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Orexin-A measurement in narcolepsy: A stability study and a comparison of LC-MS/MS and immunoassays

Mikael Lindström [a,*], Mink Schinkelshoek [b,c], Pentti J. Tienari [d,e], Jyrki P. Kukkonen [f], Risto Renkonen [a,g], Rolf Fronczek [b,c], Gert Jan Lammers [b,c], Outi Itkonen [a]

[a] HUS Diagnostic Center, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
[b] Department of Neurology, Leiden University Medical Center, Leiden, The Netherlands
[c] Sleep-Wake Centre, Stichting Epilepsie Instellingen Nederland (SEIN), Heemstede, The Netherlands
[d] HUS Neurocenter, Helsinki University Hospital, Helsinki, Finland
[e] Translational Immunology Research Program, Faculty of Medicine, University of Helsinki, Finland
[f] Department of Pharmacology, Institute of Biomedicine, University of Helsinki, Helsinki, Finland
[g] Transplantation Laboratory, Haartman Institute, University of Helsinki, Helsinki, Finland

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ABSTRACT

Background: Orexin-A and -B are neuropeptides involved in sleep–wake regulation. In human narcolepsy type 1, this cycle is disrupted due to loss of orexin-producing neurons in the hypothalamus. Cerebrospinal fluid (CSF) orexin-A measurement is used in the diagnosis of narcolepsy type 1. Currently available immunoassays may lack specificity for accurate orexin quantification. We developed and validated a liquid chromatography mass spectrometry assay (LC-MS/MS) for CSF orexin-A and B.

Methods: We used CSF samples from narcolepsy type 1 (n = 22) and type 2 (n = 6) and non-narcoleptic controls (n = 44). Stable isotope-labeled orexin-A and -B internal standards were added to samples before solid-phase extraction and quantification by LC-MS/MS. The samples were also assayed by commercial radioimmunoassay (RIA, n = 42) and enzymatic immunoassay (EIA, n = 72) kits. Stability of orexins in CSF was studied for 12 months.

Results: Our assay has a good sensitivity (10 pmol/L = 35 pg/mL) and a wide linear range (35–3500 pg/mL). Added orexin-A and -B were stable in CSF for 12 and 3 months, respectively, when frozen. The median orexin-A concentration in CSF from narcolepsy type 1 patients was <35 pg/mL (range < 35–131 pg/mL), which was lower than that in CSF from control individuals (98 pg/mL, range < 35–424 pg/mL). Orexin-A concentrations determined using our LC-MS/MS assay were five times lower than those measured with a commercial RIA. Orexin-B concentrations were undetectable.

Conclusions: Orexin-A concentrations measured by our LC-MS/MS assay were lower in narcolepsy type 1 patients as compared to controls. RIA yielded on average higher concentrations than LC-MS/MS.

1. Introduction

Orexins are essential brain peptide hormones regulating the sleep-wake cycle and appetite of many mammals including humans [1]. Orexin-A and orexin-B (also known as hypocretin-1 and -2, respectively) are derived from a single prepro-orexin polypeptide by enzymatic cleavage and post-translational modifications [1]. Biologically active orexins-A and -B are 33- and 28-amino-acid peptides, respectively. They bind to G-protein-coupled receptors called OX_1 and OX_2 receptors [2] and stimulate neuronal activity and plasticity by various mechanisms [3]. Impaired orexin function via loss of orexinergic neurons can lead to a clinical disorder of sleep such as narcolepsy [4–6]. Very low orexin concentrations are found in the cerebrospinal fluid (CSF) of patients with type 1 narcolepsy (NT1), previously classified as narcolepsy with cataplexy, while type 2 (NT2) patients without cataplexy have normal orexin-A concentrations in the CSF [7].

It has been estimated that narcolepsy affects approximately 0.02–0.05% of the population worldwide [8]. The diagnosis of

Abbreviations: LC-MS/MS, liquid chromatography tandem mass spectrometry; SPE, solid-phase extraction; CSF, cerebrospinal fluid.

* Corresponding author.
E-mail address: mikael.lindstrom@helsinki.fi (M. Lindström).

