Sleep Medicine Reviews 45 (2019) 70–87

Contents lists available at ScienceDirect

Sleep Medicine Reviews

journal homepage: www.elsevier.com/locate/smrv

PHYSIOLOGICAL REVIEW

Neurochemical features of idiopathic restless legs syndrome

Félix Javier Jiménez-Jiménez [a, *], Hortensia Alonso-Navarro [a], Elena García-Martín [b], José A.G. Agúndez [b]

[a] Section of Neurology, Hospital Universitario del Sureste, Arganda del Rey, Madrid, Spain
[b] University Institute of Molecular Pathology Biomarkers, UNEx, ARADyAL Instituto de Salud Carlos III, Cáceres, Spain

ARTICLE INFO

Article history:
Received 24 December 2018
Received in revised form
8 March 2019
Accepted 15 March 2019
Available online 21 March 2019

Keywords:
Restless legs syndrome
Neurochemistry
Neurotransmitters
Dopaminergic dysfunction
Iron deficiency
Glutamate
GABA

SUMMARY

The most important traditional hypotheses of the pathogenesis of idiopathic restless legs syndrome (iRLS) involve dopaminergic dysfunction and iron deficiency. However, a possible role of other neurotransmitter or neuromodulators, mainly glutamate, gamma-hydroxybutyric acid (GABA), and adenosine have been suggested in recent reports. Moreover, iron deficiency in experimental models (which causes sensorimotor symptoms resembling those of RLS) is able to induce changes in dopaminergic, glutamatergic and adenosinergic neurotransmission, thus suggesting its crucial role in the pathogenesis of this disease. Relationship between iRLS and opiates, oxidative stress and nitric oxide, and with vitamin D deficiency has also been reported, although data regarding these variables should be considered as preliminary. In this review, we focus on studies relating to neurochemical findings in iRLS.

Introduction

Restless legs syndrome (RLS) or Willis-Ekbom disease (WED) is a sensorimotor disorder with well established essential and supportive diagnostic criteria according to the International restless legs syndrome study group (IRLSSG) [1]. Dopaminergic dysfunction and brain iron insufficiency are thought to be the most important factors contributing to the pathophysiology of idiopathic RLS (iRLS), although this to date has not been well established. In recent years, several reports have suggested an important role of other neurotransmitters or neuromodulators. The main aim of this review is to provide an extensive descriptive review of studies reporting neurochemistry and biochemical findings on the iRLS issue, to summarize their main conclusions, and to propose suggestions for future studies with updated information [2].

Search strategy

References for this review were identified through a PubMed search which included the period from 1966 until December 12, 2018. The term "*restless legs syndrome*" was crossed with "*neurochemistry*" (1 item), "*biochemistry*" (39 items), "*neurotransmitters*" (1156 items), "*dopamine*" (1022 items), "*noradrenaline*" (13 items), "*norepinephrine*" (10 items), "*adrenaline*" (2 items), "*epinephrine*" (2 items), "*serotonin*" (73 items), "*GABA*" (132 items), "*gamma-aminobutyric acid*" (113 items), "*glycine*" (4 items), "*glutamate*" (20 items), "*neuropeptides*" (26 items), "*iron*" (583 items), "*ferritin*" (227 items), "*transferrin*" (58 items), "*white matter*" (15 items), "*gray matter*" (13 items), and "*transcranial magnetic stimulation*" (39 items). The full search retrieved 1798 references, which were examined one by one; those references strictly related with neurochemical findings in iRLS (n = 185) were selected.

Dopaminergic dysfunction

The dopaminergic dysfunction hypothesis in the pathophysiology of RLS was formulated based essentially on the symptomatic improvement of RLS symptoms achieved with dopaminergic drugs, mainly dopamine agonists. In addition, data from

* Corresponding author. C/Marroquina 14, 3º B, E-28030, Madrid, Spain. Fax: +34913280704.
E-mail addresses: fjavier.jimenez@salud.madrid.org, felix.jimenez@sen.es (F.J. Jiménez-Jiménez).

https://doi.org/10.1016/j.smrv.2019.03.006
1087-0792/

Abbreviations

ACTH	adrenocorticotropic hormone
AD	adrenaline
AD	axial diffusivity
AIF	Afferent-induced facilitation
ADH	antidiuretic hormone
A1R	adenosine receptor 1
A2AR	adenosine receptor 2A
BH4	tetrahydrobiopterin
BTBD9	BTB/POZ domain-containing protein 9
CIT	2b-carbomethoxy-3b-(4-iodophenyl) tropane
COMT	catechol-ortho-methyltransferase
CSF	cerebrospinal fluid
CxCl12	
DA	dopamine
DAT	dopamine transporter
DBH	dopamine-beta-hydroxylase
DMT1	divalent metal transporter 1
DOPA	dihydroxyphenylalanine
DOPAC	dihydroxy-phenylacetic acid
DRD1	dopamine receptor type 1
DRD2	dopamine receptor type 2
DRD2-BP	dopamine receptor type 2 binding potential
DRD3	dopamine receptor type 3
DRD4	dopamine receptor type 4
DRD5	dopamine receptor type 5
DTI	diffusion tensor imaging
ERK	Extracellular Signal-regulated Kinase
FA	fractional anisotropy
FG	fluorogold
GABA	gamma-aminobutyric acid
GABR	GABA receptor
GABRA4	GABA receptor alpha4 subunit
GABRR3	GABA receptor rho3 subunit
GFAP	glial fibrillar acid protein
GM	gray matter
GMD	gray matter density
GWAS	genome-wide association study
Hbb-b1	hemoglobin beta adult chain 1
5-HIAA	5-Hydroxyindoleacetic acid
HMOX1	Heme oxygenase 1
^{1}H-MRS	^{1}H-magnetic resonance spectroscopy
HVA	homovanillic acid
IBZM	^{123}I-S-2-hydroxy-3-iodo-6-methoxy-[(1-ethyl-2-pyrrolidinyl)methyl] benzamide
ID	iron deficiency
IPT	^{123}I-N-(3-iodopropen-2-yl)-2beta-carbomethoxy-3beta-(chloro-phenyl) tropane
iRLS	Idiopathic restless legs syndrome
IRLSSG	International restless legs syndrome study group
IRP	iron regulatory proteins
LAI	Long-latency afferent inhibition
LICI	Long interval intracortical inhibition
LOX-1	Lectin-like oxidized Low-Density Lipoprotein Receptor-1
MAO-A	monoamine oxidase A
MAO-B	monoamine oxidase B
MD	mean diffusivity
MDA	malonyl dialdehyde acid
MEIS1	meis homeobox 1 gene
MEP	motor evoked potential
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSR	monosynaptic stretch reflexes
NA	noradrenaline
NAA	N-acetylaspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NOS1	nitric oxide synthase 1
NREM	no rapid eye movements
6-OHDA	6-hydroxydopamine
3-OMD	3-orthomethyldopa
PD	Parkinson's disease
PET	Positron Emission Tomography
PLMS	periodic limb movements during sleep
PLMI	periodic limb movements index
QSM	quantitative susceptibility mapping
RD	radial diffusivity
REM	rapid eye movements
RMT	resting motor threshold
RLS	restless legs syndrome
rTMS	repetitive transcranial magnetic stimulation
SAI	Short-latency afferent inhibition
SDF-1	stromal cell-derived factor 1
SERT	serotonin transporter
SICI	Short interval intracortical inhibition
SLC1A2	solute carrier family 1 - glial affinity glutamate transporter-member 2
SLC11A2	solute carrier family 11 member 2
SPECT	single photon emission computerized tomography
T2CR	T2 change ratio
T2W	T2 weighted
tDCS	transcranial direct current stimulation
TH	tyrosine hydroxylase
TMS	transcranial magnetic stimulation
tsDCS	transcutaneous spinal direct current stimulation
VBM	voxel-based morphometry
VDR	vitamin D receptor
VMAT	vesicular monoamine transporter
WED	Willis-Ekbom disease

neuropathological, neuroimaging and biochemical studies in humans, and from experimental studies in animals, have supported the possible role of the dopaminergic system in RLS pathophysiology.

Brain post-mortem studies in humans

Few brain post-mortem studies performed in humans, summarized in Table 1, have addressed the dopaminergic system in iRLS. Most of these studies have reported similar tyrosine-hydroxylase (TH) staining in the substantia nigra [Suppl. 1, Suppl. 2] and in the diencephalic-spinal A11 system [Suppl. 4] of patients diagnosed with iRLS in comparison with healthy controls. One study described a significant increase in TH staining and active TH in the substantia nigra, and a decrease in dopamine D_2 receptors (DRD_2) in the putamen of iRLS patients [Suppl. 3].

Functional neuroimaging studies

Table 2 summarizes the results of functional neuroimaging studies reported to date comparing patients diagnosed with iRLS and healthy controls, including functional neuroimaging studies on

Table 1
Results of neuropathological studies in patients with idiopathic restless legs syndrome (iRLS) compared with controls.

	Authors, Year [Ref]	RLS/Controls	Main findings
Dopaminergic system	Connor et al., 2003 [Suppl. 1]	7 (6 females)/5 (3 females)	Non-significant differences between iRLS patients and controls in tyrosine-hydroxylase (TH) staining in the substantia nigra
	Walters et al., 2009 [Suppl. 2]	5 (females)/6 (5 females)	Non-significant differences between iRLS patients and controls in TH staining in the substantia nigra
	Connor et al., 2009 [Suppl. 3]	8 (females)/8 (females, putamen analyzed only in 7)	A significant increase in TH staining in the substantia nigra, but not in the putamen, of iRLS patients. A significant increase of phosphorylated or active TH, in the substantia nigra of iRLS patients. A significant decrease in dopamine D_2 receptors (DRD_2) in the putamen of iRLS patients, which was correlated with RLS severity. Non-significant differences between iRLS patients and controls in DRD_1 receptors, dopamine transporters and vesicular monoamine transporter (VMAT) in the putamen and in the substantia nigra
	Earley et al., 2009 [Suppl. 4]	6 (females)/6 (2 females)	Non-significant differences between iRLS patients and controls in TH staining cell volume, fractional glial fibrillary acidic protein (GFAP) staining, or general histological examination in the diencephalic-spinal A11 (hypothalamus) dopaminergic system
Iron and related substances	Connor et al., 2003 [Suppl. 1]	7 (6 females)/5 (3 females)	A marked decrease in iron-staining and H-ferritin (heavy chain subunit of ferritin) staining, mild decrease in transferrin receptor staining on neuromelanin-containing cells, and morphological differences in the cells staining for L-ferritin (light chain subunit of ferritin) in the substantia nigra of iRLS patients
	Connor et al., 2004 [Suppl. 5]	4 (females)/4 (1 female)	Decreased concentrations of ferritin, divalent metal transporter 1 (DMT1), ferroportin, and transferrin receptor, decreased activities of total iron regulatory proteins (IRP) and IRP1, decreased IRP1 protein levels, increased concentrations of transferrin, and normal IRP2 protein levels in the neuromelanin cells of the substantia nigra.
	Wang et al., 2004 [Suppl. 6]	4 (females)/4 (1 female)	Decreased concentrations of Thy1 (an adhesion molecule that plays a role in the vesicular release of neurotransmitters) to less than half in the substantia nigra of iRLS patients (this molecule is decreased in experimental animals under iron deficiency diet).
	Snyder et al., 2009 [Suppl. 7]	8 (females)/12 (3 females) (substantia nigra analyzed in 8/8)	Higher mitochondrial ferritin levels and higher cytochrome c oxidase (COX) staining (reflecting numbers of mitochondria) in the substantia nigra of iRLS patients than in controls, while these values were similar in the putamen Decreased immunostaining for cytosolic H-ferritin in iRLS patients
	Connor et al., 2011 [Suppl. 8]	14 (13 females)/18 (15 females)	Reduction of iron and H-ferritin staining and up-regulation of DMT1, ferroportin, transferrin and its receptor in the epithelial cells of choroids plexus from iRLS patients. Decreased expression of H-ferritin, transferrin and its receptor, and normality of DMT1, ferroportin, prohepcidin, mitochondrial ferritin and L-ferritin in the brain microvessels of iRLS patients.
Opiate system	Walters et al., 2009 [Suppl. 2]	5 (females)/6 (5 females)	Significant reduction of beta-endorphin and met-enkephalin cells and similar leu-enkephalin cells in the thalamus of iRLS patients compared with controls, while beta-endorphin, met-enkephalin, leu-enkephalin, and TH cells were similar in the substantia nigra of both study groups

presynaptic dopaminergic terminal and postsynaptic DRD_2. While four studies showed a slight reduction in the binding of the tracer to the presynaptic dopaminergic terminal in the striatum, or only in the caudate or the putamen [Suppl. 14, Suppl. 16-Suppl. 18], and another reported a mildly increased density in the striatum [Suppl. 13], the majority found no significant differences in the binding of the tracer [Suppl. 9–12, Suppl. 14]. Studies on post-synaptic DRD_2 performed using SPECT with ^{123}I-S-2-hydroxy-3-iodo-6-methoxy-[(1-ethyl-2-pyrrolidinyl)methyl]benzamide(IBZM) showed a similar binding in the striatum of iRLS patients and controls [Suppl. 9, Suppl. 13, Suppl. 19, Suppl. 20], with the exception of one that reported decreased binding [Suppl. 11]. However, the majority of studies on DRD_2 using the tracer ^{11}C-raclopride found decreased binding or increased availability in the entire striatum [Suppl. 16, Suppl. 21, Suppl. 23] or only in the caudate nucleus [Suppl. 22].

Cerebrospinal fluid and blood studies

The results of studies assessing cerebrospinal fluid (CSF) and blood dopamine (DA) metabolite levels in iRLS patients and controls are summarized in Table 3. CSF homovanillic acid (HVA) levels were found to be similar in iRLS patients than in controls in several studies [Suppl. 26–Suppl. 28], although others found higher CSF HVA in iRLS patients with high CSF 3-orthomethyl-DOPA(3-OMD) levels compared with patients with low CSF 3-OMD and with controls [Suppl. 30]. Poceta et al. [Suppl. 29], in a 22-h study monitoring CSF DA, HVA, dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA) from samples every 30 min, and hypocretin-1 every 60 min, found a peak for DA at 10 a.m. ($p < 0.025$) and for HVA at 2 p.m. ($p < 0.01$), but not for the other substances measured, thus suggesting the presence of a circadian rhythm for CSF DA and HVA levels in humans with higher levels in the daytime than at night. CSF 3-OMD levels in iRLS patients have been reported to be increased [Suppl. 27] or similar to those found in healthy controls [Suppl. 28]. CSF tetrahydrobyopterin (BH_4) levels have been found increased [Suppl. 26] or normal [Suppl. 27]. Allen et al. [Suppl. 30] reported higher periodic limb movements (PLMS)/hour for iRLS patients with high CSF 3-OMD levels than for those with 3-OMD, suggesting that patients with more severe iRLS showed increased DA synthesis.

