Journal of Peptide Science
J. Peptide Sci. **6**: 168–174 (2000)

GABA-induced Changes of the Tissue-specific Peptide Pool of White Rat Brain

ANDREI A. KARELIN[a,*], MARINA M. PHILIPPOVA[a], ELENA V. KARELINA[a], BORIS N. STRIZHKOV[a], IGOR V. NAZIMOV[a], VADIM T. IVANOV[a], RAISA A. DANILOVA[b] and IGOR P. ASHMARIN[b]

[a] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Ul. Miklukho-Maklaya 16/10, 117871 Moscow, Russian Federation
[b] Biological Department, Moscow State University, Vorob'evy gory, Moscow, Russian Federation

Received 15 September 1999
Accepted 20 September 1999

Abstract: Internasal administration of γ-aminobutyric acid (GABA) induced prolonged behaviour changes and the appearance of three new compounds absent in the brain extracts of control rats. Two peptides associated with GABA administration were isolated and sequenced: Thr-Tyr-Thr-Phe, which corresponds to a γ-immunoglobulin segment, and Val-Leu, which is present in a great number of proteins, hence its precursor could not be established. The third compound was not amenable to the Edman degradation technique. The data obtained show that the introduction of a neurotransmitter could cause specific changes in the levels of tissue-specific peptide components. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: γ-aminobutyric acid (GABA); brain peptides; endogenous peptides

INTRODUCTION

The systematic analysis of peptide components of bovine and rat brain has shown that brain tissue contains a limited set of components (500–800) detectable by computer analysis of the chromatograms [1,2]. The major components were isolated and identified as fragments of functional proteins. Brain tissue contains a set of fragments of neurospecific proteins (myelin basic protein and neurogranin), fragments of several cellular enzymes, the major source of the peptides being haemoglobin [1–4]. The chromatographic analysis of individual brain samples has shown that the set of brain-derived peptides is quite stable under normal conditions [1,3].

The sets or the pools of peptide components of other sources, such as rat heart, spleen and lung tissues, lysate and supernatant of human erythrocytes, being quite stable at normal conditions, significantly differ from each other and from the peptide pool of brain [5–7]. That is, the composition of the components of a tissue-specific peptide pool can be considered as an important characteristic of the tissue.

However, significant changes in the composition and the content of peptide components of brain were shown to accompany the pathologies associated with alterations of brain metabolism, such as Alzheimer's disease [3] and brain ischemia [8]. Changes in the levels of haemoglobin fragments were detected in human erythrocytes for Hodgkin's disease [9] and lymphosarcoma [10], and in lung tissue during lung carcinoma [11]. Levels of haemoglobin-derived peptides were shown to increase in human blood plasma of donors subjected to physical exercise [12]. On the basis of the data given above, the composition and the content of components of tissue-specific peptide sets were suggested to reflect both the pathological and the compensatory changes in the organism [7,13].

* Correspondence to: Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Ul. Miklukho-Maklaya 16/10, 117871 Moscow, Russian Federation; e-mail: karelin@ibch.siobc.ras.ru

This paper studies the changes in content and composition of low molecular weight components of rat brain induced by the introduction of a well-defined neuromediator, γ-aminobutyric acid (GABA).

MATERIALS AND METHODS

Preparation of Brain Specimen of Control Rats

Non-thoroughbred white rats (12 animals) were kept in groups of three for 1 week under normal conditions. After that period, the animals were killed by dislocation of the neck. Brains were removed immediately and placed in liquid nitrogen. The procedure of killing was completed within 40–50 s after removal of each rat from the cage. No animals were kept in the room where the killing took place. Investigators responsible for manipulating the specimens over the course of the study were not involved in the killing.

