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The interaction between acute oligomer $A\beta_{1-40}$ and stress severely impaired spatial learning and memory

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ABSTRACT

In this study, we investigated whether stress can enhance the toxicity of oligomer $A\beta_{1-40}$ in the mouse brain. Stress was applied to the animals, consisting of a 2-day inescapable foot shock followed by 3-weekly situation reminders (SRs). We found that stress significantly affected not only the amygdala-dependent (anxiety) but also the hippocampal-dependent (spatial learning and memory) behaviors through the oxidative damage caused in these two regions. However, oligomer $A\beta_{1-40}$ treatment alone did not induce behavioral impairment. In addition, combined oligomer $A\beta_{1-40}$ and stress treatment increased the glucocorticoid receptor (GR)/mineralocorticoid receptor (MR) ratio and the expression of corticotrophin releasing factor 1 (CRF-1) receptor in the hippocampus. Changes in the components of the hypothalamic–pituitary–adrenal (HPA) axis, such as the GR/MR ratio and CRF-1 level, were observed, accompanied by increasing Aβ accumulation, oxidative stress, nuclear transcription factor (NF-κB) hypoactivity, and apoptotic signaling in the hippocampus, and decreasing calbindin D28K and NMDA receptor 2A/2B (NR2A/2B) in the hippocampus, along with alteration of the cholinergic neurons (ChAT) in the medium septum/diagnoid band (MS/DB), noradrenergic neurons (TH) in the locus coeruleus (LC), and serotonergic neurons (5-HT) in the Raphe nucleus. Therefore, apoptosis and synaptic dysfunction in the hippocampus severely induced the impairment of spatial learning and memory. These results suggest that stress may play an important role in the early stages of Alzheimer's disease (AD), and an antioxidant strategy might be a potential therapeutic approach for stress-mediated disorders.

1. Introduction

Stress has been suggested to be one of the environmental factors that can influence the pathogenesis of Alzheimer's disease (AD) (Hasegawa, 2007; Wilson et al., 2006). Several reports have identified increased plasma corticosterone levels in patients with dementia of an Alzheimer's type (Dong et al., 2008; Peskind, Wilkinson, Petrie, Schellenberg, & Raskind, 2001; Weiner, Vobach, Olsson, Svetlik, & Risser, 1997). However, increased glucocorticoids do not absolutely lead to AD, because a number of disorders result in increased cortisol, such as Cushing's disease, and yet no evidence has been published demonstrating a link between these patients and AD (Swaab, Bao, & Lucassen, 2005). Therefore, whether an increased glucocorticoid level is a risk factor for AD needs further investigation. One recent study suggested that stress works as a co-factor to induce vulnerability in hippocampal neurons (McDonald, Craig, & Hong, 2008). Other evidences further suggest that stress increases $A\beta_{1-42}$ deposition (Dong et al., 2008; Kang, Cirrito, Dong, Csernansky, & Holtzman, 2007). Several reports have shown that, under general conditions, a higher proportion of Aβ is produced in the form of $A\beta_{1-40}$ than $A\beta_{1-42}$, and an increased $A\beta_{1-40}/A\beta_{1-42}$ ratio in cerebrospinal fluid (CSF) has been identified during the early stages of AD (Hu, Smith, Walsh, & Rowan, 2008; Kanai et al., 1998). However, whether stress also regulates $A\beta_{1-40}$ level and toxicity has not yet been elucidated in present.

The hippocampus, which is involved in central brain functions such as cognition, learning, and memory, is very sensitive to various neurological insults, such as behavioral stressors (Morra et al., 2008) and Aβ deposition in AD (Behl, 1998). There are two types of adrenal steroid receptors in the hippocampus, MR and GR. An early study showed that the balance of the GR/MR ratio regulates the homeostasis of calcium, NR2A/NR2B, NF-κB, and the considerable input mediated by biogenic amines such as acetylcholine, noradrenalin, and serotonin into hippocampal neurons (De Kloet, Vreugdenhil, Oitzl, & Joels, 1998). In addition, recent studies have also suggested that neurodegenerative and psychiatric disorders induce brain oxidative stress (Matos, Augusto, Oliveira, & Agostinho, 2008; Rammal, Bouayed, Younos, & Soulimani, 2008). Therefore, we attempted to examine the GR/MR ratio, calcium binding

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protein, $A\beta_{1-40}$ accumulation, oxidative stress, nuclear transcription factor NF-κB, NR2A/2B, and related neurotransmitters of the hippocampus in order to evaluate the effects of acute oligomer $A\beta_{1-40}$ treatment after chronic stress.

In this study, we demonstrated that mice under an inescapable electric foot shocks followed by 3-weekly situational reminders (SRs), exhibited increased levels of plasma corticosterone, $A\beta_{1-40}$ deposition in the hippocampus, and oxidative stress in the hippocampus and amygdale, as well as avoidance of the shock box, and a decreased calcium binding signal in the hippocampus and decreased related neurotransmitter projection into the hippocampus. In addition, we also found that stress affected not only amygdale-dependent but also hippocampal-dependent behaviors through oxidative damage in the hippocampus and amygdale. Therefore, the stress manipulation is reliable and provides a long-lasting stress animal model. Notably, the interactive effects of oligomer $A\beta_{1-40}$ and stress altered the components of the HPA axis, such as GR, MR, and CRF-1 in the hippocampus. Accompanying effects such as intracellular calcium dysregulation, $A\beta_{1-40}$ accumulation, oxidative stress enhancement, NF-κB hypoactivity, and apoptotic signal expression in the hippocampus were also observed in these animals. In addition to apoptosis, synaptic dysfunction may also be modified through the decreasing of NR2A/2B in the hippocampus along with the alteration of neurotransmitter systems projection into the hippocampus. Therefore, the interaction between oligomer $A\beta_{1-40}$ and stress-induced apoptosis and synaptic dysfunction in the hippocampus subsequently significantly resulted in the severe impairment of spatial learning and memory.

2. Materials and methods

2.1. Subjects

A total of 120 male C57BL/6J mice (6–8 weeks old) were purchased from the National Breeding Center for Laboratory Animals (Taipei, Taiwan). The mice were randomly divided into four groups, each containing 30 animals: (i) non-stress/CA1 administration of vehicle; (ii) non-stress/CA1 administration of oligomer $A\beta_{1-40}$; (iii) stress/CA1 administration of vehicle; and (iv) stress/CA1 administration of oligomer $A\beta_{1-40}$. The experiments were performed during the light phase between 9 AM and 5 PM. All experimental procedures involving animals were performed according to the guidelines established by the Institutional Animal Care and Use Committee of National Taiwan Normal University, Taipei, Taiwan. The mice were housed at 20–25 °C and 60% relative humidity under a 12-h light/dark cycle (light turned on at 7 AM), and food and water were made available *ad libitum*.

2.2. Experiment timeline and establishment of the stress model

The stress model was established as previously described (Li, Murakami, Wang, Maeda, & Matsumoto, 2006). As depicted in Fig. 1, the male mice ($n = 120$) were acclimatized in their home cages on days 1–5 and were handled once a day during this 5-day habituation period. Handling consisted of holding the animal in gloved hands for 2 min. After adaptation, mice received either shocks (a total of 15 intermittent inescapable electric foot shocks of 0.8 mA intensity, 10 s interval, and 10 s duration; $n = 60$) or no shocks ($n = 60$) on days 6 and 7, respectively. Prior to shock treatment, the mice were allowed a 10-s adaptation period in the shock box (the dark compartment of the light–dark transition test box). The non-stress groups received the same treatment, but with the shock mechanism inactivated. The mice were re-exposed to the same chamber but without foot shock treatment on days 8, 13, and 20 (SR 1, 2 and 3, respectively). After each SR, a blood sample was collected from each mouse and analyzed in order to measure the corticosterone level. On day 21, after SR3, 60 mice in each group were randomly assigned to receive an injection of oligomer $A\beta_{1-40}$ ($n = 30$) or vehicle ($n = 30$) in the CA1 subregion of the hippocampus. After a 5-day recovery period, mouse behavior was evaluated by locomotor, light–dark transition, and water maze tasks on days 27, 28, and 29–35, respectively. Twenty-four hours after SR4 (conducted on day 36), the mice were sacrificed for Western blot and immunohistochemistry analyses.

