Moderate Hypogonadism Enhances Hippocampal Neurotransmission, Augments Memory and Learning and Modulates Neurogenesis in Adult Male Rats.

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ABSTRACT

The study aimed to test whether the decrease in testosterone level during aging is the underlying mechanism for the deterioration in memory and cognitive functions. This was achieved through determination of hippocampal neurotransmission by in vivo determination of extracellular dopamine and serotonin in CA1 hippocampal region and brain derived neurotorphic factor (BDNF) synthesis in both normal and unilateral castrated rats (an experimental model for hypogonadism) and 2 and 4 days after single dose of exemestane (5mg/kg, p.o.). In addition, learning and memory processes were determined using Morris water maze. Results showed that unilateral castration resulted in significant decrease in testosterone level, disturbing the testosterone/estradiol ratio and enhanced hippocampal neurotransmission. Besides, these effects were accompanied with an enhanced learning and memory and significant decrease in the level of BDNF expression. Whereas, exemestane treatment increased testosterone level and inhibited DA and 5-HT release, significantly increased BDNF expression and inhibited learning and memory processes in both normal and unilateral castrated groups. The study indicated that moderate hypogonadism has a positive effect on hippocampal neurotransmission, learning and memory due to the imbalance of testosterone/estradiol in favor of estradiol. On the other hand, exemestane effects might be due to imbalance of testosterone/estradiol ratio in favor of testosterone. In addition, it seems that the beneficial effects of physiological levels of testosterone are indirectly due its conversion to estradiol. Moreover, the study indicated that moderate decline in endogenous testosterone in healthy aged individuals is not the underlying mechanism of the age-related deterioration in the cognitive function.

INTRODUCTION

Aging in men is associated with a steady decline in gonadal androgen production (hypogonadism)[1,2]. Nevertheless, unilateral castration (hemigonadectomy) provides an experimental model of hypogonadism in males[3]. Hypogonadism is associated with many symptoms and physiological changes which are similar to that occurring with normal aging[4]. Besides, several studies indicated an association between gonadal activity and cognitive task performance, where hypogonadal men had impaired cognition functions[2,5].

Hippocampus is a key brain region regulating complex cognitive

process including learning and memory as well as emotional processes[6-9]. In addition, hippocampus is a target for the neuromedulatory actions of androgens, where androgen receptors are particularly concentrated in CA1 pyramidal cells of hippocampus[10,11]. Both serotonergic and dopaminergic neurotransmission in the hippocampus have been suggested to be necessary for formation of spatial learning and memory [12,13]. Moreover, increases in hippocampal DA and 5-HT induced by selective DA and 5-HT re-uptake blockers, exerted anticonvulsant effects[14]. In addition, elevated extracellular levels of noradrenaline, serotonin and dopamine in the limbic region are associated with antidepressant- anticonvulsant actions[15].

Brain- derived neurotophic factor (BDNF), is an abundant neurotrophin that regulates the survival, growth, morphological plasticity, and synthesis of new neurons for differentiated function, gene expression, synaptic function and cognition in the adult brain [16-18]. Moreover, several studies indicated that BDNF modulates monoamines, amino acids and peptides neurotransmission[19, 20].

Aromatase is a member of the cytochrome P450 superfamily that catalyzes the conversion of androgens [C(19)], namely testosterone and androstenedione, into oestrogens [C(18)], oestradiol, and oestrone, respectively[21]. Exemestane is a reversible steroidal aromatase inhibitor currently prescribed for advanced breast cancer in postmenopausal women to inhibit estrogen synthesis[22,23]. Exemestane's effect is mechanism- based, which requires the catalytic ability of active aromatase to convert it into an active intermediate, which in turn binds irreversibly to the enzyme causing its inactivation in a time dependent manner[24]. A single 25-mg dose of exemestane suppresses estrogen and elevate testosterone levels, where the maximum change in both testosterone and estradiol levels was observed after 12 hours and lasts about 2 days and returned to baseline 3-6 days after treatment[25]. Despite its steroidal structure, exemestane does not have hormonal or anti-hormonal activity, except for a slight androgenic activity[26]. Aromatase inhibitors have been shown to modulate brain neurotransmission and cognition in both normal and ovariectomized female rats[27]. However, insufficient data are available on the influence of the aromatase inhibitors on cognitive function on males[28].

