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Altered Expression of COX-2 in Subdivisions of the Hippocampus during Aging and in Alzheimer's Disease: The Hisayama Study

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Key Words
Cyclooxygenase · Alzheimer's disease · Hippocampus

Abstract
Background: It has been reported that nonsteroidal anti-inflammatory drugs may delay the onset of Alzheimer's disease (AD). Since nonsteroidal anti-inflammatory drugs inhibit cyclooxygenase (COX), COX-2, an inducible form of COX, may be involved in the pathology of AD in association with the arachidonic acid cascade. In addition, it has been suggested that alterations in the balance of polyunsaturated fatty acids are associated with brain dysfunctions such as neurodegerative pathologies of the aging brain. ***Method:*** To explore COX-2 expression in the hippocampus, we analyzed 45 consecutive autopsy subjects without dementia and 25 AD patients derived from the town of Hisayama, Japan. ***Results:*** The neuronal expression of COX-2 in the CA3 subdivision of the hippocampus, subiculum, entorhinal cortex and transentorhinal cortex were consistently observed in both nondemented and AD brains, and COX-2 immunoreactivity correlated with age in nondemented brains. In AD patients, neurons of CA1 exhibited increased COX-2 immunoreactivity which correlated with the severity of AD pathology. This correlation was not apparent in nondemented subjects. ***Conclusion:*** These results suggest that COX-2 expression may be differentially regulated among subdivisions of the hippocampus and that elevated COX-2 expression in the CA1 of AD brains may be associated with AD pathology and thus cognitive dysfunction.

Introduction

Many epidemiological studies suggest that the use of nonsteroidal anti-inflammatory drugs delays or slows the clinical expression of Alzheimer's disease (AD) [1, 2]. The mechanism by which these drugs might affect pathophysiological processes relevant to AD remains unclear. Most nonsteroidal anti-inflammatory drugs have an inhibitory effect on cyclooxygenase (COX), an enzyme involved in the metabolism of arachidonic acid into prostanoids. There are two major known COX isoforms, the constitutively expressed COX-1 and the mitogen-inducible COX-2 [3]. While COX-1 is mainly expressed in microglia and some neuronal cells throughout the brain, COX-2 is expressed in neurons [4, 5]. It has been suggested that alterations in the balance of polyunsaturated fatty acids, including arachidonic acid and its metabolites, in the central nervous system are associated with brain dysfunction, such as in neurodegenerative pathologies of the aging

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brain [6]. Following on these epidemiological reports, several histological analyses of COX-2 expression in AD brains have been conducted [4, 7–11], but have produced conflicting results. Several studies reported increased neuronal COX-2 immunoreactivity compared to control brain tissues [5, 7]. However, in other studies, in which COX-2 expression was related to specific hallmarks of the disease, such as clinical dementia rating and Braak stage of disease, the number of COX-2-positive neurons decreased with the severity of dementia, and in the end-stage AD, COX-2-positive neurons were significantly fewer than in nondemented controls [4, 11].

Although many studies have been conducted concerning COX-2 expression not only in AD brains, but also in Parkinson disease model mice brains [12], amyotrophic lateral sclerosis brains [13] and schizophrenia brains [14], the histological analyses concerning COX-2 expression in nondemented brains are few. Now that we know that COX-2 expresses constitutively in the brain even under normal conditions [15], it is important to explore the normal COX-2 expression pattern in the brain. Without this basic knowledge, it is difficult to interpret the conflicting results of COX-2 expression in AD brains.

In this study, we investigated the neuronal expression of COX-2 in some subdivisions of hippocampi of consecutive autopsy cases without dementia. In addition, we quantified senile plaque (SP) and neurofibrillary tangle (NFT) density to assess the influence of AD pathology on neuronal COX-2 expression, and explored any differences in the pattern of COX-2 expression in these regions between nondemented subjects and AD patients.

Materials and Methods

Subjects

To minimize the selection bias of the nondemented subjects, we collected the nondemented subjects from the series of consecutive autopsy cases in the Hisayama study. The Hisayama study is a prospective population-based study in the subrural community of Hisayama, which is adjacent to the metropolitan area of Fukuoka on Kyushu Island, Japan, and it started in 1961 [16–19]. We have carried out autopsies on most deceased subjects from this region in order to confirm the cause of death and to examine brain pathology. This paradigm has allowed a reliable recruitment of nondemented subjects in which to analyze the neuronal expression of COX-2. The diagnosis of dementia was based on the guidelines of the Diagnostic and Statistical Manual of Mental Disorders, Revised Third Edition [20]. For the clinical diagnosis of AD, we used the guidelines of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association [21].

