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Levels and proteolytic processing of chromogranin A and B and secretogranin II in cerebrospinal fluid in neurological diseases

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Summary. Human cerebrospinal fluid (CSF) contains chromogranin A and B and secretogranin II which represent peptides secreted from neuronal large dense core vesicles. Within these vesicles these precursor peptides are at least partly processed to smaller peptides. We analysed the CSF levels of chromogranins/secretogranin by radioimmunoassay using specific antisera. The degree of their processing was characterized by molecular sieve column chromatography followed by radioimmunoassay.

As previously shown secretogranin II is fully processed to smaller peptides including the peptide secretoneurin, whereas processing of chromogranin A was more limited. For chromogranin B we found in this study a high degree of processing comparable to that of secretogranin II. An analysis of CSF from patients with multiple sclerosis, essential tremor, Alzheimer and Parkinson disease, did not reveal any differences in proteolytic processing of chromogranins/secretogranin when compared to control CSF. We conclude that in the four diseases investigated there is no change in the proteolytic processing of the chromogranins/secretogranin within the large dense core vesicles. The absolute levels of chromogranins/secretogranin varied in CSF collected in different hospitals, however their relative ratios were remarkable constant. We suggest to use this ratio as a parameter to standardise CSF levels of other peptides, e.g. neuropeptides. In Parkinson patients the chromogranin A/secretogranin II ratio was significantly increased whereas in Alzheimer

patients and those with essential tremor and multiple sclerosis no change of the ratios was observed. Apparently there are only limited changes in the biosynthesis, processing, secretion and CSF clearance of these peptides in pathological conditions.

Keywords: Chromogranin, secretoneurin, Parkinson's disease, Alzheimer, multiple sclerosis.

Introduction

Neurons secrete a secretory cocktail derived from two types of subcellular organelles, the small synaptic vesicles storing the classical neurotransmitters and the large dense core vesicles. These latter ones contain various neuropeptides (Hökfelt, 1991) but in addition the so called chromogranins (Blaschko et al., 1967). These peptides comprise components named chromogranin A (Schneider et al., 1967), chromogranin B (Fischer-Colbrie and Frischenschlager, 1985), secretogranin II (Rosa and Zanini, 1981; Rosa et al., 1985), secretogranin III (Dopazo et al., 1993), 7B2 (Marcinkiewicz et al., 1985) and VGF (Trani et al., 1995). They are found throughout the neuroendocrine system (see Winkler and Fischer-Colbrie, 1992; Fischer-Colbrie et al., 1995; Rosa and Gerdes, 1994). Evidence has accumulated to suggest that these proteins represent precursors of functional peptides, like pancreastatin, vasostatin and parastatin derived from chromogranin A (Iacangelo and Eiden, 1995), secretolytin from chromogranin B (Strub et al., 1995) and secretoneurin from secretogranin II (Saria et al., 1993; Reinisch et al., 1993). In accordance we have demonstrated that in brain these peptides are proteolytically processed to a high degree (Kirchmair et al., 1993, 1995; Leitner et al., 1996). Peptides derived from chromogranin A (Kirchmair et al., 1994), chromogranin B (Salahuddin et al., 1989) and secretogranin II (Kirchmair et al., 1994) were found to be present in cerebrospinal fluid in concentrations significantly higher than those of other peptides. This result made it possible to study the degree of the proteolytic processing by molecular sieve chromatography. It was found that secretogranin II was completely processed to the free peptide secretoneurin whereas the processing of chromogranin A was more limited (Kirchmair et al., 1994). We argued that analysing these peptides in CSF would allow conclusions about any disturbances of the proteolytic processing in large dense core vesicles (LDV) from which these peptides are released. In a first study on the CSF of schizophrenic patients there was no apparent change in the proteolytic processing but the chromogranin A/secretogranin II ratio was significantly elevated (Miller et al., 1996). In two recent studies it has been claimed that in Alzheimer's disease there are changes in the levels and in the processing of chromogranin A in CSF (Sekiya et al., 1994; Blennow et al., 1995). On the other hand, O'Connor et al. (1993) found an unchanged chromogranin A level in Alzheimer, but a strongly decreased one in Parkinson patients.