Mitchell et al. [Suppl. 31] found increased blood plasma DA, and normal noradrenaline (NA) and adrenaline (AD) levels in iRLS patients under dopaminergic or benzodiazepine therapy compared with unmedicated iRLS patients and with controls, and lower DRD_2 expression in lymphocytes in iRLS (both medicated and unmedicated) than in controls, suggesting a down-regulation of DRD_2 that was not reversed by medication.

Experimental models related to the dopaminergic system

Several supra-pontine structures projecting to the spinal cord, including the dopaminergic diencephalic-spinal pathway (originated in the hypothalamic A11 dopaminergic group), seem to play an important role in the pathophysiology of RLS. The existence of this pathway has been shown both in rodents (Fluoro-Gold —FG-, a fluorescent tracer, injected in the dorsolumbar spinal cord by stereotaxis, reaches the A10-A11 dopaminergic neurons in the dorsoposterior hypothalamus) [3], and in non-human primates, (unilateral injection of FG into the cervical spinal cord labels A11 neurons) [4]. Immunocytochemical studies in rats have shown that three types of neurons in the A11 region expressing calbindin, TH, or

Table 2
Results of functional neuroimaging studies using dopamine, serotonin, and opiate tracers in patients with idiopathic restless legs syndrome (RLS) compared with controls.

	Method	Authors, Year [Ref]	RLS/Controls	Main findings
Presynaptic DA terminal	^{123}I-IPT-SPECT	Eisensehr et al., 2001 [Suppl. 9]	25 (14 drug-naïve and 11 levodopa treated)/ 10	Non-significant differences between iRLS and controls
	^{123}I-IPT-SPECT	Linke et al., 2004 [Suppl. 10]	28/23	Non-significant differences between iRLS and controls
	^{123}I-beta-CIT-SPECT	Michaud et al., 2002 [Suppl. 11]	10 (drug-naïve)/10	Non-significant differences between iRLS and controls
	^{123}I-beta-CIT-SPECT	Mrowka et al., 2005 [Suppl. 12]	6/?	Non-significant differences between iRLS and controls
	^{123}I-beta-CIT-SPECT	Kim et al., 2012 [Suppl. 13]	13/12	Increased DAT density in the caudate ($P = 0.037$), posterior putamen ($P = 0.041$), and entire striatum ($P = 0.046$) in iRLS patients
	^{99m}TC-TRODAT-1	Lin et al., 2016 [Suppl. 14]	22/12	Decreased DAT binding in both caudate in iRLS patients ($P = 0.0027$ in the right and 0.0015 in the left)
	^{18}Fluoro-L-Dopa (F-DOPA) PET	Trenkwalder et al., 1999 [Suppl. 15]	4/10	Non-significant differences between iRLS and controls
	^{18}Fluoro-L-Dopa (F-DOPA) PET	Turjanski et al., 1999 [Suppl. 16]	13/14	Mild reduction of binding in the putamen ($p = 0.04$) in iRLS patients
	^{18}Fluoro-L-Dopa (F-DOPA) PET	Ruottinen et al., 2000 [Suppl. 17]	9 (drug-naïve)/27	Mild reduction of binding in caudate (88%) and putamen (89%) in iRLS patients
	^{11}C-methylphenidate PET	Earley et al., 2011 [Suppl. 18]	36/34	Decreased DAT binding in the striatum (putamen and caudate but not in the ventral striatum) both in the day and the night scans. Lack of correlation of DAT binding potentials with any clinical measures of RLS.
Postsynaptic DA terminal	^{123}I-IBZM-SPECT (striatal D_2 receptors)	Eisensehr et al., 2001 [Suppl. 9]	25 (14 drug-naïve and 11 levodopa treated)/ 10	Non-significant differences between iRLS and controls
	^{123}I-IBZM-SPECT (striatal D_2 receptors)	Tribl et al., 2002 [Suppl. 19]	14/9	Non-significant differences between iRLS and controls
	^{123}I-IBZM-SPECT (striatal D_2 receptors)	Michaud et al., 2002 [Suppl. 11]	10 (drug-naïve, all with PLMS)/10	Decreased binding in iRLS ($P = 0.006$)
	^{123}I-IBZM-SPECT (striatal D_2 receptors)	Tribl et al., 2004 [Suppl. 20]	14 (all with PLMS)/10	Non-significant differences between iRLS and controls
	^{123}I-IBZM-SPECT (striatal D_2 receptors)	Kim et al., 2012 [Suppl. 13]	13/12	Non-significant differences between iRLS and controls
	^{11}C-raclopride PET (striatal D_2 receptors)	Turjanski et al., 1999 [Suppl. 16]	13/14	Decreased binding in the caudate ($p = 0.01$) and the putamen ($p = 0.008$) in iRLS patients
	^{11}C-raclopride PET (striatal D_2 receptors)	Cervenka et al., 2006 [Suppl. 21]	16 (not treated with dopaminergic or opiate agents)/16	Increased DRD_2 availability corresponding to higher DRD_2 densities or lower levels of endogenous dopamine in iRLS patients
	^{11}C-raclopride PET (striatal D_2 receptors)	Oboshi et al., 2012 [Suppl. 22]	8 (drug-naïve)/8	Decreased DRD_2-BP in the mesolimbic dopamine region (nucleus accumbens) and caudate, and normal in the putamen in iRLS patients. Correlation between DRD_2-BP and clinical severity scores (negative) and with the degree of posttreatment improvement with pramipexole (positive) in iRLS patients.
	^{11}C-raclopride PET (striatal D_2 receptors)	Earley et al., 2013 [Suppl. 23]	31/36	Decreased DRD_2-BP in putamen and caudate but not the ventral striatum in iRLS patients. DRD_2-BP did not differ between night and day for either group. DRD_2 β (max) and K (d) did not differ significantly between patients with iRLS and controls but did show a strong and significant increase at night in the ventral striatum. Lack of correlation of DRD_2-BP with any clinical measures of RLS.
	^{11}C-FLB PET (extrastriatal D_2 receptors)	Cervenka et al., 2006 [Suppl. 21]	16 (not treated with dopaminergic or opiate agents)/16	Increased DRD_2 availability corresponding to higher DRD_2-densities or lower levels of endogenous dopamine in iRLS patients
Serotonin transporter (SERT)	^{123}I-beta-CIT-SPECT	Jhoo et al., 2010 [Suppl. 24]	16 (drug-naïve)/16	Non-significant differences in the pons and medulla between iRLS patients and controls A negative correlation between the severity of RLS and the availability of SERT
Opioid receptor	^{11}C-diprenorphine PET	Von Spiczak et al., 2005 [Suppl. 25]	15/12	Non-significant differences between iRLS patients and controls A negative correlation between ligand binding and RLS severity in the medial thalamus, amygdala, caudate nucleus, anterior cingulate gyrus, insular cortex, and orbitofrontal cortex
Regional blood flow	^{18} F-fluorodeoxyglucose PET	Trenkwalder et al., 1999 [Suppl. 15]	6/10	Non-significant differences between iRLS patients and controls

IPT N-(3-iodopropen-2-yl)-2beta-carbomethoxy-3beta-(chloro-phenyl) tropane; CIT 2β-carbomethoxy-3β-(4-iodophenyl) tropane; IBZM (S)-2-hydroxy-3-iodo-6-methoxy-[(1-ethyl-2-pyrrolidinyl) methyl] benzamide; PET Positron Emission Tomography; SPECT Single Photon Emission Computerized Tomography. DRD_2-BP Dopamine D_2 Receptor Binding Potential.

Table 3
Alterations in the CSF and blood levels of dopamine and their metabolites in patients with idiopathic restless legs syndrome (iRLS).

Biological fluid	Authors, Year [Ref]	RLS/controls	Main findings
Cerebrospinal fluid	Earley et al., 2001 [Suppl. 26]	16 (2 wk off medication)/14 (non-age-matched). (Samples obtained at 10 a.m.).	CSF homovanillic acid (HVA) and neopterin levels were similar in iRLS patients and controls after adjustment by age. Decreased CSF hydroxyindoleacetic acid (5-HIAA) ($p < 0.01$) and increased CSF tetrahydrobyopterin (BH_4) levels ($p < 0.01$) in iRLS patients after adjustment by age.
	Early et al., 2006 [Suppl. 27]	30/22 (samples obtained at 10 a.m and at 10 p.m.).	CSF HVA and BH_4 levels were similar in iRLS patients and controls. CSF 3-orthomethyl-DOPA (3-OMD) significantly higher in iRLS patients both at 10 a.m. ($p < 0.01$) and at 10 p.m. ($p < 0.05$). Higher CSF HVA/5-HIAA ratio, BH_4, and 3-OMD levels in this cohort of iRLS patients compared with those of the previous study with samples obtained at 10 a.m., suggesting changes related to the circadian rhythm
	Stiasny-Kolster et al., 2004 [Suppl. 28]	22 (drug-naïve)/11	CSF HVA, 3-OMD, BH_4, dihydrobiopterin, neopterin, and methyltetrahydrofolate levels similar in iRLS patients and controls
	Poceta et al., 2009 [Suppl. 29]	3/3 (monitorization for 22 h with samples every 30 min except for hypocretine-1 with samples every 60 min)	A peak for DA at 10 a.m. ($p < 0.025$) and for HVA at 2 p.m. ($p < 0.01$) were found, while there was no evidence of significant 24-h rhythms for dihydroxy-phenylacetic acid (DOPAC), 5-HIAA, the HVA/5-HIAA ratio, or hypocretin-1. These results demonstrate a circadian rhythm for CSF DA and HVA concentrations in humans (which could be related to the circadian rhythm of RLS symptoms), with higher levels in the daytime than at night-time.
	Allen et al., 2009 [Suppl. 30]	49/36	Increased CSF HVA levels in iRLS patients with high CSF 3-OMD ($n = 30$), and lower CSF HVA levels in RLS patients with normal CSF 3-OMD ($n = 19$), when compared with controls. Higher PLMS/hour for iRLS patients with high CSF 3-OMD (suggesting higher severity of RLS) than for those with normal CSF 3-OMD. These results suggest increased DA synthesis for more severe RLS.
Blood	Mitchell et al., 2018 [Suppl. 31]	13/12 (6 untreated patients, six treated with the dopamine agonist, 1 with a benzodiazepine)	Higher blood plasma DA and normal blood plasma NA and AD levels in medicated iRLS patients than in unmedicated iRLS patients and in controls. Dopamine receptor 2 (DRD2) expression in lymphocytes significantly lower in iRLS than in controls.

both TH and calbindin, project to the spinal cord [5]. In non-human primates, the absence of expression of DRD_1 mRNA, and expression of DRD_2 and DRD_5 mRNAs in the dorsal horn, have been observed, while DRD_3 mRNA is expressed both in the dorsal and ventral horns [4]. Several data suggest that the diencephalic-spinal DA neurons do not show presynaptic synthesis modulating D_2 autoreceptors, for instance that DRD_2 blockade with raclopride increases the DOPAC/DA ratio in the striatum and nucleus accumbens of rats, though not in the spinal cord, and the lack of prevention of increased DA concentrations in the spinal cord elicited with gamma-butyrolactone by pre-treatment with DRD_2 agonists [6].

One interesting experimental RLS model related to the dopaminergic system consists of the ***selective bilateral lesion of the A11 area in rodents*** using injections of 6-hydroxydopamine (6-OHDA). This lesion causes increased locomotor activity, which resembles in part RLS symptoms in humans [7–11], and is more marked in animals under an iron deficiency (ID) diet [8–11]. The DRD_2/DRD_3 agonist ropinirole [8,9] and pramipexole [10,11] can reverse the increased locomotor activity. Neuropathological and neurochemical changes induced by 6-OHDA lesion in the A11 area include the following:

1) Decreased of TH staining cells by 40–94% [7,9,11]. This effect is more marked in ID diet animals [9] and can be reversed by treatment with the DRD_2/DRD_3 agonist ropinirole and worsened by DRD_1 agonists [9].
2) Decreased lumbar spinal cord DA concentrations [8].
3) Decreased DRD_2/DRD_3 mRNA and protein concentrations (a change that is more marked in ID diet animals) and binding capacity in the lumbar spinal cord without inducing changes in DRD_1 [8,10].

DRD_3 knock-out mice show increased locomotor activity and increased sensory excitability to thermal stimuli [12]. Compared with wild-type, *DRD_3* knockout mice presented a significantly higher DRD_1 but similar DRD_3 protein expression in the spinal cord [12]. In the same model, low dopamine levels increased monosynaptic stretch reflexes (MSR) and longer-latency reflexes amplitude, while wild-type mice showed the opposite effect. While DRD_3 agonists and DRD_3 antagonists had no effect on MSR in *DRD_3* knockout mice, DRD_3 agonists reduced and DRD_3 antagonists increased MSR in wild-type mice [13]. These data suggest a possible role of DRD_3 in modulating spinal excitability through a reduced DRD_3 activation in the pathophysiology of RLS.

Several *MEIS1* gene variants (this gene encodes a transcription factor that has an important role in normal development) have shown a consistent association with the risk for RLS in Genome-Wide Association Studies (GWAS) [14]. The ***Meis1 knock-out mouse*** shows a pattern of circadian hyperactivity resembling that of RLS, a prepulse inhibition deficit, and hyposensitivity to the prepulse inhibition-reducing effect of the DRD_3 agonist pramipexole, which suggests a role of Meis1 in the dopaminergic system [15].

Finally, although treatment of RLS with DRD_3 agonists is initially highly effective, long-term therapy results for many patients in the augmentation phenomenon (that is, worsening of the symptoms with increasing doses). A recent report described a phenomenon resembling augmentation in a mouse model under prolonged therapy with DRD_3 agonists that was reversed by DRD_1 blockers. This suggests the presence of a hyperdopaminergic state involving DRD_1 subtype related to a DRD_1 upregulation induced by DRD_3 agonists [16].

Pharmacogenetic studies related to dopaminergic neurotransmission

Case-control association studies showed a lack of association between several variations in genes coding for enzymes and receptors related to dopaminergic neurotransmission (including *TH, dopamine-beta-hydroxylase* or *DBH*, *dopamine transporter* or *DAT*. *DRD_1*, *DRD_2*, *DRD_3*, *DRD_4*, *DRD_5*, *monoamine oxidase A* and *B* or *MAOA* and *MAOB* and *catechol-ortho-methyl-transferase* or *COMT*) and the risk of developing RLS [14].

