Objective Measurements and Characteristics of Spinal Excitability in Restless Legs Syndrome Patients

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Declaration

I, Chloe Dafkin, declare that the work contained in this thesis is my own work, except to the extent indicated in the acknowledgements or contribution sections. This thesis is being submitted for the degree of Doctor of Philosophy, at the University of the Witwatersrand, Johannesburg, South Africa.

This work has not been submitted for any other degree or examination in this or any other university.

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(signature of candidate)

Date:

April 2018

Abstract

Restless legs syndrome (RLS) is a neurological disorder that presents with sensory and motor symptoms that worsen in the evening and with rest. An evening state of spinal hyperexcitability has been proposed to be a possible cause of evening increases in RLS symptoms. Therefore the studies included in this thesis aimed to assess circadian variations in the state of spinal excitability in RLS patients in order to extend our knowledge about alterations in spinal processing and provide further elucidation of the pathophysiological mechanisms involved in RLS.

The first two studies of this thesis assessed circadian variations in spinal excitability in terms of spinal reflex responses of RLS patients compared to control participants. The plantar, flexor withdrawal, and crossed extensor reflexes all showed a circadian rhythm in RLS patients suggesting an evening increase in spinal excitability. We theorise that the circadian variation in spinal excitability in RLS patients may be due to an evening form of central sensitization particularly affecting nociceptive responses. These results reinforce the notion that there is increased spinal cord excitability in the evening in RLS patients, which corresponds to the symptomatic period of RLS.

Although RLS patients demonstrated a circadian variation in the reflex responses, indicating a possible increase in spinal excitability in RLS patients in the evening, RLS patients showed no increases in spinal excitability when compared to control participants. An unexpected finding was decreased plantar reflex responses in RLS patients compared to control participants. Excitability of both the flexor withdrawal and crossed extensor reflexes demonstrated no significant differences between RLS patients and control participants. These results do not support the theory of increased spinal excitability in RLS patients. The findings indicate that the pathophysiology of RLS is likely to involve complex spinal

alterations. The concept of global spinal hyperexcitability in RLS patients does not take into account the complex interactions of various sensory modalities in the spinal cord.

The plantar, flexor withdrawal, and crossed extensor reflexes studies in this thesis investigated spinal excitability in RLS patients in a static environment. However, dynamic sensorimotor integration is likely to play a role in RLS as inactivity brings on RLS symptoms and movement relieves RLS symptoms. Therefore, the third study of this thesis investigated temporal changes during locomotion in RLS patients using electromyography (EMG) of the muscles involved in gait in order to evaluate dynamic sensorimotor feedback in RLS patients. EMG muscle activity during the gait cycle did not show the circadian variation in RLS patients that was seen in healthy control participants. However, evening differences in gait muscle activation patterns between RLS patients and control participants were evident. These results extend our knowledge about alterations in spinal processing during gait in RLS patients. A possible explanation for these findings is central pattern generator sensitization caused by alterations in sensitivity of cutaneous afferents in RLS patients.

In conclusion, the results of these studies provide further insight into the pathophysiology of RLS, highlighting that RLS is not due to a global state of spinal hyperexcitability. As not all sensory processing is affected in the same manner the pathophysiology of RLS is likely to involve complex spinal alterations.

Acknowledgements

First and foremost I would like to thank my supervisors Dr Sam Kerr and Prof Warrick McKinon for all of their support, assistance, advice and patience. Thank you Sam for taking me under your wing and letting me be your first Padawan. Thank you for the countless hours we spent planning, discussing, and laughing. I would never have been able to do this without your support and guidance. Thank you for always checking in to see if I'm surviving and for all the motivational puppy pictures. Thank you Warrick for as always assisting me with any technical problem I may have, no matter how busy you are at the time, and for your invaluable input in getting me to this point.

Thank you to Dr Andrew Green for his presence and expertise whenever I needed him, even during my peculiar data collection times, and for listening to my crazy rants. Thank you to Prof Benita Olivier for always offering help with a smile and her unending positive attitude.

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I would like to thank all of the participants who took part in my study for happily coming to a strange place at odd hours to take part in my studies.

Lastly I dedicate my PhD thesis to my Dad, Raymond Dafkin. I love and miss you every day and I know out of everyone, you would have been the proudest.

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reported in RLS patients, showing increased spinal excitability (Bara-Jimenez *et al*., 2000; Aksu & Bara-Jimenez, 2002; Gunduz *et al*., 2017). Furthermore a decrease in RLS symptoms has been shown following transcutaneous spinal direct current stimulation (Heide *et al*., 2014). **14:** RLS patients display decreased patellar reflex responses (Kerr *et al*., 2011). **15:** Decreased Ib afferent inhibition of the H-reflex has been noted in RLS patients (Rijsman *et al*., 2005; Scaglione *et al*., 2008; Marconi *et al*., 2012). **16:** Hyperalgesia has been reported in RLS patients (Stiasny-Kolster *et al*., 2004; Bachmann *et al*., 2010; Stiasny-Kolster *et al*., 2013; Edwards *et al*., 2011). Cho and colleagues (2017) have also shown decreased C-fibre perception thresholds in RLS patients. **17:** RLS patients have been shown to have increased mechanical detection thresholds (Stiasny-Kolster *et al*., 2013). **18:** Electrophysiology studies have noted increased excitability of motor neurons in RLS patients (Isak *et al*., 2011; Congiu *et al*., 2017). Points 19-22 represent theoretical regions of the central nervous system that may be implicated in the pathophysiology of RLS. **19 & 20:** Possible alterations of adenosine A1 (A1R) and A2A (A2AR) receptor ratios in the cortex and the striatum. **21:** Possible malfunction of the A11 diencephalospinal descending dopaminergic pathway. **22:** Potential changes in dorsal horn receptors (specifically A1R:A2AR and dopamine 2 receptors) as well as a possible spinal hyperglutamatergic state.

Figure 2: Events of the normal gait cycle. Image modified from "Gait Cycle as proposed by Jaquelin Perry 1992" by chipotng [sic], retrieved from https://commons.wikimedia.org/wiki/File:GaitCycle_by_JaquelinPerry.jpg, licensed under CC-BY-SA-3.0

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Figure 1: Median and inter-quartile range of change in ankle (A) and hallux (B) angle during the flexor withdrawal reflex (FWR) for participants with restless leg syndrome (RLS) (A: n=11; B: n=12) and control participants (A: n=12; B: n=11) in the evening (PM) and morning (AM). *p<0.05 RLS PM versus RLS AM; Wilcoxon signed-rank test.

Figure 2: Median and inter-quartile range of change in ankle during the crossed extensor reflex in the evening (PM) and morning (AM) for participants with restless leg syndrome (RLS) (n=11) and control participants (n=11). *p<0.05 RLS PM versus RLS AM; Wilcoxon signed-rank test.

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Figure 1: Mean gait muscle activity curves of 14 restless legs syndrome (RLS) participants (black line) and 13 control participants (grey line) in the evening (PM – left column) and the morning (AM – right column). Data are expressed as mean and the 95% confidence interval (error bars) of the left and right legs combined for every 5% of the gait cycle. All data has been normalised to a unity average and the vertical axes have been standardised. The horizontal axis shows one complete gait cycle from heel strike (0%) to heel strike (100%). ANOVA, *$p<0.05$ RLS participants' electromyographic activation is significantly greater than control participants; †$p<0.01$ control participants' electromyographic activation is significantly greater than RLS participants. TA, *tibialis anterior*; GL, *lateral gastrocnemius*; RF, *rectus femoris*; BF, *biceps femoris*.

Figure 2: Example of a raw EMG file of two complete gait cycles showing muscle activity in the *tibialas anterior, gastrocnemius lateralis, rectus femoris* and *biceps femoris*. The dotted lines mark the percentage of the gait cycle with 0% and 100% representing heel strike. The arrows indicate the abnormal *gastrocnemius lateralis* activation during the swing phase of the gait cycle.

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List of Abbreviations

3OMD	3-ortho-methyldopa
A1R	Adenosine A1 receptor
A2AR	Adenosine A2A receptor
AIF	Afferent-induced Facilitation
AM	Morning
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BF	Biceps Femoris
CMAP	Compound Muscle Action Potential
CPG	Central Pattern Generators
CPT	Current Perception Threshold
CSF	Cerebrospinal Fluid
CSP	Cortical Silent Period
CuSP	Cutaneous Silent Period
D1R	Dopamine 1 receptor
D2R	Dopamine 2 receptor
EMG	Electromyography
fMRI	Functional Magnetic Resonance Imaging
FWR	Flexor Withdrawal Reflex
GABA	γ-aminobutyric acid
GL	Lateral Gastrocnemius
HIF	Hypoxia-induced factor
H-reflex	Hoffman reflex
Hmax	H-reflex maximum amplitude
IRLS	International Restless Legs Scale
IRLSSG	International Restless Legs Syndrome Study Group
JHRLSS	John Hopkins Restless Legs Severity Scale
LAI	Long Latency Afferent Inhibition
L-dopa	L-dihydroxyphenyalanine
MEP	Motor-evoked Potential
Mmax	M-wave maximum amplitude
MRI	Magnetic Resonance Imaging
NMDA	N-methyl-D-aspartate

NREM	Non-rapid eye movement
PLM	Periodic Limb Movements
PLMI	Periodic Limb Movements Index
PLMS	Periodic Limb Movements during sleep
PLMW	Periodic Limb Movements during wakefulness
PM	Evening
PSG	Polysomnography
QST	Quantitative Sensory Test
RII	Early component of the flexor withdrawal reflex
RIII	Late component of the flexor withdrawal reflex
REM	Rapid eye movement
RF	Rectus Femoris
RLS	Restless Legs Syndrome
RLS-6	Restless Legs Syndrome-6 Scale
SAI	Short Latency Afferent Inhibition
SF-36	Short Form 36 health questionnaire
SICF	Short Interval Intracortical Facilitation
SICI	Short Interval Intracortical Inhibition
SIT	Suggested Immobilisation Test
SIT MDS	Suggested Immobilisation Test Mean Discomfort Score
TA	Tibialis Anterior
TMS	Transcranial Magnetic Stimulation
tsDCS	Transcutaneous Spinal Direct Current Stimulation
VAS	Visual Analogue Scale
WED	Willis-Ekbom Disease

Preface

Restless legs syndrome is a common neurological disorder which has sensory and motor symptoms that follow a circadian rhythm. Both the sensory and motor symptoms of RLS severely affect a patient's quality of life. To date the pathophysiological cause of RLS is unknown, despite the large amounts of research looking into this disorder. It has been theorised that hyperexcitability of the nervous system might be involved in the pathophysiology of RLS. Additionally, despite the known circadian rhythm of RLS, most studies assessing excitability have only been done at a single time point. Therefore assessing spinal excitability at different time points in RLS patients could significantly improve our understanding of the state of spinal excitability in RLS patients. The sections that follow describe how this body of work intends to contribute to the understanding of the complex aetiology of RLS.

Chapter 1 provides a general background of RLS including how the disorder is diagnosed and its prevalence. This is followed by an examination of current literature on the aetiology of RLS. The literature review continues with a focus on excitability in RLS, both cortical and spinal. Following this is a focus on reflex studies in RLS as well as the assessment of the gait cycle in RLS. Lastly, **Chapter 1** ends with the rationale and objectives of the thesis.

Chapters 2 and 3 include two published papers focusing on the assessment of lower limb reflex responses in RLS patients at two different time points. The evaluation of reflex responses is a possible mechanism to assess changes in spinal excitability. **Chapter 2** explores the circadian variation of plantar reflex responses in RLS patients. **Chapter 3** investigates flexor withdrawal reflex and corresponding crossed extensor reflex responses in RLS patients in the evening and the morning.

Chapter 4 describes the circadian changes in muscle activity during the gait cycle in RLS patients (in a paper currently under review). Looking into the systems that control movement may aid in understanding more about the pathophysiological mechanisms of RLS because a major sensory symptom of RLS is the urge to move.

Chapter 5 summarises the results and subsequent discussions of each preceding chapter and considers these collectively in the context of the rationale for this thesis and as they relate to the field as a whole. Moreover, possible future research studies are proposed.

Chapter 6 is a complete list of all the references used in the thesis.

Chapter 7 includes the following appendices: ethical clearance certificate, list of publications co-authored by the thesis author during the period of PhD candidature, and an exert from my masters dissertation describing the use of kinematics in neurological studies.

Publications and Presentations Arising from the Work Presented in this Thesis

Publication: Dafkin C, Green A, Olivier B, McKinon W & Kerr S. (2017). Plantar reflex excitability is increased in the evening in restless legs syndrome patients. *Neuroscience Letters* **660**, 74-78. DOI: 10.1016/j.neulet.2017.09.027.

Publication: Dafkin C, Green A, Olivier B, McKinon W & Kerr S. (2017). Circadian variation of flexor withdrawal and crossed extensor reflexes in restless legs syndrome patients. *Journal of Sleep Research* (in press). DOI: 10.1111/jsr.12645.

Publication: Dafkin C, Green A, Olivier B, McKinon W & Kerr S (2018). Distal muscle activity alterations during the stance phase of gait in restless leg syndrome (RLS) patients. *Sleep Medicine* **45:** 89-93. DOI: 10.1016/j.sleep.2018.01.013.

Oral presentation: Circadian variation of flexor withdrawal and crossed extensor reflexes in restless legs syndrome. World Sleep 2017. Prague, Czech Republic.

Other publications that I co-authored during my PhD candidature, but that are not directly linked to this thesis, are listed in appendix B.

Contributions to the research

As part of the declaration of this thesis, I acknowledge contributions by various individuals as detailed below.

The experimental designs for all studies were devised by myself with the guidance of my supervisors Samantha Kerr and Warrick McKinon.

All experimental data collection was performed by myself, with assistance from my supervisors, Samantha Kerr and Warrick McKinon, as well as from Andrew Green and Benita Olivier.

All data analysis was completed by me with assistance from Andrew Green.

Algorithms for the kinematic processing were modified by myself using code written by Warrick McKinon and Andrew Green.

Drafting of the written works was undertaken by myself with guidance from my supervisors.

Chapter 1: Literature Review

1.1. Restless Legs Syndrome

Restless Legs Syndrome (RLS) is a sensory motor disorder with symptoms that worsen in the evening and with inactivity. The mechanism underlying RLS is currently unknown. A possible, and potentially important, underlying mechanism of RLS may be a state of central nervous system hyperexcitability, particularly spinal hyperexcitability. The current thesis will add to the knowledge of spinal excitability in RLS by assessing spinal reflex responses and gait neuromuscular patterns in RLS patients. As an introduction to these studies the upcoming sections will introduce RLS in terms of how the disorder is diagnosed, how the severity of RLS symptoms is measured, the prevalence of the disorder and its clinical implications. This will be followed by a summary of possible mechanisms behind the aetiology of RLS with a focus on central nervous system excitability and spinal reflexes.

1.1.1. RLS description

RLS is a neurological disorder that presents with sensory and motor symptoms. People with RLS experience an urge to move often accompanied by uncomfortable or unpleasant sensations in the legs. RLS was first noted by Sir Thomas Willis in 1685 who described RLS as "leapings and contractions" in the limbs when the patient attempts to sleep (Willis, 1685).

A more formal description of RLS was developed in 1945 by Professor Karl-Axel Ekbom. In the concluding summary of Professor Ekbom's seminal paper, Restless legs: a clinical study (Ekbom, 1945), he writes, "The paresthesias are situated inside the lower legs . . . They consist of highly disagreeable crawling, sensations (krypningar, in Swedish), hard to describe, which compel the victims to keep moving their legs or to walk about, which generally gives relief. The crawling sensations set in only when the legs have been kept still for a while, mostly in the evening, at the cinema, for example, and particularly a short while after retiral [sic] for the night."(Ekbom, 1945). Professor Ekbom noted that the essential features of the

disorder were paresthesia in the lower legs that is relieved by movement and worsens with rest, particularly in the evening. While there have been refinements over time to this description, the essential characteristic features of the disorder have not fundamentally changed from Ekbom's description in 1945.

In April 2011 the RLS foundation board of directors' attempted to rename the disorder as: Willis Ekbom disease (WED). The name is derived from the aforementioned contributors, Sir Thomas Willis and Professor Karl-Axel Ekbom, for their work in defining and understanding RLS. The new name was introduced in an attempt to eliminate inaccurate descriptors, improve diagnosis, promote cross cultural ease of use and reduce trivialisation associated with the name RLS. The name WED has not been extensively used and for the purposes of this thesis the disorder will be referred to as RLS, as is the trend in current literature.

1.1.2. Diagnosis of RLS

Currently there is no objective test available for the diagnosis of RLS. RLS is primarily diagnosed based on subjective reports by patients (Allen *et al.*, 2003). However, patients find it difficult to describe their symptoms, often using strange descriptions such as 'ants crawling in their bones' or 'soda bubbles in their veins' (Earley *et al.*, 2000*a*). A previous study conducted by my own research group demonstrated the wide variety of descriptors used by RLS patients with RLS patients using 62 different words and 39 phrases to describe their sensations (Kerr *et al.*, 2012). In addition there are disorders, such as arthritis and neuropathies, which can present with similar symptoms to RLS leading to misdiagnosis. Therefore a standard set of diagnostic criteria was needed. In 1995 the International Restless Leg Syndrome Study Group (IRLSSG) established four essential criteria to be used when diagnosing and defining RLS (Walters, 1995). These criteria were updated in 2003 (Allen *et al.*, 2003) and 2012 (Allen *et al.*, 2014*a*). The 2012 revisions kept the core features of the

original 1995 diagnostic criteria and included an additional exclusion criterion to form the list of five essential diagnostic criteria used currently. In order for a person to be diagnosed with RLS he/she must present with all five essential criteria (Allen *et al.*, 2014*a*).

The five up-to-date essential criteria as listed by Allen *et al*. (2014*a*) are:

1. An urge to move the legs usually but not always accompanied by, or felt to be caused by, uncomfortable and unpleasant sensations in the legs.

2. The urge to move the legs and any accompanying unpleasant sensations begin or worsen during periods of rest or inactivity such as lying down or sitting.

3. The urge to move the legs and any accompanying unpleasant sensations are partially or totally relieved by movement, such as walking or stretching, at least as long as the activity continues.

4. The urge to move the legs and any accompanying unpleasant sensations during rest or inactivity only occur or are worse in the evening or night than during the day.

5. The occurrence of the above features is not solely accounted for as symptoms primary to another medical or a behavioural condition (e.g. myalgia, venous stasis, leg oedema, arthritis, leg cramps, positional discomfort, habitual foot tapping) (Allen *et al.*, 2014*a*).

Ekbom's initial description of the features of RLS focused on the uncomfortable sensations (Ekbom, 1945). Since the formal diagnostic criteria were established in 1995 (Walters, 1995), the key diagnostic feature has become the urge to move the legs as described in the first criterion above. The importance of the urge to move the legs has been emphasised in the 2012 updated criteria with the addition that the urge to move may occur with or without

uncomfortable sensations (Allen *et al.*, 2014*a*). The second criterion states that the urge to move must worsen with rest or inactivity. In terms of criterion three, it is important to note that the alleviation of the urge to move and the uncomfortable sensations, if present, includes temporary relief whilst the movements are occurring and not necessarily complete relief after moving (Allen *et al.*, 2014*a*). Another fundamental feature of RLS, outlined in criterion four, is the circadian variation noted in the symptom profile of RLS patients (Allen *et al.*, 2014*a*). In some very severe cases symptoms may be constantly present, as opposed to only manifesting in the evening, however a patient can still meet the RLS diagnostic criteria provided a circadian variation in symptom severity was present at the onset of the disorder (Allen *et al.*, 2014*a*). The fifth criterion was added to mitigate against misdiagnoses due to mimicking conditions that may meet the first four essential diagnostic criteria for RLS (Hening *et al.*, 2009). RLS can occur in conjunction with the conditions mentioned in criterion five, however care must be taken to assess the differentiating symptoms of both disorders for diagnostic purposes (Allen *et al.*, 2014*a*).

In addition to the updated essential criteria in 2012, specifiers were added relating to the frequency of symptoms (chronic/persistent or intermittent) and the clinical significance of the disorder (how symptoms impact on quality of life) (Allen *et al.*, 2014*a*). The addition of specifiers allows for the creation of standardised subgroups which will hopefully aid with both research and the development of treatment protocols (Allen *et al.*, 2014*a*).

In order to improve diagnostic accuracy of RLS the essential diagnostic criteria were implemented into validated diagnostic questionnaires, the Hopkins telephone diagnostic interview and the Cambridge-Hopkins questionnaire (Hening *et al*, 2008; Allen *et al*, 2009a). In addition to covering the basic diagnostics criteria of RLS the Cambridge-Hopkins questionnaire also assesses if symptoms could be due to another disorder (Allen *et al*,

2009a). The diagnostic questionnaires have been shown to have high levels of diagnostic accuracy (Hening *et al*, 2008; Allen *et al*, 2009a).

In addition to the essential diagnostic criteria, there are non-essential supportive clinical features which can aid in confirming a diagnosis in uncertain cases (Allen *et al.*, 2003). These non-essential features include a positive family history of a first-degree relative with RLS, a positive response to dopaminergic therapy, an absence of intense daytime sleepiness in spite of poor sleep quality, and the presence of involuntary leg movements termed periodic limb movements (PLM) (discussed in section 1.1.2.1) (Allen *et al.*, 2014*a*).

Another important distinction of RLS is that it can be classified as primary or secondary RLS (Allen *et al.*, 2003). The aetiologies of primary and secondary RLS are suspected to be very different, however their physical presentation appears to be similar (Trenkwalder *et al.*, 2016). Primary RLS is idiopathic and there is strong evidence suggesting that a genetic predisposition may play a role in the development of primary RLS as more than 50% of individuals diagnosed with primary RLS present with a family history of RLS (Winkelmann *et al.*, 2007). Secondary RLS is associated with other medical conditions such as pregnancy, renal disease, peripheral neuropathy, diabetes mellitus, or can be drug-induced (Allen *et al.*, 2003; Benes *et al.*, 2007). In secondary RLS, treatment of the underlying condition will relieve or fully alleviate RLS symptoms (Benes *et al.*, 2007). In contrast however, there is currently no cure for primary RLS, highlighting the need for better insight into the aetiology of this phenomenon.

RLS is a heterogeneous disorder with different triggers and degrees of severity however with a consistent symptom profile. RLS can be classified as a primary or a secondary disorder, chronic or intermittent, mild or severe, all of which may complicate diagnosis. As RLS is diagnosed based on subjective criteria, the criteria used must be clear and stringently agreed

upon by world experts. The different phenotypes of RLS may have different aetiologies confounding research into the aetiology of RLS. Further research is needed to understand the complex mechanisms underlying this disorder. The diagnosis of RLS is primarily subjective however, objective measures such as the assessment of PLM can be used as a measure of RLS severity given the common comorbidity of RLS and PLM.

1.1.2.1. Periodic limb movements

Approximately 80% of persons with RLS have PLM (Montplaisir *et al.*, 1997; Scofield *et al.*, 2008) and therefore the presence of PLM can be considered a sensitive marker for RLS. The association between PLM and RLS was first observed by Lugaresi in 1968 (Lugaresi *et al.*, 1968). PLM are uni- or bi-lateral stereotypical leg movements characterised by fanning of the toes, dorsiflexion of the foot, and flexion of the knee and hip (Hornyak *et al.*, 2006), thought to be similar to the flexion synergy seen in the Babinski sign (Smith, 1985). The sequence of muscle activation of PLM was also noted to be similar to the flexor withdrawal response (Lugaresi *et al.*, 1968). PLM primarily occur during sleep (PLMS) but can also occur during wakefulness (PLMW). Besides the association with RLS, PLM are also associated with many other sleep disorders such as; sleep apnoea, narcolepsy, REM (rapid eye movement) sleep behaviour disorder, and are also common in adults over 45 years of age (Hornyak *et al.*, 2006; Montplaisir, 2006; Allen *et al.*, 2014*a*). Consequently the presence of PLM is not necessarily a definitive sign of RLS but can be used as supplemental evidence when diagnosing RLS in difficult cases.

PLM are conventionally identified from electromyographic (EMG) evaluation of the *tibialas anterior* muscle in conjunction with polysomnography (PSG) using a specific set of criteria (Bonnet *et al.*, 1993). The World Association of Sleep Medicine and IRLSSG latest guidelines for scoring leg movements during sleep define PLM as at least four consecutive movements

with each movement having a duration of 0.5-10 seconds, with an inter-movement interval minimum of 10 seconds and maximum of 90 seconds (Ferri *et al.*, 2016). Respiratory event associated PLM should be excluded. However, some controversy exists around the classification of respiratory event associated leg movements due to a lack of consensus defining the end of sleep-disordered breathing events and differing characteristics of leg movements depending on the type of breathing disorder (Ferri *et al.*, 2016).

The severity of PLM during sleep can be determined by calculating a PLM index (PLMI) using EMG and PSG (Bonnet *et al.*, 1993). The PLMI is an index of how many PLMS occurred per hour of sleep. A PLMI greater than five per hour is considered abnormal (Merlino *et al.*, 2007). Another method of measuring PLM is actigraphy, which records leg movements using an accelerometer attached to the ankle, foot or toe of the patient (Hening, 2004; Gschliesser *et al.*, 2009). The accelerometer data is then processed to determine if the movements follow the pattern of PLM and meet the duration criteria (Bonnet *et al.*, 1993). The PLMI calculated from actigraphy data correlates with the PLMI recorded using the gold standard EMG (Kazenwadel *et al.*, 1995; Morrish *et al.*, 2002), but is unable to exclude respiratory related movements. Measuring PLM with actigraphy is an appealing method as it is cheaper and more convenient than overnight PSG (Allen *et al.*, 2003) and thus facilitates ambulatory patient monitoring and large scale PLM studies. For these reasons a specialised PAM-RL device has been developed for the automatic detection of PLM (Sforza *et al.*, 2005).

The assessments of PLM and sleep disturbances in RLS provide objective measurements of some common RLS symptoms. However not all individuals with RLS report sleep disturbances or present with PLM and therefore assessments of the main subjective diagnostic symptoms of RLS, the urge to move and uncomfortable sensations, are still required.

1.1.3. Assessment of RLS severity

RLS is largely a subjective disorder and as such standardised methods for assessing the severity of RLS symptoms are required. Three validated severity scales have been developed in order to gauge the severity of symptoms in RLS patients; the John Hopkins Restless Legs Severity Scale (JHRLSS), the International Restless Legs Scale (IRLS) and the Restless Legs Syndrome-6 scale (RLS-6).

In 2000, Allen and Earley developed the JHRLSS (Allen and Earley, 2001*a*). The JHRLSS is a four point scale which determines the severity of RLS by focusing on one of the main aspects of diagnosis - the time of day symptoms typically begin (Allen and Earley, 2001*a*). The scale is scored as follows: 0 for no symptoms (never), 1 for symptoms occurring within an hour of going to bed (mild), 2 for symptoms occurring at or after 18:00 (moderate), 3 for symptoms occurring before 18:00 (severe), and 4 for symptoms occurring before 12:00 (very severe) (Allen and Earley, 2001*a*). However as the JHRLSS is only a 4-point scale there were some limitations in its use for assessing slight improvements following treatment and an expanded severity scale was required (Allen and Earley, 2001*a*).

In 2003, the IRLSSG created the more comprehensive IRLS. The IRLS consists of 10 questions with the answers graded from 0 (absent) to 4 (very severe) with a total score ranging from 0-40 (Walters *et al.*, 2003). The questions focus on the primary diagnostic criteria, sleep disturbances, severity of symptoms, frequency of symptoms, and impact of symptoms on daily activities and mood (Walters *et al.*, 2003). The IRLS is considered the gold standard for measuring RLS severity and is widely used both clinically and in research (Merlino *et al.*, 2007; Trenkwalder & Paulus, 2010).

In 2004 a third severity scale, the RLS-6, was developed to supplement the IRLS (Kohnen *et al.*, 2016). The IRLS does not consider the time of onset of symptoms, differences between

day-time and night-time symptoms, or differences in symptoms during rest and activity. Therefore, the primary aim of the RLS-6 scale is to fill in these gaps in symptom profiles that the IRLS does not take into consideration (Kohnen *et al.*, 2016). The RLS-6 consists of six questions each with a rating from 0 to 10 representing no symptoms at all, to very severe symptoms (Kohnen *et al.*, 2004). The questions assess sleep satisfaction, daytime sleepiness, and the severity of RLS symptoms when falling asleep, during the night, during the day at rest and during the day while engaged in physical activity (Kohnen *et al.*, 2004). As the RLS-6 is the only severity scale that assesses day-time symptoms as well as night-time symptoms it is a useful tool for investigations of treatments that focus on daytime symptoms (Kohnen *et al.*, 2016).