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narcolepsy relies on clinical symptoms and ruling out other causes of hypersomnolence. The multiple sleep latency test (MSLT) is a standard tool for diagnosing hypersomnolence, but for the time being, the only established biomarker for narcolepsy type 1 diagnosis is the lowered orexin-A level in the CSF. So far, orexin measurement has relied on immunological methods such as radioimmunoassay (RIA) [6]. These methods have claimed enough sensitivity and specificity towards CSF orexin-A, but fully clinically validated antibodies or certified reference materials for calibration are not available. The single published critical assessment of the antibody assays demonstrates that they do not produce quantitative results in CSF and probably no significant results at all in blood [9].

At the time of this study, only one RIA kit for orexin-A was commercially available from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA). Two LC-MS/MS assays have been published [10,11] reporting orexin-A concentrations of significantly different magnitudes in a limited number of clinical samples. Here we present an analytically and clinically validated liquid chromatography tandem mass spectrometry (LC-MS/MS) assay for CSF orexins. We also report orexin concentrations by our newly developed LC-MS/MS assay and by RIA in 42 samples from narcolepsy patients treated at the Helsinki University Hospital, Finland, and the Leiden University Medical Center, the Netherlands.

2. Materials and methods

2.1. Reagents and materials

Orexin stock solutions of 8.9 mg/L and 7.3 mg/L (both 2.5 μmol/L) of orexin-A and -B, respectively, Sigma-Aldrich) and corresponding stable isotope-labeled internal standards (IS; $^{13}C_6$,^{15}N-labeled orexin-A and orexin-B, Innovagen AB, Lund, Sweden) were prepared in bovine albumin solution (10 mg/L). Orexin calibrators of 35–3500 pg/mL (10–1000 pmol/L) were freshly prepared in 1:150 diluted double charcoal-stripped serum (Golden West Diagnostics, Temecula, CA, USA) and IS working solution of 10 nmol/L (35 ng/mL) in 0.1% formic acid (FA) from the stock solutions. Calibrators and QA samples were prepared on separate days and from separate stock solutions. Sample pretreatment was performed employing 96-well Oasis HLB plates (Waters, Milford, MA, USA). MS-grade methanol (MeOH), acetonitrile (ACN) and ammonium hydroxide were from Riedel-de-Haën (Seelze, Germany), and formic acid from Fischer Chemicals (Fair Lawn, NJ, USA). All reagents were of the highest analytical grade.

2.2. Samples and patients

CSF samples from five patients suspected for narcolepsy at Helsinki University Hospital were drawn into sterile cell culture tubes (Greiner Bio-One, product 163160) for orexin-A test as part of their diagnostic workup during 2016–2017. Of these, one was confirmed as NT1, two as NT2 according to the criteria formulated in the third version of the International Classification of Sleep Disorders [12], and two were considered not to have narcolepsy. CSF samples from 37 patients with a suspicion of narcolepsy at the Leiden University Medical Center were drawn for diagnostic purposes between 2008 and 2013. NT1 was diagnosed in 21 patients and NT2 in four patients according to the criteria formulated in the second version of the International Classification of Sleep Disorders [13]. As controls we used CSF samples from patients with hypersomnolence in which narcolepsy was excluded in the Netherlands (n = 12) and from Finland (n = 2) as well as left-over samples stripped of identifiers from patients suspected for neurological disorders other than narcolepsy (n = 30) in Finland. The latter ones were only assayed by LC-MS/MS and EIA. This study was approved by the ethical committee of Helsinki University Central Hospital, Finland and Leiden University Medical Center, The Netherlands. All CSF samples were kept at −80 °C until analyzed. Table 1 describes patient and control characteristics and shows measured orexin concentrations. Detailed patient information is presented in Supplementary Table 1. For quality assurance (QA) and validation, we employed control CSF spiked with 20, 60 and 120 pmol/L orexin-A and -B.

Table 1
Characteristics of the patients and non-narcoleptic controls and measured CSF orexin concentrations. HLA-DQB1*06:02 + refers to the human leukocyte antigen haplotype, which is strongly associated with narcolepsy type 1 [26]. Conversion factor for pmol/L: 1/3.561.