From October 1, 1998, to March 31, 2001, 148 Hisayama residents of varying initial ages died, 105 of whom (70.5%) underwent a postmortem examination. Consent to autopsy was unobtainable from 29.5% of the residents due to refusal, mainly on religious grounds. Of those 105 cases, 103 subjects received autopsies at the Departments of Pathophysiological and Experimental Pathology, Anatomic Pathology and Neuropathology of Kyushu University. In order to collect consecutive autopsy cases without dementia, we excluded 58 cases that were clinically diagnosed as exhibiting dementia or had some disease or condition that might influence the expression of COX-2 in the brain, such as severe chronic hepatic failure, autoimmune disease, disseminated intravascular coagulation, systemic inflammatory response syndrome, acute brain infarction, brain infection or a brain tumor. In total, 45 cases were analyzed in study A as nondemented subjects. In study B, in order to compare the nondemented subjects with AD patients, we examined all of the nondemented subjects aged 76 years or more at death of the nondemented subjects of study A and age- and sex-matched AD autopsy cases derived from Hisayama Town as the comparison group. In total, 25 nondemented subjects and 25 AD patients were analyzed in study B. We examined only cases aged 76 or more because there were few AD patients younger than 75 in the Hisayama study.

Neuropathological Assessment

Brains were weighed, evaluated for gross detectable lesions, abnormalities of the blood vessels and were fixed with 10% buffered formalin for at least 2 weeks. Brain specimens were taken following the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) guidelines and the consensus guidelines for dementia with Lewy bodies [22, 23]. Thus, the specimens in each case included the middle frontal gyrus, superior and middle temporal gyri, inferior parietal lobule, anterior cingulate gyrus, hippocampus with entorhinal cortex and transentorhinal cortex (at the level of the lateral geniculate body), calcarine cortex, basal ganglia, thalamus, substantia nigra, locus coeruleus and dorsal vagal nucleus. Samples were embedded in paraffin and cut into sections which were routinely stained using hematoxylin-eosin and a modified Bielschowsky method. Each case was also immunostained with anti-tau (polyclonal, rabbit, 1:200, Dako, Denmark), anti-ubiquitin (polyclonal, rabbit, 1:100, Dako) and anti-α-synuclein (LB509: monoclonal, mouse, 1:100, provided by Dr. Iwatubo) [24]. Immunolabeling was detected using a standard indirect immunoperoxidase method and visualized with diaminobenzidine (Dojindo, Japan). The sections were lightly counterstained with hematoxylin.

Assessment of AD Pathology

The presence of SPs was estimated by a modified Bielschowsky method. NFT presence was assessed by tau immunohistochemistry. In each case, the frequency of SPs and NFTs were evaluated and converted to a plaque score according to CERAD criteria and Braak stage for tau pathology as established by Braak and Braak [22, 25]. The CERAD score and Braak stage were combined to estimate the likelihood of AD according to the NIA-RI criteria [26]. A diagnosis of AD was made when 'definite AD' as defined by the CERAD criteria and/or a 'high-likelihood' as defined by the NIA-RI criteria were found.

In addition, SP and NFT levels in the CA1 subdivision of the hippocampus were assessed. The semiquantitative density of SPs in

CA1 was determined as being either none, sparse, moderate, or frequent, according to the guidelines established by CERAD. NFTs in CA1 were counted in 100× fields at each of three locations and the average was expressed as the NFT number per 100× field.

Immunohistochemistry and Assessment of COX-2

Immunohistochemistry was performed on 7-μm paraffin-embedded sections encompassing the hippocampus, entorhinal cortex and transentorhinal cortex (at the level of the lateral geniculate body). Sections were deparaffinized in xylene, hydrated in an ascending ethanol series and incubated in 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature to inhibit endogenous peroxidase activity. After rinsing with tap water, the sections were pretreated with 90% formic acid for 10 min and autoclaved at 121°C for 10 min in 0.01 M citrate buffer, pH 6.0, in order to enhance immunoreactivity. After washing with Tris-HCl buffer (50 mM Tris-HCl, pH 7.6), an anti-human COX-2 (polyclonal, rabbit, 1:100, Cayman Chemical Co.) was applied. The slides were incubated overnight at 4°C and then sequentially incubated for 1 h with a biotinylated secondary antibody diluted 1:200, and a peroxidase-conjugated streptavidin-biotin complex diluted 1:100 sequentially (Amersham, UK). The colored reaction product was developed with 3,3′-diaminobenzidine tetrahydrochloride solution. The sections were then lightly counterstained with hematoxylin.

