Decreased Expression of Hippocampal Cholinergic Neurostimulating Peptide Precursor Protein mRNA in the Hippocampus in Alzheimer Disease

MINA MAKI, MD, NORIYUKI MATSUWA, MD, PhD, HIROYUKI YUASA, MD, YASUSHI OTSUKA, MD, PhD, TAKAYUKI YAMAMOTO, MD, PhD, HIROYASU AKATSU, MD, PhD, TAKASHI OKAMOTO, MD, PhD, RYUZO UEDA, MD, PhD, AND KOSEI OJIKA, MD, PhD

Abstract. Hippocampal cholinergic neurostimulating peptide (HCNP) is involved in the phenotype development of the septo-hippocampal system. HCNP precursor protein (HCNP-pp) is known to interact with other molecules including phosphatidyl-ethanolamine and Raf-1 kinase, and is also known as phosphatidylethanolamine-binding protein and raf kinase-inhibitory protein. To assess whether HCNP-pp is involved in the pathogenesis of Alzheimer disease (AD), the expression levels of its mRNA in the hippocampus of autopsy brains from patients with dementia (including AD and ischemic vascular dementia) were compared with those of non-demented control subjects. The in situ hybridization analysis revealed that the expression of HCNP-pp mRNA in patients with clinically late-onset AD was decreased in the hippocampal CA1 field, but not in the CA3 field or the dentate gyrus. The early-onset AD patients showed a wide range of expression levels in the hippocampal sub-regions. Northern blot analysis of HCNP-pp mRNA in brain tissue supported these observations. Since HCNP is known to stimulate the enzymatic activity of choline acetyltransferase in neurons, its low expression in the CA1 field of AD patients may explain the downregulation of cholinergic neurons seen in these patients and may thus contribute to the pathogenic processes underlying AD.

Key Words: Alzheimer disease; Hippocampus; Hippocampal cholinergic neurostimulating peptide; HCNP; In situ hybridization; mRNA expression.

INTRODUCTION

The pathological characteristics of brains affected by Alzheimer disease (AD) include loss of neurons and gliosis associated with the accumulation of neurofibrillary tangles (NTF), senile plaques (SP), granulovacuolar degeneration (GVD), and eosinophilic rosettes (Hirano bodies: HBs) (1). Interestingly, all of these pathologic changes are typically found in the hippocampus, especially the CA1 field and the subiculum (1, 2). The molecular characterization of amyloid protein in SP (3) and tau protein in NTF (4) as well as the identification of the genetic background of AD (5) has greatly contributed to the understanding of its pathogenesis. Moreover, it has been shown that in the brain tissues of AD patients, the levels of mRNAs of brain-derived neurotrophic factor (BDNF) (6) and TrkA receptor (7) are both attenuated, although the level of nerve growth factor (NGF) is upregulated (8, 9). We previously demonstrated an interesting phenomenon that crude extracts of rat hippocampus enhanced acetylcholine synthesis and morphological development in neurons in an explant culture of medial septal nuclei (10). These effects could be ascribed, at least in part, to a novel undecapeptide purified from the hippocampus of young rats, and the peptide was thus designated hippocampal cholinergic neurostimulating peptide (HCNP) (11). The enhancing effect of HCNP on choline acetyltransferase activity was synergistic with that of NGF, suggesting that the actions of these factors are distinct from each other (12). Although HCNP-related protein and its mRNA are expressed rather ubiquitously, they are predominantly found in the hippocampus. Interestingly, HCNP is expressed during the perinatal period and it is considered to be crucial for the phenotypic development of the septo-hippocampal system (13, 14). HCNP precursor-protein (HCNP-pp) contains 186 amino acids and is cleaved by a unique chymotrypsin-like thiol protease (13, 15). HCNP-pp interacts with phosphatidylethanolamine as phosphatidylethanolamine-binding protein (PEBP) (13, 16–19), with Raf-1 kinase as raf kinase-inhibitory protein (RKIP) (20, 21), and also with serine proteases, including thrombin, neuropeptides, and chymotrypsin (22), and inhibits their enzymatic activities. Interestingly, the expression of HCNP-pp mRNA in the hippocampus is reciprocally regulated by the muncaricin cholinergic and glutamatergic neurons (23), suggesting that it may play an important role in modulating neuronal activity in the adult brain.

