Variation in cerebrospinal fluid levels of neuropeptide Y, cholecystokinin and substance P in patients with neurological disorders

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Abstract Neuropeptide Y, cholecystokinin (tetra- and octasulphated peptides) and substance P were measured in lumbar cerebrospinal fluid obtained from patients with various neurologic disorders such as Parkinson's disease, cerebrovascular disorders, multiple sclerosis, tuberculous meningitis and aseptic meningitis. These results are statistically compared with healthy results. The results accumulated showed that the data collected can provide the vital information necessary for designing drug therapy.
Introduction

The monoamines, acetylcholine and amino acids were thought to be the only classical neurotransmitters until recent years, when a large number of peptides, many of which were originally characterized in non neural tissues, have also been shown to exist in CNS [1]. During the last decades, it has become evident that numerous molecules may be involved in chemical signaling in the nervous system and that they can be divided into their different sub-classes. Thus, in addition to amino acids such as GABA and glutamate and biogenic amines such as dopamine and noradrenaline, neurons produce and release peptides. These compounds are often referred to as neuropeptides and represent a heterogeneous group of molecules, the smallest ones built up of only two amino acids with larger polypeptides consisting of 40 or more amino acids.

Receptor-active opioid peptides in human cerebrospinal fluid were demonstrated more than 20 years ago [2]. One of the major reasons for analyzing CSF peptides instead of plasma and serum is that neuropeptides do not readily cross the blood-brain barrier and CSF due to constant exchange with the nervous tissue rather than plasma can be anticipated to contain peptides which derive from the CNS [2, 3]. The changes in the CSF peptide concentration might be attributed to certain symptoms and can even be interpreted as characteristic features of the pathological conditions [4].

Neuropeptide Y (NPY), cholecystokinin (CCK) and substance P (SP) are some of the important peptides which play vital biological roles in various degenerative disorders. These peptides are known to have the ability to act as neurotransmitters. These peptides are also known to co-localize with classical neurotransmitters within a single neuron which provide a means to transmit more complex types of signals.

The peptides differ from the so-called classical transmitters since 1) they function in lower concentrations than classical transmitters, 2) synthesis of peptides is directed by mRNA in perikaryon as a part of a much larger prohormone, from which the active peptide is cleaved by peptidase whereas classical transmitters are formed from dietary sources by enzymes, 3) classical transmitters respond and adopt very rapidly to external stimuli and have their synthesis machinery with great capacity whereas neuropeptides respond slower and their synthesis machinery have a more limited capacity and speed, and 4) molecular heterogeneity of peptides can exist, the different forms have different, sometimes opposite effects [5]. Neuropeptides are widely distributed throughout the brain in specific nerve cells in coexistence with monoamines and/or other neuropeptides [6–8]. They are believed to participate in several physiological and pathophysiological processes, including pain sensation, memory, neuroendocrine functions, regulation of release of monoamine transmitters and regulation of mood [9, 10]. The effects are brought about by primary actions of neuropeptides or their modulation of the effects of monoamines transmitters.

The elucidation of peptide transmission is essential not only for our understanding of normal neuronal function, but it is highly probable that changes in the chemical transmission process may underlie or at least be related to various disease processes in the nervous system. Moreover, it is now clear that many of the common drugs used to treat various diseases in the nervous system act via interfering with the chemical transmission process [6, 11].

As discussed above, the data obtained so far in general support a role of peptides in chemical transmission auxiliary to classical transmitters. However, it is clear that in certain neuronal systems peptides play the main role. This is particularly obvious in hypothalamic neurosecretory cells which produce and release the posterior pituitary hormones vasopressin and oxytocin, or releasing and inhibitory factors such as luteinizing hormone releasing hormone (LHRH) and somatostatin. Recently, it has been shown that even these neurons have the capacity to produce and store a classical transmitter [5]. One of the first examples of coexistence in the central nervous system was the demonstration of CCK-LI in dopamine neurons in the ventral mesencephalon, especially in the ventral tegmental area [12]. It has also been shown that a small population of A10 dopamine neurons contains neurotensin [12].