Iron deficiency

Brain post-mortem studies in humans

The results of brain post-mortem studies in humans regarding iron concentrations in iRLS patients and controls are summarized in Table 1. Most of these studies show data consistent with iron deficiency in iRLS patients, which include decreased iron and ferritin (especially heavy chain ferritin of H-ferritin), divalent metal transporter 1 (DMT1), ferroportin, and transferrin receptor concentrations, decreased cytosolic H-ferritin immunostaining, decreased activities of total iron regulatory proteins (IRP) and IRP1, and increased transferrin levels and mitochondrial ferritin levels in the *substantia nigra* [Suppl. 1, Suppl. 5-Suppl. 7]. Decreased concentrations of Thy1 in the *substantia nigra* of iRLS patients, together with decreased Thy1 concentrations in ID rats, suggest a possible role of the interaction between Thy1 and iron in the compromise of dopaminergic neurotransmission and in the pathogenesis of RLS [Suppl. 6].

Blood-brain barrier and brain microvessels could play a role in the regulation of iron uptake and storage, because in patients with iRLS iron and H-ferritin staining has been found to be decreased, DMT1, ferroportin, transferrin and its receptors are up-regulated in the epithelial cells of the choroids plexus, and H-ferritin, transferrin, and its receptor expression have been found to be decreased in the brain microvessels of iRLS patients [Suppl. 8]. This role of the blood-brain barrier and blood microvessels could be related to the described association between RLS and cerebrovascular disease. RLS has been reported in 12.4% of a series of 137 patients with acute stroke and has been related with the presence of infarcts in the basal ganglia/corona radiate, pons, thalami or internal capsule [17]. Shiina et al. [18] reported RLS or RLS variants in 7.7% of 104 patients with acute stroke, which was related with lesions in the medulla, pons, corona radiata and, less frequently, in the basal ganglia and cortex. Woo et al. [19], reported 36 patients (30 of them from a literature review) with post stroke RLS or PLMS, and observed that post stroke RLS (usually bilateral) was related with lesions in the corona radiata and in basal ganglia, while lesions in the pontine base and tegmentum were associated with unilateral post stroke PLMS. Ferri et al. [20], in a study with MRI involving 97 RLS patients (44 of them with >10 y of duration of the disease) and 74 controls found a significant increase in cerebral small vessel disease in RLS patients, which was related with age and duration of the disease. In addition, a Sleep Study involving 2823 men showed an increased risk for cardiovascular disease and for stroke/transient ischemic attacks in RLS patients, which was independent on the presence of PLMS, thus suggesting a role of non-PLMS factors (sleep disturbance, genetic factors, sympathetic hyperactivity, etc) [21].

Ultrasonographic and neuroimaging studies

Ultrasonographic studies in iRLS patients have reported hypo echogenicity or reduced midbrain areas of hyper echogenicity of the substantia nigra (related with decreased iron deposition) [22–25], hypo echogenicity in the brainstem raphe [20], and hyper echogenicity of the red nucleus [25]. Although an association between RLS and Parkinson's disease (PD) has been suggested by epidemiological studies, including two recent meta-analyses [26,27], a genetic association between the two conditions has not been confirmed to date [14,27,28]. In contrast with iRLS patients, PD patients both with and without RLS show substantia nigra hyper echogenicity (likely related to neuronal loss) as a typical ultrasonographic feature of PD [27].

The results from neuroimaging studies related to iron content are summarized in Table 4. Most of these results are consistent with the presence of an iron deficiency in several brain areas of iRLS patients [Suppl. 32-Suppl. 34, Suppl. 37-Suppl. 40], with the exception of findings by Knake et al. [Suppl. 35], which failed to observe significant differences in the iron concentration after examining 12 regions of interest between iRLS patients and controls, and Margariti et al. [Suppl. 36], who presented data suggesting increased iron content in the right globus pallidus and the right subthalamic nucleus. While most studies using 1.5–3.0T MRI showed decreased iron concentration in the *substantia nigra* of iRLS patients, a recent study using 7.0T MRI reported normal iron concentrations in the *substantia nigra,* and decreased concentrations in the thalamus and dentate nuclei of the cerebellum [Suppl. 40].

CSF, plasma/serum, and lymphocyte studies of iron, ferritin, and transferrin levels

The results of case-control studies on CSF and plasma/serum levels of iron, ferritin and its subunits, and transferrin are summarized in Table 5. These studies are scarce, and the results showed high variability.

In comparison with controls, patients diagnosed with iRLS showed similar CSF iron levels [Suppl. 41, Suppl. 42], similar plasma iron in two studies [Suppl. 41, Suppl. 45], and plasma iron levels decreased by 50% in another study [Suppl. 42], normal [Suppl. 41, Suppl. 42] or increased CSF transferrin levels [Suppl. 43] and normal [Suppl. 41, Suppl. 45] or increased plasma/serum transferrin levels [Suppl. 42].

CSF ferritin levels have been found to be normal [Suppl. 42], lower [Suppl. 43], lower in iRLS patients with early-onset of symptoms [Suppl. 41], and lower in iRLS patients with high CSF 3-OMD levels [Suppl. 30]. Serum/plasma ferritin levels were found to be decreased in two studies [Suppl. 41, Suppl. 45] and normal in three others [Suppl. 41, Suppl. 43, Suppl. 46]. Plasma/serum ferritin levels had an inverse correlation with RLS severity in two studies [Suppl. 45, Suppl. 47].

A study by Early et al. [Suppl. 41] comparing CSF samples obtained at 10 a.m. with those obtained at 10 p.m., found that CSF ferritin levels were significantly lower (by 50%, $p < 0.001$), CSF transferrin levels higher (by 5-fold, $p < 0.001$), and CSF iron levels similar in RLS patients, while in controls CSF ferritin, transferrin and iron were similar, thus suggesting the presence of a circadian rhythm of CSF ferritin and transferrin in RLS patients only. These data suggest the importance of the hour of sample collection in the results of the CSF analyses, which could explain the variability among studies.

A cross-sectional study of 365 elderly German subjects (9.8% of them with iRLS) showed a lack of association between iron or ferritin deficiency and a risk for RLS (measurements included plasma iron, ferritin, transferrin, soluble transferrin receptor, and C-reactive protein levels) [29]. A prospective study involving 1100 Australian participants, which included determinations of ferritin and transferrin saturation at ages 17 and 22 y and answers to questions to determine the diagnosis of RLS according to IRRLSG criteria at age 22 y (3% of the 865 subjects with RLS questionnaire and iron stores data were diagnosed with RLS), found no relation between serum iron stores measurements and risk for RLS [30]. In a population-based study in Iceland (RLS prevalence = 18.3%) and Sweden (RLS prevalence = 11.5%), ferritin levels were significantly lower in RLS individuals ($p = 0.0002$), but statistical significance disappeared after adjustment for center, age, sex, and smoking history [31]. Earley et al. [32], reported increased levels of DMT1 protein and transferrin receptor, and normal transferrin and ferritin subtypes levels in the lymphocytes of 24 early-onset RLS women compared with 25 women controls.

Hascka et al. [Suppl. 46], in a study performed in monocytes isolated from 168 iRLS patients and 118 controls, found a significant reduction in mRNA levels of heme oxygenase 1(HMOX1) and several mitochondrial iron-related genes such as mitoferrins 1 and

Table 4
Results of neuroimaging studies related to iron concentration in patients with idiopathic restless legs syndrome (iRLS) compared with controls.

Method	Authors, year [Ref]	RLS/controls	Main findings
1.5 T MRI measurement of relaxation rates R2* (from the gradient-echo MRI) and R2 (From the spin-echo MRI)	Allen et al., 2001 [Suppl. 32]	5/5	A significant decrease of iron in R2′ MRI sequence in the substantia nigra, and to a lesser degree, in the putamen, of patients with iRLS.
1.5 T MRI measurement of the "iron index" using R2' (a mathematical derivative of the transverse relaxation time)	Earley et al., 2006 [Suppl. 33]	41 (22 early-onset, 19 late-onset)/39	A significant decrease in the mean "iron-index" from the substantia nigra in early-onset iRLS. A negative correlation between "iron-index" in the substantia nigra and RLS severity in early-onset RLS patients.
1.5 T MRI measurement of mean T2 values in 11 regions of interest	Godau et al., 2008 [Suppl. 34]	6/19	Significantly higher mean T2 values in iRLS patients (these values correlated inversely with substantia nigra echogenicity).
1.5 T MRI, measurement of T2* gradient MRI sequence in 12 regions of interest	Knake et al., 2009 [Suppl. 35]	12 (5 early-onset, 7 late-onset)/12	Non-significant differences in T2 change ratio (T2CR) between iRLS patients and controls
1.5 T MRI T2-weighted (T2W), T2 relaxometry	Margariti et al., 2012 [Suppl. 36]	11 (all early-onset, nine women)/11 (9 women)	Decreased T2 relaxation time in the right globus pallidus internal and in the right subthalamic nucleus
1.5 T MRI T2-weighted (T2W) phase sensitive imaging with a gradient echo sequence in seven regions of interest	Rizzo et al., 2013 [Suppl. 37]	15/15	Significantly higher values (indicating reduced iron content) in the substantia nigra, thalamus, putamen, and pallidus of iRLS patients
3.0 T MRI measurement of the "iron-index" using the R2′ map in five regions of interest	Moon et al., 2014 [Suppl. 38]	37 (20 early-onset, 17 late-onset)/40	A significant decrease in the mean "iron-index" from the substantia nigra significantly in late-onset RLS patients
3.0 T MRI measurement or "iron concentrations" using three relaxometry metrics (R2, R2*, and R2′) in five regions of interest	Moon et al., 2015 [Suppl. 39]	37/40	A significant decrease in the mean "iron-index" from the substantia nigra and globus pallidus in RLS patients according to the three metrics
7.0 T MRI measurement of regional brain iron concentrations using quantitative susceptibility mapping (QSM) in nine regions of interest	Li et al., 2016 [Suppl. 40]	39/29	A significant decrease in QSM in the thalamus and dentate nucleus in iRLS patients compared with controls, and non-significant differences between iRLS and controls in the substantia nigra caudate nucleus, globus pallidus, putamen, red nucleus and pulvinar. A significant correlation between QSM in the substantia nigra and PLMS in iRLS patients.

2 (which act as mitochondrial iron importers), decreased levels of mitochondrial ferritin, reduced expression of IRP2, impaired activity of mitochondrial aconitase and reduced mitochondrial superoxide formation in RLS patients, while transferrin receptor 1, heavy-chain ferritin, DMT1, and ferroportin 1 mRNA expression was similar in both study groups. These results suggest a relationship of RLS with mitochondrial iron deficiency and mitochondrial dysfunction.

Dauvilliers et al. [33] found higher serum hepcidin levels and serum hepcidin/ferritin ratio in 108 RLS drug-free patients than in 45 gender-matched controls (RLS group was 7.6 y older than controls), and a relation between serum hepcidin levels and RLS severity. Because hepcidin acts as an important regulator of the entry of iron to the circulation, the authors suggest the role of peripheral iron metabolism dysregulation in RLS.

Experimental data

Rodents under an ID diet develop sensory and motor symptoms resembling those of RLS. Moreover, as with RLS patients, ID diet is able to reduce the acute pain threshold and to increase chronic pain response. From the neuropathological point of view, ID diet rodents show an increased expression of c-Fos-immunoreactive cells in the dorsal horns [34]. Table 6 summarizes data suggesting an important interaction between ID and the dopaminergic system, which could play a role in the pathogenesis of RLS.

BTBD9 mutant mice (*BTBD9* is the gene that has been shown to have the strongest association with RLS risk in GWAS), as an important animal model of RLS, showed alterations both in serum iron levels and in monoamine neurotransmitter systems [35].

Pharmacogenetic studies related to iron

Xiong et al. [36] described a lack of association between the *solute carrier family 11 member 2* or *divalent metal ion transporter 1* gene (*DMT1* or *SLC11A2*; gene ID 4891, MIM 600423, which plays an important role in iron transport) and risk for RLS. Li et al. [37] showed a lack of association between 16 candidate genes related to iron metabolism and dopaminergic neurotransmission and RLS risk in a Chinese Han family with RLS. Oexle et al. [38] reported a lack of relation between 111 iron-related genes risk of RLS in a case-control association study (2425 RLS patients/3285 controls). In contrast, a case-control association study found a modest but significant association between the *HMOX1*rs2071746 variant (chromosome 22q13.1, gene ID 3163, MIM 141250, which plays an important role in iron metabolism) and risk of developing RLS [39].

Non-dopaminergic neurotransmitter systems, neuropeptides, and hormones

Serotoninergic system

A functional neuroimaging study showed non-significant differences in the binding of the serotonin transporter (SERT) in the pons and medulla between iRLS patients and controls. However, availability of SERT was negatively correlated with RLS severity (Table 2) [Suppl. 24]. CSF 5-HIAA levels have been found to be similar in iRLS patients to controls in three studies (Table 3) [Suppl. 26–Suppl. 28], although in one of them this value was lower after adjustment by age [Suppl. 26], and another described a higher CSF HVA/5-HIAA ratio in iRLS patients in samples obtained at 10 p.m. when compared with those obtained at 10 a.m., thus suggesting changes related with the circadian rhythm, as has been described in a previous section [Suppl. 27]. A study obtaining CSF samples every 30 min over a period of 22 h in three iRLS patients and three controls showed a lack of a circadian rhythm for CSF 5-HIAA [Suppl. 29].

Aspartate, glutamatergic and GABAergic systems

The possible implication of the main inhibitory neurotransmitter in the Central Nervous System, gamma-aminobutyric acid

Table 5
Alterations in the CSF levels of iron, ferritin, and transferrin in patients with idiopathic restless legs syndrome (iRLS) compared with controls.

