

# Dose-dependent regulation of steroid receptor coactivator-1 and steroid receptors by testosterone propionate in the hippocampus of adult male mice



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## ABSTRACT

Androgens have been proposed to play important roles in the regulation of hippocampus function either directly, through the androgen receptor (AR), or indirectly, through estrogen receptors (ERs), after aromatization into estradiol. Steroid receptor coactivator-1 (SRC-1) is present in the hippocampus of several species, and its expression is regulated by development and aging, as well as by orchidectomy and aromatase inhibitor letrozole administration, while ovariectomy only transiently downregulated hippocampal SRC-1. However, whether the expression of hippocampal SRC-1 can be directly regulated by testosterone, the principal male sex hormone, remains unclear. In the present study, we investigated the expression of hippocampal SRC-1 after orchidectomy and testosterone treatment using immunohistochemistry and Western blot analysis. We found that while hippocampal SRC-1 was significantly downregulated by orchidectomy (ORX), its expression was rescued by treatment with testosterone in a dose-dependent manner. Furthermore, we noticed that the decreased expression of hippocampal AR, ERs and the synaptic proteins GluR-1 and PSD-95 induced by ORX was also rescued by testosterone treatment in a dose-dependent manner. However, we found that hippocampal membrane estrogen receptor GPR30 and dendritic spine marker spinophilin were not altered by ORX or testosterone treatment. Together, the above results provided the first direct evidence for the androgenic regulation on hippocampal SRC-1, indicating that SRC-1 may be a direct target of androgenic regulation on the hippocampus. Furthermore, because AR and ERs can be differentially regulated by testosterone, and the transcriptional activity requires the involvement of local SRC-1, and considering the complicated regulatory pathway of each individual receptor, the converged hub regulator SRC-1 of these nuclear receptor networks is worthy of further investigation.

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## 1. Introduction

Studies have demonstrated that androgens such as testosterone and dihydrotestosterone play profound roles in the regulation of the hippocampal structure and function in both rodents and human subjects. Androgen deprivation (usually by orchidectomy, ORX) causes significant cognitive deficiency [1] and a significant decrease of hippocampal BDNF and PSD-95 [2]. Testosterone treatment significantly increased the spine synapse density in the hippocampus of rats [3] and regulated hippocampal spinogenesis

[4]. A recent study showed that testosterone in the hippocampus of male rats is protective against ORX-induced depressive-like behavior [5]. In humans, age-induced decreased levels of androgens may be one of the causative factors for heightened risk of Alzheimer's disease (AD). This is based on the fact that testosterone replacement has been shown to improve cognitive deficits in rodents [6]. However, the underlying mechanisms of androgen actions remain unclear.

In addition to its direct action pathway of binding to an androgen receptor (AR), testosterone also functions by local neural aromatization into 17β-estradiol (E2). This process is catalyzed by local aromatase (estrogen synthase), leading to a hippocampal E2 concentration of up to 8 nM for males and 0.5–2 nM for females, which is much higher than circulatory levels, as shown by hippocampal slices from adult male rats [7]. Numerous studies

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have localized aromatase mRNA and/or protein in the hippocampus. It has been detected in the hippocampus of mice [8], rats [9] and humans [10]. Over the last decade, studies have revealed a crucial role of hippocampal aromatization in the regulation of hippocampal structural and functional synaptic plasticity [11,12], as well as in spatial memory performance [13] and protective effects against depression-like behavior [5].

The actions of androgens may be achieved by either binding to AR or estrogen receptors (ERs) after conversion to E2 by aromatase, the alternative pathway for androgen action. Nuclear AR and ERs (ER $\alpha$  and ER $\beta$ ) have been detected in the hippocampus of several species [14–16]. AR and ERs show unique properties that affect the structure of the hippocampus and its function, including synaptic plasticity, learning and memory, and cognition [17–22]. Additionally, the novel membrane estrogen receptor GPR30 (also called GPER-1) has been shown to mediate rapid, non-classic, estrogenic regulation on the hippocampus [23,24]. However, the changes of these receptors after testosterone treatment have not been fully examined.

Steroid nuclear receptors require coactivators for efficient transcriptional activity. Among these coactivators, steroid receptor coactivator-1 (SRC-1; or NCoA-1) [25] is predominantly localized in the hippocampus [26,27]. Our previous studies have demonstrated that in the female rats, hippocampal SRC-1 was regulated by postnatal development and aging, but not by an ovariectomy [28,29]. We also demonstrated that SRC-1 expression is higher in the brain of male mice compared to females [30] as reported by Kerver et al. [31,32]. However, in the hippocampus, male and female mice displayed a similar postnatal developmental profile of SRC-1 expression [33], but SRC-1 remained depressed only in males after gonadectomy [34]. Recently, we found that letrozole administration and ORX induced a similar downregulation of SRC-1 immunoreactivity in specific brain regions involved in sense of smell, learning and memory, cognition, neuroendocrine, reproduction and motor control [35,36]. Although we have reported that androgen deprivation by ORX or inhibition of hippocampal E2 by letrozole administration downregulated hippocampal SRC-1 [34–36], whether hippocampal SRC-1 can be upregulated by androgens remains unclear. To explore the role of SRC-1 in the mediation of androgenic regulation on the hippocampus, we used immunohistochemistry and Western blots to investigate the expression of hippocampal SRC-1, androgen and estrogen receptors, and synaptic proteins GluR-1 and PSD-95 after the administration of testosterone.

## 2. Materials and methods

### 2.1. Animals and testosterone administration

Adult male SPF grade C57BL/6 mice (12 weeks old,  $22 \pm 2$  g) were obtained from the Experimental Animal Center of Daping Hospital, Third Military Medical University. All of the animal-related procedures were conducted in strict compliance with the Approved Institutional Animal Care and Use Protocols of this university. The animals were randomly divided into five groups. Animal surgery was carried out according to our previously reported procedures [34]. In short, mice were anesthetized with 100 mg/kg 4% chloral hydrate, the fur was clipped over the surgical area and scrubbed with Betadine and an ethanol swipe. The skin of the scrotum was then opened, the epididymis was cut and testes were completely removed, followed by suture of the wound. The testosterone treatment groups were injected with a testosterone propionate injection (H31020524, Shanghai General Pharmaceutical Co., Ltd., Shanghai, China) subcutaneously with a dose of 0.5, 1.0, or 2.0 mg/kg body weight. A sham-operation group of animals that received an injection of an equal amount of soybean oil was

used as the control group. The injection was carried out every afternoon and lasted 2 weeks.

