386	0.233
rs6265(*BDNF*)	21	11	0.980	0.432	1.672	0.145
rs6269(*COMT*)	10	18	1.184	0.320	0.925	0.467
rs1659412(*TRKB*)	28	4	1.158	0.333	0.911	0.455
rs11140778(*TRKB*)	24	8	0.587	0.662	0.349	0.833
rs4880213(*GRIN1*)	10	21	0.823	0.507	0.883	0.529
rs6293(*GRIN1*)	16	6	0.679	0.599	0.961	0.428
rs3764028(*GRIN2B*)	27	5	0.019	0.932	0.698	0.582
rs7301328 (*GRIN2B*)	26	5	0.187	0.938	0.449	0.763
rs1805247(*GRIN2B*)	26	6	0.527	0.708	1.519	0.203
rs1800497(*DRD2*)	17	15	0.609	0.649	0.949	0.435

Table 5. **Summary of F-statistics and significance of repeated measures ANOVA of log-transformed amplitudes after 1Hz rTMS for all analysed SNPs.**

5.3.3.1.2. Analysis with grouping according to grand average

Another approach was used to categorise individuals 3 groups of neurophysiological responses (inhibition, excitation, no response), as described in Chapter 4. These categories were then compared to genotype (carriers and non-carriers of the minor allele) and are shown in *Table 5.5.*. The results indicate that one SNP rs6269 from *COMT* gene obtained significance with *P*-value = 0.026. From the standard residuals analysis (*Table 5.6.*) which gives the information of how different is the outcome from the expected values and histogram (*Figure 5.6.*), I can concluded that non-carriers of G allele are more likely to be classified as non-responders, while those carrying G allele are more likely to be classified as inhibitory and excitatory outcomes.

SNP	χ^2	*P*- value
rs17789174(*GWAS*)	1.377	0.502
rs6265(*BDNF*)	0.315	0.854
rs6269(*COMT*)	**7.274**	**0.026**
rs1659412(*TRKB*)	0.768	0.681
rs11140778(*TRKB*)	0.641	0.726
rs4880213(*GRIN1*)	4.355	0.360
rs6293(*GRIN1*)	1.406	0.495
rs3764028(*GRIN2B*)	0.768	0.681
rs7301328 (*GRIN2B*)	3.241	0.198
rs1805247(*GRIN2B*)	0.210	0.901
rs1800497(*DRD2*)	7.901	0.19

Table 6. **Results of comparison genotype versus response category with Chi-square (χ^2) analysis with *P*-values and chi square values for each SNP after 1Hz rTMS.**

rs 6269 status		excitation	inhibition	non-responder
Non-carrier	Count	2	3	6
	Expected Count	3,5	4,6	2,8
	Std. Residual	-0,8	-0,8	**1,9**
Carrier G	Count	8	10	2
	Expected Count	6,5	8,4	5,2
	Std. Residual	0,6	0,6	**-1,4**

Table 5.6. **Factorial table with calculated expected count and standardized residuals for rs6269 (COMT gene) after 1Hz rTMS paradigm**.

