Central nervous system immunoreactive somatostatin, substance P and met-enkephalin concentrations in experimental hepatic encephalopathy in the rat

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Summary

Immunoreactive somatostatin, substance P and met-enkephalin concentrations were measured in various regions of the rat brain 65 hours after portacaval shunt and compared with concentrations in sham-operated animals. No significant difference was detected in any of the three peptides in the regions studied, suggesting that these peptides do not play a role in the pathogenesis of hepatic encephalopathy.

The pathogenesis of hepatic encephalopathy is unclear. Available evidence favours a defect in neurotransmission as opposed to reduced energy production. Abnormal levels of a wide variety of true and false neurotransmitters have been found in animal models of hepatic encephalopathy. Increased concentrations of brain amino acids found in these experimental animals have stimulated interest in neurotransmitters. The wide but uneven distribution of neuropeptides in the central nervous system suggests that they may fulfill neurotransmitter or neuromodulator roles. We have examined the concentrations of immunoreactive somatostatin, substance P and met-enkephalin in various areas of the brain in rats after portacaval shunt and have found that these concentrations are unchanged in this model of hepatic encephalopathy.

Material and methods

Surgical procedures

Adult male Long-Evans rats weighing 300-350 g and housed under controlled conditions of temperature, humidity and lighting were used in the study. All operations were performed under diethyl ether anaesthesia. End-to-side portacaval shunts were carried out on 10 rats as previously described. Sham operation on 10 rats consisted of laparotomy with occlusion of the inferior vena cava and portal vein for 12 minutes. The rats were killed by rapid decapitation 65 hours after operation. All rats were denied food for the 65-hour postoperative period but were allowed free access to water.

Sampling and tissue extraction

After the rats had been decapitated their brains were rapidly removed and dissected into the various regions on an ice-tray at 4°C. Immediately after dissection all tissue samples were weighed, snap-frozen in liquid nitrogen and stored in a container of solid CO2. Frozen tissues were homogenized in 2N acetic acid (5 ml per tissue sample) and separated into two equal parts. Homogenates were placed in a bath of boiling water for 20 minutes to inactivate peptidases, re-homogenized and centrifuged at 2000 g for 30 minutes at 4°C, after which the supernatant was lyophilized. Immediately before assay for somatostatin and substance P the lyophilized tissue extracts were reconstituted in 5 ml 0.01M sodium phosphate in 0.15M sodium chloride buffer, pH 7.8 (PBS), containing 0.2% bovine serum albumin (crystalline BDH) and 0.05M disodium ethylene diamine tetra-acetic acid (EDTA). For estimation of met-enkephalin levels, the lyophilized tissue extracts were reconstituted in 5 ml of the same buffer, but without the addition of EDTA. Protein concentration was measured by the method of Lowry et al. after solubilization using sodium deoxycholate. Recovery of added synthetic somatostatin prior to assay was 45% and that for substance P and met-enkephalin 85%.

Radio-immunoassays

Somatostatin immunoreactivity was assayed by radio-immunoassay as previously described. Antiserum (S,) was raised in rabbits against synthetic cyclic somatostatin conjugated by carbodiimide condensation to whelk haemocyanin. Synthetic somatostatin was labelled with 125I using chloramine-T; this was followed by purification on a Whatman CM52 cellulose column. The antiserum showed low cross-reactivity (< 0.01%) with a wide range of peptide hormones and neurotransmitters, including secretin and glucagon, which share a 10-13 tetrapeptide sequence. Studies with somatostatin fragments and analogues indicate that the antiserum binds the middle region of somatostatin. It therefore reacts poorly with somatostatin degradative products but cross-reacts with N-terminally extended forms of somatostatin. The sensitivity of the assay was 30 pg/ml with an interassay coefficient of variation of 19.2%.

Antiserum (P,) was raised in rabbits against synthetic substance P conjugated by carbodiimide condensation to whelk haemocyanin. Synthetic substance P was labelled with 125I using chloramine-T followed by centrifugation with QUSO G32 powder. The antiserum showed low cross-reactivity (< 0.01%) with a wide range of peptide hormones and neurotransmitters, and the sensitivity of the assay was 30 pg/ml with an interassay coefficient of variation of 17.5%.
Met-enkephalin was assayed using an antiserum (1952) produced in rabbits against met-enkephalin conjugated to bovine serum albumin by glutaraldehyde condensation as previously described. 11, 12 Met-enkephalin was iodinated using chloramine-T followed by Biogel-P2 purification. Since met-enkephalin undergoes varying degrees of spontaneous oxidation to the sulphoxide, and the antiserum binds avidly to this species, all met-enkephalin standards and tissue extracts were oxidized with H₂O₂ prior to assay.11, 12 Sensitivity of the radioimmunoassay was 23 pg/ml. Intra- and inter-assay coefficients of variation were 4.7% and 4.7% respectively. Cross-reactivity with leu-enkephalin was < 0.01% and with both native and oxidized endorphins was negligible. Only fragments or analogues of met-enkephalin containing the C-terminal sequence -Gly-Phe-Met- showed any cross-reactivity, indicating that the antiserum is highly specific for the C terminus. Therefore, although met-enkephalin degradative products might be quantitated by the assay, none of the C-terminally extended forms of opioid peptides are measured.

