

**ORIGINAL ARTICLE**

# The amyloidogenic potential and behavioral correlates of stress

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Observations of elevated basal cortisol levels in Alzheimer's disease (AD) patients prompted the hypothesis that stress and glucocorticoids (GC) may contribute to the development and/or maintenance of AD. Consistent with that hypothesis, we show that stress and GC provoke misprocessing of amyloid precursor peptide in the rat hippocampus and prefrontal cortex, resulting in increased levels of the peptide C-terminal fragment 99 (C99), whose further proteolytic cleavage results in the generation of amyloid- $\beta$  (A $\beta$ ). We also show that exogenous A $\beta$  can reproduce the effects of stress and GC on C99 production and that a history of stress strikingly potentiates the C99-inducing effects of A $\beta$  and GC. Previous work has indicated a role for A $\beta$  in disruption of synaptic function and cognitive behaviors, and AD patients reportedly show signs of heightened anxiety. Here, behavioral analysis revealed that like stress and GC, A $\beta$  administration causes spatial memory deficits that are exacerbated by stress and GC; additionally, A $\beta$ , stress and GC induced a state of hyperanxiety. Given that the intrinsic properties of C99 and A $\beta$  include neuroendangerment and behavioral impairment, our findings suggest a causal role for stress and GC in the etiopathogenesis of AD, and demonstrate that stressful life events and GC therapy can have a cumulative impact on the course of AD development and progression.

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## Introduction

Amyloid- $\beta$  (A $\beta$ ) peptide is generated under normal physiological conditions, but its overproduction and accumulation give rise to senile plaques that, together with aggregations of abnormally hyperphosphorylated  $\tau$  protein, constitute the main neuropathological hallmarks of Alzheimer's disease (AD). However, the amount of deposited A $\beta$  correlates poorly with the degree of cognitive impairment in AD patients,<sup>1</sup> and central administration of A $\beta$  in nongenetically modified rodents results in cognitive impairments within a relatively short period and before amyloid plaques become detectable.<sup>2</sup> These observations suggest a pivotal role for soluble nonaggregated A $\beta$  in the early stages of the disease; they are supported by data showing that soluble A $\beta$  can acutely disrupt synaptic function and cognitive and emotional behavior.<sup>3–6</sup>

A $\beta$  peptide is produced through the sequential proteolytic cleavage of amyloid precursor protein

(APP) by  $\alpha$ -,  $\beta$ - and  $\gamma$ -secretases; the last secretase is a complex of at least four proteins, with nicastrin making up the largest portion. Nicastrin may be viewed as the gatekeeper of the  $\gamma$ -secretase complex because of its essential role in the recognition of  $\gamma$ -secretase substrates.<sup>7</sup> Cleavage of APP by  $\alpha$ -secretase precludes the generation of A $\beta$ , whereas  $\beta$ -secretase ( $\beta$ -site APP-cleaving enzyme; BACE)-mediated cleavage generates the so-called C-terminal fragment 99 (C99); subsequent proteolysis of C99 by  $\gamma$ -secretase results in the generation of amyloid peptides (for review, see Bayer *et al.*<sup>8</sup>). While the neurotoxic potency of A $\beta$  is well known,<sup>9–11</sup> other studies have demonstrated the neurotoxic and cognition-impairing potential of C99.<sup>12–15</sup> Furthermore, in a vicious cascade that characterizes AD, A $\beta$  interacts with its protein precursor (APP), an interaction that is suggested to contribute to the mechanism of A $\beta$  neurotoxicity.<sup>16,17</sup>

Observations that a large percentage of AD patients display hypercortisolemia<sup>18,19</sup> suggest that glucocorticoids (GC) and stress may contribute to the development or maintenance of AD. This view recently gained support from studies in transgenic mouse models of AD in which stress or GC exacerbated AD-like neuropathology.<sup>20,21</sup> Indications of a link between stress/GC and AD may also be inferred from previous

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work in which a causal role for elevated GC levels and stressors in cognitive impairment in humans and animals was demonstrated,<sup>22,23</sup> GC-induced dendritic atrophy and synaptic loss are thought to underlie the GC-associated behavioral disruption.<sup>24,25</sup>

The present study focused on examining whether stress or elevated GC levels can drive APP metabolism toward the amyloidogenic pathway in normal, middle-aged rats, and whether stressful life events can influence the initiation/maintenance of AD-like pathology. Our studies, which focused on the hippocampus and prefrontal cortex (PFC) because they rank among the first brain areas to show AD-like neuropathology,<sup>26</sup> disclosed that chronic stress and GC similarly promote APP processing along the amyloidogenic pathway. These results provide a tentative mechanism through which stress and/or GC exert their effects on cognitive and emotional behavior. Of note is the growing recognition of the importance of emotional state in the regulation of cognition.<sup>27–29</sup> In addition, it is pertinent to mention that stress-related psychiatric disorders (for example, anxiety and major depression) have been identified as a risk for developing AD.<sup>30,31</sup>

## Materials and methods

### Animals

Male Wistar rats (Charles River, Barcelona, Spain), aged 14 months, were used, in compliance with the European Union Council's Directive (86/609/EEC) and local regulations on animal welfare. Animals were housed 4–5 per cage under standard environmental conditions (temperature 22 °C relative humidity 70%; 12 h light:dark cycle; *ad libitum* access to food and drinking solution).

### Treatments and their biological efficacy

Rats were allocated to one of two main treatment groups: stressed and unstressed. Stressed animals were exposed to a chronic, unpredictable stress paradigm;<sup>32</sup> for 4 weeks, while their unstressed counterparts were held undisturbed, under standard laboratory conditions. The stress paradigm involved random application of one of the following stressors, daily: (1) hypertonic saline (9% NaCl; 1 ml per 100 g) i.p. injection, (2) overcrowding for 1 h, (3) placement in a confined environment (30 min) and (4) placement on a vibrating/rocking platform (1 h). After 5 days, subgroups of stressed or unstressed animals were given constant-rate i.c.v. infusions of soluble A $\beta$ <sub>1–40</sub> (American Peptide Co., Inc., Sunnyvale, CA; 4.2 nmol A $\beta$ <sub>1–40</sub> per 200  $\mu$ l; 0.5  $\mu$ l h<sup>-1</sup>) or vehicle (distilled water) over a period of 14 days; half of each of these subgroups additionally received daily s.c. injections of a synthetic GC (dexamethasone (DEX; Fortecortin Merck, Darmstadt, Germany); 300  $\mu$ g kg<sup>-1</sup> body weight (BW) as an oily suspension in sesame oil (Sigma, St Louis, MI, USA)). For the i.c.v. infusions, rats were equipped with s.c. osmotic minipumps (Alzet 2002 minipumps; Alza Corp., Mountain View, CA,

