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OPIOID PEPTIDES IN CEREBROSPINAL FLUID-METHODS FOR ANALYSIS AND THEIR SIGNIFICANCE IN THE CLINICAL PERSPECTIVE

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1. ABSTRACT

The discovery of the endogenous opioid peptide systems and their subsequent identification in human cerebrospinal fluid near 30 years ago triggered an intensive research to evaluate the function of these compounds in the clinical perspective. However, for this purpose it was necessary to develop reliable techniques with high sensitivity and reproducibility. Furthermore, it was necessary to assess the chemical nature of the opioid activity present in CSF. Therefore, research on opioid peptides in CSF have to a considerable extent been directed to attempts to characterize the peptide activity present in this fluid in order to identify suitable markers of activity in any particular opioid peptide system. In the clinic these markers have been used in attempts to correlate alterations in peptide levels to various neurological diseases. This article reviews the past and ongoing research on opioid peptide systems in CSF from human with particular emphasis on their relevance in the clinical perspective.

2. INTRODUCTION-GENERAL ASPECTS OF OPIOID PEPTIDES

The endogenous opioid peptides (also known as the endorphins) constitute a large group of neuromodulators in the central nervous system (CNS). Extensive research carried out during the past decades has suggested that these compounds may have an impact on a variety of physiological functions. It has thus been argued that these compounds are involved in the control of feeding, pain perception, stress response and reproduction. In addition, alterations in the endogenous opioid peptide system have been implicated in a variety of pathological conditions. Among these are chronic pain, degenerative diseases, drug addiction and several other psychiatric disorders. Studies on the function of the endogenous opioid systems in CNS-diseases often combine screening of clinical symptoms with peptide analysis in plasma or cerebrospinal fluid (CSF). This chapter is directed to review previous and current research on opioid peptides in CSF collected from healthy individuals as well as patients with different neurological diseases. In the CNS the opioid peptides are produced and released from nerve cells before their subsequent acting in the brain and spinal cord to modulate the action of other neurotransmitters. In the periphery they are secreted from cells located e.g. in the adrenals or gastrointestinal tract. Three genetically distinct opioid peptide precursor proteins giving rise to three separate opioid peptide systems have been characterized (1). These are the so-called “classical” endogenous opioids, which consist of beta-endorphin derived from proopiomelanocortin (POMC), the enkephalins from proenkephalin (ProEnk), and the dynorphins, which are

Table 1. Stucture and receptor profile of various opioid peptides

Peptide	Structure	Receptor selectivity
Opioid peptides		
Leu-enkephalin	Tyr Gly Gly Phe Leu	delta
Dynorphin A	Tyr Gly Gly Phe Leu Arg Arg Ile Arg Pro Lys Leu Lys Trp Asp Asn Gln	kappa
Dynorphin B	Tyr Gly Gly Phe Leu Arg Arg Gln Phe Lys Val Val Thr	kappa
Beta-endorphin	Tyr Gly Gly Phe Met Thr Ser Glu Lys Ser Gln Thr Pro Leu Val Thr Leu + 9 residues	mu
Met-enkephalin	Tyr Gly Gly Phe Met	delta
Nociceptin	Phe Gly Gly Phe Thr Gly Ala Arg Lys Ser Ala Arg Lys Leu Ala Asn Gln	ORL-1
Endomorphin-1	Tyr Pro Trp Phe NH_2	mu
Endomorphin-2	Tyr Pro Phe Phe NH_2	mu
Beta-casomorphin-8	Tyr Pro Phe Val Glu Pro Ile Pro	mu
Hemorphin-7	Tyr Pro Trp Thr Gln Arg Phe	mu

derived from prodynorphin (ProDyn). The opioid peptide precursors (propeptides) are polyvalent, i.e. from each propeptide several opioid active sequences can be formed. The various opioid active peptides derived from the three different propeptides also differ regarding their receptor activation profile. Thus, the dynorphins exhibit highest affinity for the k-receptor, whereas the enkephalins preferentially bind to the delta receptor and beta-endorphin recognizes mainly the mu-opioid receptor (Table 1). Nerve cells (neurons) producing beta-endorphin are located predominately in the hypothalamus, in the brain stem and in the amygdala area. Dynorphin-containing neurons are found primarily in the hypothalamus and within limbic structures, whereas the enkephalin-producing cells are widely distributed within the CNS, including the brain stem, hypothalamus, limbic areas and the spinal cord. It is observed that neurons producing beta-endorphin or dynorphins have long axons extending to brain areas far from the cell bodies, whereas most enkephalin-containing neurons have short axons, which suggest that these peptides act close to the site of their synthesis. The biosynthesis of the opioid peptides occurs in the cell body of the peptidergic neuron (2). Following gene transcription and a subsequent translation of the transcript a large prepropeptide is formed. After removal of a signal sequence the opioid peptide precursors (propeptides) POMC, ProDyn and ProEnk are formed and stored in certain subcellular vesicular compartments. The propeptides are subsequently processed and modified by a sequence of proteolytic steps to finally yield the active peptide. Precursor processing takes place as the vesicles are transported from the cell body along the axon to reach the nerve terminal, where the matured active peptide is stored. Following release into the synaptic cleft the active opioids hit their specific receptor sites on target cells to deliver their signal. In similarity with other neuroactive peptides the action of the opioid peptides are terminated by enzymatic degradation to yield inactive fragments or free amino acids.

