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ORIGINAL RESEARCH

Upregulation of Mineralocorticoid Receptor in the Hypothalamus Associated with a High Anxiety-like Level in Apolipoprotein E4 Transgenic Mice

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Abstract Anxiety symptoms occur in a large portion of Alzheimer's disease (AD) patients. ApolipoproteinE-4 (ApoE ε4 allele), a risk factor for AD, has been recognized as an important contributor to psychiatric disorders. In the present study, we aimed to investigate the corticosterone level in relation to anxiety-like behavior changes in transgenic male mice with different glial fibrillary acidic protein (GFAP)-ApoE isoforms. GFAP-ApoE4 transgenic mice aged 3 months showed higher anxiety-like behavior in open field, light–dark box and elevated plus maze tasks compared with that of age-matched GFAP-ApoE3 mice. However, corticotropin releasing factor levels in the hypothalamus and plasma corticosterone secretion were similar in GFAP-ApoE3 and GFAP-ApoE4 transgenic male mice. Additionally, increased expression of the mineralocorticoid receptor (MR) and unchanged expression of the glucocorticoid receptor were observed in the hypothalamus of GFAP-ApoE4 mice. However, no significant differences were found in the expression levels of the MR in GFAP-ApoE3 and GFAP-ApoE4 mice at postnatal day 2. In conclusion, we found that MR upregulation rather than corticosterone level changes in the early stage of adulthood was associated with the higher anxiety-like level measured in GFAP-ApoE4 mice.

Keywords Apolipoprotein E · Mineralocorticoid receptor · Glucocorticoid receptor · Corticotropin releasing factor · Hypothalamic–pituitary–adrenal axis · Anxiety-like

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Introduction

Alzheimer's disease (AD) is one of the most common age-related neurodegenerative disorders characterized by progressive cognitive impairment, accompanied by other neuropsychiatric disturbances, such as anxiety or depression. Anxiety symptoms have been observed in a large portion of AD patients and are more prevalent among patients with a younger age of onset (de Toledo et al. 2004; Garcia-Alberca et al. 2012). However, the literature offers little information on anxiety in dementia, leaving the need to gain a better understanding of factors contributing to anxiety in AD.

Apolipoprotein E (ApoE) is a 34 kDa protein that participates in the transport of plasma lipids and the redistribution of lipoproteins and cholesterol (Swertfeger and Hui 2001). There are three alleles of ApoE in humans (ε2, ε3, and ε4) that are distinguished from each other by their cysteine/arginine content at two polymorphic sites (Liu et al. 2012). Of the three common human ApoE alleles, inheritance of ApoE ε4 allele (ApoE4) is a great genetic risk factor for the development of sporadic AD and earlier

onset of this disease, whereas the more frequent ApoE ε3 allele (ApoE3) and very rare ApoE ε2 allele (ApoE2) provide relative protection from AD (Scarmeas et al. 2002). ApoE4 might also contribute to the psychological symptoms during AD progression(Panza et al. 2012; Christie et al. 2012). Anxiety, which are more common among AD patients, is inversely related to disability in daily activities (Panza et al. 2012). AD patients with ApoE4 score higher on anxiety measures and have more sleep disturbance (Robertson et al. 2005). In probable AD patients, ApoE4 subjects show higher anxiety scores than ApoE3 subjects (Robertson et al. 2005; Pritchard et al. 2007). Apolipoprotein E from diversity of cellular origins appears to influence its effects on AD pathology (Huang et al. 2004; Xu et al. 2006). The effects of apoE on measures of anxiety have been studied in mice lacking murine apoE and expressing human apoE in astrocytes under the control of the glial fibrillary acidic protein (GFAP) promoter or in neurons under the control of the neuron-specific enolase (NSE) promoter. Consistent with human studies, similar results are also found in ApoE(-/-) mice (Robertson et al. 2005; Raber et al. 2000). In addition, GFAP-ApoE4 mice show increased anxiety-like behavior compared with GFAP-ApoE3 mice (Siegel et al. 2012).