	Controls	NT1 patients	NT2 patients
n	44(MS, EIA), 14 (RIA)	22	6
Male (n)	20	12	4
Age (yrs, median, range)	37.5, 8–83	32.3, 4–72	38.2, 21–71
Age at onset (yrs, median, range)	NA	18.0, 4–36	25.5, 12–60
Mean sleep latency on MSLT (min, median, range)	NA	3.9, 0.5–9.5	4.6, 1.5–8.5
HLA-DQB1*06:02+ (%)	NA	22/22 (100%)	5/6 (83%)
Orexin-A (pg/mL) by LC-MS/MS			
Range	<35–423	<35–131	43–125
Median	98	<35	73
IQR	57–146	<35–63	52–100
Orexin-A (pg/mL) by RIA			
Range	238–536	<66–125	296–743
Median	351	<66[a]	349
IQR	326–428	<66	325–743
Orexin-A (pg/mL) by EIA			
Range	<45–180	<45	<45–52
Median	<45[a]	<45[a]	<45 [a]
IQR	<45–56	<45	<45–52

[a] Median value was below the quantitation limit.

2.3. Sample and calibrator preparation for LC-MS/MS analysis

Calibrators, QA samples and CSF samples (500 μL) were mixed with 50 μL of IS working solution and transferred into the wells of an HLB μElution plate pre-wetted with 500 μL of MeOH and 500 μL water. The wells were washed with 500 μL of MeOH/water/ammonium hydroxide (30/65/5, vol/vol). The orexins were then eluted with 250 μL of MeOH/water/FA (80/17/3, vol/vol), dried under a flow of nitrogen, and resuspended into HPLC eluent (ACN/water/FA, 15/84.9/0.1, vol/vol).

2.4. LC-MS/Ms

Our instrument setup comprised an Agilent 1200 liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) and a TQ 5500 triple quadrupole mass spectrometer (AB Sciex, Toronto, Canada) equipped with a Turbo-V electrospray ion (ESI) source. Chromatographic separation was achieved using Polaris C18-A column (100 × 2.00 mm, 3.0 μm, Agilent) operated at 40 °C. Mobile phases were 0.1% FA in ACN (A) and in water (B). For gradient, A was kept at 15% for 1 min, then ramped to 99% in 3 min, kept at 99% for 2 min, then ramped back to 15% in 1 min and kept at 15% for 3 min. The flow rate was 300 μL/min. For MS/MS detection we followed the transitions m/z 891.1 → 854.1 and m/z 892.9 → 855.9 for orexin-A and IS, and m/z 725.9 → 684.4 and m/z 727.3 → 608.7 for quadruple charged $[M + 4H]^{4+}$ orexin-B and IS ions, respectively. Secondary, qualitative transitions for orexin-A (m/z 713.2 → 776.9) and -B (m/z 580.9 → 696.7) were also followed to monitor the ion ratios. MS instrument settings for curtain, nebulizer and heater gases were 20, 35 and 60 l/min, and collision gas setting at 8. Data were collected by the Analyst software (v. 1.6.2, AB Sciex).

2.5. Analytical validation of the LC-MS/MS method

The linear range of the assay was calculated by preparing and

analyzing 15 calibrator dilutions of 3.5–3500 pg/mL (1–1000 pmol/L) for orexin-A and of 2.9–2900 pg/mL for orexin-B on three different days. The calibration curves were derived using $1/x^2$-weighted linear least-squares regression. Relative error (RE) and the coefficient of variation (CV) were calculated. The limit of detection (LOD) was determined as the lowest concentration with a signal to noise ratio of 3. Limit of quantification (LOQ) and linear range were determined as the lowest concentration and the range, respectively, that could be measured with RE and CV < 15% (20% for the lowest concentration). Intra- and inter-assay variation were calculated from the QA sample results in a single analysis (n = 12) and on 14 separate days, respectively. Recovery of added orexin was determined using three CSF samples (endogenous orexin-A concentration 53–114 pg/mL (15–32 pmol/L), orexin-B concentration < LOD) with and without orexin-A spike of 71, 213 and 426 pg/mL (20, 60 and 120 pmol/L). Corresponding orexin-B spikes were 58, 174 and 348 pg/mL. Matrix effect was studied by extracting three CSF samples in triplicate, spiking the extracted samples with 106 and 351 pg/mL orexin-A, and with 87 and 287 pg/mL orexin-B (corresponding to 30 and 100 pmol/L, respectively) and comparing with spiked neat elution buffer. Effect on the measured signal was calculated from the peak areas. Carry-over for both orexins was determined by injecting an analyte-free blank sample after the highest calibrator and after CSF spiked at 3500 and 2900 pg/mL for orexin-A and -B, respectively.