Preparation of Brain Specimen of Rats with GABA-mediated Behaviour Reactions

GABA (0.1 ml, 22 μg per animal) was introduced internasally. The experimental group contained six animals. The physiological solution (0.1 ml) was introduced to the control animals (six rats per group). The animals were sacrificed as described above after 3 days from the beginning of the experiment. The effects of GABA were examined for in tests of locomotor and research activities and non-receptive sensitivity. The evaluation of locomotor and research activities was carried out using the automatic complex 'Rodeo'. The level of GABA-induced analgesia was determined by means of the tail flick test. The tail was placed into a reservoir with water pre-heated to 55°C. The time to elicit a tail flick response was determined.

Preparation of Rat Brain Extracts

Individual specimens of rat brain were stored in liquid nitrogen. Brain preparations were homogenized in a Potter's homogenizer in 10% acetic acid (5 ml). The mixture obtained was centrifuged at 10 000 rev/min for 8 min. The supernatant was lyophilized. All procedures were carried out within 12–15 min of starting at 0°C.

Size-exclusion Chromatography

The size-exclusion separations of individual brain specimens were performed using a liquid chromatography set (Pharmacia/LKB, Sweden) equipped with Sephadex G-25 sf column (1.0 × 85 cm) equilibrated with 0.1 M acetic acid (see Figure 1A and B). The fractions obtained were lyophilized.

Reverse-phase HPLC Separation

Analytical separation of fractions B, obtained after size-exclusion chromatography of individual brain extracts from control rats and from rats treated with GABA (Figure 1), was carried out on a Nucleosil 7μ C_8 (4.6 × 250 mm) column equilibrated with 0.1% trifluoroacetic acid (TFA).

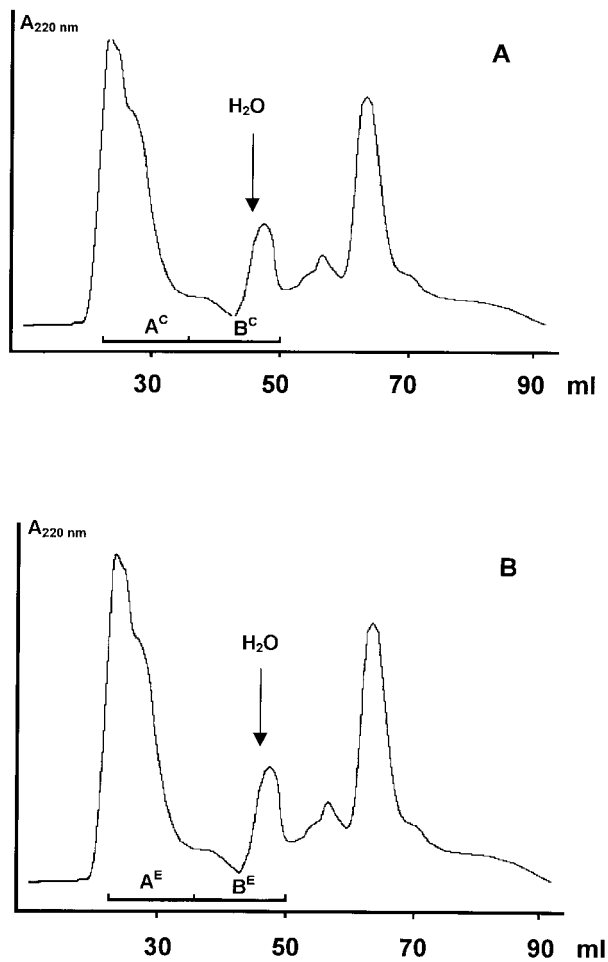

Figure 1 Size-exclusion chromatography of acidic extracts of individual rat brain preparations on Sephadex G-25 sf column (1.0 × 85 cm) equilibrated with 0.1 M acetic acid. A – typical elution profile for preparations obtained from brain tissue of intact animals; B – typical elution profile for preparations obtained from brain tissue of animals subjected to GABA administration. Collected fractions are marked.