The hypothalamic–pituitary–adrenal (HPA) axis plays an important role in emotion, cognition, and many other brain functions (Laurent and Powers 2007; Young 2014). The HPA axis regulates glucocorticoid (GC) secretion, and HPA axis hyperactivity is a well-described feature in AD, leading to increased cortisol levels in the blood and cerebrospinal fluid (CSF) (Popp et al. 2009; Csernansky et al. 2006). In the brain, the hippocampus is the major GC target and has the highest concentration of GC receptors. There are two classical receptors involved in corticosteroid feedback on the HPA axis: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). The GR is expressed throughout the brain; however, the MR is mainly expressed in the hippocampus (Kretz et al. 2001). The MR also expressed in the hypothalamus and played an important role in regulating the response to a stress event (Han et al. 2005; Berardelli et al. 2013). The MR has a ten-fold higher affinity for corticosteroid than the GR. They often work together or alone to affect cognition and emotion, and the balance between the MRs and GRs controls the HPA axis and behavior (Harris et al. 2013; Wei et al. 2004; Lai et al. 2007). In recent years, an increasing number of studies have focused on the role of MRs and GRs on anxiety and depression. The direct evidence for the involvement of GRs is that deletion of GRs in the forebrain reduces anxiety-like behavior, while forebrain-selective overexpression of GRs increases anxiety (Wei et al. 2004, 2012). In contrast, overexpression of MRs in the forebrain results in reduced anxiety-like behavior (Lai et al. 2007; Rozeboom et al. 2007). Additionally, loss of limbic MRs impairs behavioral plasticity (Berger et al. 2006). Although there was a report that overexpression of mineralocorticoid receptors did not affect anxiety-like behavior in female mice (Kanatsou et al. 2015, 2016), a series of antagonist tests in the whole brain and hippocampus showed that upregulation of MR signaling increased anxiety-like behavior in male rats (Korte et al. 1995; Bitran et al. 1998). In addition, it was reported that loss of MRs in the forebrain resulted in large differences in emotional and cognitive behaviors between female and male mice (Ter Horst et al. 2012).

Importantly, corticotropin-releasing factor (CRF) plays a major role in the regulation of the HPA axis, and HPA axis dysregulation is associated with susceptibility to anxiety (Harris et al. 2013; Rivier 2014). In addition, increased MR expression in the frontal cortex of AD patients is associated with the ApoE4 genotype(Harris et al. 2013; Rivier 2014). However, whether increased anxiety-like behavior in GFAP-ApoE4 transgenic male mice is associated with corticosterone level has not yet been illustrated. In the present study, we aimed to measure the relationship among anxiety-like behavior, corticosterone level and MR/GR expression using the GFAP-ApoE transgenic male mice.

Materials and methods

Animals

Human ApoE3 (stock number: 004633) and ApoE4 (stock number: 004631) transgenic mice (C57BL/6 J strain background) under the control of the GFAP promoter were obtained from Jackson Laboratory (Bar Harbor, Maine, USA). Three-month-old male GFAP-ApoE3 (n = 39) and GFAP-ApoE4 (n = 23) mice were used for behavioral testing. Male animals were group-housed (4 per cage) in polycarbonate cages that contained woodchip shavings for bedding and maintained with a 12-hour light/dark cycle (lights on 7:00 am), at a temperature of 22 ± 1 °C. Food and water were available ad libitum. All of the experiments were performed in accordance with the Animal Care and Use Committee of the University of Science and Technology, Anhui, P.R. China.

Behavioral tasks

All of the behavioral tasks were performed between 12:00–18:00 in a novel room that was similar to the feeding room. GFAP-ApoE3 and GFAP-ApoE4 transgenic mice were performed alternatively in the behavioral tasks to make sure that different ApoE alleles were tested at approximately the same time. Before each test, animals were given a 15-min adaptation period to explore the new

environment. Each apparatus was cleaned with 70% ethanol and dried before the next mouse was tested. All of the tasks were monitored by a video camera and analyzed by Ethovision XT software (Noldus, The Netherlands).

The open field (OF) test

An open-field procedure was designed to detect spontaneous motor activity and anxiety according to previous descriptions (Meng et al. 2011). An open, wooden box (50×50 cm) with 25-cm walls was used. The open field box was painted with water-resistant, odorless white paint. For each trial, an animal was placed into one of the four corners, facing the wall, and was permitted to explore the environment for 5 min.

The elevated plus maze (EPM) test

The EPM procedure was performed as described by Walf et al. (Walf and Frye 2007). Based on the design, the maze (made of Plexiglas) consisted of two opposite closed arms (30 cm×6 cm) that were enclosed with walls (15 cm in height) and two opposite open arms (30 cm×6 cm, without edges) that formed a plus shape. The apparatus had a central arena (6 cm×6 cm) and was elevated 80 cm above the floor. Each mouse was placed in the central arena of the maze facing an open arm and allowed to explore for 5 min.

