Chapter 31

The possible role of peptides derived from food proteins in diseases of the nervous system

W.H. Reichelt and K.L. Reichelt

Institute of Paediatric Research, University of Oslo, Rikshospitalet, N-0027 Oslo, Norway

Introduction

A role of gluten and possibly casein in schizophrenia was proposed by Dohan (1980). He found a correlation of gluten consumed to the incidence of schizophrenia of 0.96. In cultures where gluten was sparse or absent so was schizophrenia (Dohan et al., 1984).

Dietary acculturation led to a sharp increase in the incidence of schizophrenia (Lorenz, 1977). We found a large increase in peptides in the urines of schizophrenics (Reichelt et al., 1981; Reichelt et al., 1983). These peptides showed bioactivities relevant to schizophrenia (Hole et al., 1979; Reichelt et al., 1985). The opioid activity found in urine is also found in serum as part of a general hyperpeptidaemia (Drysdale et al., 1982; Idet et al., 1982; Lindstrom et al., 1986). Increased opioid receptor binding peptides were also found in the CSF (Lindstrom et al., 1986).

Hyperpeptiduria has also been found in autism (Reichelt et al., 1981; Reichelt et al., 1986; Israngkun et al., 1986; Shattock et al., 1990). Bovine casomorphin 1–8 was found in both urine and dialysis fluid of DSM III diagnosed autistic patients (Reichelt et al., 1991). Increased serum opioid activity has likewise been found in this syndrome (Gillberg et al., 1983; LeBoyer et al., 1994). In general hyperpeptidaemia is accompanied by hyperpeptiduria.

Methods

Demonstration of hyperpeptiduria

To counteract possible diurnal variations in peptide secretion, 24 h complete urines were used. Creatinine was measured twice for each urine by the Department of Clinical Chemistry, Rikshospitalet, Oslo. 0.5 ml urine was applied to Costar spin-X centrifugal cellulose acetate filters 0.22 μm and centrifuged at 2315 × g for 10 min at 4 °C.

Urine equivalent to 250 μg/ml of creatinine was then applied to C18 reverse phase columns on the HPLC system Gold for analysis. The fractionation is based on Guilemin’s method to separate peptides from amino acids and salts (Bohlen, 1980). The column was Vydac C18 250 × 4.6 mm with
the same pre-column and a column heater (BioRad Code no 1250 426) kept at 35 °C (Shattuck et al., 1990). Flow rate was 1 ml/min with detection at 215 and 280 nm and sensitivity 0.2. Buffer A was 0.1 per cent trifluoroacetic acid (TFA) and buffer B: 0.1 per cent TFA in 94.9 per cent acetonitrile and 5 per cent water. The elution was performed with: Step 1, using buffer A with 1 per cent B for 15 min to wash out most amino acids, urea, and salts; Step 2, a gradient from 1–40 per cent B was run for 60 min linearly (15–75 min); Step 3, from 75–80 min with B from 40–60 per cent; Step 4, from 80–89 min isocratically at 60 per cent buffer B; Step 5, return to 1 per cent B from 89–94 min: Step 6, re-equilibration at 1 per cent B from 89–115 min.

An automatic sample applicator (Waters 717 Autosampler from Millipore) was used, and the computer integrated the area under the curve over the peptide area on the column at 215 and 280 nm. The thymol peak eluting at 85 ml was subtracted.

**Purification of peptides**

(1) Batch isolation of peptides was done on complete 24 h urines filtered through Whatman 3MM filter after adjusting the pH to 3 and then passed through a C-18 preparative column of dimension 2.6 × 50 cm at pH 3. After washing with 100 ml 10 mM TFA the peptides were eluted in 80 per cent methanol/water by volume and rotavapored (Böhlen et al., 1980).
(2) First fractionation was a gel filtration on P 2 gels 1.6 x 90 cm in 0.5 M acetic acid with elution rate 24 ml/h and fractions of 4 ml. The application volume was 10 ml 0.5 M acetic acid at room temperature (acetic acid is a protease inhibitor). Off-line ninhydrin colour was developed after alkaline hydrolysis (Reichelt et al., 1981).