### 2.2. Tissue section preparation

Tissue section preparation was carried out according to our previous reports [14,28]. Briefly, two weeks after testosterone administration, the mice in each group ( $n=5$ ) were deeply anaesthetized with 100 mg/kg 4% chloral hydrate and perfused transcardially with saline followed by 4% paraformaldehyde in phosphate buffer (PBS, pH 7.4). The brains were carefully dissected, removed, fixed overnight with the same fixative, and transferred to freshly-prepared fixative containing 30% sucrose until they sank to the bottom of the container. Brains were then serially cut into 20  $\mu$ m-thick coronal sections with a cryostat (CM1900, Leica Microsystems, Germany). Following the principles of unbiased and systematic random sampling, the serially cut hippocampus-containing sections (between Bregma  $-0.82$  and  $-3.80$ ) were transferred into one six well plate, with every sixth section being placed in the same well.

### 2.3. Immunohistochemistry

Nickel-intensified immunohistochemistry was carried out as previously described [14]. Briefly, free-floating sections were first washed with PBS (10 mmol/L; pH 7.4), quenched for 15 min in 3% H<sub>2</sub>O<sub>2</sub>, and washed with PBS at room temperature. The sections were then incubated at 4°C overnight with the individual primary antibodies diluted in Antibody Diluent (ZLI-9028, Zhongshan Biotech, Beijing, China). After being washed, the sections were incubated with the biotinylated secondary goat-anti-rabbit antibody (1:200; ZB2010, Zhongshan Biotech) for 1 h at room temperature. The sections were washed in PBS again, incubated with the HRP-labeled streptavidin reagent (1:200; ZB2404, Zhongshan Biotech) for 1 h at room temperature and then visualized using a DAB-nickel chromogen kit (SK-4100; Vector Laboratories Inc., Burlingame, USA) for 5 min at room temperature. Finally, the sections were dehydrated, cleared and mounted.

Images of the stained sections were recorded using a digital camera (DP70, Leica Microsystems, Wetzlar, Germany) equipped with an Olympus microscope (BX60, Tokyo, Japan) as previously reported [31,32]. The average optical density of sections between Bregma  $-1.46$  and  $-2.46$  was measured using Image Pro Plus software 6.0 (Media Cybernetics, Rockville, USA), and the mean value was used to represent the regional expression level for each group.

The primary antibodies used in this study were as follows: rabbit polyclonal anti-SRC-1 (1:200; sc-8995, Santa Cruz, Dallas, USA), rabbit polyclonal anti-AR (1:50; sc-13062, Santa Cruz), rabbit polyclonal anti-ER $\alpha$  (1:50; sc-542, Santa Cruz), rabbit polyclonal anti-ER $\beta$  (1:50; sc-8974, Santa Cruz), rabbit mAb-PSD95 (1:200; 3409, Cell Signaling, Danvers, USA) and rabbit mAb-GluR-1 (1:100; 04-855, MerckMillipore, Shanghai, China).

### 2.4. Western blot

Western blot analysis was carried out according to our previous description [29,34]. Proteins of the hippocampus of the mice were extracted using a Protein Extract Kit (P0027, Beyotime Biotech; Beijing, China) and the protein concentration was determined using a BCA Assay Kit (P0010, Beyotime Biotech). Nuclear protein (for SRC-1, AR and ERs) or cytoplasmic protein (for synaptic proteins) samples were diluted in loading buffer and subjected to SDS-PAGE followed by transfer to PVDF membranes. The membranes were blocked with 5% fresh-prepared milk-TBST

overnight at 4 °C, washed with TBST and then incubated with the individual diluted primary antibodies diluted at 4 °C overnight. After TBST washes, the membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:2000, ZB-2301, Zhongshan Biotech), goat anti-mouse secondary antibody (1:2000, ZB-2305, Zhongshan Biotech), or rabbit anti-goat secondary antibody (1:2000, ZB-2306, Zhongshan Biotech), for 1 h at room temperature. The blots were finally visualized with chemiluminescent HRP Substrate (WBKLS0100, MerckMillipore) for 1 min by Western Lightning-ECL (Bio-Rad, Hercules, USA). The optical density for each band was measured using Quantity One software (Bio-Rad) and normalized to that of  $\beta$ -actin. A blank control (without the primary antibody) was used to determine the specificity of the primary antibodies and a pre-stained protein marker was employed to localize the specific band of the primary antibodies.

The antibodies used were as follows: rabbit polyclonal anti-SRC-1 (1:600; sc-8995, Santa Cruz), rabbit polyclonal anti-AR (1:50; sc-13062, Santa Cruz), rabbit polyclonal anti-ER $\alpha$  (1:50; sc-542, Santa Cruz), rabbit polyclonal anti-ER $\beta$  (1:50; sc-8974, Santa Cruz), rabbit mAb-PSD95 (1:800; 3409, Cell Signaling), rabbit mAb-GluR-1 (1:600; 04-855, MerckMillipore), mouse mAb-Actin (1:1000, AA128, Beyotime Biotech), rabbit polyclonal anti-GPR30 (1:300; sc-48525-R, Santa Cruz) and goat polyclonal anti-spinophilin (1:800; sc-14774, Santa Cruz).

### 2.5. Statistical analysis

All results are shown as the mean  $\pm$  S.E. One-way ANOVA followed by LSD-test was conducted using SPSS software (version 13.0, IBM; Chicago, USA), and  $p < 0.05$  was considered statistically significant.

