AWARD NUMBER: W81XWH-20-1-0003

TITLE: Restless Legs Syndrome and the Melanocortin System

PRINCIPAL INVESTIGATOR: Brian Koo, MD

CONTRACTING ORGANIZATION: Yale University

REPORT DATE: MARCH 2024

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
MARCH 2024	Final	1DEC2019 - 30NOV2023

4. TITLE AND SUBTITLE

Restless Legs Syndrome and the Melanocortin System

5a. CONTRACT NUMBER
W81XWH-20-1-0003

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dr. Brian Koo, MD

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Yale University
15 York Street/PO Box 208018
New Haven CT 06520

8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The objective of this proposal is to study the underlying biological mechanisms of **restless legs syndrome (RLS)**. We propose to study the hormonal **melanocortin (MC)** system in humans with RLS, based upon similarities between the MC hormones and features of RLS. Central to RLS are an urge to move associated with sensory discomfort and increased movement to alleviate symptoms. The MC hormones are well known to mediate increased locomotion and pain sensitivity in rats and cause motor restlessness when administered in humans. In this proposal, we will evaluate MC biology in humans by measuring MC hormone levels in blood and CSF of persons with RLS compared to persons without RLS. We hypothesize that MC hormone levels will be higher in persons with RLS and will correlate to the severity of RLS.

15. SUBJECT TERMS
NONE LISTED

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON USAMRDC**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. PHONE NUMBER** *(include area code)*
U	U	U	UU	**22**	

Standard Form 298 (Rev. 8-98) **Prescribed by ANSI Std. Z39.18**

TABLE OF CONTENTS

1. INTRODUCTION:

The objective of this proposal is to study the underlying biological mechanisms of **restless legs syndrome (RLS)**. We propose to study the hormonal **melanocortin (MC)** system in humans with RLS, based upon similarities between the MC hormones and features of RLS. Central to RLS are an urge to move associated with sensory discomfort and increased movement to alleviate symptoms. The MC hormones are well known to mediate increased locomotion and pain sensitivity in rats and cause motor restlessness when administered in humans. In this proposal, we will evaluate MC biology in humans by measuring MC hormone levels in blood and CSF of 40 persons with RLS compared to 40 persons without RLS. We hypothesize that MC hormone levels will be higher in persons with RLS and will correlate to the severity of RLS.

***2.* KEYWORDS:**

Restless legs syndrome, RLS; hormones, melanocortin, sleep, cerebrospinal fluid

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: To evaluate melanocortin biology by measuring α-MSH, ACTH, and POMC in serum and CSF of RLS patients and age-sex matched controls in the nighttime.

Specific Aim 2: To characterize RLS by measuring severity of RLS symptoms, physiology (sleep, PLMS, pain), and neurobehavior (mood, impulsivity, suicidality); and to correlate these changes to MC levels in RLS patients.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Major Task 1: Complete Regulatory Requirements for Study and Administrative Tasks
IRB protocol was submitted and approved within the first two month. Research staff were appropriately hired in the first two months. Amendments were appropriately submitted. Protocol deviations were not appropriately submitted which was part of the CAPA identified by the Yale IRB. Annual IRB continuing review was appropriately submitted throughout the conduct of the study.

Major Task 2: Set up Infrastructure for Research
Setting up logistics of subjects visits in the hospital research unit and Church Street research unit had been done prior to receiving funds. This includes the patient visits themselves, behavioral assessment, blood and CSF collection as well as hormone assays.

Major Task 3: Research implementation
We exceeded our original recruitment goal of 80 subjects by recruiting 87 subjects. We had submitted a request to DoD to increase our target enrollment number to 120 which was approved. Biospecimen processing was done by my research staff throughout the conduct of the study. Hormone measurements were carried out by Yale Clinical Center of Investigation and Sharon Wardlaw as stated in the grant and protocol. Data was collected and handled throughout the study. Dr. Koo performed interim analyses as appropriate as well as final data analysis. The manuscript was written in August and September of 2023 and was submitted to Annals of Neurology, where it has been reviewed and accepted for publication. Koo BB, Abdelfattah A, Eysa A, Lu L. The Melanocortin and Endorphin Neuropeptides in Patients with Restless Legs Syndrome. *Annals of Neurology* 2024; Feb 3. doi: 10.1002/ana.26876

We have included our publication of this work as an addendum to this final report. Please see figures 2 and 3 for the main results of the study which show that Plasma POMC was significantly greater in RLS than controls (17.0 ± 11.5 vs 12.7 ± 6.1fmol/ml, p = 0.048). CSF β-MSH was significantly higher in painful than nonpainful RLS or controls (48.2 ± 24.8 vs 32.1 ± 14.8 vs 32.6 ± 15.2pg/ml, analysis of variance [ANOVA] p = 0.03). CSF α-MSH was higher in RLS than controls (34.2 ± 40.9 vs 20.3 ± 11.0pg/ml, p = 0.062). CSF β-EDP was lowest in painful RLS, intermediate in nonpainful RLS, and highest in controls (8.0 ± 3.4 vs 10.8 ± 3.1 vs 12.3 ± 5.0pg/ml, ANOVA p = 0.049).

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report, although the manuscript has been published as noted above. We will also submit the results of this study to be presented at the Associated Professional Sleep Societies meeting in Houston in 2024.

What do you plan to do during the next reporting period to accomplish the goals?

We will also submit the results of this study to be presented at the Associated Professional Sleep Societies meeting in Houston in 2024.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Our major findings were that levels of alpha-MSH are higher in CSF of RLS patients than controls and that levels of beta-EDP are lower in CSF of RLS patients. When RLS patients were split into those with painful and non-painful RLS, levels of beta-EDP were lowest in those with painful RLS, intermediate in those with non-painful RLS and highest in controls.

We believe that these new findings will be highly impactful in that to date there is no explanation for what causes the idiosyncratic symptoms of RLS. In other words, we do not know the biology underlying RLS. This work is the first of its kind and highlights a hormonal system which has not been previously considered in the pathophysiology of RLS. Therefore, this work is potentially paradigm shifting. From this work could come identification of novel drug targets for RLS.

Furthermore, the finding of low B-EDP in RLS is very significant for the field and for patients. Severe medication refractory RLS is very difficult to treat. These patients often require low dose opioid medication nightly to control RLS symptoms and allow for normal sleep. In today's climate where opioid use disorder is common, physicians, pharmacists, and healthcare payers are often skeptical concerning the use of opioids to treat RLS. This research explains why such a treatment may be needed in many individuals with RLS.

We hope that our findings will have an impact on the treatment of severe RLS. Currently, RLS patients are predominantly treated with dopamine agonist medications, which often results in worsening of RLS over time in a phenomenon called augmentation. Once an RLS patient has experienced augmentation, to achieve control the patient must come off the dopamine medication. In order to do this and achieve long term control, the use of opioid medications is often needed. Our results of low beta-endorphin in CSF of RLS patients legitimizes the use of opioids to treat RLS. We hope that these results will help to validate and make acceptable the use of opioids to treat RLS.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

We hope that our findings will have an impact on the treatment of severe RLS. Currently, RLS patients are predominantly treated with dopamine agonist medications, which often results in worsening of RLS over time in a phenomenon called augmentation. Once an RLS patient has experienced augmentation, to achieve control the patient must come off the dopamine medication. In order to do this and achieve long term control, the use of opioid medications is often needed. Our results of low beta-endorphin in CSF of RLS patients legitimizes the use of opioids to treat RLS. We hope that these results will help to validate and make acceptable the use of opioids to treat RLS.

We also hope that these findings will soften the negative view of physicians, pharmacists, insurance payers, and the public in general regarding the use of opioid medications in the treatment of severe RLS.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

During study conduct, we were audited by Yale IRB on account of a patient adverse event. During the audit, the Yale IRB identified serious noncompliance and thus our protocol was suspended. We worked through the Corrective and Preventative action Plan over 7-8 months to correct each of the issues identified. Major issues included the use of expired version of informed consent and the lack of appropriate case report forms. The completed CAPA was shared with DoDIssues were corrected and measures were set to prevent similar mistakes from happening in the future. Since this time, we have been able to hire a research coordinator which has greatly aided in maintaining the appropriate records.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

As part of the IRB suspension of the protocol, there were deviations noted. These deviations included incomplete regulatory documentation of enrollment logs, delegation of authority, adverse event log, and deviation tracking. It was also identified that outdated informed consent forms were used at times and that opt in or opt out sections were sometimes not appropriately completed. These issues were addressed in a corrective and preventative action plan and submitted to Yale IRB as well as DoD.

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Koo BB, Abdelfattah A, Eysa A, Lu L. The Melanocortin and Endorphin Neuropeptides in Patients with Restless Legs Syndrome. *Annals of Neurology* 2024; Feb 3. doi: 10.1002/ana.26876

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Nothing to report

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report

What other organizations were involved as partners?