In addition to the above mentioned scales to assess the subjective severity of RLS symptoms, the suggested immobilization test (SIT) was developed in order to subjectively measure worsening of symptoms during rest (Montplaisir *et al.*, 1998). During the SIT the patient is seated with their back at a 45 degree angle and their legs outstretched. Patients are instructed to remain as still as possible for the one hour duration of the test. Electromyographic data is recorded bilaterally from the *tibialas anterior* muscles, to identify the presence of PLMW (Montplaisir *et al.*, 1998). Subjective discomfort is assessed by a visual analogue scale (VAS) anchored by 'no discomfort' and 'extreme discomfort'. The VAS is administered every five minutes for the duration of the SIT. A mean discomfort score (SIT MDS) is calculated from the 12 discomfort VAS scores collected during the SIT. The SIT MDS represents the total discomfort experienced by the patient during the SIT (Michaud *et al.*, 2002*a*). The SIT MDS can be utilised to provide supportive diagnostic criteria in uncertain cases of RLS (Merlino *et al.*, 2007).

Assessment of the severity of RLS symptoms provides a measure of the effect of RLS symptoms on daily life. Therefore assessment tools for the severity of RLS are essential in order to gauge the clinical significance of the disorder.

1.1.4. Clinical Significance of RLS and PLM

The clinical significance of RLS varies with the severity of the disorder. Up to 4.6% of the general population has RLS symptoms that are moderate to severe and have a negative impact on quality of life (Ohayon *et al.*, 2012). RLS patients have reported adverse effects on sleep quality and daily functioning. RLS is also known to be associated with increased risk of anxiety, depression and heart disease (Earley & Silber, 2010).

RLS is known to have a significant effect on sleep with more than 75% of RLS patients presenting with at least one sleep related symptom (Allen *et al.*, 2005). The most common effects on sleep in RLS patients are delayed sleep onset and poor sleep maintenance (Allen and Earley, 2001*b*; Hening *et al.*, 2004; Merlino *et al.*, 2007; Earley and Silber, 2010) and as a result sleep efficiency is severely reduced (to 65.8%) in RLS patients (Merlino *et al.*, 2007). The greater the severity of RLS the more sleep efficiency is reduced (Allen and Earley, 2001*b*). The effect of RLS on sleep is expected since the onset of RLS symptoms is often around bedtime, therefore preventing, or interfering with, sleep. In addition between 17-55% of PLMS are related to arousal (Ferri *et al.*, 2015) hence the presence of PLMS plays a role in the reduced sleep efficiency in RLS patients. A lack of sleep in RLS patients leads to difficulty concentrating and a feeling of fatigue during the day which negatively impacts quality of life (Allen *et al.*, 2005).

A significant impact on quality of life has been shown in RLS patients as measured by the short form 36 health questionnaire (SF-36). The SF-36 assesses eight aspects of health related quality of life, namely; physical functioning, physical limitations on everyday

activities, physical pain, general health, vitality, social functioning, emotional limitations on everyday activities, and mental health (Brazier *et al.*, 1992). Studies utilising the SF-36 in RLS patients have shown a decreased quality of life compared to the general population (Nichols *et al.*, 2003; Berger *et al.*, 2004; Kushida *et al.*, 2007; Allen *et al.*, 2010). SF-36 scores of RLS patients are similar to those obtained from patients with diabetes, osteoarthritis, rheumatoid arthritis, depression, congestive heart failure or polyneuropathy (Earley & Silber, 2010).

In addition to the adverse effects of RLS on sleep and quality of life, RLS patients have a higher incidence of major depressive disorder, panic disorders, and generalised anxiety disorder when compared to control participants with at least one somatic disorder (Winkelmann *et al.*, 2005). Furthermore, large scale studies have shown an increased risk of cardiovascular disorders in RLS patients compared to non-RLS participants (Ulfberg *et al.*, 2001; Ohayon & Roth, 2002; Winkelman *et al.*, 2006, 2008; Schlesinger *et al.*, 2009) however, not all studies have shown this relationship (Van Den Eeden *et al.*, 2015; Cholley-Roulleau *et al.*, 2017). The difference in results may be due to differences in methodologies, for example variations when correcting for cofounding variables. The relationship between RLS and cardiovascular disorders may be mediated through the effects of sleep fragmentation on the sympathetic nervous system (Stamatakis & Punjabi, 2010). Another possible link between cardiovascular disorders and RLS is the occurrence of blood pressure elevations accompanying PLMS as night-time variations in blood pressure are associated with cardiovascular diseases (Cassel *et al.*, 2016).

RLS symptoms are often distressing and negatively impact on quality of life or have been cited as inconsequential by some patients. The wide variations of the impact of RLS often

make determining the global impact of RLS difficult. This in turn complicates verifying the global prevalence of the disorder, which is discussed hereafter.

1.1.5. Prevalence of RLS

An estimate of the global prevalence of RLS, according to a review of numerous epidemiological studies, varies between 0.01 to 26% (Ohayon *et al.*, 2012). The disparity in prevalence statistics from different studies is probably due to the location of the populations studied, the method of diagnosis (questionnaires vs. face-to-face interviews), and the diagnostic criteria employed by each study (single question diagnosis vs. fulfilling the essential diagnostic criteria) (Ohayon *et al.*, 2012; Koo, 2015). The prevalence of RLS is highest in western populations and lowest in Asian populations (Koo, 2015) however very few studies have been conducted in South American, African and Australian populations (Koo, 2015). To achieve accurate prevalence statistics of RLS all studies should use the same diagnostic criteria however determining accurate statistics is complicated by the subjective nature of RLS diagnosis. As the criteria have been adjusted over time, and the need to exclude RLS mimics was only recently highlighted, the most recent prevalence data is likely an estimate of the true prevalence of RLS. Further studies, with homogenous methodologies, would therefore be useful to quantify the actual prevalence of RLS in different populations.

The majority of studies assessing RLS prevalence have been conducted in North American and European populations. The prevalence of RLS in these populations ranges from 0.4% (in a physician-diagnosed study in the USA (Ram *et al.*, 2010)) to 26% (in a French questionnaire based study conducted in an elderly population (Celle *et al.*, 2010)). The large range of RLS prevalence could be due to the difference in populations that were assessed as older and female populations are reported to display higher prevalence's of RLS. Further review of

studies with participants older than 18 years of age, frequency of symptoms greater than twice a week, and excluding cases of possible RLS mimics, the prevalence of RLS in North American and European populations likely to require medical management is between 1.9-4.6% (Ohayon *et al.*, 2012). Not discriminating RLS mimics has likely led to an overestimation of RLS prevalence in many epidemiological studies. Thus, the addition of the fifth essential diagnostic criterion to assist in ruling out RLS mimics (Allen *et al.*, 2014*a*) should improve the accuracy of future epidemiological studies.

Outside of western populations some studies assessing RLS prevalence have been conducted in Asia, Africa and South America. To date, a total of 13 studies have been conducted in Asian populations investigating prevalence of RLS with results ranging from 0.1 to 11.4% (Ohayon *et al.*, 2012). The majority of studies investigating the prevalence of RLS in Asian populations have been done in South Korea and Japan. A recent study conducted in India noted a fivefold increase in RLS prevalence in individuals living at high altitudes when compared to patients at low altitudes (Gupta *et al.*, 2017). These findings are consistent with another study, conducted in Ecuador, where the prevalence of RLS was 0.8% at low altitudes and 3.2% at high altitudes, and thus altitude may have an influence on the prevalence of RLS (Castillo *et al.*, 2006). The increased prevalence at higher altitudes is possibly linked to changes in ambient oxygen concentration (linked to the hypoxia aetiological hypothesis described in section 1.2.2).

In the Middle East the only RLS prevalence studies have been done in Turkish populations (Ohayon *et al.*, 2012). The results of the four Turkish studies show an RLS prevalence ranging between 3.2 to 9.7% (Ohayon, O'Hara and Vitiello, 2012). Four studies have been done looking at RLS prevalence in Africa; two in Tanzania (Winkler *et al.*, 2010*a*; Burtscher *et al.*, 2014), one in Mozambique (Ferreira *et al.*, 2013), and one in Nigeria (Fawale *et al.*, 2016).

The results of the majority of these studies demonstrated a low prevalence in Africa of only 0.01%-6.77%. The Mozambiquan study was done in patients with chronic pain and the Nigerian study was conducted on an elderly population (65-105 years of age), both populations that are typically reported to display higher prevalence's of RLS. The low prevalence reported in these African studies may be due to differences in race or socioeconomic status, as it is likely that with increased poverty people are more concerned with survival and are less likely to seek medical care for non-life threatening ailments (Winkler *et al.*, 2010*a*). As many of the African studies were not conducted on the general populations there is a need for more epidemiological investigations on the continent to elucidate the true prevalence of RLS in African populations. Possible reasons for global differences in RLS prevalence may include the limited amount of data from certain populations, cultural, environmental or genetic factors.

The prevalence of RLS increases with age in North American/European populations (Ohayon *et al.*, 2012). The same increase in prevalence with age has not been shown in Asian populations (Ohayon *et al.*, 2012). However, the increase in RLS prevalence with age may be attributable to the greater risk with increased age of medical conditions associated with secondary RLS (Garcia-Borreguero *et al.*, 2006). Familial RLS is associated with primary RLS and with earlier onset of RLS symptoms (Winkelmann *et al.*, 2000) whereas onset of RLS symptoms later in life is more likely to be associated with secondary RLS (Allen *et al.*, 2014*a*). It has been noted that the age of onset of RLS has a large range, with the commencement of symptoms occurring during childhood to over 90 years of age (Allen *et al.*, 2014*a*).

There is also a known gender bias with respect to the prevalence of RLS. Females are twice as likely to develop RLS when compared to males (Ohayon *et al.*, 2012), but this relationship

is strongly related to age (Allen *et al.*, 2014*a*). The prevalence of RLS in patients above 35 years of age is twice as high in females compared to male patients in the same age range. Conversely, in RLS patients below the age of 35, the prevalence of RLS is the same in females and males (Allen *et al.*, 2014*a*). The gender effect on RLS that is linked to age is possibly connected to pregnancy. Females that have never been pregnant have an equal prevalence of RLS when compared to males (Pantaleo *et al.*, 2010). Furthermore, RLS is the most common movement disorder related to pregnancy (Ohayon *et al.*, 2012). Temporary RLS symptoms are known to develop during the third trimester and disappear within one month postpartum (Ohayon *et al.*, 2012).

The prevalence of RLS is known to be increased in individuals with certain medical disorders such as iron deficiency, kidney disease, cardiovascular disease, migraines and neurodegenerative diseases. However, a recent study investigating the association of RLS with other major diseases reported sufficient evidence of an increased prevalence of RLS only in patients with clearly defined iron deficiency and with kidney disease (Trenkwalder *et al.*, 2016).

Iron deficiency is a known factor associated with increased risk of developing RLS (Garcia-Borreguero *et al.*, 2006). Patients with clearly defined iron deficiency have a high RLS prevalence of 25-35% (Trenkwalder *et al.*, 2016). The relationship between iron deficiency and RLS has been shown in numerous epidemiological studies and is strengthened by the alleviation of RLS symptoms after iron treatment (Trenkwalder *et al.*, 2016). Iron treatment has been shown to be effective in cases of RLS presenting with low-normal serum ferritin concentrations (O'Keeffe *et al.*, 1994). Ferritin is an intracellular protein that stores iron and thus is considered a serum marker of iron stores. Further large-scale trials in RLS patients

with normal serum ferritin levels are required to determine if the efficacy of iron treatment is also applicable in these cases (Ferré *et al.*, 2017).

Studies have also investigated the relationship between anaemia and RLS. However, the results of these studies have been inconsistent with some studies demonstrating an association between RLS and anaemia (Tasdemir *et al.*, 2010; Li *et al.*, 2012) while others have failed to find this relationship (Hadjigeorgiou *et al.*, 2007; Chen *et al.*, 2010; Kim *et al.*, 2010; Szentkiralyi *et al.*, 2014). A large scale systematic review of RLS and disease in 2016 highlighted the need to investigate iron deficiency status and not just anaemia, which is often self-reported and can occur without iron deficiency (Trenkwalder *et al.*, 2016).

The prevalence of RLS in patients with kidney disease or uraemia is increased when compared to the general population and ranges from 15% to 68% (Trenkwalder *et al.*, 2016). The link between RLS and kidney disease is strengthened by the improvement of RLS symptoms following renal transplantation (Winkelmann *et al.*, 2002). A possible reason for the increased prevalence of RLS in patients with kidney disease is due to decreased red blood cells and anaemia thus resulting in low serum iron concentrations. An association of RLS with other major diseases, as previously mentioned, may exist but further research is still needed to prove these associations (Trenkwalder *et al.*, 2016).

In summary, the most reliable estimates of RLS prevalence are from western populations indicating that 1.9 to 4.6% of the population have severe RLS symptoms. Further epidemiological studies in non-western populations taking RLS mimics into account are necessary to elucidate the accurate global impact of RLS. The prevalence of RLS has been found to increase with age, pregnancy, altitude, iron deficiency, and kidney disease.

The diagnosis of RLS is currently subjective with an emphasis placed on the key diagnostic criterion, the urge to move. The severity of RLS symptoms varies from mild, intermittent symptoms to a severe chronic disorder which has been found to have a negative effect on sleep, quality of life, and general health (Earley & Silber, 2010). This coupled with the relatively high global prevalence of RLS (Ohayon *et al.*, 2012) highlights the importance for continued research investigating the possible aetiology of RLS in order to further the understanding of this disorder and develop improved treatment options.

1.2. Aetiology of Restless legs Syndrome

RLS is a complex multifactorial disorder whose aetiology has yet to be fully elucidated (Trenkwalder & Paulus, 2004). The pathophysiological mechanism involved in RLS likely includes several areas of the central nervous system. Some of the features of RLS, such as processing of sensations, activation of movement and alterations in arousal mechanisms, involve many regions of the central nervous system including the cortex, brainstem and spinal cord (Trenkwalder & Paulus, 2010). The sections that follow will briefly discuss some of the pertinent factors that have been investigated in terms of furthering our understanding of the aetiology of RLS such as the genes associated with RLS, iron deficiency, dopamine dysfunction, glutamatergic dysfunction and a state of central nervous system hyperexcitability. In terms of possible neurotransmitter dysfunction in RLS there are other potential neurotransmitters that could be implicated. Two possibilities that have been assessed in RLS, but are not covered in this thesis, are opiate dysfunction and serotonin dysfunction (Jimenez-Jimenez *et al*., 2015).

1.2.1. Genetics

Given the considerable familial component of RLS, a large amount of work has been done in terms of investigating the pathophysiological cause of RLS through genetics. Linkage studies

in families with a known history of RLS have identified eight loci that are associated with RLS (Trenkwalder & Paulus, 2010). Genome-wide association studies have identified the following genes that are associated with risk of developing RLS; MEIS1, BTBD9, PTPRD, and MAP2K5/SKOR1 (Winkelmann *et al.*, 2007; Schormair *et al.*, 2008; Yang *et al.*, 2011). MEIS1 has been identified as the strongest genetic risk factor for developing RLS. MEIS1 is an important factor in embryonic development particularly limb, heart, and eye development (Rataj-Baniowska *et al.*, 2015). BTBD9 is involved in synaptic plasticity (Koo *et al.*, 2016), PTPRD has a regulatory role in cell growth and mitosis (Schormair *et al.*, 2008), and MAP2K5/SKOR1 is involved in cellular proliferation (Yang *et al.*, 2011). Recently data from 30,770 RLS patients has strengthened the known gene associations, and also identified 13 new risk loci for RLS (Schormair *et al.*, 2017).

It has yet to be determined if alterations to the aforementioned genes modify the symptoms of RLS. However, there are on-going studies in insect and animal knockout models of BTBD9 and MEIS1 that will further our understanding of the role these genes play in the development of the RLS phenotype (Deandrade *et al.*, 2012*a*; Freeman *et al.*, 2012). A study assessing fear and memory in mutant BTBD9 mice (a possible animal model for RLS (Li *et al.*, 2015)) has demonstrated increased long-term potentiation in the hippocampus (DeAndrade *et al.*, 2012*b*). Increased long-term potentiation represents enhanced synaptic plasticity. This state of enhanced synaptic plasticity could lead to an increased chance of developing central sensitisation as the development of central sensitisation is reliant on synaptic plasticity (Woolf, 1983). Central sensitisation could relate to the development of hyperalgesia and increased nociceptive reflex responses which have been noted in RLS patients and are discussed in more detail in section 1.2.5.2 and section 1.3.2 respectively.

1.2.2. Iron deficiency

RLS has a well-established association with iron deficiency (as discussed in section 1.1.5). A consistent abnormality noted in RLS patients is decreased brain iron concentrations which have been demonstrated in the basal ganglia (substantia nigra, putamen and caudate nucleus) and the thalamus of RLS patients (Allen *et al.*, 2001; Earley *et al.*, 2006; Godau *et al.*, 2008). The findings related to brain iron deficiency in RLS patients have been subsequently confirmed in other studies (Connor *et al.*, 2003, 2011). Furthermore, three of the main causes of secondary RLS; renal disease, iron deficiency, and pregnancy all involve dysfunctions with maintaining normal iron concentrations (Allen and Earley, 2001*b*). There is also a temporal association between iron concentrations and the symptoms of RLS. Serum iron concentrations have a circadian pattern with peak concentrations at noon and nadir between 20:00 and 24:00 (Scales *et al.*, 1988). Thus, serum iron concentrations are lowest in the evening when RLS symptoms are the most severe. The importance of iron concentrations in RLS is highlighted by the finding that iron treatment in RLS patients has been shown to alleviate symptoms in certain cases (Earley *et al.*, 2009; Allen *et al.*, 2011). Moreover, studies have noted a negative correlation between serum ferritin concentrations and indices of RLS severity (O'Keeffe *et al.*, 1994; Sun *et al.*, 1998).

Further investigations into the relationship between reduced iron concentrations and RLS have indicated that there are possible abnormalities in central iron stores in RLS patients. In the cerebrospinal fluid (CSF) of RLS patients, it was noted that ferritin concentrations were decreased with a concurrent increase in transferrin concentrations (a protein involved in the transport of iron) (Earley *et al.*, 2000*b*; Mizuno *et al.*, 2005). Correlations between CSF and serum ferritin concentrations were noted in both RLS patients and healthy controls. However, the slope of the regression line was significantly lower in RLS patients when

compared to healthy controls indicating that for any given serum ferritin concentration RLS patients will have a lower CSF ferritin concentration compared to healthy controls. These findings suggest that there is a possible deficiency in the transport of iron into the central nervous system in RLS patients (Earley *et al.*, 2000*b*). In fact RLS patients often have no evidence to indicate that peripheral concentrations of iron are reduced in spite of the fact that surrogate markers of central iron concentrations are reduced (Earley *et al.*, 2000*b*; Allen, 2015). These results have led to the hypothesis that the pathophysiological mechanisms of iron deficiency in RLS patients are related to dysfunctions in the transport of iron to the central nervous system. These changes may be a result of changes in regulatory proteins involved in iron metabolism that alter the normal transport of iron across the blood brain barrier (Allen, 2015).

Possible physiological consequences of reduced brain iron concentrations that are relevant in RLS, may involve reduced myelin and activation of hypoxia induced factor (HIF) pathways (Allen, 2015). Myelin synthesis depends on iron and as such animal models of reduced brain iron concentrations have demonstrated decreased myelin proteins (Yu *et al.*, 1986; Ortiz *et al.*, 2004). An iron dependent decrease in myelin may produce a myelin deficit in RLS patients (Allen, 2015), which may have an impact on peripheral nerve conduction. In terms of activation of HIF pathways, decreased iron concentrations can activate HIF pathways (Allen, 2015). The pathophysiological consequence of activation of HIF pathways in RLS patients is linked to dopamine, which will be discussed in the section that follows.

1.2.3. Dopamine dysfunction

Iron is a cofactor for tyrosine hydroxylase, which is the rate limiting enzyme in the conversion of L-tyrosine to L-dopa (L-dihydroxyphenyalanine), a precursor of dopamine. Thus the known iron deficiency in RLS patients could cause a corresponding dopamine

deficiency. Dopamine deficiency has been a prominent area of research in terms of elucidating the underlying causes of RLS. The importance of dopamine in RLS is furthered evidenced by the positive response of RLS patients to dopamine receptor agonist treatment (Hening *et al.*, 2004). Dopamine also has a known circadian variation, which has been demonstrated in urine, CSF and plasma concentrations of dopamine. The highest concentrations of dopamine occur in the day and the corresponding nadir in late evening/night (Kawano Y *et al.*, 1990; Hagan *et al.*, 1999) when RLS symptoms are most severe. Therefore dopamine deficiency was seen as a likely candidate for the underlying cause of RLS and warranted further investigation in RLS patients.

Despite the initial optimism at finding a dopamine linked aetiology of RLS, evidence for the expected hypo-dopaminergic state in RLS patients has proven to be elusive. Brain imaging studies in RLS patients indicated an increase in striatal dopamine concentrations; a decrease in striatal dopamine 2 receptors (D2R) and decreased membrane bound dopamine transporter (Turjanski *et al.*, 1999; Michaud *et al.*, 2002*b*; Earley *et al.*, 2011). Dopamine transporter is responsible for the reuptake of dopamine from the synapse and as such a decrease in dopamine transporter expression would result in increased extracellular dopamine concentrations. The suggestion of increased pre-synaptic dopamine activity is supported by the analysis of CSF in RLS patients (Earley *et al.*, 2001; Allen *et al.*, 2009) where a significant increase in 3-ortho-methyldopa (3OMD), a product of an alternative pathway for the metabolism of L-dopa, was noted (Allen *et al.*, 2009). The post-mortem brain analyses of RLS patients also showed a decrease in D2R expression (Connor *et al.*, 2009). These findings are supported by studies using iron deficient rats which presented with decreased D2R in the striatum (Ashkenazi *et al.*, 1982), decreased dopamine transporter, and increased extracellular dopamine (Nelson *et al.*, 1997). Hence, current data supports the

theory of increased dopamine pre-synaptically, due to a decreased reuptake of dopamine, and decreased effects of dopamine at the post-synaptic membrane due to decreased D2R (Earley *et al.*, 2017).

Further investigations were required to explain the paradoxical increase in dopamine concentrations with iron deficiency as opposed to the expected decrease. Iron deficiency causes activation of the HIF pathway. This pathway activates hypoxia-response elements on genes causing increased transcription (Earley *et al.*, 2014). In relation to dopamine, HIF increases tyrosine hydroxylase gene expression, which may produce an increase in dopamine concentrations (Bianchi *et al.*, 1999). Increased dopamine concentrations have been shown to have different effects on excitatory dopamine 1 receptor (D1R) and inhibitory D2R expression. Increased dopamine concentrations result in a degradation of D2R and recycling of D1R (Bartlett *et al.*, 2005). Hence, the overall effect of increased dopamine concentrations is a net increase in excitatory D1R at the membrane surface (Paulus & Trenkwalder, 2006). An increase in D1R may lead to a state of increased nervous system excitability which could be responsible for the generation of RLS symptoms.

A hyper-dopaminergic state in RLS patients could explain the occurrence of a treatment phenomenon known as augmentation. Augmentation is defined by an earlier onset and worsening of RLS symptoms while receiving dopaminergic treatment (Allen & Earley, 1996). Following this rationale, increased dopamine concentrations down regulate D2R expression at the post-synaptic membrane to maintain normal function during the asymptomatic phase of RLS, during the day, when dopamine levels are highest (Koo *et al.*, 2016). However, RLS symptoms may develop when dopamine levels decrease in the evening, in combination with down-regulated inhibitory D2R (Koo *et al.*, 2016). Treatment with dopamine agonists may temporarily alleviate symptoms at night. However, as the original dysfunction is due to

increased levels of dopamine, additional dopamine compounds the situation leading to augmentation (Koo *et al.*, 2016).

The A11 diencephalospinal dopaminergic pathway is the major source of spinal dopamine and has been implicated in the pathophysiology of RLS (Clemens *et al.*, 2006). The spine is a key site in the aetiology of RLS (as will be discussed in section 1.2.5.2) because the spine is the location of sensory input and motor output and, as such, dysfunction in spinal circuits could explain both sensory and motor symptoms of RLS. The A11 pathway has a regulatory effect on spinal networks; it regulates sensory input in the dorsal horn and regulates somatic motor neuron output (Clemens *et al.*, 2006). Activation of the A11 pathway has anti-nociceptive effects in the dorsal horn, decreasing the response to noxious stimuli (Fleetwood-Walker *et al.*, 1988). Clemens and colleagues therefore theorised that damage or decreased functionality of the A11 pathway would cause a loss of inhibition of sensory inputs leading to strange sensations and thereby explaining the symptoms evident in RLS patients (Clemens *et al.*, 2006). This theory is supported by evidence from animal studies demonstrating hyperactivity in rats with A11 lesions (Ondo *et al.*, 2000). Furthermore, rats with A11 lesions had decreased sensory thresholds and were more sensitive to noxious stimuli (Wang *et al.*, 2005). However, no evidence of alterations in the A11 diencephalospinal dopaminergic pathways has been found in RLS patients (Earley *et al.*, 2014).

1.2.4. Increased glutamate concentrations

A possible explanation for the positive response noted in RLS patients with respect to dopamine treatment may be changes in glutamatergic responses. Glutamate is an excitatory neurotransmitter that acts in numerous regions of the central nervous system including the spinal cord and basal ganglia (Guyton & Hall, 2006). The two most prominent receptors for

glutamate are ionotropic alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate receptor (NMDA) receptors. AMPA receptors are involved in fast excitation whereas NMDA receptors play an important role in long-term potentiation and thus have an influence on synapse plasticity (Guyton & Hall, 2006).

The initial positive response of RLS patients to dopaminergic agonists could therefore be mediated through an effect of dopaminergic agonists on post synaptic membrane sensitivity to glutamate. Animal studies have shown that dopamine binding to D2R decreases AMPA receptor responses (Cepeda & Levine, 2012) while dopamine binding to D1R increases NMDA evoked responses (Ferré *et al.*, 2017). D1R agonists strengthen the C-fibre pain response in the dorsal horn through long-term potentiation (Yang *et al.*, 2005) while D2R agonists dampen C-fibre responses in the dorsal horn (Yang *et al.*, 2005). Hence, the alleviation of symptoms in RLS patients, with D2-like receptor agonist treatment (Paulus & Trenkwalder, 2006), could be due to changes in sensory processing in the dorsal horn as a result of decreased glutamatergic responses.

Alpha-2-delta (α2δ) ligands have been introduced as an alternative treatment option for RLS due to the 50% prevalence of augmentation in RLS patients with dopaminergic treatment (Ferré *et al.*, 2017). α2δ ligands act by binding to α2δ subunits of voltage-activated calcium channels and decreasing the influx of calcium. A decrease in calcium influx decreases the release of excitatory neurotransmitters, for example glutamate, from the presynaptic terminals with a subsequent decrease in post synaptic excitability (Wijemanne & Jankovic, 2015). Treatment with α2δ ligands in RLS patients has been effective in treating RLS sensory symptoms (Hornyak *et al.*, 2014), improving sleep by increasing slow wave sleep, and decreasing arousals and sleep fragmentation (Garcia-Borreguero *et al.*, 2014). A possible mechanism of action for the symptom alleviation with α2δ ligand treatment is by decreasing

presynaptic glutamate release which decreases post synaptic excitability and thus decreases general excitability of the nervous system.

Abnormal glutamate levels have been linked to RLS, with an increase in thalamic glutamate activity noted in RLS patients compared to control participants (Allen *et al.*, 2013). Thalamic glutamate concentrations have been significantly correlated with total sleep time in RLS patients (Allen *et al.*, 2013) which is important considering the reduced sleep efficiency in these patients. This correlation between thalamic glutamate and total sleep time may be a result of an increase in thalamocortical excitation leading to increased wakefulness (Coulon *et al.*, 2012).

Given the known link between RLS and iron deficiency, the relationship between glutamate and iron has also been investigated. Ferré and colleagues have indicated that iron deficiency may produce increases in glutamate concentrations with these effects mediated through adenosine (Ferré *et al.*, 2017). Iron deficiency has been shown to cause an up regulation of adenosine A_{2A} receptors (A2AR) (Quiroz *et al.*, 2010) and down regulation of adenosine A_1 receptors (A1R) (Quiroz *et al.*, 2016). Adenosine binding to A1R inhibits glutamate release by inhibiting presynaptic calcium channels (Ferré *et al.*, 2017). Through the inhibition of glutamate, the net result of A1R activity is to promote sleep. Conversely, A2AR activation leads to an increase in glutamate and dopamine release (Ferré *et al.*, 2017). Hence, the overall increased A2AR and decreased A1R expression caused by iron deficiency results in an excitatory state. It should be noted that these findings were demonstrated in animal studies and validation of these findings is required in RLS patients.

Potential pathophysiological theories also need to account for the circadian aspect of RLS symptoms. However, there is limited evidence to suggest that there is a circadian variation of glutamatergic function. A circadian variation has been noted in glutamine synthase, which

is an enzyme that participates in glutamate metabolism, in the spinal cord of mice (Morioka *et al.*, 2012). Furthermore, in rats it has been noted that there is a circadian variation in glutamate concentrations in the brain (Marquez de Prado *et al.*, 2000*a*; Castañeda *et al.*, 2004). A circadian variation in glutamate that relates to the symptom profile in RLS patients needs to be established in human participants.