## 3. Results

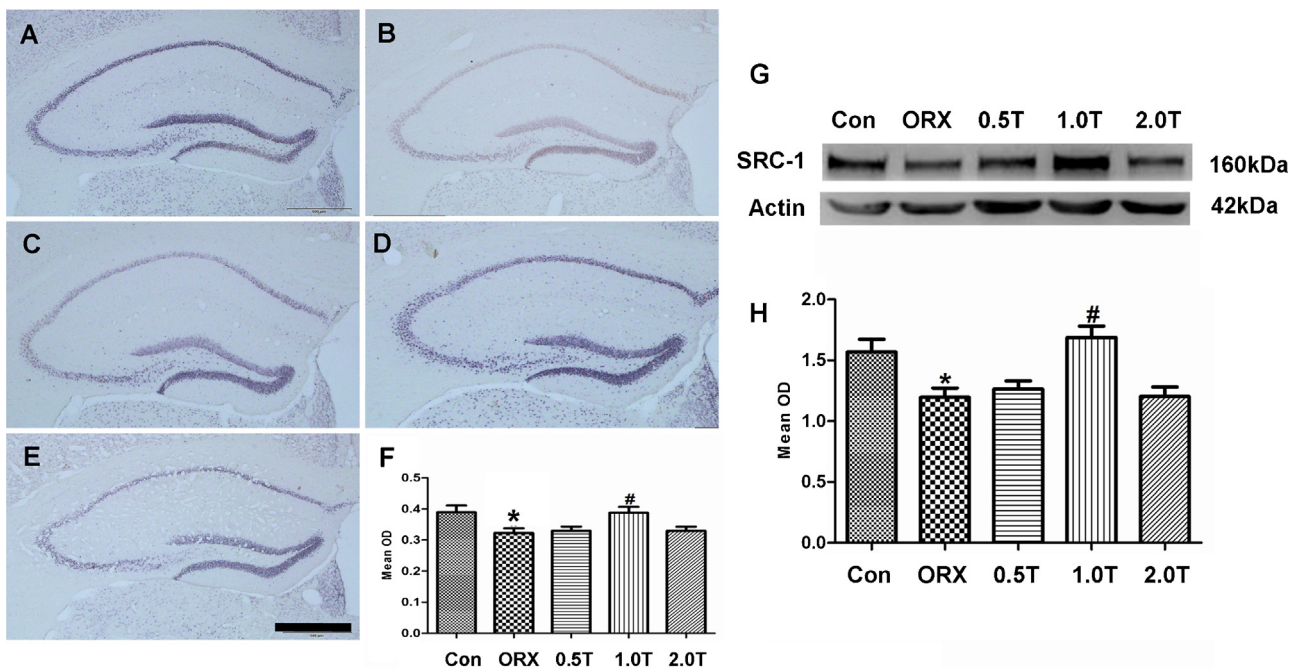
### 3.1. Testosterone reversed the ORX-induced decrease of hippocampal SRC-1

Intense SRC-1 immunoreactivity was detected in the hippocampus of adult mice, consistent with our previous reports. One-way ANOVA indicated a significant effect of the treatment ( $F_{(4,30)} = 4.065$ ,  $p < 0.01$ ). In the hippocampus, ORX induced a dramatic decrease in SRC-1 immunoreactivity when compared to that of the control (sham plus oil injection) male mice, as shown in Fig. 1A and B ( $p < 0.01$ ). This decrease was not restored by testosterone at a concentration of 0.5 mg/kg, as indicated in Fig. 1C ( $p > 0.05$ ). The levels of hippocampal SRC-1 were increased by higher concentrations of testosterone (1.0 mg/kg B.W.;  $p = 0.01$  when compared to the ORX mice) and reached the control level ( $p > 0.05$  when compared to the control mice; Fig. 1D). However, a 2.0 mg/kg B.W. injection of testosterone induced a significant decrease of hippocampal SRC-1 when compared to that of the control, or to that of mice that received a 1.0 mg/kg injection, as shown in Fig. 1E ( $p < 0.05$ ).

In the Western blot analysis, one-way ANOVA revealed a significant effect of the treatment ( $F_{(4,10)} = 7.243$ ,  $p < 0.01$ ). The results also showed that a 1.0 mg/kg testosterone injection caused a significant increase of SRC-1 when compared to that of the control or ORX-treated mice ( $p < 0.01$ ). However, 2.0 mg/kg testosterone induced a significant decrease of SRC-1 when compared to that of the control or the 1.0 mg/kg group, as shown in Fig. 1F ( $p < 0.01$ ).

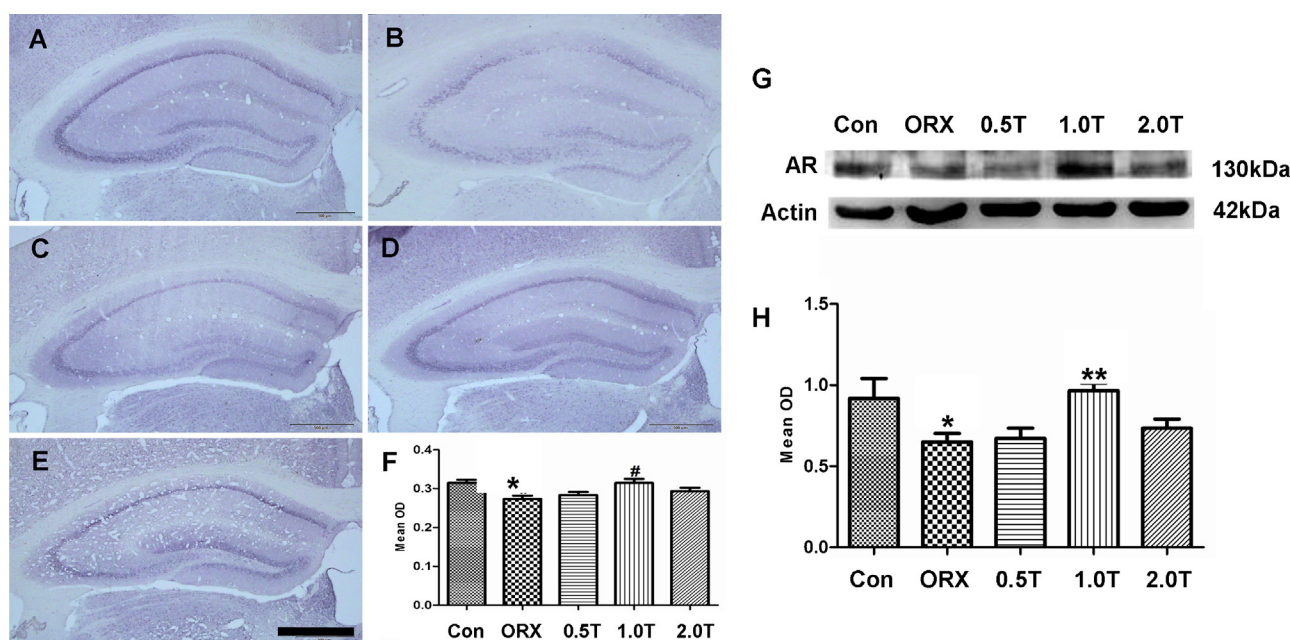
### 3.2. Testosterone restored ORX-induced decrease of hippocampal AR

One-way ANOVA for AR revealed a significant effect of the treatment ( $F_{(4,30)} = 4.441$ ,  $p < 0.01$  for immunohistochemistry and



**Fig. 1.** Testosterone restored the ORX-induced decrease of hippocampal SRC-1 in a dose-dependent manner. SRC-1 immunoreactivity can be detected in the CAs and dentate gyrus of hippocampal formation (A), and it was significantly decreased by ORX (B). This decrease was dramatically restored by 1.0 mg/kg (D) but not by 0.5 mg/kg testosterone treatment (C). However, a high concentration of testosterone (2.0 mg/kg) induced a decrease of hippocampal SRC-1 (E). Similar results were also demonstrated by Western blot (G). (F and H) Show the results of the statistical analysis. Con: control; ORX: orchidectomy; T: mg/kg testosterone. \*:  $p < 0.05$  when compared to the control mice (one-way ANOVA, LSD-test). #:  $p < 0.01$  when compared to the ORX animals. Bar = 500  $\mu$ m.