For analysis of neuronal COX-2 immunoreactivity, the mean gray values of a random-selected 5 neurons and 5 neuropil backgrounds were quantified using ImageJ 1.36b (National Institute of Health, USA) and the average was calculated. Then, we converted the mean gray value into a density using the following equation; uncalibrated density = $\log_{10}$ (255/the mean gray value). Finally, we calculated the index that the neuronal density was divided by the neuropil background density, and we considered this index as the neuronal immunostaining density of COX-2. This index was calculated in CA1, CA3, subiculum entorhinal cortex and transentorhinal cortex.

The investigator was blind to the diagnosis of each case until analysis was completed and values were assigned to each specimen.

Statistical Methods

The quantitative data obtained were compared between the groups by Mann-Whitney's U test. Statistical significance was defined as $p < 0.05$. Correlation analysis was done using the Pearson parametric and Spearman nonparametric methods.

Results

Study A

Clinico-Neuropathological Information of Nondemented Subjects

The number of the nondemented subjects was 45 (M/F: 28/17). The ages at death were between 40 and 95 years and mean age at death was 76.4 ± 12.1 years. The brain weight was 1,264.0 ± 166.8 g (mean ± SD) and postmortem time was 13.1 ± 9.3 h (mean ± SD).

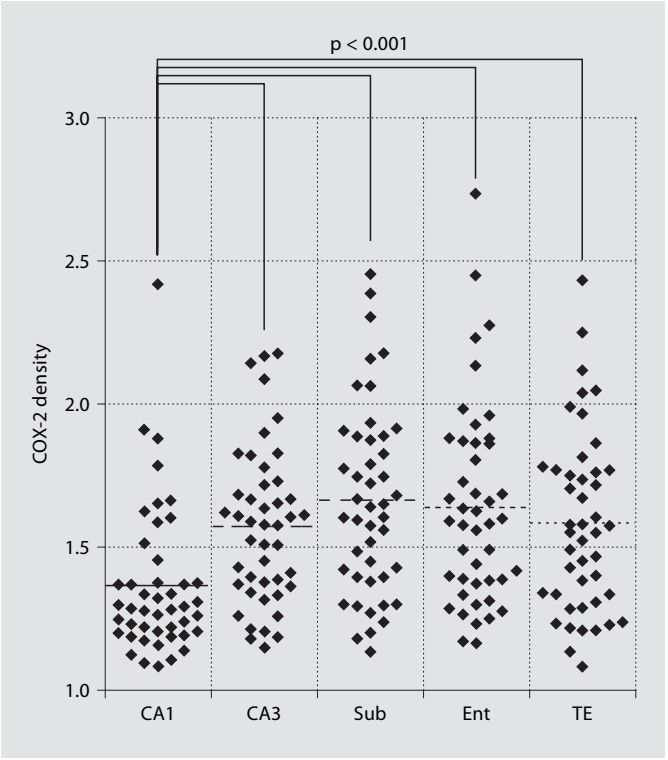

Fig. 1. Degrees of COX-2 immunoreactivity in different hippocampal subdivisions of nondemented subjects. Immunoreactivity is weak in CA1 as compared to all of the other fields examined with high statistical significance (Mann-Whitney U test, $p < 0.001$). Bars represent the mean density of neurons in each area. Sub = Subiculum; Ent = entorhinal cortex; TE = transentorhinal cortex.

COX-2 Immunoreactivity in the Hippocampus

The degree of COX-2 immunoreactivity in different hippocampal subdivisions of nondemented subjects is shown in figure 1. In nondemented subjects, the neuronal COX-2 immunoreactivity in CA3, subiculum, entorhinal cortex and transentorhinal cortex were strong (fig. 2a, d). On the other hand, the neuronal COX-2 immunoreactivity in CA1 was weak (fig. 2a, c) compared to all of the other fields examined, with high statistical significance (Mann-Whitney U test, $p < 0.001$). From these results, the constitutive expressions of COX-2 in CA3, subiculum, entorhinal cortex and transentorhinal cortex were thought to be strong, while weak in CA1.