2.6. Preanalytical validation

We studied orexin stability using CSF from control patients (n = 15) spiked with 712 pg/mL and 598 pg/mL of orexin-A and -B (both 200 pmol/L), respectively. Samples were stored at varying temperatures (room temperature, +4 °C, –20 °C and –80 °C) in parallel. Sample aliquots were analyzed on days 0–7 (daily), 14, 28 and the frozen samples additionally at 58, 94, 115, 143, 170 and 365. The effect of repeated freezing and thawing of the same aliquot was studied by analysis at 1-week intervals. Samples with < 20% change from initial concentration were considered stable. In addition, long term stability was estimated by comparing orexin-A concentration in control samples collected between 2008 and 2013 in Leiden (n = 12) and those collected between 2016 and 2017 in Helsinki (n = 32).

2.7. Orexin immunoassays

CSF orexin-A immunoassays were performed in duplicate by RIA (RK-003–30) and EIA (EKE-003–30) kits from Phoenix in Leiden and Helsinki, respectively. Samples for the EIA were concentrated four-fold by extraction according to the manufacturer's instructions. The sensitivity of the RIA is 66 pg/mL and inter- and intra-assay variation < 15%, as stated by the manufacturer (www.phoenixpeptide.com). At the Leiden University Medical Center, the observed inter-assay variation was 19% (n = 12). Therefore, reference CSF samples from Stanford Center for Sleep Sciences and Medicine (S. Nishino) were included in the assay in Leiden to adjust for inter-assay variability to previously reported values [14].

2.8. Statistical methods

We used Analyse-it for Microsoft Excel 2016 (v. 4, Analyse-it software Ltd, http://www.analyse-it.com) to run Passing–Bablok regression, Mann-Whitney and Wilcoxon rank sum tests, Bland–Altman difference analysis and receiver operating characteristic (ROC) analysis with corresponding 95% confidence intervals (CI) and interquartile ranges (IQR). A p-value < 0.05 was considered statistically significant. For statistical analysis, values of 33 pg/mL (RIA) and 18 pg/mL (LC-MS/MS) corresponding to LOQ/2 were used for patient sample results < LOQ.

3. Results

3.1. Analytical validation of the orexin assay

The retention times of orexin-A and -B were 5.8 and 5.7 min, respectively (Fig. 1). The method was linear over the concentration range 35–3500 pg/mL and 58–2900 pg/mL, the LOD was 18 and 29 pg/mL and LOQ 35 and 58 pg/mL for orexin-A and -B, respectively (Supplementary Fig. 1). Routinely, we used 35–3561 pg/mL (orexin-A) and 58–2980 (orexin-B) calibrators. The essential validation parameters are listed in Table 2. The sample matrix caused a moderate 32% signal attenuation for orexin-A and slight 7% enhancement for orexin-B, but in both cases the matrix effect was fully corrected by the added IS. We found a negligible 0.1% carry-over (peak area) for both orexins.

3.2. Preanalytical validation

Orexin-A (712 pg/mL or 200 pmol/L) spiked into CSF was stable for 12 months at –20 °C and –80 °C, for 14 days refrigerated (+4 °C) and three days at room temperature. Orexin-B (598 pg/mL or 200 pmol/L) was stable for three months frozen, seven days refrigerated and one day at room temperature (Supplementary Fig. 2). Orexin-A was stable for three freezing and thawing cycles, while orexin-B for only one cycle. No difference (p = 0.227) was found between control samples stored at −80 °C for 6–11 years (n = 12, median 84 pg/mL, range < 66–181 pg/mL) and those stored for 2–3 years (n = 32, median 103 pg/mL, range < 35–423 pg/mL).

