Neurosteroid Quantification in Human Brain Regions: Comparison between Alzheimer's and Nondemented Patients

SÉBASTIEN WEILL-ENGERER, JEAN-PHILIPPE DAVID, VÉRONIQUE SAZDOVITCH, PHILIPPE LIERE, BERNARD EYCHENNE, ANTOINE PIANOS, MICHAEL SCHUMACHER, ANDRÉ DELACOURTE, ETIENNE-EMILE BAULIEU, AND YVETTE AKWA

INSERM, U-488 (S.W.E., P.L., B.E., A.P., M.S., E.E.B., Y.A.), 54276 Le Kremlin-Bicêtre, France; Assistance Publique-Hôpitaux de Paris, Hôpital Rothschild, Service de Gériatrie (S.W.E.), 75012 Paris, France; INSERM, U-422 (J.P.D., A.D.), 59045 Lille, France; Assistance Publique-Hôpitaux de Paris, Hôpital Emile Roux, Service de Gérontologie 3 (J.P.D.), 94456 Limeil Brevannes, France; and Assistance Publique-Hôpitaux de Paris, Service d'Anatomie Pathologique Neurologique, Hôpital Pitié-Salpêtrière (V.S.), 75013 Paris, France

Some neurosteroids have been shown to display beneficial effects on neuroprotection in rodents. To investigate the physiopathological significance of neurosteroids in Alzheimer's disease (AD), we compared the concentrations of pregnenolone, pregnenolone sulfate (PREGS), dehydroepiandrosterone, dehydroepiandrosterone sulfate (DHEAS), progesterone, and allopregnanolone, measured by gas chromatography-mass spectrometry, in individual brain regions of AD patients and aged nondemented controls, including hippocampus, amygdala, frontal cortex, striatum, hypothalamus, and cerebellum. A general trend toward decreased levels of all steroids was observed in all AD patients' brain regions compared with controls: PREGS and DHEAS were significantly lower in the striatum and cerebellum, and DHEAS was also significantly reduced in the hypothalamus. A significant negative correlation was found between the levels of cortical β-amyloid peptides and those of PREGS in the striatum and cerebellum and between the levels of phosphorylated tau proteins and DHEAS in the hypothalamus. This study provides reference values for steroid concentrations determined by gas chromatography-mass spectrometry in various regions of the aged human brain. High levels of key proteins implicated in the formation of plaques and neurofibrillary tangles were correlated with decreased brain levels of PREGS and DHEAS, suggesting a possible neuroprotective role of these neurosteroids in AD. (*J Clin Endocrinol Metab* 87: 5138–5143, 2002)

A VARIETY OF STEROIDS can be synthesized in the rodent brain independently of peripheral glandular sources. Such steroids formed within the brain from cholesterol are defined as neurosteroids (1). Among them, neuroactive neurosteroids are allosteric modulators of the neurotransmitter receptor activities (2), hence regulating different aspects of animal behavior (3, 4). Particularly relevant to the aging process and Alzheimer disease (AD) are the beneficial effects of neurosteroids on memory (5) and neuroprotection against the β-amyloid peptide-induced neurotoxicity *in vitro* (6–8). In the human brain some studies have reported quantification of several neurosteroids, such as pregnenolone (PREG; 3β-hydroxy-pregn-5-ene-20-one), pregnenolone sulfate (PREGS), dehydroepiandrosterone (DHEA; 3β-hydroxy-androst-5-ene-17-one), DHEA sulfate (DHEAS), and allopregnanolone (3α,5α-THP; 3α-hydroxy-5α-pregnan-20-one). However, most of them have used subjects with a wide range of ages, between 2–85 yr (9–11), except for the one by Lacroix *et al.* (12) that used 76- to 93-yr-old subjects whose neurological status was not mentioned.