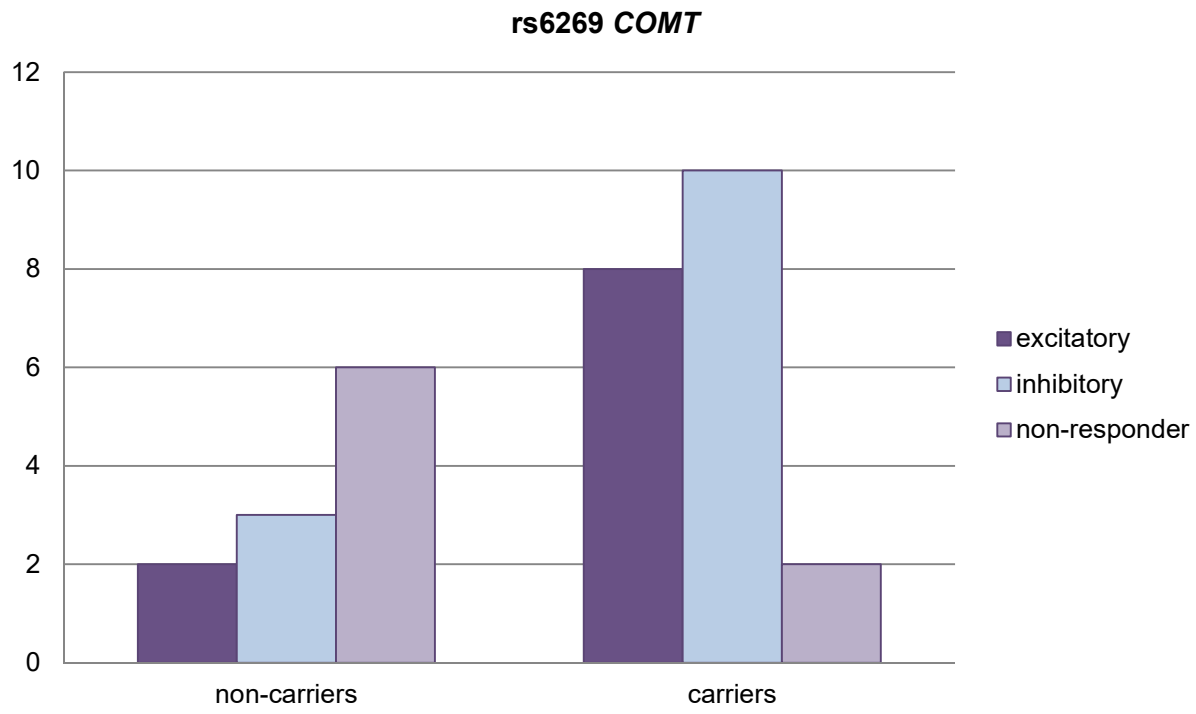

Figure 20. **Diagram shows numbers of individuals from 3 groups of outcomes** (excitatory, inhibitory and non-responders) classified according to their rs6269 status into non-carers and carers of minor allele G.

5.3.3.2. 5Hz rTMS

5.3.3.2.1. Analysis with raw data

As with 1Hz, analysis with repeated measures ANOVA with raw, transformed data from delivering 5Hz rTMS stimulation showed no association for any of analysed SNPs (*Table 5.7.*).

SNP	carriers	non-carriers	time		time*genotype	
			F	sig	F	sig
rs17789174(*KIAA0513*)	23	10	1.135	0.343	1.242	0.297
rs6265(*BDNF*)	25	8	1.204	0.310	0.919	0.453
rs6269(*COMT*)	12	7	2.310	0.056	1.720	0.144
rs1659412(*TRKB*)	29	4	1.522	0.200	0.745	0.562
rs11140778(*TRKB*)	25	8	0.859	0.488	0.909	0.459
rs4880213(*GRIN1*)	13	9	0.721	0.575	0.705	0.682
rs6293(*GRIN1*)	19	14	1.067	0.376	2.185	0.073
rs3764028(*GRIN2B*)	26	7	1.475	0.215	1.021	0.398

rs7301328 (*GRIN2B*)	26	6	1.546	0.196	1.549	0.195
rs1805247(*GRIN2B*)	26	7	0.487	0.742	1.032	0.393
rs1800497(*DRD2*)	18	15	1.308	0.270	0.621	0.649

Table 7. **Summary of F-statistics and *P*-values of repeated measures ANOVA of log-transformed amplitudes after 5Hz rTMS for all analysed SNPs.**

5.3.3.2.2. Analysis with grouping according to grand average

Analysis with Chi square indicated there was a significant difference between 5Hz rTMS outcome and one SNP - *DRD2* rs1800497 (*P*-value = 0.03). Other SNPs did not show any statistically significant results (*Table 5.8.*). According to standard residual values, differences were observed in groups of inhibitory responses and non-responders. Carriers of minor allele A from rs1800497 were more often classified in the inhibitory group and non-carers were more likely to be a non-responder (*Table 5.9.* and *Figure 5.7.*).

SNP	**χ^2**	***P*- value**
rs17789174(*KIAA0513*)	2.524	0.283
rs6265(*BDNF*)	0.579	0.749
rs6269(*COMT*)	4.764	0.092
rs1659412(*TRKB*)	0.244	0.885
rs11140778(*TRKB*)	3.508	0.173
rs4880213(*GRIN1*)	2.569	0.632
rs6293(*GRIN1*)	5.094	0.078
rs3764028(*GRIN2B*)	2.814	0.245
rs7301328 (*GRIN2B*)	4.643	0.78
rs1805247(*GRIN2B*)	0.359	0.836
rs1800497(*DRD2*)	**7.014**	**0.030**

Table 8. **Results comparison genotype versus response category with chi square analysis with *P*-values and chi square values for each SNP after 5 Hz rTMS.**

rs1800497 status		excitation	inhibition	non-responder
non carrer	Count	8	5	7
	Expected Count	7,2	8,3	4,4
	Std. Residual	0,3	**-1,2**	**1,2**
carrer A	Count	5	10	1
	Expected Count	5,8	6,7	3,6
	Std. Residual	-0,3	**1,3**	**-1,4**

Table 9. **Factorial table with calculated expected count and standardized residuals for rs1800497 (DRD2 gene) after 5 Hz rTMS paradigm.**

