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Glucocorticoids increase impairments in learning and memory due to elevated amyloid precursor protein expression and neuronal apoptosis in 12-month old mice

Wei-Zu Li, Wei-Ping Li *, Yu-You Yao, Wen Zhang, Yan-Yan Yin, Guo-Cui Wu, Hui-Ling Gong

Department of Pharmacology, Anhui Medical University, Hefei, 230032, China

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ABSTRACT

Alzheimer's disease is a chronic neurodegenerative disorder marked by a progressive loss of memory and cognitive function. Stress level glucocorticoids are correlated with dementia progression in patients with Alzheimer's disease. In this study, twelve month old male mice were chronically treated for 21 days with stress-level dexamethasone (5 mg/kg). We investigated the pathological consequences of dexamethasone administration on learning and memory impairments, amyloid precursor protein processing and neuronal cell apoptosis in 12-month old male mice. Our results indicate that dexamethasone can induce learning and memory impairments, neuronal cell apoptosis, and mRNA levels of the amyloid precursor protein, β-secretase and caspase-3 are selectively increased after dexamethasone administration. Immunohistochemistry demonstrated that amyloid precursor protein, caspase-3 and cytochrome *c* in the cortex and CA1, CA3 regions of the hippocampus are significantly increased in 12-month old male mice. Furthermore, dexamethasone treatment induced cortex and hippocampus neuron apoptosis as well as increasing the activity of caspase-9 and caspase-3. These findings suggest that high levels of glucocorticoids, found in Alzheimer's disease, are not merely a consequence of the disease process but rather play a central role in the development and progression of Alzheimer's disease. Stress management or pharmacological reduction of glucocorticoids warrant additional consideration of the regimen used in Alzheimer's disease therapies.

1. Introduction

Alzheimer's disease is a chronic neurodegenerative disorder marked by a progressive loss of memory and cognitive function. It is histopathologically characterized by amyloid β-protein deposits, neurofibrillary tangles, synaptic loss and neuronal death. In addition to the genetic factors known to be involved in early-onset familial Alzheimer's disease (i.e., linked missense mutations in amyloid precursor protein and the presenilin 1 and 2 genes), aging and environmental effects are also believed to influence the pathogenesis and the behavioral disturbances associated with Alzheimer's disease (Citron et al., 1997; Suh and Checler, 2002). Neuroendocrine malfunctions may also be involved in the Alzheimer's disease process, particularly because stress hormones can negatively affect neuronal survival (Stein-Behrens et al., 1994). Epidemiological evidence further supports the role for stress as a risk factor for Alzheimer's disease because elderly individuals prone to psychological distress are more likely to develop the disorder than age-matched, nonstressed individuals (Wilson et al., 2005).

The glucocorticoids response to stressful stimuli is regulated by the hypothalamic–pituitary–adrenal (HPA) axis, which triggers the adrenal cortex to release glucocorticoids. It has been reported that chronic stress and glucocorticoids can reduce hippocampal dendritic complexity (Kleen et al., 2006; Conrad et al., 2007) and can also promote hippocampal cell death (MacPherson et al., 2005). Since the hippocampus plays a central role in inhibiting the activity of the HPA axis, hippocampal damage could produce a repetitive cycle of increasing HPA axis dysregulation and ongoing hippocampal injury. There is ample evidence implicating HPA axis dysfunction in Alzheimer's disease, reflected by significantly elevated basal levels of circulating cortisol (Swanwick et al., 1998; Csernansky et al., 2006) and a failure to show cortisol suppression after a dexamethasone challenge (Nasman et al., 1995). Clinical assays indicate that cortisol levels expressed in elderly people without dementia and in elderly people with Alzheimer's disease are 14.26 μg/ml and 17.98 μg/ml, respectively. Plasma levels of the stress hormone, cortisol, are correlated with the rate of dementia progression in patients with Alzheimer's disease (Csernansky et al., 2006). Animal research reveals that at 9 months and older 3×Tg-AD mice have significantly higher plasma corticosterone levels than NonTg mice (Green et al., 2006). Genetic studies indicate a link between glucocorticoid function and the risk for Alzheimer's disease, due to a rare haplotype in the 5′ regulatory region of the gene encoding 11-hydroxysteroid dehydrogenase type 1, being associated with a six-fold increased risk for sporadic Alzheimer's disease (de Quervain et al., 2004). Some evidence from animal studies suggests an interaction between

* Corresponding author. Tel./fax: +86 551 5161133.
E-mail address: lwp19@126.com (W.-P. Li).

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glucocorticoids and Alzheimer's disease pathology, including amyloid precursor protein and tau pathology (Green et al., 2006). However, the interaction between amyloid precursor protein processing and neuronal cell apoptosis and learning and memory impairments induced by glucocorticiods is currently unknown.