Normal aging is accompanied by numerous physiological, behavioral, and hormonal changes. One of the most prominent endocrine changes is the continuous decrease with age in the blood levels of steroids. Besides the age-related decrease in circulating concentrations of estradiol (in menopause) and testosterone (in andropause), a significant fall also occurs in plasma in both women and men, for PREG, PREGS, DHEA, DHEAS, and 3α,5α-THP (13). Whether the profound age-related reduction in the blood concentrations of these steroids is associated with AD is still unclear. Only DHEAS and DHEA have been measured in the blood of AD patients in comparison with nondemented controls, but results were rather contradictory (14–17). To the best of our knowledge, there is no study examining the brain levels of neurosteroids in AD patients.

To gain a better knowledge of the role of neurosteroids in dementia, we compared their concentrations in several brain regions between AD patients and aged nondemented controls. We also investigated the possible relationship between the neurosteroid levels and specific structural brain abnormalities related to AD, such as the extracellular senile plaques composed of β-amyloid peptides (Aβ) and the intracellular neurofibrillary tangles (NFT) constituted of intraneuronal bundles of paired helical filaments (PHF) that result from the aggregation of pathologic tau proteins (PHF-tau) (18, 19).

Abbreviations: Aβ, β-Amyloid peptides; AD, Alzheimer's disease; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; GC-MS, gas chromatography-mass spectrometry; NFT, neurofibrillary tangles; PHF, paired helical filaments; PHF-tau, paired helical filaments that result from the aggregation of pathologic tau proteins; PREG, pregnenolone; PREGS, pregnenolone sulfate; PROG, progesterone; 3α,5α-THP, allopregnanolone.

Subjects and Methods

Subjects

Eleven patients (median age, 86.3 yr; minimum, 75.6 yr; maximum, 91.5 yr) were selected for the study. They were hospitalized and died in a geriatric unit (Service de Gérontologie 3, Hôpital Emile Roux, Limeil-Brévannes, France). Exclusion criteria were a postmortem delay beyond 24 h, steroid or benzodiazepine administration during the month before death, and prolonged hypoxemia at the time preceding death. Dementia was considered using the criteria of Diagnostic and Statistical Manual of Mental Disorders (20), and criteria of AD were those proposed by the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (21). All patients had a full clinical evaluation of neurological status and a specific complementary evaluation (cerebral tomography, electroencephalography, serological tests for syphilis and human immunodeficiency virus, and serum determinations of thyroid function, vitamin B_{12}, folates, and calcium). At the time of autopsy, one hemisphere of each brain was formalin-fixed for neuropathological examination, and the other one was deep-frozen at -80 C until biochemical analysis. Patients were included on the basis of their clinical and biological diagnoses. The diagnosis of AD was excluded for six patients (nondemented control group: two women and four men) and was sustained for five others (AD group: four women and one man). All clinical investigations described here were approved by the local committee and conducted in accordance with the Declaration of Helsinki.

Neuropathological examination of brain samples

The neuropathological features of all patients were evaluated as in a previous study (22) and are detailed in Table 1. Briefly, the presence of amyloid plaques and NFT were scored in cortical and subcortical areas according to Consortium to Establish a Registry for Alzheimer's Disease criteria (23) and Braak and Braak's stages (24), respectively. Quantification of amyloid depositions was performed by counting the number of plaques per square millimeter, using thioflavine S and antibodies against Aβ peptides, *i.e.* monoclonal Aβ 8–17 (DAKO Corp., Carpinteria, CA) or polyclonal antibodies against the N- and C-terminal partd of Aβ 1–40 and Aβ 1–42 (INSERM, U-422, Lille, France). NFT were quantified by counting the numbers per square millimeter using monoclonal antibody AD2 directed against PHF-tau (25).