COX-2 Immunoreactivity Correlates with Age in Nondemented Subjects

The correlation between neuronal COX-2 immunoreactivity and age is shown in figure 3. In the CA3 subdivi-

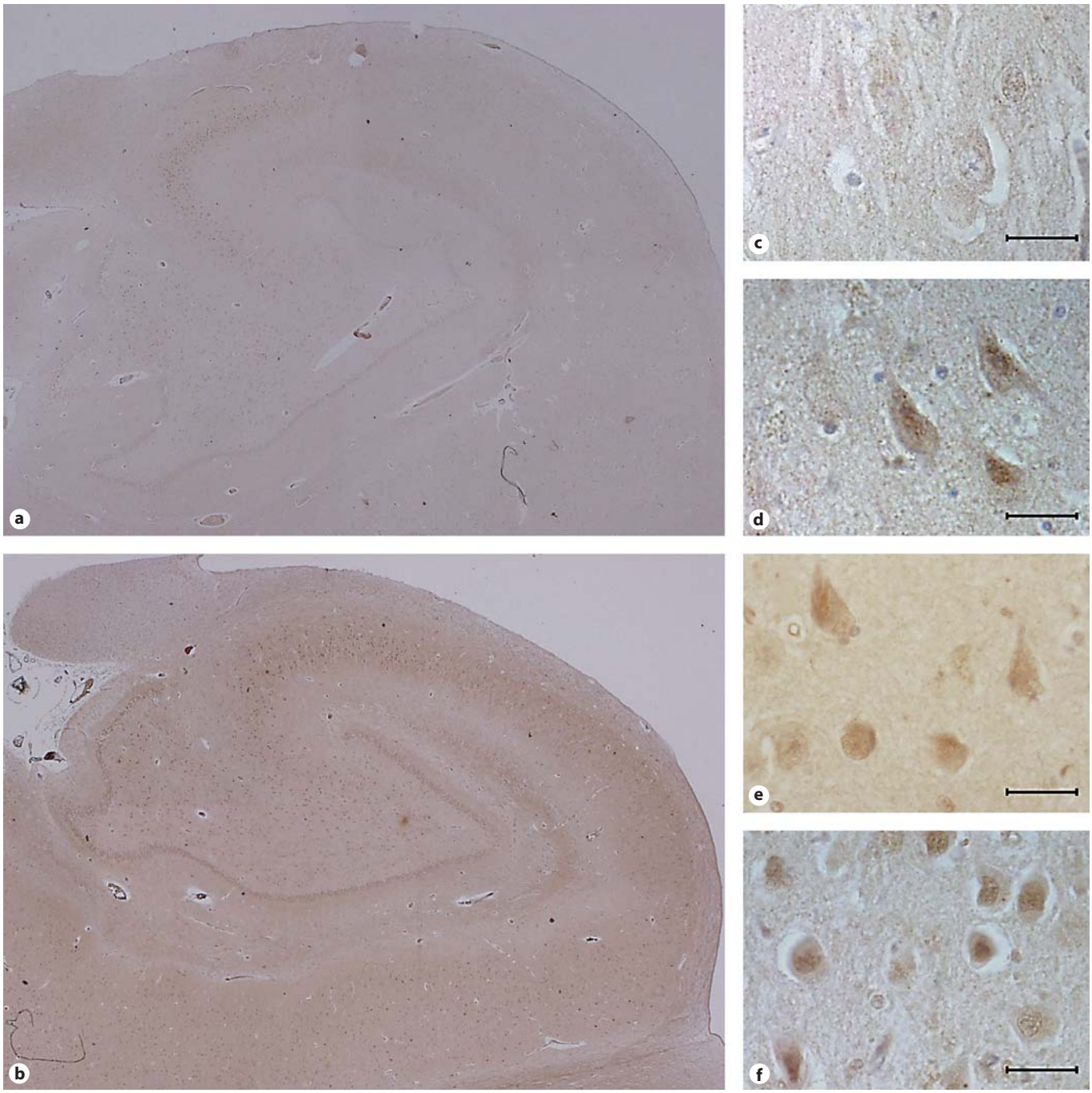

Fig. 2. COX-2 immunostaining in pyramidal neurons of the hippocampal formation in the brains of nondemented subjects (**a**, **c**, **d**) and in AD patients (**b**, **e**, **f**). **a**, **b** CA1–CA4 fields under low power. **c**, **e** Immunostaining within the CA1 field. **d**, **f** Immunostaining within CA3. Neuronal expression of COX-2 in CA3 is widely seen among both nondemented subjects and AD patients. Although neuronal expression of COX-2 in CA1 is widespread in AD patients, it is less detectable among nondemented subjects. **c–f** Bars are 30 μm.