These findings led us to investigate the possible involvement of HCNP in AD and aging. In this study, we used in situ hybridization to examine the expression levels of HCNP-pp mRNA in the brains of 13 patients with sporadic AD and we found that HCNP-pp mRNA was decreased in the CA1 field of the hippocampus, particularly in patients with late-onset AD. A possible role for HCNP-pp in the pathogenesis of AD is discussed.
TABLE
Profile of Subjects Involved in the Study

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Late AD, clinically late-onset Alzheimer disease (age ≥ 65); Early AD, clinically early onset Alzheimer disease (age < 65).
* Indicates cases omitted in statistical analysis of in situ hybridization.

MATERIALS AND METHODS
Patients and Preparation of Human Brain Tissue

Human brain tissues were obtained at autopsy in Fukushimura Hospital from 30 patients whose diagnosis was confirmed by clinical and neuropathological findings: 5 had early-onset Alzheimer disease (early AD), 12 had late-onset AD (late AD), 6 had ischemic vascular dementia (IVD), and 7 were non-demented aged individuals (NA) as controls. None of the AD patients had a family history of AD or related disorders. Early AD and late AD were defined as dementia occurring in patients under or over the age of 65, respectively, as described previously (24). The ages and gender of these patients is indicated in the Table. The clinical diagnosis of AD was based on a history of gradual and progressive intellectual deterioration without focal motor or sensory disturbance or any other causes of dementia, in accordance with DSM-IV and NINCDS-ADRDA criteria (25). The diagnosis of IVD was made when patients developed dementia associated with a sudden non-convulsive focal neurological deficit that was confirmed by the results of clinical imaging examinations including CT and/or MR imaging. The pathologic diagnoses of AD and IVD were confirmed by means of the CERAD criteria (26) and the criteria for the pathologic diagnosis of ischemic vascular dementia (27), respectively. The IVD patients had multiple small infarcts commonly in the thalamus, internal and external capsule, putamen and pallidum, but not in the medial temporal lobe, basal forebrain, or hippocampus. In this study the Gottfries, Brance, Gullberg and Steen Scale (GBS scale) for dementia syndrome was used (28). Control NA patients displayed essentially no neuritic plaques or NFT in the brain.

At autopsy each brain was divided in 2 parts: the left hemisphere was used for histopathology and the right hemisphere for Northern blot analysis. Various brain sub-regions from the left hemisphere of each patient were fixed in neutralized 10% formalin, embedded in paraffin, and then 6 μm-thick sections were stained with hematoxylin and eosin or by the methenamine-Bodian method. The right hemisphere tissue was cut into 0.5-cm-thick coronal sections containing portions of the frontal lobe, the cerebellum, and the temporal lobe (including the hippocampus at the level of the lateral geniculate body). For RNA blotting analysis, the tissue blocks were immediately frozen and 6 μm-thick tissue blocks were immersed in ethanol-acetic acid solution (3:1) for 3 days at 4°C, embedded in paraffin, and then 7-μm-thick
sections were mounted onto poly-L-lysine-coated glass slides, as described previously (29).