Columbia University New York, NY Dr. Sharon Wardlaw performed some of the hormone assays used in this study.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to report

QUAD CHARTS:

Not applicable

9. APPENDICES:

See attached paper

The Melanocortin and Endorphin Neuropeptides in Patients with Restless Legs Syndrome

Brian B. Koo, MD [1], Ahmed Abdelfattah, MD,[1] Athar Eysa, MD,[2] and Lingeng Lu, PhD[3]

Objective: Based upon similarities between the urge to move and sensory discomfort of restless legs syndrome (RLS) and properties of melanocortin hormones, including their incitement of movement and hyperalgesia, we assessed plasma and cerebrospinal fluid (CSF) α-melanocyte-stimulating hormone (α-MSH) and β-endorphin in RLS patients and controls.
Methods: Forty-two untreated moderate-to-severe RLS patients and 44 matched controls underwent venipuncture at 19:00, 20:30, and 22:00; 37 RLS and 36 controls had lumbar puncture at 21:30. CSF and plasma were analyzed for pro-opiomelanocortin (POMC), adrenocorticotropin hormone (ACTH), α-MSH, β-MSH, and β-endorphin by immunoassay. RLS severity was assessed by International RLS Study Group Severity Scale.
Results: RLS participants were 52.7 ± 12.0 years old, 61.9% were women, 21.4% had painful RLS, and RLS severity was 24.8 ± 9.0. Controls had similar age and sex. Plasma ACTH, α-MSH, and β-endorphin were similar between groups. Plasma POMC was significantly greater in RLS than controls (17.0 ± 11.5 vs 12.7 ± 6.1fmol/ml, $p = 0.048$). CSF ACTH was similar between groups. CSF β-MSH was significantly higher in painful than nonpainful RLS or controls (48.2 ± 24.8 vs 32.1 ± 14.8 vs 32.6 ± 15.2pg/ml, analysis of variance [ANOVA] $p = 0.03$). CSF α-MSH was higher in RLS than controls (34.2 ± 40.9 vs 20.3 ± 11.0pg/ml, $p = 0.062$). CSF β-EDP was lowest in painful RLS, intermediate in nonpainful RLS, and highest in controls (8.0 ± 3.4 vs 10.8 ± 3.1 vs 12.3 ± 5.0pg/ml, ANOVA $p = 0.049$). The ratio of the sum of CSF α- and β-MSH to CSF β-endorphin was highest, intermediate, and lowest in painful RLS, nonpainful RLS, and controls ($p = 0.007$).
Interpretation: CSF β-MSH is increased and CSF β-endorphin decreased in RLS patients with painful symptoms.

ANN NEUROL 2024;00:1–12

Introduction

Restless legs syndrome (RLS) is a common sensorimotor sleep disorder characterized by an unrelenting urge to move, occurring at night during periods of inactivity, when patients assume recumbency for sleep. RLS and its canonical urge to move prevent sleep by forcing the sufferer to move or walk during times of rest when sleep is most desired. RLS is the most common neurologically based sleep disorder with a prevalence of 7.2% in North American and European populations.[1] The disease is chronic and progressive and in moderate-to-severe RLS, when symptoms occur at least twice weekly, lifelong medical therapy is needed. Despite this, treatment options for RLS are finite and the medicines most commonly used to treat RLS actually cause a paradoxical worsening of RLS over months to years in the clinical phenomenon of augmentation.[2] The use of these medicines, the dopamine agonists, in part was popularized by an erroneous pathobiologic assumption of dopamine deficiency in RLS, whereas it is now known that dopamine exists in excess in the brain of RLS patients.[3,4] This clinical entity of augmentation exposes a pathobiology–treatment mismatch and underscores the critical need to further clarify the pathobiology of RLS.

RLS is characterized by an urge to move and sensory discomfort that arise at night, suggesting that a biological substance with diurnal release may underlie RLS. Hormones of the melanocortin (MC) system are produced

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.26876

Received Aug 7, 2023, and in revised form Nov 13, 2023. Accepted for publication Jan 10, 2024.

Address correspondence to Dr Koo, 575 Lambert Road, Orange, CT 06477. E-mail: brian.koo@yale.edu

From the [1]Department of Neurology, Yale University, New Haven, CT, USA; [2]Department of Internal Medicine, Morristown Medical Center, Morristown, NJ, USA; and [3]Department of Chronic Disease Epidemiology, Yale University, New Haven, CT, USA

in the hypothalamic arcuate nucleus, are released diurnally, and possess properties that parallel these core features of RLS. The MC system prohormone, pro-opiomelanocortin (POMC), is cleaved to yield adrenocorticotropin hormone (ACTH) and β-lipotropin, which subsequently are cleaved to yield the neuropeptides α-melanocyte-stimulating hormone (α-MSH) and β-endorphin (β-EDP), respectively. The main MC agonist, α-MSH, stimulates excessive locomotion and grooming in rodents as well as a state of hyperalgesia, when administered centrally.[5,6] Alternately, the actions of β-EDP oppose those of α-MSH, as it promotes passivity and analgesia.[7] Like α-MSH, ACTH administered into periaqueductal gray matter of rats provokes motor behavior and hyperalgesia.[7,8] We created a rodent RLS model through intracerebroventricular administration of α-MSH or ACTH, which caused increased locomotion and grooming, fragmented sleep, and periodic hindlimb movements during sleep, recapitulating core RLS features.[9]

Based upon similarities between core RLS features and the properties of MC peptides as well as our preliminary animal data, we aimed to test our hypothesis that changes in MC peptides, and in particular increased α-MSH and/or ACTH and decreased β-EDP, occur in patients with RLS and could underlie RLS pathophysiology. In the current study, we enrolled patients with idiopathic RLS and age-/sex-matched controls without RLS and collected blood as well as cerebrospinal fluid (CSF) in the evening/nighttime to measure levels of MC peptides, including POMC, α-MSH, ACTH, β-MSH, and β-EDP. Neuropeptide levels were measured using enzyme-linked immunoassay or radioimmunoassay. Our hypothesis is that CSF and plasma levels of POMC, ACTH, α-MSH, and β-MSH are increased whereas CSF and plasma levels of β-EDP are decreased in persons with RLS compared to age-/sex-matched controls without RLS.

Subjects and Methods

Study Cohort

This observational case–control study was conducted at Yale University's Church Street Research Units between February 2020 and September 2022 and was approved by the Yale University Institutional Review Board. Adults at least 18 years of age with moderate-to-severe RLS occurring at least twice weekly were eligible. RLS diagnosis was confirmed by the principal investigator, a board-certified sleep neurologist (B.B.K.), using International RLS Study group diagnostic criteria for RLS.[10] Individuals with ambiguous leg symptoms or symptoms suggesting an RLS mimic were excluded. Only persons with primary RLS were included, meaning persons with comorbid Parkinson disease, multiple sclerosis, renal failure, or symptomatic peripheral neuropathy were excluded. Enrolled individuals could not be taking the following medications within 7 days of sample collection: (1) dopaminergic medications, (2) opioids, (3) steroids, (4) amphetamines, or (5) alpha-2-δ ligands. Recreational drugs including opioids (heroin, morphine, methadone), cocaine, and methamphetamine were also exclusionary. RLS subjects were recruited from the Yale RLS Center of Excellence, through outreach to other Connecticut sleep providers, or through advertisement on the RLS Foundation website or RLS Facebook group sites. Persons with RLS were further divided into those with painful RLS and those with nonpainful RLS based upon their response to the following question: "Are your RLS symptoms painful? Do the feelings that you experience as RLS hurt, rather than just being uncomfortable?"

Controls were age- and sex-matched to those in the RLS cohort and must have answered "No" to the first two questions of the Cambridge-Hopkins RLS Questionnaire: (1) "Do you have, or have you had, recurrent uncomfortable feelings or sensations in your legs while you are sitting or lying down?" and (2) "Do you have, or have you had, a recurrent need or urge to move your legs while you were sitting or lying down?"[11] RLS was also excluded by personal interview with a board certified neurologist and sleep provider (B.B.K.). There were 42 RLS participants and 44 controls.

Study Visit and Biospecimen Collection

All subjects provided written informed consent as per the Declaration of Helsinki and with approval of the Yale ethical institutional review board. Enrolled subjects arrived at the clinical research unit between 18:00 and 18:30. Urine toxicology was performed to confirm that subjects were not using amphetamines, cocaine, morphine, heroin, or methadone. An intravenous catheter was placed in an antecubital vein by a trained research nurse. Blood (8ml) was collected in a purple top tube containing ethylenediaminetetraacetic acid (EDTA) at 3 different time points: 19:00, 20:30, and 22:00. To prevent further peptide breakdown, 0.6 trypsin inhibitor units (TIU) aprotinin per milliliter of blood was added. Samples were mixed and placed on ice, then promptly centrifuged at 500 × *g* and 4°C for 10 minutes. Plasma was then separated, aliquoted, and stored at −70°C for future peptide analysis. At 21:30, a lumbar puncture was performed using a 22-gauge Whitacre needle with the subject seated upright with the spine flexed. The first 0.5ml of CSF was discarded. Fourteen milliliters of CSF was collected by gravity in 2 purple top polypropylene EDTA tubes, each containing 5.0 TIU aprotinin. Tubes were mixed by vortex, placed on ice, and promptly centrifuged at 3,000 × *g* and 4°C for 5 minutes. CSF supernatant was separated, aliquoted, and stored at −70°C for future peptide analysis.

