Molecular Psychiatry (2009) 14, 95–105

www.nature.com/mp

ORIGINAL ARTICLE

The amyloidogenic potential and behavioral correlates of stress

C Catania[1,5], I Sotiropoulos[1,5], R Silva[2], C Onofri[1], KC Breen[3,4], N Sousa[2] and OFX Almeida[1]

[1]*Max Planck Institute of Psychiatry, Munich, Germany;* [2]*Life and Health Science Research Institute, University of Minho, Braga, Portugal;* [3]*Alzheimer's Disease Research Centre, University of Dundee, Dundee, UK and* [4]*Parkinson's Disease Society, London, UK*

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-β (Aβ). We also show that exogenous Aβ can reproduce the effects of stress and GC on C99 production and that a history of stress strikingly potentiates the C99-inducing effects of Aβ and GC. Previous work has indicated a role for Aβ 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, Aβ administration causes spatial memory deficits that are exacerbated by stress and GC; additionally, Aβ, stress and GC induced a state of hyperanxiety. Given that the intrinsic properties of C99 and Aβ 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.
Molecular Psychiatry (2009) **14,** 95–105; doi:10.1038/sj.mp.4002101; published online 2 October 2007

Keywords: Alzheimer's disease; amyloid precursor protein; amyloid-β; glucocorticoids; memory; anxiety

Introduction

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

Aβ peptide is produced through the sequential proteolytic cleavage of amyloid precursor protein (APP) by α-, β- and γ-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 γ-secretase complex because of its essential role in the recognition of γ-secretase substrates.[7] Cleavage of APP by α-secretase precludes the generation of Aβ, whereas β-secretase (β-site APP-cleaving enzyme; BACE)-mediated cleavage generates the so-called C-terminal fragment 99 (C99); subsequent proteolysis of C99 by γ-secretase results in the generation of amyloid peptides (for review, see Bayer *et al.*[8]). While the neurotoxic potency of Aβ is well known,[9–11] other studies have demonstrated the neurotoxic and cognition-impairing potential of C99.[12–15] Furthermore, in a vicious cascade that characterizes AD, Aβ interacts with its protein precursor (APP), an interaction that is suggested to contribute to the mechanism of Aβ neurotoxicity.[16,17]

Observations that a large percentage of AD patients display hypercortisolemia[18,19] 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.[20,21] Indications of a link between stress/GC and AD may also be inferred from previous

Correspondence: Dr OFX Almeida, Max Planck Institute of Psychiatry, Kraepelinstrasse 2-10, Munich D-80804, Germany.
E-mail: osa@mpipsykl.mpg.de
[5]These authors contributed equally to this study
Received 29 March 2007; revised 19 July 2007; accepted 31 July 2007; published online 2 October 2007

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

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,[26] 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.[27–29] 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.[30,31]

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;[32] 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 $A\beta_{1-40}$ (American Peptide Co., Inc., Sunnyvale, CA; 4.2 nmol $A\beta_{1-40}$ per 200 µl; 0.5 µl h^{-1}) 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 µg kg^{-1} 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[33]); pump and cannulae implantation was done under pentobarbital (50 mg kg^{-1}) 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 ± 65.9 and 54.7 ± 4.8 ng ml^{-1}, respectively), and thymus weights at autopsy were consistent with hypercorticalism in the stressed group (control, CON: 7.6 ± 0.9 vs stressed: 4.3 ± 0.4 vs mg kg^{-1} BW). Whereas nonstressed animals showed a net gain in BW (6.1 ± 1.3 g), stressed animals showed a significant loss of body mass (-12.1 ± 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 Aβ.

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 $MgCl_2$, 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% β-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-α-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–DPEC), sections were acetylated (0.1 M triethanolamine, 0.25% acetic anhydride in DPEC), delipidated in chloroform (5 min), dehydrated through a graded series of ethanols and air dried. A 40-mer oligonucleotide probe (GCTGGCTGCCGTCGTGGGAACTC GGACTACCTCCTCCACA) was used to detect APP695-KPI.[34,35] Slides were hybridized with an antisense or sense (control) ^{35}S-dATP-labeled oligoprobe (10^6 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_2O, 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.[25] 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 ($d.f._{6,36}$)	*APP*		*C99*	*BACE*	*Nicastrin*	
	Immature (F = *54.9*)	*Mature* (F = *132.2*)	(F = *130.3*)	(F = *25.07*)	*Immature* (F = *19.9*)	*Mature* (F = *37.2*)
Stress history	$q = 0.0336$ (NS)	$q = 1.608$ (NS)	$q = 30.03$	$q = 5.592$	$q = 8.829$	$q = 16.189$
Glucocorticoid *milieu*	$q = 13.178$	$q = 10.649$	$q = 18.231$	$q = 12.928$	$q = 2.903$ (NS)	$q = 1.293$ (NS)
Aβ	$q = 13.416$	$q = 26.671$	$q = 32.175$	$q = 5.884$	$q = 3.598$ (NS)	$q = 8.651$
Stress history in (Aβ + GC)-treated animals	$q = 9.161$	$q = 12.466$	$q = 15.490$	$q = 4.409$ (NS)	$q = 2.563$ (NS)	$q = 5.835$
PFC ($d.f._{6,36}$)	*APP*		*C99*	*BACE*	*Nicastrin*	
	Immature (F = *15.2*)	*Mature* (F = *35.4*)	(F = *26.3*)	(F = *17.7*)	*Immature* (F = *18.5*)	*Mature* (F = *15.8*)
Stress history	$q = 0.377$ (NS)	$q = 3.424$ (NS)	$q = 15.791$	$q = 4.472$	$q = 9.629$	$q = 3.575$ (NS)
Glucocorticoid *milieu*	$q = 5.131$	$q = 10.676$	$q = 7.865$	$q = 0.925$ (NS)	$q = 0.998$ (NS)	$q = 0.754$ (NS)
Aβ	$q = 8.195$	$q = 10.521$	$q = 6.042$	$q = 8.047$	$q = 1.333$ (NS)	$q = 6.948$
Stress history in (Aβ + GC)-treated animals	$q = 5.706$	$q = 7.654$	$q = 6.933$	$q = 6.719$	$q = 9.111$	$q = 8.829$