The present study represents an attempt to clarify the effect of unilateral castration (an experimental model for hypogonadism) on brain function and to clarify whether testosterone's effect is mediated by itself or indirect through its aromatization to estradiol. This was achieved through in vivo determination of the extracellular levels of dopamine and serotonin and level of expression BDNF' mRNA of hippocampus of normal and unilateral castrated adult rats alone or in combination with exemestane treatment. Besides, learning and memory were tested using Morris water maze for different groups was carried out.

MATERIALS & METHODS

Animals:

Experiments were performed on adult male Wister rats (280-300 g) purchased from Kyudo Company-Japan. Rats were housed six per cage with food and water available *ad libitum* at 12- hr light/dark cycle. The experiments and surgery were carried out according to institutional guidelines of IACUC of Graduate School of Pharmaceutical Science-Kumamoto University- Japan.

Chemicals and accessories:

Exemestane hydrochloride was purchased from Wako company-Japan. The drug was dissolved in 0.5% carboxymethyl cellulose and administered orally at a single dose of 5.0 mg/kg. Dopamine and serotonin were purchased from Sigma- Aldrich-USA. TRIzol reagent and IQ™SYBR GREEN Supermix kit for determination of BDNF channel mRNA were purchased from Invitrogen Co. and BIO-RAD Co. CA, USA. Microdialysis accessories and HPLC column (PP-ODS) were purchased from Eicom company-Japan. Elisa kits for testosterone and estradiol were purchased from GenWay Biotech, Inc. Company San Diego. CA, USA.

Animal grouping:

A total number of 48 rats were divided into equal four main groups. The first group served as sham group, the second group is unilateral castrated animals, the third group is unilateral castrated rats treated with exemestane and the fourth is exemestane treated rats. Each group is divided into two sets each of six rats, one for *in vivo* determination of hippocampal extracellular transmitters and the other for the Morris water maze task.

Unilateral castration:

The animals were anesthetized by intraperitoneal injection of pentobarbital (50.0 mg/kg). With an incision into the lower part of abdominal cavity, the testis, vas deferens, and attached testicular fat pad are pulled out of the incision. The blood vessels were closed by knotting with surgical thread. The testis, vas deferens and fatty tissues were severed just below the site of legation and the wound was sutured. Sham group was subjected to the surgical procedure without removing the testis, vas deferens and the attached parts. Two weeks after surgery, the animals were used for the experiment. Levels of testosterone and estradiol were determined according to kit's manuals and read by microplate reader at 450 nm against reference filter set at 630 nm.

In vivo determination of hippocampal DA and 5-HT

The rats were anesthetized with pentobarbital (50.0mg/kg, i.p.) and mounted on stereotaxic frame and implanted with guide canulae in CA1 in hippocampus. The stereotaxic coordinate were 3.8 mm caudal to bregma, 1.6 mm from medial- lateral, and 3.6 mm ventral from the dura surface (according to the atlas of Paxinos and Watson [29]. Two jeweler's screws were placed in the skull surrounding the cannula and cemented in place with dental acrylic. The extracellular level of CA1 hippocampal DA and 5-HT were determined in both sham and unilateral castrated animals by

perfusing the implanted probe at constant flow rate of 2µl/ min with artificial CSF (composed of 145 mM NaCl, 3 mM KCl, 126 mM $CaCl_2$, and 1mM $MgCl_2$, buffered at pH 7.4 with 2 mM sodium phosphate buffer). The perfusate was collected every six min. and automatically injected and assayed by HPLC with electrochemical detection. After 1 hour of stabilization, to reach uniform concentration of DA and serotonin in dialysate, ten samples were collected from each animal to measure the initial extracellular DA and 5-HT levels. Following determining hippocampal dopamine and serotonin, probe positioning was histologically verified on a regular basis as shown by Clinckers *et al*. [(14)].