In the present study we have now also characterized the degree of proteolytic processing of chromogranin B in CSF. We then analysed CSF from patients with various neurological diseases (Alzheimer, multiple sclerosis,

essential tremor and Parkinson) in order to detect changes in peptide levels for chromogranin A, B and secretogranin II or in the degree of their proteolytic processing.

Materials and methods

Collection of CSF

This study involved patients from 4 medical centres (Departments of Psychiatry at the University of Innsbruck and at the University of Würzburg, Saint George Hospital, Budapest, and the OPTIMA, University of Oxford). The patients and controls had the following characteristics:

- *Innsbruck:* controls (1): mean age: 53 (9 m: male, 8 f: female); controls (2): mean age: 35 (10 m, 12 f); multiple sclerosis: mean age: 39 (6 m, 10 f). For these patients CSF samples were collected as a diagnostic test. Only samples of those patients were analysed for which the diagnosis was confirmed by the presence of oligoclonal bands (isoelectric focusing) in the CSF.
- *Würzburg:* controls: mean age: 55 (12 m, 9 f); Parkinson: mean age: 63 (2 m, 1 f: three newly diagnosed cases with Hoehn-Yahr rating I to II); inflammatory diseases: mean age: 42 (6 m, 6 f).
- *Oxford:* controls: mean age: 82 (1 m, 1 f); Alzheimer: mean age: 80 (3 m, 7 f); Alzheimer plus other forms of dementia: mean age: 66 (2 m, 3 f); other forms of dementia: mean age: 73 (2 m, 3 f).
- *Budapest:* controls: mean age: 48 (7 m, 1 f); Parkinson: mean age: 63 (2 m, 3 f). These patients had the disease for 1 to 10 years (2 cases one year, one case each 3, 4 and 10 years) with the following Hoehn-Yahr ratings (I, 2xIII, IV, V). Essential Tremor (see Deuschl et al., 1987; Málly et al., 1996): mean age: 42 (1 m, 1 f).

Informed consent was obtained from all patients. Before lumbar puncture the skin of the patients was disinfected and anaesthetized with a local volatile anaesthetic. Subjects were in a sitting position and CSF was obtained from L3–L4 or L4–L5 interspaces. In the patients from the Saint George Hospital cisternal CSF was obtained. Ventricular CSF was obtained during brain operations.

Extraction of samples

CSF samples (0.5 to 1.0 ml) were boiled for 10 min and then centrifuged at 10.000 g for 20 min. The supernatants were lyophilized and used for RIA or molecular sieve chromatography.

Radioimmunoassay (RIA)

Antisera were raised against synthetic peptides representing amino acid sequences in chromogranin A (GE-25: Kirchmair et al., 1994, 1995), chromogranin B (PE11: Kroesen et al., 1996) and secretogranin II (secretoneurin: Kirchmair et al., 1993). The specificity and sensitivity of the RIA have been already described in detail (GE-25: Kirchmair et al., 1995; PE-11: Kroesen et al., 1996; secretoneurin: Kirchmair et al., 1993). It should be pointed out that the antisera against GE-25 and secretoneurin do not only react with the free peptide but also with the precursor molecules including chromogranin A and secretogranin II, whereas the antiserum against PE-11 only reacts with the free C-terminus of this peptide (see Kroesen et al., 1996). Therefore in order to detect the total immunoreactivity of this peptide sequence samples had to be digested with trypsin (for methodical details see Kroesen et al., 1996).

Molecular sieve chromatography

Lyophilized CSF-samples were dissolved in column buffer and subjected to molecular sieve chromatography (HR 10/30 gel-filtration: Pharmacia LKB, Sweden) as already described in detail (Miller et al., 1996). The eluted fractions were analysed by RIA. For some experiments the fractions were digested with trypsin (see Kroesen et al., 1996) before RIA.