Overall evidence indicates a possible state of increased glutamate concentrations in the central nervous system of RLS patients. Increased concentrations of glutamate would cause an increased state of excitability by increasing the likelihood of an action potential in the post-synaptic neuron. This state of central nervous system hyperexcitability may be a possible cause of RLS symptoms as discussed in the following section.

1.2.5. Central nervous system excitability in RLS

Increased glutaminergic activity and concurrent down regulation of D2R would lead to an increase in excitation as well as a loss of inhibition respectively which would result in cortical and spinal hyperexcitability. Many studies have been conducted assessing the central and peripheral nervous system excitability in RLS patients as a possible cause of RLS symptoms and will be discussed in the coming sections.

1.2.5.1. Cortical excitability

Recently, with advancements in technology, functional MRI (fMRI) studies have been conducted while RLS patients are experiencing symptoms to identify cortical regions that may be implicated in RLS (Bucher *et al.*, 1997; Margariti *et al.*, 2012). The areas of the brain that are activated in patients while experiencing RLS symptoms are; the putamen, pars orbicularis, anterior cingulate cortex, thalamus, pre- and post-central gyrii, prefrontal cortex, and the cerebellum (Bucher *et al.*, 1997; Margariti *et al.*, 2012). Many of these brain regions are expected to be activated by sensations, such as the thalamus and the somatosensory

cortex (post-central gyrus). Therefore activation of these aforementioned regions may occur as an epiphenomenon of the uncomfortable sensations rather than being pathophysiologically linked to RLS (Koo *et al.*, 2016). fMRI studies of PLMS have shown activity in the red nucleus, inferior olive, and cerebellum (Bucher *et al.*, 1997). These regions of the brain are important for regulating sensory input during movement and thus these brain regions could be responsible for the alleviation of RLS symptoms caused by movement (Trenkwalder & Paulus, 2004). Therefore, dysfunction in the red nucleus, inferior olive and cerebellar circuit could potentially be associated with the pathophysiology of RLS (Trenkwalder & Paulus, 2004).

The cortical involvement in PLMS, PLMW, and voluntary dorsiflexion in RLS patients has also been assessed looking at spontaneous electroencephalographic oscillations (Tyvaert *et al.*, 2009). In particular the activity of cortical motor preparation neurons represented by mu and beta event-related desynchronisation and activity of movement-related somatosensory afferents represented by mu and beta event-related synchronisation (Tyvaert *et al.*, 2009). Event-related desynchronisation occurred before PLMW and voluntary dorsiflexion; however no event-related desynchronisation was apparent before PLMS (Tyvaert *et al.*, 2009). These results suggest that there is no cortical involvement in PLMS supporting the hypothesis of a spinal generator of PLMS. This hypothesis of a spinal generator for PLMS will be discussed further in section 1.2.5.2.

The amplitude of both event-related desynchronisation and event-related synchronisation for voluntary movements were increased in RLS patients when compared to control participants, during the symptomatic phase, demonstrating potential evening hyperactivity of the sensorimotor cortices (Tyvaert *et al.*, 2009). Due to neuroplasticity, alterations in

spinal sensory processing could cause structural or chemical changes in the brain leading to the hyperactivity of the sensorimotor cortex noted in these RLS patients.

Changes in cortical plasticity and cortical excitability in RLS patients has been assessed via transcranial magnetic stimulation (TMS) as these may reveal pathophysiological mechanisms involved in the symptoms of RLS (Lanza *et al.*, 2016). In relation to RLS patients, particular focus has been given to cortical motor excitability. Variables measured using TMS include cortical silent period (CSP) duration, short interval intracortical inhibition (SICI), and short interval intracortical facilitation (SICF). CSP is recorded by TMS of the motor cortex during a voluntary tonic muscle contraction. The CSP is the period of no electrical activity seen on the EMG following the TMS and is considered a measure of intracortical inhibition in the motor cortex (Rossini *et al.*, 2015). SICI is evaluated using a subthreshold conditioning stimulus, which is too small to generate a motor evoked potential (MEP), 1 to 6ms prior to a suprathreshold stimulus that generates a MEP. This leads to an inhibition of the MEP which is highly dependent on the intensity of the conditioning stimulus (Rossini *et al.*, 2015). SICI is thought to involve activation of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) in the motor cortex. If the inter-stimulus interval between the subthreshold stimulus and the suprathreshold stimulus is 8 to 30ms it causes facilitation of the MEP, termed SICF. SICF may involve excitatory glutamatergic pathways in the motor cortex (Rossini *et al.*, 2015).

Cortical plasticity has been assessed using TMS by eliciting a MEP following exercise. Due to cortical plasticity there is normally an increase in MEP following exercise, as compared to before exercise (Samii *et al.*, 1996). Abnormalities in cortical plasticity have been shown in RLS patients by the absence of the expected increase in MEP after exercise (Scalise *et al.*, 2004, 2006, 2010). The lack of expected increase in MEP could represent alterations in motor-induced cortical plasticity. This may be due to a decrease in proprioceptive afferent

activity in RLS patients, as proprioceptive afferents are activated during movement. Decreased proprioceptive Ib afferent activity has been demonstrated in RLS patients (Rijsman *et al.*, 2005; Scaglione *et al.*, 2008; Marconi *et al.*, 2012) which may account for the absence of an increase in MEP following repetitive movements.

TMS studies assessing cortical excitability in RLS patients have noted conflicting results regarding CSP in RLS patients. Five studies (Tergau *et al.*, 1999; Quatrale *et al.*, 2003; Nardone *et al.*, 2006; Gündüz *et al.*, 2012; Lanza *et al.*, 2015) have shown no difference in CSP between RLS patients and healthy control participants. Conversely, there have been six studies (Entezari-Taher *et al.*, 1999; Stiasny-Kolster *et al.*, 2003; Kutukcu *et al.*, 2006; Scalise *et al.*, 2006, 2010; Gorsler & Liepert, 2007) that have shown decreased CSP in RLS patients when compared to control participants. The conflicting results in these studies investigating CSP in RLS patients may be due to the differences in stimulus protocols related to muscle contraction prior to TMS (Lanza *et al.*, 2016). Another reason for the inconsistency of CSP could be that most of the studies were conducted during the asymptomatic period of RLS. Differences between the asymptomatic and symptomatic periods of the disorder could contribute to the inconsistency demonstrated in the results.

A more consistent finding in RLS patients is a decrease in SICI, compared to healthy controls, demonstrating a decrease in cortical inhibition (Tergau *et al.*, 1999; Quatrale *et al.*, 2003; Scalise *et al.*, 2004, 2006, 2010; Nardone *et al.*, 2006; Rizzo *et al.*, 2010; Lanza *et al.*, 2015). Lanza *et al* (2016) has recently published an extensive review on central and peripheral excitability in RLS. The authors hypothesise that overall data from TMS studies in RLS patients support the theory of increased cortical excitability in RLS which is possibly due to alterations of cortical inhibitory circuits (Lanza *et al.*, 2016). Alternatively increased cortical excitability may be due to prolonged afferent activity caused by the constant unpleasant

sensations that are described by RLS sufferers. It has been demonstrated that prolonged afferent activity causes increased excitability of the motor cortex (Ridding *et al.*, 2001). The importance of cortical excitability in RLS is strengthened by the finding that circadian variations exist in cortical excitability. In RLS patients it has been demonstrated that there are significantly decreased motor thresholds in RLS patients compared to control participants at night (Gündüz *et al.*, 2012). The authors speculated that the decreased motor thresholds were as a result of a loss of subcortical inhibition at night (Gündüz *et al.*, 2012). As RLS symptoms present at night, the evening decreased motor thresholds may be as a result of an increase in afferent activity caused by RLS sensations. During RLS sensations activity in the somatosensory and primary motor cortices has been noted (Margariti *et al.*, 2012). Therefore the decreased motor thresholds may merely be an expected response to the RLS sensations and not representative of a cortical pathology.

In addition to cortical plasticity and excitability, sensorimotor integration can be assessed by stimulating an afferent nerve in conjunction with TMS of the motor cortex. This allows for the evaluation of the effect of sensory input on the motor cortex output (Bocquillon *et al.*, 2017). Short latency afferent inhibition (SAI) and long latency afferent inhibition (LAI) are both used in order to assess different inhibitory effects. It is thought that SAI reflects direct inhibition of the primary motor cortex while LAI is dependent on intra-cortical inhibitory connections (Rossini *et al.*, 2015). Only two studies have been conducted investigating SAI and LAI in RLS patients (Rizzo *et al.*, 2010; Bocquillon *et al.*, 2017). Rizzo et al. (2010) demonstrated a decrease in SAI in RLS patients compared to control participants, which was corrected following dopaminergic treatment (Rizzo *et al.*, 2010). The decrease in SAI demonstrates alterations in sensorimotor integration with a decrease in the normal inhibition of the motor cortex resulting from afferent activation (Rizzo *et al.*, 2010).

However, these results were not found in an investigation by Bocquillon *et al*. who noted no differences in SAI or LAI between RLS patients and control participants (Bocquillon *et al.*, 2017). Bocquillon *et al* also failed to show a circadian variation in SAI or LAI. However it was noted that control participants, but not RLS patients, experienced afferent-induced facilitation (AIF) (Bocquillon *et al.*, 2017). AIF is a facilitation of EMG muscle activity when stimulating an afferent nerve after TMS of the motor cortex at an inter-stimulus interval of 50 to 55ms (Bocquillon *et al.*, 2017). The absence of AIF in RLS patients suggests an alteration in cortical sensorimotor integration (Bocquillon *et al.*, 2017).

Another possible mechanism for the absence of AIF in RLS patients may be alterations in afferent nerve sensitivity. Further studies are required in order to confirm if there are dysfunctions in sensorimotor integration in RLS patients as currently the two investigations into this area have produced conflicting results.

As is the case for dysfunctional sensorimotor integration, studies assessing cortical excitability in RLS patients also demonstrate inconsistent conclusions. The most consistent finding is an increase in cortical excitability in RLS patients with a decrease in cortical inhibition. However some studies have shown no changes in cortical excitability between RLS patients and control participants and further research is still needed to confirm the state of cortical hyperexcitability in RLS patients. Increased cortical excitability may be merely an epiphenomenon to an increase in afferent processing due to hyperexcitability of the spinal cord. The lack of activity of cortical motor preparation neurons during PLMS further implicates changes in spinal excitability as one of the main contributing factors in the pathophysiology of RLS.

1.2.5.2. Spinal excitability

As previously emphasised, RLS is a disorder presenting with both sensory and motor symptoms, which are often manifest as an urge to move and PLM respectively (Allen *et al.*, 2014*a*). As the spinal cord is the site of sensory input and motor output it has been considered that the aetiology of RLS may be due to dysfunctions in the spinal cord (Barrière *et al.*, 2005). It is possible that RLS patients have reductions in descending spinal inhibition due to subcortical and intra-cortical dysfunction, as discussed in the previous section. The spinal cord could also be the primary site of dysfunction in RLS. A loss of supra-spinal inhibitory influence on the spinal cord or an increase in spinal excitability are plausible candidates for both RLS symptoms and PLM. Even though the presence of PLM is not a definitive criterion of RLS, approximately 80% of RLS patients experience PLM (Scofield *et al.*, 2008). Studies on spinal cord injuries and patients under spinal anaesthesia have found the presence of PLM type movements similar to those seen in RLS patients, which suggest a spinal cord origin of these involuntary movements (Yokota *et al.*, 1991; Lee *et al.*, 1996, 1997). Considerable research has been conducted on spinal excitability in RLS patients. Discussions of the salient points emanating from these investigations will be in the sections to follow.

Electrophysiological studies of spinal cord activity in RLS patients

An electrophysiological method of evaluating motor and cutaneous nerve function is by assessing the F-wave and the cutaneous silent period (CuSP) respectively. Elicitation of the F-wave is by electrical stimulation of the distal portion of a motor neuron. Measurements from the F-wave assessment are; F-wave duration and the ratio of F-wave to compound muscle action potential (CMAP) duration, which are indices of motor neuron function (Isak *et al.*, 2011). The CuSP is a brief interruption of voluntary muscle contraction due to electrically

activating a cutaneous nerve allowing for the evaluation of cutaneous nerve function and overall sensorimotor integration (Floeter, 2003) which may be dysfunctional in RLS patients.

To date there have been two studies that have investigated the F-wave and CuSP in RLS patients (Isak *et al.*, 2011; Congiu *et al.*, 2017). Both studies have demonstrated an increased F-wave duration and F-wave /CMAP duration ratio in RLS patients when compared to control participants (Isak *et al.*, 2011; Congiu *et al.*, 2017). These results suggest that there is a possible increased excitability of motor neurons in RLS patients. Furthermore, Isak and colleagues also noted decreased CuSP latency and duration in RLS patients (Isak *et al.*, 2011). The authors postulate alterations in spinal interneuron function may be the mechanism for decreased CuSP duration in RLS patients (Isak *et al.*, 2011). The decreased CuSP duration could also be a result of the increased excitability of motor neurons, as shown by the increased F-wave duration and F-wave /CMAP duration ratio in RLS patients, or as a result of a decrease in afferent neuron sensitivity. However, Congiu and colleagues did not show changes in CuSP in RLS patients (Congiu *et al.*, 2017). Further investigations are therefore required to confirm if decreased CuSP latency and duration are consistent observations in RLS patients and if this is due to changes in spinal interneuron function.

Due to the theory of RLS symptoms having a spinal origin transcutaneous spinal direct current stimulation (tsDCS) has been proposed as a treatment for RLS patients (Heide *et al.*, 2014). tsDCS is a non-invasive technique to alter spinal excitability by applying a weak current over the thoracic spinal cord via surface electrodes (Heide *et al.*, 2014). Heide and colleagues assessed the effects of tsDCS on RLS symptoms using a VAS, as a subjective measure of symptom severity. H2/H1 ratio of the soleus Hoffman reflex (H-reflex) provided an objective assessment of spinal excitability to determine if the effects of tsDCS were mediated through changes in spinal excitability. RLS patients had increased H2/H1 ratios

compared to control participants at baseline (Heide *et al.*, 2014) and following tsDCS treatment, a decrease in H2/H1 ratios was noted in RLS patients for at least 15 minutes. Furthermore, there was a significant decrease in subjective symptom severity following tsDCS treatment in RLS patients as measured using the VAS (Heide *et al.*, 2014). The effect of tsDCS on the severity of symptoms in RLS implicates spinal cord hyperexcitability in generating the urge to move in RLS patients. The important role of spinal cord hyperexcitability is strengthen by the fact that transcranial direct current stimulation of the somatosensory cortex, as opposed to stimulation of the thoracic spinal cord, had no effect on RLS symptom severity (Koo YS *et al.*, 2015).

tsDCS therapy affects spinal sensory and motor processing. The effect of tsDCS on nociception has been demonstrated by increases in pain tolerance following tsDCS treatment (Truini *et al.*, 2011). In addition to effects on afferent inputs, tsDCS also reduces post activation depression of the H-reflex (Winkler *et al.*, 2010*b*) and depresses lower limb flexion reflexes (Cogiamanian *et al.*, 2011). Thus, relief from sensory and motor RLS symptoms following tsDCS therapy may be due to tsDCS modulating spinal excitability.

The combined data from the electrophysiological evaluations of spinal cord activity in RLS patients indicate that the pathogenesis of RLS involves spinal cord hyperexcitability. Furthermore the spinal hyperexcitability may be a result of alterations in afferent neuron, motor neuron, or interneuron function. However there are methodological concerns regarding the studies assessing spinal excitability of RLS patients. None of the studies corrected for possible changes in spinal excitability due to the effects of sleep loss or the constant spinal activation during sleep due to PLMS. The increased spinal activation during sleep in RLS patients may have an effect on the spinal circuitry leading to the increased spinal excitability seen in RLS patients. In addition some of the patients in the study

conducted by Heide et al (2014) were taken off dopaminergic agonists the day before tsDCS testing. Dopaminergic agonists are known to have an effect on both cortical and spinal excitability which could be responsible for the differing responses seen in RLS patients compared to control participants. Furthermore the studies were performed at different times of day with Isak et al (2011) not even mentioning the time data was collected or if the time remained constant for all patients. For comparison of results between studies it is essential that time of day is taken into account due to the circadian nature of RLS symptoms. Nevertheless, to determine the full pathophysiological mechanism of spinal hyperexcitability in RLS patients requires further electrophysiological investigation. As RLS is characterised by sensory symptoms a large focus of previous studies has been on the evaluation of spinal sensory pathways in RLS patients.

Sensory evaluations in RLS patients

A key diagnostic feature of RLS is the urge to move accompanied by unpleasant sensations (Allen *et al.*, 2014*a*). Research has subsequently focused on spinal sensory pathways in RLS patients, in particular pain pathways, to elucidate the mechanism responsible for these unpleasant sensations. Briefly, with respect to the description of afferent nerve fibres relevant to sensory evaluations in RLS patients, A-beta fibres are large, myelinated, fast conducting fibres that relay signals from muscle spindles and cutaneous receptors. A-beta fibres relay information about proprioception, touch, and vibration. A-delta fibres are small myelinated fibres that relay nociceptive, cold, and pressure signals. C-fibres are small, unmyelinated fibres relaying information regarding nociception and heat and have a slower conduction velocity than both types of A-fibres.

Up to 80% of RLS patients describe their uncomfortable or unpleasant sensations as painful (Winkelmann *et al.*, 2000). Therefore, assessment of pain sensitivity in RLS patients could

provide information regarding the possible origin of these sensations. As stated in previous sections, there is strong evidence to indicate that there is a state of spinal hyperexcitability in RLS patients and this could result in hyperalgesia. Hyperalgesia is defined as an increased sensitivity to noxious stimuli. Stiasny-Kolster et al. assessed nociceptive pathways in RLS patients, in the evening and the morning, with the use of a punctuate mechanical stimulus. This type of mechanical stimulus activates nociceptive high threshold A-delta fibres and tests for the presence of hyperalgesia (Stiasny-Kolster *et al.*, 2004). The authors also investigated the presence of allodynia by activating low threshold mechanoreceptive A-beta fibres using a moving gentle stimulus (Stiasny-Kolster *et al.*, 2004). Allodynia is a phenomenon where normally non-noxious stimuli are perceived as painful. RLS patients had increased pain ratings in response to punctuate mechanical stimuli when compared to healthy control participants (Stiasny-Kolster *et al.*, 2004). Moreover, the increased pain ratings in RLS patients in comparison to control participants were noted in both the morning and the evening, thus excluding the possibility of a circadian influence. These findings show that there is a general state of a degree of hyperalgesia in RLS patients. As no allodynia was reported in the RLS patients, the authors concluded that the hyperalgesia was likely mediated by central sensitisation of high threshold A-delta fibre inputs (Stiasny-Kolster *et al.*, 2004).

The presence of hyperalgesia in RLS patients has been further established using the quantitative sensory testing (QST) protocol (Bachmann *et al.*, 2010; Stiasny-Kolster *et al.*, 2013). The QST protocol incorporates thermal and mechanical stimuli to assess thermal, mechanical and noxious sensitivity and thresholds (Rolke *et al.*, 2006*a*; Rolke *et al.*, 2006*b*). The QST also evaluates the presence of allodynia. Bachmann and colleagues performed the QST protocol, during the symptomatic period, on the dorsum of the feet of RLS patients and

healthy control participants (Bachmann *et al.*, 2010). RLS patients had decreased pain thresholds to both pressure and mechanical stimuli when compared to the healthy control participants. These data indicate that there was hyperalgesia to blunt pressure and pinprick pain in RLS patients. In addition, no allodynia was reported in the primary RLS patients (Bachmann *et al.*, 2010).

Another study using the QST protocol on RLS patients assessed in the morning, during the asymptomatic period, exhibited decreased mechanical pain thresholds in RLS patients however significantly increased mechanical detection thresholds compared to control participants (Stiasny-Kolster *et al.*, 2013). Furthermore, allodynia was not seen in the RLS patients or healthy control participants (Stiasny-Kolster *et al.*, 2013). The results from the Bachmann and Stiasny-Kolster studies are in agreement in that both studies indicate the presence of hyperalgesia in RLS patients and the absence of allodynia (Bachmann *et al.*, 2010; Stiasny-Kolster *et al.*, 2013). In addition, RLS patients displayed an increase in mechanical detection threshold, which is an indicator of a reduced perception of mechanical stimuli (mechanical hypoesthesia) (Stiasny-Kolster *et al.*, 2013). The difference in results regarding pain thresholds and mechanical detection thresholds indicate a more complex spinal cord dysfunction in RLS patients beyond only spinal hyperexcitability. With a global state of spinal hyperexcitability a decrease in mechanical detection thresholds, in addition to the decreased pain thresholds, would present in RLS patients. However based on the results of the QST protocol in RLS patients this is not the case.

A study in 2011 by Edwards and colleagues looked at differences in pain thresholds between RLS patients and control participants in the early afternoon by means of pressure and heat stimuli (Edwards *et al.*, 2011). Consistent with the results of previous studies, RLS patients had lower pressure pain thresholds when compared to control participants. RLS patients also

displayed greater temporal summation of heat pain (Edwards *et al.*, 2011). Temporal summation to heat pain is a measure of central pain facilitation and evaluates the subjective pain reported following a rapid series of noxious heat stimuli of equal intensity. RLS patients reported each heat stimulus as more painful than the last, despite no change in the temperature of the stimulus (Edwards *et al.*, 2011). Temporal summation corresponds to central sensitisation, hence these results support the hypothesis of a state of central sensitisation in RLS patients.

To evaluate potential mechanisms behind sensory threshold changes in RLS patients, Lim et al. (2012) investigated small fibre neuropathy by assessing warm and cold detection thresholds, and autonomic small fibre function. RLS patients had increased warm and cold detection thresholds of the feet when compared to control participants. There were no significant differences in small fibre function between RLS patients and control participants indicating that there was no presence of small fibre neuropathy in the RLS patients. These findings indicate a central, not peripheral, cause of the increased thermal detection thresholds in RLS patients (Lim *et al.*, 2012). This suggests that there is no peripheral nerve damage in RLS patients and the pathophysiology of RLS likely involves alterations in central processing in the spinal cord or the brain.

The circadian variation in sensory thresholds of A-beta, A-delta and C fibres in RLS patients has recently been evaluated by Cho and associates (2017) with the current perception threshold (CPT) test. The CPT test was performed by applying currents of different frequencies to the big toe and recording when a sensation is first reported (Cho *et al.*, 2017). Using this method Cho et al. demonstrated a circadian variation in perception thresholds of all three fibre types in RLS patients, with decreased perception thresholds in the evening compared to the morning (Cho *et al.*, 2017). The only significant difference in perception

thresholds between RLS patients and control participants was in evening C-fibre perception thresholds, which were lower in RLS patients (Cho *et al.*, 2017). Therefore RLS patients showed hyperalgesia only in small, unmyelinated nociceptive fibres. The results from this study indicate that RLS patients have increased sensitivity in afferent nerve fibres in the evening compared to the morning.

The data from studies assessing sensory evaluations in RLS patients indicate the presence of hyperalgesia. Several studies have noted that RLS patients have decreased pain thresholds compared to control participants. The presence of hyperalgesia in RLS patients suggests central sensitisation is present in the spinal cord and may be a potential mechanism that mediates the urge to move and the uncomfortable sensations. Importantly, RLS patients have hyperalgesia with an absence of allodynia indicating that the central sensitisation in the spinal cord is likely due to a different mechanism of other forms of central sensitisation seen in chronic pain conditions. In this regard, neuropathic pain has symptoms similar to RLS patients but is associated with hyperalgesia and allodynia. In terms of the circadian variation noted in the sensory pathways, RLS patients have been noted to have increased sensitivity in A-beta, A-delta and C fibres in the evening. Interestingly, hyperalgesia has been shown in RLS patients during both the symptomatic and asymptomatic periods of the disorder. These data indicate that hyerpalgesia is always present in RLS patients but the manifestation of symptoms may be subsequent to increased sensitivity in pain fibres in the evening.

In summary, the aetiology of RLS is probably complex and involves multiple systems and pathways. Figure 1 shows all the areas of the brain, brainstem and spinal cord that may be implicated in the pathophysiology of RLS. Low iron levels, specifically central nervous system iron levels, are consistently associated with RLS. A possible consequence of low iron levels is a central state of hypoxia. Hypoxia causes activation of the HIF pathway which increases

tyrosine hydroxylase gene expression leading to an increase in dopamine concentrations. Increased dopamine concentrations cause an increase in excitatory D1R expression at the membrane surface and a decrease in inhibitory D2R expression. Iron deficiency may also cause an increase in glutamate concentrations by causing an up-regulation of A2AR. Adenosine binding to A2AR causes an increase in glutamate release. In addition, the hyper-dopaminergic state and alterations in dopamine receptor ratios may also have an effect on glutamatergic activity. For example, dopamine binding to D1R increases NMDA evoked responses. An increase in glutamatergic activity coupled with a decrease in D2R expression, which causes a decrease in inhibition, would lead to a state of hyperexcitability of the nervous system. In this regard, increased cortical and spinal excitability has been shown in RLS patients. Due to the plasticity of the nervous system alterations in one system may cause changes in another. For example hyperexcitability in the spinal cord causes an increase in sensory processing which could lead to an increase in cortical excitability. Therefore the primary location for the origin of RLS symptoms is still unknown. Data from studies assessing sensory evaluations in RLS patients show enhanced spinal processing of nociceptive inputs due to spinal central sensitisation. However not all sensory modalities demonstrate an increase in sensitivity. This indicates that the alterations in nervous system excitability are likely due to complex mechanisms involving interactions between different sensory modalities and the resultant effects on motor outputs. However an explanation for the worsening RLS symptoms in the evening is still lacking. The circadian variation in symptoms may have to do with circadian fluctuations in iron or neurotransmitter concentrations. More research on RLS patients is therefore needed to determine the aetiology of this disorder. As RLS probably involves alterations in neurotransmitters and receptor expressions in the central nervous system causing alterations in nervous system excitability the evaluation of spinal excitability and sensorimotor integration in the spinal cord of RLS patients could

provide important information regarding the aetiology of RLS. A potential method for the evaluation of excitability and sensorimotor integration is through spinal reflexes. The following section will discuss spinal reflexes as well as reflex studies that have been conducted in RLS patients to date.