(3) The stepwise further purification has been extensively described for tissue specific mitosis inhibitors (Reichelt et al., 1990). Briefly the following purification sequence was used: (a) Cation exchange to separate protonable from nonprotonable mainly N-substituted peptides, (b) Anion exchange to separate out the strong anions, (c) Gel filtration on Fractogel MG 2000 from Merck in 1 M acetic and 20 mM HCl to determine size, (d) C-18 reverse phase gradient chromatography in TFA with n-propanol, methanol and acetonitrile gradients, (e) C-8 reverse phase in TFA acetonitrile gradients.

Characterization of the peptides

The complete amino acid composition was ensured by 6 M HCl hydrolysis in closed vials, evaporation of acid over KOH and P_2O_5 in vacuum and amino acid analysis on the α-plus (Pharmacia) amino acid analyser using ninhydrin technique. Sequencing was performed by manual Edman degradation (Reichelt et al., 1990).

Coechromatography with synthetic peptide in two different buffer systems and gradients on the HPLC was also taken as proof of identity.
Receptor assay of opioids

The opioid receptor displacement by material from the different peaks after C-18 peptide separation was carried out as described (Terenius & Wahlstrom, 1975) using synaptic plasma membranes. The first assays were performed by Terenius who is thanked for this help.

Immunoassay

Demonstration of binding to antibodies against bovine casomorphin was first carried out by Prof. Teschemacher in Giessen (Koch et al., 1988). He generously donated antibody and also gave training sessions to several members of our group. Cross reactivity with human casomorphins was below 0.01 per cent (Koch et al., 1988).

Table 1. Quantitative data on schizophrenia and autism

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Schizophrenics</th>
<th>Autistic individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>10</td>
<td>1250</td>
<td>913.5</td>
</tr>
<tr>
<td>95% C.I.</td>
<td></td>
<td>401–2099</td>
<td>704.5–1122.5</td>
</tr>
</tbody>
</table>

All patients were without medication. The autistic patients were obtained from seven countries. The schizophrenic patients are all Norwegian collected over three years. DSM III diagnoses were used. C.I. is Confidence Interval.
Schizophrenia

Figure 1 and 2 compared to Fig. 3 illustrates: (a) The large increase in peptides in the peptide region compared to normal; (b) the enormous heterogeneity of the patterns obtained in spite of the same diagnosis and rating (not shown). This confirms our earlier data with a different method (Reichelt et al., 1985). In Table 1 the quantitative data are given for untreated schizophrenics and autistic patients. Figure 4 shows the gradient HPLC on semipreparative C-18 reverse phase columns (Partisil M9 10/25 ODS) of the peptide peaks from the batch isolation of peptides (Bohle et al., 1980). The peaks that showed opioid receptor binding and effects are marked (Hole et al., 1979). In Table 2 the effect of diluting the test sample in the receptor assay for opioids is shown. This is quite common for peptides.

### Table 2. Opioid receptor binding

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Specific activity undiluted</th>
<th>Specific activity diluted (x10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.78</td>
<td>4.27</td>
</tr>
<tr>
<td>7</td>
<td>1.8</td>
<td>64.0</td>
</tr>
</tbody>
</table>

Specific activity = displacement of labelled dehydromorphine measured in picomoles opioid/micromole hydrolysis released amino acids in the peptide peak.

Immunoreactivity against antibodies to bovine casomorphin 1–8 is shown in Table 3 for fractions obtained from peak W in Fig. 4. The rechromatography can be seen in Fig. 5. This immunoreactivity could be diluted away and severely reduced by preincubation with cold casomorphin.