Our previous study showed that twenty one days of exposure to dexamethasone induced impairment of memory and learning, which was accompanied by severe histological damage in the CA3 fields of the hippocampus in senescent mice (Yao et al., 2007). To explore the potential mechanism of glucocorticoids on learning and memory impairments and to suggest a link between high level glucocorticoids and Alzheimer's disease progression, we investigated the pathological consequences of stress-level glucocorticoid (5 mg/kg) (Green et al., 2006) administration on learning and memory impairments, amyloid precursor protein processing and neuronal cell apoptosis in 12-month old mice. Here we report the novel findings that dexamethasone can induce learning and memory impairment, neuronal cell apoptosis, and increased mRNA levels of the β-secretase and its substrate amyloid precursor protein. Furthermore, dexamethasone treatment induced cortex and hippocampal neuron apoptosis by increasing the activity of caspase-9 and caspase-3. The present findings highlight a mechanism by which high level glucocorticoids effect Alzheimer's disease neuropathology and suggest that stress management or pharmacological reduction of glucocorticoids warrant additional consideration as treatments for Alzheimer's disease.

2. Materials and methods

2.1. Animals and treatment

All rodent experiments were performed in accordance with animal protocols approved by the local authorities in Anhui, China. We used Kunming strain mice in our study (male, 12-month old, weighing 50 ± 5 g). These animals (purchased from the Center of laboratory animals of Anhui, GradeII, Certificate No. SCXK 2005) were randomly split into two treatment groups. Animals were housed under standard conditions at 22 °C in a 12 h light/dark cycle with access to food and water ad libitum. Animals were treated with dexamethasone (Sigma, 5 mg/kg/d, ig) or vehicle for 21 days. Animals were weighed every 2 days.

2.2. Morris water maze test

The water maze apparatus, mouse handling, and general testing procedure were described elsewhere (Janus et al., 2000). All mice underwent a reference memory training protocol which included a hidden platform placed in the center of one quadrant of the pool (southeast) for 5 days (from day 17 till day 21 after dexamethasone treatment), with 4 trials (90 s per trial) per day. After the last trial on day 5, the platform was removed from the pool and each mouse received one 60 s swim probe trial. Escape latency (in seconds) was recorded using an online image video tracking system. In the probe trials, the number of passes over the platform site and swimming time in the quadrant of the platform was recorded, which expresses the spatial place preference (Gass et al., 1998). Behavioral data were analyzed using analysis of variance.

2.3. Histological examination

24 h after the final dexamethasone administration, the animals were sacrificed and the brains were removed. The brains were immediately dissected in half along the coronal line; half were frozen at −80 °C for biochemical analysis and the other half were fixed in 4% paraformaldehyde and embedded in paraffin. The brains were sliced into 5 μm sections using a microtome. The sections were stained with HE (Hematoxylin and eosin) and examined under a light microscope.

2.4. Hoechst 33258 staining

For nuclear staining, paraffin sections were deparaffinized with xylene two times for 15 min each and then rinsed with PBS. The sections were incubated with 25 mM Hoechst 33258 (ZSGB-BIO) for 15 min at 37 °C, washed with PBS, mounted onto slides using anti-fade mounting medium (Beyotime), and then examined by fluorescence microscopy (Olympus Opticals, Tokyo, Japan) (Ex/Em: 352 nm/461 nm).

2.5. RT-PCR

Total brain RNA was extracted using TRIzol (Invitrogen, Germany) according to the instructions by the manufacturer and resuspended in 20 μl of DEPC-treated water. RNA concentration was determined using a biophotometer (Shanghai Scientific China). Four micrograms of RNA underwent reverse transcription to generate cDNA using random hexamer primers and Primescript™ RTase (TaKaRa BIO). PCR was conducted using a RT-PCR kit (Promega, USA). Primer sequences (Shanghai Sangon Bio-Tech) and annealing temperatures for amyloid precursor protein, β-secretase, α-secretase, caspase-3 and β-actin PCR are listed in Table 1. PCR products and a 1 kb DNA molecular weight marker (Promega, USA) were then electrophoresed on a 1% agarose gel, and the gel was visualized and photographed using a gel imaging system (Biosens SC810X, Shanghai Bio-Tech). The gel imaging software was used for quantitative analyses.

2.6. Immunohistochemistry

Paraffin embedded brain sections were cut at 5 μm and affixed to slides to ensure adhesion. For immunohistochemistry, all sections were blocked prior to processing in dilute (3%) hydrogen peroxide to inactivate endogenous peroxidase and non-immune goat serum. In all cases the primary antibody was left overnight at 4 °C. Immunostaining was visualized by the peroxidase method with a biotinylated anti-rabbit secondary antibody and diaminobenzidine oxidation (ABC kit, BOSTER). Sections were lightly counterstained with hematoxylin and were resin-mounted. The primary antibodies were amyloid precursor protein (1:100) and cytochrome *c* (1:100) from ABZOOM and caspase-3 (1:100) from BOSTER. Analyses were carried out with an observer blinded to the experimental protocol. Four sections per group and three fields per section of the same magnification were utilized for quantitative analysis. Expression of amyloid precursor protein, cytochrome *c* and caspase-3 positive neurons in the CA1and CA3 of hippocampus and cortex were observed under the microscope. The mean optical density of amyloid precursor protein, cytochrome *c* and caspase-3 positive neurons was measured using the Image-Pro Plus 6.0 analysis system in each section.

