Cerebrospinal fluid levels of corticotropin-releasing hormone in Alzheimer's disease

UNIVERSITY OF GOTHENBURG

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Master thesis in Medicine

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative condition, in which several neurochemical abnormalities have been found. Recently, chronic stress was found to be a powerful inducer of tau pathology in two transgenic mouse models of AD, and that this effect was mediated by corticotropin-releasing factor (CRF). In this study we tested, in a series of four independent clinical materials, if the CSF levels of the human counterpart to CRF1, corticotropin-releasing hormone (CRH) were elevated in AD and if there were any correlations between CSF CRH and tau pathology in a manner similar to what has been seen in transgenic mouse models of AD.

CRH concentration in CSF was measured by radioimmunoassay (RIA). CSF T-tau, CSF T-tau and Aβ42 levels were determined using INNOTEST ELISAs (Innogenetics, Ghent, Belgium). Statistical analyses were performed using GraphPad Prism 5.

We found that CSF CRH levels were slightly elevated in AD patients compared with controls in one of the four independent case-control studies, while the other three indicated no significant change. A weak, statistically non-significant trend towards a positive correlation of CSF CRH with the tau pathology marker P-tau was seen in the first set of samples from AD patients. Taken together, the data suggest that CSF CRH is not a stable diagnostic biomarker for AD. However, we cannot exclude a role of CRH in tau hyperphosphorylation and more and larger studies are needed on this topic.

Keywords

Alzheimer’s disease, cerebrospinal fluid, corticotropin-releasing hormone, tau pathology

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Background

Alzheimer's disease (AD) is a progressive neurodegenerative condition and is the most common cause of dementia [1]. The prevalence increases with age. In its early stages, AD is characterized clinically by progressive memory loss and cognitive impairment, with gradually increasing neurological and somatic symptoms. The process is continuously progressing [2, 3].

Three neuropathological changes are typical of AD: extracellular senile plaques composed of aggregated amyloid β (Aβ), intraneuronal neurofibrillary tangles composed of hyperphosphorylated tau proteins (P-tau) and neuronal loss (Bradley et al., 2012)

Several risk factors have been associated with increased risk of sporadic AD, such as age, genetic susceptibility, head trauma, hypertension and dyslipidemia [4, 5].

Genetics of AD

Previous studies have identified numerous mutations in genes associated with rare familial forms of AD, such as mutations in amyloid precursor protein (APP), or in either of the two presenilin genes (PSEN1 and PSEN2). Presenilin genes are involved in APP metabolism [6]. Based on these findings, the 'amyloid cascade hypothesis' has been suggested. This hypothesis suggests that AD is caused by imbalance between production and clearance of Aβ that eventually leads to Aβ overload and plaque pathology, which in turn leads to tau pathology. It is widely accepted that amyloidogenic processing of APP is at the core of AD pathogenesis and that the prime target for Alzheimer-associated Aβ is the synapse, which eventually causes neuroaxonal degeneration and tau pathology [7]. However, in some disease models, it is difficult to induce tau pathology only by overexpressing Aβ and it is possible that other co-factors are needed to induce tau pathology [8].

Apolipoprotein E (APOE) plays an essential role in lipid metabolism [9]. One specific allele of APOE, ε4, has been identified as a risk factor for late onset sporadic AD [10]. The exact mechanism underlying this association is at present unknown.

Neuropathology of AD

In 1984-85 it was shown that senile plaques are composed of a 4kDa protein called amyloid β (Aβ) [11]. The protein is generated from amyloid precursor protein (APP), which is a cell membrane protein. α-, β and γ-Secretases cleave APP at various sites in APP and give rise to different fragments of the Aβ peptide ($A\beta_{1-13}$, $A\beta_{1-16}$, $A\beta_{1-17}$, $A\beta_{1-40}$ $A\beta_{1-42,}$ to mention few), which can be detected in CSF [12, 13]. γ-Secretase is an enzyme complex consisting of four components; presenilin (PSEN1), niscastrin, PSEN2, and APH1 [14]. The prime constituent of senile plaques is the 42 amino acid long and sticky $A\beta_{1-42}$ isoform. Cerebrospinal fluid (CSF) $A\beta_{1-42}$ levels are decreased in AD patients [6], which most likely reflects sequestration of the peptide in amyloid plaques in the brain.