After several chromatographic steps the peptide E showed cochromatography with synthetic bovine casomorphin 1–8 (Fig. 6). In addition to immunoreactivity it showed receptor binding to opioid
receptors. Amino acid analysis showed the same composition as the synthetic compound obtained from Novabiochem, UK. (Y 0.9; P 3.8; F 1; G 1; I 0.95). Y-P-F was the N-terminal sequence. The other immunoreactive peaks were not analysed.

**Autism**

The quantitative aspects can be seen in Table 1. Fig. 6 demonstrates the gel filtration of a peak containing an opioid binding peptide from autistic subjects (Reichelt et al., 1991). The immunoreactivity can be seen in Table 3. This peptide showed cochromatography with synthetic bovine casomorphin 1–8 (Reichelt et al., 1991) and the correct amino acid composition after hydrolysis.

**Table 3. Bovine casomorphin 1–8 immunoreactive peaks in schizophrenics (Fig. 4)**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Dilution</th>
<th>F-Moles/peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1:100</td>
<td>2001</td>
</tr>
<tr>
<td></td>
<td>1:10000</td>
<td>17</td>
</tr>
<tr>
<td>B</td>
<td>1:100</td>
<td>1330</td>
</tr>
<tr>
<td>C</td>
<td>1:100</td>
<td>1526</td>
</tr>
<tr>
<td>D</td>
<td>1:100</td>
<td>1280</td>
</tr>
<tr>
<td>E</td>
<td>1:100</td>
<td>1040</td>
</tr>
</tbody>
</table>

Controls

<table>
<thead>
<tr>
<th>Dilution</th>
<th>F-Moles/peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>0.17</td>
</tr>
<tr>
<td>1:100</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Autistic subjects (Fig. 7)

<table>
<thead>
<tr>
<th>Dilution</th>
<th>F-Moles/peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>1163.04</td>
</tr>
<tr>
<td>1:10</td>
<td>187.07</td>
</tr>
</tbody>
</table>

1/30 of each peak was tested. For autistic subjects only the peak where immunoreactivity was found is shown. Only a few examples of dilution included.
Previously published findings

We found increases over the upper normal limit in IgA antibodies against gliadin, β-lactoglobulin and casein (Reichelt & Landmark, 1995). The stable epitopes were the exorphin sequences. The
behavioural effects induced by partly purified peptide fractions from schizophrenics clearly demonstrated opioid activity (Hole et al., 1979; Drysdale et al., 1982; Idet et al., 1982). Also GABA and Glutamate releasing peptides were found (Reichelt et al., 1981, 1985).

Discussion

The presence of casomorphin antibody reactive peptides in autistic subjects (Reichelt et al., 1991) and in schizophrenics described support Dohan (1980) hypothesis. Dohan thought that schizophrenia was caused by gluten and possibly casein overloading a genetic defect in their breakdown. Schizophrenia is rare in cultures without gluten (Dohan et al., 1984; Lorenz, 1990). This view is reinforced by the immune data (Sugarman, 1982). Serum from schizophrenics inhibits the chemotactic movement of lymphocytes (Ashkenazi et al., 1979), and the gliadin peptide that causes this effect contains the gliadinmerphic sequence (Graf et al., 1987) and binds to opiate receptors. In male schizophrenics statistically significant increase in IgA antibodies against gliadin, β-lactoglobulin and casein were found (Reichelt & Landmark, 1995). In autistic subjects an increased frequency of higher than normal IgA values against casein and gliadin and gluten was also found (Reichelt et al., 1991)