USA) and cannulae (Alzet Brain Infusion Kit) that were implanted in the left lateral ventricle using the following stereotaxic coordinates: AP, -1.0 mm; DV, -2.5 mm and ML, +1.5 mm (right) with bregma as a reference (Paxinos and Watson<sup>33</sup>); pump and cannulae implantation was done under pentobarbital (50 mg kg<sup>-1</sup>) anesthesia. At the time of killing, minipumps were removed and checked for patency and functionality. Each treatment subgroup comprised 6–8 rats.

Efficacy of the stress paradigm was gauged by measuring daytime serum corticosterone (CORT) levels (Corticosterone RIA kit, ICN, Costa Mesa, CA, USA), BWs and relative thymus weights. CORT levels were significantly elevated in stressed vs nonstressed animals (384.6  $\pm$  65.9 and 54.7  $\pm$  4.8 ng ml<sup>-1</sup>, respectively), and thymus weights at autopsy were consistent with hypercorticalism in the stressed group (control, CON: 7.6  $\pm$  0.9 vs stressed: 4.3  $\pm$  0.4 vs mg kg<sup>-1</sup> BW). Whereas nonstressed animals showed a net gain in BW (6.1  $\pm$  1.3 g), stressed animals showed a significant loss of body mass (-12.1  $\pm$  2.1 g). GC-exposed rats showed biometric changes that were similar to those observed in the stressed group, whereas none of the parameters monitored differed significantly between CON and stressed animals after the superimposed treatment with A $\beta$ .

### Tissue collection

Animals were rapidly killed and their brains were immediately excised and divided along the midline. One half of each brain was immersed in 4% *p*-formaldehyde (2 days), embedded in paraffin and saved for histochemical analysis. The PFC and hippocampus were dissected out of the other half of the brain, snap-frozen in liquid nitrogen and stored at -80 °C until subsequent biochemical analyses.

### Western blot analysis

Frozen hippocampal and PFC tissues were homogenized in lysis buffer (100 mM Tris-HCl, 250 mM NaCl, 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 1% NP-40, Complete Protease Inhibitor (Roche, Mannheim, Germany) and Phosphatase Inhibitor Cocktails I and II (Sigma, St Louis, MO)) using a Dounce glass homogenizer; extracts were cleared by centrifugation (14 000 g) and their protein contents were estimated by the Lowry assay. Lysates, in Laemmli buffer (250 mM Tris-HCl, pH 6.8, containing 4% sodium dodecyl sulfate, 10% glycerol, 2%  $\beta$ -mercaptoethanol and 0.002% bromophenol blue), were thereafter electrophoresed on 8 or 10% acrylamide gels, and transferred onto nitrocellulose membranes (Protran BA 85, Schleicher & Schuell, Dassel, Germany). Membranes were blocked in Tris-buffered saline containing 5% nonfat milk powder and 0.2% Tween-20 before incubation with the following antibodies: anti-APP369 (1:5000; kindly provided by Dr Sam Gandy), anti-BACE-1 (1:500; ProSci Inc., Poway, CA, USA), anti-nicastrin (1:5000; Sigma) and anti-actin (1:2000; Chemicon, Temecula, CA, USA) or anti- $\alpha$ -tubulin (1:2000; Calbiochem,

San Diego, CA, USA). Antigens were revealed by enhanced chemiluminescence (Amersham Biosciences, Freiburg, Germany) after incubation with appropriate horseradish peroxidase-immunoglobulin G conjugates (Amersham Biosciences); blots were scanned and quantified using TINA 3.0 bioimaging software (Raytest, Straubenhardt, Germany) after ascertaining linearity. All values were normalized and expressed as percentages of control. To distinguish between mature and immature isoforms of APP, tissue lysates were digested with endoglycosidase F (Roche), according to the manufacturer's instructions.

#### Immunoprecipitation

Tissue lysates were immunoprecipitated with APP369 antibody using a mixture of Protein A and G beads (Roche) before analysis by electrophoresis on Tris-Tricine gradient gels (KMF Laborchemie, Lohmar, Germany) and immunoblotting with an APP antibody (OPA1-01132; Affinity Bioreagents, Golden, CO, USA).

#### In situ hybridization

Paraffin sections (8 µm) were deparaffinized (see above), and fixed in 4% *p*-formaldehyde in phosphate-buffered saline (PBS) containing 0.1% diethylpyrocarbonate (DEPC; Sigma). After washing (PBS-DEPC), sections were acetylated (0.1 M triethanolamine, 0.25% acetic anhydride in DEPC), delipidated in chloroform (5 min), dehydrated through a graded series of ethanols and air dried. A 40-mer oligonucleotide probe (GCTGGCTGCCGTCGTGGGAATC

GGACTACCTCCTCCACA) was used to detect APP695-KPI.<sup>34,35</sup> Slides were hybridized with an antisense or sense (control) <sup>35</sup>S-dATP-labeled oligoprobe (10<sup>6</sup> c.p.m. per slide; overnight at 42 °C), after which they were stringently washed (1 × saline sodium citrate (SSC) for 15 min at 55 °C). After sequential immersion 1 × SSC, H<sub>2</sub>O, 65% ethanol and 95% ethanol, slides were air dried and dipped in photographic emulsion (1:1 Kodak NTB2 in distilled water) and exposed for 1 month. Sections were then developed, counterstained (toluidine blue) and viewed under bright field or polarized light to view cells and silver grains, respectively. Hybridization signal on captured images (all slides) was scored by two independent observers (unaware of the treatment details) on a scale of 0–5; the raters followed common scoring criteria and the results shown in Table 1 are the means of the scores obtained by the individual raters. Representative *in situ* hybridization (ISH) images are shown in Figure 1.

#### Assays of cognitive performance and emotionality

Hippocampus-dependent spatial reference memory was assessed using the Morris water maze over 4 days (four trials per day), as previously described.<sup>25</sup> Data were recorded using a video-tracking system (Viewpoint, Champagne au Mont d'Or, France).