Biological fluid	Variable	Authors, year [Ref]	RLS/controls	Main findings
Cerebrospinal fluid	Iron	Earley et al., 2005 [Suppl. 41]	30 (15 early-onset)/22 (CSF obtained at night-time)	Normal
		Mizuno et al., 2005 [Suppl. 42]	10/10	Normal
	Ferritin	Earley et al., 2000 [Suppl. 42]	16/8	Decreased by 1.11 vs 3.5 ($p = 0.0002$)
		Earley et al., 2005 [Suppl. 43]	30 (15 early-onset)/22 (CSF obtained at night-time)	Decreased in the total RLS ($p < 0.005$) and early-onset ($p < 0.003$) but not in late-onset RLS. Strong correlation between age at onset and CSF ferritin levels (more marked in women)
		Mizuno et al., 2005 [Suppl. 44]	10/10	Normal
		Allen et al., 2009 [Suppl. 45]	49 (30 with high CSF 3-OMD/19 with normal 3-OMD	Decreased only in RLS patients with high 3-OMD.
	H-ferritin subunit	Clardy et al., 2006 [Suppl. 44]	25 (12 early-onset)/14	Decreased in the total RLS ($p < 0.05$) and early-onset RLS ($p < 0.05$) but not in late-onset RLS.
	L-ferritin subunit	Clardy et al., 2006 [Suppl. 44]	25 (12 early-onset)/14	Decreased in the total RLS ($p < 0.002$) and early-onset RLS ($p < 0.005$) but not in late-onset RLS.
	Transferrin	Earley et al., 2000 [Suppl. 43]	16/8	Increased 4-fold ($p = 0.018$)
		Earley et al., 2005 [Suppl. 41]	30 (15 early-onset)/22 (CSF obtained at night-time)	Normal
		Mizuno et al., 2005 [Suppl. 42]	10/10	Normal
Serum/Plasma	Iron	O'Keefe et al., 1994 [Suppl. 45]	18/18 (all elderly)	Normal
		Earley et al., 2005 [Suppl. 41]	30 (15 early-onset)/22 (CSF obtained at night-time)	Normal
		Mizuno et al., 2005 [Suppl. 42]	10/10	Decreased by 50% ($p < 0.05$)
		Haschka et al., 2018 [Suppl. 46]	168/119	Normal
	Ferritin	O'Keefe et al., 1994 [Suppl. 45]	18/18 (all elderly)	Decreased by 50% ($p < 0.01$). An inverse correlation with RLS severity
		Sun et al., 1998 [Suppl. 47]	27/0	An inverse correlation with RLS severity
		Earley et al., 2000 [Suppl. 43]	16/8	Normal
		Earley et al., 2005 [Suppl. 41]	30 (15 early-onset)/22 (CSF obtained at night-time)	Normal
		Mizuno et al., 2005 [Suppl. 42]	10/10	Decreased by 39% ($p < 0.01$)
		Haschka et al., 2018 [Suppl. 46]	168/119	Normal
	Transferrin	Earley et al., 2000 [Suppl. 43]	16/8	Non-significant differences between groups
		Earley et al., 2005 [Suppl. 41]	30 (15 early-onset)/22	Normal
		Mizuno et al., 2005 [Suppl. 42]	10/10	Increased 1.4-fold ($p < 0.05$)
		Haschka et al., 2018 [Suppl. 46]	168/119	Normal

(GABA), in the pathophysiology of iRLS is suggested by the improvement of RLS symptoms achieved by GABAergic drugs. The improvement of RLS by alpha-2-delta calcium-channel ligands (gabapentin and pregabalin) suggests a role of glutamate as well. Three proton MR spectroscopy (^{1}HMRS) studies assessed the status of excitatory amino acids (aspartate and glutamate) and/or GABA in iRLS, suggesting the implications of these neurotransmitters in the pathophysiology of this condition (Table 7).

Partial ablations of the descending glutamatergic pathways to the spinal cord (corticospinal tract at spinal C1 level, secondary motor cortex, somatosensory cortex or red nucleus) in rats induce RLS-like movements during sleep-wake transitions [11]. Ablation of secondary motor cortex and somatosensory cortex induces RLS-like movements during NREM and REM sleep, and bilateral ablation of the red nucleus during NREM sleep as well [11].

Yepes et al. [40], using an in vivo optogenetic-microdialysis method, showed that rats under an ID diet, compared with rats under a normal diet, had increased release of glutamate at corticostriatal glutamatergic terminals when using low frequency of optogenetic stimulation, suggesting the presence of hypersensitivity of these terminals that could be related with RLS symptoms. Glutamate release was reversed by infusion with pramipexole, ropinirole, and gabapentin.

Regarding involvement of GABA, an ^{1}HMRS study has shown an association between GABA levels and PLM indices and RLS severity (positive correlation with thalamic GABA levels and negative correlation with cerebellar GABA levels), which was interpreted by the authors as a contribution of overactivity of the cerebellum (associated with reduced GABA activity) to RLS symptoms through its influence on the striatum, via the thalamic nuclei [Suppl. 61] (Table 7). Similar findings have been reported in a PET study of patients with Tourette's syndrome, which is characterized by tics and premonitory sensory symptoms similar to those of RLS patients [41].

Regarding the possible association between glutamate-related genes and RLS risk, the *SLC1A2*rs3794987 polymorphism (*solute carrier family 1, member 2*, related to glutamate transport) showed a lack of association [42]. A recent case-control association study of the most common GABA receptors genes (*GABR*) polymorphisms showed a modest association between the *GABRR3* rs832032T allelic variant and risk for RLS, and a relation between *GABRA* rs2229940 polymorphism and age at onset of RLS symptoms in iRLS patients [43].

Opiate system

Only a few reports have addressed the possible role of the opiate system in the pathogenesis of RLS, despite the fact that opioids have shown an improvement of RLS symptoms. In a brain post-mortem study, Walters et al. [Suppl. 2] described decreased met-enkephalin and beta-endorphin cells and normal leu-enkephalin cells in the thalamus, and normal TH, met-enkephalin, leu-enkephalin, and beta-endorphin in the substantia nigra of iRLS compared with controls (Table 1).

A PET study using ^{11}diprenorphine (a marker of opiate receptor), reported a similar binding of this tracer in iRLS patients and in controls, but there was a negative correlation between RLS severity and binding of this ligand to several brain areas (Table 1) [Suppl. 25].

Table 6
Data suggesting an interaction between iron deficiency (ID) and the dopaminergic system.

DRD3 knock-out mice show more marked RLS-like symptoms than wild-type mice after exposure to ID [Suppl. 48].
Locomotor activities in mice after a bilateral lesion in the A11 nuclei with 6-OHDA are increased by ID, and this effect is reversed with DRD_2/DRD_3 agonists ropinirole [8,9] and pramipexole [10,11].
DAT density in the striatum of mice decreases both in the dark (by 28%) and in the light phases (by 20%), while striatal DOPAC/DA ratio increases in the dark phase only by the effect of ID [Suppl. 49].
The effects of ID on the caudate and in the ventral midbrain of rats include decrease in DAT mRNA levels (by 58%), DAT protein and membrane-associated (these data suggest alterations in dopamine uptake), and increase in the noradrenaline concentration; together with a decrease by 50% and a delay of the response of DA to levodopa, and an 3-fold increase of the response of noradrenalin to levodopa (these data suggest the presence of compensatory changes in noradrenalin metabolism) [Suppl. 50].
In the ventral midbrain of rodents, ID is able to reduce intracellular and increase extracellular striatal DA (these 2 effects are reversed by infusion of iron at physiological concentrations), to reduce the striatal DA uptake, and to blunt DRD_2 agonist feedback enhancement of striatal DA uptake [Suppl. 51].
Several genes in the substantia nigra of rodents can present changes in their expression related to the effect of ID. These genes include *hemoglobin beta adult chain 1* or *Hbb-b1* (these genes play functional roles in dopaminergic neurons), *SDF-1* or *Cxcl12* (*stromal cell-derived factor 1* or *C-X-C motif chemokine ligand*, which plays a role as a potent dopamine neuromodulator and a ferritin regulator), and *DRD_2* [Suppl. 52].
The density of the striatal adenosine A2A receptor (A2AR, which is related to dopaminergic signaling) in rodents is increased by the effect of ID diet [Suppl. 53, Suppl. 54], at least with severe ID diet [Suppl. 55]. The finding that in rodents under ID diet selective A2AR antagonists are able to block the effects mediated both by striatal presynaptic and postsynaptic A2AR (that is, motor output and Extracellular Signal-regulated Kinase-ERK- phosphorylation, respectively, both induced by cortical electric stimulation) raises the possibility that ID could induce a functional up-regulation of both pre- and postsynaptic striatal A2AR. This finding could be extrapolated to a model of sensory-motor disorder related with brain iron deficiency such as RLS [Suppl. 53]. Similarly, treatment of human neuroblastoma cells (SH-SY5Y) with iron chelators induces an increase in A2AR density [Suppl. 54].
Adenosine A1 receptor (A1R) density of rodents shows downregulation in the cortex and striatum by ID diet, which is concomitant to a striatal DRD_2 downregulation [Suppl. 55]. A role of this down-regulation of A1R has been suggested in PLMS and hyperarousal in RLS [Suppl. 54].
Dopamine (but not tyrosine and noradrenalin) induces modifications in cellular iron homeostasis in murine bone marrow-derived macrophages, which include increase in the uptake of non-transferrin bound iron into cells (which leads to cellular iron accumulation), increase in oxidative stress responses, and an increase in the expression of stress response genes including HMOX1 and ferroportin 1 [Suppl. 56].
ID rats develop sleep fragmentation and periodic leg movements which improve with pramipexole [Suppl. 57].
Human cells cultured under ID show a reduction in MEIS1 expression, suggesting an important role of *MEIS1* gene (implicated in the risk for RLS in GWAS) in iron metabolism [Suppl. 58].

Table 7
Results of proton MR spectroscopy (^{1}HMRS) studies related in patients with idiopathic restless legs syndrome (iRLS) compared with controls.

Method	Authors, year [ref]	RLS/controls	Main findings
1.5 T MRI High-resolution T1-weighted axial volumetric images. Acquisition of single voxel ^{1}H-MRS spectra using a point-resolved spectroscopy sequence in the medial thalamus	Rizzo et al., 2002 [Supp. 59]	23 (14 females)/19 (8 females)	Significant reduction in N-acetyl-aspartate (NAA) concentrations and in NAA/creatine ratio in the medial thalamus of iRLS patients ($p < 0.01$ for both). Association between lower NAA levels with a family history of Irls
1.5 T MRI images acquisition followed by the single-voxel ^{1}H-MRS spectra using the PROBE-P (PRESS) sequence in the right thalamus	Allen et al., 2013 [Suppl. 60]	28 (15 females)/20 (12 females)	Increased glutamate + glutamine/creatine ratio in the thalamus of iRLS patients ($p = 0.016$) Significant correlation of glutamate + glutamine/creatine ratio with several RLS-related polysomnographic sleep variables (including the wake time during sleep), with the exception of PLMS/hour.
4 T MRI High-resolution T1-weighted axial volumetric images. Acquisition of three voxels of interest with ^{1}H-MRS using a GABA-optimized MEGA-PRESS sequence (left thalamic lobe, bilateral dorsal anterior cingulate cortex and left cerebellum)	Winkelman et al., 2014 [Suppl. 61]	18 (10 females)/17 (10 females)	Non-significant differences in GABA, glutamate, and NAA concentrations in the thalamus and cerebellum between iRLS patients and controls Non-significant differences in GABA and glutamate concentrations in the thalamus and cerebellum between iRLS patients and controls Increased levels of NAA in the dorsal anterior cingulate of iRLS patients. controls ($p < 0.01$) Positive correlation of thalamic GABA levels and negative correlation of cerebellar GABA levels with PLMI and RLS severity

Finally, Sun et al. [44] reported a protective effect on neuronal damage induced by ID in cell cultures from the substantia nigra of rats by the preadministration of a delta-opioid peptide.

Adenosine

The increase in A2AR density in the striatum, and down-regulation of A1R in the cortex and striatum induced by ID diet in rodents [Supp.43-Suppl. 45], together with the effects of A1R down-regulation disrupting the adenosine-dopamine-glutamate balance in the striatum and the inhibitory effect of A1R on glutamate neurotransmission in the cortex and other non-striatal brain areas (which play an important role in hyperarousal and PLMS), led to the suggestion that adenosine neurotransmission could play a pivotal role in the pathophysiology of RLS [45]. In a recent report, Rivera-Oliver et al. [46] have shown, in slices from male mice lumbar spinal cord, the existence of A1R-DRD_1 heteromers in spinal motoneurons, which exert an important role in the control of motoneuron excitability due to tonic inhibition of DRD_1 signaling by adenosine.

Hypocretin-1 (Orexin-A)

Allen et al. [47] described increased CSF levels of hypocretin-1 (a neuropeptide which is a well-established marker of narcolepsy) in samples from patients with iRLS obtained in the evening. However,

other authors have reported similar CSF hypocretin levels in iRLS patients to controls [Suppl. 9,48,49], a lack of correlation of CSF hypocretin with severity or age at onset of RLS [48] and a lack of evidence of significant circadian rhythms for CSF hyprocretin-1 in iRLS patients [Suppl. 29].

Hormones

One study involving 10 RLS patients and eight controls found similar plasma concentrations of cortisol, growth hormone, and prolactin, and a lack of differences in the rhythms for these hormones during night-time and day-time between the two groups [50], while another study involving 73 iRLS patients and 34 controls found increased nocturnal urinary cortisol excretion in iRLS patients [51]. Urinary excretion of 6-hydroxymelatonin-sulfate has been found to be similar in iRLS patients and controls both during day-time and night-time [52].

Plasma levels of copeptin (the C-terminal fragment of antidiuretic hormone-ADH), has been found to be increased in 41 iRLS patients compared with 41 controls, although they were not associated with RLS severity [53].

Finally, central administration of adrenocorticotropic hormone (ACTH) and alpha-melanocyte stimulating hormone (alpha-MSH) in rats induced stimulation of motor activity in wake, changes in sleep architecture, and increase in PLMS, resembling those of RLS, thus suggesting their possible role in the pathogenesis of RLS [54].

Vitamin D deficiency

Because 25-hydroxyvitamin D deficiency can lead to dopaminergic dysfunction, several studies have addressed its possible role in the etiology of iRLS. Studies performed in Turkey [55,56] and Saudi Arabia [57] have found a significant decrease in serum 25-hydroxyvitamin D levels and a higher frequency of 25-hydroxyvitamin D deficiency in patients diagnosed with iRLS than in matched controls, together with an inverse correlation between serum 25-hydroxyvitamin D levels and RLS severity [55] or higher RLS severity in patients with 25-hydroxyvitamin D deficiency [57].

Another study found a significantly higher prevalence of iRLS in subjects complaining of “musculoskeletal symptoms” with low serum 25-hydroxyvitamin D levels (<20 ng/mL) than in those with high ones (>20 ng/mL) [58]. RLS incidence was also found to be higher in a cross-sectional study involving 57 vitamin D deficient subjects in comparison with 45 patients with normal levels of vitamin D [59].