2.7. Capase-3 and caspase-9 activity assay

The activity of caspase-3 and caspase-9 was measured by cleaving selective substrates acetyl-Asp-Glu-Val-Asp P-nitroanilide(Ac-DEVD-PNA) and acetyl-Leu-Glu-His-Asp P-nitroanilide(Ac-LEHD-PNA) respectively (Beyotime institute of biotechnology). Tissues were homogenized in a homogenizing buffer containing 15 mM Tris–HCl, pH 7.4, 320 mM sucrose, 1 mM EGTA, 2 mM EDTA, 50 mM NaF, 1 mM $MgCl_2$, 1 mM Na_3VO_4, and 30 mM sodium pyrophosphate plus protease inhibitors and centrifuged at 14,000×*g* for 60 min at 4 °C. Protein concentration of supernatants was measured by Bradford's method and equal amounts of proteins (10 μl) were incubated in a total volume of 100 μl comprised of 80 μl detection buffer. The reaction was started by addition of caspase-3 and caspase-9 substrates Ac-DEVD-PNA (10 μl) and Ac-LEHD-PNA (10 μl). After incubation for 60 min at 37 °C, cleavage of the substrate was detected using a MicroplateReader (SPECTRAMAX 190, USA) with a

Table 1
Oligonucleotide primers used for cDNA amplification.

mRNA	Sense primer	Antisense primer	Anneal temperature (°C)	PCR target (bp)	Cycles
APP695	5′GCGGTGAAGACAAAGTCG 3′	5′AAATGGGCGTGCTCGTTC 3′	51	289	38
APP751				457	
β-secretase	5′GGCGGGAGTGGTATTATGAA 3′	5′GTGATGCGGAAGGACTGATT 3′	52	316	38
α-secretase	5′TCCCAAGCCCAACTTTAC 3′	5′ACCAGTGACCACAATCC3′	51	395	38
Caspase 3	5′ACTGGAATGTCATCTCGCTCTG3′	5′CCACGACCCGTCCTTTGA3′	56	468	38
β-actin	5′AGCATTTGCGGTGCACGATGGAGGG3′	5′ATGCCATCCTGCGTCTGGACCTGGC3′	52	606	38

wavelength of 405 nm. Activities of caspase-3 and caspase-9 were expressed as changes in DEVDase and LEHDase activity.

2.8. Statistical analysis

Statistical differences between the groups were analyzed using either one-way ANOVA or Student's *t*-test. Results are expressed as the means ± S.D., the level of significance was $P<0.05$.

3. Results

3.1. Dexamethasone exposure accelerates behavioral impairments in 12-month old male mice

We assessed learning and memory ability by using the Morris water maze test described previously. Twenty-one day exposure to dexamethasone induced weight loss (Fig. 1) and impairment of memory and learning in mice (12-month old) (Fig. 2). In the memory training experiment, the mean escape latencies (day 2, 3, 4) were significantly different (78.22 ± 12.17 *versus* 65.64 ± 15.43 s, 72.09 ± 11.47 *versus* 54.18 ± 20.77 s and 69.905 ± 13.53 *versus* 52.25 ± 18.27 s for the dexamethasone-treated mice and vehicle-treated mice, respectively). In the probe trial the average number of mice crossing the platform site (NCP) and swimming time in the quadrant of platform (STP) were also significantly different (1.55 ± 1.13 *versus* 2.92 ± 1.73 for NCP and 13.52 ± 6.23 *versus* 22.99 ± 7.59 for STP for the dexamethasone-treated mice and vehicle-treated mice, respectively). In conclusion, a prolonged dexamethasone exposure resulted in memory impairment and decreased learning in 12-month old male mice.

3.2. Dexamethasone exposure increased neuronal degenerative changes and apoptosis in the hippocampus and cortex in 12-month old male mice

Histological examination and Hoechst 33258 staining were used to examine the neuropathology and apoptosis. No significant neuronal abnormalities were observed in either the hippocampus (CA1, CA3) or the cortex from mice in the control group, while all examined brains in the dexamethasone mice showed degeneration of neurons in the hippocampus and cortex as well as neuronal disarray. The body of neuron became short and deeply stained with dye (Fig. 3).

We further studied the nuclear morphology of hippocampal neurons and cortical cells by staining with Hoechst 33258. Hoechst 33258 binds to chromatin, allowing fluorescent visualization of normal and condensed chromatin. Morphologically, cells undergoing apoptosis show chromatin condensation, loss of nuclear envelope, and cellular fragmentation into apoptotic bodies (Qiu et al., 2000). Quantitation of apoptotic cells detected with Hoechst showed that

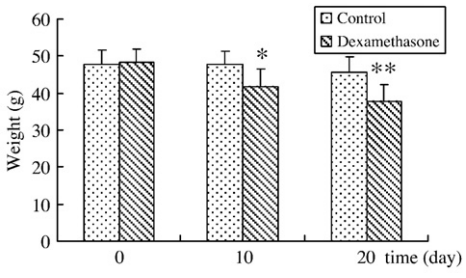

Fig. 1. Dexamethasone treatment induced weight loss in mice. Twelve-month old male mice were daily treated with 5 mg/kg dexamethasone ($n=11$) or vehicle ($n=12$) for 21 days. Dexamethasone-treated group had significant weight loss (47.58 ± 3.85 *versus* 41.82 ± 4.83 with 10 day treatment and 45.75 ± 4.14 *versus* 37.73 ± 4.52 with 20 day treatment, control group *versus* dexamethasone-treated group in both). *: $P<0.05$, **: $P<0.01$, comparison between control group and dexamethasone-treated group.