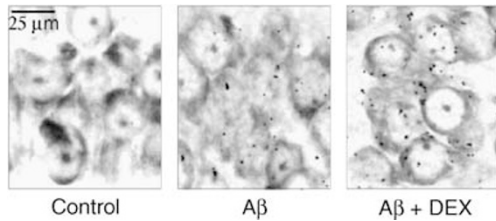
Emotional state was evaluated by monitoring locomotion, exploratory behavior and anxiety. Locomotor and exploratory behavior were assessed (total distances traveled and number and duration of rearings) online in an open-field arena (43.2 × 43.2 cm; transparent acrylic walls and white floor;

**Table 1** Overall statistical analysis of treatment (stress history, the corticosteroid *milieu*, Aβ and stress + Aβ + GC) effects on protein levels of APP, C99, BACE and nicastrin in the hippocampus and PFC

Hippocampus ( <i>d.f.</i> <sub>6,36</sub> )	APP		C99 (F = 130.3)	BACE (F = 25.07)	Nicastrin	
	Immature (F = 54.9)	Mature (F = 132.2)			Immature (F = 19.9)	Mature (F = 37.2)
Stress history	<i>q</i> = 0.0336 (NS)	<i>q</i> = 1.608 (NS)	<i>q</i> = 30.03	<i>q</i> = 5.592	<i>q</i> = 8.829	<i>q</i> = 16.189
Glucocorticoid <i>milieu</i>	<i>q</i> = 13.178	<i>q</i> = 10.649	<i>q</i> = 18.231	<i>q</i> = 12.928	<i>q</i> = 2.903 (NS)	<i>q</i> = 1.293 (NS)
Aβ	<i>q</i> = 13.416	<i>q</i> = 26.671	<i>q</i> = 32.175	<i>q</i> = 5.884	<i>q</i> = 3.598 (NS)	<i>q</i> = 8.651
Stress history in (Aβ + GC)-treated animals	<i>q</i> = 9.161	<i>q</i> = 12.466	<i>q</i> = 15.490	<i>q</i> = 4.409 (NS)	<i>q</i> = 2.563 (NS)	<i>q</i> = 5.835
PFC ( <i>d.f.</i> <sub>6,36</sub> )	APP		C99 (F = 26.3)	BACE (F = 17.7)	Nicastrin	
	Immature (F = 15.2)	Mature (F = 35.4)			Immature (F = 18.5)	Mature (F = 15.8)
Stress history	<i>q</i> = 0.377 (NS)	<i>q</i> = 3.424 (NS)	<i>q</i> = 15.791	<i>q</i> = 4.472	<i>q</i> = 9.629	<i>q</i> = 3.575 (NS)
Glucocorticoid <i>milieu</i>	<i>q</i> = 5.131	<i>q</i> = 10.676	<i>q</i> = 7.865	<i>q</i> = 0.925 (NS)	<i>q</i> = 0.998 (NS)	<i>q</i> = 0.754 (NS)
Aβ	<i>q</i> = 8.195	<i>q</i> = 10.521	<i>q</i> = 6.042	<i>q</i> = 8.047	<i>q</i> = 1.333 (NS)	<i>q</i> = 6.948
Stress history in (Aβ + GC)-treated animals	<i>q</i> = 5.706	<i>q</i> = 7.654	<i>q</i> = 6.933	<i>q</i> = 6.719	<i>q</i> = 9.111	<i>q</i> = 8.829

Abbreviations: Aβ, β-amyloid; APP, amyloid precursor peptide; BACE, β-site APP cleaving enzyme; C99, C-terminal fragment 99; GC, glucocorticoids; NS, not significant; PFC, prefrontal cortex.

Analyses were based on multiple one-way ANOVAs on ranks and Tukey's all pairwise comparison tests.



**Figure 1** APP mRNA detected by ISHH. Representative images from hippocampal CA3 cells are shown. After ISHH, silver grains were revealed by photo-emulsion dipping; sections were counterstained with toluidine blue.

MedAssociates Inc., St Albans, VT, USA) over a period of 5 min. Levels of anxiety behavior were evaluated in an elevated plus maze (EPM; a black polypropylene plus-shaped platform elevated 72.4 cm above the floor with two open arms (50.8 × 10.2 cm) and two closed arms (50.8 × 10.2 × 40.6 cm); MedAssociates Inc.). The EPM test was carried out under white light and animals were placed in the maze for a total of 5 min; times spent in the open vs closed arms were recorded online, and data were computed to yield the relative amount of time spent in the open arms of the maze; the number of open and closed arms entries were also recorded.

#### Statistical analysis

Results are expressed as group means ± s.e.m. Data were evaluated for their statistical significance using one-way analysis of variance (ANOVA), followed by Tukey's all-pairwise multiple comparison test. To avoid introducing confounds from the use of multiple one-way ANOVAs on ranks, we applied the Bonferroni correction (adjusting the *P*-values to 0.0125). For the proteins of interest in the hippocampus and PFC, multiple-way ANOVAs were performed to examine the effects of stress history, corticosteroid milieu (GC), Aβ and stress + Aβ + GC. Statistical analyses were conducted using SPSS (Chicago, IL, USA) and SigmaStat (Systat, San Jose, CA, USA) software packages; differences were considered to be significant if *P* < 0.05.

## Results

### Overall statistical analysis of treatment effects

The results of an overall analysis of the effects of stress history, the corticosteroid milieu (GC), Aβ and stress + Aβ + GC treatment on protein levels of APP, C99, BACE and nicastrin in the hippocampus and PFC are shown in Table 1.