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.

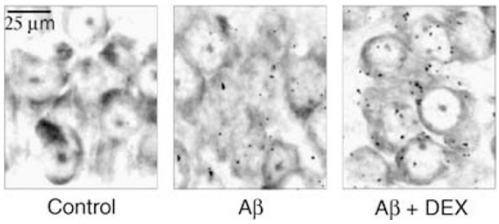

Figure 1 APP mRNA detected by ISHH. Representative images from hippocampal CA3 cells are shown. After ISHH, silver grains were revealed by photo-emulsion dipping; sections were counterstained with toluidine blue.

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 ($50.8 \times 10.2 \times 40.6$ 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,[18,19] and that stress exacerbates amyloid production in transgenic mouse models of AD,[20,21] 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 unpredictable stress protocol[32] before analysis of APP, C99, BACE-1 and nicastrin mRNA and protein levels in the hippocampus and PFC.

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.

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*.[36]).

Exogenous Aβ triggers APP misprocessing

Aβ infusion is widely used to experimentally model aspects of AD pathology in animals.[37] Since Aβ is

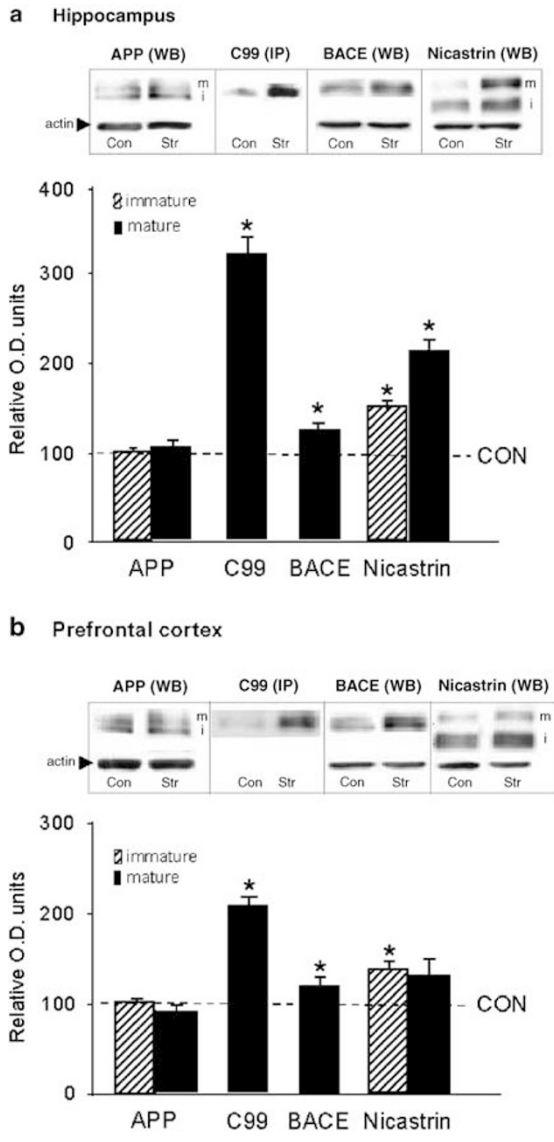

Figure 2 Stress drives amyloid precursor protein (APP) metabolism toward amyloidogenesis. Hippocampal (**a**) and prefrontal cortex (**b**) lysates from chronically stressed middle-aged rats were analyzed for their contents of APP, C99 (C-terminal fragment of APP), β-site APP-cleaving enzyme (BACE)-1 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 ± s.e.m. ($n = 6$–8 rats). Asterisks indicate $P \leqslant 0.05$ 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.