Morris Water Maze (MWM) test

The MWM test [(30)] was selected as a method of evaluation of spatial learning and memory. A circular and galvanized water tank (150 cm diameter x 50 cm height) was filled to a depth of 25 cm with water. The surface area of the tank is divided into four equal quadrants. The water was made opaque by addition of milk powder, and its temperature was maintained at 24 ± 1 °C. A hidden transparent circular platform (10 cm diameter) was placed 1.5 cm below the water surface and kept in constant position in the center of one of the four quadrants of the pool. The animals were gently released into the water, always facing the tank wall from three randomly assigned start locations (excluding the platform-containing quadrant) and allowed to search for the platform. The trial ended when an animal climbed onto the platform or when a maximum of 120 sec elapsed. When a rat mounted the platform, it was kept there for 30 sec, if the rat didn't reach the platform; it was transferred onto the platform by hand and kept there for 30 sec. Animals were trained for two days (a total of 6 trial- blocks) at the same time each day. After the training period, a probe test was administered on day 3. During this trial, the platform was removed from the pool. The latency to reach the previous location of the platform was measured during each trial. Animals were placed in a quadrant opposite to the location of the training platform and allowed to swim for 120 sec. Both the time the rat spent in searching for the platform in each quadrant and the number of times the rat entered to the quadrant of the former platform location were measured. After completion of the probe trial which assess the memory consolidation, the visible platform training was started to study possible non-spatial learning defects, i.e. sensory- motor abnormalities. During the visible platform trials, the platform was elevated 0.5 cm above the water level and marked by a flag. The location of the visible platform varied for each trial. Data collection was performed automatically by an online video-tracking device designed to track an object in a field. Escape latency (the time to climb onto the platform) and swimming speed (the distance that the animals swam divided by escape time) were determined for each trial with a behavioral tracing analyzer.

Quantitative analysis of BDNF mRNA real-time PCR

All the animals were sacrificed and hippocampus was quickly excised

on ice. Each right or left half of hippocampus was taken separately in liquid nitrogen for RNA determination. Total RNA was extracted from isolated hippocampus using a TRIzol® Reagent, (Invitrogen) according to method of Chomcsynski and Sacchi [31]. cDNA was synthesized from 200 ng of total RNA Synthesis Kit, following the suppliers protocol (Bio-Rad). Levels of BDNF mRNA were determined after reverse transcription by real-time PCR using a Chromo 4™ (Bio-Rad). The iQ™ SYBR® Green Supermix (Bio-Rad) kit was used. Primers for BDNF were constructed using Primer3 software. For standardization of quantification, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified simultaneously. The change of reporter fluorescence from each reaction tube was monitored by Chromo 4™. The threshold cycle of each gene was determined as PCR cycles at which amplification efficiency in reporter fluorescence is almost 100 % as much as possible. The level of original mRNA expression of target gene and GAPDH was calculated according to a standard curve made by total RNA extracted from whole brain. In order to reduce the variation between individuals, the expression level of target gene was normalized by that of GAPDH.

Statistical analysis:

Data reported as means ± S.E. Statistical analysis was carried out using One way ANOVA followed by post-hoc LSD test (SPSS 13.0). Probability value of $P < 0.05$ was considered significant.

RESULTS

Unilateral castration induced a significant decrease ($P < 0.001$) in the level of both testosterone and estradiol in blood in comparison to sham group. Exemestane administration to castrated group significantly ($P<0.001$) increased the level of testosterone and induced minor changes in the level of estradiol after 2 and 4 days of drug treatment, in comparison to unilateral castrated group. Exemestane treatment to normal group significantly ($P < 0.001$) increased the level of testosterone and decreased the level of estradiol after 2 days and approached the normal level after 4 days of drug treatment (Table 1).