2.3. Analysis of corticosterone level

Animals ($n = 3$ for each group) were sacrificed immediately after each SR and blood samples collected into heparin-treated tubes, which were then placed on wet ice. After centrifugation (2000*g*), blood plasma was isolated and stored at −20 °C until assaying. All experiments were performed in the morning (9–11 AM) to minimize the possibility of interference from circadian alteration in the corticosterone level. A corticosterone competitive ELISA Kit (AssayPro system, GENTAUR) was used to measure the corticosterone level as per the manufacturer's protocol.

2.4. Preparation of oligomer

$A\beta_{1-40}$ was dissolved in 1 ml of double-distilled water to a concentration of 0.23 mM and incubated at 37 °C for 7 days to allow oligomer formation, as previously described (Hoshi et al., 2003; Huang, Liang, Chen, Chen, & Hsieh-Li, 2007).

2.5. Animal surgery

To avoid the side effects caused by interaction between brain operation and shock, amyloid application was performed after SR3 (day 21), which consisted of an acute injection of Aβ into the CA1 of the mouse brain. Mice were anesthetized with avertin (0.016 ml/g, Sigma, MO, USA) and placed in a stereotaxic instrument (DKI-900, David Kopf Instruments, CA, USA). An incision was made in the scalp and a hole drilled into the skull over the injection site, and a 30-gauge-needle was then lowered into the dorsal hippocampus. The coordinates for the anterior–posterior (from bregma), medial–lateral (from midline), and dorsal–ventral (from surface of the skull) axes were −2.3, ±2.5, and −1.5 mm, respectively. Bilateral intrahippocampal infusion was administered via a 10.0-μl Hamilton microsyringe with a 30-gauge needle fitted to the arm of the stereotaxic instrument. About 0.6 μl of oligomer $A\beta_{1-40}$ or vehicle was slowly infused at a rate of 0.2 μl/min. The oligomer conformation of $A\beta_{1-40}$ was confirmed by Western blotting

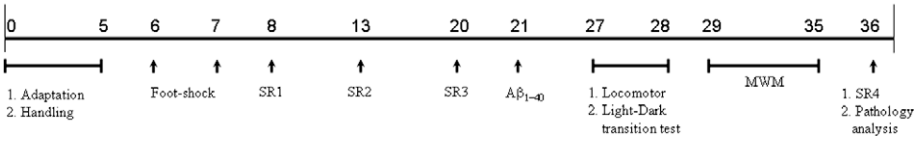

Fig. 1. The experimental timeline of this study. Mice were exposed to inescapable electric foot shocks (0.8 mA, 10 s) followed by 3-weekly SRs. After the training sessions (days 6–20), mice were submitted to a testing session composed of three different behavioral tests, motor activity test, light–dark transition test, and the Morris water maze.

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(data not shown). After an additional 5 min to ensure adequate diffusion, the needle was slowly retracted from the animal.

2.6. Locomotor

On day 27, the motor activity of the animals was recorded for 10 min. Motor tracking was performed using a video camera installed on the ceiling, which fed directly into a personal computer running a Noldus EthoVision video tracking system (Spink, Tegelenbosch, Buma, & Noldus, 2001) in subtraction mode. Prior to the start of recording, the animals were placed in Plexiglas cages ($30 \times 30 \times 30$ cm; one animal per testing cage) to which they were not habituated. The motor activity in the first and second 5-min periods was then recorded, which was the same duration as the measurement period used for the light–dark transition test.

2.7. Light–dark transition test

The apparatus, modified from a previous study (Costall, Jones, Kelly, Naylor, & Tomkins, 1989), consisted of a Plexiglas chamber subdivided into two compartments, a dark compartment ($300 \times 300 \times 350$ mm high, of the same scale, color, and odor as the chamber in which the foot shocks were delivered), and a light compartment ($450 \times 300 \times 350$ mm high, totally different from the dark compartment) illuminated by a white bulb (60 W), set 40 cm above the floor. The compartments were connected by a small divider (50×50 mm). On day 28, each animal was placed into the light compartment facing the wall opposite the divider. The latency of the first entry into the dark compartment, the time spent in the dark compartment, and the numbers of transitions were assessed for a 5-min period.

2.8. Morris water maze (MWM)

Spatial memory was evaluated using a conventional Morris water maze (MWM), a device commonly used for studying cognitive deficits in APP transgenic mice (Janus & Westaway, 2001). The water maze apparatus consisted of a circular pool (1.2 m diameter and 0.47 m high) made of white plastic; the pool was filled to a depth of 40 cm with water (24–25 °C) and made opaque by the addition of nontoxic white paint. During conventional MWM training, an escape platform (10 cm in diameter) made of white plastic, with a grooved surface for better grip, was submerged 0.5 cm underneath the water level. Cues of various types provided distal landmarks in the testing area of the room. The swim path of the mouse during each trial was recorded by a video camera suspended 2.5 m above the center of the pool and connected to a video tracking system. On the day prior to spatial training, all mice underwent pre-training in order to assess their swimming ability and to acclimatize them to the pool. Each mouse was first placed on a visible platform located at the center of the pool and allowed to stay there for 20 s. In the following three 60-s trials, the mouse was released into the water facing the wall of the pool from semi-randomly chosen cardinal compass points. If the mouse failed to swim to the platform or stay on it for 20 s, it would be placed on the platform by an experimenter. The mice were given a 4-day training session consisting of four 60-s training trials (inter-trial interval: 20–30 min) per day. The hidden platform was always placed at the same location of the pool (Northeast quadrant) throughout the training period. During each trial, from quasi-randomly chosen cardinal compass points, the mouse was released into the water facing the pool wall. After climbing onto the platform, the mouse was allowed to rest on it for 20 s. After the last training trial, all mice were given three testing trials to assess the time taken to climb onto the hidden platform. 24 h after the last testing trial, all mice were given three probe trials to evaluate their spatial memory regarding the platform.

2.9. Immunohistochemistry

Immediately after the water maze test, nine mice per group were anesthetized (avertin, 0.016 ml/g) and transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). Mouse brains were removed and post-fixed with 4% paraformaldehyde overnight and then placed in 30% sucrose in PBS for 2 days, followed by continuous serial cryostat sectioning at 30 μm. Rabbit anti-$A\beta_{1-40}$ polyclonal antibody (Chemicon, CA, 1:200 dilution) staining was used to detect the presence of Aβ. Specific antibodies were used to assess oxidative stress (MnSOD, Upstate, CA, 1:200 dilution), apoptosis (Caspase3, Chemicon, 1:40 dilution), and neurotransmitters by rabbit anti-ChAT polyclonal antibody (choline acetyltransferase, cholinergic specific, 1:500; Chemicon), rabbit anti-TH polyclonal antibody (tyrosine hydroxylase, noradrenergic-specific, 1:1000; Chemicon), and rat anti-serotonin monoclonal antibody (5-HT, serotonergic-specific, 1:100; Chemicon).

Free-floating sections were immunostained for immunohistochemical analysis. In brief, sections were washed in PBS three times (10 min/wash). Nonspecific epitopes were then blocked by incubation in 5% normal goat serum or normal rat serum and 0.1% Triton X-100 in PBS for 1 h. Sections were incubated in primary antibodies overnight at room temperature, washed with PBS, and incubated in the secondary antibodies (1:200 dilution in blocking solution, Vector Laboratories, CA, USA) for 1 h, then in an avidin–biotin complex for 1 h at room temperature. The reaction was developed using a DAB-Kit (diaminobenzidine) from Vector. All sections were mounted on coated slides and cover-slipped for light microscopy. The images obtained were loaded in a research-based digital image analysis software (Image Pro Plus, Media Cybernetics, MD, USA) and the DAB pixel counts measured by setting the threshold to the same value for each section. Pixel counts were taken as the average from three adjacent sections per animal, and the data are presented in Table 1.