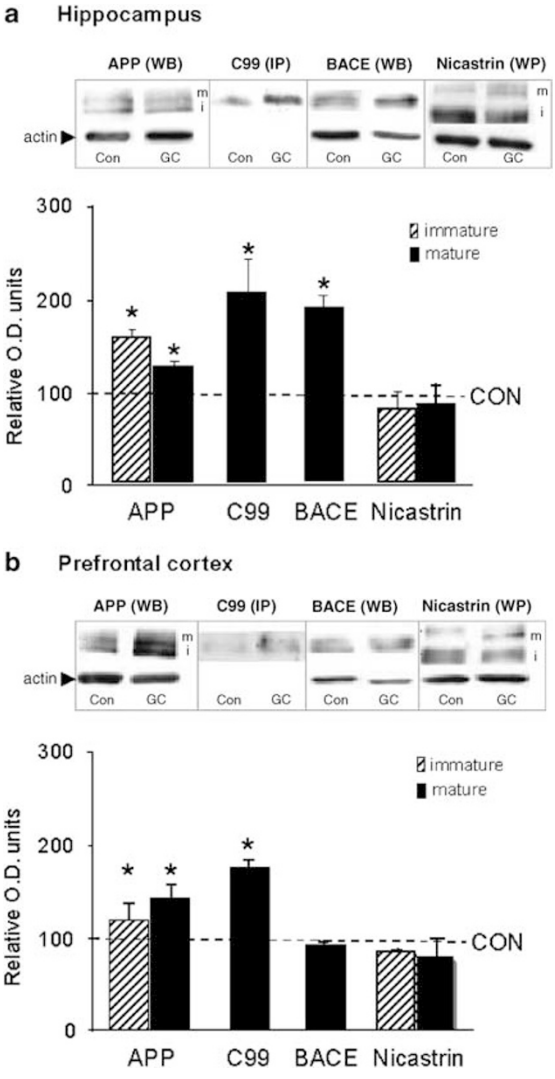

Figure 3 Glucocorticoids (GC) stimulate C-terminal fragment 99 (C99) production without upregulating nicastrin levels. Shown are the changes in amyloid precursor protein (APP), C99, β-site APP-cleaving enzyme (BACE)-1 and nicastrin expression in the hippocampus (**a**) and PFC (**b**) of middle-aged 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 ± 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 $P \leqslant 0.05$ vs control values. 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*,[38] we were interested to examine how Aβ influences APP metabolism *in vivo*.

When middle-aged rats were given constant infusions of Aβ into one lateral ventricle (Frautschy *et al*.[39]), there was a marked increase in APP mRNA signal in the hippocampi of Aβ-infused rats (semiquantitative data in Table 2). At the same time, Aβ 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 Aβ-induced increases in C99 expression were twice greater in the hippocampus than in the PFC. Together, this set of findings

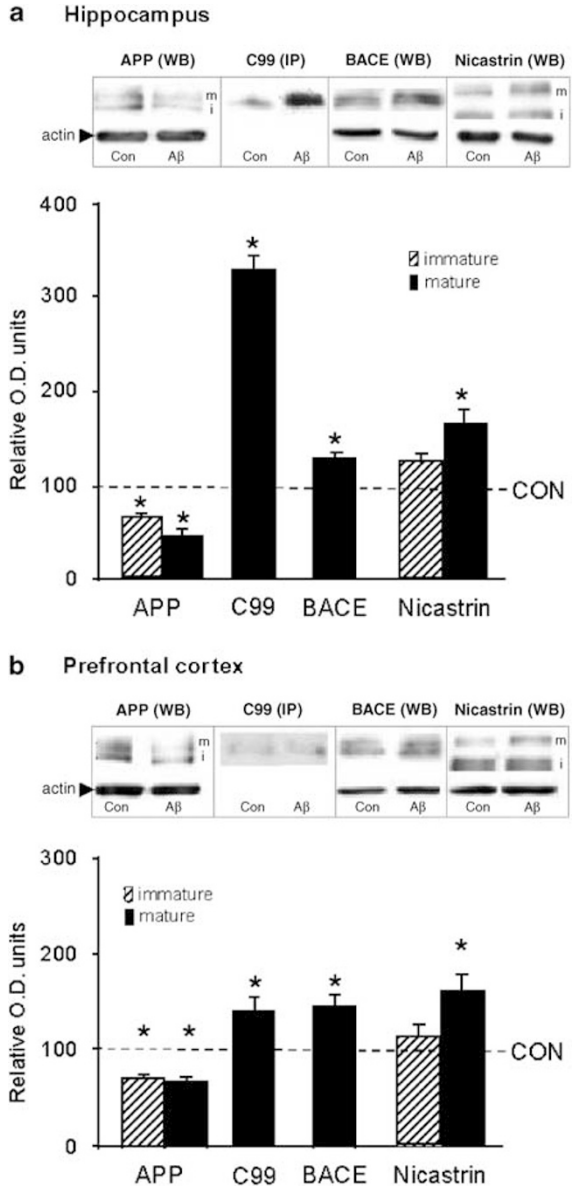

Figure 4 Amyloid-β (Aβ) stimulates amyloidogenesis. Aβ administered by chronic, constant-rate infusion into one lateral ventricle, resulted in significant increases in C-terminal fragment 99 (C99) production and, concomitantly, in nicastrin levels in the hippocampus (**a**) and PFC (**b**), suggesting 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 ± s.e.m. ($n = 6$–8 rats) are shown. Data represent optical densities of immunoreactive bands, normalized against actin or tubulin. Asterisks indicate $P \leqslant 0.05$ vs control values. In western blots, 'm' and 'i' refer to mature and immature forms of the respective proteins.