The light–dark box test

The light–dark box was established as a task for validating anxiety-like behavior in rodents. It consisted of two compartments: one light box and one dark box, and both boxes were the same size (25 cm×25 cm×25 cm), with a Plexiglas panel separating the two boxes. An arched hole was made in the center bottom of the panel to allow animals to transition from one box to the next. In each trial, mice were gently placed in the light compartment facing the wall opposite to the hole. The animals were allowed to explore in the light–dark box for 5 min.

Stress procedure

Acute restraint stress was performed using individual plastic restrainers (2.5 cm in internal diameter, 7 cm long). Mice were immobilized for 30 min and then released for recovery. Mice were sacrificed at four time points: (A) normal control group sacrificed before immobilization, (B) group immobilized for 30 min prior to sacrifice, (C) group immobilized for 30 min and released for 20 min prior to sacrifice; and (D) group immobilized for 30 min and released for 90 min prior to sacrifice. All mice were sacrificed between 9:00 and 12:00.

Plasma corticosterone measurement

To measure plasma corticosterone, blood was collected into tubes containing heparin, and centrifuged at 6000 rpm for 10 min at 4 °C. Plasma was moved to a new tube and stored at −80 °C until measurements could be taken. Plasma corticosterone was measured using a mouse plasma corticosterone ELISA kit (RapidBio, USA).

RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from the frozen hypothalamus of GFAP-ApoE mice using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The purified RNA was quantified using a UV spectrophotometer (A260/A280 = 1.9–2.0). Next, 1 μg of total mRNA was reverse transcribed using an RT kit for real-time PCR (Takara, Japan). Real-time PCR was performed using SYBR Green Mix (Takara, Japan) on an ABI Prism 7000 system. Thermo cycle conditions were as follows: 1 cycle at 95 °C for 5 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The primers used are described in Table 1. The mRNA (CRF, MR, GR, c-fos and c-jun) data are all represent values collected from animals that were sacrificed in the basal state. The relative mRNA level of the target gene was determined using the $2^{-\Delta\Delta Ct}$ method (Adnan et al. 2011).

Statistical analysis

The data are presented as the means ± standard error of the mean (SEM). All statistical analyses were performed using SPSS 11.0 software. Unpaired Student's t-test was used in the study with one exception: the latency in the light–dark

Table 1 Gene specific primers used for real time PCR

Gene	Primer sequences	Accession numbers
CRF	AGGAGGCATCCTGAGAGAAGT CATGTTAGGGGCGCTCTC	NM_205769
GR	TGCTATGCTTTGCTCCTGATCTG TGTCAGTTGATAAAACCG CTGCC	NM_008173
MR	GTGGACAGTCCTTTCACTACCG GTGGACAGTCCTTTCACTACCG	NM_001083906
c-JUN	GAAAACCTTGAAAGCGCAAAA TAGCATGAGTTGGCACCCAC	NM_010591
c-FOS	TGGTGAAGACCGTGTCAGGA GCAGCCATCTTATTCCGTTCC	NM_010234
GADPH	CATGGCCTTCCGTGTTCCTA CCTGCTTCACCACCTTCTTGAT	NM_008084

box test was analyzed using a nonparametric Mann–Whitney *U* test. P values <0.05 were considered statistically significant.

Results

GFAP-ApoE4 transgenic male mice exhibited anxiety-like behavior

An open field test was performed in the current study to measure the locomotor activity and anxiety-like behavior of the GFAP-ApoE transgenic mice. We found that locomotor activity [t(59) = 5.01, $p < 0.001$, Fig. 1c] and exploration behavior [rearing, t(59) = 4.78, $p < 0.001$, Fig. 1e] in 3-month-old ApoE4 mice were lower than that of age-matched GFAP-ApoE3 mice. Additionally, GFAP-ApoE4 male mice were more anxious to go into the inner area and traveled a shorter distance than age-matched GFAP-ApoE3 mice [t(59) = 2.60, $p < 0.05$, Fig. 1b, t(59) = 2.64, $p < 0.05$, Fig. 1d, t(59) = 3.43, $p < 0.01$ Fig. 1f]. In the elevated plus maze, 3-month-old GFAP-ApoE4 mice also showed more anxiety-like behavior, e.g., less distance traveled in the center area [t(57) = 3.21, $p < 0.01$, Fig. 2e]