2.10. Western blot analysis

Western blot analysis was conducted on proteins extracted from the hippocampus with antibodies for GR, MR, CRF-1, NF-κB, NR2A/2B, and β-actin. The antibodies used were rabbit polyclonal GR (1:200; Santa Cruz), rabbit polyclonal MR (1:100; Santa Cruz), rabbit polyclonal CRF-1 (1:100; Santa Cruz), rabbit polyclonal NF-κB (1:2000, Chemicon), rabbit polyclonal NR2A/2B (1:100; Chemicon), and mouse monoclonal β-actin (1:2000; Chemicon); the secondary antibodies used were anti-rabbit IgG HRP-linked antibody (1:10,000; Cell Signaling, USA) for GR, MR, CRF-1, NF-κB, and NR2A/2B, and anti-mouse IgG HRP-linked antibody (1:10,000; Cell signaling) for β-actin. The specific antibody–antigen complex was detected by an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The intensity of Western blots was quantified using the LAS-3000 imaging system (Fuji, Japan), and was expressed as the ratio relative to β-actin protein.

2.11. Data analysis

Data were analyzed by two-way analysis of variance (ANOVA) by group (stress and non-stress) and CA1 (vehicle and oligomer $A\beta_{1-40}$) administration, followed by Student's *post hoc t*-testing in order to compare the effects of all treatments. In addition, the light–dark transition test and Western blotting results were analyzed by nonparametric multiple independent samples testing.

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Table 1
The quantification of the immunohistochemical analyses in the different treatment mice.

	Non-stress-vehicle	Non-stress-Ap	Stress-vehicle	Stress-Ap
AP_{IJM} (CA1)	24.0 ± 3.61	242.0 ± 4.93[b,*]	122.0 ± 2.65[a,*]	552.7 ± 6.01[c,*]
MnSOD(CA1)	23.0 ± 4.04	115.0 ± 3.79[b,*]	59.3 ± 3.18[a,*]	190.0 ± 10.82[c,***]
MnSOD (Amy)	235.0 ± 3.46	259.3 ± 7.69	748.3 ± 29.87[a,***]	797.0 ± 7.93[a,*]
Caspase 3 (CA1)	0.0 ± 0.0	5.3 ± 0.45	0.0 ± 0.0	33.3 ± 4.03[c,***]
Calbindin (CA1)	175.3 ± 3.48	133.7 ± 7.22[b,*]	105.0 ± 4.04[a,*]	53.3 ± 5.04[c,***]
ChAT(MS)	193.4 ± 4.05	125.0 ± 5.03[b,*]	80.0 ± 4.93[a,*]	64.7 ± 5.46[c,***]
ChAT(DB)	164.7 ± 6.57	87.7 ± 3.28[b,*]	77.2 ± 4.09[a,*]	16.3 ± 2.60[c,***]
TH (LC)	1150.7 ± 48.35	871.7 ± 25.33[b,*]	778.3 ± 9.56[a,*]	543.7 ± 17.65[c,***]
5-HT (Raphe nucleus)	37.4 ± 4.61	18.7 ± 1.44[b,*]	22.2 ± 1.49[a,*]	0 ± 0[c,***]

Each value represent the mean ± SEM ($n = 3$ for each group).
[a] Indicated as non-stress versus stress.
[b] Indicated as Ap versus vehicle.
[c] Indicated as interaction between stress and Ap.
[*] $p < 0.05$.
[**] $p < 0.01$.
[***] $p < 0.001$.

Results are expressed as mean ± SEM. Differences were considered statistically significant if $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1. Stress enhanced the plasma corticosterone level of treated animals

The stress resulting from the shock treatment followed SRs significantly enhanced the plasma corticosterone concentration in the mice. As shown in Fig. 2, the plasma corticosterone levels were greatly increased in the stressed mice but not in the non-stressed mice ($F(1, 35) = 182.0578$, $p < 0.001$). The corticosterone levels were not significantly different between the SR ($F(3, 35) = 2.4206$, $p > 0.05$) and stress × SR interaction groups ($F(3, 35) = 0.4116$, $p > 0.05$). Furthermore, the plasma corticosterone concentration in the non-stressed mice remained at the basal morning level throughout the experiment.

3.2. Avoidance into the dark compartment increased with aversive experience in the stressed mice

Animals that had been previously exposed to stress exhibited an increase in the latency of escape from the light compartment to the dark compartment ($p < 0.05$; Fig. 3A), a decrease in the time spent in the dark compartment ($p < 0.05$; Fig. 3B), and a decrease in the number of transitions during the testing period ($p < 0.05$; Fig. 3C). These results showed that the stressed mice learned to avoid the aversive-like compartment (the dark compartment), indicating that they exhibited a fear response to the context associated with traumatic events.

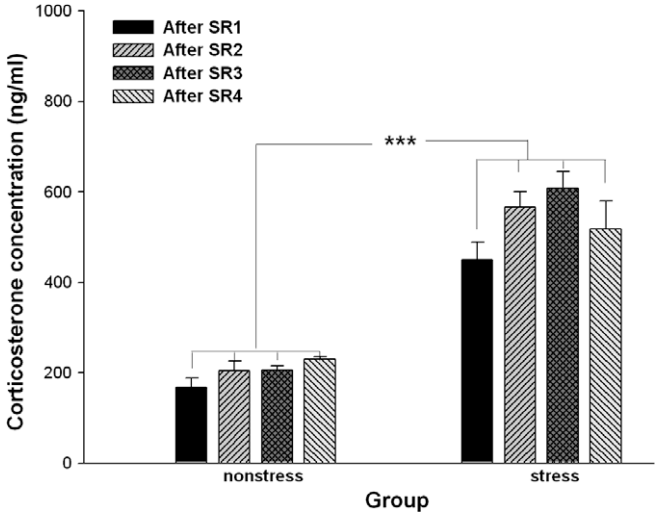

Fig. 2. Plasma corticosterone levels in the stressed and non-stressed mice. Regardless of SR, plasma corticosterone levels were significantly higher in the stressed mice than in the non-stressed mice after 24 h of SR.

3.3. Motor activity was not affected in stressed mice, regardless of oligomer $A\beta_{1-40}$ or vehicle treatment

The effects of oligomer $A\beta_{1-40}$ and vehicle on motor activity in mice with stress/non-stress were shown in Fig. 4. We used the first and second 5 min for analysis because that 5-min duration is the same as the measurement period used in the light–dark transition test. There were no significant alterations in the motor activity identified in the stressed ($F(1, 33) = 3.0382$, $p > 0.05$), oligomer-treated ($F(1, 33) = 2.2299$, $p > 0.05$), and combined stress × oligomer $A\beta_{1-40}$-treated mice ($F(1, 33) = 0.0829$, $p > 0.05$) during the first 5 min of testing (Fig. 4A). The motor activity of each group during the second 5 min of the experiment was even more constant than in the first 5 min (Fig. 4B). The statistical results for the different treatment groups are $F(1, 33) = 1.1478$ ($p > 0.05$) for the stressed group, $F(1, 33) = 3.8654$ ($p > 0.05$) for the oligomer-treated group, and $F(1, 33) = 0.0070$ ($p > 0.05$) for the combined treatment group. These results reveal that the motor activity was not affected in stressed mice.