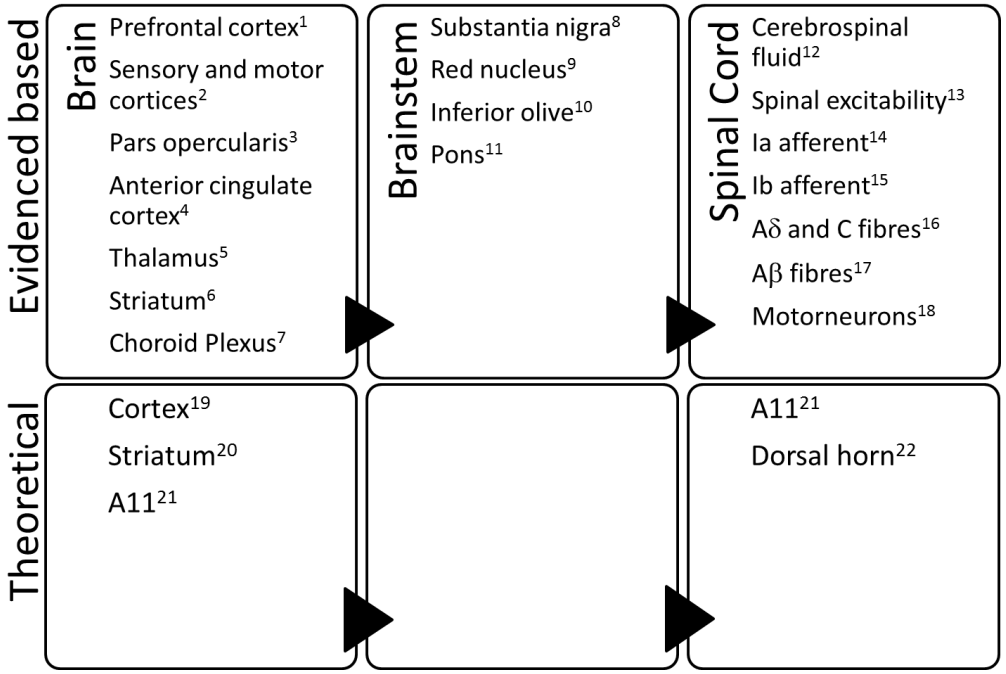

Figure 1: Schematic diagram showing the regions of the brain, brainstem and spinal cord that may be implicated in the pathophysiology of RLS as well as possible regions that could be involved in RLS pathology. **1:** Activation of the prefrontal cortex has been recorded during RLS symptoms (Margariti *et al*., 2012). **2:** Activation of both the motor and sensory cortices has been recorded during RLS symptoms (Margariti *et al*., 2012). In addition Rizzo *et al* (2010) have shown a decrease in the inhibition of the motor cortex by activation of sensory afferents and a few studies have shown decreased cortical silent periods (i.e. increased motor cortex excitability), decreased cortical inhibition and decreased motor cortex thresholds in RLS patients (Entezari-Taher *et al.*, 1999; Tergau *et al.*, 1999; Stiasny-Kolster *et al.*, 2003; Quatrale *et al.*, 2003; Scalise *et al.*, 2004, 2006, 2010; Kutukcu *et al.*, 2006; Nardone *et al.*, 2006; Gorsler & Liepert, 2007; Rizzo *et al.*, 2010; Gunduz *et al*., 2012; Lanza *et al.*, 2015). **3 & 4:** Activation of the pars opercularis and the anterior cingulate cortex has been recorded during RLS symptoms (Margariti *et al*., 2012). **5:** Activation of the thalamus has also been recorded during RLS symptoms (Bucher *et al*., 1997; Margariti *et al*., 2012). In

addition low thalamic iron levels and increased glutamate concentrations have been reported in RLS patients (Godau *et al*., 2008; Allen *et al*., 2013). **6:** Decreased dopamine 2 receptors, decreased dopamine transporter, increased dopamine concentrations and decreased iron concentrations have been shown in the striatum of RLS patients (Turjanski *et al*., 1999; Allen *et al*., 2001; Michaud *et al*., 2002; Godau *et al*., 2008; Connor *et al*., 2009; Earley *et al*., 2011). Activation of the striatum has been recorded during RLS symptoms (Margariti *et al*., 2012). **7:** Decreased choroid plexus iron concentrations have been noted in the RLS patients (Connor *et al*., 2011). **8:** Decreased iron concentrations have been reported in the substantia nigra (Allen *et al*., 2001; Connor *et al*., 2003; Earley *et al*., 2006) as well as increased tyrosine hydroxylase concentration (Connor *et al*., 2009). **9 & 10:** During periodic limb movements activation of the red nucleus and the inferior olivary nucleus has been recorded (Bucher *et al*., 1997). **11:** Changes in two pontine reflexes, the blink reflex and auditory startle reflex, have been noted in RLS patients (Frauscher *et al*., 2007; Uzun *et al*., 2016). **12:** Decreased iron and increased dopamine markers have been noted in the cerebrospinal fluid of RLS patients (Earley *et al*., 2000b; Earley *et al*., 2001; Mizuno *et al*., 2005; Allen *et al*., 2009). **13:** Increased flexor withdrawal reflex responses have been reported in RLS patients, showing increased spinal excitability (Bara-Jimenez *et al*., 2000; Aksu & Bara-Jimenez, 2002; Gunduz *et al*., 2017). Furthermore a decrease in RLS symptoms has been shown following transcutaneous spinal direct current stimulation (Heide *et al*., 2014). **14:** RLS patients display decreased patellar reflex responses (Kerr *et al*., 2011). **15:** Decreased Ib afferent inhibition of the H-reflex has been noted in RLS patients (Rijsman *et al*., 2005; Scaglione *et al*., 2008; Marconi *et al*., 2012). **16:** Hyperalgesia has been reported in RLS patients (Stiasny-Kolster *et al*., 2004; Bachmann *et al*., 2010; Stiasny-Kolster *et al*., 2013; Edwards *et al*., 2011). Cho and colleagues (2017) have also shown decreased C-fibre perception thresholds in RLS patients. **17:** RLS patients have been shown to have increased mechanical detection thresholds (Stiasny-Kolster *et al*., 2013). **18:** Electrophysiology studies have noted increased excitability of motor neurons in RLS patients (Isak *et al*., 2011; Congiu *et al*., 2017). Points 19-22 represent theoretical regions of the central nervous system that may be implicated in the pathophysiology of RLS. **19 & 20:** Possible alterations of adenosine A1 (A1R) and A2A (A2AR) receptor ratios in the cortex and the striatum. **21:** Possible malfunction of the A11 diencephalospinal descending dopaminergic pathway. **22:** Potential changes in dorsal horn receptors (specifically A1R:A2AR and dopamine 2 receptors) as well as a possible spinal hyperglutamatergic state.

1.3. Spinal Reflexes

As previously discussed, the spinal cord is a probable site of the aetiology of RLS with a possible state of spinal hyperexcitability noted in RLS patients. One possible mechanism to assess changes in spinal excitability is to evaluate reflex responses. Motor responses to sensory stimuli, such as those used to elicit a reflex response, are influenced by changes in the state of spinal excitability. A reflex response involves an afferent input, spinal integration and an efferent output. The resultant reflex response therefore is dependent on the activation threshold of the afferent neurons as well as the sensitivity of the efferent neurons responsible for the motor response.

The current thesis focuses on three lower limb reflexes in RLS patients; the plantar reflex, the flexor withdrawal reflex (FWR) and the corresponding crossed extensor reflex. Selection of these reflexes was due to the FWR and the Babinski sign, which is the pathological manifestation of the plantar reflex, having a similar flexion synergy as seen in PLM (Smith, 1985). The leg movements seen during PLM are characterised by dorsiflexion of the foot and toes and flexion of the knee and hip (Hornyak *et al.*, 2006) which are similar to the leg movements noted with the FWR and the Babinski sign (Smith, 1985). Both of these reflexes activate flexion synergy however the method of elicitation differs with the FWR being elicited by a painful stimulus and elicitation of the plantar reflex being by a moving scratching stimulus that activates other afferent receptors in addition to nociceptors. Due to the heterogeneous results from sensory evaluations in RLS patients, assessing reflexes that involve multiple afferent inputs will provide more information on the complex spinal interactions of afferent inputs in RLS patients. The sections to follow will briefly describe these reflexes and discuss the relevant studies that have been conducted assessing reflexes in RLS patients.

1.3.1. The plantar reflex

The plantar reflex involves flexion synergy of the lower limbs. The plantar reflex is elicited by running a blunt object from the heel along the lateral plantar border of the foot, before curving towards the great toe (Van Gijn, 1995). In healthy individuals, the plantar reflex involves hip, knee, ankle, and toe flexion (Van Gijn, 1995). In individuals suffering from neurological disorders that affect the corticospinal tracts, flexion synergy is exaggerated and plantarflexion of the toes is replaced by dorsiflexion (up going movement). The dorsiflexion of the toes is clinically referred to as the Babinski sign (Van Gijn, 1995). Therefore the role of the corticospinal tracts in the plantar reflex appears to be to decrease flexion synergy and inhibit toe extensors (Van Gijn, 1995).

The stimulus used to elicit the plantar reflex is unique in that it involves a moving scratching stimulus that likely causes a summation of action potentials in the neurons accountable for the response (Roby-Brami *et al.*, 1989). When eliciting the reflex the correct amount of pressure must be applied to the sole of the foot. If there is too much pressure it will result in a nociceptive withdrawal reflex. Conversely, if too little pressure is used it may lead to a withdrawal reflex due to some patients finding the sensation ticklish (Lee *et al.*, 2011). A withdrawal reflex may result in dorsiflexion of the hallux which could be mistaken for a Babinski sign.

Studies of the plantar reflex have shown that A-beta, A-delta and C fibre afferent neurons from the S1 dermatome mediate the response (Kugelberg, 1948). The afferent neurons travel to the spinal cord, synapsing in the dorsal horn and eliciting motor responses at L4, L5, S1 and S2 in the spinal cord. Thus, the plantar reflex is a polysynaptic, multi-segmental reflex. The muscles involved in the plantar reflex response include both flexor and extensor muscles (Grimby, 1965). In healthy individuals the flexor response dominates and in

individuals with damage to the pyramidal tracts the extensor response is dominant (Grimby, 1965). Muscles that are activated during the plantar reflex response include; *flexor hallucis longus, tibialis anterior, flexor digitorum longus, gastrocnemii, tensor fasciae latae* and *biceps femoris* (Kugelberg *et al.*, 1960; Van Gijn, 1975, 1976).

Assessment of the plantar reflex in RLS patients may provide insights into the possible dysfunction of lower limb spinal circuitry. The similar profile of a PLM to the Babinski sign suggests that these two responses may share spinal circuitry. The Babinski sign is thought to be present due to a loss of supraspinal inhibition. Hence, evaluation of the plantar reflex may allow for the elucidation of the changes in this spinal circuitry in RLS patients possibly involving a decrease in supraspinal inhibition. However, to date there have been no studies (current thesis excluded) that have been conducted evaluating the plantar reflex in RLS patients.

The plantar reflex is elicited by a scratching stimulus to the sole of the foot. Due to varied results regarding the sensitivity of different sensory modalities in RLS patients it is likely that reflex responses to different methods of elicitation will also vary. Assessment of another lower limb spinal reflex, such as the FWR, that has similar neuronal circuitry but is elicited by activation of nociceptors may provide more information on spinal processing in RLS patients.

1.3.2. The flexor withdrawal reflex

The FWR is a polysynaptic, multi-segmental spinal reflex elicited by a noxious stimulus (France *et al.*, 2002; Sandrini *et al.*, 2005). Noxious stimulation applied to the sole of the foot results in movement of the big toe, contraction of abdominal muscles as well as ankle, knee and hip flexion producing a fast withdrawal movement away from the noxious stimulus (Mayer, 1997). The classic example of this reflex is a person withdrawing their foot in response to stepping on a sharp object. The lower limb FWR can be elicited from numerous

stimulation sites such as the sural nerve and the sole of the foot. The most effective stimulus to evoke the FWR response is a train of electrical pulses (Andersen, 1996; Sandrini *et al.*, 2005). The FWR consists of two distinct parts, the early component (RII) and the late component (RIII). The RII has a latency (the time from the elicitation stimulus to the initiation of muscle activity) of approximately 40 to 60ms and is attributed to activation of A-beta fibres. The RIII has a latency of 90 to 180ms and is mostly ascribed to A-delta fibre activation with some contribution from C-fibres (Sandrini *et al.*, 2005). The RIII of the FWR is generally accepted as being more stable and reproducible when compared to the RII and thus is more widely used in research studies.

The level of the spinal cord where the FWR afferents enter is dependent on the stimulus site (Grimby, 1963). Stimulation of the sole of the foot will activate afferent fibres from the L4, L5, and S2 dermatomes. The afferent information converges in the spinal cord activating various interneurons which then elicit the efferent (motor) component of the reflex. The resultant withdrawal response involves flexion synergy; activation of hip, knee, and ankle flexor muscles. The parameters that are utilised in assessing FWR responses include reflex threshold, EMG latency, and amplitude (size of the response). In addition, studying the FWR allows for the non-invasive investigation of pain pathways both at the spinal level and supraspinally (Sandrini *et al.*, 2005). Furthermore, the RIII component of the FWR correlates to the perception of pain and is often considered an objective measure of pain perception (Willer, 1990).

The FWR not only serves a protective role of withdrawing a limb from a pain stimulus, but is also implicated in postural control (Spaich *et al.*, 2004). When the FWR is elicited a corresponding contralateral crossed extensor reflex occurs. The crossed extensor reflex is a postural reflex in that this response provides stability when the opposite leg withdraws. The

crossed extensor reflex spinal circuit involves multiple interneurons as the reflex response follows 200 to 500ms after the nociceptive stimulus used to evoke the FWR (Guyton & Hall, 2006). Limited research has been done on the crossed extensor reflex. Animal studies have demonstrated that GABA receptor agonists inhibit the crossed extensor reflex (Kawasaki & Matsushita, 1982). The inhibition of the crossed extensor reflex in this scenario is most possibly via supraspinal depression of the vestibulospinal tract, which has an excitatory influence on spinal extensor motor neurons (Kawasaki *et al.*, 1986, 1988).

In terms of the circadian variation of the FWR, in healthy individuals, both the RII and RIII components of the FWR have higher sensory thresholds at midnight when compared to early morning (Sandrini *et al.*, 1986). Hence, the sensitivity of the FWR may be increased in the morning compared to the evening (Sandrini *et al.*, 1986). However, the results from the Sandrini et al. study indicating a circadian variation in the FWR were obtained from a sample of only eight male participants. To establish if there is a circadian variation in the FWR in healthy individuals requires further studies especially in light of data that is contradictory to the results noted by Sandrini et al. (1986). Evidence suggests that dopamine agonists depress the FWR (Paradiso *et al.*, 2002) and it is known that dopamine concentrations are decreased in the evening. Hence, the decreased dopamine concentrations in the evening would lead to decreased inhibition of FWR responses in the evening, which is not consistent with the findings noted by Sandrini and colleagues (1986).

The FWR is a useful tool allowing for the elucidation of the pathophysiological mechanisms in RLS particularly considering that the sensations experience by RLS patients are often described as painful (Winkelmann *et al.*, 2000). Three studies have assessed the FWR in RLS patients. RLS patients exhibit increased FWR excitability compared to control participants in the investigations conducted to date (Bara-Jimenez *et al.*, 2000; Aksu & Bara-Jimenez, 2002;

Gunduz *et al.*, 2017). Moreover, during sleep both the RII and RIII components of the FWR had increased elicitation thresholds in healthy control participants and not in RLS patients (Bara-Jimenez *et al.*, 2000; Aksu & Bara-Jimenez, 2002), which indicate that RLS patients do not display the normal increased inhibition of the FWR during sleep. As the FWR resembles the movements seen in PLM, the absence of inhibition of FWR circuits during sleep in RLS patients may be the cause of PLMS. This further supports the concept of a spinal generator for PLM. The increased FWR excitability in RLS patients could be due to the nociceptive nature of the FWR. The FWR is elicited by the activation of A-delta nociceptive fibres (Sandrini *et al.*, 2005). The presence of hyperalgesia, mediated by A-delta input (Stiasny-Kolster *et al.*, 2004), has been established in RLS patients and may result in the increased excitation of the FWR response seen in RLS patients.

In addition to the FWR, other reflex studies attempting to examine the state of spinal excitability in RLS patients have been conducted. A summary of the results of these studies is in the section that follows.

1.3.3. Assessment of other reflexes in RLS

The majority of previous spinal reflexes studies in RLS patients have assessed the FWR (as discussed in section 1.3.2) and the H-reflex, with one study assessing the patellar reflex in addition to the H-reflex. A summary of the results from previous studies assessing spinal reflexes in RLS patients are shown in Table 1.

The H-reflex is a reflectory response to electrical stimulation of a nerve. The measurements recorded during an H-reflex are; the maximum H-reflex amplitude (H_{max}), the maximum M-wave amplitude (M_{max}), and the H_{max}/M_{max} ratio. No significant difference has been noted in the H_{max}/M_{max} ratios between RLS patients and control participants during the H-reflex irrespective of whether recordings were done in the symptomatic or asymptomatic periods

(Table 1). These results indicate that functioning of Ia afferents and alpha-motor neurons are not altered in RLS patients and thus supports the evidence showing no peripheral nerve conduction problems in RLS.

However, RLS patients may have altered functioning of Ib afferents. A vibratory stimulus activates Ib afferent nerves which cause presynaptic inhibition of agonist alpha-motorneurons in the spinal cord. Rijsman and colleagues assessed the effects of vibratory inhibition on the soleus H-reflex by applying a vibration stimulus to the Achilles tendon and subsequently stimulating the H-reflex. RLS patients had decreased vibratory inhibition of the soleus H-reflex when compared to control participants (Rijsman *et al.*, 2005). These results indicate decreased functioning of Ib afferents in RLS patients. The findings from the Rijsman and colleagues study were reinforced by two additional H-reflex studies in RLS patients that assessed Ib nonreciprocal inhibition of the soleus H-reflex. These investigations stimulated the *gastrocnemius medialis* nerve, as the *gastrocnemius* is an agonist muscle to the *soleus*, at varying inter-stimulus intervals before eliciting the H-reflex (Scaglione *et al.*, 2008; Marconi *et al.*, 2012). The results of both studies were consistent in demonstrating a reduction in Ib inhibition of the H-reflex in RLS patients compared to control participants (Scaglione *et al.*, 2008; Marconi *et al.*, 2012).

In addition to Ib inhibition of the H-reflex, the H2/H1 ratio has been evaluated in RLS patients. The H2/H1 ratio is the relationship between two H-reflex responses following double stimuli at varying inter-stimulus intervals, which gives an indication of spinal excitability. Heidi and colleagues noted increased H2/H1 ratios in RLS patients compared to control participants at inter-stimulus intervals of 200 to 400ms and therefore an increased facilitation of the reflex response (Heide *et al.*, 2014).

Overall, data from H-reflex studies in RLS patients indicate that there are no peripheral nerve conduction problems as evidenced by the fact that H_{max}/M_{max} ratios were not different between RLS patients and healthy participants. However, in RLS patients there is a possible alteration in spinal interneuron circuits, demonstrated by the increased facilitation (increased H2/H1 ratios), and decreased Ib inhibition.

A limitation of using the H-reflex is that an electrically induced reflex is not naturally occurring and hence the conclusions that can be drawn from these studies are limited. A biological equivalent of the H-reflex that has been assessed in RLS patients is the patellar reflex (Kerr *et al.*, 2011). The patellar reflex is a monosynaptic stretch reflex that allows for an interpretation of the effect of the muscle spindle on the reflex response, which is bypassed during the H-reflex. RLS patients had decreased patellar reflex responses when compared to control participants in the evening. Furthermore, there were no significant differences between the H_{max}/M_{max} ratio in RLS and control participants. Naturally occurring muscle spindle reflexes, like the patellar reflex, regulate motorneuron output in a phase-dependant manner (movement dependant changes in responses) (Palmieri *et al.*, 2004). Therefore, as the H-reflex bypasses the muscle spindle, the decreased patellar reflex responses, with normal H-reflex responses, in RLS patients compared to control participants may represent a phase-dependant alteration in motorneuron regulation in RLS patients.

Only one previous study assessed reflex responses during the symptomatic and asymptomatic periods (Kerr *et al.*, 2011). H-reflex responses did not differ in the morning and the evening in RLS patients. Conversely, patellar reflex responses were decreased in the evening, during the symptomatic period, compared to morning reflex responses in RLS patients (Kerr *et al.*, 2011). This was an unexpected finding as it is speculated that RLS patients have increased spinal excitability in the evening and therefore would have

increased reflex responses. The difference in circadian variations between the H-reflex and the patellar reflex in RLS patients suggest that not all spinal circuitry is affected in the same manner in RLS. Further studies assessing the circadian variation of other reflex responses are therefore necessary to provide an improved understanding of circadian changes in spinal excitability in RLS patients.

Studies have also assessed brainstem reflexes in RLS patients. The brainstem reflexes that have been evaluated in RLS patients are the blink reflex and the auditory startle reflex. RLS patients demonstrate normal blink reflexes (Bucher *et al.*, 1996; Leon-Sarmiento *et al.*, 2015; Uzun *et al.*, 2016). In terms of the circadian variation of the blink reflex, in RLS patients there was an increase in late phase recovery of the blink reflex following double stimulation in the evening. This increase in recovery shows increased excitability in the evening in RLS patients, which was in contrast to control participants who present with decreased excitability (Uzun *et al.*, 2016). Auditory startle reflexes recorded with EMG activity of the face, arms and legs in response to an unexpected loud noise showed more frequent auditory startle reflexes with larger responses and shorter latencies in RLS patients compared to control participants (Frauscher *et al.*, 2007). The changes in auditory startle reflex responses in RLS patients could be due to a decrease in descending inhibition of the lumbar-sacral spinal cord.

Table 1: Summary of spinal reflex studies that compared restless legs syndrome (RLS) patients to healthy control participants.

Study	Patients (n)	Controls (n)	Time	Reflex assessed	Response in RLS patients
Bucher et al., 1996	25	15	Asymptomatic period	Soleus H-reflex	Normal
Bara-Jimenez et al., 2000	10	10	Awake: 21:30-22:30 Asleep: NREM	Flexor reflex	Increased excitability
Aksu and Bara-Jimenez, 2002	20-sRLS	20	Awake: 21:30-22:30 Asleep: NREM	Flexor reflex	Increased excitability
Rijsman et al., 2004	9-PLMD (8 RLS)	16	Late afternoon	Soleus H-reflex Vibratory inhibition of the H-reflex	Normal Decreased vibratory inhibition
Scaglione et al., 2008	7	10	AM	Soleus H-reflex Group Ib nonreciprocal inhibition	Normal Decreased Ib inhibition
Kerr et al., 2011	10	7	PM: 17:30-18:30 AM: 08:00-9:30	Peroneal H-reflex	Normal No circadian variation
	9	9	PM: 18:30-19:30 AM: 07:00-8:00	Patellar reflex	PM decreased excitability (compared to controls and to RLS AM)
Marconi et al., 2012	9-RLS 11-sRLS	10	Afternoon	Soleus H-reflex Group Ib nonreciprocal inhibition	Normal Decreased Ib inhibition (in primary RLS only)
Heide et al., 2014	14	14	Symptomatic period (16:00-02:00)	Soleus H-reflex H2/H1 ratio	Normal Decreased inhibition
Gunduz et al., 2017	12	17	Afternoon: 13:30-15:30	Flexor reflex	Increased excitability

AM, morning; NREM, non-rapid eye movement; PLMD, periodic limb movement disorder; PM, evening; sRLS, secondary RLS.

Collectively, the assessment of brainstem reflexes in RLS patients demonstrates a decrease in descending inhibition, which is possibly only evident during the symptomatic phase of RLS.

In summary, one method to test the theory of increased nervous system excitability in RLS patients is though reflex studies. An increase in nervous system excitability would result in an increase in reflex responses in RLS patients. However the data from reflex analyses in RLS patients has failed to consistently show this expected result. Only FWR responses demonstrate increased excitability in RLS patients compared to healthy controls. H-reflex responses did not differ between RLS patients and control participants while patellar reflex responses were shown to be decreased in RLS patients. However, further investigation into spinal circuitry of H-reflexes demonstrated decreased Ib inhibition and increased facilitation of H-reflex responses. Little research has been done assessing circadian variation in reflex responses in RLS patients with the results to date showing no circadian variation in H-reflex responses and a circadian variation in patellar reflex responses. More research is still needed to confirm these results as well to determine if other reflex responses in RLS patients display a circadian variation. The increased excitability of the FWR in RLS patients may also have an effect on the gait cycle as the FWR and the corresponding crossed extensor reflex are associated with locomotion. Spinal circuits involved in these reflexes are connected to the central pattern generators (CPG) thought to be accountable for the regulation of the gait cycle.

1.4. Gait assessment

A main feature of RLS is the urge to move with the relief of symptoms with voluntary movements. Thus, the pathophysiology of RLS may involve the systems that regulate movement. A method to evaluate if there are alterations in voluntary movement patterns is though gait assessment. Gait is defined as the manner or style of locomotion for example

walking. The assessment of gait using EMG recordings allows for the evaluation of muscle activity during walking which has useful clinical and research applications. EMG recordings provide information on the functioning of muscles during the gait cycle which are activated and modified by neural impulses from the cortex, subcortex, and spinal cord. Neurological, skeletal or muscular problems can cause alterations in gait patterns. Therefore, the assessment of gait using EMG recordings is often performed in a clinical setting to provide greater understanding of disturbances in voluntary motor movements (Sutherland, 2001). The analysis of gait using EMG furthers our understanding of normal gait patterns, assists in the treatment of neuromuscular disorders such as cerebral palsy, and improves the management of patients with neuromuscular disorders (Sutherland, 2001).

The gait cycle in humans is composed of the series of events that take place between two successive heel contacts of the same foot with the ground. The cycle consists of two broad phases, the stance phase and the swing phase (Figure 1). The stance phase, which constitutes approximately 60% of the gait cycle, is when the foot of the reference limb is in contact with the ground. The stance phase consists of double stance, when both feet are in contact with the ground, and single limb support, when the contralateral limb is lifted from the ground. The last 40% of the gait cycle is the swing phase when the reference limb is not in contact with the ground.

During the gait cycle EMG activity of the muscles involved in walking follows a predictable pattern. Analysing different phases of the gait cycle is a useful way of detecting and reporting any abnormalities that may occur in this conventional pattern. Hence, the gait cycle has been further divided into eight phases. The eight phases and the approximate percentage of the gait cycle they occupy are as follows; initial contact (heel strike) (0%), loading response (foot flat) (0-12%), mid-stance (12-31%), terminal stance (heel off) (31-

50%), pre-swing (toe off) (50-62%), initial swing (62-75%), mid-swing (75-87%), and terminal swing (87-100%) (Perry, 1992) as shown in Figure 1.

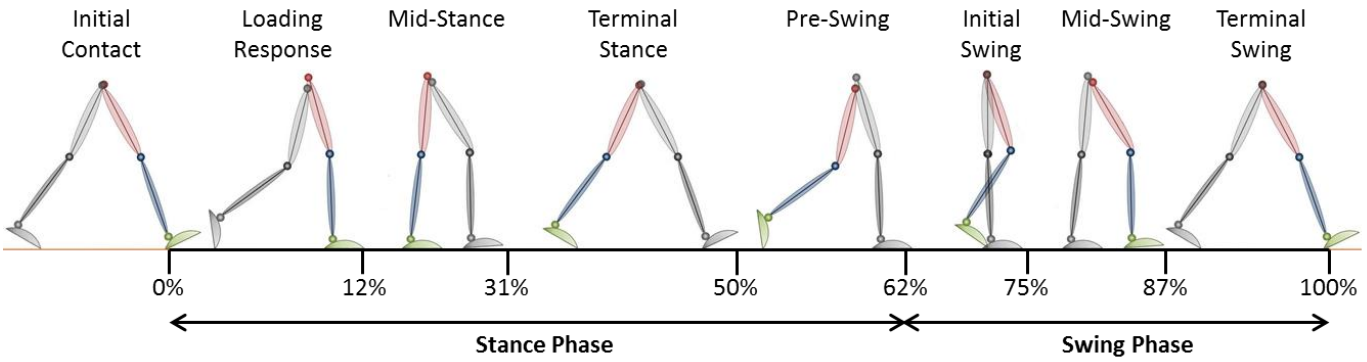

Figure 2: Events of the normal gait cycle. Image modified from "Gait Cycle as proposed by Jaquelin Perry 1992" authored by chipotng [sic], retrieved from https://commons.wikimedia.org/wiki/File:GaitCycle_by_JaquelinPerry.jpg, licensed under CC-BY-SA-3.0

In terms of the distal muscles involved in the gait cycle, during mid-stance there is controlled forward movement and the plantar flexor muscles (*gastrocnemius* and *soleus* muscles) are activated. The plantar flexor muscles remain active through the terminal stance and pre-swing phases. Following toe off, dorsiflexor (*tibialis anterior*) muscles are then activated to assist in clearing the foot from the ground. The *tibialis anterior* muscle then remains active through the swing phase, during heel contact and the loading response until activation of the plantar flexor muscles begin again during mid-stance (Sutherland, 2001). With regards to the proximal leg muscles activated during the gait cycle, knee flexor muscles (*biceps femoris, semimembranosus,* and *semitendinosus*) are activated during the swing phase. Knee extensor muscles (*quadriceps femoris*) are activated at the end of the swing phase and through the beginning half of the stance phase (Sutherland, 2001).

The normal gait cycle involves a complex system of dynamic sensorimotor interactions. Muscle activation, and the resultant movement, is regulated through afferent feedback that synapses in the spinal cord onto motorneurons, inhibitory and excitatory interneurons, and the spinal CPGs. Afferent input during the gait cycle provides crucial proprioceptive feedback regarding muscle position. Moreover, cutaneous afferent feedback provides information related to changes in the environment (Rossignol *et al.*, 2006). Proprioceptive and cutaneous afferent feedback determines the overall timing of the gait cycle by adjusting the duration of the various phases and facilitating the switch between flexor and extensor muscle activity (Rossignol *et al.*, 2006).

The influence of afferent inputs on the gait cycle may be mediated through the CPGs. The spinal CPGs, which are thought to be located in the lumbar region of the central and anterior grey matter of the spinal cord, are a specialised group of interneurons that are responsible for the regulation of the gait cycle. CPGs control the switching between flexor and extensor motor neurons during the gait cycle (Rossignol *et al.*, 2006; Guertin, 2013).

The main sensory inputs that possibly have direct access to the CPGs are proprioceptive and cutaneous afferents from mechanoreceptors and nociceptors in the foot (Van De Crommert *et al.*, 1998). Proprioceptive afferents include Ia afferents and Ib afferents from muscle spindles and golgi-tendon organs respectively, which provide information on muscle stretch and muscle tone. Most studies indicate that Ib afferents from extensor muscles have a direct inhibitory action on the flexor half of the CPG during the stance phase of gait. Stimulation of Ib afferents during the gait cycle prolongs the extensor portion of the stance phase, delaying the onset of the swing phase (Van De Crommert *et al.*, 1998). Therefore the afferent discharge from the tensed extensor muscles must fall below a certain threshold before the flexor half of the CPG can be activated and the swing phase can occur. Similarly, the

cutaneous afferents from mechanoreceptors in the foot are most active during the stance phase, particularly during the loading response, and have similarly been shown to prolong the stance phase and delay the onset of the swing phase (Van De Crommert *et al.*, 1998).

It is important to note that reflex responses may differ during different phases of movement. Reflex responses during the gait cycle are phase dependant with inhibition of statically occurring reflexes during movement and even reflex reversal (Rossignol *et al.*, 2006). Reflex reversal involves the opposite reflex response occurring from the same stimulus depending of the phase of the gait cycle (Duysens *et al.*, 1992). In this regard, the FWR during gait has differing effects depending on the phase of the gait cycle (Spaich *et al.*, 2004). Studies assessing changes in the FWR during gait have shown facilitation of *tibialas anterior* activity during the early swing phase that switches to suppression in the late swing phase (Zehr *et al.*, 2012). Similarly, eliciting a withdrawal reflex during gait causes facilitation of *soleus* activity just after heel strike and suppression after heel off (Spaich *et al.*, 2004).