However, in some cases the active peptide may be converted to an active metabolite, which interacts with receptors apart from that recognized by the parent compound. This phenomenon, known as peptide conversion, is been seen to occur in other peptide systems and has been suggested to provide a modulatory function in the actual peptide system (3). In addition to the classical endogenous opioids, peptides with opioid activity could also be released from functional proteins, such as hemoglobin and cytochrome C. From the beta-chain of hemoglobin opioid active fragments named hemorphins (4) may be enzymatically released under certain conditions and degradation of cytochrome C may give rise to so-called cytochrophins (5). From the milk protein beta-casein the beta-casomorphins (6) can be formed. A common feature among all these peptides is that they differ in structure compared to the classical endogenous opioids. Whereas the enkephalins, dynorphins and beta-endorphin share a common N-terminal unit (Tyr-Gly-Gly-Phe), essential for their opioid action, the hemorphin, beta-casomorphin and cytochrophin, as well, have the dipeptide sequence Tyr-Pro residing in their amino-terminal (Table 1). The Tyr-Pro-containing opioids have been attributed as atypical opioid peptides. They preferentially bind to and activate the mu-opioid receptor. Moreover, recently two much more potent Tyr-Pro-containing opioid peptides were identified in the brain, namely endomorphin-1 and endomorphin-2 (7, 8). The endomorphins have been characterized as the most potent endogenous ligands selective for the μ-opioid receptor. By immunochemical techniques these peptides were demonstrated to be present in pain processing areas in the brain and spinal cord (8). The mechanism for their formation in the neuron is still not clarified. Another opioid-related peptide identified during the last decade is nociceptin/orphanin (9, 10). This peptide was first believed to represent a pure opioid as it was found to produce analgesia (11). However, soon it became clear that depending on the route and site of administration this peptide exhibit both analgesic and nociceptive properties. Nociceptin binds to and activate the so-called orphanin receptor 1 (ORL-1). At present, nociceptin has attracted many scientists not only for its involvement in pain processing but also for its putative role in other CNS functions, such as memory and cognition (12). In conformity with classical opioid peptides nociceptin is released from a large propeptide (preproorphanin/FQ). Processing of this precursor may also generate an additional active peptide with antagonistic effects on the ORL-1 receptor.

3. ENDOGENOUS OPIOIDS IN CSF

In studies of opioid peptides in humans during healthy as well as pathological conditions there are

several limitations. First, there is restricted availability of human CNS tissue. For ethical reasons it is not possible to apply surgical techniques for experimental modeling like in animal experiments. Consequently, the majority of studies on opioid peptides in human CNS pathology have been limited to brain imaging and analysis of peptide levels in CSF and plasma. In this context the approach of probing peptide concentrations in CSF has several advantages since this fluid is in constant exchange with the extracellular fluid in the brain and spinal cord. Therefore, alterations in peptide levels in CSF are more likely to reflect events in the CNS than changes that may be recorded in the blood circulation. In the following this chapter will focus on detection of opioid peptides in CSF and their relevance in various pathological conditions.

3.1. The CSF compartment

The CSF is a clear fluid with comparative low concentrations of proteins and other biomolecules, as well. It originates from the blood and is secreted from the circulation into the brain ventricles by a densely vascularized tissue called choroid plexus. Epithelial cells of this tissue are the structural basis of the blood-CSF-barrier. The blood supply to this tissue emerges from small branches of the internal carotid arteries. These small blood vessels are connected to the neurons by astrocytes, which in turn support both the neurons and the blood vessels. It is believed that CSF could also enter the astrocytes at the same time as it is secreted into the lateral ventricles with respect to the choroid plexus. The entrance of neuropeptides into the neuroglia cell system may occur by a similar mechanism. As the CSF is in direct contact with the CNS substances released from central neurons that escape enzymatic degradation should appear in this fluid. Some peptides are likely to specifically be released into the CSF to use the fluid as a medium for transport to target cells. Others may passively diffuse into the CSF. In fact, most neuroactive peptides identified in CNS tissue have also been detected in the CSF.

Moreover, it also appears evident that the entrance of peptides into this fluid from the blood circulation is very restricted. Exceptions are pathological conditions connected with damage of the blood-brain barrier. Thus, the levels of the CSF peptides may vary independently from those in the circulatory system and consequently detected alterations in their CSF concentrations may be indicative of activity in the CNS. A major difficulty, however, is to relate the CSF levels of peptides to distinct nerve pathways or regions in the CNS. It is logical to anticipate that regions deep in the brain contribute a smaller proportion of CSF peptides than do regions close to the subarachnoid space. Therefore, CSF analysis may rather reveal events that occur in widespread or active systems than those that take place in small local areas. Furthermore, since CSF analysis is usually directed to samples collected at the lumbar level the detected peptide activity may rather be a more sensitive indicator of processes in the spinal cord than of those in brain. This would favor CSF analysis in conditions where mechanisms at the spinal level are likely to be essential, e.g., those involving pain. On the other hand, it has proved difficult to establish any ventricular-lumbar gradient for peptides in the CSF compartment. For instance, attempts to demonstrate a marked gradient for ß-endorphin in CSF have failed, since no difference in levels of this peptide was found comparing ventricular and lumbar CSF (13). Similar observations have been reported for other neuroactive peptides by other researchers (14). Moreover, a different origin of CSF opioid peptides was suggested from studies reported by De Riu and co-workers (15). It is thus obvious that neuropeptide levels in the CSF may reflect the rate of their release in neuronal tissue. The levels of peptides in the CSF also depend on the rate of dilution and enzymatic degradation. It is now well established that considerable proteolytic activity is present in CSF (16, 17, 18). Several proteases that have been identified in the CSF are known to act on opioid peptides. Therefore, in CSF analysis of these peptides, precautions should be taken to prevent for this phenomenon. In the clinical perspective the interest in the CSF levels of peptides is based on the assumption that these compounds serve as markers of functional activity. In fact, several laboratories have shown that activity in neurons expressing the endogenous opioids may give rise to changes in peptide levels in the CSF. For instance, high-frequency transcutanous nerve stimulation increases receptor-active as well as immunoreactive opioids in chronic pain patients (19, 20). Detectable levels of opioid peptides were also shown to increase in human ventricular CSF following focal electrical brain stimulation (21-23). An increase in CSF opioid-like material was observed in cats and rats following sciatic nerve stimulation (24).

3.2. Chemical characteristics of opioid peptides in CSF

Already at the time when the enkephalins first were discovered around 30 years ago the presence of opioid active material in human CSF was demonstrated (25). Today a large number of opioid peptides have been identified and studied in this fluid. An important issue in this context is the methods for quantification of peptides in the CSF. Also it is essential to have insight in the chemical nature of opioid peptides in CSF. It seems that the peptide activity detected by various immunological assays rather reflects prestages or fragments of the authentic peptides to be analyzed. Also, some peptides may exist in a modified or truncated form. In the following this important issue will briefly be discussed.