3.4. Alteration of cholinergic, noradrenergic, and serotonergic immunoreactive neurons in stressed and oligomer $A\beta_{1-40}$-treated animals

We observed the alteration of cholinergic immunoreactive neurons (ChAT-ir), tyrosine hydroxylase immunoreactive neurons (TH-ir), and serotonergic immunreactive neurons (5-HT-ir) in the mouse brain after different treatments (Fig. 5). The quantitative results are summarized in Table 1. Stress significantly altered ChAT-ir in the medium septum/diagnoid band (MS/DB) ($F(1, 8) = 1516.467$, $p < 0.001$), TH-ir in the locus coeruleus (LC) ($F(1, 8) = 68.2954$, $p < 0.05$), and 5-HT-ir in the Raphe nucleus ($F(1, 8) = 77.8087$, $p < 0.05$). Oligomer treatment also significantly altered the levels of ChAT-ir in the MS/DB ($F(1, 8) = 706.3953$, $p < 0.05$), 5-HT-ir in the Raphe nucleus ($F(1, 8) = 51.7464$, $p < 0.05$), and TH-ir in the locus coeruleus ($F(1, 8) = 58.5573$, $p < 0.05$). The interactive effect of stress and oligomer $A\beta_{1-40}$ treatment severely decreased the ChAT-ir in the MS/DB ($F(1, 8) = 61.3969$, $p < 0.001$), TH in the LC ($F(1,8) = 5.6313$,

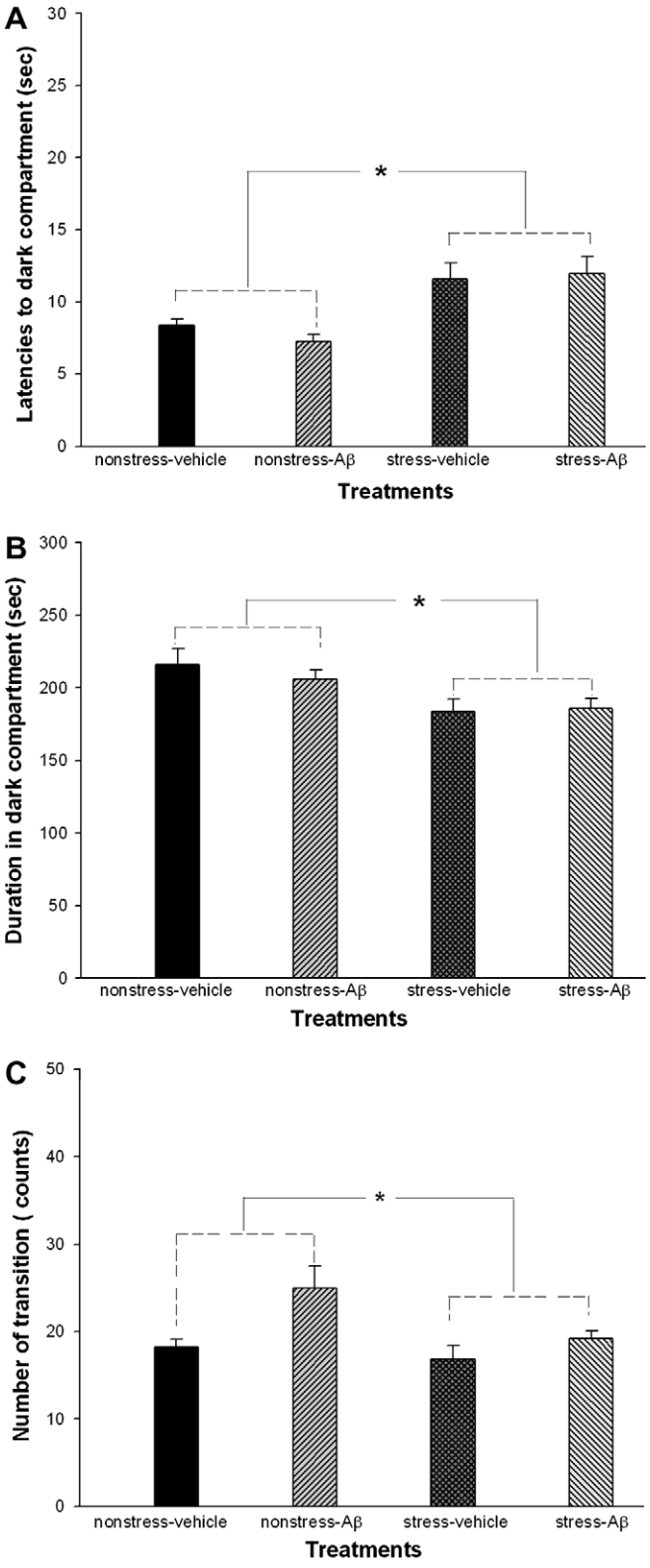

Fig. 3. Anxiety evaluation of stressed and non-stressed mice after acute CA1 administration with oligomer $A\beta_{1-40}$ and vehicle. After treatment, the mice underwent the light–dark transition test for 5 min on day 28. The latency of the first entry into the dark compartment (A), time spent in the dark compartment (B), and number of transitions (C) of the animals were recorded. These results show that the stressed mice exhibited elevated anxiety levels and specific avoidance of the context associated with stress, regardless of oligomer $A\beta_{1-40}$ or vehicle treatment.

$p < 0.001$), and 5-HT-ir in the Raphe nucleus ($F(1, 8) = 5.609$, $p < 0.001$).

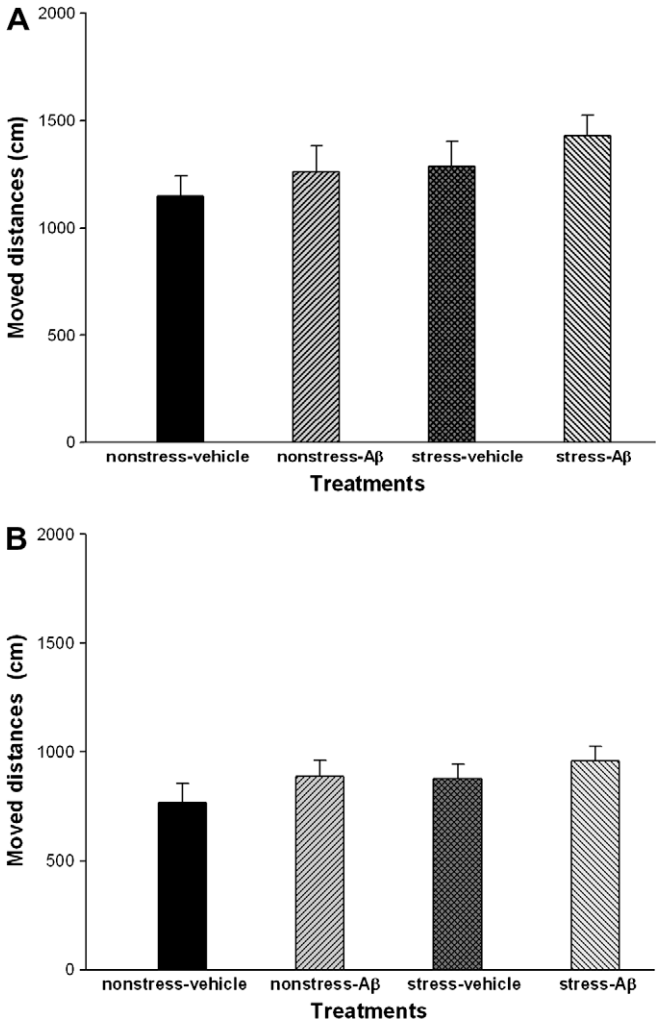

Fig. 4. The effects of oligomer $A\beta_{1-40}$ and vehicle on the motor activity in mice with stress or non-stress treatment. (A) The distance traveled within the chamber in the first 5 min of testing. (B) The distance traveled within the chamber in the second 5 min of testing. Motor activity did not differ significantly between groups in either the first or second 5-min testing period.

