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ORIGINAL PAPER

Sex-specific effects of androgen and estrogen on proliferation of the embryonic chicken hypothalamic neurons

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Abstract Effects of androgen and estrogen on proliferation of hypothalamic neurons were evaluated by a chicken hypothalamic neuron-glia coculture model. Hypothalamic cells were dispersed from 17-day-old embryos and challenged with testosterone (T) and 17β-estradiol (E_2) alone or combined with androgen receptor antagonist flutamide, estrogen receptor antagonist tamoxifen, or aromatase inhibitor letrozole for 48 h. The neuron number was counted and the proliferating cells were identified by immunocytochemistry of proliferating cell nuclear antigen (PCNA) and 5-bromo-2-deoxyuridine (BrdU) incorporation. Results showed that both E_2 and T stimulated proliferation of hypothalamic neurons. E_2 showed more intensive effect on females and this promoting effect was abrogated by tamoxifen. T played more intensive effect on males and the effect was inhibited by flutamide, tamoxifen, or letrozole. The above results indicated that E_2 stimulated neuron proliferation through estrogenic actions with more sensitive effect on females and T promoted neuron proliferation through both androgenic and estrogenic actions with more intense effect on males. These observations suggested that steroid hormones influence the proliferation of hypothalamic neurons in a sexually dimorphic manner during the development of chicken embryos.

Keywords Hypothalamic neurons · Androgen · Estrogen · Aromatase · Cell proliferation · Chicken

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Introduction

Gonadal steroids (estrogen and androgen) play important roles in the morphological and neuroendocrine sexual differentiation of mammalian central nervous system [1]. In birds, androgen is important for the expression of male phenotype and is involved in male sexual differentiation, development and maintenance of secondary characteristics, initiation and maintenance of spermatogenesis [2]. Estrogen is proposed to be a pivotal factor involved in the development of sexual differentiation, female secondary sexual characteristics, and vitellogenesis. It is now well established that the central nervous system actively synthesizes estrogen from circulating androgen. Aromatase-containing cells were found in avian brain [3]. Locally synthesized estrogen in the developing brain is thought to influence neural structures by affecting the morphology [4], survival/death, and function of estrogen target cells.

The hypothalamus is implicated in a multitude of vital physiological processes, including sexual behavior and gonadal functions. Earlier report revealed the presence of aromatase and estrogen receptors in specific regions of the brain, such as the hypothalamus, amygdale, and hippocampus, suggesting a role of estrogen in the brain [5]. Aromatase-containing cells were found in the preoptic area and hypothalamus, with a high density of positive cells being presented in the ventromedial nucleus and in the infundibulum of the Japanese quail [6]. These morphological studies indicate complicated effects of gonadal steroids on central nervous system.

In the central nervous system, sexual differentiation is initiated before birth. It is currently accepted that sex differences in brain and behavior do not only result from direct genomic actions, but also from development following early exposure to a sexually endocrine milieu. The

present study was performed to investigate the actions of gonadal steroids on the processes of neuron proliferation in the chicken embryo. Investigation of fetal brain development in vivo by maternal hormone manipulation is difficult to interpret in view of interference by the feto-placental unit, maternal metabolism, and afferents extrinsic to the fetal hypothalamus. To avoid this problem, we prepared hypothalamic cell culture from embryonic chickens. With this culture system, we demonstrated the stimulating effects of testosterone (T) and 17β-estradiol (E_2) on the hypothalamic neuron proliferation. The immunocytochemistry of the proliferating cell nuclear antigen (PCNA) and 5-bromo-2-deoxyuridine (BrdU) incorporation were conducted to assess the proliferation of cultured neurons. The mechanisms of T and E_2 on cell proliferation were revealed by respective blockade of androgen and/or estrogen receptors, or aromatase activity. Furthermore, sex differences of the promoting effect were observed in the cultured hypothalamic neurons.

Results

Morphology of hypothalamic cells in culture

The dispersed hypothalamic cell suspensions from the 17-day-old embryonic chickens contained neuronal and glial cells. Cells underwent morphological differentiation in culture. Some of them attached in clusters and extended neuritis. A monolayer of flat cells spread over the dish, forming a continuous background layer over which grew small refringent cells with an ovoid perikaryon during the first 3 days. Neurons were immunostained positively for the neuron-specific enolase (NSE) (Fig. 1), while glias were negative for NSE.