Preparation of Riboprobes

The antisense and sense riboprobes were prepared by the method described previously (29, 30). Briefly, a 230-bp fragment of human HCNP-pp cDNA and a 282-bp fragment of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were subcloned into the pGEM-3Z vector (Promega, Madison, WI). The human HCNP-pp cDNA was amplified by polymerase chain reaction using an oligonucleotide forward primer with an EcoR-I linker (5′-ATGGAATTCTAGCCGTTG GACCTCAGCAAGT) and a reverse primer with a Sph-I linker (5′-TTGTCATGCGGGTCTGTCAGGACCAAGTTG). The GAPDH cDNA was also amplified using oligonucleotide primers (forward: 5′-CTACACCATCTTCCAGGACC, reverse: 5′- TTGTCATGGAGCCCTTGGG). The antisense and sense riboprobes were obtained by in vitro transcription of a cloned plasmid (1 μg) using SP6 RNA polymerase and T7 RNA polymerase (Boehringer Mannheim, Mannheim, Germany), respectively. Digoxigenin-labeled UTP (Boehringer Mannheim) was used as the substrate for the transcription of the labeled RNA probe.

Northern Blot Analysis

Total RNA (15 μg) from the human hippocampus and frontal cortex were separated on a 1% agarose gel containing 0.16 M formaldehyde, then transferred to a nylon membrane (Hybond N; Amersham Pharmacia Biotech, Buckingham, UK), and covalently linked by means of 1,200 J/m2 UV-light irradiation. The blotted RNAs were hybridized with the digoxigenin-labeled antisense and sense riboprobes or with [α-32P] dATP-labeled full-length human HCNP-pp (13) and GAPDH (internal control) cDNA probes. The membranes were prehybridized at 65°C for 4 hours (h), and then hybridized with the probes (1 μg/ml) at 65°C for 16 h in a buffer containing 5 × saline sodium citrate (SSC), 10 × Denhardt, 10 mM Na2PO4 (pH 6.5), 0.5% sodium dodecyl sulfate (SDS), 0.1 mg/ml denatured salmon sperm DNA (ssDNA), and 50% formamide. After washing with 2 × SSC containing 0.1% SDS at 65°C, the membranes were treated with 20 μg/ml RNase A, and visualized by the alkaline phosphatase-conjugated anti-digoxigenin antibody and chemiluminescence-detection method (29, 31). The hybridization signals were compared non-parametrically between variables (HCNP-pp mRNA level, age, postmortem interval, cause of death, numbers of GVD, HBs, SP, and NFT, and GBS scale score [28]).

RESULTS

Detection of HCNP-pp mRNA by In Situ Hybridization

As demonstrated in Figure 1, in situ hybridization using the digoxigenin-labeled antisense riboprobe for buffered saline (PBS) for 10 min at room temperature (RT) and treated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 15 min at RT. Thereafter, prehybridization was carried out in a hybridization buffer containing 50% formamide, 4 × SSC, 0.1% SDS, 25 mM DTT, and 0.2 mg/ml denatured salmon sperm DNA at 50°C for 1 h. The glass plate-embedded sections were hybridized with each probe at 50°C for 18 h using 100 μl hybridization solution containing 40 ng sense or antisense riboprobe, 100 μg yeast tRNA, 1.0 M NaCl, 20 mM MgCl2, 20 mM PIPES (pH 6.4), 20 mM DTT, 0.2 μg/ml denatured salmon sperm DNA, 2 × Denhardt, 0.2 mM EDTA, 10% polyethylene glycol, and 50% formamide. The non-hybridized probes were removed by 2 successive incubations at 65°C for 30 min with buffer containing 50% formamide, 20 mM Tris-HCl (pH 7.4), 5.0 mM EDTA, 10 mM DTT, and 2 × SSC, and then the hybridized sections were subjected to RNase A treatment at 37°C for 30 min in a buffer containing 10 mM Tris-HCl (pH 7.4), 1.0 mM EDTA, 0.5 M NaCl, and 20 μg/ml RNase A. For detection of the hybridization signals, the sections hybridized with digoxigenin-labeled riboprobes were treated with alkaline phosphatase-conjugated anti-digoxigenin antibody in DIGIII buffer containing 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, and 50 mM MgCl2, and with 175 μg/ml 5-bromo-4-chloro indoxyl phosphate and 450 μg/ml nitroblue tetrazolium chloride in 10% high-molecular-weight polyvinyl alcohol. The specificity of the reaction was evaluated by incubation with the sense and antisense probes separately.