3.2.1. Authentic opioid peptides in the CSF

As mentioned above, most active peptides, which have been identified in CNS tissues, are also present in the CSF. These include beta-endorphin, dynorphin A, Leu-enkephalin, Met-enkephalin and its C-terminal extensions -Arg and -Arg-Phe (26-28). Also some atypical opioid peptides, such as beta-casomorphin-8 and hemorphin-7 (29, 30) have been detected in CSF. Recently the opioid related peptide nociceptin/orphanin FQ was found in detectable levels in this fluid (31). A major part of these identified peptides, however, are referred as to immunoreactive-like peptides as no structure confirmation has been accomplished. However, in several cases the immunoassays

were combined with high performance liquid chromatography (HPLC) or mass spectrometry. In this way structure confirmation have been carried out for dynorphin A (32), met-enkephalin-Arg-Phe (33), beta-casomorphin-8 (29) and hemorphin-7 (30). Recent studies have revealed that levels of opioid peptides in CSF may display age-related changes. For instance, a study comparing the concentrations of beta-endorphin-like immunoreactivity in the CSF specimens obtained from 39 neurologically normal children, aged 1 month to 10 years of age, and in 9 adult controls demonstrated levels of this opioid peptide with a peak during the first year of life, and subsequently a negative correlation with increasing age (34).

3.2.2. High molecular weight opioid peptides in CSF

Around 20 years ago our laboratory demonstrated the existence of high molecular weight forms in human CSF of the enkephalins (35) as well as dynorphin A (32). Moreover, it appeared that the major fraction of opioid peptides was due to these extensions of the active authentic entities (36). These studies have now been followed up by similar observations in other laboratories. For instance, studies have shown that the unprocessed precursor molecule POMC seems to represent the predominant peptide of the POMC family in human CSF (37, 38). Also with regard to proenkephalin it has been shown that partially processed peptides are present in concentrations exceeding those of the active pentapeptides. This is in contrast with what is found in CNS tissues, where the completely processed and active peptide predominates (36). Prestages or precursors of atypical opioid peptides such as beta-casomorphin (29, 39) and hemorphins have also been found in human CSF (4, 30, 40). Reports that several products of the orphanin/FQ precursor can be detected in CSF are present in the literature (31). The presence in CSF of N-acetylated opioid peptides has also been reported (41). In addition, some CSF peptides containing amino acid residues methionine or tryptophan may undergo modifications due to oxidative processes. E.g. Methionine enkephalin may appear with an additional negative charge, which will affect its interaction with its antibody in immunoassays. This process is most likely to occur after CSF sampling. Other events that may affect the detection of CSF peptides include the presence proteolytic enzymes. Several proteases, more or less specific towards opioid peptides, have been identified in human CSF. Among them are aminopeptidases, angiotensin-converting enzyme (ACE), enkephalinase or neutral endopeptidase (NEP), and several dynorphin convertases (DCE) (for reviews, see 16-18).

3.3. Assessment of opioid peptide concentrations in CSF

Various approaches have been applied in attempt to assess the level of opioid peptides in samples of human CSF. Most procedures developed for quantification of CSF opioids are based on immunological techniques. These include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) or radiometric assays (IRMA). Moreover, opioid activity in human CSF has frequently been probed by radioreceptor assay (RRA). This procedure was in frequent use before individual opioid peptides were identified in the fluid. In recent years, attempts to analyze opioid peptides CSF using mass spectrometry (MS) or MS combined with some chromatographic techniques have been described (40).

3.3.1. Radioimmunoassay

The assay most frequently used for measuring opioid peptides in CSF is the RIA. Over the years this technique has proven to be very sensitive and reproducible (for an extensive review, see 42). It can detect peptides at levels down to some fmol per milliliter CSF and allows screening of a large number of samples within a comparatively short time interval. A major problem with the RIA emerged from the fact that the antibodies used may cross-react with molecules of similar structure to the antigen and furthermore, salt and proteins present in the sample may also interfere in the assay. To overcome this problem many RIA-procedures include a pre-separation step using mini-columns with reversed phase silica gels or with an anion exchanger. An additional disadvantage with the RIA method is that due to use of different antibodies and different procedures for pre-separation of the CSF samples it significantly varies between different laboratories. E.g. the level recorded for the enkephalins may vary from 5 to 200 fmol/ml CSF. The corresponding figures for ß-endorphins are 1 to 100 fmol/ml CSF. In recent years several attempts to improve the pre-separation or pre-extraction procedures have been reported. We recently introduced a pre-extraction method for recovery of peptides in CSF samples, which is based on a system with 3-step liquid-liquid extraction procedure using various organic solvents (33). This approach allowed us to recover a peptide fraction suitable for evaporation and analysis with a subsequent RIA at yields up to 90%. This improvement was typified in a study of CSF samples collected from patients with fibromyalgia (FS) and matched controls (33). By this procedure it was thus possible to confirm a decrease in the level of the opioid heptapeptide Met-enkephalin-Arg-Phe in FS patients (33), a feature, which we failed to confirm in an earlier study (44). This improved pre-extraction procedure was also suitable to use for measurements of nociceptin (45, 46) and other non-opioid related peptides (47).

3.3.2. Radioreceptor assay

In addition to RIA, CSF opioids have also been quantified by radioreceptor assay (RRA). The RRA has the advantage that it may detect all opioid activity in a given sample and thereby increase the possibility to observe any significant alteration. On the other hand, it does not discriminate between different opioid systems and therefore it is not possible to clarify if any particular peptide is changed. Nevertheless, the RRA procedure was useful in attempts to correlate alterations in opioid activity with the symptoms of various neurological or psychiatric disorders (48). Studies have also confirmed a correlation between certain fractions of receptor active CSF opioid peptides with certain diagnosis of chronic pain (49).

3.3.3. Chromatographic and mass spectrometric procedures

On-line coupled analytical techniques can also be of advantage in the assay of peptides in the CSF. A recent