3.3. Orexin in CSF samples

The median orexin-A concentration in CSF samples from control individuals was 98 pg/mL (n = 44, range < 35–423 pg/mL) by LC-MS/MS, and 351 pg/mL (n = 14, range 238–536 pg/mL) by RIA (Table 1). Two results were < 35 pg/mL by the LC-MS/MS assay. In the CSF samples from 22 NT1 patients, the median orexin-A concentration by LC-MS/MS was < 35 pg/mL (range < 35–131 pg/mL). Twelve results

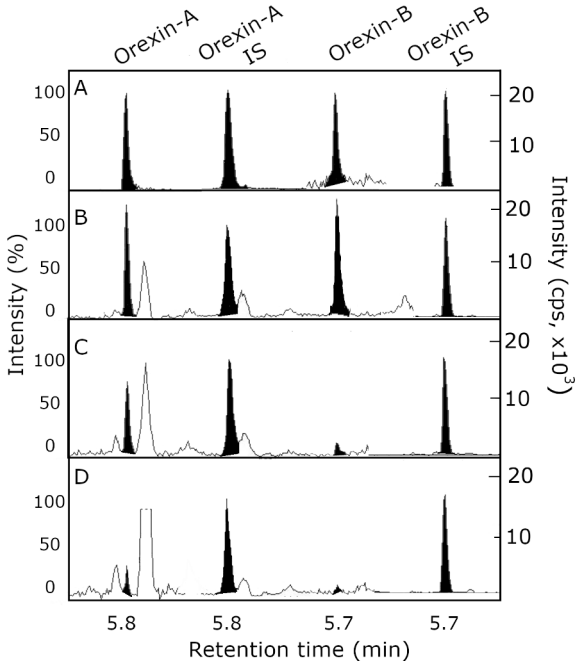

Fig. 1. Chromatograms of (A) orexin calibrators with 356 pg/mL of orexin-A and 290 pg/mL orexin-B (both 100 pmol/L), (B) QA sample containing 427 pg/mL orexinA and -B, (C) a control CSF sample with 267 pg/mL (75 pmol/L) orexin-A and (D) a patient sample with 89 pg/mL (25 pmol/L) of orexin-A.

Table 2
Analytical validation parameters of the orexin LC-MS/MS assay. Conversion factors for pmol/L: orexin-A 1/3.561, orexin-B 1/2.899.

Parameter	Orexin-A	Orexin-B
Linear range (pg/ml)	35–3500	58–2900
LOD (pg/ml)	18	27
LOQ (pg/ml)	35	58
Recovery of added orexin A (71/213/426 pg/ml) B (58/174/348 pg/ml)	99/92/97%	105/92/97%
Matrix effect	–32% (CV 6.8%)	+7% (CV 5.3%)
Intra-assay CV of orexin A (106/351 pg/mL) and B (87/287 pg/ml)	12.6/9.7%	13.1/11.9%
Inter-assay CV of orexin A (106/351 pg/mL) and B (87/287 pg/ml)	17.2/11.5%	21.4/20.4%

(54%) were < 35 pg/mL and in eleven samples orexin-A was non-detectable (<18 pg/mL). By the RIA, only three results (13%) were > LOQ of 66 pg/mL, *i.e.* 72, 110 and 125 pg/mL. We had CSF samples from six NT2 patients. The median orexin-A concentration in these was 73 pg/mL (range 43–125 pg/mL), and 351 pg/mL (range 296–743 pg/mL) by LC-MS/MS, and RIA, respectively. The difference between the assays and between patient and control samples was statistically significant ($p < 0.001$, Fig. 2). By EIA, the median concentration in the control samples was < 45 pg/mL (n = 44, range < 45–180 pg/mL), 23 of 44 (52%) being below the assay LOQ. In patient samples, all NT1 samples and 66% of NT2 samples had an orexin-A concentration too low to quantitate (Table 1). Passing–Bablok regression revealed Y (RIA) = 5.1 (95%CI 3.4–9.8)X (LC-MS/MS) –58 (95%CI –28 – –284) (n = 42, Fig. 3). Bland-Altman difference plot showed a 72.7% (95% CI −68.77 – 214.0%) mean difference between the assays. Spearman's coefficient of rank correlation 0.58 indicates a moderate association between the LC-MS/MS and RIA assays.

When comparing the diagnostic performance between the assays in differentiating NT1 vs. other patients, ROC analysis produced areas under the curve (AUCs) of 0.86 (CI 0.77–0. 95) for LC-MS/MS and 0.97 (CI 0.90–1.0) for RIA, respectively. The AUC for EIA was 0.66 (CI 0.60–0.73). However, the difference between LC-MS/MS and RIA AUCs was not statistically significant (p = 0.30), while EIA AUC was different against both (p = 0.01). Optimal cut-off values for the LC-MS/MS and RIA assays were 48 pg/mL and 238 pg/mL, the sensitivities at these values were 70%, and 75%, while specificities were 86% and 93%, respectively. Values could not be reliably calculated for EIA.