A proteomic study of CSF in a small sample (5 early-onset RLS patients/5 controls) showed increased CSF vitamin D binding protein levels as a possible biomarker for RLS [60]. Regarding vitamin D-related genes, our group reported decreased risk for iRLS in carriers of the rs731236A allelic variant of the *vitamin D receptor* (*VDR*) gene, while *VDR* rs2228570 polymorphism showed a lack of relation with RLS risk [61].

A recent report by Wali et al. [62], in a 12-week randomized, placebo-controlled trial involving 35 RLS patients (vitamin D and placebo groups did not differ in age, sex, RLS severity, or vitamin D levels) showed a lack of efficacy of vitamin D supplements in improving RLS symptom severity.

Nitric oxide

Several data suggest that oxidative stress and nitric oxide (NO) could play a role in the pathophysiology of RLS:

1) In an immunohistochemical study, expression of the synthesizing enzyme of NO, NO synthase 1 (NOS1, neuronal NOS of nNOS) in substantia nigra was found to be increased in four out of six iRLS patients but not in six healthy controls [63].
2) Serum/plasma levels of malonaldehyde (MDA, an important marker of lipid peroxidation) and of advanced oxidation protein products have been reported to be increased, and those of nitrites (markers of NO) and thiol (an antioxidant molecule) decreased in iRLS patients (22 iRLS patients/20 age- and sex-matched controls) [64].
3) DRD_3 mice, compared with wild-type mice, showed increased NOS expression in the thoracic intermediolateral nucleus of the spinal cord [65].

Although a three-stage design association study showed an association between the *NOS1* rs7977109 polymorphism and the risk for RLS [66], a further replication study did not find such an association [67].

Other substances

Serum or plasma levels of vitamin B_6 [68], vitamin B_{12} [Suppl. 45,68,69], folate [Suppl. 45,68,69], and homocysteine [68] have been found to be similar in iRLS patients and in controls, with the exception of one study that reported lower serum vitamin B_{12} levels in iRLS patients [56]. Patients diagnosed with iRLS have been observed to have similar CSF and plasma levels of magnesium than controls [70].

Recent studies describe decreased serum or plasma levels of LOX-1 (Lectin-like oxidized Low-Density Lipoprotein Receptor-1, a proatherogenic substance) [69], apelin (an antioxidant and anti-inflammatory molecule secreted by adipose tissue with a protective effect on neuronal and cardiac tissue) [71], and endocan (a marker of endothelial dysfunction) [56] in iRLS patients compared with controls.

Finally, Schulte et al. [72], in a study quantifying 456 metabolites in serum samples of the KORA cohort (consisting of 1272 controls belonging to the general population) and 82 RLS patients, showed increased levels of medium chain fatty acids with 7–11 carbon atom backbones and an increase in two isomers of inositol in the RLS group.

Gray matter density (GMD), white matter (WM) density and cortical thickness studies

Gray matter

Table 8 summarizes the results of studies addressing gray matter (GM) density (GMD) or volume in iRLS patients and healthy controls, most of them using 1.5 or 3 T MRI T1-weighted images and processing with Voxel-based morphometry (VBM) [Suppl. 36, Suppl. 59,Suppl. 62–Suppl. 70], with the exception of one study which used a surface-based technique [Suppl. 71]. While several studies described increased density in the pulvinar nuclei of the thalamus [Suppl. 62], left occipital region [Suppl. 58], bihemispheric primary somatosensory cortex [Suppl. 65], and left hippocampal gyrus, parietal lobes, medial frontal areas and cerebellum [Suppl. 69], respectively, the majority of these studies found no significant differences between iRLS patients and controls in any of the brain areas analyzed [Suppl. 36, Suppl. 59, Suppl. 66, Suppl. 69, Suppl. 71]. Pan et al. [Suppl. 69] reported non-significant differences in GMD in the anterior cingulate cortex between their overall series of iRLS patients (all drug-naïve) and controls, but the subgroup of iRLS patients with depression showed a significant reduction in GMD in that area in comparison with non-depressed iRLS patients and with controls.

White matter

The results of studies addressing WM volume or integrity, summarized in Table 7, include VBM and diffusion tensor imaging studies (DTI). Two studies using VBM have reported non-significant differences between iRLS patients and controls [Suppl. 36, Suppl. 59], while another found a slight decrease in WM volume in iRLS patients [Suppl. 73]. Studies using DTI, all of them measuring fractional anisotropy (FA, a marker of white matter integrity) [Suppl. 59, Suppl. 66, Suppl. 70, Suppl. 72, Suppl. 74, Suppl. 75], and others measuring mean diffusivity (MD [Suppl. 59, Suppl. 66], axial diffusivity (AD) [Suppl. 59, Suppl. 66, Suppl. 70, Suppl. 72], and radial diffusivity (RD) [Suppl. 59, Suppl. 66, Suppl. 70, Suppl. 74], present varying results (Table 7). Interestingly, two studies show significantly lower FA values in the brainstem [Suppl. 70, Suppl. 75] and one in the cervical spinal cord at C2-C3 vertebrae levels [Suppl. 75].

Finally, a neuropathological study involving 11 iRLS patients and 11 controls shows decreased expression the of myelin basic protein (by approximately 25%), proteolipid protein, and the oligodendrocyte-specific 3′5′-cyclic nucleotide phosphohydrolase, and decreased amounts of H-ferritin and transferrin in the myelin of iRLS patients [Suppl. 73].

Cortical thickness

A recent report has shown similar cortical thickness in iRLS patients and controls with the exception of a slight decrease by 7.5% in the bilateral postcentral gyrus in iRLS patients [Suppl. 71]. This study also found a substantial decrease in thickness of the corpus callosum posterior midbody in iRLS [Suppl. 71] (Table 8).

Transcranial magnetic stimulation (TMS) and direct current stimulation (DCS) studies

Transcranial magnetic stimulation (TMS) is a non-invasive neurophysiological technique which is used for assessing the excitability of the primary motor cortex and the cortical-spinal tract. Several recent studies have addressed the effects of TMS in iRLS patients compared with healthy controls [73]. The main findings of these studies are summarized in Table 9. Data from TMS suggest the presence of reduced intracortical inhibition (mediated by GABA) and reduced cortical plasticity related to the dopaminergic system in iRLS patients. Direct current stimulation (DCS) is a non-invasive form of neurostimulation that uses constant low direct current, delivered on the head (transcranial DCS or tDCS) or in the spinal cord (transcutaneous spinal DCS or tsDCS). The main findings from DCS are summarized in Table 9. Both high-frequency TMS over the primary motor area [Suppl. 95, Suppl. 96], low-frequency TMS over primary somatosensory or primary motor areas [Suppl. 97], and tsDCS over thoracic spinal cord [Suppl. 100] have shown a symptomatic improvement of RLS symptoms, while tDCS did not [Suppl. 99]. It has been suggested that the lack of efficacy of tDCS could be related to a relatively low intensity of the stimulus [74].

Correlation between neurochemical data and RLS duration and/or severity

A limited number of studies have addressed the issue of a possible correlation between the neurochemical features of RLS with disease duration or severity. Most of these studies are included in Tables 4, 5, 7 and 8. The data, summarized, are the following:

1) Neuropathological studies: correlation between a decrease in dopamine D_2 receptors (DRD_2) in the putamen of iRLS patients with RLS severity [Suppl. 3].
2) Functional neuroimaging studies: lack of correlation of DAT binding potentials (using ^{11}C-methylphenidate PET) in the striatum of RLS patients with severity and duration of the disease, PLMS and sleep efficiency [Suppl. 18]; a negative correlation between DRD_2-BP and clinical severity scores and a positive correlation with the degree of posttreatment improvement with pramipexole (positive) in iRLS patients in one study using ^{11}C-raclopride PET [Suppl. 22], and a lack of correlation of this value with severity and duration of the disease, PLMS and sleep efficiency in another study [Suppl. 23]; a negative correlation between the severity of RLS and the availability of SERT in the pons and medulla (measured with ^{123}I-beta-CIT-SPECT) [Suppl. 24]; and a negative correlation between ^{11}C-diprenorphine binding (by PET) and RLS severity in the medial thalamus, amygdala, caudate nucleus, anterior cingulate gyrus, insular cortex and orbitofrontal cortex [Suppl. 25]
3) Measures of iron content: a negative correlation between “iron-index” in the substantia nigra and RLS severity in early-onset RLS patients. [Suppl. 33]; an inverse correlation between mean T2 values with substantia nigra echogenicity [Suppl. 34]; and a significant correlation between quantitative susceptibility mapping (QSM) in substantia nigra with PLMS [Suppl. 40].
4) Serum/plasma and CSF ferritine levels: inverse correlation of serum/plasma ferritin levels with RLS severity [Suppl. 45, Suppl. 47], a correlation between CSF ferritin levels and age at onset of RLS (more marked in women) [Suppl. 43], and a decrease of CSF H-ferritin and L-ferritin levels in early-onset RLS [Suppl. 44].
5) Serum/plasma levels of other substances: serum hepcidin levels were correlated with age at onset and severity of RLS [33], plasma copeptin levels were not related with RLS severity [53], and serum 25-hydroxyvitamin D levels were inversely correlated with RLS severity [55].
6) CSF 3-OMD levels: higher PLMS/hour for iRLS patients with high CSF 3-OMD levels (suggesting higher severity of RLS) [Suppl. 30].
7) CSF hypocretine levels: a lack of correlation with age at onset and severity of RLS [Suppl. 29].
8) ^{1}HMRS studies: a correlation of thalamic glutamate + glutamine/creatine ratio with several RLS-related polysomnographic sleep variables (including the wake time during sleep), with the exception of PLMS/hour [Suppl. 60], and a positive correlation of thalamic GABA levels and a negative correlation of cerebellar GABA levels with PLMI and RLS severity [Suppl. 61].
9) White matter density studies: a correlation of GM volume in the bihemispheric primary somatosensory cortex and in left-sided primary motor areas with RLS severity and duration [Suppl. 65]; a correlation of MD and AD values with RLS severity in the WM of the left sensory-motor area, and splenium of corpus callosum, and between MD, AD, and RD values and duration of RLS in the right prefrontal WM [Suppl. 66]; a negative correlation of FA and a positive correlation of AD in the frontal WM adjacent to the inferior frontal gyrus with RLS severity [Suppl. 74]; and a lack of correlation of FA values in the brainstem and in the cervical spinal cord (C2-C3) with RLS severity and duration [Suppl. 75]

Table 8
Results of neuroimaging studies related to gray and white matter density and cortical thickness in patients with idiopathic restless legs syndrome (iRLS) compared with controls.

Method	Authors, Year [Ref]	RLS/controls	Main findings
1.5 T MRI T1-weighted acquisition, and processed with VBM	Etgen et al., 2005 [Suppl. 62]	51 (38 women)/51 (38 women)	A significant increase of GMD in both thalamic pulvinar nuclei
1.5 T MRI T1-weighted acquisition, and processed with VBM	Hornyak et al., 2007 [Suppl. 63]	14 (untreated, nine women)/14 (9 women)	Slight increase of GMD in the left ventral hippocampus and in the right middle orbitofrontal gyrus in iRLS patients. Non-significant differences of GMD in the thalami between iRLS patients and controls.
1.5 T MRI T1-weighted acquisition, and processed with VBM	Celle et al., 2010 [Suppl. 64]	17 (16 women)/54 (34 women)	Non-significant differences of GMD in both thalami, hippocampus and frontal areas. Non-significant trend towards higher GM volume in the left occipital region and smaller GM volume in the right superior temporal area in RLS patients
1.5 T MRI T1-weighted acquisition, and processed with VMB	Unrath et al., 2007 [Suppl. 65]	63 (45 women)/40 (29 women)	Significant regional decreases of GM volume in the bihemispheric primary somatosensory cortex and in left-sided primary motor areas. GM volume was correlated with RLS severity and with disease duration
1.5 T MRI T1-weighted acquisition, and processed with VBM	Rizzo et al., 2012 [Suppl. 66]	20 (13 women)/20 (10 women)	Non-significant differences of GMD and WM volume in any brain areas between iRLS patients and controls.
1.5 T MRI T1-weighted acquisition, and processed with VBM	Comley et al.. 2012 [Suppl. 67]	16 (drug-naïve, eight women)/16 (8 women)	Non-significant differences in the global GMD (including cortical and deep gray matter structures and cerebellum) between iRLS and controls.
1.5 T MRI T1-weighted acquisition, and processed with VBM	Margariti et al., 2012 [Suppl. 36]	11 (all early-onset, nine women)/11 (9 women)	Non-significant differences in GM volume and WM volume in any brain areas between iRLS patients and controls.
1.5 T MRI T1-weighted acquisition, and processed with VBM	Rizzo et al., 2012 [Suppl. 59]	23(14 women)/19 (8 women)	Non-significant differences in the GMD in left and right medial thalamus between iRLS patients and controls.
3.0 T MRI T1-weighted acquisition, and processed with VBM	Pan et al., 2014 [Suppl. 68]	34 (drug-naïve, 16 with depression, 28 women)/16 (12 women)	Non-significant differences of GMD in the anterior cingulate cortex between iRLS patients and controls. The subgroup of iRLS with depression showed a significant reduction in the GMD in both anterior cingulate cortex in comparison with iRLS patients without depression and with healthy controls
3.0 T MRI T1-weighted acquisition, and processed with VBM	Chang et al., 2015 [Suppl. 69]	46 (32 women)/46 (28 women)	Regional decreases of GM volume in several areas of iRLS patients, including the left hippocampal gyrus, both parietal lobes, medial frontal areas, and cerebellum.
1.5 T MRI T1-weighted acquisition, and processed with VBM	Belke et al., 2015 [Suppl. 70]	12 (9 women)/12 (9 women)	Non-significant differences of GMD in any brain areas between iRLS patients and controls.
3.0 T MRI T1-weighted acquisition, and processed with a surface-based technique	Lee et al., 2018 [Suppl. 71]	28 (17 women)/51 (32 women)	Non-significant differences of GM volume in caudate, putamen, globus pallidus, accumbens nuclei and thalamus between iRLS patients and controls.
1.5 T MRI single-shot SE-EPI sequence acquisition, processed with Voxel-wise analysis of DTI. Measurement of FA	Unrath et al., 2008 [Suppl. 72]	45 (32 women)/30 (21 women)	Significantly reduced FA in frontal and parietal WM from both hemispheres in the right hemispheric thalamus (posterior ventral lateral nucleus), in motor projection fibers and adjacent to the left anterior cingulus in iRLS patients.
3 T MRI T1-weighted acquisition, and processed with VMB	Connor et al., 2011 [Suppl. 73]	23 (15 women, 18 drug-naïve)/23 (15 women)	Significant regional reductions of WM volumes in iRLS patients in the corpus callosum, anterior cingulum (bilaterally) and with matter regions adjacent to precentral gyrus (bilaterally).
1.5 T MRI single-shot SE-EPI sequence acquisition, processed with Voxel-wise analysis of DTI. Measurement of MD, AD, RD and FA	Rizzo et al., 2012 [Suppl. 66]	20 (13 women)/20 (10 women)	Non-significant differences of MD, AD, RD, and FA in any brain region between iRLS patients and controls, with the exception of decreased FA and increased RD in right peridentate WM of patients with RLS Correlation of MD and AD values with RLS severity in the WM of the left sensory-motor area, and splenium of corpus callosum, and between MD, AD, and RD values and duration of RLS in the right prefrontal WM.
1.5 T MRI single-shot SE-EPI sequence acquisition, processed with Voxel-wise analysis of DTI. Measurement of MD, AD, RD, and FA	Rizzo et al., 2012 [Suppl. 59]	23(14 women)/19 (8 women)	Non-significant differences in any of the values in left and right medial thalamus between iRLS patients and controls.
3.0 T MRI single-shot SE-EPI sequence acquisition, processed with Voxel-wise analysis of DTI. Measurement of AD, RD and FA	Chang et al., 2014 [Suppl. 74]	22 (19 women)/22 (10 women)	A significant decrease of FA and increase of AD and RD in the genu of the corpus callosum, putamen and frontal WM adjacent to the inferior frontal gyrus. A negative correlation of FA and positive correlation of AD in the frontal WM adjacent to the inferior frontal gyrus with RLS severity
1.5 T MRI single-shot SE-EPI sequence acquisition, and processed with Voxel-wise analysis of DTI. Measurement of AD, RD, and FA	Belke et al., 2015 [Suppl. 70]	12 (9 women)/12 (9 women)	Significantly lower FA values in iRLS patients in the brainstem, olfactory and cerebellum areas, and higher FA values in temporal, lingula/occipital and right internal capsule. Significantly higher AD values in iRLS patients in right crus cerebri, right pallidum, right cerebellum, and right subcortical WM. Significantly higher RD values in iRLS patients in left and right cerebellum, right and left subcortical WM, right parietal and corpus callosum.
1.5 T MRI single-shot SE-EPI sequence acquisition, and processed with Voxel-wise analysis of DTI in the brainstem and slicewice analysis in cervical spinal cord. Measurement of FA	Lindeman et al., 2016 [Suppl. 75]	25 (17 women)/25 (16 women)	Reduced FA values in two clusters at the brainstem and in the cervical spinal cord at the C2 and C3 vertebrae levels (the latter did not survive multiple comparison analysis). Lack of correlation of FA values with RLS severity and duration
3.0 T MRI T1-weighted acquisition, and processed with a surface-based technique	Lee et al., 2018 [Suppl. 71]	28 (17 women)/51 (32 women)	Decrease by 7.5% in the cortical thickness in the bilateral postcentral gyrus in iRLS patients, and non-significant differences of cortical thickness in other cortical regions between iRLS patients and controls. A substantial decrease in the corpus callosum posterior midbody thickness in iRLS patients ($p < 0.008$)