study based on size-exclusion chromatography combined with reversed phase (RP) liquid chromatography allowed to detect synthetic enkephalins piked in CSF samples (50, 51). Although this technique appears promising it has not yet been successfully used for assessing levels of naturally occurring enkephalins in CSF samples. We have used an RP-HPLC system to quantify levels of hemorphin-7 and fragments thereof in CSF from patients with cerebrovascular bleedings (52). By this approach we could detect the actual peptides in nmol levels. However, CSF levels of the hemorphins in this group of patients highly exceed those of enkephalins. To make the on-line separation technique suitable for quantification it needs to be combined with an additional analytic procedure such as RIA or MS. Actually, during the past decade extensive research has been directed to attempts to develop techniques for quantification of CSF peptides, based on MS or MS combined with some chromatographic technique. We developed a rapid technique to analyze LVV-hemorphin-7 in CSF fluid from a patient with cerebrovascular bleedings using a combination of size-exclusion chromatography and electrospray ionization mass spectrometry (40). The analysis utilized small quantities of CSF (0.3-0.5 ml) and was completed within a few hours. Keeping in mind that in patients with cerebral bleeding the actual hemorphin-7 extension is present in CSF at comparatively high concentration, further refinements of the technique are necessary before it could also apply for other endogenous peptides present in CSF at relatively low concentrations. Nevertheless, this experiment could be considered as an important step in progressive work to find the ultimate MS-based procedure for direct measurement of endogenous opioid peptides in small volumes of body fluids. In addition, studies focused on non-opioid CSF peptides have also contributed to forward the MS-technique further to the goal having the MS technique as an established instrument for routine analysis of endogenous peptides in CSF. For instance, a combined liquid chromatography/mass (LC/MS) analytical procedure, using a single column for sample clean-up, enrichment and separation, was developed (53). This procedure was applied for the determination of a peptide in monkey CSF. Collected samples were injected and analyzed using a polymer-coated mixed-function HPLC column with gradient elution and application of a timed valve-switching event. In this case the mass spectrometer was operated in the positive electrospray ionization mode with single ion recording at the m/z associated with this compound. However, as the lower limit of quantitation of this system did not reach levels below 1 nmol/ml CSF it would not apply for opioid peptide analysis in its present mode. According to the above-mentioned observations that the major fraction of opioid-related material in human CSF is represented by high molecular weight peptides it should be considered that these large extensions of the peptide sequences could serve as markers of opioid activity, as well. Therefore, progress in the development of techniques allowing analysis of polypeptides and proteins may be of value also for studies directed to the opioid peptide systems. A recently described technique allowing analysis of 2D-gel-separated CSF proteins by tandem high resolution MS might be useful also for studies of opioid peptide precursors in this fluid (54). Moreover, studies addressing the various opioid peptide systems also include the assessment of the CSF activity of enzymes responsible for biotransformation and degradation of the endogenous opioids. In this context the MS technique has been of potential value. For instance, HPLC combined with MS was applied in studies of dynorphin-converting enzyme in human spinal cord (55). A study on proenkephalin A processing enzymes in human lumbar cerebrospinal fluid also utilized the MS-technique (56, 57) and the activity of an enkephalin degrading enzyme in CSF from chronic pain patients (58) was also probed by MS-analysis. Electrospray ionization MS combined with LC-MS appeared to apply well for studies on the metabolism of dynorphin A peptides in brain tissue *in vitro* and *in vivo* (59). Very recently the cystatin C content in cerebrospinal fluid (CSF) during active pain states was measured by surface enhanced laser desorption ionization (SELDI) MS (60). Cystatin C is a secreted cysteine protease inhibitor involved in inflammatory responses. In this case it was possible to discern a difference in the peptide content in women at term pregnancy in labor pain from a matched group of pregnant women who were not in pain. SELDI MS is an interesting technique for peptide analysis and it may be further developed to allow CSF analysis also of endogenous opioids. However, although the MS, LC-MS or MS-MS techniques have been shown to be powerful with regard to structure identification, analysis of peptide pattern and in case of high concentration also assessment of peptide or protein amounts in the CSF, an additional breakthrough regarding the sensitivity of these techniques seems necessary before they can be used for routine analysis of opioid peptides in this fluid.

4. CSF OPIOIDS IN THE CLINICAL PERSPECTIVE

Soon after the discovery of opioid peptides in human CSF attempts to probe these compounds in samples of clinical relevance were initiated. A particular focus was directed to studies of CSF opioids in patients with neurological and psychiatric disorders. The early studies focused on the bulk of opioid activity in CSF and probed by receptor assays have now been followed up by studies directed to measurements of individual peptides. A favorite peptide to be analyzed at the different laboratories is beta-endorphin. This compound with high affinity for the mu-opioid receptor has been examined in CSF specimens collected from patients suffering from a variety of disorders. Other endorphin peptides, which have attracted investigators in many clinical laboratories are dynorphin A and its fragment dynorphin A (1-8), as well as Met-enkephalin and the C-terminal extension of this pentapeptide, Met-enkephalin-Arg-Phe. All these peptides are present in RIA-detectable levels. A few recent studies dealing with CSF measurments of nociceptin have been carried out. Some studies addressing assessment of CSF levels of the atypical opioid peptides such as beta-casomorphins and hemorphins have been published but so far no studies on the endomorphins in human CSF have yet been reported.

4.1. Opioid peptides during pregnancy

For ethical reasons it has been difficult to collect

CSF from pregnant women and therefore studies on opioids in this material are limited. However, in connection with spinal anesthesia applied to some women prior to cesarean section it has been possible to get access to this fluid. Also, in a study on the effect of acupuncture at term pregnancy some individuals volunteered and participated in a study. An early study on receptor-active endorphins indicated that the level of the endogenous opioids is enhanced at term pregnancy (61). Using an enzymatic-RIA procedure it was possible to record an increase in prodynorphin-derived peptides at late pregnancy (62). However it was not possible to distinguish alteration in any individual opioid peptide. An interesting observation from these studies was the observed correlation between the CSF concentration of dynorphin A and choice of analgetic assistance (63). Moreover, at term pregnancy the milk-derived atypical opioid beta-casomorphin-8 was found to be elevated and this elevation increased further six months later in the puerperal period (29). Today these studies appear to be quite unique, as they have not yet been followed up by similar approaches by other laboratories. One very recent study assessing the CSF levels of beta-endorphin was carried out on obstetrical patients at term. The peptide concentration was examined in one group of patients in severe pain during labor and compared with levels recorded in patients elected for caesarean section and were not in pain. However, no difference in their level of immunoreactive beta-endorphin was found (60). Also, a study on enkephalin immunoreactivity in CSF from pregnant women failed to demonstrate any alteration compared to those from non-pregnant in samples collected prior to cesarean section (64). A recent study (31) measured plasma and cerebrospinal fluid (CSF) concentrations of nociceptin, the newly identified endogenous agonist of the ORL-1 receptor. Patients included in this study were presented for elective Caesarean section (control) or in established labour and requiring combined spinal epidural anaesthesia for pain relief. Nociceptin-like immunoreactivity was detected in all CSF samples with mean concentrations, which were significantly higher than plasma concentrations and there were no differences between the two groups (31). These data report the first measurements of CSF levels of nociceptin in man and show no association with the acute pain of labour. A more recent study investigating nociceptin-LI in patients with orthopedic pain demonstrated that no correlation between various pain states and nociceptin could be observed (46).