Effects of E_2 and T on proliferation of hypothalamic neurons

E_2 (10^{-8}–10^{-6} M) significantly increased the number of chicken hypothalamic neurons after 48 h treatment in both males and females, and the female showed higher responsiveness to E_2 stimulation than the male ($P < 0.05$, Fig. 2A). Likewise, treatment of T (10^{-7}–10^{-6} M) also increased the number of hypothalamic neurons in both male and female chickens. On the contrary to E_2 stimulation, the male displayed a higher sensitivity to T than the female ($P < 0.05$, Fig. 2B).

Effects of tamoxifen on E_2-stimulated proliferation of hypothalamic neurons

There was no significant change in the number of hypothalamic neurons after treatment with tamoxifen (10^{-8}–10^{-6} M) alone. However, combined treatment of tamoxifen (10^{-8}–10^{-6} M) and E_2 (10^{-6} M) significantly depressed the E_2-stimulated proliferation of hypothalamic neurons in a dose-dependent manner ($P < 0.05$, Fig. 3).

Effects of flutamide, tamoxifen, and letrozole on T-stimulated proliferation of hypothalamic neurons

No significant change in the number of hypothalamic neurons was observed after treatment with flutamide (3.62×10^{-7}–3.62×10^{-6} M), tamoxifen (10^{-8}–10^{-6} M), and letrozole (10^{-8}–10^{-6} M) alone, but these two receptor antagonists or aromatase inhibitor depressed the proliferation of hypothalamic neurons that was induced by T. The number of neurons was markedly decreased in the combined groups, compared with the group treated with T alone at 10^{-6} M ($P < 0.05$, Fig. 4). Consistent changes of PCNA immunocytochemistry and BrdU incorporation were demonstrated in which flutamide, tamoxifen, or letrozole decreased T-stimulated increase in PCNA labeling index and BrdU-positive cell percentage (Figs. 5 and 6).

Discussion

In the absence of maternal and placental influence, avian embryo provides a good model system to study the role of hormones during fetal development [7, 8]. In this study, we

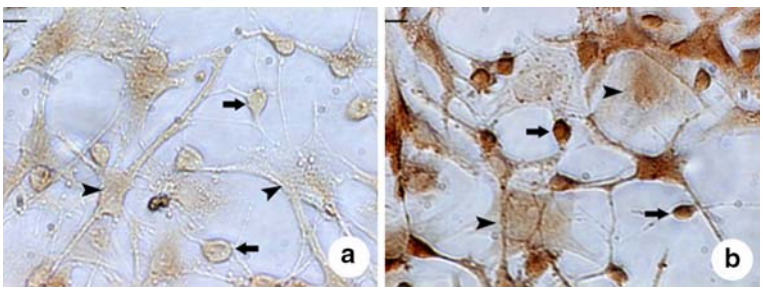

Fig. 1 Immunocytochemical characterization of neurons by NSE. →:Neurons; ➤: glial cells. (**a**) Negative; (**b**) Positive. Scale bar: 10 μm

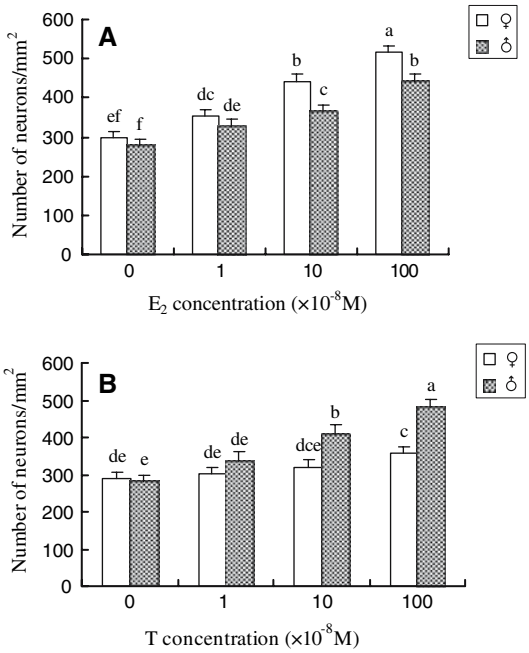

Fig. 2 Effects of E_2 (**A**) and T (**B**) on proliferation of embryonic chicken hypothalamic neurons after 48 h treatment in serum-free medium. Values are the means ± SEM (n = 4). Bars with different superscripts are statistically different ($P < 0.05$)