Collection of brain regions

Upon thawing of one cerebral hemisphere at –10 C, large portions of hippocampus, amygdala, frontal cortex, striatum, hypothalamus, and cerebellum were dissected out, and representative fractions of 100 mg of each brain structure were collected and immediately stored at –80 C. From a total of 66 samples (11 patients × 6 brain regions) to be collected,

TABLE 1. Neuropathological features of the aged nondemented and Alzheimer's patients

Group	Gender	Age (yr)	Histological classification	
			Amyloid plaques	NFT
Nondemented control	Woman	87.9	0	III
	Woman	90.3	A	III
	Man	75.6	0	II
	Man	80.9	0	I
	Man	86.3	A	II
	Man	91.5	A	I
Alzheimer's patients	Woman	81.4	C	V
	Woman	85.5	C	IV
	Woman	88.0	C	VI
	Woman	91.1	B	IV
	Man	86.0	C	V

Histological classification as evaluated previously (22). Amyloid plaques and NFT were classified in cortical areas according to the Consortium to Establish a Registry for Alzheimer's Disease criteria (23) and Braak and Braak's classification (24), with respect to the location and the density of amyloid plaques (0–C) and NFT (I–VI).

7 samples (1 hippocampus and 2 amygdala from controls as well as 2 amygdala and 1 hypothalamus from AD patients) were not included in the study because of difficulties in definite identification.

Biochemical analysis of Aβ and PHF-tau proteins

The levels of Aβ were quantified in homogenates of frontal cortex as in a previous study (22). Briefly, Aβ was identified by dot blot using the same immunological probes as those used for amyloid plaque detection, according to the procedure developed by Permanne *et al.* (26) and was quantified using the ImageMaster program developed by Amersham Pharmacia Biotech (Piscataway, NJ).

The levels of PHF-tau proteins were determined in all the brain regions collected. PHF-tau proteins were immunodetected by Western blot using monoclonal antibody AD2 as described for NFT detection, according to the method reported by Sergeant *et al.* (27). They were quantified, using the ImageMaster program, by measuring the area of the peaks corresponding to tau 55, 64, and 69, which were then scored by comparison with a temporal cortex homogenate from a patient with early-onset AD, considered as a positive internal standard, as previously described (28).

Biochemical analysis of steroids

The method used for steroid analysis was previously described and validated (29). Briefly, after adding 3β,5β-tetrahydroandrostenedione (Sigma-Aldrich, St. Louis, MO) as the internal standard for PREG, DHEA, progesterone (PROG), 3α,5α-THP, and [^{2}H$_4$]PREGS ([17,21,21,21-^{2}H]PREGS, trimethyl ammonium salt, prepared by Dr. R. Purdy, La Jolla, CA) as the internal standard for PREGS and DHEAS, the steroids were extracted by methanol 100%. The sulfated and unconjugated steroids were separated and purified by chromatography on AMPREP silica minicolumns C_{18} (Amersham International, Little Chalfont, UK) using solutions of MeOH-H_2O [40:60, (vol/vol) and 85:15 (vol/vol), respectively]. The sulfated steroid fraction was solvolysed with ethyl acetate containing 0.2% 0.5 M H_2SO_4 (30) at room temperature for 1 h. The unconjugated steroid fraction was submitted to HPLC. The HPLC system from Thermoquest (San Jose, CA) consisted of a P1000XR quaternary pump and an AS 100 XR TSP autoinjector. HPLC was achieved with a Lichrosorb Diol column (25 cm × 4.6 mm, 5 μm). The solvent system consisted of hexane and mixture A (90:10, vol/vol), the latter composed of hexane-isopropanolol (85:15, vol/vol). The elution was performed at a flow rate of 1 ml/min. The elution profiles of PREG, DHEA, PROG, and 3α,5α-THP were identical to those previously reported (29). The unconjugated steroids from the HPLC fractions and those obtained after solvolysis were then derivatized separately with anhydre heptafluorobutyric acid (Pierce Chemical Co., Rockford, IL) before injection into the gas chromatography-mass spectrometry (GC-MS) system. GC was performed in the splitless mode with a GC 8000 Top gas chromatograph (Carlo Erba, Milan, Italy). The mass spectrometer (model 150, Finnigan Automass, Argenteuil, France) was operated in the electron impact mode. Identification was performed in the full-scan mode, and quantification was performed in the single ion monitoring mode. Each GC-MS measurement was made in duplicate.