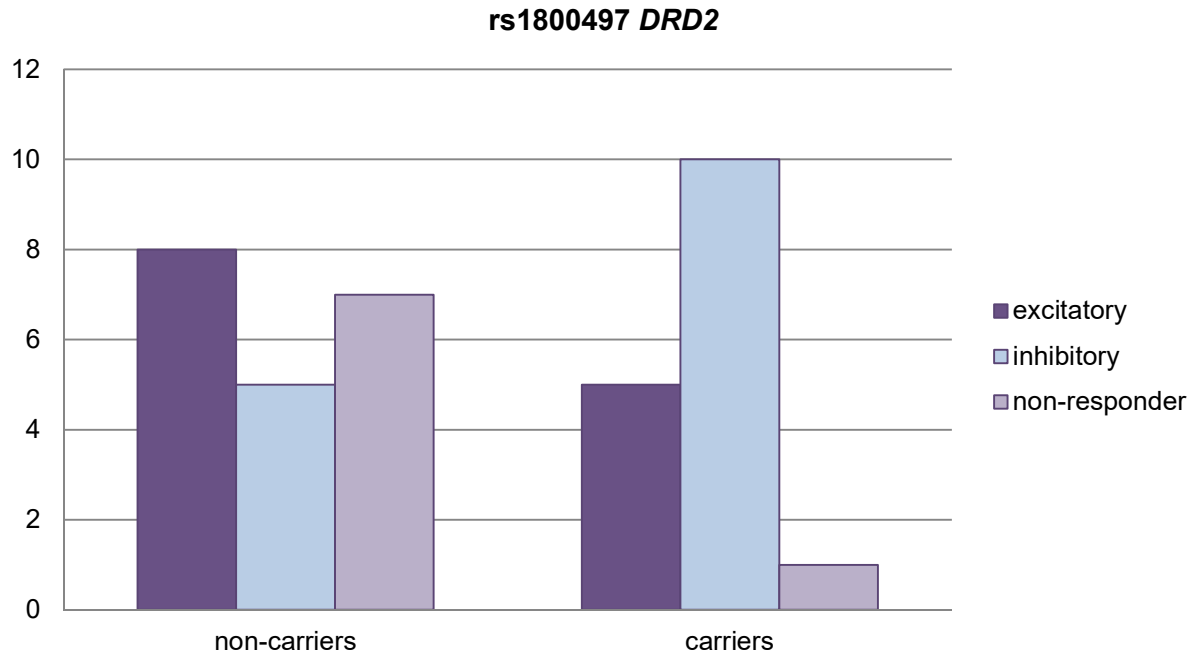

Figure 21. **Diagram shows numbers of individuals from 3 groups of outcomes** (excitatory, inhibitory and non-responders) classified according to their rs1800497 status into non-carriers and carriers of minor allele A.

5.3.3.3. Analysis for both paradigms with expected and unexpected outcome

The Chi Square test did not show any significant differences between genetic variants and neurophysiological outcomes from the two groups of expected and non-expected values (*Table 5.10.*).

SNP	χ^2	*P*- value
rs17789174(*KIAA0513*)	0.034	0.854
rs6265(*BDNF*)	0.471	0.492
rs6269(*COMT*)	0.185	0.667
rs1659412(*TRKB*)	0.849	0.357
rs11140778(*TRKB*)	0.797	0.372
rs4880213(*GRIN1*)	0.978	0.613
rs6293(*GRIN1*)	1.914	0.166
rs3764028(*GRIN2B*)	1.052	0.305
rs7301328 (*GRIN2B*)	1.368	0.242
rs1805247(*GRIN2B*)	1.886	0.170
rs1800497(*DRD2*)	1.213	0.271

Table 10. **Results of cross-tabulation analysis with *P*-values and chi square (χ^2) values for each SNP after both 1Hz and 5Hz rTMS paradigms.**

5.4. Discussion

This chapter presents results of exploring associations between neurophysiological outcomes from two rTMS paradigms delivered over the pharyngeal motor cortex and 12 SNPs from selected genes (*KIAA0513, COMT, BDNF, TRKB, GRIN1* and *GRIN2B* and *DRD2*). Analysis of the raw amplitudes of the pharyngeal MEPs did not show any significant associations for both rTMS paradigms. No statistically significant results were also observed in the analysis of groups according to the GA into expected vs non-expected outcomes from the combined 1Hz and 5Hz paradigms when considered as three groups (non-response; inhibitory and excitatory) showed significant associations for two SNPs: rs6269 from *COMT* gene (after 1Hz rTMS) rs1800497 from the *DRD2* gene (after 5Hz rTMS) when the GA was applied to the separate paradigms.

The first statistically significant association was found between the outcome from 1Hz rTMS paradigm (grouped according to GA) and rs6269 from *COMT* gene. According to the analysis, carriers of the minor allele (homo- or heterozygotes) were more likely to have either excitatory or inhibitory outcomes. One of the possible explanations of this result might be that the presence of minor allele in rs6269 could influence higher reactivity of the pharyngeal motor cortex and non-carriers of this allele are less likely to respond to the rTMS paradigm. There is an emerging literature from different scientific areas on the effect of *COMT* individual genotype and swallowing performance or overall motor cortical reactivity [165-168].

In the recent paper Nimmons *et al.* [165], showed an association between SNPs from *COMT* (rs165599, rs10835211) and the *BDNF* (rs1083521) genes and presence of self-reported swallowing impairments within a cohort of community dwelling elderly individuals. Positive association between phenotype and *COMT* rs165599 was detected only in the presence of *BDNF* rs1083521 status. Heterozygotes for rs10835211 depending on rs165599 status showed either protective or harmful effects on developing swallowing impairment in the same cohort. *BDNF* rs1083521 did not show statistically significant result when analysed alone, similar to this study. Unfortunately, my study did not have the sample size to support an interaction analytical approach.