4.2. Opioid peptides in patients with chronic pain disorders

One of the first studies carried out to measure opioid activity in CSF were directed to patients with various syndromes of chronic pain. A fraction of receptor-active opioids was found to display a decrease compared to control subjects. The majority of these patients suffered from so-called neurogenic pain such as neuralgia and causalgia. This observation was followed by a series of studies on patients with various pain diagnoses (for review, see 49). An interesting observation that was made is that patients with neurogenic pain, who also exhibit resistance to common analgesics did respond to treatment for one week with transcutaneous electric nerve stimulation (TENS). This treatment also increased their CSF opioids to normal level (19). These studies have later been followed up by studies of individual peptides derived from the ProEnk and ProDyn systems. Thus, low frequency TENS (2 Hz) produced a significant increase in the CSF level of the enkephalin heptapeptide Met-enkephalin-Arg-Phe, but did not affect the concentration of dynorphin A. In contrast, high frequency TENS (100 Hz) induced a significant elevation of the dynorphin A level but had no effect on Met-enkephalin-Arg-Phe (20). This study reporting that peripheral stimulation depending on frequency may induce a differential release of peptides from two distinct opioid peptide system provided an important contribution to the understanding of opioid mediating effects by electro-acupuncture. Substantial evidences, that emerged from various clinics and laboratories have now been accumulated that even needle acupuncture has prominent analgesic effect (65, 66). Many of these evidences are based on results indicating that the endogenous opioid peptides participate in this form of pain treatment. Quantitative analysis of beta-endorphin-like immunoreactivity (beta-End-LI) in CSF has been carried out in various diseases including chronic pain. beta-endorphin is involved in the endogenous descending pain inhibitory system. Activity in a brain pathway producing POMC-related peptides and innervating the periaqueductal grey (PAG) area in the brain stem may affect the CSF levels of beta-endorphin. The results reported on CSF-studies of this peptide have not been consistent. A quite recent study investigated whether or not the CSF content of beta-End-LI demonstrated any potential for assessing the degree of subjective pain in various spinal diseases. The peptide was measured in CSF from patients with lumbar disc herniation (LDH), with lumbar canal stenosis (LCS) and with cergical myelopathy (CM), and also controls. The severity of pain was self-evaluated by each patient using a linear visual analogue scale (VAS). However, in this study beta-endorphin was not correlated with the VAS. It was concluded that the measurement of the beta-End-LI level in CSF does not appear to have any potential for assessing the severity of pain associated with various spinal diseases (67). On the other hand, it was earlier shown (68) that in patients with intractable chronic pain, deep brain stimulation releasing beta-endorphin into CSF relieved pain symptoms. A significant correlation was also found between VAS ratings and levels of beta-End-LI (68). The involvement of beta-End in pain related to perioperative conditions was examined in a recent study (69). The peptide was analyzed in CSF from patients undergoing orthopedic surgery and samples were collected before surgery, after completed surgery and general anesthesia but still under spinal anesthesia, on occurrence of postoperative pain and 1 day after the operation. The levels of beta-End-LI in CSF after surgery, but still under spinal anesthesia were significantly higher than levels determined at other times. However, no correlation between levels of beta-End-LI and pain severity was found at any time point. The significance of this observation remains to be elucidated.

Among the various chronic pain disorders attracting for CSF analysis of opioid peptides has the fibromyalgia syndrome (FS) received attention. This

disease, which mainly occurs in women is characterized by widespread pain, morning stiffness, sleep disturbances and also depressive symptoms. However, the major syndrome in FS patients is chronic pain. Studies on CSF levels of beta-End in FS have failed to discern any significant difference as compared to control subjects without pain (70, 71). A similar observation was made in studies on CSF levels of Dynorphin A and the enkephalin heptapeptide Met-enkephalin-Arg-Phe (44). However, in a recent study a significant decrease was found in levels this heptapeptide in FS patients (33). The latter study used a new approach to assay the enkephalin peptide. The decrease in the enkephalin was compatible with a concomitant increase in the CSF levels of substance P (33).

4.3. Opioid peptides in CSF in trauma and stress

Several studies have suggested that endogenous opioids may be involved in the mechanism underlying the pathophysiology of disorders related to trauma and stress. In this context the POMC derived opioid beta-End has received particular interest. Projections from the PAG to the brainstem and spinal cord receiving inputs from amygdala and hypothalamus may activate antinociceptive mechanisms involving beta-End during anxiety or stress. POMC neurons in the arcuate nucleus of the hypothalamus may give rise to release of beta-End in PAG and other caudal brain areas including the spinal cord. The level of CSF beta-End was reported to display a significant increase in human after an acute cerebral ischemic insult (72). Also, in following up studies Nappi and co-workers (73) observed significant elevated levels of beta-End in CSF but not in plasma in patients suffering from acute brain ischemia. This finding was later supported by data obtain by animal studies (15, 74) indicating independent origin of the peptide in these two compartments. A study on beta-End-LI in CSF from combat veterans with post-traumatic stress disorder confirmed a significant elevation of the peptides in these patients compared to control subjects (75). Opioid peptides other than those derived from the POMC system, have also been analyzed in the CSF in patients with CNS trauma. For instance, the concentrations of both Met- and Leu-enkephalin were examined in CSF from patients after severe head injury using a techniques based on the combination of column chromatography and RIA (76). The study investigated the levels of these peptides over time for diagnostic reasons and it was found that constantly elevated Met-enkephalin levels were paralleled with decreasing levels of Leu-enkephalin. The authors suggested a different role of these two opioids during the actual pathophysiological conditions (76). Furthermore, in an additional work (77) significant changes in CSF beta-End levels were detected in patients with a wide range of head trauma (from minor head trauma to severe injury). However, in this study the increased CSF beta-End levels were not correlated to the early prognosis of the patients. Also, in an early study beta-End-LI was measured in CSF of patients with acute head injury and compared to controls. The mean values of beta-End-LI in CSF of controls and patients with moderate and severe acute head injury were 52 pg/ml, 111 pg/ml, and 174 pg/ml respectively, with significant difference between them. This results clearly demonstrated that beta-End-LI is enhanced in CSF of acute head injury patients (78). Although peptides derived from the ProDyn system have been implicated in a role during traumatic conditions there is a lack of studies on these compounds in CSF from human subjects with trauma to the CNS. A number of animal studies have demonstrated that dynorphin A and particular C-terminal fragment thereof may be involved in the outcome of spinal cord injury (79-84). Block of kappa-receptors (85) and NMDA receptors (86) or topical application of antibodies against dynorphin A (87) may modulate the trauma-induced pathophysiology.