Tau is an axonal protein that plays a central role in stabilizing microtubules, and is expressed predominantly in unmyelinated axons, primarily in the areas of the brain responsible for memory processing [15]. Alternative splicing of MAPT (microtubule-associated protein tau) gene transcripts gives rise to six different isoforms of tau protein [16]. Tau regulates the stability of microtubules through the formation of the different isoforms and through varying degrees of phosphorylation of its amino acids. Tau phosphorylation is regulated by the balance between several different kinases and phosphatases [17]. Several phosphorylation sites have been identified in tau, but the degree of phosphorylation is mainly quantified at Thr181 or Thr231 [18, 19]. Hyperphosphorylated tau has a reduced capacity to assemble and stabilize microtubules, and

this affects the stability of the structure, which in turn ultimately leads to impaired axonal transport, neuronal dysfunction and neuroaxonal degeneration. Abnormal hyperphosphorylation of tau protein causes accumulation and aggregation of the protein in structures called paired helical filaments (PHFs); a process that eventually leads to formation of larger deposits called neurofibrillary tangles. Tau in the brain of AD patients has been shown to be hyperphosphorylated compared with tau in healthy adults. Tau pathology in AD patients is characterized by elevated CSF T-tau and CSF P-tau levels due to cortical neuronal decay [6, 17, 20, 21]

CSF total tau (T-tau) reflects the intensity of neuronal degeneration, and particularly high levels of this biomarker have been reported in conditions such as Creutzfeldt-Jakob disease. [21, 22]. Positive correlations between P-tau and T-tau in AD patients have been reported in several studies [6]. CSF P-tau levels reflect the degree of tau phosphorylation in brain tissue and formation of neurofibrillary tangles. AD patients exhibit an increase in CSF P-tau and CSF T-tau compared with neurologically healthy individuals [6].

Biomarkers for AD

During the last decades, scientists have tried to identify biomarkers related to AD, which could be used for efficient diagnosis of AD, and also detect patients in different stages of the disease such as mild cognitive impairments (MCI). Treatments in animal models have been reported to be most effective if initiated at an early stage of the disease [23, 24].

So far, three AD-related biomarkers in CSF, which have attracted great interest among clinicians and researchers have been identified; CSF $A\beta_{1-42}$, CSF T-tau and CSF P-tau. Previous studies suggest that AD patients exhibit elevated levels of CSF T-tau and CSF P-tau and reduced levels of CSF $A\beta_{1-42}$. Together, these three biomarkers have high sensitivity and specificity to identify

AD patients with dementia and also AD patients in the mild cognitive impairment stage of the disease [6].

Corticotropin Releasing Hormone (CRH)

Corticotropin releasing hormone (CRH) is a peptide consisting of 41 amino acids and is produced mainly by the neurons in hypothalamus, but it can also be produced in placenta, testicles, etc [25]. The hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system are the two biological systems that are activated during stress. The paraventricular nucleus (PVN) of the hypothalamus synthesizes and releases CRH, which regulates adrenocorticotropic hormone (ACTH) secretion via CRH receptors in the pituitary gland [26]. Three neurotransmitters, namely GABA, glutamate and norepinephrine regulate CRH [27]. CRH affects various biological systems in the body, such as the central nervous system (CNS), gastrointestinal- , endocrine- and immune systems, and female reproduction. CRH increases the uptake of several biologically active factors; adrenaline, noradrenalin, glucose, glucagon and vasopressin etc, and decreases heart rhythm [25, 26, 28, 29]. Two different types of CRH receptors have been identified, CRHr1 and CRHr2, the first of which regulates ACTH during stress, whereas the second regulates stress behaviors such as anxiety and agitation [30].

ACTH is a multipotent glycoprotein that mainly exerts its effect on adrenal cortex, including increased production and secretion of glucocorticoids. Glucocorticoids, through negative feedback, regulate CRH secretion from the hypothalamus. This hormone system forms the HPA-axis [26, 31]. HPA axis dysfunction is relatively common in AD patients and stress related disorders [32]. Chronic stress is characterized by elevated glucocorticoid levels, which has been associated with cognitive impairments in AD patient [33].

Previous studies show varying results in terms of CSF CRH in AD patients. Most have concluded that CSF CRH is reduced in AD patients compared with sex - and age matched neurologically healthy controls [34-36]. Other studies show that there are no significant differences in CSF CRH between AD patients and neurologically healthy controls [37-39].