As discussed is section 1.2.3, RLS was believed to be a hypo-dopaminergic disorder due to the positive response of RLS patients to dopamine agonist treatment. Alterations in dopamine levels would have consequences on voluntary movements as seen in Parkinson's disease, a hypo-dopaminergic disorder. Therefore, Paci *et al*. theorised that RLS patients may show minor alterations in the gait cycle that can only be detected during gait analysis. Paci and colleagues thus conducted the only study to date that assesses gait in RLS patients. RLS patients had abnormal activation of the gastrocnemii during the swing phase of the gait cycle where there is normally no activity in these muscles (Paci *et al.*, 2009). The gait assessments in these participants were however conducted between 10:00 and 11:00, which is during the non-symptomatic phase of RLS. Further studies are required in order to confirm if gastrocnemii activity during the swing phase of the gait cycle is a consistent pathological

finding in RLS. Furthermore, the circadian influence on the gait cycle in RLS patients still needs to be investigated.

1.5. Motion Analysis

Parts of the data collected in the studies of this thesis have made use of techniques not conventionally used in the neurological study of patients, namely kinematics. For my masters' dissertation, I described the use of these kinematic techniques in neurological studies and supply my description in appendix C.

1.6. Rationale for the investigations undertaken in the current thesis

Research has shown that the symptoms of RLS are most likely caused by abnormalities of the central nervous system such as hyperexcitability of the spinal cord and/or decreased cortical and spinal inhibition. Nevertheless, the exact pathophysiological mechanisms involved in the aetiology of RLS have yet to be fully elucidated. Furthermore, an essential diagnostic criterion required for the diagnosis of RLS is a circadian variation in the symptom profile. However, few studies assessing hyperexcitability of the nervous system in RLS patients fully investigated if there were circadian variations in their results. As such, the novel investigations included in this thesis focused on circadian changes in spinal excitability, a key pathophysiological focus of RLS, in order to confirm if changes in spinal excitability are related to the aetiology of RLS.

The assessment of spinal reflexes provides a non-invasive mechanism to assess changes in spinal excitability as the resultant reflex responses are influenced by changes in the state of spinal excitability. Therefore the first two studies of my thesis focused on spinal excitability in RLS patients by kinematically and electromyographically assessing reflex responses of three lower limb reflexes; the plantar reflex, the FWR and the crossed extensor reflex. The reason the studies in the current thesis used kinematics in conjunction with

electrophysiology, is that it allowed for the measurement of both movement and muscle activity produced by these reflexes. This is a novel approach in the field however it allowed for an objective repeatable measurement of the physical movement of the limbs during the reflex responses, thus improving accuracy and removing bias compared to subjectively assessing the reflex movements. In addition, the essential diagnostic criterion of circadian variation in the RLS symptom profile was also taken into account and the three reflexes were assessed at two different times of the day: the asymptomatic morning and symptomatic evening periods.

The initial study, designed to tackle the above research goal evaluated the plantar reflex response in RLS patients and control participants during the symptomatic and asymptomatic phases. The plantar reflex was chosen as the movements seen during a PLM have been noted to be similar to the movements of the Babinski sign, which is the pathological manifestation of the plantar reflex (Smith, 1985). Since Smith documented this similarity more than thirty years ago, no subsequent studies have been done assessing plantar reflex responses in RLS patients. A loss of supraspinal inhibition is thought to cause a Babinski sign. The similarity of movements during a PLM and the Babinski sign indicate that these two responses may share spinal circuitry. Hence, by evaluating if there are any circadian changes in plantar reflex responses in RLS patients greater understanding of the changes in this spinal circuitry possibly involving an evening decrease in supraspinal inhibition, may be possible.

The second study assessed the circadian variation in FWR and crossed extensor reflex responses in RLS patients and control participants. The FWR reflex response, like the Babinski sign, has been noted to resemble the movements during PLM and therefore may also share common spinal circuitry. Unlike the plantar reflex, which has not been assessed in RLS patients, increased excitability of the FWR has been demonstrated in RLS patients.

However, to date the circadian variation in FWR responses in RLS patients has not been assessed. Additionally, in previous studies assessing the FWR in RLS patients, the corresponding contralateral crossed extensor reflex has not been considered. The crossed extensor reflex is elicited simultaneously with the FWR. Thus by assessing the FWR in combination with the corresponding crossed extensor reflex in this thesis provided an additional avenue for evaluating potential variations in spinal circuitry in RLS patients.

A possible reason for the increased excitability of the FWR in RLS patients may be due to the hyperalgesia shown in RLS patients. The FWR is used as a non-invasive assessment for the investigation of the functional status of nociceptive pathways. In this regard the FWR is elicited by a nociceptive stimulus. Therefore an additional reason for assessing the plantar reflex and the FWR in RLS patients is due to the heterogeneous results from sensory evaluations in RLS patients. The plantar reflex is elicited using a scratching stimulus or a moving pressure and the FWR is elicited by a nociceptive stimulus. Therefore by assessing reflexes that involve multiple afferent inputs, but share similar spinal circuitry, provided more information on the complex spinal interactions of afferent inputs in RLS patients.

By assessing plantar reflex, FWR, and crossed extensor reflex responses in RLS patients the first two studies of my thesis provided information on circadian variations in spinal excitability in a static environment. However, a key diagnostic feature of RLS involves movement – the urge to move, the onset of sensations at rest and the relief of the sensations with movement. Investigations into the systems that drive movement may be crucial in determining the pathophysiological mechanisms underlying RLS. A potential technique that may be utilised to evaluate movement in RLS patients is the analysis of gait. Reflex responses elicited from the sole of the foot are implicated in locomotion as the spinal networks involved in these reflexes play a role in the complex generation and regulation of

gait. However, responses to afferent input are phase-dependent and may differ in dynamic versus static conditions. Therefore as alterations in afferent processing have been documented in RLS patients, these alterations may cause variations in the gait cycle of RLS patients that can only be detected using sophisticated gait analysis. Hence, my third study investigated circadian variations of neuromuscular aspects of gait in patients with RLS in order to examine circadian variations in dynamic spinal excitability in RLS patients.

Therefore the main aim of the studies that comprise this thesis was to investigate circadian variations in spinal excitability in RLS patients both statically and dynamically.

1.7. Aims

The specific aims for the studies conducted in this thesis, which follow from the rationale above, are as follows:

1. To assess the circadian variation in the state of spinal excitability in RLS patients utilising electromyographic and kinematic measurements of the plantar reflex.
2. To further evaluate the the circadian variation of the state of spinal excitability, and to determine the effect of different afferent inputs, in RLS patients utilising electromyographic and kinematic measurements of the FWR and corresponding crossed extensor reflex.
3. To assess the neuromuscular profile of the gait cycle in RLS patients to determine if there are circadian variations or disturbances in the gait cycle in RLS patients and thus in dynamic spinal excitability.

Chapter 2: Plantar reflex excitability is increased in the evening in restless legs syndrome patients.

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2.1. Abstract

Objective: To investigate if diurnal changes in spinal excitability (plantar reflex) occur in restless legs syndrome (RLS) participants compared to healthy matched controls.

Methods: Thirteen RLS participants and 13 healthy control participants' plantar reflex responses were evaluated in the evening (PM) and the morning (AM). Plantar reflex responses were assessed electromyographically, using motion analysis (kinematically) and by subjective nociception (Visual Analogue Scale).

Results: RLS participants showed a circadian variation in plantar reflex responses whilst control participants did not. Evening ankle angle changes were larger and faster in RLS participants compared to morning responses. In addition RLS participants displayed significantly smaller change in ankle angle and significantly slower ankle movements in the evening and the morning as well as significantly lower *lateral gastrocnemius* maximum amplitude in the compared to control participants.

Conclusion: The findings of the current study support the theory of RLS circadian fluctuations in spinal excitability. An unexpected finding was decreased plantar reflex responses in RLS participants compared to healthy control participants. However this finding supports the theory of mechanical hypoesthesia in RLS. The results of this study provide further insight into the pathophysiology of RLS, highlighting that not all sensory processing is affected in the same manner.

2.2. Introduction

Restless legs syndrome (RLS) is a neurological disorder presenting with sensorimotor symptoms that are most pronounced in the evening (Walters, 1995; Hening *et al.*, 1999; Trenkwalder *et al.*, 1999). A motor symptom commonly associated with RLS is involuntary leg movements, called periodic limb movements (PLM) (Montplaisir *et al.*, 1997; Scofield *et al.*, 2008). PLM are characterised by dorsiflexion of the foot and toes, and flexion of the knee and hip (Hornyak *et al.*, 2006), thought to be similar to the flexion synergy seen in the pathological plantar reflex response; a positive Babinski sign (Smith, 1985). A positive Babinski sign is caused by a loss of supraspinal inhibition caused by a lesion to the pyramidal tracts (Van Gijn, 1995). The resemblance of PLM to a positive Babinski sign suggests that RLS symptoms may be caused by a decrease in supraspinal inhibition causing a state of spinal hyperexcitability (Rijsman *et al.*, 2005).

Previous studies have suggested a state of spinal hyperexcitability in RLS patients (Wechsler *et al.*, 1986; Bara-Jimenez *et al.*, 2000; Rijsman *et al.*, 2005); however details of the aetiology of RLS are still largely unknown. Since the spine is a key site of interest in the aetiology of RLS, the study of reflex responses is an ideal tool to assess whether there is an altered state of spinal excitability in RLS.

Although various spinal reflexes have previously been assessed in RLS patients, few studies have assessed a circadian variation in RLS spinal excitability. An evening state of spinal hyperexcitability, for example, may be the cause of an evening increase in RLS symptoms. A study looking at the patellar reflex showed decreased patellar reflex responses during the symptomatic phase compared to the asymptomatic phase in RLS participants (Kerr *et al.*, 2011). Data from several studies have shown normal H-reflex responses in RLS patients during the asymptomatic phase of the disorder (Bucher *et al.*, 1996; Scaglione *et al.*, 2008;

Marconi *et al.*, 2012). Bara-Jimenez *et al*. (2000) and Gündüz *et al*. (2017) demonstrated increased flexor withdrawal reflex response in RLS individuals during the symptomatic and asymptomatic phases respectively. Despite these findings, to date, no studies have investigated the plantar reflex in RLS.

Currently, very little evidence from reflex based studies support the concept of RLS circadian variation in spinal excitability. Further studies of reflexes are thus necessary to provide more information on RLS pathology. To address this gap in knowledge, this paper will examine the excitability state of the spinal cord of RLS participants using the currently uninvestigated plantar reflex as it may possibly provide evidence of decreased supraspinal inhibition in RLS. Due to the known circadian variation in RLS symptoms, the plantar reflex will be investigated at two different times of the day in order to establish if there is a circadian variation of the plantar reflex, and thus spinal excitability, in RLS participants. We hypothesised that due to the circadian rhythm of RLS symptoms, the plantar reflex response would be increased in the evening compared to the morning in RLS participants. Additionally, due to the theorised spinal hyperexcitability in RLS participants, we hypothesised that RLS participants would display increased plantar reflex responses in comparison to healthy control participants.

2.3. Materials and Methods

2.3.1. Participants

Thirteen RLS participants were recruited through local advertisements. A comprehensive screening questionnaire, including the essential diagnostic criteria for RLS along with questions to rule out mimics, was completed by each participant. All participants who met the updated International Restless Legs Syndrome Study Group (IRLSSG) diagnostic criteria (Allen *et al.*, 2014) and had no other sleep or neurological disorders were included in the study. RLS participants completed the IRLSSG severity scale (Walters *et al.*, 2003). Thirteen

healthy age and gender matched control participants, who did not have any sleep or neurological disorders, were also recruited. Prior to participating in the study all participants signed a written informed consent sheet. Ethical clearance for the current study was obtained from the human research ethics committee of the associated tertiary institution (M140322). Anthropometric data was obtained for all participants.

2.3.2. Study procedure

The plantar reflex was elicited bilaterally by stroking the lateral plantar surface of the foot of participants in the supine position using a specialised Babinski reflex hammer designed to resemble the reflex hammer used in clinical practice while allowing for the measurement of the force that was exerted on the foot to be recorded (as described in Dafkin *et al*. (2014)). A 10cm visual analogue scale (VAS) (anchors: “no pain” and “most severe pain ever felt”) was completed by the participant after each plantar reflex to determine the subjective pain experienced by the participant during the elicitation of the plantar reflex. Plantar reflex testing was done between 20:00PM and 22:00PM and repeated between 6:30AM and 8:00AM the following morning.

The movements of the feet and legs were recorded kinematically with 18 Optitrack high speed cameras (Natural Point, Oregon, USA) at 100Hz. Calibration and 3D tracking was performed using AMASS (C-Motion Germantown, Maryland, USA). Calibration accuracy was calculated to be sub-millimetre.In order to record joint movements, retro-reflective markers were placed on both feet and legs of each participant at specific anatomical landmarks. These landmarks were: head of proximal phalanges and distal phalanges of the hallux (first digit), third and fifth digits, tuberosity of the fifth metatarsal, plantar surface of the heel and apex of the arch of the foot; lateral and medial malleoli (ankle), midpoint of the tibia, medial

and lateral epicondyles of the femur (knee), midpoint of the femur, greater trochanter (hip), anterior superior iliac spine and the iliac crest.

Trigno Wireless Electromyograph (EMG) sensors (Delysys, Natick Massachsetts, USA) connected to a PowerLab (ADI Instruments, 26T, Sydney, Australia) were used to record muscle activity simultaneously with the kinematic data. Muscle activity was recorded bilaterally in the following leg muscles: *tibialis anterior*, *lateral gastrocnemius*, *rectus femoris* and *biceps femoris* by placing one wireless EMG sensor on the belly of each muscle according to the SENIAM guidelines.

2.3.3. Data analysis

The variables measured from the Babinski reflex hammer were the average force (Newtons) and maximum force (Newtons) exerted by the reflex hammer on the foot. All biomechanical variables were calculated using raw marker trajectories in MatLab 7 (Mathworks, Natick, Massachusetts, USA) with custom written algorithms. The hallux, ankle and knee change in angles (°) during the plantar reflex were calculated from initial position to the maximum hallux plantarflexion angle, ankle dorsiflexion angle and knee flexion angle respectively. The angular velocities (°/s) of the knee, ankle and hallux during the plantar reflex were calculated by dividing the change in angle by the time taken to reach the maximum angle. Muscle activity was reported as the maximum amplitude of all muscle contractions (mV) calculated with LabChart7 (ADI Instruments, Sydney, Australia).

Statistical package GraphPad Prism 5 (San Diageo, USA) was used for statistical analysis. Kinematic, EMG and VAS data were non-parametric and as such are expressed as median and inter-quartile range. Characteristics of participants, meanstroke force and maximum stroke force were parametric and are reported as mean ± standard deviation. As there was no difference between the right and left sides, for simplicity of reporting, data from both

legs were combined. T-tests assessed the difference in anthropometric measurements of RLS and control participants. Paired T-tests compared the evening and morning average and maximum hammer forces while T-tests were performed to assess average and maximum hammer force differences between RLS participants and controls. Wilcoxon signed rank tests compared the kinematic, EMG and VAS variables between the evening and morning reflexes. Kinematic, EMG and VAS variables between RLS participants and control participants at each time point were compared by means of Mann Whitney U-tests.

2.4. Results

Table 1 presents demographic and anthropometric data as well as RLS characteristics of all participants. There were no significant anthropometric differences between RLS and control participants (Table 1). All participants with RLS were treatment naïve except for one who discontinued dopaminergic treatment (Pexola) three days before reflex testing.

Table 1: Characteristics of restless leg syndrome (RLS) participants and control participants.

	RLS	Controls	*p-value*
Number (male:female)	13 (4:9)	13 (4:9)	
Age (years)	45.5±14.5	42.1±14.5	*0.56*
Height (m)	1.7±0.1	1.7±0.1	*0.45*
Weight (kg)	70.0±14.9	76.0±24.1	*0.45*
BMI (kg/m^2)	25.3±4.0	25.8±4.4	*0.75*
Age of onset of RLS (years)	28.4±14.2	N/A	
Duration of RLS (years)	16.6±14.0	N/A	
RLS severity (IRLSSG severity scale score)	17.2±7.3	N/A	
Family history of RLS (%)	31	N/A	

Data is expressed as mean±SD. BMI, body mass index; IRLSSG, international restless legs syndrome study group.

There were no significant differences for maximum stroke force between evening and morning (RLS PM: 3.0±0.7N, AM: 3.0±0.8N p=0.99; control PM: 3.4±1.1N, AM: 3.3±0.9N p=0.88) or between RLS participants and controls (PM: p=0.36; AM: p=0.39). Mean stroke force did not differ significantly between evening and morning for RLS (PM: 1.7±0.5N, AM:

1.7±0.6N; p=0.69) or controls participants (PM: 1.3±0.7N, AM: 1.1±0.7N; p= 0.37); or between RLS participants and control in the evening (p= 0.06). However, in the morning, the mean stroke force needed to elicit the reflex response in the RLS participants was significantly greater than for the control participants (p= 0.02).

Kinematic, EMG and VAS statistical results are summarised in Table 2. The reflex responses of the RLS participants displayed a circadian variation in ankle angle and the angular velocity of the ankle with both being significantly increased in the evening compared to the morning (Table 2). No circadian variation was shown for control participants (Table 2). However both in the evening and the morning control participants had a significantly greater change in ankle angle and significantly quicker ankle movements compared to RLS participants (Table 2). The maximum amplitude of the *lateral gastrocnemius* of the control participants was significantly increased compared to the RLS participants' response in the morning but not the evening (Table 2). There were no other significant differences for plantar reflex responses between RLS and control participants or between the evening and the morning (Table 2). None of the recorded reflexes showed hallux dorsiflexion (a positive Babinski sign). Due to markers not being visible or detachment of EMG sensors from the participant, not all participants' biomechanical or EMG data were analysed thus leading to the differences in n-values seen in Table 2.

Table 2: Restless legs syndrome (RLS) participants and control participants plantar reflex parameters in the evening (PM) and the morning (AM).

		Time	RLS	Controls	*p-value*
Change in angle (°)	Knee	PM	0.79(0.45-2.23)	1.86(0.61-2.81)	*0.26*
		AM	0.58(0.44-1.18)	2.35(0.59-4.71)	*0.06*
		p-value	*0.38*	*0.09*	
	Ankle (RLS n= 12)	PM	7.91(4.27-19.08)	25.66(6.80-39.63)	*0.04***
		AM	6.79(2.40-11.29)	20.96(8.41-34.38)	*0.02***
		p-value	*0.03**	*0.79*	
	Hallux (Control n=12)	PM	11.21(3.84-22.50)	19.50(11.35-21.29)	*0.15*
		AM	10.22(6.76-16.49)	17.29(5.52-20.94)	*0.24*
		p-value	*0.50*	*0.27*	
Angular velocity (°/s)	Knee	PM	0.25(0.18-1.00)	0.48(0.18-1.10)	*0.57*
		AM	0.22(0.12-0.34)	0.63(0.17-1.71)	*0.09*
		p-value	*0.09*	*0.17*	
	Ankle (RLS n= 12)	PM	2.40(1.18-5.62)	6.68(2.23-12.81)	*0.04***
		AM	2.14(0.81-3.22)	6.42(2.54-11.99)	*0.01***
		p-value	*0.00**	*0.59*	
	Hallux (RLS n= 12; control n=12)	PM	3.64(1.33-9.30)	4.86(3.21-8.44)	*0.34*
		AM	3.25(1.85-6.12)	4.24(1.61-7.76)	*0.47*
		p-value	*0.23*	*0.11*	
Maximum Amplitude (mV)	Tibialis anterior	PM	0.45(0.08-0.74)	0.46(0.21-1.22)	*0.47*
		AM	0.18(0.06-0.84)	0.44(0.26-0.65)	*0.31*
		p-value	*0.45*	*0.17*	
	Lateral gastrocnemius	PM	0.05(0.02-0.10)	0.07(0.04-0.17)	*0.11*
		AM	0.03(0.02-0.06)	0.08(0.03-0.21)	*0.04***
		p-value	*0.83*	*0.79*	
	Rectus femoris (RLS n=12)	PM	0.05(0.03-0.11)	0.12(0.06-0.23)	*0.15*
		AM	0.06(0.04-0.12)	0.10(0.04-0.21)	*0.50*
		p-value	*0.27*	*0.64*	
	Biceps femoris (RLS n=11)	PM	0.06(0.04-0.09)	0.05(0.04-0.15)	*0.64*
		AM	0.06(0.04-0.10)	0.06(0.03-0.14)	*0.86*
		p-value	*0.76*	*0.68*	
VAS (mm)		PM	20.50(3.00-40.50)	8.00(5.00-22.00)	*0.64*
		AM	26.25(3.50-32.00)	7.50(2.88-20.75)	*0.54*
		p-value	*0.18*	*0.96*	

Data represented as median and interquartile range. Wilcoxon signed-rank tests were used to compare between PM and AM. Comparisons between RLS participants and control participants were done with Mann-Whitney U-tests.

*significant difference between RLS PM and RLS AM.

**significant difference between control participants and RLS participants. VAS, visual analogue scale. n=13 for both groups unless indicated otherwise.

2.5. Discussion

The first hypothesis of the current study was that the plantar reflex response displays circadian variation in RLS participants. The current study showed, in agreement with the hypothesis, that there is a circadian variation of the plantar reflex response in RLS participants. This circadian variation in RLS participants was shown by an increased plantar reflex responses in the evening compared to the morning. The increase in the evening is very slight and is unlikely to have been felt by the participants however it may provide information about the aetiology of RLS. In contrast; control participants exhibited no significant difference between evening and morning plantar reflex responses. An unexpected finding of the current study was that the plantar reflex responses of RLS participants were significantly decreased compared to control participants both in the evening and the morning.

A circadian variation in the degree of movement of the foot during the plantar reflex was shown in RLS participants; however there was no corresponding significant circadian variation in EMG amplitude. The lack of agreement in results between physical movement and EMG activity could be due to kinematics being a more sensitive measure than single site EMG and thus revealing more subtle variations. Another explanation for this difference could be a possible circadian variation in muscle activity in other muscles involved in dorsiflexion of the ankle, for example the *extensor digitorum longus* muscle, that were not assessed in the current study. The EMG amplitude of the *lateral gastrocnemius* (partially responsible for foot movements) was significantly smaller in RLS participants compared to control participants in the morning. A possible reason for the lack of significant EMG differences in the evening could be due to the large inter-individual variation in EMG amplitudes (shown by the wide interquartile ranges) and as such showing a significant

difference in EMG amplitude is difficult. The kinematic analysis of the present study showed only significant changes in ankle angles and not knee or toe angles. This is likely due to the greater range of motion of the ankle during the plantar reflex than the smaller, and less detectable, movements of the knee and toe during the reflex response.

None of the RLS participants exhibited positive Babinski responses either in the evening or the morning. As a positive Babinski sign results from a loss of descending spinal inhibition (Van Gijn, 1995), the lack of positive Babinski responses in RLS participants show that there is not a decrease in supraspinal inhibition, caused by pyramidal tract dysfunction, in RLS. This is supported by previous research which has shown normal maximum H-reflex to maximum M response ratios (H/M ratios) during the H-reflex in RLS participants (Bucher *et al.*, 1996; Rijsman *et al.*, 2005; Marconi *et al.*, 2012) as increased H/M ratios are evidence of pyramidal tract dysfunction (Rijsman *et al.*, 2005).

In reviewing the literature, no previous studies focussing on the plantar reflex in RLS participants were found. The circadian variation in RLS participants plantar reflex responses seen in the present study further supports the idea, suggested by Bara-Jimenez *et al*. (2000), of increased spinal excitability in RLS participants, specifically during the symptomatic phase of the disorder. Previous studies looking at other spinal reflexes in RLS participants show conflicting results in that some found increased reflex responses in RLS participants compared to control participants (Bara-Jimenez *et al.*, 2000; Gunduz *et al.*, 2017) and others showed no difference between RLS and control participants (Bucher *et al.*, 1996; Rijsman *et al.*, 2005; Marconi *et al.*, 2012). A possible reason for the discrepancy in results may be due to the reflexes being assessed either during the symptomatic phase (Bara-Jimenez *et al.*, 2000) or the asymptomatic phase (Bucher *et al.*, 1996; Rijsman *et al.*, 2005; Marconi *et al.*, 2012; Gunduz *et al.*, 2017) of the disorder and not during both phases. Kerr *et al*. (2011)

showed a circadian variation in the patellar reflex however they found decreased patellar reflex responses in the evening compared to the morning in RLS participants. A study done looking at the circadian variation of perception thresholds of A-beta, A-delta and C fibers in RLS participants, showed decreased thresholds in all three fiber types in the symptomatic phase compared to the asymptomatic phase (Cho *et al.*, 2017). Considering that during elicitation of the plantar reflex A-beta fibers, A-delta fibers and C-fibers are activated (Kugelberg, 1948), the evening decrease in nerve fiber activation thresholds shown by Cho *et al*. (2017) may provide a possible explanation for the RLS participants increased evening plantar reflex responses compared to the morning as shown in the current study.

While the plantar reflex response within RLS participants is consistent with the literature regarding the increased response in the evening, the comparison to control participants is less consistent. Surprisingly, the results of the current study demonstrated that RLS participants had decreased plantar reflex responses in comparison to control participants during both symptomatic and asymptomatic periods. Previous studies, particularly focusing on the flexor withdrawal reflex, have shown increased reflex responses in RLS participants compared to control participants (Bara-Jimenez *et al.*, 2000; Gunduz *et al.*, 2017). Both the flexor withdrawal reflex and the plantar reflex involve stimulation of plantar afferents as well as activation of flexor leg muscles (Binder *et al.*, 2009); however the method of eliciting the plantar reflex differs from that of the flexor withdrawal reflex. The plantar reflex is elicited using a scratching stimulus or a moving pressure (Roby-Brami *et al.*, 1989) whereas the flexor withdrawal reflex is elicited by a nociceptive stimulus (Binder *et al.*, 2009). In both the previous studies looking at flexor withdrawal reflex responses in RLS participants the flexor withdrawal reflex was elicited with electrical stimulation; eliciting electrodes were positioned on the plantar surface of the foot (Bara-Jimenez *et al.*, 2000; Gunduz *et al.*, 2017).

In the present study the plantar reflex was elicited by stroking the lateral plantar surface of the foot.

A possible explanation for the decreased plantar reflex responses in RLS participants, seen in the current study, might be that control participants were withdrawing from the stimulus thus leading to the quicker and larger foot movements compared to RLS participants. The maximum stroke force used to elicit the reflex on RLS participants and control participants did not differ significantly however the average stroke forces were lower for control participants in the morning and the evening, though only significantly lower in the morning. The average stroke forces in the evening were not significantly different ($p=0.06$) however with a larger sample size it is possible that the average stroke forces would also be significantly lower in the evening. Therefore, it is possible that the smaller average force used to elicit the plantar reflex in control participants is due to control participants pulling their foot away from the stimulus. Control participants withdrawing from the stimulus and RLS participants not withdrawing may possibly be explained by RLS participants having tactile hypoesthesia.

Tactile hypoesthesia (increased mechanical detection thresholds) has previously been shown in RLS participants (Stiasny-Kolster *et al.*, 2013) suggesting that RLS participants need more mechanical stimulation to feel a sensation than do healthy control participants. Control participants in the current study might have found the sensation of eliciting the plantar reflex ticklish which may have caused them to withdraw (Lee *et al.*, 2011). The low VAS scores seen in the present study imply that neither the RLS nor control participants found the elicitation of the reflex painful. Therefore it is unlikely that the control participants' withdrawal was from pain. RLS participants may have an increased mechanical detection threshold and therefore did not experience the same ticklish sensation and did not withdraw

from the stimulus. Stiasny-Kolster *et al*. (2013) hypothesised the reason for mechanical hypoesthesia in RLS is possibly that a constant state of hyperalgesia results in concurrent mechanical hypoesthesia as a consequence of continuous nociceptive fiber activation causing presynaptic inhibition of mechanoreceptive fibers (Jänig & Zimmermann, 1971; Magerl & Treede, 2004; Geber *et al.*, 2008). In agreement with the findings of the present study, the decreased patellar reflex responses shown by Kerr *et al*. (2011) could in part be due to mechanical hypoesthesia in RLS participants.

A limitation of the current study is the small sample size. Fortunately we were still able to show significant differences between RLS participants and controls. Further research on the plantar reflex in RLS patients, using a larger sample size, is recommended to confirm the reproducibility of these findings. Another limitation is the use of surface EMG and thus not being able to assess muscle activity in deep set muscles.

In conclusion, the present study set out to assess the circadian variation of the plantar reflex in RLS participants. A major finding of the current study was an evening increase in plantar reflex responses in RLS participants, thus implying a circadian rhythm in spinal excitability in RLS. Another major finding of the current study was decreased plantar reflex responses in RLS participants compared to healthy control participants. This finding was surprising and supports the theory of mechanical hypoesthesia in RLS proposed by Stiasny-Kolster *et al*. (2013). The findings of this research provide insights to the growing understanding of RLS pathophysiology by supporting the theory of circadian fluctuations in spinal excitability whilst also suggesting possible alterations in sensory processing in RLS patients.