4.4. Opioid peptides in neurodegenerative disorders

Studies on CSF levels of opioid peptides have also been addressed to patients suffering from various types of neurodegenerative disorders and in particular in Alzheimer's disease and in Parkinsonism. Senile dementia of Alzheimer type (SDAT) is the most common demense disorder during aging life and this disease is the major cause of mortality in the geriatric population. Previous research has suggested that dysfunctions in some neuropeptide systems may underlie some symptoms of SDAT. In this context neuroactive peptides involved in learning and behavior or memory have received particular interest. Among these are somatostatin, substance P, nociceptin and opioid peptides. Several clinical studies on SDAT patients have focused on measurements of opioid active peptides in the CSF. For instance, CSF beta-End-LI was observed to decrease in patients with dementia (88). The greatest decrease was seen in patients with presenile and senile dementia of SDAT. The recorded activity significantly correlated with psychological functions when examined using a dementia rating scale and it was suggested that beta-End is associated with the pathophysiology of dementia. This original study was followed by several others indicating decreased CSF levels of beta-End in SDAT (89-96). In studies of beta-End in CSF from both SDAT and patients with multi-infarct dementia it was found that the peptide level was decreased in both groups of patients suggesting that low CSF ß-End level may be generally related to dementia (89). In contrast, Nappi *et al.* reported reduced CSF level of beta-End-LI in SDAT but not in samples from patients with multi-infarct dementia (92). A study on beta-End in CSF from SDAT by Raskind and co-workers (97) did not report any differences between the diseased group and controls. However, it appears that most studies demonstrate reduced content of CSF beta-End-LI in SDAT patients and a proposed cause for this alteration is an abnormal processing of POMC (91, 94). Indeed, the processing of POMC peptides was investigated post-mortem at the pituitary and hypothalamic level in a patient with SDAT and in a control subject (91). The results obtained in this approach indicated that defects in the axonal transport and/or secretion rather than synthesis could account for the abnormalities of POMC peptides in the CSF. A more recent study showed that beta-End is not only markedly decreased in CSF from SDAT patients, the levels of the peptide also correlated negatively with degree of dementia within the patient population (96). In addition to beta-End other opioid peptides present in CSF have also been investigated in SDAT patients. One example of these is the dynorphin A fragment Dyn A (1-8). The level of this octapeptide with affinity for k-opioid

receptors was examined in CSF from a group of nine patients with SDAT. The Alzheimer patients revealed a 40% decrease in the CSF Dyn A (1-8) compared to controls. This finding was further supported when an additional 20 SDAT patients with similar clinical backgrounds also showed reduced CSF level of the opioid octapeptide (95). The measured CSF level of Dyn A (1-8) did not correlate significantly with clinical variables or CSF measures of monoamine metabolites. According to a previous published finding of increased k-binding throughout limbic areas in the brain of SDAT patients the authors suggested that the reduced content of the dynorphin-related peptide is associated with this up-regulation of the k-opioid receptor.

It appears clearly documented that neurotransmitters or neuromodulators other than dopamine, including neuropeptides, could have important pathophysiologic and therapeutic roles in Parkinson's disease. Since there is evidence suggesting that opiates and opioid peptides affect the activity in the dopamine system and particular in the striatum, the opioid peptides have received interest in studies on Parkinson's disease (98). It is well known that both Met-enkephalin, one important peptide neuromodulator of the striatopallidal pathway, and dynorphin, which is shown to modulate the striatonigral pathway display complex anatomic and biochemical interactions with the basal ganglionic dopamine system. Therefore several studies on CSF opioids in Parkinson's disease have focused on these endogenous opioids. The levels in lumbar CSF of Met-enkephalin, and extended forms of enkephalin were examined in nine patients undergoing elective surgical procedures (used as controls) and in eight patients with advanced Parkinson's disease, prior and subsequent to autologous transplantation of adrenal medullary fragments into the right caudate nucleus. The levels of CSF Met-enkephalin and its extensions before surgery in patients with Parkinson's did not demonstrated any alteration (99). However, a later study (100) investigating the CSF content of the ProEnk derived octapeptide, Met-enkephalin-Arg-Gly-Leu (MERGL), demonstrated significantly low concentrations of this opioid in parkinsonian patients following overnight withdrawal of all medications compared with control subjects. The level of MERGL did not exhibit any change after at least 16 h of steady-state, optimal doses of levodopa infusion intravenously. However, MERGL levels increased with advancing age among normal individuals but not among patients with Parkinson's disease. In contrast dynorphin A (1-8) levels were not different between the two study groups and did not change with levodopa therapy. Furthermore, no correlation with age or any indices of disease progression was found with respect to dynorphin A (1-8). It was suggested that the abnormality of the enkephalin system in Parkinson disease in the primary pathologic process is due to the involvement of striatal neurons (100). A currently used treatment for the suppression of parkinsonian tremor is the high frequency electrical stimulations of thalamic nuclei. In order to provide some insight into the mechanisms behind this treatment Met-enkephalin-LI was measured in ventricular CSF of patients with Parkinson's disease and subjected to thalamic electrical stimulation (101). In stimulated patients, a relative increase in Met-enkephalin-LI was observed in the CSF sample taken after a 30-minute stimulation as compared to the sample taken immediately before stimulation was applied. In contrast, the levels of the dopamine metabolite 5-HIAA remained unaffected by the stimulation. These data were suggested to confirm the existence of negative interactions between dopaminergic and enkephalinergic systems in man. In addition, they were considered to indicate that alterations in dopaminergic or enkephalinergic neurotransmission might be involved in the therapeutic action of thalamic electrical stimulation in patients with parkinsonian symptoms (101).