demonstrates that Aβ treatment triggers BACE-1-mediated APP misprocessing into C99 which can be potentially cleaved by γ-secretase into amyloid peptides. These results show that the actions of Aβ 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 Aβ deposition and induces cognitive deficits,[20] and that elevated GC exacerbate amyloidogenesis.[21] Given that the organism experiences stressful events throughout its lifetime, and aged individuals tend to show exaggerated GC secretory responses to stress,[22] we here examined the sequential effects of stress and GC on AD-like pathology by simultaneously treating previously stressed and nonstressed rats with Aβ (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: $F = 6.9$, $P \leqslant 0.003$; mature APP: $F = 11.1$, $P \leqslant 0.0001$; C99: $F = 224.2$, $P \leqslant 0.0001$; immature nicastrin: $F = 3.5$, $P \leqslant 0.043$; mature nicastrin: $F = 11.9$, $P \leqslant 0.001$) and PFC (immature APP: $F = 8.9$, $P \leqslant 0.001$; mature APP: $F = 10.7$, $P \leqslant 0.0001$; C99: $F = 11.5$, $P \leqslant 0.0001$; BACE: 6.6, $P \leqslant 0.004$; immature nicastrin: $F = 8.9$, $P \leqslant 0.001$; mature nicastrin: $F = 10.7$, $P \leqslant 0.0001$). Briefly, while stress did not influence the effects of subsequently applied Aβ on APP metabolism (data not shown), the amyloidogenic effects of Aβ + GC were further accentuated in rats that had been previously exposed to stress. Specifically, as shown in Figure 5, concomitant treatment with Aβ 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 Aβ + 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 Aβ.

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 Aβ, impair spatial reference memory to similar extents (Table 3). A tendency for further impairment of spatial reference memory was observed when Aβ and GC were applied concomitantly, and even more pronounced deficits were seen in animals that had been exposed to stress before receiving Aβ + GC (Table 3).