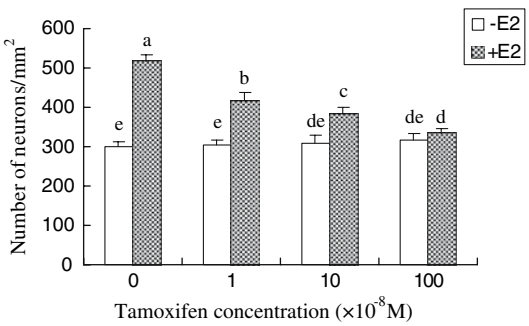

Fig. 3 Effects of tamoxifen on E_2 (1 μM)-stimulated increase in hypothalamic neuron number of female embryonic chickens after 48 h treatment. Values are the means ± SEM (n = 4). Bars with different superscripts are statistically different ($P < 0.05$)

established a neuron-glia coculture model in which neurons could grow upon or among glial cells and manifest increased proliferation in response to gonadal steroids. We distinguished neuronal from glial cells with both cell morphology and NSE immunocytochemistry. The proliferating effects of gonadal steroids on hypothalamic neurons were elucidated by changes in the cell number and immunocytochemical study of PCNA and BrdU incorporation. In addition, sex differences of the stimulating effects were explored in which T displayed more sensitive effect on the male while E_2 imposed more sensitive effect on the female.

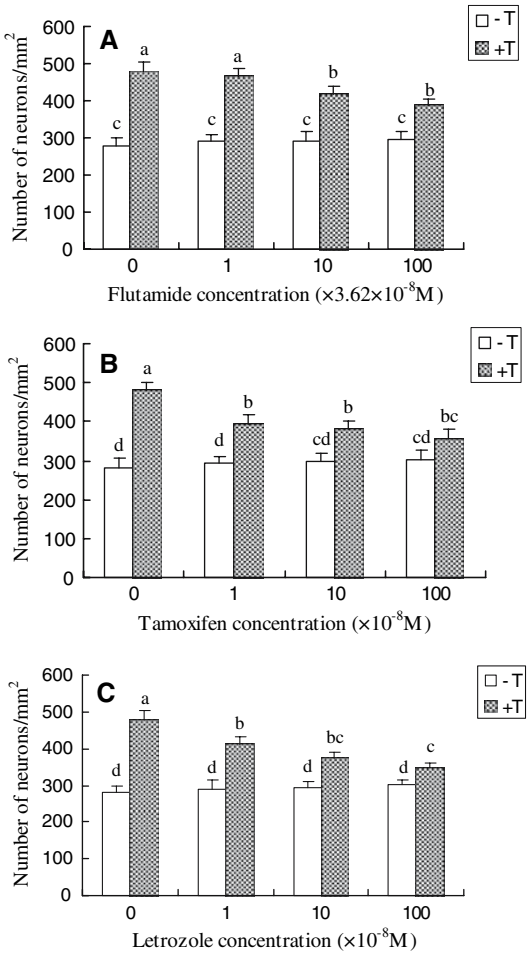

Fig. 4 Changes in hypothalamic neuron number in male embryonic chickens after treatment with T (10^{-6} M) alone and combined with flutamide (**A**), tamoxifen (**B**) or letrozole (**C**) for 48 h culture. Values are the means ± SEM (n = 4). Bars with different superscripts are statistically different