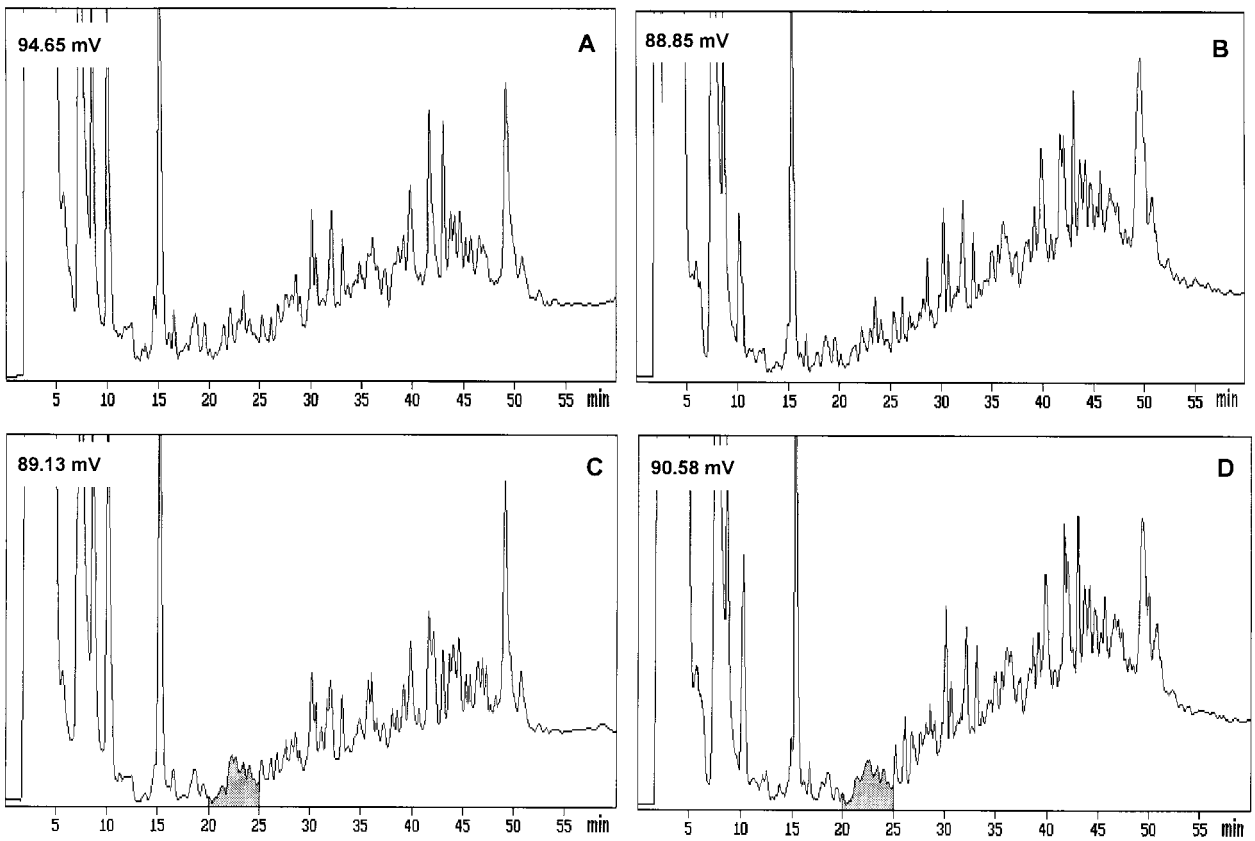

Figure 2 RP-HPLC of fractions B^C (control animals) and B^E (experimental animals) (Figure 1) obtained after size-exclusion chromatography of acidic extracts of individual rat brain specimens on Nucleosil 7μ C_8 column (4.6 × 250 mm) equilibrated with 0.1% TFA. Elution was performed by acetonitrile concentration gradient 0–50%; duration: 60 min; flow rate: 0.8 ml/min); wavelength detection: 226 nm (1800 mV = 2.56 AUFS); sample size: 1/2 from total fractions B. A and B — elution profiles for preparations obtained from brain tissue of intact animals (Figure 1A); C and D — elution profiles for preparations obtained from brain tissue of animals after GABA administration (Figure 1B). Elution zones differing in the brain samples of the control and the experimental animals are marked.