Orexin-B concentration was below the LOQ of our LC-MS/MS assay in all measured CSF samples. The EIA and RIA kits employed in this study were not designed to measure orexin-B.

4. Discussion

We have developed and validated a new LC-MS/MS assay for CSF orexin-A and -B. The assay achieved 35 pg/mL sensitivity (orexin-A) and is linear over the concentration range of 35–3500 pg/mL. Due to the very low expected orexin concentrations, calibrators of 35–1750 pg/mL were used for clinical validation. The reproducibility for orexin-A is good, with both intra- and inter-assay CVs below 20% (Table 2). The assay is robust with long term (12 mo) variation < 15%. Although quantitatively fully compensated by the use of IS, the observed 32% raw signal attenuation for orexin-A, that could be due to the *e.g.* peptide adhering to plastic surfaces. Earlier MS assays [10,11] report similar CVs with a lower LOQ of 3.6–7.2 pg/mL, but overall, our assay has a good performance and compares well with them. We compared our LC-MS/MS assay with the widely used RIA and EIA kits for orexin-A by Phoenix. The lowest calibrator in these assays is 10 pg/mL, and according to the kit insert, the linear ranges are 10–1280 pg/mL and 0–100 pg/mL, respectively. However, on the homepage (www.phoenixpeptide.com), the reported LOQs are 66 pg/mL and 180 pg/mL, respectively. This detail is easy to miss. Because of the poor sensitivity of the EIA towards crude CSF, we used an additional sample extraction step as suggested by the manufacturer, resulting in four-fold concentration of the samples. It is not clear in published reports [10,11,15–19], how the LOQ of the immunoassays employed has been taken into consideration.

The median CSF orexin-A concentration by our LC-MS/MS assay was 98 pg/mL in samples from control individuals (n = 44), <35 pg/mL in samples from NT1 patients (n = 22) and 73 pg/mL in samples from NT2 patients (n = 6). In 52% of the NT1 patient samples, the orexin-A results were <LOQ. Somewhat surprisingly orexin-A measured by LC-MS/MS was <35 pg/mL in two control individual samples. These samples were anonymized left-overs from patients suspected for neurological disorders other than narcolepsy. The preanalytical process of these control samples was somewhat different than that of the orexin-A samples, as they were first directed to oligoclonal IgG assay at HUS Diagnostic Center before they were frozen at −80 °C. Orexin-A in these two samples may have been degraded. On the other hand, no reference limits for orexin-A have been established, so these results may in fact be very low. NT1 in these patients cannot be excluded, though it is unlikely.

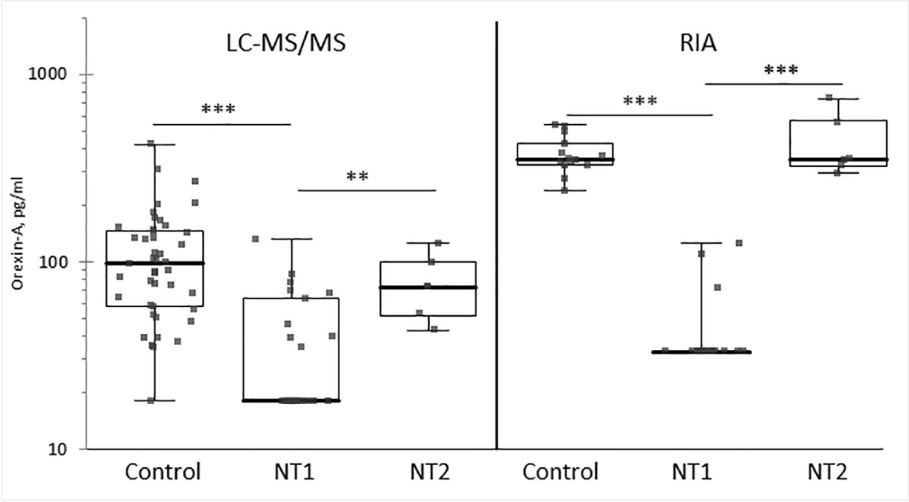

Fig. 2. Comparison of orexin-A concentrations in control and patient CSF samples quantified by LC-MS/MS and RIA, presented as median (line) and interquartile ranges (box) and minimum/maximum ranges (whiskers). Concentrations < LOQ were given a value of LOQ/2. P-values are denoted as ** and *** correspond to < 0.01 and < 0.001, respectively.