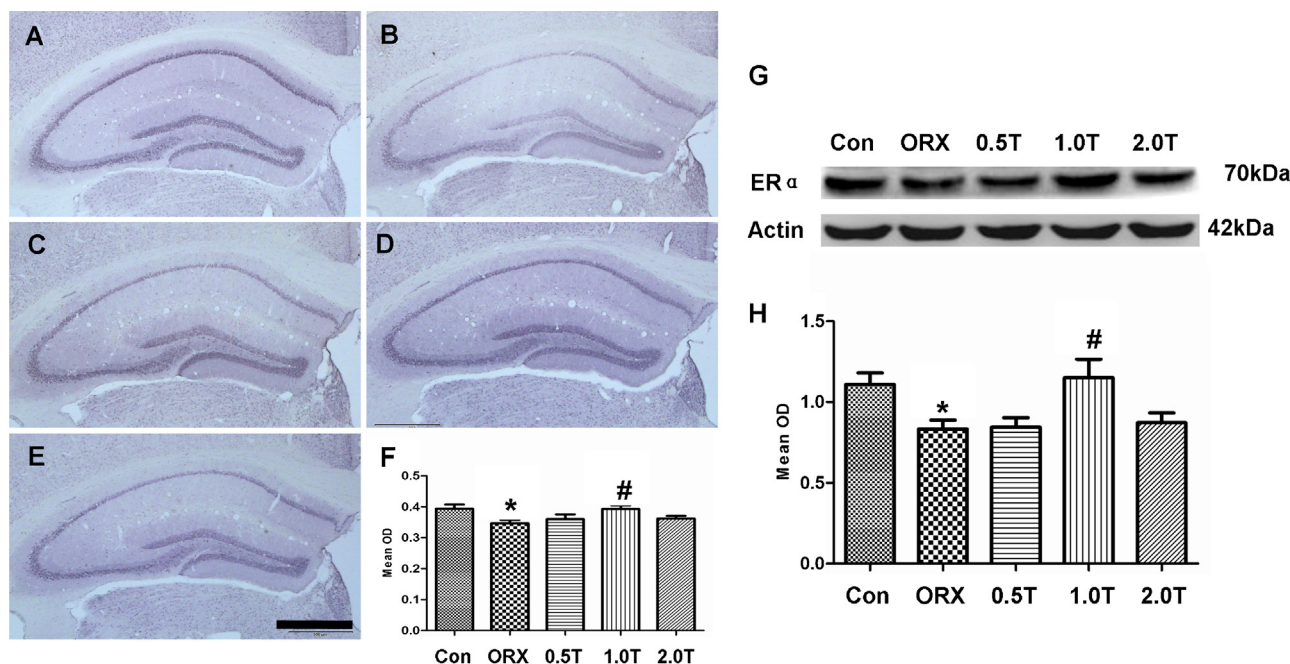




**Fig. 2.** Testosterone restored the ORX-induced decrease of hippocampal AR dose-dependently. AR immunoreactivity (A) was significantly decreased by ORX (B). This decrease was dramatically restored by 1.0 mg/kg (D) but not by 0.5 mg/kg testosterone treatment (C). High concentration of testosterone (2.0 mg/kg) did not change the expression of hippocampal AR (E). Similar results were also demonstrated by Western blot (G). (F and H) Show the results of the statistical analysis. Con: control; ORX: orchidectomy; T: mg/kg testosterone. \*:  $p < 0.01$  when compared to the control mice; #:  $p < 0.05$  when compared to the ORX animals; \*\*:  $p < 0.01$  when compared to the ORX animals (one-way ANOVA, LSD-test). Bar = 500  $\mu$ m.

$F_{(4,10)} = 3.862$ ,  $p < 0.05$  for Western blot, respectively). The immunolocalization profile of AR in the hippocampus of adult male mice was similar to that of SRC-1. The levels of AR immunoreactivity were dramatically decreased by ORX when compared to that of the control mice ( $p < 0.01$ ), as shown in Fig. 2A and B. This decrease was not restored by 0.5 mg/kg testosterone ( $p > 0.05$ ), as shown in Fig. 2C. However, it was significantly restored by 1.0 mg/kg

testosterone ( $p < 0.01$  when compared to the ORX mice or to the 0.5 mg/kg testosterone treated group; Fig. 2D). Additionally, levels of hippocampal AR did not show any difference at 2.0 mg/kg testosterone treatment when compared to the ORX animals as shown in Fig. 2E ( $p > 0.05$ ). Similar results were also demonstrated by Western blot analysis, as shown in Fig. 2F, which showed that the ORX-induced significant decrease of AR was upregulated by



**Fig. 3.** Testosterone restored the ORX-induced decrease of hippocampal ERα in a dose-dependent manner. ERα immunoreactive materials (A) were significantly decreased by ORX (B). This decrease was dramatically restored by 1.0 mg/kg (D) but not by 0.5 mg/kg testosterone treatment (C). A high concentration of testosterone (2.0 mg/kg) did not change the expression of hippocampal ERα (E). Similar results were also demonstrated by Western blot (G). (F and H) Show the results of the statistical analysis. Con: control; ORX: orchidectomy; T: mg/kg testosterone. \*:  $p < 0.05$  when compared to the control mice; #:  $p < 0.05$  when compared to the ORX, 0.5T and 2.0T animals (one-way ANOVA, LSD-test). Bar = 500  $\mu$ m.

1.0 mg/kg testosterone when compared with that of the ORX and 0.5 mg/kg testosterone treated group ( $p < 0.05$ ).