Chapter 3: Circadian variation of flexor withdrawal and crossed extensor reflexes in restless legs syndrome patients.

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3.1. Abstract

An evening state of spinal hyperexcitability has been proposed to be a possible cause of evening increases in restless legs syndrome (RLS) symptoms. Thus the objective of the current study was to assess the circadian variation in spinal excitability in RLS patients based on flexor withdrawal reflex (FWR) and crossed extensor reflex responses. The reflexes were elicited on 12 RLS participants and 12 healthy control participants in the evening (PM) and the morning (AM). Reflex response magnitudes were measured electromyographically and kinematically. Both the reflexes showed a circadian rhythm in RLS participants but not in control participants. Changes in ankle (median FWR PM: 16.0° vs AM: 2.8°, p=0.042; crossed extensor reflex PM: 0.8° vs AM: 0.2°, p=0.001) angle was significantly larger and ankle angular velocity (median FWR PM: 38.8°.s^{-1} vs AM: 13.9°.s^{-1}, p=0.049; crossed extensor reflex PM: 2.4°.s^{-1} vs AM: 0.5°.s^{-1}, p=0.002) was significantly faster in the evening compared to the morning in RLS participants, for both reflexes. RLS participants evening change in hallux angle was significantly larger than morning responses (median PM: 5.0° vs AM: 1.3°, p=0.012). No significant differences for any of the electromyographic or kinematic variables were observed between RLS participants and controls. The FWR and the crossed extensor reflex show a circadian rhythm in RLS participants suggesting an evening increase in spinal excitability. We hypothesize the circadian variation in spinal excitability may be due to a possible nocturnal form of afferent circuitry central sensitization in the dorsal horn of the spinal cord in RLS patients.

3.2. Introduction

A circadian fluctuation is evident in restless legs syndrome (RLS) with the pronounced appearance of RLS symptoms in the evening (Walters, 1995; Hening *et al.*, 1999; Trenkwalder *et al.*, 1999). The sensorimotor nature of symptoms in RLS patients implicates the spinal cord as a central site for dysfunction due to the spinal cord being the location of incoming sensory information and outgoing motor signals (Paulus & Schomburg, 2006). RLS symptoms could therefore be due to hyperexcitability at the level of the spinal cord (Kerr *et al.*, 2011). The responses to stimuli, such as those used to elicit a reflex response, are influenced by changes in the state of spinal excitability. Previous research suggests a state of spinal hyperexcitability in RLS patients based on increased excitability of the flexor withdrawal reflex (FWR) (Bara-Jimenez *et al.*, 2000). The diurnal variation in symptoms, in turn, may be explained by a circadian variation in hyperexcitability of neurones within the spinal cord.

The theory of RLS spinal hyperexcitability in the evening is not fully supported by the evidence of current reflex derived findings and knowledge gained from further study of other reflexes may provide enhanced theories of RLS pathology. The FWR, which is used to investigate the functional status of nociceptive spinal pathways (Sandrini *et al.*, 2005), is of particular interest due to the hyperalgesia shown in RLS patients (Stiasny-Kolster *et al.*, 2004, 2013; Cho *et al.*, 2017). In previous studies of the flexor withdrawal reflex in RLS patients (Bara-Jimenez *et al.*, 2000; Gunduz *et al.*, 2017), the corresponding contralateral crossed extensor reflex has not been assessed. As the crossed extensor reflex is elicited simultaneously with the FWR, assessing both reflex responses may aid in understanding more about potential variations in spinal circuitry which may be associated with RLS.

Most research on reflexes in RLS patients has been carried out during the asymptomatic (daytime) phase of the disorder. In order to account for the known circadian rhythm of RLS and to include the understudied crossed extensor reflex, the current study assessed the FWR and the crossed extensor reflex during the symptomatic phase (evening) as well as the asymptomatic phase (morning) of the disorder. We hypothesised that both the FWR and the crossed extensor reflex would be increased in the evening compared to the morning in RLS participants, thus exhibiting spinal hyperexcitability in the evening.

3.3. Materials and methods

Ethical clearance for this study was obtained from the Human Research Ethics Committee of the associated tertiary institution (M140322). All participants signed a written informed consent sheet prior to participating in the study. Twelve RLS participants were recruited through local advertisements and completed a comprehensive screening questionnaire which included the essential diagnostic criteria for RLS as well as questions to rule out mimics. The principle investigator (CD) administrated the questionnaire to all participants to ensure that all participants understood the questions. In order to be included in the study all participants experienced uncomfortable sensations in their legs which caused urges to move the legs. The sensations must worsen in the evening and with inactivity and are partially or totally relieved by movement. The sensations could also not solely be accounted for as symptoms primary to another condition and thus all participants met the updated International Restless Legs Syndrome Study Group (IRLSSG) diagnostic criteria (Allen *et al.*, 2014). RLS participants completed the IRLSSG severity scale (Walters *et al.*, 2003). The recruited participants also participated in a study investigating the plantar reflex (Dafkin *et al.*, 2017). Twelve healthy age and gender matched control participants were also recruited through local advertisements. All control participants did not have any sleep or neurological

disorders and answered no to the essential diagnostic criteria of RLS. Blood samples were analysed from all RLS participants to assess serum ferritin levels.

3.3.1. Reflex evaluation

Reflex testing was performed in the evening (between 20:00 and 22:00) and in the morning (between 6:30 and 8:00) applying the same procedure. The FWR was elicited on the right leg of all participants resulting in a withdrawal response of the right leg and a crossed extensor reflex response of the left leg. Participants lay supine on a plinth and the FWR was elicited through two electrodes placed on the sole of the right foot using a constant current stimulator (Stimsola linear isolated stimulator, Biopac, California, USA) with a train of four individual 3ms square wave pulses at 100Hz. The current amplitude was adjusted for each participant starting from 1mA and increased in 1mA intervals until the participant felt the stimulus. The stimulus intensity first felt by the participant was recorded as the participant's detection threshold and the reflex was elicited at 3x the detection threshold (adapted from Bara-Jimenez *et al*., 2000). Detection threshold was obtained immediately before FWR stimulation.

Both the FWR of the right leg and the corresponding crossed extensor reflex of the left leg were recorded kinematically and electromyographically. Kinematic data was recorded using 18 Optitrack (Natural Point, Oregon, USA) high speed cameras that record at 100 Hz. These cameras record the movements of retro-reflective markers that were positioned on both legs and feet at specific anatomical landmarks. The anatomical landmarks on the legs were: the iliac crest, the anterior superior iliac spine (ASIS), the greater trochanter (hip), the medial and lateral epicondyles of the femur (knee) and on the lateral and medial malleoli (ankle). Markers were also placed midway between the ankle and knee markers and midway between the knee and hip markers. The anatomical landmarks on the plantar surface of the

feet were: the heel, the apex of the arch, the tuberosity of the fifth metatarsal, the head of the proximal phalanges as well as on the head of the distal phalanges of the first (hallux), third and fifth digits. Muscle activity was simultaneously recorded using Trigno Wireless EMG sensors (Delysys, Natick Massachusetts, USA) connected to a PowerLab (ADI Instruments, 26T, Sydney, Australia). Muscle activity for the FWR was recorded in the following muscles of the right leg: tibialis anterior, lateral gastrocnemius, rectus femoris and biceps femoris. For the crossed extensor reflex muscle activity was recorded from the extensor muscles of the left leg, tibialis anterior and rectus femoris. One wireless EMG sensor was placed on the body of each muscle according to the SENIAM guidelines.

3.3.2. Data analysis

Kinematic data was analyzed with AMASS (AMASS, C-Motion Germantown, Maryland, USA) and Matlab7 (Mathworks, Natick, USA). Leg joint (knee and ankle) and hallux angles were calculated as per previously published conventions (Kadaba *et al.*, 1990; Dafkin *et al.*, 2014). The variables calculated from this data, for the FWR, were change in angle (degrees) measured from the initial position to the maximum angle of knee flexion, ankle dorsiflexion and hallux plantar flexion. For the crossed extensor reflex the variables calculated were change in angle (degrees) measured from the initial position to the maximum angle of knee extension and ankle dorsiflexion [anatomical extension of the foot (Kendall *et al.*, 2005)]. The angular velocity (calculated from the change in angle divided by the time taken to reach the maximum angle) of the knee and ankle, for both reflexes, and the hallux for the FWR ($°.s^{-1}$) was calculated. The maximum amplitudes (mV) and latencies (ms) of all muscles were calculated from the EMG data in LabChart7 (ADI Instruments, 26T, Sydney, Australia).

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego California USA).The distribution of the data was assessed using a D'Agostino and Pearson

omnibus normality test. All the data, with the exception of the characteristics of the participants, were non-parametric and as such are expressed as median and interquartile range. The participant characteristics are presented as mean ± standard deviation. The kinematic variables, EMG variables and detection thresholds were compared with Wilcoxon signed-rank tests between the evening (PM) and morning (AM) reflexes. Mann Whitney U-tests were used to compare kinematic variables, EMG variables and detection thresholds between RLS participants and controls at each time point.

3.4. Results

The characteristics of the RLS participants and the control participants are described in Table 1. One participant stopped Pramipexole (dopamine agonist) treatment a week prior to reflex testing. All other participants were treatment-naïve. Seven of the RLS participants described their uncomfortable sensations in their legs as painful.

Table 1: Characteristics of participants with RLS and control participants.

	RLS participants	Control participants	p-value
Number (male:female)	12(4:8)	12(4:8)	
Age (years)	45.8±13.8	43.0±14.7	0.63
Height (m)	1.7±0.1	1.7±0.1	0.46
Weight (kg)	69.5±15.0	77.8±24.3	0.33
BMI ($kg.m^{-2}$)	24.9±3.6	26.2±4.3	0.43
Age of onset of RLS (years)	28.6±14.3	N/A	
Duration of RLS (years)	16.7±14.7	N/A	
Family history of RLS (%)	33	N/A	
RLS severity (IRLSSG severity scale score)	17.7±7.2	N/A	
Serum ferritin ($ug.\ L^{-1}$)	130.2±90.1	N/A	

BMI, body mass index; IRLSSG, international restless legs syndrome study group; RLS, restless legs syndrome

There were no differences in detection thresholds between PM and AM for both RLS participants (median: PM 2.5mA, AM 2.0mA; p=0.59) and control participants (median: PM 2.0mA, AM 2.0mA; p=0.35) or between RLS participants and control participants (PM p=0.41; AM p=0.35). Changes in both ankle and hallux angles were significantly larger in the evening compared to the morning in RLS participants, but not in control participants, for the FWR (Figure 1). Of the RLS participants 73% and 75% showed greater evening changes in ankle and hallux angles respectively during the FWR. RLS participants evening change in ankle angle was significantly larger compared to the morning for the crossed extensor reflex (Figure 2). Larger evening changes in ankle angles during the crossed extensor reflex were seen in 82% of RLS participants.

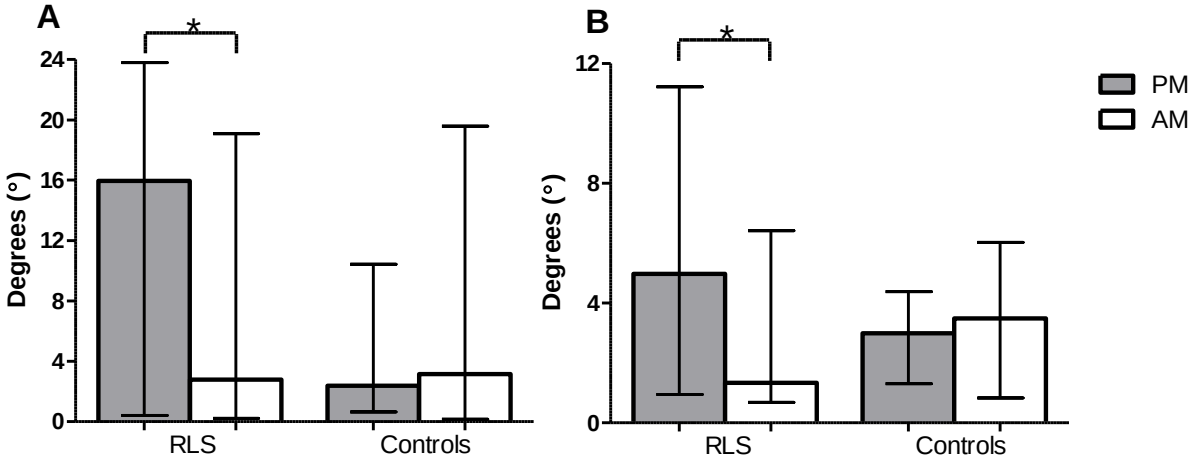

Figure 1: Median and inter-quartile range of change in ankle (A) and hallux (B) angle during the flexor withdrawal reflex (FWR) for participants with restless leg syndrome (RLS) (A: n=11; B: n=12) and control participants (A: n=12; B: n=11) in the evening (PM) and morning (AM). *p<0.05 RLS PM versus RLS AM; Wilcoxon signed-rank test.

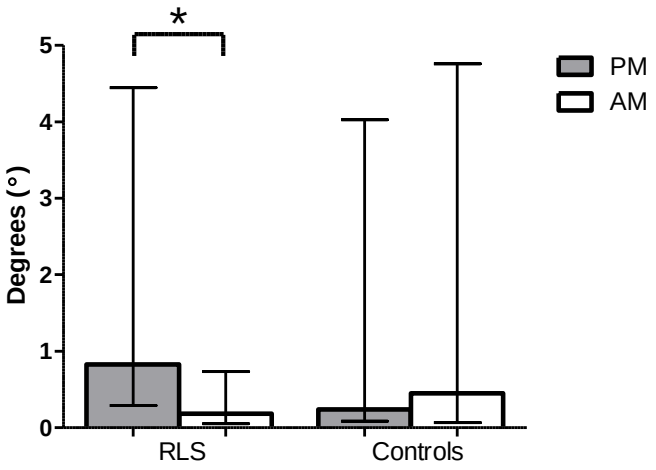

Figure 2: Median and inter-quartile range of change in ankle during the crossed extensor reflex in the evening (PM) and morning (AM) for participants with restless leg syndrome (RLS) (n=11) and control participants (n=11). *p<0.05 RLS PM versus RLS AM; Wilcoxon signed-rank test.

No significant differences were found for change in knee flexion angle for the FWR or for change in knee extension angle for the CER between the evening and morning reflexes. Angular velocities, EMG maximum amplitudes and latencies of the FWR and crossed extensor reflex are shown in Table 2 and Table 3 respectively. Only ankle angular velocity of RLS participants was significantly faster between the evening and the morning for the FWR (Table 2) and the crossed extensor reflex (Table 3). No significant differences were shown for EMG measurements for either the FWR (Table 2) or the crossed extensor reflex (Table 3) between the evening and morning. No significant differences for any of the assessed variables were observed between RLS participants and controls, either in the evening or the morning. The difference in n-values seen in Table 2 and Table 3 is due to instances where markers were not visible or EMG sensors detached from the participant, and thus not all participants' biomechanical or EMG data were analysed for both reflexes.

Table 2: FWR parameters of the right leg for participants with RLS and control participants in the evening (PM) and the morning (AM).

		Time of day	RLS	Controls	*p-value*
Angular	Knee	PM	4.66(0.25-27.50)	1.14 (0.21-12.36)	*0.340*
velocity	(RLS n=11)	AM	1.04(0.16-24.18)	1.85(0.25-26.96)	*0.782*
($°.s^{-1}$)		*p-value*	*0.083*	*0.677*	
	Ankle	PM	38.75(3.78-78.41)	8.23 (1.73-36.73)	*0.156*
	(RLS n=10;	AM	13.91(0.41-51.66)	13.30(0.49-43.29)	*0.974*
	control n=11)	*p-value*	*0.049**	*0.380*	
	Hallux	PM	11.53(2.17-18.70)	8.48(4.21-19.08)	*0.948*
	(RLS n=11)	AM	5.20(2.21-26.24)	11.24(4.07-14.69)	*0.470*
		p-value	*0.413*	*0.898*	
Maximum	TA	PM	0.32(0.04-1.04)	0.16(0.05-0.46)	*0.559*
Amplitude	(RLS n=11)	AM	0.24(0.03-0.56)	0.12(0.03-1.18)	*0.782*
(mV)		*p-value*	*0.240*	*0.638*	
	GL	PM	0.07(0.03-0.11)	0.03(0.02-0.07)	*0.204*
		AM	0.02(0.02-0.22)	0.03(0.02-0.17)	*0.840*
		p-value	*0.733*	*1.000*	
	RF	PM	0.17(0.04-4.90)	0.04(0.02-1.16)	*0.073*
		AM	0.13(0.03-0.34)	0.04(0.01-0.21)	*0.204*
		p-value	*0.204*	*0.733*	
	BF	PM	0.17 (0.03-0.52)	0.02 (0.02-0.15)	*0.121*
	(RLS n=10)	AM	0.03 (0.02-0.18)	0.03 (0.01-0.37)	*0.767*
		p-value	*0.275*	*0.413*	
Latency (ms)	TA	PM	132.00(110.00-182.00)	100.00(95.00-125.00)	*0.108*
	(RLS n=11)	AM	138.00(105.00-185.00)	127.50(96.25-183.80)	*0.805*

		p-value	*0.894*	*0.646*	
	GL	PM	125.00(109.50-181.50)	123.00(101.30-172.30)	*0.644*
		AM	164.50(120.00-213.50)	106.50(91.25-183.80)	*0.094*
		p-value	*0.158*	*0.594*	
	RF	PM	207.50(119.80-233.00)	135.00(110.50-183.80)	*0.194*
		AM	162.50(123.50-214.80)	146.50(93.50-196.50)	*0.507*
		p-value	*0.142*	*0.922*	
	BF	PM	162.50(116.50-216.30)	129.00(112.00-153.00)	*0.409*
	(RLS n=10)	AM	124.00(99.00-200.00)	140.00(98.00-187.50)	*0.843*
		p-value	*0.307*	*0.666*	

Comparisons between PM and AM were done with Wilcoxon signed-rank tests. Mann-Whitney U-tests were used to compare between participants with RLS and control participants. *significant difference between PM and AM for RLS participants. Data represented as median and interquartile range. n=12 unless indicated otherwise.
BF, biceps femoris*;* FWR, flexor withdrawal reflex; GL, lateral gastrocnemius; RF, rectus femoris; RLS, restless legs syndrome; TA, tibialis anterior.

Table 3: Crossed extensor reflex parameters of the left leg for participants with RLS and control participants in the evening (PM) and the morning (AM).

		Time of day	RLS	Controls	*p-value*
Angular	Knee	PM	1.72(0.22-2.54)	0.56(0.16-0.98)	*0.358*
velocity	(RLS n=11;	AM	0.13(0.07-2.89)	0.48(0.10-3.19)	*0.393*
(°.s^{-1})	control n=11)	*p-value*	*0.123*	*0.700*	
	Ankle	PM	2.37(0.69-9.45)	0.65(0.25-16.78)	*0.325*
	(RLS n=11;	AM	0.50(0.17-2.13)	0.91(0.11-10.13)	*0.470*

	control n=11)	*p-value*	*0.002**	*0.898*	
Maximum	TA	PM	0.05(0.03-0.21)	0.03(0.02-0.16)	*0.194*
Amplitude	(RLS n=11)	AM	0.06(0.02-0.22)	0.03(0.02-0.22)	*0.470*
(mV)		*p-value*	*0.301*	*0.556*	
	RF	PM	0.04(0.03-0.15)	0.02(0.01-0.12)	*0.126*
		AM	0.04(0.02-0.10)	0.04(0.02-0.25)	*0.751*
		p-value	*0.151*	*0.176*	
Latency (ms)	TA	PM	186.50(109.50-233.80)	139.50(102.50-183.00)	*0.312*
	(RLS n=11)	AM	190.00(131.00-210.00)	125.00(112.50-218.00)	*0.175*
		p-value	*0.388*	*0.456*	
	RF	PM	185.00(137.50-264.80)	152.50(91.00-173.80)	*0.069*
		AM	151.00(105.50-200.00)	172.50(114.50-228.30)	*0.623*
		p-value	*0.209*	*0.091*	

Mann-Whitney U-tests were used to compare variables of participants with RLS and control participants. Wilcoxon signed-rank tests were done to compare between PM and AM. *significant difference between PM and AM for RLS participants. Data represented as median and interquartile range. n=12 unless indicated otherwise.
RF, rectus femoris; RLS, restless legs syndrome; TA, tibialis anterior.

3.5. Discussion

The major findings of this study demonstrate a circadian variation of both the FWR and the crossed extensor reflex in RLS participants. The degree of movements of the ankle and the angular velocity of the ankle (showing the speed of the reflex) were increased for both reflexes in RLS participants in the evening compared to the morning. The same circadian fluctuation of reflex responses was not evident in the control participants. The results, therefore agree with the hypothesis, showing a circadian variation of excitability of spinal

cord reflexes in RLS with an increase in excitability during the symptomatic period (the evening). There were no significant differences in detection thresholds, and therefore stimulation intensities, between the evening and morning reflex testing.

Despite demonstrating an increase in the degree of movement of the limbs during the reflexes there was no corresponding significant increase in EMG amplitude. The difference in results between the physical movement and the EMG activity could be due to kinematics being a more sensitive measure and thus detecting more subtle variations. Another explanation for this difference could be the increase in the number of muscles involved in the reflex response at night (Bara-Jimenez *et al.*, 2000). An increase in the number of muscles involved in the response would explain the increased degree of movement seen at night but would not necessarily increase the amplitude in one specific muscle.

To our knowledge the crossed extensor reflex in RLS patients has not been assessed previously. The results of this study indicate that with primary RLS there is a circadian variation of the crossed extensor reflex, with increased crossed extensor reflex responses in the evening compared to the morning. This shows a possible increase in spinal excitability in the evening in RLS. Due to the relationship between the crossed extensor reflex and the FWR, both reflexes are elicited by the same stimulus, comparing the responses of both reflexes provides a greater representation of what is occurring in the spinal cord in RLS patients. The spinal excitability circadian variation within this group of RLS patients was previously demonstrated testing the plantar reflex (Dafkin *et al.*, 2017). The FWR and plantar reflex aid in assessing lumbar spinal circuits. However, the method of eliciting the reflexes differs significantly. The FWR is elicited by painful stimulus to cause withdrawal of the limb whilst the plantar reflex is elicited by mechanical stimulation (scratching stimulus). Hence, the FWR and plantar reflex allow for the assessment of different receptors and afferent

neurons. By assessing variances between different lower limb reflex responses it allows for further elucidation of the underlying pathophysiological mechanisms associated with RLS.

Previous studies looking at the FWR in RLS patients have shown increased excitability of the reflex by describing decreased reflex stimulus thresholds (with no difference in detection thresholds), increased spatial spread but with no apparent differences in EMG latencies (Bara-Jimenez *et al.*, 2000), decreased tibialis anterior EMG latency, increased duration of tibialis anterior muscle activity but no difference in tibialis anterior EMG amplitude (Gunduz *et al.*, 2017) of the FWR in RLS participants compared to healthy controls. The current study showed no significant difference in tibialis anterior EMG latency or amplitude between RLS and control participants or between the two different times of day. A possible explanation for the lack of significant EMG findings in the current study is the large inter-individual variation in reflex responses. However, the current study also examined the degree of physical movement in addition to the EMG measures of leg muscle activity during the FWR and the circadian changes of these responses. The degree of movement of the reflexes was greater in the evening compared to the morning for the RLS participants (Figures 1 and 2), indicating a circadian variation of the spinal excitability in RLS participants. In contrast previous studies of awake FWR responses were recorded at a single time only, between 21:00 and 24:00 (Bara-Jimenez *et al*., 2000) and between 13:30 and 15:30 (Gunduz *et al*., 2017).

Although a circadian variation of the FWR in RLS patients has not previously been assessed, a circadian variation in pain sensitivity has been shown in RLS patients (Cho *et al*., 2017). Cho *et al* (2017) showed that RLS patients had a lower current perception threshold in A-delta and C fibers (an increase in pain sensitivity) in the evening compared to the morning. A possible reason that the current study did not show a difference in perception threshold

could be due to the differing frequencies and location of stimulus used by Cho *et al* (2017) in comparison to the current study. An increase in pain sensitivity could be a sign of central sensitization which would result in hyperexcitability of nociceptive spinal reflexes (Woolf, 1983). The FWR is a nociceptive reflex involving activation of flexor reflex afferents (A-delta and C pain fibers) leading to the resultant contraction of limb flexor muscles (Binder *et al.*, 2009). As the FWR is nociceptive pain sensitivity could play a role in changes to the reflex response. Several other studies have assessed pain sensitivity in RLS patients with an overall finding of an increase in pain sensitivity in RLS patients at various times of the day compared to healthy controls (Stiasny-Kolster *et al.*, 2004, 2013; Bachmann *et al.*, 2010; Edwards *et al.*, 2011).

The FWR and the crossed extensor reflex share afferent circuitry; they are elicited by activation of the same afferents. The current study has shown that these reflexes exhibit similar increased excitability in the evening (increased amplitude of foot movements as well as the increased speed of the reflex). A possible explanation for the circadian variation of spinal excitability is a circadian dysfunction in the afferent circuitry involved in the FWR and the crossed extensor reflex in RLS patients. This dysfunction could be due to increased excitatory neurotransmitters (glutamate or substance P) being released from the afferent neuron, an increase of the number of receptors or an increase in sensitivity of receptors [NMDA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), Neurokinin 1 (NK-1)] on the post synaptic membrane, decreased descending inhibition in the dorsal horn or a combination of the aforementioned mechanisms.

The loss of descending dopaminergic spinal inhibition (in the dorsal columns) from A11 neurons is a prevalent theory for the pathophysiology of RLS (Clemens *et al.*, 2006). This loss of inhibition, or the theorised increase in excitation in the dorsal horn that we suggest, could

cause a state of central sensitization in RLS which has been proposed by Stiansy-Kolster *et al* (2004). With central sensitization there would be hyperexcitability (Woolf, 1983), as seen with alterations in reflex responses shown in previous studies (Bara-Jimenez *et al.*, 2000; Isak *et al.*, 2011; Congiu *et al.*, 2017; Gunduz *et al.*, 2017) as well as in the current study, and hyperalgesia (Woolf, 1983), which has also been shown in RLS patients (Stiasny-Kolster *et al.*, 2004, 2013; Bachmann *et al.*, 2010; Edwards *et al.*, 2011; Cho *et al.*, 2017).

Limitations of the current study include the small sample size as well as the large amount of inter-individual variation. Between groups (RLS vs. control) differences may have been detected had a larger sample size been available. Fortunately, paired comparisons were made within groups which removed the effect of inter-individual variation. A further limitation of the study is the use of kinematics to assess the reflex which is not a standard measure making the comparison with previous studies difficult. However in the current study the reflex responses were also assessed electromyographically as has been done in previous studies. Moreover there is no standard measure to assess the physical movement of the limbs during the reflex response. During a neurological assessment reflex responses are evaluated subjectively by the health practitioner. Kinematic assessment allows for an objective, repeatable measurement of the movement which improves accuracy and removes bias. RLS participants possibly having concurrent neuropathy is a limitation of the current study as this could have affected the results. As almost all of the RLS participants in the study were treatment naïve the results reported may be specific to an RLS subtype with mild manifestations. The participants being treatment naïve could also be due to a lack of knowledge about treatments for the disorder and thus participants' not seeking treatment.

3.6. Conclusion

The aim of the present study was to assess the circadian variation of the FWR and crossed extensor reflex in RLS. The results of the current study suggest an evening increase in spinal excitability in RLS participants. The increase in both FWR and crossed extensor reflex responses indicate the afferent circuitry in the dorsal horn of the spinal cord as a possible site of dysfunction in RLS. These findings contribute to the understanding that RLS pathophysiology could be based in spinal central sensitization with a marked increase in spinal excitability in the evening.

Chapter 4: Distal muscle activity alterations during the stance phase of gait in restless leg syndrome (RLS) patients.

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4.1. Abstract

Objective: To assess if there is a circadian variation in electromyographical (EMG) muscle activity during gait in restless legs syndrome (RLS) patients and healthy control participants.

Methods: Gait assessment was done in 14 RLS patients and 13 healthy control participants in the evening (PM) and the morning (AM). Muscle activity was recorded bilaterally from the *tibialis anterior* (TA), *lateral gastrocnemius* (GL), *rectus femoris* (RF) and *biceps femoris* (BF) muscles.

Results: A circadian variation during the stance phase in only TA (PM>AM, $p<0.005$) and BL (PM<AM, $p=0.008$) activity was observed in control participants. Conversely no circadian variation was seen in any muscles in the RLS patients. RLS patients had an increased TA and GL activity (RLS>Controls, $p<0.05$) during early stance and decreased GL activity (RLS<Controls, $p<0.01$) during terminal stance in comparison to control participants in the evening. No other significant differences were noted between RLS patients and control participants. Activation of GL during the swing phase was noted in 79% of RLS patients and in 23% of control participants in the morning compared to 71% and 38% in the evening, respectively.