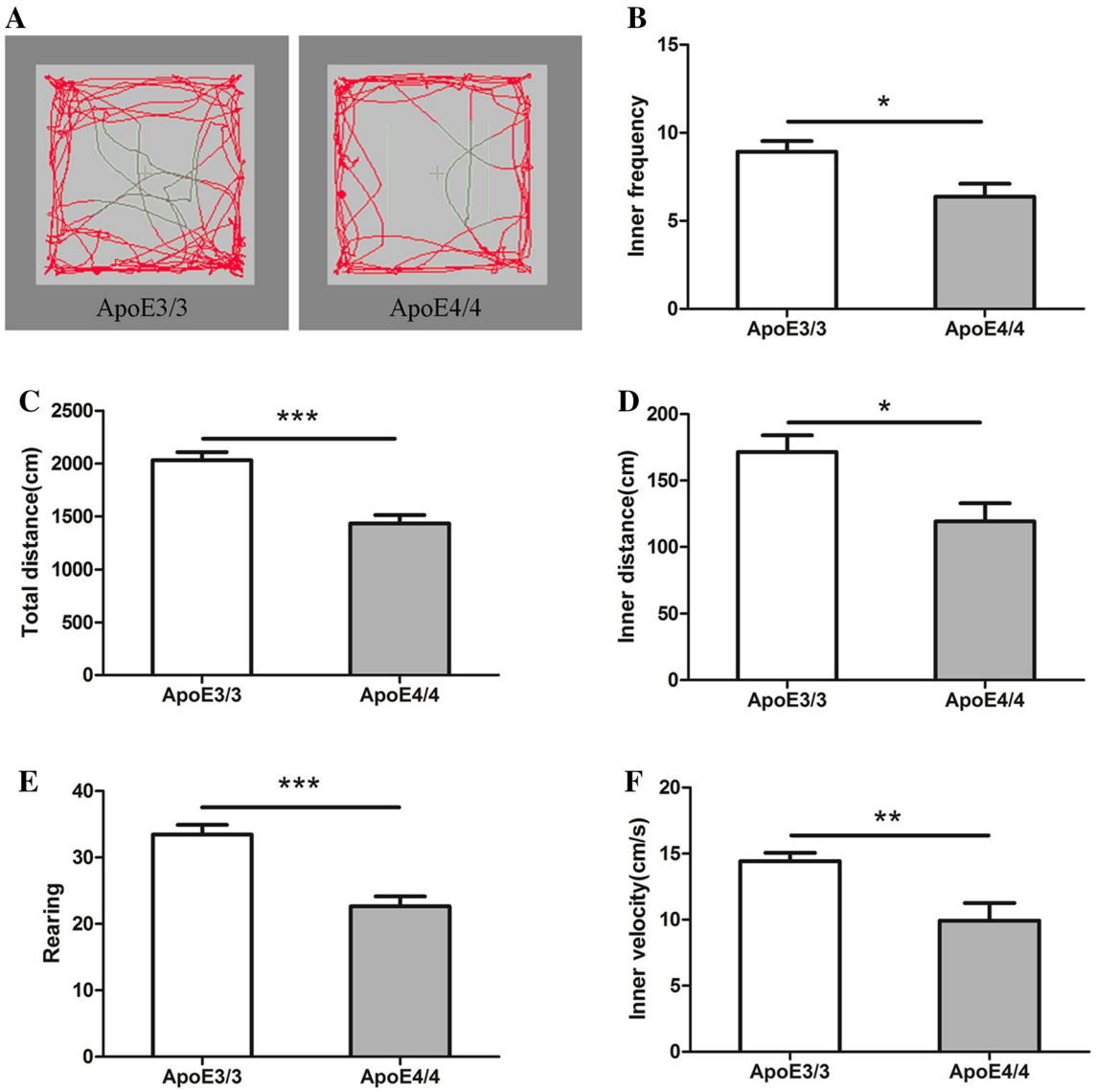

Fig. 1 The locomotor activity and anxiety-like behavior of the GFAP-ApoE3 and GFAP-ApoE4 transgenic male mice based on the open field test. **a** Representative activity traces for GFAP-ApoE3 and GFAP-ApoE4 transgenic mice during a 5-min trial. The total distance traveled (**c**) and rearing (**e**) represent locomotor activity and exploration behavior. Frequency in inner area (**b**), inner distance (**d**) and inner velocity (**f**) represent anxiety-like behavior. Values are expressed as the means ± SEM. Unpaired Student's *t*-test, $*p < 0.05$; $**p < 0.01$ $***p < 0.001$; n(3/3) = 39 mice, n(4/4) = 23 mice