Results

Characterization of PE-11 immunoreactivity in CSF

PE-11 represents an amino acid sequence present in the chromogranin B molecule. The antiserum used only reacts with the free C-terminal end of this peptide (see Kroesen et al., 1996). When the immunoreactive material present in human CSF was subjected to molecular sieve chromatography followed by a PE-11 RIA only one major immunoreactive peak was found (see Fig. 1) irrespective of the source of the CSF (lumbar, cisternal or ventricular). However, this peak did not elute in the position of the free peptide as shown by the addition of exogenous PE-11 (see Fig. 2). The endogenous peptide eluted

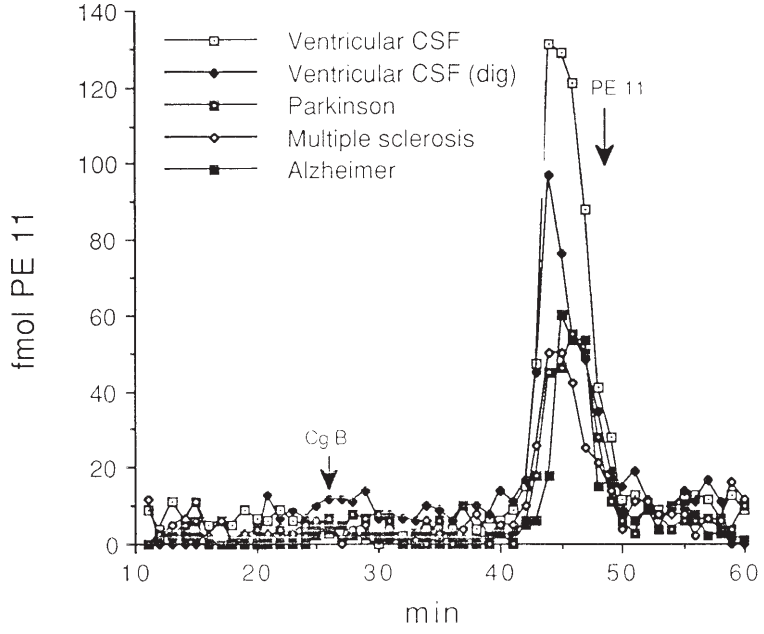

Fig. 1. Characterization of PE-11 immunoreactivity in CSF by molecular sieve chromatography. Samples of CSF from controls (ventricular CSF) and patients with multiple sclerosis (lumbar CSF), Alzheimer (lumbar CSF) or Parkinson (lumbar CSF) were subjected to molecular sieve chromatography followed by RIA for PE-11. A representative result is shown for each case. For each curve shown an analogous result (not shown) was obtained in a second experiment (with the exception of the Alzheimer CSF which was only analyzed in one experiment). One major immunoreactive peak was eluted after 44 min whereas the eluting position of the free peptide (PE-11) is at 49 min. Trypsin digestion of the eluted fraction (ventricular CSF dig.) did not reveal any additional immunoreactive peaks