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
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<td>CSF</td>
<td>Cerebro-Spinal Fluid</td>
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<td>NPY</td>
<td>Neuro-Peptide Y</td>
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<td>SP</td>
<td>Substance P</td>
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<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
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<tr>
<td>CVD</td>
<td>Cerebro-Vascular Disorder</td>
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<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
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<tr>
<td>AM</td>
<td>Aseptic Meningitis</td>
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<tr>
<td>TBM</td>
<td>Tuberculous Meningitis</td>
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<tr>
<td>CT-scan</td>
<td>Computerized Tomography</td>
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<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<td>HS</td>
<td>Healthy Subjects</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>L4/L5</td>
<td>Lumbar vertebra 4 and 5</td>
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<tr>
<td>CBF</td>
<td>Cerebral Blood Flow</td>
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On the basis of co-existence with monoamine transmitters and distinct regional distribution, assumptions have been made that neuropeptides play a role in CNS disorders [4, 13–15]. In this study, CSF analyses of neuropeptides, such as cholecystokinin, substance P and neuropeptide Y, in various neurologic disorders have been shown. These results are compared with healthy subjects.

**Material and Methods**

*Patients and healthy subjects*

All patients were recruited from the Department of Neurology, Huddinge University Hospital, Sweden, except patients with tuberculous meningitis who were recruited from the Department of Neurology, the Aga Khan University Hospital, Karachi, Pakistan. CSF samples were obtained by lumbar puncture within 4–8 hours of admission. 20 Parkinson’s disease (PD) patients (7 females, with mean age 72±12 years), 16 patients (6 females, mean age 62±4 years) with ischemic stroke or cerebrovascular disorders (CVD), 20 patients (8 females, mean age, 54±6 years) with definite multiple sclerosis (MS), 18 patients (9 females, mean age, 46±5 years) with aseptic meningitis (AM) and 14 patients (5 females, mean age 51±4 years) with tuberculous meningitis (TBM) were included in this study. The diagnosis of PD was made according to established clinical criteria [16]. None of these patients exhibited clinical indications of symptomatic parkinsonism or depression and all had shown clear response to L-DOPA. Among PD patients, 6 patients were included who did not receive L-DOPA and other DAergic drugs. For CVD patients, computerized tomography (CT scan) and/or MRI confirmed the evidence of stroke and its site. All the strokes were in the territory around the middle cerebral artery. Ten of the patients had hemiplegia and 6 had hemiparesis at the time of admission. All the patients showed normal CSF cell count while CSF protein and IgG were significantly higher (Table 1). Multiple sclerosis was diagnosed according to Schaumacher criteria [17]. 9 of the MS patients had exacerbation, 5 were in remission, while 6 had chronic progressive form of MS at the time of sampling. Exacerbation was defined as sudden appearance of new signs and symptoms or sudden worsening of previous signs and symptoms lasting more than 24 hours. All of these patients had oligoclonal bands in their CSF. Routine CSF examination showed mononuclear pleocytosis (> 5x10^6/liter) in all patients. Since, there was no difference in the levels of CSF neuropeptides in exacerbation, remission and chronic progressive form, all MS patients are considered in one group.

Slightly raised CSF/serum albumin ratio reflecting low-grade blood-brain barrier damage was present in MS patients, and elevated IgG Index [18] was observed. For meningitis patients, the diagnosis of the disease was based on standard diagnosis criteria [19]. Besides, aseptic meningitis was diagnosed on the basis of clinical features, culture, sensitivity tests and laboratory investigations of CSF at the time of admission to the hospital. Subsequent examination of CSF was carried out the basis of clinical and therapeutic considerations. The diagnosis of tuberculous meningitis was established on clinical and CSF findings, positive cerebrospinal Gram stain and latex agglutination and positive presence of growth of pathogenic bacteria. CSF samples were also collected from 14 healthy individuals (5 females, mean age 53±5 years) with complaints of muscular tension headache. These individual were considered as healthy subjects (HS) because CSF routine analysis as well as blood complete examination, liver function test, electrolytes, ESR were within normal limits. Their general physical examination, neurological

