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EXPRESSION AND DISTRIBUTION OF CARBOXYPEPTIDASE B IN THE HIPPOCAMPAL SUBREGIONS OF NORMAL AND ALZHEIMER'S DISEASE BRAIN

HENRIETTA PAPP,[1] I. TÖRÖK,[2] A. MATSUMOTO,[3] T. ENOMOTO,[4] S. MATSUYAMA[5] and P. KÁSA[1]*

[1]Alzheimer's Disease Research Centre, University of Szeged, H-6720 Szeged, Hungary; [2]Elisabeth City Hospital, Hódmezővásárhely, Hungary; [3]Division of Cell Biology, Kobe University Graduate School of Medicine, Kobe, Japan; [4]Division of Sciences for Natural Environment, Faculty of Human Development, Kobe University, Kobe, Japan; [5]Division of Molecular Pharmacology, Kobe University Graduate School of Medicine, Kobe, Japan

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Earlier neurochemical studies suggested that human brain carboxypeptidase B may play a significant role in the degradation of amyloid-β1-42 in the brain. Using an immunohistochemical technique we report here on the neuronal expression and distribution of this enzyme in the segments (CA1a, CA1b and CA1c) of the CA1 subfield and in area CA4 of the hippocampus in normal and Alzheimer's disease brain samples. Its distribution was compared with the appearance of neurofibrillary tangles in the same brain sample. For immunohistochemical localization of carboxypeptidase B, a specific C14-module antibody was applied, together with the Gallyas silver impregnation technique for the demonstration of neurofibrillary tangles. The results revealed that, in the control samples, most of the immunoreactivity appeared in segment CA1a in the pyramidal cells, less in segment CA1b and least in segment CA1c. In the Alzheimer's disease samples, there was no particular immunostaining in the neurons, but, a large number of silver-impregnated degenerated neurons appeared. The results support the suggestion that carboxypeptidase B may play a significant role in elimination of the intracellular accumulation and toxicity of amyloid-β in the human brain and thereby protect the neurons from degeneration.

Keywords: Carboxypeptidase B – Alzheimer's disease – hippocampus – neurofibrillary degeneration – amyloid-β

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia among people aged 65 or older. It has been suggested that the accumulation of amyloid-β1-42 (Aβ1-42), which is present in small amounts in the normal brain, may play a significant role in the aetiology and neuropathology of the disease. Earlier neurochemical studies revealed that the neurotoxic Aβ1-42 is a cleavage product of the amyloid precursor protein. It is generated by two enzymes, β-secretase and γ-secretase. α-Secretase was at one time thought to be responsible for the degradation of Aβ1-42. Recently, however, it was demonstrated that neprilysin [9, 18, 20] and carboxypeptidase B [15, 16]

* Corresponding author; e-mail: kp@comser.szote.u-szeged.hu

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might also be involved in the metabolic regulation of brain Aβ. If the cellular levels of these enzymes are reduced, the intracellular accumulation of Aβ increases and neurofibrillary degeneration and neurofibrillary tangle (NFT) formation may occur as neurotoxic consequences. Indeed, it has been shown that there is a relationship between Aβ generation and the reduced level of neprilysin in the AD brain [1]. Similarly, carboxypeptidase B has been found to be reduced in the neurons in AD samples [14]. There are no data on the distribution of carboxypeptidase B in the subfields of the hippocampus in control and AD brain samples. Our aim in this investigation, therefore, was to establish the subregional distribution of this enzyme in comparison with the appearance of NFTs in the normal and AD brain samples.

MATERIALS AND METHODS

Materials

Post mortem brain samples were obtained from 7 neurologically healthy controls (age range 61–85 years) and 8 individuals with AD (age range 76–88 years). The diagnosis of AD was based on neuropathological observations of grossly evident brain atrophy, as well as the presence or absence of NFTs [13]. The post mortem delay ranged from 2 to 4 h. Brain tissues (1 cm in thickness) were fixed in 4% paraformaldehyde, and after 2 days, were transferred to a 30% buffered sucrose solution for cryoprotection until the samples sank to the bottom of the glass container. The samples were cut at a thickness of 40 μm on a freezing microtome.