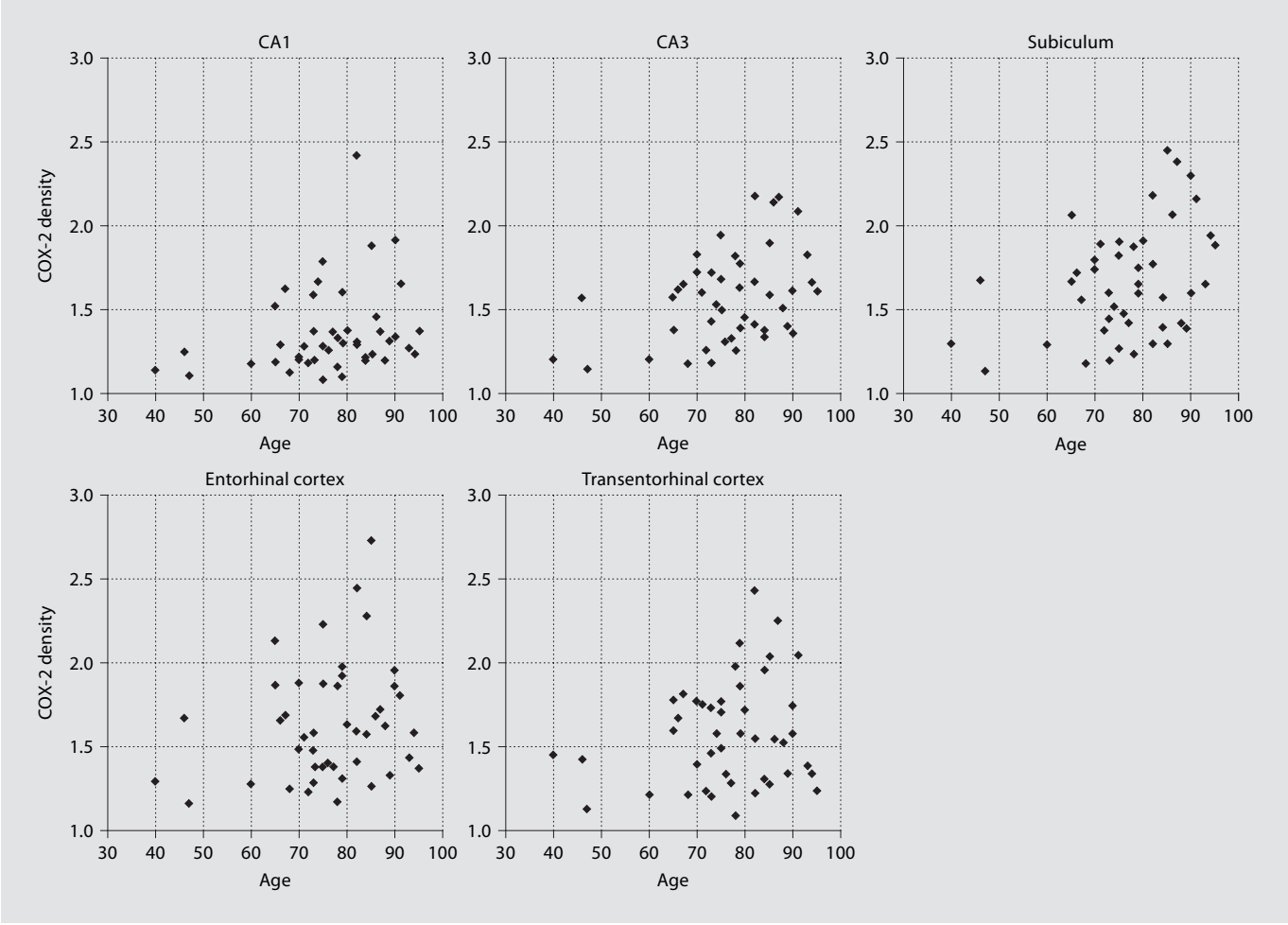

Fig. 3. A significant correlation exists between aging and COX-2 immunoreactivity in CA3 and the subiculum of nondemented subjects. In nondemented subjects, aging correlates with COX-2 immunoreactivity in CA3 and the subiculum (Pearson's correlation coefficient test, r = 0.399, p = 0.007; r = 0.380, p = 0.010, respectively).

sion of the hippocampus and subiculum of nondemented subjects, COX-2 immunoreactivity correlated with age (Pearson's correlation coefficient test, r = 0.399, p = 0.007; r = 0.380, p = 0.010, respectively) and this correlation was not evident in the CA1 subdivision of the hippocampus, entorhinal cortex or transentorhinal cortex (Pearson's correlation coefficient test, r = 0.268, p = 0.078; r = 0.220, p = 0.147; r = 0.194, p = 0.202, respectively).