Proliferating effects of steroid hormones on hypothalamic neurons

Previous studies elucidated extensive effects of gonadal steroids on different organs. At the gonadal level, estrogens stimulated proliferation of ovarian germ cells [9] and androgen promoted proliferation of testicular germ cells [10] in embryonic chickens. Gonadal steroids also play an essential role in the development of central nervous system, as well as in maintaining normal brain function in adulthood. During the fetal development, gonadal steroids influence the sexual differentiation of the central nervous system and behaviors [11, 12]. In addition, estradiol influenced cell proliferation in the adult brain under normal physiological conditions [13]. In this study, we revealed that the number of neurons was increased by E_2 and T treatment in both males and females. Consistent with these findings, we found that T increased the number of PCNA or

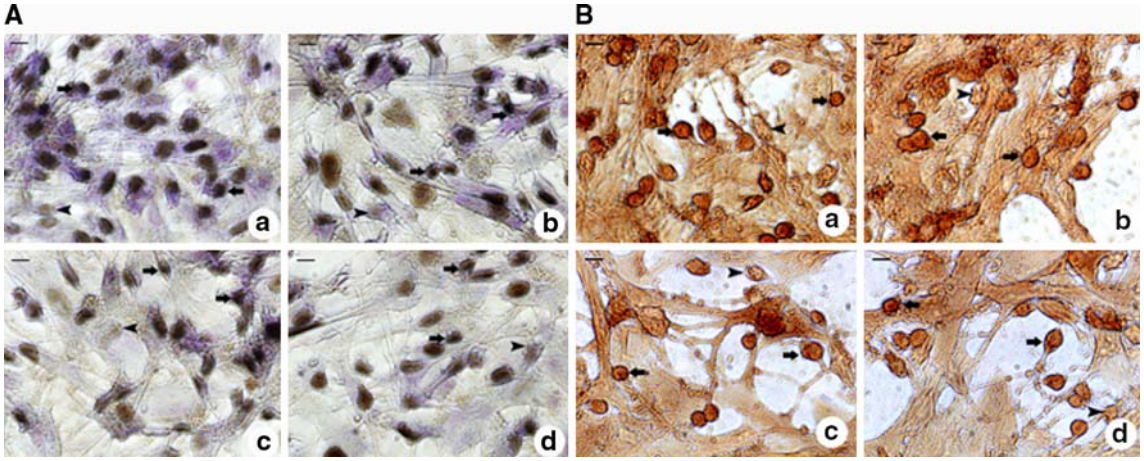

Fig. 5 Demonstration of proliferating neurons by immunocytochemical staining of PCNA (**A**) and BrdU (**B**) in hypothalamic neurons of male embryonic chickens after 48 h treatment. (**a–d**) indicated hypothalamic cells treated with T (10^{-6} M), T + flutamide (3.62×10^{-6} M), T + tamoxifen (10^{-6} M), T + letrozole (10^{-6} M), respectively. *Arrows* show PCNA or BrdU-positive neurons and arrowheads show the negative neurons. Scale bar: 10 μm

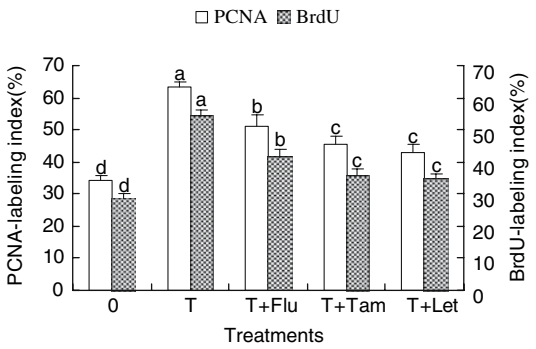

Fig. 6 Quantitative changes in PCNA and BrdU labeling index in hypothalamic neurons from embryonic chickens after treatment with T (10^{-6} M) alone or in combinations with flutamide (Flu, 3.62×10^{-6} M), tamoxifen (Tam, 10^{-6} M), or letrozole (Let, 10^{-6} M). Values are the means ± SEM ($n = 4$). Bars with different superscripts are statistically different ($P < 0.05$)

BrdU-labeled cells. These results indicated that androgen and estrogen promoted the proliferation of hypothalamic neurons.