There are a number of studies exploring effects of *COMT* gene and increased activity within the hand motor cortex areas following non-invasive brain stimulation. Witte *et al.* [168] study explored the impact of Paired Associated Stimulation (PAS) on 32 health young female on the hand motor cortex facilitation and the influence of *BDNF* and *COMT* rs4680. Both motor and grammar learning were accessed during the study. All individuals were pre-screened for *BDNF* and *COMT* genotype. *BDNF* alone did not have an influence on the neurophysiological outcome, however homozygotes Met/Met for *COMT* genotype along with *BDNF* Val/Val homozygotes showed an increase in hand MEPs immediately after PAS. Again no association was found between both SNPs alone.

Another study explored the influence of *COMT* rs4680 on cortical plasticity of hand movement [166]. Continuous Theta Burst Stimulation (cTBS) was delivered to 18 elderly individuals, mostly females to decrease neuronal excitability of responses. Interestingly a decrease in the hand MEPs amplitudes was observed only after 10 min post intervention followed by high facilitation after 40 minutes post-intervention. Non-carriers of the minor allele had significantly reduced hand MEPs immediately after stimulation comparing to carriers of minor allele [167]. The researchers concluded that cTBS-induced motor excitability was inhibited in the *COMT* minor allele non-carriers.

The above presented evidence could be argued to be supportive of findings of this doctoral research about possible effects of *COMT* gene, not only with respect to swallowing but also in controlling excitability of the motor cortex. *COMT* inactivates catecholamines and catechol drugs such as L-dopa used in treatment of Parkinson's disease [175]. SNP rs6269 at codon 158 results in a valine to methionine substitution. *COMT* gene has a susceptibility to neurological disorders such as schizophrenia [176], obsessive-compulsive disorder [177] and anorexia nervosa [178]. These results should be interpreted with caution however, as different SNPs from *COMT* gene were analysed and different study designs were used. It is possible that there is a complicated network of interactions within the *COMT* gene as well as between other genes such as *BDNF* in these neurological pathways.

The second positive result from the above analysis was found between SNP rs1800497 from *DRD2* gene and the grouped phenotype of 5Hz rTMS stimulation. In my study rs1800497 predisposed carriers of minor allele to be classified in the inhibitory group of outcomes. Substitution of a minor allele causes an amino acid change from glutamic acid to lysine. *DRD2* gene is associated with neuromodulation of dopaminergic system [174].

In the study exploring genetic variants on neuromodulation by L-dopa in healthy, young individuals, Pearson-Fuchrop *et al.* [167] used TMS to measure cortical excitability with hand MEPs and rs1800497. The study showed effects of rs1800497 on motor learning (*P*-value = 0.02) and its modulation by L-Dopa (*P*-value = 0.0001), but not with any TMS measures.

Interestingly both SNPs rs6269 and rs1800497 are located within the genes which modulate L-Dopa pathways. *COMT* inhibitors are used as therapy of Parkinson's disease in combination with dopamine replacement therapies. Patients with Parkinson's disease have high prevalence of neurogenic swallowing impairments, with some studies suggesting up to 83% [5]. Therefore further studies on exploring effects of both genes *COMT* and *DRD2* should be performed to study dopamine neurotransmission and its effects on swallowing performance.

No association was found between other genes nor were there any other statistical associations with cortical excitability of the pharyngeal motor area. Explanations for this might include the

lack of sufficient power, the experimental paradigm used to study swallowing and the selection of SNPs, which represent only part of the genetic architecture of that gene.

SNP rs17601696 which achieved statistical significance in SNP-based GWAS studies (Chapter 2) from the SNP-based GWAS studies did not meet HWE conditions and had to be withdrawn from further analysis. The minor allele frequency (MAF) of this SNP from the 1000 Genome project is also very low (MAF**=** 0.03 (T)). Despite the low MAF I wanted to replicate the finding, if possible, therefore I have included rs17601696 in the TMS work. One of the possible explanations for this low amount of minor allele carriers in the analysed cohort is that rs17601696 might be a single nucleotide variant with increased frequency in this cohorts of elderly individuals strengthened by survival bias.

In contrast to findings from Jayasekeran's studies, no association between rTMS outcome and *BDNF* status were found. This discrepancy might be caused by differences in the study design, differences in age (the group studied by Jayasekeran *et al*., were significantly older), lower number of subjects and pre-screening of individuals. Another difference in results could be related to the measured outcome of Jayasekeran's *et al.* studies*,* were clear excitation was observed after applying 5Hz rTMS and clear inhibition after 1Hz rTMS. In the cohort used for my doctoral research, the phenotype was much more complex, without clear pathways of responses. Therefore different statistical methods were used to analyse both neurophysiological and genetic data.