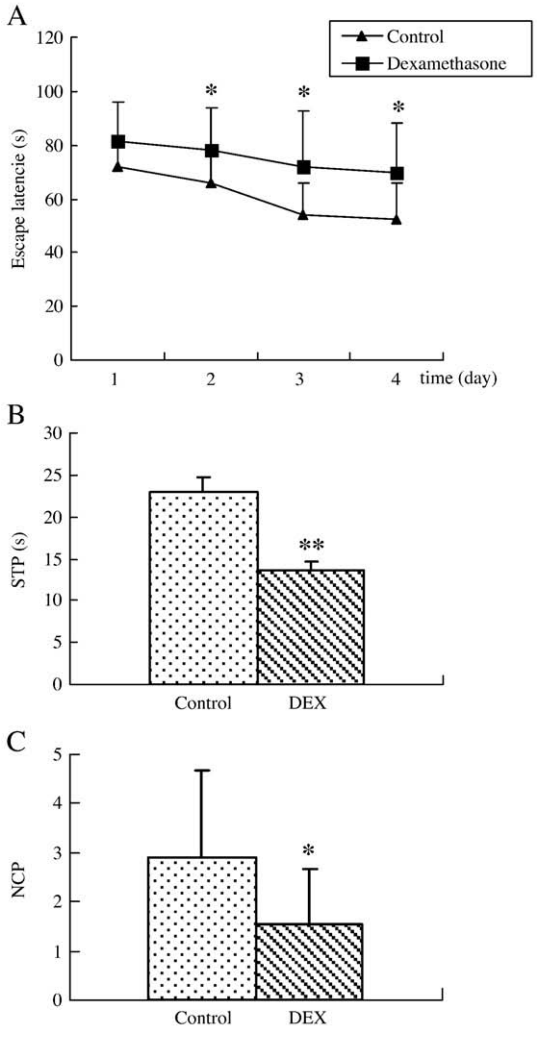

Fig. 2. Dexamethasone (DEX) treatment resulted in an impairment of memory and learning in 12-month old male mice. The mean escape latency represents the time for mice to find the platform that was placed in a fixed location in the pool. A: The latency presented is the sum of four trials per day per group in memory training experiment. B: Swimming time in the quadrant of platform (STP) in the probe trial in Morris water maze test. C: The average number of crossing the platform site (NCP). Dexamethasone group, $n=11$; Control group, $n=12$. *: $P<0.05$; **: $P<0.01$, Dexamethasone *versus* control group.

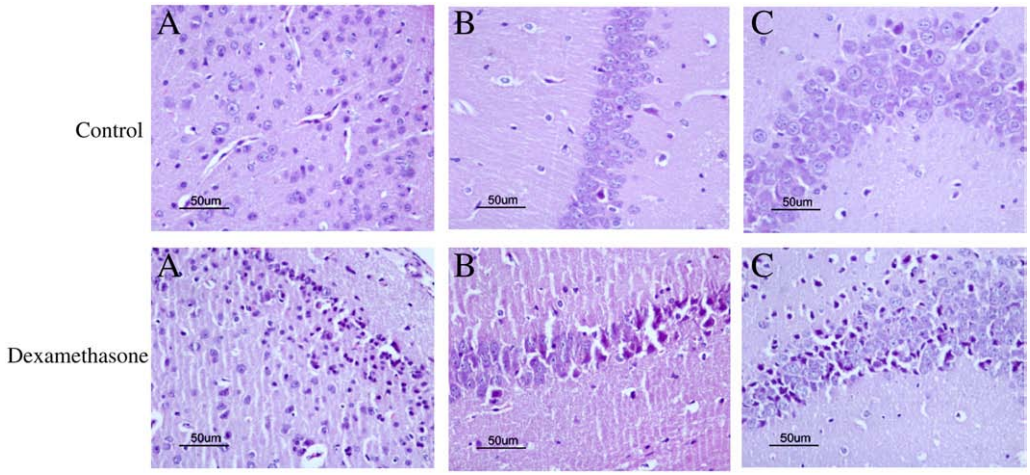

Fig. 3. Histopathological observation of the mouse brain hippocampus and cortex (HE stain, 400×). No remarkable neuronal abnormalities in the hippocampus and cortex of the control group were observed, while Dexamethasone treated group mice showed degeneration of neurons in the hippocampus and cortex and disorder of the array of neurons. (A) Cortex; (B) CA1; (C) CA3.

dexamethasone model mice have a significant increase in the number of apoptotic cells in both the hippocampus and the cortex. (Fig. 4).

