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Hypocretin measurement: shelf age of radioimmunoassay kit, but not freezer time, influences assay variability

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Abstract

Background—The hypothalamic peptide hypocretin 1 (orexin A) may be assayed in cerebrospinal fluid to diagnose narcolepsy type 1. This testing is not commercially available, and factors contributing to assay variability have not previously been comprehensively explored.

Methods—In the present study, cerebrospinal fluid hypocretin concentrations were determined in duplicate in 155 patient samples, across a range of sleep disorders. Intra-assay variability of these measures was analyzed.

Results—Inter-assay correlation between samples tested at Emory and at Stanford was high ($r = 0.79$, $p < 0.0001$). Intra-assay correlation between samples tested in duplicate in our center was also high ($r = 0.88$, $p < 0.0001$); intra-assay variability, expressed as the difference between values as a percentage of the higher value, was low at 9.4% (SD = 7.9%). Although both time the sample spent in the freezer ($r = 0.16$, $p = 0.04$) and age of the kit used for assay ($t = 3.64$, $p = 0.0004$) were significant predictors of intra-kit variability in univariate analyses, only age of kit was significant in multivariate linear regression ($F = 4.93$, $p = 0.03$).

Conclusion—Age of radioimmunoassay kit affects intra-kit variability of measured hypocretin values, such that kits closer to expiration exhibit significantly more variability.

Keywords

cerebrospinal fluid; narcolepsy; immunoassay; sleep wake disorders; assay

Introduction

Narcolepsy is a neurologic disorder caused by immune-mediated loss of hypocretin-producing neurons in the hypothalamus [1]. Its onset, especially in children, has been associated with use of adjuvant-containing H1N1 vaccine [2, 3]. The key biomarker for

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narcolepsy is the cerebrospinal fluid (CSF) hypocretin 1 (HCT1; orexin A) level, which can be used to distinguish narcolepsy with cataplexy from other disorders of hypersomnolence that share similar symptomatology [4]. Radioimmunoassay (RIA) kits are commercially available to determine CSF HCT1 concentrations, but assays are not routinely offered by human diagnostic testing laboratories, and only scant data on factors impacting intra-assay variability have been published. In this report, we present systematically collected data on HCT1 assay variability derived from human CSF.

Materials and Methods

We examined CSF samples collected from 155 subjects between the years of 2010 to 2016. Of these, 6 received a clinical diagnosis of narcolepsy type 1, whereas the others were diagnosed with a broad range of disorders, including narcolepsy type 2 (*i.e.*, narcolepsy without cataplexy; non-hypocretin deficient narcolepsy; n = 22), idiopathic hypersomnia (n = 48), obstructive sleep apnea with sleepiness (n = 34), and Kleine-Levin syndrome (n = 1). Forty subjects reported problematic sleepiness but did not meet electro-diagnostic criteria for any of the central disorders of hypersomnolence and four subjects had lumbar punctures performed for indications other than excessive sleepiness.

Samples were obtained by lumbar puncture with a 22-gauge sterile needle entered at the L4-5 or L3-4 level, with an average volume of CSF obtained of 12 mL (SD = 3.9). Samples were collected between 8 am and 6pm and were immediately refrigerated; further detail on time of collection was not available. They were aliquoted into 1 mL portions within 1-4 hours of collection and transferred to a -80° degree freezer for long-term storage.

HCT1 levels were measured in crude (unextracted) CSF using highly sensitive commercially available 125-I RIA kits (Orexin A RIA kits; Phoenix Pharmaceuticals, Burlingame, CA, USA). All samples were blindly measured in 100 μl duplicates for intra-assay reliability within the same kit. The standard curve range was 10-1280 pg/mL. Assay quality was controlled by the positive control sample included in the assay kit. In addition, two reference samples representing low (<110 pg/mL) and normal (approximately 350-400 pg/mL) HCT1 levels were included. The low and normal reference samples were chosen from a group of 26 randomly selected patients whose samples were assessed independently by RIA at the Stanford Sleep and Molecular Analysis Laboratory (E. Mignot, Director), substituting a different rabbit polyclonal anti-sera for that available in the same Phoenix Pharmaceuticals assay kits (personal communication, L. Lin, 2016).

Intra-assay variability was calculated by dividing the absolute value of the difference between measured HCT1 values on the two tubes from the same kit by the higher of the two values (*i.e.*, |tube1value – tube2value|/highesttubevalue). Time each sample had been frozen prior to testing was treated as a continuous variable in correlation analyses. Because there were only 6 different kit ages, the age of kit variable was dichotomized at the median of days to kit expiration. Age of kit effect was tested via Student t-test, corrected for unequal variances when appropriate. Multiple linear regression was performed to assess relative contribution of significant factors, as well to assess for possible interactions between significant factors, on amount of intra-assay variability. The model was checked for

violations of model assumptions (*i.e.*, linearity, homoscedasticity, collinearity). When necessary, arithmetic transformation of variables was attempted, followed by conversion to categorical variables. This research was approved by the Emory University Institutional Review Board and all subjects provided written informed consent.