Study B

Information of Nondemented Subjects and AD Patients

In study B, in order to compare the nondemented subjects with AD patients, we examined 25 nondemented subjects aged 76 years or more from study A and we collected another 25 age- and sex-matched AD autopsy cases derived from Hisayama Town (table 1). All of the AD patients were free of other types of dementia.

COX-2 Immunoreactivity in the Hippocampus

The degrees of COX-2 immunoreactivity in different hippocampal subdivisions of nondemented subjects and AD patients are shown in figure 4. The immunoreactivity in CA1 was increased in AD patients as compared to nondemented subjects with high statistical significance (Mann-Whitney U test, p = 0.001; fig. 2c, e). On the other hand, the differences between the nondemented subjects and AD patients were small in CA3, subiculum, entorhinal cortex and transentorhinal cortex (Mann-Whitney U test, p = 0.171, p = 0.467, p = 0.712, p = 0.621,

Table 1. Subjects of study B

Nondemented subjects			Age- and sex-matched AD patients		
ID	Sex	Age	ID	Sex	Age
22735	F	76	23297	F	77
22907[C]	F	77	21363	F	78
22880	F	78	22598	F	79
22939[C]	M	78	23373	M	79
22739	F	79	23114	F	79
22910[C]	F	79	23392	F	79
22819	M	79	20565	M	80
22803	M	80	23185	M	81
22933	M	82	20189	M	83
22828[C]	F	82	20617	F	82
22892	M	82	20316	M	83
22772[C]	F	84	20461	F	84
23015[C]	F	84	22502	F	84
23061	M	85	20706	M	85
22906	F	85	23018	F	84
22950	F	86	20240	F	87
22795	M	87	21501	M	86
22976[C]	F	88	23334	F	88
22798	M	89	22156	M	90
22767	F	90	20748	F	90
22992	F	90	23289	F	90
23021[C]	M	91	23028	M	92
22955[C]	F	93	23377	F	93
23055[C]	F	94	22661	F	94
22896[C]	F	95	23269	F	95

We studied all subjects aged >76 years of the 45 nondemented subjects of study A. As a comparison, we collected 25 age- and sex-matched AD patients. The subjects whose IDs are marked with 'C' are nondemented subjects with AD pathology (CERAD: moderate or frequent, with Braak and Braak stage 4, 5 or 6) and are the subjects of study C.

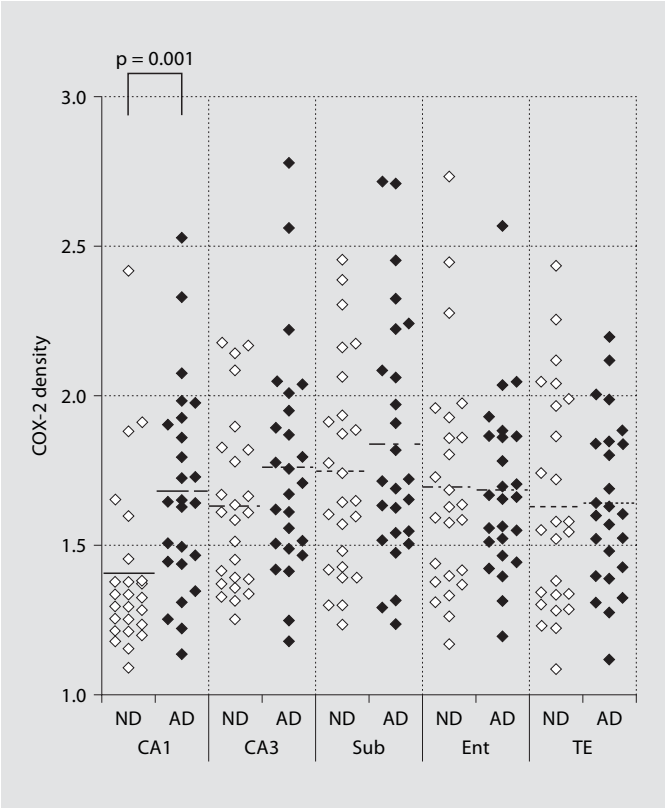

Fig. 4. Degrees of COX-2 immunoreactivity in different hippocampal subdivisions of nondemented subjects and AD patients. Immunoreactivity is increased in AD patients as compared to nondemented subjects, and the increase is observed in almost all hippocampal fields and reached statistical significance in the CA1 field (Mann-Whitney U test, p = 0.001). Bars represent the mean density of neurons in each area.

respectively). From these results, COX-2 immunoreactivity in CA1 has presumably been induced along with the development of AD; therefore, we assessed the influence of AD pathology on neuronal COX-2 expression in CA1.