3.5. Increasing Aβ accumulation, oxidative stress, and apoptotic signals in the stressed and oligomer $A\beta_{1-40}$-treated mice

Immunohistochemical analyses of $A\beta_{1-40}$ accumulation, oxidative stress, and apoptotic signals are shown in Fig. 6 and Table 1. We observed that stress, oligomer treatment, and combined interaction (stress × oligomer $A\beta_{1-40}$) significantly enhanced the accumulation of $A\beta_{1-40}$ in the CA1 of the hippocampus ($F(1, 8) = 2076.072$, $p < 0.05$; $F(1, 8) = 5230.547$, $p < 0.05$; and $F(1, 8) = 562.2155$, $p < 0.001$, respectively). In addition, we also found that stress significantly enhanced the oxidative stress signal MnSOD in the CA1 of the hippocampus ($F(1, 8) = 78.5606$, $p < 0.05$) and the basolateral part of the amygdale (BLA) ($F(1, 8) = 1076.376$, $p < 0.001$); however, oligomer treatment significantly induced oxidative stress only in the CA1 ($F(1, 8) = 314.2423$, $p < 0.05$), and not in the BLA ($F(1, 8) = 5.1928$, $p > 0.05$). Significant oxidative stress was also identified in the CA1 ($F(1, 8) = 9.4761$, $p < 0.001$), but not in the BLA ($F(1, 8) = 0.577$, $p > 0.05$), induced by combined stress × oligomer $A\beta_{1-40}$ treatment. In addition, all the treatments significantly induced the apoptotic signal caspase 3 in the CA1: for stress, ($F(1, 8) = 37.5319$, $p < 0.001$); for oligomer treatment, $F(1, 8) = 71.5745$, $p < 0.001$; and for stress × oligomer $A\beta_{1-40}$ interaction, $F(1, 8) = 37.5319$, $p < 0.001$. However, only in stress-treated oligomer $A\beta_{1-40}$ mice

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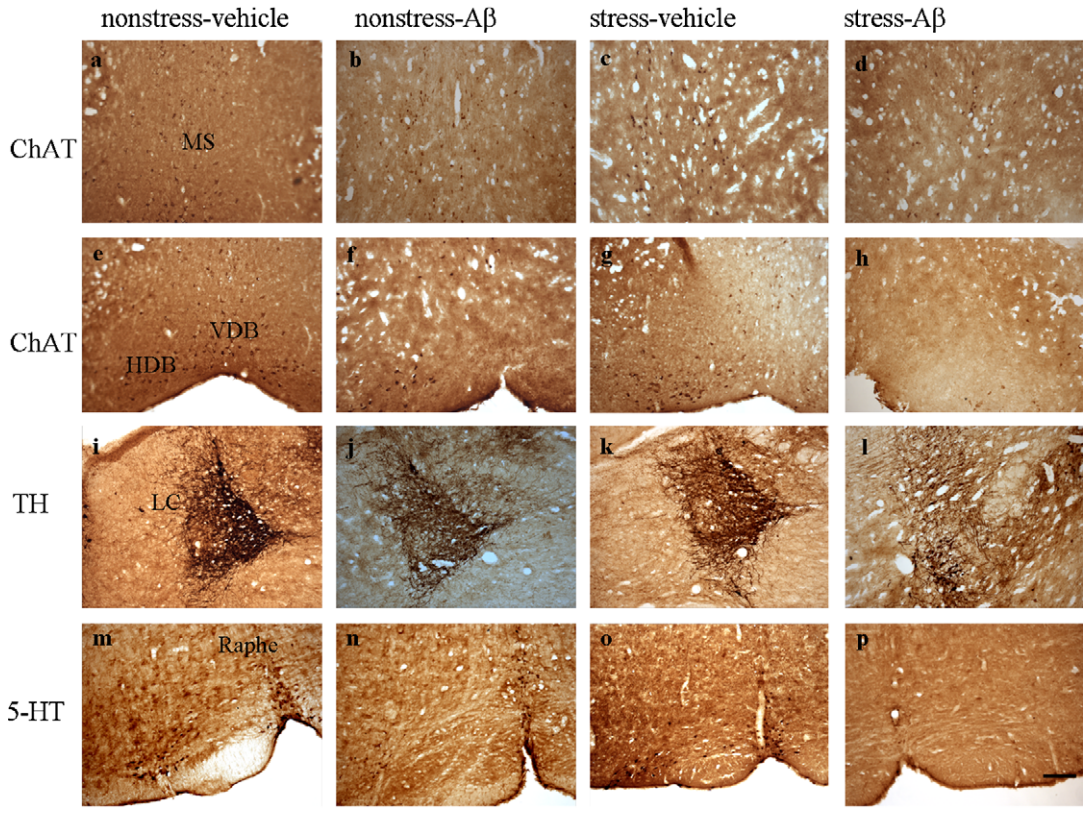

Fig. 5. Reduction of cholinergic, noradrenergic, and serotoninergic immunoreacitve neurons in treated mice. Representative immunostaining results of ChAT in the MS (a–d) and VDB/HDB (e–h), TH in the LC (i–l), and 5-TH in the Raphe nucleus (m–p). Whether combined with oligomer $A\beta_{1-40}$ treatment or not, stress treatment caused a significant reduction in immunoreactive neurons in the MS/DB, LC, and Raphe nucleus; however, combined treatment induced an even more severe reduction in immunoreactive neurons in these regions of treated mice ($n = 3$ for each group). Scale bar = 50 μm.

was a significant difference in apoptosis of the CA1 induced as compared with the other three groups, from the *post hoc* Student's *t*-test analysis.

3.6. Reduction of calbindin immunoreactive neurons in stress and oligomer $A\beta_{1-40}$-treated mice

The calbindin immunoreactive neurons were significantly reduced in the CA1 of the hippocampus in the stress- ($F(1, 8) = 294.2402$, $p < 0.05$; Fig. 7 and Table 1) and oligomer $A\beta_{1-40}$-treated mice ($F(1,8) = 123.9265$, $p < 0.05$; Fig. 7 and Table 1); a significant decrease in the stress × oligomer $A\beta_{1-40}$ interaction group ($F(1, 8) = 5.6667$, $p < 0.001$; Fig. 7 and Table 1) was also observed. In addition, from the *post hoc* Student's *t*-test, the stress and oligomer $A\beta_{1-40}$-treated mice exhibited the most severe loss of calbindin immunopositive neurons in the CA1 of the hippocampus as compared with the other three groups.

3.7. Increases in the GR/MR ratio and CRF-1 expression, and decreases in NF-κB and NR 2A/2B expression were observed in the hippocampus of the combined stress and oligomer $A\beta_{1-40}$-treated mice

We examined the GR/MR ratio, CRF-1, NF-κB, and NR2A/2B expression in the whole hippocampus in the animals ($n = 9$ for each group), and found a significant alteration in the GR/MR ratio, CRF-1, NF-κB, and NR2A/2B expression in the hippocampus of the combined-treated mice ($p < 0.05$; Fig. 8D). However, no significant effect on the GR/MR ratio, CRF-1, NF-κB, or NR2A/2B expression was identified in animals that received stress or oligomer Aβ treatment alone ($p > 0.05$; Fig. 8).