4.5. Opioid peptides in adolescent and childhood diseases

There are at least five different diseases occurring in children and adolescents that have received special interest in research focused on opioid peptides in CSF. Among them are infantile autism, Rett's and Tourette's syndrome, respiratory disorder and anorexia. Autism, which is a disease of unknown etiology, is characterized by reduced responsiveness to pain. As the endogenous modulation of pain at several CNS levels involves opioid peptides there has been a natural interest to assess CSF levels of these compounds in this particular disease. Most CSF studies on opioid peptides in autistic children have been directed to attempts to assess the levels of beta-End. Treatment with the amphetamine-like drug fenfluramine has been shown to improve behavior in infantile autism and has been suggested to induce a decrease in abnormally elevated blood serotonin content. However, primary central effects of this drug have not been proved to be serotonergic. It rather seems that other brain modulators such as beta-End is involved in the anorexic effect of fenfluramine and may play a role in autism. In an early study beta-End-LI was determined in lumbar CSF of autistic patients during and before or after treatment with fenfluramine and then was compared to normal controls (102). The opioid peptide was significantly increased in the baseline autistic group and was reduced toward control values during fenfluramine treatment. These results are consistent with a role for beta-End in infantile autism but also in the mechanism of fenfluramine treatment. An additional study on beta-End in CSF from autistic children indicated lower levels in beta-End compared to children with other types of childhood disorders, such as infantile spasm (103, 104), whereas in some studies no alteration in CSF beta-End compared to control was found (105). Moreover, in the latter study there was no significant correlation between CSF levels of the opioid peptide and clinical symptoms, including self-injurious behavior, pain insensitivity, and stereotyped movement.

Another childhood disorders is the Rett syndrome. This is a postnatal development and neurological disorder seen only in girls (106). The Rett syndrome is characterized by symptoms of autistic-like behaviour. microcephaly, progressive loss of motor, cognitive, and language skills, stereotyped hand movements, respiratory abnormalities, and seizures. Many of these symptoms are analogues with effects seen

following administration of opioids. Therefore, CSF levels of beta-End have been measured in girls suffering from Rett's syndrome and results confirmed significant increases in the level of this peptide in samples collected from these subjects (107-111). Nielsen and co-workers found increased levels of beta-End-LI in CSF in a group of girls with Rett's syndrome but the severity of symptoms was not found to be related to the level of this opioid. Moreover, another study reported reduced level of beta-End in CSF collected from girls with the Rett syndrome (112). The conflicting findings were suggested to be a result of differences between control groups (111).

Gilles de la Tourette's syndrome is characterized by multiple motor phonic tics and various behavioral problems, including some forms of obsessive-compulsive disorder (113). Since the pathology of this disorder may include dysfunction in the central dopaminergic system, which in turn is influenced by the endogenous opioids, studies on CSF endorphins in Tourette's have attracted several researchers. Indeed, alterations in CSF opioids have been observed in these patients although some conflicting results have been reported (114- 116).

As it is well known that opioids are capable of causing respiratory depression in man several studies on CSF opioids in apnea and other respiratory disorders have been carried out. From the pioneer work by Brandt and co-workers in 1980 (117) studying a child with necrotizing encephalomyelopathy to the recent work by Storm and co-workers (118). Several reports indicating elevated levels of endogenous opioids in CSF in respiratory depression have appeared in the literature. Previous studies have indicated increased activity of beta-End-LI in the CSF of infants under two years of age with apnea. To assess the role of endogenous opioids in the pathogenesis of apnea in children, the effect of oral treatment with the opioid antagonist naltrexone has been studied in apneic infants, as well as in older apneic children, with demonstrated increases in CSF levels of beta-End-LI (109). In these groups of apneic infants with elevated beta-End-LI in lumbar CSF, with one exception, no further apnea occurred during naltrexone therapy. However, apnea reoccurred in some patients after attempts to discontinue naltrexone treatment. Moreover, three children with Leigh's syndrome had elevated levels of beta-End-LI and their apnea also responded to naltrexone. It was concluded that elevated endogenous opioids contribute to the pathogenesis of apnea in children and may even in some cases result in physical dependence. More recent studies have confirmed a role of beta-End in respiratory disorder, including sleep apnea and sudden infant death syndrome (118, 119, 120). It appears that assessment of opioid peptides in CSF may serve as a useful indicator of respiratory dysfunction, which in certain cases could be subjected to opioid antagonist therapy.

A particular interest has been addressed to studies on opioid peptides in anorexia nervosa. This disorder occurs primarily in adolescent girls and is characterized by severe self-induced weight loss, an intense fear of becoming obese, disturbed body image and depression (121). Several of the observed symptoms in anorexia is associated with excessive production of endogenous opioids (122) and it was early reported that there seems to exist a significant correlation between CSF opioids and the severity and acuteness of this disorder (123). Later studies suggested that this activity was not associated with the POMC system (124) but rather with peptides derived from ProDyn (125). Evidence that changes in CSF opioid may be associated with the clinical expression of dissociation in patients with eating disorders during the acute phase of their illness have been described in more recent studies (126).

4.6. Opioid peptides in Psychiatric disorders

Already at the time when the presence of endogenous opioids in the CSF first was confirmed, studies addressing questions on the possible involvement of opioid peptides in psychiatric disorders were initiated. During the following decade a number of reports on CSF endorphins and their link to psychiatric symptoms appeared in literature (for review, see 48). For instance, in patients diagnosed with schizophrenia an observed elevation of CSF opioids followed by studies showing an antihallucinatory effect of naloxone in certain cases of this disorder. Elevated content of CSF opioid peptides in schizophrenic patients was also seen in subsequent studies and neuroleptic therapy was found to reduce this enhancement. Studies also suggested that the elevated opioid activity in CSF from schizophrenic subjects was rather due to abnormally processed opioid peptide precursors than rises in individual maturated peptides (48). In later studies assessments of individual opioid peptides in CSF from patients suffering from schizophrenia have been carried out. A RIA procedure was used to measure the CSF activity of dynorphin A-LI in a group of schizophrenic patients before and after neuroleptic treatment and compared to that recorded in nonpsychiatric surgical controls (127). The mean concentration of dynorphin-A-LI found in the schizophrenic group on admission was significantly elevated compared the nonpsychiatric controls. However, the CSF concentration of this peptide remained almost unaltered after 4 weeks treatment with a neuroleptic drug (zuclopenthixol). This occurred despite a significant reduction in overt psychopathological symptoms assessed by means of the Brief Psychiatric Rating Scale (BPRS). After inclusion of an additional group of psychiatric patients to increase the number of samples, a significant correlation between the CSF level of dynorphin A-LI and the BPRS total score was observed (127). During the past ten years very few studies on CSF opioids in patients with schizophrenia or depressive disorders have appeared in the literature. This could reflect the increased interest of directing these kind of studies, e.g. to the area of image analysis, genetics and proteomics. Further CSF studies of endogenous opioids in these disorders may be re-uptaken at the time when new better markers have been identified and techniques for their assessment have been fully developed.