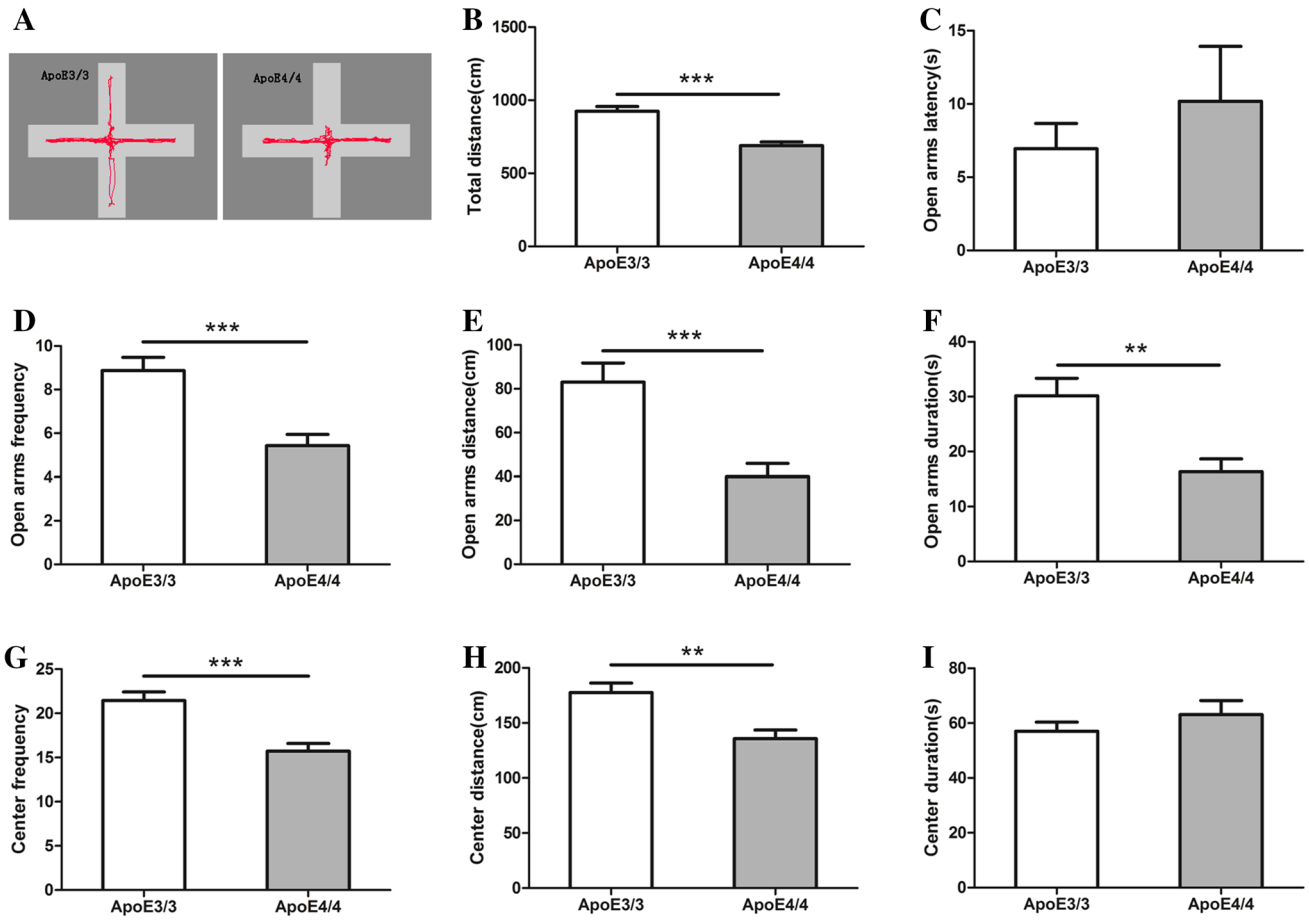

Fig. 2 The anxiety-like level of GFAP-ApoE3 and GFAP-ApoE4 transgenic mice based on the elevated plus maze task. **a** Representative activity traces for ApoE mice during a 5-min trial. Total distance traveled (**b**) in the elevated plus maze represents the locomotor activity. Open arm latency (**c**), open arm frequency (**d**), open arm distance (**e**) and open arm duration (**f**) represent activity and anxiety-like behavior in the open arms. Center frequency (**g**), center distance (**h**) and center duration (**i**) represent activity and anxiety-like behavior in the center area. Values are expressed as the means ± SEM. Unpaired Student's *t*-test, $*p < 0.05$; $**p < 0.01$ $***p < 0.001$; n(3/3) = 38 mice, n(4/4) = 21 mice

and open arms [t(57) = 3.41, $p < 0.01$, Fig. 2h] and fewer entries into the center [t(57) = 3.88, $p < 0.001$, Fig. 2g] and open arms [t(57) = 3.80, $p < 0.001$, Fig. 2d] than the GFAP-ApoE3 mice. To further confirm our findings, we chose another anxiety-based task, the light–dark box test. We found that GFAP-ApoE4 mice took less time to enter into the dark box than age-matched GFAP-ApoE3 mice