Quantification of the levels of mRNA expression in the neurons of the hippocampal CA1 and CA3 fields and the dentate gyrus was carried out using a computer-assisted image-analyzer (Nikon Instech, Japan). The hybridization signal was then digitized automatically with respect to intensity and density using image-analysis software (ATTO Densitograph, Japan) (23). In order to compare the hybridization intensity among brain tissue samples containing different numbers of neurons, the total hybridization signal obtained for each section was divided by the total area occupied by neurons there. The total area was estimated using an ATTO Densitograph employing a manual procedure in which every neuron on the screen was outlined in each section. The feasibility of semi-quantification of the hybridization signal using the non-isotopic digoxigenin system was validated by examining the features of the signal generated using the same probe with an increasing alkaline phosphatase reaction time. The reproducibility of the quantification of patient samples with various HCNP-pp mRNA contents was confirmed by multiple independent experiments.

Statistical Analysis

The digitized data were subjected to statistical evaluation. The hybridization signals were compared non-parametrically using Mann-Whitney U-test (for comparison of 2 groups). Simple linear-regression analysis was used to assess relationships between variables (HCNP-pp mRNA level, age, postmortem interval, cause of death, numbers of GVD, HBs, SP, and NFT, and GBS scale score [28]).
HCNP-pp produced clear hybridization signals, mainly in the pyramidal cells and granular cells of the hippocampus. No hybridization signal was observed when the labeled sense riboprobe was used (Fig. 1E). This hybridization was compatible with the results of Northern blot analysis using the antisense riboprobe, which revealed a single 1.4-kb band indicating HCNP-pp mRNA, whereas the sense riboprobe showed no band (Fig. 1A). Although quantification of hybridized signals following in situ hybridization using a non-isotope-labeled riboprobe has been reported (23, 32–34), the validity of the quantitative determination of the HCNP-pp mRNA level using a non-isotopic digoxigenin system has never been documented. Therefore, we first determined the optimal hybridization conditions and examined signal increase with respect to the alkaline phosphatase reaction time. As shown in Figure 2A–F, in cerebellar tissue sections from 1 of the AD patients, prominent hybridization signals were detected in Purkinje cells. After hybridization with the HCNP-pp riboprobe, alkaline phosphatase-conjugated anti-digoxigenin antibody was added, and the alkaline phosphatase reaction was carried out for 1 to 8 h. The signal intensity increased from 1 to 5 h and then reached a plateau (Fig. 2G), indicating that a reaction-time of 5 to 6 h would be appropriate for semi-quantitative determination of HCNP-pp mRNA by in situ hybridization and for comparison of the levels of HCNP-pp mRNA among different brain tissues from various patients and control subjects.

To further evaluate the reproducibility and examine the reliability of the quantification data for HCNP-pp mRNA levels, we determined the inter-experimental variation in the in situ hybridization study. As shown in Figure 3, three consecutive sections from 8 representative cases of NA, IND, late AD, and early AD were subjected to in situ hybridization (on 3 different occasions) and the signal intensity in CA1 pyramidal cells was determined. The HCNP-pp mRNA determination was carried out on cerebellar Purkinje cells in 1 early AD case (Fig. 3, case no. 9717c). Although the determinations were carried out on separate occasions, the mean inter-experimental variation (the average of the standard deviation [SD] values divided by the mean of the HCNP-pp mRNA signal intensities) was 17.7% (Fig. 3), indicating that the results obtained in this analysis were sufficiently reliable for further analysis to be carried out in this study.