Several recently published studies draw the conclusions that chronic stress exacerbates tau pathology in animal models, via CRH-receptor-dependent mechanism, and thus contributes to the pathogenesis in AD [40-42].

Recently, Carroll and co-workers showed that chronic stress was a powerful inducer of tau pathology in two transgenic (tg) mouse models of AD, and that this effect was mediated by the mouse counterpart of CRH, corticotropin-releasing factor (CRF) [40].

Aim

Stress is a hot topic in the health sciences, and is related to a variety of medical conditions, such as visceral fat and cardiovascular disease [26]. The above mentioned study on transgenic (tg) AD mice [40] has aroused interest to investigate whether there are any correlations between stress and AD in humans. The increased understanding of different pathogenic factors related to the disease could be used as a tool to fight this dreaded condition. Here we test the hypothesis that CSF levels of the human counterpart to CRF1, CRH are elevated in AD and if there were any correlations between CSF T-tau, CSF P-tau and tau pathology in a manner similar to what has been seen in tg mouse models of AD.

Method

Patients and samples

The study was approved by the ethics committee at Karolinska University Hospital, Huddinge, Stockholm and Kuopio University Hospital, Finland. CSF samples were collected by lumbar puncture through the L3/L4 or L4/L5 interspace. The first 12 mL of CSF was collected in a polypropylene tube, immediately transported to the local laboratory for centrifugation at 2000 x g at 4°C for 10 min. The supernatant was pipetted off, gently mixed to avoid possible gradient effects, and aliquoted in 0.5-2 mL portions that were stored at -80°C pending testing.

The samples were collected in four sets. Set A samples were from patients who sought medical advice because of cognitive impairment. Patients were designated as normal (non-AD) or AD according to CSF biomarker levels using cutoffs that are 90% specific for AD (Hansson et al., 2006): total tau (T-tau) >350 ng/L, P-tau>80 ng/L and Aβ42<530 ng/L. None of the biochemically normal subjects fulfilled these criteria.

Set B-D patients received a diagnosis of AD using the DSM-IIIR (Diagnostic and Statistical Manual of Mental Disorders, third edition, revised) (American Psychiatric Association, 1987) and National Institute of Neurological and Communicative Disorders and Stroke- Alzheimer's Disease and Related Disorders Association (McKhann et al., 1984) criteria of dementia and probable AD, respectively. Mini- Mental State Examination (MMSE) score was used as a global measure of functioning (Folstein et al., 1975). Inclusion criteria for controls were that they should be physically and mentally healthy and not experiencing or exhibiting any cognitive impairment. All controls were thoroughly interviewed about their somatic and mental health by a

research nurse before inclusion in the study and were cognitively stable over at least 2 years after the initial examination.

Biochemical analyses

CSF T-tau, CSF T-tau and Aβ42 levels were determined using INNOTEST ELISAs (Innogenetics, Ghent, Belgium) (Hulstaert et al., 1999; Vanmechelen et al., 2000).

CRH concentration in CSF was measured by radioimmunoassay (RIA) in which the analyte competes with radio-labeled synthetic CRH in binding to an antiserum specific against the peptide (Fig.1A). Radioactive ligand was synthesised using a modified chloramine-T method (Hunter and Greenwood, 1962), purified with RP-HPLC using a µ-Bondapak C18 column (3.9 x 300 mm, 125 Å, 10 µm, Waters Code no. 27324), diluted 1/10 in 0.05 M sodium phosphate buffer containing, 0.25% bovine serum albumin, pH 7.4 and stored at -20°C until use. Synthetic CRH (human) was used both as a calibrator and tracer (^{125}I-CRH) (Ekman et al., 1993). The radioimmunoassay was performed with triples of calibrators and duplicates of samples. Calibrators of 100 µL, samples and controls of 200 µL were incubated with 200 µL CRH-rabbit antiserum (IPN B2 860611), final dilution 1/40,000 in assay buffer (0.15 M sodium phosphate buffer, pH 7.4, 0.1% bovine serum albumin and 0.1 % Triton X-100) at 21ºC for 24 hours. A second incubation in the same manner was performed after addition of 200 µL ^{125}I-[Tyr^{0}]-CRH (diluted to 10 000 cpm ±10% in assay buffer). Free and bound tracer was separated using 100 µL anti-rabbit IgG (AA-Sac1 from IDS) (Fig.1A), incubation at room temperature for 30 min, addition of 1 mL deionised water and centrifugation (2500 x g, 21°C, 5 min). The supernatant was discarded and the precipitate measured in a gamma counter (Wizard 1470) connected with

an immunoassay software programme (MultiCalc Advanced, Wallac Oy, Finland) (Fig.1B). Intra-assay and inter-assay coefficients of variation for controls at 6 pmol/L were <6%.