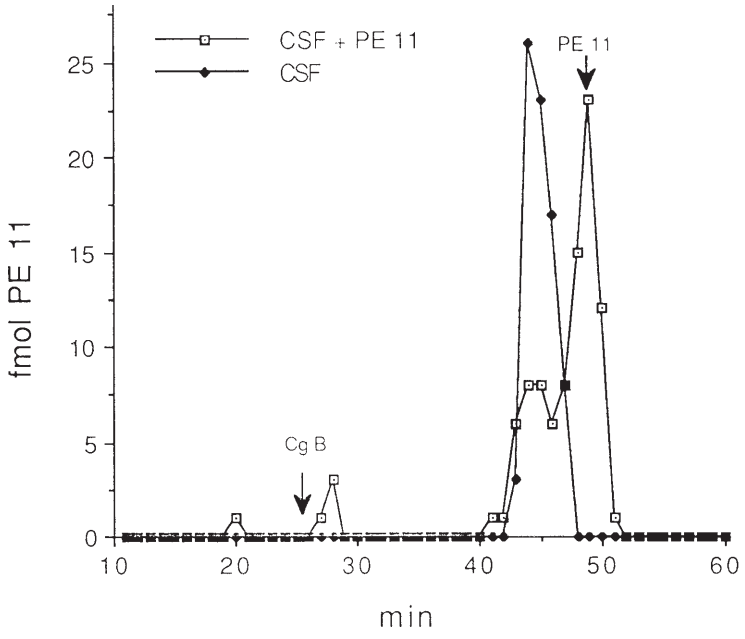

Fig. 2. Comparison of CSF-PE11 immunoreactivity with the free peptide. A CSF sample with and without the addition of the free peptide PE-11 was subjected to molecular sieve chromatography followed by RIA for PE-11. The free peptide added to the CSF elutes about 5 min after the immunoreactive peak present in the CSF sample

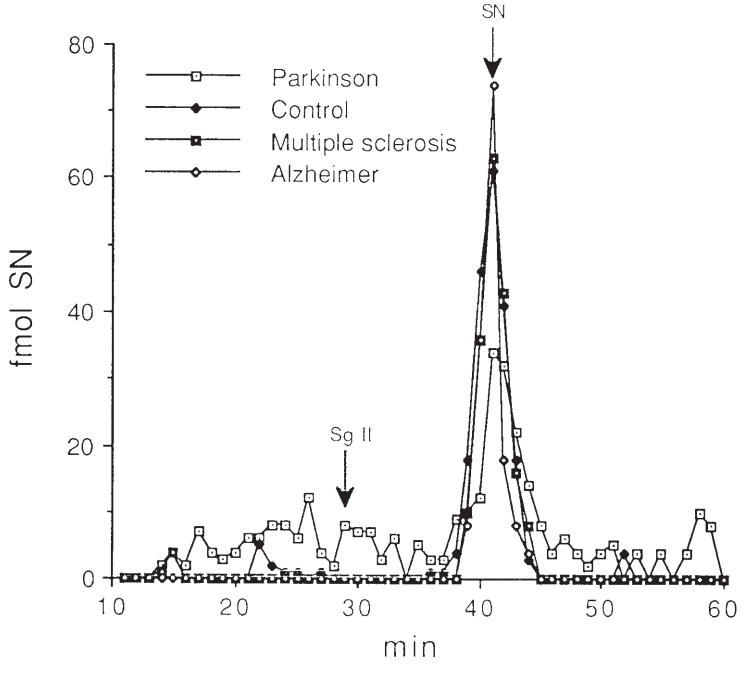

Fig. 3. Characterization of secretoneurin immunoreactivity in CSF by molecular sieve chromatography. Samples of CSF (lumbar: control, multiple sclerosis and Alzheimer, cisternal: Parkinson) were subjected to molecular sieve chromatography followed by RIA for secretoneurin. Representative results are shown for each case, analogous results were obtained for 5 other Alzheimer CSF's, for three control samples and for one other Parkinson CSF