The following can be deduced from the autistic and schizophrenic patterns (Figs. 1 and 2): (a) There is an enormous quantitative spread in the level of peptides secreted in the urine (Reichelt et al., 1985). (b) There are large variations in the pattern of peptide peaks obtained. This must mean that autism and schizophrenia are syndromes caused by different enzymes in different patients ending up with peptides of different chain lengths but the same bioactivity. The multiple peaks immunoreactive to casomorphin (bovine) 1–8 antibodies (Fig. 4; Table 3) confirm that opioids of different chain lengths are present as with most bioactive peptides. (c) The opioid activity found shows bell-shaped dose–response curves (increasing receptor binding on dilution). This may explain the contradictory data on opioids in schizophrenia and autism. Very concentrated opioid solutions do not bind to the receptor (Table 2). It is therefore not surprising that the effect of naloxone and naltrexone varies too (Gunne et al., 1977; Janowsky et al., 1977). Peptides very frequently show bell-shaped dose–response curves (Moon, 1988; Pincus et al., 1990; Reichelt et al., 1990). It is therefore quite important to test out isolated peptide factors over a very wide range of concentrations.

Because peptiduria is caused by defects in peptide metabolism (Abassi et al., 1992; Watanabe et al., 1993), it is reasonable to expect that different peptidases in different families may be the cause. Because peptidases are regulated by and often inhibited by hormones (Griffiths, 1975) like testosterone and cortisone; the relationship of disease to puberty and stress in schizophrenia is understandable.

Increased uptake from the gut of exorphins can furthermore be caused by peptidase defects or inhibition (Mahe et al., 1989) but also by trans sulphation defects (Waring & Nung, 1993). Uptake of intact and bioactive proteins (Husby et al., 1986; Jakobsson et al., 1986) may be the cause of multiple sequences of opioids present in one molecule of protein (Pukudome & Yoshikawa, 1991), causing large peptide increases if poorly broken down. Uptake of intact proteins is confirmed by finding intact and dietary proteins in mother’s milk (Stuart et al., 1984; Troncone et al., 1987). Botulinum toxin is a case of such small but tragic uptake.

Sufficiently prolonged treatment with gluten-free diet has therapeutic effects in at least some schizophrenics (Dohan & Grasberger, 1973; Singh & Kay, 1976; Reichelt et al., 1990b), and in autistic individuals (Reichelt et al., 1991; Knivsberg et al., 1995). Because the improvements progressed over four years in autistic children (Knivsberg et al., 1995) this probably excludes placebo effects. Those that quit the diet showed regression (Reichelt et al., 1991, Knivsberg et al., 1995).

Finally the long term effect of neuroleptics supports a peptidase involvement, because they induce
peptidases (Traficante & Turnbull, 1982; Koning et al., 1990; Konkoy et al., 1993). Neuroleptics cause decrease in peptides found in the urine (Reichelt & Teigland-Gjerstad, 1995). Furthermore Lithium in therapeutic doses directly stimulates pyroglutamyl aminopeptidase (DeGandaries et al., 1994). Because about half the peptides in the urine are pyrogluuropeptides (Reichelt et al., 1985), this direct enzyme activation may also be important.

**Consequences of opioid accumulation**

Opioid increases would inhibit the normal maturation of the CNS. (Zagon & McLaughlin, 1987). Inhibited maturation is found in schizophrenia (Crow, 1994; Roberts & Bruton, 1990). Opioids may well also interfere with the important maturational pruning process (Feinberg, 1982/83) and thus cause progressive dysfunction. Casomorphins are known to be the mediators of the dramatic state of post-partum psychosis (Lindstrom et al., 1984).

Opioids furthermore inhibit socialization and inhibit separation distress calls in chicken, puppies and kittens (Panksepp et al., 1978; Panksepp, 1978). It is therefore possible that opioids may be the mediators of the social isolation typical of both schizophrenia and autism. This has been proposed (Panksepp et al., 1980).

**Conclusions**

Immune data and the high levels of peptides indicate a dietary source of hyperpeptidemia and hyperpeptiduria. The effect of diet and the isolation of probable exorphins from the urine and dialysis fluid all support Dohan's hypothesis (Dohan, 1980).

Because opioids can enter the cerebrospinal fluid, the inhibition of maturation of the CNS may be ascribed to such compounds. Opioids furthermore may cause social isolation of significance to both autism and schizophrenia.

**References**


236
Chapter 31 Role of peptides in diseases of the nervous system


237