Other SNPs from selected gene showed no association in the study *(KIAA0513, TRKB, GRIN1, GRIN2B*). This might again be explained by insufficient power or too small genetic effects of candidate genes. It may also be the case that *TRKB, GRIN1* and *GRIN2B* genes might influence very specific motor pathways of limb movements and not control neuromuscular reflexes in swallowing.

The results of my study pose a number of other questions which could be further investigated in future research. Whether polymorphisms in *COMT* and *DRD2* influence neuroplasticity via synaptic mechanisms or changes in neural morphological changes? Do these SNPs affect pharyngeal responses in the same manner with multiple sessions for the same individuals? Further replication will also need to be performed in different cohorts of individuals with different ages and then in individuals with pathological conditions like Parkinson's disease and Stroke. Indeed, the clinical significance of applying rTMS as treatment of neurogenic dysphagia is likely determined by multiple biological factors. Two genetic polymorphisms from genes *COMT* and *DRD2*, if evaluated in more details, might be in future used as genetic markers predisposing an individual's responsiveness to specific neurostimulation paradigms in populations such as dysphagic stroke patients or patients with Parkinson's disease.

5.5. Conclusions

In conclusion, there is an evidence of genetic association with the neuromuscular control of swallowing affected by rTMS paradigms and two SNPs from *COMT* and *DRD2* genes. The association depends of the paradigm applied. Further research is needed to replicate and unravel the potential mechanisms of cortical control of swallowing. This work opens up new approaches to stratified medicine in the field of dysphagia therapy.

5.6.Supplementary material

Supplementary Table 5.1. Results of *t*-test values for each SNP after both 1Hz (A) and 5Hz (B) rTMS paradigms.

A)

SNP	**t-test**	***P*- value**
rs17789174(*GWAS*)	*t*(34) = 0.151	0.881
rs6265(*BDNF*)	*t*(33) = 0.106	0.916
rs6269(*COMT*)	*t*(29) = 0.047	0.962
rs1659412(*TRKB*)	*t*(33) = 0.454	0.635
rs11140778(*TRKB*)	*t*(33) = 0.144	0.887
rs4880213(*GRIN1*)	*t*(33) = 0.558	0.581
rs6293(*GRIN1*)	*t*(33) = 1.124	0.269
rs3764028(*GRIN2B*)	*t*(33) = 0.937	0.356
rs7301328 (*GRIN2B*)	*t*(32) = 0.737	0.466
rs1805247(*GRIN2B*)	*t*(33) = 0.389	0.699
rs1800497(*DRD2*)	*t*(33) = 0.917	0.366

B)

SNP	**t-test**	***P*- value**
rs17789174(*GWAS*)	*t*(34) = -1.140	0.262
rs6265(*BDNF*)	*t*(34) = -0.221	0.826
rs6269(*COMT*)	*t*(30) = -1.771	0.87
rs1659412(*TRKB*)	*t*(34) = 0.737	0.466
rs11140778(*TRKB*)	*t*(34) = -0.146	0.885
rs4880213(*GRIN1*)	*t*(34) = 0.996	0.327
rs6293(*GRIN1*)	***t*(34) = -2.312**	**0.027**
rs3764028(*GRIN2B*)	*t*(34) = -1.003	0.232
rs7301328 (*GRIN2B*)	*t*(33) = 0.948	0.35
rs1805247(*GRIN2B*)	*t*(34) = -0.204	0.84
rs1800497(*DRD2*)	*t*(34) = 0.837	0.408

Supplementary material 5.1.

Group Statistics

		N	Mean	Std. Deviation	Std. Error Mean
GA	non carriers	19	-11.9159	25.22691	5.78745
	carriers	17	9.0722	29.23429	7.09036

This study found that carriers of G allele in SNP rs6293 had statistically significantly higher mean grand average (9.07 ± 29.23) compared non- carriers (-11.91 ± 25.23), t(34) = -2.312, p = 0.027 after delivering the 5Hz rTMs paradigm.

Chapter 6

Discussion

6.1. Summary of chapters

In the 1st Chapter on my thesis I presented some background information on swallowing anatomy, physiology and neurophysiology. Further, I provided an explanation of swallowing impairment, also known as dysphagia, followed by description of neurogenic dysphagia and therapies including non-invasive brain stimulation. I then included a short literature review that highlighted existing evidence of possible genetic underpinnings of swallowing impairments. In the last part of Chapter 1, I proposed two approaches to examine the genetic background of swallowing impairments. Firstly I described a global genome-wide association study (GWAS) approach; secondly I presented a replication of the GWAS findings and previously reported genetic markers in a neurophysiological study design.

The 2nd Chapter described the study from the field of genetic epidemiology used to examine multiple genetic loci in a genome wide approach which might be associated with swallowing impairments. Here, I explored a possible association between self-reported swallowing impairment in the cohort of elderly community-dwelling individuals and over 500 000 of single nucleotide polymorphisms. After a detailed statistical analysis and applying strict criteria for multiple testing one SNP rs17601696 showed genome-wide statistical significance.