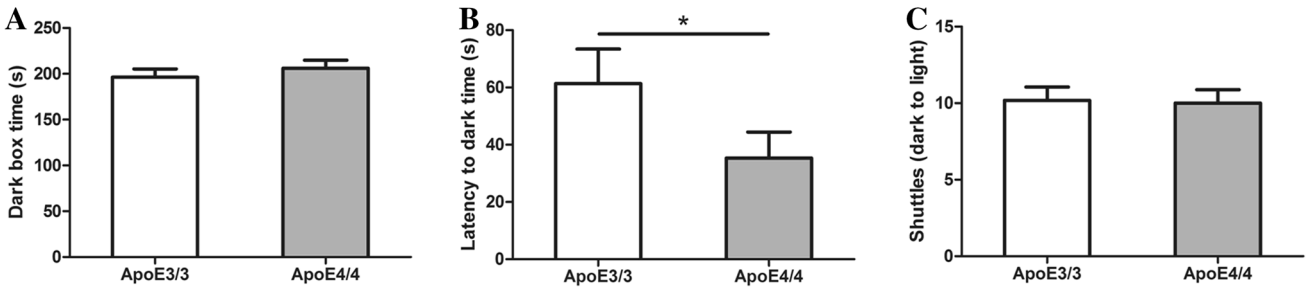

Fig. 3 The light–dark box test showed an inherent preference in the GFAP-ApoE3 and GFAP-ApoE4 transgenic male mice. Time in the dark box (**a**), latency to enter the dark box (**b**) and number of dark to light box shuttles (**c**) were analyzed in the task. Values are expressed as the means ± SEM. Nonparametric Mann–Whitney *U* test, $*p < 0.05$. n(3/3) = 39 mice, n(4/4) = 23 mice

(Fig. 3b), but there was no significant difference in the times spent in the dark box (Fig. 3a).

Basal plasma corticosterone level and normal response to restraint stress

Basal plasma corticosterone levels were detected to assess the role of corticosterone level in anxiety-like behavior in the different GFAP-ApoE transgenic mice. No significant difference was found in basal plasma corticosterone levels between GFAP-ApoE3 and GFAP-ApoE4 mice aged 3-months (26.7 ± 14.9 and 33.4 ± 28.3 ng/ml, respectively). Acute restraint stress is a sensitive procedure to assess plasma corticosterone secretion to stress of different GFAP-ApoE transgenic mice. After 30 min of restraint stress, the plasma corticosterone secretion level was markedly increased in both GFAP-ApoE3 and GFAP-ApoE4 mice, but no significant difference was observed in the plasma corticosterone level between the two groups (178.1 ± 30.4 and 174.3 ± 35.6 ng/ml, respectively). After a 20 or 90-min recovery period following the restraint stress, the plasma corticosterone secretion level returned to the normal level. Likewise, no significant difference was found in the plasma corticosterone level between GFAP-ApoE3 and GFAP-ApoE4 mice (Fig. 4b).

CRF, MR and GR expression in the hypothalamus in GFAP-ApoE transgenic mice

To explore the possible relationship between the ApoE genotype and the regulation of HPA axis activity, we measured the expression of the CRF, MR and GR genes in the hypothalamus in the GFAP-ApoE transgenic male mice aged 3-months using q-PCR. No significant difference was found in basal CRF mRNA levels in the hypothalamus between GFAP-ApoE3 and GFAP-ApoE4 mice (Fig. 4a). Interestingly, the GR expression level was similar between GFAP-ApoE4 and GFAP-ApoE3 transgenic mice in the hypothalamus; however, GFAP-ApoE4 transgenic mice expressed the MR at a higher level than GFAP-ApoE3 transgenic mice aged 3 months [$t(10) = 2.86$, $p < 0.05$, Fig. 5]. Expression levels of early stress response genes (c-fos and c-jun) in the hypothalamus were also measured. C-fos expression was higher in the hypothalamus of GFAP-ApoE4 transgenic mice than in that of GFAP-ApoE3 transgenic mice, but no significant difference was observed in hypothalamus expression levels of c-jun between GFAP-ApoE4 and GFAP-ApoE3 transgenic mice (Fig. 6).