3.3. Effect of dexamethasone exposure on the mRNA levels of amyloid precursor protein, α-secretase, β-secretase and caspase-3

RT-PCR was used to investigate whether dexamethasone exposure affected amyloid precursor protein processing in 12-month old male mice. Furthermore, we also monitored caspase-3 mRNA levels after dexamethasone treatment. We found that 21 days dexamethasone treatment significantly increased amyloid precursor protein (APP, 696, 751) mRNA (0.719 ± 0.080 *versus* 0.453 ± 0.081 and 0.513 ± 0.081 *versus* 0.298 ± 0.075 for the dexamethasone-treated mice and control group mice) as well as β-secretase mRNA levels (0.788 ± 0.082 *versus* 0.473 ± 0.075 for the dexamethasone-treated mice and control group mice), and significantly decreased the α-secretase mRNA level (0.494 ± 0.086 *versus* 0.757 ± 0.097 for the dexamethasone-treated mice and control group mice). Concurrently, we also found that the caspase-3 mRNA levels were significantly increased after dexamethasone treatment (0.774 ± 0.088 *versus* 0.547 ± 0.071 for the dexamethasone-treated mice and control group mice). (Fig. 5A and B).

3.4. Effect of dexamethasone exposure on amyloid precursor protein, cytochrome c, and caspase-3 immunoreactivity in hippocampus and cortex cells

We investigated the influence of dexamethasone on amyloid precursor protein, cytochrome *c* and caspase-3 immunoreactivity in the hippocampus and cortex cells in mice brains. There were few amyloid precursor protein immunoreactive neuronal cells in the hippocampus and cortex of control group mice. There were fewer positively stained cells and the staining intensity was decreased. In the hippocampal CA1, CA3 region and cortex of dexamethasone treated mice the number of amyloid precursor protein immunoreactive cells increased significantly in comparison with their controls (Fig. 6 APP).

The results of cytochrome *c* and caspase-3 immunochemical staining showed that the hippocampal CA1, CA3 region and cortex of control group mice contained fewer positively stained cytochrome *c* and caspase-3 immunoreactive cells. In the dexamethasone-treated mice, the number of cytochrome *c* and caspase-3 immunoreactive cells was more than that of control group mice (Fig. 6 cytochrome *c* and caspase-3). The mean optical density of amyloid precursor protein, cytochrome *c*

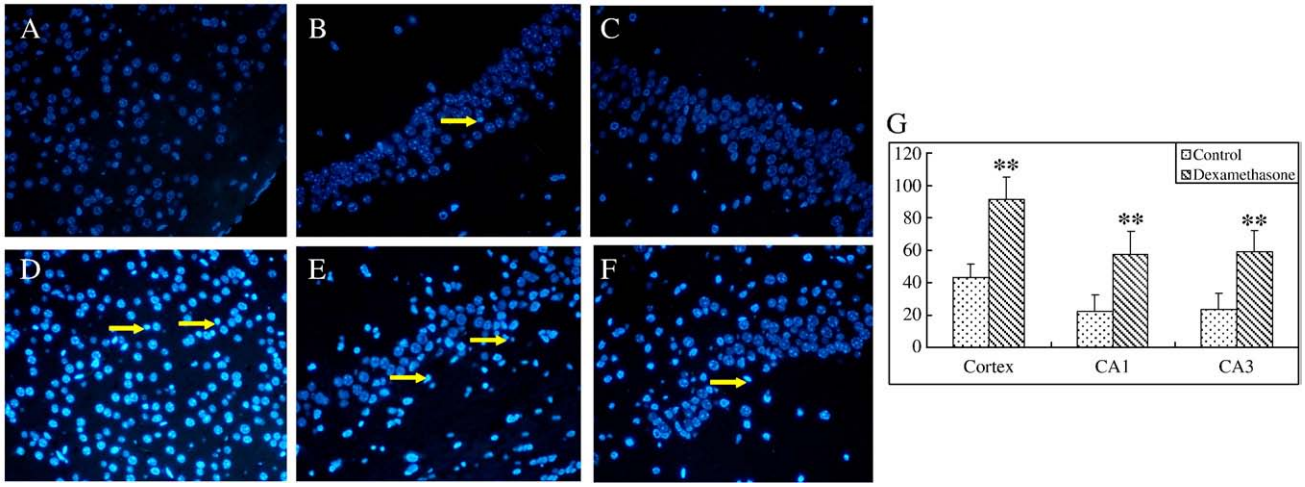

Fig. 4. Photomicrographs of hippocampal neurons and cortical cells staining with Hoechst 33258 after treatment with dexamethasone. Nuclear condensation was examined under a fluorescence microscope (Ex/Em: 352 nm/461 nm). Nuclear condensation and fragmentation were prominent 21 days after treatment with 5 mg/kg dexamethasone (D, E, F) when compared with control group (A, B, C). (A, D) Cortex; (B, E) CA1; (C, F) CA3. 400×. The average number of apoptotic cells in cortex, CA1 and CA3, which appear smaller than normal and in which the chromatin appears condensed, were counted ($n = 4$). Compared with control group, dexamethasone treatment significantly increased the average number of apoptotic cells (G, $P < 0.01$).