The 3rd Chapter provided a description of neurophysiological studies with repetitive Transcranial Magnetic Stimulation (rTMS). This study was conducted on healthy young volunteers to allow more specific phenotype to further genetic analysis. Two rTMS paradigms were used, previously described as being inhibitory (1Hz) and excitatory (5Hz). Interestingly the results showed high variability in individual responses. Repeated-measures ANOVA did not show any common trends in responses, which indicated the need to consider other analytical approaches.

In the 4th Chapter I made an attempt to group the outcomes from rTMS paradigms by different categories in order to simplify the phenotype for further genetic analysis. Here, I decided to use 3 approaches of grouping: first the analysis of results from 1Hz and 5Hz rTMS alone, and then combining the two paradigms. In the first two approaches I used normalized to baseline data expressed as the grand average of 5 time points of percentage of change of the MEPs amplitudes. According to this grouping, individuals were put into excitatory, inhibitory and non-responders group. For combined 1Hz and 5Hz paradigms, I classified individuals into two groups: those with expected outcomes (inhibition after 1Hz and excitation after 5Hz) and with unexpected responses. Finally, I repeated the analysis of demographic characteristics of the group to identify any associations with these core data.

In Chapter 5 I analysed a possible link between specifically chosen SNPs and phenotypes from previously identified groups described in Chapter 4 as well as raw data described in Chapter 3. Fifteen SNPs were initially selected in order to replicate findings from Chapter 2 (rs17601696 and one SNP from *KIAA0513* rs17789174) and a number of other SNPs identified from the

literature review from Chapter 1. After quality control procedures 11 SNPs from 7 genes (*KIAA0513*, *BDNF, COMT, TRKB, GRIN1, GRIN2B* and *DRD2*) were used for further analysis. Two SNPs showed statistical significance: rs6269 from *COMT* gene in the 1Hz paradigm and rs1800497 from *DRD2* from the 5Hz rTMS paradigm. No other associations were found in the analysis.

Because each chapter had its own discussion, in the Discussion Chapter 6, I brought together the results from each section, providing an overview of the work in this thesis. In the last part of the discussion I suggest possible future application of the results in treatments of neurogenic dysphagia.

6.2. Overview of discussion points in the thesis

6.2.1. Novel findings

This study was the first to provide possible evidence of involvement of genes and swallowing impairments, where two physiologically different swallowing phenotypes were used. In the first part, self-reported swallowing impairments phenotype was used to study the association with strategically selected SNPs from the whole genome. These findings were then replicated along with a number of SNPs from genes highlighted in the literature, in a specific neurophysiological phenotype representing swallowing brain function.

The GWAS studies identified one significant SNP rs17601696, not previously described in the literature.

The neurophysiological rTMS studies which explored human swallowing neurophysiology were conducted on the largest to date cohort of young individuals. The results provided clear evidence of high variability in responses after delivering 1Hz and 5Hz paradigm over the pharyngeal motor cortex. This finding stands in the line with studies conducted on the hand motor cortex and other non-invasive brain stimulations.

Two SNPs from genes *COMT* and *DRD2* were directly associated with response after 1Hz rTMS and 5Hz rTMS paradigms, respectively. Both genes play a role in the dopamine metabolism and neuroplasticity mechanisms which is believed to be a key driver in recovery from swallowing impairments in stroke patients.

SNPs identified with studies from my two approaches (GWAS and rTMS) and lack of replication between two phenotypes confirms the likely hypothesis that neurogenic swallowing impairments are most likely controlled by multiple genes with small genetic effects and complex interactions between them.

6.2.2. General discussion

One of the most important issues of the presented doctoral research is the lack of replication of significant SNPs between the two experiments. The GWAS could not be replicated due to lack of compatible phenotypic data with Genome Wide genotyping measures. A number of possible explanations for the different results from these two approaches will be highlighted below.

The first reason for no association between a novel SNP rs17601696 and statistically suggestive SNPs may related to large discrepancies between phenotypes and cohorts used in the study design (*Figure 6.1.*). Self-reported swallowing impairments might have different aetiologies, not necessarily caused by neurological damage. On the other hand, experiments with rTMS used very specific outcomes exploring one neuromuscular pathway involved in swallowing processes. In the first approach clinically relevant changes in swallowing performance were tested while in the TMS studies only the amplitude of pharyngeal MEPs (PMEPS) was measured. Perhaps the lack of association comes from the difference that these phenotypes represent, one relatively general, the other more specific.

Another important difference was the age of subjects used in the first and the second study. Advanced age of subjects used in the GWAS studies increased the risk of bias of the results. Firstly, through the presence of coexisting other diseases or 'silent aspiration' which could not be detected by using self-reported questionnaires. Comparatively, young volunteers could not provide statistically significant results for SNPs generated in the analysis of cohorts of elderly subjects because some of the changes in neuronal excitability of the pharyngeal motor cortex could be related to chronological age. Secondly, changes in the excitability of the pharyngeal motor cortex were induced artificially which might be controlled by different mechanisms compared to any pathological neuronal changes observed in patients.