MRI magnetic resonance imaging, VBM voxel-based morphometry, GM gray matter, GMD gray matter density, WM white matter, DTI diffusion tensor imaging, FA fractional anisotropy, MD mean diffusivity, AD axial diffusivity, RD radial diffusivity.

Table 9
Summary of results of transcranial magnetic stimulation (TMS) and direct current stimulation studies in patients with iRLS.

	Variable	Definition	Main results	Interpretation
Single-pulse TMS variables	Motor evoked potentials (MEP)	MEP recorded in the contralateral target muscles.	No significant differences between iRLS patients and controls in most studies [Suppl. 76–Suppl. 82]. Lin et al. [Suppl. 83]: normal unconditioned MEP amplitude in abductor pollicis brevis and increased amplitude in tibialis anterioris in iRLS patients.	Integrity of the cortical-spinal pathway in iRLS patients suggested by most studies
	Resting motor threshold (RMT).	Minimum stimulus intensity required to obtain an MEP with a minimum of amplitude in 5 or more of 10 consecutive stimuli at rest.	No significant differences between iRLS patients and controls in most studies [Supp. 77–Suppl. 88] Stiasny-Kolster et al. [Suppl. 76] found increased active RMT in the tibialis anterioris muscle. Salas et al. [Suppl. 89] described normal RMT in the 1st dorsal interosseus muscle and decreased RMT in the tibialis anterioris. Gündüz et al. [Suppl. 90] described decreased active RMT from 1st dorsal interosseous exclusively during night-time.	RMT is decreased in situations hyperexcitability of cortical-spinal system, and increased in cortical-spinal tract lesions. The integrity of cortical-spinal pathway in RLS patients is suggested by the normality of RMT found in most studies addressing this value
	Cortical silent period (CSP).	Transient suppression of the electromyographic activity following the MEP after delivering a single magnetic pulse during a voluntary contraction of the contralateral muscle	Several studies have shown a marked shortening of CSP in iRLS patients [Suppl. 76-Suppl. 78, Suppl. 81, Suppl. 85, Suppl. 87], which can be reversed by the effect of dopaminergic drugs [Suppl. 76, Suppl. 80, Suppl. 87]. Many other reports described normality of CSP in patients diagnosed with iRLS [Suppl. 77, Suppl., 79, Suppl. 82-Suppl. 84, Suppl. 90-Suppl. 92].	CSP indicates suppression of the cortical-spinal system. The results in iRLS patients are not conclusive
Paired-pulse TMS variables	Short interval intracortical inhibition (SICI)	SICI should reflect the excitability of GABAergic inhibitory cortical neurons. It is associated with a low intensity conditioned stimulus which produces shorter periods of cortical inhibition.	Markedly decreased in iRLS patients, even more in the most affected side [Suppl. 77-Suppl. 79, Suppl. 81, Suppl. 82, Suppl. 85, Suppl. 88, Suppl. 93] which could be reversed by dopaminergic therapy [Suppl. 79, Suppl. 82, Suppl. 83]. Salas et al. [Suppl. 89] described normal SICI in RLS patients both in 1st dorsal interosseous and tibialis anterioris muscles.	Reduced intracortical inhibition in iRLS patients.
	Long interval intracortical inhibition (LICI)	LICI should reflect the excitability of GABAergic inhibitory cortical neurons. LICI is associated with a high intensity conditioned stimulus which produces longer periods of cortical inhibition.	Salas et al. [Suppl. 89] described a significant decrease of LICI in tibialis anterioris but normality of this value in 1st dorsal interosseous muscle in iRLS patients, and they found that this value was even lower in RLS patients with augmentation than in those not experiencing this phenomenon	Reduced intracortical inhibition in iRLS patients.
	Intracortical facilitation (ICF)	ICF is likely related to the activity of intracortical glutamatergic excitatory circuits.	ICF has been reported to be normal in iRLS patients in three studies [Supp.79, Suppl. 82, Suppl. 83], but 2 other studies showed a hyper-facilitation in iRLS [Suppl. 77, Suppl. 84]. Salas et al. [Suppl. 89] described a significant decreased of ICF in the 1st dorsal interosseus but normality of this value in tibialis anterioris muscle in iRLS patients.	Results in iRLS patients are not conclusive
TMS measures of sensorimotor variables	Short-latency afferent inhibition (SAI)	Suppression of the amplitude of MEP induced by an electrical stimulus applied to a peripheral nerve 20 ms before TMS of the corresponding area of the contralateral motor cortex	Rizzo et al. [Supp.88] described decreased SAI in iRLS patients, an effect that was reversed by dopaminergic therapy. Bocquillon et al. [Suppl. 94] report similar values in iRLS and controls.	Normality of SAI is likely to reflect the integrity of cholinergic neural circuits. Results in iRLS are not conclusive
	Long-latency afferent inhibition (LAI).	LAI is likely related to cortical-cortical connections involving the motor cortex and primary and secondary somatosensory cortical areas.	Two studies reported non-significant differences in this value between iRLS patients and controls [Supp. 88, Suppl. 94], although one of them showed the presence of LAI in the morning and absence in the evening in both healthy controls and iRLS patients [Suppl. 94]. Lin et al. [Suppl. 83] described decreased LAI of the median nerve at 150 and 250 interstimulus intervals, but normal LAI values upon peroneal nerve stimulation, in iRLS patients.	Results in iRLS patients are not conclusive

Table 9 (*continued*)

	Variable	Definition	Main results	Interpretation
	Afferent-induced facilitation (AIF)	AIF represents the net increase in motor excitability that can be observed 45–60 ms after a peripheral stimulus, and it is likely to reflect sensorimotor integration processes in the long cortico-cortical pathways.	Bocquillon et al. [Suppl. 94] described lack of AIF in the upper limbs of iRLS patients, while it was present in healthy controls both in the morning and in evening sessions.	The result of a single study suggests impairment of sensorimotor integration in iRLS.
Plasticity-related TMS measures	Postexercise facilitation	Evaluation of motor cortical excitability changes occurring at various times after repetitive finger movement or bimanual motor tasks	Untreated iRLS patients have shown lack of postexercise facilitation in response to TMS [Suppl. 78, Suppl. 81, Suppl. 93], delayed facilitation being reversed by dopamine agonists [Suppl. 81].	Cortical plasticity related to dopaminergic system is reduced in RLS patients
Repetitive TMS (rTMS)	High-frequency rTMS	Non-invasive procedure in which a changing magnetic field is used to cause the electric current to flow in a small targeted region of the brain via electromagnetic induction	Marked improvement in RLS symptoms [Suppl. 95, Suppl. 96], sleep disturbances and anxiety in RLS patients [Suppl. 95] Clinical improvement is related to an increase of functional activity, measured as the amplitude of low-frequency fluctuations with functional MRI, in the sensorimotor and occipital regions (this value is decreased in iRLS patients as compared with controls) [Suppl. 96].	It has been suggested that rTMS induces focal release of dopamine within the ipsilateral striatum (likely by activation of corticostriatal projections), which could enhance descending inhibition and prevent abnormal somatosensory processing [Suppl. 96]
	Low-frequency rTMS	Non-invasive procedure in which a changing magnetic field is used to cause the electric current to flow in a small targeted region of the brain via electromagnetic induction	Low-frequency rTMS over primary sensorimotor and primary motor areas improved the sensorimotor symptoms of RLS patients, and caused shortening of the CSP, while rTMS over primary somatosensory area decreased RMT (although lesser than in controls) and had no effect on MEP latency and central motor conduction time in iRLS patients [Suppl. 97]. Low-frequency rTMS over primary motor area did not induce changes in basal RMT and MEPs amplitude [Suppl. 97, Suppl. 98], but caused a significantly lower reduction of MEPs amplitudes in RLS patients than in controls [Suppl. 98]	It has been suggested that inhibitory rTMS causes an impairment of the long-term depression-like mechanisms and a possible role of GABA in the pathophysiology of RLS [Suppl. 98].
Direct current stimulation	Transcranial direct current stimulation (tDCS)	Non-invasive form of neurostimulation that uses constant, low direct current delivered via electrodes on the head	Lack of improvement in IRLSSGRS scores, CGI-I, PGI scale, Pittsburgh Sleep Quality Index, Medical Outcome Study sleep subscale, and Beck depression inventory in a 2-week, double-blind, randomized, sham-controlled involving 33 drug-naïve females with RLS using cathodal, anodal, and sham-tCDS with electrodes on the sensorimotor cortex, making assessments at baseline, 3 d and 13 d [Suppl. 99].	Hyperexcitability of primary sensorimotor cortex would be an epiphenomenon rather than a cause of iRLS [Suppl. 99]
	Transcutaneous spinal direct current stimulation (tsDCS)	Non-invasive neuromodulatory intervention that has been shown to modify excitability in spinal and supraspinal circuits in animals and humans.	Short-lasting clinical improvement in RLS symptoms (measured by a visual analog scale) by anodal and cathodal stimulation of the thoracic spinal cord, and decrease in H2/H1 ratios (of two H-reflex responses to double stimuli) by anodal stimulation (these ratios are increased in RLS patients during the symptomatic phase in the evening), in a double-blinded, placebo-controlled study involving 20 iRLS patients and 14 healthy subjects using cathodal, anodal and sham stimulation of the thoracic spinal cord for 15 min (2.5 mA) each [Suppl. 100]	These results support the patho-physiological concept of increased spinal cord hyperexcitability in primary RLS [Suppl. 100]

Specific considerations on periodic limb movements during sleep (PLMS), augmentation and other symptoms associated with RLS

Periodic limb movements during sleep (PLMS)

PLMS is a frequent finding in polysomnographic studies, ranging from 4 to 11% in adults; it occurs in most RLS patients and is considered as a symptom of the disease [75]. However, data on the neurochemistry of PLMS have been addressed in only a few studies, most of them in the context of RLS neurochemistry:

1) Functional neuroimaging studies: a lack of correlation of DAT binding potentials (using ^{11}C-methylphenidate PET) [Suppl. 18] and of DRD_2-BP (using ^{11}C-raclopride PET) [Suppl. 22] in the striatum of RLS patients with PLMS.
2) CSF studies: iRLS patients with higher CSF 3-OMD levels (related to increased dopamine synthesis) showed higher PLMS/hour than with normal CSF 3-OMD levels [Suppl. 30].
3) Serum ferritin studies: a study involving a cohort of 801 individuals who used a PLM automatic detector reported an association between PLMI higher than 15 PLMS/hour and serum ferritin levels <50 ng/mL [76], while a cross-sectional study

involving 100 patients with RLS showed similar PLMI in 28 patients with serum ferritin levels <50 ng/mL compared with 64 patients with >75 ng/mL [77].
4) Measures of iron content: a significant correlation between quantitative susceptibility mapping (QSM) in substantia nigra with PLMS [Suppl. 40].
5) 1HMRS studies: PLMS/hour were not correlated with thalamic glutamate + glutamine/creatine ratio [Suppl. 60]; there was a positive correlation with thalamic and a negative correlation with cerebellar GABA levels [Suppl. 61].
6) Transcranial magnetic stimulation: although RLS patients showed reduced CSP which normalized with dopaminergic treatment, CSP duration was not correlated with PLMI [78].
7) Experimental models: lesions of the A11 dopaminergic nuclei with 6-OHDA in rats induce RLS and PLMS [79]; ID rats develop PLMS which improve with pramipexole [Suppl. 57], and central administration of ACTH and alpha-MSH are able to induce PLMS in rats [45].