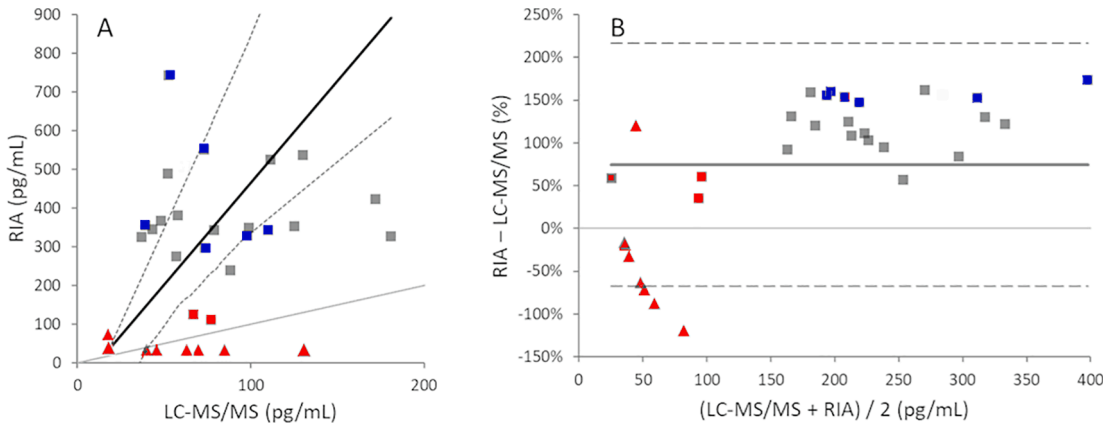

Fig. 3. Passing–Bablok regression analysis (A) and Bland–Altman difference plot (B) of the orexin-A concentrations in patients suspected for narcolepsy (■, n = 42) by LC-MS/MS and RIA. Confirmed cases marked in red for NT1 (n = 22) and blue for NT2 (n = 6). In A, grey line represents identity, black the slope and dashed lines the CI. In B, thick line represents the mean difference of the assays and the dashed lines the CI. Concentrations < LOQ (▲) were given a value of LOQ/2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Unfortunately, orexin-A result in these two samples by RIA was not available for comparison.

Two other LC-MS/MS assays for CSF orexin-A have been published [10,11]. Our findings are in line with those reported by Hirtz *et al.* [10], with median orexin-A of 105 pg/mL (IQR 80–144 pg/mL) in samples from healthy controls (n = 22), and 4 pg/mL (IQR 3–12 pg/mL) in NT1 patients (n = 22). However, Bårdsen *et al.* [11] reported markedly lower median concentrations of 11 pg/mL (IQR 8–14 pg/mL) in control CSF (n = 22) and 2 pg/mL (IQR 1–3 pg/mL) in NT1 patient (n = 9) CSF. Both studies report orexin-A concentrations below the respective assay LOQ of 3.6 pg/mL and 7.2 pg/ml. Nevertheless, they both distinguish between healthy controls and NT1 patients.

No reference standard or a commutable calibrator for CSF orexin-A exists and the measured differences are most likely calibration-dependent. We used orexin-A peptide from Sigma-Aldrich in 150-fold diluted double charcoal-stripped serum. Hirtz *et al.* [10] and Bårdsen *et al.* [11] used orexin-A from Phoenix Scientific diluted in water/acetonitrile/formic acid or 1% newborn calf serum, respectively. Bårdsen *et al.* [11] also used orexin-A from Peptide Institute (Osaka, Japan) and Hirtz [10] used the standard Stanford reference sample. Different sample preparation procedures, chromatographic conditions and column chemistry may affect the assessed concentrations. Preparation of in-house calibrators is critical, especially when external quality assurance scheme, reference measurement procedure and reference standard are missing. Median concentrations in patient samples may also be biased due to the small number of samples in these studies. Our study included 72 clinical samples as compared to 44 and 31 in those by Hirtz *et al.* [10] and Bårdsen *et al.* [11], respectively. However, this cannot explain the discrepancy between the concentrations measured by Bårdsen *et al.* [11] as compared to those by us or by Hirtz *et al.* [10].