Swallowing Phenotype	**GWAS**	**rTMS**
How it is collected?	Sydney Swallow Questionnaire	Electromyography, the catheter with bipolar electrodes
Primary outcome	Answers to 17 questions	Pharyngeal motor evoked potentials
Assessment	Self-reported	researcher
Age Outcome	Old, healthy Clinical	Young healthy Artificially induced

.

Table 11. **Comparison between the phenotypes in GWAS from the field of genetic epidemiology and rTMS studies neurophysiological studies**

As described in the previous research, genes such as *BNDF* and *APOE* were commonly studied in swallowing neurophysiology and pathology [108, 110, 165].

BDNF was not associated with swallowing outcome in both GWAS and neurophysiological experiments. There are several probable causes for the lack of association between *BDNF* and swallowing phenotype. *BDNF* is only a one gene in multiple pathways of many genes involved in brain control of swallowing; therefore its effects could be masked. Additionally, I have tested only one SNP rs6265 within *BDNF* with the TMS protocol which again might not give sufficient information to find the effects. It should be noted that in the Jayasekeran's *et al*. studies [108], association between excitability of the pharyngeal motor cortex and *BDNF* was found. In my rTMS protocols, I used the same parameters for both 1Hz and 5Hz rTMS. The main difference between our studies were using different cohorts (Jayasekeran *et al.* used mainly older individuals) and different number of people (I used the largest to date cohort). Another difference was that I did not select volunteers for my studies based on genotype , unlike in Jayasekeran's *et al.* Results of my neurophysiological studies showed much more complicated set of individual outcomes without common pathway of responses unlike in Jayasekeran *et al.* studies where clear facilitation after 1Hz and clear inhibition after 5Hz were observed. It is not clear why the two studies show these response differences in heterogeneity, although differences in selection and sample size are discussed. This in part forced me to change my approach for the analysis and find a way to deal with phenotypic heterogeneity.

As for *APOE* there was no association between two SNPs and swallowing phenotype with both study designs. Plausible explanations for this were discussed including the association of homozygous *APOE* status in previous work not used in my work. GWAS studies showed no association with the swallowing phenotype with another common gene *COMT*. Surprisingly studies conducted on the same cohort of healthy community dwelling individuals from the Dyne Steel cohort showed effects of interaction between these two genes. One of the reasons why my studies did not confirm these findings were different statistical methods and the study design. Study by Mentz *et al.* analysis approach showed significant association only with homozygosity of allele E4 while I have used Genome Wide association using an additive model and slightly smaller subsample of the same cohort. Lack of replication is very common in studies from the field of genetic epidemiology. Even stronger power and multi sectional population studies struggle with replication of results, which does not necessarily means that the association is invalid but may also suggest a linkage with different trait than previously expected [179].

The variability of the outcomes reported in Chapter 3 is not an uncommon observation and it has been already reported by Paine *et al.* in 2006 [180] in the studies on the MEPs of the oesophageal muscles involved in swallowing. Recent studies with other non-invasive brain stimulations delivered over the hand motor cortex where higher numbers of individuals were studied also report intra- and inter-subject variability [158-160, 162-164, 181, 182]. The commonest statistical approach in studies with high heterogeneity in outcomes is to group individuals according to grand average of percentage of change from the baseline (as it was presented in Chapter 4). However we still do not know which threshold over or under which the

response is classified as clinically significant. Depending on the study design some researchers use thresholds between 10% and -10% or 20% and -20% of the grand average of responses [163, 181], while others evaluating every response with positive values as excitatory and negative values as inhibitory [159]. However we considered the 20% threshold to be too conservative for our data, and as an a priori decision used the 10% threshold in grand average of percentage of change from the baseline as the most appropriate approach. In fact more studies should be performed to explore the boundaries according to which we can classify responses as a response.

Both high and low frequencies were used in clinical trials to affect the swallowing cortex excitability [95, 96] [91, 102, 104, 105]. Of course the above mentioned clinical trials typically had different study designs, but all showed increase in motor excitability of the swallowing muscles. Like in our cohort after applying inhibitory paradigm (1Hz rTMS) some showed excitation which might indicate that the brain immediately compensates possible harmful effects of inhibition. From the molecular point of view these discrepancies might be explained by specificity of LTP- and LTD-like plasticity, when only a certain amount of stimuli determined the amount of neurotransmitters released through the presynaptic membrane [73, 74].

Other explanation for high heterogeneity observed in our neurophysiological studies with rTMS could possibly be caused by individual anatomical properties. It has been discovered that the strongest response of TMS stimuli is generated when stimulation is applied to the top of the gyri of the cerebral cortex [183]. Skull shape might also affect the coil orientation which is also a very important factor influencing responses [184]- 45 degrees orientation of the coil from the scalp causes the strongest MEPs of the stimulated area.