Unilateral castration in rats induced significant increase ($P<0.001$) in extrcellular level of both dopamine and serotonin in CA1 hippocampal region in comparison to sham group. Exemestane treatment to both castrated and sham groups significantly ($P<0.001$) depressed dopamine and serotonin release to a level lower than the sham group. There was no significant difference in extracellular dopamine and serotonin levels between exemestane- castrated and exemestane –sham groups (table 2).

During the hidden platform phase in the first and second day, castrated rats exhibited least latency to reaching the platform compared with sham and exemestane- treated groups. Exemestane treatment showed highest latency to reaching the platform in comparison to castrated and sham animals (Figure 1). The working memory test (the probe test) showed

that castrated rats spent high percentage time in the target quadrant within 120 sec. compared with sham and exemestane-treated groups. On the other hand, exemestane groups (castrated- and sham-exemestane) spent the lower percentage time in the target quadrant within 120 sec in comparison to castrated and sham animals (Figure 2). There was no difference in the performance between different groups measured on visibility test (Figure 3).

In addition, unilateral castrated animals exhibited a depressed expression level of BDNF in the hippocampal region in comparison to control (sham) group. Meanwhile, exemestane treatment significantly decreased the expression level of BDNF in control group and didn't change the depressive effect of castration in castrated group. (Figure 4).

Table 1: Effect of Exemestane Administration on Testosterone and β Estradiol Serum Levels in Both Normal and Castrated Adult Male Rats.

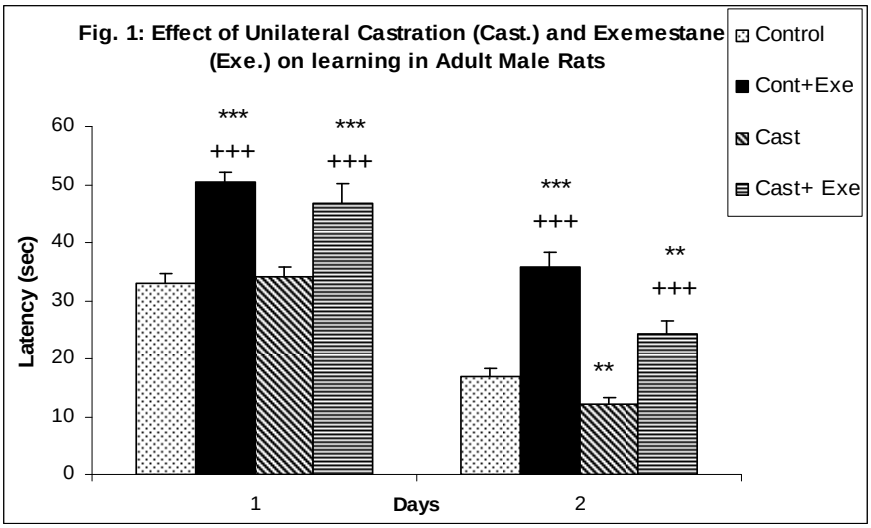
		Before Exemestane Treatment	After Exemestane Treatment	
Testosterone (ng/ml)			**2 days**	**4 days**
	Control	7.675 ± 0.346	15.127±0.611***,+++	6.500±0.327*, +++
	Castrated	3.625±0.263 ***	5.750±0.250***, +++	4.125±0.295***
β Estradiol (pg/ml)	Control	61.375±3.041	48.375±2.061***	56.750±2.541 +++
	Castrated	44.750±1.709***	47.000±2.079***	50.125±1.913***

* P<0.05, ** P<0.01, *** P<0.001 Significant different compared to control group,
\+ P<0.05, ++P<0.01, +++P<0.001 Significant different compared to castrated group,

Table 2: Effect of Exemestane Administration on Extracellular Levels of Dopamine and Serotonin in CA1 hippocampal Region in Both Normal and Castrated Adult Male Rats.