### Chronic stress drives APP processing along the amyloidogenic pathway

In light of clinical reports of GC hypersecretion in AD patients,<sup>18,19</sup> and that stress exacerbates amyloid production in transgenic mouse models of AD,<sup>20,21</sup> it was of interest to examine whether stress stimulates the amyloidogenic pathway in normal, middle-aged rats. To this end, rats were exposed to a chronic

**Table 2** Semiquantitative evaluation of silver grains representing APP mRNA in the CA1 and CA3 subfields of the hippocampus of rats exposed to various treatments involving exposure to stress, Aβ, GC or Aβ + GC, or a combination thereof

Treatment	Hippocampus	
	CA1	CA3
Control	+	+
Glucocorticoid (GC)	+ / + +	+ / + +
Amyloid beta (Aβ)	+ + + +	+ + + +
Aβ + GC	+ + + + +	+ + + +
Stress	+ +	+ +
Stress + Aβ + GC	+ + +	+ + +

*In situ* hybridization histochemistry was performed as described in 'Materials and methods', using a 40-mer oligo probe designed to recognize APP695-KPI.

unpredictable stress protocol<sup>32</sup> before analysis of APP, C99, BACE-1 and nicastrin mRNA and protein levels in the hippocampus and PFC.

Exposure to stress resulted in a slight increase in the expression levels of APP mRNA in the CA1 and CA3 subfields of the hippocampus (see Table 2 for semiquantitative evaluation), but did not alter levels of immature and mature APP protein in either the hippocampus (Figure 2a) or PFC (Figure 2b). However, exposure to stress resulted in a significant increase in levels of the C99 fragment of APP in both brain regions, with the magnitude of changes being greater in the hippocampus than the PFC. Consistent with the alterations in the levels of C99, stress was associated with increased hippocampal and PFC levels of BACE-1 and nicastrin. Thus, stress was shown to drive APP processing along the amyloidogenic pathway.

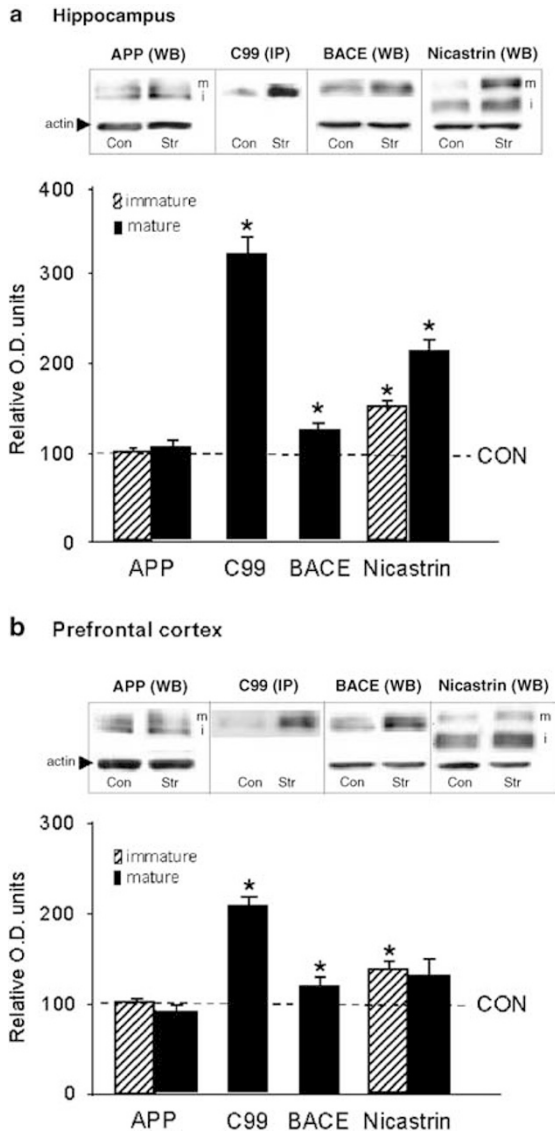
### Proamyloidogenic actions of GC

Glucocorticoid secretion represents the major physiological response to stress. Therefore, we next asked whether GC mediate the above-reported effects of stress on APP metabolism. This question was addressed by administering rats with daily injections of the synthetic glucocorticoid receptor (GR) agonist, DEX, for a period of 14 days.

As shown in Table 2, GC treatment resulted in increased APP mRNA levels (see semiquantitative results in Table 2) and hippocampal and PFC levels of APP and C99 (Figures 3a and b). BACE-1 expression was, however, only significantly elevated in the hippocampus (Figure 3a); nicastrin levels were not altered by GC treatment (Figures 3a and b), suggesting that signals, other than GC, are responsible for mediating the above-reported ability of stress to increase nicastrin levels (cf. Ni *et al.*<sup>36</sup>).

### Exogenous Aβ triggers APP misprocessing

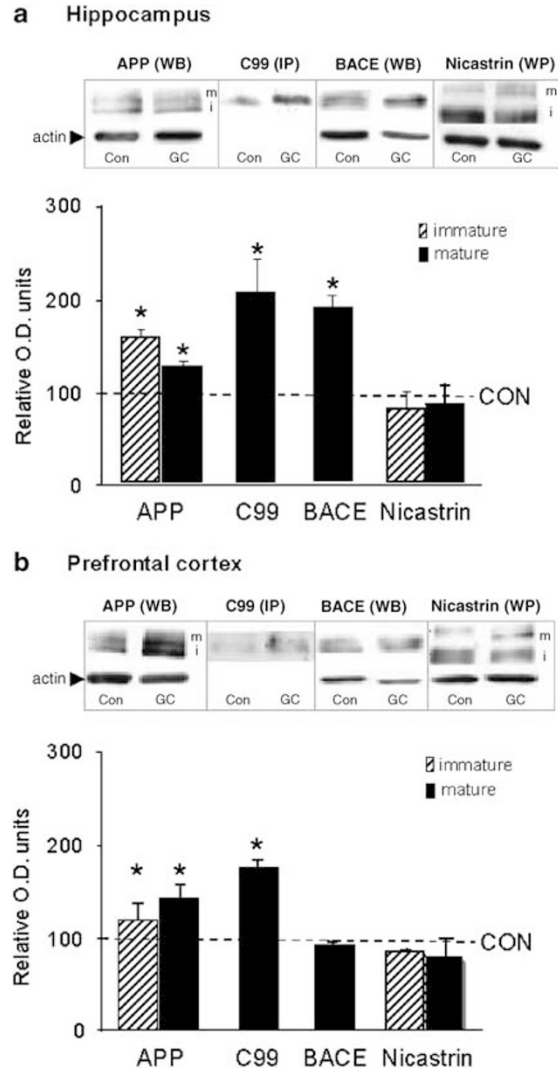
Aβ infusion is widely used to experimentally model aspects of AD pathology in animals.<sup>37</sup> Since Aβ is



**Figure 2** Stress drives amyloid precursor protein (APP) metabolism toward amyloidogenesis. Hippocampal (a) and prefrontal cortex (b) lysates from chronically stressed middle-aged rats were analyzed for their contents of APP, C99 (C-terminal fragment of APP),  $\beta$ -site APP-cleaving enzyme (BACE)-1 and nicastrin, by immunoprecipitation and western blotting. Representative blots from each brain region are shown alongside the respective numerical data; the latter are based on optical density evaluations, normalized against actin or tubulin and are depicted relative to control (CON) values as means  $\pm$  s.e.m. ( $n=6-8$  rats). Asterisks indicate  $P \leq 0.05$  vs CON values. Stressors were applied over a period of 4 weeks. In both brain areas, nicastrin was upregulated by stress, indicating the potential for C99 to be further processed to amyloid peptide. In western blots, 'm' and 'i' refer to mature and immature forms of the respective proteins.