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

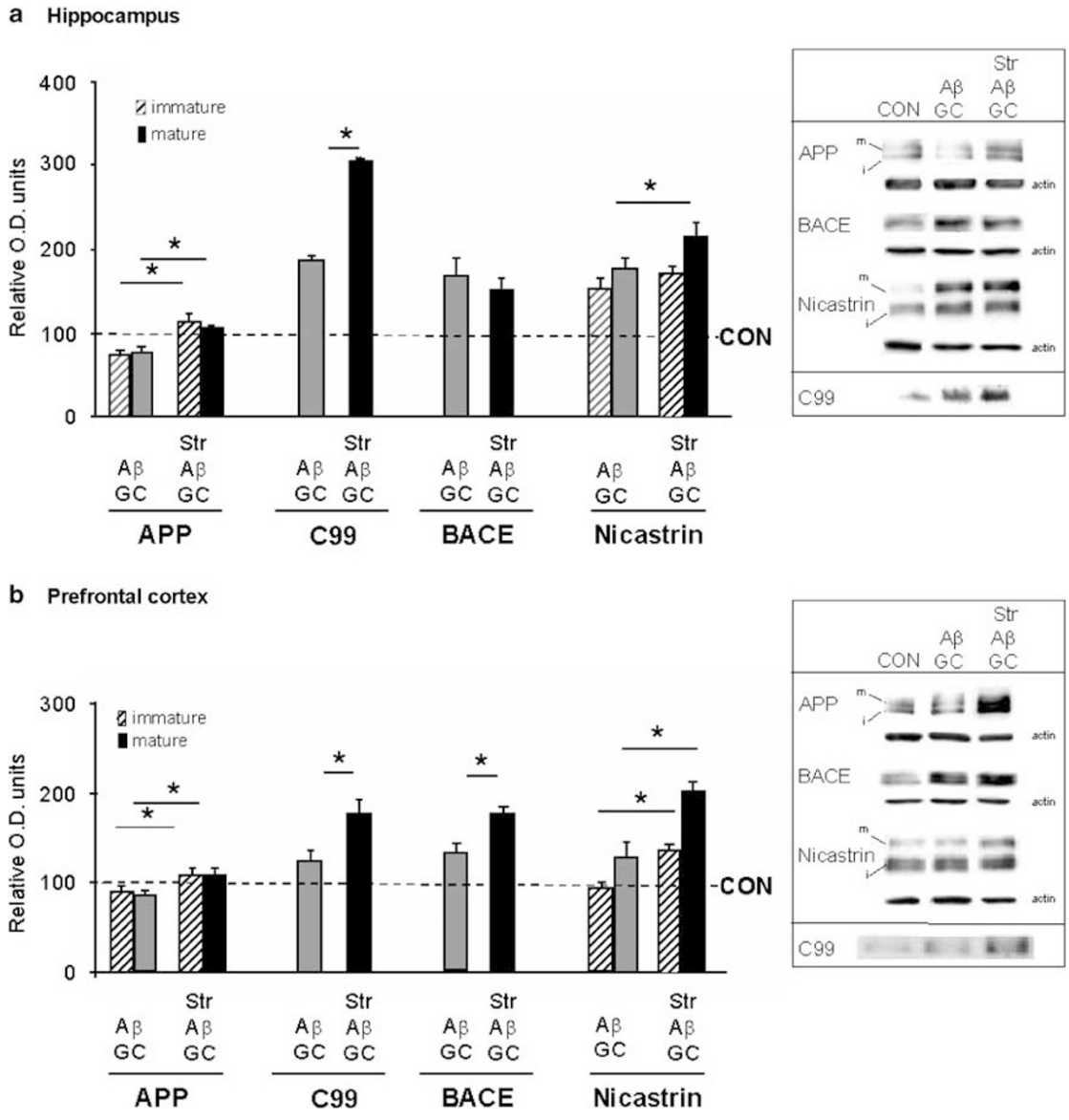

Figure 5 Stress history exacerbates the amyloidogenic effects of amyloid-β (Aβ) + glucocorticoids (GC) treatment. Rats that had been preexposed (4 weeks) to a chronic unpredictable stress paradigm responded to the coadministration of Aβ + GC (14 days) with markedly increased levels of C-terminal fragment 99 (C99), β-site APP-cleaving enzyme (BACE)-1 and nicastrin in the hippocampus (**a**) and PFC (**b**). Means ± s.e.m. are depicted ($n = 6$–8 rats). Numerical data refer to optical density readings from immunoreactive bands, normalized against actin. Asterisks indicate $P \leqslant 0.05$. 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 Aβ 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 Aβ and GC appeared to be resistant to the anxiogenic effects of GC and Aβ (cf. data for Aβ 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. Aβ-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*	*Aβ*	*Aβ + DEX*	*Stress + Aβ*	*Stress + Aβ + DEX*
Spatial memory (Morris water maze)							
Mean escape latency (s; trials 2–4)	7.41±0.8	15.36±1.7*	15.73±4.4*	20.87±2.7*	21.19±3.3*	19.04±4.7*	25.72±5.5*‡
Locomotor and exploratory behavior (open field)							
Total distance (cm)	287.0±76.3	278.7±11	252.0±42.5	256.7±42.9	233.0±46.5	232.5±44.5	209.0±69.8
No. of rearings/extensions	21.0±4.0	12.1±2.0*	10.0±1.5*	9.5±2.6*	9.8±1.4*	7.7±1.7*	7.2±2.3*
Anxiety level (elevated plus maze)							
Time in open arms (%)	26.1±6.7	14.9±3.3*	8.8±2.8*	8.3±2.0*	4.4±3.0*	10.0±1.9*	11.2±2.0*

Abbreviations: Aβ, β-amyloid; CON, control; DEX, dexamethasone.
Data shown are means±s.e.m. ($n=6$ rats per treatment group).
*$P \leqslant 0.05$ vs CON; ‡$P \leqslant 0.05$ vs stress + Aβ.

primary physiological response to stress. Both stress and GC can induce neurodegenerative changes in the hippocampus and PFC.[23,40–45] These brain regions express GRs;[46] they are important for cognition and are affected early in the development of AD.[47] Clinical reports of hypercortisolism in a significant number of AD patients[18,19] 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.[48,49] 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.[47] In addition, we show that Aβ 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 Aβ 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.[26] Misprocessing of APP commences with its endocytic cleavage by β-secretase (BACE) to yield the βC-terminal fragment (βCTF), C99. Subsequent cleavage of C99 by γ-secretase results in the generation of Aβ peptides. While much emphasis has been placed on examining the role and mechanisms of action of Aβ 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[12] and causes synaptic degeneration;[13] furthermore, C99 can impair long-term potentiation[14] and cognition.[15]