Groups Parameters	**Control**	**Control-exemestane**	**Castrated**	**Castrated-exemestane**
DA (pg/12 µl)	0.171±0.003	0.090±0.003***,+++	0.235±0.013***	0.086±0.007***, +++
% of control	100.0	52.6%	137.4%	95.6%
5-HT(pg/12µl)	0.375±0.019	0.219±0.013***,+++	1.262±0.099 ***	0.233±0.016***, +++
% of control	100.0	58.4%	336.5%	62.1%

* P<0.05, ** P<0.01, *** P<0.001 Significant different compared to control group,
\+ P<0.05, ++P<0.01, +++P<0.001 Significant different compared to castrated group,



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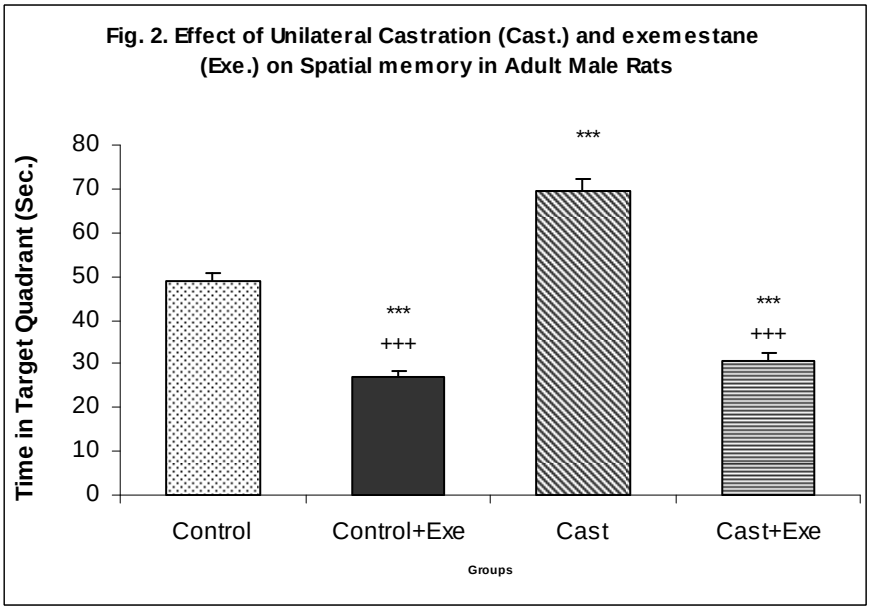

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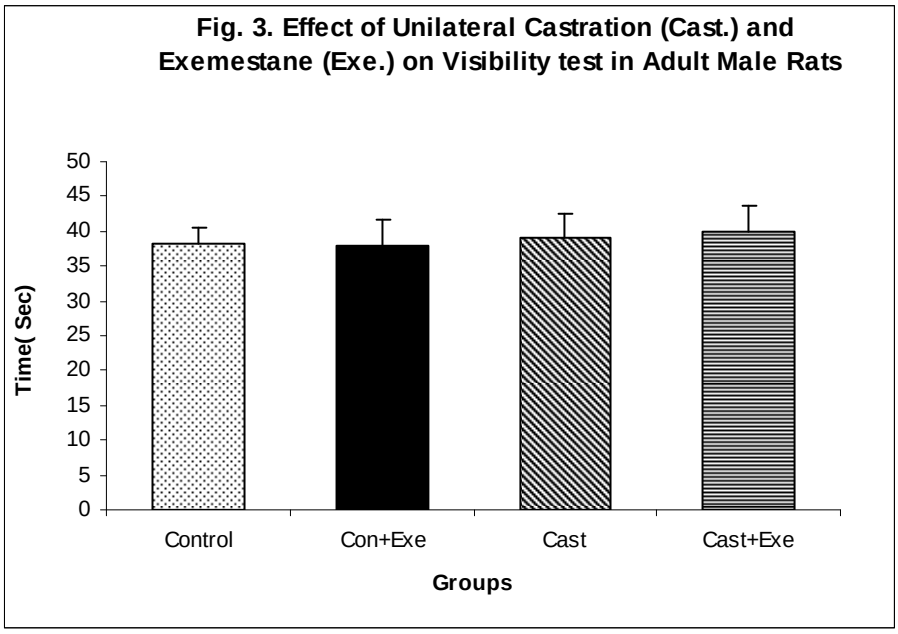