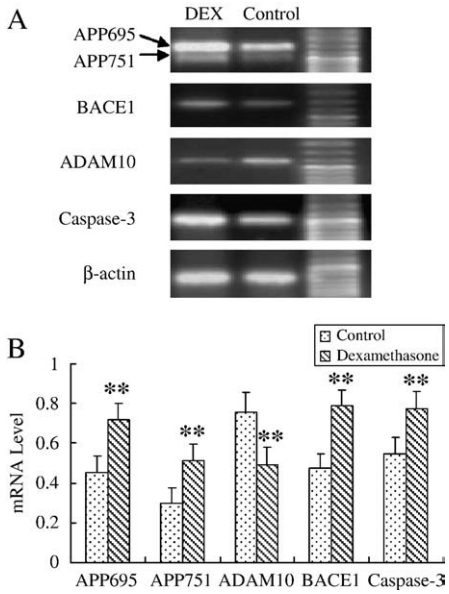

Fig. 5. Effect of dexamethasone (DEX) exposure on mRNA levels of amyloid precursor protein (APP, 696, 751), α-secretase (ADM10), β-secretase (BACE1) and caspase-3 in the brain of mice. A: A representative experiment, showing the mRNA expression levels of amyloid precursor protein (696, 751), α-secretase, β-secretase and caspase-3 in dexamethasone-treated and control mice. B: Quantitative mRNA expression levels of amyloid precursor protein (696, 751), α-secretase, β-secretase and caspase-3, normalized to β-actin. $n=5$ in all groups. **: $P<0.01$ *versus* control.

and caspase-3 positive neurons was measured using the Image-Pro Plus 6.0 analysis system in each section (Fig. 6D, E, F).

3.5. Effect of dexamethasone exposure on the activity of caspase-3 and caspase-9

We next investigated whether dexamethasone administration would increase the activity of caspase-9 and caspase-3. Our results revealed a marked increase in both caspase-9 and caspase-3 in the dexamethasone treated group (18.83 ± 4.99 *versus* 12.12 ± 3.44 and 13.85 ± 3.09 *versus* 8.96 ± 3.44 for dexamethasone treated group and control group respectively). (Fig. 7).

4. Discussion

Stress is an unavoidable condition of the human experience and includes both major life events and the problems associated with daily life (McEwen, 2002). Chronic psychosocial stressors trigger increases in the levels of glucocorticoid stress hormones that, in turn, have deleterious effects on the structure and function of CNS structures, especially the hippocampus (Hibberd et al., 2000; Csernansky et al., 2006). The hippocampus is a target for glucocorticoid stress hormones, which promote hippocampal aging (Sapolsky, 1994; Reagan and McEwen, 1997). Moreover, the hippocampus is a recognized vulnerable region in Alzheimer's disease. Hippocampal atrophy induced by corticosteroids may play an important role in the pathogenesis of Alzheimer's disease (Hoschl and Hajek, 2001). It has been proposed that elevated levels of glucocorticoids may be neurotoxic (Kleen et al., 2006; Conrad et al., 2007) and can even cause hippocampal cell death. Increasing evidence indicates that escalations in HPA axis activity have also been directly associated with Alzheimer's disease. Increases in plasma cortisol levels have been reported in individuals with probable Alzheimer's disease and have been generally interpreted as evidence that the disease process (i.e., Alzheimer's-induced hippocampal degeneration) leads to disinhibition of the HPA axis (Rasmussen et al., 2002; Csernansky et al., 2006). In line with this hypothesis, correlations have been reported between increases in HPA axis activity and dementia severity (Miller et al., 1998) or hippocampal volume loss in individuals with probable Alzheimer's disease. The present study was therefore designed to explore the mechanism by which high level glucocorticoids could accelerate the Alzheimer's disease process.

Aging is an important risk factor of Alzheimer's disease and often associate with altered function of the HPA axis. Base levels of glucocorticoids are known to be incessantly elevated in aging people (Lupien et al., 1998; Csernansky et al., 2006). Additionally, it has been reported that the glucocorticoid responsive element binding activity after dexamethasone administration was decreased by 30% in 3 month old male mice. Glucocorticoid responsive element binding activity was increased in the group of middle-aged (12 months) and aged (24 months) rats after dexamethasone treatment, indicating that aged animals are more susceptible to this hormone in terms of impaired glucocorticoid-negative feedback (Terzic et al., 2003). Dexamethasone has no known significant effect on learning and memory impairments in young mice (Yao et al., 2007). So in our present study, 12-month old mice were used to investigate the link between high level glucocorticoids and the genesis of Alzheimer's disease.

We demonstrated that increased glucocorticoid levels may increase amyloid β-protein and neuronal apoptosis in hippocampus and cortex which could have an impact on the Alzheimer's disease genesis. To our knowledge, this is the first reported data indicating that elevated levels of stress hormones might promote Alzheimer's disease genesis in 12-month nontransgenic mice. The current study demonstrates that twenty one days after exposure to dexamethasone induced impairment of learning and memory, accompanied by severe histological damage and neuronal apoptosis in the CA1, CA3 regions of the hippocampus and cortex neurons in 12-month old mice. The mRNA levels of the amyloid precursor protein, β-secretase and caspase-3 are also selectively increased after dexamethasone administration. The amyloid precursor protein, caspase-3 and cytochrome *c* in the cortex and CA1, CA3 regions of the hippocampus are significantly increased. These findings suggests that long-term glucocorticoid elevation may mediate the exacerbation of hippocampus and cortex damage, which may have an important role in Alzheimer's disease genesis.