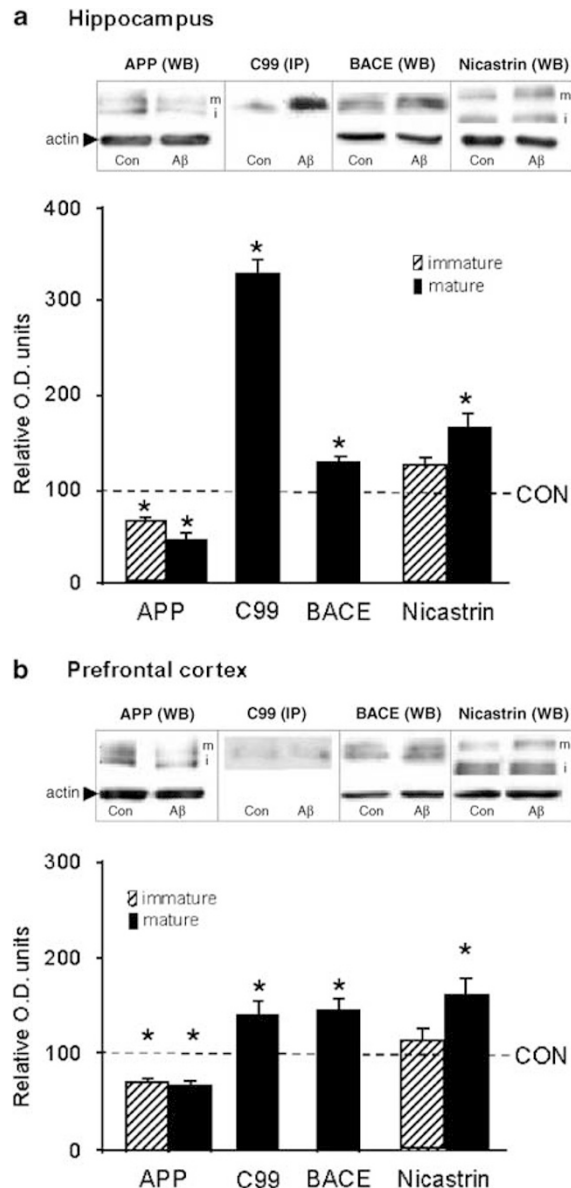
known to induce AD pathology and to stimulate its own production *in vitro*,<sup>38</sup> we were interested to examine how A $\beta$  influences APP metabolism *in vivo*.

When middle-aged rats were given constant infusions of A $\beta$  into one lateral ventricle (Frautschy



**Figure 3** Glucocorticoids (GC) stimulate C-terminal fragment 99 (C99) production without upregulating nicastrin levels. Shown are the changes in amyloid precursor protein (APP), C99,  $\beta$ -site APP-cleaving enzyme (BACE)-1 and nicastrin expression in the hippocampus (a) and PFC (b) of middle-aged rats that had been treated with DEX, a synthetic GC, for 14 days. Note that nicastrin levels were not increased in either brain area. Means  $\pm$  s.e.m. are depicted ( $n=6-8$  rats). The data emerged from semiquantitative assessment (optical densities) of immunoreactive bands, normalized against actin or tubulin. Asterisks indicate  $P \leq 0.05$  vs control values. In western blots, 'm' and 'i' refer to mature and immature forms of the respective proteins.

*et al.*<sup>39</sup>), there was a marked increase in APP mRNA signal in the hippocampi of A $\beta$ -infused rats (semiquantitative data in Table 2). At the same time, A $\beta$  treatment produced significant reductions in the levels of immature and mature APP in the hippocampus (Figure 4a) and PFC (Figure 4b), changes that were paralleled by increases in C99, BACE-1 and the mature form of nicastrin; the A $\beta$ -induced increases in C99 expression were twice greater in the hippocampus than in the PFC. Together, this set of findings



**Figure 4** Amyloid- $\beta$  (A $\beta$ ) stimulates amyloidogenesis. A $\beta$  administered by chronic, constant-rate infusion into one lateral ventricle, resulted in significant increases in C-terminal fragment 99 (C99) production and, concomitantly, in nicastrin levels in the hippocampus (**a**) and PFC (**b**), suggesting the potential for further processing of C99 to amyloid peptides(s). The treatment resulted in significant reductions in amyloid precursor protein (APP) levels, most likely due to its rapid processing into C99. Means  $\pm$  s.e.m. ( $n=6-8$  rats) are shown. Data represent optical densities of immunoreactive bands, normalized against actin or tubulin. Asterisks indicate  $P \leq 0.05$  vs control values. In western blots, 'm' and 'i' refer to mature and immature forms of the respective proteins.

demonstrates that A $\beta$  treatment triggers BACE-1-mediated APP misprocessing into C99 which can be potentially cleaved by  $\gamma$ -secretase into amyloid peptides. These results show that the actions of A $\beta$  closely resemble those of stress and GC.