Chromatographic conditions are given in the legend to Figure 2.

Semi-preparative separation of the combined fractions B was carried out on a Nucleosil 7μ C_8 (10 × 250 mm) column. Chromatographic conditions are given in the legends to Figures 3–5.

The elution profiles were obtained using an integrator (Ampersend, Russian Federation) at 226 nm. The optical density values given in Figures 2–5 correspond to the formula: 2.56 OD = 1800 mV. Analysis of the elution profiles was performed with the Multi-Chrom V2.60 program (Ampersend, Russian Federation).

The stability and reproducibility of the data obtained were checked by analysis of elution profiles from three independent experiments. Both the comparative analysis of elution profiles and the sequencing data were used to verify the reproducibility of the results.

Peptide Sequencing

Amino acid sequences were determined by means of a gas-phase sequencer (Model 477A, Applied Biosystems).

Sequence Identification

Identification of the sequences established was performed using the PIR databank, version 38.

RESULTS AND DISCUSSION

Internasal administration of GABA (22 μg per animal) induced reliable changes in the behaviour of

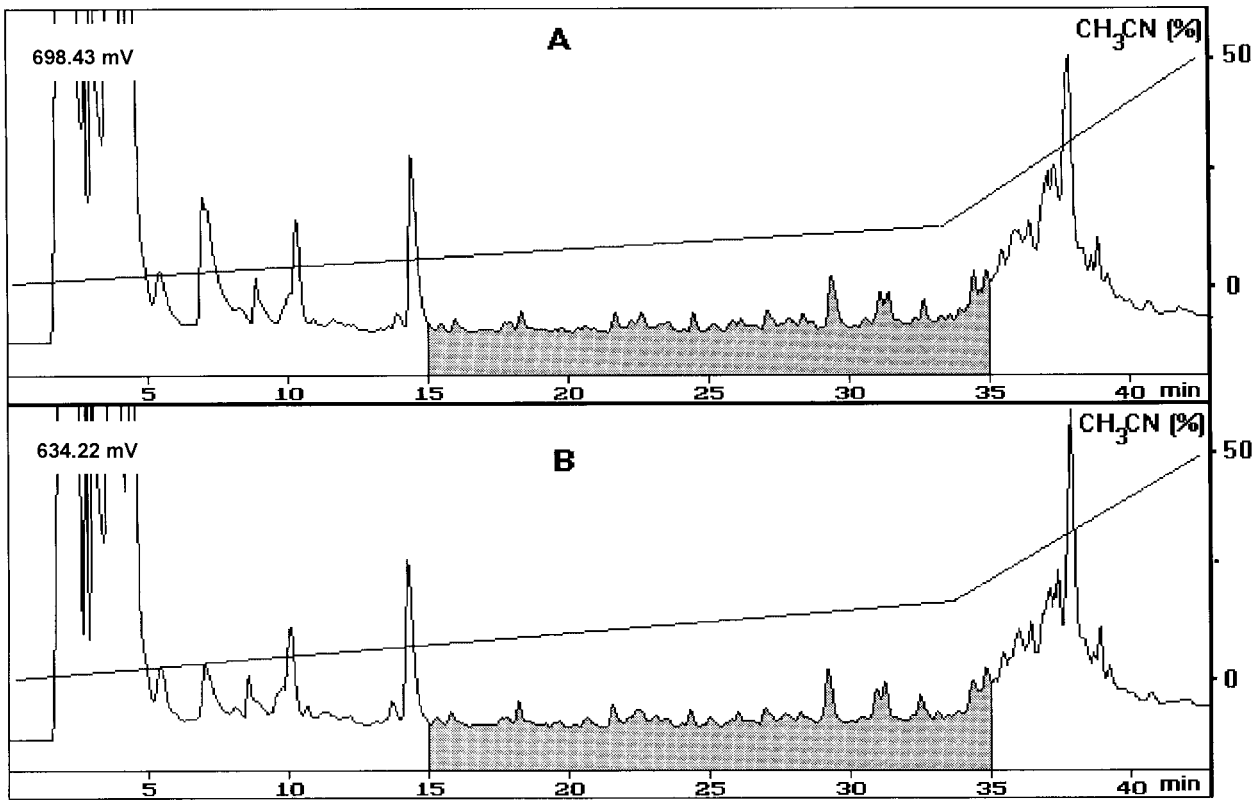

Figure 3 RP-HPLC of combined fractions B (Figure 1) on Nucleosil 7μ C_8 (10 × 250 mm) column equilibrated with 0.1% TFA. Gradient conditions are given. Flow rate: 2.5 ml/min; wavelength detection: 226 nm (1800 mV = 2.56 AUFS). A – fractions B^C corresponding to control animals; B – fractions B^E corresponding to experimental animals. Elution zones with detectable differences are marked.