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 pardigm 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,[50] (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 Aβ.[51–53] 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,[12–15] 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 γ-secretase complex;[54,55] because of its role in the recognition and presentation of substrate (C99) to presenilin-1 and -2, nicastrin is crucial for the generation of Aβ.[7,56] 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 Aβ production (cf. Ni *et al*.[36]) On the other hand, the possibility that GC can eventually generate Aβ (for example, by acting on other members of the γ-secretase complex) cannot be excluded; technical limitations of existing assays unfortunately precluded direct measurements of rat Aβ in the present study.

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

Interactions between endogenous and exogenous factors (for example, age, mutations and Aβ 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 Aβ increases with age.[62] The results of recent studies in transgenic animal models showed that chronic stress potentiates Aβ deposition and induces cognitive deficits[21] and that amyloid production is exacerbated when GC levels are elevated.[20] 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 Aβ. The results clearly demonstrate that stress history exacerbates the APP misprocessing effects of Aβ 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 Aβ 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.[63] Based on this and previous studies,[64,65] we suggest that stress (and GC) make neurons more vulnerable to the pathological actions of Aβ, including Aβ-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 Aβ, and (2) that stress history 'primes' the brain to generate greater amounts of amyloidogenic peptide(s) upon subsequent exposures to GC and Aβ. These findings prompt the hypothesis that the effects of stress/GC on neuronal atrophy may be mediated by C99 and/or Aβ, and that they may ultimately be causally related to disruptions of behavior that are typical of AD. The observation that Aβ, like stress and GC, can increase emotionality is interesting in view of the increasing recognition of reciprocal regulatory relationships between cognition and emotion,[27–29] reports that many AD patients are hyperanxious,[66] and the implication of stress and GC in disorders of anxiety[67] and cognition.[22,59] Importantly, together with previously established links between depression and the increased risk for developing AD,[31] the present findings suggest that Aβ 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 anamnesic data. Lastly, our results reiterate the need for judicial use of GC therapy in older subjects, especially in light of the poor efficacy of GC to slow the progression of AD.[68,69]

Acknowledgments

We thank Dieter Fischer and Rainer Stoffel for excellent technical assistance, Carola Hetzel for administrative help, and Drs Keiro Shirotani and

Ayako Yamamoto for helpful advice. Isabel Matos and Lucilia Pinto are thanked for help with histological preparation and scoring, Dr Sam Gandy for providing the 369 antibody and Dr Alexandre Patchev for help with image preparation. CC and IS were supported by stipends from the Max Planck Society and EU Marie Curie Training Fellowships (at University College London, UK). The collaboration between the German and Portuguese laboratories was supported through the German–Portuguese Luso-Alemas Program (DAAD/GRICES). This study was conducted within the framework of the EU-supported integrated project 'CRESCENDO' (Contract FP6-018652).