Pathways of estrogenic and androgenic actions

In the present study tamoxifen inhibited the stimulating action of T/E_2 on neuron proliferation. Estrogen receptors were expressed in a large quantity in avian hypothalamic cells [14]. By acting via its receptor, estrogen could elicit direct transcriptional effects to regulate gene expression [15]. According to these analyses, both E_2 and T (converted into estrogen by aromatase) may exert the promoting effects through binding to the estrogen receptor directly on hypothalamic neurons or indirectly on glial cells. Furthermore, lower concentration of tamoxifen could inhibit the E_2-stimulited neuron proliferation. Borgna and Rochefort found that many estrogen target tissues in chickens and rodents were able to convert tamoxifen into 4-hydroxytamoxifen in vitro and in vivo [16]. 4-Hydroxytamoxifen was the highest-affinity compound and was 100-fold more active than tamoxifen in MCF7 cells [17]. On the other hand, the metabolic pathway of estradiol to estrone appeared to be favored in microsomes prepared from liver, intestine, stomach, kidney, brain, and heart [18]. Though E_2 is the most active endogenous estrogen, the metabolism of E_2 into estrone or estriol will greatly decrease its activity. According to these analyses, we speculate that E_2 may be partly converted to its metabolites in chicken hypothalamic cells to greatly abate its function; Meanwhile, tamoxifen may be converted to 4-hydroxytamoxifen to greatly increase its activity to antagonize E_2.

Our result also indicated that flutamide which was used as antagonists in chicken [19] restrained proliferation action that was induced by T. In the avian brain, androgen receptors have been observed predominantly in the hypothalamus [20]. Androgen receptors are believed to mediate many cellular responses to androgen in neurons. Our results suggested that the stimulating effect of T on neurons might also be via its direct binding to androgen receptor. Based on these results, the inhibitory effect of tamoxifen on the E_2-induced proliferation of hypothalamic neurons confirmed the role of estrogen on stimulating hypothalamic neurons through estrogen receptor-mediated mechanism. Moreover, the inhibitory effect of tamoxifen and flutamide on T-induced proliferation of hypothalamic neurons indicated the mechanism was through both androgen receptor-mediated direct action and estrogen receptor-mediated

indirect action as a precursor for estrogen. Since T could also be converted to dihydrotestosterone (DHT) by 5α-reductase, a non-aromatizable androgen that can also bind to androgen receptor, further study is required to identify whether the binding was T or DHT with androgen receptor.

Involvement of aromatase in the action of androgen induced neuron proliferation

T is metabolized locally to either E_2 via aromatase. The conversion of T into estrogen plays a limiting role in the activation of many aspects of reproductive behavior, as indicated by the facts that non-aromatizable androgens are often ineffective in stimulating these behaviors and aromatase inhibitors partly or completely block the action of T [21]. Besides influencing the reproductive behavior in adult, T had an effect on neuron development of the chicken embryo. In this study, the combined treatment of letrozole, a specific aromatase inhibitor, prevented the T-induced increase in neuron proliferation. This result implies that the conversion of T into estrogen by aromatase attributed partly to the action of T on neuron proliferation.

Sexually dimorphic pattern of hormonal effects on proliferation of hypothermic neurons

In this study, gonadal hormone-stimulated proliferation of hypothalamic neurons followed a sexually dimorphic pattern. The male neurons were much more responsive to T and female neurons showed higher responsiveness to E_2. Sexual differentiation of the brain occurs during the steroid-sensitive phases in early development, and is affected particularly by exposure to estrogens formed in the brain by aromatization of androgen [22]. This effect resulted in the male-specific neuronal morphology and regulation of reproductive behaviors. Furthermore, T induces hypothalamic aromatase activity more efficiently in male Japanese quail than in females [23]. From this experiment, we found that T played more sensitive effect on males. The difference was probably induced by the aromatase system that was highly expressed in the male. Based on these findings, we speculate that T played a crucial role on the process of neuron proliferation in the male brain development. While in the female, considering the higher level of E_2 in circulation and the lower activity of aromatase compared with the male, circulating E_2 may played a more important role on neuron proliferation.