However, as described above, to further clarify it should be mentioned that RIA measures CRH-like immunoreactivity (CRH-LI) in CSF, not the CRH peptide itself.

Figur 1A

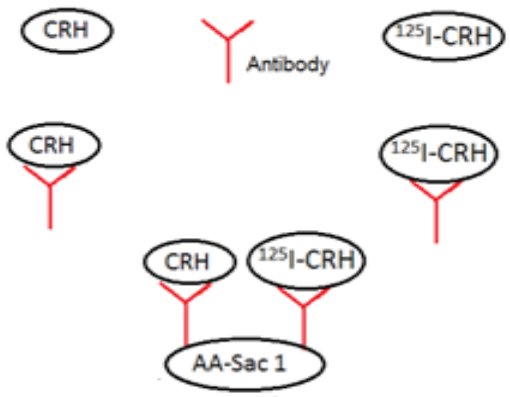

Fig 1A. Schematic representation of the RIA method showing labeled and unlabeled CRH, as well as the first and second antibody used to precipitate bound CRH. The second antibody is coated to a cellulose solid phase (the AA-Sac1 system).

Figur 1B

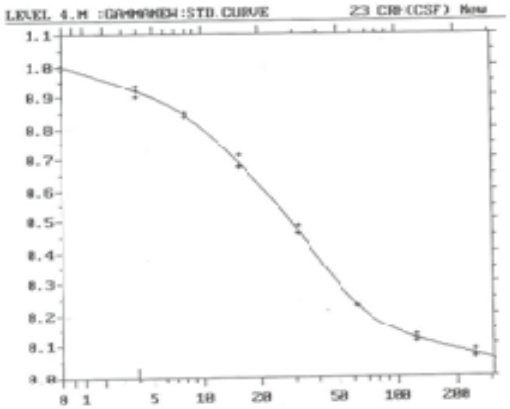

Fig 1B. Example of a CRH standard curve. The Y axis shows the proportion of precipitated labeled CRH tracer. The X axis shows CRH concentrations in the standard sample series.

Statistical Analysis

Differences in biomarker distributions among groups were analyzed with t-tests in Excel and in a scientific graphing program, GraphPad Prism 5. The Pearson correlation coefficient was used for correlation analyses. Statistical significance was set to $P<0.05$. Mean value of each group and standard deviations within group were calculated and diagrams were produced in GraphPad Prism and Excel, to thoroughly examine if there were any correlations between different groups.

Results

We tested four independent clinical materials, namely Set A-D. The result of each Set is presented separately.

Set A

A statistically non-significant trend towards a positive correlation of CSF CRH with P-tau and T-tau was seen in the AD group (Fig. 2A, 2B). CSF CRH levels were significantly higher in the AD group (22 patients) (11.56 ± 4.0 pmol/L) compared with the non-AD group (18 samples) (8.66 ± 2.6 pmol/L, p=0.01) (Fig. 2C, Table 1).