Following the first blood collection at 19:00, the first suggested immobilization test (SIT) was performed. SIT was performed to assess the propensity to RLS symptom onset and was also used to provoke RLS symptoms; the SIT test was conducted as previously described.[12] Subjects (both RLS and controls) were instructed to sit on a hospital bed with knees flexed at 60–70°. The head of the bed was elevated for comfort. Windows were covered with opaque shading material to account for seasonal

variability of outside light. Overhead lights were turned off, and portable ambient lighting was used to light the room at approximately 20–50 lux. Patients were instructed to maintain wakefulness and not to move, save for slight adjustments to avoid discomfort or slight movements of the feet. Large movements of the body or legs were discouraged. Discomfort from RLS was rated on a Likert scale from 0 to 10, 10 being the most severe, at 10 minutes and every 10 minutes thereafter until the final 60-minute time point. Following the first SIT test, the subject was given a 10-minute break, and then the second SIT test was performed in an identical fashion. RLS symptom severity during each SIT was expressed as the mean of the 6 reported RLS symptom severity ratings. Lumbar puncture was then performed at 21:30 and the final blood draw at 22:00. Finally, pain sensitivity was measured by digital algometry (FPIX; Wagner, Greenwich, CT). The algometer was affixed with a $1cm^2$ rubber tip and measured pressure applied from this tip to a skin area. Pressure was delivered with increasing levels from 0 to 10lbs at ~1lb/s to the anterior forearm, dorsal hand, and shin, until the subject reported feeling pain. This was repeated 3 times per site, and pressure readings were averaged for each site and then averaged overall to give a total algometer score from 0 to 10lbs. The assessment of different sites with 3 repetitions at a constant rate of force has been previously described.[13]

Immunoassays

Plasma was assessed for POMC, ACTH, α-MSH, and β-EDP, and CSF was assessed for POMC, ACTH, α-MSH, β-MSH, and β-EDP. POMC was measured using a custom 2-site enzyme-linked immunosorbent assay (ELISA) with the capture antibody directed to $ACTH_{10–18}$ and the detection antibody directed to γ-MSH research resource identifier (RRID: AB_2756529 and AB_2756530).[14] Antibodies have no cross-reactivity with ACTH, α-MSH, β-MSH, or β-EDP. Affinity-purified human 31 K POMC peptide with a sensitivity of 8fmol/ml was used as a standard. α-MSH was measured using a commercially available ELISA kit for human α-MSH (EKX-KWLBSG; Nordic Biosite, Täby, Sweden), with a sensitivity of 7.5pg/ml and range of 12.5 to 800pg/ml with an intra-assay precision coefficient of variation (CV) < 8%. β-MSH was measured by radioimmunoassay, using human β-MSH antiserum and isolated human β-MSH peptide for tracer iodination and standards (Phoenix Pharmaceuticals, Burlingame, CA); there is 10% cross-reactivity with POMC. β-EDP was measured using an ELISA kit for human β-EDP (EKH1392, Nordic Biosite), with a sensitivity of 9.375pg/ml and range of 15.625 to 1,000pg/ml with an intra-assay precision CV < 8%. ACTH was assayed by double-antibody radioimmunoassay using ^{125}I (hACTH Double Antibody RIA kit, 07–106,102; MP Biomedicals, Solon, OH) as per the manufacturer's protocol. All assays were performed in duplicate, and there was >90% agreement between measurements.

Questionnaires

As an assessment of sleep quality, the Insomnia Severity Index (ISI) was administered to all participants; the ISI is a 7-question scale validated to assess sleep difficulty was scored from 0 to 28. ISI questions assess, in the past 2 weeks, difficulty falling asleep, difficulty staying asleep, sleep satisfaction, and disruption of life quality from sleep difficulty, scored from 0 to 4 as "non," "mild," "moderate," "severe," and "very severe," respectively; 0–7 = no clinically significant insomnia, 8–14 = subthreshold insomnia, 15–21 = moderate clinical insomnia, and 22–28 = severe clinical insomnia. The Barratt Impulsiveness Scale-11 (BIS) was administered to all participants; the BIS is a 30-item scale validated to assess the level of impulsiveness and impulsive personality traits. Items included statements like "I plan tasks carefully" or "I do things without thinking." Answer choices were "rarely," "occasionally," "often," and "almost always." Choices were scored from 1 to 4, with greater impulsiveness indicated by higher scores (range = 30–120).

For participants with RLS, RLS severity was assessed using the International RLS Study Group Severity Rating Scale (IRLS), which assesses severity of RLS-related symptoms over the past 2 weeks.[15] The IRLS contains 10 questions that are answered with a score between 0 and 4 for none, mild, moderate, severe, or very severe, respectively. Representative questions include: "Overall, how would you rate the RLS discomfort in your legs or arms?"; "Overall, how severe is your sleep disturbance from your RLS symptoms?"; and "How often do you get RLS symptoms?" (answered as 0 = never, 1 = 1 day per week or less, 2 = 2–3 days per week, 3 = 4–5 days per week, 4 = 6–7 days per week). IRLS scores 1–10, 11–20, 21–30, and 31–40 are considered as mild, moderate, severe, and very severe RLS, respectively.

Statistical Analysis

Descriptive statistics were conducted to describe categorical variables as number and percentage and continuous variables as mean and standard deviation. Characteristics were compared between the RLS and control groups with χ^2 tests for categorical variables and Student t tests for comparison of continuous variables between RLS and control groups and analysis of variance for comparison among painful RLS, nonpainful RLS, and control groups; statistical significance was considered as a 2-sided p value < 0.05. If comparison among the groups with ANOVA indicated a statistically different result ($p < 0.05$), then post hoc testing was carried out with Student t tests between the different groups. Although there were no data from CSF α-MSH in RLS patients to perform a power analysis, one study of 14 healthy adult women showed a mean ± standard deviation CSF α-MSH of 25.5 ± 2.5pg/ml.[16] Estimating conservatively that we would find a 20% increase in CSF α-MSH in RLS patients with a standard deviation of 7.5pg/ml, 35 subjects would be needed in each group to find a statistically significant difference. With 36 subjects in each group, we would have 81.2% power to detect a 5.1pg/ml difference with a standard deviation of 7.5pg/ml and a significance level of 0.05. Statistical analyses were performed with the R statistical package 4.1.3 (Auckland, New Zealand).

Results

Characteristics of RLS and Control Groups

A total of 86 individuals participated in the study, 51 being female (42 RLS and 44 control participants). Subjects were middle-aged, with an average age of 51.5 ± 12.8 years. RLS and control cohorts were comparable in age, sex distribution, and body mass index (BMI; Table 1) as were controls, those with painful RLS, and those with nonpainful RLS. Those with RLS scored significantly higher on the ISI, falling in the moderate clinically significant insomnia range compared to controls, who generally did not have clinically significant insomnia. Pain sensitivity as measured by algometry and impulsiveness as measured by the BIS were similar among the groups.

On average, participants with RLS scored in the severe range on the IRLS, with an average score of 24.8 ± 9.0; IRLS was similar between those with painful RLS and nonpainful RLS. Nine of the RLS subjects or 21.4% described their RLS symptoms as painful. Those with painful RLS did not differ from those with nonpainful RLS or controls in terms of age, sex distribution, or BMI. Pain sensitivity as measured by algometry did not differ in the RLS group compared to controls (8.7 ± 3.0 vs 8.5 ± 2.3, $p = 0.84$), nor did it differ between those with painful RLS and nonpainful RLS (7.8 ± 1.7 vs 8.9 ± 3.3, $p = 0.26$).

Plasma Hormone Measurements

Table 2 shows the average plasma levels of ACTH, α-MSH, and β-EDP for 3 different times points of 19:00, 20:30, and 22:00 and for plasma POMC at 22:00. There was no difference in levels of ACTH, α-MSH, or β-EDP between RLS and control participants at any of the time points. This is also shown graphically in Figure 1A–C. Similarly, there were no differences in levels of ACTH, α-MSH, or β-EDP at any time point among the painful RLS, nonpainful RLS, and control groups (data not shown). Plasma POMC levels in femtomoles per milliliter at 22:00 were significantly greater in RLS (n = 39) than in control (n = 39) subjects (17.0 ± 11.5 vs 12.7 ± 6.1, $p = 0.048$). Plasma POMC levels were significantly different among those with nonpainful RLS (n = 30), those with painful RLS (n = 9), and controls (18.7 ± 12.1 vs 11.2 ± 7.4 vs 12.7 ± 6.1, ANOVA $p = 0.01$) with levels being significantly higher in those with non-painful RLS compared to RLS patients with painful RLS (18.7 ± 12.1 vs 11.2 ± 7.4, $p = 0.04$) and also compared to controls (18.7 ± 12.1 vs 12.7 ± 6.1, $p = 0.02$; Fig 2A,B).