Immunohistochemical demonstration of carboxypeptidase B

The presence or absence of carboxypeptidase B in the various subfields of the hippocampal area and within it, in the different neurons, was investigated by an immunohistochemical technique described in detail by Matsumoto et al. [14]. In brief: to eliminate the endogenous peroxidase, the sections were treated for 20 min by immersion in 3% hydrogen peroxide solution. The samples were then incubated overnight at 4 °C in a moist chamber with the primary antibody, using an anti-C14-module polyclonal antibody at a dilution of 1 : 1000, which recognizes the C-terminal peptide unique to human brain carboxypeptidase B (HBCPB). The characterization of this antibody is described in detail in [14].

Histochemical demonstration of the neurofibrillary degeneration

To demonstrate neurofibrillary degeneration, a widely used silver impregnation technique described in detail by Gallyas [6] was applied. In brief: human brain tissue samples were washed in 1% Na-acetate and thereafter treated with 5% periodic acid

for 30 min. After washing, the sections were incubated for 30 min at room temperature in a solution containing 4% sodium hydroxide, 10% potassium iodide and 0.03% silver nitrate. After a brief rinse in 1% sodium carbonate, the samples were transferred to a physical developer. Finally, the sections were washed, first in 1% acetic acid and thereafter in double distilled water. After mounting and dehydration in a series of alcohol, the sections were covered with DPX.

RESULTS

Distribution of carboxypeptidase B-immunoreactive neurons in the hippocampal area

The regional distributions of the HBCPB-immunoreactive neurons and the silver-stained NFT-positive structures were observed in the various segments (CA1a, CA1b and CA1c) of subfield CA1 and in the area CA4 of the hippocampal formation of the normal and AD brain samples, as demonstrated in Fig. 1. In this immunohistochemical study, it was observed that on the basis of the distribution of HBCPB-positive neurons, subfield CA1 can be divided into smaller segments. In the control samples, HBCPB immunoreactivity was localized to the perikarya of the pyramidal cells in the various segments of subfield CA1. Significant differences were observed between the different subfields. Most of the stained neurons appeared to be present in subfield CA1a (Fig. 2A), less in CA1b (not demonstrated) and the least in CA1c (Fig. 2B). In area CA4 area, only some of the multipolar neurons were immunopositive only (Fig. 2C).

In the AD brain samples, the immunohistochemical staining was reduced or had even disappeared from most of the neurons in all segments studied. No or merely very light staining was present in areas CA1a (Fig. 2D), CA1c (Fig. 2E) and CA4 (Fig. 2F).

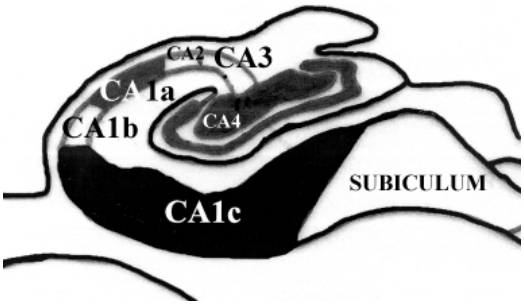

Fig. 1. Diagram showing the various subfields (CA1, CA2, CA3 and CA4) and subsectors (CA1a, CA1b and CA1c) in subfield CA1 in the hippocampal formation