Augmentation

Augmentation of RLS is an iatrogenic worsening of symptoms that follows initial improvement on dopaminergic therapy. With augmentation, RLS symptoms become more intense, they begin earlier in the day, they have shorter latency of the symptoms at rest, duration of the relief with treatment is shorter, and they spread to body parts previously unaffected [80]. Although the pathophysiology of augmentation is not well understood, the main neurochemical data are the following:

1) RLS patients suffering from augmentation present lower serum ferritin levels than those without this phenomenon [81,82], and augmentation improves with intravenous iron therapy [83].
2) Mice under prolonged therapy with DRD_3 agonists showed a phenomenon resembling augmentation, which was reversed by DRD_1 blockers [16]. This suggests the presence of a hyperdopaminergic state involving DRD_1 subtype related to a DRD_1 up regulation induced by DRD_3 agonists [16]. However, prolonged DRD_3 agonist therapy did not cause either augmentation or changes in the expression of DRD_2 [84].

Other symptoms associated with RLS

Patients with iRLS and depression presented a significant bilateral reduction in GMD in anterior cingulate cortex compared with patients with iRLS without depression and with healthy controls in a VBM study [Suppl. 68]. A 2-week, double-blind, randomized, sham-controlled study with tCDS involving 33 RLS patients did not show improvement in a depression inventory [Suppl. 99].

Interactions between neurotransmitter systems and their relationship with neurophysiological mechanisms of RLS

Lanza & Ferri [74] recently reported an interesting integrative scheme on the role of alterations in the main neurotransmitter systems implicated in the pathophysiology of RLS in susceptible individuals and their relationship with neurophysiological mechanisms. In summary, brain iron deficiency would be related with dopaminergic impairment, adenosine (hypoadenosinergic state) and opiate involvement, and a hyperglutamatergic stage (causing disinhibition of ascending arousal system with hyperarousal/hyperexcitability state). Adenosine and opiate systems alterations would contribute to dopaminergic impairment and to hyperglutamatergic state. Dopaminergic impairment would lead to dysfunction of the cortical-striatal-thalamic-cortical network (responsible for PLMS and akatisia) and to a deficit in GABA inhibition (which would lead to disinhibition of ascending arousal system and hyperarousal/hyperexcitability state). Both PLMS/akatisia and hyperarousal/hyperexcitability should cause sleep loss and fragmentations and clinical symptoms [74]. Although PLMS can cause arousals, the hyperarousal state seems to be independent of PLMS, since PLMS are suppressed by pramipexole without affecting arousals, whereas clonazepam has no effects on PLMS and reduces arousals, both drugs being effective in sensory symptoms of RLS [85].

Conclusions

The possible role of dopaminergic dysfunction in iRLS is mainly supported by the good response of RLS symptoms to drug enhancing dopaminergic neurotransmission and by data obtained from experimental models such as selective stereotactic bilateral lesions in the A11 area in rodents, i.e., the *DRD_3* knock-out mouse, and the *Meis1* knockout mouse. Data derived from neuropathological studies are scarce and based on small size samples, but results suggest the possibility of dopaminergic dysfunction and its relationship with iron insufficiency [Suppl. 1, Suppl. 3, Suppl. 5, Suppl. 8]. Functional neuroimaging studies on pre- and post-synaptic dopaminergic terminals (Table 2) gave controversial results: although the majority of studies showed normality of the presynaptic dopaminergic terminal in iRLS patients, several studies were consistent with a mild impairment; and most of the studies of the post-synaptic dopaminergic terminal using IBZM showed similar results in iRLS patients and control, while studies using ^{11}C-raclopride were consistent with decreased binding or increased availability in the striatum. Several studies with large sample sizes found decreased DRD_2 binding potentials in the striatum (suggesting an increase in synaptic dopamine and a hyperdopaminergic state) [Suppl. 23] and decreased DAT binding both in day and in night scans in iRLS patients [Suppl. 18]. The results of studies on CSF DA metabolite concentration are also controversial because they are based on studies with small sample sizes. Two potentially interesting findings are the possibility of a circadian rhythm for CSF levels of DA and HVA concentrations [Suppl. 27, Suppl. 29], and the possible relationship of CSF levels of 3-OMD both with RLS severity and with CSF HVA levels [Suppl. 30], but these results should be confirmed by studies with larger samples.

It is likely that brain iron deficiency plays a crucial role in the pathogenesis of iRLS. This hypothesis is supported by neuropathological, neuroimaging and transcranial sonographic studies in iRLS patients and controls. The results of studies on CSF and serum or plasma concentrations of iron, ferritin and transferrin concentrations in iRLS patients are controversial, are based on small size samples, and may be influenced by the hour of the samples collection [Suppl. 41]. Interestingly the existence of a rodent model of ID diet causing sensory-motor symptoms resembling those of RLS, the interactions between iron and the dopaminergic system, and the effects of ID on adenosine system, leading to the previously mentioned disruption of the adenosine-dopamine-glutamate (that is, a hypoadenosinergic-hyperdopaminergic-hyperglutamatergic state), gives important support to this hypothesis.

In addition to data on the role of dopaminergic dysfunction and iron deficiency as the main pathogenic hypotheses of iRLS, the possible implication of other neurotransmitters or neuromodulators, mainly glutamate and GABA, has been raised by recent studies with ^{1}HMRS and TMS in humans and in experimental models. A possible role of oxidative stress and NO, and the possibility of vitamin D deficiency in the pathogenesis of this disease, has been suggested by preliminary works but requires confirmation.

Practice points

1. Brain iron regional deficiencies seem to be the most important factor in the pathophysiology of iRLS, which could trigger changes in several neurotransmitters, including the dopaminergic, glutamatergic and adenosinergic systems.
2. Dopaminergic dysfunction (probably as a hyperdopaminergic state), in interaction with iron deficiency, plays an important role in the pathophysiology of iRLS.
3. The role of a hyper-glutamatergic state in iRLS is suggested by recent reports including results of neuroimaging studies in humans and studies in experimental models.
4. Adenosine neurotransmission also seems to play a role in the pathophysiology or iRLS, suggested by the down-regulation of A1R in the cortex and striatum found in experimental models of ID, as a hypo-adenosinergic state.
5. A role in the pathophysiology of GABAergic transmission is suggested by the correlation between thalamic GABA levels and clinical variables of RLS and data from TMS studies.sss
6. The role, if any, of vitamin E deficiency and oxidative stress in the pathogenesis of iRLS remains to be determined.

Research agenda

Ideally, futures studies aiming to establish the neurochemical features of RLS (specifically iRLS) should fulfill, at least, the following conditions:

1. The design of these studies should be prospective and multicentre.
2. Follow-up of patients should be long-term.
3. Patients should be included if they are diagnosed with iRLS according to standardized criteria [1], regardless of the duration of the disease, and have a positive family history of RLS. Healthy controls involved in these studies should not fulfill clinical criteria for the diagnosis of RLS and should not have a family history of RLS.
4. Periodic clinical evaluations, including the International RLS group rating scale (IRLSGRS), should be performed on the iRLS patients included in the study. Subjects included as healthy controls should also be evaluated for the eventual development of RLS symptoms.
5. Periodic studies with MRI, transcranial ultrasonography, DAT-SPECT, IBZM or ^{11}C raclopride PET, and ^{1}HMRS should be performed at baseline and during the follow-up period in iRLS patients.
6. It would be desirable to collect CSF and plasma/serum samples in iRLS patients and controls at different hours [Suppl. 29] (previous to starting treatment for RLS in the patient's group) for the measurement of multiple potential biological markers.
7. Obtention of blood DNA from the iRLS patients and controls for genetic studies related to potential biomarkers would be also desirable.
8. It would be desirable to obtain permission to perform a neuropathological examination of the brain of patients and controls who died during the study interval.

Conflicts of interest

The authors do not have any conflicts of interest to disclose.

Acknowledgments

Sources of funding: We recognize the effort of the personnel of the Library of Hospital Universitario “Príncipe de Asturias”, Alcalá de Henares (Madrid, SPAIN), who achieved an important number of papers for us. Professor James McCue revised in detail the quality of the English language. The work of the authors was supported in part by Grants PI15/00303 and RETICS RD12/0013/0002 from Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Madrid, Spain and GR15026 and IB16170 from Junta de Extremadura, Mérida, Spain. Partially funded with FEDER funds.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.smrv.2019.03.006.

References

[1] Allen RP, Picchietti DL, Garcia-Borreguero D, Ondo WG, Walters AS, Winkelman JW, et al. Restless legs syndrome/Willis-Ekbom disease diagnostic criteria: updated International Restless Legs Syndrome Study Group (IRLSSG) consensus criteria - history, rationale, description, and significance. Sleep Med 2014;15:860–73.
[2] Jiménez-Jiménez FJ, Alonso-Navarro H, García-Martín E, Agúndez JAG. Neurochemistry of idiopathic restless legs syndrome. Eur Neurol Rev 2015;10:35–44. Link, http://www.touchneurology.com/articles/neurochemistry-idiopathic-restless-legs-syndrome.
*[3] Qu S, Ondo WG, Zhang X, Xie WJ, Pan TH, Le WD. Projections of diencephalic dopamine neurons into the spinal cord in mice. Exp Brain Res 2006;168:152–6.
*[4] Barraud Q, Obeid I, Aubert I, Barrière G, Contamin H, McGuire S, et al. Neuroanatomical study of the A11 diencephalospinal pathway in the non-human primate. PLoS One 2010;5:e13306.
[5] Ozawa H, Yamaguchi T, Hamaguchi S, Yamaguchi S, Ueda S. Three types of A11 neurons project to the rat spinal cord. Neurochem Res 2017;42:2142–53.
[6] Pappas SS, Behrouz B, Janis KL, Goudreau JL, Lookingland KJ. Lack of D2 receptor mediated regulation of dopamine synthesis in A11 diencephalospinal neurons in male and female mice. Brain Res 2008;1214:1–10.
*[7] Ondo WG, He Y, Rajasekaran S, Le WD. Clinical correlates of 6-hydroxydopamine injections into A11 dopaminergic neurons in rats: a possible model for restless legs syndrome. Mov Disord 2000;15:154–8.
[8] Zhao H, Zhu W, Pan T, Xie W, Zhang A, Ondo WG, et al. Spinal cord dopamine receptor expression and function in mice with 6-OHDA lesion of the A11 nucleus and dietary iron deprivation. J Neurosci Res 2007;85:1065–76.
[9] Qu S, Le W, Zhang X, Xie W, Zhang A, Ondo WG. Locomotion is increased in a11-lesioned mice with iron deprivation: a possible animal model for restless legs syndrome. J Neuropathol Exp Neurol 2007;66:383–8.
[10] Luo F, Li C, Ondo WG, Xu P, Xie W, Le W. The long-term effects of the dopamine agonist pramipexole in a proposed restless legs syndrome animal model. Sleep Med 2011;12:41–6.
*[11] Guo CN, Yang WJ, Zhan SQ, Yang XF, Chen MC, Fuller PM, et al. Targeted disruption of supraspinal motor circuitry reveals a distributed network underlying Restless Legs Syndrome (RLS)-like movements in the rat. Sci Rep 2017;7:9905.
[12] Meneely S, Dinkins ML, Kassai M, Lyu S, Liu Y, Lin CT, et al. Differential dopamine D1 and D3 receptor modulation and expression in the spinal cord of two mouse models of restless legs syndrome. Front Behav Neurosci 2018;12:199.
[13] Clemens S, Hochman S. Conversion of the modulatory actions of dopamine on spinal reflexes from depression to facilitation in D3 receptor knock-out mice. J Neurosci 2004;24:11337–45.
[14] Jiménez-Jiménez FJ, Alonso-Navarro H, García-Martín E, Agúndez JAG. Genetics of restless legs syndrome: an update. Sleep Med Rev 2018;39:108–21.
[15] Salminen AV, Garrett L, Schormair B, Rozman J, Giesert F, Niedermeier KM, et al. Meis1: effects on motor phenotypesand the sensorimotor system in mice. Dis Model Mech 2017;10:981–91.
[16] Dinkins ML, Lallemand P, Clemens S. Long-term treatment with dopamine D3receptor agonists induces a behavioral switch that can be rescued by blocking the dopamine D1 receptor. Sleep Med 2017;40:47–52.

* The most important references are denoted by an asterisk.