CSF Hormone Measurements

Lumbar puncture was performed at 21:30, and CSF was collected for 37 RLS and 36 control participants. CSF was analyzed for ACTH, α-MSH, β-MSH, β-EDP, and POMC (Table 3). Levels of CSF ACTH (pg/ml) were similar between those with RLS and controls (22.0 ± 10.6 vs 24.4 ± 14.0, $p = 0.42$) and among controls, painful RLS sufferers, and nonpainful RLS sufferers. CSF α-MSH trended toward being higher in RLS patients compared to controls (34.2 ± 40.9 vs 20.3 ± 11.0, $p = 0.062$). CSF α-MSH was highest in nonpainful RLS sufferers, intermediate in those with painful RLS, and lowest in controls, although this was not statistically

TABLE 1. Patient Demographics and Characteristics

Characteristic	Controls, n = 44	Nonpainful RLS, n = 33	Painful RLS, n = 9	p[a]
Age, mean ± SD	50.4 ± 13.5	53.8 ± 12.3	50.8 ± 11.2	0.52[b]
BMI, mean ± SD	26.8 ± 5.0	25.7 ± 3.6	28.7 ± 5.1	0.20[b]
Female, n (%)	25 (56.8)	20 (60.1)	6 (66.7)	0.84[c]
ISI	5.3 ± 6.4	14.4 ± 6.7	16.8 ± 5.8	<0.0001[b]
Algometer, lbs	8.5 ± 2.1	8.9 ± 3.3	7.8 ± 1.7	0.63[b]
Barratt scale	66.0 ± 10.2	67.7 ± 8.1	69.9 ± 6.5	0.50[b]
IRLS	—	24.3 ± 9.5	26.3 ± 7.3	0.50[d]

BMI = body mass index in kg/m^2; IRLS = International RLS Study Group Severity Rating Scale; ISI = Insomnia Severity Index; RLS = restless legs syndrome.

[a]Comparing RLS patients to controls; superscript letter indicates type of test used.

[b]Analysis of variance.

[c]Chi-squared test.

[d]Student t test.

TABLE 2. Plasma Hormone Levels in Controls and RLS Patients

Plasma Hormone	Sample Size (controls, RLS)	Controls	RLS	*p*
Plasma $ACTH_{19:00}$, pg/ml	(40, 42)	87.2 ± 67.2	75.9 ± 32.0	0.33
Plasma $ACTH_{20:30}$, pg/ml	(40, 42)	82.4 ± 68.2	77.8 ± 46.4	0.72
Plasma $ACTH_{22:00}$, pg/ml	(39, 41)	101.0 ± 71.9	122.8 ± 99.4	0.27
Plasma α-$MSH_{19:00}$, pg/ml	(40, 41)	259.6 ± 155.5	254.2 ± 161.0	0.62
Plasma α-$MSH_{20:30}$, pg/ml	(38, 40)	261.9 ± 151.9	231.4 ± 143.5	0.88
Plasma α-$MSH_{22:00}$, pg/ml	(38, 39)	262.9 ± 151.9	225.7 ± 128.7	0.26
Plasma β-$EDP_{19:00}$, pg/ml	(38, 43)	434.6 ± 180.5	409.0 ± 172.7	0.52
Plasma β-$EDP_{20:30}$, pg/ml	(41, 44)	428.7 ± 187.5	412.1 ± 154.8	0.66
Plasma β-$EDP_{22:00}$, pg/ml	(40, 43)	450.5 ± 195.2	444.4 ± 144.1	0.87
Plasma $POMC_{22:00}$, fmol/ml	(39)	12.7 ± 6.1	17.0 ± 11.5	0.048[a]

ACTH = adrenocorticotropin hormone; POMC = pro-opiomelanocortin; RLS = restless legs syndrome; α-MSH = α-melanocyte-stimulating hormone; β-EDP = β-endorphin.
[a]$p < 0.05$.

significant (36.0 ± 45.3 vs 28.5 ± 23.0 vs 20.3 ± 11.0, ANOVA $p = 0.14$; Fig 3A–C).

CSF β-MSH (pg/ml) was similar between RLS subjects and controls (36.2 ± 18.9 vs 32.6 ± 15.2, $p = 0.38$), but when RLS subjects were subclassified, CSF β-MSH was higher in those with painful RLS than in nonpainful RLS or in controls (48.2 ± 24.8 vs 32.1 ± 14.8 vs 32.6 ± 15.2, ANOVA $p = 0.03$; Figure 3). CSF β-EDP (pg/ml) trended toward being lower in the RLS compared to the control cohort (10.3 ± 3.2 vs 12.3 ± 5.0, $p = 0.059$), and was significantly different among the different cohorts, with those with painful RLS having the lowest, nonpainful RLS having intermediate, and controls having the highest CSF β-EDP (8.0 ± 3.4 vs 10.8 ± 3.1 vs 12.3 ± 5.0, ANOVA $p = 0.049$; Figure 3). With post hoc testing, those with painful RLS had significantly lower CSF β-EDP than controls (8.0 ± 3.4 vs 12.3 ± 5.0, $p = 0.02$). In the overall cohort, severity of RLS as determined by the IRLS did not correlate significantly to levels of any hormone. In women with RLS, severity of RLS by IRLS correlated positively with CSF β-MSH ($r = +0.57$, $p = 0.007$) and marginally with CSF ACTH ($r = +0.40$, $p = 0.059$), but not levels of other CSF hormones. Also in women, CSF β-MSH correlated with severity of RLS symptoms in the first SIT ($r = +0.48$, $p = 0.04$) but not the second SIT ($r = +0.31$, $p = 0.19$). In men with RLS, RLS severity by IRLS correlated negatively with CSF POMC ($r = -0.55$, $p = 0.0501$) but not other CSF hormones.

Ratios of the MSH hormones to β-EDP were calculated and compared among the groups. The ratio of α-MSH to β-EDP was significantly higher for those with RLS than for controls (3.45 ± 3.8 vs 1.90 ± 1.72, $p = 0.03$). The ratio of β-MSH to β-EDP was similar in RLS and control cohorts, but was significantly higher in those with painful RLS than those with nonpainful RLS or controls (6.87 ± 3.63 vs 3.18 ± 1.77 vs 3.18 ± 2.08, ANOVA $p = 0.0004$). The ratio of the sum of α-MSH and β-MSH to β-EDP was significantly higher in those with RLS compared to controls (7.66 ± 5.14 vs 5.15 ± 2.95, $p = 0.02$) and was highest in painful RLS, intermediate in nonpainful RLS, and lowest in controls (10.6 ± 6.18 vs 6.77 ± 4.56 vs 5.15 ± 2.95, $p = 0.007$).

Discussion

This is the first study to evaluate the potential involvement of the MC system in humans with RLS. POMC-derived peptides were measured in blood and CSF to determine whether the MSH peptides are increased and β-EDP decreased in RLS patients compared to age-/sex-matched controls. The major findings demonstrated higher CSF α-MSH and lower CSF β-EDP in RLS patients, although results did not differ with statistical significance. Following RLS subtyping, those with painful RLS had the lowest, nonpainful RLS intermediate, and controls the highest CSF β-EDP levels. CSF β-MSH was significantly higher in those with painful RLS than in