rats. The behavioural characteristics were determined for different time intervals after administration (from 1 h to 14 days). As a result, GABA was shown to cause a pronounced stimulation of locomotor and research activities. The effect was detected within 7–10 days of introduction, with maximal activity at days 3–5. Internasal administration of GABA also caused a reliable increase in the time required to elicit a tail flick response, i.e. it induces an analgesic effect.

The aim of the study was to determine the possible changes in composition of peptides induced by the introduction of a neuromediator. The animals were separated into small groups and kept under normal conditions. The brain preparations were frozen in liquid nitrogen immediately after the decapitation procedure. The method of killing excludes prolonged stress action. Extracts of individual specimens of rat brain were obtained after homogenization in 10% acidic acid at 4°C within 5–7 min. The obtained extracts were centrifuged and then lyophilized. The conditions applied ensured minimal post-mortem proteolysis of brain components [2,4,14].

Brain extracts corresponding to individual animals were separated using size-exclusion chromatography. Chromatographic separation was carried out under standard conditions. All extracts gave highly reproducible elution profiles. Typical profiles corresponding to control (A) and experimental (B) animals are given in Figure 1. Comparative analysis revealed no reliable differences between the elution profiles of brain preparations within the control group and the experimental animals.

Fractions A and B corresponding to individual animals were subjected to RP-HPLC. The portions corresponding to a half of the material comprising fractions A and B were analysed. The elution profiles of fractions A^C (1.0–4.5 kDa) (not shown) and B^C (containing the compounds with molecular weight < 1 kDa and the sorbed material) (Figure 2A and B) from the control animals were, as expected [1], highly reproducible.