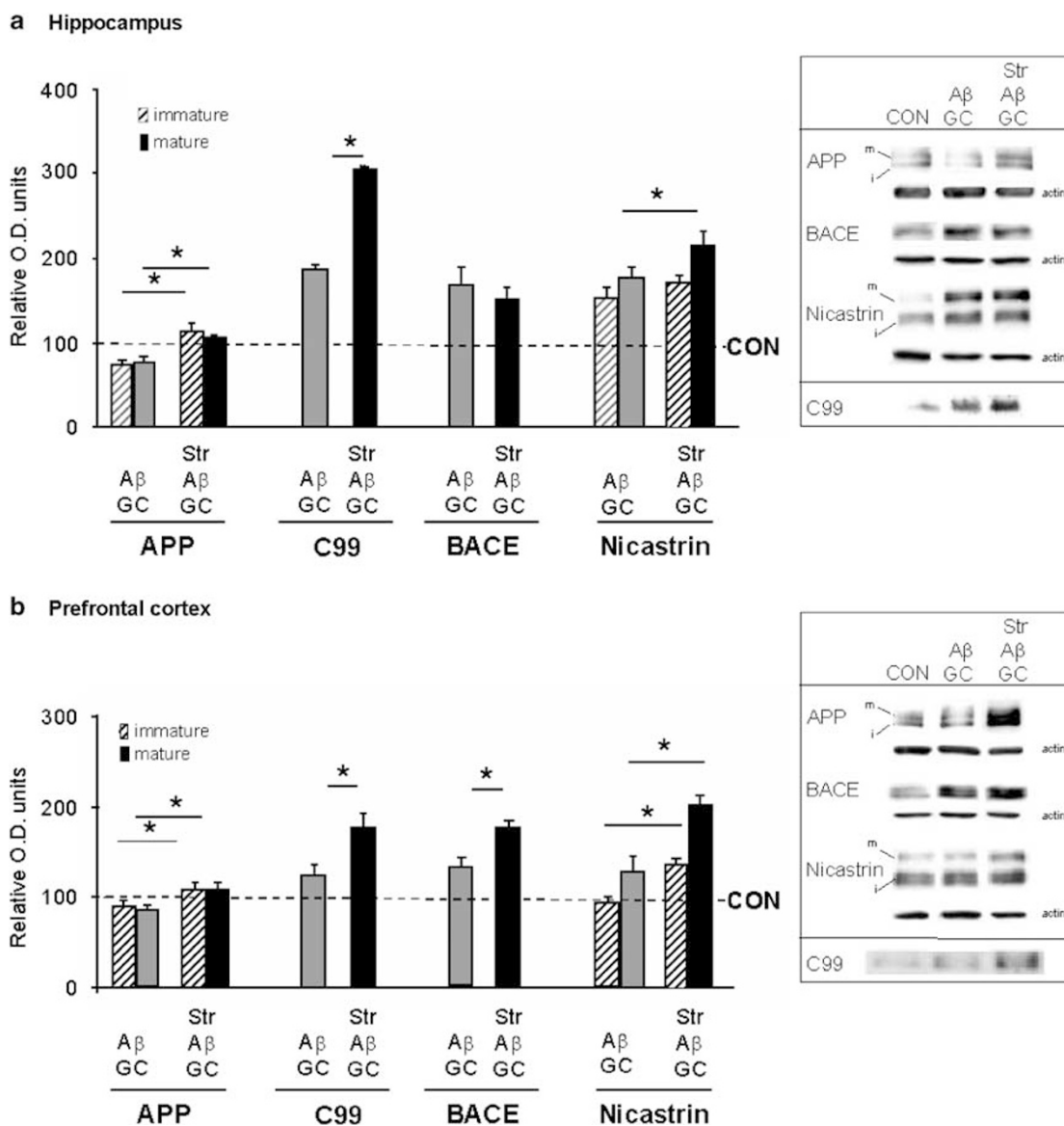
### Stress history exacerbates APP misprocessing

It was recently shown in transgenic mouse models of AD that chronic stress potentiates A $\beta$  deposition and induces cognitive deficits,<sup>20</sup> and that elevated GC exacerbate amyloidogenesis.<sup>21</sup> Given that the organism experiences stressful events throughout its lifetime, and aged individuals tend to show exaggerated GC secretory responses to stress,<sup>22</sup> we here examined the sequential effects of stress and GC on AD-like pathology by simultaneously treating previously stressed and nonstressed rats with A $\beta$  (i.c.v. infusions) and/or GC (s.c. injections). Multiple-way ANOVA analysis revealed that stress history plays a significant role in determining subsequent treatment effects on responses of the APP processing in the hippocampus (immature APP:  $F=6.9$ ,  $P \leq 0.003$ ; mature APP:  $F=11.1$ ,  $P \leq 0.0001$ ; C99:  $F=224.2$ ,  $P \leq 0.0001$ ; immature nicastrin:  $F=3.5$ ,  $P \leq 0.043$ ; mature nicastrin:  $F=11.9$ ,  $P \leq 0.001$ ) and PFC (immature APP:  $F=8.9$ ,  $P \leq 0.001$ ; mature APP:  $F=10.7$ ,  $P \leq 0.0001$ ; C99:  $F=11.5$ ,  $P \leq 0.0001$ ; BACE:  $F=6.6$ ,  $P \leq 0.004$ ; immature nicastrin:  $F=8.9$ ,  $P \leq 0.001$ ; mature nicastrin:  $F=10.7$ ,  $P \leq 0.0001$ ). Briefly, while stress did not influence the effects of subsequently applied A $\beta$  on APP metabolism (data not shown), the amyloidogenic effects of A $\beta$ +GC were further accentuated in rats that had been previously exposed to stress. Specifically, as shown in Figure 5, concomitant treatment with A $\beta$  and GC triggered APP misprocessing (into C99) in both the hippocampus and PFC of nonstressed rats, an effect that was significantly exacerbated in previously stressed animals. BACE-1 levels in A $\beta$ +GC-treated nonstressed and stressed rats revealed increases that were only significant in the PFC (Figure 5b). Mature nicastrin levels were significant in both hippocampus and PFC, indicating the greater likelihood of C99 processing along the amyloidogenic pathway. In summary, the results reported in this section demonstrate that a history of stress increases vulnerability to subsequent exposures to GC and A $\beta$ .

### Behavioral performance

Impairments in learning and memory are the primary behavioral manifestations of AD. Accordingly, it was considered important to evaluate the extent to which the various treatments used in the present study impacted on cognitive performance. Examination of escape latencies in the Morris water maze revealed that stress and GC, as well as A $\beta$ , impair spatial reference memory to similar extents (Table 3). A tendency for further impairment of spatial reference memory was observed when A $\beta$  and GC were applied concomitantly, and even more pronounced deficits were seen in animals that had been exposed to stress before receiving A $\beta$ +GC (Table 3).