Statistical analysis

Nonparametric tests were used. The Kruskal-Wallis test was used for overall comparisons between the two groups of patients and followed, when significant, by the Mann-Whitney test applied region by region. The Friedman test was used for overall comparisons between regions and followed, when significant, by the Wilcoxon test. Statistical associations between two sets of independent and continuous data were assessed by the Spearman test. Data are presented as the mean ± SEM. Significance was defined at $P < 0.05$.

Results

Age and sex characteristics

Six patients were assigned to the control group, and five patients constituted the AD group. In the control and AD groups, the median ages were 87.1 and 86.0 yr, respectively,

and there was no significant difference in age ($P > 0.999$) or in sex ratio (by Fisher's exact test, $P = 0.242$) between the two groups. Women were slightly older than men (median, 87.9 and 86.0 yr, respectively), but no significant difference was found with age according to sex ($P = 0.361$).

Steroid quantification in brain regions

As indicated in Figs. 1–3 the steroids found at the highest concentrations were in decreasing order (PREG > DHEA > PROG, > PREGS > DHEAS ≫ $3\alpha,5\alpha$-THP) in all regions of both groups. No difference was noted between regions in the AD group. In contrast, a significant overall difference between regions was observed in the control group for each steroid measured (PREGS, $P < 0.05$; other steroids, $P < 0.01$), except for $3\alpha,5\alpha$-THP ($P = 0.097$). In this group, regions containing the highest levels of steroids were, in decreasing order, hypothalamus > striatum > frontal cortex > cerebellum > amygdala ≈ hippocampus. Region by region comparisons in the control group revealed that the levels of all steroids measured, except $3\alpha,5\alpha$-THP, were always significantly lower in the hippocampus and frontal cortex than in the hypothalamus ($P < 0.05$) and also in the striatum for the sulfated steroids ($P < 0.05$). Moreover, free steroids (*i.e.* PREG, DHEA, and PROG) exhibited a significantly lower level in the cerebellum than in the hypothalamus ($P < 0.05$).

A general trend toward lower levels of steroids was observed in the six brain regions of the AD patients compared
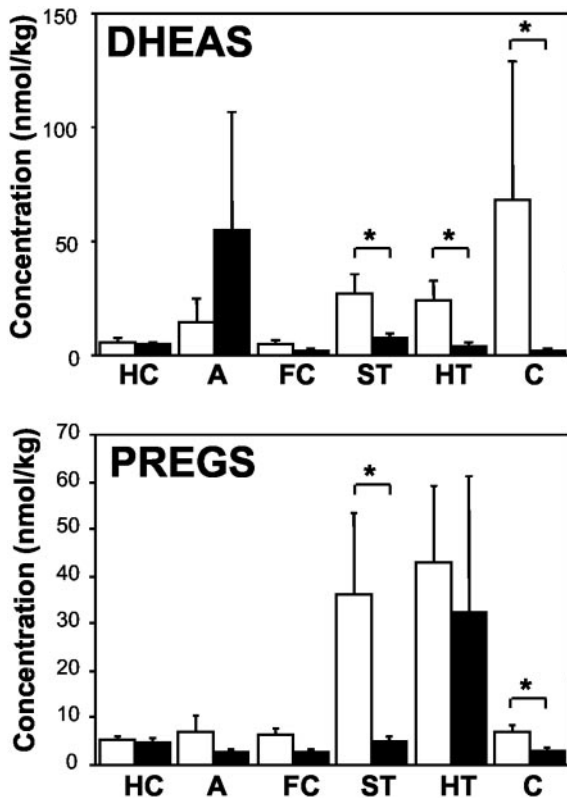