Figure 2A

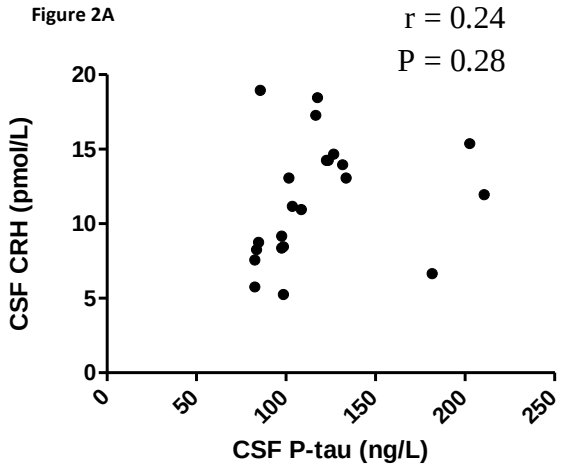

Figure 2B

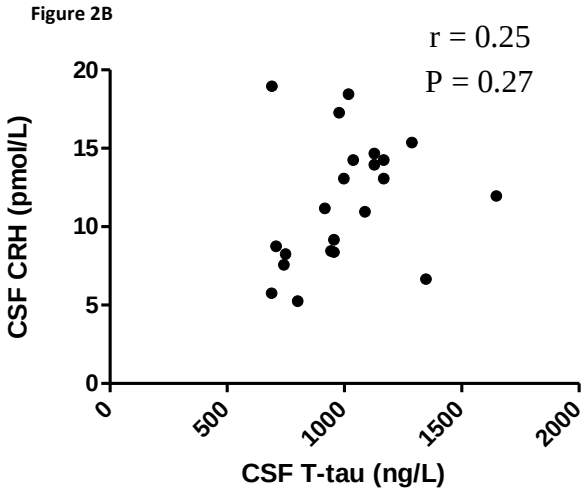

Figure 2C

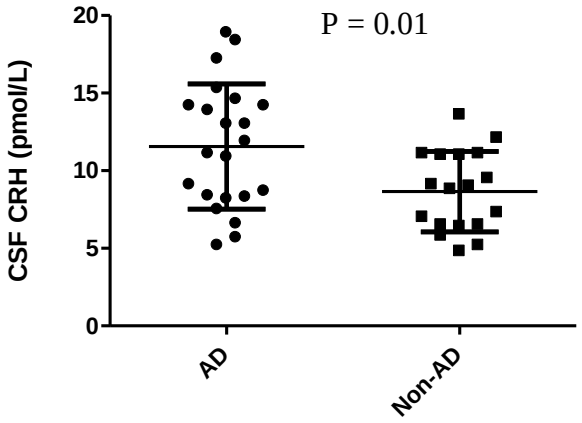

Figure 2A and 2B shows a statistically non-significant trend towards a positive correlation of CSF CRH with CSF P-tau respectively CSF T-tau. Figure 2C shows elevated levels of CSF CRH in the AD group compared with non-AD group and in addition illustrates mean and standard deviation (SD) in each group.

Set B

A slight statistically significant difference in CRH levels was seen between AD (24 patients) and healthy controls (28 controls) (Fig. 2D) using t-test (p-value 0.04). Mean CRH levels were lower in the AD group (12.76 ± 3.4 pmol/L) compared with the control group (14.92 ± 4.0 pmol/L) (Table 1). CSF CRH levels did not correlate with CSF P-tau (Fig. 2E) or T-tau (data not shown).

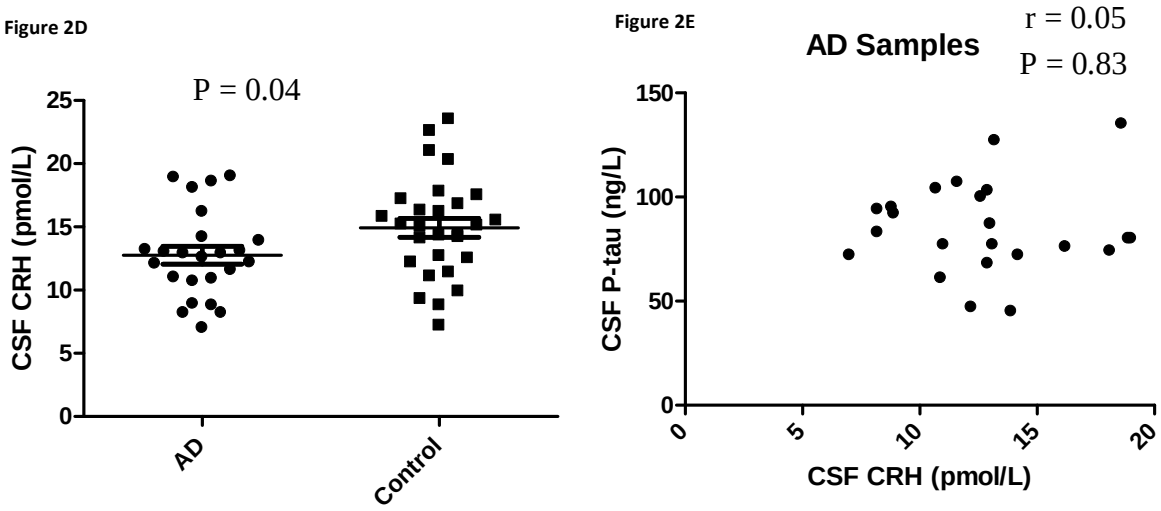

Figure 2D shows CSF CRH levels between the AD and control groups. Figure 2E illustrates that no correlation between CSF CRH and CSF P-tau was seen.