3.8. Spatial reference learning and memory was severely impaired in the stress and oligomer $A\beta_{1-40}$-treated mice

During the testing phase, the stressed mice took a significantly longer time than the non-stressed mice to reach the hidden platform ($F(1, 36) = 31.3427$, $p < 0.001$; Fig. 9A), especially the oligomer $A\beta_{1-40}$-treated mice ($F(1, 36) = 6.5382$, $p < 0.05$; Fig. 9A). However, no significant effect was identified in the combined stress × oligomer $A\beta_{1-40}$-treated animals ($F(1, 36) = 3.1349$, $p > 0.05$; Fig. 9A). During the training phase, a substantial delay in learning was observed in the stressed mice as compared with the mice in the non-stressed groups (Fig. 9B). On training day 1 of the spatial learning task, stress was found to significantly damage the acquisition of spatial learning ($F(1, 36) = 4.2773$, $p < 0.05$), as did oligomer treatment ($F(1, 36) = 4.0673$, $p < 0.05$), but no significant interaction was found in the combined stress × oligomer $A\beta_{1-40}$-treated mice ($F(1, 36) = 0.8443$, $p > 0.05$). However, on training days 2–4, significant impairment in the spatial learning task was observed in the stressed ($p < 0.05$), oligomer-treated ($p < 0.05$), and combined stress × oligomer $A\beta_{1-40}$-treated ($p < 0.05$) mice. The acquisition of spatial learning was not impaired in the non-stressed groups, although the oligomer $A\beta_{1-40}$-treated mice needed more time to search for the platform than the vehicle-treated mice ($p < 0.05$; Fig. 9B). By two-factor ANOVA analysis, no treatment effect ($F(3, 244) = 0.6129$, $p > 0.05$; Fig. 9C) but a significant quadrant effect ($F(3, 244) = 27.4603$, $p < 0.001$; Fig. 9C) was identified, and significant interaction between the different treatments and quadrants ($F(9, 244) = 5.6336$, $p < 0.001$; Fig. 9C) was noted. *Post hoc* comparison showed a significant decrease in the target quadrant as compared with the other three quadrants for the combined stress and oligomer $A\beta_{1-40}$-treated mice. Furthermore, the swimming velocity

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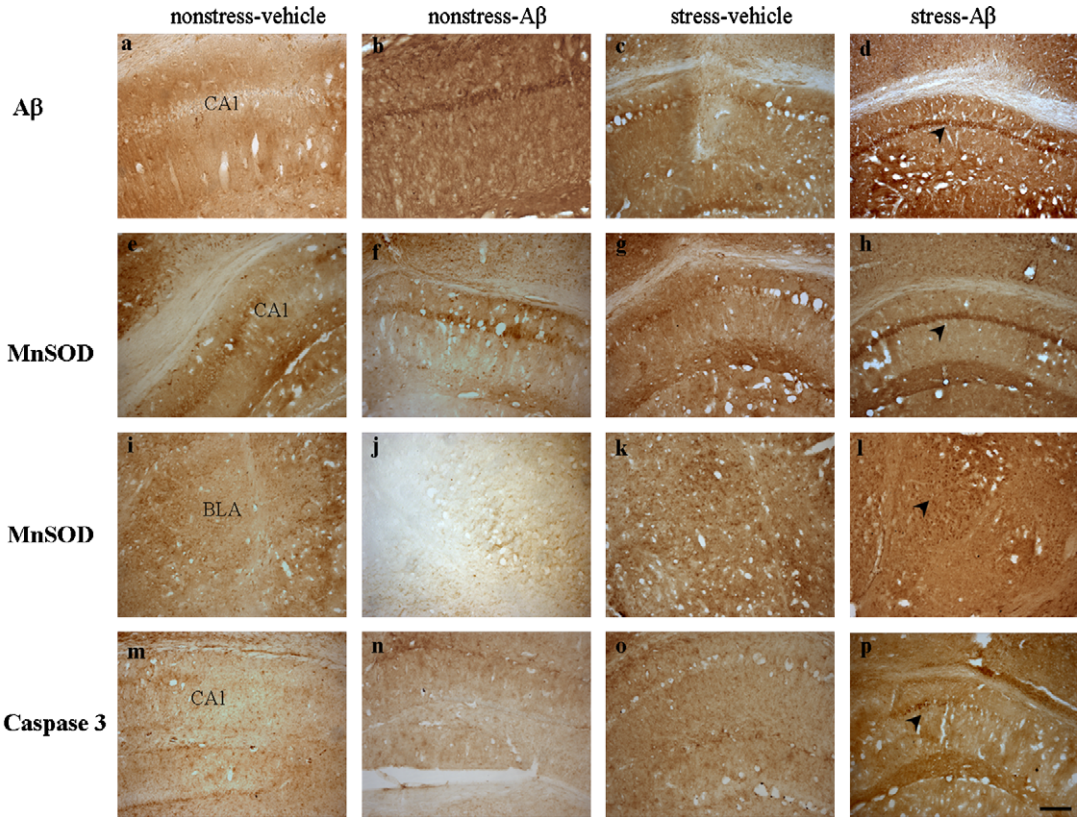

Fig. 6. Immunohistochemistry of $A\beta_{1-40}$ accumulation, oxidative stress and apoptosis in treated mice. Representative immunostaining results of $A\beta_{1-40}$ immunoreactive neurons in the CA1 (a–d), MnSOD in the CA1 (e–h), and BLA (i–l) and caspase 3 in the CA1 (m–p). Oligomer $A\beta_{1-40}$ treatment induced $A\beta_{1-40}$ accumulation and oxidative stress in the CA1; stress induced $A\beta_{1-40}$ accumulation in the CA1, and oxidative stress in the CA1 and BLA. Furthermore, the interaction between acute administration of oligomer $A\beta_{1-40}$ and stress accelerated $A\beta_{1-40}$ accumulation, oxidative stress, and apoptosis in the CA1. Arrows indicate signals. Scale bar = 50 μm.

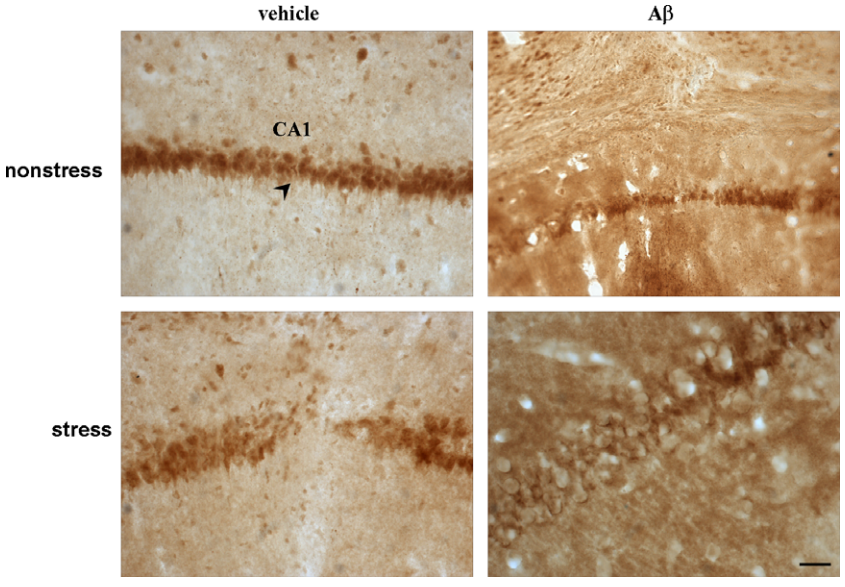

Fig. 7. Calbindin expression in the CA1 subregion of the hippocampus in treated mice. Mice subjected to oligomer $A\beta_{1-40}$, stress or combined treatment exhibited significantly reduced calbindin immunopositive neurons as compared with the non-stressed and vehicle-treated mice. Arrowheads indicate signals. Scale bar = 50 μm.

in the probe test was not significantly different between the groups of mice (data not shown).