FIG. 1. Concentrations of PREGS and DHEAS in six brain regions of nondemented (□) and AD (■) patients. HC, Hippocampus; A, amygdala; FC, frontal cortex; ST, striatum; HT, hypothalamus; C, cerebellum. Values are the mean ± SEM. *, $P < 0.05$ (by Mann-Whitney test).
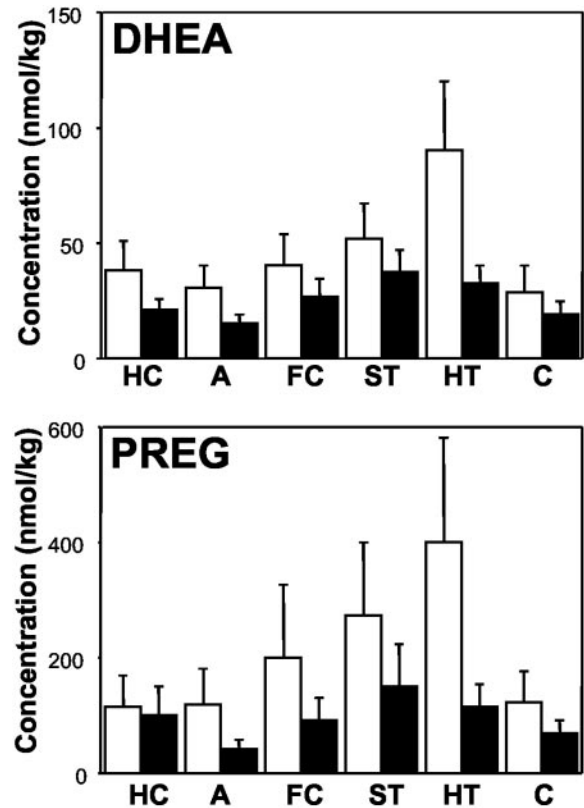

FIG. 2. Concentrations of PREG and DHEA in six brain regions of nondemented (□) and AD (■) patients. HC, Hippocampus; A, amygdala; FC, frontal cortex; ST, striatum; HT, hypothalamus; C, cerebellum. Values are the mean ± SEM.

with the controls. An overall significant regional difference was found between the two groups for PREGS ($P < 0.001$) and DHEAS ($P < 0.001$; Fig. 1). PREGS was significantly lower in the striatum ($P < 0.05$) and cerebellum ($P < 0.05$) of AD patients than in controls and was close to a significant level of difference in the frontal cortex ($P = 0.068$). DHEAS was significantly lower in the AD group compared with controls in the striatum ($P = 0.029$), cerebellum ($P = 0.011$), and hypothalamus ($P = 0.019$).

Relationship among the levels of brain steroids, PHF-tau proteins, and Aβ

The levels of PHF-tau proteins were significantly higher in the AD group compared with the controls in all brain regions ($P < 0.05$) except the amygdala, for which the difference was just above the level of significance ($P = 0.077$) despite the four samples missing, and the cerebellum, in which PHF-tau proteins were undetectable in all patients (Fig. 4). A significant negative correlation was observed between the PHF-tau proteins and DHEAS levels in the hypothalamus ($P < 0.05$; $\rho = -0.717$), and no relationship was found for the other steroids analyzed.