Conclusion: EMG muscle activity shows no circadian variation in RLS patients. Evening differences in gait muscle activation patterns between RLS patients and control participants are evident. These results extend our knowledge about alterations in spinal processing during gait in RLS. A possible explanation for these findings is central pattern generator sensitization caused by increased sensitivity in cutaneous afferents in RLS patients.

4.2. Introduction

Restless legs syndrome (RLS) is a neurological disorder that presents with both sensory and motor symptoms (Walters, 1995; Hening *et al.*, 1999). RLS patients experience uncomfortable sensations in their legs which are alleviated by movement (Allen *et al.*, 2014), which indicate that the pathophysiological mechanisms underlying RLS could be associated with sensory and motor pathways. A possible method to investigate both sensory and motor pathways simultaneously is in the analysis of gait as the coordination of movement is partly regulated by sensory feedback (Rossignol *et al.*, 2006). In this regard, the investigation of the gait cycle in RLS patients may offer further elucidation of the pathophysiological mechanisms involved in RLS.

Paci and colleagues have assessed gait in RLS patients and noted an abnormal activation of the gastrocnemii during the swing phase of the gait cycle where there is normally no activity in these muscles (Paci *et al.*, 2009). The gait assessments in these participants were however conducted between 10:00 and 11:00, which is during the non-symptomatic phase of RLS. Due to the known circadian variation in RLS (Trenkwalder *et al.*, 1999), with symptoms presenting in the evening, the investigators have suggested that the abnormal gastrocnemii activation may be more pronounced in the evening in RLS patients.

To date, the investigation conducted by Paci and colleagues has been the only study that has analysed gait in RLS patients (Paci *et al.*, 2009). The aim of the current study was to further assess gait in RLS patients, by extending the investigation to both the morning and evening periods, as well as confirm the finding of gastrocnemius activation in RLS patients during the swing phase. The evening and morning periods incorporate the two time points where RLS symptoms are at their most and least prominent respectively. Given the known circadian variation in RLS patients, the current study aimed to investigate if this variation could be

attributed to differences in motor and sensory pathways as assessed by analysing the activity pattern of leg muscles involved in gait.

4.3. Materials and methods

4.3.1. Participants

Ethical clearance for this study was obtained from the Human Research Ethics Committee of the associated tertiary institution (M140322). All participants signed written informed consent prior to participating in the study. RLS patients and healthy participants were recruited through local advertisements. All participants completed a comprehensive screening questionnaire including the essential diagnostic criteria for RLS as well as questions to rule out mimics. The RLS group consisted of 14 participants. To be included in the RLS group, participants had to meet the updated International Restless Legs Syndrome Study Group (IRLSSG) diagnostic criteria (Allen *et al.*, 2014) and whose RLS symptoms could not be explained by any other sleep or neurological disorders. The RLS participants completed the IRLSSG severity scale (Walters *et al.*, 2003). One RLS patient stopped dopaminergic treatment a week before the gait assessment. All other RLS patients were treatment-naïve. The control group consisted of 13 healthy, age and gender matched participants. Control participants were included in the study if they answered no to the essential RLS diagnostic criteria and they did not have any sleep or neurological disorders.

4.3.2. Gait assessment

The gait cycle was assessed electromyographically (EMG) in the evening (19:30-20:30) and again in the morning (6:30-7:30). Each participant walked 12 to15 steps barefoot at a comfortable self-selected walking pace. Only data from the middle steps were analysed, excluding data from the initiation and the termination of movement. The complete gait cycle is divided into eight phases, represented on a scale from 0 to 100%; heel strike (0%), loading

response (0-12%), mid-stance (12-31%), terminal stance (31-50%), pre-swing (50-62%), initial swing (62-75%), mid-swing (75-87%), and terminal swing (87-100%).

EMG of muscle activity was performed using Trigno wireless EMG sensors (Delysys, Natick Massachsetts, USA). Trigno EMG sensors were placed bilaterally on the bodies of the *tibialis anterior* (TA), *lateral gastrocnemius* (GL), *rectus femoris* (RF) and *biceps femoris* (BF) muscles according to the SENIAM guidelines. The EMG sensors were connected to a PowerLab system with LabChart7 software to record the output from the sensors (ADI Instruments, 26T, Sydney, Australia). Heel strike and toe off were obtained kinematically. This was done by placing reflective markers bilaterally on the heel and the base of the first hallux. Markers were tracked using 18 Optitrack (Natural Point, Oregon, USA) high speed cameras recording at 100 Hz and the recorded data was analysed with AMASS (AMASS, C-Motion Germantown, Maryland, USA).

4.3.3. Data analysis

Kinematic data of foot placement was analyzed with AMASS (AMASS, C-Motion Germantown, Maryland, USA). All EMG data were high pass filtered at 30Hz to decrease motion artifacts (Miller *et al.*, 1996). The maximum amplitudes (mV) of all muscles were calculated for every 5% of the gait cycle in LabChart7 and compressed to a standard length [from heel strike (0%) to heel strike (100%) for each step]. Data at each time point were normalised to a unity average (Yang & Winter, 1984) in order to allow comparisons between participants and between time points.

Statistical analyses were performed with Statistical Analysis Software Version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Data are expressed as mean ± standard deviation unless otherwise stated. Age between groups was compared using an unpaired t-test. No significant difference was found between the right and left sides ($p>0.05$ for all four muscles

at all points of the gait cycle in each participant) and therefore for ease of reporting, data from both legs were combined. A two-way repeated measures ANOVA was performed to assess the impact of group (RLS vs. control) and time (evening (PM) vs. morning (AM)) and the group time interaction on the muscle activation of the various muscles during the gait cycle. Tukey post-hoc tests were performed to identify differences at specific stages of the gait cycle. The probability of muscle activation in one group at a specific time point was calculated using a Chi-squared approach with Yates correction.

4.4. Results

4.4.1. Participant characteristics

The characteristic of the RLS and control groups were as follows. The RLS and control groups were well matched in terms of gender (M/F: RLS 5/11, control 4/9) and age (RLS: 43.4±14.4 years old, control 42.1±14.5 years old; p=0.82). In terms of RLS criteria; RLS patients had mean IRLSSG scores of 18.9±6.7, RLS duration of 15±13.5 years, and age of RLS onset of 28.4±13.1 years. A family history of RLS was reported in 43% of the RLS participants.

4.4.2. Evening (PM) versus morning (AM) muscle activity

Figure 1 demonstrates the activity in the TA, GL, RF, and BF muscles recorded during the gait cycle. No significant differences were noted between evening and morning muscle activation patterns in the four muscles in RLS patients ($p>0.05$ at all points of the gait cycle for all muscles). Control participants exhibited significant evening vs. morning differences in two of the four muscles. In this regard, a significant difference was noted at 10% and 15% of the gait cycle in TA activity ($p<0.005$), with increased TA activity in the morning compared to the evening. Furthermore, in the control group, BF activity was significantly greater at 10% of the gait cycle in the evening compared to the morning (p=0.0077).

4.4.3. RLS patients versus control participants muscle activity

RLS patients had significantly increased TA activity at 10% of the gait cycle in the evening when compared to control participants. Conversely, in the morning period, TA activity was decreased at 10% and 15% of the gait cycle in RLS patients when compared to control participants (Figure 1). In the evening, GL activity was increased at 15% of the gait cycle in RLS patients when compared to control participants, and decreased at 45 and 50% of the gait cycle in comparison to control participants (Figure 1). No differences in RF and BF muscle activity were observed between RLS patients and control participants (Figure 1).

Assessment of raw EMG data demonstrated an abnormal activation of GL at 70-90% of the gait cycle in RLS patients. Figure 2 displays an example of the abnormal activation of GL in a single participant. In the morning, the probability of abnormal GL activity was greater in the RLS patients when compared to control participants (11 out of 14 RLS patients vs. 3 out of 13 control participants, Chi-square with Yates correction = 6.24, p=0.01). However, in the evening no significant difference was found in the probability of abnormal GL activation occurring in RLS patients and control participants (Chi-square with Yates correction =1.78, p=0.18). In the evening, abnormal GL activation was seen in 5 out of 13 control participants and in 10 out of 14 RLS patients.

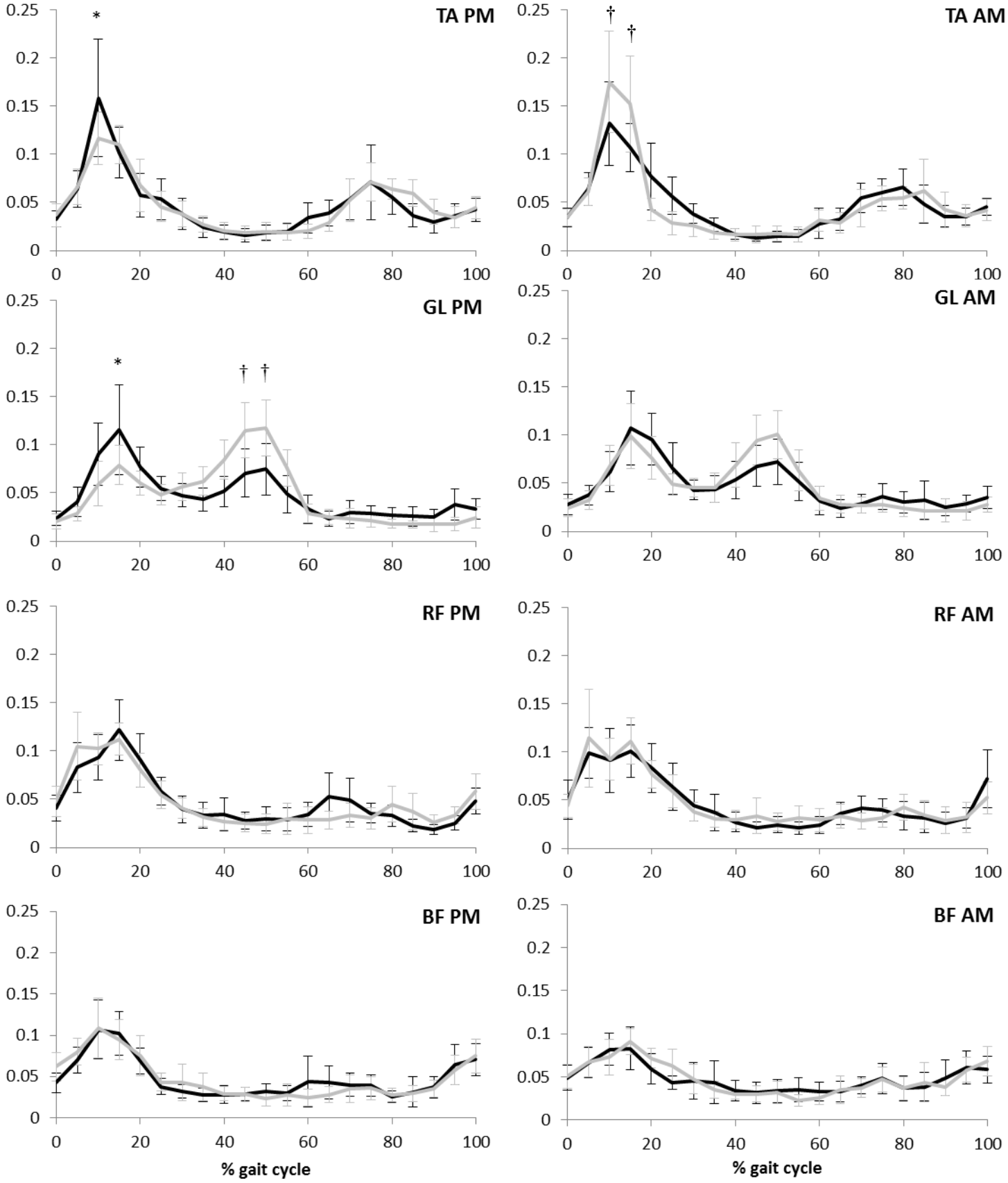

Figure 1: Mean gait muscle activity curves of 14 restless legs syndrome (RLS) participants (black line) and 13 control participants (grey line) in the evening (PM – left column) and the morning (AM – right column). Data are expressed as mean and the 95% confidence interval

(error bars) of the left and right legs combined for every 5% of the gait cycle. All data has been normalised to a unity average and the vertical axes have been standardised. The horizontal axis shows one complete gait cycle from heel strike (0%) to heel strike (100%). ANOVA, *p<0.05 RLS participants' electromyographic activation is significantly greater than control participants; †p<0.01 control participants' electromyographic activation is significantly greater than RLS participants. TA, *tibialis anterior*; GL, *lateral gastrocnemius*; RF, *rectus femoris*; BF, *biceps femoris*.

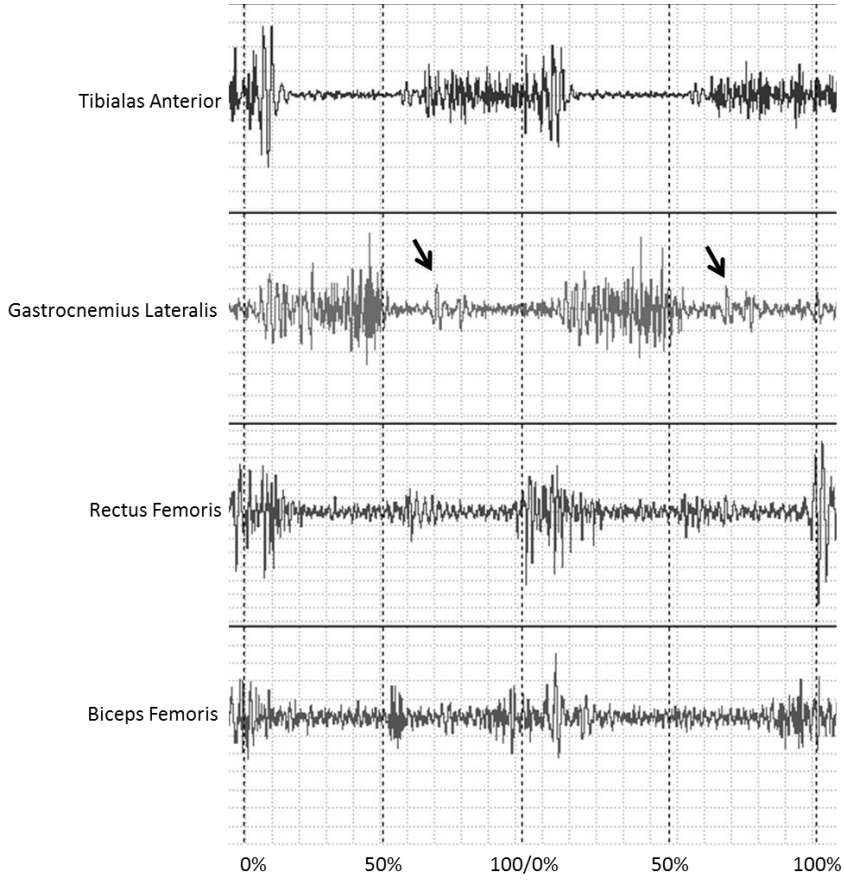

Figure 2: Example of a raw EMG file of two complete gait cycles showing muscle activity in the *tibialas anterior, gastrocnemius lateralis, rectus femoris* and *biceps femoris*. The dotted lines mark the percentage of the gait cycle with 0% and 100% representing heel strike. The arrows indicate the abnormal *gastrocnemius lateralis* activation during the swing phase of the gait cycle.

4.5. Discussion

The results of this study show time-dependant variation in TA and BF muscle activity during the loading phase of the gait cycle in control participants. Contrary to expectation, no circadian variation in muscle activity was seen in RLS patients. There were however time- and phase-dependant differences noted in muscle activity during gait when comparing the RLS patients and control participants. In this regard, RLS patients had increased TA and GL activity during the loading phase and a decreased degree of GL activation during terminal stance in the evening when compared to control participants. No significant differences between RLS patients and control participants with respect to the activation of proximal muscles (RF and BF) were noted. A possible reason for the differences only being found in the distal muscles is that RLS symptoms tend to be more prominent in the lower leg (Allen *et al.*, 2014) and thus pathophysiological findings may be more pronounced in muscles of the lower leg.

The results from the present study are congruent with the findings of Paci *et al* in that abnormal GL activity was seen in 79% of RLS patients in the morning (comparable to 77% in the Paci study) (Paci *et al.*, 2009). However, contrary to the hypothesis that abnormal activation of GL would be more pronounced in the evening in RLS patients, the probability of abnormal activity of the GL muscle occuring was greater only in the morning and not in the evening in RLS patients when compared to control participants. In the present study a small percentage of control participants also displayed abnormal GL activation in the morning and the evening, which was not a finding noted by Paci and colleagues (2009). This may be due to the smaller sample size of the control group in the study conducted by Paci and colleagues (n=8). The results from the present study emphasise that time of day is an important factor that needs to be taken into consideration when assessing GL activity in RLS patients as this may be more pronounced in the morning period. Furthermore, the results

from this study indicate that GL activity was present during the swing phase in healthy participants and therefore may not be of any clinical relevance in terms of assessing RLS. Hence, the notion that the presence of abnormal GL activity can be used as a supportive diagnostic feature (Paci *et al.*, 2009) requires further investigation.

The increased activation of TA and GL muscles during the loading response of the gait cycle (10-15%) noted in RLS patients when compared to control participants in the evening indicate that perhaps differences in afferent activation from the sole of the foot are responsible for these findings. Specifically, during the loading response the foot is in full contact with the ground and is subjected to the full weight of the body. Afferent feedback plays a vital role in modulating spinal central pattern generators, and thus gait responses during movement (Rossignol *et al.*, 2006). Central pattern generators may be altered in RLS patients owing to the fact that they display a large amount of plasticity (Dietz, 2003; Guertin, 2013). Hyperalgesia is present in RLS patients (Stiasny-Kolster *et al.*, 2004; Bachmann *et al.*, 2010) as well as an evening decrease in current perception threshold in cutaneous afferent fibers (Cho *et al.*, 2017). Consistent with this, gait muscle activation in RLS patients may be altered due to constant or increased input from cutaneous afferents leading to changes in central pattern generators with a subsequent increase in response to cutaneous afferents during gait. An increase in response to cutaneous afferents would be most prominent during the loading response of the gait cycle when the foot comes into contact with the ground and the cutaneous afferents from the sole of the foot are activated (Van De Crommert *et al.*, 1998), which is what has been observed in the present investigation. Therefore when cutaneous afferents are activated it causes an increased response due to possible sensitization of the central pattern generators. This notion is further reinforced by the observation that GL muscle activation was decreased in RLS patients during terminal stance

(40-50%) at which point the heel is lifted from the ground and the body weight is distributed across both legs reducing the load present on the sole of the foot.

The decrease in GL muscle activity noted during the terminal stance, when the heel is lifted off the ground, could be due to reflex reversal. Reflex reversal is a phenomenon whereby an opposite response occurs from the same stimulus depending of the phase of the gait cycle (Duysens *et al.*, 1992). Studies assessing changes in the withdrawal reflex, elicited by electrically activating cutaneous afferent fibers, during gait have shown facilitation of TA and soleus (an agonist of GL) muscle activities just after heel contact and conversely suppression of soleus muscle activity after heel off (Spaich *et al.*, 2004) and suppression of GL muscle activity during midstance (Duysens *et al.*, 1996). Extensor muscles (soleus and GL) reflex reversal from facilitation just after heel contact to suppression at midstance and after heel off, due to activation of cutaneous afferents (which may have enhanced sensitivity in RLS patients), may explain the decrease in GL activity found during terminal stance of the gait cycle in the current study in RLS patients.

A circadian variation in the loading phase of the gait cycle in control participants was evidenced by an evening increase in BF (extensor) muscle activity and an evening decrease in TA (flexor) muscle activity. A plausible explanation for the circadian variation could be a result of increased evening sensitivity in triceps surae muscles 1a afferents, which are activated due to stretching of the muscles during the loading phase (Pierrot-Deseilligny *et al.*, 1981). An increase in extensor muscles 1a afferent sensitivity results in an increase in 1a afferent activity which will stimulate contraction of agonist muscles (BF), thus increasing the muscle activity, and inhibition of antagonist muscles (TA), decreasing the muscle activity. No circadian variation was noted in RLS patients, which may be related to a lack of increase, or a decrease, in 1a afferents sensitivity in the evening. Indeed, a previous study has showed a

possible evening decrease in 1a afferents sensitivity in RLS patients demonstrated by a decrease in patellar reflex responses in RLS patients compared to control participants in the evening (Kerr *et al.*, 2011).

In terms of study limitation, the limited sample size of the current investigation must be taken into consideration. Nevertheless, even with a small sample size significant differences were noted between the RLS and control groups and in the evening and morning periods. Future studies with a larger sample size are required in order to reinforce the findings of the current study.

The present study aimed to assess if there are differences in muscle activity during the gait cycle in RLS patients compared to control participants. The results indicate that there are differences in the evening, the symptomatic phase of the disorder, in gait muscle activation patterns when comparing RLS and control participants. Moreover, the results from this study are consistent with previous research demonstrating abnormal activation of GL in RLS patients during the swing phase of the gait cycle (Paci *et al.*, 2009). These findings extend our knowledge about alterations in spinal processing in RLS. A possible explanation for these findings is sensitization of central pattern generators caused by increased activity, due to increased sensitivity, in cutaneous afferents in RLS patients.

Chapter 5: Summary and Conclusions

RLS is a neurological disorder that has accepted subjective diagnostic criteria which include an urge to move, symptoms brought on by inactivity, relief of symptoms with movement, and a marked circadian variation in symptom profile. The aetiology of RLS is, however, still unknown. A pathophysiology of the nervous system is thought to be involved in the generation of symptoms in RLS due to the key features of the disorder, processing of sensations and the activation of movement, involving the nervous system. Current neurotransmitter evidence in RLS patients indicates decreased D2R as well as a possible increase in glutamatergic activity (Turjanski *et al.*, 1999; Michaud *et al.*, 2002*b*; Connor *et al.*, 2009; Allen *et al.*, 2013). Taken together, the neurotransmitter data indicate a possible state of nervous system hyperexcitability in RLS patients. To date alterations in cortical (Entezari-Tergau *et al.*, 1999; Taher *et al.*, 1999; Quatrale *et al.*, 2003; Stiasny-Kolster *et al.*, 2003; Kutukcu *et al.*, 2006; Nardone *et al.*, 2006; Scalise *et al.*, 2004, 2006, 2010; Gorsler & Liepert, 2007; Tyvaert *et al*., 2009; Rizzo *et al.*, 2010; Lanza *et al.*, 2015) and spinal (Bara-Jimenez *et al.*, 2000; Aksu & Bara-Jimenez, 2002; Rijsman *et al.*, 2005; Scaglione *et al.*, 2008; Isak *et al.*, 2011; Kerr et al., 2011; Marconi *et al.*, 2012; Heide *et al.*, 2014; Congiu *et al.*, 2017; Gunduz *et al.*, 2017) excitability have been shown in RLS patients but, despite such evidence, the basis of the circadian rhythm of RLS symptoms is still unknown (Earley et al., 2014). Further research is still needed to determine the aetiology of this disorder in order to improve diagnoses and treatment protocols. Evaluation of circadian variations in spinal excitability and spinal sensorimotor integration of RLS patients could provide important information regarding the aetiology of RLS. Therefore the current thesis consists of three novel studies designed to further evaluate the circadian variations in spinal excitability in RLS patients.

The main aims of this thesis were: to evaluate circadian variations in static spinal excitability in RLS patients by assessing circadian variations in spinal reflex excitability; and to determine

if there are circadian variations in dynamic spinal excitability in RLS patients by evaluating circadian variations in the neuromuscular profile of the gait cycle. The sections to follow will discuss the data from the investigations conducted to fulfil the aforementioned aims within the context of the current understanding of RLS from published literature.

5.1. Spinal reflex excitability

5.1.1. Spinal excitability in RLS patients compared to control participants

The concept of a state of nervous system hyperexcitability in RLS patients is supported by previous literature, which is discussed in Chapter 1. Therefore it was expected in the current investigation that RLS patients would display spinal hyperexcitabiltiy when compared to healthy control participants. However, contrary to the expected hypothesis, RLS patients showed no increases in spinal excitability when compared to control participants. Plantar reflex responses in RLS patients were decreased compared to control participants, both in the evening and the morning. No significant differences were noted in the indices of spinal excitability in the FWR and crossed extensor reflex when comparing the RLS and control groups. The plantar reflex results indicate that despite the theory that a state of global spinal hyperexcitability exists in RLS patients, this may not be true for all sensory modalities. The interpretation of these results should be within the context that there is a large degree of inter-individual variation in reflex responses. Hence, while the absolute indices of spinal excitability were not increased in the inter-group analyses of RLS patients and control participants, these results may not be a true reflection of these biological relationships.

There are conflicting data with respect to the analyses of the FWR in RLS patients. The results from the present investigations demonstrated no significant differences between RLS and control groups in both EMG amplitude and latency for any of the assessed muscles of the FWR; *tibialis anterior, lateral gastrocnemius, rectus femoris* or *biceps femoris*. Consistent with the

results from the current investigations, previous studies analysing the FWR between RLS patients and healthy controls have not noted differences in EMG latency (Bara-Jimenez *et al.* 2000) and amplitude (Gunduz *et al.* 2017) of the FWR. However, Bara-Jimenez *et al*. (2000) did demonstrate increased FWR responses with decreased stimulus thresholds and increased spatial spread (number of muscles involved in the reflex response) in RLS patients when compared to control participants. Similarly, Gunduz *et al.* (2017) showed increased durations of *tibialis anterior* muscle contraction and decreased *tibialis anterior* latency in RLS patients compared to controls, thus showing quicker and longer reflex responses. It should be noted that EMG latency is dependent on stimulus intensity (Bara-Jimenez *et al.*, 2000) hence making inter-group and -study comparisons difficult. A possible factor that could also mask detection of a potential increase in FWR and crossed extensor reflex responses in the study in Chapter 3 may be the large inter-individual variation in reflex responses. To confirm whether the FWR and crossed extensor responses are different in RLS patients when compared to healthy controls requires large scale studies that mitigate the considerable inter-individual variations in reflex responses. Collectively, from the results available to date, it is difficult to conclude if there are alterations in the FWR in RLS patients.

The plantar reflex response was significantly decreased in RLS patients compared to control participants. No previous studies have assessed the plantar reflex response in RLS patients. The decreased responses noted in RLS patients with respect to the plantar reflex in Chapter 2 may be due to activation of A-beta fibres, which are not involved in the FWR. The FWR and the plantar reflex are activated by nociceptive afferent fibres (A-delta and C-fibres), but the plantar reflex is also activated by low threshold mechanoreceptive A-beta afferent fibres. Hence, a possible explanation for the decreased plantar reflex responses in RLS patients compared to control participants, and no difference in the FWR responses, may be due to

alterations in A-beta fibre activity in RLS patients. Another possibility is that there is an excitatory ceiling effect of certain spinal circuits in RLS patients that negatively feeds back on other spinal circuits.

With regards to A-beta fibre activity mechanical detection thresholds can be used as a marker of A-beta fibre thresholds. A previous study utilising the QST protocol demonstrated increased mechanical detection thresholds in RLS patients (Stiasny-Kolster *et al.*, 2013). However Cho *et al*. noted no difference in A-beta current perception threshold between RLS patients and control participants (Cho *et al.*, 2017). The inconsistency in these results may be due to differences in methodologies. Stiasny-Kolster *et al.* (2013) assessed mechanical detection thresholds of the dorsum of the foot with von Frey filaments (small nylon 50mm rods of varying diameters used to assess mechanical sensitivity), and Cho *et al*. (2017) assessed the threshold of A-beta fibres utilising an electric current (2000Hz) on the hallux. Hence, the alterations in A-beta fibre sensitivity may be location specific or may be dependent on spinal interneuronal circuits that are activated by mechanical stimulation. As the plantar reflex was elicited via a mechanical scratching stimulus, and not an electrical stimulus, increased mechanical detection thresholds in RLS patients could be the reason for the decreased plantar reflex responses noted in Chapter 2.

A possible mechanism for the increased mechanical detection thresholds in RLS patients may be due to nociceptive fibres inhibiting A-beta fibres. The increased mechanical detection thresholds, coupled with the consistent finding of hyperalgesia in RLS patients with the absence of allodynia (Stiasny-Kolster *et al.*, 2004, 2013; Bachmann *et al.*, 2010), shows an increase in noxious responses with a possible corresponding decrease in mechanoreceptive responses. Stiasny-Kolster and colleagues postulated that the constant state of a degree of hyperalgesia in RLS patients causes continuous nociceptive fibre activation which

presynaptically inhibits mechanoreceptive A-beta fibres. The inhibition of mechanoreceptive A-beta fibres results in increased mechanical detection thresholds.