### 3.3. Testosterone differentially affected hippocampal ERs

For ER $\alpha$ , one-way ANOVA indicated a significant effect of treatment ( $F_{(4,30)} = 2.486$ ,  $p < 0.05$  for immunohistochemistry and  $F_{(4,10)} = 4.226$ ,  $p < 0.05$  for Western blot). The levels of ER $\alpha$  immunoreactivity were dramatically decreased by ORX when compared to that of the control mice, as shown in Fig. 3A and B ( $p < 0.01$ ). This decrease was not restored by 0.5 mg/kg testosterone, as indicated in Fig. 3C ( $p > 0.05$ ). However, it was significantly restored by 1.0 mg/kg testosterone ( $p < 0.01$  when compared to the ORX mice; Fig. 3D). Additionally, 2.0 mg/kg testosterone injection did not cause any increase in the hippocampal ER $\alpha$  when compared to that of other groups, as shown Fig. 3E ( $p > 0.05$ ). Similar results were also detected with Western blot analysis, as shown in Fig. 3F, but the 2.0 mg/kg testosterone treated mice showed a significant decrease of ER $\alpha$  when compared with that of the 1.0 mg/kg testosterone-treated mice ( $p < 0.05$ ).

One-way ANOVA revealed a significant effect of treatment on ER $\beta$  ( $F_{(4,30)} = 2.988$ ,  $p < 0.05$  for immunohistochemistry and  $F_{(4,10)} = 4.799$ ,  $p < 0.05$  for Western blot). ER $\beta$  expression was dramatically decreased by ORX when compared to that of the control mice ( $p < 0.01$ ), as shown in Fig. 4A and B. This decrease was significantly restored by 0.5 mg/kg testosterone, as indicated in Fig. 4C ( $p < 0.05$ ). A testosterone injection of 1.0 mg/kg or 2.0 mg/kg did not cause an additional increase of hippocampal ER $\beta$  ( $p > 0.05$  when compared to the 0.5 mg/kg testosterone treated mice; Fig. 4D and E). Similar results were also detected by Western blot analysis, as shown in Fig. 4F; however, 2.0 mg/kg-treated mice showed a significant decrease of ER $\beta$  when compared to that of the 0.5 mg/kg-treated mice ( $p < 0.05$ ; one-way ANOVA, LSD-test).

For GPR30, one-way ANOVA revealed no significant effects of the trial ( $F_{(4,10)} = 2.194$ ,  $p > 0.05$ ). Western blot analysis showed that

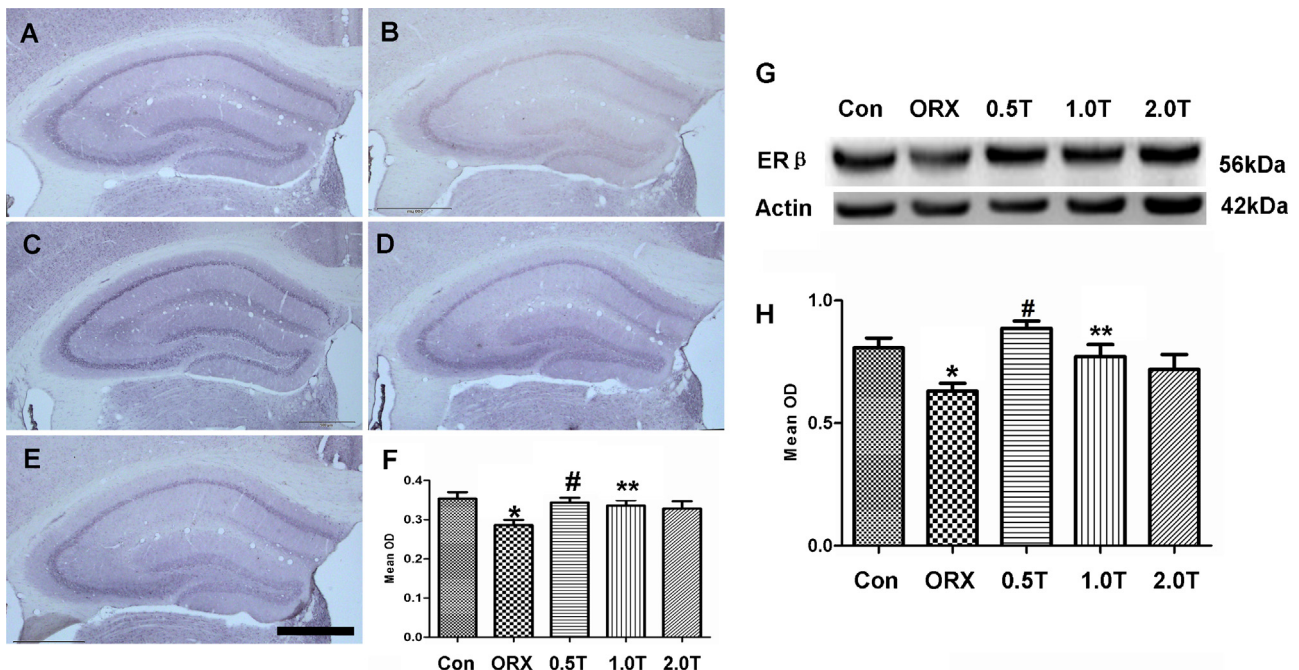
expression of GPR30 was not significantly affected by ORX or the 2-week testosterone administration at the tested doses ( $p > 0.05$ , Fig. 7A and B).

### 3.4. Testosterone differentially affected hippocampal synaptic proteins

One-way ANOVA for GluR-1 revealed a significant effect of the treatment ( $F_{(4,30)} = 3.800$ ,  $p < 0.05$  for immunohistochemistry and  $F_{(4,10)} = 3.507$ ,  $p < 0.05$  for Western blot). Levels of GluR-1 immunoreactivity were dramatically decreased by ORX when compared to that of the control mice ( $p < 0.01$ ) as shown in Fig. 5A and B. This decrease was not restored by a 0.5 mg/kg testosterone injection, as shown in Fig. 5C ( $p > 0.05$ ). However, it was significantly restored by a 1.0 mg/kg testosterone injection ( $p < 0.01$  when compared to the ORX or 0.5 mg/kg testosterone injection mice but  $p > 0.05$  when compared to that of the control mice; Fig. 5D). Additionally, 2.0 mg/kg of testosterone did not cause additional changes in hippocampal GluR-1 when compared to that of other groups, as shown in Fig. 5E ( $p > 0.05$ ). Western blot analysis (Fig. 5F) showed that a 0.5 mg/kg testosterone injection could induce a significant increase of GluR-1 ( $p < 0.05$  when compared to that of the ORX group), and this was further increased by 1.0 mg/kg testosterone.