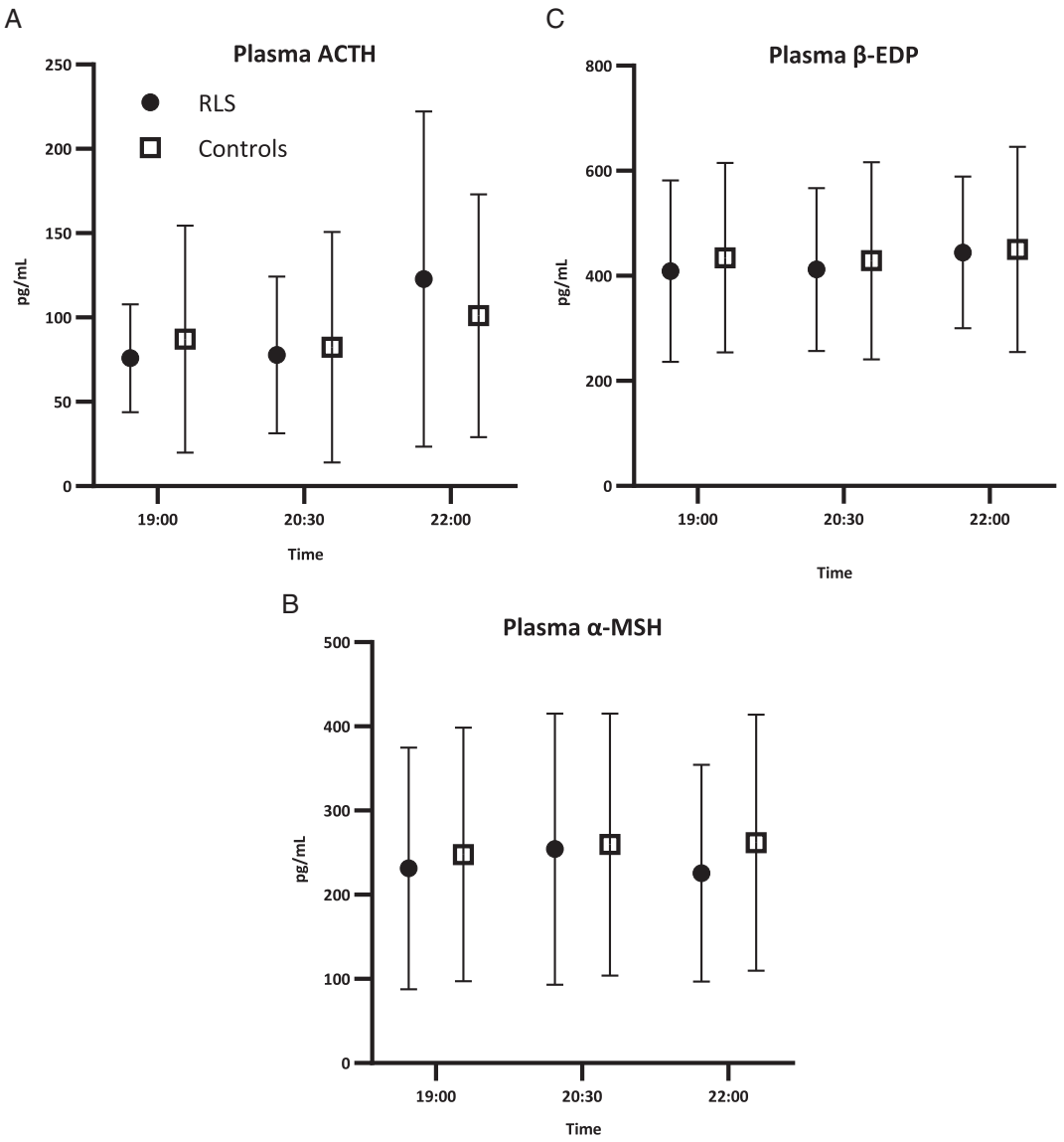

FIGURE 1: Nighttime plasma melanocortin levels in restless legs syndrome (RLS) and control cohorts are shown as mean and standard deviation in picograms per milliliter of (A) adrenocorticotropin hormone (ACTH) in RLS (n = 40, 40, 39) compared to controls (n = 42, 42, 41), (B) α-melanocyte-stimulating hormone (α-MSH) in RLS (n = 41, 38, 38) compared to controls (n = 40, 40, 39), and (C) β-endorphin (β-EDP) in RLS (n = 38, 41, 40) compared to controls (n = 43, 44, 43) at 17:00, 20:30, and 22:00 in RLS (*black circles*) and controls (*open squares*; n = $N_{17:00}$, $N_{20:30}$, $N_{22:00}$). There were no differences between RLS and control groups at any time point for plasma ACTH, α-MSH, or β-EDP. Error bars indicate standard deviation.

those with nonpainful RLS or controls. There were statistically significant differences in the ratios of CSF β-MSH to CSF β-EDP and of CSF α-MSH + β-MSH to CSF β-EDP among groups, with ratios being highest in painful RLS. In plasma, ACTH, α-MSH, and β-EDP were similar between groups at all time points. Plasma POMC at 22:00 was significantly higher in RLS than controls. Considering these data as a whole, nonpainful RLS is associated with increased plasma POMC and increased CSF α-MSH, perhaps reflecting increased MC tone. Painful RLS is associated with normal plasma POMC, yet increased CSF β-MSH and decreased CSF β-EDP, not reflecting increased MC tone, yet a shift toward β-MSH and away from β-EDP, which both arise from β-lipotropin cleavage.

This study provides evidence that MC hormone levels may be altered in RLS. These findings should be interpreted with caution, as it is the first case–control study to assess CSF levels of the MC hormones in patients with RLS. Furthermore, RLS is a very complex phenotype, with numerous genes influencing its expression and different behavioral traits, such as impulsivity, anxiety, and poor sleep, which are difficult to control for. Thus, in a relatively small number of patients with RLS, it is

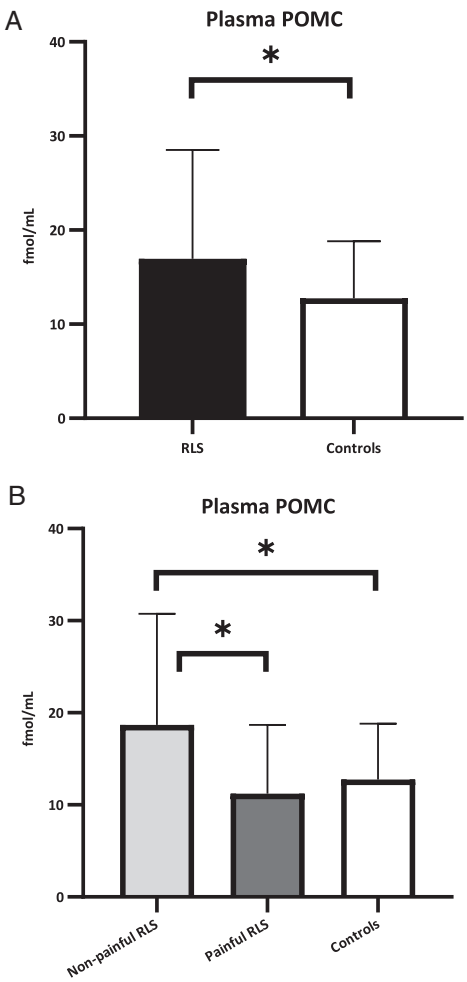

FIGURE 2: Plasma prohormone pro-opiomelanocortin (POMC) levels are shown in femtomoles per liter at 22:00 for (A) restless legs syndrome (RLS; black; n = 39) and controls (white; n = 39) and (B) nonpainful RLS (light gray; n = 30), painful RLS (dark gray; n = 9), and controls (white; n = 39). (A) Plasma POMC levels are significantly higher in RLS patients compared to controls (17.0 ± 11.5 vs 12.7 ± 6.1, $p = 0.048$). (B) Plasma POMC levels are significantly different among RLS patients with nonpainful RLS, RLS patients with painful RLS, and controls (18.7 ± 12.1 vs 11.2 ± 7.4 vs 12.7 ± 6.1, analysis of variance $p = 0.01$). Plasma POMC levels are significantly higher in RLS patients with nonpainful RLS compared to RLS patients with painful RLS (18.7 ± 12.1 vs 11.2 ± 7.4, $p = 0.04$) and also compared to controls (18.7 ± 12.1 vs 12.7 ± 6.1, $p = 0.02$). *$p < 0.05$; error bars indicate standard deviation.

possible that controls and/or the RLS subtype groups differed in one or more particular genes or traits and that this group difference accounted for the observed changes in CSF hormones observed in the study. A future study should take into account these aspects of the disease when aiming to confirm these findings. Nevertheless, the observed alterations in MC hormones in RLS patients, when combined with similarities between MC properties and RLS features, suggest that the MC system may play an important role in RLS pathogenesis. Furthermore, the biology of the MC system may provide a clue as to what may cause the symptoms of RLS, namely the urge to move and sensory discomfort, which we discuss below.