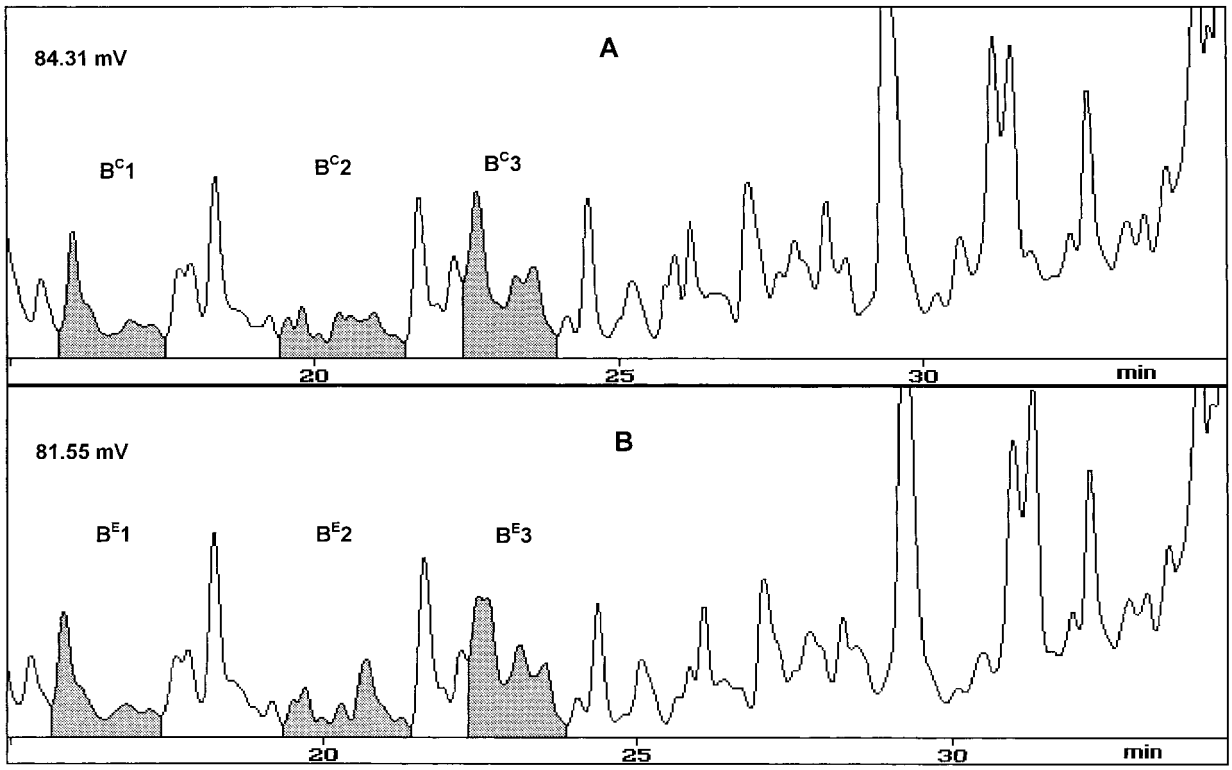

Figure 4 Enlarged elution profiles marked in Figure 3. Elution zones with detectable differences are shaded and marked as B^C2 and B^E2; B^C3 and B^E3, respectively. The corresponding fractions containing no detectable differences are shaded and marked as B^C1 and B^E1.

In the case of the experimental animals, no detectable changes in the set of components of high molecular weight fractions A^C and A^E were found (not shown), while local changes were observed in the elution profile of fraction B^E (Figure 2C and D). Zones of the elution profiles differing in the groups of the control and the experimental animals are shaded.

The high reproducibility of the elution profiles in the groups of control and experimental animals allowed the combination of peptide material present in fractions B. The resultant portions contain the material corresponding to all remaining brain extracts of the control or the experimental rats. The combined fractions B were subjected to a standard chromatographic procedure (Figure 3A and B). Elution zones differing in the brain preparations of the control and the experimental group of animals are marked. As seen from the expanded fragments of elution profiles given in Figure 4, only two fractions corresponding to the elution zone given in Figure 3, i.e., B^C2 and B^C3, B^E2 and B^E3, show detectable differences due to GABA introduction. In contrast, elution zones corresponding to fractions B^C1 and B^E1 were identical.

The fractions corresponding to elution zones B^C1, B^C2, B^C3, B^E1, B^E2 and B^E3 were subjected to further separation (Figure 5). Comparative analysis of the profiles obtained has demonstrated that the detected differences result from the presence of two novel substances in fraction B^E2 (Figure 5B and E) and a single substance in fraction B^E3 (Figure 5C and F). To confirm the similarity of the elution profiles of fractions B^C1 and B^E1, the latter were subjected to chromatographic separation under the same conditions and were found to have no detectable differences. The substances corresponding to the peaks marked in Figure 5E and F were isolated to homogeneity and subjected to automatic Edman degradation, giving rise to the amino acid sequences Thr-Tyr-Thr-Phe and Val-Leu, shown in Figure 5E and F. The third shaded peak (Figure 5E) contained material for which the amino acid sequences could not be determined. This component had an absorbance maximum at 268 nm, i.e. this substance is not of peptide nature and does not

