Hypocretin Measurements in the CSF, and Blood and Brain Tissue
Basic and Clinical Applications

Seiji Nishino, MD, PhD

1. INTRODUCTION

After the discovery of narcolepsy genes in dogs (hypocretin receptor 2) and mice (prepro-orexin) (1,2), establishing functional assays for hypocretin/orexin status in human cases became a high priority. It is unlikely that these high-penetrant hypocretin-related genes found in animals are involved in most human narcoleptic cases (this was later confirmed by mutation screenings in human narcoleptic subjects including high-risk cases; 3), but functional loss/impairment of hypocretin neurotransmission might be involved.

We therefore initiated hypocretin measures in the blood and cerebrospinal fluid (CSF) using commercially available 125I radioimmunoassay (RIA) kits and found that hypocretin-1 can be reliably measured in human CSF but not in blood (see ref. 4). The inhibitory concentration of 90% (IC90) of the RIA was 4–8 pg/tube, and the antibody used did not crossreact to other neuropeptides such as growth hormone-releasing factor (GRF), glucagon, vasoactive intestine peptide (VIP), secretin for the secretin family peptides, bombesin, gastrin-releasing peptide (GRP), and neuromedin C and B for the bombesin family peptides (unpublished data). Using these RIA kits, we subsequently found that most human narcolepsy–cataplexy subjects (as well as sporadic cases of canine narcolepsy) had undetectably low CSF hypocretin levels (4–8).

This finding in human CSF was immediately confirmed by several other investigators; undetectably low CSF hypocretin levels were observed in 90–95% of narcolepsy–cataplexy subjects in several ethnic groups (7,9–11). Because the specificity of low CSF hypocretin levels in neurologic and sleep disorders is high (7,8,12), CSF hypocretin measures will be included in the diagnostic criteria for narcolepsy in the second version of International Classification of Sleep Disorders (ICSD). Narcolepsy is currently diagnosed mostly by clinical observation and polysomnographic findings (shorter sleep latencies and sleep onset REM periods during multiple sleep latency tests) with the aid of human leukocyte antigen (HLA) typing (HLA DQb1*0602 positive; see ref. 13). However, the sensitivity and specificity of these findings for narcolepsy are not high, and the final diagnosis is often delayed for several years after disease onset. With this new discovery, many patients are likely to receive immediate benefit from this new diagnostic test. The results of CSF hypocretin measures also addressed nosological issues regarding classification of narcolepsy/excessive daytime...
sleepiness (EDS) disorders, since most cases of “narcolepsy without cataplexy” (as well as idiopathic narcolepsy) were found to have normal CSF hypocretin levels \(7,9–11\), suggesting an etiological difference between “narcolepsy–cataplexy” and “narcolepsy without cataplexy” and other primary EDS disorders. CSF hypocretin measures are also useful for selecting treatment, especially if hypocretin replacement therapies become available.

Hypocretin measures in the CSF and extracellular fluid are also useful in basic neuroscience research to study the roles of the hypocretin system in regulation of sleep and other hypothalamic functions. This is especially important since changes in mRNA signals are relatively slow and do not vividly reflect synaptic releases of hypocretin peptides (hypocretin neuronal activities; see Chap. 13, Hypocretin/Orexin Tonus and Vigilance Control).

In this chapter, technical concerns regarding hypocretin measures, especially their usage and limitations, are discussed.

2. GENERAL USAGE AND LIMITATIONS OF HYPOCRETIN MEASURES

Over the past 4 yr, about 50 papers have reported the results of CSF hypocretin measures, and 20 papers have reported on blood hypocretin levels in relation to hypocretin status in various physiological and pathological conditions. However, the results (especially hypocretin levels in the blood; see Subheading 5 below) vary significantly depending on the assay conditions used. This has made some of the major findings of the studies controversial (see refs. 9 and 14–16). Therefore, it is strongly recommended that investigators (if they are not familiar with RIA/enzyme immunoassay [EIA] techniques) refer to specific handbooks, such as Radioimmunoassay of Gut Regulatory Peptides by Bloom and Long (17), for details on the uses, limitations, and technical problems of RIA/EIA.

Currently, RIA and EIA are the two major methods for measuring hypocretin peptides. Most authors measure hypocretin peptides by \(^{125}\text{I} \) RIA using polyclonal antibodies either with extraction (brain, CSF, and blood) or without extraction (CSF and blood). Bioassays, such as \(\text{Ca}^{2+}\) mobilization using a hypocretin receptor 2-expressing cell line, have also been used to quantify CSF hypocretin levels (18), but the availability of these cell lines and the equipment needed for the assay are limited to a few laboratories. In any case, these microassays, including \(\text{Ca}^{2+}\) mobilization, are “relative measures” and do not give us the absolute values of the substances measured even if respective standards are included in each assay. The values reported by RIA/EIA depend on the characteristics of the antibodies, the radioligand (RIA), the reagents, and many other factors that also influence the immunoreaction and the final values (17).

Tables 1 and 2 present a typical example of measured values of the same CSF samples using RIA and different antibodies and radioligands. Duplicates of 100 μL of CSF (without extraction) were applied to the respective RIA (i.e., direct assay), and the results clearly show the nature of the “relative measures” of the immunoassay. This reminds us that we cannot compare values if they are obtained with different assay settings. Although RIA is a well-established and reliable microassay that is widely used (quantification in the fmol range, with a small intraassay variation), the intraassay variation is sometimes large even if we use the same antibody, mostly because of variation in the specific activity of the radioligand and its quality (see paragraph below). Thus, appropriate adjustments should be made if the values are obtained from multiple assays.

Compared with \(^3\text{H}\) labeling, the specific activity of \(^{125}\text{I} \) (radioactivity that labels 1 mol of substance) is much larger, and thus \(^{125}\text{I} \) RIA, in general, is more sensitive (17). EIAs for hypocretin measures are also available commercially or in several laboratories. EIA, a less expensive and environmentally safer assay system (no radioactive ligand required, and thus no radioactive