6.2.3. Limitations of the research techniques

The biggest disadvantage of GWAS studies was lack of replication in other cohorts and low numbers of individuals. I made an attempt to contact other cohorts to increase the power and provide a replication of the results (The Lothian Birth cohort and TwinsUK cohort), however swallowing phenotype is not routinely collected by physicians and researchers.

A self-reported questionnaire is not ideal tool to collect swallowing phenotypic information. Swallowing questionnaires might omit individuals with silent aspirations and is not sensitive enough to give the information on the number of individuals that are really affected by swallowing impairments: this figure might be higher or lower.

Combination of tools with completely different experimental protocols even though it provides information from different perspectives might be a disadvantage of the study, because phenotypes could be too distinct from each other.

Even though TMS is believed to have a focal effect, the signal can spread across the scalp. Because hand motor threshold is close (hot spots for these two muscles lay close to each other, often around 0.5 to 2 cm apart). Perhaps this is the reason of high variability in outcomes in hand MEPs. Excitation and inhibition observed in the thenar motor thresholds might be an effect of spreading the signal of rTMS. Therefore some might argue that different muscles could be targeted as controls.

Another limitation of the study was inability to look at gene-gene interaction effects between genes in both experimental protocols. However, such an approach would require a larger sample size than available.

The issue of volunteers' age cohort differences which is influenced by older adults having greater variability in comorbidities, medicines that may affect the responses and survival selection.

6.3. Directions for future research

6.3.1. Exploring genetics of dysphagia

Considering the GWAS study, having demonstrated significant association with a SNP in this work, the opportunity exists for other groups with population cohorts to consider including swallowing questionnaires in their field work. Encouraging researchers to collect swallowing information from subjects participating in large genetic epidemiological studies provides an opportunity to replicate findings of my doctoral research. There is also the potential to establish a consortia to study the genetics of swallowing using metanalysis of GW data. Sufficient power of further studies could also allow the researchers to explore whole pathways and gene-gene interactions instead of analysis of single genes/SNPs.

Another area to replicate findings is in animal studies with accurate phenotypes of neurogenic dysphagia. The field of rodent animal studies gives this opportunity by recently developed videofluoroscopic swallowing assessment [185]. Knock-out (for certain genes of interest) mice could also undergo artificially induced stroke to study influence of these genes in the disease model [186]. As for neurophysiological studies, further groups should be performed with higher numbers, sham stimulation and then on pathological cohorts.

Non-invasive brain stimulations have a growing potential and may be more routinely used in research. If this can be combined with collection of suitable material (such as saliva sample DNA in this study) then further investigation of the effects of individual genetic predispositions for certain responses after delivering for non-invasive stimulation paradigms over the swallowing motor cortex could be forthcoming. Results could be replicated in both healthy volunteers (young vs elderly) and pathological cohort (dysphagic stroke, Parkinson's patients or others) to study mechanisms underlying neurogenic dysphagia.

6.3.2. Treatment of neurogenic dysphagia and stimulus induced variability

The results of the following studies showed that variability rises along with increasing sample size. This might be a subject of importance in the future assessment of patients who could be carefully classified into certain treatment therapies. If rTMS will be routinely used as a treatment therapy alone or with combination of other techniques, clinicians should be aware of high variability in the responses which is present also in cohorts of young volunteers. Perhaps rTMS interventions which were described as being either inhibitory or excitatory do not have to cause the same effects in all patients. Therefore more studies on inter- and intra-subject variability are needed.

6.3.3. Potential of genetic markers

Selected genetic changes (either SNPs or whole genes) have the potential to be used as genetic markers of swallowing disorders. Stratified medicine is a growing field which should enable researchers to adopt specific treatment therapies to specific patients in order to maximize their effectiveness.

Further work is required to establish reliable genetic markers used in clinical practice such as using various study designs, different swallowing phenotypes and other techniques. Perhaps genetic results should be also combined with other techniques such as neuroimaging to test cortical excitability. Swallowing disorders should be assessed with more caution in epidemiological studies to give robust results. Research questions that should be also asked include exploring functional perspectives of gene regulation and expression such as epigenetic changes which can affect neuronal excitability [187]. Other experimental models such as molecular imaging with ligands related to reported genetic markers, or blood markers that can be related to neurochemistry might provide another source of information regarding neuromolecular changes within the brain.

6.4. Conclusions

In this doctoral research I have provided evidence from two novel study designs, alongside existing literature on the genetic underpinnings of swallowing neurophysiology and pathology. My work supports further research on genetic markers of dysphagia. Exploring molecular mechanisms of neurological control of swallowing as well as developing potential genetic markers of swallowing impairments will further assist in developing new types of studies with various study designs. The evidence of strong variability in responses after rTMS intervention in healthy young volunteers shows the complex nature of applying rTMS as treatment therapies in clinical practice. The variability needs to be further researched to understand its basis and possible relevance to therapy.

The work supports the hypothesis that a personalised stratified medicines approach is important. Greater emphasis on individual clinical or biological characteristics including genetic may be the most promising way forward in human research in health and disease.

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