Set C

Mean CSF CRH in 15 AD patients (6.81 ± 1.5 pmol/L) tended to be lower compared with 8 neurologically healthy controls (8.30 ± 1.9 pmol/L) (Fig.2F), although no significant difference between the groups was seen (p-value 0.051). CSF T-tau and P-tau levels were not measured in this set and therefore analyses of correlations between these biomarkers and CRH were not made.

Figure 2F

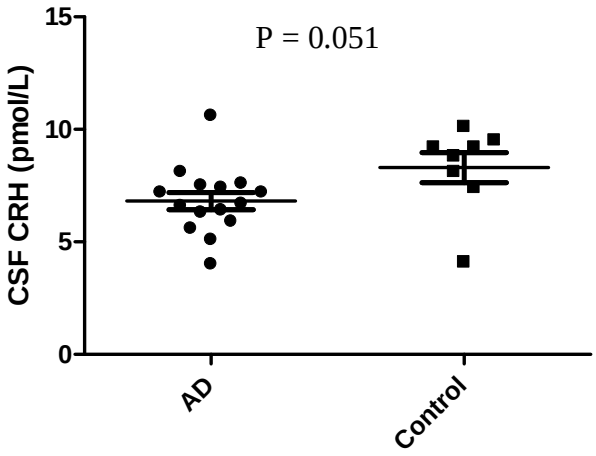

Figure 2F illustrates that CSF CRH levels were slightly elevated in control group compared with AD group.

Set D

No statistically significant difference in CRH levels was seen between 17 AD patients and 14 healthy controls (p-value 0.45) (Table 1, Fig 2G). The AD group showed slightly elevated mean levels of CSF CRH (9.22 ± 1.8 pmol/L), compared with controls (8.64 ± 2.4 pmol/L), but the difference was not statistically significant. CSF T-tau and P-tau levels were not measured in this set and therefore analyses of correlations between these biomarkers and CRH were not made.

Figure2G

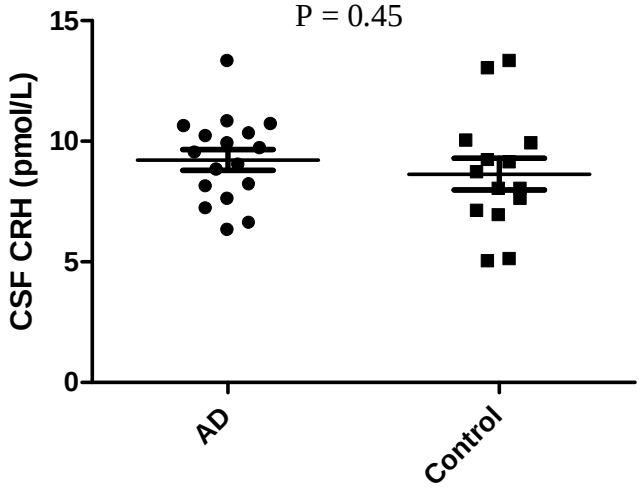

Figure 2G shows that there was no significant difference in CSF CRH levels between control group and AD group.

Table 1

	CRH (AD)	CRH (C/N)	P-value AD vs C/N
Set A	11.56	8.66	0.01
Set B	12.76	14.92	0.04
Set C	6.81	8.30	0.051
Set D	9.22	8.64	0.45

Table 1 shows mean values of CSF CRH and all concentrations are in pmol/L. P-value of each set indicates if there are any statistically differences in CSF CRH levels between AD group and Non-AD or Control group. Statistical significance was set to $P<0.05$. Alzheimer's Disease (AD). Control/Normal (C/N).

Discussion

Based on the original article [40] and our hypothesis, Set A showed promising results. A statistically non-significant trend towards a positive correlation was seen between CSF CRH and CSF P-tau and CSF T-tau respectively, which was partly in line with the study by Carrol et al, who had found that chronic stress, mediated via CRH- receptor dependent mechanism, contributed to tau pathology in a mouse model of AD. In addition, elevated CSF levels of CRH in the AD group compared to non-AD group were seen. We therefore wanted to go ahead and confirm the results in several independent clinical materials.

We examined three additional case-control cohorts. One of these showed a non-significant trend towards elevated CRH levels in AD CSF but the other two showed non-significant and slightly significant respectively opposite trends, i.e., reduced CSF CRH levels in AD. Previous studies

have shown different results in terms of CSF CRH in AD patients, where most have reported that the levels are reduced [34, 36], but few studies have reported that no significant changes could be seen [37, 39].