POMC undergoes various cleavage steps to yield the peptides γ-MSH, ACTH, and β-lipotropin. ACTH is further cleaved to α-MSH, whereas β-lipotropin is cleaved to β-MSH and β-EDP (Fig 4). POMC is highly conserved across higher vertebrate species and underlies basic life behaviors/functions, which include feeding, sex, movement, and pain sensitivity.[17] Study of MC-related behavioral effects date back to 1955 and descriptions of the ACTH-induced stretching and yawning syndrome.[18] In this syndrome, animals appear tired, while stretching and yawning repeatedly, reminiscent of the tiredness and desire to stretch that RLS sufferers report. Yawning and stretch are also induced by intracerebral α-MSH or β-MSH.[19] The most extensively described of the MC-related behaviors is excessive grooming and locomotion, caused by central α-MSH, β-MSH, or ACTH administration.[9,20,21] Excessive locomotion is the animal corollary of the urge to move, which compels the RLS sufferer to walk at night, giving them the colloquial title, "nightwalker." Sleep following this initial state of α-MSH-induced hyperactivity displays periodic cortical arousal and periodic hindlimb movements,[9,22] similar to the RLS-related phenomena of periodic limb movements during sleep and arousal.[23] Centrally administered ACTH and α-MSH result in a decreased time to tail-flick in response to a painful thermal stimulus, reflecting hyperalgesia.[6,8] RLS is experienced as painful in approximately 20% of cases[24] and dysesthetic in the majority of cases. Moreover, persons with RLS exhibit hyperalgesia to pinprick.[25]

Although the MSH peptides and β-EDP arise from the same prohormone, POMC, their actions are opposing. The MSH peptides promote movement and pain hypersensitivity, whereas β-EDP decreases locomotion and pain.[26] Intracerebroventricular β-EDP and nucleus solitarius microinjection enhance slow wave sleep[27,28] and produce analgesia, inhibiting ACTH-/α-MSH-induced hyperalgesia.[26,29,30] Thus, CSF β-EDP being low in RLS and lowest in painful RLS is fitting, as are findings of high CSF α-MSH in RLS given the promotor properties of MC agonists. In nonpainful RLS, both plasma POMC and CSF α-MSH were increased, which may reflect generally increased MC activity. In painful RLS, neither plasma POMC nor CSF α-MSH were increased; rather, CSF β-MSH was increased and CSF β-EDP was decreased. Both β-MSH and β-EDP are β-lipotropin cleavage products, so the abnormality in painful RLS may be a shift toward β-MSH at the expense of β-EDP, not an overall increase in MC activity.

TABLE 3. CSF Hormone Levels in Controls and RLS Patients

CSF Analyte	Sample Size (controls, RLS, nonpainful RLS, painful RLS)	Controls, n = 36	All RLS, n = 37	p[a]	Nonpainful RLS	Painful RLS	p[b]
ACTH	(35, 37, 28, 9)	24.4 ± 14.0	22.0 ± 10.6	0.42	21.4 ± 11.1	24.0 ± 9.1	0.62
α-MSH	(34, 34, 26, 8)	20.3 ± 10.9	34.2 ± 40.9	0.062	36.0 ± 45.3	28.5 ± 23.0	0.14
β-MSH	(35, 35, 26, 9)	32.6 ± 15.2	36.2 ± 18.9	0.38	32.1 ± 14.8	48.2 ± 24.8	0.03[c]
β-EDP	(34, 35, 27, 7)	12.3 ± 5.0	10.3 ± 3.2	0.059	10.8 ± 3.1	8.0 ± 3.4	0.049[c]
POMC	(36, 37, 28, 9)	241.9 ± 108	225.5 ± 69	0.44	228.5 ± 68	216 ± 74.0	0.70
α-MSH:β-EDP	(33, 32, 25, 7)	1.90 ± 1.72	3.45 ± 3.8	0.03[c]	3.37 ± 3.93	3.73 ± 3.53	0.09
β-MSH:β-EDP	(33, 32, 25, 7)	3.18 ± 2.08	4.00 ± 2.71	0.19	3.18 ± 1.77	6.87 ± 3.63	0.0004[d]
α-MSH+β-MSH: β-EDP	(32, 30, 23, 7)	5.15 ± 2.95	7.66 ± 5.14	0.02[c]	6.77 ± 4.56	10.6 ± 6.18	0.007[e]

":" indicates ratio.

ACTH = adrenocorticotropin hormone; CSF = cerebrospinal fluid; POMC = pro-opiomelanocortin; RLS = restless legs syndrome; α-MSH = α-melanocyte-stimulating hormone; β-EDP = β-endorphin.

[a]Student *t* test comparing means for all RLS patients to controls.

[b]Analysis of variance comparing means among nonpainful RLS, painful RLS, and controls.

[c]$p < 0.05$.

[d]$p < 0.001$.

[e]$p < 0.01$.

As the MSH peptides and β-EDP originate from the same transcript, one could ask, "Why are the MSH peptides increased and β-EDP decreased in RLS?" The answer may come through consideration of POMC processing. POMC undergoes extensive post-translational processing in the hypothalamic arcuate nucleus and pituitary.[31] At the cellular level, POMC is ferried to the endoplasmic reticulum, where it is amidated and marked for transport to the trans-Golgi network.[31,32] Here, vesicles bud to produce secretory granules, where proteolytic processing occurs by diverse regulatory mechanisms, yielding varying degrees of ACTH and β-lipotropin, then secretion of variable amounts of MSH peptide and β-EDP.[33] In this way, both increased and decreased levels of different POMC-derived peptides could occur in the same individual and in RLS a favoring toward MC and away from β-EDP activity. Our data reflect this, as ratios of α-MSH + β-MSH to β-EDP are increased in RLS.

The ratio of CSF α-MSH + β-MSH to β-EDP was highest in painful RLS, intermediate in nonpainful RLS, and lowest in controls. This result was driven by 3 factors. First, CSF β-EDP was lowest, intermediate, and highest in painful, nonpainful RLS, and controls, respectively; second, CSF α-MSH was higher in RLS than controls; and third, CSF β-MSH was higher in painful than nonpainful RLS or controls. Whereas CSF α-MSH was similar in RLS subtypes, CSF β-MSH was higher in painful than nonpainful RLS. Biochemically, both α-MSH and β-MSH show affinity for the MC-1 and MC-4 receptors, the activity of which underlies hyperalgesia and non-neuropathic and neuropathic pain, respectively.[34–37] We focused on α-MSH and β-MSH, but not γ-MSH, as the latter MSH peptide has little pain-modulating activity.[34] Given their pain-modulating involvement, α- and β-MSH may act synergistically, with pain sensitivity being greatest when both MSH peptides are elevated, in particular when β-EDP is low. As reflected in an increased ratio of CSF α-MSH + β-MSH to β-EDP, an increase in α-MSH and β-MSH and decreased β-EDP would favor motor activity and hyperalgesia over passivity and analgesia.[7]

RLS pharmacology is also consistent with MC-endorphin RLS pathobiology. RLS treatment with L-dopa was described in 1982.[38] Dopamine inhibits ACTH and α-MSH secretion from the pituitary, and inhibits α-MSH acetylation to active α-MSH,[39,40] thereby decreasing MSH tone and treating RLS. Opioid medications are used mostly in medication-refractory RLS.[41] Like β-EDP, opioids bind μ-opioid receptors to produce analgesia and passivity.[42,43]