Results

Comparison of HCT1 values from the 26 patients sent for blind evaluation at Stanford showed a highly significant inter-assay correlation ($r = 0.79$, $p < 0.0001$) without a significant difference in means (Emory mean = 230.1 pg/mL, Stanford mean = 228.8 pg/mL, $t = -0.07$, $p = 0.94$), suggesting comparability of assay procedures across laboratories. Mean HCT1 values for all 155 patients was 267.5 pg/mL (SD = 72.3). Low (< 110 pg/ml) mean HCT1 values were obtained for 6 patients, compatible with their clinical diagnoses of narcolepsy type 1. Intermediate values of HCT1 (*i.e.*, 110-200 pg/mL) were obtained on an additional 7 patients. Other than those with narcolepsy type 1, there were no significant differences in HCT1 values by major diagnosis categories (idiopathic hypersomnia mean = 274.9 pg/mL, SD = 48.5; narcolepsy type 2 mean = 280.3 pg/mL, SD = 61.9; sleep apnea mean = 276.6 pg/mL, SD = 53.2; other hypersomnolence mean = 279.3 pg/mL, SD = 50.5; $F = 0.08$, $p = 0.97$).

Intra-Assay Variation

Considering intra-assay variation, *i.e.*, from blinded duplicate testing of each of 155 samples in two different tubes on the same kit, there was a strong and highly significant correlation between paired samples ($r = 0.88$, $p < 0.0001$), without a significant difference in means (tube 1 mean = 269.3 pg/mL, SD = 73.1; tube 2 mean = 265.8 pg/mL, SD 76.2; $t = 1.18$, $p = 0.24$). Average intra-assay variability was 9.4% (SD = 7.9%). Intra-assay variation for the 6 cases whose mean HCT1 values fell below the clinically significant value of 110 pg/ml was slightly higher (15.7%, SD = 7.7). Bland-Altman analyses (Figure 1) indicated that intra-kit variability was not strongly influenced by HCT1 level.

Average time that samples were in the freezer between collection and measurement of hypocretin was 606.3 days (SD = 343.4 days, range 19 to 1821 days). Considering the group of 155 subjects as a whole, there was a weak negative correlation between time in freezer and measured hypocretin levels ($r = -0.17$, $p = 0.03$). This correlation was not apparent for the 149 samples with hypocretin values > 110 pg/mL ($r = 0.009$, $p = 0.92$). Because samples from patients with known narcolepsy with cataplexy tended to have been stored for longer periods, this potential reduction in HCT values by longer storage time is likely a spurious observation. In contrast to the mean hypocretin levels, intra-assay variability was minimally but significantly correlated with time in freezer for both the group as a whole ($r = 0.16$, $p = 0.04$) and for those with hypocretin values > 110 pg/mL ($r = 0.19$, $p = 0.02$), suggesting a weak effect of freezer time on variability.

Because samples were run in batches as CSF samples were acquired, the age of each kit (*i.e.*, the time remaining before kit expiration) differed by batch. Across all 155 samples, the average number of days to kit expiration at the time the RIA was performed was 25.6 (SD = 7.3, median 23.0) days. When dividing the age of kit by those > 23 days to expiration (*i.e.*,

newer) versus those ≤ 23 days (*i.e.*, older), there was no significant difference in measured HCT1 level (t = -0.36, p = 0.72). However, intra-assay variability was significantly higher in kits that were within 23 days of expiration (mean variability in kits ≤ 23 days to expiration = 11.2% versus 6.8% in kits > 23 days to expiration, t = 3.64, p = 0.0004).

Linear regression was performed to model the effects of time in freezer, time to kit expiration, measured hypocretin level (as an average between two tubes), and a possible interaction between time in freezer and age of kit, on intra-assay variability. Because of the inclusion of both time in freezer and hypocretin level, this model was limited to the 149 subjects with measured hypocretin > 110 pg/mL. Time in freezer was converted to a categorical variable by tertiles (less than or equal to 500 days, 501 to 670 days, and greater than 670 days). In multivariate analyses, only age of kit was a significant predictor of intra-assay variability (F = 4.93, p = 0.03).

In all six cases with low average hypocretin values, both measurements were < 110 pg/mL, with the mean difference between pairs of measurements being 2.3 pg/mL. Five of these six cases were measured using older kits. Among those 149 subjects with average hypocretin levels > 110 pg/mL, eleven subjects had paired samples whose results were discordant, i.e., one value fell within the intermediate range of 110-200 pg/mL and the other value fell in the normal range > 200 pg/mL. The mean difference between values in discordant cases was 48.1 pg/mL. Of these 11 discordant pairs, 9 were performed using older kits. Taken together, these results suggest that kit age is likely to play a role in a small number of cases with intermediate values (7.1%), but not for cases clearly in the normal or abnormal range.