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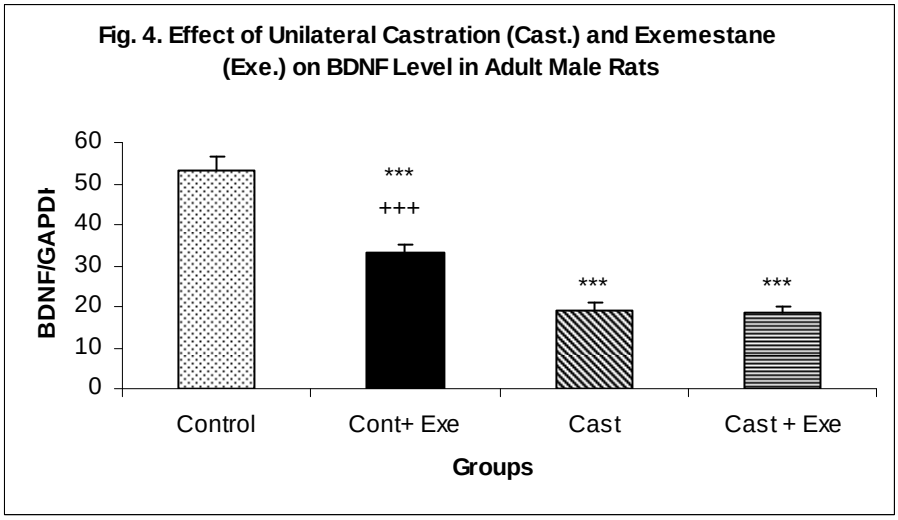

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DISCUSSION

The present data showed that unilateral castration- as an experimental model for hypogonadism- induced significant decrease in testosterone and estradiol levels and disturbed testosterone/ estradiol ratio in favor of estradiol. It is suggested that unilateral castration with the subsequent reduction in the testosterone level concomitantly with the uninterrupted synthesis and secretion of estradiol from extra-gonadal sources, might offset the physiologic equilibrium between testosterone and estradiol in favor of estradiol. In accordance to this interpretation, aromatase enzyme exists in various tissues in both females and males, thus oestrogens are produced not only in gonads but also in extra-gonadal localizations such as brain, adipose tissue, breast, skin, and bone[21]. On the other hand, exemestane treatment decreased estradiol level and increased testosterone level in both sham and castrated group due to its inhibitory effect on aromatase enzyme. The present finding is in accordance with Mauras *et al*.[25].

In addition, unilateral castration elevated the extracellular level of both dopamine and serotonin in CA1 region of hippocampus compared to normal group. This might indicate that hypogonadism induced activation in the transmitters' release, reflecting an enhancement of both dopaminergic and serotonergic neurotransmission in the hippocampus. On the other hand, exemestane treatment in both castrated and sham animals induced significant reduction in the extracellular level of both dopamine and serotonin in CA1 region of hippocampus, which might indicate a reduction in the transmitters' release, reflecting an inhibition of both dopaminergic and serotonergic neurotransmission in the hippocampus. It seems that there is a correlation between castration with the subsequent change in testosterone/ estradiol ratio in favor of estradiol and the enhanced neurotransmission in the hippocampus. The elevation of testosterone level resulting from the inhibition of estradiol synthesis under exemestane treatment concomitant with the noticeable inhibition in the transmitters release might support this opinion. In agreement, Bimonte-Nelson *et al*. [32] reported that the ratio of estradiol to testosterone or the actions of aromatase enzyme may be the responsible for the effects of testosterone on memory and cognition.