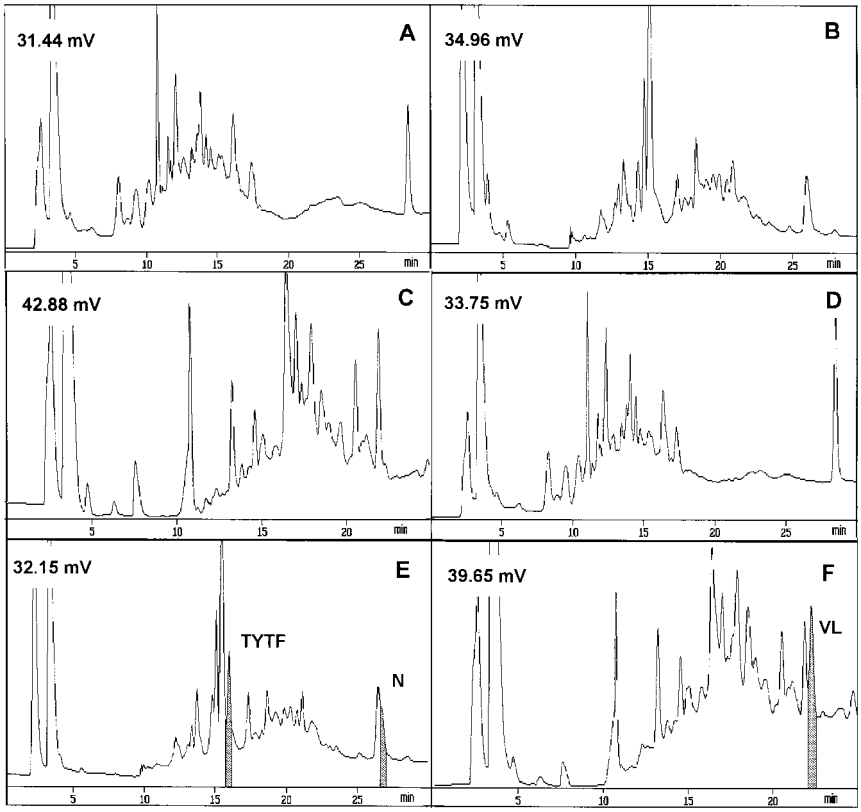

Figure 5 RP-HPLC of fractions B^C1 (A), B^E1 (D), B^C2 (B), B^E2 (E), B^C3 (C) and B^E3 (F) (Figure 4) on Nucleosil 5μ C_{18} (4.0×250 mm) cartridge equilibrated with 0.05 mM ammonium–acetate buffer (pH 4.8). Elution was performed by acetonitrile concentration gradient 0–10%; duration: 30 min; flow rate: 0.75 ml/min); wavelength detection: 215 nm (1800 mV = 2.56 AUFS). The fractions containing the substances detected after GABA administration are marked and respective amino acid sequences are given.

contribute to changes in the brain peptide pool caused by GABA administration. The content of pure material was not sufficient for more detailed structural analysis. The peptide Thr-Tyr-Thr-Phe corresponds to a γ-immunoglobulin ε-chain segment. The dipeptide Val-Leu might be derived from a great number of protein precursors. Considering that this sequence is repeated six times in rat haemoglobin, and taking into account the high content of the dipeptide in brain tissue (more than 0.3 nmol/g tissue), we suggest that the Val-Leu dipeptide might be of haemoglobin origin. The data obtained show that the introduction of a neurotransmitter might cause relatively prolonged and specific changes in the levels of tissue-specific peptide components. These peptides could be involved in prolonged behaviour changes induced by GABA introduction.

The comparative analysis of peptide components of individual samples of brain of the control rats has demonstrated that the peptide set of brain tissue is highly stable if animals are kept under normal conditions. These results support our previous data on the stability of peptide pools of different tissues under normal conditions.