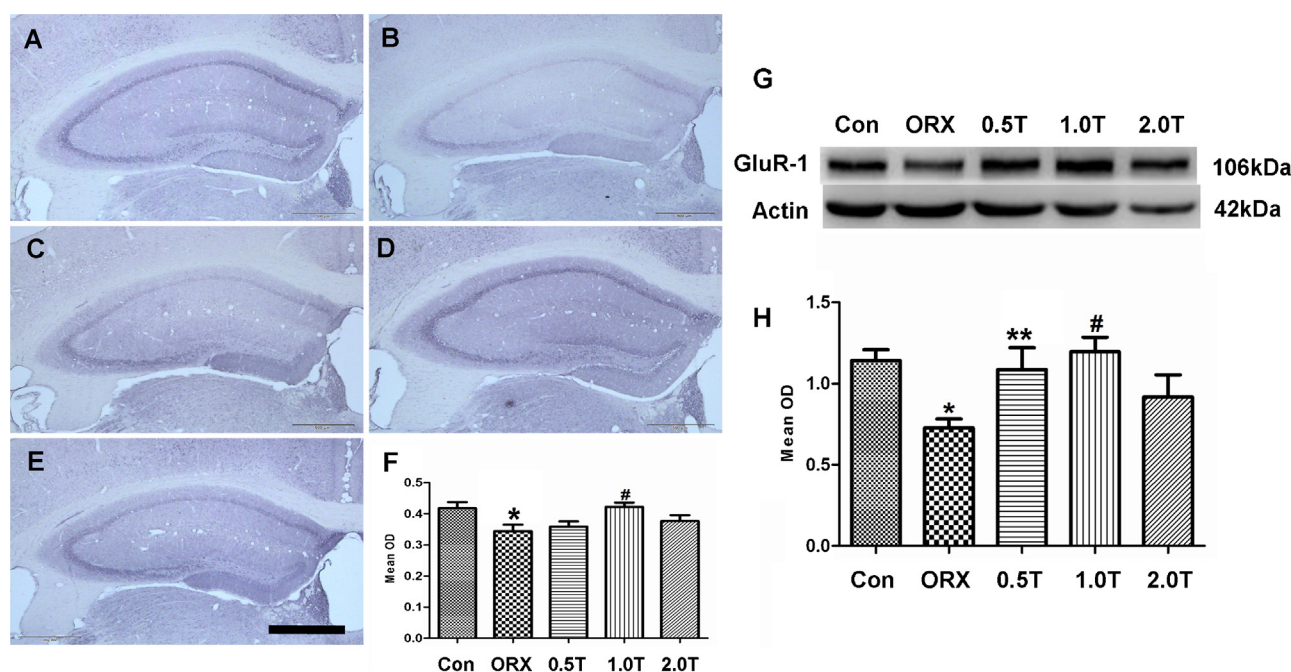
As for PSD-95, one-way ANOVA revealed a significant effect of ORX ( $F_{(4,30)} = 2.728$ ,  $p < 0.05$ ). PSD-95 expression was dramatically decreased by ORX when compared to that of the control mice ( $p < 0.01$ ), as shown in Fig. 6A and B. This decrease was not restored by 0.5 mg/kg testosterone ( $p > 0.05$ ), as indicated in Fig. 6C. However, it was significantly restored by a 1.0 mg/kg injection of testosterone ( $p < 0.05$  when compared to the ORX mice; Fig. 6D). Additionally, a 2.0 mg/kg B.W. ( $p < 0.05$ ) testosterone injection did not induce further changes of hippocampal PSD-95 when compared to that of the control mice, as shown Fig. 6E ( $p > 0.05$ ).

However, one-way ANOVA revealed a nonsignificant effect of the trial on spinophilin expression ( $F_{(4,10)} = 0.130$ ,  $p > 0.05$ ). Western blot analysis showed that expression of spinophilin was not



**Fig. 4.** Testosterone restored the ORX-induced decrease in hippocampal ER $\beta$  in a dose-dependent manner. ER $\beta$  immunoreactive materials (A) were significantly decreased by ORX (B). This decrease was dramatically restored by 0.5 mg/kg (C) and 1.0 mg/kg testosterone treatment (D). There were no significant differences between 1.0 and 2.0 mg/kg testosterone treatment (E). Similar results were also demonstrated by Western blot (G). (F and H) Show the results of the statistical analysis. Con: control; ORX: orchidectomy; T: mg/kg testosterone. \*:  $p < 0.01$  when compared to the control mice; #:  $p < 0.05$  when compared to the ORX animals; \*\*:  $p < 0.05$  when compared to the ORX animals (one-way ANOVA, LSD-test). Bar = 500  $\mu$ m.





**Fig. 5.** Testosterone restored the ORX-induced decrease in hippocampal GluR-1 in a dose-dependent manner. GluR-1 immunoreactivity (A) was significantly decreased by ORX (B). This decrease was dramatically restored by 1.0 mg/kg (D) but not by 0.5 mg/kg testosterone treatment (C). Levels of GluR-1 did not show any differences between 2.0 mg/kg testosterone treatment (E) and other groups. Similar results were also demonstrated by Western blot (G). (F and H) Show the results of the statistical analysis. Con: control; ORX: orchidectomy; T: mg/kg testosterone. \*:  $p < 0.01$  when compared to the control mice; #:  $p < 0.01$  when compared to the ORX or the 0.5 mg/kg treated animals; \*\*:  $p < 0.05$  when compared to the ORX animals (one-way ANOVA, LSD-test). Bar = 500  $\mu\text{m}$ .

significantly affected by ORX or the 2-week testosterone administration at the tested doses ( $p > 0.05$ , Fig. 7C and D).