COX-2 Immunoreactivity in CA1 Correlates with SP and NFT Density in AD Patients

In the CA1 subdivision of the hippocampi of AD patients, COX-2 immunoreactivity correlated with the semiquantification of SPs (Spearman's rank correlation test, $\rho = 0.500$, $p = 0.001$; fig. 5a) and with the number of NFTs (Pearson's correlation coefficient test, $r = 0.536$, $p = 0.003$; fig. 5b). On the other hand, in the CA1 subdivision of the hippocampi of nondemented subjects, COX-2 immunoreactivity did not correlate with either the semiquantification of SPs (Spearman's rank correlation test, $\rho = 0.013$, $p = 0.949$) or the number of NFTs (Pearson's correlation coefficient test, $r = -0.141$, $p = 0.501$).

Study C

In the Hisayama study, we sometimes encountered autopsy cases that were cognitively normal though exhibited severe Alzheimer type pathology in their brains. Therefore, in study C, we compared the immunoreactivity of COX-2 in CA1 of 11 nondemented subjects with AD pathology (CERAD: moderate or frequent, with Braak and Braak stage 4, 5 or 6; table 1) with that of 11 age- and sex-matched AD patients. The immunoreactivity of COX-

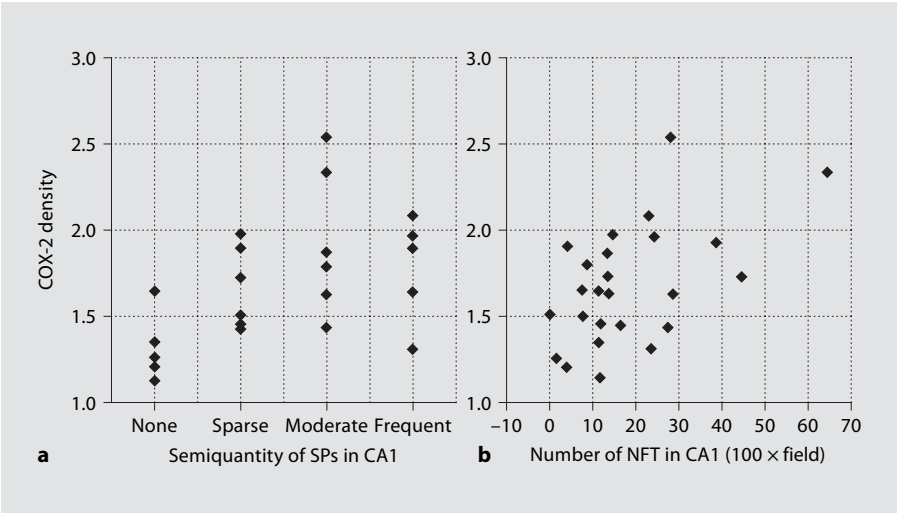

Fig. 5. COX-2 immunoreactivity in CA1 correlates with SP (**a**) and NFT (**b**) density in AD patients. The semiquantitative density of SPs in CA1 was determined using guidelines established by CERAD. In AD patients, a correlation between COX-2 immunoreactivity and the semiquantitative density of SPs in CA1 was statistically significant (Spearman's rank correlation test, $\rho = 0.500$, $p = 0.001$). Also, a correlation between COX-2 immunoreactivity and the density of NFT in CA1 was statistically significant (Pearson's correlation coefficient test, $r = 0.536$, $p = 0.003$).

2 in CA1 of AD patients was obviously stronger than in nondemented subjects with AD pathology (Mann-Whitney U test, $p < 0.001$; fig. 6).

Discussion

To our knowledge, this is the first report that explores the regional distribution of COX-2 immunoreactivity in the hippocampi of nondemented subjects of a general population. We found that among nondemented subjects, COX-2 immunoreactivity in CA3, subiculum, entorhinal cortex and transentorhinal cortex was widespread, suggesting that COX-2 is constitutively expressed in these subdivisions of the hippocampus, whilst weak immunoreactivity was observed in CA1. In addition, in the CA3 subdivision of the hippocampi and subiculum of nondemented subjects, COX-2 immunoreactivity correlated with age, suggesting that the COX-2 expression in this region was augmented with aging in the normal condition.