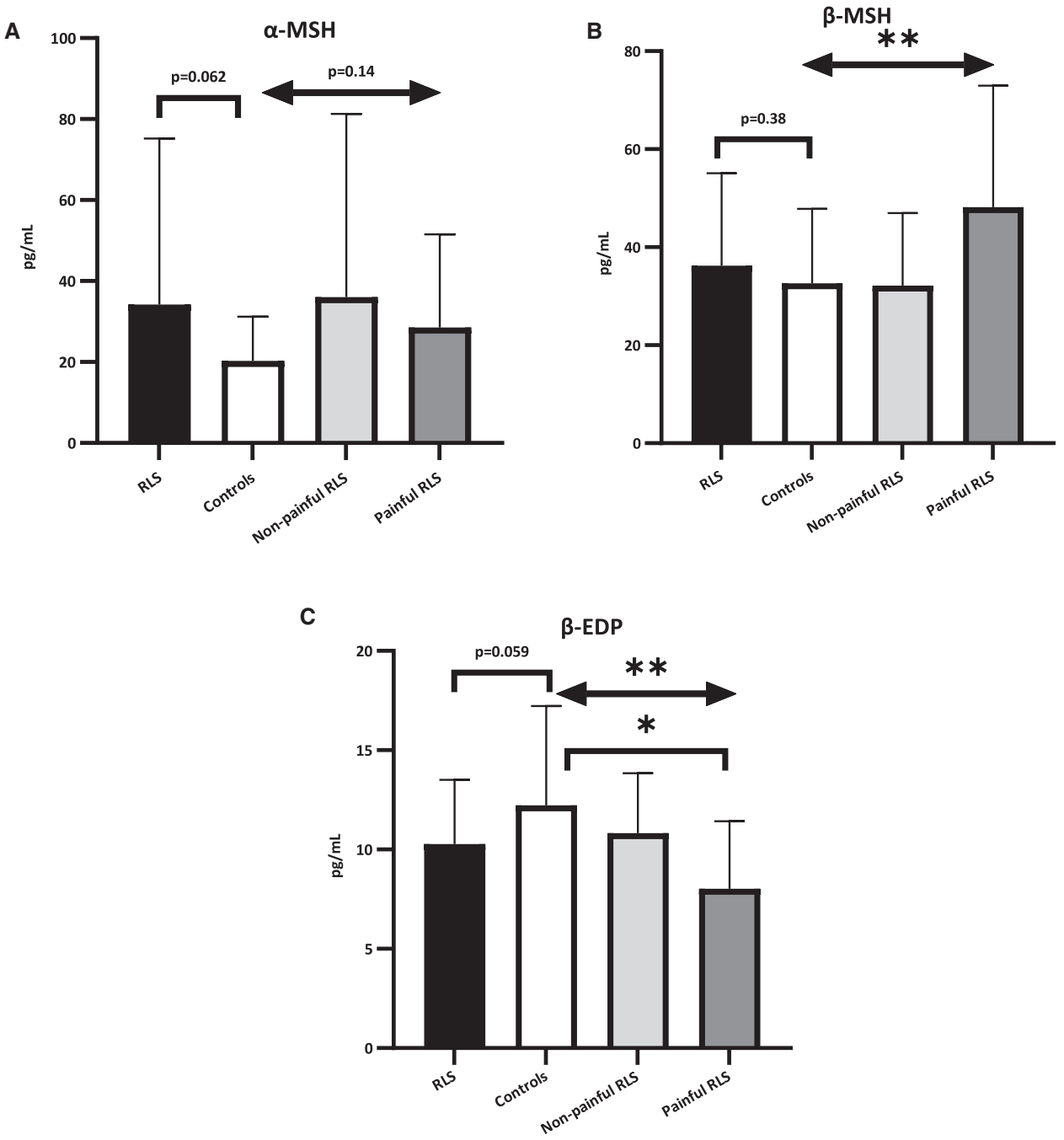

FIGURE 3: Cerebrospinal fluid (CSF) α-melanocyte-stimulating hormone (α-MSH), β-MSH, and β-endorphin (β-EDP) levels are shown in picograms per milliliter in restless legs syndrome (RLS; black), painful RLS (dark gray), nonpainful RLS (light gray), and controls (white). (A) CSF α-MSH was higher in RLS patients than controls (34.2 ± 40.9 vs 20.3 ± 11.0, $p = 0.062$). CSF α-MSH was highest in nonpainful RLS (n = 26), intermediate in painful RLS (n = 8), and lowest in controls (n = 34), although this was not statistically significant (36.0 ± 45.3 vs 28.5 ± 23.0 vs 20.3 ± 11.0, analysis of variance [ANOVA] $p = 0.14$). (B) CSF β-MSH was similar between RLS and controls (36.2 ± 18.9 vs 32.6 ± 15.2, $p = 0.38$). CSF β-MSH was higher in painful RLS (n = 9) than in nonpainful RLS (n = 26) or in controls (n = 35; 48.2 ± 24.8 vs 32.1 ± 14.8 vs 32.6 ± 15.2, ANOVA $p = 0.03$). (C) CSF β-EDP was lower in the RLS compared to the control cohort (10.3 ± 3.2 vs 12.3 ± 5.0, $p = 0.059$). CSF β-EDP was lowest in painful RLS (n = 7), intermediate in nonpainful RLS (n = 27), and highest in controls (n = 34; 8.0 ± 3.4 vs 10.8 ± 3.1 vs 12.3 ± 5.0, ANOVA $p = 0.049$). With post hoc testing, those with painful RLS had significantly lower CSF β-EDP than controls (8.0 ± 3.4 vs 12.3 ± 5.0, $p = 0.02$). *$p < 0.05$ between 2 groups using Student *t* test; **over the <–> refers to $p < 0.05$ among 3 groups using ANOVA test. Error bars indicate standard deviation.

In our study, CSF β-EDP was lowest in painful RLS. Given that β-EDP deficiency may be a cause of RLS, it is understandable why opioid medications treat RLS.

In addition to being the first study to assess MC hormones in RLS patients, our study has other strengths. Only patients with moderate-to-severe RLS were included to

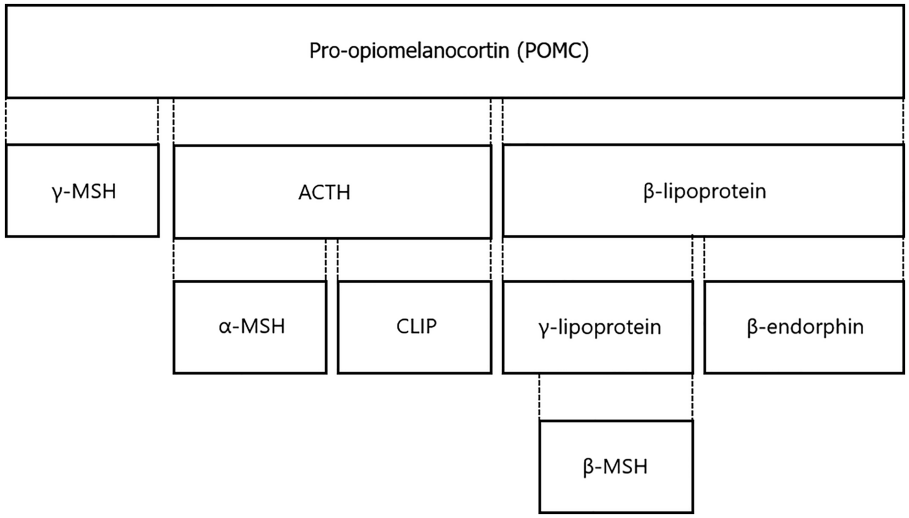

FIGURE 4: Processing of the prohormone pro-opiomelanocortin (POMC). The prohormone POMC undergoes extensive post-translational processing and is cleaved to yield γ-melanocyte-stimulating hormone (γ-MSH), adrenocorticotropin hormone (ACTH), and β-lipotropin. ACTH is cleaved to give α-MSH and corticotropinlike intermediate peptide (CLIP), whereas β-lipotropin is broken down into γ-lipotropin and β-endorphin. Finally, γ-lipotropin is cleaved into β-MSH.

increase chances of seeing hormonal abnormalities. Patients taking RLS medicines, specifically dopamine agonists, alpha-2 delta ligands, or opioids, were ineligible or had to stop medications for 7 days, as these medicines could influence MC hormone levels. It should be noted that only 2 RLS patients had to be tapered from an opioid medication, one from the painful RLS group and the other from the nonpainful RLS group. Our CSF sample size of 37 RLS and 36 control subjects was large and based upon CSF α-MSH results in healthy women and a conservative 20% α-MSH change estimate.[16] We inquired about painful RLS, which accounts for approximately 20% of RLS.[24] This subclassification produced arguably the most interesting finding that CSF β-EDP was lowest in painful RLS, intermediate in nonpainful RLS, and highest in controls. Whereas CSF β-EDP levels differed among the groups, this was not true for plasma β-EDP. Research in other human disease has also shown discordance of plasma and CSF β-EDP. One study of US veterans showed that those with post-traumatic stress disorder had significantly higher CSF β-EDP than veterans without the disorder, whereas levels of plasma β-EDP were similar between the groups. This discordance between CSF and plasma β-EDP was similarly seen in our study.

There are important limitations to note. CSF was collected at one time point, as the principal investigator, a neurologist, had no experience with lumbar drains. Thus, we approximated a time when many prepare for sleep and experience RLS, and chose 21:30. Eight control and 5 RLS subjects were enrolled in whom lumbar puncture was unsuccessful. The number of samples successfully assayed for various plasma/CSF hormones differed slightly on account of varying sample numbers available at times assays were performed as well as rare assay failure. There were many statistical comparisons conducted for different peptides between groups, which increased potential for type I errors. However, directionality of the differences, increased MSH and decreased β-EDP in RLS, and the hormone level gradient in RLS subtypes make it more likely that findings are real. Nevertheless, it is possible that the observed findings occurred by chance or that significant changes were the result of other factors not necessarily related to RLS; thus, it is important to reproduce these findings in other studies. There is great complexity in the RLS phenotype, wherein the expression of the disease is influenced by numerous risk genes and differs based upon predilection to different behavioral traits, such as anxiety, poor sleep, and impulsivity. These complexities challenge the selection of a truly representative group of individuals with RLS.

The ISI was used rather than the Pittsburgh Sleep Quality Index and may not have adequately assessed sleep quality.

In summary, our study represents a first step toward integration of the POMC-derived peptides into the pathogenetic RLS framework. For the first time, there is a biological explanation as to what may cause the sensorimotor idiosyncrasies of RLS. These results should be interpreted with caution and need replication. Future studies should explore how the MC system interacts with iron and dopamine systems in idiopathic RLS, focusing on the painful subtype. As a field, there is much to learn and study regarding the involvement of MSH peptides and β-EDP in RLS. Investigation into the behavioral effects of MCs

in humans is needed. A study conducted in 1968 to investigate effects of intravenous α-MSH on menstruation noted that 3 of 6 women experienced "severe motor restlessness."[44] Replication and further characterization of the behavioral effect of MC agonism and the hormonal profile in patients with RLS should be a future goal.

Acknowledgments

This work was supported by a Department of Defense Discovery Award grant, part of the Peer Reviewed Medical Research Program and the Congressionally Directed Medical Research Program. The grant was entitled "Restless Legs Syndrome and the Melanocortin System" (grant number W81XWH2010003).