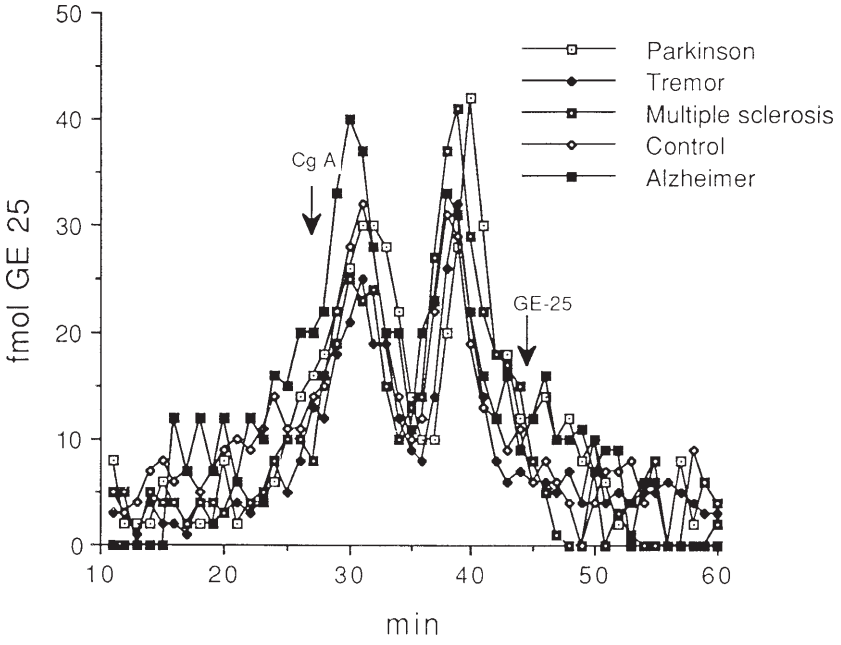

Fig. 4. Characterization of GE-25 immunoreactivity in CSF by molecular sieve chromatography. Samples of CSF (lumbar: control, multiple sclerosis, cisternal: Parkinson) were subjected to molecular sieve chromatography followed by RIA for GE-25. Representative results are shown for each case, analogous results were obtained for several further CSFs (2 for Parkinson, 3 for tremor and multiple sclerosis, 4 for Alzheimer and 7 for controls)

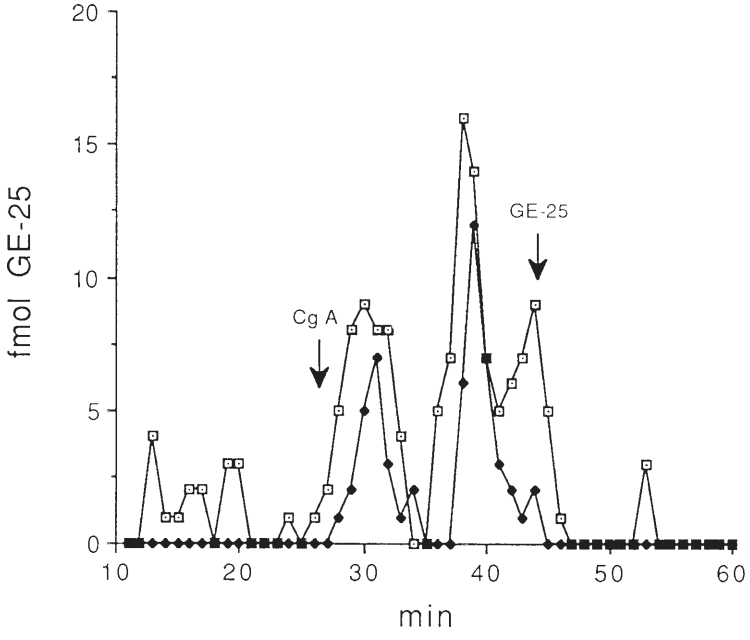

Fig. 5. Characterization of GE-25 immunoreactivity in ventricular CSF. Samples of ventricular CSF were subjected to molecular sieve chromatography followed by RIA for GE-25. Two representative analyses are shown demonstrating that in half of the samples the free peptide GE-25 was apparently present

earlier indicating the presence of a N-terminally elongated form of PE-11. Peptides eluting in this position are about 25 amino acids long (compare GE-25, see Fig. 4).

In order to demonstrate whether larger peptides were present which contained the PE-11 sequence in a form not recognised by the antibody fractions from the HPLC column were digested with trypsin. This treatment (see Fig. 1) did not reveal any hidden immunoreactivity.