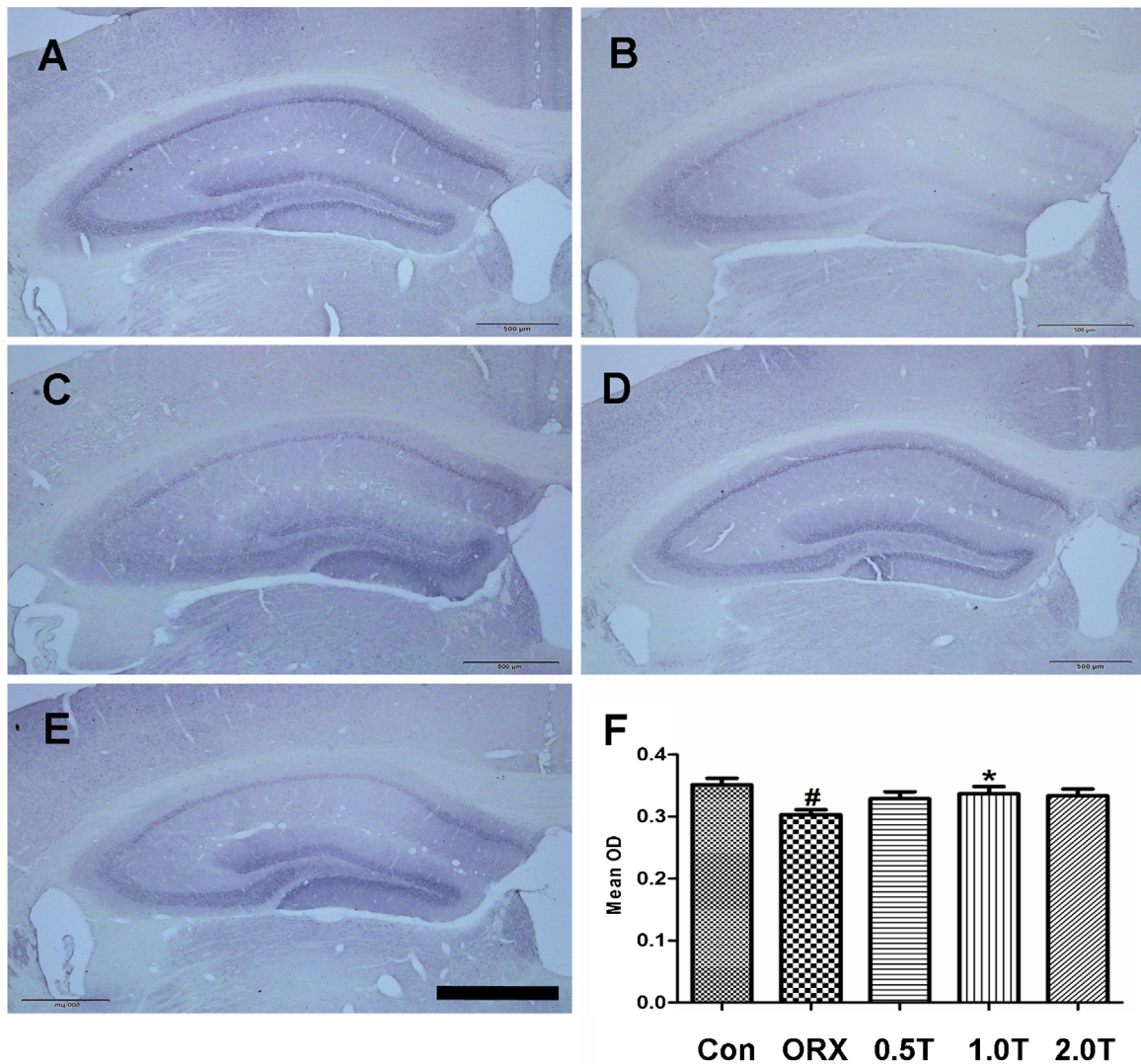
#### 4. Discussion

The structure and function of the hippocampus, including the expression of synaptic proteins, spinogenesis, synaptogenesis, functional plasticity LTP and LTD, learning and memory, and cognition are profoundly affected by sex steroids [37]. However, the underlying mechanisms of this relationship remain largely unclear. It is known that androgens exert their function through either androgen receptors and/or estrogen receptors, and SRC-1 has been shown to coactivate the transcription activities of these nuclear receptors. We previously reported that hippocampal SRC-1 is regulated by androgens, and it may be involved in the modulation of hippocampal synaptic plasticity. Therefore, in the present study, we investigated the effects of testosterone treatment on the expression of hippocampal SRC-1, AR and ERs as well as some synaptic proteins. We found that expression of hippocampal SRC-1 in adult male mice was upregulated by testosterone in a dose-dependent manner. This upregulation was accompanied by a similar profile of nuclear AR and ERs (ER $\alpha$  and ER $\beta$ ). Additionally, we noticed that levels of the synaptic proteins GluR-1 and PSD-95 were also upregulated by testosterone in a dose-dependent manner. However, expression of the membrane estrogen receptor GPR30 and the dendritic spine marker spinophilin did not change with ORX or testosterone treatment.

We have demonstrated the first direct evidence that hippocampal SRC-1 expression can be significantly upregulated by testosterone administration (1.0 mg/kg). The expression of hippocampal SRC-1 has been previously reported. Meijer et al. first reported SRC-1 transcripts in the hippocampus of mature Wistar rats using *in situ* hybridization [38]. Ogawa et al. first reported strong SRC-1-immunoreactivity in the pyramidal cells and the granular cells of the hippocampus [26]. This was confirmed by our previous studies [28–30,33–36]. However, only limited studies

have reported the regulation of SRC-1 in specific brain regions. Tetel et al. reported that in the bed nucleus of the stria terminalis and medial amygdala of Siberian hamster brain, expression of SRC-1 was decreased by short days [39]. Charlier et al. reported that in the hypothalamus of male Japanese quail, expression of SRC-1 was upregulated by testosterone [40]. In our previous studies, we showed that hippocampal SRC-1 was regulated by postnatal development and aging [28,29,33], indicating that developmental- and aging-related alterations of circulating and/or hippocampal sex hormones may be closely related to the changes in SRC-1. Furthermore, a significant male-predominance of hippocampal SRC-1 was observed in the brains of mice [30], which was significantly downregulated by ORX [34,35] and letrozole administration [36], but not by OVX [34]. These findings indicated a potential role of androgens in the regulation of hippocampal SRC-1. In the present study, using testosterone administration, we further demonstrated that hippocampal SRC-1 was indeed upregulated by testosterone in a dose dependent manner; this finding is in agreement with our previous results that androgens and their aromatization may be the main regulator of local SRC-1.