Discussion

Our intra-assay variability is similar to that described by other researchers using this assay [5, 6]. The novelty of our finding is that this variability is partially explained by the age of the kit used, but not by the time the sample was stored frozen at -80C. Because hypocretin testing is not a commercially available clinical laboratory service, understanding the factors that influence variability in this research assay is important. Our results, pending confirmation, imply that use of kits with at least several weeks remaining until expiration would be ideal to reduce the magnitude of such variability.

Use of cerebrospinal fluid biomarkers for the definition of neurologic disorders of excessive sleepiness is expanding to include not only measures of hypocretin but also novel endogenous sleep-promoting substances that may bear diagnostic and clinical relevance [7]. Such markers, including hypocretin, cannot at present be reliably tested within plasma or serum, which underscores the continued relevance of cerebrospinal fluid testing in these disorders. The development of newer hypocretin assays, *e.g.*, using mass spectrometry, may hold promise and offer some advantages over traditional radioimmunoassays [8].

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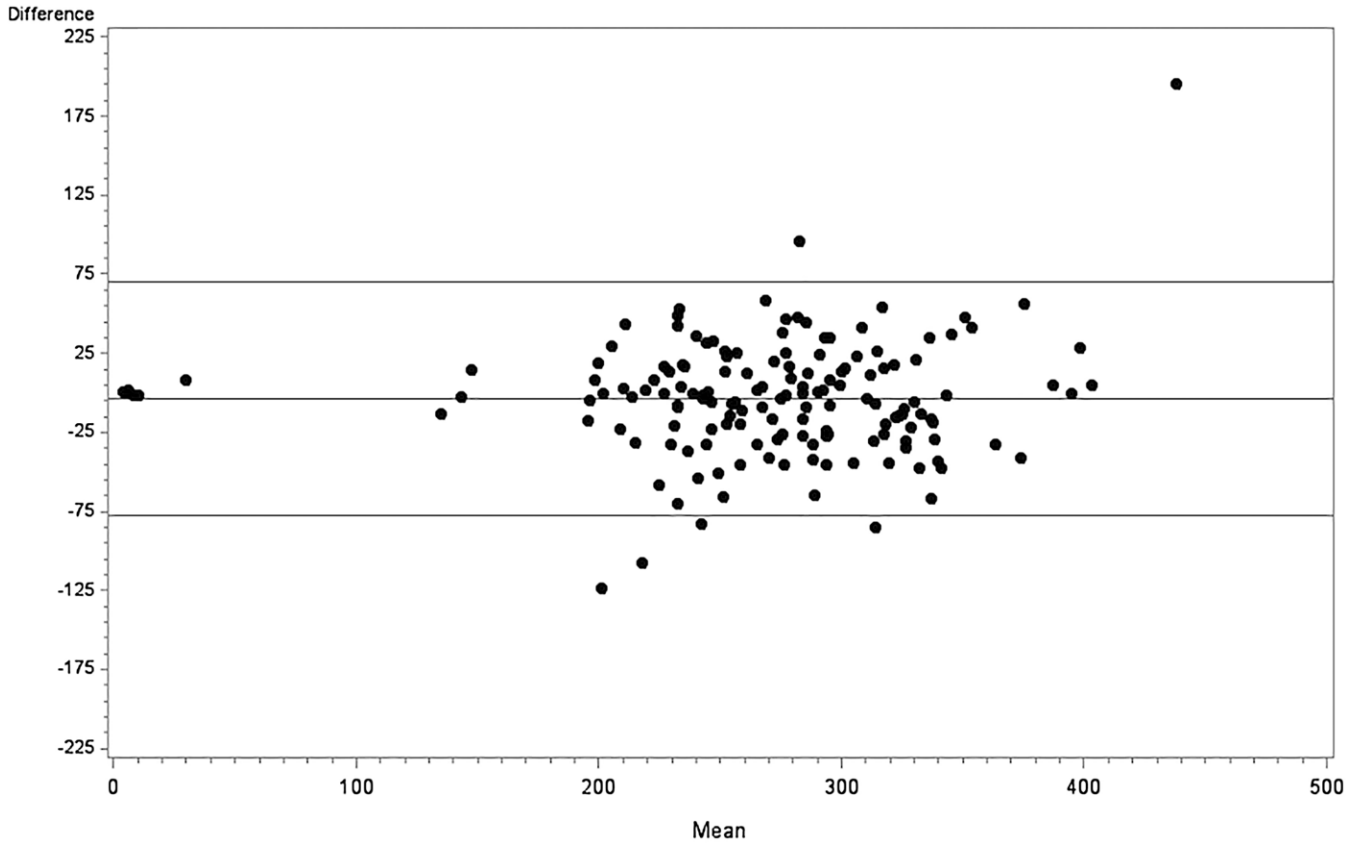

Fig. 1. Bland-Altman comparison of average hypocretin versus difference in measured hypocretin between tubes