Immunoreactivity of GE-25, PE-11 and secretoneurin in various diseases

The immunoreactivities were characterized by HPLC. It should be pointed out that the antisera against GE-25 and secretoneurin also react with the precursor proteins. Thus even without trypsin digestion all peptides containing the respective sequences are detected. Secretoneurin immunoreactivity eluted always in one peak irrespective whether lumbar, cisternal or ventricular CSF were analyzed (representative examples are shown in Fig. 3). For GE-25 there was no evidence for the presence of the free peptide in lumbar and cisternal CSF (Fig. 4), since only two intermediate immunoreactive peaks

Table 1. Levels of chromogranins in CSF. The results (fmol/ml ± S.E.M. are grouped according to the source of the CSFs (number of patients in parenthesis). All samples were collected from the lumbar site with the exception of those from Budapest and CSF samples from the ventricles collected in Innsbruck. The asterisks indicate a significant difference versus the respective controls: * $p < 0.05$ ** $p < 0.001$ (student t-test)

	Cg A	Sg II	Cg A/Sg II	Cg B	Cg B/Sg II
Würzburg					
Controls	1084 ± 127 (21)	1309 ± 151 (21)	0.86 ± 0.06 (21)	117 ± 16 (14)	0.11 ± 0.02 (14)
Parkinson	1905 ± 318 (3)	1576 ± 242 (3)	1.20 ± 0.02 (3)*	107 ± 21 (3)	0.07 ± 0.02 (3)
Inflammatory diseases	1099 ± 272 (12)	1160 ± 189 (12)	1.04 ± 0.22 (12)	106 ± 18 (9)	0.11 ± 0.03 (9)
Innsbruck					
Controls 1	992 ± 91 (16)	1398 ± 146 (17)	0.79 ± 0.04 (16)	135 ± 11 (7)	0.14 ± 0.02 (7)
Controls 2	1295 ± 164 (22)	1836 ± 193 (22)	0.73 ± 0.05 (22)	150 ± 21 (7)	0.08 ± 0.01 (7)
Multiple sclerosis	909 ± 83 (16)	1504 ± 170 (16)	0.66 ± 0.06 (16)	165 ± 29 (7)	0.10 ± 0.02 (7)
Ventricular	487 ± 127 (7)	686 ± 234 (7)	0.84 ± 0.16 (7)	60 ± 13 (4)	0.09 ± 0.02 (3)
Oxford					
Controls	1662 ± 25 (2)	1949 ± 339 (2)	0.88 ± 0.17 (2)	184 ± 3 (2)	0.10 ± 0.02 (2)
Alzheimer	1540 ± 312 (10)	1747 ± 316 (10)	0.91 ± 0.05 (10)	98 ± 15 (6)	0.07 ± 0.02 (6)
Alzheimer + Dementia	1242 ± 170 (5)	1691 ± 422 (5)	0.90 ± 0.19 (5)	104 ± 25 (5)	0.07 ± 0.02 (5)
Dementia	1368 ± 453 (5)	1784 ± 594 (5)	0.78 ± 0.06 (5)	73 ± 20 (4)	0.05 ± 0.01 (4)
Budapest (cistemal)					
Controls	1044 ± 133 (8)	1591 ± 243 (8)	0.69 ± 0.06 (8)	164 ± 30 (5)	0.11 ± 0.01 (5)
Parkinson	962 ± 188 (5)	896 ± 199 (5)	1.11 ± 0.10 (5)**	92 ± 17 (4)	0.13 ± 0.02 (4)
Tremor	1842 ± 446 (2)	1868 ± 194 (2)	0.96 ± 0.14 (2)		

were eluted. In the cisternal CSF results were variable, since in 3 out of 6 cases the free peptide was found (in Fig. 5 two representative analyses one with the free peptide and one without are shown).

As shown in Fig. 4 for GE-25, for secretoneurin in Fig. 3 and for PE-11 in Fig. 1 the elution pattern of the immunoractive material in the various diseases were practically identical to control patients.