<table>
<thead>
<tr>
<th>Patients</th>
<th>n</th>
<th>Females</th>
<th>Age (years)</th>
<th>CSF-albumin</th>
<th>CSF-IgG</th>
<th>IgG-index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects</td>
<td>14</td>
<td>5</td>
<td>53±5</td>
<td>218±17</td>
<td>34±4</td>
<td>0.44±0.01</td>
</tr>
<tr>
<td>PD</td>
<td>20</td>
<td>7</td>
<td>72±12</td>
<td>332±42*</td>
<td>51±7**</td>
<td>0.43±0.01</td>
</tr>
<tr>
<td>CVD</td>
<td>16</td>
<td>6</td>
<td>70±10</td>
<td>313±31*</td>
<td>46±6*</td>
<td>0.42±0.01</td>
</tr>
<tr>
<td>MS</td>
<td>20</td>
<td>8</td>
<td>54±6</td>
<td>188±14</td>
<td>65±7***</td>
<td>1.03±0.14**</td>
</tr>
<tr>
<td>AM</td>
<td>18</td>
<td>9</td>
<td>46±5</td>
<td>412±53***</td>
<td>83±12***</td>
<td>0.68±0.06**</td>
</tr>
<tr>
<td>TBM</td>
<td>14</td>
<td>5</td>
<td>51±4</td>
<td>442±61**</td>
<td>88±11***</td>
<td>0.73±0.11**</td>
</tr>
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*p<0.05,**p<0.01,***p<0.001
observation and CT scan of the head were also normal. The clinical data on the healthy subjects and patient groups are presented in Table 1. All values are expressed as mean ± Standard Error Mean (SEM).

**Lumbar puncture and routine CSF analysis**

10–12 ml cerebrospinal fluid (CSF) was collected from each patient and healthy subject in a sitting position at the L4–L5 levels. Blood samples were collected by venipuncture. The basic CSF analyses included: cell counting by phase-contrast microscopy (Sörnäs, 1967), determination of CSF/serum albumin ratio and CSF/immunoglobulin G (IgG) index [20] as well as isoelectric focusing for detection of oligoclonal IgG band. Serum and CSF albumin and IgG were determined using Hitachi 737 Automatic Analyzer (Naka Works, Hitachi Ltd., Tokyo, Japan). CSF and serum samples were kept at −70°C if not analyzed immediately.

**Analysis of neuropeptides**

CCK-8s and CCK-4 were measured using previously described HPLC technique with electrochemical detection (Qureshi et al., 1993). For measurement of CCK-peptides, the chemical identity of these peptides was ensured using a novel HPLC system for micropuriﬁcation (SMART) and subsequent fast atom bombardment mass spectrometry [22]. The detection limit for CCK-4 and CCK-8s was 0.2 and 1.0 pmol/l respectively.

The quantitation of NPY was done according to the previously described radio-immunoassay [23]. The detection limit was between 0.2–0.5 pmol/l.

SP-L1 was determined using SP2 antisera according to the previously described method [24] based on RIA procedure. The limit of detection using this procedure was 0.1–0.2 pmol/l.

**Analysis of data**

Data are presented as mean ±SEM differences in concentration of CCK-4, CCK-8s, NPY and SP were analyzed with ANOVA and group comparison were made with t-test. A p-value less than 0.05 was considered significant.

**Results**

Figure 1 shows the CSF levels of NPY in healthy subjects (HS) and patients with neurological disorders. As compared to HS, NPY levels were decreased significantly from 29.2±3.6 pmol/l to 24.1±2.0 (p<0.05), 12.3±1.4 (p<0.01), 23.3±3.0 (p<0.05) and 21.7±2.2 (p<0.05) pmol/l in CVD, MS, TBM and AM patients and increased significantly to 37.2±4.9 (p<0.01) pmol/l in PD patients.

Figure 2 shows the CSF levels of CCK-4 and CCK-8s where CCK-4 levels were significantly increased from 5.2±0.9 pmol/l to 7.1±0.33 (p<0.001) and 6.2±0.22 (p<0.01) in TBM and AM patients respectively and decreased significantly to 3.8±0.15 (p<0.05) and 2.2±0.2 (p<0.001) in PD and MS patients respectively. The levels of CCK-8s were decreased significantly from 20.9±1.3 pmol/l to 11.2±0.5 (p<0.001), 13.3±0.31 (p<0.01) and 16.3±0.46 (p<0.05) pmol/l in PD, CVD and MS patients respectively whereas significant increased levels of 35.3±1.3 (p<0.001) and 29.8±1.35 (p<0.001) in TBM and AM patients respectively were found.