Finally, in light of the evidence for interactions between emotion and cognition<sup>27-29</sup> and evidence that AD patients show hyperanxiety,<sup>30,31</sup> we monitored the emotional state of animals subjected to the various treatment regimens described above. Locomotor and



**Figure 5** Stress history exacerbates the amyloidogenic effects of amyloid- $\beta$  (A $\beta$ ) + glucocorticoids (GC) treatment. Rats that had been preexposed (4 weeks) to a chronic unpredictable stress paradigm responded to the coadministration of A $\beta$  + GC (14 days) with markedly increased levels of C-terminal fragment 99 (C99),  $\beta$ -site APP-cleaving enzyme (BACE)-1 and nicastrin in the hippocampus (a) and PFC (b). Means  $\pm$  s.e.m. are depicted ( $n=6-8$  rats). Numerical data refer to optical density readings from immunoreactive bands, normalized against actin. Asterisks indicate  $P \leq 0.05$ . In western blots, 'm' and 'i' refer to mature and immature forms of the respective proteins.

exploratory behavior were assessed in an open-field setup, and anxiety-like behavior in an EPM; the results are summarized in Table 3. Stressed and GC-treated rats were found to be more anxious than controls; animals given A $\beta$  also displayed increased signs of anxiety when compared to controls. Unexpectedly, animals that were exposed to stress before being treated with either GC or the combination of A $\beta$  and GC appeared to be resistant to the anxiogenic effects of GC and A $\beta$  (cf. data for A $\beta$  and GC in nonstressed animals). Importantly, this anxious phenotype cannot be attributed to locomotor deficits as none of the experimental procedures produced

differences in total distances traveled in the open field Table 3 or in the number of closed arm entries in the EPM (data not shown). Interestingly, there were impairments in exploratory behavior as assessed by rearing activity. Briefly, stressed and GC-treated rats displayed fewer rearings than controls. A $\beta$ -treated rats also displayed decreased exploratory behavior when compared to controls.

### Discussion

Stress and GC secretion may be regarded as inseparable phenomena since GC secretion represents a

**Table 3** Tests of spatial memory and emotionality (exploratory and anxiety-like behavior)

	CON	Stress	DEX	A $\beta$	A $\beta$ + DEX	Stress + A $\beta$	Stress + A $\beta$ + DEX
<i>Spatial memory (Morris water maze)</i>							
Mean escape latency (s; trials 2–4)	7.41 $\pm$ 0.8	15.36 $\pm$ 1.7*	15.73 $\pm$ 4.4*	20.87 $\pm$ 2.7*	21.19 $\pm$ 3.3*	19.04 $\pm$ 4.7*	25.72 $\pm$ 5.5*†
<i>Locomotor and exploratory behavior (open field)</i>							
Total distance (cm)	287.0 $\pm$ 76.3	278.7 $\pm$ 11	252.0 $\pm$ 42.5	256.7 $\pm$ 42.9	233.0 $\pm$ 46.5	232.5 $\pm$ 44.5	209.0 $\pm$ 69.8
No. of rearings/extensions	21.0 $\pm$ 4.0	12.1 $\pm$ 2.0*	10.0 $\pm$ 1.5*	9.5 $\pm$ 2.6*	9.8 $\pm$ 1.4*	7.7 $\pm$ 1.7*	7.2 $\pm$ 2.3*
<i>Anxiety level (elevated plus maze)</i>							
Time in open arms (%)	26.1 $\pm$ 6.7	14.9 $\pm$ 3.3*	8.8 $\pm$ 2.8*	8.3 $\pm$ 2.0*	4.4 $\pm$ 3.0*	10.0 $\pm$ 1.9*	11.2 $\pm$ 2.0*

Abbreviations: A $\beta$ ,  $\beta$ -amyloid; CON, control; DEX, dexamethasone.Data shown are means  $\pm$  s.e.m. ( $n = 6$  rats per treatment group).\* $P \leq 0.05$  vs CON; † $P \leq 0.05$  vs stress + A $\beta$ .

primary physiological response to stress. Both stress and GC can induce neurodegenerative changes in the hippocampus and PFC.<sup>23,40–45</sup> These brain regions express GRs;<sup>46</sup> they are important for cognition and are affected early in the development of AD.<sup>47</sup> Clinical reports of hypercortisolism in a significant number of AD patients<sup>18,19</sup> suggest a causal role for GC in AD, a view supported by studies in laboratory rodents that have shown that GC participate in the regulation of APP levels.<sup>48,49</sup> Using middle-aged rats, we now show that (1) stress and GC can drive APP metabolism toward amyloidogenesis, (2) a history of stress, involving hypersecretion, biases APP processing toward the amyloidogenic pathway and (3) that the proamyloidogenic effects of stress/GC are more pronounced in the hippocampus than in the PFC, a finding that is consistent with the temporal pattern of neuropathological events in the AD brain.<sup>47</sup> In addition, we show that A $\beta$  induces APP misprocessing in a manner similar to that observed after exposure to stress or elevated GC levels. Lastly, we show that stress, GC and A $\beta$  produce similar, as well as additive, impairments in cognition and emotional behavior.

A considerable body of evidence indicates that the amyloidogenic (mis)processing of APP plays a central role in the neurodegenerative changes and behavioral deficits that characterize AD.<sup>26</sup> Misprocessing of APP commences with its endocytic cleavage by  $\beta$ -secretase (BACE) to yield the  $\beta$ C-terminal fragment ( $\beta$ CTF), C99. Subsequent cleavage of C99 by  $\gamma$ -secretase results in the generation of A $\beta$  peptides. While much emphasis has been placed on examining the role and mechanisms of action of A $\beta$  in AD, there is growing evidence that C99 also makes a substantial contribution to AD pathology. In this context, it is worth noting that C99 has intrinsic neurotoxic properties<sup>12</sup> and causes synaptic degeneration;<sup>13</sup> furthermore, C99 can impair long-term potentiation<sup>14</sup> and cognition.<sup>15</sup>