Strong expression of COX-2 in the CA3 subdivision of the hippocampus and expression of COX-2 in the subiculum was reported previously in the normal rat brain [27]. Also, an immunohistochemical study of the human hippocampus showed more COX-2 positive neurons to be present in the CA3 than in the CA1 or CA2 field, and this difference became clearer when analysis was limited to dense staining [11]. These results are compatible with our data and indicate that our staining and assessment of COX-2 are reasonable and also suggest the constitutive expression of COX-2 in these subdivisions of the hippocampus.

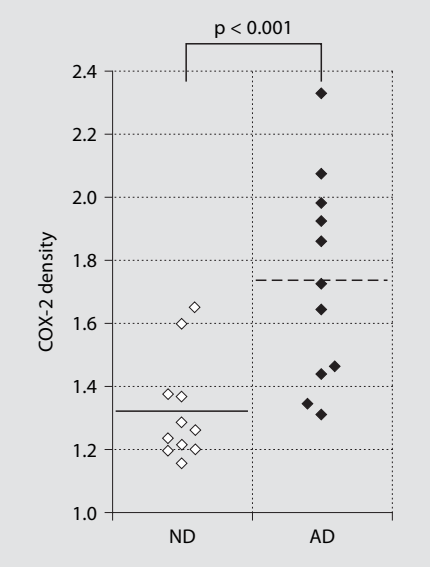

Fig. 6. Degrees of COX-2 immunoreactivity in the CA1 subdivision of the hippocampus of nondemented subjects with AD pathology and AD patients. COX-2 immunoreactivity is increased in AD patients as compared to nondemented subjects (Mann-Whitney U test, $p < 0.001$). Bars represent the mean density of neurons in each area.

The enhanced expression of COX-2 with age was reported in a study examining aged mouse macrophages [28], but little is known about changes in COX-2 expression in the human brain during aging. Now that several inflammatory processes are thought to play a critical role in brain aging and to be associated with an increased vul-

nerability to neurodegeneration [29], we think that our finding provides a new point of view in regard to the aging brain. For example, a COX inhibitor was reported to protect mice from age-associated cognitive impairment under normal conditions [30]. These studies may illustrate a new 'anti-aging' treatment for the human brain.

In addition, we assessed neuropathologically confirmed AD patients in order to appreciate the influence of AD pathology on COX-2 expression within the hippocampus. We found that COX-2 immunoreactivity was increased in AD patients as compared to nondemented subjects and this change was observed in almost all hippocampal fields, reaching statistical significance in the CA1 field. This difference remained statistically significant even when we limited the nondemented subjects to those with AD pathology. In other words, the upregulation of COX-2 in the CA1 field in conjunction with AD pathology may be one of the important factors for developing AD. Over the last 10 years, several studies have examined the expression of COX-2 in postmortem AD brain tissues but have yielded conflicting results. One of the problems in these studies is in the method of selecting the nondemented group used for comparison with the AD group. As we mentioned, there is a possibility that COX-2 expression may be augmented with age; therefore, in a study of aged subjects, the difference of COX-2 expression between the AD and the nondemented group may become small and the opposite may also be true. Making the matter complicated, the degree of this augmentation may differ in each area.

We found that COX-2 immunoreactivity in CA1 correlates with AD pathology in AD patients. A previous immunohistochemical study reported a similar result [8], and recently the possibility of a direct interaction between human Aβ and COX-2 being mediated by peroxidase activity was reported [31]. Why the correlation between COX-2 immunoreactivity in the CA1 field and AD pathology was observed only in AD patients and not in nondemented subjects is still unclear and further examination is required.

Here, we assessed the relationship between the neuronal expression of COX-2 and AD. It has recently been reported that the neuronal expression of COX-2 may play an important role in other types of neurodegenerative disorders. For instance, neuronal COX-2 expression in all CA fields of the hippocampus was significantly upregulated in ALS patients as compared to control subjects [13] and COX-2 was upregulated in brain dopaminergic neurons of both Parkinson's disease and MPTP-treated mice [12]. Upregulation of neuronal COX-2 expression may be a common pathway that a variety of neurodegenerative disorders exhibit.

In conclusion, in this study we have explored the regional expression of COX-2 in the hippocampus of the normal brain, and based on this result we have also explored the correlation between AD pathology and COX-2 expression in this region. While COX-2 expression appears to be differently regulated amongst hippocampal subdivisions and its presence may be augmented with age in the cognitively normal brain, COX-2 expression within the CA1 field of AD brains may be associated with the cognitive decline to some extent.

Acknowledgments

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