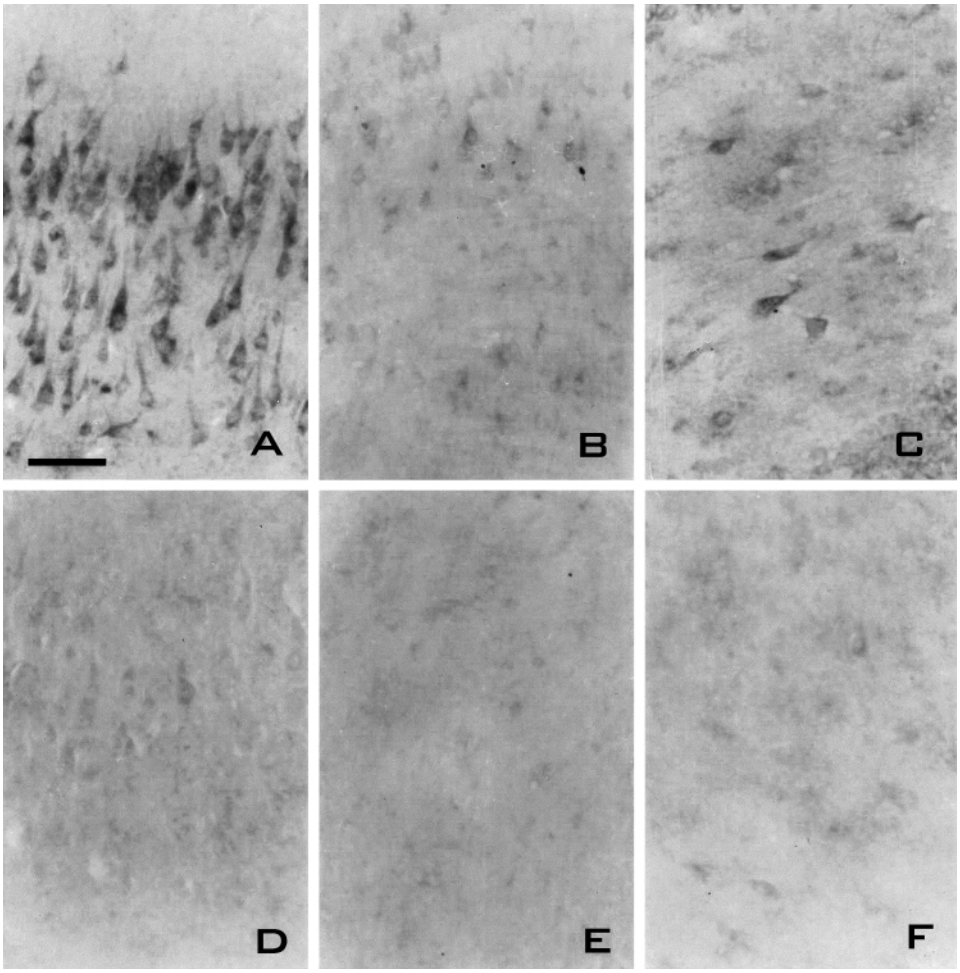

Fig. 2. Immunohistochemical demonstration of carboxypeptidase B in areas CA1a (A), CA1c (B) and CA4 (C) in the hippocampus of the control brain, and the reduced enzyme levels in areas CA1a (D), CA1c (E) and CA4 (F) in Alzheimer's disease brain samples. Scale bar = 100 μm

Distribution of neurofibrillary tangles in the hippocampal area

To reveal the presence or absence of NFTs in the samples in which the distribution of the HBCPB immunoreactivity was observed, the samples were stained for the degenerated neurons. The Gallyas technique [6] for the demonstration of the NFTs in the various hippocampal areas revealed that NFTs appeared very rarely in the control samples. With this silver staining a pretangled neuron could be observed very seldom in segment CA1a in the control sample (Fig. 3A), while no NFTs were present in