We are grateful to the many research assistants who helped with the recruiting of research subjects, processing of biosamples, and input of data. We are appreciative of the immunoassay work provided by C. Todeasa and S. Wardlaw. We are grateful to the research nurse, E. O'Neill and C. Smith, and of course we are grateful to all the patients with RLS and controls without RLS who participated in the study.

Author Contributions

B.B.K., A.A., and L.L. contributed to the conception and design of the study. B.B.K., A.A, and A.E. contributed to the acquisition and analysis of data. All authors contributed to the drafting and editing of the manuscript.

Potential Conflicts of Interest

Nothing to report.

Data Availability

Data reported in this article are available from the corresponding author upon reasonable request.

References

1. Allen RP, Walters AS, Montplaisir J, et al. Restless legs syndrome prevalence and impact: REST general population study. Arch Intern Med 2005;165:1286–1292.
2. Garcia-Borreguero D. Dopaminergic augmentation in restless legs syndrome/Willis-Ekbom disease: identification and management. Sleep Med Clin 2015;10:287–292. xiii.
3. Earley CJ, Kuwabara H, Wong DF, et al. Increased synaptic dopamine in the putamen in restless legs syndrome. Sleep 2013;36: 51–57.
4. Connor JR, Wang XS, Allen RP, et al. Altered dopaminergic profile in the putamen and substantia nigra in restless leg syndrome. Brain 2009;132:2403–2412.
5. Torre E, Celis ME. Alpha-MSH injected into the substantia nigra or intraventricularly alters behavior and the striatal dopaminergic activity. Neurochem Int 1986;9:85–89.
6. Sandman CA, Kastin AJ. Intraventricular administration of MSH induces hyperalgesia in rats. Peptides 1981;2:231–233.
7. Jacquet YF. Opiate effects after adrenocorticotropin or beta-endorphin injection in the periaqueductal gray matter of rats. Science 1978;201:1032–1034.
8. Bertolini A, Poggioli R, Ferrari W. ACTH-induced hyperalgesia in rats. Experientia 1979;35:1216–1217.
9. 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: 1234–1242.
10. Allen RP, Picchietti DL, Garcia-Borreguero D, 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;15:860–873.
11. Allen RP, Burchell BJ, MacDonald B, et al. Validation of the self-completed Cambridge-Hopkins questionnaire (CH-RLSq) for ascertainment of restless legs syndrome (RLS) in a population survey. Sleep Med 2009;10:1097–1100.
12. Garcia-Borreguero D, Kohnen R, Boothby L, et al. Validation of the multiple suggested immobilization test: a test for the assessment of severity of restless legs syndrome (Willis-Ekbom disease). Sleep 2013;36:1101–1109.
13. Melia M, Schmidt M, Geissler B, et al. Measuring mechanical pain: the refinement and standardization of pressure pain threshold measurements. Behav Res Methods 2015;47:216–227.
14. Page-Wilson G, Meece K, White A, et al. Proopiomelanocortin, agouti-related protein, and leptin in human cerebrospinal fluid: correlations with body weight and adiposity. Am J Physiol Endocrinol Metab 2015;309:E458–E465.
15. Walters AS, LeBrocq C, Dhar A, et al. Validation of the international restless legs syndrome study group rating scale for restless legs syndrome. Sleep Med 2003;4:121–132.
16. Nam SY, Kratzsch J, Kim KW, et al. Cerebrospinal fluid and plasma concentrations of leptin, NPY, and alpha-MSH in obese women and their relationship to negative energy balance. J Clin Endocrinol Metab 2001;86:4849–4853.
17. Krieger DT. Brain peptides: what, where, and why? Science 1983; 222:975–985.
18. Ferrari WFE, Paulesu F. Su di una particolare, imponente sintomalogia prodotta nel cane dall'ACTH iniettato nella cisterna magna. Bolletin della Società Italiana Biologica Sperimentale 1955; 31:862.
19. Barbeau A, Gonce M, Kastin AJ. Neurologically active peptides. Pharmacol Biochem Behav 1976;5:159–163.
20. Gispen WH, Wiegant VM, Greven HM, de Wied D. The induction of excessive grooming in the rat by intraventricular application of peptides derived from ACTH: structure-activity studies. Life Sci 1975;17: 645–652.
21. Vécsei L, Bollók I, Telegdy G, Schally AV. Comparative studies with beta-(Tyr9)melanotropin-(9-18) and amphetamine on open-field behavior and striatal dopamine uptake and release. Pharmacol Res Commun 1984;16:689–697.
22. Concu AFW, Gessa GL, Mereu GP, Tagliamonte A. In: Levin PKW, ed. *EEG changes induced by the intraventricular injection of ACTH in cats*. Basel, Switzerland: Karger Publishers, 1974.
23. Montplaisir J, Boucher S, Poirier G, et al. Clinical, polysomnographic, and genetic characteristics of restless legs syndrome: a study of 133 patients diagnosed with new standard criteria. Mov Disord 1997;12:61–65.
24. Cho YW, Song ML, Earley CJ, Allen RP. Prevalence and clinical characteristics of patients with restless legs syndrome with painful symptoms. Sleep Med 2015;16:775–778.

25. Stiasny-Kolster K, Pfau DB, Oertel WH, et al. Hyperalgesia and functional sensory loss in restless legs syndrome. Pain 2013;154:1457–1463.
26. Fratta W, Rossetti ZL, Poggioli R, Gessa GL. Reciprocal antagonism between ACTH1-24 and beta-endorphin in rats. Neurosci Lett 1981; 24:71–74.
27. Firemark HM, Weitzman RE. Effects of beta-endorphin, morphine and naloxone on arginine vasopressin secretion and the electroencephalogram. Neuroscience 1979;4:1895–1902.
28. Reinoso-Barbero F, de Andrés I. Effects of opioid microinjections in the nucleus of the solitary tract on the sleep-wakefulness cycle states in cats. Anesthesiology 1995;82:144–152.
29. Kastin AJ, Jemison MT, Coy DH. Analgesia after peripheral administration of enkephalin and endorphin analogues. Pharmacol Biochem Behav 1979;11:713–716.
30. Williams DW Jr, Lipton JM, Giesecke AH Jr. Influence of centrally administered peptides on ear withdrawal from heat in the rabbit. Peptides 1986;7:1095–1100.
31. Harno E, Gali Ramamoorthy T, Coll AP, White A. POMC: the physiological power of hormone processing. Physiol Rev 2018;98:2381–2430.
32. Schnabel E, Mains RE, Farquhar MG. Proteolytic processing of pro-ACTH/endorphin begins in the Golgi complex of pituitary corticotropes and AtT-20 cells. Mol Endocrinol. 1989;3:1223–1235.
33. Tanaka S, Yora T, Nakayama K, et al. Proteolytic processing of pro-opiomelanocortin occurs in acidifying secretory granules of AtT-20 cells. J Histochem Cytochem 1997;45:425–436.
34. Mountjoy KG. Distribution and function of melanocortin receptors within the brain. Adv Exp Med Biol 2010;681:29–48.
35. Chu H, Xia J, Yang Z, Gao J. Melanocortin 4 receptor induces hyperalgesia and allodynia after chronic constriction injury by activation of p38 MAPK in DRG. Int J Neurosci 2012;122:74–81.
36. Starowicz K, Bilecki W, Sieja A, et al. Melanocortin 4 receptor is expressed in the dorsal root ganglions and down-regulated in neuropathic rats. Neurosci Lett 2004;358:79–82.
37. Delaney A, Keighren M, Fleetwood-Walker SM, Jackson IJ. Involvement of the melanocortin-1 receptor in acute pain and pain of inflammatory but not neuropathic origin. PloS One 2010;5:e12498.
38. Akpinar S. Treatment of restless legs syndrome with levodopa plus benserazide. Arch Neurol 1982;39:739.
39. Fischer JL, Moriarty CM. Control of bioactive corticotropin release from the neuro-intermediate lobe of the rat pituitary in vitro. Endocrinology 1977;100:1047–1054.
40. Jenks BG, Verburg van Kemenade BM, Tonon MC, Vaudry H. Regulation of biosynthesis and release of pars intermedia peptides in Rana ridibunda: dopamine affects both acetylation and release of alpha-MSH. Peptides 1985;6:913–921.
41. Winkelman JW, Purks J, Wipper B. Baseline and 1-year longitudinal data from the National Restless Legs Syndrome Opioid Registry. Sleep 2021;44:zsaa183.
42. Lord JA, Waterfield AA, Hughes J, Kosterlitz HW. Endogenous opioid peptides: multiple agonists and receptors. Nature 1977;267: 495–499.
43. Wüster M, Schulz R, Herz A. Specificity of opioids towards the mu-, delta- and epsilon-opiate receptors. Neurosci Lett 1979;15:193–198.
44. Kastin AJ, Kullander S, Borglin NE, et al. Extrapigmentary effects of melanocyte-stimulating hormone in amenorrhoeic women. Lancet 1968;1:1007–1010.