Figure 3 shows the levels of CSF SP, where significant increased levels from 3.2±0.2 pmol/l to 4.4±0.15 (p<0.05), 5.6±0.13 (p<0.001) and 4.9±0.11 (p<0.01)
pmol/l were found in CVD, TBM and AM patients respectively whereas a significant deceased level of 2.6±0.19 (p<0.01) was found in MS patients. The CSF level of SP in PD patients remained unchanged.

Discussion

Neuropeptide Y (NPY) is a peptide consisting of 36 amino acid residues and has been shown to modulate a number of functions of CNS [15]. NPY has several physiological effects [25] of which the most potent are an increase in vasoconstriction [26] and an increase in food intake [27]. NPY is described as the most potent appetite stimulator known [25]. It is found in high concentrations in several regions of the brain including nuclei of brain stem and nerve fibers surrounding cerebral vessels, and it has been proposed to play a role in regulating cerebral blood flow (CBF) and systemic vegetative functions. Vascular inflammation resulting in spasm and thrombosis has been documented during meningitis by histopathology and angiography and is likely to lead to focal reductions of CBF [28]. Clinical and experimental studies have further documented that autoregulation of CBF is lost during meningitis, making CBF directly dependent on cerebral perfusion pressure [29]. Both occurrence of vasospasm and the loss autoregulation suggest that the regulation of cerebral vascular tone may be disrupted during meningitis. The coexistence of NPY with NA has also been demonstrated [30]. NPY plays an important role in anxiety and depression besides eating disorders. Depression is a multifactorial pro-
cesses [7] therefore, it is of interest to study the coexistence of NPY with NA, 5-HT and DA systems relevant to depression since various neurologic patients such as MS show signs of depression commonly. Low levels of NPY in CSF have been reported in depressed patients, particularly in anxiety [31]. Among our patients only PD patients showed increased CSF levels of NPY whereas all other groups showed significantly decreased levels (Fig. 1). The observed reduced NPY concentrations in CSF of MS, CVD, and meningitis patients are of interest since the infection of the CNS due to meningitis and their potential relationship to the local and systematic consequences could be the reason for this change in NPY levels in infection disorder whereas the reduction, as observed in CVD and MS patients, could be attributed to the reduction in CBF. In larger vessels, NPY has been shown to exert powerful vasoconstrictive properties.

The CSF concentration of NPY has been less extensively investigated as compared to other neuropeptides. Few studies have shown decreased levels of NPY in Alzheimer’s disease [34], MS [34, 35] and increased levels in patients with subarachnoid hemorrhage [36].

Treatment with a specific blocker of the NPYY1 receptor resulted in an anxiety-type behavior in rats [38]. Thus, a link between anxiety and NPY seems to exist. There are several lines of evidence which suggest that NPY is a neurotransmitter or a neuromodulator since it causes contraction of cerebral arteries [39]. NPY in the brain is localized mainly to the large dense core vesicles within nerve terminals [40]. The lack of a gradient might suggest that the spinal cord contributes to the concentration of the peptide in lumbar CSF. Similar results for other peptides including neurotensin, CCK and VIP have been reported [41]. Studies in humans have shown [42] significant concentrations of NPY at all levels of the spinal cord. Thus the lack of a CSF rostro-caudal gradient for NPY indicates a spinal cord or peripheral origin for a fraction of CSF NPY levels.

Cholecystokinin (CCK) peptides constitute a family of homogenous peptides, 4-58 amino acids in length, and derived from their precursor, preprocholecystokinin. CCK is a neuroactive peptide which exists in various forms and exhibits a variety of peripheral and CNS actions [43]. Besides, CCK-peptides attract considerable interest in research due to their part in termination of food intake, dopamine regulated functions and pain mechanism [44, 45]. While some of these effects are mediated by the CCK octapeptide (CCK-8), other CCK peptides have different effects. CCK-8 is believed to be the most prevalent form of CCK in the CNS. It appears that CCK-8 and various analogs may modulate CNS dopamine neurotransmission and have been studied as potential antipsychotic agents [46]. Among the other CCK peptides, its tetrapeptide (CCK-4) is anxiogenic in humans and a considerable amount of data supports its role in anxiety with panicogenic properties with minimal gastrointestinal effects [44].