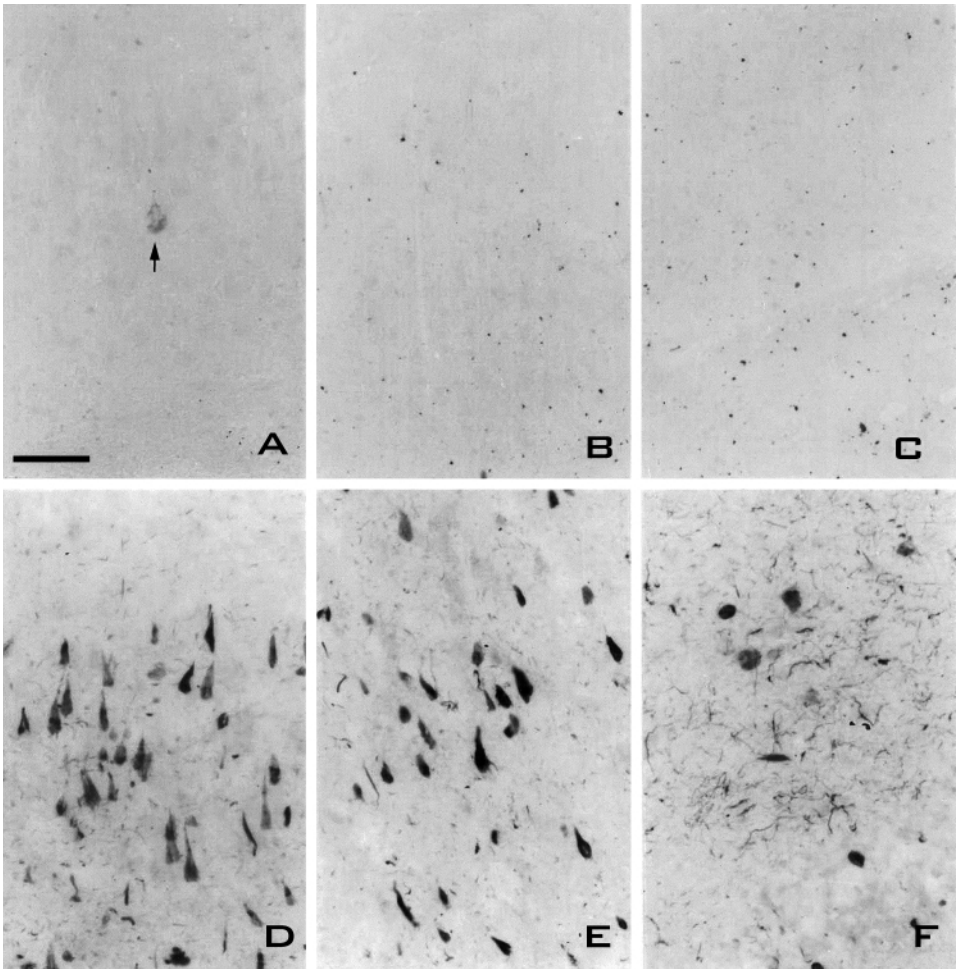

Fig. 3. Gallyas silver-impregnated samples for the demonstration of neurofibrillary tangles in the hippocampal formation. In CA1c (A) only a tangle-like (arrow) neuron was visible, while in sectors CA1c (B) and CA4 (C) no degenerated cells could be revealed in the control brain. In the Alzheimer's disease brain samples, a large number of neurofibrillary tangles appear in subfields CA1a (D), CA1c (E), but less in CA4 (F). Scale bar = 100 μm

areas CA1c (Fig. 3B) and CA4 (Fig. 3C). In contrast, in those samples where the HBCPB staining was reduced or had disappeared, a large number of NFTs were observed in hippocampal areas CA1a (Fig. 3D), CA1c (Fig. 3E) and CA4 (Fig. 3F). This finding is in agreement with the suggestion that a reduced level of HBCPB may cause the accumulation of intraneuronal Aβ and consequently, the evolution of NFTs.

DISCUSSION

In most immunohistochemical and histochemical studies dealing with the distribution of a given enzyme/protein, the hippocampal area in the human brain is subdivided areas (CA1, CA2, CA3 and CA4) [2, 4, 11, 12, 17]. However, in this study it was observed that, with the demonstration of HBCPB, area CA1 of the hippocampus could be further divided into smaller segments, such as CA1a, CA1b and CA1c. In control brains, both anti-prepro-HBCPB and anti-C14 module [14] immunoreactivities were detected in the neuronal perikarya of the hippocampal pyramidal cells. No stained cells were demonstrated in the granule cells of the dentate gyrus [14]. Since neurochemical studies revealed that carboxypeptidase B plays a role in the degradation of Aβ, it was interesting to ascertain whether the protein can be localized in the same cells or not. To analyse the relationship between Aβ peptides and HBCPB, the hippocampal samples were double-stained with anti-human Aβ1-40 and anti-C14 antibodies.