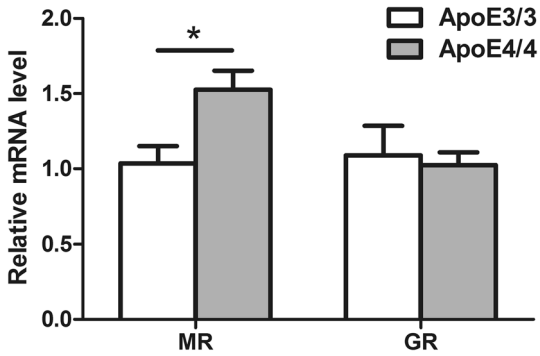

Fig. 5 The cortisol receptor levels in the hypothalamus in GFAP-ApoE transgenic mice at the age of 3 months. Values are expressed as the means ± SEM. Unpaired Student's *t*-test, $*p < 0.05$; n = 6 per group

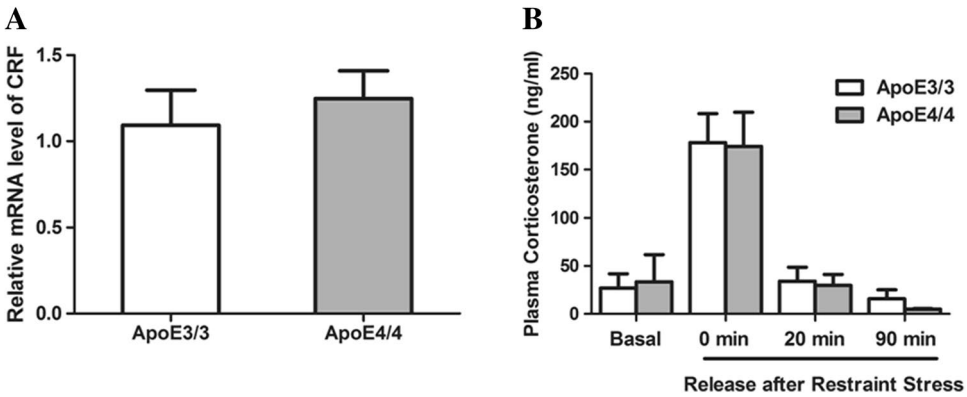

Fig. 4 The HPA axis function measured in the GFAP-ApoE transgenic male mice. The levels of CRF in the hypothalamus (**a**) and corticosterone secretion at baseline and after acute restraint stress (**b**) were detected in GFAP-ApoE transgenic mice at the age of 3 months. R30, restraint stress for 30 min; R30 + 20, restraint stress for 30 min and release for 20 min; R30 + 90, restraint stress for 30 min and release for 90 min. Value are expressed as the means ± SEM, n(A) = 6–8, n(B) = 4 mice per group

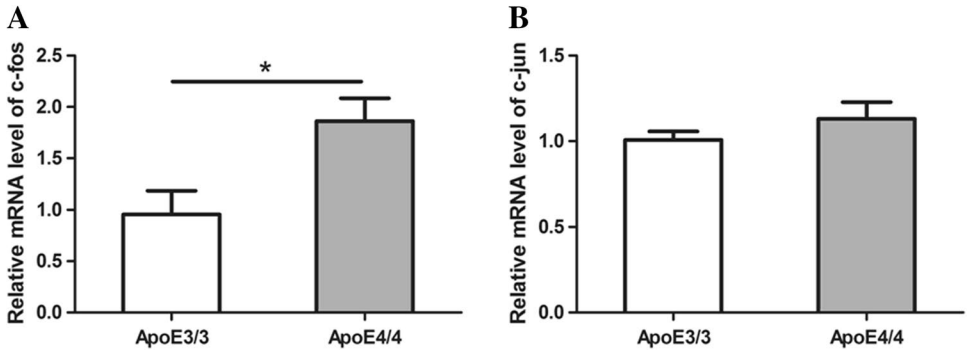

Fig. 6 The levels of c-fos and c-jun expression in the hypothalamus. Values are expressed as the means ± SEM. Unpaired Student's *t*-test, $*p < 0.05$; n = 6 per group

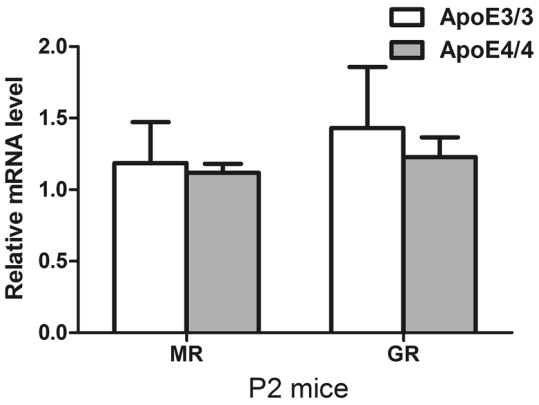

Fig. 7 The MR and GR expression levels in the hypothalamus of GFAP-ApoE transgenic mice at postnatal day 2 (P2). Values are expressed as the means ± SEM. Unpaired Student's *t*-test, $*p < 0.05$; n = 6 per group