4. Discussion

The aim of this study was to evaluate the effects of stress, acute oligomer $A\beta_{1-40}$ and the combined interaction of these two factors in C57BL/6J male mice by assessing plasma corticosterone concentration, serial behavioral, immunohistochemical data and western blot analyses results. First, a stress paradigm was established by administering 2 days of inescapable foot shocks followed by repeated SRs. After SR administration, we found that the plasma corticosterone level in the stressed mice was significantly enhanced, a result consistent with those of previous studies (Bland et al., 2005;

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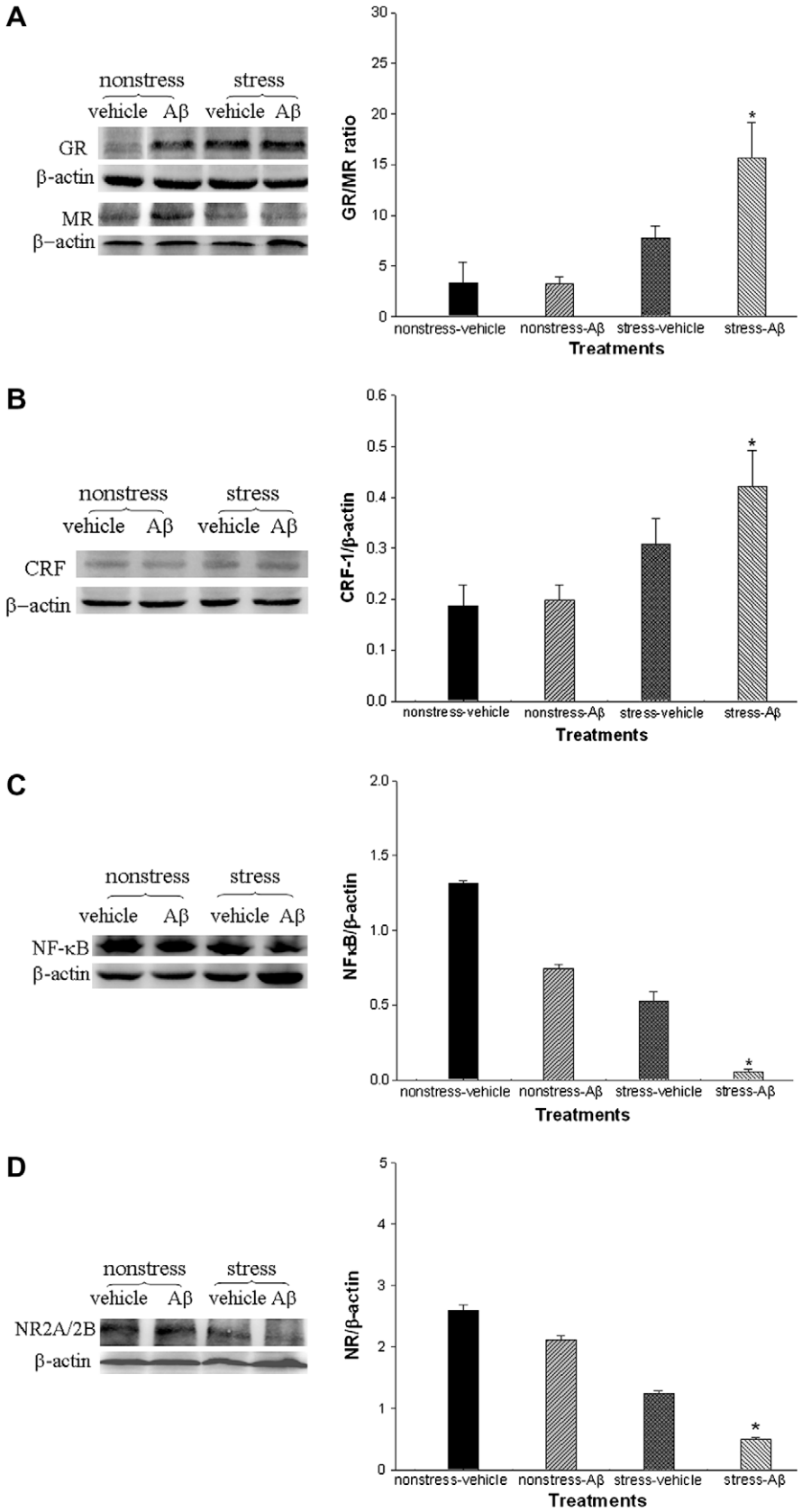

Fig. 8. GR/MR ratio, CRF-1, NF-κB, and NR2A/2B expression in the hippocampus of the treated mice. (A) Expression and quantification of the GR/MR ratio in the whole hippocampus. (B) Expression and quantification of CRF-1 in the whole hippocampus. (C) Expression and quantification of NF-κB in the whole hippocampus. (D) Expression and quantification of NR2A/2B in the whole hippocampus. After stress, mice treated with oligomer $A\beta_{1-40}$ exhibited a significant change in the GR/MR ratio, CRF-1, NF-κB, and NR2A/2B expression in the hippocampus as compared with the other three groups. To confirm equal protein loading, the same blots were hybridized with antibodies specific for β-actin.

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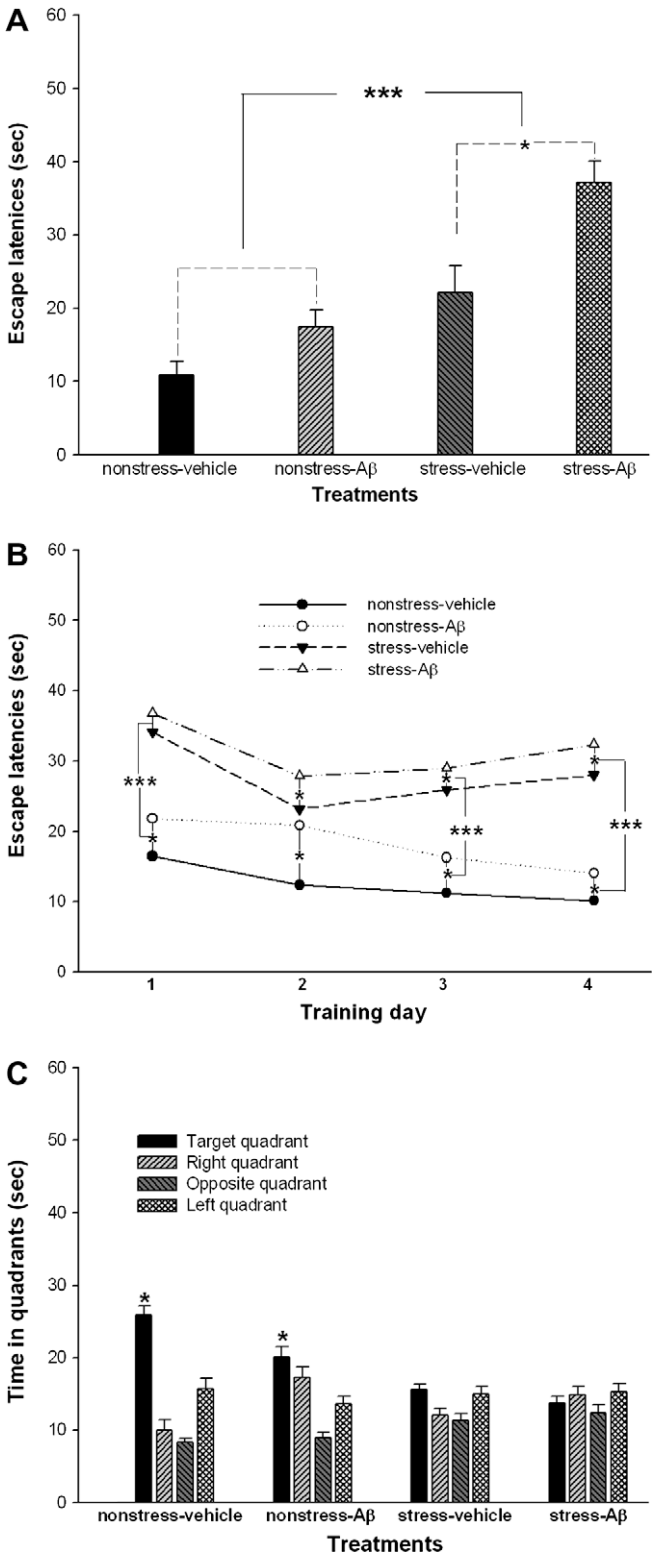

Fig. 9. Interactive effects of oligomer $A\beta_{1-40}$ and stress on mouse performance in the Morris water maze task. After four training sessions on days 30–33, three testing trials were carried out on day 34, and the probe trial was performed 24 h after the last testing trial. (A) Task acquisition in the stressed mice was significantly slower than in the non-stressed mice. Acquisition of the task in the oligomer $A\beta_{1-40}$-treated mice was also significantly slower than in the vehicle-treated mice. (B) Oligomer $A\beta_{1-40}$ treatment in association with stress severely induced impairment in the spatial learning task. (C) The stressed mice spent less time in the target quadrant than the non-stressed mice, especially under oligomer $A\beta_{1-40}$ treatment.