Expression of HCNP-pp mRNA in the Hippocampus

Hippocampal tissues from AD, IVD, and control subjects were examined for the gene expression of HCNP-pp by in situ hybridization using digoxigenin-labeled antisense riboprobes. Abundant HCNP-pp mRNA was detected in the pyramidal cells of CA1 to CA4, as well as in the granular cells of the dentate gyrus in 21 of the 30 cases examined (Fig. 4). Nine cases were omitted as no hybridization signal was detected by either HCNP-pp or GAPDH probes (Table). The distribution of HCNP-pp
Fig. 2. HCNP-pp mRNA detected by in situ hybridization using cerebellar tissue sections from an AD patient (case no. 9716; Table). Prominent hybridization signals were detected in Purkinje cells. A, 1 h; B, 2 h; C, 3 h; D, 4 h; E, 6 h; F, 8 h (alkaline phosphatase reaction time). G: Time-dependent signal intensity for Purkinje cells (quantified as digitized optical density per square plotted against time). The signal intensity increased from 1 to 5 h and then reached a plateau.

mRNA, but not that of GAPDH, showed significant intergroup differences; the HCNP-pp mRNA expression in CA1 was highest in control subjects and lowest in late AD patients (Fig. 4a–h). It was noted that the HCNP-pp mRNA expression was particularly low in the dentate of both late AD and IVD patients, with the latter even lower than the former. In some late AD cases, although HCNP-pp mRNA was decreased in CA1, no decrease was detected in the dentate gyrus.

In Figure 5, the HCNP-pp mRNA levels in the pyramidal cells of hippocampal CA1 and CA3, and in the granular cells of the dentate gyrus are quantified for 21 autopsy cases: 4 early AD (mean age 73.5 ± 8.5 yr), 9 late AD (84.8 ± 6.1), 4 IVD (75.5 ± 10.6), and 4 NA control subjects (89.5 ± 7.5) (Table). The expression of HCNP-pp mRNA was decreased by a factor of 3 in the CA1 of late AD patients as compared with NA controls (p < 0.01). In IVD cases, a decreased HCNP-pp mRNA level was observed in both dentate gyrus (p < 0.05) and CA1 (p < 0.05). The level of GAPDH mRNA (internal control) showed little difference from NA control except in the CA3 of the IVD cases, possibly indicating a more extensive pathologic involvement in IVD. There was no significant relationship between the HCNP-pp mRNA level and the GAPDH mRNA level. We also assessed the relationships between the HCNP-pp mRNA level and various pathologic findings (including GVD, HBs, SP, and NFT), clinical stage of dementia (including GBS scale score [31]), disease duration, age of onset, age at death, and postmortem interval. We assessed these relationships totally and separately for each group of disease, however, no evident relationship was revealed in these assessments (data not shown).

Finally, Northern blot analysis was performed using total RNA samples obtained from the hippocampus of the above 21 cases (Fig. 6). Although the yield of total RNA...
preparation from the hippocampus was variable because of the difficulty of obtaining a sufficient amount of brain tissue in some cases, we found a significant decrease in the expression of HCNP-pp mRNA in both late AD (p < 0.01) and early AD (p < 0.05) patients as compared with NA controls. No such significant decrease in HCNP-pp mRNA level was observed in IVD patients.

**DISCUSSION**

In this study, we examined the level of HCNP-pp mRNA in patients with various clinical diagnoses who all developed dementia. We found that the gene expression of HCNP-pp was significantly decreased in patients with late AD, particularly in the hippocampal CA1 field. However, it was not significantly decreased in the hippocampal CA3 field or in the dentate gyrus of brain tissues from these late AD patients. Since a wide variety of characteristic AD neuropathologies has typically been demonstrated in the hippocampal CA1 field in late AD cases (1, 2), the decrease in HCNP-pp gene expression in this region (Fig. 5) may suggest a causal relationship with the pathogenesis of late AD. In fact, a loss of cholinergic neurons innervating the hippocampus is observed in the brain tissues of AD patients (35). However, a decrease in HCNP-pp mRNA was not found in early AD (Fig. 5), possibly indicating different pathophysiologic processes.