The influence of ApoE Genotype on MR and GR mRNA levels

To further confirm that the changes in MR and GR expression level were dependent on the ApoE genotype, MR and GR mRNA levels were detected in GFAP-ApoE transgenic mice at postnatal day 2 (P2). Unexpectedly, no significant difference was found in the expression of MR or GR in the hypothalamus between these newborn GFAP-ApoE3 and GFAP-ApoE4 mice (Fig. 7).

Discussion

In the present study, we found that MR upregulation rather than corticosterone level changes in the early stage of adulthood was related with the higher anxiety-like level measured in GFAP-ApoE4 mice, which suggested that receptor changes may have predated the change of corticosterone level.

GFAP-ApoE4 mice and NSE-ApoE4 transgenic mice have been reported to show high anxiety-like levels in the elevated plus maze, and reduced activity in the open field (Raber et al. 2000), which is consistent with our results seen in GFAP-ApoE4 mice. Furthermore, our behavioral results showed that GFAP-ApoE4 mice behaved anxiously at an age as young as 3 months. In addition, it has been reported that ApoE4 mutants had a different stress reactivity and corticosterone feedback. However, the age of ApoE4 mutants in these studies is different from our GFAP-ApoE transgenic mice (Raber 2007; Grootendorst et al. 2004). Our results move the time at which increased anxiety-like behavior can be observed in GFAP-ApoE4 mice forward from 6 to 3 months. There was no significant difference in CRF expression in the PVN and basal plasma corticosterone levels in mice aged 3 months, which indicated that there was no impairment in HPA axis function at the age of 3 months. The data were consistent with previous reports that ApoE-/- and ApoE4 mice exhibited normal basal plasma corticosterone levels and a normal reaction to restraint stress at 2–4 months old (Raber et al. 2000). Previous studies showed that the MR in the hypothalamus played a major role in regulating the response to stress (Han et al. 2005; Berardelli et al. 2013). Intracerebroventricular (ICV) administration of an MR antagonist induced a decrease in anxiety-like behavior, suggesting that increased MR activity might induce anxiety-like behavior (Korte et al. 1995). Wang et al. also found that MR expression was increased in the PVN in patients with depression (Wang et al. 2008), which supports our findings. In addition, MR mRNA levels were shown to be inversely correlated between the human paraventricular nucleus (PVN) and dorsal lateral prefrontal cortex (DLPFC) and might play opposing roles in the two regions (Qi et al. 2013). Increased anxiety-like behavior in GFAP-ApoE4 mice might result from upregulated MR expression in the hypothalamus in GFAP-ApoE4 transgenic mice. Furthermore, Considering a higher affinity of corticosteroid to the MR than the GR and a higher response of MR to stress (Kalman and Spencer 2002), it was suggested that upregulated MR signaling contributed to anxiety-like behavior in the GFAP-ApoE4 transgenic mice.

We investigated the relationship between the MR/GR expression level and ApoE genotype further in our experiments. No significant difference was observed in MR and

GR expression levels between GFAP-ApoE4 and GFAP-ApoE3 transgenic mice at postnatal day 2, indicating that the upregulation of the MR expression level was not due to the inheritance of ApoE4 isoform or it was not yet manifested at postnatal day 2. Furthermore, the results from the ApoE3- and ApoE4-expressing cell models showed no direct regulation of ApoE4 on the expression of MR (data not shown). Thus, there could be a cofactor or effector that was expressed in a cell/tissue/developmental-specific way. The ability of the ApoE4 genotype to alter the vulnerability to environmental factors such as stress might account for this discrepancy (de Kloet et al. 2002; Grootendorst et al. 2002). Therefore, expression of MR, the first regulator in the stress response, is changed in early adulthood.

In conclusion, we found that GFAP-ApoE4 transgenic mice showed enhanced anxiety-like behavior at a younger age than previously reported. Upregulation of MR signaling in GFAP-ApoE4 transgenic male mice provided a possible mechanism for the cause of the anxiety-like behavior, which might afford a new therapeutic approach for affective disorders based on MR.

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Compliance with ethical standards

Conflict of interest Fan-Tao Meng, Jun Zhao, Hui Fang, Li-Feng Zhang, Hui-Mei Wu, and Ya-Jing Liu declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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