References

1 Guillozet AL, Weintraub S, Mash DC, Mesulam MM. Neurofibrillary tangles, amyloid, and memory in aging and mild cognitive impairment. *Arch Neurol* 2003; **60**: 729–736.
2 Cleary JP, Walsh DM, Hofmeister JJ, Shankar GM, Kuskowski MA, Selkoe DJ *et al.* Natural oligomers of the amyloid-beta protein specifically disrupt cognitive function. *Nat Neurosc* 2005; **8**: 79–84.
3 Almeida CG, Tampellini D, Takahashi RH, Greengard P, Lin MT, Snyder EM *et al.* Beta-amyloid accumulation in APP mutant neurons reduces PSD-95 and GluR1 in synapses. *Neurobiol Dis* 2005; **20**: 187–198.
4 Roselli F, Tirard M, Lu J, Hutzler P, Lamberti P, Livrea P *et al.* Soluble beta-amyloid1-40 induces NMDA-dependent degradation of postsynaptic density-95 at glutamatergic synapses. *J Neurosci* 2005; **25**: 11061–11070.
5 Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R *et al.* Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Ann Neurol* 1991; **30**: 572–580.
6 Olariu A, Tran MH, Yamada K, Mizuno M, Hefco V, Nabeshima T. Memory deficits and increased emotionality induced by β-amyloid (25–35) are correlated with the reduced acetylcholine release and altered phorbol dibutyrate binding in the hippocampus. *J Neural Transm* 2001; **108**: 1065–1079.
7 De Strooper B. Nicastrin: gatekeeper of the gamma-secretase complex. *Cell* 2005; **122**: 318–320.
8 Bayer TA, Wirths O, Majtenyi K, Hartmann T, Multhaup G, Beyreuther K *et al.* Key factors in Alzheimer's disease: beta-amyloid precursor protein processing, metabolism and intraneuronal transport. *Brain Pathol* 2001; **11**: 1–11.
9 Lorenzo A, Yankner BA. Beta-amyloid neurotoxicity requires fibril formation and is inhibited by congo red. *Proc Natl Acad Sci USA* 1994; **91**: 12243–12247.
10 Weldon DT, Rogers SD, Ghilardi JR, Finke MP, Cleary JP, O'Hare E *et al.* Fibrillar beta-amyloid induces microglial phagocytosis, expression of inducible nitric oxide synthase, and loss of a select population of neurons in the rat CNS *in vivo*. *J Neurosci* 1998; **18**: 2161–2173.
11 Mattson MP. Pathways towards and away from Alzheimer's disease. *Nature* 2004; **430**: 631–639.
12 Yankner BA, Dawes LR, Fisher S, Villa-Komaroff L, Oster-Granite ML, Neve RL. Neurotoxicity of a fragment of the amyloid precursor associated with Alzheimer's disease. *Science* 1989; **245**: 417–420.
13 Oster-Granite ML, McPhie DL, Greenan J, Neve RL. Age-dependent neuronal and synaptic degeneration in mice transgenic for the C terminus of the amyloid precursor protein. *J Neurosci* 1996; **16**: 6732–6741.
14 Nalbantoglu J, Tirado-Santiago G, Lahsaini A, Poirier J, Goncalves O, Verge G *et al.* Impaired learning and LTP in mice expressing the carboxy terminus of the Alzheimer amyloid precursor protein. *Nature* 1997; **387**: 500–505.
15 Berger-Sweeney J, McPhie DL, Arters JA, Greenan J, Oster-Granite ML, Neve RL. Impairments in learning and memory accompanied by neurodegeneration in mice transgenic for the carboxyl-terminus of the amyloid precursor protein. *Mol Brain Res* 1999; **66**: 150–162.
16 Lorenzo A, Yuan M, Zhang Z, Paganetti PA, Sturchler-Pierrat C, Staufenbiel M *et al.* Amyloid beta interacts with the amyloid precursor protein: a potential toxic mechanism in Alzheimer's disease. *Nat Neurosci* 2000; **3**: 460–464.
17 Heredia L, Lin R, Vigo FS, Kedikian G, Busciglio J, Lorenzo A. Deposition of amyloid fibrils promotes cell-surface accumulation of amyloid beta precursor protein. *Neurobiol Dis* 2004; **16**: 617–629.
18 Hartmann A, Veldhuis JD, Deuschle M, Standhardt H, Heuser I. Twenty-four hour cortisol release profiles in patients with Alzheimer's and Parkinson's disease compared to normal controls: ultradian secretory pulsatility and diurnal variation. *Neurobiol Aging* 1997; **18**: 285–289.
19 Elgh E, Lindqvist Astot A, Fagerlund M, Eriksson S, Olsson T, Nasman B. Cognitive dysfunction, hippocampal atrophy and glucocorticoid feedback in Alzheimer's disease. *Biol Psychiatry* 2006; **59**: 155–161.
20 Green KN, Billings LM, Roozendaal B, McGaugh JL, LaFerla FM. Glucocorticoids increase amyloid-beta and tau pathology in a mouse model of Alzheimer's disease. *J Neurosci* 2006; **26**: 9047–9056.
21 Jeong YH, Park CH, Yoo J, Shin KY, Ahn SM, Kim HS *et al.* Chronic stress accelerates learning and memory impairments and increases amyloid deposition in APPV717I-CT100 transgenic mice, an Alzheimer's disease model. *FASEB J* 2006; **20**: 729–731.
22 Lupien SJ, Fiocco A, Wan N, Maheu F, Lord C, Schramek T *et al.* Stress hormones and human memory function across the lifespan. *Psychoneuroendocrinology* 2005; **30**: 225–242.
23 Cerqueira JJ, Pego JM, Taipa R, Bessa JM, Almeida OF, Sousa N. Morphological correlates of corticosteroid-induced changes in prefrontal cortex-dependent behaviors. *J Neurosci* 2005; **25**: 7792–7800.
24 Sousa N, Almeida OFX. Corticosteroids: sculptors of the hippocampal formation. *Rev Neurosci* 2002; **13**: 59–84.
25 Cerqueira JJ, Taipa R, Almeida OFX, Sousa N. Specific configuration of dendritic degeneration in pyramidal neurons of the medial prefrontal cortex induced by differing corticosteroid regimen. *Cereb Cortex* 2006; **17**: 1998–2006.
26 Selkoe DJ. Alzheimer's disease: genotypes, phenotypes, and treatments. *Science* 1997; **275**: 630–631.
27 Dolan RJ. Emotion, cognition, and behavior. *Science* 2002; **298**: 1191–1194.
28 Ochsner KN, Gross JJ. The cognitive control of emotion. *Trends Cogn Sci* 2005; **9**: 242–249.
29 Phelps EA. Emotion and cognition: insights from studies of the human amygdala. *Annu Rev Psychol* 2006; **57**: 27–53.
30 Ownby RL, Harwood DG, Barker WW, Duara R. Predictors of anxiety in patients with Alzheimer's disease. *Depress Anxiety* 2000; **11**: 38–42.
31 Ownby RL, Crocco E, Acevedo A, John V, Loewenstein D. Depression and risk for Alzheimer disease: systematic review, meta-analysis, and metaregression analysis. *Arch Gen Psychiatry* 2006; **63**: 530–538.
32 Sousa N, Almeida OFX, Holsboer F, Paula-Barbosa MM, Madeira MD. Maintenance of hippocampal cell numbers in young and aged rats submitted to chronic unpredictable stress. Comparison with the effects of corticosterone treatment. *Stress* 1998; **2**: 237–249.
33 Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*,4th edn. Academic Press: San Diego, 1998.
34 Solà C, Mengod G, Probst A, Palacios KM. Differential regional and cellular distribution of â-amyloid precursor RNAs containing and lacking the Kunitz protease inhibitor domain in the brain of human, rat and mouse. *Neuroscience* 1993; **53**: 267–295.
35 Panegyres PK. The effects of excitotoxicity on the expression of the amyloid precursor protein gene in the brain and its modulation by neuroprotective agents. *J Neural Transm* 1998; **105**: 463–478.
36 Ni Y, Zhao X, Bao G, Zou L, Teng L, Wang Z *et al.* Activation of beta(2)-adrenergic receptor stimulates gamma-secretase activity and accelerates amyloid plaque formation. *Nat Med* 2007; **12**: 1390–1396.
37 Stephan A, Phillips AG. A case for a non-transgenic animal model of Alzheimer's disease. *Genes Brain Behav* 2005; **4**: 157–172.