The potential occurrence of hyperalgesia with hypoesthesia (decreased tactile sensitivity) in RLS patients could be due to central plasticity of afferent spinal input (Geber *et al.*, 2008). It is theorised that the hypoesthesia is as a result of direct inhibition of A-beta fibres by activated C-fibres (Geber *et al.*, 2008). This theory is similar to that of the gate control theory of pain proposed by Melzack and Wall (1965). The gate control theory of pain is the process of non-noxious stimuli inhibiting pain signals at the level of the spinal cord (Melzack & Wall, 1965). However, it is possible that noxious stimuli could influence the processing of non-noxious stimuli as well. This has been demonstrated by a depression of activity in A-fibres when C-fibre activation precedes A-fibre activation (Zimmermann, 1968; Jänig & Zimmermann, 1971). In RLS patients the decreased thresholds of noxious afferents may lead to increased C-fibre activity which then depresses activation of A-beta fibres.

Hypoesthesia may also be as a result of an increase in inhibitory interneuron activity that suppresses non-noxious afferent fibres (Geber *et al.*, 2008). The theory of alterations in spinal interneuron function in RLS patients has been postulated previously as a possible cause for increased facilitation and decreased inhibition during H-reflex testing in RLS patients (Rijsman *et al.*, 2005; Scaglione *et al.*, 2008; Wall-Scheffler *et al.*, 2010; Marconi *et al.*, 2012; Heide *et al.*, 2014). Furthermore, alterations in interneuron function are thought to cause decreased CuSP following activation of a cutaneous nerve seen in RLS patients (Isak *et al.*, 2011). Thus alterations in interneuron function in RLS patients may be affecting the processing of non-painful sensations.

Collectively, data from comparisons of spinal reflex excitability between RLS patients and control participants indicate alterations in sensory processing with A-delta and C-fibre

changes differing to those seen in A-beta fibres in RLS patients. The state of hyperalgesia in RLS patients may lead to a depression of A-beta fibres causing a decrease in plantar reflex responses.

Contrary to the notion that RLS patients have a state of increased global spinal excitability, RLS patients have decreased plantar reflex responses compared to control participants, both in the evening and the morning. The unexpected plantar reflex results between RLS and control participants indicate that the pathophysiology of RLS is likely to involve complex spinal alterations. The concept of purely increased excitability in the spinal cord of RLS patients fails to acknowledge the complex interaction of various sensory modalities in the spinal cord. The pathophysiology of RLS likely involves changes to spinal circuits encompassing interactions between the various afferent inputs, interneurons and motor neurons.

5.1.2. Circadian variations of spinal reflex excitability in RLS

One of the key diagnostic criteria of RLS is a circadian variation in the symptom profile. Therefore it is important to take circadian variation into account when assessing possible pathophysiological mechanisms. In this regard, spinal reflex excitability in Chapters 2 and 3 was assessed both in the symptomatic and asymptomatic periods of the disorder. It was hypothesised that RLS patients would exhibit an increase in spinal excitability in the evening compared to the morning. The results from Chapters 2 and 3 of this thesis demonstrated an evening increase in assessed reflex responses in RLS patients and therefore, in agreement with this hypothesis, possibly an evening increase in spinal excitability. The RLS patients exhibited a greater degree of change in ankle angle as well as quicker movement around the ankle joint for the plantar reflex, the FWR and the crossed extensor reflex in the evening compared to morning. Although the differences are significant, the sample size was small

and these results still need to be replicated in a larger sample. Importantly, no circadian variation in the plantar reflex, FWR, or crossed extensor reflex responses were noted in healthy control participants. Collectively, these results indicate that there is a possible increase in spinal excitability in RLS patients in the symptomatic evening period, which may provide further insight into the possible mechanism underlying the symptoms of RLS.

A previous study assessing the circadian variation in spinal reflex responses in RLS patients noted conflicting results to those shown in Chapters 2 and 3. An evening decrease in patellar reflex responses was demonstrated with no circadian variation in H-reflex responses (Kerr *et al.*, 2011). The difference in results is possibly due to differences in the reflexes that were assessed. The H-reflex and patellar reflex are mediated by proprioceptive afferents, while the plantar reflex is mediated by nociceptive and mechanoreceptive afferents and the FWR is mediated by nociceptive afferents. Therefore, a possible theorised mechanism for the circadian variation in spinal excitability, and by inference the symptoms of RLS, may be due to time-dependent dysfunction in nociceptive afferent circuitry.

The nociceptive afferent circuitry plays a role in all three of the reflexes (plantar reflex, FWR and crossed extensor reflex) assessed in this thesis. The common features of the plantar reflex, FWR and crossed extensor reflex are activation of A-delta and C fibre high threshold nociceptors in order to elicit a reflex response. The increased evening reflex responses noted in RLS patients could therefore be due to time-dependent increases in excitatory neurotransmitters being released from the A-delta and C-fibre presynaptic nociceptive afferent neurons. There is currently no direct evidence to support the theory of increased excitatory neurotransmitters causing nociceptive circuitry dysfunction in RLS patients and further investigations are required. However, there is previous research that provides strength to this theory and is discussed below.

The positive effects of α2δ ligand treatment in alleviating symptoms in RLS patients indicate a possible dysfunction in excitatory neurotransmitter release (Allen *et al.*, 2014*b*; Wijemanne & Jankovic, 2015). The α2δ ligands act by binding to α2δ subunits of voltage-activated calcium channels and decreasing the release of neurotransmitters from the presynaptic terminals, which decreases post synaptic excitability (Wijemanne & Jankovic, 2015). These α2δ containing calcium channels are found in the spinal cord dorsal horn (Ferré *et al.*, 2017) and the action of α2δ ligands has been demonstrated in the spinal cord (Thorpe *et al.*, 2011). Thus, the beneficial effects of α2δ ligands in the treatment of RLS (Ferré *et al.*, 2017) may be due to a decreased release of excitatory neurotransmitters at the dorsal horn of the spinal cord (Keeler *et al*., 2012). Therefore, the positive effects of α2δ ligand treatment in RLS patients gives credence to the theory that afferent circuitry dysfunction mediated by inappropriate neurotransmitter release could be present in patients with RLS.

The notion of increased excitatory neurotransmitters causing afferent circuitry dysfunction in RLS patients is further supported by previous research conducted on the neurotransmitter glutamate. In this regard, an increase in thalamic concentrations of the excitatory neurotransmitter glutamate has been noted in RLS patients (Allen *et al.*, 2013). Furthermore, decreased iron concentrations correlate with increased brain glutamate concentrations in iron deficient rodents (Ill *et al.*, 2006; Ward *et al.*, 2007) thus secondary RLS associated with iron deficiency, such as anaemia and end stage renal disease, may have an effect on glutamate levels. Patients in end stage renal disease have increased concentrations of serum glutamate when compared to healthy controls however the presence of RLS in these patients was not reported (Rogachev *et al.*, 2012). Collectively, there is some evidence to support the theory that overall glutamate concentrations may be increased in RLS patients. These increases in glutamate concentrations may correspond with increased spinal

glutamate concentrations in RLS patients and therefore could result in sensitising afferent circuitry to nociceptive stimuli. Further investigations are however still required to provide direct evidence to support the theory of increased spinal glutamate concentrations in RLS patients.

Given that the circadian variation of RLS symptoms is a key diagnostic criterion in the diagnosis of the disorder and the evening increase in spinal reflex excitability shown in Chapters 2 and 3, the plausibility of increased glutamate concentrations as a mediating factor in RLS needs to be discussed within the context of the circadian variation of RLS. Currently, there is limited evidence with regards to circadian variations of spinal glutamate concentrations or spinal glutamate receptors in humans. Glutamine synthase, which participates in glutamate metabolism, has been shown to be increased during the active period compared to the rest period in the lumbar spinal cord of mice (Morioka *et al.*, 2012). Rats demonstrate a significant circadian rhythm of brain glutamate, with increased glutamate concentrations noted at night, the active period of rodents (Marquez de Prado *et al.*, 2000*b*; Castañeda *et al.*, 2004). However no circadian variation in the glutamate NMDA receptor expression has been noted in mice (Morioka *et al.*, 2012). These data indicate that there may be a circadian variation in glutamate concentrations in rodents that peaks during the active phase, equivalent to the asymptomatic period of RLS. However, further research is required to confirm if the circadian variation profile of glutamate concentrations, or associated receptors, noted in rodents is also present in human RLS patients.

The mechanisms accounting for the increased glutamate concentrations in patients with RLS may be related to the theory of changes in A1R and A2AR ratios in RLS as discussed in section 1.2.4. Ferré and colleagues (2017) have speculated that the increase in glutamate concentrations in RLS patients may be as a result of an increase in A2AR and a decrease in

A1R caused by an iron deficiency. Therefore, possible alterations in A1R and A2AR ratios in the dorsal horn of the spinal cord in RLS patients may be the cause of increased glutamate concentrations and mediate a state of spinal hyperexcitability. Moreover, activation of spinal A1R have anti-nociceptive properties, and have been shown to reverse hyperalgesia (Hayashida *et al.*, 2005). The hyperalgesia demonstrated in RLS patients (Stiasny-Kolster *et al.*, 2004, 2013; Bachmann *et al.*, 2010; Edwards *et al.*, 2011) may develop as a result of the theorised decreased expression of A1R receptors via increased release of glutamate from nociceptive afferents and thus subsequently increasing reflex responses to nociceptive stimuli. Furthermore, adenosine concentrations are known to increase during prolonged wakefulness, producing sleep-inducing effects (Basheer *et al.*, 2004). Therefore it is possible that increased adenosine concentrations in the evening, combined with the theorised decrease in A1R and increased A2AR in RLS patients, causes an evening increase in glutamate release leading to increased evening spinal reflex excitability in RLS patients (as seen in Chapters 2 and 3).

In addition to, or as a result of, dysfunctions in afferent nociceptive circuits, spinal central sensitisation may mediate the increased reflex responses noted in the RLS patients in Chapters 2 and 3. Decreased descending inhibition at the dorsal horn of the spine could result in an increased sensitivity to afferent stimuli in RLS patients. Impaired descending dopaminergic spinal inhibition, in the dorsal columns, from A11 diencephalospinal pathway neurons theorised in RLS patients (Clemens *et al.*, 2006) may give rise to increased excitability in the dorsal horn and a subsequent state of central sensitisation in RLS (Stiasny-Kolster *et al.*, 2004). In addition, it has been noted that RLS patients have a down-regulation of D2R in the brain (Connor *et al.*, 2009; Allen, 2015). If a corresponding spinal down regulation of D2R is present in RLS patients this may lead to increased NMDA-induced

responses in the spinal cord possibly resulting in central sensitisation. Furthermore, the aforementioned increase in afferent nociceptive circuits' excitability could also result in central sensitisation in RLS patients.

Central sensitisation would result in hyperexcitability (Woolf, 1983), as seen with the increased reflex responses shown in previous studies (Bara-Jimenez *et al.*, 2000; Gunduz *et al.*, 2017) and reinforced in Chapters 2 and 3. Furthermore, central sensitisation would also be associated with hyperalgesia (Woolf, 1983), which has also been noted in RLS patients (Stiasny-Kolster *et al.*, 2004, 2013; Bachmann *et al.*, 2010; Edwards *et al.*, 2011). The state of central sensitisation in RLS patients is further supported by an increased temporal heat summation shown in RLS patients (Edwards *et al.*, 2011), as temporal summation is considered to be a surrogate marker of central sensitisation (Edwards *et al.*, 2011). Central sensitisation of the spinal cord may cause a loss of discrimination between harmless, minor sensations, and normal sensory input (Ashmawi & Freire, 2016); and subsequently result in the uncomfortable and unpleasant sensations reported in RLS. Abnormalities in the processing of these sensations may be involved in the release of motor programs resulting in the urge to move and PLM.

The circadian changes noted in RLS patients may be due to an altered state in RLS. RLS patients may have a greater sensitivity to natural circadian changes in spinal excitability as compared to control participants. Very few studies have been conducted assessing the circadian rhythm of spinal reflex responses in humans. Studies that have been done show greater stretch reflex responses in the morning compared to the evening (Lombard, 1887; Toft *et al*., 1991) as well as larger FWR responses in the morning compared to the evening (Sandrini *et al*., 1986). To my knowledge no studies have been done assessing the circadian rhythm of the plantar reflex. Therefore, based on the observations noted in previous

studies, the increased evening reflex responses seen in RLS patients in the current thesis is opposite to what would be expected if RLS patients were more sensitive to the normal circadian rhythm of spinal reflex responses.

Overall, the investigations of the plantar reflex, FWR and crossed extensor reflex in the current thesis indicated that there is a circadian variation in the reflex responses in RLS patients. These results reinforce the notion that there is increased spinal cord excitability in the evening, which corresponds to the symptomatic period of RLS. These findings may be mediated through a possible time-dependant state of central sensitisation, particularly affecting nociceptive neurons.

5.2. Muscle activity during gait analysis and the circadian variation thereof in RLS

The plantar reflex, FWR and crossed extensor reflex investigated in this thesis have also been associated with the regulation of gait. The FWR and crossed extensor reflexes are implicated in locomotion as the spinal networks involved in these reflexes play a role in the complex generation and regulation of gait (Spaich *et al.*, 2004). The plantar reflex, though not proven to play a role in the regulation of the gait cycle, is elicited by activation of cutaneous afferents on the sole of the foot which are known to play an important role in the regulation of the gait cycle (Rossignol *et al.*, 2006). Having demonstrated differences in reflex responses and circadian variation of reflex responses in RLS patients and healthy participants, possible effects on locomotion were assessed by comparing muscle activity during the gait cycle.

The analyses of the gait cycle in Chapter 4 have demonstrated that RLS patients have an evening increase in *tibialis anterior* and *gastrocnemius lateralis* EMG amplitude during the loading response of the gait cycle when compared to control participants. Furthermore, an evening decrease in *gastrocnemius lateralis* activation during terminal stance was noted in RLS patients when compared to control participants. In addition to the muscle amplitude

differences, circadian differences in the activity of muscles involved in gait between RLS patients and control participants were also noted. Control participants, but not RLS patients, demonstrated an evening decrease in *tibialis anterior* amplitude and increase in *biceps femoris* amplitude during the gait cycle. To date, this is the first study to investigate possible circadian variations in muscle activity during the gait cycle and further studies to confirm these results and to explain the underlying mechanisms are required. These mechanisms underlying the potential differences in the circadian variation and muscle activity between healthy individuals and RLS patients may play a role in the development of RLS symptoms.

Differences in muscle activity during gait between RLS patients and control participants occurred during the loading response and terminal stance phases of the gait cycle. Therefore, the differences may be due to alterations in foot sole cutaneous afferent fibre sensitivity in RLS patients. Results from previous investigations indicate that cutaneous A-delta and C fibre sensitivity may be increased in RLS patients (Stiasny-Kolster *et al.*, 2004, 2013; Bachmann *et al.*, 2010; Edwards *et al.*, 2011). During the loading response of the gait cycle cutaneous afferents from the sole of the foot are most active. Therefore, the increased sensitivity in the A-delta and C fibres during the loading phase may cause an increased excitation of motorneurons leading to the evening increase in *tibialis anterior* and *gastrocnemius lateralis* activity noted in RLS patients when compared to healthy controls.

Similarly alterations in sensitivity of cutaneous afferent nerve fibres may be a potential mechanism for the different circadian profiles, between RLS patients and healthy control participants, of the muscles activated during the gait cycle. Chapters 2 and 3 of this thesis demonstrated a possible increase in afferent nerve fibre sensitivity in the evening in RLS patients evidenced by an increase in plantar reflex, FWR and crossed extensor reflex responses in the evening when compared to the morning. An evening increase in afferent

fibre sensitivity is supported by the findings of Cho et al. (2017) who noted decreased current perception thresholds in the evening for A-beta, A-delta and C-fibres compared to morning perception thresholds in RLS patients (Cho *et al.*, 2017). The circadian differences in muscle activity involved in the gait cycle in control participants were noted during the loading response of the gait cycle. As previously mentioned this is the phase of the gait cycle when the sole of the foot is in full contact with the ground and cutaneous afferents are most active. Therefore, an evening increase in afferent nerve fibre sensitivity from the sole of the foot in RLS patients may be masking the normal evening decrease in *tibialis anterior* muscle activation noted in control participants, resulting in no significant change in RLS patients' *tibialis anterior* muscle activation.

Another potential explanation for both the differences in muscle activity noted between RLS patients and control participants and the differences in circadian variation during the gait cycle might be a result of fluctuations in proprioceptive afferent sensitivity. Proprioceptive feedback plays a major role in coordinating gait. Previous studies assessing proprioceptive afferent function in RLS patients have been done via the assessment of Ib inhibition during the H-reflex (Rijsman *et al.*, 2005; Scaglione *et al.*, 2008; Marconi *et al.*, 2012) and evaluating Ia afferent fibre activity by assessing the patellar reflex (Kerr *et al.*, 2011). Both Ia and Ib proprioceptive afferents have decreased excitability in RLS patients (Rijsman *et al.*, 2005; Scaglione *et al.*, 2008; Kerr *et al.*, 2011; Marconi *et al.*, 2012). Ib afferent activity during the stance phase prolongs stance and delays the onset of the swing phase (Van De Crommert *et al.*, 1998). Therefore, if the excitability of Ib afferents is decreased in RLS patients, then it would be expected that there would be decreased inhibition of muscle activity and thus greater muscle activity in RLS patients compared to control participants. However, during terminal stance, when Ib proprioceptive afferents would be active, RLS patients

demonstrated decreased muscle activity in the *gastrocnemius lateralis* compared to control participants. A possible explanation for these paradoxical results is due to the fact that H-reflex studies demonstrating decreased Ib afferent activity in RLS patients were conducted while patients were stationary. The decrease in muscle activity during terminal stance of the gait cycle may be caused by the phenomenon of reflex reversal. Reflex reversal is defined as the opposite response occurring to the same stimulus in order to maintain posture during movements. In the context of the current results, reflex reversal may cause Ib afferents to facilitate muscle activity as opposed to inhibiting it (Pierrot-Deseilligny *et al.*, 1981; Duysens *et al.*, 1992). Therefore, the possible decreased excitability of Ib afferents in RLS patients would cause a decrease in facilitation of muscle activity leading to the decrease in muscle activity during terminal stance that has been noted in RLS patients when compared to healthy controls (in Chapter 4).

Circadian variations in extensor muscle proprioceptive sensitivity may cause circadian changes to muscle activity during the gait cycle. All the studies assessing Ib afferent excitability were conducted at a single time point and therefore it is not known if Ib afferent excitability varies throughout the day in RLS patients. However, the patellar reflex study of Kerr and colleagues (Kerr *et al.*, 2011) was conducted in the morning and evening and noted decreased reflex responses in the evening compared to the morning in RLS patients, possibly demonstrating evening decreased Ia afferent sensitivity. Thus, the possible evening decrease in Ia afferent sensitivity in RLS patients could mask the normal circadian alterations in afferent fibre activity that cause changes in muscle activity patterns during the gait cycle in healthy control participants.

The results obtained from the assessment of muscle activity during gait in RLS patients demonstrate possible circadian changes in both cutaneous and proprioceptive afferent fibre

excitability in RLS patients. However there is currently no evidence to support the theory of afferent circuitry dysfunction in RLS patients causing alterations in muscle activity during gait and further investigations are required. In the spinal cord afferent inputs from different sensory modalities are constantly adjusted in order to generate appropriate motor outputs as different sensory inputs have differing effects on motor outputs. Therefore, sensorimotor interaction in the spinal cord varies dependent on the necessary motor output. Responses to afferent input differ depending on whether a person is stationary or whether they are moving. The phase-dependent inhibition of afferent inputs during movement may play a role in relieving RLS symptoms as it decreases higher order processing of sensory information, hence alleviating the sensations associated with RLS. Further investigations into the systems that drive movement are needed to provide more insight into the physiological mechanisms of how movement alleviates RLS symptoms.

5.3. Conclusions and future studies

The main findings from the studies included in my thesis indicate that plantar reflex responses are significantly decreased in RLS patients compared to control participants. However, no significant differences in FWR or crossed extensor reflex excitability exist between RLS patients and control participants. Therefore the pathophysiology of RLS probably does not encompass a global increase in spinal excitability but involves complex changes to spinal circuits including interactions between different sensory modalities. Circadian variation in spinal reflex excitability is present in RLS patients, with an increase in excitability occurring in the evening. The evening increase in spinal reflex responses may be facilitated by a possible time-dependant state of central sensitisation, particularly affecting nociceptive afferent inputs. The reflexes assessed in the current body of work, the plantar, flexor withdrawal and crossed extensor reflexes, are mediated through proprioceptive

pathways. The study of these reflexes in RLS patients may provide answers to the pathological mechanism behind the urge to move. Activation of the FWR and crossed extensor reflex, and possibly the plantar reflex, involve the spinal CPG circuits (Spaich *et al.*, 2004) which are responsible for regulating leg movements during walking. Abnormalities in the processing of inputs to the spinal CPG circuits may be responsible for generating the sensation of needing to move. The relief of symptoms following movement may occur due to the completion of the CPG circuits, from the inappropriate afferent inputs to the activation of the efferent neurons controlling the muscles of the legs.

Furthermore, evening differences in gait muscle activation patterns between RLS patients and control participants were evident during the loading response and terminal stance phases of the gait cycle. As afferent inputs have regulating effects on gait the differences between RLS patients and control participants in muscle activation during gait are possibly due to alterations in afferent nerve excitability. Additionally EMG muscle activity during gait did not show the same circadian variation in RLS patients that was seen in healthy control participants. The mechanisms underlying the differences in the circadian variation of muscle activity during gait between healthy individuals and RLS patients may play a role in the development of RLS symptoms.

It should however be noted that there are some limitations of the studies that make up this thesis. Firstly there was no control for the possible effects of sleep loss or increased spinal activation due to increased voluntary evening activity and the presence of leg movements during sleep on the spinal excitability of RLS patients. As the above mentioned aspects were not controlled for the increased spinal excitability in the evening noted in RLS patients may be due to the loss of sleep and/or increased spinal activation and not primarily owing to the RLS pathology. Secondly multiple statistical comparisons were made in the studies that make

up the current thesis without a correction for multiple comparisons being done. As such the statistical results may be due to chance and future larger scale studies are needed to validate the results reported in the current thesis.

The studies in this thesis are the first to assess circadian variations in the plantar reflex, FWR, crossed extensor reflex, and gait in RLS patients. Further investigations are therefore needed to corroborate these findings as well as more in-depth studies to probe the nature of spinal excitability in RLS patients. Future studies should also attempt to control for confounding variables such as differences in quality of sleep between RLS patients and control participants as well as the possible effects PLMS and increased voluntary evening activity may have on spinal excitability. Possible future work includes assessing reflex responses at multiple times throughout a 24hr cycle in RLS patients to establish a circadian rhythm in spinal excitability; and assessing reflex responses in RLS patients during different phases of the gait cycle in order to ascertain any phase-dependant changes in reflex responses during movement. Further studies should also attempt to assess the sensitivity of afferent nerve fibres in isolation in order to confirm the hypotheses emerging from this thesis of heterogeneous alterations in afferent nerve fibre sensitivity in RLS patients.

In conclusion, the data emanating from the studies included in my thesis provide further insight into the pathophysiology of RLS by indicating that there may be changes to RLS spinal excitability, both at varying times within RLS patients and between RLS patients and control participants. However, these changes are heterogeneous as not all afferent input is affected in the same manner. The results from this thesis therefore suggest a phase-dependent and modality-dependent state of central sensitisation in RLS patients and, consequently, the theory of global hyperexcitability of RLS patients' needs to be reassessed.

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Chapter 7: Appendices

Appendix A: Ethical clearance certificate

R14/49 Ms Chloe Dafkin et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140322

NAME: (Principal Investigator)	Ms Chloe Dafkin et al
DEPARTMENT:	School of Physiology University of the Witwatersrand
PROJECT TITLE:	Objective and Subjective Measurements and Characteristics of Spinal Excitability and Periodic Limb Movements in Restless Leg Syndrome Patients
DATE CONSIDERED:	28/03/2014
DECISION:	Appproved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Samantha Kerr
APPROVED BY:	Professor P Cleaton-Jones, Co-Chairperson, HREC (Medical)
DATE OF APPROVAL:	02/07/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature Date 04/07/2014

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B: List of publications co-authored by the thesis author during the period of PhD candidature

1. Dafkin C, Green A, Kerr S, Raymond A, Veliotes D, Elvin A, Olivier B and McKinon W (2014). Kinematic and kinetic analysis of the inter- and intra-applicator assessment of the Babinski reflex. *Neurophysiologie Clinique/Clinical Neurophysiology* **44(5)**, 471-477.

2. Green A, Dafkin C, Kerr S, McKinon W (2015). The effects of walking on golf drive performance in two groups of golfers with different skill levels. *Biology of Exercise* **11(1)**, 13-25.

3. Green A, Dafkin C, Kerr S, McKinon W (2015). The relationships between physical and biomechanical parameters and golf drive performance. *South African Journal for Research in Sport, Physical Education and Recreation* **37(3)**, 83-95.

4. Dafkin C, Green A, Kerr S, Veliotes D, Olivier B and McKinon W (2016). The interrater reliability of subjective assessments of the Babinski reflex. *Journal of Motor Behavior* **48(2)**, 116-121.

5. Green A, Kerr S, Dafkin C & McKinon W. (2016). The calibration and application of an individual scrummaging ergometer. *Sports Engineering* **19(1)**, 59-69.

6. Kerr S, Olivier B, Green A, Dafkin C, Wood S, Woodiwiss A & Mckinon W. (2016). Analysis of balance and body positioning in ballerinas with different levels of skill: sport science. *African Journal for Physical Activity and Health Sciences (AJPHES)* **22(Issue-32)**, 883-895.

7. Green A, Kerr S, Olivier B, Dafkin C & McKinon W. (2016). The relationships between rugby ground pass accuracy and kinematic variables resulting from two different pelvic orientations. *South African Journal of Sports Medicine* **28(2)**, 51-54.

8. Green A, Kerr S, Olivier B, Dafkin C & McKinon W. (2016). The trade-off between distance and accuracy in the rugby union place kick: a cross-sectional, descriptive study. *Kineziologija* **48(2)**, 11-12.

9. Green A, Kerr S, Dafkin C, Olivier B & McKinon W. (2017). A lower body height and wider foot stance are positively associated with the generation of individual scrummaging forces in rugby. *International Journal of Performance Analysis in Sport* **17(1-2)**, 177-189.

10. Green A, Kerr S, Dafkin C & McKinon W. (2017). Combined individual scrummaging kinetics and muscular power predict competitive team scrum success. *European Journal of Sport Science* **30**, 1-10.

11. Green A, Kerr S, Olivier B, Meiring R, Dafkin C & McKinon W. (2017). A simulated rugby match protocol induces physiological fatigue without decreased individual scrummaging performance. *South African Journal of Sports Medicine* **29(1)**.

Appendix C: Exert from my masters dissertation describing the use of kinematics in neurological studies.

Even though objective recording of reflex responses have been made since Charcot in the late 1800's (as per Cartwright, 1992), it wasn't until the recent development of kinematics within the field of biomechanics that true objective measurements of movement could be made. Biomechanics is the study of the mechanics of living organisms. The division of biomechanics which is relevant to this review is kinematics. Kinematics looks at the motion of an object or segment in time and space (Kreighbaum *et al*, 1996). The way in which kinematics of a movement are captured is through the use of high speed cameras and strategically positioned retro-reflective markers. The markers are placed on joints of a limb segment and through the use of specialised computer code a three dimensional model of the limb can be reconstructed. From this the degree of motion and the speed of movement can be calculated from the model. This provides an objective measure of movement of a specific limb or a combination of body segments.

Currently the only accepted objective measurement of reflexes is EMG of the muscles involved. Neurological examinations are mostly done subjectively by the physician reporting what he/she has seen and by the use of subjective rating scales. It is important to decrease any subjective variability found when assessing reflexes in order to create more accurate ratings of reflexes which will lead to more precise diagnoses and improved treatments. Biomechanics is ideal for the objective assessment of reflexes. Recently, biomechanical assessment has been used as a tool to objectively assess neurological reflexes however this is only in research studies and not yet in clinical practice. The withdrawal reflex has been looked at kinematically by Benz *et al* (2005). They assessed the changes in angles for the ankle, knee and hip (Benz *et al*, 2005). Other reflexes that have been looked at biomechanically include the patellar reflex (Tham *et al*, 2010, Dafkin *et al*, 2012 and Dafkin *et al*, 2013) and ankle clonus (Benz *et al*, 2005). To date the Babinski reflex has not been assessed with kinematics.

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Cartwright L (1992). "Experiments of destruction": cinematic inscriptions of physiology. *Representations* **40**, 129-152.

Dafkin C, Green A, Kerr S, and McKinon W (2012). The patellar reflex: does activity of quadriceps femoris muscles reflect leg movement? *Neurol Res* **34**, 623-626.

Dafkin C, Green A, Kerr S, Veliotes D and McKinon W (2013). The accuracy of subjective clinical assessments of the patellar reflex. *Muscle Nerve* **47(1)**, 81-88.

Kreighbaum E and Barthels KM (1996). Biomechanics. A Qualitative Approach for Studying Human Movement. Allyn and Bacon, Needham Heights, MA.

Tham LK, Osman NAA, Lim KS, Pingguan-Murphy B and Wan Abas WAB (2010). Assessment of deep tendon reflexes by motion analysis: a preliminary study. *IFMBE Proceedings* **31**, 251-253.