CSF levels of GE-25, PE-11 and secretoneurin in various diseases

Table 1 presents the values obtained for CSF from various sources. The concentration of the chromogranins in the ventricular CSF is clearly lower than in lumbar or in cisternal CSF. In controls the absolute levels of the three peptides exhibit some variation (for GE-25 in lumbar CSF e.g. from 992 fmol/ml to 1662 fmol/ml), however, the ratios of two respective peptides show excellent agreement irrespective of the source of the CSF (ventricular, cisternal or lumbar). For these ratios significant differences between patients and controls were only found for the two groups of Parkinson patients.

Discussion

We have previously demonstrated that in human brain and CSF chromogranin A and secretogranin II are processed to smaller peptides (Kirchmair et al., 1994). As also shown again in this study, secretogranin II appears completely processed to secretoneurin, whereas for chromogranin A intermediate breakdown products predominate. We have now extended this to chromogranin B by using an antiserum against PE-11 representing an 11 amino acid long sequence in this molecule.

After molecular sieve chromatography only one immunoreactive peak representing an elongated form of this peptide was found. Since the antiserum only reacts with the free C-terminal end of PE-11 (see Kroesen et al., 1996) we had to incubate the fractions eluted from the column with trypsin in order to discover also any PE-11 sequences hidden within a larger molecule. However, although this approach revealed the precursor peptides in adrenal medulla (see Kroesen et al., 1996) in human CSF no additional peptide was discovered. Thus we can conclude that in human brain the precursor chromogranin B is fully processed to a slightly elongated form of the peptide PE-11. In rat brain with the same antibody as used in the present study we found complete processing of chromoganin B to the free peptide PE-11 (Kroesen et al., 1996). Apparently in rat the dibasic cleavage site on the N-terminal end of PE-11 can be cleaved, whereas in human brain this appears not possible. Thus the cleavage occurs further up the C-terminal end. The most likely cleavage site is a monobasic one at position 537 (Benedum et al., 1987), which would yield a peptide of 25 amino acids. Interestingly like in human brain also in bovine tissues (Wolkersdorfer et al., 1996) this elongated peptide appears to be predominant.

To what degree do the peptides found in CSF reflect their occurrence in brain? We have already pointed out previously (Kirchmair et al., 1994) that the concentrations of the chromogranins in CSF are significantly higher (e.g.

for secretoneurin 170 times) than in serum. Thus serum chromogranins are unlikely to contribute to CSF levels. For secretogranin II we have already shown that in both brain and CSF only the free peptide secretoneurin can be found (Kirchmair et al., 1994). For chromogranin A there was a discrepancy between the peptides present in brain and CSF, since in brain two intermediate peptides and free GE-25 were found whereas in CSF the free peptide GE-25 was practically absent (Kirchmair et al., 1994). One of the explanations we offered at that time was that the free peptide was preferentially removed from the CSF. Our present results seem to support this suggestion since at least in ventricular CSF (which is likely to represent secretory material close to the release site from brain) free GE-25 was present in half of the samples. For chromogranin B no data on human brain are available, however, in rat brain this peptide appears also completely processed. Thus we can conclude that in brain secretogranin II and chromogranin B are fully processed to small peptides whereas for chromogranin A processing is more limited. Furthermore analyses of the secreted peptides in CSF yield results representative for brain tissue. Therefore by analysing CSF peptides we can draw conclusions about processing of the chromogranins in the LDV of brain from which they are released and finally reach the CSF.