Amyloid plaques involving deposits of β-amyloid (Aβ), are defining lesions in the Alzheimer's disease brain (Selkoe, 2000). Aβ is a proteolytic product of the amyloid precursor protein and is generated through sequential cleavages by β- and γ-secretase enzymes. β-Secretase was identified as the transmembrane aspartic protease β-site APP cleaving enzyme (BACE) (Haniu et al., 2000; Zhao et al., 2007). Since β-Secretase initiates formation of Aβ, factors that elevate β-Secretase levels may promote Alzheimer's disease. Recent studies demonstrate that β-Secretase levels and activity are increased in postmortem Alzheimer's disease brains (Fukumoto et al., 2002; Harada et al., 2006), suggesting a important role in Alzheimer's disease. Another enzyme, α-secretase, cleaves between residues 16 and 17, precluding Aβ formation. It has been reported that administering stress-level glucocorticoids (5 mg/kg) to aged 3×Tg-AD mice increased the insoluble Aβ load. Additionally, elevated glucocorticoids levels increase Aβ production by augmenting steady state levels of amyloid precursor protein and β-Secretase in just 7 day (Green et al., 2006). Elevated glucocorticoid levels were associated with decreased degradation of Aβ in aged macaques, through a downregulation of insulin-degrading enzyme (Kulstad et al., 2005). In our previous study, we revealed that dexamethasone could strongly increase the vulnerability of the hippocampal neuron to amyloid β-protein in vitro (Yao et al., 2007). Our present results showed that 21 days after dexamethasone administration significantly increased amyloid precursor protein mRNA and β-secretase mRNA levels, and decreased α-secretase mRNA level in 12-month old nontransgenic mice. We further investigated