Alternatively, we might speculate that testosterone and estradiol play antagonizing actions and the maintenance of their ratio is very important for the normal brain neurotransmission. Thus, testosterone through its binding to the androgenic receptors exerts a braking effect on both serotonergic and dopaminergic neurotransmission in the normal state. Whereas, in hypogonadism, the braking effect of testosterone diminishes and the enhancing effect of estradiol prevails leading to an activation of the dopamine and serotonin neurotransmission. In accordance to this interpretation, intra- CA1 hippocampal injection of testosterone impaired learning and memory acquisition, consolidation

and retrieval in adult male rats [33]. Moreover, Martin *et al*. [34] indicated that higher levels of endogenous testosterone, particularly in the elderly, may have deleterious effects on cognitive functioning in men.

In accordance, gonadectomy has been shown to increase the activity of brain monoaminergic neurons and reduced 5-HT depletion-induced disinhibitory behavior in shock-induced behavioral inhibition model (punished conflict model), whereas testosterone substitution antagonize this effect, which might be related to serotonin release[35]. Moreover, Aydin *et al.*[27] indicated that administration of an aromatase inhibitor, letrozole (1mg/kg, p.o.) significantly decreases dopamine and noradrenaline in the hippocampus. It is worthy to note that there exists a correlation between the elevated level of testosterone with the lower serotonergic activity measured by low cerebrospinal fluid 5-hydroxyindolacetic acid form one side and appearance of behavioral abnormalities like aggression, anxiety and depression from the other side [36]. Consistently, Keleta *et al*.[37] indicated that repeated testosterone treatment significantly reduced levels of 5-HT and 5-HIAA in most brain regions except frontal cortex.

Morris water maze task

Hidden platform test indicated that castrated rats exhibited enhanced learning ability (least latency to reaching the platform) compared with sham and exemestane- treated groups. Whereas exemestane treatment induced significant inhibition in learning ability in comparison to castrated and sham animals, which might indicate the suppressive effect of elevated unconvertible testosterone on the learning process. The performance in visibility test showed no significant difference between the different groups which might indicate that unilateral castration and exemestane administration didn't cause abnormalities in sensory processes, motivations, locomotor activity, or coordination in comparison to sham group. In accordance to this explanation, Sarnyai *et al*.[38] reported that increased escape latency in visibility test indicates the occurrence of sensory–motor coordination impairment.

Interestingly, males have notably stable and abundant levels of substrate (testosterone) for conversion to estradiol and exhibit higher levels of brain aromatase compared to females. Thus, it has been speculated that testosterone may play an indirect role through its aromatization to estradiol[25].

The observation that exemestane treatment induced moderate decrease in BDNF expression while unilateral castration inhibited hippocampal neurogenesis decrease might indicate the indirect importance of testosterone through its conversion to estradiol. Consequently, the change in the physiological level of testosterone due to castration or exemestane administration, along with changes in the hippocampal neurotransmission might affect the expression of BDNF mRNA. Alternatively, the change on the testosterone/estradiol balance due to castration or exemestane administration might directly modify the expression of BDNF which in turn modulates serotonin

neurotransmission by inhibiting 5-HT transporter expression. In accordance to this interpretation, Ottem *et al*.[39] indicated that testosterone maintains a BDNF signaling that may underlie the maintenance of dendritic structure and synaptic signaling. Moreover, several studies revealed that changing the levels of BDNF expression modulates the serotonergic neurotransmission[40,41]

It's worthy to note that expression of BDNF mRNA is an activity dependent, undergoes regulation during development, and also shows marked and transient changes in response to a number of neuronal insults and pharmacological manipulations[42]. Moreover, regulation of BDNF mRNA levels under basal conditions, as well as in response to different insults, involves a complex interplay between different neurotransmitter systems[42]. Interestingly, neurons in the adult rat forebrain of both sexes, particularly the pyramidal cells of CA3 and CA1 regions of hippocampus, coexpress estrogen and neurotrophin receptors and are the sites of estrogen and neurotrophin synthesis [43]. Consistently, Zhou et al.[44] demonstrated that 17 beta-estradiol increased BDNF expression in CA1 and CA3 regions of hippocampus of ovariectomized rats. The observation that supraphysiological levels of testosterone initiate the apoptotic cascade leading to neuronal cell death [45] might suggest that beneficial effect of testosterone is due to its conversion to estradiol [46]. In accordance, Johnson *et al*.[47] indicated that androgens decreases dopamine neurons survival in rat midbrain.