Is there any change in proteolytic processing in various diseases? The simple answer is apparently not, at least not for essential tremor, for Parkinson, for multiple sclerosis and for Alzheimer and as previously shown by us (Miller et al., 1996) for schizophrenic patients. For Alzheimer patients Sekiya et al. (1994) reported a difference in the degree of processing of chromogranin A with an antiserum against pancreastatin (a chomogranin A derived peptide: see Winkler and Fischer-Colbrie, 1992). In these patients the immunoreactive material in the CSF appeared of slightly larger molecular size than in control patients. Unfortunately the antiserum used reacted only with the C-terminal glycine amide of pancreastatin and therefore not with larger molecules. In our study, where we detected all GE-25 containing peptides derived from chromogranin A, there was no evidence for a change in processing in Alzheimer patients. However, we can of course not exclude that during the processing of chromogranin A to pancreastatin the proteolytic cleavage is changed only at a site affecting pancreastatin but not GE-25 formation. The same argument may also apply to the very recent finding (Strittmatter et al., 1997), that in CSF of Alzheimer patients there is a relative lack of somatostatin-14 compared to somatostatin-25/28. In fact, it has been shown that the endoproteases likely to be involved in neuropeptide processing have quite a varying activity on different dibasic cleavage sites (Seidah et al., 1994). One might of course argue for all the diseases investigated that a change in proteolytic processing appears unlikely. This certainly applies to the possibility that there is a genetic change in processing, however, it has recently been shown that the prohormone convertases responsible for neuropeptides and chromogranin processing are up- and downregulated by stimulation of neurons through hormones and by neuronal activity (Bloomquist et al., 1991; Day et al., 1992; Meyer et al., 1996). Thus in diseases affecting so profoundly the central nervous system a change in the activity of these enzymes and conse-

quently a change in chromogranin and neuropeptide processing might appear quite possible, however our present study now clearly argues against it, at least as far as the chromogranins are concerned. Of course our data for the chomogranins which are widespread in brain do not exclude a change in processing confined to a small subregion of this organ.

The levels of the chromogranins were lower in ventricular CSF when compared with cisternal and lumbar CSF which is in agreement with O'Connor et al. (1993). Apparently chromogranins are released throughout the brain and therefore accumulate from the ventricles to the cisterna.

The absolute levels of the chromogranins in cisternal and lumbar CSF varied to some degree. This may be due to differences in sampling technique and other factors. However we already pointed out previously that the ratio of chromogranin A to secretogranin II showed much less interindividual variation (Kirchmair et al., 1994) and this is borne out again by the present results. Thus the chromogranin A/secretogranin II ratio from the controls in three different centres were all within the narrow range of 0.73 to 0.88. Therefore we consider changes in these ratios as more likely to be relevant than those in absolute levels. In Alzheimer patients this ratio was not changed. This is in agreement with O'Connor et al. (1993) who found normal chromogranin A levels in these patients. On the other hand, Sekiya et al. (1994) reported, that the level of pancreastatin, a peptide derived from chromogranin A was lower in the CSF of Alzheimer patients, whereas Blennow et al. (1995) found a lower chromogranin A level only in a subgroup of these patients. In Parkinson patients the chromogranin A/secretogranin II ratio was significantly increased. Since an analogous change was observed in the patients from Würzburg and in those from Budapest the results appear reliable although the number of patients was small. This result is in apparent contradiction to the findings of O'Connor et al. (1993) who reported very low levels of chromogranin A in Parkinson patients. However, their cases were patients at a very late stage of the disease prior to adrenal to caudate autotransplantation. On the other hand our patients were in early or at the most intermediate stages of the disease. One might therefore suggest that at the beginning of the disease there is an overproduction or oversecretion of chromogranin A followed later by a degeneration of chromogranin A producing or secreting neurones. Obviously, a further study including a larger number of Parkinson patients at various stages of the disease seems warranted. Why the chromogranin A/secretogranin II ratio should be high in patients in earlier stages of Parksinson disease is difficult to interpret. A similarly high ratio was found previously for drug free schizophrenic patients with their first attack (Miller et al., 1996). There is no obvious common feature of these two diseases and therefore it seems at present prudent to abstain from complicated speculations.

Numerous papers have reported changes in neuropeptide levels in various neurologic and psychiatric diseases (see e.g. for schizophrenic patients Liebermann and Koreen, 1993). As shown in the present study absolute values for peptides like the chromogranins are variable. It might be advisable for future studies to relate neuropeptide levels to those of the chromogranins

or their ratios in order to reduce interindividual variation which might then help to pinpoint true changes in peptide levels.

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