The levels of Aβ in the frontal cortex were significantly higher in the AD patients compared with the control group (respectively, 67.8 ± 20.8 and 2.2 ± 2.0 nmol/kg brain tissue, respectively; $P = 0.008$). A significant negative correlation was found between the levels of cortical Aβ and PREGS in
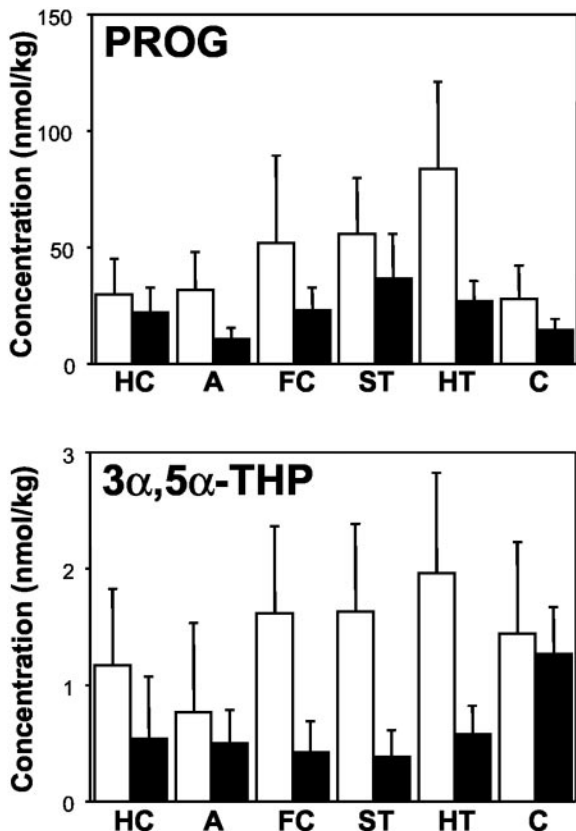
FIG. 3. Concentrations of PROG and $3\alpha,5\alpha$-THP in six brain regions of nondemented (□) and AD (■) patients. HC, Hippocampus; A, amygdala; FC, frontal cortex; ST, striatum; HT, hypothalamus; C, cerebellum. Values are the mean ± SEM.
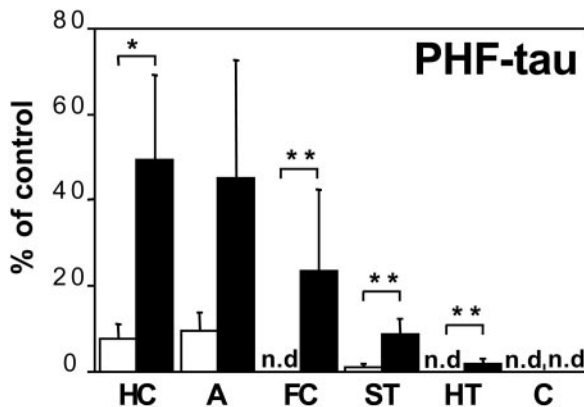

FIG. 4. Concentrations of PHF-tau in six brain regions of nondemented (□) and AD (■) patients, expressed as a percentage of the positive control value (22). HC, Hippocampus; A, amygdala; FC, frontal cortex; ST, striatum; HT, hypothalamus; C, cerebellum; n.d., not detected. Values are the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$ (by Mann-Whitney test).

the striatum ($P < 0.05$; $\rho = -0.718$) and cerebellum ($P < 0.05$; $\rho = -0.729$). DHEAS concentrations were also negatively correlated with Aβ levels in these two structures, but were at the threshold of significance ($P = 0.094$ and $P = 0.055$, respectively). In the other brain regions studied, there were no significant correlations between the levels of Aβ and those of DHEAS, PREGS, or any other steroid.

Discussion

The present study is the first report, to the best of our knowledge, of a comparative analysis of the concentrations of several neurosteroids, determined by GC-MS, in various brain regions between aged AD patients and aged nondemented controls. Compared with RIA, the technique employed here, coupling HPLC and GC-MS, offers the advantages of high sensitivity and specificity and allows the simultaneous quantification of different steroids in individual tissue samples (29). This methodology allowed us to compare the levels of various neurosteroids in specific brain regions between AD patients and nondemented controls. Despite the rise in interindividual variability that occurs during aging, differences in brain steroid levels between the AD group and controls were observed.

In the human brain, numerous steroids have already been quantified, such as PREG and DHEA (12, 31), PREGS and DHEAS (31), PROG (10, 11, 31), and $3\alpha,5\alpha$-THP (10). To date, no study has used GC-MS technology to quantify steroids in the human brain, thus making difficult the comparison of our GC-MS values with the previously published RIA measurements. In addition, only a few studies have analyzed more than three human brain regions, and the regions that were chosen were generally different from one study to another (9, 10, 12, 31).