In these studies it was found that the two immunoreactivities were colocalized as granules in the perikarya of the pyramidal cells [14]. Our observations established, however, that most of the immunohistochemically positive pyramidal neurons were present in segment CA1a, less in CA1b and the least in CA1c. This finding supports the earlier finding that most NFTs can be found in sector CA1c, where the accumulation of intracellular Aβ is high. This finding again argues for in favour of the toxic role of the intracellularly accumulated Aβ and of a decreased level of HBCPB in the area where a large extent of neurofibrillary degeneration occurs.

With another Aβ-degrading peptidase, neprilysin, it was demonstrated by immunohistochemical means that the immunoreactivity was particularly rich in the caudate nucleus, putamen, globus pallidus and substantia nigra. The paucity of neprilysin, a putative Aβ degrading enzyme [18, 21], in the neuropil of the hippocampus, may explain the vulnerability of this area to Aβ deposition [1]. In support of this report, in animal studies a neprilysin deficiency resulted in defects in both the degradation of exogenously administered Aβ and the metabolic suppression of the endogenous Aβ levels in a gene dose-dependent manner. This suggested that even a partial down-regulation of neprilysin activity could contribute to AD development by promoting the accumulation of Aβ [9, 10]. In contrast with the decreased levels of HBCPB and neprilysin, increased activities of cathepsin D (CD), cathepsin B (CB) and major lysosomal proteases were demonstrated in the neurons in AD. Immunohistochemically, the presence of CD and CB has been observed in senile plaques, and it has been suggested that abnormal distribution of these enzymes in the patients may also be involved in primary and/or secondary manner in the development of AD [17].

In AD, neuronal loss and NFT formation are usually most marked in the hippocampal area [2, 3, 5]. Various silver impregnation techniques [6, 7, 19] have clearly documented [see for ref. 8] that the clinical expression of AD correlates with the presence and accumulation of NFTs in various areas in the human brain, including the subfields of the hippocampus. This report lends further support to the suggestion

that, besides neprilysin and the cathepsins, the decreased level of HBCPB in the pyramidal cells in the various segments of the hippocampus may induce an increase in the number of NFTs and promote the development of AD.