We found marked differences in orexin-A concentrations measured by our LC-MS/MS and by commercial RIA and EIA. Orexin-A concentrations in CSF by LC-MS/MS were on average 3.3-fold and 4.8-fold lower than those obtained by the commercial RIA in CSF from control individuals and NT2 patients, respectively. This confirms previous findings [10,11]. On contrary to RIA, orexin-A concentrations in samples from NT1 patients and controls by LC-MS/MS overlapped. The antibody employed by the RIA may detect fragments of the orexin-A peptide [20], immature or alternatively post-translationally modified orexin-A, or prepro-orexin, which could explain the higher concentrations obtained. The currently implemented LC-MS/MS assays only target the intact, mature orexin-A. The reported poor precision of RIA at very low concentrations [6,21] may also contribute to the difference between the LC-MS/MS and RIA results. The majority of the NT1 patient sample results were below the LOQ of each assay. We observed that orexin-A by one commercial EIA resulted in very low measured concentrations. Even though the RIA and EIA kits employed are from the same manufacturer, there was on average a 10-fold difference between the measured concentrations. Few clinical studies have employed the EIA kit, but compared to our results, Liguori *et al.* [15] reported median orexin-A concentrations two- to three-fold higher in 16 patient and 16 control samples using the Phoenix EIA kit. Also in this study, the majority of the reported values were below the kit LOQ. Ono *et al.* have reported that orexin-A in CSF samples from 80 patients with various hypersomnolence conditions were four-fold lower when measured by an EIA assay from Wako (Wako Pure Chemical Industries, Osaka, Japan) as compared to the Phoenix RIA [22]. Harmonization of orexin-A assays is thus warranted, as also previously noted for the RIA assay [22].

ROC analysis revealed AUCs of 0.97 and 0.86 for the RIA and LC-MS/MS assay, respectively. It is important to realize, that the pre-confirmed narcolepsy diagnosis for the NT1 and NT2 patients was partly based on their orexin-A RIA result. Therefore, one cannot judge diagnostic accuracy of the assays based on ROC analysis. Instead, it can be concluded that there is no marked difference in the performance between the RIA and our LC-MS/MS assay. AUC of the EIA assay was 0.66.

We found that orexin-B spiked into CSF was less stable than orexin-A under all storage conditions. Orexin-A can be stored frozen for a year, refrigerated for two weeks and at room temperature for three days. Thus, samples for orexin-A assay can be sent at room temperature to the laboratory. However, we suggest immediate freezing whenever possible. Long-term storage requires freezing, and repeated analysis is possible for up to three freeze–thaw cycles. Unfortunately, the concentrations spiked into our stability samples were high as compared to those found in patient samples. Therefore, these results need to be confirmed. Recently Keating *et al.* [23] found a weak negative correlation between time in freezer (19–1821 days) and measured orexin-A concentrations only for samples with orexin-A < 110 pg/mL. We found no difference in orexin-A concentrations in control samples stored at −80 °C for 2–3 and for 6–11 years. The two intramolecular disulphide bridges of orexin-A are likely to stabilize the peptide and render it less prone to *e.g.* proteolytic enzymes than orexin-B. Orexin-B, when spiked into CSF, suffers from instability during storage. It was only stable when frozen, and even then, for three months only. However, no orexin-B was detected in fresh control CSF samples, suggesting concentrations below the assay LOQ or rapid decomposition as found in spiked CSF. Quantitative studies assessing orexin-B [17,24,25] have reported values below the RIA LOQ of 50 pg/mL (www.phoenixpeptide.com).

In conclusion, we have validated a new LC-MS/MS assay for orexins -A and -B in CSF. We analyzed a relatively large number of clinical samples and our results confirm previous findings that orexin-A

concentration is decreased in CSF of NT1 patients. We found a marked variation between various commercial and previously published assays for orexin-A, and assay harmonization is thus warranted. Orexin-A in CSF is stable for three days at room temperature and for prolonged storage at –20 °C. Orexin-B was not detected in the CSF samples, and when spiked, showed poor stability. Taken together, we describe an analytically, preanalytically and clinically validated orexin-A assay that can be used for laboratory diagnosis of narcolepsy.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2021.01.009.

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