The study indicated that hypogonadism has a positive effect on hippocampal neurotransmission and cognitive functions, at the short term, due to the imbalance of testosterone/estradiol in favor of estradiol and exerts adverse effect on hippocampus neurogensis. In addition, it seems that testosterone's effects are due to its conversion to estradiol rather than to testosterone itself. Consistently, exemestane induced-negative effects might be due to imbalance of testosterone/estradiol ratio in favor of testosterone. The study speculates that declines in endogenous testosterone in healthy middle- aged individuals are just co-occurred physiological declines along with age-related deterioration in cognition and memory. Moreover, the long term effect of hyopgonadism on cognitive functions and neurogenesis merits other studies.

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قصور الغدد التناسلية المعتدل ينشط النقل العصبي في الهيبوكامبس، تقوي الذاكرة والتعلم وينظم تكوين الخلايا العصبية في الفئران الذكور البالغة.

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تهدف الدراسة إلى اختبار ما إذا كان الانخفاض في مستوى هرمون التستوستيرون خلال الشيخوخة هي الآلية الكامنة وراء تدهور في الذاكرة والوظائف الإدراكية. وقد تم دراسة ذلك من تقدير مستوي كلا من الناقل العصبي الدوبامين والسيروتونين خارج الخلية في منطقة CA1 بالهيبوكامبس بالمخ و كذلك تقدير (عامل التغذية العصبية مخي المنشأ) (BDNF) في كل من الفئران العادية والمخصي من جانب واحد (نموذج تجريبي لقصور الغدد التناسلية) و بعد ٢ و ٤ أيام من الحقن بجرعة واحدة من عقار exemestane (٥ مجم/ كجم/ عن طريق الفم). وبالإضافة إلى ذلك، تم دراسة التعلم والذاكرة باستخدام متاهة موريس المائية. وأظهرت النتائج أن الإخصاء من جانب واحد أدى إلى انخفاض كبير في مستوى هرمون التستوستيرون، و أدت الي اختلال نسبة التستوستيرون الى الاستراديول و كذلك الى تنشيط النقل العصبي في منطقة الهيبوكامبسمن خلال زيادة معدل انطلاق كلا من الدوبامين و السيروتونين، بالاضافة الي تحسن في القدرة على التعلم والذاكرة وانخفاض ملحوظ في مستوى التعبير لعامل التغذية العصبية. في حين أدت المعالجة بعقار exemestane الى زيادة مستوى هرمون التستوستيرون، ونقص في مقدار انطلاق كلا من الناقل العصبي الدوبامين و السيروتونين ، زيادة كبيرة لعامل التغذية العصبية و تدهور القدرة على التعلم والذاكرة في كلا من المجموعة العادية ومجموعة قصور الغدد التناسلية المعتدل. وأشارت الدراسة إلى أن قصور الغدد التناسلية المعتدل له تأثير إيجابي على النقل العصبي في منطقة الهيبوكامبس، و زيادة القدرة على التعلم والذاكرة نتيجة اختلال نسبة هرمون التستوستيرون / الاستراديول لصالح الاستراديول، في حين يسبب عقار exemestane خلل في نسبة التستوستيرون/ الاستراديول لصالح من هرمون التستوستيرون. وبالإضافة إلى ذلك، يبدو أن الآثار المفيدة لمستويات هرمون التستوستيرون الفسيولوجية تكون مفيدة بشكل غير مباشر بسبب تحولها إلى استراديول. وعلاوة على ذلك، أشارت الدراسة إلى أن الانخفاض المعتدل لهرمون التستوستيرون لدى الأفراد الاصحاء المسننين ليست الآلية الأساسية التي تفسر تدهور الحالة الادراكية المرتبطة بالشيخوخة.