There are many reasons for the divergent results in different studies. CSF CRH levels may vary at different stages of the disease. Patients with end-stage AD (i.e., severely demented with profound neuronal loss) may have lost their ability to produce CRH, whereas patients in the MCI stage of AD may have elevated levels, if stress contributes to initiation of the disease. Further, different methods may measure different forms of the CRH protein. For example, a method based on two antibodies, one to the N-terminal part and another to the C-terminal part, would measure full-length CRH, whereas our method also recognizes cleaved forms. To ensure the relationship between AD and CSF CRH further studies are required, where one may measure specific isoforms of CRH instead of CRH-IL. One should note that it is difficult to distinguish with certainty AD patients from patients in different stages of MCI or even from neurochemically normal individuals who exhibit clinical key features of AD.

Another reason for the differences in results between the different sample sets is that set A was grouped on the basis of the neurochemical AD biomarker profiles, whereas sets B-D were grouped according to clinical status.

Today, there is unfortunately no other method that measures CSF CRH other than RIA, but it would be interesting to develop a more sensitive and specific method to measure this peptide and repeat the analyses, as RIA with its around 10% variation tendency is not optimal.

Conclusions

One of the four independent case-control studies showed an elevated CSF CRH levels in AD patients compared with controls, while one showed the opposite trend, i.e., slightly reduced CSF CRH levels in AD, and the other two indicated no significant changes. A weak positive but non-significant trend towards a correlation of CSF CRH with the tau pathology marker P-tau was seen in Set A samples from AD patients. We conclude that CSF CRH is no stable diagnostic biomarker for AD. A positive correlation of CRH with P-tau in one of the sample sets would be in agreement with the animal model-derived hypothesis that CRH-mediated signaling may induce tau pathology, but we only detected a trend towards a correlation in one of the examined data sets and more studies are needed on the topic.

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Populärvetenskaplig sammanfattning

Alzheimers sjukdom och Stress

Alzheimers sjukdom (AD) är ett progressiv neurodegenerativ tillstånd och är den vanligaste orsaken till demens. Förekomsten ökar med åldern. Kliniskt kännetecknas AD av progressiva minnessvårigheter och kognitiva funktionsnedsättningar, med gradvis ökande neurologiska och somatiska symtom. Tre neuropatologiska förändringar är typiska för AD: extracellulära senila plack som består av aggregerade amyloid β (Aβ), intraneuronala neurofibrillary tangles bestående av hyperfosforylerat tau-proteiner (P-tau) och neurodegeneration. Tau är ett axonalt protein som har en central roll i stabiliseringen av mikrotubuli och abnorma hyperfosforylerade tau proteiner korrelerar väl med graden av AD. Stress har föreslagit öka risken för AD och i en musmodell för AD har man sett ett positivt samband mellan en corticotropin-releasing hormone (CRH)-aktiverad pathway och tau patologi.

I denna studie ville vi undersöka om patienter med AD hade ökade nivåer av corticotropin-releasing hormone (CRH) i cerebrospinalvätskan (CSF, den vätska som omsluter hjärnan och återspeglar dess ämnesomsättning), och om det fanns några korrelationer mellan detta och graden av tau patologi. Vi mätte total-tau (T-tau), fosfo-tau (P-tau) och amyloid beta ($A\beta_{1-42}$) i CSF med hjälp av INNOTEST ELISA hos AD patienter och kontroller. CSF CRH mättes med hjälp av radioimmunoassy (RIA). Statisktiska analyser utfördes i Graphpad Prism för att utvärdera resultaten.

Vi fann överlag inga signifikanta skillnader i CSF CRH nivåer mellan AD-patienter och neurologiskt friska kontroller. Endast en av våra fyra oberoende studieserier visade ökade nivåer av CRH hos AD-patienter jämfört med kontroller. Resultaten talar om att CSF CRH inte är en

pålitlig biomarkör för AD, men att det kan finnas ett svagt samband mellan CRH och tau-patologi. Bättre och mer specifika metoder att mäta CRH och CRH-reglerade proteiner i CSF behöver utvecklas och det skulle även vara intressant att undersöka AD-patienter i olika stadier av sjukdomen. Kanske spelar CRH en roll i tidiga faser av sjukdomen innan neurodegenerationen hunnit bli för omfattande?