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REFERENCES

1. Akiyama, H., Kondo, H., Ikeda, K., Kato, M., McGeer, P. L. (2001) Immunohistochemical localization of neprilysin in the human cerebral cortex: inverse association with vulnerability to amyloid β-protein (Aβ) deposition. *Brain Res. 902,* 77–281.
2. Bobinski, M., Wegiel, J., Wisniewski, H. M., Tarnawski, M., Bobinski, M., Reisberg, B., De Leon, M. J., Miller, D. C. (1996) Neurofibrillary pathology – Correlation with hippocampal formation atrophy in Alzheimer disease. *Neurobiol. Aging 17,* 909–919.
3. Braak, H., Braak, E. (1998) Evolution of neuronal changes in the course of Alzheimer's disease. *J. Neural. Transm. 53,* 127–140.
4. Brady, D. R., Mufson, E. J. (1991) Alz-50 immunoreactive neurophil differentiates hippocampal complex subfields in Alzheimer's disease. *J. Comp. Neurol. 305,* 489–507.
5. Fukutani, Y., Kobayashi, K., Nakamura, I., Watanabe, K., Isaki, K., Cairns, N. J. (1995) Neurons, intracellular and extracellular neurofibrillary tangles in subdivisions of the hippocampal cortex in normal ageing and Alzheimer's disease. *Neurosci. Lett. 200,* 57–60.
6. Gallyas, F. (1971) Silver staining of Alzheimer's neurofibrillary changes by means of physical development. *Acta Morphol. Acad Sci. Hung. 19,* 1–8.
7. Haga, C., Ikeda, K., Iwabuchi, K., Akiyama, H., Kondoh, H., Kosaka, K. (1994) Methenamine-silver staining: a simple and sensitive staining method for senile plaques and neurofibrillary tangles. *Biotechnic Histochem. 69,* 295–300.
8. Iqbal, K., Alonso, A. C., Gong, C. X., Khatoon, S., Pei, J. J., Wang, J. Z., Grundke-Iqbal, I. (1998) Mechanisms of neurofibrillary degeneration and the formation of neurofibrillary tangles. *J. Neural. Transm. Suppl. 53,* 169–180.
9. Iwata, N., Tsubuki, S., Takaki, Y., Watanabe, K., Sekiguchi, M., Hosoki, E., Kawashima-Morishima, M., Lee, H. J., Hama, E., Sekine-Aizawa, Y., Saido, T. C. (2000) Identification of the major $A\beta_{1-42}$-degrading catabolic pathway in brain parenchyma: Suppression leads to biochemical and pathological deposition. *Nat. Med. 6,* 143–150.
10. Iwata, N., Tsubuki, S., Takaki, Y., Shirotani, K., Lu, B., Gerard, C., Hama, E., Lee, H. J., Saido, T. C. (2001) Metabolic regulation of brain Aβ by neprilysin. *Science 292,* 1550–1552.
11. Kása, P., Papp, H., Kása, P. jr, Török, I. (2000) Donepezil dose-dependently inhibit acetylcholinesterase activity in various areas and in the presynaptic cholinergic and the postsynaptic cholinoceptive enzyme-positive structures in the human and rat brain. *Neuroscience 1001,* 89–100.
12. Kása, P., Rakonczay, Z., Gulya, K. (1997) The cholinergic system in Alzheimer's disease. *Prog. Neurobiol. 52,* 511–535.
13. Markesbery, W. R. (1997) Neuropathological criteria for the diagnosis of Alzheimer's disease. *Neurobiol. Aging 18,* S13–S19.
14. Matsumoto, A., Itoh, K., Matsumoto, R. (2000) A novel carboxypeptidase B that processes native β-amyloid precursor protein is present in human hippocampus. *Eur. J. Neurosci. 12,* 227–238.
15. Matsumoto, A., Itoh, K., Seki, T., Motozaki, K., Matsuyama, S. (2001) Human brain carboxypeptidase B, which cleaves β-amyloid peptides in vitro is expressed in the endoplasmic reticulum of neurons. *Eur. J. Neurosci. 13,* 1653–1657.

16. Matsumoto, A., Motozaki, K., Seki T., Sasaki, R., Kawabe, T. (2001) Expression of human brain carboxypeptidase B, a possible cleaving enzyme for β-amyloid precursor protein, in peripheral fluids. *Neurosci. Res. 39,* 313–317.
17. Nakamura, Y., Takeda, M., Suzuki, H., Hattori, H., Tada, K., Hariguchi, S., Hashimoto, S., Nishimura, T. (1991) Abnormal distribution of cathepsins in the brain of patients with Alzheimer's disease. *Neurosci. Lett. 130,* 195–198.
18. Shirotani, K., Tsubuki, S., Iwata, N., Takaki, Y., Harigaya, W., Maruyama, K., Kiryu-Seo, S., Kiyama, H., Iwata, H., Tomita, T., Iwatsubo, T., Saido, T. C. (2001) Neprilysin degrades both amyloid beta peptides 1–40 and 1–42 most rapidly and efficiently among thiorphan- and phosphoramidon-sensitive endopeptidases. *J. Biol. Chem. 276,* 21895–21901.
19. Yamaguchi, H., Haga, C., Hirai, S., Nakazato, Y., Kosaka, K. (1990) Distinctive, rapid, and easy labeling of diffuse plaques in the Alzheimer brain by new methenamine silver stain. *Acta Neuropathol. 79,* 569–572.
20. Yasojima, K., McGeer, E. G., McGeer, P. L. (2001) Relationship between beta amyloid peptide generating molecules and neprilysin in Alzheimer disease and normal brain. *Brain Res. 919,* 115–121.
21. Yasojima, K., Akiyama, H., McGeer, E. G., McGeer, P. L. (2001) Reduced neprilysin in high plaque areas of Alzheimer brain: a possible relationship to deficient degradation of β-amyloid peptide. *Neurosci. Lett. 297,* 97–100.