Because androgens can function either by direct binding to AR or by aromatization into E2 then through binding to ER $\alpha$ , ER $\beta$  and/or GPR30, we examined the changes of these receptors after testosterone treatment. We found that AR and ER $\alpha$  were significantly regulated by 1.0 mg/kg testosterone treatment, and the levels of ER $\beta$  were significantly regulated by 0.5 mg/kg testosterone treatment. These results were in general agreement with previous studies that reported the involvement of AR and nuclear ERs in the mediation of androgens/aromatization action on hippocampus. For example, the AR antagonist flutamide was capable of blocking the action of testosterone on spatial memory during cognitive tests [41], ER $\alpha$  may mediate letrozole-induced alterations of hippocampal synaptic plasticity [42] and ER $\beta$  may, at least in part, mediate the effects of androgens on learning enhancement [43]. However, few studies have addressed the direct evidence for regulation by androgens on these nuclear



**Fig. 6.** Testosterone restored the ORX-induced decrease in hippocampal PSD-95 in a dose-dependent manner. Dense PSD-95 immunoreactive materials can be detected in the hippocampus (A); it was significantly decreased by ORX (B). This decrease was dramatically restored by 1.0 mg/kg (D) but not by 0.5 mg/kg testosterone treatment (C). Levels of PSD-95 did not show any differences between 2.0 mg/kg testosterone treatment (E) and other groups. (F) Shows the results of the statistical analysis. Con: control; ORX: orchidectomy; T: mg/kg testosterone. #:  $p < 0.01$  when compared to the control and 1.0T mice; \*:  $p < 0.01$  when compared to the ORX (one-way ANOVA, LSD-test). Bar = 500  $\mu$ m.

receptors, and some of these results were indeed controversial. For example, some early reports did not detect any effect of testosterone or castration on the expression of AR in the hippocampus [41,43]; however, later studies reported that hippocampal AR was reduced by castration and restored by androgen/testosterone replacement [44–46]. Our recent studies reported that in the hippocampus, levels of AR, as well as ER $\alpha$  and ER $\beta$ , decreased significantly after letrozole injection [36]. In the present study, we found that expression of AR, ER $\alpha$  and ER $\beta$  was significantly upregulated by testosterone treatment at various levels. These results strongly suggest that both the direct AR-binding pathway and indirect nuclear ER pathway after aromatization into E2 are involved in the androgenic regulation of the hippocampus. A recent finding reported that in the hippocampus slices from young and adolescent male rats, LTD and depotentiation depend on AR, while LTP depends on ERs in both age groups. This also indicates that AR and ERs may be collaborative in determining hippocampal synaptic plasticity in male rats [47].

Furthermore, we also noticed that ER $\alpha$  and ER $\beta$  showed different responses to testosterone treatment, such as the

inverted-U response of ER $\beta$  to testosterone. The reason for this finding may be that ER $\alpha$  and ER $\beta$  could function differentially in the regulation of hippocampal synaptic plasticity [48]. For example, Mukai H et al. reported that slow actions of 17 $\beta$ -estradiol occur via classical nuclear ER $\alpha$  or ER $\beta$ , while rapid E2 actions occur via synapse-localized ER $\alpha$  or ER $\beta$  [7]. The most convincing evidence is from Chamniansawat and Chongthammakun, who reported that the aromatase inhibitor anastrozole suppressed ER $\beta$  but not ER $\alpha$  in hippocampal H19-7 cells [49], indicating the susceptibility of ER $\beta$  under steroid action (downregulated by aromatase inhibition or higher doses of testosterone). Another unexpected result in the present study was that hippocampal GPR30 was not regulated by ORX or testosterone treatment. To our knowledge, only one study has shown that activation of GPR30 could decrease testosterone levels in the rat and human testis [50], and no studies reported the regulation of GPR30 by testosterone in any tissues. The expression of GPR30 may remain unchanged after ORX or testosterone treatment because testosterone regulation does not involve this receptor. Alternately, this lack of change may be due to the relatively long duration of treatment



**Fig. 7.** Effects of ORX and testosterone treatment on hippocampal GPR30 and spinophilin. (A and B) Expression of GPR30 was not affected by ORX or 0.5–2.0 mg/kg testosterone; (C and D) Expression of spinophilin was not affected by ORX or 0.5–2.0 mg/kg testosterone. Con: control; ORX: orchidectomy; T: mg/kg testosterone; Spino: spinophilin.

in our studies, since GPR30 mediates rapid non-genomic effects of estrogens, while the treatment time in our studies lasted two weeks.

Because the close relationship between SRC-1 and synaptic proteins, we examined the expression of GluR-1, PSD-95 and spinophilin under androgen manipulation. We found that the ORX-induced decrease of the first two proteins could be restored by testosterone replacement. These results were in agreement with previous findings that modulation of ER $\beta$  influences hippocampal GluR-1 and PSD-95 [22], an ER $\alpha$  agonist induces increased hippocampal ER $\alpha$  [51], ORX induces a decrease in hippocampal GluR-1 and PSD-95 in the adult male mice [34], and testosterone regulates the expression of hippocampal PSD-95 in the male mice [2]. Our latest finding showed that when SRC-1 was inhibited, levels of hippocampal neuronal PSD-95 were significantly down-regulated [52]. These results again strongly suggest a potential role of SRC-1 in the mediation of androgenic regulation in the hippocampus. However, we also found that another synaptic protein, spinophilin, was not regulated by ORX and testosterone treatment. This result is in general agreement with that of Todd et al., who reported that in the ventromedial nucleus of the hypothalamus, spinophilin was upregulated only in females by estradiol treatment [53]. This result, together with our findings, indicates that male spinophilin may not be regulated by steroids. However, the above results still need further verification.

Accumulating studies from rodents and humans have shown that androgens may improve hippocampal structure and function, such as synaptic plasticity, learning and memory as well as cognitive performance. However, these effects and the mechanisms of androgens are not well understood. In the present study, we reported that in the hippocampus of male mice, SRC-1 and synaptic proteins, as well as AR and nuclear ERs could be upregulated by testosterone treatment in a dose-dependent manner. These results strongly indicate that, in the male hippocampus, SRC-1 might mediate androgenic action on hippocampal structure and function through nuclear steroid receptors. However, the exact role (collaborative, antagonistic or compensational) of individual receptors in the androgenic regulation of the hippocampus still needs further investigation. Because many receptors are involved in androgenic actions, such as AR, ER $\alpha$  and

ER $\beta$  and even the novel membrane estrogen receptor GPR30 [54], it is challenging to effectively and comprehensively study the actions of these individual receptor networks. Therefore, because it is a downstream hub factor, the expression and function of hippocampal SRC-1 is interesting. Furthermore, we also noticed that a relatively higher dose of testosterone usually induced a decrease of these factors, indicating that androgens can be neuroprotective or neurotoxic according to the concentration [55]. This should be considered when androgens are used for the prevention and/or treatment of Alzheimer's dementia, although it has been shown that significant age-related decreases of serum androgen levels may be one of the risk factors for Alzheimer's dementia, and androgen treatment can delay the onset and improve the symptoms of dementia [56].

### Conflict of interest

The authors declare that they have no conflicts of interest.

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