[17] Lee SJ, Kim JS, Song IU, An JY, Kim YI, Lee KS. Poststroke restless legs syndrome and lesion location: anatomical considerations. Mov Disord 2009;24:77–84.
[18] Shiina T, Suzuki K, Okamura M, Matsubara T, Hirata K. Restless legs síndrome and its variants in acute ischemic stroke. Acta Neurol Scand 2019;139:260–8.
[19] Woo HG, Lee D, Hwang KJ, Ahn TB. Post-stroke restless leg syndrome and periodic limb movements in sleep. Acta Neurol Scand 2017;135:204–10.
[20] Ferri R, Cosentino FI, Moussouttas M, Lanuzza B, Aricò D, Bagai K, et al. Silent cerebral small vessel disease in restless legs syndrome. Sleep 2016;39:1371–7.
[21] Winkelman JW, Blackwell T, Stone K, Ancoli-Israel S, Redline S. Associations of incident cardiovascular events with restless legs syndrome and periodic leg movements of sleep in older men, for the outcomes of sleep disorders in older men study (MrOS sleep study). Sleep 2017;40(4). https://doi.org/10.1093/sleep/zsx023.
[22] Schmidauer C, Sojer M, Seppi K, Stockner H, Högl B, Biedermann B, et al. Transcranial ultrasound shows nigral hypoechogenicity in restless legs syndrome. Ann Neurol 2005;58:630–4.
[23] Ryu JH, Lee MS, Baik JS. Sonographic abnormalities in idiopathic restless legs syndrome (RLS) and RLS in Parkinson's disease. Park Relat Disord 2011;17:201–3.
[24] Godau J, Manz A, Wevers AK, Gaenslen A, Berg D. Sonographic substantia nigra hypoechogenicity in polyneuropathy and restless legs syndrome. Mov Disord 2009;24:133–7.
[25] Godau J, Wevers AK, Gaenslen A, Di Santo A, Liepelt I, Gasser T, et al. Sonographic abnormalities of brainstem structures in restless legs syndrome. Sleep Med 2008;9:782–9.
[26] Yang X, Liu B, Shen H, Li S, Zhao An R, et al. Prevalence of restless legs syndrome in Parkinson's disease: a systematic review and meta-analysis of observational studies. Sleep Med 2018;43:40–6.
[27] Alonso-Navarro H, García-Martín E, Agúndez JAG, Jiménez-Jiménez FJ. Association between restless legs syndrome and other movement disorders. Neurology 2019 (in press).
[28] Vilariño-Güell C, Soto AI, Young JE, Lin SC, Uitti RJ, Wszolek ZK, et al. Susceptibility genes for restless legs syndrome are not associated with Parkinson disease. Neurology 2008;71:222–3.
[29] Berger K, von Eckardstein A, Trenkwalder C, Rothdach A, Junker R, Weiland SK. Iron metabolism and the risk of restless legs syndrome in an elderly general population-the MEMO-Study. J Neurol 2002;249:1195–9.
*[30] Lammers N, Curry-Hyde A, Smith AJ, Eastwood PR, Straker LM, Champion D, et al. Are serum ferritin and transferrin saturation risk markers for restless legs syndrome in young adults? Longitudinal and cross-sectional data from the Western Australian Pregnancy Cohort (Raine) Study. J Sleep Res 2018 Jul 31:e12741. https://doi.org/10.1111/jsr.12741 [Epub ahead of print].
[31] Benediktsdottir B, Janson C, Lindberg E, Arnardóttir ES, Olafsson I, Cook E, et al. Prevalence of restless legs syndrome among adults in Iceland and Sweden: lung function, comorbidity, ferritin, biomarkers and quality of life. Sleep Med 2010;11:1043–8.
[32] Earley CJ, Ponnuru P, Wang X, Patton SM, Conner JR, Beard JL, et al. Altered iron metabolism in lymphocytes from subjects with restless legs syndrome. Sleep 2008;31:847–52.
[33] Dauvilliers Y, Chenini S, Vialaret J, Delaby C, Guiraud L, Gabelle A, et al. Association between serum hepcidin level and restless legs syndrome. Mov Disord 2018;33:618–27.
[34] Dowling P, Klinker F, Amaya F, Paulus W, Liebetanz D. Iron-deficiency sensitizes mice to acute pain stimuli and formalin-induced nociception. J Nutr 2009;139:2087–92.
[35] DeAndrade MP, Johnson Jr RL, Unger EL, Zhang L, van Groen T, Gamble KL, et al. Motor restlessness, sleep disturbances, thermal sensory alterations and elevated serum iron levels in Btbd9 mutant mice. Hum Mol Genet 2012;21:3984–92.
[36] Xiong L, Dion P, Montplaisir J, Levchenko A, Thibodeau P, Karemera L, et al. Molecular genetic studies of DMT1 on 12q in French-Canadian restless legs syndrome patients and families. Am J Med Genet B Neuropsychiatr Genet 2007;144B:911–7.
[37] Li J, Hu LD, Wang WJ, Chen YG, Kong XY. Linkage analysis of the candidate genes of familial restless legs syndrome. Yi Chuan Xue Bao 2003;30:325–9.
[38] Oexle K, Schormair B, Ried JS, Czamara D, Heim K, Frauscher B, et al. Dilution of candidates: the case of iron-related genes in restless legs syndrome. Eur J Hum Genet 2013;21:410–4.
[39] García-Martín E, Jiménez-Jiménez FJ, Alonso-Navarro H, Martínez C, Zurdo M, Turpín-Fenoll L, et al. Heme oxygenase-1 and 2 common genetic variants and risk for restless legs syndrome. Medicine (Baltimore) 2015;94:e1448.
*[40] Yepes G, Guitart X, Rea W, Newman AH, Allen RP, Earley CJ, et al. Targeting hypersensitive corticostriatal terminals in restless legs syndrome. Ann Neurol 2017;82:951–60.
[41] Lerner A, Bagic A, Simmons JM, Mari Z, Bonne O, Xu B, et al. Widespread abnormality of the γ-aminobutyric acid-ergic system in Tourette syndrome. Brain 2012;135:1926–36.
[42] Jiménez-Jiménez FJ, Alonso-Navarro H, Martínez C, Zurdo M, Turpín-Fenoll L, Millán-Pascual J, et al. The solute carrier family 1 (glial high affinity glutamate transporter), member 2 gene, SLC1A2, rs3794087 variant and assessment risk for restless legs syndrome. Sleep Med 2014;15:266–8.
[43] Jiménez-Jiménez FJ, Esguevillas G, Alonso-Navarro H, Zurdo M, Turpín-Fenoll L, Millán-Pascual J, et al. Gamma-aminobutyric acid (GABA) receptors genes polymorphisms and risk for restless legs syndrome. Pharmacogenomics J 2018;18:565–77.
[44] Sun YM, Hoang T, Neubauer JA, Walters AS. Opioids protect against substantia nigra cell degeneration under conditions of iron deprivation: a mechanism of possible relevance to the Restless Legs Syndrome (RLS) and Parkinson's disease. J Neurol Sci 2011;304:93–101.
*[45] Ferré S, Quiroz C, Guitart X, Rea W, Seyedian A, Moreno E, et al. Pivotal role of adenosine neurotransmission in restless legs syndrome. Front Neurosci 2018;11:722.
[46] Rivera-Oliver M, Moreno E, Álvarez-Bagnarol Y, Ayala-Santiago C, Cruz-Reyes N, Molina-Castro GC, et al. Adenosine A1-dopamine D1 receptor heteromers control the excitability of the spinal motoneuron. Mol Neurobiol 2019;56:791–811.
[47] Allen RP, Mignot E, Ripley B, Nishino S, Earley CJ. Increased CSF hypocretin-1 (orexin-A) in restless legs syndrome. Neurology 2002;59:639–41.
[48] Stiasny-Kolster K, Mignot E, Ling L, Möller JC, Cassel W, Oertel WH. CSF hypocretin-1 levels in restless legs syndrome. Neurology 2003;61:1426–9.
[49] Mignot E, Lammers GJ, Ripley B, Okun M, Nevsimalova S, Overeem S, et al. The role of cerebrospinal fluid hypocretin measurement in the diagnosis of narcolepsy and other hypersomnias. Arch Neurol 2002;59:1553–62.
[50] Wetter TC, Collado-Seidel V, Oertel H, Uhr M, Yassouridis A, Trenkwalder C. Endocrine rhythms in patients with restless legs syndrome. J Neurol 2002;249:146–51.
[51] Schilling C, Schredl M, Strobl P, Deuschle M. Restless legs syndrome: evidence for nocturnal hypothalamic-pituitary-adrenal system activation. Mov Disord 2010;25:1047–52.
[52] Tribl GG, Waldhauser F, Sycha T, Auff E, Zeitlhofer J. Urinary 6-hydroxymelatonin-sulfate excretion and circadian rhythm in patients with restless legs syndrome. J Pineal Res 2003;35:295–6.
[53] Topaloglu Tuac S, Ozben S, Koseoglu Bitnel M, Koksal A, Kucuksayan E, Hanikoğlu A, et al. Plasma copeptin levels in patients with restless legs syndrome. Acta Neurol Scand 2018;137:316–20.
[54] Koo BB, Feng P, Dostal J, Strohl KP. Alpha-melanocyte stimulating hormone and adrenocorticotropic hormone: an alternative approach when thinking about restless legs syndrome? Mov Disord 2008;23:1234–42.
[55] Balaban H, Yıldız ÖK, Çil G, Şentürk İA, Erselcan T, Bolayır E, et al. Serum 25-hydroxyvitamin D levels in restless legs syndrome patients. Sleep Med 2012;13:953–7.
[56] Celik K, Cikrikcioglu MA, Halac G, Kilic E, Ayhan S, Ozaras N, et al. Serum endocan levels in women with restless legs syndrome. Neuropsychiatr Dis Treat 2015;11:2919–25.
*[57] Wali S, Alsafadi S, Abaalkhail B, Ramadan I, Abulhamail B, Kousa M, et al. The association between vitamin D level and restless legs syndrome: a population-based case-control study. J Clin Sleep Med 2018;14:557–64.
[58] Oran M, Unsal C, Albayrak Y, Tulubas F, Oguz K, Avci O, et al. Possible association between vitamin D deficiency and restless legs syndrome. Neuropsychiatr Dis Treat 2014;10:953–8.
[59] Çakır T, Doğan G, Subaşı V, Filiz MB, Ülker N, Doğan ŞK, et al. An evaluation of sleep quality and the prevalence of restless leg syndrome in vitamin D deficiency. Acta Neurol Belg 2015;115:623–7.
[60] Patton SM, Cho YW, Clardy TW, Allen RP, Earley CJ, Connor JR. Proteomic analysis of the cerebrospinal fluid of patients with restless legs syndrome/Willis-Ekbom disease. Fluids Barriers CNS 2013;10:20.
[61] Jiménez-Jiménez FJ, García-Martín E, Alonso-Navarro H, Martínez C, Zurdo M, Turpín-Fenoll L, et al. Association between vitamin D receptor rs731236 (Taq1) polymorphism and risk for restless legs syndrome in the Spanish caucasian population. Medicine (Baltimore) 2015;94:e2125.
[62] Wali SO, Abaalkhail B, Alhejaili F, Pandi-Perumal SR. Efficacy of vitamin D replacement therapy in restless legs syndrome: a randomized control trial. Sleep Breath 2018 Nov 14. https://doi.org/10.1007/s11325-018-1751-2 [Epub ahead of print].
[63] Patton SM, Ponnuru P, Snyder AM, Podskalny GD, Connor JR. Hypoxia-inducible factor pathway activation in restless legs syndrome patients. Eur J Neurol 2011;18:1329–35.
[64] Baskol G, Korkmaz S, Erdem F, Canikliogl A, Kocyigit M, Aksu M. Assessment of nitric oxide, advanced oxidation protein products, malondialdehyde, and thiol levels in patients with restless legs syndrome. Sleep Med 2012;13:414–8.
*[65] Clemens S, Sawchuk MA, Hochman S. Reversal of the circadian expression of tyrosine-hydroxylase but not nitric oxide synthase levels in the spinal cord of dopamine D3 receptor knockout mice. Neuroscience 2005;133:353–7.
[66] Winkelmann J, Lichtner P, Schormair B, Uhr M, Hauk S, Stiasny-Kolster K, et al. Variants in the neuronal nitric oxide synthase(nNOS, NOS1) gene are associated with restless legs syndrome. Mov Disord 2008;23:350–8.
[67] Jiménez-Jiménez FJ, Alonso-Navarro H, Martínez C, Zurdo M, Turpín-Fenoll L, Millán-Pascual J, et al. Neuronal nitric oxide synthase(nNOS, NOS1) rs693534 and rs7977109 variants and risk for restless legs syndrome. J Neural Transm 2015;122:819–23.
[68] Bachmann CG, Guth N, Helmschmied K, Armstrong VW, Paulus W, Happe S. Homocysteine in restless legs syndrome. Sleep Med 2008;9:388–92.
[69] Halac G, Kilic E, Cikrikcioglu MA, Celik K, Toprak-Erek A, Keskin S, et al. Serum soluble lectin-like oxidized low-density lipoprotein receptor-1 levels in patients with restless legs syndrome. Bratisl Lek Listy 2016;117:316–20.

[70] Walters AS, Elin RJ, Cohen B, Moller JC, Oertel W, Stiasny-Kolster K. Magnesium not likely to play a major role in the pathogenesis of Restless Legs Syndrome: serum and cerebrospinal fluid studies. Sleep Med 2007;8:186–7.
[71] Korkmaz S, Aksu M, Baskol G. Plasma apelin level in patients with restless legs syndrome and its association with periodic leg movements. Sleep Breath 2017;21:19–24.
[72] Schulte EC, Altmaier E, Berger HS, Do KT, Kastenmüller G, Wahl S, et al. Alterations in lipid and inositol metabolisms in two dopaminergic disorders. PLoS One 2016;11:e0147129.
*[73] Lanza G, Cantone M, Lanuzza B, Pennisi M, Bella R, Pennisi G, et al. Distinctive patterns of cortical excitability to transcranial magnetic stimulation in obstructive sleep apnea syndrome, restless legs syndrome, insomnia, and sleep deprivation. Sleep Med Rev 2015;19:39–50.
*[74] Lanza G, Ferri R. The neurophysiology of hyperarousal in RLS: hints for a role of glutamate/GABA. Adv Pharmacol 2019 (in press), https://doi.org/10.1016/bs.apha.2018.12.002.
[75] Hornyak M, Feige B, Riemann D, Voderholzer U. Periodic leg movements in sleep and periodic limb movement disorder: prevalence, clinical significance and treatment. Sleep Med Rev 2006;10:169–77.
[76] Li J, Moore H, Lin L, Young T, Finn L, Peppard PE, et al. Association of low ferritin with PLM in the Wisconsin sleep cohort. Sleep Med 2015;16:1413–8.
[77] Heidbreder A, Stefani A, Mitterling T, Weiss P, Brandauer E, Högl B. Do periodic leg movements differ between restless legs syndrome patients with low versus normal iron stores? Sleep Med 2017;32:271.
[78] Kutukcu Y, Dogruer E, Yetkin S, Ozgen F, Vural O, Aydin H. Evaluation of periodic leg movements and associated transcranial magnetic stimulation parameters in restless legs syndrome. Muscle Nerve 2006;33:133–7.
[79] Lopes C, Esteves AM, Frussa-Filho R, Tufik S, de Mello MT. Evaluation of periodic limb movements in a putative animal model of restless leg syndrome. Mov Disord 2012;27:413–20.
[80] García-Borreguero D, Williams AM. Dopaminergic augmentation of restless legs syndrome. Sleep Med Rev 2010;14:339–46.
[81] Trenkwalder C, Högl B, Benes H, Kohnen R. Augmentation in restless legs syndrome is associated with low ferritin. Sleep Med 2008;9:572–4.
[82] Frauscher B, Gschliesser V, Brandauer E, El-Demerdash E, Kaneider M, Rücker L, et al. The severity range of restless legs syndrome (RLS) and augmentation in a prospective patient cohort: association with ferritin levels. Sleep Med 2009;10:611–5.
[83] LaBarbera V, Trotti LM, Rye D. Restless legs syndrome with augmentation successfully treated with IV iron. Neurol Clin Pract 2017;7:e26–8.
[84] Oliveira de Almeida WA, Maculano Esteves A, Leite de Almeida-Júnior C, Lee KS, Kannebley Frank M, Oliveira Mariano M, et al. The effects of long-term dopaminergic treatment on locomotor behavior in rats. Sleep Sci 2014;7:203–8.
[85] Manconi M, Ferri R, Zucconi M, Bassetti CL, Fulda S, Aricò D, et al. Dissociation of periodic leg movements from arousals in restless legs syndrome. Ann Neurol 2012;71:834–44.