At the same time, the introduction of GABA gives rise to at least three new compounds in brain tissue, two of them being of peptide nature. These changes are highly reproducible and non-dependent on individual differences of the animals. That is, homeostatic changes induced by GABA introduction include distinct changes of the composition of brain-derived peptides. Analogous to the changes in peptide sets of tissues associated with the pathologies, we believe them to participate in or to result from the compensatory processes at tissue and cellular levels [8,13]. Although the mechanisms of these alterations are not yet established, the data obtained suggest an essential *in vivo* regulatory role of the fragments of functional proteins.

REFERENCES

1. Slemmon JR, Flood DG. Profiling of endogenous brain peptides and small proteins: methodology, computer-assisted analysis, and application to aging and lesion models. *Neurobiol. Aging* 1992; **13**: 649–660.
2. Ivanov VT, Karelin AA, Mikhaleva II, Vaskovsky BV, Sviryaev VI, Nazimov IV. Isolation, structure and propoties of novel endogenous peptides. *Soviet J. Bioorgan. Khim.* 1993; **18**: 677–702.
3. Slemmon JR, Hughes CM, Cambell GA, Flood DG. Increased levels of hemoglobin-derived and other peptides in Alzheimer's disease cerebellum. *J. Neurosci.* 1994; **14**: 2225–2235.
4. Karelin AA, Philippova MM, Karelina EV, Strizhkov BN, Grishina GA, Nazimov IV, Ivanov VT. Peptides from bovine brain: structure and biological role. *J. Peptide Sci.* 1997; **4**: 210–225.
5. Ivanov VT, Karelin AA, Philippova MM, Nazimov IV, Pletnev VZ. Hemoglobin as a source of endogenous bioactive peptides. A concept of tissue specific peptide pool. *Biopolymers* 1997; **43**: 171–188.
6. Karelin AA, Blishchenko EYu, Ivanov VT. Peptide pools: species conservative and tissue specific. In *Peptides 1998*, Bajusz S, Hudecz F (eds). Akademiai kiado: Budapest, 1999; 726–727.
7. Karelin AA, Blishchenko EYu, Ivanov VT. A novel system of peptidergic regulation. *FEBS Lett.* 1998; **428**: 7–12.
8. Slemmon JR, Wengenack TM, Flood DG. Profiling of endogenous peptides as a tool for studying development and neurological disease. *Biopolymers* 1997; **43**: 157–170.
9. Pivnik AV, Rasstrigin NA, Philippova MM, Karelin AA, Ivanov VT. Alteration of intraerythrocytes proteolytic degradation of hemoglobin during Hodgkin's disease. *Leukemia Lymphoma* 1996; **22**: 345–349.
10. Philippova M, Blishchenko E, Karelin A, Ivanov V, Pivnik A, Moiseeva T, Karpova I. Alteration of intraerytrocyte proteolytic degradation of hemoglobin during Hodgkin's disease and lymphosarcoma. *Leukemia Lymphoma* 1998; **29**: 111.
11. Zhu YX, His KL, Chen ZG, Zhang HL, Wu SX, Zhang SY, Fang PF, Guo SY, Kao YS, Tsou K. Neo-kyotorphin, an analgesic peptide isolated from human lung carcinoma. *FEBS Letts.* 1986; **208**: 253–257.
12. Nyberg F, Sanderson K, Glamsta E-L. The hemorphins: a new class of opioid peptides derived from blood protein hemoglobin. *Biopolymers* 1997; **43**: 147–156.
13. Karelin AA, Blishchenko EYu, Ivanov VT. Fragments of functional proteins: role in endocrine regulation. *Neurochem. Res.* 1998; **24**: 1119–1126.
14. Yatskin ON, Philippova MM, Blishchenko EYu, Karelin AA, Ivanov VT. LVV- and VV-hemorphins: comparative levels in rat tissues. *FEBS Letts.* 1998; **428**: 286–290.