The results from this study show that stress upregulates steady-state APP mRNA levels without inducing any change in APP protein levels. These findings, together with our observation that stress causes a significant increase in nicastrin levels, suggest that stress promotes APP misprocessing. On the other hand, our GC treatment paradigm seemed to be a less potent inducer of APP misprocessing insofar that while it led to increased expression of both APP mRNA and protein, it did not elevate nicastrin levels. Consistent with this interpretation are our findings that both, stress and GC, upregulate BACE-1 levels as well as those of its cleavage product, C99. A glucocorticoid response element in the promoter region of the APP and BACE genes has been described,<sup>50</sup> (DK Lahiri, personal communication), making it likely that GC and GR mediate the regulatory actions of stress on APP and BACE-1 expression. BACE-1 is essential for C99 production, and previous studies have shown that even slight increases in this enzyme result in the generation of high levels of A $\beta$ .<sup>51–53</sup> Although both GC and stress stimulate APP processing to C99, we found that whereas stress significantly



increases nicastrin levels, GC actions are limited to the point of C99 production. Given the adverse actions of C99 on neuronal function and survival, as well as behavior,<sup>12–15</sup> the potential importance of GC-induced increases in C99 production for AD-like pathology is nevertheless significant.

Nicastrin, and in particular its glycosylated mature form, is an essential component of the  $\gamma$ -secretase complex,<sup>54,55</sup> because of its role in the recognition and presentation of substrate (C99) to presenilin-1 and -2, nicastrin is crucial for the generation of A $\beta$ .<sup>7,56</sup> Our observations that stress (but not GC) increases nicastrin levels, especially those of the mature form, suggest that stress can recruit additional mechanisms that allow it to carry C99 metabolism through to A $\beta$  production (cf. Ni *et al.*<sup>36</sup>) On the other hand, the possibility that GC can eventually generate A $\beta$  (for example, by acting on other members of the  $\gamma$ -secretase complex) cannot be excluded; technical limitations of existing assays unfortunately precluded direct measurements of rat A $\beta$  in the present study.

Since increased A $\beta$  production is causally related to AD pathology, exogenous administration of A $\beta$  peptides into the brain has been extensively used in normal rodents and primates to reproduce the neuropathological and behavioral features of early stage AD.<sup>37,39,57</sup> Using the A $\beta$  i.c.v. infusion paradigm in middle-aged rats, we here observed that A $\beta$  upregulates APP mRNA levels, but reduces APP protein levels; at the same time, A $\beta$  treatment results in increased tissue levels of C99 and nicastrin. Thus, A $\beta$  appears to accelerate APP metabolism, increasing the potential for amyloid peptide generation. The resemblance of the A $\beta$ -induced changes to those found after exposure to stress and GC supports the view that stress and GC may contribute to the etiopathogenesis of AD. Moreover, since A $\beta$ , stress and GC all have negative effects on cognition,<sup>21,22,58</sup> it would appear that A $\beta$  contributes to the molecular signaling machinery that mediates the behavioral actions of stress and GC. Lastly, it should be noted that the A $\beta$  infusion protocol used here appropriately models the preclinical stages of AD insofar that the pathobiochemical changes observed here occurred in a gradient manner, being stronger in the hippocampus than in the PFC (cf. Braak and Braak<sup>47</sup>). While our soluble A $\beta$  infusion paradigm did not result in detectable amyloid depositions, it should be noted that the role of A $\beta$  plaques in the pathogenesis of AD is contentious.<sup>59,60</sup> Recent work indicates a strong correlation between soluble A $\beta$  levels and the severity of memory deficits in AD patients.<sup>61</sup> Importantly, soluble A $\beta$  is known to trigger synapse loss,<sup>4</sup> an early event in the pathogenesis of AD.

Interactions between endogenous and exogenous factors (for example, age, mutations and A $\beta$  production on the one hand, and stress on the other) are important determinants of the onset and progress of AD. The organism experiences intermittent stressors throughout life, and its endogenous production of A $\beta$  increases with age.<sup>62</sup> The results of recent studies

in transgenic animal models showed that chronic stress potentiates A $\beta$  deposition and induces cognitive deficits<sup>21</sup> and that amyloid production is exacerbated when GC levels are elevated.<sup>20</sup> In the present work, we attempted to mimic typical lifetime events by exposing rats to a chronic unpredictable stress paradigm before subsequent treatment with GC and/or central infusions of A $\beta$ . The results clearly demonstrate that stress history exacerbates the APP misprocessing effects of A $\beta$  and GC, and, strikingly, also activated the amyloidogenic pathway in the PFC, an area that had otherwise proven to be relatively resilient to the individual stimuli. Importantly, the combinatorial treatment resulted in a deterioration of spatial reference memory that was greater than that induced by the individual treatments. Thus, stress history/previous exposure to high GC levels can markedly worsen the effects of subsequent exposures to stress/GC and A $\beta$  on AD-like biochemical and behavioral pathology; accordingly, stress may be an important precipitating and exacerbating factor in AD. The present findings provide experimental support for the implication of stress as a contributory factor in early AD disease.<sup>63</sup> Based on this and previous studies,<sup>64,65</sup> we suggest that stress (and GC) make neurons more vulnerable to the pathological actions of A $\beta$ , including A $\beta$ -induced stimulation of APP misprocessing.

To sum up, we have demonstrated that (1) stress/GC can contribute to AD pathology by driving APP metabolism toward the production of C99 and, eventually, of A $\beta$ , and (2) that stress history 'primes' the brain to generate greater amounts of amyloidogenic peptide(s) upon subsequent exposures to GC and A $\beta$ . These findings prompt the hypothesis that the effects of stress/GC on neuronal atrophy may be mediated by C99 and/or A $\beta$ , and that they may ultimately be causally related to disruptions of behavior that are typical of AD. The observation that A $\beta$ , like stress and GC, can increase emotionality is interesting in view of the increasing recognition of reciprocal regulatory relationships between cognition and emotion,<sup>27–29</sup> reports that many AD patients are hyperanxious,<sup>66</sup> and the implication of stress and GC in disorders of anxiety<sup>67</sup> and cognition.<sup>22,59</sup> Importantly, together with previously established links between depression and the increased risk for developing AD,<sup>31</sup> the present findings suggest that A $\beta$  may be a common denominator underlying these various stress-related disorders, and indicate the potential importance of including histories of stress and GC therapy in anamnestic data. Lastly, our results reiterate the need for judicious use of GC therapy in older subjects, especially in light of the poor efficacy of GC to slow the progression of AD.<sup>68,69</sup>

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