Since CCK-ergic neurotransmission can be detected throughout the brain, it is not surprising that such an interaction occurs with many other neurotransmitter systems and that CCK has a variety of functional implications. Since the first years of research on CCK in the CNS, much attention has already been drawn to the interactions between CCK and dopamine [47], and the role for CCK in schizophrenia has been suggested. Interactions with dopamine and with several other neurotransmitters such as GABA, serotonin and noradrenaline are also of interest with regard to anxiogenic-like effects, their involvement in cognitive processes and the CCK-ergic modulation of opioid actions, and of pain perception. Central CCK (octapeptide, CCK-8s) has also been implicated in other functional roles such as a mediator of satiety responses, a regulator of sexual and maternal behavior and of seizure activity. As may be expected from the involvement of CCK in several of these processes, CCK has also been shown to interact with other peptides and steroid hormones, CCK apparently has a role as a modulator of learning and memory [48]. The involvement of CCK (tetrapeptide, CCK) in anxiety responses is supported by numerous reports based on animal studies as well clinical observation [49]. Previously, we have shown [35] decreased levels of CCK-4 and CCK-8s in MS patients; however, the results in patients with PD, mood disorders, eating disorders have indicated inconsistent observations [44]. Our present study shows decreased levels of CCK-4 in PD and MS patients whereas increased levels in TBM and AM patients. The level of CCK-8s is decreased in PD, CVD and MS patients and increased in TBM and AM patients (Figure 2).

Substance P (SP) is an undecapeptide which derives from alpha, beta and gamma preprotachykinin gene transcripts and is a neurotransmitter or neuromodulator of primary nociceptive afferents [50]. SP is the most well known tachykinin and was, for many years, the only one known to exist in mammalian tissues. SP has received great attention due to its interaction with classical transmitters. In the CNS, SP is found in most regions with the highest levels in the substantia nigra and in the dorsal horns of the spinal cord. In the raphe nuclei in the brain.
stem SP is known to coexist with serotonin in neurons that project to the ventral spinal cord [51]. SP is an undecapeptide with an almost established role as neurotransmitter or neuromodulator [52]. The interaction between SP and serotonin is of importance in view of many studies linking serotonin to depressive illness [11]. In pathways descending from the raphe nuclei, SP is found in the same neurons as serotonin [1]. In accordance with the observation in rat, the basal ganglia are the regions in human brain most abundantly containing SP-L1 with the substantia nigra having the highest level. Very few studies are directed to compare the levels of SP in neurodegenerative diseases; however in Huntington’s disease, patients showed [53] that SP-L1 levels in caudal nucleus and putamen are unchanged while those in globus pallidus and substantia nigra are substantially reduced. Brains from patients suffering from Parkinson’s disease also seem to have decreased levels of SP in globus pallidus and substantia nigra as compared to healthy controls [54]. Unilateral injection of SP into substantia nigra of the rat evolves dose-dependent contralateral motoric behavior, possibly indicating activation of the ipsilateral nigro-striatal dopamine pathway [55]. Furthermore, the behavioral excitation induced by a bilateral injection of SP into substantia nigra is abolished in rats with 6-OH DA lesion of the nigro-striatal dopamine pathway suggesting a possible excitatory effect of SP on DA neurons [56]. Very few studies are conducted which study the levels of SP in CSF from the patients with neurologic disorders, however, since SP plays a very important role in the CNS, it is very likely that CSF would provide us with its role in various neurological disorder. However, since SP plays a very important role in the CNS, it is very likely that CSF would provide us with its role in various neurological disorders. Our results indicate an increased level of CSF SP in CVD, TBM and AM patients and a decreased level in MS patients (Figure. 3).

From this study, mapping of neuropeptide genes and the studies of their linkage could provide clues to genetic factors involved in these diseases. On the basis of future results on degenerative disorders, the role of various neurotransmitters and the role of neuropeptides could be clearly defined in order to develop drug therapy.

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