4.7. Opioid peptides in addictive diseases

The role of opioid peptides in addiction to alcohol and other drugs of abuse have for long been the subject in research focused on drug tolerance and dependence. The neurobiology underlying the development

of drug addiction is gradually being clarified. Anatomic pathways in the brain of primary drug reinforcement or reward and the molecular architecture of the receptors, on which addictive drugs act, have been revealed (128). It appears that all addictive drugs in some way mimic (or occasionally block) the actions of authentic neurotransmitters or neuromodulators involved in reinforcement and reward. In the case of heroin or methadone, they act analogous with endogenous opioids, such as beta-End or enkephalins. As for alcohol it seems that this drug produces reinforcement through a mechanism that involves the release of beta-End.

4.7.1. Alcohol addiction

A clear link between the endogenous opioid system and excessive alcohol consumption has been confirmed (129, 130). It has thus been shown that acute or light alcohol intake induces a release of opioid peptides (e.g. beta-End) in brain regions that are involved in reinforcement and reward. This release of ß-End is considered to mediate, at least in part, the euphoric and reinforcing effects of alcohol. On the other hand, chronic heavy alcohol consumption causes a deficiency in the central opioid systems, which may result in withdrawal to endogenous opioids and thereby give rise to continuous alcohol consumption through the mechanisms of negative reinforcement. The involvement of the endogenous opioids in alcohol dependence is further supported by the effectiveness of opioid receptor antagonists (e.g. naltrexone) in reducing alcohol consumption in alcohol addictive subjects. CSF studies of endogenous opioids in people with alcohol dependence have mainly been directed to the assessment of beta-End levels in this fluid. An early study of ß-End in the plasma and CSF of alcohol addicts compared these values with those found in normal volunteers. Results showed that alcohol addicts exhibit CSF levels of beta-End-LI that were 3- fold lower than those of the controls. This finding indicated that alcohol addiction is associated with a marked alteration in the CSF content of the POMC-related opioid peptide, which was suggested to play a role in alcohol-seeking behavior typical of this syndrome. The alteration in the ß-End concentration during alcohol dependence have been followed-up by several other studies (131, 132). A recent study compared the levels of CSF mono-amines with those of beta-End in samples from early-onset as well as late-onset of alcohol-dependent patients and healthy controls (133). It also examined whether the recorded CSF measures predicted the degree of craving experienced in response to an alcohol cue. However, from this study it was evident that the used CSF markers did not predict the precue levels of craving, or the increase in craving after alcohol cue exposure (133). A more recent study on plasma beta-End in alcohol dependent individuals was conducted in order to evaluate the hypothesis whether beta-End is associated with the expression of anxiety, depression and craving during acute alcohol withdrawal. The result showed that in accordance with prior studies, beta-End-LI was significantly lowered on day 1 and day 14 of alcohol withdrawal compared to controls. Self-rated anxiety, depression, and alcohol craving decreased significantly between day 1 and day 14. Levels of beta-End-LI were inversely correlated with anxiety on day 1 and day 14. In addition, a significant inverse relationship was found between beta-End and craving on day 14. No correlation between beta-End levels and depression was detected. It was concluded that this study provided the first evidence that reduced beta-End levels during alcohol withdrawal may possible contribute to anxiety as a common disturbance during this condition (134).

4.7.2. Opioid addiction

The assumption by Dole, Kreek and Nyswander that opioid addiction should be considered as a concrete neurochemical abnormality of the endogenous opioid system has lead to the development of programmes for treatment of heroin addicts using methadone maintenance. However, it also initiated an intensive research on endorphin peptides in opioid dependent individuals. Some of these studies have been directed to analysis of opioid peptides in plasma and CSF. There are at least two studies focused on the assessment of beta-End in CSF from methadone-maintained addicts (135, 136). Although both studies indicated alterations in the level of beta-End-LI the observed changes appeared to diverge to opposite directions. On the other hand, these two studies differed in their design regarding the doses of methadone and the duration of treatment. In more recent work beta-End-LI in CSF has been studied in heroin addicts subjected to treatment with electroacupuncture (137). This therapy has proven to successfully reduce the expression of opioid withdrawal. As mentioned above, low frequency electroacupuncture (2 Hz) is known to produce release of enkephalins, whereas high frequency electroacupuncture (100 Hz) elicits release of dynorphins. The alternating high and low frequency stimulation produced the most significant improvement on the opioid withdrawal syndrome.

5. CONCLUDING REMARKS

It is evident from this review that there is a broad clinical applicability of CSF analysis of opioid peptides. Although these analyses have provided a broad insight with regard to the involvement of the endogenous opioids in various CNS disorders it is obvious that there are limitations. A major difficulty is connected with limited amount of CSF that could be withdrawn from each patient. The restrictions to collect CSF specimens in the case of human due to ethical reasons also contribute some limitation. Moreover, most studies mentioned here have used the RIA technique for peptide detection and, as mentioned earlier, this technique also has its limitation due to cross-reaction and sensitivity. Another thing to discuss is the choice of markers for probing activity in a particular peptide system. In the majority of clinical studies cited in this chapter the active product of a particular opioid peptide system has been chosen as measure but there is evidence that the main part of peptide material from any opioid peptide system present in CSF is rather due to unprocessed high molecular weight polypeptides (see e.g. 36). Moreover, it is also evident that some proteolytic activity responsible for opioid peptide degradation or conversion may also represent markers of activity in a particular

peptide system (see 17-19). However, it should be noted that due current progress in genetic and proteomic research new relevant markers of activity in the opioid peptide systems might arise. Moreover, the recent progress in the development of new techniques for peptide analysis in biological samples may lead to new procedures, which may simplify analysis of CSF peptides. In this context it is assumed that e.g. the new mass spectrometric instrumentation may provide powerful tools for peptide analysis with high sensitivity, reproducibility and precision in various body fluids, CSF included.

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