Conclusions

This study revealed the proliferating effects of gonadal steroid hormones on embryonic chicken hypothalamic neurons in culture. E_2 promoted proliferation of neurons through estrogenic action and T stimulated neuron proliferation via its direct androgenic action and indirect estrogenic action. In addition, these effects were dose-dependent and sex-specific in which T played more sensitive effect on the male while E_2 showed more sensitive effect on the female.

Materials and methods

Animals

Fertilized Arbor Acres broiler chicken eggs were obtained from a commercial hatchery and incubated at 38.5°C and 60% humidity till 17-day-old.

Hypothalamic cell culture

Primary culture of embryonic hypothalamic cells was prepared according to the method by Pérez-Martínez et al. [24] with modifications. The embryos were sexed by gonadal inspection and the hypothalamus was dissected and placed in Ca^{2+} and Mg^{2+} free Hank's solution. The minced tissue pieces were dissociated in a solution of 0.5 mg/ml trypsin and 0.5 mg/ml collagenase at 37°C in a shaking water bath for 30 min with frequent pipetting to facilitate cell dissociation. The dispersed cell aggregates were filtered through a 50-μm mesh, and then washed with DMEM (Hyclone, Utah, USA). The cells were seeded in collagen-coated 96-well plastic culture plates (Nunc, Denmark) at a density of 10^5/well in 200 μl DMEM supplemented with 2.5% dextran-coated charcoal-stripped fetal bovine serum (GIBCO-BRL), 2 mM glutamine, 1.75 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated at 39°C in a water-saturated atmosphere of 95% air and 5% CO_2 for 3 days before treatment of chemicals.

Treatment of hypothalamic cells with chemicals

After 3 days in culture, the medium was refreshed with serum-free DMEM supplemented with 10 μg/ml insulin, 3×10^{-8} M selenite, and 5 μg/ml transferrin (Sigma, St. Louis, MO) as the ITS medium. The chemicals were dissolved in ethanol and then diluted with medium. Hypothalamic cells were treated with T (10^{-8}–10^{-6} M) or E_2 (10^{-8}–10^{-6} M). Cells were also challenged with flutamide (3.62×10^{-8}–3.62×10^{-6} M), tamoxifen (10^{-8}–10^{-6} M, Sigma), and letrozole (10^{-8}–10^{-6} M,Jiangsu Hengrui Medicine Co., China) alone or in combinations with T or E_2 (10^{-6} M). Controls received vehicles only. The final concentration of ethanol in the medium is $\leq 0.1\%$.

Morphological study

Morphological changes of hypothalamic cells were observed under an IX70 phase contrast microscope (Olympus, Japan). The images of cultured cells were captured with a digital video camera (Pixera Pro 150ES, USA). The neuron number was counted in each image with Simple PCI Advanced Imaging Software (Compix Inc., USA).

Immunocytochemistry of NSE and PCNA

The cultured cells were fixed with 4% polyformaldehyde and treated with 3% H_2O_2 to quench endogenous peroxidase. Cells were incubated with blocking buffer (PBS containing 10% neonate bovine serum), then with rabbit anti-NSE polyclonal antibody (1:400 dilution, Sigma) or mouse anti-PCNA polyclonal antibody (1:200 dilution, Boster Co., Wuhan, China) overnight at 4°C. The secondary antibody was biotin conjugated goat anti-rabbit/mouse IgG. The binding was visualized with a PicTure-Pius Kit (Zymed Laboratories, CA). Nuclei that were brown to black were counted as positive cells. The PCNA-labeling index was determined as the percentage of the neuron number with positively stained nuclei to the total number of neurons in the same fields.

BrdU incorporation

The incorporation of BrdU into replicating DNA was used to label proliferating cells. The cultured cells were incubated with 10 ng/ml BrdU for 6 h before the end of chemical treatments. Next steps were similar with NSE immunocytochemistry except that the first antibody was mouse anti-BrdU antibody (Sigma) with a 1:100 dilution.

Statistics

The experiments were repeated three times and each treatment included four wells. Data were expressed as the mean ± SEM and analyzed by ANOVA and Duncan's multiple range tests using the SAS 6.12 software. $P < 0.05$ was considered significantly different.

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