Jin, Jin, Zhou, Li, & Chi, 1997; Louvart et al., 2006; Ottenweller, Natelson, Pitman, & Drastal, 1989). In terms of behavioral analysis, stress not only induced anxiety, as shown in previous studies (Koba et al., 2001; Li et al., 2006; Louvart et al., 2006), but also impaired spatial learning and memory, as reported in an early study (Ladera-Fernandez, 2001). In addition, we also found that stress had no influence on motor activity, as found in previous studies (Li et al., 2006; Pynoos, Ritzmann, Steinberg, Goenjian, & Prisecaru, 1996; Yan et al., 2004), although some early evidence suggested that animals exhibit hypoactivity after exposure to electrical foot shocks (Van den Berg, Lamberts, Wolterink, Wiegant, & Van Ree, 1998; van Dijken, Mos, van der Heyden, & Tilders, 1992). The reason for these contradictory results may be the different experimental paradigms, animal strains, or shock intensities used in each study. From the immunostaining results, it was found that stress significantly increased $A\beta_{1-40}$ deposition in the CA1 subregion of the hippocampus, and oxidative stress in the CA1 subregion and the BLA, and decreased 5-HT-ir in the Raphe nucleus, ChAT-ir in the MS/DB, TH-ir in the LC, and calbindin D28K in the CA1 subregion. Previous studies have reported that stress enhanced the Aβ level (Dong et al., 2008; Hasegawa, 2007), oxidative stress (Ng, Berk, Dean, & Bush, 2008; Zafir & Banu, 2008), and dysregulation in neurotransmitter regulation in serotonergic, cholinergic and noradrenergic neurons (Berridge & Waterhouse, 2003; Brand, Groenewald, Stein, Wegener, & Harvey, 2008; Forster, Schoenfeld, Marmar, & Lang, 1995; Helm, Ziegler, & Gallagher, 2004), and decreased calcium binding protein calbindin D28K (Keifer, Brewer, Meehan, Brue, & Clark, 2003). Therefore, these data suggest that inescapable foot shock treatment followed by SRs is a reliable and long-lasting stress animal model,

In this study, acute oligomer $A\beta_{1-40}$ treatment alone induced $A\beta_{1-40}$ accumulation, oxidative stress, calcium binding signal reduction in the CA1, and related neurotransmitter dysregulated projection into the hippocampus; however, it did not induce any behavioral impairment in the animals. These results are consistent with those of recent studies (Huang et al., 2007; Schmid et al., 2008). Recent evidence also suggests that alterations in neurotransmitters alone cannot induce alteration of behavior (Alves et al., 2008; Lamour, Bassant, Potier, Billard, & Dutar, 1994). Taken together, these results suggest that acute oligomer $A\beta_{1-40}$ treatment alone is not sufficient to induce behavioral impairment; however, interaction with other factors, such as stress, as shown in our study, is sufficient to impair spatial learning and memory.

The different physiological responses exhibited by mice receiving stress and acute oligomer $A\beta_{1-40}$ treatment were evidenced by the level of plasma corticosterone and the target sites of oxidative stress. In this study, we found that stress, and not acute oligomer $A\beta_{1-40}$ treatment, induced anxiety and the impairment of spatial learning and memory through oxidative stress in the amygdala and hippocampus. Several pieces of evidence have shown that reduction in the activities of some antioxidant enzymes can be directly caused by high levels of corticosterone in the brain (McIntosh, Hong, & Sapolsky, 1998; Zafir & Banu, 2008). In addition, an oxidative pathophysiology in stress disorders is strongly supported by animal models and human biochemical data (Ng et al., 2008). Recent evidence further shows that dysfunction in the connection between the hippocampus and amygdale induces modification of synaptic plasticity in the hippocampus (Hegde et al., 2008; Reagan, Grillo, & Piroli, 2008; Tsoory et al., 2008). These data suggest that oxidative mechanisms may be involved in the common pathogenic pathways of stress, which implies new targets for the development of therapeutic interventions for stress-mediated disorders.

In addition, we found that elevated corticosteroid levels dramatically increased the toxic effect of oligomer $A\beta_{1-40}$ in the

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hippocampus, such as accumulation of Aβ, the level of oxidative stress, the GR/MR ratio, the apoptosis signals, expression of CRF-1, NR2A/2B, NF-κB, calbindin, cholinergic neurons in the MS/DB, serotonergic neurons in the Raphe nucleus, and noradrenergic neurons in the LC, which subsequently induced a severe impairment of spatial learning and memory. Recent studies have noted that corticosterone exacerbates the accumulation of the Aβ in Tg2576 Alzheimer's transgenic mice (Green, Billings, Roozendaal, McGaugh, & LaFerla, 2006; Lee et al., 2009). Therefore, we suggest that stress enhances the toxicity of oligomer $A\beta_{1-40}$. In addition, the GR/MR ratio, CRF-1 expression, Aβ accumulation, and oxidative stress levels were increased after combined stress and oligomer $A\beta_{1-40}$ treatment. A previous study suggested that changes in the components of the HPA axis induce Aβ accumulation (Dong et al., 2008); recent evidence further implies that accumulation of oxidative stress and elevation of Aβ level may exacerbate a vicious cycle in progressive Aβ accumulation (Misonou, Morishima-Kawashima, & Ihara, 2000; Shen et al., 2008; Tabner et al., 2005; Tamagno et al., 2005; Tong et al., 2005). Therefore, we suggest that positive feedback may be established in the relationships between the corticosterone concentration, GR/MR ratio, CRF-1 receptor, Aβ accumulation, and oxidative stress. In this study, we also found that the interactive effect of stress and oligomer $A\beta_{1-40}$ induced NF-κB hypoactivity and apoptotic signaling in the hippocampus. This result was consistent with that of a recent study, which found that intracerebroventricular Aβ injection downregulated NF-κB and induced apoptosis in the hippocampus (Ji et al., 2008). With the exception of apoptosis, our results further indicated that expression of NR2A/2B and calbindin in the hippocampus was significantly decreased by oligomer $A\beta_{1-40}$ treatment on previous aversive experience. These results were consistent with previous evidence that has shown that stress increases the accumulation of Aβ and oxidative stress and further induces deregulation of NMDA receptors (Harvey, Oosthuizen, Brand, Wegener, & Stein, 2004; Parameshwaran, Dhanasekaran, & Suppiramaniam, 2008). Therefore, these results demonstrated that positive feedback among corticosterone concentration, GR/MR ratio, CRF-1 expression, Aβ accumulation and oxidative stress-induced apoptosis through the inhibition of NF-κB activity in the hippocampus and synaptic dysfunction through decreasing the NR2A/2B and calcium binding protein levels in the hippocampus, which eventually severely induced impairment of spatial learning and memory.

In summary, the results of this study first indicate that 2 days of inescapable foot shock treatment followed by 3-weekly SRs is a reliable and long-lasting stress animal model, in which an increased plasma corticosterone level and avoidance of the shock compartment were observed. In addition, we found that oxidative damage in the CA1 and BLA was induced by stress, and propose that oxidative damage may be involved in the common pathogenic pathways of stress. Furthermore, interaction between stress and oligomer $A\beta_{1-40}$ induced a positive feedback relationship among corticosterone concentration, GR/MR, CRF-1, $A\beta_{1-40}$ accumulation, oxidative stress, and NF-κB, which resulted in apoptosis in the hippocampus. Finally, the interaction severely impaired hippocampal-dependent behavior, in addition to decreasing NR2A/2B, calcium-binding signaling, and the projection of related neurotransmitters into the hippocampus. Therefore, our findings suggest that stress may play an important role in the early stages of Alzheimer's disease (AD), and antioxidant therapy might attenuate stress and the impairment of spatial learning and memory.

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