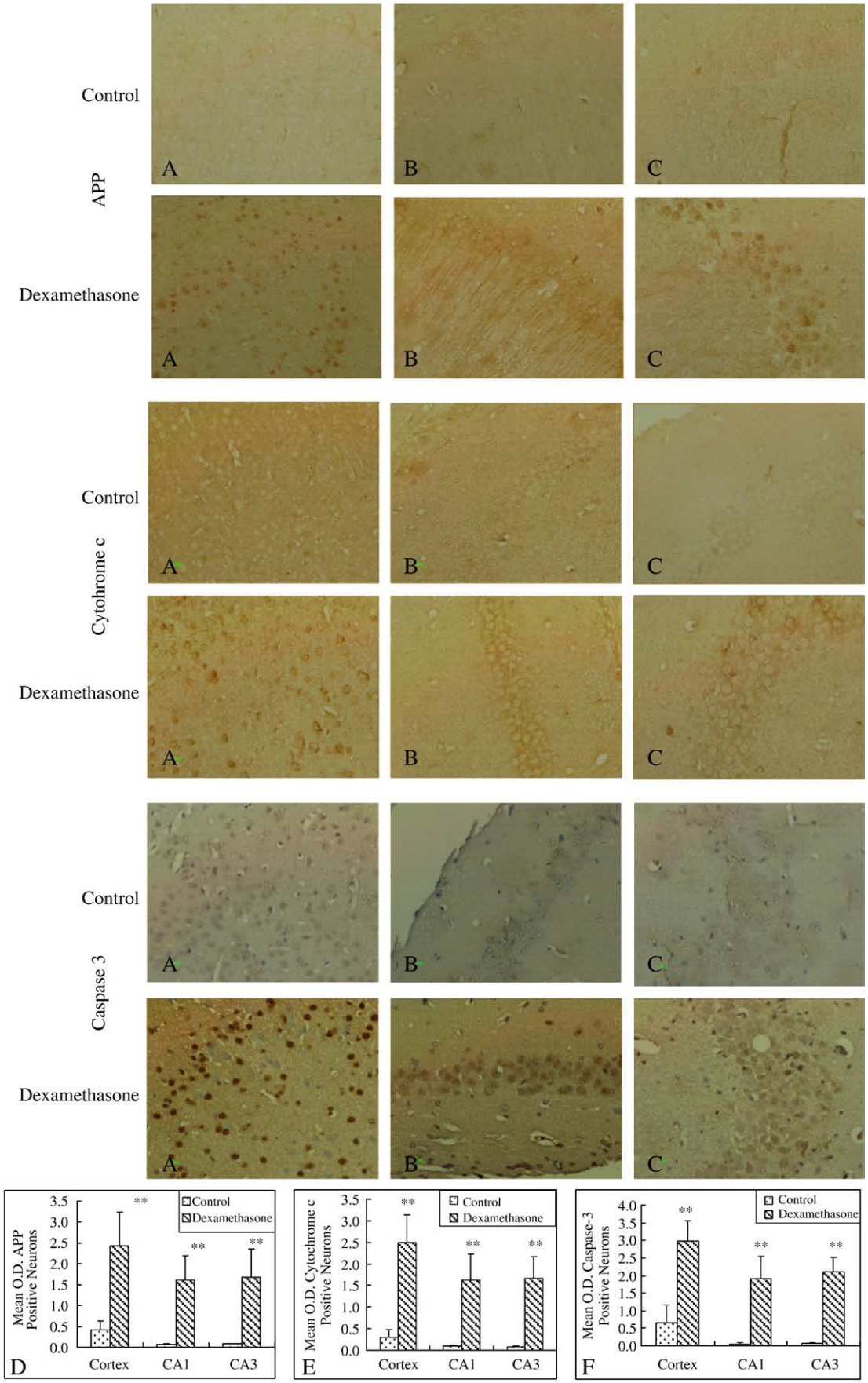

Fig. 6. Effect of dexamethasone exposure on amyloid precursor protein (APP), cytochrome *c* and caspase-3 immunoreactive cells in the cortex and hippocampal CA1, CA3 region of mice (400×, $n=4$). A, cortex; B, CA1; C, CA3. The immunoreactive cells are abundant and brown-stained. The mean optical density was analyzed in three fields of the same magnification with Image-Pro Plus 6.0. **: $P<0.01$ *versus* the control. D, APP; E, cytochrome *c*; F, caspase-3.

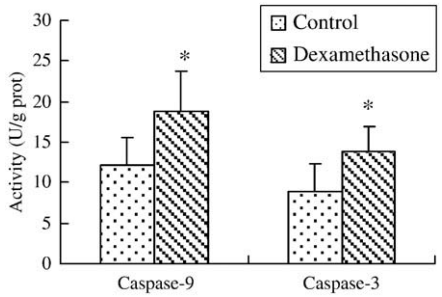

Fig. 7. The activity of caspase-9 and caspase-3 was measured by cleaving acetyl-Asp-Glu-Val-Asp P-nitroanilide (Ac-DEVD-PNA) and acetyl-Leu-Glu-His-Asp P-nitroanilide (Ac-LEHD-PNA), respectively. Compared with control group, dexamethasone exposure significantly increased the activity of caspase-9 and caspase-3 in the brain of mice. ($n=6$, *: $P<0.05$ *versus* control).

the amyloid precursor protein expression by immunohistochemistry. Compared with control groups, 21 days after dexamethasone significantly increased amyloid precursor protein expression in the cortex as well as in the hippocampus. Increases in amyloid precursor protein and β-secretase lead to increased processing of amyloid precursor protein to C99 by β-secretase, which is consequently cleaved by the γ-secretase to release Aβ. These results show that stress level glucocorticoids may increase formation of Aβ and promote Alzheimer's disease genesis.

Neuronal death is another important characteristic of Alzheimer's disease. So we further investigated the effects of stress level glucocorticoid on neuronal apoptosis in the hippocampus and cortex. Neuronal cell death is the final pathological consequence of many central nervous system diseases, including Alzheimer's disease. Apoptosis is a subtype of cell death that is involved in diverse physiological and pathological processes, including Alzheimer's disease (Yang et al., 2008). In the present study, histological examination showed that dexamethasone treatment induced degeneration of neurons in the hippocampus and cortex. The neuronal cell body became short and deeply stained with dye. Nuclear staining with Hoechst 33258 showed nuclear condensation and fragmentation present in dead cells. These results indicate that 21 days of dexamethasone treatment can induce neurons apoptosis.

Various stimuli that induce apoptosis lead to the release of cytochrome *c* from mitochondria, which plays a key role in a common activation pathway of caspases (Mancini et al., 1998; Mulugeta et al., 2007). Cytosolic cytochrome *c* can bind Apaf-1 and subsequently trigger the sequential activation of caspase-9 and caspase-3 (Mancini et al., 1998). Activation of caspase-3 has been recently shown to be a key step in the execution process of apoptosis, and its inhibition can block apoptotic cell death. Activated caspases cleave a variety of target proteins, thereby disabling important cellular processes and leading to the breakdown of structural components within the cell, such as lamin, and eventually causing cell death (Thornberry et al., 1997). We investigated the influence of dexamethasone on immunoreactivities of cytochrome *c* and caspase-3. In our study, the immunoreactivities of cytochrome *c* and caspase3 in the hippocampus and cortex were found to be extensively elevated in the dexamethasone treated group, and this group was also found to have higher caspase-3 mRNA levels *versus* the control group. We further investigated the activities of caspase-3 and caspase-9 in the brain homogenate. The results showed that the activities of caspase-3 and caspase-9 were significantly increased in the dexamethasone treated group. Thus, these findings are consistent with our immunoreactivity data and suggest that the activities of caspase-3 and caspase-9 are significantly activated by glucocorticoid treatment.

Overall, this study demonstrates that 21 days of increased exposure to stress level glucocorticoids accelerates cognitive impairments and neurodegeneration. Our findings provide support for the hypothesis that elevated glucocorticiods increase formation of Aβ and promote neuronal apoptosis which play a significant causal role in the development of Alzheimer's disease.

Acknowledgments

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