According to previous measurements of neurosteroids in the human brain using RIA, only the study by Lanthier *et al.* (31) has provided data on the levels of PREGS and DHEAS in brain regions of five patients. These researchers also assayed PREG and DHEA, as did Lacroix *et al.* (12), specifically in the cortex and cerebellum of 10 subjects. Comparisons between our data and those of the cited studies revealed that the differences in the concentrations of neurosteroids (PREG, DHEA, PREGS, and DHEAS) in control patients are limited to the hypothalamus, where our values were 2- to 4-fold higher than those found by the others. Apart from this difference, our steroid measurements in the frontal cortex, amygdala, and hippocampus are in the same range and, to the best of our knowledge, we are the first to describe the concentrations of PREG, DHEA, PREGS, and DHEAS in the striatum. In the previous reports old subjects were used, but their neurological status was not described despite the high prevalence of neurological diseases in this age class. Significant differences between the two groups of patients were found in several regions for DHEAS and PREGS. It is of importance to highlight that these two steroids are those that have shown the greatest evidence for a neuroprotective effect (8, 32–35). Cerebral concentrations of DHEA and PREG in the present study were much higher (in the range of 5.69–201.99 nmol/kg for DHEA and 11.09–1011.33 nmol/kg for PREG, depending on the brain regions) than those reported in the blood of very old patients (13, 36, 37), consistent with the postulate of local production of DHEA and PREG in the human brain.

Measurements of PROG in specific regions of the brain of elderly subjects have been reported, but a great heterogeneity could be observed among the values. Overall values from the control patients of our study are either clearly higher than some of those previously reported (10–12, 31) or very similar

to those of the oldest subgroup (women; median age, 75 yr) of subjects in the study by Bixo *et al.* (10). To date, quantification of $3\alpha,5\alpha$-THP in the human brain has only been reported in one study (10). In that work, conducted upon much younger subjects than those in our study, the $3\alpha,5\alpha$-THP values were 20 times higher in the cortex and hypothalamus and 30 times higher in the striatum and cerebellum compared with those in the corresponding brain areas in the controls of the present study.

AD is the most common cause of dementia in the elderly. One line of research to explain the pathogenesis of this neurological disorder is currently oriented toward examination of the possible relationships between specific structural, metabolic, and neurochemical brain abnormalities (for review, see Ref. 38). Natural progression of AD involves first the hippocampus and neocortex, but also other areas of the limbic system, such as the amygdala and olfactory bulbs (39), and extends progressively to cortical brain areas during the course of AD (22). The involvement of basal nuclei, such as hypothalamus or striatum, and the cerebellum occurs later in the progression of the disease (40, 41).

We demonstrate here for the first time that two biochemical hallmarks of AD, namely PHF-tau and Aβ, are correlated with the levels of the neurosteroids PREGS and DHEAS in distinct brain regions. Our results revealed that PHF-tau levels were significantly and negatively correlated with the DHEAS concentration in the hypothalamus. They also indicated that the levels of cortical Aβ were significantly and negatively correlated with PREGS levels and to some extent with DHEAS in the striatum and cerebellum. It is known that a widespread neuronal loss occurs during the course of AD that could be in part causal or consecutive to the lower steroid levels observed in AD brains. The absence of a relationship between the levels of PHF-tau and steroids, particularly in the hippocampus and cerebral cortex, is consistent with the fact that NFT, which are the histological hallmark of high levels of PHF-tau, are known to always be present in the hippocampus and entorhinal cortex of very old patients regardless of their state of dementia (22, 42, 43). These findings are supportive of the role of DHEAS in the protection against Aβ-induced neurotoxicity (7, 8) and in the enhancement of Amyloid precursor protein production (44).

In conclusion, this study provides reference values, determined by GC-MS, for steroid concentrations in various regions of the aged human brain. High levels of key proteins implicated in the formation of plaques and NFT were correlated with decreased brain levels of PREGS and DHEAS, thus suggesting a possible protective role for these neurosteroids in the dementia related to AD.

Acknowledgments

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