HCNP has been shown to have a neurotrophic action on cholinergic neurons (10–13), and although CA3 also receives a dense cholinergic innervation from the basal forebrain (36), the downregulation of HCNP-pp gene expression together with selective neuronal damage in CA1...
suggests that glutamatergic neurons that produce HCNP and are predominantly present in CA1 may be the primary target of the disease process in late AD. Although most CA1 neurons express both NMDA and AMPA receptors, HCNP production is stimulated only through AMPA receptors (23). Furthermore, continuous NMDA-receptor activation leads to a downregulation of HCNP-pp mRNA expression in the hippocampal CA1 field (23).

We postulate that an imbalance in the usage of NMDA and AMPA receptors, such as continuous NMDA signaling and/or a blockade of AMPA signaling, may predispose individuals to late AD. In the hippocampus of AD patients, although the expression of NMDAR1 (a major subunit of the NMDA receptor) remains unaffected in CA1 pyramidal neurons (37, 38), the loss of an AMPA receptor subunit, GluR2(3), precedes the formation of NFT in the CA1/subiculum (39). Interestingly, HCNP and HCNP-pp mRNA are increased in the CA1 of senescence-accelerated prone mice, SAMP8, as compared with senescence-accelerated resistant mice, SAMR1 (28). SAMP8 show both an early onset of impaired learning and memory (40) and reduced NMDA receptor activity in the hippocampus (41). Although the histopathologic features of SAMP8 differ from those of AD (42), increased amyloid-beta precursor-protein and its mRNA have been shown in the hippocampus of SAMP8, as they have in AD (43, 44). A recent study has reported alterations in the AD-related expressions of bcl-2α, presenilin-1, 2, and tau mRNA in the hippocampus of SAMP8 (45). We can explain these data by supposing that pathogenic changes in the hippocampus of SAMP8 and late AD are different, however, disorder of glutamatergic input followed by altered HCNP-pp mRNA expression may potentially be involved in impaired learning and memory in both AD patients and SAMP8.

The biological significance of the decrease in HCNP-pp mRNA expression in specific hippocampal sub-regions (CA1 and dentate gyrus, but not CA3) in IVD patients is as yet uncertain. However, the hippocampus is an important region receiving a variety of neuronal inputs from other brain areas via the entorhinal cortex (46, 47), and widespread vascular lesions in multiple brain areas in IVD patients could lead to a decrease in hippocampal
Fig. 6. A: Northern blot analysis performed with total RNA extracted from human hippocampus (21 cases) using \( ^{32}P \) dATP-labeled HCNP-pp cDNA. B: Slight, but significant, decreases in relative expression of HCNP-pp mRNA with respect to GAPDH mRNA were revealed in both late AD and early AD. Asterisk and double asterisks indicate significant difference (\( p < 0.05 \) and \( p < 0.01 \), respectively) from NA (Mann-Whitney U-test). No significant decreases were seen in IVD cases.

Afferent neuronal traffic. In patients with IVD, an attenuation of such afferent neuronal traffic may be responsible for the decrease in the expression of HCNP-pp mRNA in the dentate gyrus and CA1 field, but not in CA3. In this context, it is important to note the decrease in GAPDH mRNA expression in CA3 in IVD patients. Neurons in CA3 are known to be relatively resistant to ischemic stress in the gerbil hippocampus (48). Recent data demonstrate that GAPDH overexpression participates in the neuronal apoptosis induced by various insults, and its pharmacologic suppression has a neuroprotective effect in vitro (49, 50). Thus, we suggest the possibility that a decrease in GAPDH mRNA expression may reflect a protective response in CA3 neurons in the pathogenesis of IVD.

Finally, HCNP-pp/phosphatidylethanolamine-binding protein has been shown to act as a potent serine-protease inhibitor in the brain (22) and to inhibit Raf-1 kinase (as raf kinase-inhibitory protein) (20). It is yet to be clarified whether such biochemical activities associated with HCNP-pp could explain some of the neurophysiologic actions of HCNP. Further studies are needed before we can understand the link between a decrease in HCNP-pp gene expression and the neuropathophysiology of late AD.

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