Identification of peptides in brain extracts

Studies directed at the partial identification of substance P, somatostatin and met-enkephalin were undertaken. Dilution curves of tissue extracts were compared with those of synthetic standard peptides for parallelism and the elution characteristics of extracted and synthetic peptides compared on Biogel-P2 and Sephadex G-25 permeation chromatography.

Results

Identification of peptides

Met-enkephalin immunoreactivity in a variety of tissue extracts diluted out in parallel with the synthetic peptide. Regression analysis of the logit-log-transformed data exhibited no significant differences (P > 0.8).11 An extract of rat brain emerged as two major immunoreactive peaks on Sephadex G-25 chromatography with antiserum 1952, which recognizes met-enkephalin and the oxidized form11 (Fig. 1). The second peak co-eluted with met-enkephalin and the first peak was tentatively identified as somatostatin-enkephalin sulphoxide. This identification is further supported by the demonstration that the material in the first peak did not interact with the antiserum 1954, which binds the oxidized form poorly12 (Fig. 1). Only very low levels of immunoreactivity were detectable in the elution position of the endorphins.

Somatostatin immunoreactivity from hypothalamic extracts diluted out in parallel with the synthetic peptide using this antiserum.9 On Sephadex G-25 and cellulose CM-32 cation exchange chromatography, however, several immunoreactive peaks were detected. One of these is identifiable as somatostatin-28. Since this peptide also yields parallel displacement curves it is clear that somatostatin immunoreactivity of extracts of brain regions represents the sum of a number of related somatostatin species.

Substance P immunoreactivity from hypothalamic and spinal cord diluted out in parallel with a synthetic standard10 and extracts of this region co-eluted as a single peak on Sephadex G-25(1) chromatography in the same region as synthetic substance P.9

Neuropeptide concentrations in brain regions.

Brain concentrations of somatostatin (Table I), substance P (Table II) and met-enkephalin (Table III) after portacaval shunt did not differ significantly from those found in sham-operated rats.

| Table I. Immunoreactive Somatostatin Concentrations (ng/mg Protein) (Mean ± SEM) |
|---------------------------------|-----------------|-----------------|
| Brain region                    | Sham            | PCS             |
| Olfactory lobe                  | 12.08 ± 2.09 (10)* | 12.45 ± 2.15 (9) |
| Septum and pre-optic area       | 40.12 ± 4.66 (9) | 30.26 ± 6.09 (10) |
| Striatum                        | 15.98 ± 1.56 (9) | 14.32 ± 1.88 (10) |
| Hypothalamus                    | 97.25 ± 17.67 (10) | 63.76 ± 13.00 (10) |
| Cortex                          | 8.25 ± 0.71 (8)  | 9.15 ± 0.94 (9)  |
| Thalamus                        | 6.16 ± 0.30 (10) | 5.28 ± 0.50 (10) |
| Midbrain                        | 9.88 ± 0.88 (10) | 7.47 ± 0.84 (10) |
| Brainstem                       | 9.49 ± 0.86 (9)  | 8.94 ± 0.92 (9)  |
| Spinal cord                     | 16.48 ± 2.64 (9) | 14.65 ± 3.53 (9) |

*The number of rats in each group is shown in parentheses. | Sham = sham-operated rats, PCS = portacaval-shunted rats. No significant differences between the two groups could be detected.

The immunoreactive concentrations of somatostatin were lower in the experimental group than in sham-operated animals, but no differences reached statistical significance. Although the trend in immunoreactive concentrations of substance P is similar to those of somatostatin, concentrations of I-substance P were higher in brainstem and spinal cord of the experimental group; these differences, however, were not statistically significant. The
On the other hand, immunoreactive somatostatin is clearly comprised of a number of species, and we have recently shown that N-terminally extended forms (e.g. somatostatin-28) are the major forms stored and secreted in the hypothalamus. In the present study we have not positively identified the nature of the immunoreactive material quantitated and have therefore used the term 'immunoreactive' peptide.

Since the pathogenesis of hepatic encephalopathy is likely to be multifactorial and since abnormal levels of peptide neurotransmitters have been claimed to play a role in the causation of liver coma, we have measured the concentrations of the inhibitory peptide somatostatin and the excitatory peptide substance P as well as the concentrations of the putative neuromodulator met-enkephalin in the brain of rats subjected to portacaval shunt.

Somatostatin, substance P and met-enkephalin immunoreactivity showed no statistically significant alteration in the brain regions in the rats studied after portacaval shunt, suggesting that these neuropeptides do not play a role in the causation of hepatic encephalopathy. However, the possibility of changes in neuropeptide concentration in more discrete regions than those dissected in this study cannot be excluded. Furthermore, changes in the release and turnover of these neuropeptides might not be reflected in alterations in tissue concentration.

It is therefore premature to conclude from our studies on neuropeptide concentrations in brain regions that changes in neuropeptide biosynthesis, secretion and turnover do not play a part in hepatic encephalopathy. Clearly more extensive investigations of these aspects are required before a definitive conclusion can be drawn.

We wish to thank the South African Medical Research Council, the South African Atomic Energy Board and University of Cape Town Staff Research Fund for financial assistance and J. King, M. S. Hendricks and G. Engelbrecht for technical assistance.

**REFERENCES**