38 Davis-Salinas J, Saporito-Irwin SM, Cotman CW, Van Nostrand WE. Amyloid beta-protein induces its own production in cultured degenerating cerebrovascular smooth muscle cells. *J Neurochem* 1995; **65**: 931–934.
39 Frautschy SA, Yang F, Calderon L, Cole GM. Rodent models of Alzheimer's disease: rat A beta infusion approaches to amyloid deposits. *Neurobiol Aging* 1996; **17**: 311–321.
40 Magarinos AM, Verdugo JM, McEwen BS. Chronic stress alters synaptic terminal structure in hippocampus. *Proc Natl Acad Sci USA* 1997; **94**: 14002–14008.
41 Lupien SJ, de Leon M, de Santi S, Convit A, Tarshish C, Nair N *et al.* Cortisol levels during human aging predict hippocampal atrophy and memory deficits. *Nat Neurosci* 1998; **1**: 69–73.
42 Sousa N, Paula-Barbosa MM, Almeida OFX. Ligand and subfield specificity of corticoid-induced neuronal loss in the rat hippocampal formation. *Neuroscience* 1999; **89**: 1079–1087.
43 Wellman CL. Dendritic reorganization in pyramidal neurons in medial prefrontal cortex after chronic corticosterone administration. *J Neurobiol* 2001; **49**: 245–253.
44 Cerqueira JJ, Catania C, Sotiropoulos I, Schubert M, Kalisch R, Almeida OFX *et al.* Corticosteroid status influences the volume of the rat cingulate cortex—a magnetic resonance imaging study. *J Psychiatr Res* 2005; **39**: 451–460.
45 Radley JJ, Morrison JH. Repeated stress and structural plasticity in the brain. *Ageing Res Rev* 2005; **4**: 271–287.
46 McEwen BS, De Kloet ER, Rostene W. Adrenal steroid receptors and actions in the nervous system. *Physiol Rev* 1986; **66**: 1121–1188.
47 Braak H, Braak E. Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol (Berl)* 1991; **82**: 239–259.
48 Islam A, Kalaria RN, Winblad B, Adem A. Enhanced localization of amyloid beta precursor protein in the rat hippocampus following long-term adrenalectomy. *Brain Res* 1998; **806**: 108–112.
49 Budas G, Coughlan CM, Seckl JR, Breen KC. The effect of corticosteroids on amyloid beta precursor protein/amyloid precursor-like protein expression and processing *in vivo*. *Neurosci Lett* 1999; **276**: 61–64.
50 Sambamurti K, Kinsey R, Maloney B, Ge YW, Lahiri DK. Gene structure and organization of the human beta-secretase (BACE) promoter. *FASEB J* 2004; **18**: 1034–1036.
51 Haass C. Take five—BACE and the gamma-secretase quartet conduct Alzheimer's amyloid beta-peptide generation. *EMBO J* 2004; **23**: 483–488.
52 Johnston JA, Liu WW, Todd SA, Coulson DT, Murphy S, Irvine GB *et al.* Expression and activity of beta-site amyloid precursor protein cleaving enzyme in Alzheimer's disease. *Biochem Soc Trans* 2005; **33**: 1096–1100.
53 Li Y, Zhou W, Tong Y, He G, Song W. Control of APP processing and Abeta generation level by BACE1 enzymatic activity and transcription. *FASEB J* 2006; **20**: 285–292.
54 Yang DS, Tandon A, Chen F, Yu G, Yu H, Arawaka S *et al.* Mature glycosylation and trafficking of nicastrin modulate its binding to presenilins. *J Biol Chem* 2002; **277**: 28135–28142.
55 Herreman A, Van Gassen G, Bentahir M, Nyabi O, Craessaerts K, Mueller U *et al.* γ-secretase activity requires the presenilin-dependent trafficking of nicastrin through the Golgi apparatus but not its complex glycosylation. *J Cell Sci* 2003; **116**: 1127–1136.
56 Shah S, Lee SF, Tabuchi K, Hao YH, Yu C, LaPlant Q *et al.* Nicastrin functions as a gamma-secretase-substrate receptor. *Cell* 2005; **122**: 435–447.
57 Geula C, Wu CK, Saroff D, Lorenzo A, Yuan M, Yankner BA. Aging renders the brain vulnerable to amyloid beta-protein neurotoxicity. *Nat Med* 1998; **4**: 827–831.
58 Starkman MN, Giordani B, Berent S, Schork MA, Schteingart DE. Elevated cortisol levels in Cushing's disease are associated with cognitive decrements. *Psychosom Med* 2001; **63**: 985–993.
59 Lue LF, Kuo YM, Roher AE, Brachova L, Shen Y, Sue L *et al.* Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. *Am J Pathol* 1999; **155**: 853–862.
60 Edison P, Archer HA, Hinz R, Hammers A, Pavese N, Tai YF *et al.* Amyloid, hypometabolism, and cognition in Alzheimer disease: an [11C]PIB and [18F]FDG PET study. *Neurology* 2007; **68**: 501–508.
61 McLean CA, Cherny RA, Fraser FW, Fuller SJ, Smith MJ, Beyreuther K *et al.* Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. *Ann Neurol* 1999; **46**: 860–866.
62 Braak H, Braak E. Diagnostic criteria for neuropathologic assessment of Alzheimer's disease. *Neurobiol Aging* 1997; **18**(Suppl 4): S85–S88.
63 Swanwick GR, Kirby M, Bruce I, Buggy F, Coen RF, Coakley D *et al.* Hypothalamic–pituitary–adrenal axis dysfunction in Alzheimer's disease: lack of association between longitudinal and cross-sectional findings. *Am J Psychiatry* 1998; **155**: 286–289.
64 Behl C, Lezoualch F, Trapp T, Widmann M, Skutella T, Holsboer F. Glucocorticoids enhance oxidative stress-induced cell death in hippocampal neurons *in vitro*. *Endocrinology* 1997; **138**: 101–106.
65 Sapolsky RM. The possibility of neurotoxicity in the hippocampus in major depression: a primer on neuron death. *Biol Psychiatry* 2000; **48**: 755–765.
66 Grossberg GT. Diagnosis and treatment of Alzheimer's disease. *J Clin Psychiatry* 2003; **64**(Suppl 9): S3–S6.
67 Tatsch MF, Bottino CM, Azevedo D, Hototian SR, Moscoso MA, Folquitto JC *et al.* Neuropsychiatric symptoms in Alzheimer disease and cognitively impaired, nondemented elderly from a community-based sample in Brazil: prevalence and relationship with dementia severity. *Am J Geriatr Psychiatry* 2006; **14**: 438–445.
68 in 't Veld BA, Ruitenberg A, Hofman A, Launer LJ, van Duijn CM, Stijnen T *et al.* Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease. *N Engl J Med* 2001; **345**: 1515–1521.
69 Aisen PS. The potential of anti-inflammatory drugs for the treatment of Alzheimer's disease. *Lancet Neurol* 2000; **1**: 279–284.